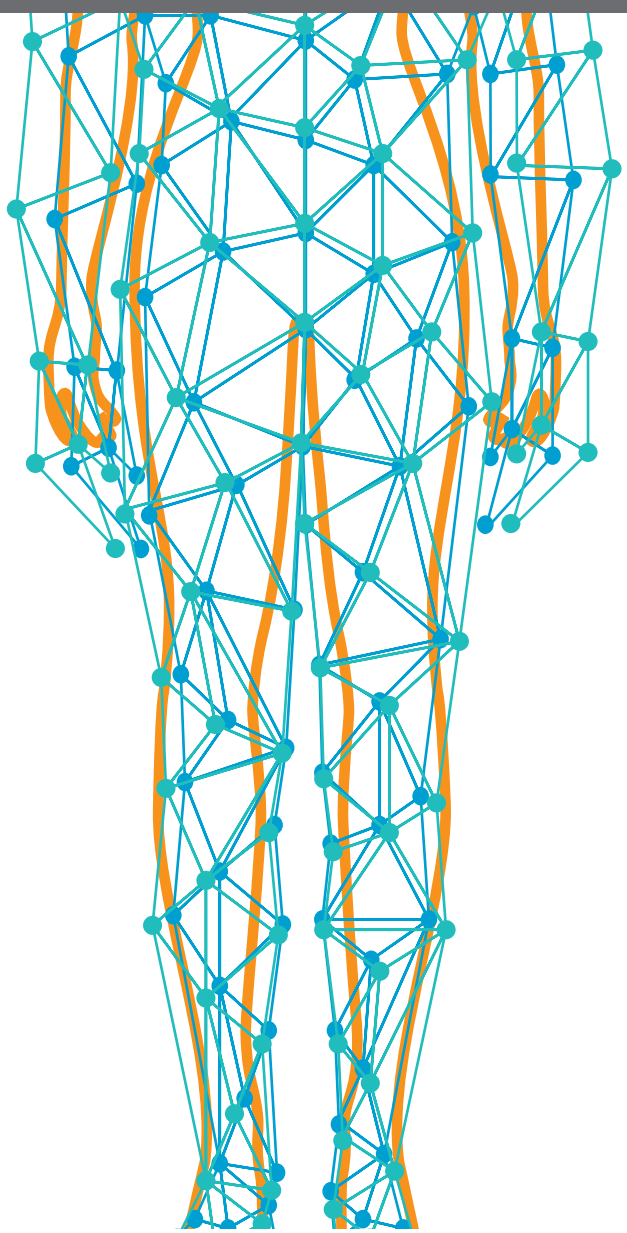
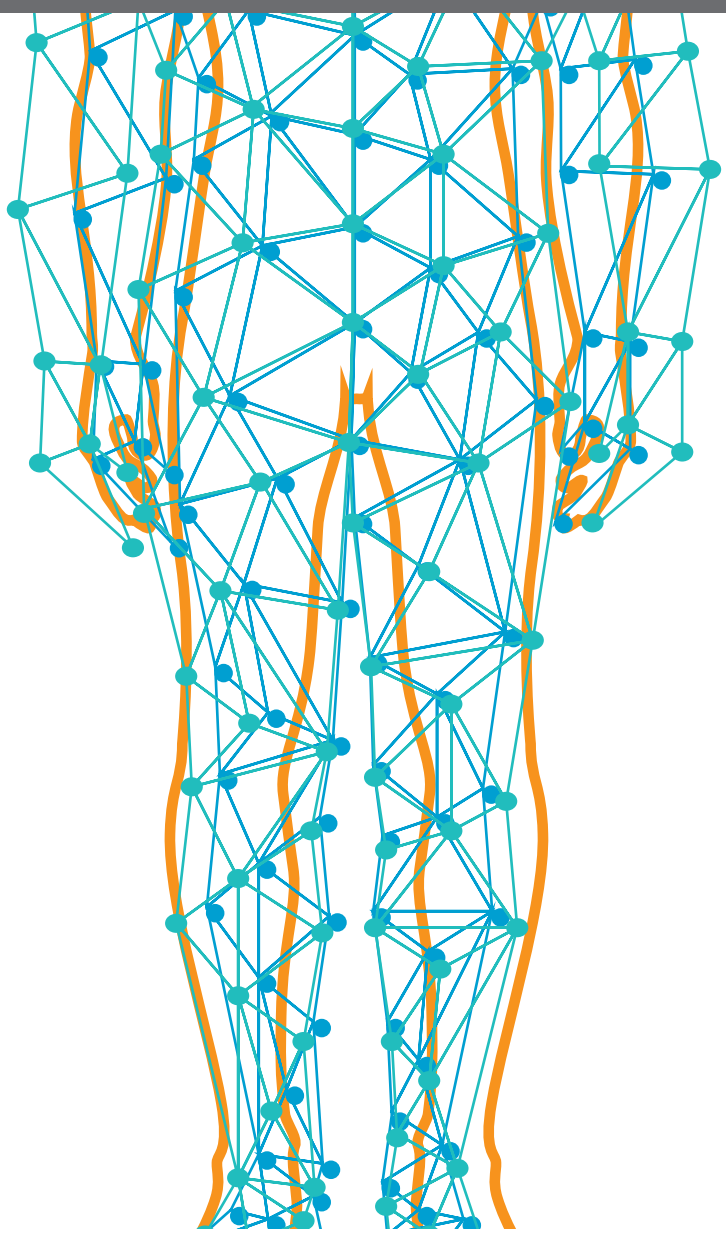
WOMEN IN SCIENCE - HEMATOLOGY 2021

EDITED BY: Eleni Gavriilaki, Chien-Ling Huang and Lalitha Nayak
PUBLISHED IN: Frontiers in Medicine





frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88976-523-2
DOI 10.3389/978-2-88976-523-2

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

WOMEN IN SCIENCE - HEMATOLOGY 2021

Topic Editors:
Eleni Gavriilaki, University of Cagliari, Italy
Chien-Ling Huang, Hong Kong Polytechnic University, SAR China
Lalitha Nayak, Case Western Reserve University, United States

Citation: Gavriilaki, E., Huang, C.-L., Nayak, L., eds. (2022). Women in Science - Hematology 2021. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88976-523-2

Table of Contents

frontiers | Frontiers in Medicine

EDITORIAL
published: 13 June 2022
doi: 10.3389/fmed.2022.926204

Editorial: Women in Science—Hematology 2021

Eleni Gavriilaki[1]*, *Chien-Ling Huang*[2] and *Lalitha Nayak*[3]

[1] Hematology Department—Bone Marrow Transplantation Unit, G Papanicolaou Hospital, Thessaloniki, Greece, [2] Department of Health Technology and Informatics, The Hong Kong Polytechnic University, Hong Kong, Hong Kong SAR, China, [3] Division of Hematology and Oncology, University Hospitals, Case Western Reserve University, Cleveland, OH, United States

Keywords: medicine, hematology, STEM, histo-hematology, molecular-hematology, UNESCO

Editorial on the Research Topic

Women in Science—Hematology 2021

Although the proportion of women and men in science, technology, engineering, and mathematics (STEM) at undergraduate level is relatively equal, there is a lack of representation of women in senior positions in public health. According to the UNESCO Institute for Statistics (UIS) data in 2016, <30% of researchers in STEM are women. In the field of hematology, there are many highly influential and successful women who are contributing to the field and tackling important questions. Yet, female scientists are still under-represented in various aspects of academic life. More importantly, the COVID-19 (coronavirus disease 2019) pandemic had a significant impact on female scientists and especially early career researchers in hematology (1, 2). In this context, maternity issues remain an important aspect, especially pregnancy and lactation. Since the latter by no means can be shared with husband/spouse, some female colleagues consider them as a privilege, whereas others as an issue that significantly affects their career.

Several initiatives have been recently created to increase the visibility of women in science, such as awards for women in STEM and diversity initiatives like those of the European Hematology Association (EHA). However, evidence indicates that a gender bias is still present throughout many scientific disciplines.

This Research Topic highlighted female contributions to medicine, specifically in the field of hematology, aiming to delineate:

- General perspectives on a specific field of research inspired, started, or sparked by a woman
- Articles celebrating outstanding female researchers and their contributions to computer science and public health
- Public health studies led by women researching technology and health.

To be considered for this Research Topic, the first or last authors were female researchers, and we recommend early career researchers to team up with senior female colleagues. All articles submitted to us for this Research Topic underwent a rigorous peer review process. Ultimately, 11 articles were published.

(i) The Spanish group led by Ceballos et al. published a brief research report on coagulation markers in COVID-19 suggesting that lower levels at diagnosis might be associated with higher morbidity and mortality.

(ii) In the context of COVID-19, Anipindi et al. documented an interesting case report of cerebral venous sinus thrombosis.

(iii) In a collaboration of first and last female authors, Vanegas et al. provided a mini review article on unrelated umbilical cord blood graft vs. haploidentical donor transplantation.

OPEN ACCESS

Edited and reviewed by:
Argiris Symeonidis, University of Patras, Greece

***Correspondence:**
Eleni Gavriilaki
elenicelli@yahoo.gr

Specialty section:
This article was submitted to Hematology, a section of the journal Frontiers in Medicine

Received: *22 April 2022*
Accepted: *02 May 2022*
Published: *13 June 2022*

Citation:
Gavriilaki E, Huang C-L and Nayak L (2022) Editorial: Women in Science—Hematology 2021. Front. Med. 9:926204. doi: 10.3389/fmed.2022.926204

(iv) In the life-threatening field of thrombotic microangiopathies, Pang et al. identified a rare case of thrombotic microangiopathy induced by remethylation disorders.
(v) In their very interesting paper, Fenoglio et al. from Italy, report on a prospective single study evaluating the clinical presentation and effects of rituximab in patients with non-HCV-related cryoglobulinemic syndrome.
(vi) This study from Palestine, led by Aldwaik et al. describes the characteristics and evaluates hematological, biochemical, and hormonal findings in β-thalassemia patients in the West Bank and highlights the importance of establishing patient-tailored comprehensive assessment and follow-up protocols with emphasis on blood transfusion and iron-chelation practices for the management of this disease.
(vii) Xu et al. demonstrate how the addition of two condition-specific bolt-on items can increase performance on the EQ-5D-5L in patients with hemophilia.
(viii) Patterson et al. sought to assess thalassemia patients' knowledge of transfusion, complications, and guidelines. Their results suggest that the necessity of increased patient education on terminologies pertaining to red cell transfusion and implementation of a nationwide registry to make transfusion data available to providers are some of the actions that could help reduce transfusion complications,
(ix) Zhang et al. show that the presence of circulating nucleated red blood cells (NRBCs) in patients of hemorrhagic fever with renal syndrome is associated with disease severity. This study provides further insights on the role of and pathological changes in NRBCs during Hantaan virus infection.
(x) An interesting case report, published by Sun et al., reported on gestational psittacosis concomitant with secondary hemophagocytic syndrome. Metagenomic next-generation sequencing is recommended to provide a clear diagnosis in addition to hematological examinations.
(xi) In a novel methods article, a modified preparation method of an ideal platelet-rich fibrin matrix from whole blood has been described (Reksodiputro et al.).

Taking into account the multi-disciplinary character of this Research Topic, we hope that it will inspire female researchers and clinicians to continue their explorations into novel advances in their fields.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

EG was supported by the ASH Global Research Award. C-LH was supported by the Health and Medical Research Fund Commissioned Research on COVID-19 (COVID1903007). LN was supported by a National Heart, Lung, and Blood Institute grant HL121131-01.

REFERENCES

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

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.

frontiers in Medicine

ORIGINAL RESEARCH
published: 25 May 2021
doi: 10.3389/fmed.2021.665410

The Presence of Circulating Nucleated Red Blood Cells Is Associated With Disease Severity in Patients of Hemorrhagic Fever With Renal Syndrome

Jingang Zhang[1,2], Kang Tang[1], Yun Zhang[1], Ying Ma[1], Chunmei Zhang[1], Haifeng Hu[3], Xiaozhou Jia[4], Ran Zhuang[1], Boquan Jin[1], Meng Wang[1], Xiyue Zhang[1], Dalu Liu[5] and Yusi Zhang[1]**

[1] Department of Immunology, Basic Medicine School, Air Force Medical University, Xi'an, China, [2] Brigade of Cadet, Air Force Medical University, Xi'an, China, [3] Center for Infectious Diseases, Second Affiliated Hospital of Air Force Medical University, Xi'an, China, [4] Department of Infectious Disease, Xi'an Eighth Hospital, Xi'an, China, [5] Department of Radiation Medicine and Protection, Ministry of Education Key Lab of Hazard Assessment and Control in Special Operational Environment, School of Public Health, Air Force Medical University, Xi'an, China

OPEN ACCESS

Edited by:
Chien-Ling Huang,
Hong Kong Polytechnic University,
Hong Kong

Reviewed by:
Lih-Chyang Chen,
Mackay Medical College, Taiwan
Kelvin Ka-lok Wu,
Hong Kong Polytechnic University,
Hong Kong

***Correspondence:**
Yusi Zhang
zhangyusi_scu@163.com
Dalu Liu
dl_neurosci@126.com

Specialty section:
This article was submitted to Hematology, a section of the journal Frontiers in Medicine

Received: 08 February 2021
Accepted: 19 April 2021
Published: 25 May 2021

Citation:
Zhang J, Tang K, Zhang Y, Ma Y, Zhang C, Hu H, Jia X, Zhuang R, Jin B, Wang M, Zhang X, Liu D and Zhang Y (2021) The Presence of Circulating Nucleated Red Blood Cells Is Associated With Disease Severity in Patients of Hemorrhagic Fever With Renal Syndrome. Front. Med. 8:665410. doi: 10.3389/fmed.2021.665410

Hemorrhagic fever with renal syndrome (HFRS) is a regional infectious disease of epidemic potential caused by the Hantaan virus (HTNV). Red blood cells (RBCs) are the major components of peripheral blood. However, pathological changes in RBCs and the underlying mechanisms during HTNV infection remain largely unclear. Therefore, this study sought to explore changes in RBCs in the peripheral blood of HFRS patients. We isolated PBMCs from HFRS patients and performed single-cell RNA sequencing. The results showed that clusters of RBCs in the peripheral blood of HFRS could be classified as nucleated red blood cells (NRBC) based on their cellular components, gene expression profiles and cell surface markers. In addition, it was shown that the higher the count of NRBC in peripheral blood, the more severe the disease status was. Moreover, hematological indices related to RBCs were analyzed and the results showed that impairment in the folate pathway might be the possible reason behind the presence of NRBCs. This study, for the first time showed that the presence of NRBCs in the peripheral blood of HFRS patients was associated with disease severity. This was also the first study to show that infection with the HTNV virus hindered the maturation of RBCs. Therefore, this work provides further insights on the role of and pathological changes in RBCs during HTNV infection.

Keywords: HTNV, HFRS, NRBC, vitamin B12 (B12), folic acid (B9)

INTRODUCTION

Hemorrhagic fever with renal syndrome (HFRS) is caused by infection with the Hantaan virus (HTNV) and has resulted to epidemics throughout Eurasia. Notably, China has the highest number of HFRS patients worldwide (1). Moreover, the phenotypic and functional characteristics of peripheral blood mononuclear cells (PBMCs) including T cells, B cells, dendritic cells and natural

killer cells have been well-studied during HTNV infection (2–6). However, responses in red blood cells (RBCs), a major component of blood to HTNV infection are yet to be reported.

RBCs are developed within the bone marrow through the process of erythropoiesis which involves a series of maturation steps. During this process, multipotent hematopoietic stem cells differentiate into erythroid progenitor cells. Thereafter, nucleated precursors differentiate from proerythroblasts into basophilic, polychromatic and orthochromatic erythroblasts (7, 8). These stages result to the accumulation of hemoglobin (HGB) (9). Additionally, when reticulocytes enter peripheral circulation, they undergo enucleation, loss of organelles and subsequent maturation into RBCs (10). Therefore, given the maturation process of RBCs, the nucleated red blood cells (NRBCs) should remain in the bone marrow of healthy adults except for certain physiological or pathological conditions where they become visible.

Studies on intensive care unit (ICU) patients reported that the appearance of NRBC in circulation was a predictor of increased mortality and poor prognosis (11–13). Moreover, the role of NRBCs in some infectious diseases such as HIV was reported. The immature RBCs ($CD71^+$erythroid cells) were found to contribute to the persistence and transmission of HIV (14). However, none of these studies explored the gene expression profile of the NRBCs and their underlying physiological functions. Furthermore, little is known about the causes of the presence of NRBCs in peripheral blood under disease conditions.

In order to address these gaps, the present study for the first time performed single-cell RNA sequencing (scRNA-seq) to profile the PBMCs of HFRS patients. The results showed that NRBCs were present in circulation during infection with HTNV. Moreover, the study established that the presence of NRBCs was associated with disease severity by evaluating the cell counts, clusters distributions and gene expression of the cells. Furthermore, reasons behind the appearance of NRBCs were explored and the study established that disorders in the folate pathway might contribute to the appearance of the cells in the peripheral blood of HFRS patients. Therefore, this study not only depicts the cellular components and gene expression profiles of NRBCs in the circulating blood of HFRS patients but also explores the possible reasons behind these phenomena. Consequently, the results will help in understanding how the erythroid cell lineage copes with the challenge of HTNV infection.

MATERIALS AND METHODS

Sample Collection

All the HFRS patients enrolled in this study were recruited from the Tangdu Hospital of the Fourth Military Medical University (Xi'an, China) and Xi'an Eighth Hospital from 2019 to 2021. Clinical diagnosis of HFRS was confirmed serologically through the detection of specific IgM and IgG antibodies against the HTNV-nucleocapsid protein (NP). The peripheral whole blood samples were collected using vacuum-based blood collectors with ethylenediaminetetraacetic acid (EDTA) from 82 HFRS patients (62 males and 20 females within the age range of 16–85 years) during hospitalization. Following centrifugation at 3,000 rpm for 15 min, the plasma layer was aliquoted and stored at −80°C until use. Additionally, PBMCs were isolated by standard Ficoll density gradient centrifugation and frozen in liquid nitrogen with RPMI/10% DMSO as the freezing solution and used within 2 weeks. The viral load in each sample was measured according to a method previously established in our lab (15). All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation [Xijing Hospital, First Affiliated Hospital of Fourth Military Medical University, Xi'an, China, (NO. KY20173177-1)] and with the Helsinki Declaration of 1975, as revised in 2008. Informed consent was obtained from all patients for being included in the study. One child patient was included with the written consent obtained from a parent and assent obtained from the child patient herself. All the data were analyzed anonymously.

Single-Cell RNA Sequencing

All the sequencing was done by the Novel Bioinformatics Co., Ltd, Shanghai, P. R. China. Briefly, the single-cell RNA sequencing (scRNA-Seq) libraries were generated using the 10X Genomics Chromium Controller Instrument and Chromium Single Cell 5' library & gel bead kit (10X Genomics, Pleasanton, CA). The cells were then concentrated to 1,000 cells/μL and ∼6,000 cells were loaded into each channel to generate single-cell gel bead-in-emulsions (GEMs). Following the RT step, GEMs were broken and the barcoded-cDNA purified and amplified. The amplified barcoded cDNA was then used to construct 5' gene expression libraries. Afterwards, the amplified barcoded cDNA was fragmented, A-tailed, ligated with adaptors and index PCR amplified. The final libraries were quantified using the Qubit High Sensitivity DNA assay (Thermo Fisher Scientific) and the size distribution of the libraries determined using a High Sensitivity DNA chip on the Bioanalyzer 2,200 (Agilent). All libraries were sequenced using the illumina sequencer (Illumina, San Diego, CA) on a 150 bp paired-end run.

Single-Cell RNA Statistical Analysis

scRNA-seq data analysis was performed by the Novel Bioinformatics Co on the NovelBrain Cloud Analysis Platform. The adaptor sequence was filtered and low-quality reads removed to obtain clean data. Afterwards, the feature-barcode matrices were obtained by aligning reads to the human genome (GRCh38 Ensemble: version 91) using cellranger v3.1.0. Cells that contained over 200 expressed genes and had a mitochondria UMI rate below 20% passed the cell quality filtering and mitochondrial genes were removed from the expression table.

Moreover, the Seurat package (version: 2.3.4) was used for cell normalization. Regression was based on the expression table according to the UMI counts of each sample and percent of mitochondrial rate to obtain the scaled data. Principal component analysis (PCA) was constructed based on the scaled data with the top 2,000 highly variable genes. In addition, the top 10 principals were used for constructing the t-distributed stochastic neighbor embedding (tSNE) graph. Using the graph-based cluster method, the unsupervised cell cluster result was

obtained based on the PCA top 10 principals. Moreover, the marker genes were calculated using the Find All Markers function and the wilcox rank sum test algorithm utilizing the following criteria: 1. lnFC > 0.25; 2. *P*-value<0.05; 3. min.pct > 0.1. Finally, in order to obtain details on the cell types, clusters of the same cell type were selected for re-tSNE analysis, graph-based clustering and marker analysis.

Pseudotime Analysis

Single-cell trajectories analysis was performed through Monocle2 (http://cole-trapnell-lab.github.io/monocle-release) using the DDR-Tree and default parameters. Before Monocle analysis, the marker genes from the Seurat clustering results were selected and raw expression counts of the cell passed filtering. Based on pseudotime analysis, branch expression analysis modeling (BEAM Analysis) was applied for branch fate determined gene analysis.

Co-regulated Gene Analysis

The find_gene_modules function of Monocle3 was used with the default parameters to discover the gene co-regulation network.

In vitro HTNV Infection

Peripheral whole blood samples were collected using vacuum-based blood collectors with EDTA from 4 health donors. Following centrifugation at 3,000 rpm for 15 min, the plasma layer was aliquoted and stored at −80°C until use. The whole blood cells were resuspended in RPMI 1,640 with 10% FBS.

HTNV strain 76–118 was stored and frozen at −80°C in the Department of Microbiology, Fourth Military Medical University. Mock HTNV control was prepared by incubating HTNV at 60 °C for 30 min. For the infection, virus was allowed to adsorb to the cells at multiplicity of infection (MOI) of approximately 1 for 2 h at 37 °C. The cells were then washed and afterwards incubated in RPMI 1,640 with 10% FBS. After 72 h post infection, the cells were collected.

Flow Cytometry Analysis

For PBMCs surface staining, 4×10^6 freshly isolated PBMCs from 40 HFRS patients in different disease severity and 7 health donors were used. For whole blood cell staining, 100 μl whole blood cells from 4 health donors were used. The cells were stained with anti-CD71 antibody (eBioscience, clone: OKT9) in staining buffer (PBS containing 3% FCS and 0.01% NaN3) for 30 min on ice at first place. After washing the cells with staining buffer once, the Alexa fluor 488-conjugated goat anti-mouse antibody (Invitrogen) was used as a secondary antibody. After incubating for 30 min on ice, the cells were washed again with staining buffer and stained with the PE-CD235a (BioLegend, clone: H1264), APC-Cy7-CD45 (BioLegend, clone: 2D1) and Alexa fluor 647-αvβ3 (BioLegend, clone: 23C6) were added and incubated for another 30 min on ice. The cells were then washed once with cold staining buffer before re-suspending in staining buffer. The intracellular staining of HTNV-NP was performed as follows. 100 μl whole blood cells were performed surface staining with APC-Cy7-CD45, APN-CD71 (BioLegend, Clone: CY1G4) and PE-CD235a firstly. Intracellular staining with FITC-NP (clone: 1A8, produced by Department of Microbiology, Fourth Military Medical University) (16) was performed after fixating and permeabilizing the cells using an intracellular staining kit (eBioscience Fixation/Permeabilization kit). All procedures were performed according to the manufacturer's instructions. Flow cytometry was conducted on an ACEA *Novo* Express system (Agilent Bio) and data analyzed using the FlowJo software (TreeStar). Results were expressed as a percentage of positive cells.

Enzyme-Linked Immunosorbent Assay (ELISA)

Competitive ELISA assays for vitamin B12 (Catalog No. EHC9140) and folate (Catalog No. EHC9139) were performed using ELISA kits from Neobioscience according to the manufacturer's protocol. Absorbance at 450 nm was then obtained using the SpectraMax Reader (Molecular devices).

RNA Extraction and Real-Time PCR

The RBCs were enriched by sorting $CD235a^+$ cells. The total RNA of RBCs was extracted using TRIzol (Invitrogen) according to the manufacturer's protocol and 1 μg was used for cDNA synthesis (Takara, Japan). Quantitative analysis of mRNA expression was done by quantitative Real-time PCR using the SYBR Green detection method. The specific primers for *NP* and *β-actin* were as follows. *NP* (F: TACAGAGGGAAA TCAATGCC; R: TGTTCAACTCATCTGGATCCTT), *β-actin* (F: CATGTACGTTGCTATCCAGGC; R: CTCCTTAATGTC ACGCACGAT) Reactions were analyzed using a BIO-RAD system (CFX96 Real-Time System). The delta ct method was used to calculate. Then, *NP* was normalized to the housekeeping gene *β-actin* and was presented as fold changes of untreated cells.

Statistical Analysis

Prior to the analyses, all the data was tested for normality using the Shapiro-Wilks test and homogeneity of variance using the Levenes test. Thereafter, One-way ANOVA and student *t*-test were used for comparison between the groups. In addition, correlation coefficients were computed using Pearson correlation or Spearman correlation. All the statistical tests were two sided and *P*-values < 0.05 were considered significant.

RESULTS

Red Blood Cells Shown in PBMCs of HFRS Patients

In order to profile the peripheral immune response to HTNV, scRNA-seq was performed on isolated PBMCs from two healthy donors and six hospitalized patients. The patients had been diagnosed as HFRS positive using the anti-HTNV IgM. The demographics and clinical features of the patients are listed in **Figure 1A**. All the six patients profiled were male and aged between 26 and 52 years. On the other hand, the healthy donors consisted of individuals working in our lab. One was a 29-year-old male while the other was a 33-year-old female. The samples were collected within 10 days after the onset of disease to ensure that all the patients were in the acute phase of HFRS which

included fever, shock, and diuretic stages. With regard to severity, patients P04, P15 and P26 were diagnosed as critical while the other three, P06, P07 and P16 were found to have moderate severity (**Figure 1A**).

The cells sequenced in each sample are summarized in **Supplementary Table 1**. The tSNE graph partitioned cells into 19 clusters (**Figure 1B**). In addition, the regular PBMCs were identified as T cells (Cluster 0, 1, 3–5, 8, 12, 17, and 18), B cells (cluster 6), monocytes (cluster 2, 9, and 13), plasma cells (cluster 7), NKT cells (cluster 11), macrophages (cluster 14) and megakaryocyte (cluster 15). Erythrocytes (red blood cells, RBCs, cluster 10 and 16) were also identified (**Figures 1B,C**). The characteristic genes of RBCs in cluster 10 were hemoglobin B (*HBB*) and hemoglobin A2 (*HBA2*) while those in cluster 16 were *HBA1*, hemoglobin A2 (*HBA2*) and *HBB* (**Figure 1D**). The other marker genes expressed in cluster 10 and cluster 16 are shown in **Supplementary Figures 1, 2**.

The distribution of RBCs in each studied sample was then examined. It was observed that RBCs were absent in the two healthy donors and P07, one of the HFRS patients with moderate severity (**Figure 2A**). The other two samples from moderate HFRS patients, P06 and P16, contained RBCs majorly in cluster 10. Additionally, the percentage of absolute cell counts of RBCs in the total sequenced cells were calculated (**Figure 2B**). There is a trend that the critical HFRS patients had more RBCs in PBMCs (3.3–17.6%) than those with moderate HFRS (1.5∼2.1%) and healthy donors (0%) (**Figure 2B**). Moreover, the percentages of total sequenced cells in cluster 10 and 16 in HFRS patients with different levels of severity are shown for comparison. Clusters10 and 16 both had critical HFRS patients while only cluster 10 had HFRS patients with moderate severity (**Figure 2C**). Therefore, these results indicated that the RBCs mainly appeared in the critical HFRS patients. However, although some of the moderate HFRS patients had RBCs, most of the cells were classified under cluster 10.

The RBCs Were Characterized as Nucleated Cells

A more specific analysis on RBCs was then conducted and they were further separated into seven new clusters (**Figure 3A**). The heatmap in **Supplementary Figure 3** shows the signature genes in each cluster. Clusters that had similar gene expression profiles were grouped close together. Generally, clusters 0, 1, 4 and 5 were grouped as a module while clusters 2, 3, and 6 were classified as another module (**Supplementary Figure 4**). Clusters 0, 1, 4, and 5 expressed genes related to the hematopoietic system (such as *TMSB4X*), nuclear functions (such as *GAPDH*) and BCR or TCR rearrangement (such as *IGLV2-8*) (**Figure 3B**). On the other hand, clusters 2, 3, and 6 had genes related to the functioning of mature erythrocytes (such as *HBA1*) and intermediate stages during normal erythroid cell development (such as *AHSP* and *SLC4A1*) (17) (**Figure 3B**). Moreover, expression of *CD47*, which is considered to be a cell surface marker of primitive erythrocytes (17), was distributed in each cluster (**Supplementary Figure 5**). These results indicated that the cells in all the clusters were gradually transitioning through different stages of erythropoiesis. Furthermore, gene ontology (GO) analysis of biological process (BP) illustrated that cluster 0, 1, 4, and 5 had nuclear and ribosomal functions, including transcription and translation. However, the oxygen transport process was only available in cluster 2, 3, and 6. Cluster 6 additionally had heme synthesis and erythrocytes development processes (**Figure 3C**). Therefore, these findings revealed that the RBCs not only had nuclei but also other organelles. Based on these outcomes, we considered that the RBCs in the PBMCs from HFRS included NRBCs.

To further confirm these RBCs are nucleated erythroid cells, the flow cytometry was also applied to detect $CD71^{+}CD235a^{+}$ cells, which are the erythroid lineage markers as previously reported (14), in PBMCs from 52 HFRS samples (40 HFRS patients including 29 critical HFRS samples, and 23 moderate HFRS samples) and 7 health donors (setting as normal control, NC). The results showed that the critical HFRS patients had significantly higher percentage of $CD71^{+}CD235a^{+}$ cells in their acute phase. While, the PBMCs from moderate HFRS patients and health donors did not have a discernible $CD71^{+}CD235a^{+}$ population (**Figures 4A,B**). The total cell counts of NRBCs were calculated by counting the PBMCs total cell numbers in 10 ml whole blood and evaluating the proportion of NRBCs in PBMCs determined by flow cytometry. As shown in **Figure 4C**, the samples from critical/severe patients had more NRBCs cell counts/μl whole blood than moderate/mild patients. It is also demonstrated that in acute phase of HFRS, including both critical/severe and moderate/mild patients, the proportion (**Figure 4D**) and total cell counts of NRBCs (**Figure 4E**) were higher than that in convalescent phase. In our previous study, we have demonstrated the HTNV viral load are correlated with disease severity (15). To further verify the relationship between NRBCs and HFRS severity, the correlation between the viral load and the percentage of NRBCs were calculated. As expected, the percentage (**Figure 4F**) and the cell counts (**Figure 4G**) of NRBCs were positively correlated with the HTNV viral load.

From these, we concluded that the NRBCs were present in the PBMCs of HFRS patients and were correlated with disease severity.

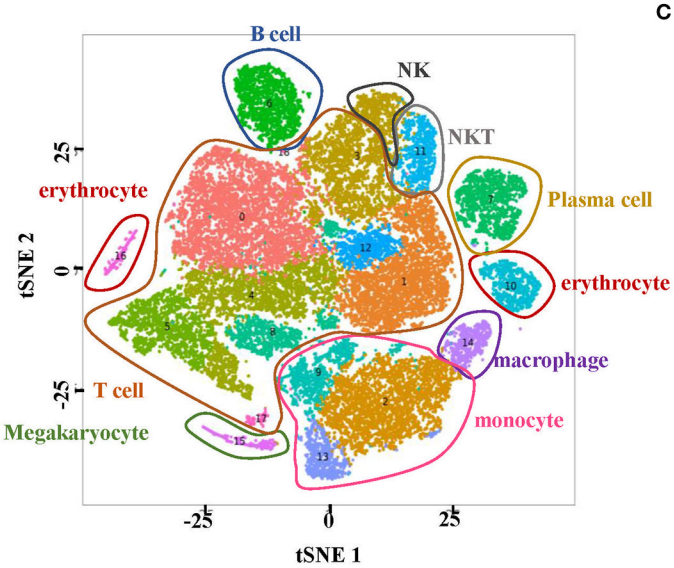
The Distributions of NRBCs Clusters Were Associated With Disease Severity

The distribution of these NRBCs in each studied sample was observed (**Figures 5A,B**). Notably, the tSNE graph showed that critical HFRS patients had more cell counts of NRBCs compared to those with moderate severity (**Figure 5A**). The bar graph shows the percentage of each cluster in each sample and cluster 6 was absent in the moderate HFRS patients. In addition, the percentages of cluster 0 and cluster 1 were higher in critical HFRS patients. On the contrary, clusters 4 and 5 had a higher percentage in the moderate HFRS patients (**Figure 5B**). Since the NRBCs are thought to be erythroblasts, the study further investigated the details of their development. Monocle2 was used to perform a pseudotime analysis to determine trajectories of differentiation by ordering cells according to their transcriptional changes. The results showed that cluster 5 was positioned at the root of the pseudotime trajectory, while cluster 6 was assigned to the end of a

A

Sample No.	age	sex	Days onset	Disease phase	Disease severity	Viral load (copies/ml)	Clinical outcome
P04	52	male	9	oligouria	critical	2.04×10^{8}	Discharged home
P06	34	male	9	oligouria	moderate	1.04×10^{8}	Discharged home
P07	49	male	5	fever	moderate	5.26×10^{7}	Discharged home
P15	29	male	7	Fever, shock, and oligouria	critical	5.24×10^{8}	Discharged home
P16	37	male	8	fever	moderate	2.36×10^{7}	Discharged home
P26	26	male	7	Fever, and shock	critical	2.35×10^{9}	Discharged home

B



C

Cluster	CellType
0	T cell
1	T cell
2	monocyte
3	T cell
4	T cell
5	T cell
6	B cell
7	Plasma cell
8	T cell
9	monocyte
10	erythrocyte
11	NKT
12	T cell
13	monocyte
14	macrophage
15	megakaryocyte
16	erythrocyte
17	T cell
18	T cell

D

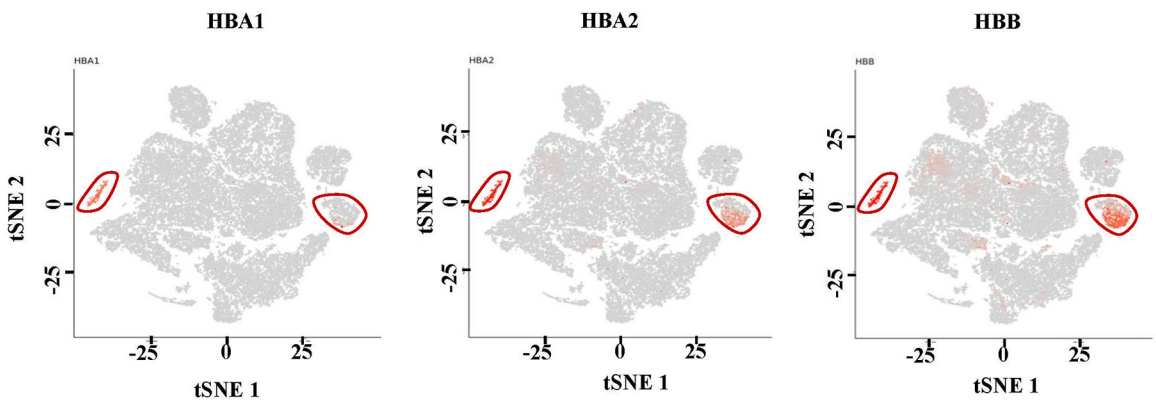


FIGURE 1 | Single-cell RNA sequencing analysis of PBMCs from HFRS patients. **(A)** Characteristics of the samples and the disease course of patients with HFRS. **(B)** The tSNE presentation of the cell types and **(C)** associated clusters in PBMC from HFRS patients. **(D)** The expression of HBA1, HBA2 and HBB in cluster 10 and 16 defined the two clusters as erythrocytes.

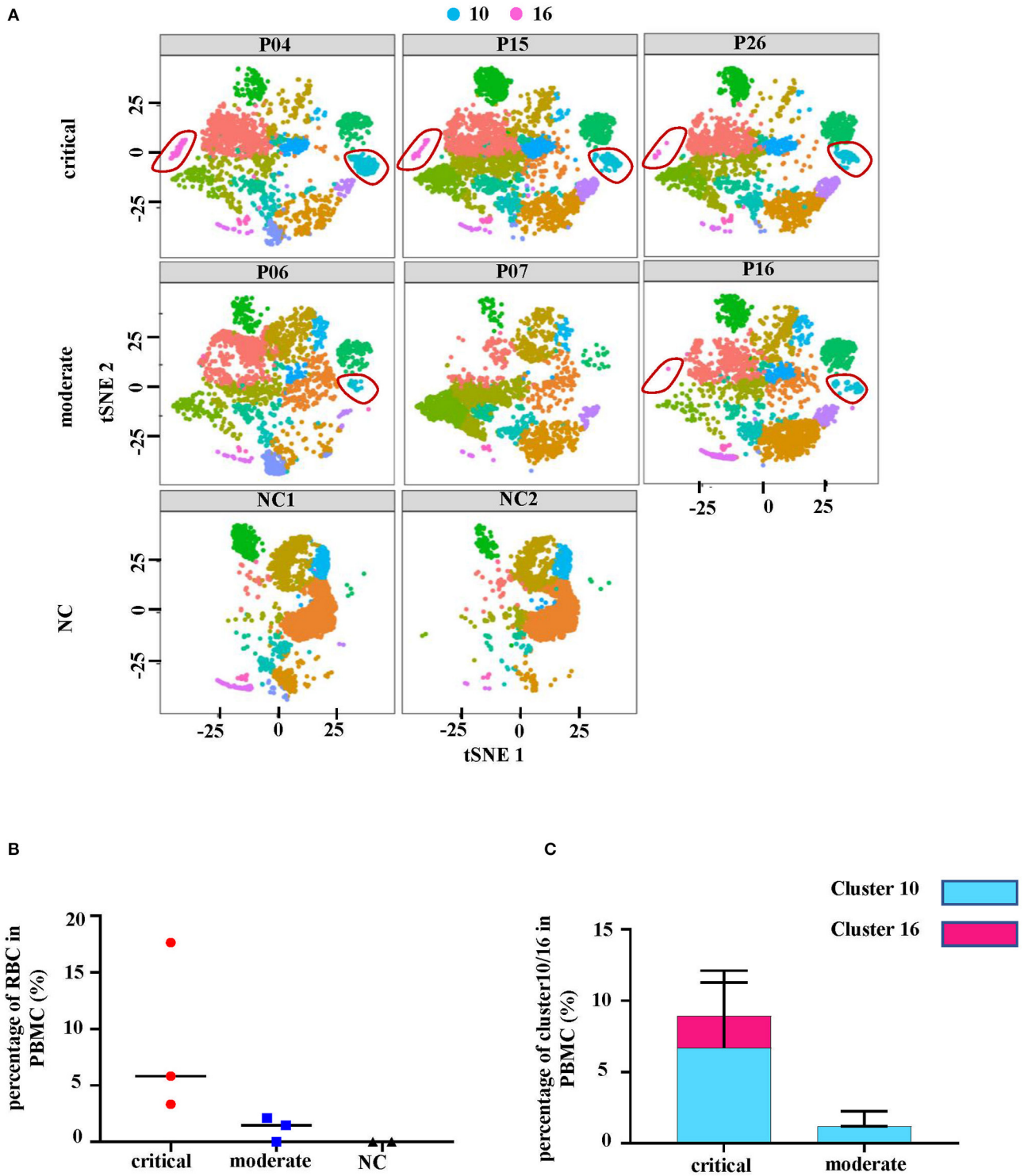


FIGURE 2 | The number of erythrocytes was associated with disease severity. **(A)** The tSNE plots of each studied subject, including 3 critical cases (P04, P15 and P26), 3 moderately severe cases (P06, P07, and P16) and 2 controls (NC1 and NC2). **(B)** The percentages of erythrocytes (red blood cells, RBC) in all the cells as shown in different levels of disease severity. **(C)** The comparison of cluster 10 and cluster 16 proportions in different levels of disease severity.

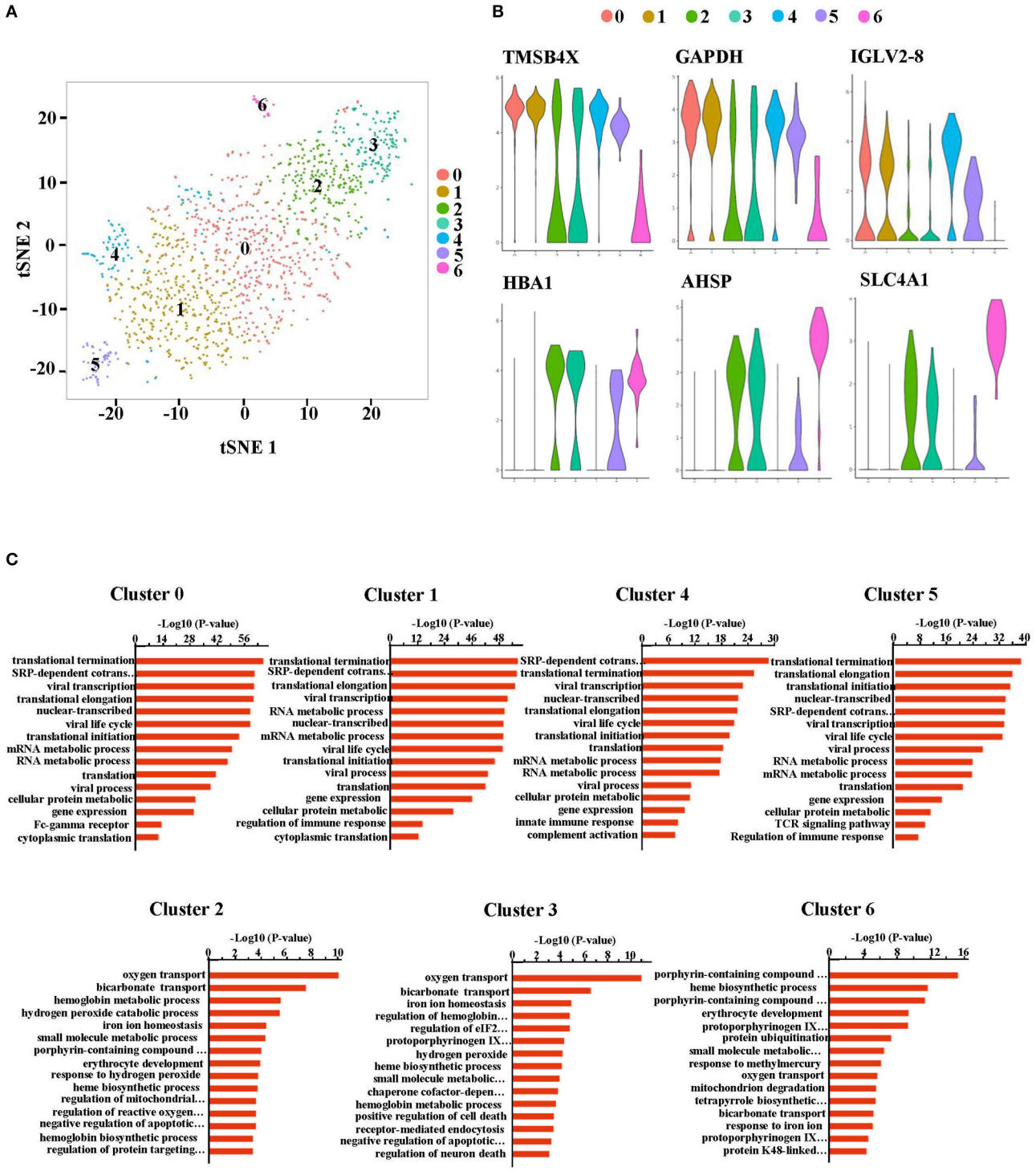


FIGURE 3 | The RBCs in the peripheral blood from HFRS patients were defined as nucleated red blood cells (NRBCs). **(A)** Erythrocytes (cluster 10 and cluster 16) were taken out and reanalyzed. tSNE projection revealed 7 new clusters (cluster 0–6). **(B)** Representative violin plot of single-cell expression levels of the characteristic genes in each cluster. The progenitor-like genes were enriched in cluster 0, 1, 4, and 5 (upper). The erythroblast-related genes were enriched in the cluster 2, 3, and 6 (lower). **(C)** Bar charts depicting gene ontology (BP) enrichment analysis (biological process: BP). Each row represents a pathway.

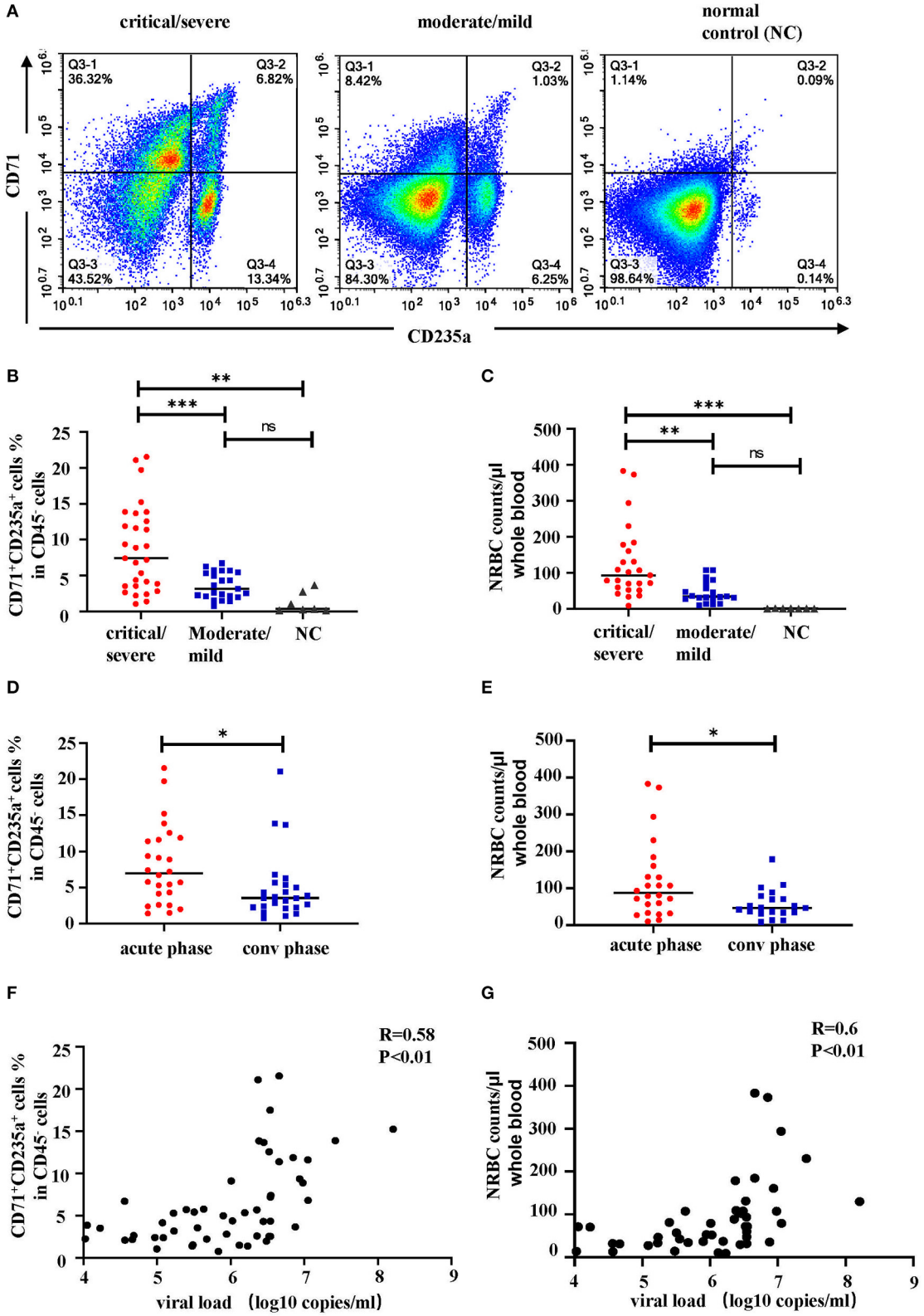


FIGURE 4 | The percentage and cell counts of NRBCs detected by flow cytometry were correlated with HFRS disease severity. Representative flow cytometric graphs **(A)** and cumulative results **(B)** calculating percentage of $CD71^{+}CD235a^{+}$ cells (NRBCs) in $CD45^{-}$ cells. Data was analyzed using One-way ANOVA. Results

(Continued)

FIGURE 4 | are presented as means ± SEM. **(C)** Comparison of NRBC total cell counts per μl whole blood in different disease severity. Data was analyzed using One-way ANOVA. Results are presented as means ± SEM. Comparison of **(D)** percentage and **(E)** cell counts of NRBCs in acute phase and convalescent (conv) phase in HFRS patients. Data was analyzed using Student *t*-test. Results are presented as means ± SEM. Analysis of correlation between viral load and percentage **(F)** or cell counts **(G)** of NRBCs by Spearman correlation. The r and *P*-values are indicated on the graph. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns means no significant, N number equals 52 samples from 40 HFRS patients including 32 males and 8 females, N (NC) number equals 7.

differentiation trajectory (**Figure 5C**). Additionally, pseudotime analysis in each sample showed that the NRBCs in critical HFRS patients were distributed from the early stage to the end stage during the development process. However, in moderate HFRS patients, the cells were clustered around the early stage of NRBCs development (**Figure 5D**). These findings therefore indicated that HTNV infection affects the development of RBCs from the early to the end stage, especially in critical HFRS patients.

NRBCs Expressed Genes Associated With Antiviral Response

The study then discussed the role of the NRBCs. Given that the normal immune related functions of mature RBCs were impaired in NRBCs, whether the cells could exert some antiviral effects still needs to be discussed. Therefore, we profiled the expression of genes encoding antiviral markers. Notably, NRBCs from HFRS patients had higher levels of encoded genes related to CTL associated markers, such as *CD3E*, granulysin (*GNLY*), granzyme A (*GZMA*), granzyme B (*GZMB*), granzyme H (*GZMH*) and granzyme K (*GZMK*) (**Figure 6A**). Apart from these cytotoxic mediators, the NRBCs also encoded type-I interferon response genes including *IRF1*, *IFITM2*, *IFITM3*, *FAM46C* and *LY6E* (**Figure 6B**). Zheng *et. al* demonstrated that IFITM3 could inhibit HTNV infection (18). Moreover, the HLA class I and II molecules were widely expressed in the NRBCs (**Figure 6C**). Nombela also described in his review that RBCs from rainbow trout expressed MHC-I molecules on their surface. Additionally, piscine orthoreovirus infection and poly I:C were reported to stimulate the upregulation of GO pathways related to antigen processing, antigen presentation and MHC-I receptor activity (19). According to **Figure 3C**, GO enrichment analysis of biological processes related to viral transcription, viral life cycle and viral process were upregulated in clusters 0, 1, 4, and 5. Therefore, this data not only proved that these RBCs had nuclei but also indicated that the NRBCs might be infected by HTNV and had antiviral activity. All in all, the results suggested that NRBCs in the PBMCs from HFRS patients displayed antiviral responses.

HTNV Infection Contributed to the Erythropoiesis Disorders in HFRS

Indicators of hematopoiesis, i.e., RBC, HGB and mean corpuscular volume (MCV) were then analyzed from 40 HFRS patients. It was shown that levels of RBCs (**Figure 7A**) and HGB (**Figure 7B**) gradually decreased during the HFRS process. The RBC counts in the oliguria and diuretic/convalescent stages were (3.4 ± 0.5) $\times 10^{12}$/L and (3.3 ± 0.6) $\times 10^{12}$/L, respectively. These were lower than that in the fever/shock stage (4.3 ± 0.7) $\times 10^{12}$/L and below the lower limit of the normal range (4∼5.5 $\times 10^{12}$/L) (**Figure 7A**). Similar to the RBCs, the levels of HGB (normal range 131–150 g/L) also decreased from 137.5 ± 20.7 g/L in the fever/shock stage to 104.0 ± 16.2 g/L in the oliguria stage and 106.0 ± 19.0 g/L in the diuretic/convalescent stage, respectively (**Figure 7B**). Since a lot of factors might result to the presence of NRBC and a decrease in RBC and HGB, the study then explored the possible pathways that could cause the anemia. As a result, MCV and RBC were further analyzed. Although the MCV of HFRS patients did not exceeded 100 fl (femtoliter, 1×10^{-15}L), it was observed that the levels increased as infection progressed. The MCV in the fever/shock stage was 87.8 ± 4.4 fl then increased to 90.8 ± 4.8 fl in the oliguria stage and 92.0 ± 4.8 fl in the diuretic/convalescent stage, respectively (**Figure 7C**). Moreover, the critical/severe HFRS patients had both lower RBC counts (**Figure 7D**) and HGB levels (**Figure 7E**) but higher levels of MCV (**Figure 7F**) compared to the moderate/mild HFRS patients. Additionally, the Pearson correlation results showed that MCV was negatively correlated with RBC and HGB, i.e., the lower the RBC counts and HGB levels, the higher the levels of MCV (**Figures 7G,H**). These results indicated that HFRS patients, especially those with critical/severe disease exhibited a trend of megaloblastic anemic features.

Based on the above analysis of clinical data, HFRS patients may have erythropoiesis disorders. According to the GO analysis of RBCs in **Figure 3C**, which suggested "viral life cycle and viral process" were upregulated in RBCs, we inferred that RBCs might be the target of HTNV infection. For further validation, we detected the expression of αvβ3, which was the pathogenic receptor of HTNV (20), on the surface of RBCs. As shown in **Figures 8A,B**, around 10% of $CD71^{+}CD235a^{+}$ NRBCs and around 6% of $CD71^{-}CD235a^{+}$ RBCs were $\alpha v\beta 3^{+}$. *In vitro* infection study indicated that RBCs could be infected by HTNV. The flow cytometry results suggested that 72h after infection, more than 10% of $CD235a^{+}$ RBCs had NP in the cells (**Figures 8C,D**). The following qPCR results also demonstrated that the NP mRNA level was increased after HTNV infection compared to the mock virus infection (**Figure 8E**). It is noteworthy that *in vitro* HTNV infection could increase the percentage of $CD71^{+}CD235a^{+}$ NRBCs directly (**Figures 8F,G**). Based on the above results, we suggested that HTNV infection on RBCs might lead to erythropoiesis disorders in HFRS patients.

