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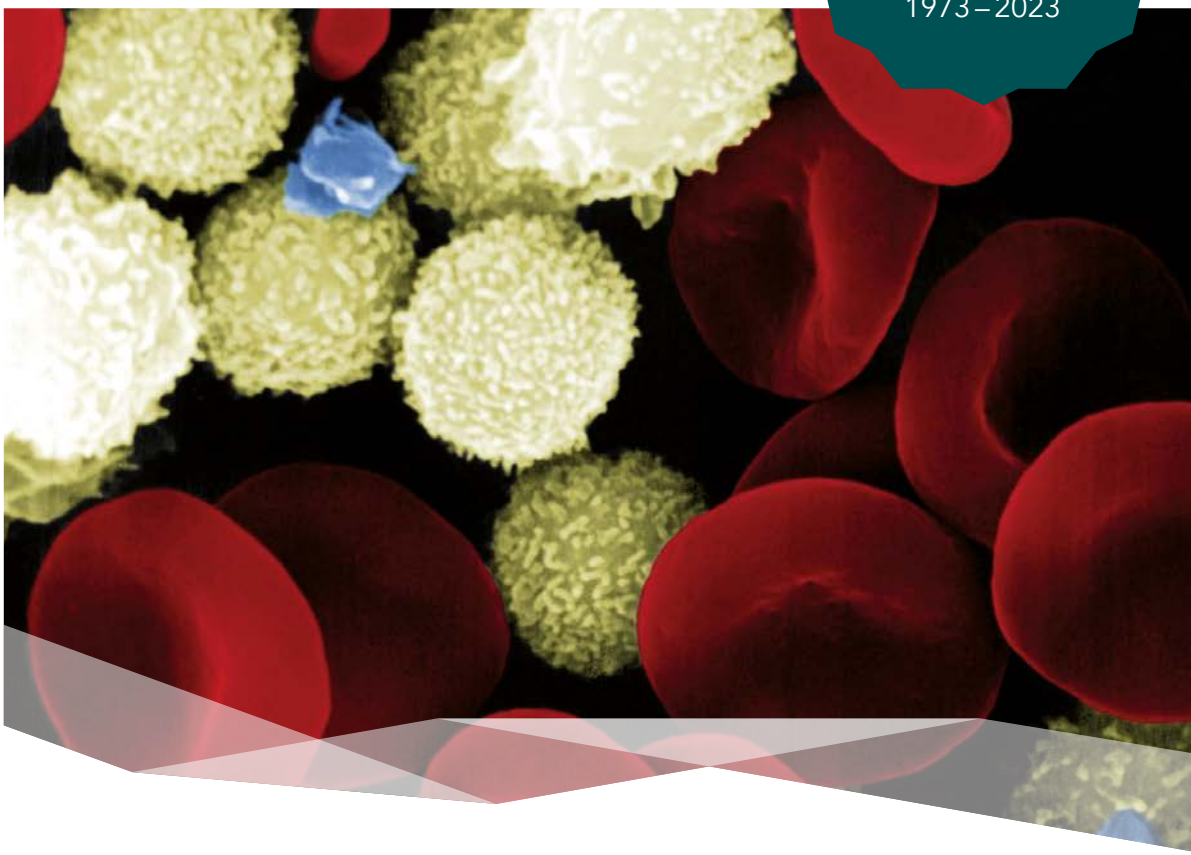

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This journal is devoted to all areas of transfusion medicine. These include the quality and security of blood products, therapy with blood components and plasma derivatives, transfusion-related questions in transplantation, stem cell manipulation, therapeutic and diagnostic problems of hemostasis, immuno-hematological investigations, and legal aspects of the production of blood products as well as hemotherapy. Both comprehensive reviews and primary publications that detail the newest work in transfusion medicine and hemotherapy promote the international exchange of knowledge within these disciplines. Consistent with this goal, continuing clinical education is also specifically addressed.

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Evaluation of Single Nucleotide Variants in Intron 1 of the ABO Gene as Diagnostic Markers for the A_1 Blood Group

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Keywords

ABO genotyping · ABO blood group · Diagnostic marker · Sensitivity · Specificity

Abstract

Introduction: The molecular diagnosis of the A_1 blood group is based on the exclusion of *ABO* gene variants causing blood groups A_2, B, or O. A specific genetic marker for the A_1 blood group is still missing. Recently, long-read ABO sequencing revealed four sequence variations in intron 1 as promising markers for the *ABO*A1* allele. Here, we evaluated the diagnostic values of the 4 variants in blood donors with regular and weak A phenotypes and genotypes. ***Methods:*** ABO phenotype data (A, B, AB, or O) were taken from the blood donor files. The *ABO* genotypes (low resolution) were known from a previous study and included the variants c.261delG, c.802G>A, c.803G>C, and c.1061delC. *ABO* variant alleles (*ABO*AW.06, *AW.08, *AW.09, *AW.13*, **AW.30*, and **A3.02*) were identified in weak A donors by sequencing the *ABO* exons before. For genotyping of the *ABO* intron 1 variants rs532436, rs1554760445, rs507666, and rs2519093, we applied TaqMan assays with endpoint fluorescence detection according to a standard protocol. Genotypes of the variants were compared with the ABO phenotype and genotype. Evaluation of diagnostic performance included sensitivity, specificity, positive (PPV), and negative predictive value (NPV). ***Results:*** In 1,330 blood donors with regular ABO phenotypes and genotypes, the intron 1 variants were significantly associated with the proposed A_1 blood group. In 15 donors, we found discrepancies to the genotype of at least one of the 4 variants. For the diagnosis of the ABO*A1 allele, the variants showed 98.79–99.48% sensitivity, 99.66–99.81% specificity, 98.80–99.31% PPV, and 99.66–99.86% NPV. Regarding the A phenotype, the diagnostic values were 99.02–99.41% sensitivity, 99.63–99.76% specificity, 99.41–99.61% PPV, and 99.39–99.63% NPV. The **A1* marker allele of all intron 1 variants was also associated with the **AW.06*, **AW.13*, and **AW.30* variants. Samples with **AW.08*, **AW.09*, and **A3.02* variants lacked this association. ***Conclusion:*** The *ABO* intron 1 variants revealed significant association with the *ABO*A1* allele and the A phenotype. However, the intron 1 genotype does not exclude variant alleles causing weak A phenotypes. With the introduction of reliable tag, single nucleotide variants for the A_1, A_2, B, and O blood groups and the genotyping instead of phenotyping of the ABO blood group are getting more feasible on a routine basis.

Introduction

The ABO blood group system (ISBT 001) includes the two major antigens A and B. The further antigens A, B, and A1 are less common. The major phenotypes A, B, AB, and O are defined by the presence or absence of the A and B antigens [1]. The A phenotype is divided into A_1 and A_2 with approximately 5 times more A epitopes per red cell for A_1. Furthermore, a significantly lower number of epitopes per red cell is the most important cause of additional phenotypes such as weak A and weak B.

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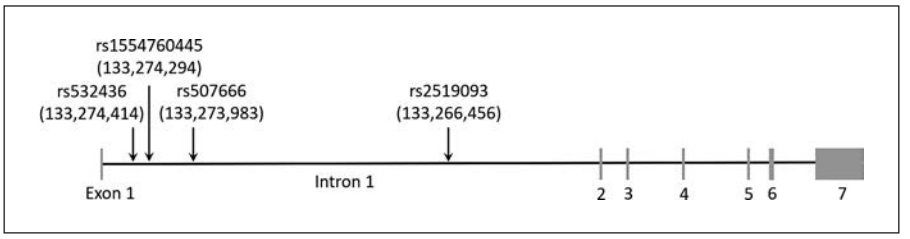

Fig. 1. Genomic structure of the *ABO* gene on chromosome 9 and location of rs532436, rs1554760445, rs507666, and rs2519093. Chromosomal position of the variations is given according to the reference genome GRCh38.p13.

The *ABO* gene located on the long arm of human chromosome 9 encodes the ABO blood group system. Compared to the reference allele *ABO***A1.01*, most of the sequence variations were identified in exon 6 and 7, encoding the catalytic domain of the glycosyltransferase [2, 3]. According to the ISBT allele table (version 1.2, released on October 21, 2017), 84 alleles are associated with the A_1, A_2, and weak A phenotypes [4]. Most of the **A2* alleles for the A_2 phenotype have the deletion of C at position c.1061 in common. The 49 listed alleles for the B and weak B phenotypes are defined by specific sequence variations such as c.803G>C compared to the *ABO***A1.01* reference allele. The O phenotype is most commonly caused by *ABO* alleles with deletion of G at position c.261. More than 40 deletional *ABO***O.01* alleles with sequence variations in addition to c.261delG have been described. The most common non-deletional allele *ABO***O.02* is characterized by the missense variation c.802G>A in exon 7. In summary, genotyping of the most relevant ABO sequence variations c.261delG, c.802G>A, c.803G>C, and c.1061delC enabled diagnosis of the A_2, B, and O phenotypes [5]. Exclusion of these proposed the **A1* allele for the A_1 phenotype. This low-resolution genotype does not consider rare variants with mutations at other positions.

In a recent study by Gueuning et al. [6], long-read sequencing of the entire *ABO* gene led to the identification of four sequence variations in intron 1 significantly associated with the *ABO***A1* allele. These variants represent promising diagnostic targets for the A_1 blood group. Here, we genotyped the four intron 1 variants in a representative sample of 1,330 blood donors with known ABO phenotype and genotyped for **A2*, **B*, **O.01*, and **O.02* alleles. The diagnostic values were calculated for each variant with regard to the identification of the **A1* allele and the A blood group.