Depending on the presence of immature NRBCs and the increase in MCV, the folate pathway, which was associated with megaloblastic anemic, was then investigated for metabolic hematopoietic abnormalities in HFRS patients. Therefore, the values of vitamin B12 (cobalamin) and B9 (folic acid), the key regulators of the folate pathway, were measured in the plasma of HFRS patients. The bar graph showed that both vitamin B12 (**Figure 9A**) and B9 (**Figure 9D**) were downregulated in

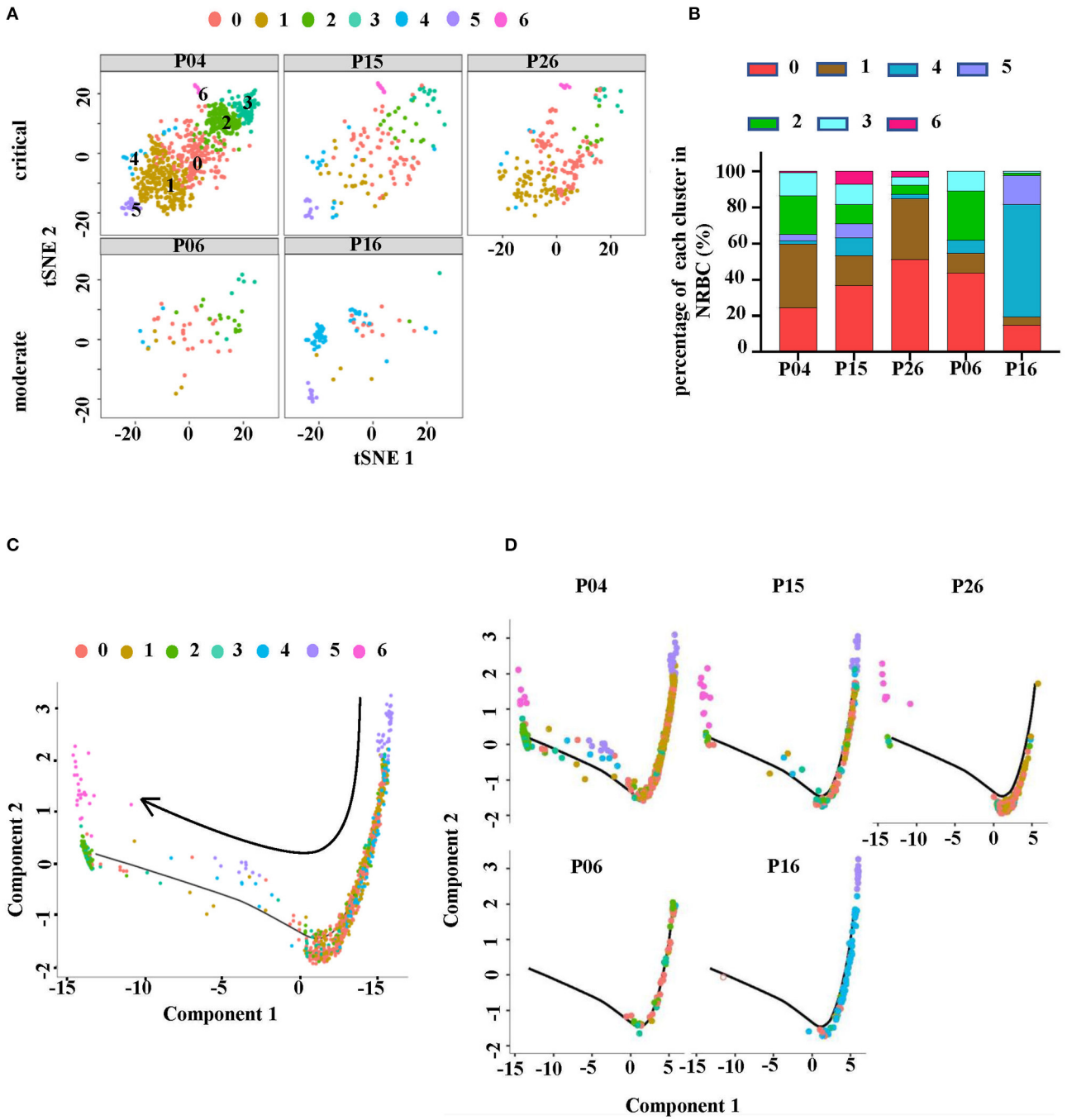


FIGURE 5 | NRBC cluster distributions were different in each sample. **(A)** The tSNE plots showing the RBCs in each studied sample. The upper row represents critical HFRS patients while the lower one represents moderate HFRS patients. **(B)** A comparison of the proportion of each cluster in each sample. **(C)** The Monocle pseudotime trajectory showed the differentiation of NRBC, from the beginning on the top right and advancing as cells approached the left. **(D)** The display of pseudotime trajectory in each sample with the upper level from critical HFRS patients and lower level from moderate HFRS patients.

HFRS patients. The study then analyzed the levels of the vitamins in each stage during HTNV infection. The findings showed that both vitamin B12 (**Figure 9B**) and B9 (**Figure 9E**) were significantly reduced in the acute phase which includes fever, shock, and oliguria in critical/severe HFRS patients. Although vitamin B12 (**Figure 9C**) and B9 (**Figure 9F**) were also decreased in the acute phase of moderate/mild HFRS patients, the differences were not significant. This data therefore suggested

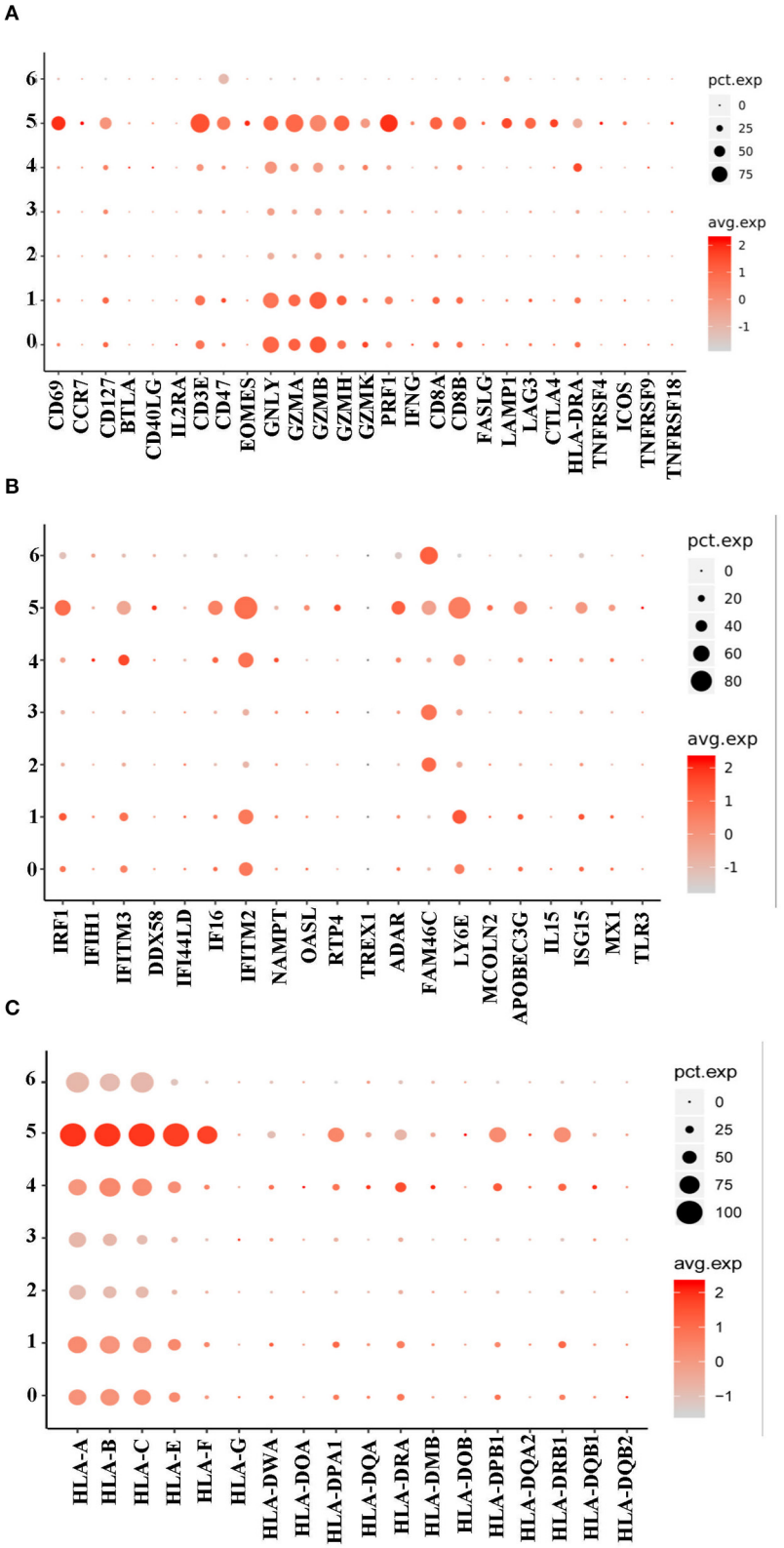


FIGURE 6 | A dot plot showing that the NRBCs encoded antiviral related genes. **(A)** Cluster 0, 1, and 5 encoded higher levels of CTL related genes. **(B)** Cluster 0, 1, 4, and 5 encoded type I IFN related genes. **(C)** All the 7 clusters encoded MHC-I genes while cluster 0, 1, 4, and 5 had MHC-II genes.

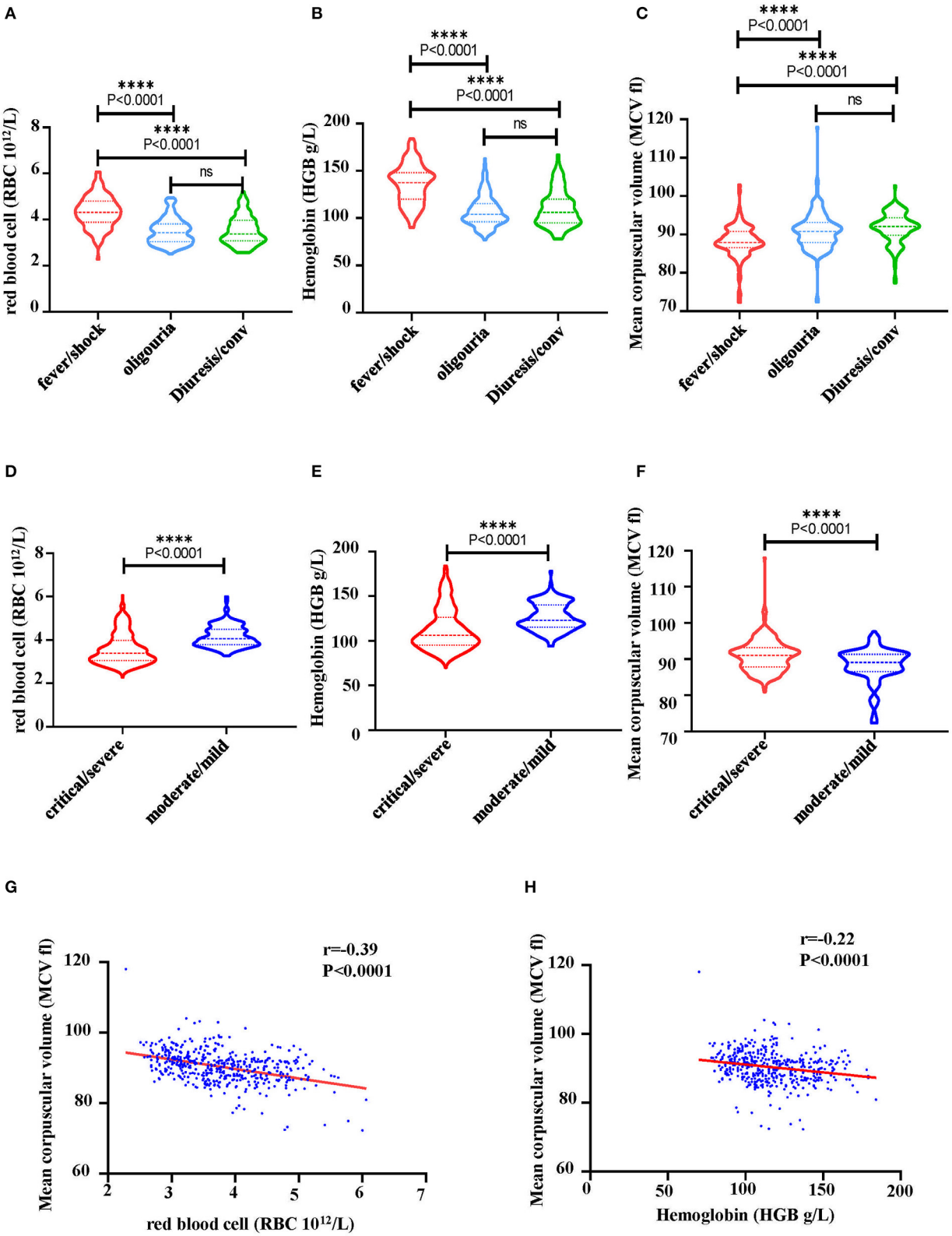


FIGURE 7 | Characteristics of hematological markers in HFRS patients. **(A)** A comparison of RBC counts, **(B)** hemoglobin (HGB) and **(C)** mean corpuscular volume (MCV) at different stage of HFRS in patients. **(D)** A comparison of RBC counts, **(E)** HGB and **(F)** MCV between critical/severe and moderate/mild severe HFRS. The correlation between **(G)** RBC and MCV, **(H)** HGB and MCV was determined through Pearson correlation. The r and *P*-values are indicated on the graph. ****$P <$ 0.0001; ns, no significance. N number equals 42 HFRS patients' samples including 30 males and 12 females.

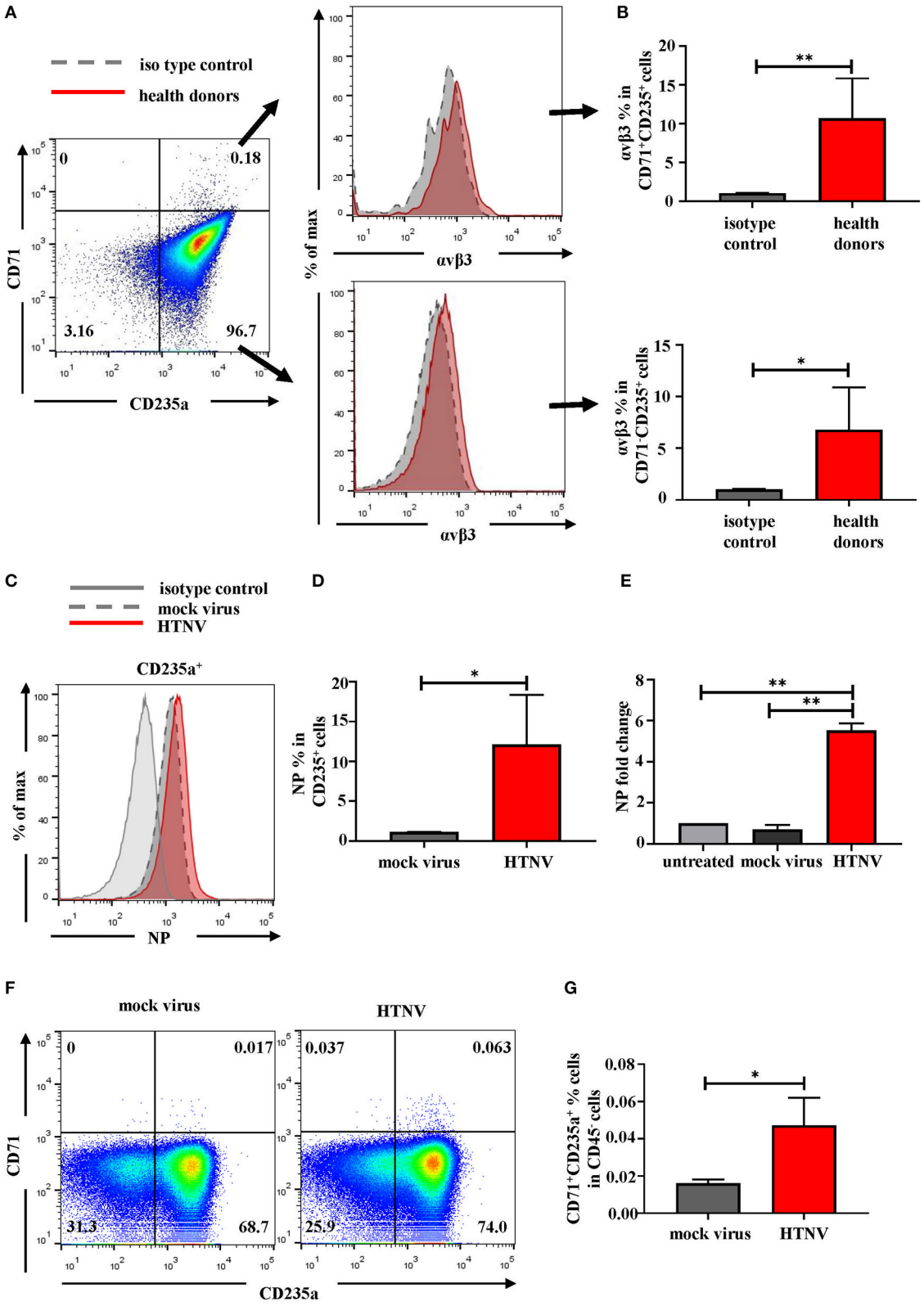


FIGURE 8 | RBCs are the targets of HTNV infection. **(A)** Representative flow cytometric graphs and **(B)** cumulative results calculating percentage of αvβ3 on the surface of $CD71^{+}CD235a^{+}$ cells (upper) and $CD71^{-}CD235a^{+}$ cells (lower) from health donors. Data was analyzed using Student *t*-test. **(C)** The expression of NP in

(Continued)

FIGURE 8 | the RBCs after 72 h post infection *in vitro*. **(D)** The statistical analysis of the percentage of NP^+ RBCs. Data was analyzed using Paired Student *t*-test. **(E)** The mRNA level of NP in the RBCs was quantified by qPCR. Data was analyzed using One-way ANOVA. Results are presented as means ± SEM. **(F)** Representative flow cytometric graphs of $CD71^+CD235a^+$ cells (NRBCs) and **(G)** comparison the percentage of NRBCs under mock infection and HTNV infection. Data was analyzed using Paired Student *t*-test. $^*P < 0.05$, $^{**}P < 0.01$, N number equals 4.

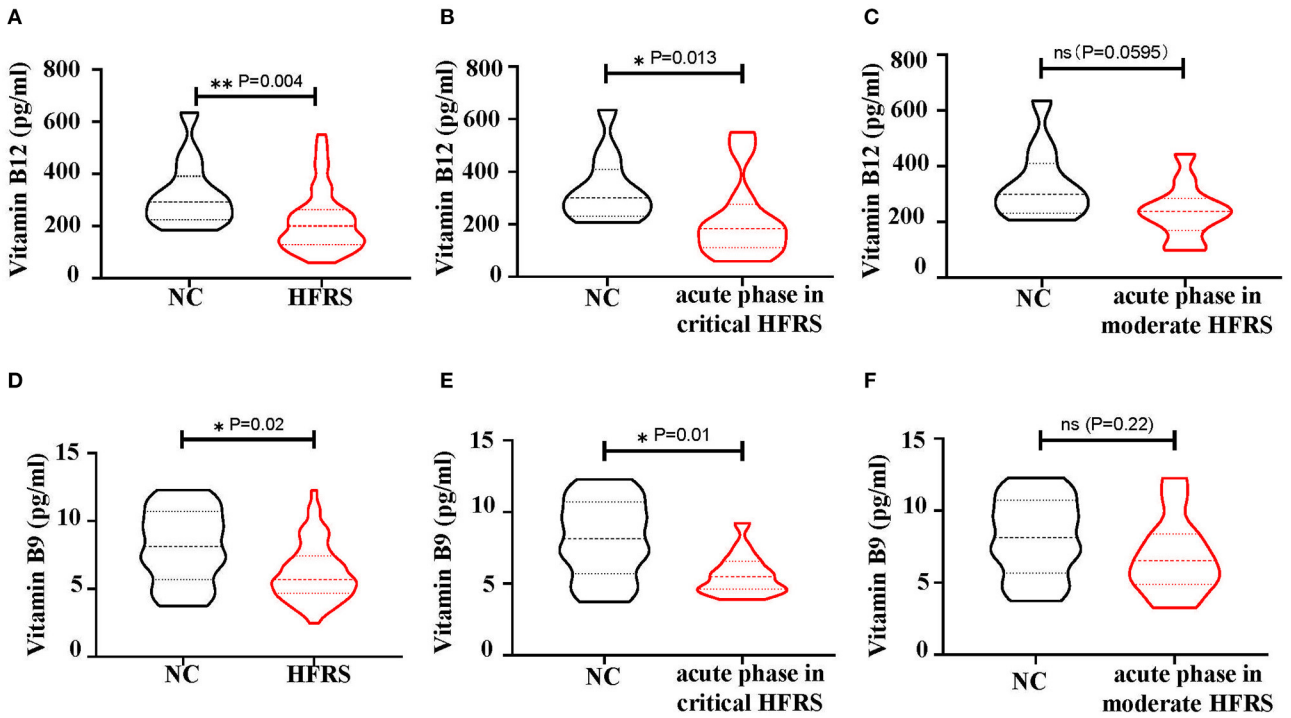


FIGURE 9 | The levels of vitamin B12 and vitamin B9 in the plasma of HFRS patients. **(A)** The level of vitamin B12 and **(D)** vitamin B9 in Normal Control (NC) vs. HFRS. **(B)** The level of vitamin B12 and **(E)** vitamin B9 in NC vs. acute phase in critical HFRS. The level of **(C)** vitamin B12 and **(F)** vitamin B9 in NC vs. acute phase in moderate HFRS. The *P*-value is indicated on the graph. $^*P < 0.05$, $^{**}P < 0.01$; ns, no significance. N number equals 58 samples from 29 HFRS patients including 21 males and 8 females, N (NC) number equals 12.

that HFRS patients were deficiencies in vitamin B12 and B9. The folate pathway might be a therapeutic target for treatment of the erythropoiesis disorders in HFRS patients.

DISCUSSION

A recent study that used scRNA-seq to profile PBMCs from COVID-19 patients suggested that RBCs were found in peripheral blood during infection (21). The study however found no significant difference in the RBCs of healthy individuals and COVID-19 patients. Notably, only one patient with acute respiratory distress syndrome (ARDS) had over 50% RBC counts, consistent with results from the present study. Additionally, patients who suffered from more severe disease had more RBCs in PBMCs. Contrary to our findings, the work on COVID-19 additionally found RBCs and neutrophils in PBMCs obtained from healthy donors. Although improper manipulation during the isolation of the PBMCs from whole blood might cause RBCs' contamination in PBMCs, the step of RBCs lysis before scRNA-seq made the results reliable. Previous studies that detected NRBCs in the whole blood of ICU patients used mechanical blood analyzers (12, 13). In these reports, patients with NRBC counts over 2,000/μl died. Therefore, the higher NRBC count, the higher mortality rate was for the ICU patients. Our study used flow cytometry to count the NRBCs, their counts could be estimated as 8–380/μl based on the percentages of NRBCs and the total PBMCs counts in whole blood.

Erythropoiesis is regulated by multiple factors including erythropoietin (EPO), iron and the folate pathway. EPO is a cytokine synthesized in the kidney to regulate erythropoiesis through binding with the EPO receptor (EPOR) expressed on the erythroid nucleated precursors (22). Therefore, deficiency of EPO is the major cause of anemia in chronic kidney disease (23). On the other hand, iron is an essential functional component of erythrocyte hemoglobin (24) and its deficiency can result in microcytic hypochromic anemia with decreased levels of RBCs, MCV < 80 fl and decreased amount of HGB (25). Moreover, vitamin B12 and vitamin B9 are the two important coenzymes for DNA synthesis in the folate pathway and deficiency of both may cause megaloblastic anemia (26). In this study, anemia [HGB < 120 g/L in premenopausal females and HGB < 130 g/L in postmenopausal females as well as males of all ages (27)] accompanied with an increasing level of MCV was observed (**Figures 7G,H**). However, the levels of MCV did not meet the

diagnostic criteria for megaloblastic anemia. Consequently, the appearance of NRBCs in HFRS patients were related to the deficiency of vitamin B12 and/or folic acid based on the signs of megaloblastic anemia.

Furthermore, the distribution of EPOR on the NRBCs was analyzed using the NovelBrain Cloud Analysis Platform. The low expression of EPOR indicated that EPO may not have major role on the impairment of erythropoiesis during the HTNV infection process. Further investigation on the EPO level in the circulation should provide more evidence on its role in erythropoiesis during HTNV infection. Additionally, since the high expression of HBB and HBA were found at the end stage of erythrocytes and MCV did not display a declining trend, iron deficiency could not have been the main cause of impairment in erythrocyte maturation. Apart from the factors mentioned above, inflammatory cytokines such as TNF-α, IL-6 and IL-12p70 could also exert their effects on erythropoiesis (28, 29). Therefore, further studies on the role of inflammatory cytokines in erythropoiesis are needed.

Although we suggested that NRBCs in the circulating blood of HFRS patients might have antiviral effects through the expression of cytotoxic mediators, IFN response genes and MHC molecules, the role of NRBCs during infection is still unclear. Nombela et al. reported that NRBCs had immune properties including phagocytosis, antigen presentation and interleukin-like production (19). Their study also demonstrated that NRBCs could generate immune responses against viruses regardless of whether they were infected (19). However, given that CR1 was absent in the NRBCs (data not shown), it is possible that they were unable to clear the virus as normal erythrocytes, which exerted immune-clearance ability via CR1 (30). Other studies also suggested that NRBCs had immunosuppressive function during infection (14, 31). More studies are still needed to clarify the role of NRBCs in HTNV infection. In our study, we found the appearance of NRBCs during HTNV infection accompanied by the deficiency of vitamin B12 and vitamin B9 in HFRS patients. According to the previous studies that during HCV infection, vitamin B12 could inhibit HCV IRES-dependent translation and improve the rates of sustained viral response (32, 33), we suggested that the administration of vitamin supplements may confer beneficial effects to HFRS patients.

Despite the insightful findings, this study had a number of limitations. First, the sample size used for sRNA-seq was too small making it difficult to deduce statistical significance. Second, the samples were limited to the acute phase of HFRS although this is the stage where more obvious changes occur. However, changes that occurred during the convalescent phase are still unknown. Therefore, given that there is no HFRS disease model, further studies are needed to explore in detail erythropoiesis disorders in the bone marrow smears of HFRS patients.

To the best of our knowledge, this is the first study to describe changes in the RBCs of HFRS patients. It is also the first one to depict the cellular components and gene expression profiles of NRBCs in the peripheral blood of HFRS patients. Therefore, this study on RBCs may provide novel insights on adjuvant therapy and monitoring the status of patients during HTNV infection.

DATA AVAILABILITY STATEMENT

The authors declare that all data supporting the findings of this study are available within this article and its **Supplementary Information** files, or from the corresponding author upon reasonable request. Single-cell RNAseq gene expression data have been deposited in the Gene Expression Omnibus database (GSE161354).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Xijing Hospital, First Affiliated Hospital of Fourth Military Medical University, Xi'an, China, (NO. KY20173177-1). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

YusZ, DL, and BJ: conceived and designed the experiments. KT, HH, XJ, YM, CZ, YunZ, and RZ: recruited patients and collected and processed samples. JZ, YusZ, DL, KT, HH, YM, MW, XZ, and CZ performed the experiments. JZ, YusZ, DL, YM, and CZ: analyzed the data. DL, KT, HH, XJ, YM, YunZ, RZ, and BJ: contributed reagents/materials/analysis tools. YusZ, DL, and BJ: wrote the paper. YunZ and BJ: verified the underlying data. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by grants from the Nation Natural Science Foundation of China (81701555, 81771705, 32000876, 81871239, and 81901600) and the Natural Science Basic Research Plan in Shaanxi Province of China (Program No. 2018JQ8060 and 2019JQ-437).

ACKNOWLEDGMENTS

We thank all the volunteers who generously participated in this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.665410/full#supplementary-material

Supplementary Figure 1 | Expression of genes in cluster 10 of PBMCs.

Supplementary Figure 2 | Expression of genes in cluster 16 of PBMCs.

Supplementary Figure 3 | A heatmap indicating the gene expression profile in cluster 0-6 of red blood cells.

Supplementary Figure 4 | A heatmap indicating the gene expression module of red blood cells.

Supplementary Figure 5 | Expression of CD47 in each cluster.

Supplementary Table 1 | Absolute cell numbers sequenced in each sample.

Supplementary Table 2 | Characteristics of enrolled subjects in testing vitamin B9/B12.

REFERENCES

1. Tian H, Yu P, Bjornstad ON, Cazelles B, Yang J, Tan H, et al. Anthropogenically driven environmental changes shift the ecological dynamics of hemorrhagic fever with renal syndrome. *PLoS Pathog.* (2017) 13:e1006198. doi: 10.1371/journal.ppat.1006198
2. Ma Y, Yuan B, Zhuang R, Zhang Y, Liu B, Zhang C, et al. Hantaan virus infection induces both Th1 and ThGranzyme B+ cell immune responses that associated with viral control and clinical outcome in humans. *PLoS Pathog.* (2015) 11:e1004788. doi: 10.1371/journal.ppat.1004788
3. Xie M, Dong Y, Zhou Y, Ren H, Ji Y, Lv S. Levels of HTNV-specific CD8+ T lymphocytes in PBMC from the patients with hemorrhagic fever with renal syndrome. *Intern Emerg Med.* (2013) 8:503–8. doi: 10.1007/s11739-011-0633-4
4. Gu XS, You ZQ, Meng GR, Luo FJ, Yang SH, Yang SQ, et al. Hemorrhagic fever with renal syndrome. Separation of human peripheral blood T and B cells and detection of viral antigen. *Chin Med J.* (1990) 103:25–8.
5. Raftery MJ, Kraus AA, Ulrich R, Kruger DH, Schonrich G. Hantavirus infection of dendritic cells. *J Virol.* (2002) 76:10724–33. doi: 10.1128/JVI.76.21.10724-10733.2002
6. Braun M, Bjorkstrom NK, Gupta S, Sundstrom K, Ahlm C, Klingstrom J, et al. NK cell activation in human hantavirus infection explained by virus-induced IL-15/IL15Ralpha expression. *PLoS Pathog.* (2014) 10:e1004521. doi: 10.1371/journal.ppat.1004521
7. Orkin SH. Diversification of haematopoietic stem cells to specific lineages. *Nat Rev Genet.* (2000) 1:57–64. doi: 10.1038/35049577
8. Zivot A, Lipton JM, Narla A, Blanc L. Erythropoiesis: insights into pathophysiology and treatments in 2017. *Mol Med.* (2018) 24:11. doi: 10.1186/s10020-018-0011-z
9. Granick S, Levere RD. Heme synthesis in erythroid cells. *Prog Hematol.* (1964) 4:1–47.
10. Gifford SC, Derganc J, Shevkoplyas SS, Yoshida T, Bitensky MW. A detailed study of time-dependent changes in human red blood cells: from reticulocyte maturation to erythrocyte senescence. *Br J Haematol.* (2006) 135:395–404. doi: 10.1111/j.1365-2141.2006.06279.x
11. Schwartz SO, Stansbury F. Significance of nucleated red blood cells in peripheral blood; analysis of 1,496 cases. *J Am Med Assoc.* (1954) 154:1339–40. doi: 10.1001/jama.1954.02940500019007
12. Stachon A, Kempf R, Holland-Letz T, Friese J, Becker A, Krieg M. Daily monitoring of nucleated red blood cells in the blood of surgical intensive care patients. *Clin Chim Acta.* (2006) 366:329–35. doi: 10.1016/j.cca.2005.11.022
13. Desai S, Jones SL, Turner KL, Hall J, Moore LJ. Nucleated red blood cells are associated with a higher mortality rate in patients with surgical sepsis. *Surg Infect.* (2012) 13:360–5. doi: 10.1089/sur.2011.089
14. Namdar A, Dunsmore G, Shahbaz S, Koleva P, Xu L, Jovel J, et al. CD71(+) erythroid cells exacerbate HIV-1 susceptibility, mediate trans-infection, and harbor infective viral particles. *Mbio.* (2019) 10:1–24. doi: 10.1128/mBio.02767-19
15. Yi J, Xu Z, Zhuang R, Wang J, Zhang Y, Ma Y, et al. Hantaan virus RNA load in patients having hemorrhagic fever with renal syndrome: correlation with disease severity. *J Infect Dis.* (2013) 207:1457–61. doi: 10.1093/infdis/jis475
16. Xu Z, Wei L, Wang L, Wang H, Jiang S. The *in vitro* and *in vivo* protective activity of monoclonal antibodies directed against hantaan virus: potential application for immunotherapy and passive immunization. *Biochem Biophys Res Commun.* (2002) 298:552–8. doi: 10.1016/S0006-291X(02)02491-9
17. Zhao Y, Li X, Zhao W, Wang J, Yu J, Wan Z, et al. Single-cell transcriptomic landscape of nucleated cells in umbilical cord blood. *Gigascience.* (2019) 8:1–15. doi: 10.1093/gigascience/giz047
18. Xu-Yang Z, Pei-Yu B, Chuan-Tao Y, Wei Y, Hong-Wei M, Kang T, et al. Interferon-Induced transmembrane protein 3 inhibits hantaan virus infection, and its single nucleotide polymorphism rs12252 influences the severity of hemorrhagic fever with renal syndrome. *Front Immunol.* (2016) 7:535. doi: 10.3389/fimmu.2016.00535
19. Nombela I, Ortega-Villaizan MDM. Nucleated red blood cells: immune cell mediators of the antiviral response. *PLoS Pathog.* (2018) 14:e1006910. doi: 10.1371/journal.ppat.1006910
20. Song JW, Song KJ, Baek LJ, Frost B, Poncz M, Park K. *In vivo* characterization of the integrin beta3 as a receptor for hantaan virus cellular entry. *Exp Mol Med.* (2005) 37:121–7. doi: 10.1038/emm.2005.16
21. Wilk AJ, Rustagi A, Zhao NQ, Roque J, Martinez-Colon GJ, McKechnie JL, et al. A single-cell atlas of the peripheral immune response in patients with severe COVID-19. *Nat Med.* (2020) 26:1070–76. doi: 10.1038/s41591-020-0944-y
22. Sulahian R, Cleaver O, Huang LJ. Ligand-induced EpoR internalization is mediated by JAK2 and p85 and is impaired by mutations responsible for primary familial and congenital polycythemia. *Blood.* (2009) 113:5287–97. doi: 10.1182/blood-2008-09-179572
23. Babitt JL, Lin HY. Mechanisms of anemia in CKD. *J Am Soc Nephrol.* (2012) 23:1631–4. doi: 10.1681/ASN.2011111078
24. Kautz L, Nemeth E. Molecular liaisons between erythropoiesis and iron metabolism. *Blood.* (2014) 124:479–82. doi: 10.1182/blood-2014-05-516252
25. Provan D. Mechanisms and management of iron deficiency anaemia. *Br J Haematol.* (1999) 105 (Suppl. 1):19–26.
26. Lindenbaum J, Healton EB, Savage DG, Brust JC, Garrett TJ, Podell ER, et al. Neuropsychiatric disorders caused by cobalamin deficiency in the absence of anemia or macrocytosis. *N Engl J Med.* (1988) 318:1720–8. doi: 10.1056/NEJM198806303182604
27. Freeman AM, Rai M, Morando DW. Anemia screening. In: *StatPearls*. (Treasure Island, FL: StatPearls Publishing) (2020).
28. Stachon A, Bolulu O, Holland-Letz T, Krieg M. Association between nucleated red blood cells in blood and the levels of erythropoietin, interleukin 3, interleukin 6, and interleukin 12p70. *Shock.* (2005) 24:34–9. doi: 10.1097/01.shk.0000164693.11649.91
29. Trey JE, Kushner I. The acute phase response and the hematopoietic system: the role of cytokines. *Crit Rev Oncol Hematol.* (1995) 21:1–18. doi: 10.1016/1040-8428(94)00141-3
30. Davies KA, Hird V, Stewart S, Sivolapenko GB, Jose P, Epenetos AA, et al. A study of *in vivo* immune complex formation and clearance in man. *J Immunol.* (1990) 144:4613–20.
31. Elahi S, Ertelt JM, Kinder JM, Jiang TT, Zhang X, Xin L, et al. Immunosuppressive CD71+ erythroid cells compromise neonatal host defence against infection. *Nature.* (2013) 504:158–62. doi: 10.1038/nature12675
32. Lott WB, Takyar SS, Tuppen J, Crawford DH, Harrison M, Sloots TP, et al. Vitamin B12 and hepatitis C: molecular biology and human pathology. *Proc Natl Acad Sci USA.* (2001) 98:4916–21. doi: 10.1073/pnas.081072798
33. Rocco A, Compare D, Coccoli P, Esposito C, Di Spirito A, Barbato A, et al. Vitamin B12 supplementation improves rates of sustained viral response in patients chronically infected with hepatitis C virus. *Gut.* (2013) 62:766–73. doi: 10.1136/gutjnl-2012-302344

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.

frontiers in Medicine

ORIGINAL RESEARCH
published: 05 August 2021
doi: 10.3389/fmed.2021.707998

Investigating the Added Value of the EQ-5D-5L With Two Bolt-On Items in Patients With Hemophilia

Richard Huan Xu[1†], *Dong Dong*[2,3*†], *Nan Luo*[4], *Renchi Yang*[5], *Junshuai Liu*[6] *and Shuyang Zhang*[7*]

[1] Department of Rehabilitation Sciences, The Hong Kong Polytechnic University, Kowloon, Hong Kong, [2] JC School of Public Health and Primary Care, The Chinese University of Hong Kong, Shatin, Hong Kong, [3] Shenzhen Research Institute, The Chinese University of Hong Kong, Shenzhen, Guangdong, China, [4] Saw Swee Hock School of Public Health, National University of Singapore, Singapore, Singapore, [5] Thrombosis and Hemostasis Center, Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin, China, [6] Beijing Society of Rare Disease Clinical Care and Accessibility, Beijing, China, [7] Department of Cardiology, Peking Union Medical College Hospital, Peking Union Medical College and Chinese Academy of Medical Science, Beijing, China

OPEN ACCESS

Edited by:
Lalitha Nayak,
Case Western Reserve University,
United States

Reviewed by:
Chaojie Liu,
La Trobe University, Australia
Magdy El Ekiaby,
Independent Researcher, Giza, Egypt

***Correspondence:**
Dong Dong
dongdong@cuhk.edu.hk
Shuyang Zhang
shuyangzhang103@nrdrs.org

[†]*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: *11 May 2021*
Accepted: *07 July 2021*
Published: *05 August 2021*

Citation:
Xu RH, Dong D, Luo N, Yang R, Liu J and Zhang S (2021) Investigating the Added Value of the EQ-5D-5L With Two Bolt-On Items in Patients With Hemophilia. Front. Med. 8:707998. doi: 10.3389/fmed.2021.707998

Objective: This study examined the impact of adding two condition-specific bolt-on items to the EQ-5D-5L and assessed their psychometric properties in patients with hemophilia.

Methods: The data were obtained from a nationwide cross-sectional online survey of patients with hemophilia in China. Self-reported and proxy-reported data were analyzed separately. Ceiling effect, informativity, and discriminatory power of the EQ-5D-5L with two bolt-on items, dignity (DG), and bleeding (BL), were examined. Spearman's rank correlation (rho) was used to assess the associations of the EQ-5D-5L and two bolt-on items with the Hemophilia Quality of Life Questionnaire for Adults (Haem-A-QoL) and SF-12. Multiple regression analysis was performed to evaluate the explained variance of the EQ-5D-5L and bolt-on items in predicting EQ-VAS scores.

Results: A total of 895 patients and 222 caregivers completed the questionnaire. The ceiling effect decreased from 1.9 to 0.6% and 5.9 to 0.9% when using the EQ-5D-5L and the EQ-5D-5L with two bolt-on items among participants with both self- and proxy-completed questionnaires. Both DG and BL were strongly correlated with Haem-A-QoL sum score [rho: DG = 0.64 (patient) vs. 0.66(proxy); BL = 0.49 (patient) vs. 0.31 (proxy)], SF-12 mental component [rho: DG = −0.36 (patient) vs. −0.41 (proxy); BL = −0.53 (patient) vs. −0.57(proxy)], and SF-12 physical component [rho: DG = −0.61 (patient) vs. −0.61 (proxy); BL = −0.35 (patient) vs. −0.39 (proxy)]. Known-group comparisons confirmed that the two bolt-on items had satisfactory discriminatory power. Multiple regression analysis indicated that adding two bolt-on items significantly increased the ability to predict EQ-VAS scores. The adjusted R^2 increased by 8.2 and 8.8% for reports completed by the patients or patients' proxy respondents, respectively.

Conclusion: Adding the DG and BL bolt-on items can increase performance on the EQ-5D-5L in patients with hemophilia. A future valuation study will be carried out.

Keywords: EQ-5D, health-related quality of life, bolt-on items, psychometric properties, hemophilia

INTRODUCTION

Hemophilia, which has two main types (A and B), is a rare hematological disease that primarily affects males (1). Globally, hemophilia affects approximately 400,000 people, with an estimated prevalence of 1 in 5,000 male live births for type A and 1 in 30,000 live births for type B (2). Hemophilia undoubtedly has a negative impact on patients' health-related quality of life (HRQoL) and psychological well-being due to its chronicity, symptoms, and complications (3). Life expectancy in hemophilia varies, depending on whether patients receive appropriate treatment. Overall, the mortality rate of male patients is about twice the rate of healthy men; whereas for those with severe hemophilia, the rate could be four to six times higher (4). In addition, comorbidity with other diseases is also regarded as a potential but uncertain factor that may affect the mortality rate of patients with hemophilia as well.

Bleeding episodes produced by abnormal clotting factors are hallmarks of hemophilia. The severity of hemophilia varies, from bleeding after surgery to spontaneous bleeding, and depends on the level of clotting factors (5). It is usually internal and causes joint and muscle damage and pain (1). In some cases, repeated bleeding in the same body part can cause chronic inflammation and reduce joint flexibility and muscle mobility, causing permanent disability (6, 7). Previous studies have shown that bleeding disorders lead to a significantly poor physical HRQoL, and alleviating these symptoms is highly likely to improve patients' physical HRQoL and well-being (8–10).

Survival rates for patients with hemophilia have increased significantly (11); however, longer life expectancy has increased the risk of developing some chronic psychiatric, psychological, and social support problems, all of which can lead to loss of dignity (12, 13). One previous study indicated that a large number of patients with hemophilia are disabled to some extent due to the complications from treatment. As a result, these patients cannot live in accordance with their standards and values, and the loss of self-esteem jeopardizes their HRQoL and well-being (14). Ganzini et al. also found that loss of dignity is one of the main reasons that the family of hemophiliacs have advocated for legalized physician-assisted death (15, 16). Further studies have demonstrated that low dignity is strongly associated with poor HRQoL (17, 18). However, few clinical or social intervention studies have been carried out on the topic.

The EQ-5D, one of the most widely used generic preference-based measures (PBMs) worldwide (19–22), is increasingly used to measure HRQoL in different patient groups (23, 24). It can generate a summarized utility score that aids decision-makers in allocating scarce healthcare resources (25). Unlike condition-specific PBMs, such as the Hemophilia Quality of Life Questionnaire for Adults (Haem-A-QoL), generic PBMs are intended for use across conditions and treatments and to provide consistency and comparability for economic evaluations (26). Although the use of the EQ-5D in hemophilia is growing, there are notable gaps.

The EQ-5D was designed for both simplicity and comprehensiveness in the measurement of HRQoL (27). To ensure simplicity, the descriptive system comprises only five items to reflect different dimensions of HRQoL, four of which measure the physical aspect of HRQoL and one of which measures the mental aspect of HRQoL. For comprehensiveness, the EQ-5D tries to cover all the dimensions of health in general, rather than specific aspects. Hence, it can provide consistency and comparability within and between different populations and settings. However, a growing number of studies have indicated that the EQ-5D lacks sensitivity and appropriateness for measuring changes in HRQoL in some specific patient groups (26, 28–30). To increase the ability of the EQ-5D to capture the important variations of HRQoL in patients with specific conditions, adding condition-specific bolt-on items were introduced. The EQ-5D with the two bolt-on items has been shown to provide valid and reliable results that may increase the sensitivity of the EQ-5D to capture the condition-specific changes in HRQoL in a specific patient group (31–33). Using the bolt-on method, the integral structure of the EQ-5D descriptive system can be maintained, and simultaneously, the predictive ability to estimate the change in HRQoL in a subpopulation is improved.

For patients with hemophilia, the EQ-5D may not be able to detect certain changes in HRQoL due to symptoms unique to hemophilia or side effects resulted from its treatment. Using the bolt-on approach can ensure comparability, transparency, and consistency when measuring HRQoL across different hemophilia-related interventions. Previous studies indicated that adding condition-specific bolt-on items to the EQ-5D may undermine the cross-program comparability because different interventions measure different dimensions of health (34, 35), and a new algorithm including the bolt-on items is therefore needed to calculate utility scores to support cost-utility analysis. However, when performing economic evaluations, the so-called orphan drugs and products for rare diseases (RDs) including hemophilia are often found not being cost-effective if measured under standard thresholds (36). Hence, without disease-specific bolt-on items, the risks of not responding to the patients' needs will be increased and equal access to medical care will be hindered (37).

To develop an independent value set that considers specific symptoms of hemophilia and side-effects caused by its treatment, which is vital to assess the cost-effectiveness of different hemophilia-related interventions, it is important firstly to confirm the validity of the additional dimensions added to the EQ-5D so that a local value set can be developed based on this expanded instrument. In this study, two hemophilia-specific bolt-on items were added to the EQ-5D: bleeding (BL) and dignity (DG). They were identified and developed through a literature review, focus group interviews, and expert discussions aimed at specifically measuring the changes in physical and mental HRQoL for hemophiliacs that might be insufficiently captured by the original EQ-5D. Thus, the aims of this exploratory study were to examine the impact of adding two bolt-on items to the EQ-5D and to assess the psychometric properties of the EQ-5D with bolt-on items in a sample of Chinese patients with hemophilia.

METHODS

Research Population and Data Collection

The data used in this study were obtained from an online nationwide cross-sectional survey to investigate the health and socioeconomic status of patients living with hemophilia in China. The survey was conducted between August 2019 and December 2019. Research team had collaborated with the "*Home of Hemophilia*," the biggest hemophilia patient organization in China, to perform data collection. All the participants were recruited via the patient organization's internal network. A recruitment advertisement was sent to its registered members via its internal member management platform. Inclusion criteria were: (1) ≥18 years; (2) no cognitive problems (screened by the patient organization and self-reported by the patients); (3) able to provide informed consent. Interested and eligible members (assessed by the patient organization) were invited to join in an online "surveying group" and a link of questionnaire was provided in that group. Information on patients' demographics, socioeconomic status, diagnosis, and treatment status, HRQoL, and access to and use of healthcare services were collected. Survey procedure, implementation, and quality control were defined and monitored by the survey committee, which was composed of medical specialists dealing with hemophilia, leaders of patient organizations, and our research team. The first page of the online questionnaire was the consent form, and all the participants were forced to read through it. The survey would not begin until participants clicked the "Agree" button at the end of that page. They were also provided an option on "Do not agree and leave." Since some patients might not be able to complete the questionnaire all by themselves due to poor health status, their main caregivers would be recruited to complete the survey for them. At the beginning of the survey, participants were therefore required to indicate their identity as a patient or a caregiver. Then patients and caregivers were asked to complete different versions of the questionnaire, and their responses were coded as either self- or proxy-completed data. The Institutional Review Board Ethics Committee of the Chinese University of Hong Kong and Peking Union Medical College Hospital approved the study protocol (Ref no.: SBRE-18-268 and SK814). This study was carried out in accordance with the 1964 Declaration of Helsinki and its later amendments. Written informed consent was provided by all participants.

HRQoL Measurement

EQ-5D-5L

In this study, we used the five-level version of the EQ-5D (EQ-5D-5L). It has two sections: a descriptive system section and a visual analog scale (EQ-VAS). The descriptive system section consisted of five items, one on each of the following: mobility (MO), self-care (SC), usual activities (UA), pain/discomfort (PD), and anxiety/depression (AD). For each item, responses are based on a 5-point scale, ranging from "no problem" (1) to "extreme problems/" (5). A profile of "11111" indicates that the patient has no problems in all five items and is the best possible health state. The EQ-VAS reflects a person's overall health on a vertical visual analog scale, ranging from 0 to 100, with a higher score indicating a better imagined health state (38).

Bolt-On Items to the EQ-5D-5L

Two condition-specific bolt-on items to the EQ-5D-5L, the DG and BL, were developed based on a sophisticated process including literature review, patient focus group interview, and expert discussion. Two bolt-on items were designed to reflect general changes in psychosocial and physical HRQoL in hemophiliacs, respectively. Dignity is a complicated concept that comprises a number of related issues, such as self-respect, self-concept, self-confidence, and self-esteem (39–42). Given that patients with RDs, including hemophilia, have to deal with dignity-related issues both during doctor visits and daily life, we decided to follow the Dixon et al.'s work (42) and developed this bolt-on item to directly ask patients about their experiences and feelings about DG in general. Bleeding was defined as patients experiencing all types of bleeding, including bleeding into the joints, skin, and mouth; bleeding of the mouth and gums; bleeding after circumcision; bleeding after receiving shots; blood in the urine or stool; and hard-to-stop nosebleeds (43). Patients were asked to indicate how severe the problem of bleeding they experience in daily life on the day of survey, regardless the type or source of bleeding or control effectiveness. Both the DG and BL items were framed the same as the other items of the EQ-5D-5L, with the same number of response options. Responses to the DG item was worded as follows (Chinese version was presented to all participants): I live with full dignity," "I live with many dignities," "I live with some dignities," "I live with few dignities," and "I live with no dignity." Responses to the BL item were as follows: "I have no bleeding problems," "I have slight bleeding problems," "I have moderate bleeding problems," "I have severe bleeding problems," and "I have extreme bleeding problems."

Haem-A-QoL

The Haem-A-QoL is a commonly used instrument to assess the HRQoL for adult patients with hemophilia (44–47). It consists of 46 items grouped into 10 dimensions. The scale specifically assesses HRQoL of patients with hemophilia (48). These include physical health (PHYS, 5 items), feelings (FEEL, 4), view of self (VIEW, 5), sports and leisure (SPORT, 5), work and school (WORK, 4), dealing with hemophilia (DEAL, 3), treatment (TREAT, 8), future (FUTURE, 5), family planning (FAMPL, 4), and partnership and sexuality (SEXUAL, 3). The sum score of the Haem-A-QoL ranges from 0 to 100 and is obtained by summing up the scores of all 10 subscales, with a higher score indicating poorer HRQoL.

SF-12

The SF-12 is one of the most widely used generic non-preference based measure, which has been used to assess HRQoL in patients with hemophilia and proved having satisfactory performance (49, 50). It consists of 12 questions on 8 dimensions of physical and mental health: general health (GH), physical functioning (PF), role physical (RP), and body pain (BP), vitality (VT), social functioning (SF), role emotional (RE), and mental health (MH).

Scores are reported as physical and mental component summary scores (PCS and MCS) (51).

Statistical Analysis

Self-completed and proxy-completed data were analyzed separately. Descriptive analysis was used to describe the participants' characteristics. The proportion of participants' responses on each level of the EQ-5D-5L and bolt-on items are presented as percentages. We calculated the proportion of participants with the best health state, as measured with the EQ-5D-5L and EQ-5D-5L with two bolt-on items. For the EQ-5D-5L, the best health state was indicated by a profile of "11111." For the EQ-5D+DG and EQ-5D+BL, it was indicated by a profile of "111111." For the EQ-5D+DG+BL, it was indicated by a profile of "1111111."

Convergent validity was determined by examining the correlations between the EQ-5D-5L with two bolt-on items and the Haem-A-QoL and SF-12. Spearman rank correlation (*rs*) was used to confirm the strength of the associations, where $0.25 < rs < 0.5$, and $rs \geq 0.5$ were identified as moderate and strong correlations, respectively (52). We assumed that the EQ-5D-5L with bolt-on items would positively correlate with the Haem-A-QoL sum score, but negatively correlate with PCS and MCS of the SF-12. We further posited that the DG item would show a stronger association with MCS than PCS, and that the BL item would show a stronger association with PCS than MCS. Known-group validity was assessed by testing the priori hypotheses that patients with poorer health status had more problems with dignity and bleeding. A chi-squared test was used to assess the discriminatory ability of the DG and BL items to differentiate patients known to differ in terms of severity of hemophilia, disabling levels, and comorbidity.

We performed both univariable and multivariable linear regression analysis to compare the exploratory power of the EQ-5D-5L and EQ-5D-5L with two bolt-on items. The EQ-VAS was used as the dependent variable in all the models. For univariable analysis, five items of the EQ-5D-5L and another two bolt-on items were analyzed separately (seven models). For multivariable analysis, another five models were developed. (a) DG+BL model, (b) EQ-5D model (MO+SC+UA+PD+AD), (c) EQ-5D+DG model, (d) EQ-5D+BL model, and (e) full model (MO+SC+UA+PD+AD+DG+BL). *R*-squared (R^2) and adjusted *R*-squared (adjusted R^2) were used to determine the exploratory ability of the models. The Shannon index ($H^{'}$) and the Shannon evenness index ($J^{'}$) were used to assess the classification efficiency of the EQ-5D-5L and EQ-5D-5L with two bolt-on items, respectively. They provided information to assess the ability of the measurements to gauge the diversity of patients (53). All analyses were performed using R software (R Foundation, Vienna, Austria). Statistical significance was set at $p \leq 0.05$.

RESULTS

Participant Characteristics

Data from 895 patients and 222 caregivers who completed the survey were included in our analysis. Approximately 85.8% of self-completed questionnaires and 85.4% of proxy-completed questionnaires were from type A patients. Approximately 72.1% of patients who self-completed their questionnaire were aged between 21 and 40 years, and 46.9% completed secondary or above education. More than 60% were rural residents, and nearly 70% reported a family income of <50,000 CNY ($7,800 USD) per year. For participants whose questionnaires had been completed by proxy, more than 70% were aged under 30 years, and 53.6% were rural residents (**Table 1**).

Frequency of Health States and Ceiling Effects

Table 2 demonstrates that among patients who self-completed their questionnaire, only 15% and 2% responded that they had no problems for the DG and BL items, respectively, which indicated an acceptable ceiling effect. For the EQ-5D-5L, 1.9% of patients reported a perfect health state (i.e., "11111"); however, the proportion was lower, at 0.6%, for the EQ-5D+DG+BL (i.e., "1111111"). For patients whose questionnaire had been completed by proxy, a total of 5.9% of participants reported a perfect health state on the EQ-5D-5L, whereas the proportion was lower, at 0.9%, for the EQ-5D+DG+BL.

Convergent Validity

The correlations between the EQ-5D-5L and bolt-on items are shown in **Figure 1**. For patients who self-completed their questionnaire, the associations of the EQ-5D-5L with the DG and BL items ranged from 0.29 to 0.57 and 0.36 to 0.53, respectively. For patients who had completed their questionnaire by proxy, the associations of the EQ-5D-5L with the DG and BL items were slightly stronger than those of patients who had self-completed their questionnaires. **Table 3** demonstrates the convergent validity of the EQ-5D-5L with two bolt-on items. The results confirmed our hypotheses that the EQ-5D-5L with two bolt-on items show a positive relationship with the Haem-A-QoL total score [range: 0.2 to 0.64 (self); 0.27 to 0.64 (proxy)], but a negative relationship with PCS [−0.64 to −0.29 (self); −0.65 to −0.4 (proxy)], and MCS [−0.62 to −0.3 (self); −0.61 to −0.35 (proxy)]. Additionally, the DG item was strongly and significantly correlated with MCS, whereas the BL item showed a stronger correlation with PCS.

Exploratory Power

Univariable and Multivariable linear regression analysis was used to examine the performance of the models in predicting the VAS scores. The data in **Table 4** reveal that the EQ-5D+DG+BL model showed better performance than the other models on the basis of adjusted R^2. Compared with the EQ-5D, adding two bolt-on items increased the explanatory power from 0.294 to 0.318 for patients who self-completed their questionnaires and 0.432 to 0.47 for patients whose questionnaires had been completed by proxy. In other words, the two bolt-on items have significantly increased the instrument's ability to predict EQ-VAS scores. The adjusted R^2 increased by 8.2 and 8.8% for reports completed by the patients or patients' proxy respondents, respectively. In the comparison of the performance of the EQ-5D+DG and the EQ-5D+BL, the former combination explained

TABLE 1 | Respondents' characteristics.

	Overall respondents	Self-completed respondents		Proxy-completed respondents		*p*-value
		n	%	*n*	%	
Sex						
Male	1,117	895	100	222	100	–
Ethnic group						
Han	1,064 (95.3)	853	95.3	211	95.0	0.87
Others	53 (4.7)	42	4.7	11	5.0	
Age						
≤20	164 (14.7)	72	8.0	92	41.4	<0.001
21–30	427 (38.2)	362	40.4	65	29.3	
31–40	324 (29)	287	32.1	37	16.7	
41–50	141 (12.6)	125	14.0	16	7.2	
≥51	61 (5.5)	49	5.5	12	5.4	
Education						
No/Primary	581 (52)	475	53.1	106	47.7	0.06
Secondary	399 (35.7)	305	34.1	94	42.3	
Tertiary or above	137 (12.3)	115	12.8	22	9.9	
Family register						
Urban resident	451 (40.4)	348	38.9	103	46.4	0.05
Rural resident	666 (59.6)	547	61.1	119	53.6	
Family income						
≤10,000	183 (17)	151	17.5	32	14.7	0.9
10,001–30,000	328 (30.4)	260	30.2	68	31.3	
30,001–50,000	257 (23.8)	203	23.6	54	24.9	
50,001–80,000	120 (11.1)	94	10.9	26	12.0	
80,001–100,000	98 (9.1)	78	9.1	20	9.2	
≥10,0001	92 (8.5)	75	8.7	17	7.8	
Type						
Type A	946 (85.8)	759	85.8	187	85.4	0.43
Type B	150 (13.6)	122	13.8	28	12.8	
Others/uncertain	4 (0.4)	2	0.2	2	0.9	

Family income per year (Chinese Yuan).

more variations in VAS scores than the latter, which substantially reduced the adjusted R^2 of 3.2 and 3.7% both patients who self-completed their questionnaire and patients whose questionnaires were completed by proxy, respectively.

Known-Group Validity

For both patients who self-completed their questionnaire and patients whose questionnaires were completed by proxy, those who reported hemophilia-related disability, poorer perceived health status, and comorbidity were highly likely to report having extreme problems with both dignity and bleeding. Patients who self-completed their questionnaire showed a higher proportion of reporting extreme problems with dignity and bleeding compared with patients whose questionnaires were completed by proxy. The results confirmed that the two bolt-on items had good discriminatory ability to differentiate patients with different health statuses. The results of the analysis of known-group validity of the HRQoL measurements are shown in **Table 5**.

Classification Efficiency

The values of $H^{'}$ for the EQ-5D+DG+BL were higher than those for the EQ-5D-5L, which indicate that the EQ-5D-5L with two bolt-on items generated a larger amount of information. However, the values of $J^{'}$ for the EQ-5D+DG+BL were lower than those for the EQ-5D-5L. These findings suggest that more information is captured by adding two more items to the EQ-5D-5L, but the observed gain in discrimination in both self- and proxy-completed samples is relatively low due to the increase in classification options (**Table 6**).

DISCUSSION

Principal Findings

This study assessed the impact of adding two condition-specific bolt-on items to the EQ-5D-5L in a sample of Chinese patients with hemophilia and examined the psychometric properties of the measure. Compared with the EQ-5D-5L, a more satisfactory performance of the EQ-5D-5L with two bolt-on items was

TABLE 2 | Proportion of EQ-5D-5L items and the two bolt-on items.

	%						
	EQ-5D-5L descriptive system					EQ-5D-5L bolt-on items	
	MO	SC	UA	PD	AD	DG	BL
SELF-COMPLETED							
No problem	15.5	54.5	22.8	7.1	17.9	15.0	2.0
Slight problems	34.1	25.8	38.1	38	37.3	24.6	18.5
Moderate problems	26.6	13.5	23.6	33.2	26.5	22.2	40.9
Severe problems	16.6	4.1	11.6	13.8	10.1	24.7	31.8
Unable/extreme problems	7.2	2.1	3.9	7.9	8.1	13.5	6.8
The best health (11111)	1.9						
The best health including DG (111111)	1.6						
The best health including BL (111111)	0.7						
The best health including both (1111111)	0.6						
PROXY-COMPLETED							
No problem	27.0	54.5	31.1	9.0	22.5	19.4	1.8
Slight problems	33.3	25.2	40.5	39.2	45.0	33.8	24.3
Moderate problems	19.8	13.5	16.2	28.4	19.4	19.8	41.4
Severe problems	11.7	2.3	7.2	13.5	9.9	18.0	28.8
Unable/extreme problems	8.1	4.5	5.0	9.9	3.2	9.0	3.6
The best health (11111)	5.9						
The best health including DG (111111)	3.2						
The best health including BL (111111)	1.4						
The best health including both (1111111)	0.9						

MO, mobility; SC, self-care; UA, usual activates; PD, pain/discomfort; AD, anxiety/depression; DG, dignity; BL, bleeding.

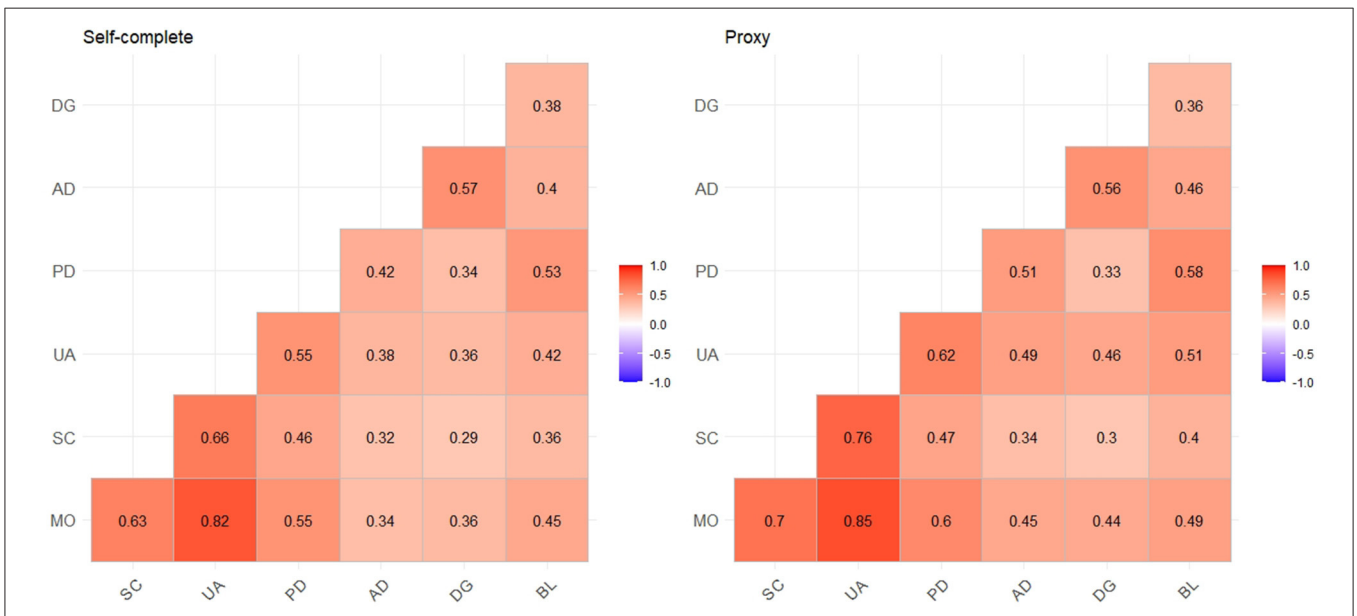


FIGURE 1 | Correlation between EQ-5D items and bolt-on items; MO, mobility; SC, self-care; UA, usual activates; PD, pain/discomfort; AD, anxiety/depression; DG, dignity; BL, bleeding; all the coefficients are statistically significant.

confirmed in both the self- and proxy-completed samples. We found that, compared with the EQ-5D-5L, adding two items significantly decreased the ceiling effects (68.4 and 84.7% for self- and proxy-completed samples) and significantly increased the discriminative power to differentiate patients with different health statuses. As expected, the DG item strongly correlated

TABLE 3 | Correlation between EQ-5D-5L, bolt-on items and the Haem-A-QoL and the SF-12.

	SPEARMAN CORRELATION COEFFICIENT					
	Self-completed			Proxy-completed		
	Haem-A-QoL	PCS	MCS	Haem-A-QoL	PCS	MCS
MO	0.38***	−0.64***	−0.32***	0.64***	−0.65***	−0.49***
SC	0.2**	−0.47***	−0.3***	0.47**	−0.47***	−0.35***
UA	0.31***	−0.61***	−0.35***	0.54**	−0.62***	−0.47***
PD	0.42***	−0.51***	−0.36***	0.37*	−0.53***	−0.41***
AD	0.59***	−0.29***	−0.62***	0.27	−0.4***	−0.58***
DG	0.64***	−0.36***	−0.61***	0.66***	−0.41***	−0.61***
BL	0.49***	−0.53***	−0.35***	0.31*	−0.57***	−0.39***

*MO, mobility; SC, self-care; UA, usual activates; PD, pain/discomfort; AD, anxiety/depression; DG, dignity; BL, bleeding; PCS, physical component summary score; MCS, mental component summary score. *p < 0.05; **p <0.01; ***p <0.001.*

TABLE 4 | Regression analysis evaluating the impact of adding bolt-on items in predicting EQ-VAS score.

	R^2	Adjusted R^2	*F*-statistics	*p*-Value
SELF-COMPLETED				
MO	0.196	0.192	54.11	<0.001
SC	0.144	0.14	37.49	<0.001
UA	0.178	0.175	48.35	<0.001
PD	0.191	0.188	52.65	<0.001
AD	0.154	0.15	40.45	<0.001
DG	0.163	0.159	43.31	<0.001
BL	0.152	0.148	39.9	<0.001
DG+BL	0.23	0.22	33.06	<0.001
MO+SC+UA+PD+AD	0.31	0.294	19.70	<0.001
MO+SC+UA+PD+AD+DG	0.331	0.312	17.97	<0.001
MO+SC+UA+PD+AD+BL	0.321	0.302	17.18	<0.001
MO+SC+UA+PD+AD+DG+BL	0.339	0.318	15.93	<0.001
PROXY-COMPLETED				
MO	0.341	0.325	22.32	<0.001
SC	0.228	0.21	12.76	<0.001
UA	0.367	0.352	25.03	<0.001
PD	0.264	0.247	15.52	<0.001
AD	0.267	0.25	15.73	<0.001
DG	0.272	0.255	16.16	<0.001
BL	0.243	0.226	13.88	<0.001
DG+BL	0.37	0.35	15.86	<0.001
MO+SC+UA+PD+AD	0.484	0.432	9.41	<0.001
MO+SC+UA+PD+AD+DG	0.519	0.461	8.87	<0.001
MO+SC+UA+PD+AD+BL	0.504	0.444	8.35	<0.001
MO+SC+UA+PD+AD+DG+BL	0.537	0.470	7.99	<0.001

MO, mobility; SC, self-care; UA, usual activates; PD, pain/discomfort; AD, anxiety/depression; DG, dignity; BL, bleeding.

with the AD item of the EQ-5D-5L, whereas the BL item strongly correlated with the PD item. Both showed a statistically significant association with the Haem-A-QoL sum score, but the impact on mental and physical HRQoL (SF-12) was different.

Regression models revealed that the EQ-5D-5L with two bolt-on items showed an improvement in the ability to predict VAS scores. The value of the adjusted R^2 increased from 0.294 to 0.318 for patients who self-completed their questionnaires and 0.432 to 0.47 for patients whose questionnaires had been completed by proxy. When comparing the predictive ability of the two bolt-on items, the DG model outperformed the BL model. The adjusted R^2 increased by 6.1% when the DG item

TABLE 5 | Summary of known-groups validity testing of the two bolt-on items.

	%										
	Overall										
		DG					**BL**				
		No problem	**Slight problem**	**Moderate problem**	**Severe problem**	**Unable/Extreme**	**No problem**	**Slight problem**	**Moderate problem**	**Severe problem**	**Unable/Extreme**
SELF-COMPLETED											
Disability											
No	37.1	22.8	28.0	21.8	17.3	10.0	3.8	27.0	44.6	20.4	4.2
Yes	62.9	10.6	22.3	22.1	29.2	15.7	1.2	13.7	38.9	37.0	9.2
p-value		<0.001					<0.001				
Severity											
Minor	27.4	22.0	35.0	20.3	16.3	6.5	5.3	32.5	52.8	8.9	0.4
Moderate	26.5	14.7	22.7	29.8	25.2	7.6	1.3	13.9	48.7	32.8	3.4
Severe	46.1	11.1	19.6	18.8	29.5	21.0	0.5	12.8	29.2	44.9	12.6
p-value		<0.001					<0.001				
Comorbidity											
No	58.0	18.8	27.8	23.4	20.7	9.2	2.5	21.3	40.3	31.3	4.6
Yes	42.0	9.8	20.2	20.4	30.2	19.4	1.3	14.6	41.6	32.6	9.8
p-value		<0.001					0.003				
PROXY-COMPLETED											
Disability											
No	53.9	27.3	36.4	15.5	12.7	8.2	2.7	33.6	40.0	21.8	1.8
Yes	46.1	13.8	30.9	23.4	24.5	7.4	1.1	12.8	46.8	34.0	5.3
p-value		0.03					0.004				
Severity											
Minor	25.7	29.8	33.3	15.8	19.3	1.8	3.5	36.8	54.4	5.3	0
Moderate	23.0	15.7	27.5	25.5	23.5	7.8	3.9	27.5	45.1	23.5	0
Severe	51.4	15.8	36.8	19.3	14.9	13.2	0	16.7	33.3	43.0	7.0
p-value		0.06					<0.001				
Comorbidity											
No	64.9	25.7	38.2	17.4	13.2	5.6	2.8	29.2	43.8	22.2	2.1
Yes	35.1	14.0	25.6	24.4	26.9	15.4	0	15.4	37.2	41.0	6.4
p-value		<0.001					0.004				

DG, dignity; BL, bleeding.

TABLE 6 | Shannon index (H′) and Shannon Evenness index (J′) of EQ-5D and bolt-on items.

	H′	J′
SELF-COMPLETE		
EQ-5D	8.29	0.71
EQ-5D+DG	8.48	0.66
EQ-5D+BL	8.49	0.66
EQ-5D+DG+BL	8.64	0.62
PROXY		
EQ-5D	6.89	0.59
EQ-5D+DG	7.07	0.55
EQ-5D+BL	7.08	0.55
EQ-5D+DG+BL	7.24	0.52

DG, dignity; BL, bleeding.

(proxy: 6.7%) was added and 2.7% (proxy: 2.8%) when the BL item was added. One possible reason for this finding may be that the DG item was designed to capture the variation of mental HRQoL among patients, which provided extra information that might not have been possible to capture, or fully capture, with the EQ-5D-5L only. The BL item was used to measure the patients' physical HRQoL. Given that four of the five items of the EQ-5D-5L measure physical HRQoL, it is likely that the information gathered with the BL item overlapped with other physical HRQoL items, which strengthened its predictive ability. Moreover, Neufeld et al. found that there are other factors that significantly affect patients' overall HRQoL and day-to-day variability in the non-bleeding state (54).

Comparison With Previous Studies

Although no studies have targeted hemophilia, adding bolt-on items to the EQ-5D to improve its sensitivity in capturing the variations of HRQoL in different patient groups have been studied before. For example, Gandhi et al. indicated that the bolt-on item of "vision" could increase the responsiveness of the EQ-5D to detect changes in health outcomes among patients undergoing cataract surgery (33). Geraerds et al. also found that adding a cognition item to the EQ-5D could improve its explanatory power and informativity among patients with traumatic brain injury (53). Kangwanrattanakul et al. demonstrated that the bolt-on item of "activities related to bending knees" could improve the sensitivity of the EQ-5D questionnaire and better measure health status among Thai people (31).

In the present study, both the BL and DG items were found to substantially increase the predictive ability of the EQ-5D-5L, and the DG item showed a larger increase than the BL item. This suggests that, besides regaining physical health from the treatment, living with independence, dignity, and self-esteem may be more important among patients with hemophilia (40). Previous studies in the other patient groups showed similar findings. One German study found that low sense of dignity in patients with cancer was significantly associated with psychological distress (55). Baillie and Llott also found that patients feel vulnerable when they experience loss of control, lack of privacy, and insecurity (56). Another qualitative study conducted in the United Kingdom demonstrated that patients with spinal injury must be treated with respect and dignity as long as they need (57). Adding a dignity dimension to the EQ-5D is important in measuring HRQoL, considering that it is a chronic, rather than instantaneous, need of patients. The performance of a dignity item in the EQ-5D in patients with other RDs warrants further investigation.

We noted that the strength of the correlation between the DG and BL items was not very strong, which indicates that patients who feel the least dignity are not those who have the most severe bleeding problems. This is in line with the findings of some cancer studies that have indicated that loss of dignity may be a broader concern among medically ill people and is not limited to patients with very severe conditions or at the end of life (58–60). It is important to note that dignity is a multi-faceted concept; for example, for end-of-life patients, dignity involves quality care to achieve a sense of spiritual peace and well-being (61). However, for patients with moderate or mild conditions, showing them dignity could be more general, for example, sharing decision-making with them, respecting their preferences, protecting their privacy, etc. (62).

Few studies have compared the performance of bolt-on items from the perspective of patients and caregivers. Our findings were consistent with those of previous studies that suggest that patients are likely to overestimate the severity of their health states compared with caregivers or the general public (63). We further found that, compared with caregivers, patients reported suffering more extreme problems on the BL item (47%) than on the DG item (33%). This finding suggests that caregivers, to some extent, are more likely to underestimate the physical HRQoL of hemophilia patients than mental HRQoL. Further, we also find that the EQ-5D items and bolt-on items tended to be statistically insignificant in predicting the Haem-A-QoL scores in the sample of caregiver (**Supplementary Table A1**). The utility score of the EQ-5D elicited from the perspective of the public or patients is a long-term controversy. The current literature mainly supports that patients tend to assign higher utilities compared with members of the public (64–66). However, few alternative utility algorithms were produced when bolt-on items were included in the EQ-5D (32), which limited our knowledge on the impact of bolt-on items on the values for EQ-5D health states. Further research is needed to develop the utility values of the EQ-5D+BL+DG.

STRENGTHS AND LIMITATIONS

This was the first study to develop bolt-on items to improve the sensitivity of the EQ-5D-5L to measure HRQoL in patients with hemophilia worldwide. In addition, this study was one of the very few worldwide, and the first in China, to present and compare the impact and psychometric properties of the bolt-on items to the EQ-5D-5L from the perspectives of patients and proxies. Third, in this study, the development of the bolt-on items to the EQ-5D-5L followed a solid process, which included a literature review,

patient interviews, expert discussions, and cognitive debriefing. Finally, we found that dignity is an important issue affecting mental HRQoL and social well-being in patients with RDs. We confirmed that the EQ-5D-5L with dignity showed satisfactory psychometric properties among hemophiliacs, which suggests a further application in the other RD patient groups.

This study also had several limitations that should be addressed. First, data used in this study was collected via an online survey, the patients who were unable to approach Internet were excluded from the survey, which may lead to some selection bias. Additionally, no response rate can be calculated based on the online survey, because only information of successfully submitted respondents were recorded. Neither can we rule out the possibility that some of the participants might have cognitive problems, even though this was one of the eligibility criteria. Second, all the participants in this study were recruited from a national hemophilia patient group, even though it is the largest patient group for hemophilia patients in China, selection bias might have been introduced. Last, in this study, caregivers completed the self-completed rather than proxy version of the EQ-5D, which may weaken the validity of our findings. Finally, all the data were self-reported, which might have caused recall bias, which could limit the generalizability of our findings.

CONCLUSIONS

The EQ-5D-5L with two bolt-on items showed good psychometric properties among Chinese patients with hemophilia in our study. Both items improved the sensitivity and exploratory power of the EQ-5D-5L. The DG item was strongly correlated with hemophilia psychological HRQoL, whereas the BL captured more information related to patients' physical HRQoL. A higher convergent and known-group validity of the EQ-5D-5L was observed in the patients who had self-completed their questionnaire compared with patients who had completed their questionnaire by proxy, suggesting that the additional value of condition-specific items to the EQ-5D-5L was more relevant in the self-completed sample. This study was designed as the first stage in an assessment of the influence of bolt-on items on health states covering a range of RDs in China. The second stage of the research would be to undertake a valuation study to facilitate an estimate of the value algorithm for the EQ-5D-5L with bolt-on items. Further, the performance of the dignity will be investigated in patients with the other RDs.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the institutional review board ethics committee of the Chinese University of Hong Kong and Peking Union Medical College Hospital approved the study protocol and informed consent (Ref no.: SBRE-18-268 and SK814). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RX: material preparation, data collection, draft preparation, data analysis, and manuscript revision. DD: study conception and design, material preparation, data collection, data analysis, manuscript revision, and supervision. NL: data analysis and manuscript revision. RY and JL: manuscript revision. SZ: study conception and design, manuscript revision, supervision, and funding. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Key Research and Development Program of China (Grant No. 2016YFC0901500) and the Center for Rare Diseases Research, Chinese Academy of Medical Sciences, Beijing, China (Grant No. 2016ZX310174-4).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.707998/full#supplementary-material

REFERENCES

1. Srivastava A, Brewer AK, Mauser-Bunschoten EP, Key NS, Kitchen S, Llinas A, et al. Guidelines for the management of hemophilia. *Haemophilia.* (2013) 19:e1–47. doi: 10.1111/j.1365-2516.2012.02909.x
2. Stoffman J, Andersson NG, Branchford B, Batt K, D'Oiron R, Escuriola Ettingshausen C, et al. Common themes and challenges in hemophilia care: a multinational perspective. *Hematology.* (2018) 24:39–48. doi: 10.1080/10245332.2018.1505225
3. Trindade GC, Viggiano LG de L, Brant ER, Lopes CAO, Faria ML, Ribeiro PHNS, et al. Evaluation of quality of life in hemophilia patients using the WHOQOL-bref and Haemo-A-Qol questionnaires. *Hematol Transfus cell Ther.* (2019) 41:335–41. doi: 10.1016/j.htct.2019.03.010
4. *Hemophilia Prognosis and Life Expectancy*. Available online at: https://hemophilianewstoday.com/hemophilia-prognosis-life-expectancy/ (accessed June 15, 2021).
5. Mayo Clinics. *Hemophilia - Symptoms and Causes*. Available online at: https://www.mayoclinic.org/diseases-conditions/hemophilia/symptoms-causes/syc-20373327 (accessed March 1, 2021).
6. O'Hara J, Walsh S, Camp C, Mazza G, Carroll L, Hoxer C, et al. The impact of severe haemophilia and the presence of target joints on health-related quality-of-life. *Health Qual Life Outcomes.* (2018). 16:84. doi: 10.1186/s12955-018-0908-9
7. Mulder K, Llinas A. The target joint. *Haemophilia.* (2004) 10:152–6. doi: 10.1111/j.1365-2516.2004.00976.x
8. McLaughlin JM, Munn JE, Anderson TL, Lambing A, Tortella B, Witkop ML. . Predictors of quality of life among adolescents and young adults

with a bleeding disorder. *Health Qual Life Outcomes.* (2017) 15:67–9. doi: 10.1186/s12955-017-0643-7
9. Scalone L, Mantovani LG, Mannucci PM, Gringeri A; COCIS Study Investigators. Quality of life is associated to the orthopaedic status in haemophilic patients with inhibitors. *Haemophilia.* (2006) 12:154–62. doi: 10.1111/j.1365-2516.2006.01204.x
10. Elander J, Morris J, Robinson G. Pain coping and acceptance as longitudinal predictors of health-related quality of life among people with haemophilia-related joint pain. *Eur J Pain.* (2013) 17:929–38. doi: 10.1002/j.1532-2149.2012.00258.x
11. Saba HI, Tran DQ Jr. Challenges and successes in the treatment of hemophilia: the story of a patient with severe hemophilia A and high-titer inhibitors. *J Blood Med.* (2012) 3:17–23. doi: 10.2147/JBM.S30479
12. von der Lippe C, Diesen PS, Feragen KB. Living with a rare disorder: a systematic review of the qualitative literature. *Mol Genet genomic Med.* (2017). 5:758–73. doi: 10.1002/mgg3.315
13. Weill A. Haemophilia treatment for all and the role of tolerance, difference and education. *Haemophilia.* (2017) 23:341–3. doi: 10.1111/hae.13213
14. Barclay L. In sickness and in dignity: a philosophical account of the meaning of dignity in health care. *Int J Nurs Stud.* (2016) 61:136–41. doi: 10.1016/j.ijnurstu.2016.06.010
15. Ganzini L, Goy ER, Dobscha SK. Why oregon patients request assisted death: family members' views. *J Gen Intern Med.* (2008) 23:1296. doi: 10.1007/s11606-008-0542-z
16. Chochinov HM, Kristjanson LJ, Hack TF, Hassard T, McClement S, Harlos M. Burden to others and the terminally ill. *J Pain Symptom Manage.* (2007) 34:463–71. doi: 10.1016/j.jpainsymman.2006.12.012
17. Chochinov HM. Dignity-conserving care–a new model for palliative care: helping the patient feel valued. *JAMA.* (2002) 287:2253–60. doi: 10.1001/jama.287.17.2253
18. Albers Gwenda Ms, Pasman H. Roeline W. P, Deliens Luc P, et al. Does Health status affect perceptions of factors influencing dignity at the end of life? *J Pain Symptom Manage.* (2013). 45:1030–8. doi: 10.1016/j.jpainsymman.2012.06.012
19. Ramos-Goñi JM, Oppe M, Slaap B, Busschbach JJV, Stolk E. Quality control process for EQ-5D-5L valuation studies. *Value Heal.* (2017) 20:466–73. doi: 10.1016/j.jval.2016.10.012
20. Sullivan PW, Ghushchyan VH. EQ-5D Scores for diabetes-related comorbidities. *Value Heal.* (2016) 19:1002–8. doi: 10.1016/j.jval.2016.05.018
21. Rencz F, Gulácsi L, Drummond M, Golicki D, Prevolnik Rupel V, Simon J, et al. EQ-5D in Central and Eastern Europe: 2000–2015. *Qual Life Res.* (2016) 25:2693–710. doi: 10.1007/s11136-016-1375-6
22. Rowen D, Azzabi Zouraq I, Chevrou-Severac H, van Hout B. International regulations and recommendations for utility data for health technology assessment. *Pharmacoeconomics.* (2017) 35:11–9. doi: 10.1007/s40273-017-0544-y
23. Soucie JM, Grosse SD, Siddiqi AE, Byams V, Thierry J, Zack MM, et al. The effects of joint disease, inhibitors and other complications on health-related quality of life among males with severe haemophilia A in the United States. *Haemophilia.* (2017). 23:e287–93. doi: 10.1111/hae.13275
24. Carroll L, Benson G, Lambert J, Benmedjahed K, Zak M, Lee XY. Real-world utilities and health-related quality-of-life data in hemophilia patients in France and the United Kingdom. *Patient Prefer Adherence.* (2019) 13:941–57. doi: 10.2147/PPA.S202773
25. Abdin E, Subramaniam M, Vaingankar JA, Luo N, Chong SA. Measuring health-related quality of life among adults in Singapore: population norms for the EQ-5D. *Qual Life Res.* (2013) 22:2983–91. doi: 10.1007/s11136-013-0405-x
26. Brazier JE, Rowen D, Lloyd A, Karimi M. Future directions in valuing benefits for estimating QALYs: is time up for the EQ-5D? *Value Heal.* (2019) 22:62–8. doi: 10.1016/j.jval.2018.12.001
27. Brazier J. *Measuring and Valuing Health Benefits for Economic Evaluation*. 2nd ed. Oxford : Oxford University Press. (2007).
28. Tosh J, Brazier J, Evans P, Longworth L. A review of generic preference-based measures of health-related quality of life in visual disorders. *Value Heal.* (2012) 15:118–27. doi: 10.1016/j.jval.2011.08.002
29. Brazier J, Yang Y, Tsuchiya A, Rowen DL. A review of studies mapping (or cross walking) non-preference based measures of health to generic preference-based measures. *Heal Econ Prev Care.* (2010) 11:215–25. doi: 10.1007/s10198-009-0168-z
30. Brazier J, Connell J, Papaioannou D, Mukuria C, Mulhern B, Peasgood T, et al. A systematic review, psychometric analysis and qualitative assessment of generic preference-based measures of health in mental health populations and the estimation of mapping functions from widely used specific measures. *Health Technol Assess.* (2014). 18:vii–viii, xiii–xxv, 1–188. doi: 10.3310/hta18340
31. Kangwanrattanakul K, Gross CR, Sunantiwat M, Thavorncharoensap M. Exploration of a cultural-adaptation of the EQ-5D for Thai population: a "bolt-on" experiment. *Qual Life Res.* (2019) 28:1207–15. doi: 10.1007/s11136-018-2072-4
32. Yang Y, Rowen D, Brazier J, Tsuchiya A, Young T, Longworth L. An exploratory study to test the impact on three "bolt-on" items to the EQ-5D. *Value Heal.* (2015) 18:52–60. doi: 10.1016/j.jval.2014.09.004
33. Gandhi M, Ang M, Teo K, Wong CW, Wei YC, Tan RL, et al. A vision 'bolt-on' increases the responsiveness of EQ-5D: preliminary evidence from a study of cataract surgery. *Eur J Heal Econ.* (2020) 21:501–11. doi: 10.1007/s10198-019-01156-w
34. Brazier J, Tsuchiya A. Preference-based condition-specific measures of health: what happens to cross programme comparability? *Health Econ.* (2010) 19:125–9. doi: 10.1002/hec.1580
35. Finch AP, Brazier JE, Mukuria C. Selecting bolt-on dimensions for the EQ-5D: examining their contribution to health-related quality of life. *Value Heal.* (2019) 22:50–61. doi: 10.1016/j.jval.2018.07.001
36. Pearson I, Rothwell B, Olaye A, Knight C. Economic modeling considerations for rare diseases. *Value Heal.* (2018) 21:515–24. doi: 10.1016/j.jval.2018.02.008
37. Czech M, Baran-Kooiker A, Atikeler K, Demirtshyan M, Gaitova K, Holownia-Voloskova M, et al. A review of rare disease policies and orphan drug reimbursement systems in 12 Eurasian countries. *Front Public Heal.* (2020) 7:416. doi: 10.3389/fpubh.2019.00416
38. Herdman M, Gudex C, Lloyd A, Janssen M, Kind P, Parkin D, et al. Development and preliminary testing of the new five-level version of EQ-5D (EQ-5D-5L). *Qual Life Res.* (2011) 20:1727–36. doi: 10.1007/s11136-011-9903-x
39. Beach MC, Sugarman J, Johnson RL, Arbelaez JJ, Duggan PS, Cooper LA. Do patients treated with dignity report higher satisfaction, adherence, and receipt of preventive care? *Ann Fam Med.* (2005) 3:331–8. doi: 10.1370/afm.328
40. Jacelon CS, Connelly TW, Brown R, Proulx K, Vo T. A concept analysis of dignity for older adults. *J Adv Nurs.* (2004) 48:76–83. doi: 10.1111/j.1365-2648.2004.03170.x
41. Baillie L, Gallagher A, Wainwright P. *Defending Dignity – Challenges and Opportunities for Nursing*. Royal College of Nursing (2008).
42. Dixon S, Palfreyman S, Shackley P, Brazier J. What is dignity? A literature review and conceptual mapping (2011). Available online at: http://eprints.whiterose.ac.uk/43280/ (accessed March 1, 2021).
43. Centers for Disease Control and Prevention. *What is Hemophilia.* Centers for Disease Control and Prevention. (2020). Available online at: https://www.cdc.gov/ncbddd/hemophilia/facts.html (accessed March 1, 2021).
44. Mackensen S, Catalani O, Asikanius E, Paz-Priel I, Lehle M, Trask P. Determining meaningful health-related quality-of-life improvement in persons with haemophilia A using the Haemophilia Quality of Life Questionnaire for Adults (Haem-A-QoL). *Haemophilia.* (2020) 26:1019–30. doi: 10.1111/hae.14184
45. Xu RH, Dong D, Luo N, Wong EL, Wu Y, Yu S, et al. Evaluating the psychometric properties of the EQ-5D-5L and SF-6D among patients with haemophilia. *Eur J Heal Econ.* (2021) 22:547–57. doi: 10.1007/s10198-021-01273-5
46. Mackensen S, Eldar-Lissai A, Auguste P, Krishnan S, von Maltzahn R, Yu R, et al. Measurement properties of the Haem-A-QoL in haemophilia clinical trials. *Haemophilia.* (2017) 23:383–91. doi: 10.1111/hae.13140
47. Mackensen S V, Gringeri A. Development and pilot testing of a disease-specific quality of life questionnaire for adult patients with haemophilia (Haem-A-QoL). *Blood.* (2004). 104:608A—9A. doi: 10.1182/blood.V104.11.2214.2214
48. Von Mackensen S, Czepa D, Herbsleb M, Hilberg T. Development and validation of a new questionnaire for the assessment of subjective physical performance in adult patients with haemophilia – the HEP-Test-Q. *Haemophilia.* (2010) 16:170–8. doi: 10.1111/j.1365-2516.2009.02112.x

49. Shah RM, Banahan BF III, Holmes ER, Patel AS, Barnard M, Khanna R, et al. An evaluation of the psychometric properties of the sf-12v2 health survey among adults with hemophilia. *Health Qual Life Outcomes.* (2018) 16:229. doi: 10.1186/s12955-018-1059-8
50. Poon J-L, Doctor JN, Nichol MB. Longitudinal changes in health-related quality of life for chronic diseases: an example in hemophilia A. *J Gen Intern Med.* (2014) 29(Suppl 3):S760–6. doi: 10.1007/s11606-014-2893-y
51. Ware JE, Kosinski M, Keller SD. A 12-item short-form health survey: construction of scales and preliminary tests of reliability and validity. *Med Care.* (1996) 34:220–33. doi: 10.1097/00005650-199603000-00003
52. DeVellis RF. *Scale Development : Theory and Applications*. 4th ed. Los Angeles, CA: SAGE. (2017).
53. Geraerds A, Bonsel G, Janssen M, de Jongh MA, Spronk I, Polinder S, et al. The added value of the EQ-5D with a cognition dimension in injury patients with and without traumatic brain injury. *Qual Life Res.* (2019) 28:1931–9. doi: 10.1007/s11136-019-02144-6
54. Neufeld EJ, Recht M, Sabio H, Saxena K, Solem CT, Pickard AS, et al. Effect of acute bleeding on daily quality of life assessments in patients with congenital hemophilia with inhibitors and their families: observations from the dosing observational study in hemophilia. *Value Heal.* (2012) 15:916–25. doi: 10.1016/j.jval.2012.05.005
55. Oechsle K, Wais MC, Vehling S, Bokemeyer C, Mehnert A. Relationship between symptom burden, distress, and sense of dignity in terminally ill cancer patients. *J Pain Symptom Manage.* (2014) 48:313–21. doi: 10.1016/j.jpainsymman.2013.10.022
56. Baillie L, Ilott L. Promoting the dignity of patients in perioperative practice. *J Perioper Pract.* (2010) 20:278–82. doi: 10.1177/175045891002000802
57. Davis RE, Vincent C, Henley A, McGregor A. Exploring the care experience of patients undergoing spinal surgery: a qualitative study. *J Eval Clin Pract.* (2013) 19:132–8. doi: 10.1111/j.1365-2753.2011.01783.x
58. Solomon BK, Wilson KG, Henderson PR, Poulin PA, Kowal J, McKim DA. Loss of dignity in severe chronic obstructive pulmonary disease. *J Pain Symptom Manage.* (2015) 51:529–37. doi: 10.1016/j.jpainsymman.2015.11.007
59. Koppelman ER. Dementia and dignity: towards a new method of surrogate decision making. *J Med Philos.* (2002) 27:65–85. doi: 10.1076/jmep.27.1.65.2971
60. Horton R. Rediscovering human dignity. *Lancet.* (2004) 364:1081–5. doi: 10.1016/S0140-6736(04)17065-7
61. Chochinov HM, Hack T, Hassard T, Kristjanson LJ, McClement S, Harlos M. Dignity therapy: a novel psychotherapeutic intervention for patients near the end of life. *J Clin Oncol.* (2005) 23:5520–5. doi: 10.1200/JCO.2005.08.391
62. Bagheri H, Yaghmaei F, Ashktorab T, Zayeri F. Patient dignity and its related factors in heart failure patients. *Nurs Ethics.* (2012) 19:316–27. doi: 10.1177/0969733011425970
63. Peeters Y, Vliet Vlieland TPM, Stiggelbout AM. Focusing illusion, adaptation and EQ-5D health state descriptions: the difference between patients and public. *Heal Expect.* (2012) 15:367–78. doi: 10.1111/j.1369-7625.2011.00667.x
64. Bremner KE, Chong CA, Tomlinson G, Alibhai SM, Krahn MD. A review and meta-analysis of prostate cancer utilities. *Med Decis Mak an Int J Soc Med Decis Mak.* (2007) 27:288–98. doi: 10.1177/0272989X07300604
65. Peeters Y, Stiggelbout AM. Health state valuations of patients and the general public analytically compared: a meta-analytical comparison of patient and population health state utilities. *Value Heal.* (2010) 13:306–9. doi: 10.1111/j.1524-4733.2009.00610.x
66. De Wit GA, Busschbach JJ V, De Charro FT. Sensitivity and perspective in the valuation of health status: whose values count? *Health Econ.* (2000). 9:109–26. doi: 10.1002/(SICI)1099-1050(200003)9:2<109::AID-HEC503>3.0.CO;2-L

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.

frontiers in Medicine

METHODS
published: 13 August 2021
doi: 10.3389/fmed.2021.724488

A Modified Preparation Method of Ideal Platelet-Rich Fibrin Matrix From Whole Blood

Mirta Hediyati Reksodiputro[1]*, *Alida Roswita Harahap*[2], *Lyana Setiawan*[3] and *Mikhael Yosia*[4]

[1] Division of Facial Plastic & Reconstructive Surgery, Department of Otorhinolaryngology-Head and Neck Surgery, Faculty of Medicine, Universitas Indonesia, Dr. Cipto Mangunkusumo Hospital, Jakarta, Indonesia, [2] Department of Clinical Pathology, Faculty of Medicine, Universitas Indonesia, Dr. Cipto Mangunkusumo Hospital, Jakarta, Indonesia, [3] Department of Clinical Pathology, Rumah Sakit Dharmais Pusat Kanker Nasional, Jakarta, Indonesia, [4] Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia

One bioproduct that is widely used in the wound healing process is platelet-rich plasma (PRP). PRP is a liquid solution with high autologous platelet concentration, making it a good source of growth factors to accelerate wound healing. Recent development in PRP had created a new product called platelet-rich fibrin matrix (PRFM), which has a denser and more flexible structure. PRFM is the newest generation of platelet concentrate with a fibrin matrix that holds platelet in it. The key concept in creating PRFM from PRP is the addition of $CaCl_2$ followed by centrifugation, which converts fibrinogen to fibrin, and the fibrin cross-links to form a matrix that contains viable platelets. There are many commercially available kits to create PRFM, but they are often expensive and uneconomical. This research will test a modified method of making ideal PRFM from PRP without any commercial kits. The modified method will include determining the minimum level of $CaCl_2$ used, the type of centrifuge, and the speed and duration of centrifugation. By performing a modified preparation method on five samples of whole blood, it was found that the ideal PRFM could be made by mixing PRP with 25 mM $CaCl_2$ and centrifuging it at a speed of 2,264 $\times$ *g* for 25 min at room temperature. The PRP and PRFM platelet counts of this method tend to be lower than the platelet counts found in other studies. Although visually comparable, further study is needed to compare the performance of PRFMs made with this method and PRFMs made with commercial kits.

Keywords: platelet concentrate, fibrin matrix, platelet rich plasma, platelet rich fibrin matrix, $CaCl_2$, centrifugation

OPEN ACCESS

Edited by:
Robert Campbell,
The University of Utah, United States

Reviewed by:
Navin Mishra,
Indira Gandhi Institute of Medical Sciences, India
Jose Fabio Lana,
Instituto do Osso e da Cartilagem, Brazil

***Correspondence:**
Mirta Hediyati Reksodiputro
citamirta@yahoo.com

Specialty section:
This article was submitted to Hematology, a section of the journal Frontiers in Medicine

Received: *13 June 2021*
Accepted: *07 July 2021*
Published: *13 August 2021*

Citation:
Reksodiputro MH, Harahap AR, Setiawan L and Yosia M (2021) A Modified Preparation Method of Ideal Platelet-Rich Fibrin Matrix From Whole Blood. Front. Med. 8:724488. doi: 10.3389/fmed.2021.724488

INTRODUCTION

Otorhinolaryngology specialists in plastic reconstruction have reported successful use of exogenous growth factors and PRP in clinical settings. Sclafani reported that the release of growth factors in wound healing was primarily carried out by platelets, echoing that PRP plays a vital role in wound healing. The influence of growth factors on endothelial cells and fibroblasts will increase within 7 days after injury and disappear after 14 days (1). Other studies have also reported that PRP can increase and accelerate wound healing by 80% in ulcers compared to placebo (2, 3). In clinical settings, people often use PRP in the form of a solution or gel to facilitate tissue repair. The solution or gel form of conventional PRP sometimes poses clinical challenges because it cannot be fixated adequately in certain conditions, such as deep diabetic ulcers with exposed bones and minimal soft tissue availability for PRP injections.

Platelet-rich fibrin matrix (PRFM) is the latest generation of platelet concentrates with simple preparation without biochemical ingredients (bovine thrombin). PRFM is a slow polymerization of fibrin in PRP, resulting in a PRFM structure that resembles natural fibrin (4). This specific structure will play an essential role in increasing cell migration, cell proliferation, and cyclic formation. Through this polymerization process, all platelets in PRP will be deposited between the PRFM fibrin fibers. According to that logic, levels of platelet (and subsequently, growth factor) in PRFM are expected to be equivalent to PRP.

Laboratory experiments looking at usage of PRFM in specific media show that there was an increase in levels of platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and transforming growth factor beta (TGFβ) on the first day, followed by a gradual decrease on the next day (1, 5). This characteristic was not observed in PRP since most of the growth factors in PRP were released on the first day of application to the wound. PRFM also has fibrin characteristics with a more natural platelet distribution that mimics the body's response to injury and a denser and more flexible macroscopic structure (6, 7). These factors theoretically make PRFM superior to use for wound healing.

Commercial kits for preparing PRFM had been available and widely used in clinical settings. One such example is the FIBRINET tubes which can produce PRFM from whole blood through the addition of $CaCl_2$ and centrifugation at 1,100 × *g* for 6 min (5). However, the use of the aforementioned commercial tools has several drawbacks, including (1) high prices, rendering the use of PRFM in a clinical setting to be economically dubious; and (2) unknown concentration of platelet in PRFM, increasing the possibility of not achieving the creation of ideal PRFM. This study aims to overcome the problems mentioned above and cover the weaknesses of the existing invention by proposing a modified method to produce PRFM.

In order to guarantee the production of ideal PRFM, the modified method will ensure that platelet-poor plasma (PPP), a by-product in PRFM production, has a platelet content of 0/μl, representing the fact that all platelets are attached to the PRFM fibrin matrix at the bottom of the tube. Measurement of TGFβ1 will be conducted to reassure that growth factors are still available and viable inside the end product. The modified method will also try to make PRFM production more economical by creating PRFM from PRP through improvised methods without using a commercial PRFM production kit. At the same time, this study will determine the minimum amount of $CaCl_2$ needed to produce an ideal PRFM.

This study will carry out trials processing human whole blood, making PRP and adding in 1 M of calcium chloride ($CaCl_2$) before centrifuging the solution to form the PRFM. The trials will evaluate whether the modified method can prepare ideal PRFM to accelerate the wound healing process. Improvement in wound healing is essential in reconstructive surgery and will affect the successes of operations both aesthetically and functionally.

TABLE 1 | Profile of volunteers whose blood was used in the study.

Subject	Gender	Age (years)	Hematological profile and CBC
1	Male	30	Between normal limits
2	Male	36	Between normal limits
3	Male	41	Between normal limits
4	Female	29	Between normal limits
5	Male	39	Between normal limits

CBC, Complete Blood Count.
Hematologic profile examinations (platelet count, PT/APTT, bleeding time, clotting time).

MATERIALS AND METHODS

This study is conducted in Indonesia and approved by the Ethical Clearance Committee from the Faculty of Medicine, University of Indonesia (202/H2.F1/ETIK/2013). The study design and protocol are made and implemented following the Helsinki Declaration. Volunteers recruited from the study were given an explanation, given an information sheet, and signed the informed consent form before participating in the study.

Whole Blood Collection From Participants

Whole blood samples (8 ml per tube) were collected from five healthy volunteers (**Table 1**). The volunteers are non-smokers, with no history of chronic diseases, and with normal hematologic profile (including normal thrombocyte count, PT/APTT). Blood is collected using the 10 ml vacuum tube with cell selector gel from the PRP kit (RegenKit® A-PRP®; Regen Lab, Le Mont-sur-Lausanne, Switzerland). A small amount of whole blood was also taken to analyze thrombocyte count with the Celtac-a automatic cell counter (Automated Hematology Analyzer MEK-6450; Nihon Kohden, Tokyo, Japan). The kit from RegenLab is designed to produce 4–6 mL of PRP from every tube of blood collected. The RegenKit® vacuum tube was inverted back and forth three times after blood collection is finished.

Preparation of PRP and Calculation of Thrombocyte Count From PRP-PPP (Platelet-Poor Plasma)

This study utilizes a commercial PRP kit, the RegenKit® A-PRP® (Regen Lab, Le Mont-sur-Lausanne, Switzerland). This instruction directly follows the protocol presented in the kit. PRP can also be produced using other PRP kits or methods. Blood that had been taken from volunteers were immediately centrifuged using RegenLab 642VFD PRP Fixed Angle Centrifuge (Regen Lab, Le Mont-sur-Lausanne, Switzerland). The centrifuge from RegenLab was designed to prepare PRP and was programmed to run at 1,500 × *g* for 5 min at room temperature. The centrifuge process resulted in three separate layers: a clear yellowish plasma thrombocyte on top (consisting of a PPP layer on top and a PRP layer in the bottom), a cell selector gel layer with leucocyte in the middle, and a red blood cell layer on the bottom. A small amount of PPP and PRP was taken from each specimen to analyze thrombocyte count using Celtac-a automatic cell counter. PRP process is determined to be successful when the thrombocyte

FIGURE 1 | (A) RegenLab PRP-Centri CH-1052 centrifuge used in processing the PRP in cylinder tubes and **(B)** Beckman CS-6R centrifuge used in processing PRP in a Wheaton bottle.

count in PPP is 0/μl. The tube was then inverted gently back and forth three times to mix the plasma, thrombocyte, and leucocyte, forming the final PRP. PPP was mixed with PRP to ensure a larger volume of end product used for PRFM preparation. A total of five PRP specimens will be produced at the end of this step and will be used to produce PRFM.

Preparation of PRFM and Calculation of Thrombocyte Count From PRFM-PPP

This experiment concentrates on creating PRFM using methods proposed by O'Connel, which is a modified Fibrinet (Cascade Medical Enterprise, Wayne, NJ, USA) PRFM kit method (8). Orthopedic surgeons with more extensive wounds or operating areas usually utilize Fibrinet. Centrifuge from RegenLab was used, as it is the ideal centrifuge to produce PRFM with minimal addition of $CaCl_2$. The maximal speed of this centrifuge is 4,500 RPM (2,264 × *g*). Six milliliters of PRP were taken from four different PRP specimens using a micropipette (extra care was given to avoid taking the red blood cell layer) and moved to four separate 10 ml cylindrical centrifuge tubes (Pyrex®, Staffordshire, England). Each tube was then added with $CaCl_2$ 1 M until a final concentration of 70, 45, 25, or 15 mM of $CaCl_2$ was achieved in each tube. All four tubes were then centrifuged at 2,264 × *g* for 25 min at room temperature. The resulting product will consist of two layers, the PRFM and PPP.

PPP from all four PRFM specimens with different concentrations of $CaCl_2$ was then analyzed using the Celtac-a automatic cell counter. As previously mentioned, an ideal PRFM is the one with a 0/μl thrombocyte count in their PPP. The lowest concentration of $CaCl_2$ that produces an ideal PRFM was then determined as the minimum recommended concentration of $CaCl_2$.

After the minimum amount of $CaCl_2$ needed had been obtained from the previous steps, the fifth PRP specimen was assigned to an alternative PRFM preparation method to produce a coin-shaped PRFM. This particular shape was sought after because in several reconstructive surgery, a round coin-shaped PRFM with larger surface area is practical in certain clinical conditions. Six milliliters from the fifth PRP specimen was taken using a micropipette and put into a wide-mouth Wheaton bottle (diameter 30 mm), followed by addition of the $CaCl_2$. The Wheaton bottle was then centrifuged with a Beckman CS-6R centrifuge at 3,800 RPM (4,043 × *g*) for 25 min (**Figure 1**). The resulting product consisted of two layers, the PRFM and PPP. The PRFM and PPP were taken for thrombocyte count analysis using the Celtac-a automatic cell counter.

Analysis of the PRFM Characteristic With a Scanning Electronic Microscope (SEM)

The PRFMs are trimmed into equal sizes, washed using phosphate-buffered saline (PBS), and fixated in 2.5% glutaraldehyde in PBS for 1 h at 4°C. The samples were then washed for the second time with 0.1 M cacodylate buffer (pH 7.3) followed by fixation with 1% osmium tetroxide (OsO4) and 0.1 M cacodylate buffer for 1 h at room temperature (22°C ± 2°C). The samples were then dehydrated in serial ethanol and dried before being placed on an aluminum sheet with silver adhesive paint coated with a layer of 4 nM of gold inside an Edward S150B argon atmosphere apparatus (Crawley, West Sussex, UK). The samples were then observed at 0° with SEM Stereoscan 200 (Cambridge, UK) at 20 kV. Three different fields of view were examined for each sample, and the diameter of thrombocyte and the size of fibrin fibers were examined.

Analysis of TGFβ1 in PRFM

TGFβ1 in PRFM were analyzed using a TGFβ1 immunoassay kit (Quantikine R&D Systems; Thermo Fisher Scientific, Portsmouth, NH, USA). The immunoassay kit works by identifying reactions of growth factors in the sample with a TGFβ1 monoclonal antibody in the microtiter plate wells. The bond between TGFβ1 and anti-TGFβ1F was identified via a TGFβ1 polyclonal antibody that is labeled with an enzyme.

TABLE 2 | Thrombocyte count (per μl) for whole blood, PRP, and PPP after preparation of PRP.

	Thrombocyte count (/μl)		
Sample	**Whole blood**	**PRP**	**PRP-PPP**
Subject 1	313.000	52.000	0
Subject 2	414.000	64.000	0
Subject 3	205.000	66.000	0
Subject 4	280.000	121.000	0
Subject 5	393.000	186.000	0

Note that the thrombocyte count for PPP is 0/μl for all the samples, indicating a successful creation of ideal PRP.

With additional substrates, these reactions will produce a varying intensity of colors identified with spectrophotometry at 450 nm. The intensity of the color is directly correlated to the concentration of protein analyzed. Compared to a standard solution with a known concentration, the concentration of TGFβ1 in the PRFM sample can be analyzed.

PRFM specimens analyzed were put into the microtiter plate wells coated with TGFβ1 monoclonal antibody and incubated. TGFβ1 in the sample will bind to the antibody inside the well. After washing the well to remove excess substances, the TGFβ1 polyclonal antibody with a horseradish peroxidase (HRP) label was added. Second incubation was done, during which the polyclonal antibody will bind to the anti-TGFβ1*TGFβ1 complex that formed in the first incubation forming a sandwich of anti-TGFβ1*TGFβ1*anti-TGFβ1.HRPO. A substrate is added to form a blue color that will change into yellow after a stop solution is added. The intensity of color will correlate with the amount of TGFβ1 inside the sample.

RESULTS

Volunteer Baseline Data and Examination of PRP

Blood was drawn from all volunteers and was processed right away using the methods above. All five volunteers had a normal hematologic profile, no chronic comorbidities, and normal CBC (hemoglobin, hematocrit, thrombocyte, leucocyte, differential counts) examinations. Comparison of the amount of thrombocyte in whole blood, PRP, and PPP in the PRP specimen can be seen in **Table 2**; note that a decrease of thrombocyte count is expected.

Determination of the Minimum Recommended Concentration of $CaCl_2$ for Ideal PRFM

Four PRP specimen were assigned to different amounts of $CaCl_2$ to determine the minimal amount in which ideal PRFM (the ones with 0 μl of thrombocyte in its PPP). The schematic shown in **Figure 2** shows that a concentration of 25 mM of $CaCl_2$ is the minimum amount needed to produce ideal PRFM. The fifth specimen was used to produce a coin-shaped PRFM to represent diversity in the practical application of PRFM in different clinical conditions (**Figure 3**). Both PRP specimen (disc and dome shaped) centrifuged with 25 mM $CaCl_2$ had PPP of 0/μl.

PRFM Analysis With SEM

Measurement of fibrin fiber and platelet diameter in PRFM specimens were performed using SEM. PRFM from five volunteers was prepared and fixated to produces slices that were thin enough to be analyzed by SEM. It was found that PRFM had platelets scattered among the fibrin fibers (**Figure 4**). The average diameter size (from three measurements in each PRFM specimens) of the subject's platelets and fibrin fiber matrix can be seen in the table below (**Table 3**). PRFM from Subject 1 dried out during the preparation and fixation process, rendering it inappropriate for analysis.

TGFβ1 in PRFM

The average TGFβ1 rate from PRFM was 37,497 pg/mg, with the highest rate being 42,147 pg/mg and the lowest being 31,849 pg/mg (**Table 4**). PRFM from Subject 1 dried out during the preparation and fixation process, rendering it inappropriate for analysis.

DISCUSSION

Currently, several methods and commercial kits are available for the preparation of PRP. Most of these methods will produce an end product in liquid or gel form. Due to these mechanical properties, conventional PRP is often impractical in clinical settings that require secure implantation in a specific site or where released growth factors could be washed out during an operation.

The latest development in PRP use for wound healing involves altering its physical property through plasma and platelet stimulation. Alteration of PRP physical properties can be achieved by adding calcium ($CaCl_2$) and centrifugation to produce PRFM without the need for additional exogenous thrombin. The addition of $CaCl_2$ and centrifugation to PRP will convert fibrinogen to fibrin, and the fibrin cross-links to form a matrix that contains viable platelets (**Figure 4**). The PRFM preparation process creates a gel-like matrix containing high concentrations of non-activated, functional, intact platelets within a fibrin matrix. These platelets had been proven to release a relatively constant concentration of growth factors over 7 days (9).

The resulting PRFM is a thin sheet with a more robust physical structure than the liquid PRP. The PRFM can replicate the effect of a natural wound healing response (i.e., the three-dimensional formation of a cross-linked fibrin matrix). This scaffold-like fibrin matrix is essential as a place for platelet adhesion. This scaffolding helps localize platelets and ultimately increases the concentration of growth factors to the desired point or location for tissue regeneration (10).