Materials and Methods

Blood Donors

The geographical origin of blood donors of our transfusion service is the southwestern part of Germany. A DNA bank was established as a representative sample of our blood donor cohort and encompasses 1,330 blood donors with a mean age of 46.8 ± 15.3 years (range 18.0–68.8 years) and 1:1 gender distribution. DNA was isolated from buffy coats of CPD-anticoagulated whole blood donations using a commercial system (FlexiGene DNA Kit; Qiagen, Hilden, Germany). Data about ABO blood groups were taken from the blood donor files, and all donors had regular ABO blood groups (A, B, AB, or O) without discrimination of the A_1 and A_2 phenotypes. The low-resolution *ABO* genotypes were determined in a previous study [7] and included the variants c.261delG (for deletional **O.01* alleles), c.802G>A (for the non-deletional O alleles **O.02*), c.803G>C (for **B* alleles), and c.1061delC (for **A2* alleles). The A_1, A_2, B, and O phenotypes were deduced from the low-resolution genotype. Furthermore, DNA samples from blood donors and patients with weak A phenotype and *ABO* variant alleles previously identified by sequencing exons 1 to 7 were included. All donors gave written consent to use their blood samples for research purposes. The anonymous DNA bank was approved by the Ethics Committee of the Heidelberg University, Medical Faculty Mannheim.

Genotyping of ABO Intron 1 Variants

According to the database (dbSNP), the four variants are located in intron 1 of the *ABO* gene (Fig. 1). The rs1554760445 is a CA>T insertion/deletion (INDEL) variant, whereas rs532436 (G>A), rs507666 (G>A), and rs2519093 (C>T) are single nucleotide variants (SNVs). Allelic discrimination was achieved by endpoint fluorescence detection using custom TaqMan™ SNP genotyping assays (assay IDs: AN9HYTF for rs2519093; ANAAP46 for rs532436; ANCFJP3 for rs507666; and ANDKEAZ for rs1554760445) designed and produced by Thermo Fisher Scientific (Darmstadt, Germany). For all variants, the minor allele was the proposed tag for the *ABO***A1* allele.

Results

Allelic discrimination was achieved for all variants and enabled determination of the genotypes in most of the 1,330 samples (Fig. 2). Genotyping failed for rs532436 in 3 samples, for rs1554760445 in 4 samples, for rs507666 in 8 samples, and for rs2519093 in 3 samples. The minor allele frequencies were 0.2163 for rs532436, 0.2177 for rs1554760445, 0.2179 for rs507666, and 0.218 for rs2519093.

For most of the samples, the genotype of the intron 1 variants corresponded to the low-resolution *ABO* genotype and the phenotype. The proposed **A1* markers showed false positive or false negative results in 15 samples (Table 1). In 9 samples, at least one of the intron 1 variants was wrong, and in 6 samples, all 4 markers showed a genotype not corresponding to the proposed *ABO* genotype. With regard to the diagnosis of blood

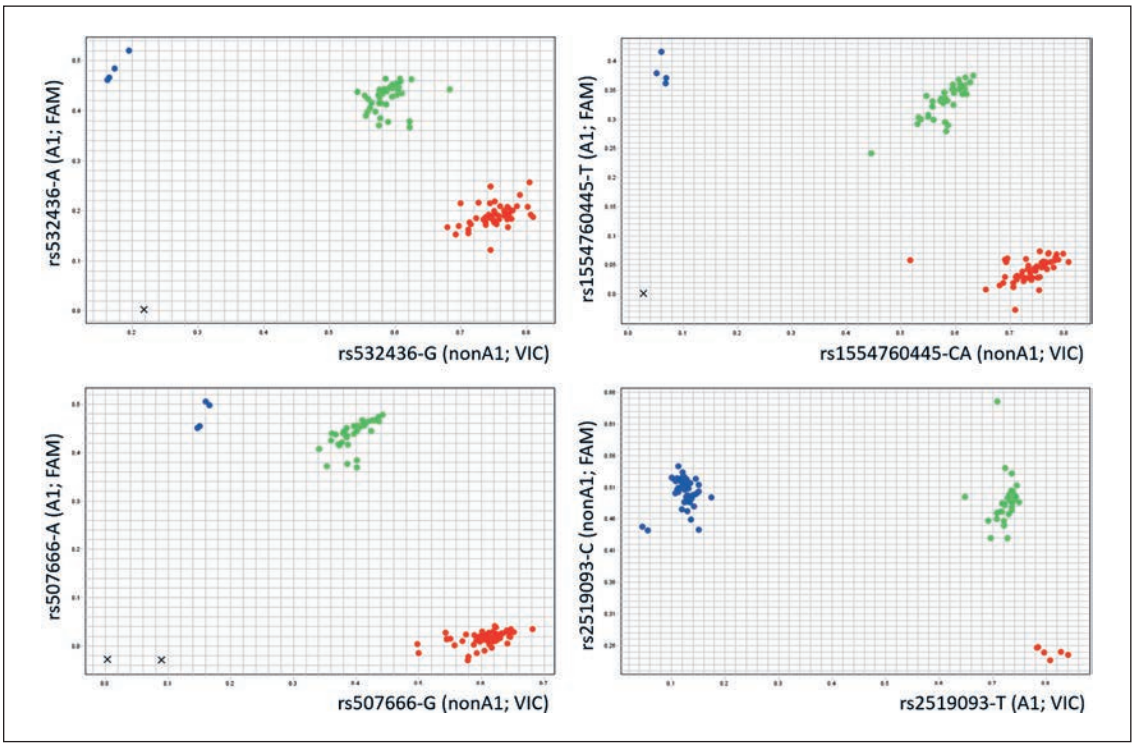

Fig. 2. Representative results from genotyping of 95 DNA samples for rs532436, rs1554760445, rs507666, and rs2519093 by using TaqMan™ assays. Allelic discrimination was achieved by endpoint fluorescence detection of FAM- and VIC-labeled allele-specific probes. All samples group into homozygous for VIC allele (red dots), homozygous for FAM allele (blue dots), and heterozygous for both (green dots).

Table 1. In 15 samples, the genotypes of the intron 1 variants revealed discrepancy to the ABO phenotype or low-resolution genotype

Sample	Phenotype*	Genotype	Deduced phenotype#	rs532436	rs1554760445	rs507666	rs2519093
M01E10	**AB**	***A1/*B**	**A1B**	**nonA1 (FN)**	**A1/nonA1**	**A1 (FP)**	**A1/nonA1**
M02B09	**A**	***O.01*A2**	**A2**	**A1/nonA1 (FP)**	**A1/nonA1 (FP)**	**A1/nonA1 (FP)**	**nonA1**
M03F08	**A**	***O.01/*A1**	**A1**	**nonA1 (FN)**	**nonA1 (FN)**	**nonA1 (FN)**	**nonA1 (FN)**
M05C04	**A**	***O.01/*A1**	**A1**	**nonA1 (FN)**	**nonA1 (FN)**	**nonA1 (FN)**	**nonA1 (FN)**
M05G09	A	**A2/*A2*	A_2	A1/nonA1 (FP)	A1/nonA1 (FP)	A1/nonA1 (FP)	A1/nonA1 (FP)
M07C07	A	**A1*	A_1	A1/nonA1 (FN)	A1/nonA1 (FN)	A1/nonA1 (FN)	A1
M07G07	A	**A1*	A_1	A1/nonA1 (FN)	A1/nonA1 (FN)	A1/nonA1 (FN)	A1
M10A06	A	**O.02/*A1*	A_1	A1/nonA1	A1/nonA1	A1/nonA1	A1 (FP)
M10F09	**A**	***O.01/*A1**	**A1**	**nonA1 (FN)**	**nonA1 (FN)**	**A1/nonA1**	**A1/nonA1**
M11B01	**0**	***O.01**	**0**	**A1/nonA1 (FP)**	**A1/nonA1 (FP)**	**A1/nonA1 (FP)**	**A1/nonA1 (FP)**
M11D12	**0**	***O.01**	**0**	**A1/nonA1 (FP)**	**A1/nonA1 (FP)**	**A1/nonA1 (FP)**	**A1/nonA1 (FP)**
M12E12	A	**O.02/*A1*	A_1	A1/nonA1	A1/nonA1	A1/nonA1	A1 (FP)
M13D08	**AB**	***A/*B**	**A1B**	**nonA1 (FN)**	**nonA1 (FN)**	**nonA1 (FN)**	**nonA1 (FN)**
M13G10	A	**O.01/*A1*	A_1	A1/nonA1	A1/nonA1	A1/nonA1	A1 (FP)
M14G12	A	**O.02/*A1*	A_1	A1/nonA1	A1/nonA1	A1/nonA1	A1 (FP)

* ABO phenotype from the donor files without discrimination of A_1 and A_2 phenotype. # ABO phenotype deduced from the low-resolution genotype; gray background indicates false *ABO* genotype proposed from the genotype of the intron 1 variants: FN, false negative; FP, false positive for the A1 allele; in 8 samples given in bold letters the genotypes of the intron 1 variants indicated a wrong A phenotype.

group A, the intron 1 variants failed in 8 of the 15 samples (Table 1). In two samples with blood group O, all intron 1 variants were heterozygous indicating blood group A_1. In three samples with blood group A, the absence of the **A1* allele at all intron variants revealed false negative results. In all 15 samples sequencing of the *ABO* exons, 1 to 7 confirmed the supposed presence or absence of the **A1.01* allele.