In this experiment, we obtained a formula for making PRFM without using a costly reagent kit. The ideal PRFM requires that maximal platelets are trapped in the PRFM; the PPP platelet count of 0/ml can prove this. This experiment also found the

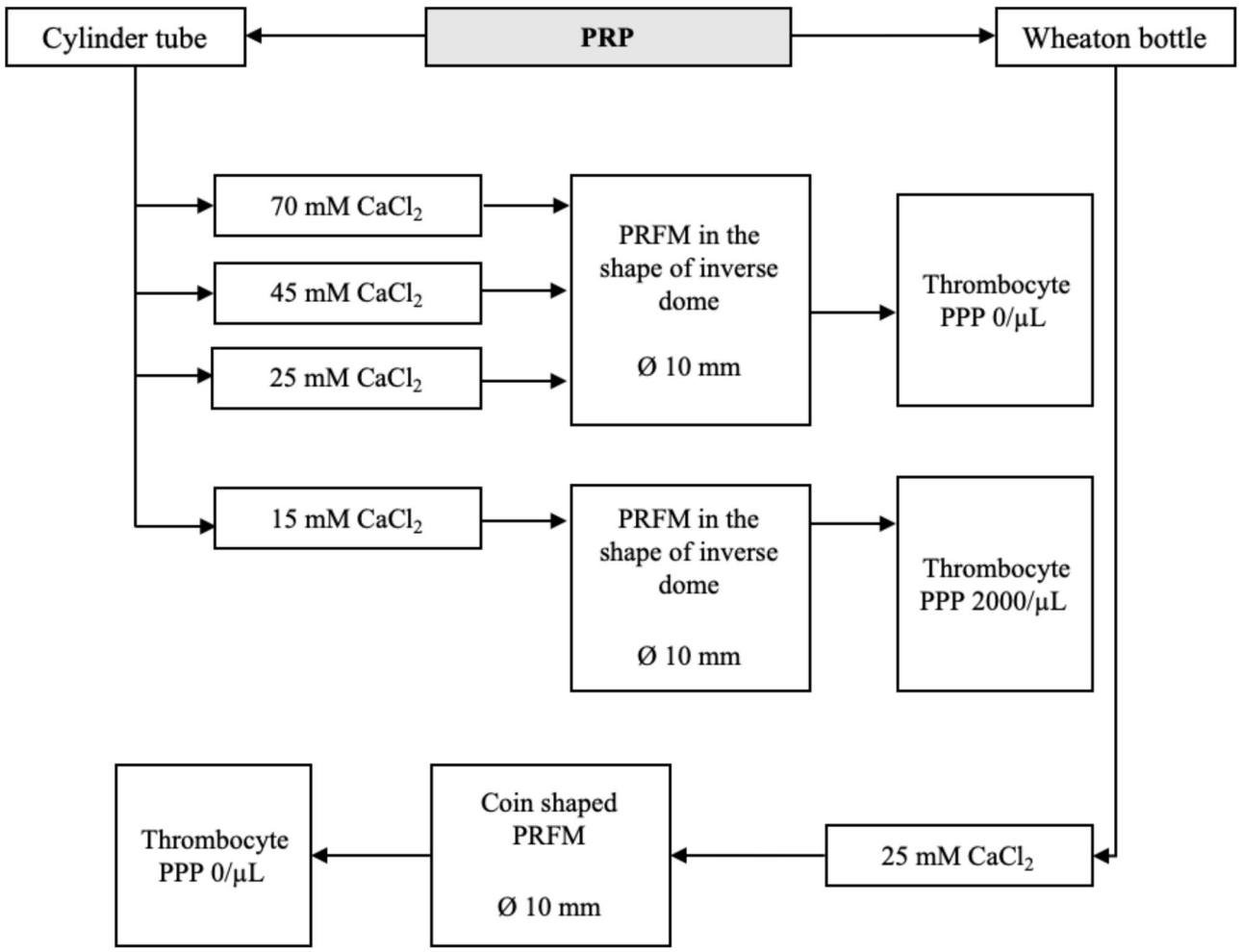


FIGURE 2 | Finding the minimum recommended concentration of $CaCl_2$ for ideal PRFM. Regardless of the shape, 25 mM of $CaCl_2$ is determined to be the minimum concentration needed to produce PRFM with 0/µl of thrombocyte in its PPP.

minimum amount of $CaCl_2$ and the centrifuge settings needed to obtain the ideal PRFM. To achieve ideal PRFM, PRP obtained was mixed with 25 mM $CaCl_2$, then centrifuged again at a speed of 2,264 × *g* for 25 min at room temperature. The centrifugation will result in two layers, the PPP and PRFM. The platelet level in PPP was 0/µl; thus, it can be assumed that this method produces ideal PRFM with all the platelets from PRP adhering to the matrix fibrin. Commercial kits usually include $CaCl_2$ solutions in their package, though most choose not to disclose the amount or concentration of $CaCl_2$ used in their set.

The PRFM protocol for this study is based on a previous experiment by O'Connell, which breaks down the creation of PRFM from whole blood. O'Connell created PRFM by inserting PRP and $CaCl_2$ in a Wheaton bottle before starting the centrifugation. In his study, 18 ml of whole blood can create 7–8 ml of PRP, which in turn yields a 35 mM round PRFM membrane (8). In our study, 8 ml of whole blood produces 6 ml of PRP, which then yields a 10 mm round PRFM membrane. A suspected vital difference in methodology lies during the PRP creation in which tubes containing a thixotropic polyester separator gel was used. These separator gels contain different properties depending on the PRP kit's brand (O'Connell used tubes from the Cascade Autologous System, while this study used one from RegenLab). Differences in the separator gel may affect performance in isolating both the platelets and plasma (containing fibrinogen) from the packed red and white cell fraction (8, 11).

The platelet count results for whole blood, PRP and PPP after the first centrifugation showed that the platelet count in PRP was lower than that of the whole blood (**Table 2**). In general, the preparation of PRP is followed by volume adjustment by removing the PPP, resulting in a platelet concentration 2.5–8 times higher than that found in whole blood (12). This study's median PRP platelet count was 97,800 (52,000–186,000)/ml—up to 50% loss of platelet, which is smaller than the typical PRP platelet count (13). The low platelet count is probably caused

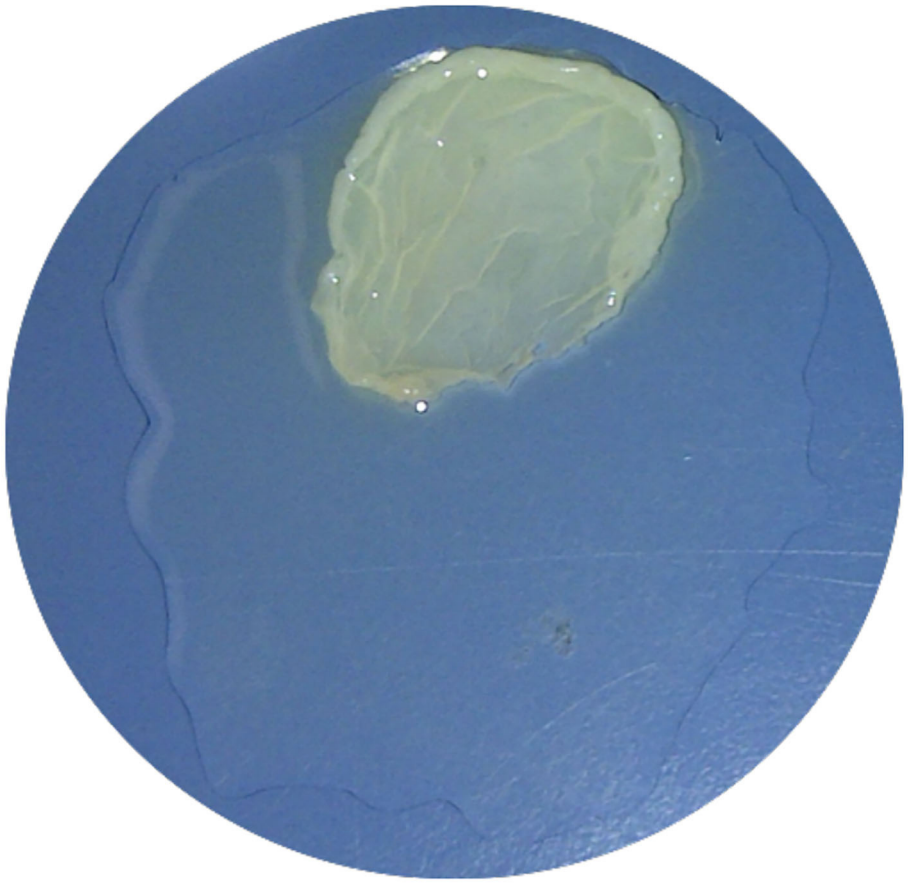


FIGURE 3 | Coin-shaped PRFM produced via centrifuging PRP in a Wheaton bottle.

TABLE 3 | Average fibrin size and platelet diameter in PRFM analyzed through SEM.

PRFM	Fibrin fiber size (nm)	Platelet diameter (nm)
Subject 1	*	*
Subject 2	446.7	670.1
Subject 3	403.6	638.2
Subject 4	360.2	625.4
Subject 5	428.1	638.2

**PRFM dried out during specimen preparation.*

TABLE 4 | Level of TGFβ1.

PRFM	TGFβ1 (pg/mg)
Subject 1	*
Subject 2	39,796
Subject 3	34,128
Subject 4	39,564
Subject 5	31,849

**PRFM dried out during specimen preparation.*

by the trapping of platelets in the RegenKit gel tube or due to erythrocyte deposits (**Figure 5**). Platelet yield must be taken into account in assessing the PRP creation methods used, including standardized methods (commercial kits). Ideally, efforts are made so that minimal platelets are lost since these platelets are a source of growth factors.

Studies that looked upon platelet, leucocyte, and erythrocyte yield on PRP production shed more light on the conundrum regarding the number of platelets being yielded. A review by Marxref concluded that a platelet count of 1 million in a 6-ml aliquot could be considered a benchmark for therapeutic PRP, again showing that the amount of platelet inside the PRP in our experiment falls short in terms of platelet counts to be considered as benchmark PRP (14). It seems that particular emphasis needs to be put on centrifuge force used to yield optimal PRP. Recommendation of a far less intense centrifugal force: 900 × *g* for 5 min for separating centrifuge and 1,500 × *g* for 15 min is best to produce optimal PRP (15). The low platelet yield had been one of the primary concerns in this

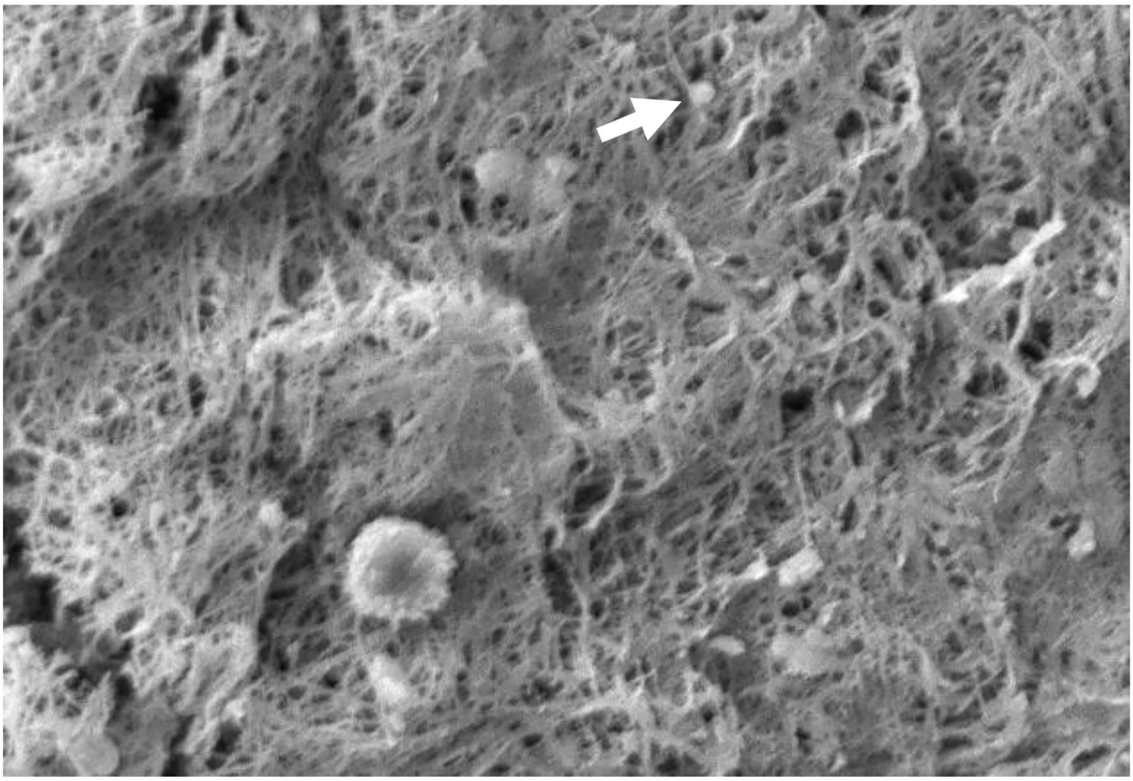


FIGURE 4 | The appearance of fibrin and platelet fibers on PRFM by SEM examination. The white arrows show the platelets between the fibrin fiber matrix. It appears that the fibrin fibers are evenly distributed like a mesh. 1,000× magnification.

study; through omitting the dilution of the PRP solution and optimizing centrifugal force, this problem should be resolved. It should be noted, however, that the procedure of creating PRP in this study followed protocols available with the PRP kit; recreation of PRP with other kits or methods may also alleviate this problem.

Based on SEM examination, it can be seen that the PRFM obtained has a microscopic fibrin fiber matrix resembling a mesh (**Figure 4**). All platelets derived from PRP are evenly distributed among the fibrin fiber matrix. Thus, it can be concluded that this PRFM preparation method consistently produces microscopic fibrin fiber matrix with identical platelet distribution present in ideal PRFM (10). The resulting PRFM is denser and more flexible, resembling a fascial layer that can be sewn, identical to the PRFM preparation resulting from a commercial kit (5).

Other properties analyzed in the PRFM produced are TGFβ1, a growth factor responsible for controlling and promoting cell growth, proliferation, and differentiation (16). Contrary to popular belief, usually, the TGFβ1 level in PRP is higher than the PRFM. The difference in TGFβ1 level can occur due to the activation of platelets by exogenous factors during PRP and PRFM preparation. These exogenous factors can occur in the process of venous blood collection, during pipetting, or in the centrifugation process (17–19). Activated platelets will release granules along with their contents, including growth factors such as TGFβ1 and PDGF, which will then be dissolved in the plasma. During PRFM preparation, the centrifugation process at 1,800 × *g* for 60 min will precipitate platelets and the formed fibrin polymer. The dissolved protein will remain in the plasma, resulting in TGFβ1 and the activated platelets not precipitating in PRFM. It is known that both cytokines and growth factors secreted from cells have a short half-life, which means that a higher level of TGFβ1 in PRP does not mean that PRP is better than PRFM (20). Comparison of TGFβ1 between PRP and PRFM might not yield any meaningful, practical result, yet it would be interesting to look upon the correlation of the initial amount of TGFβ1 in PRFM and the amount of TGFβ1 released after the PRFM had been administered to a media or wound site.

The weakness of this study is a lack of actual observation and comparison toward actual clinical use of the PRFM produced through the modified method proposed. A follow-up study looking at how the PRFM produced through this method fares compared to PRFM produced by commercial kit would further raise the credibility of using the proposed method as an alternative way of preparing PRFM, especially in limited-resource settings. A continuation of the study with more extensive and more varied samples may also yield valuable knowledge and

FIGURE 5 | RegenKit tube with separator gel in the middle. Platelet might be trapped in the gel tube thus resulting in lower platelet count in the resulting PRP.

further understanding of the procedure. Further modification and adjustment in the PRP creation method also need to be done to improve platelet yield during PRP production; as previously stated, omitting dilution, changing the centrifugation speed, or using alternative PRP creation kits or methods might counter this problem. Nevertheless, the study had proven that this modified method could produce ideal PRFM with somewhat comparable quality to the ones produced using a commercial kit.

CONCLUSION

The proposed modified method by mixing PRP with 25 mM $CaCl_2$ and centrifuging at a speed of 2,264 × *g* for 25 min at room temperature can reliably produce ideal PRFM comparable in quality to the commercial kit. Further follow-up study is needed to compare the performance of PRFM produced by the modified method to those produced with commercial kits.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

MR: study concept, design, ethical submission, data collection, data analysis, interpretation, and writing the paper. AH: study concept, design, lab examination, data analysis, and interpretation. LS: study concept, design, lab examination, and data analysis. MY: data analysis and interpretation and writing the paper and submission. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

The authors would like to thank friends and fellow staff in the University of Indonesia for supporting the study.

REFERENCES

1. Sclafani AP. Safety, efficacy, and utility of platelet-rich fibrin matrix in facial plastic surgery. *Arch Facial Plast Surg.* (2011) 13:247–51. doi: 10.1001/archfacial.2011.3
2. Greer N, Foman NA, MacDonald R, Dorrian J, Fitzgerald P, Rutks I, et al. Advanced wound care therapies for nonhealing diabetic, venous, and arterial ulcers: a systematic review. *Ann Intern Med.* (2013) 159:532–42. doi: 10.7326/0003-4819-159-8-201310150-00006
3. Thomas CS, Rao SN, Soni R. The use of autologus platelet-rich plasma in treatment of chronic ulcers and wound. *MedPulse Int J Surg.* (2020) 13:76–9. doi: 10.26611/10613212
4. Choukroun J, Diss A, Simonpieri A, Girard MO, Schoeffler C, Dohan SL, et al. Platelet-rich fibrin (PRF): a second-generation platelet concentrate. Part V: histologic evaluations of PRF effects on bone allograft maturation in sinus lift. *Oral Surg Oral Med Oral Pathol Oral Radiol Endodontol.* (2006) 101:299–303. doi: 10.1016/j.tripleo.2005.07.012
5. Lucarelli E, Beretta R, Dozza B, Tazzari PL, O'Connell SM, Ricci F, et al. A recently developed bifacial platelet-rich fibrin matrix. *Eur Cells Mater.* (2010) 20:13–23. doi: 10.22203/eCM.v020a02
6. Kobayashi E, Flückiger L, Fujioka-Kobayashi M, Sawada K, Sculean A, Schaller B, et al. Comparative release of growth factors from PRP, PRF, and advanced-PRF. *Clin Oral Investig.* (2016) 20:2353–60. doi: 10.1007/s00784-016-1719-1
7. Gole PV, Muhammed N, Patadia SR. Efficacy of autologous platelet rich fibrin matrix in the management of non-healing ulcers. *Int J Res Dermatol.* (2019) 5:686. doi: 10.18203/issn.2455-4529.IntJResDermatol20194566
8. O'Connell SM, Impeduglia T, Hessler K, Wang XJ, Carroll RJ, Dardik H. Autologous platelet-rich fibrin matrix as cell therapy in the healing of chronic lower-extremity ulcers. *Wound Repair Regen.* (2008) 16:749–56. doi: 10.1111/j.1524-475X.2008.00426.x
9. Carroll RJ, Arnoczky SP, Ms SG, Ascp MT, Connell SMO. Characterization of autologous growth factors in Cascade ® Platelet-Rich Fibrin Matrix (PRFM). *Processing.* (2005) 4–5. https://www.pulsevet.com/Research/PRP/ResearchArticles/Carroll_Growth%20Factor_Release_WhitePaper.pdf
10. Roy S, Driggs J, Elgharably H, Biswas S, Findley M, Khanna S, et al. Platelet-rich fibrin matrix improves wound angiogenesis via inducing endothelial cell proliferation. *Wound Repair Regen.* (2011) 19:753–66. doi: 10.1111/j.1524-475X.2011.00740.x
11. O'Connell SM, Hessler K, Dardik H. Cascade ® autologous system platelet-rich fibrin matrix in the treatment of chronic leg ulcers. *Adv Wound Care.* (2012) 1:52–5. doi: 10.1089/wound.2011.0290
12. Cole BJ, Seroyer ST, Filardo G, Bajaj S, Fortier LA. Platelet-rich plasma: where are we now and where are we going? *Sports Health.* (2010) 2:203–10. doi: 10.1177/1941738110366385

13. Straum OK. The optimal platelet concentration in platelet-rich plasma for proliferation of human cells *in vitro*-diversity, biases, and possible basic experimental principles for further research in the field: a review. *PeerJ.* (2020) 8:e10303. doi: 10.7717/peerj.10303
14. Marx RE. Platelet-rich plasma: evidence to support its use. *J Oral Maxillofac Surg.* (2004) 62:489–96. doi: 10.1016/j.joms.2003.12.003
15. Delong JM, Russell RP, Mazzocca AD. Platelet-rich plasma: the PAW classification system. *Arthrosc J Arthrosc Relat Surg.* (2012) 28:998–1009. doi: 10.1016/j.arthro.2012.04.148
16. Massagué J, Chen YG. Controlling TGF-β signaling. *Genes Dev.* (2000) 14:627–44. doi: 10.1101/gad.14.6.627
17. Eppley BL, Woodell JE, Higgins J. Platelet quantification and growth factor analysis from platelet-rich plasma: implications for wound healing. *Plast Reconstr Surg.* (2004) 114:1502–8. doi: 10.1097/01.PRS.0000138251.07040.51
18. Bagamery K, Kvell K, Barnet M, Landau R, Graham J. Are platelets activated after a rapid, one-step density gradient centrifugation? Evidence from flow cytometric analysis. *Clin Lab Haematol.* (2005) 27:75–7. doi: 10.1111/j.1365-2257.2004.00662.x
19. Laffan MA, Manning RA. Investigation of haemostasis. In: *Dacie and Lewis Practical Haematology*, 12th ed. London: Churchill Livingstone (2017). p. 366–409.
20. Ren X, Zhao M, Lash B, Martino MM, Julier Z. Growth factor engineering strategies for regenerative medicine applications. *Front Bioeng Biotechnol.* (2020) 7:469. doi: 10.3389/fbioe.2019.00469

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.

frontiers in Medicine

BRIEF RESEARCH REPORT
published: 30 September 2021
doi: 10.3389/fmed.2021.718053

Are Reduced Levels of Coagulation Proteins Upon Admission Linked to COVID-19 Severity and Mortality?

Francisco C. Ceballos[1], Pablo Ryan[2], Rafael Blancas[3], María Martin-Vicente[1], Erick Joan Vidal-Alcántara[1], Felipe Peréz-García[4], Sofía Bartolomé[1], Juan Churruca-Sarasqueta[5], Ana Virseda-Berdices[1], Oscar Martínez-González[3], Oscar Brochado-Kith[1], Marta Rava[6], Carolina Vilches-Medkouri[7], Natalia Blanca-López[8], Ignacio Ramirez Martinez-Acitores[3], Patricia Moreira-Escriche[9], Carmen De Juan[9], Salvador Resino[1], Amanda Fernández-Rodríguez[1*†] and María Ángeles Jiménez-Sousa[1†]*

[1] Unit of Viral Infection and Immunity, National Center for Microbiology (CNM), Health Institute Carlos III (ISCIII), Madrid, Spain, [2] Department of Infectious Diseases, Hospital Universitario Infanta Leonor, Madrid, Spain, [3] Critical Care Department, Hospital Universitario del Tajo, Aranjuez, Spain, [4] Clinical Microbiology Department, Hospital Universitario Príncipe de Asturias, Alcalá de Henares, Spain, [5] Haematology and Haemostasis Department, University Hospital Infanta Leonor, Madrid, Spain, [6] Unit AIDS Research Network Cohort (CoRIS), National Center of Epidemiology (CNE), Health Institute Carlos III (ISCIII), Madrid, Spain, [7] Clinical Analysis Service, Hospital Universitario Príncipe de Asturias, Alcalá de Henares, Spain, [8] Allergology Department, University Hospital Infanta Leonor, Madrid, Spain, [9] Department of Infectious Diseases, Hospital Universitario Severo Ochoa, Madrid, Spain

Background: The link between coagulation system disorders and COVID-19 has not yet been fully elucidated.

Aim: Evaluating the association of non-previously reported coagulation proteins with COVID-19 severity and mortality.

Design: Cross-sectional study of 134 COVID-19 patients recruited at admission and classified according to the highest COVID-19 severity reached (asymptomatic/mild, moderate, or severe) and 16 healthy control individuals.

Methods: Coagulation proteins levels (antithrombin, prothrombin, factor_XI, factor_XII, and factor_XIII) and CRP were measured in plasma by the ProcartaPlex Panel (Invitrogen) multiplex immunoassay upon diagnosis.

Results: We found higher levels of antithrombin, prothrombin, factor XI, factor XII, and factor XIII in asymptomatic/mild and moderate COVID-19 patients compared to healthy individuals. Interestingly, decreased levels of antithrombin and factors XI, XII, and XIII were observed in those patients who eventually developed severe illness. Additionally, survival models showed us that patients with lower levels of these coagulation proteins had an increased risk of death.

Conclusion: COVID-19 provokes early increments of some specific coagulation proteins in most patients. However, lower levels of these proteins at diagnosis might "paradoxically" imply a higher risk of progression to severe disease and COVID-19-related mortality.

Keywords: SARS-CoV-2, coagulation factors, COVID-19, mortality, SARS

OPEN ACCESS

Edited by:
Alec Schmaier, Beth Israel Deaconess Medical Center and Harvard Medical School, United States

Reviewed by:
Pavan Bendapudi, Beth Israel Deaconess Medical Center and Harvard Medical School, United States
Bipin P. Kulkarni, Indian Council of Medical Research, India

***Correspondence:**
Amanda Fernández-Rodríguez
amandafr@isciii.es
Salvador Resino
sresino@isciii.es

[†] 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: *31 May 2021*
Accepted: *03 September 2021*
Published: *30 September 2021*

Citation:
Ceballos FC, Ryan P, Blancas R, Martin-Vicente M, Vidal-Alcántara EJ, Peréz-García F, Bartolomé S, Churruca-Sarasqueta J, Virseda-Berdices A, Martínez-González O, Brochado-Kith O, Rava M, Vilches-Medkouri C, Blanca-López N, Ramirez Martinez-Acitores I, Moreira-Escriche P, De Juan C, Resino S, Fernández-Rodríguez A and Jiménez-Sousa MÁ (2021) Are Reduced Levels of Coagulation Proteins Upon Admission Linked to COVID-19 Severity and Mortality? Front. Med. 8:718053. doi: 10.3389/fmed.2021.718053

HOW THIS FITS IN

COVID-19 causes an early increase in coagulation proteins in most patients, even in those asymptomatic or with mild symptoms. Although not reflected in routine tests such as PT and aPTT, patients who lately advanced to severe disease, showed low levels of antithrombin, prothrombin, and factors XI, XII, and XIII at disease diagnosis. These reduced levels were associated for the first time with a higher COVID-19-related mortality.

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is associated with a significant activation of the coagulation cascade. While thrombosis has been classically described in acute and chronic infections including respiratory diseases (1), thrombotic risk appears to be higher in COVID-19 (2). Consequently, thromboembolic complications are common in hospitalized patients, especially among those in intensive care units (ICUs) (3). In this sense, several mechanisms of coagulation activation have been postulated (4) and large dynamic fluctuations in coagulation and fibrinolysis laboratory parameters have been described during disease course (5). Development of overt disseminated intravascular coagulation (DIC) seems to be rare and to follow a different pattern from other infection-derived DIC (5–7), but it has been reported in up to 71% of fatal cases as a late and ominous sign (8).

Additionally, both venous and arterial thrombotic events have been independently associated with mortality (9). Several hemostatic-system abnormalities such as thrombocytopenia, elevated D-dimer levels, prolonged prothrombin time (PT) or activated partial thromboplastin time (aPTT), decreased factor V activity, hypofibrinogenemia, and reduced levels of natural anticoagulants (e.g., antithrombin) appear with increasing disease severity and have been linked to death (6, 8, 10). However, the bidirectional relationship between SARS-CoV-2 and the coagulation system is still not completely understood (4). A predominant increase of D-dimer is typical, and its presence on admission has been repeatedly described as significantly higher in non-survivors (11) but scarce or no abnormalities in PT and aPTT are usually found at disease onset (5, 12). To date, coagulation markers measured in the early phase of COVID-19 have evidenced a complex scenario and elucidation of the pathophysiology of immunothrombosis is evolving. Therefore, to continue unraveling the insights of COVID-19-induced coagulopathy, we evaluate several coagulation proteins at an early stage of disease and their association with disease severity and mortality.

METHODS

Design and Study Population

In this cross-sectional study, 128 COVID-19 non-selected patients consecutively admitted to three different hospitals in Madrid (Infanta Leonor University Hospital, Aranjuez University Hospital, and Príncipe de Asturias University Hospital), with an available plasma sample, were enrolled from March to September 2020.

Patients were classified according to their highest severity grade during the course of COVID-19 (**Figure 1**): (1) Severe: (i) death during hospitalization, (ii) ICU admission, (iii) invasive mechanical ventilation, or (iv) presence of bilateral pulmonary infiltrates, mechanical ventilation, and oxygen saturation ($Sat0_2$) $\leq$93. (2) Moderate: the remaining hospitalized patients who did not fulfill the severe COVID-19 criteria; (3) asymptomatic/mild (AM): individuals who had minor or no COVID-19 symptoms; (4) a control group of 16 pre-pandemic healthy controls without any known infection was included, they were age- and sex-matched with COVID-19 groups to limit confounding factors. The STROBE-ID checklist was used to strength the design and conduct the study.

Clinical Data and Samples

Epidemiological, clinical, and disease evolution data, as well as laboratory parameters such as PT, international normalized ratio (INR), and aPTT were collected from clinical records using an electronic case report form (eCRF) which was built using REDCap (13). Plasma samples were obtained after centrifugation of blood in EDTA tubes at hospital admission (median = 2 days, IQR = 4 days). Samples were processed at the National Center for Microbiology (Majadahonda), Institute of Health Carlos III (Madrid, Spain).

Coagulation Proteins

Coagulation proteins (antithrombin, prothrombin, factor XI, factor XII, and factor XIII) and C-reactive protein (CRP) were measured by a Human Coagulation 6-PLEX ProcartaPlex Panel (Invitrogen), and D-dimer and IL-6 were measured by a multiplex immunoassay using a Bio-Plex 200^{TM} system (Bio-Rad) according to the manufacturer's specifications. Coagulation proteins and their relationship in the coagulation cascade are shown in **Figure 1**.

Statistical Analysis

As outcome variables, the highest COVID-19 severity and mortality were considered. For descriptive data, differences between groups were tested using Chi^2 or Fisher's exact test with a Monte Carlo-simulated *p*-value for categorical variables and Kruskal-Wallis test for continuous variables. The association between coagulation proteins, measured in the first days of disease, and the severity were explored using generalized linear mixed models (GLMMs). This analysis allows us to test the pairwise difference among disease severity classes by grouping the four coagulation proteins analyzed in this study. We fit a model where the protein was considered a random effect:

$$Y_{Severity\ class\ A,\ severity\ class\ B} = \left(\beta_0 + b_{p,0p}\right) + \beta_1 X_i \\ \beta_2 X_j + \beta_3 X_k + e_{pijk} \quad (1)$$

Where bp, 0p is the random effect of each coagulation protein, β1Xi is the fixed effect of the protein levels, β2Xj is the fixed effect of the age, and β3Xk is the fixed effect of the sex. By using this model, we tested whether we were able to

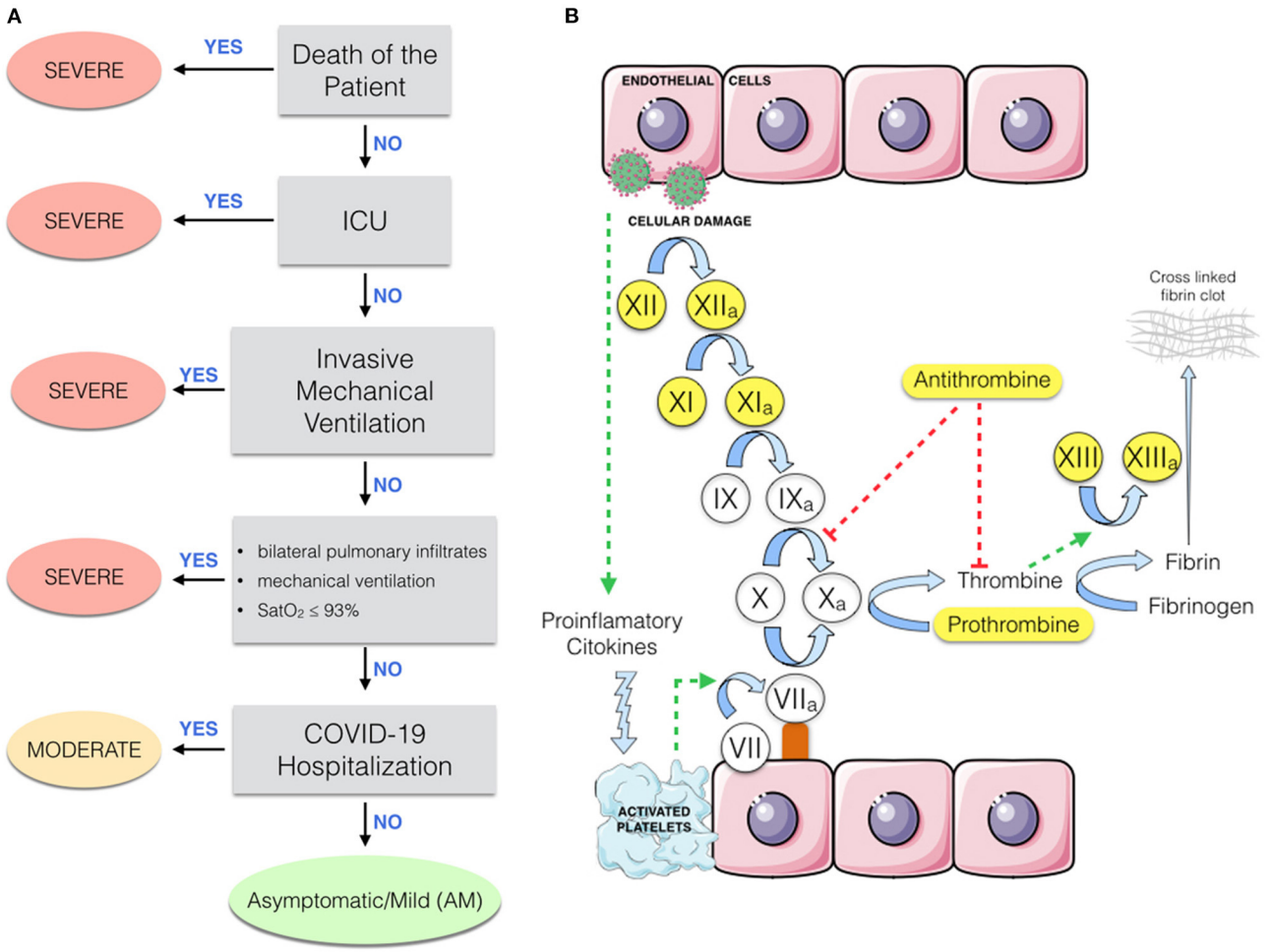


FIGURE 1 | (A) Flowchart describing the classification of patients with respect to COVID-19 severity. ICU, intensive care unit; ARDS, acute respiratory distress syndrome; AM, asymptomatic/mild patients. **(B)** Partial representation of the coagulation cascade. Proteins analyzed by this study are highlighted in yellow.

detect general effects between disease severity classes. We were also interested in studying the behavior of each coagulation protein. Pairwise comparisons between disease severity classes, for each protein, were carried out using multivariable logistic regressions. Sex and age were included as covariables in the multivariable analysis.

Survival time was defined as time between hospitalization and death, and individuals alive at 90 days were considered censored data. Survival curves were modeled using the Kaplan Meier method and a log rank test was performed to assess univariate differences in survival time according to coagulant proteins tertile levels. Death risk was estimated with the Cox proportional-hazard and Aalen's additive models. Age and sex were included as covariables. The survival Cox proportional-hazard model's goodness of fit was evaluated by the Harrel's concordance index (C-index). This index ranges from 0 to 1 and the intuition behind it is that, if the risk model is good, patients who had shorter times-to-death should have higher risk scores. Values of C-index near 0.5 indicate that the risk score predictions are not better than chance in determining which individual will die first. Coagulation proteins discrimination capabilities were measured by the area under the receiver operating characteristic (ROC) curve.

Two-sided tests were used for all statistical methods. Analyses were performed using the R 4.0.3 software.

RESULTS

Clinical and Epidemiological Data of Patients

Patient's characteristics are shown in **Table 1**. Coagulation proteins were measured before therapy administration, and no associations were found with comorbidities, chronic medications, or COVID-19 presentation (**Supplementary Table 1**; **Supplementary Figure 1**), showing no prior bias. Additionally, aPTT and PT (INR) did not show differences among the three groups of COVID-19 patients (**Supplementary Figure 2**).

TABLE 1 | Patient's characteristics.

	COVID-19 severity			
	Healthy	**Asymptomatic/mild**	**Moderate**	**Severe**
Demographics				
N	16	13	68	47
Age	58.8 ± 12.3	64.3 ± 18.1	61.1 ± 15.3	62.1 ± 18.1
Gender (male)	9/16 (56.2%)	5/13 (38.4%)	38/68 (55.8%)	33/47 (70.2%)
BMI ≥ 25	8/11 (72.7%)	2/12 (16.6%)	11/68 (16.1%)	13/47 (27.6%)
Smoke status (Yes)	NA	2/13 (15.3%)	3/68 (4.4%)	3/47 (6.3%)
Former smoker	NA	4/13 (30.7%)	8/68 (11.7%)	11/47 (23.4%)
Comorbidities				
Hypertension	NA	5/13 (38.4%)	30/68 (44.1%)	23/47 (48.9%)
Cardiopathy	NA	4/13 (30.7%)	12/68 (17.6)	8/47 (17.0%)
Chronic pulmonary disease	NA	0/13 (0%)	8/68 (11.7%)	10/47 (21.2%)
Chronic kidney disease	NA	2/13 (15.3%)	4/68 (5.8%)	9/47 (19.1%)
Chronic liver disease	NA	NA	4/65 (6.2%)	2/47 (4.2%)
Chronic neurological disease	NA	0/13 (0%)	9/67 (13.4%)	8/47 (17.0%)
Neoplasia	NA	NA	3/63 (4.8%)	4/47 (8.5%)
Diabetes	NA	2/13 (15.3%)	10/68 (14.7%)	11/47 (23.4%)
Chronic inflammatory disease	NA	NA	2/63 (3.1%)	5/47 (10.6%)
Autoimmune disease	NA	NA	1/63 (1.5%)	4/47 (8.5%)
Therapy				
Chronic medications				
NSAIDs	NA	0/13 (0%)	0/68 (0%)	7/47 (14.9%)
ACE inhibitors	NA	0/13 (0%)	15/68 (22.3%)	11/47 (23.4%)
ARBs	NA	1/13 (7.6%)	7/68 (10.4%)	8/47 (17.0%)
Corticosteroids	NA	NA	6/68 (9.5%)	6/47 (12.8%)
HIV antiretrovirals	NA	NA	1/68 (1.5%)	1/47 (2.1%)
Treatment				
Chloroquine and Hydroxychloroquine	0/16 (0%)	0/13 (0%)	62/63 (98.4%)	33/47 (70.2%)
Tocilizumab	0/16 (0%)	0/13 (0%)	10/67 (15.0%)	20/47 (42.5%)
Corticosteroids	0/16 (0%)	0/13 (0%)	26/67 (38.8%)	40/47 (85.1%)
COVID-19 related symptoms				
Dyspnea	0/16 (0%)	0/13 (0%)	43/68 (63.2%)	41/47 (87.2%)
Cough	0/16 (0%)	0/13 (0%)	48/63 (76.1%)	34/47 (72.3%)
Headache	0/16 (0%)	0/13 (0%)	24/63 (38.1%)	10/47 (21.2%)
Diarrhea or abdominal pain	0/16 (0%)	0/13 (0%)	33/63 (52.4%)	19/47 (40.4%)
Hospitalization				
Length of stay (days)	0/16 (0%)	0/13 (0%)	10.7 ± 8.6	22.1 ± 15.6
Maximum temperature	NA	NA	37.8 ± 0.8	38.2 ± 0.8
Oxygen therapy	0/16 (0%)	0/13 (0%)	39/68 (57.3%)	47/47 (100%)
Non-invasive mechanical ventilation	0/16 (0%)	0/13 (0%)	3/67 (4.5%)	20/47 (38.99%)
Invasive mechanical ventilation	0/16 (0%)	0/13 (0%)	0/67 (0%)	18/47 (38.2%)
Pulmonary infiltrates	0/16 (0%)	0/13 (0%)	60/68 (88.2%)	47/47 (100%)
ICU				
ICU length of stay	0	0	0	6.61 ± 13.5
Death	0/16 (0%)	0/13 (0%)	0/68 (0%)	18/47 (38.3%)

Patients were classified according to the highest disease severity reached during the COVID-19 evolution. Denominator indicates number of patients with available data. ***Statistics****: Individual characteristics were summarized using standard descriptive statistics: mean ± standard deviation for continue variables and count (percentage) for categorical variables. Differences between groups were tested using Fisher's exact test. No statistically significant association was found between covariates and disease severity with the exception of the following treatments: Chloroquine/hydroxychloroquine (Fisher's exact test = $1.6e^{-05}$), Tocilizumab (Fisher's exact test = $8.1e^{-05}$), corticosteroids (Fisher's exact test = $5.7e^{-08}$), and the use of supplemental oxygen (Fisher's exact test = $3.2.e^{-07}$). NA, non-available; BMI, body mass index; NSAIDs, non-steroidal anti-inflammatory drugs; ACE, angiotensin-converting-enzyme; ARBs, angiotensin II inhibitors; HIV, human immunodeficiency virus; ICU, intensive care unit.*

Coagulation Proteins Association With Severity

Distribution of antithrombin, prothrombin, factor XI, factor XII, and factor XIII protein levels for each disease severity group is shown in **Figure 2A**. Statistical differences between severity groups are shown through generalized linear models in **Figure 3**. AM, moderate, and severe individuals had higher levels of coagulation proteins compared to healthy individuals. Also, we detected a significant reduction of factor XI, factor XII, and factor XIII levels in severe patients compared to moderate individuals (**Supplementary Table 2**). These pairwise differences between COVID-19 severity groups were also detected at the individual protein level (**Figure 3**; **Supplementary Table 3**).

Additionally, a traditional coagulation activation marker such as D-dimer and surrogate markers of inflammation such as CRP and IL-6 were also assessed. Statistical differences were observed mainly between moderate and severe groups, except for CRP, for which extreme groups showed the highest differences (**Supplementary Table 4**; **Supplementary Figure 3**). D-dimer showed significant correlation with prothrombin ($R^2 = 0.698$, $P = 2.1\text{E}^{-22}$) and factor XIII ($R^2 = -0.216$, $P = 3.7\text{E}^{-03}$).

Survival Analysis

Kaplan-Meier analyses showed that patients with lower levels of the analyzed proteins had an increased risk of death during hospitalization (**Figure 2B**). For antithrombin, prothrombin, and factors XI and XIII, this increase was more accentuated in men (**Supplementary Figure 4**). The effect of proteins levels on survival was confirmed by the Cox proportional-hazard models and Aalen's additive regression (**Supplementary Table 5**). Both models found a significant effect of the different coagulation proteins on survival. These models found a negative correlation between the proteins' levels and the time of survival (**Supplementary Table 5**). A reduction in the coagulation proteins levels involves a higher risk of death, however, the effect size was slight for each individual protein (**Supplementary Table 5**). ROC curves showed that the addition of the coagulation proteins to the basic model composed by sex and age slightly improved the survival prediction although it was not significant (**Supplementary Figure 5**). We also performed survival analysis with aPTT and INR using different statistical models, but no significant association was observed (data not shown).

DISCUSSION

Our study shows that coagulation protein levels are affected at the first stages of COVID-19 and that these early changes already reflect disease severity. We report increased levels of antithrombin, prothrombin, factor XI, factor XII, and factor XIII in AM and moderate patients, compared to healthy individuals. In contrast, significantly decreased levels of antithrombin and factors XI, XII, and XIII were found at presentation in patients who followed a severe course with respect to moderate patients. By considering the coagulation protein as a random effect (in a logistic mixed model context), we could suggest a general activation of the coagulation cascade between healthy individuals and COVID-19 patients, and also a general decrease in protein levels in severe COVID-19 patients. As expected, D-dimer and IL-6 were significantly higher in severe patients than moderate patients. Moreover, CRP showed higher levels in moderate and severe patients than healthy and AM individuals. Additionally, aPTT and PT (INR) did not show differences among the three groups of COVID-19 severity, neither with survival. When we explored the effects of the coagulant proteins' levels on survival defined as time-to-death, we observed that patients with lower levels of the analyzed proteins had an increased risk of death during hospitalization.

Finally, we describe a sex-specific effect in further analyses, showing differences in the coagulation cascade regulation in both men and women. Variable sex was significant in the general mixed model and survival analysis. The negative association between coagulation proteins and disease severity was, in general, more pronounced in men. Our results seem to be in agreement with the clinical experience that found that men hospitalized with COVID-19 had more severe thrombosis than women (14).

Strength and Limitations

We present the evaluation of coagulation factors underrepresented in the literature as an additional approach to study coagulopathy in COVID-19 disease. In the results we report new associations of some protein factors with COVID-19 severity and include specific groups of patients with scarce coagulation data, such as asymptomatic patients. We also add healthy non-hospitalized individuals to address for collider bias (15). Furthermore, we were able to detect a sex-specific effect of SARS-CoV-2 on coagulation protein levels. Two main limitations should be considered. First, the limited sample size of healthy and asymptomatic cases, which could have limited the possibility of finding statistically significant differences for some comparisons. Secondly, we analyzed five coagulation proteins not routinely used in clinical practice; as coagulation cascade is extremely complex, further studies should consider additional factors to fully describe COVID-19 effects over the entire coagulation cascade. However, these additional factors have been extensively studied, and we analyzed those that were not previously addressed.

Comparison With Existing Literature

Since its worldwide outbreak in the first trimester of 2020, it has been systematically reported that COVID-19 is associated with a significant activation of the coagulation cascade. Our results are in accordance with previous studies (16) that show a consumption of coagulation proteins among COVID-19 non-survivors, or a reduction in abundance of prothrombin correlated with disease severity (17). Several recently published meta-analyses and reviews have shown significantly higher levels of D-dimer, fibrinogen, and fibrin in severe COVID-19 patients in comparison to non-severe (18–23).

However, as far as we know this is the first report studying coagulation factors XI, XII, and XIII in COVID-19, as most of the published studies have focused in other parameters such as

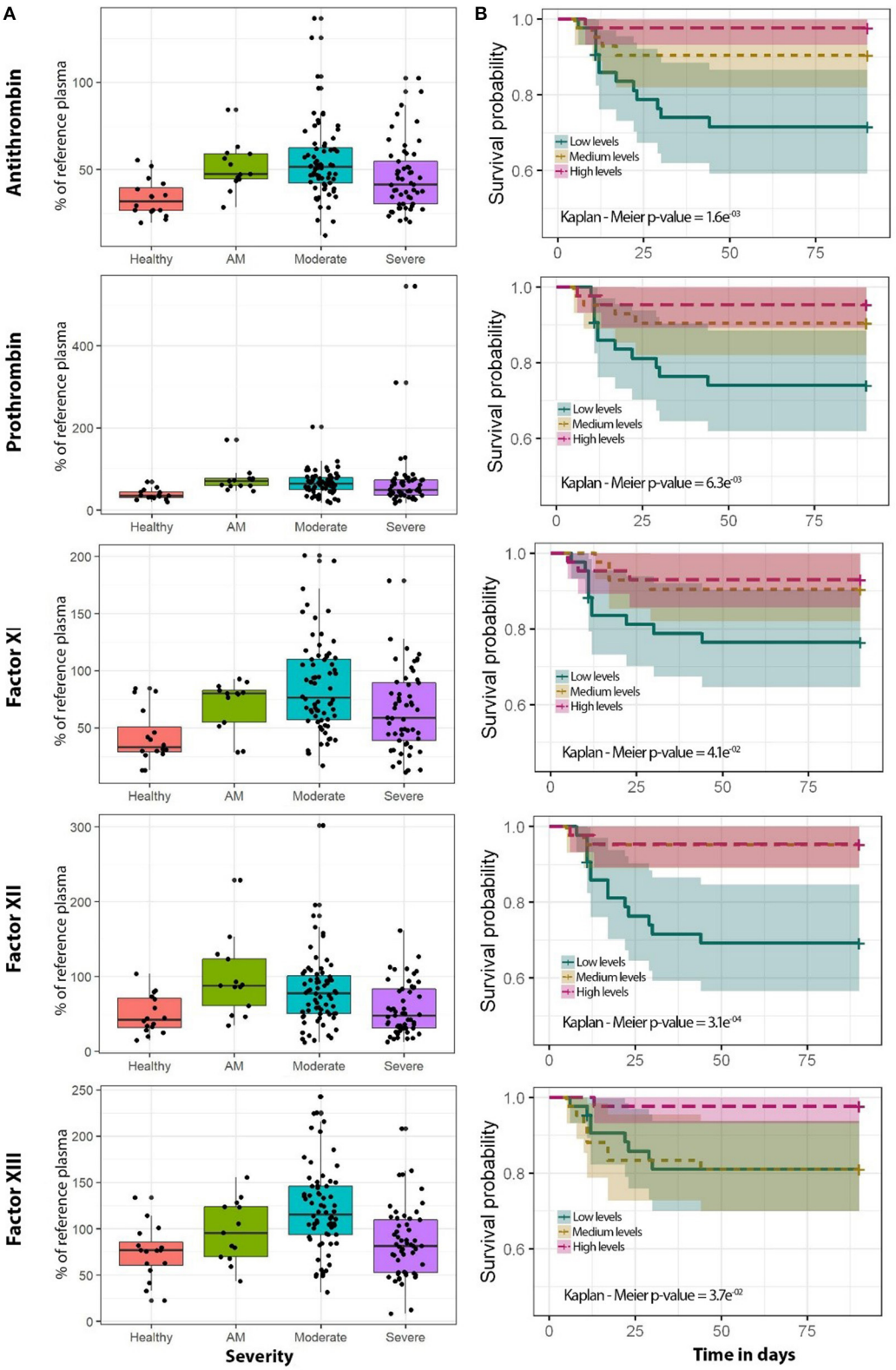


FIGURE 2 | Coagulation protein levels regarding COVID-19 severity and survival analysis for each of the coagulation proteins analyzed in this study. **(A)** Boxplots of healthy (purple, $n = 16$), AM (blue, $n = 13$), moderate (green, $n = 68$), and severe (yellow, $n = 47$) individuals.

(Continued)

FIGURE 2 | (B) Kaplan-Meier plot. The cutoffs of coagulation proteins for the Kaplan-Meier plot were obtained using three quantiles to get low (blue), medium (yellow), and high (pink) percentages of the reference plasma. The specific categorical levels by using tertiles were as follows: Antithrombin: low (12.14–41.3), medium (41.3–54.1), high (54.1–136.7). Prothrombin: low (16.3–48.7), medium (48.7–72.2), high (72.2–545). Factor XI: low (10.8–56.4), medium (56.4–90.1), high (90.1–200.9). Factor XII: low (11.7–47.8), medium (47.8–87.1), high (87.1–301.9). Factor XIII: low (8.3–82.6), medium (82.6–118.3), high (118.3–242.7). AM, asymptomatic/mild patients; OLR, ordinal logistic regression.

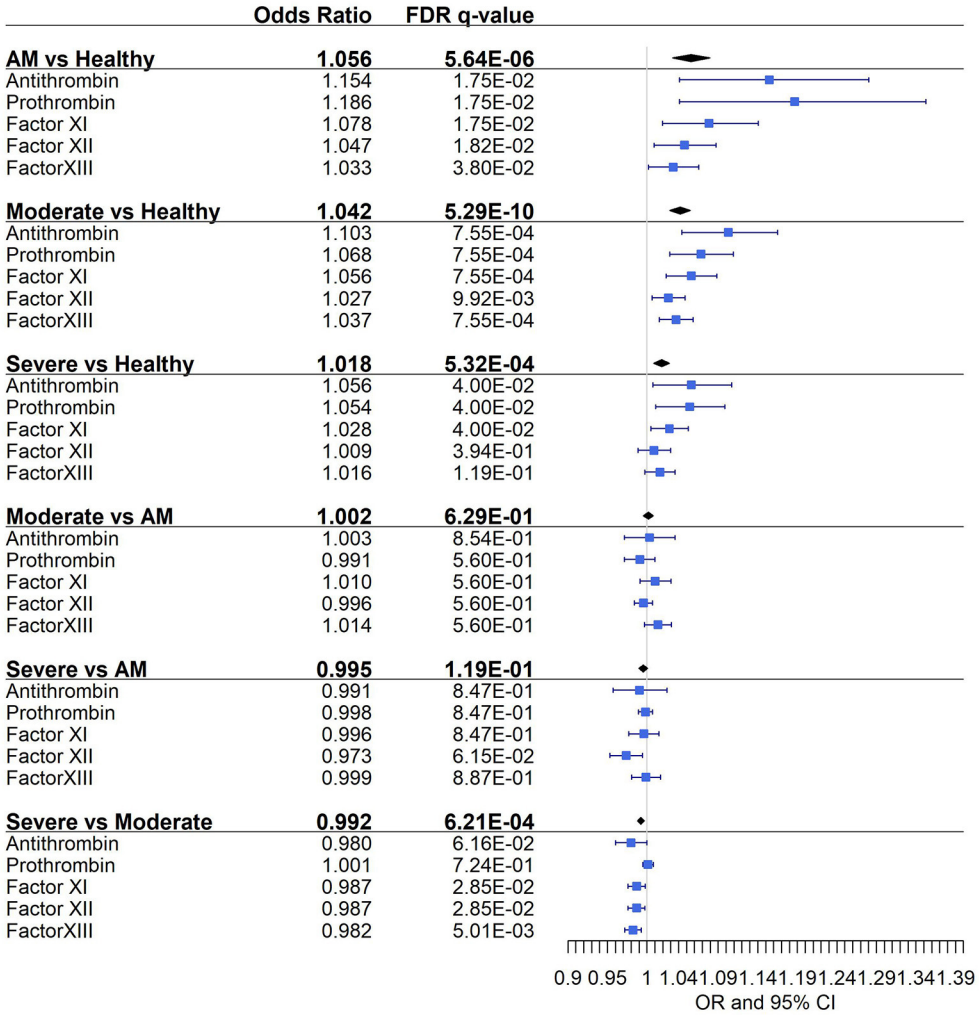


FIGURE 3 | Pairwise association between coagulation protein levels and the different severity classes. Statistics: Pairwise comparisons between COVID-19 severity classes were obtained using a logistic mixed model where the protein was considered a random effect. Pairwise comparisons within each protein were obtained using multiple logistic regression analysis. The False Discovery Rate or FDR was used to cope with multiple testing, q-values are provided. AM, asymptomatic/mild patients.

platelets, D-dimer, fibrin, fibrinogen, aPTT, PT, and even factor VIII and thrombin (18, 22, 23).

The increase in natural anticoagulant and procoagulant proteins in COVID-19 has been attributed to the thromboinflammatory response caused by SARS-CoV-2, which provokes endotheliitis and increases the hepatic production of factors (24). This could explain the increases of clotting proteins found in our study even in early and mild stages.

Additionally, we showed that this negative association was more pronounced in men.

We observed that at the time of admission, there were no differences in aPTT and TP between survivors or non-survivors. These results are also in line with previous studies (2), where normal to slightly elevated aPTT or PT have been described in most COVID-19 patients at presentation. The INR, measured at the acute phase of the disease, seems to be elevated in non-survivors compared to survivors (8), however, no correlation was evident between COVID-19 severity and other DIC indicators like prolonged aPTT (16). Therefore, we suggest that an early reduced production or, more likely, an increased consumption

(due to pulmonary or systemic coagulopathy) of clotting proteins could predict a worse prognosis.

The survival results were in accordance with previous studies (15) that show a consumption of coagulation proteins among COVID-19 non-survivors, however, we cannot rule out an impaired production of these coagulation proteins.

Implications for Research and/or Practice

In conclusion, our results indicate that: (1) COVID-19 causes an early increase of some specific coagulation proteins such as antithrombin, prothrombin, contact factors, and factor XIII in most patients, even in those who will not suffer from clinically significant disease, suggesting that commonly elevated D-dimer levels are driven by an initial enhanced procoagulant state and not just by hyperfibrinolysis; (2) although not reflected in routine tests such as PT and aPTT, and despite common initial hyperfibrinogenemia, patients who will eventually advance to severe disease show early decreased levels of these anticoagulant and procoagulant markers, suggesting either consumption or impaired production, and these levels were associated with higher COVID-19-related mortality. Evolving investigations will allow us to better clarify the crosstalk between the immune and clotting systems in this pandemic disease.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Institute of Health Carlos III (PI 33_2020-v3) and the Ethics Committee of each hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AF-R and MJ-S: funding body, supervision, and visualization. AF-R, MJ-S, and FC: study concept and design, statistical analysis, interpretation of data, and writing of the manuscript. PR, RB, MM-V, AV-B, OB-K, FC, FP-G, OM-G, CV-M, NB-L, and IR: patients' selection and clinical data acquisition. MM-V, AV-B, EV-A, SB, and OB-K: sample preparation and biomarker analysis. FC, AF-R, and MJ-S: writing of the manuscript. PR, RB, FP-G, JC-S, OM-G, MR, PM-E, CD, and SR: critical revision of the manuscript for relevant intellectual content. All authors read and approved the final manuscript.

FUNDING

This study was supported by grants from Instituto de Salud Carlos III [ISCIII; Grant Number COV20/1144 (MPY224/20) to AF-R/MJ-S]. AF-R, MJ-S, and MR are Miguel Servet researchers supported and funded by ISCIII (Grant Numbers: CP14CIII/00010 to AF-R, CP17CIII/00007 to MJ-S, and CP19CIII/00002 to MR).

ACKNOWLEDGMENTS

This study would not have been possible without the collaboration of all the patients, their families, medical and nursery staff, and data managers who have taken part in the project.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.718053/full#supplementary-material

REFERENCES

1. Grimnes G, Isaksen T, Tichelaar YIGV, Brækkan SK, Hansen JB. Acute infection as a trigger for incident venous thromboembolism: results from a population-based case-crossover study. *Res Pract Thromb Haemost.* (2018) 2:85–92. doi: 10.1002/rth2.12065
2. Hadid T, Kafri Z, Al-Katib A. Coagulation and anticoagulation in COVID-19. *Blood Rev.* (2020) 47:100761. doi: 10.1016/j.blre.2020.100761
3. Poissy J, Goutay J, Caplan M, Parmentier E, Duburcq T, Lassalle F, et al. Pulmonary embolism in patients with COVID-19: awareness of an increased prevalence. *Circulation.* (2020) 142:184–6. doi: 10.1161/CIRCULATIONAHA.120.047430
4. Iba T, Connors JM, Levy JH. The coagulopathy, endotheliopathy, and vasculitis of COVID-19. *Inflamm Res.* (2020) 69:1181–9. doi: 10.1007/s00011-020-01401-6
5. Asakura H, Ogawa H. COVID-19-associated coagulopathy and disseminated intravascular coagulation. *Int J Hematol.* (2020) 7:1–13. doi: 10.1007/s12185-020-03029-y
6. Helms J, Tacquard C, Severac F, Leonard-Lorant I, Ohana M, Delabranche X, et al. High risk of thrombosis in patients with severe SARS-CoV-2 infection: a multicenter prospective cohort study. *Intensive Care Med.* (2020) 46:1089–98. doi: 10.1007/s00134-020-06062-x
7. Muñoz-Rivas N, Abad-Motos A, Mestre-Gómez B, Sierra-Hidalgo F, Cortina-Camarero C, Lorente-Ramos RM, et al. Systemic thrombosis in a large cohort of COVID-19 patients despite thromboprophylaxis: a retrospective study. *Thromb Res.* (2021) 199:132–42. doi: 10.1016/j.thromres.2020.12.024
8. Tang N, Li D, Wang X, Sun Z. Abnormal coagulation parameters are associated with poor prognosis in patients with novel coronavirus pneumonia. *J Thromb Haemost.* (2020) 18:844–7. doi: 10.1111/jth.14768
9. Bilaloglu S, Aphinyanaphongs Y, Jones S, Iturrate E, Hochman J, Berger JS. Thrombosis in hospitalized patients with COVID-19 in a New York City Health System. *JAMA.* (2020) 324:799–801. doi: 10.1001/jama.2020.13372
10. Stefely JA, Christensen BB, Gogakos T, Cone Sullivan JK, Montgomery GG, Barranco JP, et al. Marked factor V activity elevation in severe COVID-19 is associated with venous thromboembolism. *Am J Hematol.* (2020) 95:1522–30. doi: 10.1002/ajh.25979
11. Zhang L, Yan X, Fan Q, Liu H, Liu X, Liu Z, et al. D-dimer levels on admission to predict in-hospital mortality in patients with Covid-19. *J Thromb Haemost.* (2020) 18:1324–9. doi: 10.1111/jth.14859

12. Levi M, Iba T. COVID-19 coagulopathy: is it disseminated intravascular coagulation? *Intern Emerg Med.* (2020) 16:309–12. doi: 10.1007/s11739-020-02601-y
13. Harris PA, Taylor R, Minor BL, Elliott V, Fernandez M, O'Neal L, et al. The REDCap consortium: building an international community of software platform partners. *J Biomed Inform.* (2019) 95:103208. doi: 10.1016/j.jbi.2019.103208
14. Wilcox T, Smilowitz N, Berger J. Age and sex differences in incident thrombosis in patients hospitalized with COVID 19. *J Am Coll Cardiol.* (2021) 77:1826. doi: 10.1016/S0735-1097(21)03182-X
15. Griffith GJ, Morris TT, Tudball MJ, Herbert A, Mancano G, Pike L, et al. Collider bias undermines our understanding of COVID-19 disease risk and severity. *Nat Commun.* (2020) 11:5749. doi: 10.1038/s41467-020-19478-2
16. Martín-Rojas RM, Pérez-Rus G, Delgado-Pinos VE, Domingo-González A, Regalado-Artamendi I, Alba-Urdiales N, et al. COVID-19 coagulopathy: an in-depth analysis of the coagulation system. *Eur J Haematol.* (2020) 105:741–50. doi: 10.1111/ejh.13501
17. Overmyer KA, Shishkova E, Miller IJ, Balnis J, Bernstein MN, Peters-Clarke TM, et al. Large-scale multi-omic analysis of COVID-19 severity. *Cell Syst.* (2021) 12:23–40.e7. doi: 10.1016/j.cels.2020.10.003
18. Lin J, Yan H, Chen H, He C, Lin C, He H, et al. COVID-19 and coagulation dysfunction in adults: a systematic review and meta-analysis. *J Med Virol.* (2021) 93:934–44. doi: 10.1002/jmv.26346
19. Xiang G, Hao S, Fu C, Hu W, Xie L, Wu Q, et al. The effect of coagulation factors in 2019 novel coronavirus patients: a systematic review and meta-analysis. *Medicine.* (2021) 100:e24537. doi: 10.1097/MD.0000000000024537
20. Rahi MS, Jindal V, Reyes SP, Gunasekaran K, Gupta R, Jaiyesimi I. Hematologic disorders associated with COVID-19: a review. *Ann Hematol.* (2021) 100:309–20. doi: 10.1007/s00277-020-04366-y
21. Gu SX, Tyagi T, Jain K, Gu VW, Lee SH, Hwa JM, et al. Thrombocytopathy and endotheliopathy: crucial contributors to COVID-19 thromboinflammation. *Nat Rev Cardiol.* (2021) 18:194–209. doi: 10.1038/s41569-020-00469-1
22. Ortega-Paz L, Capodanno D, Montalescot G, Angiolillo DJ. Coronavirus disease 2019-associated thrombosis and coagulopathy: review of the pathophysiological characteristics and implications for antithrombotic management. *J Am Heart Assoc.* (2021) 10:e019650. doi: 10.1161/JAHA.120.019650
23. Lazzaroni MG, Piantoni S, Masneri S, Garrafa E, Martini G, Tincani A, et al. Coagulation dysfunction in COVID-19: the interplay between inflammation, viral infection and the coagulation system. *Blood Rev.* (2021) 46:100745. doi: 10.1016/j.blre.2020.100745
24. Wise J. Covid-19 and thrombosis: what do we know about the risks and treatment? *BMJ.* (2020) 369:m2058. doi: 10.1136/bmj.m2058

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.

frontiers in Medicine

CASE REPORT
published: 15 October 2021
doi: 10.3389/fmed.2021.741594

Case Report: Cerebral Venous Sinus Thrombosis and COVID-19 Infection

Manasa Anipindi, Amanda Scott, Li Joyce, Salman Wali and Mark Morginstin**

Einstein Healthcare Network, Philadelphia, PA, United States

Coronavirus disease-2019 is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2 virus). Coronavirus disease-2019 (COVID-19) was declared a pandemic in March 2020 and has changed our lives in many ways. This infection induces a hypercoagulable state leading to arterial and venous thrombosis, but the exact pathophysiology of thrombosis is unknown. However, various theories have been postulated including excessive cytokine release, endothelial activation, and disseminated intravascular coagulation (DIC). We present a patient diagnosed with cerebral venous sinus thrombosis (CVST) with COVID-19 infection. A 66-year-old man presented to a hospital for evaluation of persistent headaches. He tested positive for COVID-19, and MRI of the brain and CT venogram revealed CVST. He was started on heparin drip in the hospital and transitioned to oral anticoagulants at the time of discharge. His headaches improved with treatment. Even though headache is the most frequent and initial symptom of cerebral venous thrombosis, it is rarely the only symptom. A high index of suspicion is therefore required to diagnose CVST especially if the patient presents with a simple complaint like a headache. Common complaints can delay the diagnosis leading to disease progression. Considering the high mortality rates in patients diagnosed with CVST, we suggest the importance of knowing the association between COVID-19 infection and CVST, especially in susceptible patients.

Keywords: case report, cerebral venous sinus thrombosis (CVST), COVID-19, headache, anticoagulant

OPEN ACCESS

Edited by:
Eleni Gavriilaki, G. Papanikolaou General Hospital, Greece

Reviewed by:
Bipin P. Kulkarni, Indian Council of Medical Research, India
Maria Gavriilaki, University General Hospital of Thessaloniki AHEPA, Greece

****Correspondence:***
Manasa Anipindi
AnipindM@einstein.edu
Mark Morginstin
morginsm@einstein.edu

Specialty section:
This article was submitted to Hematology, a section of the journal Frontiers in Medicine

Received: *15 July 2021*
Accepted: *15 September 2021*
Published: *15 October 2021*

Citation:
Anipindi M, Scott A, Joyce L, Wali S and Morginstin M (2021) Case Report: Cerebral Venous Sinus Thrombosis and COVID-19 Infection. Front. Med. 8:741594. doi: 10.3389/fmed.2021.741594

INTRODUCTION

The coronavirus disease-2019 (COVID-19) pandemic has affected nearly every individual across the world. Thus far, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) virus infection has infected approximately 180 million individuals and has resulted in 4 million deaths. The transmission of the virus is through direct contact and by airborne droplets (1). The virus attaches to a site on the ACE2 receptor and then replicates in the cytoplasm of the cell, producing progeny virions (2, 3). These virions are released from the cell into surrounding tissues of the respiratory tract (4). They are usually shed by coughing or sneezing and the incubation period of the infection is between 4.5 and 5.8 days (5). ACE2 receptors are present in many organs and tissues including the lungs, trachea, bronchi, stomach, small intestine, sweat glands, parathyroid, pituitary, pancreas, and cerebellar endothelial cells (6, 7).

Clinical manifestations of COVID-19 range from an asymptomatic state to multiorgan dysfunction (8). COVID-19 affects the respiratory system causing progressive respiratory failure and organ dysfunction, leading to generalized coagulopathy (9–11). Most commonly, infection manifests with symptoms including cough, fever, diarrhea, fatigue, headaches, and myalgias.

Shortness of breath due to pneumonia is the most common symptom of hospital admission (12, 13). Respiratory failure, acute respiratory distress syndrome (ARDS), cardiac arrhythmias, coagulopathy, and shock are delayed manifestations of severe disease (14). Severe disease and progression are secondary to the release of inflammatory cytokines including interleukins (IL-2, IL-6, IL 7, IL 10), Granulocyte Colony Stimulating Factor (G-CSF), Interferon gamma-induced protein 10 (IP-10), MCD 1, M1 P18, and TNF alpha (15, 16).

Autopsies of infected patients have shown that the virus can cause microvascular injury in the brain (17). The mechanism by which the virus spreads to the brain is via the cribriform plate into areas surrounding the olfactory bulb, olfactory nerve, and through the blood-brain barrier (18–20). Neurological manifestations of Covid-19 range from headache to encephalopathy. The symptoms include headaches, dizziness, ageusia, weakness, and confusion. Patients with central nervous system involvement can be diagnosed with acute encephalopathy, acute cerebrovascular problems, acute ischemic stroke, hypoxia, Guillain-Barré syndrome (GBS), ataxia, olfactory disorders, gustatory dysfunction, seizures, psychosis, and cerebral venous sinus thrombosis (CVST) (21, 22).

CEREBRAL VENOUS SINUS THROMBOSIS

Cerebral venous sinuses are an uncommon location of venous thrombosis and are mostly observed in women and the younger population (23–25). Common causes of CVST include hereditary prothrombotic conditions, antiphospholipid antibody syndrome (APLS), cancer, pregnancy, autoimmune diseases, and infections (26). It can lead to increased intracranial pressure, encephalopathy, strokes, cranial nerve palsies, seizures, and headaches (27). The most common presentation of CVST is a headache and it can be the only symptom in some instances (26, 28–31). The etiology of headache is likely secondary to the compression of nerves within veins (32), increased intracranial pressure, or, sometimes, venous infarct (33, 34). It is often difficult to differentiate CVST-induced headaches from other primary headache disorders. Most headaches caused by CVST are subacute in onset, causing diffuse throbbing pain. Other associated neurological findings include neurologic deficits, photophobia, or signs of increased intracranial pressure (35–38). Sometimes CVST can also lead to damage of brain parenchyma (39).

Diagnosing CVST can be very difficult due to differences in clinical presentation and imaging findings. Only about 25% of patients with CVST can be diagnosed by unenhanced head CT. CT venography and MRI can be used if there is a high suspicion after unenhanced head CT. The European Academy of Neurology recommends intravenous heparin or subcutaneous low molecular weight heparin first followed by oral anticoagulation for at least 3 to 12 months depending on the etiology of CVST (31).

Abbreviations: CVST, Cerebral venous sinus thrombosis; GERD, Gastroesophageal reflux disease; DVT, Deep vein thrombosis; APLS, Antiphospholipid syndrome; BMI, Basal Metabolic Index; CBC, Complete Metabolic Panel; BMP, Basal Metabolic Panel; LDH, Lactate dehydrogenase; CRP, C-Reactive protein; MRI, Magnetic resonance imaging; CT, Computed tomography.

CVST AND COVID-19

Coronavirus disease-2019 infection causes a hypercoagulable state, resulting in increased inflammatory markers like D-dimer, Lactate dehydrogenase (LDH), ferritin, and C-reactive protein (CRP). It also causes an increase in clotting times (40–44). Usually, prothrombotic events including deep vein thrombosis (DVT) and pulmonary embolism occur later as the disease progresses. Many studies have shown an association of COVID-19 infection with CVST (45–48). The most common location of CVST secondary to COVID-19 infection is the transverse sinus followed by the sigmoid sinus (49). The severe microvascular injury in COVID-19 infection is suggested to be due to the combined effect of complement activation and hypercoagulability, leading to microvascular thrombosis. The cerebrovascular effects also seem to be secondary to hypercoagulability and endothelial injury resulting from the release of pro-inflammatory cytokines. Some patients diagnosed with CVST in COVID-19 infection present elevated anticardiolipin IgM antibody and lupus anticoagulant. They also had increased fibrinogen levels, inflammatory markers, and prothrombin time (50–57).

CASE DISCUSSION

A 66-year-old man with a past medical history significant for GERD, hypothyroidism, previous head and neck cancer status, post-resection history chemotherapy, and radiation presented to the hospital for evaluation of headaches. The patient reported waking from sleep with a pounding headache two to three times per month over a 6-month period. The headaches were associated with palpitations, dizziness, and diaphoresis. He denied any complaints of blurred vision, slurred speech, weakness, numbness, or tingling of his extremities, nausea, and vomiting. He reported no complaints of shortness of breath, cough, nasal congestion, fatigue, or diarrhea. His pain was reportedly resolved by two to three tablets of ibuprofen 200 mg. While his headaches did not significantly impact his activities of daily living, they did prompt discussion with his primary care physician. An outpatient MRI brain was performed, which revealed findings suspicious of CVST. Thus, he was sent to the emergency department for further evaluation.

Three months prior to admission, the patient was tested for SARS-CoV-2 virus infection from a potential exposure at work. He tested positive and exhibited symptoms of cough, headache, and nasal congestion. He never exhibited significant shortness of breath or pleuritic chest pain. His symptoms improved with basic medical management. On his current admission, he was again tested for COVID-19 and tested positive. He denied a history of smoking, alcohol abuse, or recreational drug use. He had a past medical history of cancer on the base of the left tongue

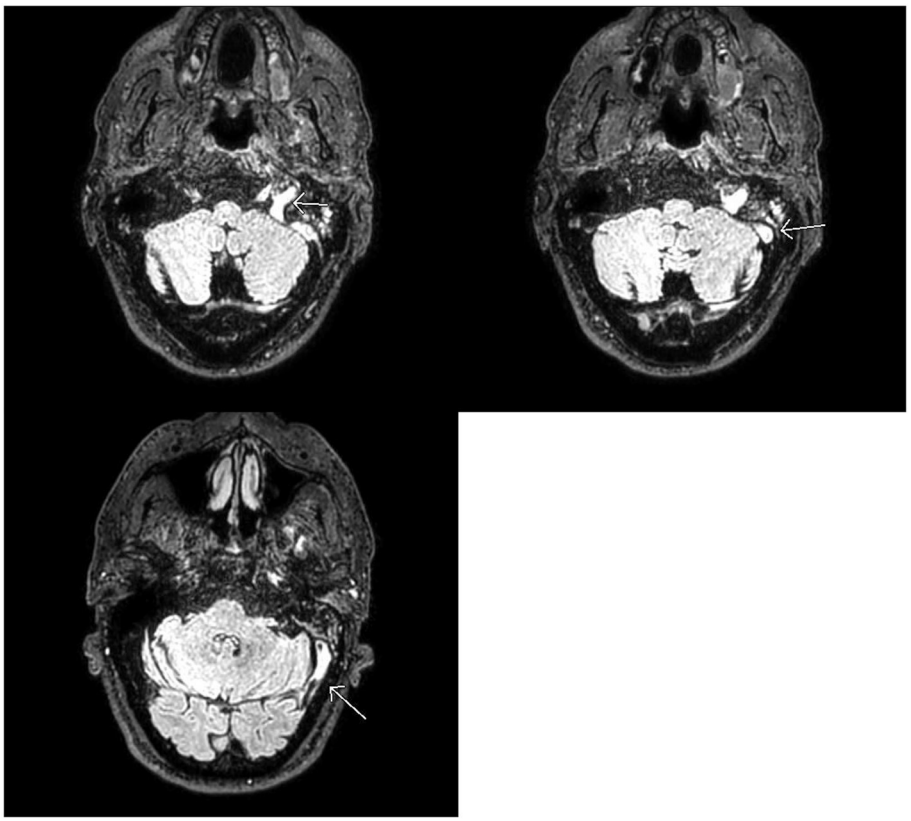


FIGURE 1 | MRI Brain: Abnormal FLAIR signal in the distal left transverse and sigmoid sinuses as well as the jugular bulb with loss of normal flow void. No corresponding signal abnormality on T1 or gradient weighted sequences (not shown). Findings favored to be slow flow but can be seen with dural venous sinus thrombosis in the setting of headaches. If there is clinical concern for dural venous sinus thrombosis, CT, or MR venogram should be considered for definitive diagnosis.

involving the left tonsils and left neck lymph nodes which was treated by surgery, radiation, and chemotherapy 11 years prior. He never presented any signs or symptoms of recurrence. Family history was not significant. His body mass index at the time of admission was 28 kg/m^2, vital signs were within normal limits, and he was saturating well on room air. Physical examination including complete neurological examination was within normal limits. CBC revealed hemoglobin of 12.3, BMP revealed mild elevation of potassium was 5.2 mg/dl, TSH was within normal limits. His IgG, IgM, and IgA beta-2 glycoprotein antibodies were all within normal limits. IgG, IgA cardiolipin antibodies were within normal limits, but IgM was mildly positive at 21. Factor V Leiden and prothrombin gene mutations were negative. On telemetry, the patient had a pattern of bigeminy and trigeminy. He had no complaints of chest discomfort; there were no signs of ischemia, and a subsequent two-dimensional echo was within normal limits. CT brain imaging without contrast revealed no acute intracranial hemorrhage, midline shift, or mass effect but showed parenchymal volume loss, which was a probable sequelae of chronic small vessel ischemic change, as expected for age of the patient. There was no evidence of recurrence of his known cancer. MRI brain findings showed an abnormal FLAIR signal with loss of flow void in the distal left transverse sinus, sigmoid sinus, and jugular bulb (**Figure 1**). This was followed by a CT venogram, which revealed a filling defect in the left sigmoid sinus, jugular bulb, and visualized left internal jugular vein compatible with dural venous sinus thrombosis (**Figure 2**). He was started on intravenous heparin for dural sinus venous thrombosis. Prior to discharge, he has transitioned to Rivaroxaban 20 mg with anticipated treatment lasting 6 months. His symptoms had resolved at the follow-up appointment 3 months later.

DISCUSSION

Coronavirus disease-2019 usually manifests as fever, dyspnea, cough, diarrhea, and fatigue. The most common thromboembolic complications with COVID-19 infection are DVT and pulmonary emboli (47, 58). CVST is a rare thromboembolic event in the setting of COVID-19 disease, but the neurologic complications can sometimes be very severe if not diagnosed and treated early in the disease course (59, 60). The amount of CVST burden in hospitalized patients with SARS-CoV-2 infection was 0.08% with a 95% CI of 0.01 to −0.5

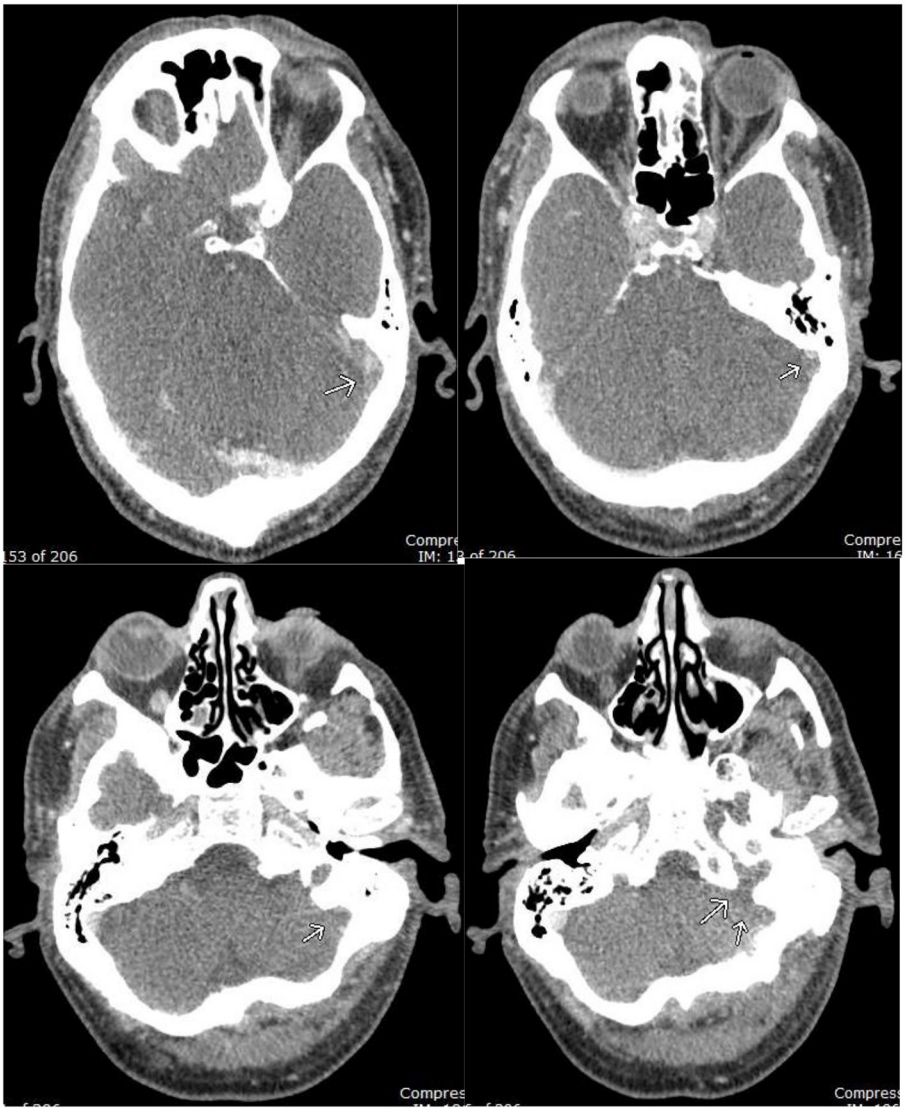


FIGURE 2 | CT Venogram: filling defect in the left distal transverse sinus, sigmoid sinus, and jugular bulb, compatible with occlusive dural venous sinus thrombosis.

in a meta-analysis. It was also noted in the same study that risk factors other than SARS-CoV-2 infection were present in 31% of these individuals (59). Most patients presenting with CVST do not have a history of thrombotic disorders (58). Hereditary and acquired prothrombotic conditions like factor V Leiden, G20210A prothrombin gene mutation, malignancy, oral contraceptive use, pregnancy, and infection increase the risk of CVST compared to the general population (23). CVST complication in COVID-19 positive patients can present as loss of consciousness (37, 61), upward gaze (37, 61), visual deficits (60), convulsions (62), hemiparesis (62), headache (62, 63), executive dysfunction (63), dyspraxia (63), tongue biting (61), altered mentation (64), and aphasia (64). However, headache is the most common presenting symptom in patients with CVST (48). With advances in neuroimaging modalities, diagnosing CVST has become straightforward, however, early recognition of CVST is often obscured due to a wide variety of symptoms. Our case report presents headache as a manifestation of CVST associated with COVID-19. CVST can have symptoms from very mild to very severe. Headache is a common symptom in most viral illnesses and headache syndromes, therefore, a thorough evaluation is needed to rule out common problems before proceeding with expensive imaging studies. But new-onset recurring headaches need a high index of suspicion for treatable conditions like CVST as we can improve outcomes in such patients. Our patient had a previous history of cancer, and about 7–10% of patients diagnosed with CVST have cancer (65). Even though it is unlikely that his treated cancer which is in remission caused CVST, it is important to note that cancer increases the risk

of thrombosis (66). The mortality rate of CVST associated with COVID-19 virus infection is significantly high (67, 68). Thus, in this pandemic era, even a symptom as common as a new-onset persistent headache should be investigated further as it may sometimes represent a medical emergency. In conclusion, timely recognition and awareness of this association enable prompt diagnosis and treatment with good clinical outcomes.

DATA AVAILABILITY STATEMENT

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

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

MA wrote the case report under the guidance of MM. MM is the Hematologist who treated the patient. SW is the Neurologist involved in the care of the patient. LJ and AS from radiology were responsible for the interpretation of images. All authors were involved in making appropriate changes as needed and approved the final case report.

REFERENCES

1. Subbarao K, Mahanty S. Respiratory virus infections: understanding COVID-19. *Immunity.* (2020) 52:905–9. doi: 10.1016/j.immuni.2020.05.004
2. Cuervo NZ, Grandvaux N. ACE2: Evidence of role as entry receptor for SARS-CoV-2 and implications in comorbidities. *Elife.* (2020) 9:e61390. doi: 10.7554/eLife.61390
3. Shang J, Ye G, Shi K, Wan Y, Luo C, Aihara H, et al. Structural basis of receptor recognition by SARS-CoV-2. *Nature.* (2020) 581:221–4. doi: 10.1038/s41586-020-2179-y
4. Fehr AR, Perlman S. Coronaviruses: an overview of their replication and pathogenesis. Coronaviruses. (2015) 1–23. doi: 10.1007/978-1-4939-2438-7_1
5. Lauer SA, Grantz KH Bi Q, Jones FK, Zheng Q, Meredith HR, Azman AS, et al. The incubation period of coronavirus disease (2019). (COVID-19) from publicly reported confirmed cases: estimation and application. *Annals of Internal Medicine.* (2020) 172:577–82. doi: 10.7326/M20-0504
6. Ding Y, He LI, Zhang Q, Huang Z, Che X, Hou J, et al. Organ distribution of severe acute respiratory syndrome (SARS) associated coronavirus (SARS-CoV) in SARS patients: implications for pathogenesis and virus transmission pathways. *J Pathol.* (2004) 203:622–30. doi: 10.1002/path.1560
7. Hamming I, Timens W, Bulthuis ML, Lely AT, Navis GV, van Goor H. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. *J Pathol.* (2004) 203:631–7. doi: 10.1002/path.1570
8. Centers for Disease Control and Prevention (2021). Available online at: https://stacks.cdc.gov/view/cdc/89980
9. Li X. Ma X. Acute respiratory failure in COVID-19: is it "typical" ARDS?. *Critical Care.* (2020) 24:1–5. doi: 10.1186/s13054-020-02911-9
10. Zaim S, Chong JH, Sankaranarayanan V, Harky A. COVID-19 and multi-organ response. *Curr Probl Cardiol.* (2020) 28:100618. doi: 10.1016/j.cpcardiol.2020.100618
11. Al-Samkari H, Karp Leaf RS, Dzik WH, Carlson JC, Fogerty AE, Waheed A, et al. COVID-19 and coagulation: bleeding and thrombotic manifestations of SARS-CoV-2 infection. *Blood.* (2020) 136:489–500. doi: 10.1182/blood.2020006520
12. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, et al. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet.* (2020) 395:1054–62. doi: 10.1016/S0140-6736(20)30566-3
13. Wang D, Hu B, Hu C, Zhu F, Liu X, Zhang J, et al. Clinical characteristics of 138 hospitalized patients with 2019 novel coronavirus–infected pneumonia in Wuhan, China. *Jama.* (2020) 323:1061–9. doi: 10.1001/jama.2020.1585
14. Guo T, Fan Y, Chen M, Wu X, Zhang L, He T, et al. Cardiovascular implications of fatal outcomes of patients with coronavirus disease 2019 (COVID-19). *JAMA cardiology.* (2020) 5:811–8. doi: 10.1001/jamacardio.2020.1017
15. Singhal T. A review of coronavirus disease-2019 (COVID-19). *Indian J Pediatr.* (2020) 87:281–6. doi: 10.1007/s12098-020-03263-6
16. Moore JB, June CH. Cytokine release syndrome in severe COVID-19. *Science.* (2020) 368:473–4. doi: 10.1126/science.abb8925
17. Lee MH, Perl DP, Nair G, Li W, Maric D, Murray H, et al. Microvascular injury in the brains of patients with Covid-19. *N Engl J Med.* (2021) 384:481–3. doi: 10.1056/NEJMc2033369
18. Cheng Q, Yang Y, Gao J. Infectivity of human coronavirus in the brain. *EBioMedicine.* (2020) 56:102799. doi: 10.1016/j.ebiom.2020.102799
19. Baig AM, Khaleeq A, Ali U, Syeda H. Evidence of the COVID-19 virus targeting the CNS: tissue distribution, host–virus interaction, and proposed neurotropic mechanisms. *ACS Chem Neurosci.* (2020) 11:995–8. doi: 10.1021/acschemneuro.0c00122
20. Meinhardt J, Radke J, Dittmayer C, Franz J, Thomas C, Mothes R, et al. Olfactory transmucosal SARS-CoV-2 invasion as a port of central nervous system entry in individuals with COVID-19. *Nat Neurosci.* (2021) 24:168–75. doi: 10.1038/s41593-020-00758-5
21. Zubair AS, McAlpine LS, Gardin T, Farhadian S, Kuruvilla DE, Spudich S. Neuropathogenesis and neurologic manifestations of the coronaviruses in the age of coronavirus disease 2019: a review. *JAMA Neurol.* (2020) 77:1018–27. doi: 10.1001/jamaneurol.2020.2065
22. Wildwing T, Holt N. The neurological symptoms of COVID-19: a systematic overview of systematic reviews, comparison with other neurological conditions and implications for healthcare services. *Ther Adv Chronic Dis.* (2021) 12:2040622320976979. doi: 10.1177/2040622320976979
23. Ageno W, Beyer-Westendorf J, Garcia DA, Lazo-Langner A, McBane RD, Paciaroni M. Guidance for the management of venous thrombosis in unusual sites. *J Thromb Thrombolysis.* (2016) 41:129–43. doi: 10.1007/s11239-015-1308-1
24. Coutinho JM, Zuurbier SM, Aramideh M, Stam J. The incidence of cerebral venous thrombosis: a cross-sectional study. *Stroke.* (2012) 43:3375–7. doi: 10.1161/STROKEAHA.112.671453
25. Silvis SM, Lindgren E, Hiltunen S, Devasagayam S, Scheres LJ, Jood K, et al. Postpartum period is a risk factor for cerebral venous thrombosis: a case-control study. *Stroke.* (2019) 50:501–3. doi: 10.1161/STROKEAHA.118.023017
26. Green M, Styles T, Russell T, Sada C, Jallow E, Stewart J, et al. Non-genetic and genetic risk factors for adult cerebral venous thrombosis. *Thromb Res.* (2018) 169:15–22. doi: 10.1016/j.thromres.2018.07.005
27. Ferro JM, Canhão P, Stam J, Bousser MG, Barinagarrementeria F. Prognosis of cerebral vein and dural sinus thrombosis: results of the International Study on Cerebral Vein and Dural Sinus Thrombosis

(ISCVT). *Stroke.* (2004) 35:664–70. doi: 10.1161/01.STR.0000117571.76197.26
28. Zuurbier SM, Middeldorp S, Stam J, Coutinho JM. Sex differences in cerebral venous thrombosis: a systematic analysis of a shift over time. *Int J Stroke.* (2016) 11:164–70. doi: 10.1177/1747493015620708
29. Zuurbier SM, Hiltunen S, Lindgren E, Silvis SM, Jood K, et al. Cerebral venous thrombosis in older patients. *Stroke.* (2018) 49:197–200. doi: 10.1161/STROKEAHA.117.019483
30. Ameri A, Bousser MG. Cerebral venous thrombosis. *Neurol Clin.* (1992) 10:87–111. doi: 10.1016/S0733-8619(18)30235-4
31. Ferro JM, Bousser MG, Canhão P, Coutinho JM, Crassard I, Dentali F, et al. European Stroke Organization guideline for the diagnosis and treatment of cerebral venous thrombosis–endorsed by the European Academy of Neurology. *Eur Stroke J.* (2017) 2:195–221. doi: 10.1177/2396987317719364
32. Mahale R, Mehta A, John AA, Buddaraju K, Shankar AK, Javali M. et al. Acute seizures in cerebral venous sinus thrombosis: what predicts it? *Epilepsy Res.* (2016) 123:1–5. doi: 10.1016/j.eplepsyres.2016.01.011
33. Kalita J, Misra UK, Singh VK, Dubey D. Predictors and outcome of status epilepticus in cerebral venous thrombosis. *J Neurol.* (2019) 266:417–25. doi: 10.1007/s00415-018-9145-8
34. Kowoll CM, Kaminski J, Weiß V, Bösel J, Dietrich W, Jüttler E, et al. Severe cerebral venous and sinus thrombosis: clinical course, imaging correlates, and prognosis. *Neurocrit Care.* (2016) 25:392–9. doi: 10.1007/s12028-016-0256-8
35. Lee KR, Subrayan V, Win MM, Mohamad NF, Patel D. ATRA-induced cerebral sinus thrombosis. *J Thromb Thrombolysis.* (2014) 38:87–9. doi: 10.1007/s11239-013-0988-7
36. Coutinho JM, Zuurbier SM, Gaartman AE, Dikstaal AA, Stam J, Middeldorp S, et al. Association between anemia and cerebral venous thrombosis: case–control study. *Stroke.* (2015) 46:2735–40. doi: 10.1161/STROKEAHA.115.009843
37. Buyck PJ, De Keyzer F, Vanneste D, Wilms G, Thijs V, Demaerel P, et al. density measurement and H: H ratio are useful in diagnosing acute cerebral venous sinus thrombosis. *Am J Neuroradiol.* (2013) 34:1568–72. doi: 10.3174/ajnr.A3469
38. Rodallec MH, Krainik A, Feydy A, Hélias A, Colombani JM, Jullès MC, et al. Cerebral venous thrombosis and multidetector CT angiography: tips and tricks. *Radiographics.* (2006) 26:S5–18. doi: 10.1148/rg.26si065505
39. Fu FW, Rao J, Zheng YY, Song L, Chen W, Zhou QH, et al. Perimesencephalic nonaneurysmal subarachnoid hemorrhage caused by transverse sinus thrombosis: a case report and review of literature. *Medicine.* (2017) 96:e7374. doi: 10.1097/MD.0000000000007374
40. Lippi G, Favaloro EJ. D-dimer is associated with severity of coronavirus disease 2019: a pooled analysis. *Thromb Haemost.* (2020) 120:876. doi: 10.1055/s-0040-1709650
41. Xie Y, Wang X, Yang P, Zhang S. COVID-19 complicated by acute pulmonary embolism. *Radiology: Cardiothoracic Imaging.* (2020) 2:e200067. doi: 10.1148/ryct.2020200067
42. Yin S, Huang M, Li D, Tang N. Difference of coagulation features between severe pneumonia induced by SARS-CoV2 and non-SARS-CoV2. *J Thromb Thrombolysis.* (2021) 51:1107–10. doi: 10.1007/s11239-020-02105-8
43. Guan WJ Ni ZY, Hu Y, Liang WH, Ou CQ, He JX, et al. Clinical characteristics of coronavirus disease 2019 in China. *N Engl J Med.* (2020) 382:1708–20. doi: 10.1056/NEJMoa2002032
44. Kuba K, Imai Y, Rao S, Gao H, Guo F, Guan B, et al. A crucial role of angiotensin converting enzyme 2 (ACE2) in SARS coronavirus–induced lung injury. *Nat Med.* (2005) 11:875–9. doi: 10.1038/nm1267
45. Cavalcanti DD, Raz E, Shapiro M, Dehkharghani S, Yaghi S, Lillemoe K, et al. Cerebral venous thrombosis associated with COVID-19. *Am J Neuroradiol.* (2020) 41:1370–6. doi: 10.3174/ajnr.A6644
46. Abdalkader M, Shaikh SP, Siegler JE, Cervantes-Arslanian AM, Tiu C, Radu RA, et al. Cerebral Venous Sinus Thrombosis in COVID-19 Patients: A Multicenter Study and Review of Literature. *J Stroke Cerebrovascular Dis.* (2021) 4:105733. doi: 10.1016/j.jstrokecerebrovasdis.2021.105733
47. Al-Mufti F, Amuluru K, Sahni R, Bekelis K, Karimi R, Ogulnick J, et al. Cerebral venous thrombosis in COVID-19: A New York Metropolitan Cohort Study. *Am J Neuroradiol.* (2021) 42:1196–200. doi: 10.3174/ajnr.A7134
48. Dakay K, Cooper J, Bloomfield J, Overby P, Mayer SA, Nuoman R, et al. Cerebral venous sinus thrombosis in COVID-19 infection: a case series and review of the literature. *J Stroke Cerebrovascular Dis.* (2020) 6:105434. doi: 10.1016/j.jstrokecerebrovasdis.2020.105434
49. Tu TM, Goh C, Tan YK, Leow AS, Pang YZ, Chien J, et al. Cerebral venous thrombosis in patients with COVID-19 infection: a case series and systematic review. *J Stroke Cerebrovascular Dis.* (2020) 6:105379. doi: 10.1016/j.jstrokecerebrovasdis.2020.105379
50. Teuwen LA, Geldhof V, Pasut A, Carmeliet P. COVID-19: the vasculature unleashed. *Nature Rev Immunol.* (2020) 20:389–91. doi: 10.1038/s41577-020-0343-0
51. Libby P, Lüscher T. COVID-19 is, in the end, an endothelial disease. *Eur Heart J.* (2020) 41:3038–44. doi: 10.1093/eurheartj/ehaa623
52. Middleton EA, He XY, Denorme F, Campbell RA, Ng D, Salvatore SP, et al. Neutrophil extracellular traps contribute to immunothrombosis in COVID-19 acute respiratory distress syndrome. *Blood.* (2020) 136:1169–79. doi: 10.1182/blood.2020007008
53. Panigada M, Bottino N, Tagliabue P, Grasselli G, Novembrino C, Chantarangkul V, et al. Hypercoagulability of COVID-19 patients in intensive care unit: a report of thromboelastography findings and other parameters of hemostasis. *J Thrombosis Haemostasis.* (2020) 18:1738–42. doi: 10.1111/jth.14850
54. Ranucci M, Ballotta A, Di Dedda U, Bayshnikova E, Dei Poli M, Resta M, et al. The procoagulant pattern of patients with COVID-19 acute respiratory distress syndrome. *J Thrombosis Haemostasis.* (2020) 18:1747–51. doi: 10.1111/jth.14854
55. Maier CL, Truong AD, Auld SC, Polly DM, Tanksley CL, Duncan A. COVID-19-associated hyperviscosity: a link between inflammation and thrombophilia? *Lancet.* (2020) 395:1758–9. doi: 10.1016/S0140-6736(20)31209-5
56. Medicherla CB, Pauley RA, de Havenon A, Yaghi S, Ishida K, Torres JL. Cerebral venous sinus thrombosis in the COVID-19 pandemic. *J Neuro-Ophthalmol.* (2020) 40:457–62. doi: 10.1097/WNO.0000000000001122
57. Gavriilaki E, Anyfanti P, Gavriilaki M, Lazaridis A, Douma S, Gkaliagkousi E. Endothelial dysfunction in COVID-19: lessons learned from coronaviruses. *Curr Hypertens Rep.* (2020) 22:1–2. doi: 10.1007/s11906-020-01078-6
58. Lodigiani C, Iapichino G, Carenzo L, Cecconi M, Ferrazzi P, Sebastian T, et al. Venous and arterial thromboembolic complications in COVID-19 patients admitted to an academic hospital in Milan, Italy. *Thromb Res.* (2020) 191:9–14. doi: 10.1016/j.thromres.2020.04.024
59. Baldini T, Asioli GM, Romoli M, Carvalho Dias M, Schulte EC, Hauer L, et al. Cerebral venous thrombosis and severe acute respiratory syndrome coronavirus-2 infection: A systematic review and meta-analysis. *Eur J Neurol.* (2021) 28:3478–90. doi: 10.1111/ene.14727
60. Abouhashem S, Eldawoody H, Taha MM. Cerebral venous sinus thrombosis in patients with COVID-19 infection. *Interdisciplinary Neurosurgery.* (2021) 24:101091. doi: 10.1016/j.inat.2021.101091
61. Hemasian H, Ansari B. First case of Covid-19 presented with cerebral venous thrombosis: a rare and dreaded case. *Rev Neurol.* (2020) 176:521. doi: 10.1016/j.neurol.2020.04.013
62. Thompson A, Morgan C, Smith P, Jones C, Ball H, Coulthard EJ, et al. Cerebral venous sinus thrombosis associated with COVID-19. *Pract Neurol.* (2021) 21:75–6. doi: 10.1136/practneurol-2020-002678
63. Hughes C, Nichols T, Pike M, Subbe C, Elghenzai S. Cerebral venous sinus thrombosis as a presentation of COVID-19. *Eur J Case Rep Intern Med.* (2020) 7:001691. doi: 10.12890/2020_001691
64. Ulivi L, Squitieri M, Cohen H, Cowley P, Werring DJ. Cerebral venous thrombosis: a practical guide. *Pract Neurol.* (2020) 20:356–67. doi: 10.1136/practneurol-2019-002415
65. Xian Z, Chen Y, Chen L, Lu Q, Huang G, Qin Q, et al. A clinical research on the potential pathogenesis of somatic cancer related cerebral venous sinus thrombosis. *Medicine.* (2019) 98:e15134. doi: 10.1097/MD.0000000000015134
66. Elyamany G, Alzahrani AM, Bukhary E. Cancer-associated thrombosis: an overview. *Clinical Medicine Insights: Oncology.* (2014). 8:CMO-S18991. doi: 10.4137/CMO.S18991

67. Ostovan VR, Foroughi R, Rostami M, Almasi-Dooghaee M, Esmaili M, Bidaki AA, et al. Cerebral venous sinus thrombosis associated with COVID-19: a case series and literature review. *J Neurol.* (2021) 22:1–2. doi: 10.1007/s00415-021-10450-8
68. Borhani-Haghighi A, Ghannadi S, Safari A, Neydavoudi M, Poursadeghfard M, Ashjazadeh N, et al. The study of intermediate-term survival of the patients with cerebral venous sinus thrombosis. *Neurology Asia.* (2020) 25:453–7. Available online at: https://www.neurology-asia.org/articles/neuroasia-2020-25(4)-453.pdf

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.

frontiers in Medicine

MINI REVIEW
published: 28 October 2021
doi: 10.3389/fmed.2021.749810

Clinical Outcomes of Unrelated Umbilical Cord Blood Graft vs. Haploidentical Donor Transplantation: Critical Issues for an Adequate Comparison

Diana Vanegas[1], *Laura Niño-Quiroga*[1], *Mauricio Chaparro*[2], *Bernardo Camacho-Rodríguez*[1], *Marcela Estupiñán*[2]* and *Ana-María Perdomo-Arciniegas*[1]*

[1] Cord Blood Bank, Instituto Distrital de Ciencia, Biotecnología e Innovación en Salud, Bogota, Colombia, [2] Unidad de Trasplante, Fundación HOMI-Hospital de la Misericordia, Bogota, Colombia

OPEN ACCESS

Edited by:
Marcos De Lima,
The Ohio State University,
United States

Reviewed by:
Anna Sureda,
Catalan Institute of Oncology, Spain
Aiming Pang,
Chinese Academy of Medical Sciences and Peking Union Medical College, China

***Correspondence:**
Marcela Estupiñán
mestupinanp@homifundacion.org.co
Ana-María Perdomo-Arciniegas
amperdomo@idcbis.org.co

Specialty section:
This article was submitted to Hematology, a section of the journal Frontiers in Medicine

Received: 30 July 2021
Accepted: 27 September 2021
Published: 28 October 2021

Citation:
Vanegas D, Niño-Quiroga L, Chaparro M, Camacho-Rodríguez B, Estupiñán M and Perdomo-Arciniegas A-M (2021) Clinical Outcomes of Unrelated Umbilical Cord Blood Graft vs. Haploidentical Donor Transplantation: Critical Issues for an Adequate Comparison. Front. Med. 8:749810. doi: 10.3389/fmed.2021.749810

Unrelated umbilical cord blood (UCB) and haploidentical grafts have been used for allogeneic hematopoietic stem and progenitor cell (HSPC) transplantation in patients without a related or non-related human leukocyte antigen (HLA)-matched donor. The less stringent HLA-matching requirement in both sources raises an important possibility for patients in need of urgent transplantation to treat any hematological disease. Selection of the best alternative donor is a difficult task that will depend on donor criteria, center experience, patient disease conditions, and risk, among others. Most comparisons available in scientific publications between both graft sources are obtained from retrospective analysis in wide time windows and a heterogeneous number of patients, types of disease, disease stages, previous treatments, graft source, conditioning regimen, graft vs. host disease (GVHD) approach, and evaluable endpoints. There is also an evident impact of the economic traits since low-income countries must consider less expensive treatments to satisfy the needs of the patients in the most effective possible path. Therefore, haploidentical transplantation could be an appealing option, even though it has not been completely established if any chronic treatment derived from the procedure could become a higher cost. In Colombia, there is a huge experience in UCB transplantation especially in units of pediatric transplantation where benign indications are more common than in adults. Due to the availability of a public UCB bank and HLA high-resolution typing in Colombia, there is a wider inventory of cord blood donors. Unfortunately, we do not have an unrelated bone marrow donor registry, so UCB is an important source along with haploidentical transplantation to consider in decision-making. This minireview focuses on comparing the main issues associated with the use of both HSCP sources and provides tools for physicians who face the difficult decision between these alternative donor sources.

Keywords: hematopoietic stem cell transplantation, cord blood, haploidentical, HLA matching, clinical outcomes

INTRODUCTION

Hematopoietic stem and progenitor cell (HSPC) transplantation is a potentially curative treatment that has been used for different disorders that affect hematopoiesis (1, 2). Depending on the disease, transplantation may be autologous when the HSPCs are obtained from the patient or allogeneic when the graft comes from a match-related or unrelated donor. Since the transplantation involves a bone marrow reconstitution in the patient, matching of human leukocyte antigen (HLA) between donor and recipient is probably the most considered and debating topic in the literature (3–6). However, according to some clinical practice guidelines, there are several additional factors that must be considered for the donor and HSPC source selection, such as the urgency of the transplantation, status and risk of the disease, donor criteria, and transplantation center experience (1, 7, 8).

It is known that only 30% of the patients who require an HSPC transplant have a fully HLA-matched sibling donor (6, 9, 10) and therefore, an alternative graft source, such as a haploidentical donor and UCB units, turns out very important for the clinical practice. However, there is huge variability in the clinical populations in which both graft sources are currently used, such as adult and pediatric patients, malignant and non-malignant disease, previous relapses or refractory disease, conditioning regimen, and prophylaxis treatment, among others. Therefore, we focused on verifying the information reported in the literature only for malignant disease in which umbilical cord blood and haploidentical transplantation are compared simultaneously as a two-arm design, retrospective or prospective, with overall survival or progression-free survival (PFS) evaluated as clinical endpoints and including information regarding the time of engraftment. Although there are several reports of the clinical results and experience with each graft source separately, we did not include them, considering the aforementioned variability.

There is an important experience in Colombia with UCB transplantation for pediatric patients and a higher inventory of cord blood donors with HLA high-resolution typing available in the public UCB bank which started 5 years ago (11). Since there is no unrelated bone marrow donor registry in the country, UCB turns out an important source along with haploidentical donors for patients who require transplantation. The purpose of this work is to verify the clinical outcomes in malignant disease treated with transplantation from these two sources and to consider the additional conditions that may impact not only the results but the selection of one source over the other.

HAPLOIDENTICAL DONOR TRANSPLANTATION RATE INCREASE

Over the past 5 years, some authors have been pointing out an evident increase of haploidentical donor transplantation procedures and a significant decrease in UCB for allogeneic transplants in Europe and the United States (10, 12–14). In 2018, Weisdorf (14) reported that although the total number of HSPC transplantations per year in the US has been stable, UCB use has declined from 800 to 600 per year, while haploidentical has raised from 500 to 1,200 by the end of 2016. In the European scenario, UCB use has decreased from over 800 transplantations per year in 2010 to <500 in 2016, and it appears to be restricted to the pediatric population. Haploidentical transplantation numbers have been rising, mainly in the less wealthy countries, suggesting an economic effect on the donor selection process (12, 13).

There are some particularities in Latin America regarding this issue. Although HSPC transplantation has been performed for the last 50 years in Europe and the US (4), in Latin American countries, its practice barely started in the 1980s only in Brazil and Argentina and has been increasing from that moment in the other countries (15). However, the available information regarding the number of hematopoietic transplants performed in the 28 countries from the Latin American region is scarce and probably underestimated because of non-reporting transplantation centers (16). From the obtained data until 2012, Jaimovich et al. reported a 20% increase in allotransplants from 2009, mainly as a treatment for leukemia. There was also a relation between gross national income (GNI) per capita and transplantation activity, which was completely absent in countries with <3.3 million people or GNI per capita <US$3,400. However, the transplantation rate did not exhibit a clear trend related to GNI per capita only, and some high-income countries bear a low transplantation rate. It is, however, clear that by 2012, the Latin American transplant rate was from 5- to 8-fold lower than North America and Europe, and the density of transplant teams in proportion with the population was also lower, only considering data from the reporting countries, without any auditing (16).

There is also an underestimated genetic variability in most of the countries and regions in Latin America. The European colonization, African slavery, native American populations, and different migration processes from the Middle East and Asia have established a mixed gene pool in the region, including HLA genes, and therefore, probably impacting the search for a suitable allogeneic donor in European or North American Bone marrow (BM) donor registries. Registries available in Latin American countries are considerably smaller, the searching activities are less organized and take longer times, which is critical for patients with malignant diseases (16). Since haploidentical transplantation (T cell repleted, without any *ex vivo* manipulation) solves the problem of finding an HLA-matching donor and its associated high costs, it represents an appealing option in developing countries and its use is rapidly increasing, as reported in the US and Europe (17).

HAPLOIDENTICAL DONOR AND CORD BLOOD UNIT SELECTION INEQUALITY

The main question would be whether haploidentical transplantation exhibits superiority in terms of clinical outcomes compared to UCB transplantation. As previously mentioned, a haploidentical donor could be guaranteed to almost every transplant candidate and it is economically beneficial. The first decades of haploidentical transplantation performance did not

exhibit favorable clinical results, mainly due to the poor quality of life and high morbimortality rates in the patients, caused by the incidence of GVHD. These clinical problems were partially surpassed using *in vivo* and *ex vivo* T-cell depletion techniques (9), which altogether with higher cellular doses initially favored clinical results of HSPC transplantation of haploidentical donors compared to UCB grafts.

On the other hand, it had been stated that, unlike other sources, UCB did not require an allele-level matching in all five or six HLA loci (HLA-A, -B, -C, -DRB1, -DQB1, and DPB1) and that cell dose comprises a more impacting factor than HLA matching on clinical outcomes (3, 5). However, recent evidence suggests that allele-level HLA matching improves clinical outcomes using UCB (18–21). It is also a matter of concern that an important proportion of patients previously transplanted with UCB, matched by low-resolution typing in HLA-I, has one or more additional HLA mismatches when allele-level typing was retrospectively analyzed (22). Therefore, many UCB-transplanted patients with a previously stated 4/6 HLA match with the graft have in fact less than a 3/6. This is a clear disadvantage for UCB compared to haploidentical transplantation, which starts with at least a 6/12 match.

In a different scenario, a recent report shows that not only the traditional matching of HLA class I sequences in the peptide-binding region could drive the risk of GVHD or even impact the results of the transplantation. A dimorphism in HLA-B exon 1, which produces two different leader peptides, could provide information regarding relapse and non-relapse mortality risk after UCB transplantation. This confirms the importance and potential of high-resolution HLA typing in UCB graft selection and transplantation (23).

Current HLA-matching protocols could be unbalanced if a comparison is pretended between UCB and haploidentical transplantation clinical outcomes. A low- and intermediate-resolution HLA typing is enough for the latter case, due to the inherent relation between donor and patient. On the other hand, and according to the previously mentioned evidence, allelic-level HLA typing would be more appropriate in a non-related UCB setting, not only for HLA-DRB1 but for all HLA genes. There is a potential improvement of the UCB procedure in this area, which could lead to the obtention of better clinical outcomes if additional characteristics are included in the donor selection process.