Table 2. Calculation of the diagnostic values for the intron 1 variants with regard to the diagnosis of the *ABO*A1* allele and the A phenotype

	Intron 1 variants			
Diagnosis of the *ABO*A1* allele	rs532436	rs1554760445	rs507666	rs2519093
True positive, *n*	570	573	571	574
False positive, *n*	4	4	5	7
True negative, *n*	2,073	2,069	2,063	2,070
False negative, *n*	7	6	5	3
Sensitivity, %	98.79	98.96	99.13	99.48
Specificity, %	99.81	99.81	99.76	99.66
PPV, %	99.30	99.31	99.13	98.80
NPV, %	99.66	99.71	99.76	99.86
Diagnosis of the A phenotype				
True positive, *n*	505	507	504	505
False positive, *n*	2	2	3	2
True negative, *n*	815	813	812	816
False negative, *n*	5	4	3	4
Sensitivity, %	99.02	99.22	99.41	99.21
Specificity, %	99.76	99.75	99.63	99.76
PPV, %	99.61	99.61	99.41	99.61
NPV, %	99.39	99.51	99.63	99.51

Sensitivity: true positive/(true positive + false negative); specificity: true negative/(true negative + false positive); positive predictive value (PPV): true positive/(true positive + false positive); negative predictive value (NPV): true negative/(true negative + false negative).

Table 3. Association of intron 1 variants with *ABO* variant alleles causing the weak A phenotype

Variant*	Samples, *n*	Genotype	rs532436	rs1554760445	rs507666	rs2519093
**AW.06*	12	**O.01/*AW.06*	A1/nonA1	A1/nonA1	A1/nonA1	A1/nonA1
	1	**AW.06/*B.01*	A1/nonA1	A1/nonA1	A1/nonA1	A1/nonA1
**AW.08*	10	**O.01/*AW.08*	nonA1	nonA1	nonA1	nonA1
**AW.09*	4	**O.01/*AW.09*	nonA1	nonA1	nonA1	nonA1
**AW.13*	11	**O.01/*AW.13*	A1/nonA1	A1/nonA1	A1/nonA1	A1/nonA1
	4	**AW.13/*B.01*	A1/nonA1	A1/nonA1	A1/nonA1	A1/nonA1
	2	**AW.13/*B.01*	nonA1	nonA1	nonA1	nonA1
**AW.30*	25	**O.01/*AW.30*	A1/nonA1	A1/nonA1	A1/nonA1	A1/nonA1
	1	**O.02/*AW.30*	A1/nonA1	A1/nonA1	A1/nonA1	A1/nonA1
	1	**AW.30/*B.01*	A1/nonA1	A1/nonA1	A1/nonA1	A1/nonA1
**A3.02*	5	**O.01/*A3.02*	nonA1	nonA1	nonA1	nonA1
	1	**A3.02/*B.01*	nonA1	nonA1	nonA1	nonA1

ABO variants defined by mutations: c.502C>G (**AW.06*), c.488C>T and c.526C>G (**AW.08*), c.46G>A (**AW.09*), c.2T>C (**AW.13*), c.646T>A (**AW.30* and others), and c.829G>A (**A3.02*).

The diagnostic values including sensitivity, specificity, positive (PPV), and negative predictive value (NPV) were calculated for the intron 1 variants (Table 2). Overall, the diagnostic values for the 4 intron 1 variants were similar. For the diagnosis of the *ABO*A1* allele, the intron 1 variants showed a sensitivity of 98.79–99.48%, a specificity of 99.66–99.81%, a PPV of 98.80–99.31%, and a NPV of 99.66–99.86%. For the diagnosis of the A phenotype, we found 99.02–99.41% sensitivity, 99.63–99.76% specificity, 99.41–99.61% PPV, and 99.39–99.63% NPV. None of the intron 1 variants showed complete association with the *ABO*A1* allele or the A phenotype.

Furthermore, 77 samples with known *ABO* variant alleles causing a weak A phenotype were genotyped for the intron 1 variants (Table 3). The **AW.06* and **AW.30* variants were associated with the **A1* allele of all intron 1 variants in 13 and 27 samples, respectively. For the **AW.13* variant, we found an association with the **A1* al-

lele of the intron 1 variants in 15 of 17 samples. Two samples with **AW.13* and all samples with **AW.08*, **AW.09*, and **A3.02* variants lacked this association.

Discussion

The identification of sequence characteristics of the *ABO* gene as specific markers for the A, B, and O blood groups enabled the molecular diagnosis of ABO phenotypes. Because of the lack of a specific marker, the A_1 phenotype was diagnosed by exclusion of alleles encoding A_2, B, or O phenotypes. Based on the genotyping of the 4 major variants c.261delG, c.802G>A, c.803G>C, and c.1061delC, a genotype-phenotype correlation was achieved for 99.7% of the samples [7]. However, the significant diversity of the *ABO* gene and the increasing number of mutations associated with aberrant blood group phenotypes impeded the molecular diagnosis. The introduction of additional DNA markers, especially for the A_1 blood group, is important to increase the specificity and sensitivity of the molecular diagnosis of the ABO blood group.

The rs507666 and rs2519093 have been used as a marker for the A_1 blood group in studies on the association of the *ABO* locus with ICAM-1 plasma levels and trisk of venous thrombosis, respectively [8, 9]. Further analyses linking the lead SNVs to *ABO* allele groups have not been undertaken in these studies. Thus, proof of the diagnostic value of the markers as tags for blood group A_1 was lacking. Long-read sequencing of the *ABO* locus uncovered putatively *ABO*A1* diagnostic variants in the intron 1 region [6]. Three SNVs (rs532436, rs1554760445, and rs507666) are located in close proximity within a 431 bp region at the 5' end of intron 1. The rs2519093 is located more than 7.5 kbp downstream from rs507666. In many of the samples with discordant results, we observed a linkage of the first 3 SNVs, i.e., concordant genotypes, whereas rs2519093 revealed a different genotype. This could indicate recombination events in intron 1 as a cause of discordant genotyping results. A multi-ethnic validation approach indicated that the three SNV-based variants rs532436, rs507666, and rs2519093 significantly tagged the *ABO*A1* allele across ethnicities. The INDEL variant rs1554760445 specifically tagged the *ABO*A1.01* allele in populations outside Africa. The sensitivities ranged from 97.55% (rs507666) to 97.99% (rs2519093) and the specificities from 99.41% (rs532436) to 99.72% (rs1554760445). The geographic origin of the blood donors in our study is Southwestern Germany; however, the ethnicity is not known in detail. It can be assumed that the majority of the donors are European, and only a minor proportion is of African descent.

We evaluated the performance of the intron 1 variants for the diagnosis of the *ABO*A1* allele. All 4 variants showed a strong but not complete association with the **A1* allele in 1,330 samples with regular ABO phenotypes. For the diagnosis of the *ABO*A1* allele, we found sensitivities ranging from 98.79% (rs532436) to 99.48% (rs2519093) and specificities from 99.66% (rs2519093) to 99.81% (rs532436), similar to the values reported previously [6]. For the diagnosis of the A blood group, the rs507666 showed the highest sensitivity (99.41%), whereas, rs532436 and rs2519093 had the highest specificity (99.76%). In 6 of the 1,330 samples (0.45%), none of the 4 intron 1 variants showed correct tagging of the **A1* allele with 3 false negative and 3 false positive results. The combination of all 4 intron variants did not increase the diagnostic performance. According to our data from blood donors with regular ABO phenotypes, the genotyping of the intron 1 variants would lead to a wrong phenotype prediction in 0.6% of the donors. To increase the diagnostic reliability, ABO genotyping should include the markers for **A2*, **B*, **O.01*, and **O.02* alleles in addition to the **A1* markers in intron 1.

Further limitation of ABO genotyping is given by the large number of variant alleles. Most of these variants are very rare (<0.01%), and the association with the intron 1 variants is not known. In our study, we found that the rare *ABO*AW.06*, **AW.13*, and **AW.30* alleles causing a weak A phenotype were associated with the **A1* markers, i.e., the intron 1 variants indicated a regular A_1 phenotype. Other variant alleles such as **AW.08*, **AW.09*, and **A3.02* lacked this association, i.e., intron 1 genotyping indicated the absence of a regular A_1 phenotype. The prevalence of variant alleles could be different in the populations. The **AW.09* allele tagged by c.49G>A (rs55917063) is rare in Europeans (0.02%) but frequent in Africans (2%) [10]. The frequency of the **AW.08* allele tagged by c.488C>T (rs55756402) is 0.1% in Europeans and South Asians but not observed in Africans. Thus, depending on the population, the association of the **A1* markers with rare *ABO* gene variants could significantly delimit the correct diagnosis of the A_1 blood group.

Blood group genotyping projects usually include numerous clinically relevant blood group systems but not ABO. This is because ABO phenotyping is cost effective and well established in routine labs. The diagnosis of the ABO blood group based on genotyping is getting more relevant since molecular techniques are developed toward high-throughput screening. For example, the genotype screening project at our institute includes 37 blood groups as well as platelet and neutrophil antigens [11]. The inclusion of *ABO* genotyping with tag SNVs could be cost and time effective. Thereby, the availability of reliable diagnostic markers for the main phenotypes is an important prerequisite. The genotyping platform for blood donors introduced by the Blood Transfusion Genomics Consortium (BGC) comprised a large number of

determinants of blood group, platelet, and leukocyte antigens as well as variants in genes relevant to antigen expression [12]. The genotyping results demonstrated concordance of 99.91% with antigen typing data for 28 blood group antigens including ABO. In 7 of 7,449 samples (0.09%), the ABO genotyping results were discordant with the phenotype.