OUTCOMES OF HAPLOIDENTICAL TRANSPLANTATION VS. UMBILICAL CORD BLOOD

Pediatric Patients

Although the reduction trend in UCB use aligns with the haploidentical transplantation increase, there are not many studies comparing both HSPC sources in terms of clinical efficacy. In pediatric patients, only two publications were found (24, 25) while in adults, including some studies with double cord transplant, there are five published studies (26–30), all of them in malignant disease, retrospective, and non-randomized. Recent work published a comparison of pooled data from these trials in terms of GVHD and relapse incidence, non-relapse-associated mortality, and 2-year disease-free survival (DFS), in which no statistically significant differences were found between the two sources (31). The obtention of conclusive results from these comparisons is a complicated task, mainly because of patient heterogeneity in terms of disease stages, previous treatments, conditioning regimen, conditioning intensity, GVHD prophylaxis, and variability in clinical outcomes definition.

However, pediatric UCB-transplanted patients showed a statistically significant (<0.001) delayed neutrophil recovery (determined as the first out of 3 consecutive days with absolute count $\geq 0.5 \times 10^9$/L) in both studies, with a median time of 20 and 16 days compared to haploidentical transplanted patients, with a median time of 13 days in both reports (29, 30). Both studies had identical endpoint definitions, all patients had malignant diseases (acute lymphoblastic leukemia and acute myeloid leukemia), although there were some differences in the conditioning regimen and GVHD prophylaxis treatment for all groups (24, 25).

Adult Patients

Published studies in adults exhibited less consistent results. The median time of neutrophil reconstitution was significantly different in three out of five papers comparing UCB and haploidentical transplantation (T-cell repleted) (26, 29, 30). The neutrophil median recovery time was 21 days for UCB and 18 days for haploidentical transplantation. The median time of platelet reconstitution was only reported in three studies (27, 28, 30), and there is an observable delay in UCB (41 and 38 days) compared to haploidentical transplantation (27 and 24 days). However, it is not possible to determine if these differences may be impacting any of the clinical outcomes, such as overall survival, PFS, or GVHD incidence, evaluated by Li et al. (31). Infections were reported by some authors as the cause of transplant-related death independently from the graft source (26, 29). Interestingly, there were no differences in the incidence of infections between both groups, although the neutrophil and platelet reconstitution delay found in UCB may lead to the longest hospitalization times, more transfusions, and a higher probability of complications (27). A recent meta-analysis and systematic review of haploidentical and non-related UCB donor transplantation of a relatively large population of adult and pediatric patients found no differences when comparing chronic GVHD incidence and DFS at 2 years but found a statistically significant higher risk for acute GVHD in the haploidentical adult transplantation group (32).

The website www.clinicaltrials.gov was consulted to verify if any ongoing clinical trial was comparing outcomes of transplantation from haploidentical and non-related UCB donors. Three interventional, open-label studies were found, two for hematological malignancies and one for ß-thalassemia. The latter lacks information since 2015 and its current state is unknown. The studies in hematological malignancies were

designed to compare in parallel groups double UCB vs. haploidentical transplantation. One of those was terminated due to slow accrual—with only two enrolled patients—while the third one, a phase III active study with 368 enrolled patients, was recently completed and published (33). This is the only non-retrospective, randomized, and stratified study found so far comparing these two HSPC sources for transplantation. The study was performed in adults from 18 to 70 years of age, with high-risk acute leukemia or lymphoma (Hodgkin or no-Hodgkin) and two sources of HSPC available: first, two UCB units matched at least 4/6 each one with a cell dose $> 1.5 \times 10^7$ and second, a haploidentical bone marrow donor matched for at least one allele per loci (HLA-A, -B, -C, DRB1) at allele-level. After randomization, 172 patients received double-UCB transplantation, and 153 patients were transplanted from a haploidentical donor in a post-transplant cyclophosphamide setting. All patients received a reduced intensity conditioning regimen (33).

As opposed to the previously mentioned studies, the median time of neutrophil recovery in this trial was lower for double UCB (15 days) than for haploidentical donors (17 days) and is even statistically significant when comparing the cumulative incidence at day 56, although it is not completely clear if this variable assessment is identical to the previous studies mentioned. Platelet recovery, however, remains slower in double UCB (median 42 days) than in haploidentical donors (median 28 days). As for GVHD, there were no significant differences between treatments either in the acute or chronic frame. There were no statistically significant differences between the two treatments in the probability of 2-year PFS (primary endpoint) and 2-year incidence of relapse/progression; nonetheless, the 2-year incidence of non-relapse mortality was significantly higher in double-UCB-transplanted patients, which leads to a lower 2-year overall survival than the haploidentical graft receptors (secondary endpoints). In this case, the most common cause of death in both groups was a recurrent disease, followed by infection. Although no analysis regarding this data is provided there is a difference of only 2% in deaths proportion by infection in UCB transplantation compared to haploidentical (9.15 vs. 7.1%) (33).

DISCUSSION

To determine which is the most favorable donor alternative between unrelated UCB and haploidentical donors for pediatric and adult HSPC transplantation candidates with any hematological disease is not an easy task, considering the diversity of the results. To the best of our knowledge, there are no published comparisons between the two sources in the treatment of non-malignant diseases. There is also an unexplored possibility comparing haploidentical transplantation with a higher matching UCB donor, considering four HLA loci in high-resolution typing, a condition that may improve the neutrophil reconstitution time, as described by Eapen et al. (18). It is possible that upgrading the matching of UCB units with the patient leads to a better clinical outcome in terms of immune reconstitution and consequently in overall survival or PFS even in the setting of haplo-cord transplantation.

Although there is not enough evidence favoring haploidentical over UCB transplantation from the clinical perspective, there is an increase of haploidentical transplantation vs. UCB (10, 13, 14). This trend may not only be caused by the higher possibility of finding a suitable donor and the lower costs but also because the neutrophil engraftment delay could turn into early complications and longer hospitalization care. It is not known whether the UCB scenario globally represents higher costs for health services compared to haploidentical transplantation, or if the latter might imply even higher long-term costs considering the required treatment for chronic GVHD. More research is required to improve and expand possibilities of HSPC graft source for patients that do not have an identical family donor.

Finally, it is important to note that just as haploidentical transplantation has improved over time, UCB transplantation may reach better results upgrading the clinical criteria and practice, especially because it has gone through a shorter path in history. Even though the comparisons so far have not been equitable between both sources in terms of HLA matching, which is one of the main success indicators, the lack of statistically significant differences in clinical outcomes strongly suggests that improving the UCB donor selection could definitively raise a new scenario for this source, adding possibilities for those patients lacking a related donor and joining forces with haploidentical transplantation possibilities. It is possible that the reduction of UCB use as an HSPC source compared to the haploidentical donor is not essentially based on research data, but it could be also driven by other conditions, such as the knowledge and skills in the clinical practice in some countries, economic issues, and difficulties of finding a donor.

AUTHOR CONTRIBUTIONS

DV and A-MP-A contributed to the initial conception of comparing the two sources for transplantation reviewing the available bibliography. LN-Q, MC, ME, and BC-R contributed with the clinical perspective for comparison. DV wrote the first draft of the manuscript, A-MP-A wrote sections of the manuscript. BC-R had financial support. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This work was supported by the Sistema General de Regalías. Fondo de CTeI, Colombia and Fondo Financiero Distrital de Salud (FFDS). Secretaría Distrital de Salud, Bogotá DC, Colombia. BPIN: 2016000100035.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.749810/full#supplementary-material

REFERENCES

1. Majhail NS, Farnia SH, Carpenter PA, Champlin RE, Crawford S, Marks DI, et al. Indications for autologous and allogeneic hematopoietic cell transplantation: guidelines from the american society for blood and marrow transplantation. *Biol Blood Marrow Transplant.* (2015) 21:1863–9. doi: 10.1016/j.bbmt.2015.07.032
2. Wynn R. Stem cell transplantation in inherited metabolic disorders. *Hematol Am Soc Hematol Educ Program.* (2011) 2011:285–91. doi: 10.1182/asheducation-2011.1.285
3. Baron F, Storb R. Allogeneic hematopoietic cell transplantation as treatment for hematological malignancies: a review. *Springer Semin Immunopathol.* (2004) 26:71–94. doi: 10.1007/s00281-004-0165-3
4. 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
5. Little AM, Green A, Harvey J, Hemmatpour S, Latham K, Marsh SGE, et al. BSHI Guideline: HLA matching and donor selection for haematopoietic progenitor cell transplantation. *Int J Immunogenet.* (2016) 43:263–86. doi: 10.1111/iji.12282
6. Ballen KK, Koreth J, Chen Y Bin, Dey BR, Spitzer TR. Selection of optimal alternative graft source: mismatched unrelated donor, umbilical cord blood, or haploidentical transplant. *Blood.* (2012) 119:1972–80. doi: 10.1182/blood-2011-11-354563
7. Xu L, Chen H, Chen J, Han M, Huang H, Lai Y, et al. The consensus on indications, conditioning regimen, and donor selection of allogeneic hematopoietic cell transplantation for hematological diseases in China - Recommendations from the Chinese Society of Hematology. *J Hematol Oncol.* (2018) 11:1–17. doi: 10.1186/s13045-018-0564-x
8. Ciurea SO, Al Malki MM, Kongtim P, Fuchs EJ, Luznik L, Huang XJ, et al. The European Society for Blood and Marrow Transplantation (EBMT) consensus recommendations for donor selection in haploidentical hematopoietic cell transplantation. *Bone Marrow Transplant.* (2020) 55:12–24. doi: 10.1038/s41409-019-0499-z
9. Khan MA, Bashir Q, Chaudhry Q-N, Ahmed P, Satti TM, Mahmood SK. Review of haploidentical hematopoietic cell transplantation. *J Glob Oncol.* (2018) 4:1–13. doi: 10.1200/JGO.18.00130
10. Liu JH, Kanakry CG, Luznik L. Have haploidentical transplants replaced umbilical cord transplants for acute leukemias? *Curr Opin Hematol.* (2018) 25:103–11. doi: 10.1097/MOH.0000000000000412
11. Vanegas D, Galindo CC, Páez-Gutiérrez IA, González-Acero LX, Medina-Valderrama PT, Lozano JC, et al. Human leukocyte antigen and red blood cells impact umbilical cord blood CD34+ cell viability after thawing. *Int J Mol Sci.* (2019) 20:4875. doi: 10.3390/ijms20194875
12. Passweg JR, Baldomero H, Bader P, Bonini C, Cesaro S, Dreger P, et al. Hematopoietic SCT in Europe 2013: recent trends in the use of alternative donors showing more haploidentical donors but fewer cord blood transplants. *Bone Marrow Transplant.* (2015) 50:476–82. doi: 10.1038/bmt.2014.312
13. Passweg JR, Baldomero H, Bader P, Basak GW, Bonini C, Duarte R, et al. Is the use of unrelated donor transplantation leveling off in Europe? The 2016 European society for blood and marrow transplant activity survey report. *Bone Marrow Transplant.* (2018). 53:1139–48. doi: 10.1038/s41409-018-0153-1
14. Weisdorf D. Can haploidentical transplantation meet all patients' needs? *Best Pract Res Clin Haematol.* (2018) 31:410–3. doi: 10.1016/j.beha.2018.09.012
15. Eckrich M, Pasquini M. Hematopoietic cell transplantation in Latin America. *Hematology.* (2012) 17(Suppl. 1):S189–91. doi: 10.1179/102453312X13336169157059
16. Jaimovich G, Martinez Rolon J, Baldomero H, Rivas M, Hanesman I, Bouzas L, et al. Latin America: the next region for haematopoietic transplant progress. *Bone Marrow Transplant.* (2017) 52:671–7. doi: 10.1038/bmt.2016.361
17. Arrieta-Bolaños E, Oliveira DC, Barquera R. Differential admixture, human leukocyte antigen diversity, and hematopoietic cell transplantation in Latin America: challenges and opportunities. *Bone Marrow Transplant [Internet].* (2020) 55:496–504. doi: 10.1038/s41409-019-0737-4
18. Eapen M, Klein JP, Ruggeri A, Spellman S, Lee SJ, Anasetti C, et al. Impact of allele-level HLA matching on outcomes after myeloablative single unit umbilical cord blood transplantation for hematologic malignancy. *Blood.* (2014) 123:133–40. doi: 10.1182/blood-2013-05-506253
19. Lee SJ, Klein J, Haagenson M, Baxter-Lowe LA, Confer DL, Eapen M, et al. High-resolution donor-recipient HLA matching contributes to the success of unrelated donor marrow transplantation. *Blood.* (2007) 110:4576–83. doi: 10.1182/blood-2007-06-097386
20. Eapen M, Wang T, Veys PA, Boelens JJ, St Martin A, Spellman S, et al. Allele-level HLA matching for umbilical cord blood transplantation for non-malignant diseases in children: a retrospective analysis. *Lancet Haematol.* (2017) 4:e325–33. doi: 10.1016/S2352-3026(17)30104-7
21. Kögler G, Enczmann J, Rocha V, Gluckman E, Wernet P. High-resolution HLA typing by sequencing for HLA-A, -B, -C, -DR, -DQ in 122 unrelated cord blood/patient pair transplants hardly improves long-term clinical outcome. *Bone Marrow Transplant.* (2005) 36:1033–41. doi: 10.1038/sj.bmt.1705189
22. Cornetta K, Laughlin M, Carter S, Wall D, Weinthal J, Delaney C, et al. Umbilical cord blood transplantation in adults: results of the prospective cord blood transplantation (COBLT). *Biol Blood Marrow Transplant.* (2005) 11:149–60. doi: 10.1016/j.bbmt.2004.11.020
23. Petersdorf EW, Gooley T, Volt F, Kenzey C, Madrigal A, McKallor C, et al. Use of the HLA-B leader to optimize cord-blood transplantation. *Haematologica.* (2020) 105. doi: 10.3324/haematol.2020.264424. [Epub ahead of print].
24. Mo XD, Tang BL, Zhang XH, Zheng CC, Xu LP, Zhu XY, et al. Comparison of outcomes after umbilical cord blood and unmanipulated haploidentical hematopoietic stem cell transplantation in children with high-risk acute lymphoblastic leukemia. *Int J Cancer.* (2016) 139:2106–15. doi: 10.1002/ijc.30249
25. González-Vicent M, Molina B, Andión M, Sevilla J, Ramirez M, Pérez A, et al. Allogeneic hematopoietic transplantation using haploidentical donor vs. unrelated cord blood donor in pediatric patients: A single-center retrospective study. *Eur J Haematol.* (2011) 87:46–53. doi: 10.1111/j.1600-0609.2011.01627.x
26. Giannotti F, Labopin M, Shouval R, Sanz J, Arcese W, Angelucci E, et al. Haploidentical transplantation is associated with better overall survival when compared to single cord blood transplantation: An EBMT-Eurocord study of acute leukemia patients conditioned with thiotepa, busulfan, and fludarabine. *J Hematol Oncol.* (2018) 11:1–10. doi: 10.1186/s13045-018-0655-8
27. El-Cheikh J, Crocchiolo R, Furst S, Bramanti S, Sarina B, Granata A, et al. Unrelated cord blood compared with haploidentical grafts in patients with hematological malignancies. *Cancer.* (2015) 121:1809–16. doi: 10.1002/cncr.29271
28. Brunstein CG, Fuchs EJ, Carter SL, Karanes C, Costa LJ, Wu J, et al. Alternative donor transplantation after reduced intensity conditioning: Results of parallel phase 2 trials using partially HLA-mismatched related bone marrow or unrelated double umbilical cord blood grafts. *Blood.* (2011) 118:282–8. doi: 10.1182/blood-2011-03-344853
29. Ruggeri A, Labopin M, Sanz G, Piemontese S, Arcese W, Bacigalupo A, et al. Comparison of outcomes after unrelated cord blood and unmanipulated haploidentical stem cell transplantation in adults with acute leukemia. *Leukemia.* (2015) 29:1891–900. doi: 10.1038/leu.2015.98
30. Raiola AM, Dominietto A, di Grazia C, Lamparelli T, Gualandi F, Ibatici A, et al. Unmanipulated haploidentical transplants compared with other alternative donors and matched sibling grafts. *Biol Blood Marrow Transplant.* (2014) 20:1573–9. doi: 10.1016/j.bbmt.2014.05.029
31. Li D, Li X, Liao L, Li N. Unrelated cord blood transplantation versus haploidentical transplantation in adult and pediatric patients with

hematological malignancies-A meta-analysis and systematic review. *Am J Blood Res*. (2020) 10:1–10.
32. Li D, Li X, Liao L, Li N. Unrelated cord blood transplantation versus haploidentical transplantation in adult and pediatric patients with hematological malignancies-A meta-analysis and systematic review. *Am J Blood Res*. (2020).
33. Fuchs EJ, Donnell PVO, Eapen M, Ph D, Antin JH, Dawson P, et al. Double unrelated umbilical cord blood versus HLA-haploidentical bone marrow transplantation BMT CTN 1101 trial. *Blood*. (2021) 137:420–8. doi: 10.1182/blood.2020007535

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.

frontiers in Medicine

CASE REPORT
published: 18 November 2021
doi: 10.3389/fmed.2021.755669

Gestational Psittacosis With Secondary Hemophagocytic Syndrome: A Case Report and Literature Review

*Li Sun†, Pulin Li†, Bo Pang, Peipei Wu and Ran Wang**

Department of Respiratory and Critical Care Medicine, The First Affiliated Hospital of Anhui Medical University, Hefei, China

Gestational psittacosis and hemophagocytic syndrome (HPS) are rare clinical diseases. In this article, a case of gestational psittacosis concomitant with secondary HPS was reported. An analysis was performed on the clinical characteristics, signs, laboratory findings, progression, diagnosis, and treatment of a patient with gestational psittacosis concomitant with secondary HPS. Besides, the literature with respect to this disease was reviewed. This patient was definitively diagnosed through metagenomic next-generation sequencing techniques, bone marrow puncture and smear examination, and the determination of sCD25 level and natural killer (NK) cell activity. Anti-infectives such as doxycycline and etoposide combined with hormone chemotherapy achieved significant improvement in cough and expectoration, a return to normal temperature, and a significant improvement in oxygenation index. In addition, chest computed tomography revealed obvious absorption of lung lesions and a return of NK cell activity and sCD25 levels to normal ranges. *Chlamydia psittaci* pneumonia requires a clear determination of etiology, while HPS requires bone marrow puncture and smear examination, together with the determination of sCD25 level and NK cell activity in the blood. The findings of this study suggest that metagenomic next-generation sequencing is an effective instrument in clearly identifying pathogens that cause lung infection. Clinicians should consider atypical pathogens of lung infection in patients with poor response to empirical anti-infectives, and strive to design an effective treatment strategy as per an accurate diagnosis based on the etiology. As for patients suffering from long-term high fever and poor temperature control after broad-spectrum antibiotic treatment, non-infectious fever should be taken into account. A rapid and clear diagnosis would significantly improve patient prognosis.

Keywords: gestational psittacosis, hemophagocytic syndrome, metagenomic next-generation sequencing, gestational psittacosis with secondary hemophagocytic syndrome, long-term high fever

OPEN ACCESS

Edited by:
Chien-Ling Huang,
Hong Kong Polytechnic University,
Hong Kong SAR, China

Reviewed by:
Ching-Ying Kuo,
National Taiwan University, Taiwan
Wenjie Qi,
Capital Medical University, China

***Correspondence:**
Ran Wang
ranwangtjmu@hotmail.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: *09 August 2021*
Accepted: *07 October 2021*
Published: *18 November 2021*

Citation:
Sun L, Li P, Pang B, Wu P and Wang R (2021) Gestational Psittacosis With Secondary Hemophagocytic Syndrome: A Case Report and Literature Review. Front. Med. 8:755669. doi: 10.3389/fmed.2021.755669

INTRODUCTION

Intimate contact with birds is usually associated with a risk of suffering from anthropozoonosis caused by *Chlamydia psittaci*. C. *psittaci* pneumonia is a rare clinical disease, with an incubation period of 1–4 weeks. The clinical manifestations of this disease include influenza and high fever with a temperature more than 40°C, accompanied by headache, myalgia, joint pain, and other symptoms, including cough, expectoration, and bloody sputum occurring ∼1 week after the

infection. *C. psittaci* infection can involve multiple systems, among which the pulmonary organs are the main targets. Therefore, respiratory failure may occur in severe cases (1). Hemophagocytic syndrome (HPS), also known as hemophagocytic lymphohistiocytosis (HLH), is a type of excessive inflammatory response syndrome caused by primary or secondary immune abnormalities. Most cases would develop disease secondary to infection, tumor(s), and/or autoimmune diseases (2). Infection can be caused by bacteria, parasites, and viral infections, including Epstein–Barr virus and cytomegalovirus. HPS is characterized by acute onset, rapid progress, difficult to treat, and high mortality. *C. psittaci*, which belongs to the genus *Chlamydia*, together with *Chlamydia pneumoniae* and *Chlamydia trachomatis*, can survive for several months at room temperature, and is an anthropozoonosis (3). Gestational psittacosis with secondary HPS is even more rare. To our knowledge, there is currently no report on gestational psittacosis concomitant with secondary HPS. Therefore, a case of gestational psittacosis with secondary HPSs is expounded in this article.

CASE REPORT

In this article, a case involving a 27-year-old woman with gravida 2, para 1, and gestational weeks of 26 was reported. This patient suffered from a fever shortly after she became soaked in the rain 6 days previously, with the highest temperature of 40°C. Then, she had cough 3 days previously, with white phlegm and occasional bloody sputum, combined with chest tightness and asthma. These symptoms were not improved after the administration of mezlocillin sodium and sulbactam sodium as anti-infectives in the outpatient department. Subsequently, she visited the emergency department of the First Affiliated Hospital of Anhui Medical University (Anhui, China) on October 22, 2020, where she was admitted to the hospital with "fever and pregnancy status (26 weeks pregnant)." This patient developed intermittent diarrhea in the previous 3 days, with the clinical manifestations of yellow watery stool and dark urine. The patient was previously in good condition and had no other diseases. Through auscultation, breathing sounds in both lungs were thick, with obvious moist rales. On the admission day, the temperature of the patient was 38.5°C, with a heart rate of 150 beats/min, a blood pressure of 105/69 mmHg, and a respiratory rate of 35 breaths/min. The findings of laboratory examinations were as follows: hemoglobin, 88 g/L; C-reactive protein (CRP), 145.46 mg/L; white blood cell count (WBC), 14.73×10^9/L; platelet count, 189×10^9/L; procalcitonin (PCT), 19.36 ng/mL; D-dimer, 12.85 μg/mL; glutamic-oxaloacetic transaminase, 125 U/L; and creatinine, 39.3 μmol/L. Chest X-ray (**Figure 1A**) revealed bilateral lung inflammation, lung abscess in the superior lobe of the left lung, and right pleural effusion. The preliminary differential diagnosis included severe pneumonia, acute respiratory distress syndrome, septic shock, and pregnancy. Although meropenem and oseltamivir were administered to resist infection, the patient's dyspnea symptoms persisted, and her high-flow oxygenation index was < 100 mmHg. After endotracheal intubation, the patient was transferred to the intensive care unit (ICU). On October 23, 2020, the patient was examined through fiberoptic bronchoscopy, sputum and alveolar lavage fluid in the left and right bronchi were aspirated, and meropenem, vancomycin, azithromycin, and oseltamivir were administered with mechanical-assisted ventilation. On October 25, 2020, the patient received metagenomic next-generation sequencing (mNGS) (**Figures 2A,B**) of alveolar lavage fluid and blood, with both results indicating *C. psittaci*. Therefore, the diagnosis of *C. psittaci* pneumonia was confirmed, and the antibiotic regimen was adjusted to include doxycycline, cefoperazone sodium and sulbactam sodium, and moxifloxacin. Symptomatic treatment, including mechanical ventilation, anti-shock, protection of important organs, nutritional support, sedation, and analgesia, was actively administered. On October 29, 2020, bronchoscopy was performed again, with the results of metagenomic next-generation sequencing (mNGS) of alveolar lavage fluid and blood continuing to show *C. psittaci*. However, the level of *C. psittaci* (**Figures 2C,D**) in the alveolar lavage fluid decreased compared to the earlier analysis, although the level of *C. psittaci* in the blood increased. On October 31, 2020, the oxygenation index of the patient reached 280 mmHg, which was significantly improved, the temperature of the patient was 40°C, with a heart rate of 105 beats/min and a blood pressure of 120/60 mmHg. The findings of laboratory examinations were as follows: hemoglobin, 93 g/L; CRP, 80.19 mg/L;WBC, 14.36 $\times 10^9$/L; platelet count, 140 $\times 10^9$/L; PCT, 4.87 ng/mL; D-dimer, 8.35 μg/mL; glutamic-oxaloacetic transaminase, 54 U/L; and creatinine, 26.7 μmol/L. Chest X-ray revealed improved bilateral lung inflammation and lung lesions compared with the condition at admission. However, the peak temperature of this patient was higher than before. As for those patients suffering from long-term high fever, if the temperature is not effectively controlled after extensive coverage with broad-spectrum and powerful antibiotics, non-infectious fever should be taken into account at the same time. In other cases, non-infectious fever is common in the rheumatic blood system and tumor diseases. Bone marrow puncture and smear examination were performed on November 2, 2020, and the tumor index of the patient was elevated, which was considered to be related to pregnancy and hypoproteinemia. Sputum and blood cultures showed *Acinetobacter baumannii*, and sputum and urine cultures showed *Candida albicans* and *Candida tropicalis*. Therefore, the antibiotic regimen was adjusted to include polymyxin + cefoperazone sodium and sulbactam sodium + doxycycline + teicoplanin + voriconazole on November 9, 2020. On November 15, 2020, ultrasound indicated that the umbilical cord was wound around the neck of the fetus for 2 weeks, the volume of amniotic fluid was decreased, and fetal kidney parenchyma echo was enhanced. After communicating with the family members of the patient and obstetrics physicians, rivanol was injected into the amniotic cavity to induce labor. On November 19, 2020, the patient became conscious, and her breathing and circulation were stable; however, she developed a cough reaction, and tried to break away from the ventilator and remove tracheal intubation. Pulmonary imaging revealed mitigated fever (temperature > 38.5°C), reduced red blood

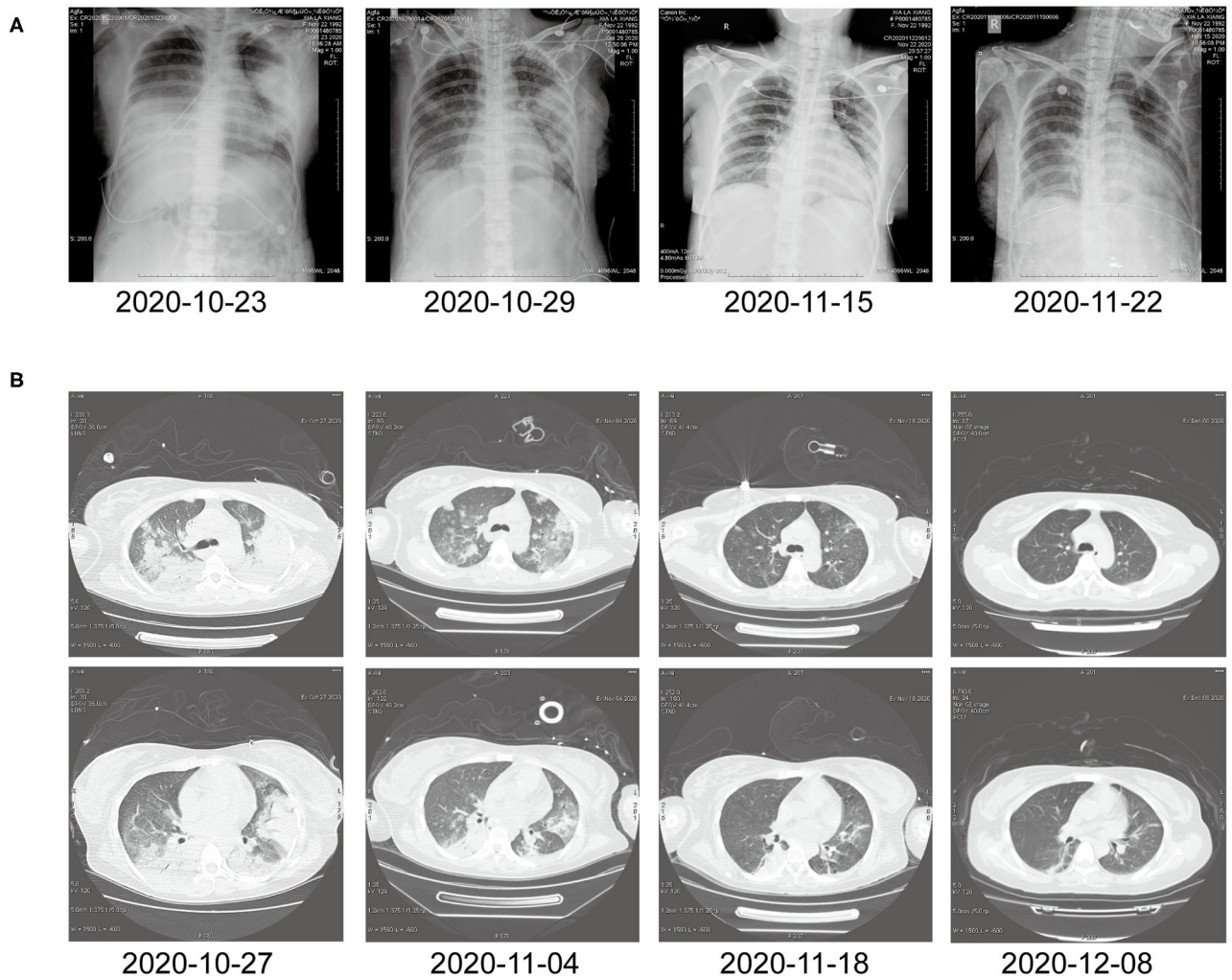


FIGURE 1 | (A) Chest X-ray before and after treatment. **(B)** Chest computed tomography at different time points after treatment.

cell (RBC) count (1.99 × 10^{12}/L) and platelet count (111 × 10^9/L), hypertriglyceridemia (triglycerides, 6.21 mmol/L), and hyperferritinemia (ferritin, 2,284 μg/L). Phagocytic cells were apparent on bone marrow smear and HPS could not be excluded. Besides, natural killer (NK) cell activity and soluble CD25 levels were examined, and the CD107a excitation test was improved. These results indicated that the level of sCD25 increased, and NK cell activity decreased. Therefore, the diagnosis of HPS caused by infection was confirmed. The HLH-2004 regimen, recommended by the International Histocyte Association, was applied in the treatment of HPS. Etoposide combined with hormone therapy can significantly mitigate—if not eliminate—symptoms, and immunosuppressants, such as cyclosporine A (CSA) and anti-thymocyte globulin (ATG), could be added according to the condition (4). On November 20, 2020, VP-16 (150 mg) and dexamethasone (10 mg) were administered to eliminate symptoms. On November 22, 2020, the temperature of the patient returned to 36.8°C.On November 27, 2020, the temperature of the patient was basically maintained within the normal range. The finding of laboratory examinations were as follows: hemoglobin, 92 g/L; CRP, 7.48 mg/L;WBC, 11.43 × 10^9/L; platelet count, 103 × 10^9/L; PCT, 0.11 ng/mL; D-dimer, 0.93 μg/mL; glutamic-oxaloacetic transaminase, 62 U/L; creatinine, 13.3 μmol/L; RBC count, 2.83 × 10^{12}/L; hypertriglyceridemia (triglycerides, 5.68 mmol/L); and hyperferritinemia (ferritin, 2,212 μg/L). The indices of ferritin and triglycerides for the patient did not decrease significantly compared with the previous time, and HPS had not been completely controlled. On November 27, 2020, etoposide (100 mg) was administered on the basis of dexamethasone to control the primary disease. The findings of laboratory examinations on December 3, 2020, were as follows: WBC, 6.55 × 10^9/L; platelet count, 155 × 10^9/L; triglycerides, 2.34 mmol/L; ferritin, 1,807 μg/L; and glutamic-oxaloacetic transaminase, 14 U/L. Etoposide (100 mg) was administered for the third time on December 6, 2020. On December 10, 2020, NK cell activity

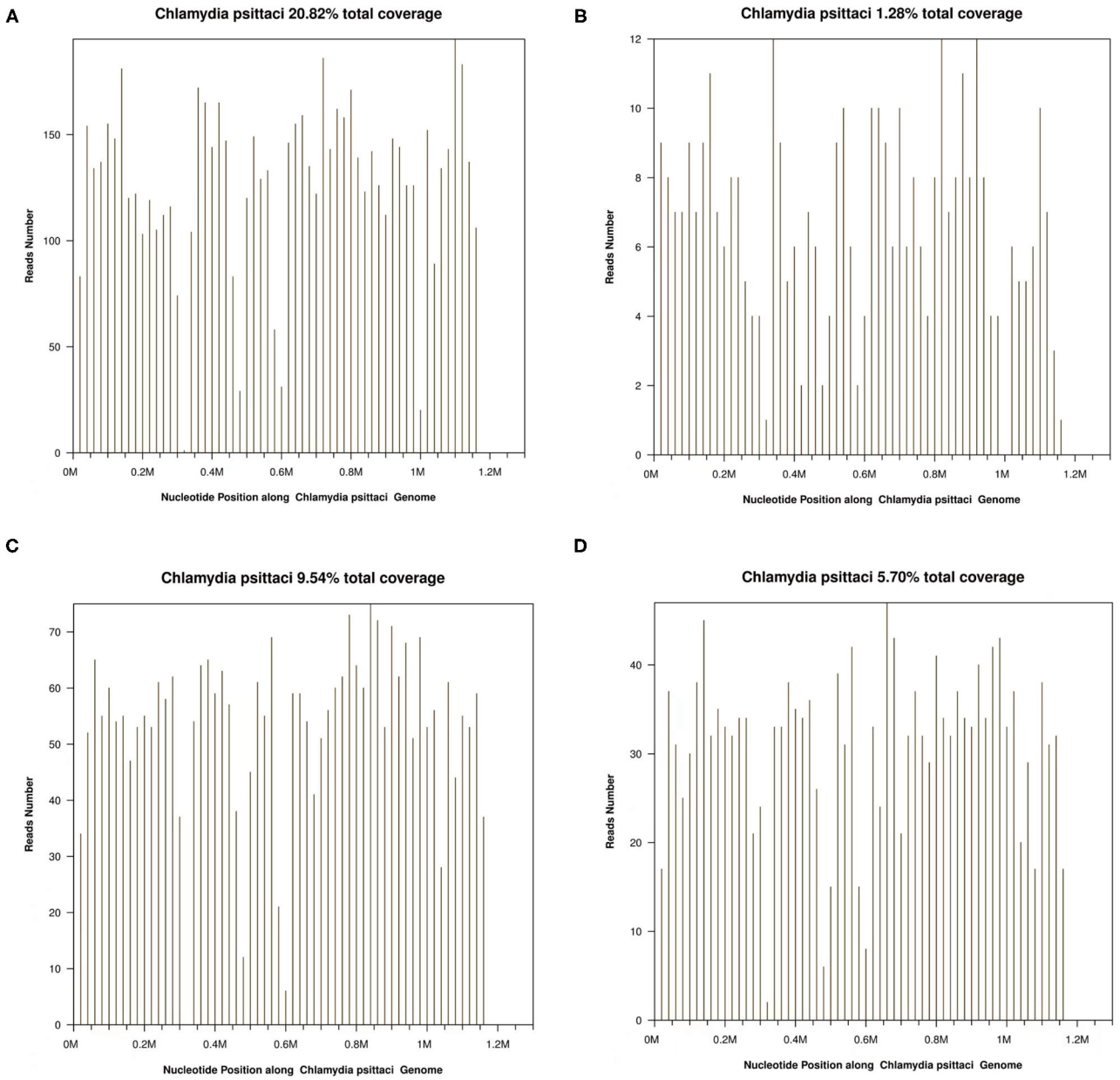


FIGURE 2 | (A) *Chlamydia psittaci* detection in bronchoalveolar lavage fluid (BALF) using metagenomic next-generation sequencing (mNGS) on October 25, 2020. **(B)** *C. psittaci* in blood detected by mNGS on October 25, 2020. **(C)** *C. psittaci* detected in BALF using mNGS on November 4, 2020. **(D)** *C. psittaci* in blood detected by mNGS on November 4, 2020.

and sCD25 levels were re-examined, with the results showing that NK cell activity did not decrease and the level of sCD25 returned to the normal range. Therefore, it was not necessary to continue the treatment with etoposide, and dexamethasone was gradually decreased. On December 23, 2020, the fever, cough, and expectoration were obviously mitigated and breath sounds in both lungs became clear. Chest CT (**Figure 1B**) revealed that the lung lesions were absorbed, and the level of sCD25 and NK cell activity suggested that the condition of the patient was basically stable; therefore, the patient was discharged from the hospital. The telephone follow-up 1 week later revealed that the patient had no complaints of discomfort, fever, cough, or expectoration, and she was advised to conduct outpatient reviews regularly (**Figure 3**).

DISCUSSION

The pregnant woman reported that she had a history of contact with poultry a week before the onset of illness. Also, it was found from the results of mNGS sent by bronchoalveolar lavage fluid (BALF) that she was infected with *C. psittaci*.

22/10/2020	Admitted to hospital with "fever, and pregnancy status (26 weeks pregnant)". Meropenem and oseltamivir.
25/10/2020	mNGS of alveolar lavage fluid and blood, all indicated with both results indicating C. psittaci the diagnosis of C. psittaci pneumonia was confirmed. Doxycycline, cefoperazone sodium and sulbactam sodium.
09/11/2020	Polymyxin + cefoperazone sodium and sulbactam sodium + doxycycline + teicoplanin + voriconazole.
15/11/2020	Rivanol was injected into the amniotic cavity to induce labor.
20/22/2020	VP-16 (150 mg) and dexamethasone (10 mg) .
27/11/2020	Etoposide 150mg QW, DXM 10mg QD.
06/12/2020	Etoposide (100 mg) was used administered for the third time.
23/12/2020	The patient was discharged from hospital.

FIGURE 3 | Timetable used to show the progress of the disease and the patient's treatment.

After active anti-infective treatment, the lung lesions of the patient were significantly absorbed. However, she still had a high fever, reduced RBC and platelet count, hypertriglyceridemia, hyperferritinemia, apparent phagocytic cells on bone marrow smear, increased level of sCD25, and decreased activity of NK cells, and hence the diagnosis of gestational psittacosis with secondary HPS could be confirmed.

Chlamydia psittaci infections occur mainly in the autumn, and almost all relevant cases are associated with a fever. There are also other symptoms, including chills, myalgia, headache, diarrhea, dyspnea, and hemoptysis. *C. psittaci* pneumonia accounts for ∼1% of community-acquired pneumonias (5). Sixty percent of patients with *C. psittaci* pneumonia exhibit dysfunction of the liver (6), as did the patient described in this article. The findings of laboratory examinations on October 25, 2020, were as follows: alanine aminotransferase (ALT), 88 U/L; aspartate aminotransferase (AST), 433 U/L; direct bilirubin, 22.6 μmol/L; and indirect bilirubin 38.3 μmol/L. After treatment, laboratory examinations were performed on November 2, 2020, with the results were as follows: ALT, 14 U/L; AST, 30 U/L; direct bilirubin 10.7 μmol/L; and indirect bilirubin 14.2 μmol/L. Ito et al. drew a comparison between mycoplasma pneumonia, *C. pneumonia*, and *C. psittaci* pneumonia, and they found that *C. psittaci* infection was dominated by frosted glass shadow bilateral lung lesions which were more common, and ∼8% of patients exhibited pleural effusion (7). In this case, the patient exhibited bilateral lung disease with right pleural effusion. It has been demonstrated in some studies that the level of D-dimer in patients with *C. psittaci* pneumonia concomitant with single or multiple lung lobe involvement is significantly higher than that in those with simple lung segment lesions, and the level of D-dimer can be regarded as a prognostic factor, which can be employed to reveal the changes in the condition of patients (8). There were also significant changes in the level of D-dimer which was evident in the present case, with the value exceeding 20 μg/mL at the initial stage of the disease. After the treatment with doxycycline, moxifloxacin, and other antibacterial drugs, the lung lesions in the patient decreased, and the level of D-dimer decreased to 4.17 μg/mL on November 2, 2020. Severe *C. psittaci* pneumonia is similar to *Legionella pneumonia* with regard to extrapulmonary manifestations, biological characteristics, and prognosis. The difference is that *C. psittaci* pneumonia is associated with a history of exposure to sick birds and a prolonged duration of symptoms before admission (9). In this case, the patient (a 27-year-old woman) had a history of contact with poultry 1 week before the onset of the disease.

At present, the diagnosis of *C. psittaci* pneumonia is mainly based on the contact history with sick birds, and pathogens in bronchial secretions. Currently, the detection methods include pathogen isolation and identification, immunofluorescence, complement fixation test, ELISA, routine PCR, and fluorescence PCR. The micro-immunofluorescence method is a common method to detect *C. psittaci*, and requires the sample collection twice at the acute and recovery stages. It is a retrospective diagnosis, which can be employed to determine the disease condition and evaluate the exposure situation. However, it is difficult to implement the culture technology for *C. psittaci* and excessive handling of samples may lead to contamination. Therefore, the high-level biosafety is required, which further limits clinical application. The detection of *C. psittaci* nucleic acid in respiratory secretions, blood, and tissues of patients with suspected acute-stage infection is a rapid and reliable diagnostic method with high sensitivity and specificity (10, 11). Moreover, nucleic acid detection can also provide strain genotyping and avoid cross-reactions with serological diagnosis (12). The recently developed mNGS is a novel technology to detect/identify microorganisms, and features high pathogen-positive detection rate, rapidity, wide pathogen coverage, and high sensitivity. It has been widely applied in the field of metagenomic detection of unknown pathogens, including hereditary diseases, tumors, infectious diseases, human leukocyte antigen analysis, and non-invasive prenatal screening (13). At present, mNGS is a favorable method for detecting unknown pathogens in pulmonary infections. The patient in this report suffered from severe infection, but no abnormality was found through routine detection of sputum etiology, and the empirical anti-infection treatment took no effect, in combination with the contact history of the patient with poultry 1 week before the onset of this disease. Bronchoscopy and alveolar lavage were utilized to collect BALF, and blood was collected for

mNGS. The results identified *C. psittaci* as the pathogen, which provided clear direction for subsequent treatment. Currently, there are a few reports on the detection of *C. psittaci* through second-generation sequencing methods (14, 15). However, it has been reported in some studies that tetracyclines are the first-choice treatment for patients with *C. psittaci* infection (16). Azithromycin has also exhibited favorable activity against *C. psittaci*, while fluoroquinolones have a weak inhibitory effect, but can be used as an alternative (15). In this case, after 2 days of treatment with doxycycline, the temperature and oxygenation levels of the patient exhibited no significant improvement. Considering the poor anti-infective effect on the patient and the favorable therapeutic effect of moxifloxacin on *C. psittaci* pneumonia (16), antibiotic treatment was replaced with doxycycline, cefoperazone sodium, and sulbactam sodium combined with moxifloxacin. After 1 week of treatment, the condition of the patient was significantly improved, with specific manifestations of decreased inflammation index and absorbed lung lesions as per the chest CT results.

Hemophagocytic syndrome is a syndrome involving multiple systems, and it can be caused by the abnormal proliferation of activated macrophages and histiocytes (17). Its common clinical manifestations include high fever, enlarged liver, splenic lymph nodes, pancytopenia, low fibrinogen level, hypertriglyceridemia, abnormal liver enzyme levels, poor prognosis, and a high mortality rate (18). In this case, the patient also had a fever, with the temperature reaching 40°C, RBC count decreasing to 1.67×10^{12}/L, platelet count decreasing to 75×10^{9}/L, triglyceride levels reaching 4.38 mmol/L, and abnormal liver enzyme levels.

Hemophagocytic syndrome is associated with many diseases, and its etiology is particularly important in the diagnosis due to the fact that these potentially relevant diseases would exert significant impacts on the treatment and prognosis of individuals with HPS (19). HPS occurs rarely in pregnant women, and there are only a few studies about pregnancy-related HPS. In these published cases, it is unclear whether the cause of HPS was related to pregnancy itself or viral infection, or both (20). HPS in the patient was considered to be secondary to severe *C. psittaci* pneumonia. The pathophysiology of HPS remains unclear; however, a "cytokine storm" has been assumed as a cause in one theory (21, 22). Currently, there is no specific diagnostic method for HPS, although the diagnostic standard revised by the Histiocyte Society in 2004 has been widely adopted (4). The diagnosis can be made according to any of the following.

Molecular diagnosis: At present, it is known that related genes, such as *PRF1, UNC13D, STX11, STXBP2, Rab27a, LYST,* and *SH2D1A*, have pathological mutations; and meet five of the following eight standards: fever, temperature > 38.5°C, duration > 7 days; spleen, hypocytopenia (two or three series of peripheral blood); hemoglobin < 90 g/L, PLT $< 100 \times 10^{9}$/L, and GR $< 1.0 \times 10^{9}$/L, and these are not caused by the decreased hematopoietic function of bone marrow; hypertriglyceridemia and/or hypoproteinemia: triacylglycerol > 3 mmol/L, fibrinogen < 1.5 g/L; phagocytic blood cells found in bone marrow, spleen, or lymph nodes; elevated serum ferritin ≥ 500 μg/L; decreased level or lack of NK cell activity; and an increase in the level of sCD25.

Although the general prognosis for HPS is poor, the prognosis for HPS caused by bacterial infection is better. However, the prognosis for HPS caused by Epstein–Barr virus is the worst. The mortality of HPS caused by other viruses is generally ∼50%, and the mortality of tumor-related HPS is almost 100%. The appearance of HPS indicates the deterioration of this disease. The main causes of death include hemorrhage, infection, multiple organ failure, and disseminated intravascular coagulation. Factors associated with poor prognosis include low albumin and/or high lactate dehydrogenase levels, severe hemorrhage and uncontrolled infection, thrombocytopenia, liver function damage, and aging (23). HPS therapies can be divided into two categories: induced remission and etiological therapies. Induced remission therapy mainly refers to controlling excessive inflammation, while the objective of etiological therapy is to correct immunodeficiency by actively treating the primary disease (24). In this case, after the treatment with etoposide and hormone, the temperature of the patient rapidly returned to normal ranges, and the related indices were improved. In summary, the clinical manifestations of HPS are complex and diverse, and the disease progresses rapidly and the prognosis is poor. Therefore, it is significant to make early diagnosis for this disease and treat it correctly.

Although HPS has been investigated in some studies around the world, cases of *C. psittaci* pneumonia concomitant with secondary HPS during pregnancy have not, to our knowledge, been reported. Katsura et al. reported a case of psittacosis during pregnancy, and reviewed the research about 23 cases of psittacosis during pregnancy from 1967 to date (25). The mortality rates for fetuses and pregnant women were 82.6% (19/23) and 8.7% (2/23), respectively. The primary reason for the death of pregnant women and fetuses was that *C. psittaci* infection was not diagnosed in time during the progression of the disease. In this case, although the fetus was subjected to labor induction, the reason for the better prognosis of the patient was that the diagnosis of *C. psittaci* pneumonia was confirmed by mNGS in the early stage of the disease, which provided a basis for the selection of effective antibiotics to quickly control lung lesions. In those patients with a persistent high fever after pulmonary lesions are obviously mitigated, HPS is diagnosed by bone marrow biopsy, smear, NK cell activity, soluble CD25 level, and a CD107a challenge test. The condition of the patient was improved after the active treatment with etoposide and hormone therapy according to the HLH-94 and HLH-2004 guidelines, and all indicators gradually returned to normal ranges.

Through this study, it can be recognized that the clinical application of mNGS should be actively pursued for pulmonary infections with unknown etiology.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Review Board at First affiliated hospital of Anhui Medical University. The patients provided their written informed consent to participate in this study. Written informed consent was obtained from the individual for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

RW and LS conception and design of the study. LS, BP, and PW provided clinical treatment for the patient and manuscript preparation. RW performed manuscript review. All authors have read and approved the content of the manuscript.

FUNDING

The present study was supported by a fund from the Natural Science Foundation of China (No. 81970051). Excellent Top Talent Cultivation Project of Anhui Higher Education Institutions (gxgwfx2021014).

ACKNOWLEDGMENTS

The authors thank the patient for her cooperation during the diagnostic process. The authors thank the Intensive Care Unit of the First Affiliated Hospital of Anhui Medical University for its great help in the treatment of the patient.

REFERENCES

1. Heddema ER, van Hannen EJ, Duim B, de Jongh BM, Kaan JA, van Kessel R, et al. An outbreak of psittacosis due to *Chlamydophila psittaci* genotype A in a veterinary teaching hospital. *J Med Microbiol.* (2006) 55:1571–5. doi: 10.1099/jmm.0.46692-0
2. Janka GE. Familial and acquired hemophagocytic lymphohistiocytosis. *Eur J Pediatr.* (2007) 166:95–109. doi: 10.1007/s00431-006-0258-1
3. Cong W, Huang SY, Zhang XX, Zhou DH, Xu MJ, Zhao Q, et al. *Chlamydia psittaci* exposure in pet birds. *J Med Microbiol.* (2014) 63:578–81. doi: 10.1099/jmm.0.070003-0
4. Henter JI, Horne A, Arico M, Egeler RM, Filipovich AH, Imashuku S, et al. HLH-2004: diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. *Pediatr Blood Cancer.* (2007) 48:124–31. doi: 10.1002/pbc.21039
5. Hogerwerf LB, Baan BW. *Chlamydia psittaci* (psittacosis) as a cause of community-acquired pneumonia: a systematic review and meta-analysis. *Epidemiol Infect.* (2017) 145:3096–105. doi: 10.1017/S0950268817002060
6. Branley JM, Weston KM, England J, Dwyer DE, Sorrell TC. Clinical features of endemic community-acquired psittacosis. *New Microbes New Infect.* (2014) 2:7–12. doi: 10.1002/2052-2975.29
7. Itoh I, Ishida T, Hashimoto T, Arita M, Osawa M, Tachibana H, et al. Chest radiograph of atypical pneumonia: comparison among *Chlamydia pneumoniae*. Pneumonia, ornithosis, and *Mycoplasma pneumoniae* pneumonia. *Kansenshogaku Zasshi.* (2000) 74:954–60. doi: 10.11150/kansenshogakuzasshi1970.74.954
8. Querol-Ribelles JM, Tenias JM, Grau E, Querol-Borras JM, Climent JL, Gomez E, et al. Plasma d-dimer levels correlate with outcomes in patients with community-acquired pneumonia. *Chest.* (2004) 126:1087–92. doi: 10.1378/chest.126.4.1087
9. Gacouin A, Revest M, Letheulle J, Fillatre P, Jouneau S, Piau C, et al. Distinctive features between community-acquired pneumonia (CAP) due to *Chlamydophila psittaci* and CAP due to *Legionella pneumophila* admitted to the intensive care unit (ICU). *Eur J Clin Microbiol Infect Dis.* (2012) 31:2713–8. doi: 10.1007/s10096-012-1618-6
10. Fukumoto H, Sato Y, Hasegawa H, Saeki H, Katano H. Development of a new real-time PCR system for simultaneous detection of bacteria and fungi in pathological samples. *Int J Clin Exp Pathol.* (2015) 8:15479–88.
11. Benitez AJ, Thurman KA, Diaz MH, Conklin L, Kendig NE, Winchell JM. Comparison of real-time PCR and a microimmunofluorescence serological assay for detection of *Chlamydophila pneumoniae* infection in an outbreak investigation. *J Clin Microbiol.* (2012) 50:151–3. doi: 10.1128/JCM.05357-11
12. Nieuwenhuizen AA, Dijkstra F, Notermans DW, van der Hoek W. Laboratory methods for case finding in human psittacosis outbreaks: a systematic review. *BMC Infect Dis.* (2018) 18:442. doi: 10.1186/s12879-018-3317-0
13. Langelier C, Zinter MS, Kalantar K, Yanik GA, Christenson S, O'Donovan B, et al. Metagenomic sequencing detects respiratory pathogens in hematopoietic cellular transplant patients. *Am J Respir Crit Care Med.* (2018) 197:524–8. doi: 10.1164/rccm.201706-1097LE
14. Wu XD Li YY, Zhang M, Li MM, Zhang R, Lu X, et al. Etiology of severe community-acquired pneumonia in adults based on metagenomic next-generation sequencing. A prospective multicenter study. *Infect Dis Ther.* (2020) 9:1003–15. doi: 10.1007/s40121-020-00353-y
15. Paul L, Comstock J, Edes K, Schlaberg R. Gestational psittacosis resulting in neonatal death identified by next-generation RNA sequencing of postmortem, formalin-fixed lung tissue. *Open Forum Infect Dis.* (2018) 5:ofy172. doi: 10.1093/ofid/ofy172
16. Mandell LA, Wunderink RG, Anzueto A, Bartlett JG, Campbell GD, Dean NC, et al. Infectious Diseases Society of America/American Thoracic Society consensus guidelines on the management of community-acquired pneumonia in adults. *Clin Infect Dis.* (2007) 44:S27–72. doi: 10.1086/511159
17. Basarab M, Macrae MB, Curtis CM. Atypical pneumonia. *Curr Opin Pulm Med.* (2014) 20:247–51. doi: 10.1097/MCP.0000000000000048
18. Janka GE. Familial and acquired hemophagocytic lymphohistiocytosis. *Annu Rev Med.* (2012) 63:233–46. doi: 10.1146/annurev-med-041610-134208
19. Fraeyman A, Boel A, Van Vaerenbergh K, De Beenhouwer H. Atypical pneumonia due to *Chlamydophila psittaci*: 3 case reports and review of literature. *Acta Clin Belg.* (2010) 65:192–6. doi: 10.1179/acb.2010.040
20. Haddad E, Sulis ML, Jabado N, Blanche S, Fischer A, Tardieu M. Frequency and severity of central nervous system lesions in hemophagocytic lymphohistiocytosis. *Blood.* (1997) 89:794–800. doi: 10.1182/blood.V89.3.794
21. Rousselin A, Alavi Z, Le Moigne E, Renard S, Tremouilhac C, Delluc A, et al. Hemophagocytic syndrome in pregnancy: case report, diagnosis, treatment, and prognosis. *Clin Case Rep.* (2017) 5:1756–64. doi: 10.1002/ccr3.1172
22. Chmait RH, Meimin DL, Koo CH, Huffaker J. Hemophagocytic syndrome in pregnancy. *Obstet Gynecol.* (2000) 95:1022–4. doi: 10.1016/S0029-7844(00)00834-6
23. Parrott J, Shilling A, Male HJ, Holland M, Clark-Ganheart CA. Hemophagocytic lymphohistiocytosis in pregnancy: a case series and review of the current literature. *Case Rep Obstet Gynecol.* (2019) 2019:9695367. doi: 10.1155/2019/9695367
24. Jawad S, Dhariwal A, Ellis L, Cosgrove C, Reyal Y. Haemophagocytic lymphohistiocytosis in pregnancy. *Lancet Haematol.* (2020) 7:e498. doi: 10.1016/S2352-3026(20)30101-0

25. Katsura D, Tsuji S, Kimura F, Tanaka T, Eguchi Y, Murakami T. Gestational psittacosis: a case report and literature review. *J Obstet Gynaecol Res.* (2020) 46:673–7. doi: 10.1111/jog.1421

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.

frontiers in Medicine

ORIGINAL RESEARCH
published: 20 December 2021
doi: 10.3389/fmed.2021.788758

Health Status of Patients With β-Thalassemia in the West Bank: A Retrospective-Cohort Study

*Reem Aldwaik[1], Tamara Abu Mohor[1], Israa Idyabi[1], Salam Warasna[1], Shatha Abdeen[1], Bashar Karmi[2] and Rania Abu Seir[1]**

[1] Department of Medical Laboratory Sciences, Al-Quds University, Jerusalem, Palestine, [2] Thalassemia Patients' Friends Society, Ramallah, Palestine

Management of β-thalassemia in developing countries is demanding in the absence of available therapies rather than recurrent transfusions. This study describes the characteristics and evaluates the hematological, biochemical, and hormonal findings of patients with β-thalassemia in the West Bank. We conducted a retrospective cohort study between January 2017 and December 2018. Data were collected through medical files of the patients with β-thalassemia from eight primary healthcare clinics, nine emergency departments, and 11 governmental hospitals across the West Bank. Results of the hematological, biochemical, and hormonal evaluations, in addition to demographic data and the use of iron chelation were included in the study and analyzed. A total of 309 patients with β-thalassemia were included with a male-to-female ratio of 1:1 and an average age of 23.4 ± 10.4 years. The anemic presentation was reported in 78.6% of the patients as indicated by hemoglobin level (mean ± SD = 8.4 ± 1.4 g/dl), and 73.1% had iron overload with serum ferritin (SF) levels ≥ 1,000 μg/L (mean ± SD = 317.8 ± 3,378.8 μg/L). Evaluation of the liver function tests showed that alanine transaminase (ALT) and aspartate transaminase (AST) levels were high among 38.1 and 61.2% of the patients, respectively. ALT and AST showed significant positive correlations with SF levels, while the kidney tests did not. As for iron chelation medications, patients receiving deferoxamine (26.5%) showed significantly higher SF levels compared with patients receiving deferasirox (73.5%). This study highlights the importance of establishing patient-tailored comprehensive assessment and follow-up protocols for the management of β-thalassemia with an emphasis on blood transfusion and iron chelation practices.

Keywords: β-thalassemia (β-thal), blood transfusion—adverse effects, iron overload, chelation therapy, thalassemia management

OPEN ACCESS

Edited by:
Lalitha Nayak,
Case Western Reserve University, United States

Reviewed by:
Yasser Wali,
Sultan Qaboos University, Oman
Bahador Bagheri,
Semnan University of Medical Sciences, Iran
Farzana Sayani,
University of Pennsylvania, United States

***Correspondence:**
Rania Abu Seir
rabusear@staff.alquds.edu

Specialty section:
This article was submitted to Hematology, a section of the journal Frontiers in Medicine

Received: *03 October 2021*
Accepted: *22 November 2021*
Published: *20 December 2021*

Citation:
Aldwaik R, Abu Mohor T, Idyabi I, Warasna S, Abdeen S, Karmi B and Abu Seir R (2021) Health Status of Patients With β-Thalassemia in the West Bank: A Retrospective-Cohort Study. Front. Med. 8:788758. doi: 10.3389/fmed.2021.788758

INTRODUCTION

β-thalassemia is a recessively autosomal inherited blood disorder characterized by anomalies in the production of the hemoglobin (Hb) beta chain resulting in variable degrees of hemolysis, chronic anemia, and ineffective erythropoiesis (1, 2). The broad spectrum of clinical picture of the patients ranges from the silent asymptomatic state to the lifelong transfusion-dependent anemic state, with its related complications (3). The main treatment option for most patients is supportive care consisting of blood transfusion and iron chelation therapy. Blood transfusion is used to reduce

FIGURE 1 | Map of Palestine showing the geographical distribution of patients with β-thalassemia registered in the Thalassemia Patients' Friends Society (TPFS), 2018.

anemia complications, while chelation reduces iron overload caused by chronic blood transfusions. Nevertheless, despite the significant improvements in the management of β-thalassemia, it remains a challenge, especially in low-resource countries, where the burden of thalassemia is the highest (2).

In Palestine, it was estimated that the prevalence of thalassemia carriers in Palestine was around 4% (4). Records from Thalassemia Patients' Friends Society (TPFS) showed that the prevalence of thalassemia was 17.4 per 100,000 population in 2018, with a total number of symptomatic thalassemia patients of 847 including both the West Bank and Gaza Strip. In addition, records have shown that two-thirds of the Palestinian patients were from the West Bank. Males and females had similar disease rates, and 73% of them were between 10 and 30 years old. Furthermore, among patients from the West Bank, the largest proportion was from the northern governorates (47%), while only 9% were from the central governorates, and 12% were from the south (**Figure 1**). TPFS data show that even though bone marrow transplantation is the only available curative therapy, only 4% of patients with thalassemia in Palestine underwent it (5).

Like the other developing countries, β-thalassemia is a growing health and economic problem. Consequently, significant efforts must be invested in improving the medical care of thalassemia patients to prolong and improve the quality of their life. In this study, we aim to describe the characteristics and evaluate the hematological, biochemical, and hormonal findings of patients with β-thalassemia in the West Bank, in addition to investigating the correlation between serum ferritin (SF) level, iron chelation therapy, and these parameters in order to reflect on the disease management.

MATERIALS AND METHODS

A retrospective cohort study was conducted between January 2017 and December 2018. The study utilized data from the medical files of the Ministry of Health (MOH) for 309 patients with β-thalassemia covering eight primary healthcare clinics, nine emergency departments, and 11 governmental hospitals across the West Bank. Inclusion criteria of patients in this study included diagnosis with β-thalassemia major or intermedia and receiving regular or occasional blood transfusion during the study period.

The retrieved data included the demographic characteristics of the patients, laboratory test results, and iron chelation treatment. Demographic characteristics included age, sex, and place of the treatment. Results of laboratory tests included hematological tests (Hb); biochemical tests (serum creatinine, blood urea nitrogen (BUN), alanine aminotransferase (ALT), aspartate aminotransferase (AST), serum total calcium, and SF); and hormonal tests [thyroid-stimulating hormone (TSH), total triiodothyronine (TT3), free thyroxine (FT4), and parathyroid hormone (PTH)]. Test results were categorized as normal or high according to the reference ranges reported in the Tietz Fundamentals of Clinical Chemistry and Molecular Diagnostics (6). SF and Hb levels were categorized according to guidelines of the Thalassemia International Federation (TIF) for the management of transfusion-dependent thalassemia (TDT) (7). We also retrieved data regarding the intake of iron chelation therapy. The available iron chelators in MOH are deferoxamine (DFO) and deferasirox (DFX).

TABLE 1 | Demographic characteristics and iron chelation therapy status of β-thalassemia patients.

Variable	Category	Frequency (%)
Sex	Male	154 (49.8)
	Female	155 (50.2)
Age (years)	0–9	20 (8.2)
	10–19	69 (28.3)
	20–29	110 (45.0)
	30–39	28 (11.5)
	≥ 40	17 (7.0)
Region[a]	North	197 (63.8)
	Middle	31 (10.0)
	South	81 (26.2)
Chelation status	Receiver	225 (72.8)
	Non-receiver	84 (27.2)
Type of iron chelator	Deferasirox	175 (77.8)
	Deferoxamine	63 (28.0)

[a] *North: Jenin, Tubas, Tulkarm, Nablus, Qalqiliya and Salfit; Middle: Ramallah and Jericho; South: Bethlehem and Hebron.*

The data were arranged, coded, and analyzed using the IBM Statistical Package of Social Sciences (IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.). Frequencies and percentages were calculated for the categorical variables. The continuous measures were presented as means, SD, and ranges. An independent sample *t*-test was used to measure the significance of differences between DFO and DFX receivers in terms of Hb, SF, BUN, serum creatinine, ALT, and AST. Furthermore, Pearson correlation coefficients were calculated to measure the association between Hb, SF, BUN, serum creatinine, ALT, AST, and SF levels. A $p < 0.05$ was considered statistically significant.

The study was approved by the Research Ethics Committee at Al-Quds University and the Palestinian MOH under the reference number 162/1075/2019. The data were previously anonymized and no private information were collected as part of this study; therefore, informed consent from patients was waived for this study.

RESULTS

A total of 309 patients with β-thalassemia were included in this study with a male-to-female ratio of 1:1 and an average age of 23.4 ± 10.4 years ranging from 2 to 68 years. **Table 1** shows the baseline characteristics of patients with β-thalassemia. Most of the patients were from the northern West Bank governorates (63.8%), while 26.2% were from the southern governorates. As for treatment with iron chelation therapy, 72.8% of the patients were under chelation therapy; 77.8% of those patients received DFX, while 28% received DFO (**Table 1**).

TABLE 2 | The biochemical, hematological, and hormonal baseline characteristics of β-thalassemia patients.

Indicator	Number of patients (n)	Category	Frequency *n* (%)	Mean ± SD	Range
Hemoglobin (g/dL)	285	<6	2 (0.8)	8.4 ± 1.4	5.7–16.8
		6–9	200 (77.8)		
		>9	55 (21.4)		
Serum ferritin (μg/L)	266	250– <1,000	68 (26.9)	3,175.8 ± 3,378.8	75.5–17,450.0
		1,000–2,500	84 (33.2)		
		>2,500	101 (39.9)		
Serum creatinine (μmol/L)	277	Normal	253 (99.2)	44.2 ± 17.7	17.7–123.8
		High	2 (0.8)		
BUN[a] (mmol/L)	269	Low	5 (2.0)	5.1 ± 2.4	1.3–16.2
		Normal	197 (79.8)		
		High	45 (18.2)		
ALT[b] (IU/L)	257	Normal	148 (61.9)	41.8 ± 36.5	5.0–208.3
		High	91 (38.1)		
AST[c] (IU/L)	255	Normal	92 (38.8)	52.0 ± 40.6	11.2–369.0
		High	145 (61.2)		
Serum total calcium (mmol/L)	65	Low	9 (14.0)	2.3 ± 0.3	1.4–2.6
		Normal	54 (84.4)		
		High	1 (1.6)		
PTH[d] (pmol/L)	53	Low	4 (8.0)	620.9 ± 440.6	20.0–28,00.7
		Normal	25 (50.0)		
		High	21 (42.0)		
TT3[e] (nmol/L)	71	Normal	47 (66.2)	2.9 ± 1.4	1.1–5.9
		High	24 (33.8)		
FT4[f] (pmol/L)	80	Low	3 (3.8)	3.7 ± 3.7	0.5–13.8
		Normal	50 (63.3)		
		High	26 (32.9)		
TSH[g] (mIU/L)	165	Low	1 (0.7)	3.0 ± 1.6	0.1–9.3
		Normal	133 (84.7)		
		High	23 (14.6)		

[a]BUN, blood urea nitrogen; [b]ALT, alanine transaminase; [c]AST, aspartate transaminase; [d]PTH, parathyroid hormone; [e]TT3, total triiodothyronine; [f]FT4, free thyroxine; [g]TSH, thyroid-stimulating hormone.

Table 2 demonstrates the hematological, biochemical, and hormonal baseline characteristics of patients with β-thalassemia. Among the enrolled patients, the mean SF level was 3,175.8 ± 3,378.8 μg/L, ranging from 75.5 to 17,450.4 μg/L. The majority of the patients (73.1%) had SF levels ≥ 1,000 μg/L. Among these patients, 54.6% had SF levels > 2,500 μg/L. The mean Hb level was 8.4 ± 1.4 g/dL. The majority of the patients (78.6%) showed anemic presentation with Hb levels ≤ 9 g/dL. The mean serum creatinine was 44.2 ± 17.7 (μmol/L), whereas the mean of BUN was 5.1 ± 2.4 mmol/L. In addition, we found that 99.2% of the patients had a serum creatinine level within the normal reference range, while 18.2% of the patients had high BUN levels. Evaluating liver function using test results of ALT and AST, elevated ALT levels were observed in 38.1% of the patients, and elevated AST levels were observed among 61.2%. The number of patients who had test results for serum total calcium, PTH, TT3, FT4, and TSH during the 2 years of the study was insufficient to provide a reliable estimation of the hormonal abnormalities or perform any further comparisons.

Classification of the hematological, biochemical, and hormonal baseline characteristics of patients with β-thalassemia by age showed that while patients between the ages of 20 and 29 years had the highest SF levels, most patients in all the age groups had an anemic presentation with Hb levels ≤9 g/dL. Furthermore, abnormal laboratory findings were the most commonly observed among older patients; however, this was not true in patients ≥40 years old (**Table 3**).

Table 4 demonstrates a comparison in the means of the hematological and biochemical parameters by the type of chelation therapy (DFX vs. DFO). The mean SF level of DFO receivers (6,272.8 ± 5,781.3 μg/L) was significantly higher than the mean SF level of DFX receivers (2,965.9 ± 2,755.8 μg/L), with a $p = 0.009$. Furthermore, the comparison between males and females in SF levels showed no significant statistical difference ($p = 0.973$).

Finally, we measured the correlation between hematological and biochemical parameters and SF levels using Pearson correlation. We found a significant positive correlation between

TABLE 3 | The biochemical, hematological, and hormonal baseline characteristics of patients with β-thalassemia by age.

Indicator	Category	Age group (years) *n* (%)				
		0–9	10–19	20–29	30–39	≥40
Hemoglobin (g/dL)	<6	0 (0.0)	0 (0.0)	2 (2.1)	0 (0.0)	0 (0.0)
	6–9	16 (80.0)	55 (83.3)	72 (74.2)	22 (81.5)	14 (82.4)
	>9	4 (20.0)	11 (16.7)	23 (23.7)	5 (18.5)	3 (17.6)
Serum ferritin (μg/L)	250–<1,000	6 (33.3)	15 (24.6)	21 (23.3)	5 (22.7)	5 (31.3)
	1,000–2,500	6 (33.3)	23 (37.7)	23 (25.6)	13 (59.1)	5 (31.3)
	>2,500	6 (33.3)	23 (37.7)	46 (51.1)	4 (18.2)	6 (37.4)
Serum creatinine (μmol/L)	Normal	16 (100.0)	62 (100.0)	83 (100.0)	19 (95.0)	15 (93.8)
	High	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.0)	1 (6.2)
BUN[a] (mmol/L)	Low	1 (5.9)	1 (1.7)	1 (1.2)	0 (0.0)	1 (6.2)
	Normal	14 (82.3)	49 (84.5)	61 (74.4)	14 (70.0)	12 (75.0)
	High	2 (11.8)	8 (13.8)	20 (24.4)	6 (30.0)	3 (18.8)
ALT[b] (IU/L)	Normal	10 (58.8)	40 (67.8)	38 (49.4)	14 (82.4)	10 (62.5)
	High	7 (41.2)	19 (32.2)	39 (50.6)	3 (17.6)	6 (37.5)
AST[c] (IU/L)	Normal	4 (23.5)	24 (40.7)	28 (36.4)	8 (47.1)	6 (40.0)
	High	13 (76.5)	35 (59.3)	49 (63.6)	9 (52.9)	9 (60.0)
Serum total calcium (mmol/L)	Low	0 (0.0)	2 (12.5)	4 (19.0)	1 (12.5)	1 (33.3)
	Normal	4 (80.0)	14 (87.5)	17 (81.0)	7 (87.5)	2 (66.7)
	High	1 (20.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
PTH[d] (pmol/L)	Low	0 (0.0)	0 (0.0)	2 (14.1)	1 (50.0)	1 (50.0)
	Normal	2 (100.0)	9 (64.3)	6 (42.9)	1 (50.0)	0 (0.0)
	High	0 (0.0)	5 (35.7)	6 (42.9)	0 (0.0)	1 (50.0)
TT3[e] (nmol/L)	Normal	3 (100.0)	11 (73.3)	17 (81.0)	2 (66.7)	2 (100.0)
	High	0 (0.0)	4 (26.7)	4 (19.0)	1 (33.3)	0 (0.0)
FT4[f] (pmol/L)	Low	0 (0.0)	1 (5.3)	1 (4.2)	0 (0.0)	1 (50.0)
	Normal	4 (100.0)	17 (89.4)	17 (70.8)	5 (100.0)	1 (50.0)
	High	0 (0.0)	1 (5.3)	6 (25.0)	0 (0.0)	0 (0.0)
TSH[g] (mIU/L)	Low	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Normal	8 (80.0)	32 (86.5)	44 (84.6)	11 (78.6)	10 (90.9)
	High	2 (20.0)	5 (13.5)	8 (15.4)	3 (21.4)	1 (9.1)

[a]BUN, blood urea nitrogen; [b]ALT, alanine transaminase; [c]AST, aspartate transaminase; [d]PTH, parathyroid hormone; [e]TT3, total triiodothyronine; [f]FT4, free thyroxine; [g]TSH, thyroid-stimulating hormone.

TABLE 4 | Means and SDs of all hematological and biochemical parameters for deferasirox receivers and deferoxamine receivers.

Indicator	Deferasirox (DFX)	Deferoxamine (DFO)	*P*-value
Serum ferritin (μg/L)	2,965.9 ± 2,755.8	6,272.8 ± 5,781.3	0.009*
Hemoglobin (g/dL)	8.1 ± 0.8	8.4 ± 1.2	0.152
BUN[a] (mmol/L)	5.7 ± 2.5	4.3 ± 0.04	0.597
ALT[b] (IU/L)	46.1 ± 35.6	55.3 ± 32.6	0.289
AST[c] (IU/L)	52.9 ± 30.3	71.8 ± 77.8	0.311
Serum creatinine (μmol/L)	35.4 ± 8.8	44.2 ± 8.8	0.597

*[a]BUN, blood urea nitrogen; [b]ALT, alanine transaminase; [c]AST, aspartate transaminase. *P < 0.05.*

SF and ALT ($r = 0.527$, $p < 0.0001$) and SF and AST ($r = 0.254$, $p < 0.0001$). On the contrary, we did not find any significant correlations between serum creatinine, BUN, Hb, and SF levels ($p = 0.553$, 0.280, and 0.057, respectively) (**Table 5**).

DISCUSSION

Managing β-thalassemia syndrome in the developing countries poses a major challenge to the healthcare system and a burden on the public health resources (8). Therefore, understanding the current situation should be a high priority, particularly with an emphasis on blood transfusion therapy, iron chelation therapy, and the management of associated comorbidities. Previously, a cross-sectional study conducted in Gaza Strip revealed deteriorated hematological and biochemical statuses of patients with β-thalassemia (9). This study assessed the health status of patients with β-thalassemia in the West Bank with a representative sample size in terms of age, sex, and geographical distribution. The outcome of this evaluation study could be used

TABLE 5 | The correlation between serum ferritin levels and all the hematological and biochemical parameters of patients with β-thalassemia.

Indicator	Correlation coefficient (r)	*P*-value
Hemoglobin (g/dL)	−0.125	0.057
BUN[a] (mmol/L)	−0.072	0.280
ALT[b] (IU/L)	0.527	<0.0001*
AST[c] (IU/L)	0.254	<0.0001*
Serum creatinine (μmol/L)	−0.039	0.553

[a]*BUN, blood urea nitrogen;* [b]*ALT, alanine transaminase;* [c]*AST, aspartate transaminase.*
**P < 0.05.*

to modify the existing policies in the management of patients with β-thalassemia, emphasizing blood transfusion, and iron chelation therapy practices in Palestine. In addition, our findings could roadmap the bridge between researchers and healthcare professionals to ensure the best potential patient outcomes.

The advent of safe blood transfusion with the adjuvant of the chelation therapy has significantly increased life expectancy of the patients, with the close monitoring and following-up programs of the patients with β-thalassemia can now survive into the fourth and fifth decades of their lives (10, 11). In this study, a total of 309 patients with β-thalassemia representing the West Bank were included. Comparing our sample demographics to other areas, the average age of patients with β-thalassemia in the neighborhood countries such as Lebanon was similar to ours (23.4 ± 10.4 years in the West Bank compared with 21.0 ± 11.0 years in Lebanon) (12). On the contrary, a better life expectancy was reported in Taiwan, with an average age of 37.8 ± 23.7 years (10). Furthermore, the high burden of β-thalassemia in the West Bank, especially in the north, could be explained by the preference for traditional consanguineous marriages and the large family sizes associated with social and cultural considerations (13, 14).

Blood transfusion is lifesaving for patients with β-thalassemia. The main goal of blood transfusion is the correction of anemia, and the decision to start transfusion therapy should be mainly based on the assessment of Hb levels (2). For accurate monitoring of the effectiveness of transfusion therapy, continuous follow-up is required at each transfusion for pre- and post-transfusion Hb, hematocrit of the blood unit, daily Hb fall, and the transfusional interval (2). According to recommendations of TIF, the current acceptable mean Hb is 12 g/dl with a post-transfusion Hb of 14–15 g/dl and a pretransfusion Hb of 9–10.5 g/dl (7). Among the patients in our study, an anemic presentation was widely observed with a mean Hb during the 2-year follow-up of 8.4 ± 1.4 g/dl. These low levels of Hb indicate poor management of transfusion treatment regimens, lack of adherence to international guidelines, and possibly failure to achieve a regular adherence to the treatment by patients. Similar to our findings, low Hb levels were seen among patients with β-thalassemia in Egypt (8.2 ± 1.8 g/dl) (15), Jordan (8.9 ± 2.4 g/dl) (16), and Pakistan (7.7 ± 0.1 g/dl) (17).

Even though blood transfusion is lifesaving for patients with β-thalassemia, it burdens the body with excess iron that ultimately results in hemosiderosis and other related comorbidities leading to consequential and irrevocable biological damages (9, 18). Based on Guidelines for managing TDT of TIF, monitoring body iron levels by measuring liver iron concentration through MRI-based methods is recommended to maintain adequate and safe management (7). However, MRI-based methods are not yet available in the West Bank. On the other hand, serial measurement of SF concentration is the most commonly reliable non-invasive method used to monitor iron load. Therefore, it is used as a reasonable estimate of the total body iron. However, as a single value, SF could be influenced by factors such as liver disease, inflammatory disorders, and cancer, and it is not a good indicator of myocardial iron overload (2, 19). Nevertheless, SF might be the only feasible test for measuring iron burden and efficacy of chelation therapy in the developing countries (20).

In this study, the mean SF level of our patients with β-thalassemia was markedly higher than the cutoff point recommended by the Guidelines of TIF for the management of TDT (7), which must be maintained below 1,000 μg/L (3,175.8 ± 3,378.8 μg/L). In addition, SF did not vary significantly in terms of sex. Similar to our findings, high SF levels were reported among Pakistani patients with thalassemia 3,087.6 ± 1,625.0 μg/L (21). In contrast, in the developed countries such as France, better SF levels were reported among their patients with β-thalassemia (1,240 μg/L) (22). High SF levels indicate improper or unmonitored blood transfusion and/or therapeutic chelation practices (23, 24).

With the support of blood transfusion, iron chelation therapy is the best feasible choice to decrease comorbidities and increase life expectancy of the patients (25). Worldwide, the available iron chelators are DFO, DFX, and DFP. In the West Bank, only DFO and DFX are included in the Essential Drug List; however, availability of drugs in MOH is such a dilemma due to the high cost; therefore, these drugs are usually provided as donations from other countries. As a result, drug availability is an important factor affecting the choice of drug of the clinicians and the provision of the prescribed medication for the patients on time to use (26). In this study, we found that most of the patients (72.8%) received chelation therapy during the study period. Among those, 77.8% were prescribed DFX and 28% were prescribed DFO. Comparison of SF levels among our patients revealed that the mean SF level for DFO receivers (6,272.8 ± 5,781.3 μg/L) was significantly higher than the mean SF level for DFX receivers (2,965.9 ± 2,755.8 μg/L).

In comparison, DFO is the standard iron chelator in Gaza, and dramatically higher SF levels were reported 7,115.6 ± 3,561.7 μg/L (9). Studies have shown that DFO, administered subcutaneously or intravenously, has a short half-life. In addition, DFO has the lower efficacy with a low-compliance rate because of the dose-related toxicity and the associated discomfort during administration (20, 27). DFX has a favorable safety profile since it is an oral suspension with less toxicity and higher compliance and efficacy compared to DFO, but it is an expensive chelator (28). Experimental trials showed that using DFX and DFO combined results in excreting more iron without any noticeable significant complications (29). Thus, this approach

can be promising, especially in the poor-developing countries with the limited resources such as Palestine. However, further research is required to understand the factors affecting response to iron chelation therapy, including indications for starting and adjusting treatment regimen, dosage, drug availability, and compliance of the patient.

In transfusion-dependent patients with β-thalassemia, the liver is the first affected organ by the toxic effect of excess iron on hepatocytes. The prevalence of the impaired liver function is estimated at 40.5% (30, 31). In our study, liver dysfunction was manifested by the elevated levels of ALT and AST among our patients. This elevation was significantly correlated with an increase in SF levels. A previous study conducted in India reported similar findings. The study showed that once SF levels increase above 1,000 ng/ml and the total number of blood transfusions goes beyond 30 times, the liver impairment starts (30). Therefore, monitoring and evaluating hepatic iron overload is vital to protect the liver and the rest of the body (32). In addition to iron overload, elevation in the levels of liver enzymes in both the transfusion-dependent and transfusion-independent patients with β-thalassemia might be attributed to other factors such as viral infection (hepatitis B and C viruses), hepatic siderosis, bile obstruction, portal fibrosis, and even cirrhosis (30).

The renal dysfunctions, including the renal tubular and glomerular injuries were reported to be the fourth most common morbidity of β-thalassemia (4%) (31). Several studies suggested the involvement of iron chelators in the pathophysiology of the renal disease among patients with thalassemia (31). In our study, most of the enrolled patients had normal serum creatinine levels, and only 18.2% of them had a slight increase in BUN levels. In addition, there was no statistically significant association between SF levels and BUN or serum creatinine. Similarly, the reported BUN and serum creatinine levels were within the normal ranges in a study conducted in Iran, indicating that the function of the renal tubular and glomerular was not adversely affected by iron overload (33).

Endocrinopathies are the most common complications of β-thalassemia syndrome and iron overload, with a prevalence of 44.7% (34). Endocrinopathies could result in the delayed puberty and hypogonadism (40.5%), short stature and retarded growth (30.8%), impaired glucose tolerance, and diabetes mellitus (9.9%), hypoparathyroidism (6.9%), hypothyroidism (3.2%), and adrenal insufficiency (7). Despite the high rates of endocrinopathies and the broad spectrum of associated comorbidities, we found that a small proportion of patients with β-thalassemia in West Bank had their endocrine functions evaluated. In addition, among the patients who underwent testing for endocrinopathies, the performed tests were not enough to make an interpretation or to establish any diagnosis. Although this study did not provide enough data to estimate the burden of endocrinopathies among patients in West Bank, the absence of regular endocrine assessment and follow-up was highlighted as one of the significant gaps in the management of β-thalassemia. The continuous evaluation of the toxic effect of the iron overload on endocrinopathies could be accomplished through regular monitoring of signs and symptoms of endocrine complications. Together with the appropriate iron chelation practice, continuous monitoring could ensure early diagnosis, prevention, and timely treatment of such complications (7, 35).

Classification of the hematological, biochemical, and hormonal baseline characteristics of patients with β-thalassemia by age showed that the older patients generally had a higher prevalence of complications and worse profiles. However, this was not true among patients ≥40 years old, which could be because patients with less severe phenotypes of thalassemia survive longer. Furthermore, poor management of transfusion therapy and persistent anemia at the young ages would affect growth of children, resulting in the worst disease outcomes later in the life.

In summary, this study showed that regardless of the provision of blood transfusion services and iron chelation therapies, the lack of standardized protocols for the management of thalassemia is a major problem in West Bank. For the majority of the patients, Hb levels were far below the international recommendations, and uncontrolled SF levels were found, reflecting the absence of evidence-based administration of blood transfusion and iron chelation therapy. Furthermore, the data indicated improper assessment and follow-up for comorbidities associated with iron overload. It is also important to remember that blood transfusion is associated with other adverse outcomes such as transfusion reactions; the development of allo- and autoantibodies, which can further complicate thalassemia management; and blood-borne infectious diseases such as hepatitis and HIV.

The limitations of this study were mainly derived from the lack of adequate care. Furthermore, we did not include patients from Gaza Strip in this study. The management of patients with thalassemia in Gaza Strip is affected by a broader range of factors, including the political and economic situation of the area limiting their access to healthcare and access to testing and treatment. Moreover, a major limitation in this study was the incomplete patient information on the health information system, which is mainly attributed to the fragmentation of the healthcare system. The fragmentation of the healthcare information system poses a serious challenge to managing patients with thalassemia in Palestine.

CONCLUSIONS

In conclusion, these findings highlight the need for the comprehensive assessment protocols and evidence-based practices as they are crucial for the reduction of β-thalassemia-associated morbidity and mortality. Therefore, regular evaluation and follow-up with emphasis on blood transfusion and iron chelation practices are highly recommended to improve clinical picture of the patients, life expectancy, and quality of life. Also, the treatment has to be tailored for each patient due to the wide heterogeneity of β-thalassemia. In addition, despite being the only available curable treatment, stem cell transplantation is not feasible in our country. Thus, establishing advocacy programs aiming to increase adherence of the patients to treatment and follow-up programs is a high priority. This could be achieved through the collaboration of a national multidisciplinary team

consisting of the hematologists, endocrinologists, cardiologists, ophthalmologists, dentists, psychologists, social workers, and dietitians. Finally, it is noteworthy to mention that the absence of evidence-based transfusional regimens and follow-up protocols are not the only gaps in the management of patients with thalassemia. Furthermore, although genetic testing of thalassemia is becoming important, especially in complex atypical thalassemias (36), molecular diagnosis is rarely performed. This service is not provided in MOH facilities, which is another gap in managing patients with thalassemia in West Bank.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Research Ethics Committee (REC), Al-Quds University, Jerusalem, Palestine. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

RAb conceived and designed the study and reviewed the final draft. BK facilitated the process of data collection through the MOH and contributed to confirming the cases selected in the study and interpretation of the results. RAl, TA, II, SA, and SW were responsible for the data collection, entry, analysis, and interpretation. RAl drafted the manuscript. All the authors contributed to manuscript revision, read, and approved the submitted version.

ACKNOWLEDGMENTS

We gratefully acknowledge the Palestinian Ministry of Health and the Thalassemia Patients' Friends Society for providing permission to medical files of patients, and for providing the updated demographic statistics of the Palestinian patients with β-thalassemia.

REFERENCES

1. Martin A, Thompson AA. Thalassemias. *Pediatr Clin North Am.* (2013) 60:1383–91. doi: 10.1016/j.pcl.2013.08.008
2. Galanello R, Origa R. Beta-thalassemia. *Orphanet J Rare Dis.* (2010) 5:11. doi: 10.1186/1750-1172-5-11
3. Tarazi I, Al Najjar E, Lulu N, Sirdah M. Obligatory premarital tests for β-thalassaemia in the Gaza Strip: evaluation and recommendations. *Int J Lab Hematol.* (2007) 29:111–8. doi: 10.1111/j.1751-553X.2006.00836.x
4. Al Sabbah H, Khan S, Hamadna A, Abu Ghazaleh L, Dudin A, Karmi BA. Factors associated with the continuing emergence of beta-thalassemia major despite prenatal testing: a cross-sectional survey. *Int J Womens Health.* (2017) 9:673–9. doi: 10.2147/IJWH.S141936
5. (TPFS) TPFS. *Thalassemia Patients in 2019* (2019). Available online at: https://tpfs.ps/lang/ar (accessed December 18, 2020).
6. Burtis C, Bruns D. *Tietz Fundamentals of Clinical Chemistry and Molecular Diagnostics, 7th ed.* Amsterdam: Elsevier (2015).
7. Cappellini MD, Cohen A, Porter J, Taher A, Viprakasit V. *Guidelines for the Management of Transfusion Dependent Thalassaemia (TDT)*. Nicosia, Cyprus: Thalassaemia International Federation (2014).
8. Rahman Mu, Lodhi Y. Prospects & future of conservative management of beta thalassemia major in a developing country. *Pak J Med Sci.* (2004) 20:105–12. Available online at: http://pjms.com.pk/issues/aprjun04/pdf/article4.pdf
9. Ayyash H, Sirdah M. Hematological and biochemical evaluation of β-thalassemia major (βtm) patients in gaza strip: a cross-sectional study. *Int J Health Sci.* (2018) 12:18.
10. Tang C-H, Furnback W, Wang BC, Tang J, Huang VW-H, Tang D, et al. *Greater Red Blood Cell Transfusion Burden Is Associated with More Healthcare Resource Utilization in Patients with Beta-Thalassemia*. Washington, DC: American Society of Hematology (2019).
11. Poggiali E, Cassinerio E, Zanaboni L, Cappellini MD. An update on iron chelation therapy. *Blood Transfus.* (2012) 10:411.
12. Inati A, Zeineh N. Isma'Eel H, Koussa S, Gharzuddine W, Taher A. *β*-Thalassemia: the lebanese experience. *Clinic Lab Haematol.* (2006) 28:217–27. doi: 10.1111/j.1365-2257.2006.00792.x
13. Williams TN, Weatherall DJ. World distribution, population genetics, and health burden of the hemoglobinopathies. *Cold Spring Harb Perspect Med.* (2012) 2:a011692. doi: 10.1101/cshperspect.a011692
14. Rajech S, Esmael A, Mohamad K, Ahmad S-M, Abdussalam A, Muhammad M. consanguinity and its effect on morbidity and congenital disorders among arabs in Israel. *Human Genetic Diseases IntechOpen.* (2011) 11:23970. doi: 10.5772/23970
15. Hassan MAM, Tolba OA. Iron chelation monotherapy in transfusion-dependent beta-thalassemia major patients: a comparative study of deferasirox and deferoxamine. *Electron Physic.* (2016) 8:2425. doi: 10.19082/2425
16. Shams S, Ashtiani MTH, Monajemzadeh M, Koochakzadeh L, Irani H, Jafari F, et al. Evaluation of serum insulin, glucose, lipid profile, and liver function in β-thalassemia major patients and their correlation with iron overload. *Lab Med.* (2010) 41:486–9. doi: 10.1309/LMS0EOOUZSII2BNE
17. Asif M, Manzoor Z, Farooq MS, Kanwal A, Shaheen U, Munawar SH, et al. correlation between serum ferritin level and liver function tests in thalassemic patients receiving multiple blood transfusions. *Int J Res Med Sci.* (2014) 2:988. doi: 10.5455/2320-6012.ijrms20140853
18. Habibzadeh F, Yadollahie M, Merat A, Haghshenas M. Thalassemia in Iran; an overview. *Arch Iran Med.* (1998) 1:27–33.
19. Paul A, Thomson VS, Refat M, Al-Rawahi B, Taher A, Nadar SK. Cardiac involvement in beta-thalassaemia: current treatment strategies. *Postgrad Med.* (2019) 19:1–7. doi: 10.1080/00325481.2019.1608071
20. Hoffbrand AV, Taher A, Cappellini MD. How I treat transfusional iron overload. *Blood.* (2012) 120:3657–69. doi: 10.1182/blood-2012-05-370098
21. Rehim MH, Mustafa S, Rizvi SKA. Correlation between serum TSH and serum ferritin in patients of beta thalassemia major. *Pak J Med Health Sci.* (2017) 11:266–9. Available online at: https://www.pjmhsonline.com/2017/jan_march/pdf/266.pdf
22. Thuret I, Pondarré C, Loundou A, Steschenko D, Girot R, Bachir D, et al. Complications and treatment of patients with β-thalassemia in France: results of the National Registry. *Haematologica.* (2010) 95:724–9. doi: 10.3324/haematol.2009.018051
23. Mentzer WC, Kan YW. Prospects for research in hematologic disorders: sickle cell disease and thalassemia. *JAMA.* (2001) 285:640–2. doi: 10.1001/jama.285.5.640
24. Al Haddad RM. "Molecular, Biochemical and Hematological Investigations of b-Thalassemic Children in Gaza Governorate," In: *Molecular, Biochemical and Hematological Investigations of b-Thalassemic Children in Gaza Governorate* (2012).

25. Rachmilewitz EA, Giardina PJ. How I treat thalassemia. *Blood.* (2011) 118:3479–88. doi: 10.1182/blood-2010-08-300335
26. Pharmacy GDo. *Iron Accumulation in Thalassemic Patients: State of Palestine Ministry of Health.* (2015). Available online at: http://pharmacy.moh.ps/index/ArticleView/ArticleId/132/Language/ar (accessed December 20, 2020).
27. Saliba AN, Harb AR, Taher AT. Iron chelation therapy in transfusion-dependent thalassemia patients: current strategies and future directions. *J Blood Med.* (2015) 6:197–209. doi: 10.2147/JBM.S72463
28. Cappellini MD. Exjade®(deferasirox, ICL670) in the treatment of chronic iron overload associated with blood transfusion. *Ther Clin Risk Manag.* (2007) 3:291. doi: 10.2147/tcrm.2007.3.2.291
29. Arandi N, Haghpanah S, Safaei S, Zahedi Z, Ashrafi A, Eatemadfar P, et al. Combination therapy–deferasirox and deferoxamine–in thalassemia major patients in emerging countries with limited resources. *Transfus Med.* (2015) 25:8–12. doi: 10.1111/tme.12188
30. Suman L, Sanadhya A, Meena P, Goyal S. Correlation of liver enzymes with serum ferritin levels in β-thalassemia major. *Int J Res Med Sci.* (2016) 4:3271. doi: 10.18203/2320-6012.ijrms20162278
31. Demosthenous C, Vlachaki E, Apostolou C, Eleftheriou P, Kotsiafti A, Vetsiou E, et al. Beta-thalassemia: renal complications and mechanisms: a narrative review. *Hematology.* (2019) 24:426–38. doi: 10.1080/16078454.2019.1599096
32. Soliman A, Yassin M, Al Yafei F, Al-Naimi L, Almarri N, Sabt A, et al. Longitudinal study on liver functions in patients with thalassemia major before and after deferasirox (DFX) therapy. *Med J Hematol Infect Dis.* (2014) 6:25. doi: 10.4084/mjhid.2014.025
33. Nickavar A, Qmarsi A, Ansari S, Zarei E. Kidney function in patients with different variants of beta-thalassemia. *Iran J Kidney Dis.* (2017) 11:132. Available online at: http://www.ijkd.org/index.php/ijkd/article/view/2865/909
34. Mokhtar GM, Gadallah M, El Sherif NH, Ali HT. Morbidities and mortality in transfusion-dependent beta-thalassemia patients (single-center experience). *Pediatr Hematol Oncol.* (2013) 30:93–103. doi: 10.3109/08880018.2012.752054
35. Filosa A, Di Maio S, Aloj G, Acampora C. Longitudinal study on thyroid function in patients with thalassemia major. *J Pediatr Endocrinol Metabol.* (2006) 19:1397–404. doi: 10.1515/JPEM.2006.19.12.1397
36. Brancaleoni V, Di Pierro E, Motta I. Cappellini MD. Laboratory diagnosis of thalassemia. *J Lab Heamtol.* (2016) 38:32–40. doi: 10.1111/ijlh.12527

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.

frontiers in Medicine

BRIEF RESEARCH REPORT
published: 01 March 2022
doi: 10.3389/fmed.2022.772886

Transfusion Complications in Thalassemia: Patient Knowledge and Perspectives

Sashoy Patterson[1]*, *Ashley Singleton*[1], *Jane Branscomb*[1], *Vivien Nsonwu*[2] and *Regena Spratling*[3]

[1] Georgia Health Policy Center, Andrew Young School of Policy Studies, Georgia State University, Atlanta, GA, United States, [2] School of Medicine, Meharry Medical College, Nashville, TN, United States, [3] Byrdine F. Lewis College of Nursing and Health Professions, Georgia State University, Atlanta, GA, United States

Chronic transfusion, used to manage clinically significant forms of thalassemia, carries complication risks including iron overload, alloimmunization, and allergic or hemolytic reactions. Dissemination and implementation of evidence-based guidelines for minimizing these risks are complicated by the small numbers and increasing dispersion of the thalassemia population. This elevates patients' role in understanding and communicating with providers about treatment. The present study sought to assess thalassemia patients' knowledge of transfusion, complications, and guidelines; their experience of clinicians' following guidelines; and their perspectives on ways to store and share personal transfusion data. A convenience sample of 32 patients participated in structured interviews. Most, if not all, understood iron overload, chelation therapy, blood typing, and cross-matching. Awareness of each of five of evidence-based transfusion guidelines that were discussed ranged from 72 to 88%. Patients expressed privacy concerns about each of three data storage options, slightly less for a registry than for a wallet card or smartphone app. The registry also avoided concerns that the other options placed extra burden on patients. Recommendations include increased education on the meaning and significance of packed, washed red blood cells, leuko-reduction, and detailed cross-matching, and implementation of a nationwide registry to make transfusion data available to providers anywhere at the point of care. Registry implementation should be sensitive to patients' privacy and security concerns, but also help them appropriately weigh those against safety benefits. These actions could help reduce transfusion complications in thalassemia by improving patient efficacy and increasing adherence to evidence-based guidelines.

Keywords: thalassemia, transfusion, transfusion guidelines, transfusion reactions, transfusion complications, patient knowledge

OPEN ACCESS

Edited by:
Robert W. Maitta,
Case Western Reserve University,
United States

Reviewed by:
Aranzazu Sancho-Lopez,
Hospital Universitario Puerta de Hierro Majadahonda, Spain
Mohamed A. Yassin,
Hamad Medical Corporation, Qatar

***Correspondence:**
Sashoy Patterson
spatterson8@gsu.edu

Specialty section:
This article was submitted to Hematology, a section of the journal Frontiers in Medicine

Received: *04 October 2021*
Accepted: *19 January 2022*
Published: *01 March 2022*

Citation:
Patterson S, Singleton A, Branscomb J, Nsonwu V and Spratling R (2022) Transfusion Complications in Thalassemia: Patient Knowledge and Perspectives. Front. Med. 9:772886. doi: 10.3389/fmed.2022.772886

INTRODUCTION

Thalassemia describes a group of inherited blood disorders associated with the alpha and beta globulins of hemoglobin. Alpha thalassemia and beta thalassemia result from the reduction of either alpha or beta globulins, respectively. Both types of thalassemia result in moderate to severe anemia and extramedullary hematopoiesis, leading to signs and symptoms such as poor growth and

development; skeletal deformities; thrombosis; pain in the head, back, and legs; impaired cardiac function; hepatosplenomegaly; non-transfusional iron overload; and conditions such as hydrops fetalis (1–4). It is estimated that 300,000 to 400,000 babies are born worldwide with hemoglobinopathies, with approximately 23,000 per year characterized as thalassemia major, the most severe form of thalassemia (5). Though cases of thalassemia worldwide typically concentrate around the "thalassemia belt," immigration into the United States from Italy, Greece, Pakistan, Iran, India, and particularly central and southeast Asian countries has markedly increased the prevalence of thalassemia in this country (6–8).

Alpha thalassemia major is often incompatible with life. In cases of severe anemia in beta thalassemia major, individuals are usually diagnosed within the first 2 years of life and may survive with chronic, life-long blood transfusions (1, 3, 5, 9, 10). Unfortunately, transfusions have risks and complications. Iron overload and resulting endocrine complications, erythrocyte alloimmunization, transfusion reactions, and infections are all risks for thalassemia patients who are dependent on blood transfusions (5, 8). These complications, however, are largely preventable (11). If patients know their personal transfusion histories and are aware of key clinical practice recommendations, they can contribute to reducing their complication risk through their actions and through their communications with providers (3, 4, 8).

Little literature exists on thalassemia patients' self-reported knowledge and experience regarding transfusion complications and practices to lower risk. One study described the physical, psychosocial, and economic impacts of transfusion reported by 32 thalassemia patients, as well as challenges those patients encountered during transfusion procedures (12). The authors did not identify any studies addressing patient knowledge specific to transfusion complications, related practice guidelines, or patients' preference of data storage methods to improve transfusion safety.

The current study addresses these gaps in evidence, seeking to (1) describe knowledge and experience of transfusion among transfused thalassemia patients to inform patient education and outreach; (2) characterize patients' transfusion experience in relation to providers' adherence to recommended practices to inform provider education and outreach; and (3) assess patients' preferences regarding personal data tracking to inform methods for ensuring that providers have access to current, accurate transfusion histories to inform real-time decisions. The study's aim is to improve dissemination and implementation of best practices for reducing the risk of transfusion complications in thalassemia.

METHODS

Study Design

This descriptive study collected data from individuals with thalassemia in the United States who received prior therapeutic transfusions. Structured interviews included questions eliciting participants' knowledge and experience of transfusions; their experience of providers' performance of key actions recommended in applicable guidelines published by the Thalassemia International Federation [TIF; (13)]; and their preferences and feedback regarding possible ways to store and share their personal transfusion data.

The interview guide (see **Supplementary Material**) was developed by an interdisciplinary team including thalassemia and transfusion medicine clinical specialists, qualitative researchers, and thalassemia patient advocates. It consisted of three parts: (1) six open-ended questions about patient knowledge and experience of transfusions; (2) patient review and reflection on a brief, lay-language summary of key TIF recommendations for transfusion in thalassemia; and (3) reaction to three methods for storing and sharing personal transfusion data: a smartphone app, a wallet card, or a patient or transfusion registry. These options were described on the handout with practice recommendations. To minimize the time burden on participants and staff, and to support participants' confidence in their anonymity, thus improving candor and reducing social desirability bias, the interview guide did not collect demographic information.

The same interdisciplinary team translated relevant TIF guidelines into the short, lay-language handout mentioned above (see **Supplementary Material**), which was shared with participants before asking questions about their experience of providers' following those practices. Accessibility to a wide patient audience was validated by a Flesch-Kincaid Grade level of 8.7. Medical accuracy was confirmed by clinical specialists in thalassemia and transfusion on the team. Patient advocates helped assure patient-centered language. The study was approved for human subject research through the Georgia State University Institutional Review Board and all participants gave informed consent.

Recruitment

Inclusion criteria, all confirmed by self-report, included a thalassemia diagnosis, being at least 13 years of age, and having received at least one blood transfusion as a treatment for thalassemia. These criteria were chosen to capture a wide range of participants who could reflect on and communicate their experiences. The Cooley's Anemia Foundation, a nationwide thalassemia patient advocacy group, assisted in recruiting a convenience sample of participants meeting inclusion criteria. The Foundation shared the approved recruitment flier through its social media channels and email listserv. Interested individuals followed an embedded link to an online form where they provided availability and contact information. A researcher followed up to schedule the interview at a mutually convenient time. Consent was obtained at the time of the telephone interview. If the participant was a minor, a parent or guardian was required to be present for the interview and provide consent, while assent was obtained from the minor. Participants received a $25 gift card in consideration of their time.

Data Collection

Data collection took place in June and July 2018. All interviews were conducted one-on-one in English, over the telephone from the work office of one of three researchers. The goals of the study were explained prior to the commencement of the interview,

with consent obtained to continue. Interviews took ∼30–40 min and were audio-recorded. Recorded interviews were transcribed verbatim and de-identified for review and analysis. Interview recordings and transcripts with personal identifiers were stored on a secured server in password protected files accessible only to IRB-approved researchers.

Analysis

Data analysis was guided by the "Sort and Sift: Think and Shift" method (14, 15). This qualitative approach combines tenets of phenomenology, grounded theory, and narrative inquiry, all of which use an iterative process for data analysis that facilitates the merging of findings among the research team. The analysis followed five steps: (1) create a neutral domain name corresponding to each interview question; (2) create a summary template for use by the team; (3) assess the summary template; (4) establish consistency in template use across the team; and (5) transfer summaries into a matrix with rows for each respondent and columns for each domain to synthesize results (14).

Researchers created short domain names corresponding to each interview question. For example, the question, "What information have you been given about transfusions?" was represented by the domain name "transfusion information received." Next, an interview summary template was drafted to include each domain name. Team members used this template to code an initial set of transcripts. The team met to determine whether the domain names were intuitive, accurate, and complete; to assess the ease of use of the template and length of time required; and to reach consensus on guidelines for consistent coding and template use–for example, the type and length of quotes to include on the form. Finally, the team coded the remaining transcripts and compiled the results into a single document for synthesis. Results were summarized for each domain, noting both common and unique responses to reflect the breadth of perspective and experience across the sample.

RESULTS

Sample Characteristics

Thirty-two individuals participated in the study. Though weighted to the East and West coasts of the United States, the sample included participants from across the country: Alaska, Arizona, California, Florida, Kansas, Maryland, Massachusetts, Michigan, Missouri, Nevada, New Jersey, New York, North Carolina, Pennsylvania, Tennessee, and the District of Columbia. State of residence and spontaneous comments indicate that the sample included participants from both high- and low-population density areas. All participants were able to communicate effectively in English. One participant was a minor, who participated with a parent present; all other participants were adults.

The Transfusion Experience

Respondents shared reflections on their experience before, during, and after receiving a blood transfusion. All participants were able to describe the frequency of their transfusions, the blood typing and crossmatching process prior to transfusion, and at least some potential transfusion complications, most notably iron overload. They described the process as lengthy, while also acknowledging the importance of transfusion for their well-being.

Frequency

Most participants stated that they receive transfusions every 2–4 weeks, although one participant described receiving transfusions when they feel like they need to or when they are "low on blood". All participants managed the scheduling of their transfusions themselves, in consultation with their provider.

Blood Typing and Crossmatching

On average, participants described receiving blood typing and crossmatching 1–3 days before the transfusion. One participant explained, "I go into the hospital, and they already have the blood ready for me, because I was typed and crossed 2 days prior". Another participant said they "go into the lab 2 days prior to my transfusion to get my blood drawn so they can crossmatch me, because I have antibodies in my blood and it's harder for them to find blood to match. So they need that extra day for me." Another participant, who reported having received blood transfusions for 29 years, described a specific schedule: "I go on Tuesdays for...crossmatching, and then Fridays for the blood".

Length

Most participants described long wait times during the transfusion: "They should get me a chair and it's about three and a half hours, I sit there, and I get the blood", said one participant. Another said, "It's time consuming. I have to make sure... I have the day off, or I can...be able to be in the hospital all day." A participant who reported receiving transfusions from the same hospital since the age of five described planning for activities to do during the transfusion. Their suggestion: "Bring like fun things to do, because you're... just gonna sit in a bed or a chair for the next 6 h, and it's a little boring." Respondents mentioned doing work, watching TV, and "hanging out" as ways they pass the time during transfusion.

Complications

All participants acknowledged potential risks of receiving blood transfusions, most commonly iron overload. One explained, "Some of the bad things are iron overload. I already have a huge iron overload in my body, and so, you know, getting transfusions is just adding to it". Some participants suggested ways to improve the transfusion process to address safety or convenience issues, though acknowledging that some of the ideas might not be feasible. Suggestions included improving tests for viruses in donor blood; transfusing with blood that has the iron removed; transfusing with blood that has extended blood cell life; receiving transfusions at home; and any methods that could shorten the transfusion process.

Importance

Most participants expressed that for them, transfusions are essential for staying alive. One stated, "You have to get blood. You don't have a choice, you know... If you don't get blood, eventually your blood count will go so low that, you know, you

will die without the blood." A few participants also described feeling better after individual transfusions, as in this comment: "Before transfusion I get really tired and barely can focus; and after I get transfusion, I'm more energized and I can focus better".

Advice and Concerns

All participants shared advice on what a patient should know before receiving a transfusion. Most said that patients should be informed about the process, possible complications, and what the patient can do to avoid complications. A participant noted, "It's an important part that every patient knows what's happening, what pre-medications are you getting, what type of blood are they getting, just be willing to ask questions. And then, also, to know their own blood type and their own orders, so that if anything is different from what they were expecting, again, they can ask the question." Other recurring sentiments were that a patient should "just kind of relax," "be prepared to be there for a long time," and again, "Don't be afraid to ask questions".

Some respondents expressed satisfaction with their care and viewed their experiences positively. Others shared concerns about a lack of specialized medical providers and resources, particularly in some geographic areas. One participant said that this makes it even more important for the patient to be involved in their care by asking important questions. "I would be worried getting a transfusion at a small-town hospital. I guess those people, if they're doing that, they just really need to ask those questions that you asked me: Are they processing it this way and that way? And they need to know what diseases they're checking the blood for". A few participants suggested a need for more providers who are knowledgeable about thalassemia. One said, "It's the subpar training of nurses or physicians. If you're in a smaller town, and I'm sure it's even worse in towns that are smaller than mine, just a lack of knowledge that people, you know, have had about this condition means you have to be your own health care advocate during blood transfusion".

Knowledge of Transfusion Recommendations

All participants were asked about five key TIF guidelines for reducing transfusion complications in transfusion-dependent thalassemia. The lay language versions of these are shown in **Table 1** along with summarized results of patient knowledge of and experience with each. Most, but not all, participants were knowledgeable and aware of all five guidelines. Recommendations 3 and 5 were the least well recognized among the participants. Nearly one-third of participants (9 of 32) were unfamiliar with or unclear on the specifications "leukocytes removed," "washed," and "packed" red blood cell units. Some participants expressed a belief that recommendations 1 and 2 are standard procedure in all transfusion settings; however, the literature does not corroborate this (3, 16).

Perspectives on Data-Tracking Approaches

Study participants were asked to consider three general methods of data tracking: a smartphone app, a wallet card, or a transfusion registry. Confidentiality and privacy were common concerns; however, they led respondents to different conclusions. Some expressed worry about their wallet or phone being lost or stolen or of the app being hacked. Some wondered about the security of a registry; one expressed the opinion that a registry might be more secure than a smart-phone app. Specific feedback on tracking methods is presented in **Table 2**.

DISCUSSION

Implications

The purpose of this study was to describe thalassemia patents': (1) knowledge and experience of transfusions and their complications; (2) knowledge of and experience in relation to their providers' adherence to recommended transfusion practices; and (3) preferences regarding possible methods to store and share personal transfusion data. Observed knowledge gaps, reported experiences, and the perspectives patients shared all suggest opportunities for intervention to improve transfusion safety.

Transfusion Complications

Nearly all participants were aware of iron overload as a potential transfusion complication, and of chelation therapy to manage it. The clarity displayed by this majority about the importance of adherence to prescribed chelation therapy suggests that patients could be powerful spokespersons for delivering this message to peers. At the same time, the fact that a minority in the sample knew of this serious complication but not of its management indicates a need for increased education on chelation. Patients were familiar with concepts of mild transfusion reactions; but less mention was made regarding hemolytic transfusion reactions. This may suggest a need for more education among thalassemia patients about the potential for alloimmunization and the goals of antibody screens and crossmatching. Some concern was expressed about infection, a relatively low risk, suggesting that a realistic understanding of the degree of safety of the blood supply continues to be important for the thalassemia community, as it is for the public at-large.

Practice Guidelines

In rare diseases such as thalassemia, patient knowledge and self-advocacy can be important factors in receiving appropriate care. This makes translation of practice guidelines into lay language for affected populations a vital component of guideline dissemination and implementation for such conditions. Participants in the current study noted, for example, that those in small towns, where providers familiar with thalassemia may be far away, have increased burden for understanding their condition, knowing their medical history, and asking the right questions. Telementoring by peers could be a valuable resource for such individuals.

Most patients in the current study were aware of the five practice guidelines. Their misconceptions about the universal practice of the first two, as noted above, suggests that patients be encouraged to remain vigilant about them. Of all five recommendations, patients were most well-versed in the fourth one, regarding pre-medication to avoid allergic reactions. They

TABLE 1 | Knowledge of transfusion guidelines.