In conclusion, with the introduction of reliable molecular markers, genotyping instead of phenotyping of the ABO blood group is getting more feasible also on a routine basis. This could be reached by a combination of SNVs that tag the A_1, A_2, B, and O phenotypes and the exclusion of *ABO* variants encoding aberrant phenotypes. However, as long as discordance of genotypes is observed, the antibody-based ABO phenotyping remains the only method to ensure ABO-matched blood transfusion.

Statement of Ethics

This study was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. The donors gave written consent to provide blood samples for research purposes. The anonymous DNA bank was approved by the Ethics Committee of the Heidelberg University, Medical Faculty Mannheim (Ref. 87/04).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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No funding was received for this study.

Author Contributions

Peter Bugert coordinated the study, analyzed the data, and wrote the manuscript. Gabi Rink performed genotyping and summarized the data. Harald Klüter coordinated the study and wrote the manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

References

1 Storry JR, Olsson ML. The ABO blood group system revisited: a review and update. Immunohematology. 2009;25(2):48–59.
2 Yamamoto F, McNeill PD, Hakomori S. Genomic organization of human histo-blood group ABO genes. Glycobiology. 1995;5(1):51–8.
3 Chester MA, Olsson ML. The ABO blood group gene: a locus of considerable genetic diversity. Transfus Med Rev. 2001;15(3):177–200.
4 The International Society of Blood Transfusion. ABO (ISBT 001) blood group allele table.
5 Gassner C, Schmarda A, Nussbaumer W, Schonitzer D. ABO glycosyltransferase genotyping by polymerase chain reaction using sequence-specific primers. Blood. 1996;88(5):1852–6.
6 Gueuning M, Thun GA, Wittig M, Galati AL, Meyer S, Trost N, et al. Haplotype sequence collection of ABO blood group alleles by long-read sequencing reveals putative A1-diagnostic variants. Blood Adv. 2022 Sep 21:bloodadvances.2022007133.
7 Bugert P, Rink G, Kemp K, Klüter H. Blood group ABO genotyping in paternity testing. Transfus Med Hemother. 2012 Jun;39(3):182–6.
8 Paré G, Chasman DI, Kellogg M, Zee RY, Rifai N, Badola S, et al. Novel association of ABO histo-blood group antigen with soluble ICAM-1: results of a genome-wide association study of 6, 578 women. PLoS Genet. 2008 Jul 4;4(7):e1000118.
9 Goumidi L, Thibord F, Wiggins KL, Li-Gao R, Brown MR, van Hylckama Vlieg A, et al. Association between ABO haplotypes and the risk of venous thrombosis: impact on disease risk estimation. Blood. 2021 Apr 29;137(17):2394–402.
10 gnomAD database (accessed December 06, 2022).
11 Portegys J, Rink G, Bloos P, Scharberg EA, Klüter H, Bugert P. Towards a regional registry of extended typed blood donors: molecular typing for blood group, platelet and granulocyte antigens. Transfus Med Hemother. 2018 Oct;45(5):331–40.
12 Gleadall NS, Veldhuisen B, Gollub J, Butterworth AS, Ord J, Penkett CJ, et al. Development and validation of a universal blood donor genotyping platform: a multinational prospective study. Blood Adv. 2020 Aug 11;4(15):3495–506.

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ECIL: European Conference on Infections in Leukaemia; **NUB:** Neue Untersuchungs- und Behandlungsmethoden.

Referenzen:
1. Ljungman P et al. Guidelines for the management of cytomegalovirus infection in patients with haematological malignancies and after stem cell transplantation from the 2017 European Conference on Infections in Leukaemia (ECIL 7). Lancet Infect Dis. 2019;19(8):e260 – e272. **2.** Einsele H et al. How I treat CMV reactivation after allogeneic hematopoietic stem cell transplantation. Blood. 2020;135(19):1619 – 1629. **3.** Derigs P et al. Letermovir prophylaxis is effective in preventing cytomegalovirus reactivation after allogeneic hematopoietic cell transplantation: single-center real-world data. Ann Hematol. 2021;100(8):2087 – 2093. **4.** Unveröffentlichte Daten bei MSD.

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Effects of Different Hemoglobin Levels on Near-Infrared Spectroscopy-Derived Cerebral Oxygen Saturation in Elderly Patients Undergoing Noncardiac Surgery

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Keywords

Regional cerebral oxygen saturation · Near-infrared spectroscopy · Red blood cell transfusion

Abstract

Background: Near-infrared spectroscopy (NIRS) is a commonly used technique to evaluate tissue oxygenation and prevent harmful cerebral desaturation in the perioperative setting. The aims of the present study were to assess whether surgery-related anemia can be detected via NIRS of cerebral oxygen saturation and to investigate the effects of different perioperative transfusion strategies on cerebral oxygenation, potentially affecting transfusion decision-making. ***Study Design and Methods:*** Data from the ongoing multicenter LIBERAL-Trial (liberal transfusion strategy to prevent mortality and anemia-associated ischemic events in elderly noncardiac surgical patients, LIBERAL) were used. In this single-center sub-study, regional cerebral oxygenation saturation (rSO_2) was evaluated by NIRS at baseline, pre-, and post-RBC transfusion. The obtained values were correlated with blood gas analysis-measured Hb concentrations. ***Results:*** rSO_2 correlated with Hb decline during surgery ($r = 0.35$, $p < 0.0001$). Different RBC transfusion strategies impacted rSO_2 such that higher Hb values resulted in higher rSO_2. Cerebral desaturation occurred at lower Hb values more often. ***Discussion:*** Cerebral oxygenation monitoring using NIRS provides noninvasive rapid and continuous information regarding perioperative alterations in Hb concentration without the utilization of patients' blood for blood sampling. Further investigations are required to demonstrate if cerebral rSO_2 may be included in future individualized transfusion decision strategies.

Introduction

Transfusion of allogeneic red blood cell (RBC) units is frequently required during major surgery with elderly patient being at risk due to higher incidence of comorbidities including preoperative anemia and vascular disease predisposing to myocardial and cerebral ischemia [1–3]. Transfusion of RBC is the standard therapy of perioperative anemia with a broad Hb treatment range between 6 and 10 g/dL. However, existing evidence does not support a single criterion for transfusion and justification should depend on patients comorbidities, potential or actual on-

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going bleeding (rate and magnitude), volume status, cardiopulmonary reserve, and most notably signs of organ ischemia resulting in impaired tissue oxygenation [4].

Near-infrared spectroscopy (NIRS) is a commonly applied, noninvasive technique that uses the different absorption and dispersion spectrum of near infrared light to distinguish between oxygenated and deoxygenated hemoglobin, indicative of tissue oxygenation, in different tissues approximately 25 mm below the skull [5]. Therefore, any given NIRS presents a relative value that reflects a balance between oxygen demand and supply, within the evaluated tissue at current conditions. Furthermore, the assessed value depended on patient's individual baseline but also affected by various covariates including perfusion, oxygenation, and hemoglobin concentration [6]. While tissue perfusion is influenced by blood pressure and cardiac function, oxygenation is controlled during general anesthesia through the ventilatory settings. Hemodynamics and oxygenation are continuously monitored and kept within predefined ranges during anesthesia. Although hemoglobin concentrations commonly change during the course of major surgery with increased probability of blood loss, affecting tissue oxygenation, noninvasive techniques continuously evaluating hemoglobin concentration tend to be inferior to invasive blood gas analyses (BGAs). However, BGA is usually performed in response to clinical signs including blood loss and volume compensation and thus provides only intermittent information.

Most studies investigating the perioperative impact of cerebral oxygenation saturation (rSO_2) on postoperative outcome are performed in cardiac or vascular surgery and report a correlation between rSO_2 and central venous oxygenation saturation. Current guidelines recommend monitoring of cerebral oxygenation during cardiac surgery to detect cerebral desaturation early, to increase oxygen supply, and thereby to improve neurological outcome [7]. Although, investigation showed that NIRS can be used for the detection of anemia in intensive care patients, no study has yet investigated the perioperative usage of NIRS for the detection of intraoperative bleeding and consecutive anemia and deoxygenation to potentially consider transfusion of RBC in adult patients undergoing major noncardiac surgery. Therefore, we hypothesize that anemia occurring during noncardiac surgery can be detected via NIRS evaluation of cerebral oxygen saturation and RBC transfusion improves cerebral oxygenation, potentially affecting transfusion decision-making (TDM) in elderly patients.

Material and Methods

Study Design

The present analysis contains data of 67 consecutive patients, undergoing spine or hip surgery, recruited within the ongoing LIBERAL-Trial from 2020 to 2021. The LIBERAL-Trial is a prospective, multicenter, randomized, open, phase 4 trial, investigating the effects of a liberal transfusion strategy of RBCs on mortality and anemia-associated ischemic events in elderly patients undergoing noncardiac surgery [8]. In brief, patients ≥70 years scheduled for intermediate- or high-risk noncardiac surgery were included. Hemoglobin levels were monitored during the surgery and daily after surgery. If Hb level dropped below ≤9.0 g/dL during surgery or in the postoperative phase until postoperative day 3, patients were randomized either to the LIBERAL (receiving one single RBC unit each time Hb reaches ≤9.0 g/dL) or restrictive transfusion regime (a single RBC unit each time Hb reaches ≤7.5 g/dL). To investigate the impact of anemia severity on RBC transfusion-induced changes of cerebral NIRS, we grouped patients according to their pre-transfusion Hb concentration irrespective of their randomization group assignment: Hb <7.5 g/dL, Hb 7.5–8.5, and Hb ≥8.5 g/dL. This sub-study did neither unblind any data nor analyze any group differences of the large LIBERAL trial.

Anesthesia Protocol

Pulse oximetry, electrocardiogram, noninvasive blood pressure measurement, and a peripheral line were established upon arrival at the introduction room. Anesthesia was induced using fentanyl, propofol (2 mg/kg), and rocuronium (0.5 mg/kg). Following intubation, ventilation was adjusted to 8–10 mL/kg and respiratory rate was set to maintain $etCO_2$ at 35–40 mm Hg. Subsequently, the radial artery was cannulated for invasive blood pressure measurement and BGAs. Oxygen concentration was adjusted to pO_2 between 120 and 150 mmHg to maintain SpO_2 above 95%. Anesthesia was maintained using propofol and sedation dept was monitored using bi-spectral index monitoring (XP-sensor, Covidien plc, Dublin, Ireland) and bi-spectral index values were kept between 40 and 60, ensuring an appropriate anesthesia level. During the surgery, phenylephrine infusion has been used to maintain mean arterial blood pressure within 20% of pre-anesthesia level. To assess regional tissue oxygenation, the probe of an INVOS™ 5100C (Medtronic Inc., Minneapolis, MN, USA) was placed on the right forehead and cerebral oxygenation saturation (rSO_2) was continuously monitored throughout the surgery. Anesthesia regime and NIRS evaluation was performed in the present sub-study according to institutional standard and not specified in the LIBERAL-Trial study protocol [8]. Patients with carotid artery stenosis were not included due to its potential impact on cerebral perfusion. rSO_2 values were assessed through NIRS prior to and 10 min after transfusion of each RBC unit to investigate the impact of RBC transfusion on cerebral tissue oxygenation at different Hb concentrations.

Statistical Analyses

Statistical analyses were performed using Graph Pad PRISM 8. Data are expressed as mean ± standard deviation for normally distributed continuous variables. Differences between groups were determined using one-way ANOVA and Bonferroni post hoc analyses. Baseline, pre-, and post-transfusion differences were analyzed using two-way ANOVA and Bonferroni post hoc analyses.

Results

The present study included 67 patients that received RBC transfusion during elective hip or spine surgery. Age, height, weight, and BMI were not different between groups (Table 1).

Table 1. Patients characteristics were analyzed using one-way ANOVA with Bonferroni post hoc analyses and are expressed as mean ± SEM ($p < 0.01$)

	<7.5 g/dL	7.5–8.5 g/dL	>8.5 g/dL	*p* value
Age, years	79±1	79±1	78±1	ns
Height, cm	170±2	167±2	171±2	ns
Weight, kg	78±4	72±3	81±4	ns
BMI, kg/m^2	27±1	26±1	27±1	ns

Cerebral regional oxygen saturation (rSO_2) and Hb concentrations were evaluated after induction of anesthesia, prior to RBC transfusion, and 10 min after transfusion. As both hemoglobin concentrations and NIRS values changed during the surgery, the association between cerebral rSO_2 values and Hb concentrations at baseline, pre-transfusion, and post-transfusion were analyzed using Spearman's correlation. Our data indicate a positive correlation between Hb concentration and cerebral tissue oxygenation ($r = 0.35$, $p < 0.0001$) (Fig. 1).

Hb concentrations increased in all groups after transfusion of 1 RBC unit. However, post-transfusion Hb concentration remained lower in patients with pre-transfusion Hb concentrations <7.5 g/dL compared to 7.5–8.5 g/dL and >8.5 g/dL groups. Statistical analyses indicated effects of pre-transfusion Hb concentrations, transfusion, and an interaction of pre-transfusion Hb and RBC transfusion on post-transfusion Hb concentrations (Fig. 2).

Cerebral rSO_2 was lower prior to transfusion compared to baseline in patients transfused at Hb concentrations <7.5 g/dL, while there was no difference observed in other groups prior to transfusion compared to baseline values. Pre- to post-transfusion increases in rSO_2 were observed in all groups. Furthermore, rSO_2 was increased after transfusion compared to baseline values in 7.5–8.5 g/dL and >8.5 g/dL groups, while there was no difference observed in patients transfused at Hb concentrations <7.5 g/dL rSO_2 was not different between groups at baseline or post-transfusion. However, rSO_2 was significantly lower in patients transfused at Hb concentrations <7.5 g/dL compared to patients transfused at Hb concentrations >8.5 g/dL prior to transfusion. Two-way ANOVA indicated effects of transfusion, Hb concentrations pre-transfusion, and an interaction on rSO_2 (Fig. 3).

Delta rSO_2 baseline to pre-transfusion values were negative and significantly lower in patients transfused at Hb concentrations <7.5 g/dL compared to other groups. rSO_2 reduction from baseline values prior to transfusion was 12% in <7.5 g/dL, while 3% in 7.5–8.5 g/dL and >8.5 g/dL groups. Furthermore, delta pre- to post-transfusion rSO_2 was greater in patients transfused at Hb concentrations <7.5 g/dL compared to other groups ($p < 0.001$). However, neither baseline to pre- nor pre- to post-transfusion rSO_2 values were different between patients transfused at 7.5–8.5 g/dL and >8.5 g/dL (Fig. 4).

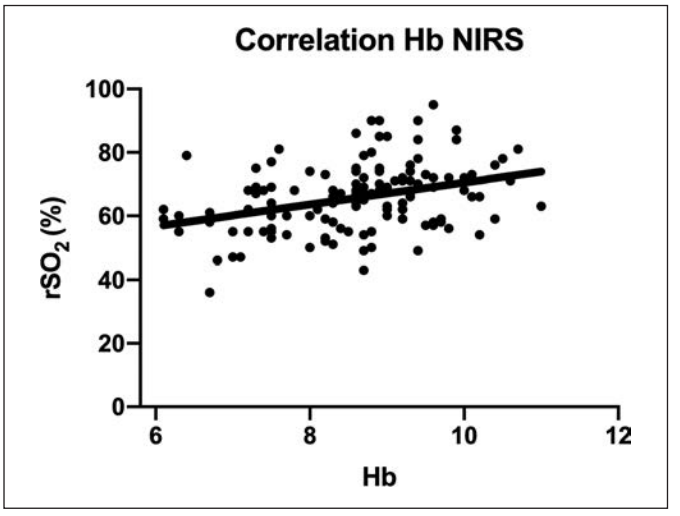

Fig. 1. Correlation between Hb concentration and NIRS-derived rSO2 measured after anesthesia induction, pre-transfusion, and posttransfusion. Data were analyzed using Spearman *r* test ($n = 129$, $r = 0.3479$, $p < 0.0001$).

Oxygenation parameters including arterial pO_2 and pCO_2 were not different between subgroups at any timepoint. Blood pressures were not different between groups but were higher after transfusion in patients transfused at Hb concentrations >8.5 g/dL (Fig. 5). Norepinephrine dosages were not different between groups pre- and post-transfusion (Table 2).

Discussion

The findings of the present study indicate a correlation between cerebral tissue oxygenation and blood loss-induced anemia and the subsequent effects of transfusion-induced changes of Hb concentrations in elderly patients undergoing intermediate- or high-risk noncardiac surgery. Substantial hemorrhage can occur during spine and hip surgeries.

Blood loss needs to be closely monitored through observation of the surgical field and calculations based on used sponges and collected volumes, but assessments are highly variable and affected by individual estimation [9]. Our study provides evidence that blood loss during hip and spine surgery and subsequent volume resuscitation

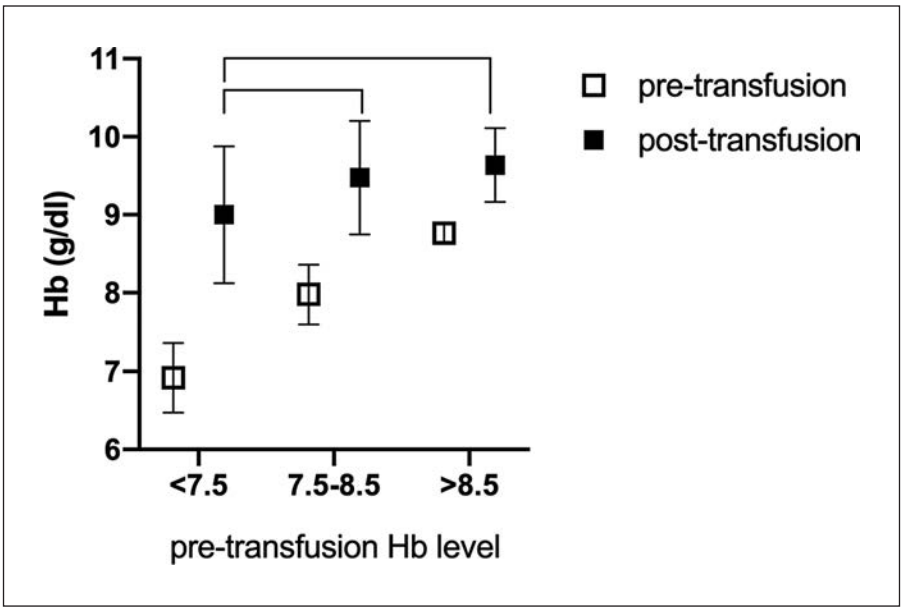

Fig. 2. Pre- and post-transfusion Hb concentrations in patients; RBC transfusion starting at Hb <7.5 g/dL, $n = 21$; 7.5–8.5 g/dL, $n = 21$; or >8 g/dL, $n = 25$. Data were analyzed using two-way ANOVA with Bonferroni post hoc analyses and expressed as mean ± SD. Lines indicate significant post-transfusion differences between groups ($p < 0.01$).

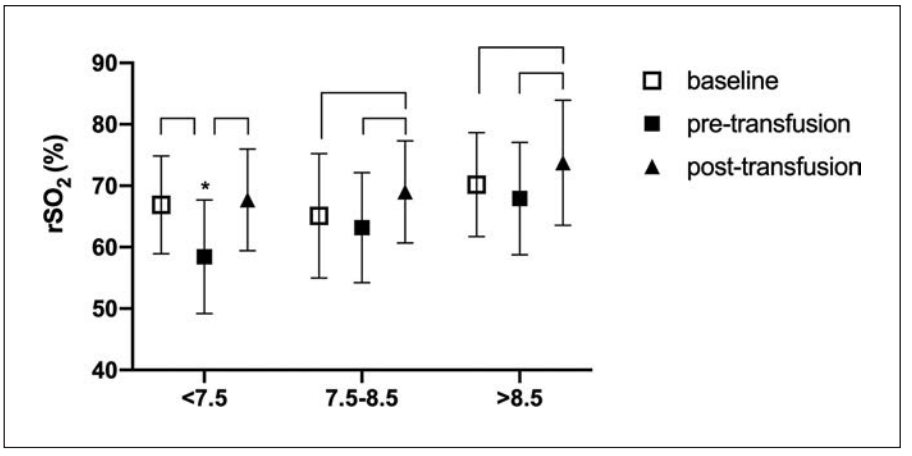

Fig. 3. rSO_2 values at baseline, pre-, and post-transfusion in patients; RBC transfusion starting at pre-transfusion Hb <7.5 g/dL, 7.5–8.5 g/dL, or >8.5 g/dL. Data were analyzed using two-way ANOVA with Bonferroni post hoc analyses and expressed as mean ± SD (<7.5 g/dL, $n = 21$; 7.5–8.5 g/dL, $n = 21$; >8.5 g/dL, $n = 25$; $p < 0.01$; *compared to >8.5 g/dL pre-transfusion; lines indicate significant post-transfusion differences between groups).

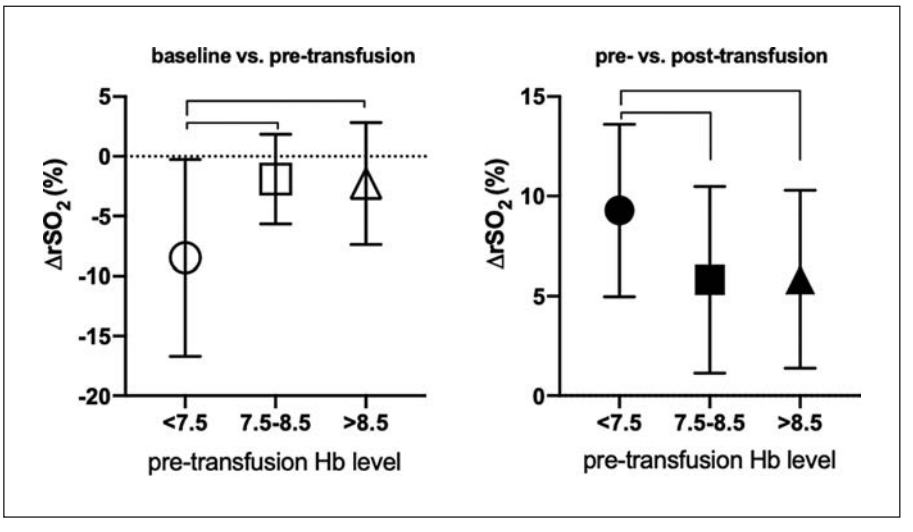

Fig. 4. rSO_2 changes from baseline to pre-transfusion (left panel) and pre- to post-transfusion (right panel) in patients receiving RBC transfusion starting at Hb <7.5 g/dL, 7.5–8.5 g/dL, or >8.5 g/dL. Data were analyzed using one-way ANOVA and expressed as mean ± SD (<7.5, $n = 21$; 7.5–8.5, $n = 21$; >8.5, $n = 25$; lines indicate significant post-transfusion differences between groups; $p < 0.01$).

using the corresponding volume led to a reduction of Hb, resulting in moderate to severe anemia that correlated with a reduction of NIRS-evaluated cerebral oxygen saturation.

TDM is based on the occurrence of transfusion triggers such as hemodynamic instability, lactemia, reduced mixed venous oxygenation, and reduced perfusion or deoxygenation of vital organs. However, anemia monitor-

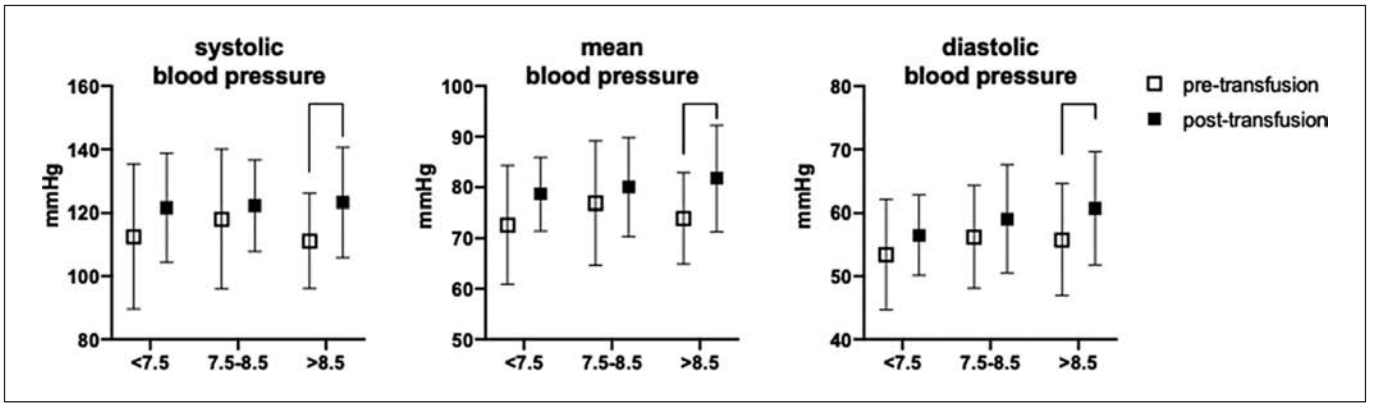

Fig. 5. Systolic, mean, and diastolic blood pressures assessed pre- and post-transfusion in patients receiving RBC transfusion starting at Hb <7.5 g/dL, 7.5–8.5 g/dL, or >8.5 g/dL. Data were analyzed using two-way ANOVA with Bonferroni post hoc analyses and expressed as mean ± SD (<7.5 g/dL, $n = 21$; 7.5–8.5 g/dL, $n = 21$; >8.5 g/dL, $n = 25$; lines indicate significant differences between pre- and post-transfusion; $p < 0.01$).

Table 2. Phenylephrine dosages in microgram per kilogram body weight pre- and post-transfusion were analyzed using one-way ANOVA with Bonferroni post hoc analyses and expressed as mean ± SEM ($p < 0.01$)

	<7.5 g/dL	7.5–8.5 g/dL	>8.5 g/dL	*p* value
Pre-transfusion norepinephrine dosage, µg/kg BW	0.08±0.02	0.10±0.03	0.08±0.02	ns
Post-transfusion norepinephrine dosage, µg/kg BW	0.06±0.01	0.07±0.02	0.08±0.02	ns

ing includes hemoglobin/hematocrit measurements prior to transfusion that is performed in a point-of-care fashion using BGAs that provide stable Hb and Hct values using 1-2 mL blood volume [4, 10]. Although Hb can be frequently determined during major surgery with high amounts of blood loss, BGA are still irregularly performed upon clinical assessment. In contrast, NIRS-based perfusion and oxygenation monitoring provides a continuous and high granular evaluation of cerebral tissue oxygenation in the absence of any blood utilization. Most notably, information regarding the oxygenation status of this crucial tissue may help TDM and in the absence of cerebral desaturation and subsequent transfusion triggers potentially prevent unnecessary transfusion. High expenses of the NIRS optodes compared to BGAs need to be addressed. However, tissue oxygenation monitoring may be a great adjunct to Hb concentration in making a clinical decision for RBC transfusion, or most importantly against a transfusion within currently recommended limits. Prevention of RBC transfusion reduces the risk for postsurgical complications, and improves outcomes, all according to current WHO recommendations on PBM [11]. Subsequent studies are required to evaluate the impact of tissue oxygenation monitoring on the reduction of perioperative RBC transfusion.

While baseline NIRS values were not different between groups, cerebral oxygenation was significantly declined in patients with pre-transfusion Hb <7.5 g/dL compared to 7.5–8.5 and >8.5 g/dL. After transfusion, rSO_2 increased and no differences were observed between subgroups. These data suggest that a restrictive transfusion strategy in elderly patients may result in minor cerebral deoxygenation episodes prior to RBC transfusion while patients receiving RBC transfusion at higher pre-transfusion Hb levels did not show rSO_2 decrease. These findings are in accordance with previous studies performed in neonatal critical care and perioperative pediatric patients that report a RBC transfusion-induced increase in cerebral oxygenation and correlation between Hb concentration and tissue oxygenation [12, 13]. Alternatively, Aktas and colleagues did not observe a transfusion-induced increase in cerebral oxygenation in neonatal intensive care patients, while abdominal and renal rSO_2 values increased after transfusion [14]. However, according to their study protocol NIRS values were assessed 24 h after transfusion and compared to pre-transfusion values, and cerebral changes could be more pronounced in the acute setting. Also, compensatory mechanisms may initiate within hours that maintain cerebral oxygenation at lower level. Our subanalysis focused on acute anemia due to acute surgery-related blood loss and transfusion in the perioperative setting where observed differences in cerebral oxygenation are likely to occur before compensatory mechanism is effective. Similar to the studies in neonatal intensive care

patients [14, 15], the most pronounced transfusion-induced increases in cerebral oxygenation were observed in patients with lower pre-transfusion Hb level <7.5 g/dL, potentially indicating physiologic severe anemia levels.

The observation that reduction in blood pressure was accompanied by a reduction in cerebral oxygenation has been reported in previous studies and the autoregulatory mechanisms of cerebral perfusion may be involved in this observation [16]. Furthermore, vasopressor treatment affects hemodynamics and tissue oxygenation in anesthetized patients [17]. However, the herein used norepinephrine dosages were not different between groups pre- and post-transfusion and are therefore unlikely to be responsible for rScO2 differences, but the assessment of physiological mechanisms of cerebral autoregulation is beyond the scope of our investigation.

NIRS evaluation of rScO2 provides an individual trend of tissue oxygen availability. Although the individual trend is precise, interindividual measurements are markedly variable; thus, this method has its limitations in the ratio of oxygen supply to oxygen consumption that need to be addressed. However, NIRS-evaluated tissue oxygenation has been proposed as an alternative method for TDM in pediatric intensive care patients [18]. However, the impact of NIRS on tissue oxygenation in the elderly patients has not yet been evaluated. Although noninvasive methods for Hb measurement tend to be inferior to invasive BGA, our data indicate that NIRS provides continuous information of cerebral oxygenation and has the potential to detect rapid changes during major surgery potentially being related to a critical Hb concentration without frequent utilization of patients' blood. TDM is an individual assessment that is based on the occurrence of distinct transfusion triggers and should not be made based on Hb values only [19, 20]. Therefore, a continuous monitoring that is not only providing changes in Hb concentration but also postulating information concerning the oxygen saturation and more important critical desaturation of a vulnerable tissue such as the brain seems reasonable as an individualized perioperative RBC transfusion strategy.

While acute severe normovolemic anemia at hemoglobin level between 6 and 5 g/dL is associated with cognitive dysfunction [21], intraoperative decreases in NIRS have also been shown to correlate with postoperative cognitive dysfunction, and a patient-specific algorithm that incorporates cerebral NIRS monitoring and a restrictive RBC transfusion threshold improve clinical outcomes while reducing costs [22, 23]. Further studies are required to investigate the potential of perioperative NIRS evaluation and prevention of cerebral deoxygenation through RBC transfusion on postoperative cognitive dysfunction in elderly patients undergoing major noncardiac surgery.

There are some limitations that need to be addressed. First of all, this is a sub-study to the ongoing multicenter LIBERAL-Trial. Therefore, the sample size was not calculated for the primary outcome and our results are exploratory. Second, the data are just from one single center and the number of patients is limited. However, our results show a stable and significant effect and to the best of our knowledge this is the first study reporting a correlation between perioperative anemia and NIRS in elderly patients undergoing noncardiac surgery. Further studies are required to investigate the potential prevention of postoperative cognitive dysfunction through the prevention of perioperative cerebral desaturation.

Conclusions

In conclusion, cerebral oxygenation monitoring by NIRS indicates cerebral desaturation, but also provides valuable information regarding perioperative alterations in Hb concentration, implying blood loss-induced anemia, potentially providing the clinician information when anemia-related impaired oxygenation is still compensated or transfusion may be indicated, in the context of individualized medicine. Transfusion-induced increases in cerebral oxygenation are more pronounced at lower pre-transfusion Hb level. Further studies are required to investigate the impact of cerebral oxygenation measurement on individual TDM in the perioperative setting, potentially preventing postoperative cognitive dysfunction.

Statement of Ethics

This study protocol was reviewed and approved by the Institutional Review Board of the Rheinische Friedrich-Wilhelms-University Bonn (Ref: 096/17-AMG) and the federal authority (Paul-Ehrlich-Institute) LIBERAL-Trial (NCT03369210). Written informed consent was obtained from all participants before being included into the study.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Conceptualization: Patrick Meybohm, Markus Velten, and Maria Wittmann; data curation: Achilles Delis, Derek Bautz, Thomas M. Randau, Andreas C. Strauss, Ivana Habicht, and Erdem Güresir; formal analysis: Heidi Ehrentraut, Karin Doll, Hol-

ger Bogatsch, and Peter Kranke; investigation and visualization: Achilles Delis and Derek Bautz; project administration: Patrick Meybohm, Markus Velten, Maria Wittmann, Holger Bogatsch, and Peter Kranke; supervision: Heidi Ehrentraut, Karin Doll, and Holger Bogatsch; writing – original draft: Markus Velten and Patrick Meybohm; and writing – review and editing: Maria Wittmann, Achilles Delis, Derek Bautz, Thomas M. Randau, Andreas C. Strauss, Ivana Habicht, Erdem Güresir, Heidi Ehrentraut, Karin Doll, Holger Bogatsch, and Peter Kranke.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

References

1 Anderson SA, Menis M, O'Connell K, Burwen DR. Blood use by inpatient elderly population in the United States. Transfusion. 2007 Apr;47(4):582–92.
2 Cobain TJ, Vamvakas EC, Wells A, Titlestad K. A survey of the demographics of blood use. Transfus Med. 2007 Feb;17(1):1–15.
3 Khanna MP, Hébert PC, Fergusson DA. Review of the clinical practice literature on patient characteristics associated with perioperative allogeneic red blood cell transfusion. Transfus Med Rev. 2003 Apr;17(2):110–9.
4 American Society of Anesthesiologists Task Force on Perioperative Blood Management. Practice guidelines for perioperative blood management: an updated report by the American society of anesthesiologists task force on perioperative blood management. Anesthesiology. 2015 Feb;122(2):241–75.
5 Ikeda K, MacLeod DB, Grocott HP, Moretti EW, Ames W, Vacchiano C. The accuracy of a near-infrared spectroscopy cerebral oximetry device and its potential value for estimating jugular venous oxygen saturation. Anesth Analg. 2014 Dec;119(6):1381–92.
6 Marin T, Moore J. Understanding near-infrared spectroscopy. Adv Neonatal Care. 2011 Dec;11(6):382–8.
7 Habicher M, Zajonz T, Heringlake M, Böning A, Treskatsch S, Schirmer U, et al. S3 guidelines on intensive medical care of cardiac surgery patients: hemodynamic monitoring and cardiovascular system-an update. Anaesthesist. 2018 May;67(5):375–9.
8 Meybohm P, Lindau S, Treskatsch S, Francis R, Spies C, Velten M, et al. Liberal transfusion strategy to prevent mortality and anaemia-associated, ischaemic events in elderly non-cardiac surgical patients: the study design of the LIBERAL-Trial. Trials. 2019 Feb 4;20(1):101.
9 Gerdessen L, Meybohm P, Choorapoikayil S, Herrmann E, Taeuber I, Neef V, et al. Comparison of common perioperative blood loss estimation techniques: a systematic review and meta-analysis. J Clin Monit Comput. 2021 Apr;35(2):245–58.
10 Tanner L, Lindau S, Velten M, Schlesinger T, Wittmann M, Kranke P, et al. Factors influencing the bias between blood gas analysis versus central laboratory hemoglobin testing. A secondary analysis of a randomized controlled trial. PLoS One. 2020;15(10):e0240721.
11 Schmitt E, Meybohm P, Neef V, Baumgarten P, Bayer A, Choorapoikayil S, et al. Preoperative anaemia and red blood cell transfusion in patients with aneurysmal subarachnoid and intracerebral haemorrhage: a multicentre subanalysis of the German PBM Network Registry. Acta Neurochir. 2022 Apr;164(4):985–99.
12 Goldstein GP, Rao A, Ling AY, Ding VY, Chang IJ, Chock VY. Influence of enteral feeding and anemia on tissue oxygen extraction after red blood cell transfusion in preterm infants. Transfusion. 2020 Mar;60(3):466–72.
13 Whitehead HV, Vesoulis ZA, Maheshwari A, Rao R, Mathur AM. Anemia of prematurity and cerebral near-infrared spectroscopy: should transfusion thresholds in preterm infants be revised? J Perinatol. 2018 Aug;38(8):1022–9.
14 Aktas S, Ergenekon E, Ozcan E, Aksu M, Unal S, Hirfanoglu IM, et al. Effects of blood transfusion on regional tissue oxygenation in preterm newborns are dependent on the degree of anaemia. J Paediatr Child Health. 2019 Oct;55(10):1209–13.
15 Koyano K, Kusaka T, Nakamura S, Nakamura M, Konishi Y, Miki T, et al. The effect of blood transfusion on cerebral hemodynamics in preterm infants. Transfusion. 2013 Jul;53(7):1459–67.
16 Liu L, Qiang Z, Zhang J, Ren Y, Zhao X, Fu W, et al. Effect of hemoglobin content on cerebral oxygen saturation during surgery for scoliosis in pediatric patients. BMC Anesthesiol. 2021 Jun 1;21(1):165.
17 Meng L, Cannesson M, Alexander BS, Yu Z, Kain ZN, Cerussi AE, et al. Effect of phenylephrine and ephedrine bolus treatment on cerebral oxygenation in anaesthetized patients. Br J Anaesth. 2011 Aug;107(2):209–17.
18 Markham C, Small S, Hovmand P, Doctor A. Transfusion decision making in pediatric critical illness. Pediatr Clin North Am. 2017 Oct;64(5):991–1015.
19 Causey MW, Miller S, Foster A, Beekley A, Zenger D, Martin M. Validation of noninvasive hemoglobin measurements using the masimo radical-7 SpHb station. Am J Surg. 2011 May;201(5):592–8.
20 Lamhaut L, Apriotesei R, Combes X, Lejay M, Carli P, Vivien B. Comparison of the accuracy of noninvasive hemoglobin monitoring by spectrophotometry (SpHb) and HemoCue® with automated laboratory hemoglobin measurement. Anesthesiology. 2011 Sep;115(3):548–54.
21 Weiskopf RB, Kramer JH, Viele M, Neumann M, Feiner JR, Watson JJ, et al. Acute severe isovolemic anemia impairs cognitive function and memory in humans. Anesthesiology. 2000 Jun;92(6):1646–52.
22 Ellis L, Murphy GJ, Culliford L, Dreyer L, Clayton G, Downes R, et al. The effect of patient-specific cerebral oxygenation monitoring on postoperative cognitive function: a multicenter randomized controlled trial. JMIR Res Protoc. 2015 Dec 18;4(4):e137.
23 Soenarto RF, Hidayat JK, Eureka O, Auerkari AN. Can Near-Infrared Spectroscopy (NIRS) monitoring prevent post-operative cognitive dysfunction following open- heart surgery? J Pak Med Assoc. 2021 Feb;71(Suppl 22):S10–3.

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Predicting Individual Patient Platelet Demand in a Large Tertiary Care Hospital Using Machine Learning

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Keywords

Patient individual · Platelet prediction · Machine learning · Blood transfusion · Donor management · Platelet · Platelet concentrates

Abstract

Introduction: An increasing shortage of donor blood is expected, considering the demographic change in Germany. Due to the short shelf life and varying daily fluctuations in consumption, the storage of platelet concentrates (PCs) becomes challenging. This emphasizes the need for reliable prediction of needed PCs for the blood bank inventories. Therefore, the objective of this study was to evaluate multimodal data from multiple source systems within a hospital to predict the number of platelet transfusions in 3 days on a per-patient level. ***Methods:*** Data were collected from 25,190 (42% female and 58% male) patients between 2017 and 2021. For each patient, the number of received PCs, platelet count blood tests, drugs causing thrombocytopenia, acute platelet diseases, procedures, age, gender, and the period of a patient's hospital stay were collected. Two models were trained on samples using a sliding window of 7 days as input and a day 3 target. The model predicts whether a patient will be transfused 3 days in the future. The model was trained with an excessive hyperparameter search using patient-level repeated 5-fold cross-validation to optimize the average macro F2-score. ***Results:*** The trained models were tested on 5,022 unique patients. The best-performing model has a specificity of 0.99, a sensitivity of 0.37, an area under the precision-recall curve score of 0.45, an MCC score of 0.43, and an F1-score of 0.43. However, the model does not generalize well for cases when the need for a platelet transfusion is recognized. ***Conclusion:*** A patient AI-based platelet forecast could improve logistics management and reduce blood product waste. In this study, we build the first model to predict patient individual platelet demand. To the best of our knowledge, we are the first to introduce this approach. Our model predicts the need for platelet units for 3 days in the future. While sensitivity underperforms, specificity performs reliably. The model may be of clinical use as a pretest for potential patients needing a platelet transfusion within the next 3 days. As sensitivity needs to be improved, further studies should introduce deep learning and wider patient characterization to the methodological multimodal, multisource data approach. Furthermore, a hospital-wide consumption of PCs could be derived from individual predictions.

Introduction

It is an ever-increasing worldwide challenge to supply blood products to patients. In Germany, around 15,000 blood cell units are required every day, and about 45,000 blood products are transfused annually at our clinic, of which approximately 30,000 are red blood cell concen-

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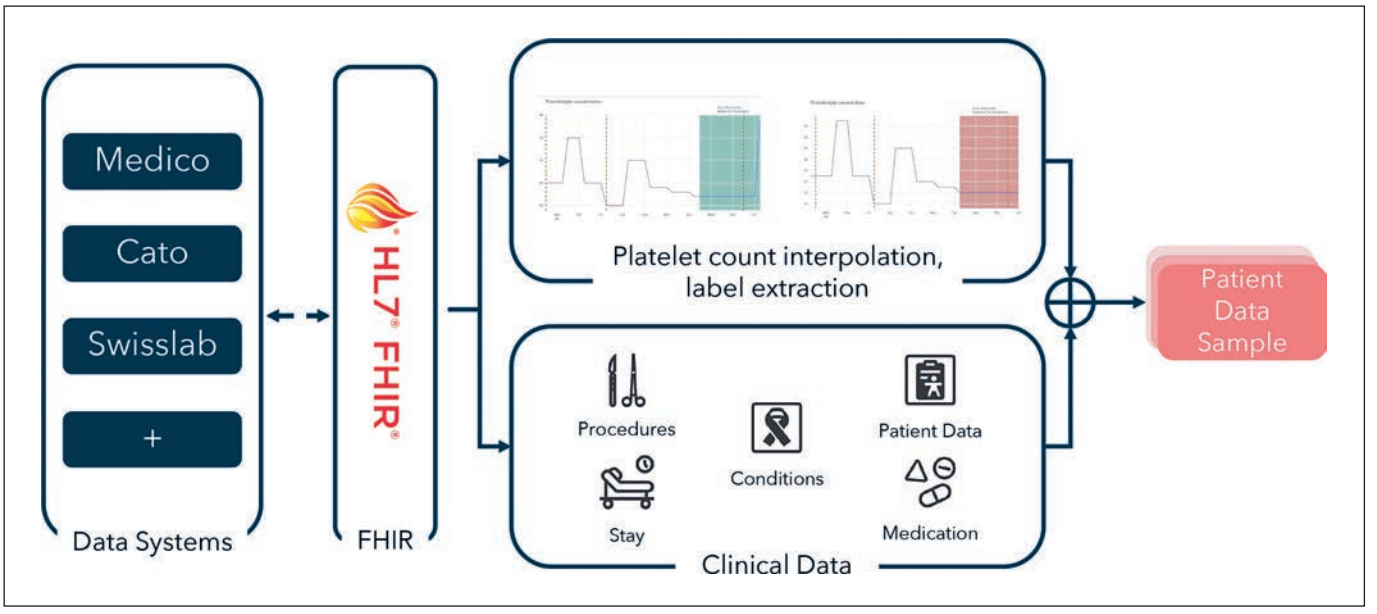

Fig. 1. Data source to patient data pipeline (taking multiple hospital systems into account) followed by the HL7 FHIR server, which extracts and stores the data, then the extraction process, and, finally, the per-patient storing of all relevant patients.

trates, 10,000 are platelet concentrates (PCs), and 5,000 are fresh frozen plasmas [1]. Furthermore, the ongoing demographic change is expected to increase the relative shortage of blood donors and thus increase the shortage of stored blood products [2–4]. Therefore, optimal use of this valuable resource is becoming increasingly important from a medical, ethical, and economic point of view. To solve these problems, improving logistics management in transfusion medicine is crucial.

Thus, coordinating the inventory, expiry, and consumption of PCs plays a vital role. A PC typically has a shelf life of 4 days after testing and screening procedures on the production day [5]. The transfusion of different patient collectives treated in a university hospital for hematology and oncology, transplantation, cardiac, and thoracic surgery leads to dramatic fluctuations in the consumption of PCs [6–8]. Therefore, storing PCs in line with requirements is challenging: on the one hand, a PC shortage is unacceptable, while on the other hand, it is necessary to minimize the wasteful disposal of blood donations.

According to the Paul Ehrlich Institute, 575,608 PCs were produced in Germany in 2020 [1]. From a total of 255,722 pool PCs, 48,467 PCs (19%) expired at the producer. From a total of 319,886 apheresis PCs, 22,411 (7%) expired at the producer [1]. Taking their market value into account, the disposal of unused PCs results in an economic loss of more than 30 million Euros per year. Thus, reducing the forfeiture rate by just two percent would save more than 5.5 million Euros annually [9].

The high expiration rates indicate that a patient-specific prediction of PC consumption is required to optimize logistics management. At present, however, the patient- and hospital-specific data already available in most hospitals in Germany are not sufficiently utilized for this purpose.

This retrospective study aimed to develop a novel AI-based approach to improve the prediction of individual PC demand. Because donation appointments must be scheduled and the donation products tested and screened, predictions for patient transfusion needs at the end of 3 days are critical. The objective of this study was, therefore, to create a machine learning-based model which predicts whether a patient requires a platelet transfusion 3 days into the future. This is achieved by extracting, transforming, and loading multimodal data from multiple source systems within the hospital.

Materials and Methods

Data Sources