Guideline (lay version)	Summary of findings	Example quotes
1) Wherever you go for a transfusion, doctors should have access to a record of your transfusion history. It should show up-to-date information on how often you get transfused, your blood type, and any antibodies or transfusion reactions you have developed.	Five of 32 study participants (16%) were unfamiliar with this recommendation. They agree that it should be practiced, with the understanding that it would be beneficial for both the patient and the provider. Many participants stated that the facility they go to for transfusions keeps a record of their history.	"No, I'm not [familiar with this recommendation]. I think that, of course, I think that should happen…" "I have to tell [my hemoglobin and ferritin levels] to my doctor. She doesn't see it on her own. Right now, I'm vocal, but, God forbid there is something too severe that happens …and if I'm not aware of all that, this is something they might not be seeing."
2) Before each transfusion, they should run a full crossmatch and screen your blood for new antibodies.	Five of 32 study participants (16%) were unfamiliar with this recommendation and were unclear about what a full crossmatch is, or if it is practiced by their usual care facility.	"I know they do a cross match. I don't know when they do a cross match, is it a full cross match." "I haven't [heard of this recommendation], but that would be good because I do have a lot of antibodies."
3) You should be given packed red blood cells with leukocytes removed.	Nine of 32 participants (28%) were unfamiliar with this recommendation. They were unsure of what packed red blood cells are or whether that is what they receive; and/or they were unfamiliar with the term "leukocytes" and why they should be removed.	"Um I know I get washed, I don't know if I get packed. …But I don't know about the leukocytes." "I'm not necessarily familiar enough to know in certainty that it, on, on the leukocyte piece- what the benefits are, are, would be to remove them or not remove them."
4) If you have a fever or allergic reaction during a transfusion, then in the future when you get transfusions the doctor should give you acetaminophen (Tylenol) or diphenhydramine (Benadryl) first.	Only four of 32 study participants (13%) were unaware of this recommendation. One participant suggested that premedication should be given by discretion; that isolated reactions should not dictate perennial premedication.	"I would like, see how you go on your next transfusion. Go very slow in the beginning, see if you're making reactions. And then, from there just… Because, there's been times that I've had reactions, and isolations, and the majority of times I don't."
5) If you have a severe allergic reaction, they should give you washed, packed red blood cell units any time you get transfusions again.	This recommendation was unfamiliar to nine of 32 (28%) participants, who did not know what "washed, packed, red blood cell units" are.	"That I didn't know. I didn't know there was washed red blood cells... If you had a reaction. I did not know that." "I'm not familiar with that. I don't think I've experienced it....What are 'washed RBCs?"

TABLE 2 | Perspectives on data-tracking methods.

Method	Summary of findings	Example quotes
Phone app	Participants generally expressed approval or interest in a phone app used to store personal blood and transfusion information. Some participants did, however, express concern about the extent of confidentiality and security of the app. Others saw value in having easy access to their information for themselves and their medical providers.	"So, a lot of times I will have to go to [a hospital]...but they don't necessarily know anything about me, and my record. And if it's late at night I can't get in touch with my doctor, you know, something like an app or something where you do have your medical history on hand is very important." "Some people don't know what their blood type is or how many times they get transfusions. So, I think it would just be a good idea for them if they have the information on their phones."
Wallet card	Participants expressed no uniform opinions about a wallet card having their blood and transfusion information. There was concern about losing a wallet card but overall, participants valued the role that a wallet card could play in case of emergency.	"In an emergency... that information would be available to an emergency responder. So, if someone is... in a car accident, and needed blood transfusions, I think the responding team or the ER knowing what type of blood that particular patient could or could not accept would be extremely important."
Registry	Participants generally expressed approval or interest in an electronic registry used for storing medical information. Participants were told that, ideally, patient information would be accessible online from any location and they could choose to share their information with any health care provider. Participants supported having their information available so that they could receive proper treatment anywhere, especially when traveling. However, there was no consensus on whether the registry could be trusted to keep medical information confidential	"It's not that difficult to get into someone's cell phone… As opposed to a registry I think would have more security." "As long as there's not, like, social security number or anything like that, I wouldn't worry about anybody getting into that."

were least well-versed in recommendations 3 and 5 regarding blood specifications. The need for education on these may reflect the technical terminology involved, as patients expressed unfamiliarity with "washed, packed" units as well as "leukocytes" and "leukocyte-reduced" units. The possible gaps in provider practices that are reflected in patients' reported experience are assessed more directly elsewhere, with provider-focused intervention recommendations (3, 16).

Data Tracking

The transfusion guidelines referenced in this study point to the need for providers to have accurate, up-to-date transfusion history for the patient on which to base clinical decisions. In the U.S., there is no health record universally accessible to providers across health systems. Providers seeing patients outside of patients' usual places of care must rely on patient report or real-time outreach to their prior provider. Data

tracking approaches that improve on those two, flawed options could increase adherence to practice guidelines and reduce transfusion complications. A national antibody registry is the recommendation based on this study's findings. Privacy and security concerns were lowest for this option; it avoids the patient burden of keeping up with a wallet card or smartphone and installed app; and it would be fully accessible even in the event of a patient being unable to speak or operate their smartphone. Patients affirmed the urgency of closing the data gap, expressing doubt about the sufficiency of medical training in thalassemia; worry on behalf of patients in less-populated areas with even less access to knowledgeable providers; and acknowledgment of risk when receiving a transfusion away from the known provider. Developers of solutions should note the common concern for privacy among patients and account for this in both the design and the implementation of data sharing strategies.

Limitations

Thalassemia is a rare disorder, with a relatively small patient population in the United States. While the present sample included individuals from multiple regions of the country and from both high- and low-population density areas, its size limits its representativeness of the overall study population. The absence of demographic data precludes characterization of the sample's representativeness across race, ethnicity, sex, and age. Because the thalassemia population in the U.S. includes both multi-generation Americans and more recent immigrants from a wide range of countries, the English proficiency of the current sample may introduce bias as well. Finally, recruitment through the Cooley's Anemia Foundation mailing list and inclusion by voluntary response may mean the sample skews toward being better informed on thalassemia and more engaged in personal care than the overall population of individuals with thalassemia receiving therapeutic blood transfusions. Despite these limitations, the range of responses and their convergent and divergent aspects offer findings that may be useful as a starting point for further studies or pilot interventions.

Conclusions

The findings of this study can contribute to health education and other patient interventions, as well as future research initiatives, for reducing complications of therapeutic transfusions in individuals with thalassemia. While the study sample encompassed a generally well-informed group, gaps in knowledge and understanding emerged. These include the meaning and implications of terms such as "leukocyte-reduced" and "washed-packed" red blood cells; the recommended extent of antigen matching; and the balance between privacy concerns and life-threatening risk that attach to decisions about data storage to ensure accurate transfusion history for providers at the point of care. Increased education on these topics is recommended. Peer mentoring strategies that connect geographically isolated patients with others who have access to more robust thalassemia care and resources could help bridge an indicated gap. A national antibody registry is likely to find the greatest acceptance among patients, but care should be taken to address specific patient concerns in its design and implementation. Finally, a dissemination and implementation strategy for best practices and clinical guidelines—particularly in the case of a rare disease such as thalassemia—would do well to include parallel, patient-focused strategies.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because names and other identifying information will only be found on contact sheets, consent/assent forms, and gift card receipts, all of which will be accessible only to focus group facilitators and kept in locked offices at Augusta University and Georgia State University. Recordings will be stored on password-protected hard drive (R-drive) and GSU's password-protected OneDrive. The recordings on both drives will only be accessible to research staff with proper IRB approval. Research materials will be kept in locked offices or, if electronic, the Augusta University R-drive (hard drive) and GSU's password-protected OneDrive. Requests to access the datasets should be directed to Sashoy Patterson spatterson8@gsu.edu.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Georgia State University Institutional Review Board. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

AS, SP, and JB: study design. SP and VN: data collection. SP, VN, RS, and JB: analysis and writing. All authors contributed to the article and approved the submitted version.

FUNDING

This work was made possible by cooperative agreement number NU58DD001138 from the Centers for Disease Control and Prevention. The content is the responsibility of the authors and does not necessarily reflect the official policies of the CDC or the Department of Health and Human Services.

ACKNOWLEDGMENTS

The authors wish to acknowledge the support of the Cooley's Anemia Foundation, in particular Craig Butler and Sandy Gilbert, for their generous assistance with protocol development and participant recruitment.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.772886/full#supplementary-material

REFERENCES

1. Bayanzay K, Alzoebie L. Reducing the iron burden and improving survival in transfusion-dependent thalassemia patients: current perspectives. *J Blood Med.* (2016) 7:159. doi: 10.2147/JBM.S61540
2. Chonat S, Quinn CT. Current standards of care and long-term outcomes for thalassemia and sickle cell disease. *Adv Exp Med Biol.* (2017) 59–87. doi: 10.1007/978-1-4939-7299-9_3
3. Goss C, Giardina P, Degtyaryova D, Kleinert D, Sheth S, Cushing M. Red blood cell transfusions for thalassemia: results of a survey assessing current practice and proposal of evidence-based guidelines. *Transfusion.* (2014) 54:1773–81. doi: 10.1111/trf.12571
4. Tubman VN, Fung EB, Vogiatzi M, Thompson AA, Rogers ZR, Neufeld EJ, et al. Thalassemia clinical research network. Guidelines for the standard monitoring of patients with thalassemia: report of the thalassemia longitudinal cohort. *J Pediatr Hematol Oncol.* (2015) 37:e162. doi: 10.1097/MPH.0000000000000307
5. De Sanctis V, Soliman AT, Elsedfy H, Skordis N, Kattamis C, Angastiniotis M, et al. Growth and endocrine disorders in thalassemia: The international network on endocrine complications in thalassemia (I-CET) position statement and guidelines. *Indian J Endocrinol Metab.* (2013) 17:8. doi: 10.4103/2230-8210.107808
6. Milman N. Anemia—still a major health problem in many parts of the world! *Ann Hematol.* (2011) 90:369–77. doi: 10.1007/s00277-010-1144-5
7. Vichinsky EP. Changing patterns of thalassemia worldwide. *Ann N Y Acad Sci.* (2005) 1054:18–24. doi: 10.1196/annals.1345.003
8. Vichinsky E, Neumayr L, Trimble S, Giardina PJ, Cohen AR, Coates T, et al. Transfusion complications in thalassemia patients: a report from the Centers for Disease Control and Prevention. *Transfusion.* (2014) 54:972–81. doi: 10.1111/trf.12348
9. Abdelrazik AM, Elshafie SM, El Said MN, Ezzat Ahmed GM, Al-Gamil AK, El Nahhas MG, et al. Study of red blood cell alloimmunization risk factors in multiply transfused thalassemia patients: role in improving thalassemia transfusion practice in Fayoum, Egypt. *Transfusion.* (2016) 56:2303–7. doi: 10.1111/trf.13695
10. Azarkeivan A, Ansari S, Ahmadi MH, Hajibeigy B, Maghsudlu M, Nasizadeh S, et al. Blood transfusion and alloimmunization in patients with thalassemia: multicenter study. *Pediatr Hematol Oncol.* (2011) 28:479–85. doi: 10.3109/08880018.2011.568595
11. Perrotta PL, Snyder EL. Non-infectious complications of transfusion therapy. *Blood Reviews.* (2001) 15:69–83. doi: 10.1054/blre.2001.0151
12. Arbabisarjou A, Karimzaei T, Jamalzaei A. The perception of biological experience in patients with major thalassemia: A qualitative research. *Glob J Health Sci.* (2015) 7:79. doi: 10.5539/gjhs.v7n1p79
13. Cappellini M, Cohen A, Eleftheriou A, Piga A, Porter J, Taher A. *Guidelines for the Clinical Management of Thalassaemia*. Nicosia: Thalassaemia International Federation (2008). Available online at: https://www.ncbi.nlm.nih.gov/books/NBK173968/
14. Beebe J. Rapid assessment process: An introduction. *Rowman Altamira.* (2001).
15. Maietta RC. State of the art: Integrating software with qualitative analysis. Improving aging and public health research: Qualitative and mixed methods. (2006) p. 117-39.
16. Fasano RM, Meyer EK, Branscomb J, White MS, Gibson RW, Eckman JR. Impact of red blood cell antigen matching on alloimmunization and transfusion complications in patients with sickle cell disease: a systematic review. *Transfusion Medicine Reviews.* (2019) 33:12–23. doi: 10.1016/j.tmrv.2018.07.003

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.

frontiers in Medicine

CASE REPORT
published: 02 March 2022
doi: 10.3389/fmed.2022.837253

Case Report: A Rare Case of Thrombotic Microangiopathy Induced by Remethylation Disorders

*Lu Pang[1], Jian Chen[1], Haiyan Yu[1], Haiming Huang[1], Bo Jin[1], Xin Wang[2] and Haixia Li[1]**

[1] Department of Clinical Laboratory, Peking University First Hospital, Beijing, China, [2] Department of Pathology, National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China

In this research, we described a very rare case of thrombotic microangiopathy induced by remethylation disorders. A 16-year-old boy presented to the emergency department with 5 months of weakness and fatigue. He was diagnosed with thrombotic microangiopathy based on clinical manifestation and laboratory information, which showed microangiopathic hemolytic anemia, renal impairment, and thrombocytopenia. After a complex diagnostic workup, the metabolite screening parameters and sequencing results guided us toward the diagnosis of remethylation disorders. The patient was diagnosed with thrombotic microangiopathy induced by remethylation disorders (cblC).

Keywords: hemolytic uremic syndrome, schistocytes, remethylation disorders, cblC, *MMACHC*

OPEN ACCESS

Edited by:
Roberta Fenoglio, Ospedale San Giovanni Bosco, Italy

Reviewed by:
Ioanna Sakellari, G. Papanikolaou General Hospital, Greece
Emanuele De Simone, Azienda Sanitaria Locale "Città di Torino", Italy

****Correspondence:***
Haixia Li
bdyylhx@126.com

Specialty section:
This article was submitted to Hematology, a section of the journal Frontiers in Medicine

Received: *16 December 2021*
Accepted: *31 January 2022*
Published: *02 March 2022*

Citation:
Pang L, Chen J, Yu H, Huang H, Jin B, Wang X and Li H (2022) Case Report: A Rare Case of Thrombotic Microangiopathy Induced by Remethylation Disorders. Front. Med. 9:837253. doi: 10.3389/fmed.2022.837253

INTRODUCTION

A hemolytic uremic syndrome is a form of thrombotic microangiopathy. Hemolytic uremic syndrome (HUS) encompasses several disorders: shiga toxin-induced HUS, and pneumococcus-induced HUS, HUS associated with complement dysregulation, HUS related to cobalamin C (cblC) defect, and HUS secondary to a heterogeneous group of causes (infections, drugs, cancer, and systemic diseases). Among these causes, cblC defect is one of the combined remethylation disorders with a genetically heterogeneous disorder of cbl metabolism. In this study, we presented a rare case with thrombotic microangiopathy (TMA) induced by remethylation disorders (cblC).

CASE DESCRIPTION

A 16-year-old boy presented to the emergency department with 5 months of weakness and fatigue. The patient was in good general health, had no relevant medical history, no abdominal pain, and diarrhea, and had a normal physical examination. Laboratory evaluation showed thrombocytopenia (platelet count, 58 $\times$ 10^9/L) and hemolytic anemia (hemoglobin, 77 g/L; reticulocyte, 199.9 $\times$ 10^9/L; lactate dehydrogenase, 609 IU/L). Additional laboratory tests showed an elevated creatinine level of 2.6 mg/dL and elevated 24 h proteinuria of 5.66 g/24 h. Serum vitamin B12 (348 nmol/L), folate (20.31 nmol/L), ferritin (169.2 ng/mL), iron (14.1 μmol/L), complement 3 (0.733 g/L), and complement 4 (0.325 g/L) were all within the normal range. Wright-stained peripheral smear (PS) showed schistocytes (**Figure 1**; original magnification $\times$ 1,000). Level of factor H and ADAMTS13 activity were normal. Anti-factor H-antibody and Coombs's test were negative. The autoantibodies, such as an anti-nuclear antibody, anti-dsDNA antibody, anti-phospholipase A2 receptor antibody, anti-phospholipid antibody, and anti-neutrophil

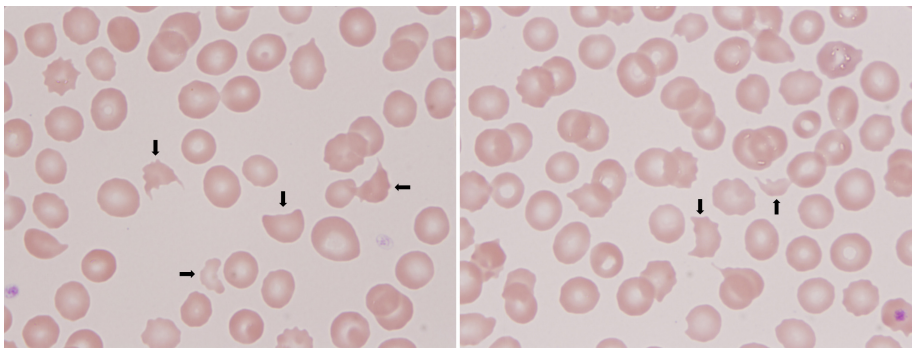


FIGURE 1 | Wright-stained peripheral smear (PS) showed schistocytes. The arrows mean schistocytes.

cytoplasmic antibody were all negative. Renal pathology suggested that there were severe TMA with swelling endothelial cells, thickening arterial wall, thrombus in a capillary lumen, and IgM and C3 deposition. By screening for metabolic diseases, we found that the concentration of plasma total homocysteine (tHcy, 247.84 μmol/L), urinary methylmalonic acid (MMA, 62.29 mmol/mol creatinine), and blood propionyl carnitine (C3, 8.67 μmol/L) were increased obviously, and plasma methionine (Met, 7.8 μmol/L) was decreased. The whole exon sequencing and Sanger sequencing revealed a compound heterozygous *MMACHC* mutation (**Figure 2**; c.1A > G and c.80A > G).

The patient was diagnosed as TMA induced by remethylation disorders (cblC) according to the guideline (1). The patient was treated with betaine, hydroxycobalamin, and folic acid. After treatment, the tHcy level was normalized but hemolysis, thrombocytopenia, and acute kidney failure persisted. Because plasma exchange was ineffective, he required continued renal replacement treatment (CRRT) three times a week due to renal injury of TMA.

The study was in compliance with the Declaration of Helsinki and approved by the ethics committee of Peking University First Hospital. The significance of the research was explained to the patient and informed consent was obtained.

DISCUSSION

Remethylation disorders are rare inherited disorders in which impaired remethylation of homocysteine (Hcy) to Met. Met leads to accumulation of tHcy. Remethylation disorders can be classified into three groups (1). Combined remethylation disorders (cblC, cblD-MMA/HC, cblF, and cblJ) are alterations of the transport and intracellular metabolism of cobalamins, which causes a defect in the synthesis of the two functional forms of cobalamin (cbl; vitamin B12): methylcobalamin, cofactor of methionine synthase, and adenosylcobalamin, a cofactor for methylmalonyl-CoA mutase (MCM). Isolated remethylation disorders (cblD-HC, cblE, and cblG) are isolated deficits in the production of methylcobalamin. 5,10-methylenetetrahydrofolate reductase (MTHFR) deficiency is an abnormality of the folate cycle.

The clinical diagnosis of remethylation disorders can be easily and rapidly diagnosed by biochemical investigation. Serum vitamin B12, serum folate, plasma tHcy, plasma Met, blood acylcarnitine profile, plasma, or urinary MMA are required. Combined remethylation disorders are associated with increased tHcy and MMA (2). In this case, Serum Vitamin B12 and folate were within the normal range. The concentration of plasma tHcy, urinary MMA, and blood C3 were increased obviously. The plasma Met was decreased. Therefore, this case was diagnosed as a combined remethylation disorder.

Combined remethylation disorders are genetically heterogeneous disorders of cbl metabolism. Different forms of the disorder have been classified as cblC, cblD-MMA/HC, cblF, and cblJ. Among these, cblC type is most frequent and caused by a mutation in the *MMACHC* gene on chromosome 1p34.1. The three most common mutations are c.271dupA, c.394C > T, and c.331C > T, However, in Chinese patients, the c.609G > A mutation accounts for 85% of the identified alleles in *MMACHC* mutation (1). In this case, heterozygous mutations of *MMACHC* (c.1A > G and c.80A > G) were found, which have been reported before (3), although the mutations are uncommon in Chinese patients.

cblC defect usually occurs during the 1st year of life, and late-onset is applicable to children older than 4 years. There is great variability in the clinical presentation of cblC defect, and more common in the blood and nervous system (3). In this study, we focus on the TMA, which is a rarely late-onset manifestation in cblC defect.

Hemolytic uremic syndrome (HUS) is a form of TMA, characterized by microangiopathic hemolytic anemia, renal impairment, and thrombocytopenia (4). Another form of TMA is thrombotic thrombocytopenic purpura (TTP) defined by a severe deficiency in ADAMTS13, the specific von Willebrand factor (vWF)-cleaving protease (5). It is worth noting that schistocytes

Abbreviations: aHUS, atypical hemolytic uremic syndrome; C3, propionyl carnitine; cbl, cobalamin; HUS, hemolytic uremic syndrome; Met, methionine; MMA, methylmalonic acid; MTHFR, 5,10-methylenetetrahydrofolate reductase; PS, peripheral smear; STEC, shiga toxin-producing Escherichia coli; tHcy, total homocysteine; TMA, thrombotic microangiopathy; TTP, thrombotic thrombocytopenic purpura; vWF, von Willebrand factor.

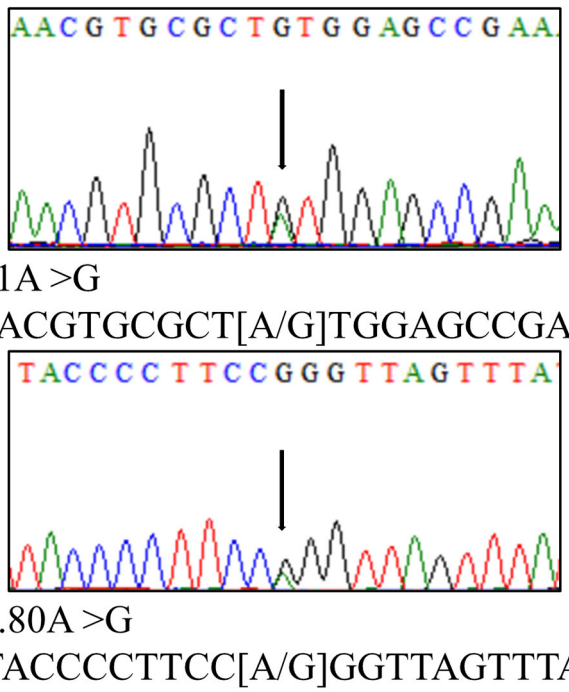


FIGURE 2 | Genetic analysis revealed a compound heterozygous *MMACHC* mutation (c.1A > G and c.80A > G).

are fragments of red blood cells produced by extrinsic mechanical damage and a diagnostic feature of TMA (6). A percentage of schistocytes above 1% is a robust indicator for the diagnosis of TMA (7). In this case, the thrombocytopenia, hemolytic anemia, renal dysfunction, schistocytes, and normal ADAMTS13 activity all support the diagnosis of TMA.

The HUS encompasses a heterogeneous group of disorders, including typical HUS due to an infection from shiga toxin-producing Escherichia coli (STEC), and atypical hemolytic uremic syndrome (aHUS), caused by dysregulation of the alternative pathway of complement secondary to complement gene mutations or anti-complement factor H antibodies (8). cblC is a genetic disease that can lead to HUS (9). So far, only a few cases of HUS have been triggered by a metabolic disorder of cbl (10). Although HUS can be caused by cblC defect accompanied by complement dysregulation (11), dysregulation of the complement alternative pathway was not found with the whole-exome sequencing and ADAMTS13 test in this case. This patient is not complement-driven and, therefore, cannot benefit from complement inhibitors (12). Based on the diagnoses of cblC defect, the patient was diagnosed as TMA induced by combined remethylation disorders (cblC).

In this study, we presented a case of combined remethylation disorder, which is cblC defect and *MMACHC*-related, is the most common inborn error of cbl metabolism and it is a rare cause of TMA. Combined remethylation disorders should be considered in those patients with unclear microangiopathic hemolytic anemia. Therefore, plasma tHcy and MMA measurement should be included in the workup of renal TMA children. Plasma tHcy is readily available in clinical laboratories and easily ruled the suspicion of a cbl metabolism defect, so it is the first biochemical parameter to assess when a remethylation disorder is suspected. Thereafter, the combination of methylmalonic aciduria, homocystinuria, and normal serum vitamin B12 concentration distinguish patients with a genetic defect of Cbl from vitamin B12 deficiency due to nutritional deficiency or acquired malabsorption (1).

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Peking University First Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

LP, JC, and HL conceived the manuscript, performed analysis, and wrote the manuscript. HY, HH, BJ, XW, LP, and HL performed interpretation of clinical data and contributed to manuscript writing. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Huemer M, Diodato D, Schwahn B, Schiff M, Bandeira A, Benoist JF, et al. Guidelines for diagnosis and management of the cobalamin-related remethylation disorders cblC, cblD, cblE, cblF, cblG, cblJ and MTHFR deficiency. *J Inherit Metab Dis.* (2017) 40:21–48. doi: 10.1007/s10545-016-9991-4
2. Huemer M, Diodato D, Martinelli D, Olivieri G, Blom H, Gleich F, et al. Phenotype, treatment practice and outcome in the cobalamin-dependent remethylation disorders and MTHFR deficiency: data from the E-HOD registry. *J Inherit Metab Dis.* (2019) 42:333–52. doi: 10.1002/jimd.12041
3. Chen RY, Li XZ, Lin Q, Zhu Y, Shen YY, Xu QY, et al. Proteinuria as a presenting sign of combined methylmalonic acidemia and homocysteinemia: case report. *BMC Med Genet.* (2020) 21:183. doi: 10.1186/s12881-020-01122-x
4. Cody EM, Dixon BP. Hemolytic uremic syndrome. *Pediatr Clin North Am.* (2019) 66:235–46. doi: 10.1016/j.pcl.2018.09.011
5. Joly BS, Coppo P, Veyradier A. An update on pathogenesis and diagnosis of thrombotic thrombocytopenic purpura. *Expert Rev Hematol.* (2019) 12:383–95. doi: 10.1080/17474086.2019.1611423
6. Bhandari S, Kumar R. Thrombotic thrombocytopenic purpura. *N Engl J Med.* (2019) 380:e23. doi: 10.1056/NEJMicm1813768
7. Zini G, d'Onofrio G, Briggs C, Erber W, Jou JM, Lee SH, et al. ICSH recommendations for identification, diagnostic value,

and quantitation of schistocytes. *Int J Lab Hematol.* (2012) 34:107–16. doi: 10.1111/j.1751-553X.2011.01380.x
8. Fakhouri F, Zuber J, Fremeaux-Bacchi V, Loirat C. Haemolytic uraemic syndrome. *Lancet.* (2017) 390:681–96. doi: 10.1016/S0140-6736(17)30062-4
9. Adrovic A, Canpolat N, Caliskan S, Sever L, Kiykim E, Agbas A, et al. Cobalamin C defect-hemolytic uremic syndrome caused by new mutation in MMACHC. *Pediatr Int.* (2016) 58:763–5. doi: 10.1111/ped.12953
10. Martinez de Companon Z, Poblet-Puig M, Valles G, Del Toro M, Vilalta R, Moreno A, et al. Cobalamin disorder CblC presenting with hemolytic uremic syndrome and pulmonary hypertension. *Nefrologia.* (2018) 38:333–5. doi: 10.1016/j.nefro.2017.03.019
11. Barlas UK, Kihtir HS, Goknar N, Ersoy M, Akcay N, Sevketoglu E. Hemolytic uremic syndrome with dual caution in an infant: cobalamin C defect and complement dysregulation successfully treated with eculizumab. *Pediatr Nephrol.* (2018) 33:1093–6. doi: 10.1007/s00467-018-3941-3
12. Gavriilaki E, Peffault de Latour R, Risitano AM. Advancing therapeutic complement inhibition in hematologic diseases: PNH and beyond. *Blood.* (2021) 2021:12860. doi: 10.1182/blood.2021012860

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.

frontiers in Medicine

ORIGINAL RESEARCH
published: 28 March 2022
doi: 10.3389/fmed.2022.819320

Non HCV-Related Mixed Cryoglobulinemic Vasculitis With Biopsy-Proven Renal Involvement: The Effects of Rituximab

Roberta Fenoglio, Savino Sciascia, Daniela Rossi, Carla Naretto, Mirella Alpa and Dario Roccatello*

Nephrology and Dialysis Unit (The European Rare Kidney Disease Reference Network, The European Reference Network on Rare and Complex Connective Tissue and Musculoskeletal Diseases, and the European Reference Network That Aims at Improving the Care of Patients With Rare Immunological Disorders), Center of Research of Immunopathology and Rare Diseases- Coordinating Center of the Network for Rare Diseases of Piedmont and Aosta Valley, Department of Clinical and Biological Sciences, University of Turin and S. Giovanni Bosco Hub Hospital, Turin, Italy

In the countries where HCV infection is still endemic, about 90% of subjects with mixed cryoglobulinemia had previously been infected with HCV and about 80% are RNA positive. Remarkable results in severe HCV-related cryoglobulinemic vasculitis have been obtained with Rituximab. Details of the clinical characteristics and effective treatment of non HCV-related cryogloulinemic syndromes are presently lacking. This paper reports on a prospective single-Center open study aimed at evaluating the clinical presentation and effects of Rituximab administered alone in patients with severe non HCV-related cryoglobulinemic syndrome. The study group included 11 patients followed for at least 6 months. Three patients had type I cryoglobulinemia, 6 had type II and the remaining 2 patients had type III. Mean cryocrit was 2.5%. Four out of 11 patients had symptomatic sicca complex with anti-SSA (Ro)/anti SSB (La) antibodies. All 11 patients presented with biopsy-proven renal involvement, 4 out of 11 with leukocytoclastic vasculitis, and 8 with involvement of the peripheral nervous system. Renal biopsy revealed diffuse membranoproliferative glomerulonephritis (MPGN) in 9 out of 11 patients. Extracapillary proliferation and necrosis of the glomerular tuft was observed in 1 of these 9 cases. Interstitial nephritis together with mesangial expansion and capillary immune deposits were observed in 1 patient. Prevalent interstitial fibrosis and glomerular sclerosis were detected in the remaining case. Patients underwent treatment with rituximab alone. After 6 months we observed a remarkable improvement in the necrotizing skin ulcers and a substantial amelioration of the electrophysiological parameters of motor and sensory peripheral neuropathy. Improvement in both renal function (from 2.8 to 1.4 mg/dl, $p < 0.001$) and proteinuria (from 4.2 g/24 to 0.4 g/24 h, $p < 0.001$) was found in 10 out of 11 patients, while 1 could not be fully treated because of a severe infusion reaction and sudden development of anti-Rituximab antibodies. Good renal response was confirmed at the end of follow-up (38.4 months). Three patients had a relapse at 6, 12, and 48 months, respectively. In our cohort the administration of 4 once-weekly infusions of Rituximab followed by 2 more infusions after 1 and 2 months proved to be effective in the management of these rare patients.

Keywords: essential mixed cryoglobulinemia, hematological disease, autoimmune disease, corticosteroids, Rituximab, renal biopsy

OPEN ACCESS

Edited by:
Lalitha Nayak,
Case Western Reserve University,
United States

Reviewed by:
Mohsen Saleh Elalfy,
Ain Sham University, Egypt
Jan Willem Cohen Tervaert,
University of Alberta, Canada

****Correspondence:***
Roberta Fenoglio
roberta.fenoglio@unito.it

Specialty section:
This article was submitted to Hematology, a section of the journal Frontiers in Medicine

Received: *21 November 2021*
Accepted: *03 March 2022*
Published: *28 March 2022*

Citation:
Fenoglio R, Sciascia S, Rossi D, Naretto C, Alpa M and Roccatello D (2022) Non HCV-Related Mixed Cryoglobulinemic Vasculitis With Biopsy-Proven Renal Involvement: The Effects of Rituximab. Front. Med. 9:819320. doi: 10.3389/fmed.2022.819320

INTRODUCTION

Cryoglobulinemia is a condition characterized by the presence of immunoglobulins (Igs) that reversibly precipitate in serum, form a gel at temperatures below 37°C and re-dissolve upon re-warming (1). Cryoglobulinemia classification is based on Ig composition and includes three subgroups (2). Monoclonal cryoglobulinemia (type I) involves a single type of monoclonal Ig. It accounts for 10–15% of all cases of cryoglobulinemia and is often related to hematological disorders such as Waldenstrom's macroglobulinemia, multiple myeloma, or chronic lymphocytic leukemia (3). Mixed cryoglobulinemia (MC) consists of a mixture of either polyclonal IgG and monoclonal IgM (type II), or of polyclonal IgG and polyclonal IgM (type Ill), both sharing rheumatoid factor (RF) activity. In HCV-negative patients, Type II cryoglobulinemia may be associated with hepatitis B virus or HIV (4, 5), autoimmune diseases (mainly systemic lupus erythematosus, Sjögren's syndrome) (6), and lymphoproliferative disorders. Type III MC is commonly detected in a great deal of infectious or autoimmune disorders. Type II, and less commonly type III MC, may result in a distinct disorder, which can be classified among the systemic vasculitis affecting small vessels, including glomeruli, vasa nervorum and dermal small vessels. Biochemical analyses usually reveal type II cryoglobulins (IgM–k, polyclonal IgG), positive rheumatoid factor, and low values of C3, C4, and C1q. C4 levels are usually very low, and sometimes even undetectable, and represent a valid surrogate marker of cryoglobulinemia. Despite the fact that in some countries such as Netherlands HCV infection is rare (7) in other countries where it is still endemic, about 90% of subjects with MC had previously been infected with HCV and about 80% are RNA positive (8–10). Despite the availability of direct antiviral agents, millions of people still have a HCV infection, but MC is a rare disease affecting <1 patient/2,000 individuals, and cryoglobulinemic nephritis is much rarer. However, a certain percentage of cases have no identifiable disease association, and hence the cryoglobulinemia is called "essential". Previous studies have reported treatment with corticosteroids (CS) and immunosuppressants (IS) as not being satisfactory, especially in severe forms (11, 12). Today there is not a unique approach of treatment of these patients (13). Remarkable results in severe HCV-related cryoglobulinemic vasculitis (CV) have been obtained with Rituximab (RTX) (14). The lymphoma protocol, consisting of 4 once-weekly infusions of 375 mg/m^2, was found to be effective in some life-threatening conditions. Two more doses at 1-month intervals have also been used in the so-called "4 plus 2" or "improved protocol" which was found to improve or cure HCV-related cryoglobulinemic glomerulonephritis in 75–90% of cases (15, 16). A complete description of the clinical characteristics and effective treatment of non HCV-related cryoglobulinemic syndromes are presently lacking. This paper reports on a prospective single-Center open study aimed at evaluating the clinical presentation and effects of Rituximab given alone in patients with severe, non HCV-related cryoglobulinemic syndrome.

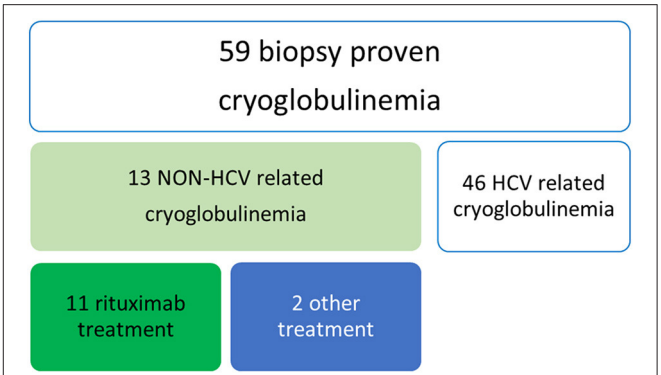


FIGURE 1 | Algorithm for patient selection.

MATERIALS AND METHODS

We retrospectively analyzed all patients with a previous diagnosis of non HCV-related cryoglobulinemia and biopsy-proven renal involvement (**Figure 1**). Inclusion criteria for the study were the presence of cryoglobulins (≥0.5% cryocrit) on at least 2 determinations and the absence of HCV infection (negative serology and viral load by polymerase chain reaction). Cryoglobulins were classified according to the method described by Brouet et al. (2). For each patient, the following data were collected: age at diagnosis, sex, possible cause (infections, connective tissue disease, hematologic disorder), clinical manifestations (if not explainable by another cause) and outcome. Laboratory assessment at the time of therapy with RTX, 6 months later and at the end of the follow up included cryoglobulin type, serum levels of complement C3 and C4 fractions (g/L), RF activity (UI/mL), serum creatinine (mg/dl) and proteinuria (gr/day). All patients underwent renal biopsy.

They were all treated with 4 once-weekly doses of Rituximab (375 mg/m^2) plus two more doses, administered 1 and 2 months later (improved protocol) (16). Seven patients received previous treatment with prednisone (2 out of 7 in association with methotrexate) at the dosage of 0.3–0.8 mg/Kg for 1–5 months. Each infusion of rituximab was preceded by premedication with methylprednisolone 100 mg, paracetamol 1,000 mg and cetirizine 10 mg. No patients received steroids following rituximab therapy.

Complete renal response was defined as primary efficacy renal response, i.e., ratio of protein-to-creatinine ratio (PCR) of ≤0.7 and eGFR that was either improved or stabilized as compared to baseline values. Relapse has been defined as re-appearance of proteinuria > 50% of the lowest value observed.

RESULTS

Demographic, clinical and immunological features are summarized in **Tables 1**–**3**. The study group included 4 females and 7 males, mean age 63.8 years (range 54–79), followed for at least 6 months. Three had type I cryoglobulinemia, 6 had type II and the 2 remaining patients had type III. Mean cryocrit

TABLE 1 | Detailed data about demographic and clinical characteristics and follow-up of the 11 individual patients.

	Sex	Age	Type Cryo	Sicca	Kidney	Skin	PNS	Previous treatment	Basal sCr (mg/dl)	Last sCr (mg/dl)	Basal uPt (g/day)	Last uPt (g/day)	Relapse (months)
Pt 1	M	75	I	No	Yes	No	No	No	5.8	1.3	5	0.4	No
Pt 2	M	58	II	Yes	Yes	No	No	Prednisone	2.8	1.3	9	0.6	No
Pt 3	M	72	II	No	Yes	Yes	Yes	Colchicine	3.2	1.5	1	0.4	No
Pt 4	M	65	II	No	Yes	Yes*	Yes	Prednisone	2.5	1.8	4.4	0.4	No
Pt 5	F	74	II	No	Yes	No	Yes	Pred/Meto	1.4	1.0	8	1	48
Pt 6	M	54	I	No	Yes	No	Yes	No	1.6	1.4	0.5	0.2	6
Pt 7	F	64	III	Yes	Yes	No	Yes	No	2.3	0.9	1.6	0.2	No
Pt 8	M	55	II	No	Yes	No	Yes	Prednisone	2.4	2.1	1.9	0.6	No
Pt 9	F	70	II	No	Yes	Yes	Yes	Prednisone	3	1.7	6	0.1	12
Pt 10	F	78	I	No	Yes	Yes	Yes	Pred/meto	2.8	1.4	4.2	0.4	No
Pt 11	M	52	III	Yes	Yes	No	No	Prednisone	3.5	–	6	–	–

PNS, peripheral nervous system; sCr, serum creatinine; uPt, proteinuria; Pred, prednisone; Meto, methotrexate.
**Pt 4 required autologous skin transplantation. Pt 11 developed anti-Rituximab antibodies.*

TABLE 2 | Clinical and at the time of Rituximab.

	At baseline
Age at diagnosis (years)	63.8 (54–79)
Female/male	4/7
Type I	3
Type II	6
Type III	2
Previous treatment:	
- Prednisone[#]	5/11
- Prednisone + methotrexate	2/11
- Colchicine 3 mesi	1/11
- No treatment	3/11
Renal biopsy	
- Diffuse Membranoprolipherative GN	11/11
- Mesangial GN	9/11*
- Tubulo-Interstitial nephritis	1/11
	1/11

**With Extracapillary proliferation and necrosis of the glomerular tuft in 1 pt.*
[#]0.3–0.8 mg /kg day for 1–5 months.

TABLE 3 | Clinical and laboratory improvements at 6 months and at last follow up compared to baseline figures in 10 pts given at least 1 complete cycle of Rituximab.

	Before Rituximab	6 months follow up	End of follow-up (mean 38.4 months)
Skin involvement	4/10	1/10	1/10
Kidney involvement	10/10	0/10	0/10
PNS involvement	8/10	2/10	1/10
RF	467 (98–1280)	181.4 (35–650)	76 (14–202)
Cryocrit (%)	2.5 (0.5–7)	0.8 (0–3)	0.5 (0–1)
C4 (mg/dl)	4.9 (1–26)	8.4 (1.5–18)	11 (2–19)
C3 (mg/dl)	71 (44–104)	88.3 (68–102)	87.4 (66–100)
sCr (mg/dl)	2.8 (1.4–5.8)	1.3 (0.9–2.1)	1.5 (0.9–1.7)
uPt (g/day)	4.2 (0.5–9)	0.4 (0.1–1)	0.49 (0.1–3.3)

was 2.5% (range 0.5–7%). As shown in **Table 2**, no associated disease was identified in 6 patients (54.5%): they were classified as having true "essential" cryoglobulinemia. One of the 3 patients with type I cryoglobulinemia had a low grade lymphocytic lymphoma. The other 2 patients with type I cryoglobulinemia had a normal bone marrow biopsy. Four out of 11 patients (36.4%) had symptomatic sicca complex with anti-SSA (Ro)/anti SSB (La) antibodies, one of whom was found to have myeloid hyperplasia with mild plasmocytosis at bone marrow biopsy. Four out of 11 (36.4%) had leukocytoclastic vasculitis, 8 (72.8%) had involvement of the peripheral nervous system, and all 11 presented with biopsy-proven renal involvement. Renal biopsy revealed that 9 out of 11 patients had diffuse membranoproliferative glomerulonephritis (MPGN) with proliferation and expansion of the mesangium, duplication of the glomerular basement membrane, interposition by mesangial cells and monocyte/macrophages, subendothelial and mesangial deposition of immune reactants, and intracapillary leukocyte accumulation with endoluminal hyaline pseudothrombi. Extracapillary proliferation and necrosis of the glomerular tuft was observed in 1 of these 9 cases. Mesangial glomerulonephritis with moderate interstitial inflammation and capillary immune deposits were observed in 1 patient. In these 10 cases cryoglobulin deposits were detected as organized in curved, short, thick-walled tubular structures which appear circular on cross sections. All patients had a dominant IgM deposition. Prevalent interstitial fibrosis and glomerular sclerosis were detected in the remaining case.

The improved protocol was used in all patients. Results at 6 months (**Table 3**) were as follows: remarkable improvement of the necrotizing skin ulcers in 3 of the 4 affected patients, while 1 required autologous skin transplantation; substantial amelioration of the electrophysiologic parameters of motor

and sensory peripheral neuropathy in 6 out of 8 patients; improvement of both renal function (from 2.8 mg/dl, range 1.4–5.8, to 1.34 mg/dl, range 0.9–2.1, $p < 0.001$) and proteinuria (from 4.2 g/24 h, range 0.5–9.0, to 0.4 g/24 h, range 0.1–1.0, $p < 0.001$) in 10 out of 11 patients; 1 patient could not be fully treated because of a severe infusion reaction and a sudden development of anti-Rituximab antibodies. One patient experienced a severe infection (pneumonia) which resolved with a course of i.v. antibiotic therapy. The mean time of follow-up was 38.4 months (range 6–144 months). Apart from the patient who developed anti-Rituximab antibodies, complete and persistent (after 6 months) CD20 depletion was achieved in all cases. They invariably had a complete renal response (**Table 3**). Three patients relapsed at 6, 12, and 48 months, respectively. Two of them received another full course of re-induction with Rituximab followed by a single infusion of 375 mg/m^2 every 3 months for 2 years, the remaining patient was treated with Abatacept. Every patient re-treated with RTX experienced a long lasting clinical remission. The patient treated with Abatacept had an initial renal response, but was lost from follow-up after 3 months.

Three patients died at 14, 24, and 144 months of follow-up due to cardiovascular events (two for an ischemic heart attack, while the remaining patient had a stroke).

DISCUSSION AND CONCLUSIONS

The landscape of MC is changing, with HCV-related incident cases dropping continuously due to DAA agent. In the last years we observed an inversion in the underlying causes of MC. Patients with cryoglobulinemia with no evidence of causative factors are still classified as having essential MC. The prevalence of essential MC, previously estimated to be about 1:100,000 by medical reports (17), is now increasing. Essential MC encompasses a heterogeneous group of individuals and represents a very challenging disease variant with regard to both etiopathogenetic and therapeutic concerns (18).

The clinical manifestations are variable, ranging from mild symptoms (fatigue, purpura, arthralgia) to serious complications (widespread vasculitis, glomerulonephritis). Renal involvement has been reported in 18–40% of patients with CV and it has been associated with a worse prognosis (19). In non-HCV cryoglobulinemia, age > 65 yrs and involvement of the lungs, kidneys or gastrointestinal tract have been reported as negative prognostic factors (20). The most frequent presentation (55%) (21) is proteinuria with microscopic hematuria and a variable degree of renal insufficiency; however in 5% of patients with renal involvement, acute oliguric kidney failure is the first manifestation of kidney disease. Only few studies have reported biopsy-proven renal involvement, especially in essential MC.

When compared with HCV-related cryoglobulinemia, data on the pathogenetic mechanisms underlying the development of mixed cryoglobulinemia in the context of other disorders are scarce (18). In HCV-related MC, antiviral therapy combined with rituximab proved to be very effective (15, 16, 22). However, to date, a definitive therapeutic strategy has not been established for essential non HCV MC. Low dose CSs have been proposed as an option (23). Severe manifestations require the use of IS. Today therapy of this rare disorder is mostly "eminence-based".

Most studies concerning rituximab have focused on HCV-associated mixed cryoglobulinemia. Retrospective studies showed that in non-infectious mixed CryoVas, rituximab plus corticosteroids had greater therapeutic efficacy compared to corticosteroids alone (24) or alkylating agents plus corticosteroids in achieving complete clinical, renal and immunological responses. However, the authors reported a greater number of severe infections, particularly when high doses of corticosteroids were used (12, 25), and similar death rates compared to a previously reported French cohort (25), our patients with non HCV infectious mixed CryoVas were older (63.8 vs. 51.9 years), and were also more frequently men (63.6 vs. 36.4%). Previous data reported less severe clinical manifestations and higher C4 levels compared to HCV-related cryoglobulinemia. These data were not confirmed in our cohort of patients in whom symptoms and laboratory values were comparable to those reported in patients with HCV-related cryoglobulinemia. However, compared to previous cohorts, all our patients had biopsy-proven renal involvement with immune-complex MPGN as the main histological manifestation. Renal response was achieved in 100% of patients, but one pt who was excluded due to the appearance of anti-RTX antibodies which prevented the continuation of treatment.

The findings observed in our cohort can not be considered as the results of epidemiological study however, they expand insights in the topic of HCV-negative cryoglobulinemia. As previously reported non HCV-cryoglobulinemia shows a lower percentage of the typical clinical triad (26).

A major point of our results relies on the use of rituximab administered alone without corticosteroids. This choice is in line with our previous experience with the treatment of patients with HCV-associated cryoglobulinemia with membranoproliferative glomerulonephritis (27). These results have been reported by another independent group (28).

Among the three patients two died from an ischemic heart attack, while the last patient had a stroke. With improvement in therapeutics to control acute vasculitis leading to longer survival, cardiovascular morbidity and mortality has emerged as the leading cause of death for vasculitis patients (29). The putative role of an accelerated arteriosclerosis cannot be excluded in these three patients.

In conclusion, in our cohort of non-HCV patients with cryoglobulinemia, the administration of 4 once-weekly infusions of Rituximab followed by 2 more infusions after 1 and 2 months (the so-called "4 + 2" or "improved protocol") proved to be as effective as it is in HCV-associated cryoglobulinemia. The absence of significant infections is probably due to the absence of corticosteroids.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

RF and DRoc designed the study, collected the data, drafted the manuscript, and participated in the data analysis. SS, CN, and DRos participated in the clinical evaluation, patient selection and data collection, and critically reviewed the manuscript. SS, RF, and DRoc performed the laboratory investigations and critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Ramos-Casals M, Stone JH, Cid MC, Bosch X. The cryoglobulinaemias. *Lancet.* (2012) 379:348–60. doi: 10.1016/S0140-6736(11)60242-0
2. Brouet JC, Clauvel JP, Danon F, Klein M, Seligmann M. Biologic and clinical significance of cryoglobulins. A report of 86 cases. *Am J Med.* (1974) 57:775–88. doi: 10.1016/0002-9343(74)90852-3
3. Néel A, Perrin F, Decaux O, Dejoie T, Tessoulin B, Halliez M, et al. Long-term outcome of monoclonal (type 1) cryoglobulinemia. *Am J Hematol.* (2014) 89:156–61. doi: 10.1002/ajh.23608
4. Ferri C, Sebastiani M, Giuggioli D, Cazzato M, Longombardo G, Antonelli A, et al. Mixed cryoglobulinemia: demographic, clinical, and serologic features and survival in 231 patients. *Semin Arthritis Rheum.* (2004) 33:355–74. doi: 10.1016/j.semarthrit.2003.10.001
5. Scotto G, Cibelli DC, Saracino A, Prato R, Palumbo E, Fazio V, et al. Cryoglobulinemia in subjects with HCV infection alone, HIV infection and HCV/HIV coinfection. *J. Infect.* (2006) 52:294–9. doi: 10.1016/j.jinf.2005.05.025
6. Terrier B, Marie I, Lacraz A, Belenotti P, Bonnet F, Chiche L, et al. Non HCV-related infectious cryoglobulinemia vasculitis: results from the French nationwide CryoVas survey and systematic review of the literature. *J Autoimmun.* (2015) 65:74–81. doi: 10.1016/j.jaut.2015.08.008
7. Cohen Tervaert JW, Van Paassen P, Damoiseaux J. Type II cryoglobulinemia is not associated with hepatitis C infection: the Dutch experience. *Ann N Y Acad Sci.* (2007) 1107:251–8. doi: 10.1196/annals.1381.027
8. Ferri C, Greco F, Longombardo G, Palla P, Moretti A, Marzo E, et al. Association between hepatitis C virus and mixed cryoglobulinemia. *Clin Exp Rheumatol.* (1991) 9:621–24.
9. Disdier P, Harlé JR, Weiller PJ. Cryoglobulinaemia and hepatitis C infection. *Lancet.* (1991) 338:1151–2. doi: 10.1016/0140-6736(91)92014-S
10. Arribas JR, Barbado FJ, Zapico R, Sendino A, Gonzalez I, Vazquez JJ. Association between hepatitis C virus and mixed cryoglobulinemia. *Rev Infect Dis.* (1991) 13:770–1. doi: 10.1093/clinids/13.4.770-a
11. Perez-Alamino R, Espinoza LR. Non-infectious cryoglobulinemia vasculitis (CryoVas): update on clinical and therapeutic approach. *Curr Rheumatol Rep.* (2014) 16:420. doi: 10.1007/s11926-014-0420-0
12. Damoiseaux J, Cohen Tervaert JW. Diagnostics and treatment of cryoglobulinaemia: it takes two to tango. *Clin Rev Allergy Immunol.* (2014) 47:299–310. doi: 10.1007/s12016-013-8390-y
13. Desbois AC, Cacoub P, Saadoun D. Cryoglobulinemia: an update in 2019. *Joint Bone Spine.* (2019) 86:707–13. doi: 10.1016/j.jbspin.2019.01.016
14. Rossi D, Sciascia S, Fenoglio R, Ferro M, Baldovino S, Kamgaing J, et al. Cryoglobulinemic glomerulonephritis: clinical presentation and histological features, diagnostic pitfalls and controversies in the management. State of the art and the experience on a large monocentric cohort treated with B cell depletion therapy. *Minerva Med.* (2021) 112:162–74. doi: 10.23736/S0026-4806.20.07076-7
15. Roccatello D, Baldovino S, Rossi D, Giachino O, Mansouri M, Naretto C, et al. Rituximab as a therapeutic tool in severe mixed cryoglobulinemia. *Clin Rev Allergy Immunol.* (2008) 34:111–7. doi: 10.1007/s12016-007-8019-0
16. Roccatello D, Sciascia S, Baldovino S, Rossi D, Alpa M, Naretto C, et al. Improved (4 Plus 2) rituximab protocol for severe cases of mixed cryoglobulinemia: a 6-year observational study. *Am J Nephrol.* (2016) 43:251–60. doi: 10.1159/000445841
17. Gorevic PD, Kassab HJ, Levo Y, Kohn R., Meltzer. M, Prose P, et al. Mixed cryoglobulinemia: clinical aspects and long-term follow-up of 40 patients. *Am J Med.* (1980) 69:287–308. doi: 10.1016/0002-9343(80)90390-3
18. Roccatello D, Saadoun D, Ramos-Casals M, Tzioufas AG, Fervenza FC, Cacoub P, et al. Cryoglobulinaemia. *Nat Rev Dis Primers.* (2018) 4:11. doi: 10.1038/s41572-018-0009-4
19. Tarantino A, Campise M, Banfi G, Confalonieri R, Bucci A, Montoli A, et al. Long-term predictors of survival in essential mixed cryoglobulinemic glomerulonephritis. *Kidney Int.* (1995) 47:618–23. doi: 10.1038/ki.1995.78
20. Terrier, B, Semoun O, Saadoun D, Sène D, Resche-Rigon M, et al. Prognostic factors in patients with hepatitis C virus infection and systemic vasculitis. *Arthritis Rheum.* (2011) 63:1748–57. doi: 10.1002/art.30319
21. Cacoub P, Comarmond C, Domont F, Savey L, Saadoun D. Cryoglobulinemia vasculitis. *Am J Med.* (2015) 128:950–5. doi: 10.1016/j.amjmed.2015.02.017
22. Ferri C, Cacoub P, Mazzaro C, Roccatello D, Scaini P, Sebastiani M, et al. Treatment with rituximab in patients with mixed cryoglobulinemia syndrome: results of multicenter cohort study and review of the literature. *Autoimmun Rev.* (2011) 11:48–55. doi: 10.1016/j.autrev.2011.07.005
23. Muchtar E, Magen H, Gertz MA. How I Treat cryoglobulinemia. *Blood.* (2017) 129:289–98. doi: 10.1182/blood-2016-09-719773
24. Fuentes A, Mardones C, Burgos PI. Understanding the Cryoglobulinemias. *Curr Rheumatol Rep.* (2019) 21:60. doi: 10.1007/s11926-019-0859-0
25. Terrier B, Krastinova E, Marie I, Launay D, Lacraz A, Belenotti P, et al. Management of non infectious mixed cryoglobulinemia vasculitis: data from 242 cases included in the CryoVas survey. *Blood.* (2012) 119:5996–6004. doi: 10.1182/blood-2011-12-396028
26. Mascia MT, Ferrari D, Campioli D, Sandri G, Mussini C, Ferri C. Non HCV-related mixed cryoglobulinemia. *Dig Liver Dis.* (2007) 39:S61–4. doi: 10.1016/S1590-8658(07)80013-2
27. Roccatello D, Baldovino S, Rossi D, Mansouri M, Naretto C, Gennaro M, et al. Long-term effects of anti-CD20 monoclonal antibody treatment of cryoglobulinaemic glomerulonephritis. *Nephrol Dial Transplant.* (2004) 19:3054–61. doi: 10.1093/ndt/gfh469
28. Quartuccio L, Soardo G, Romano G, Zaja F, Scott CA, De Marchi G, et al. Rituximab treatment for glomerulonephritis in HCV-associated mixed cryoglobulinaemia: efficacy and safety in the absence of steroids. *Rheumatology.* (2006) 45:842–6. doi: 10.1093/rheumatology/kel004
29. Clifford AH, Cohen Tervaert JW. Cardiovascular events and the role of accelerated atherosclerosis in systemic vasculitis. *Atherosclerosis.* (2021) 325:8–15. doi: 10.1016/j.atherosclerosis.2021.03.032

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.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership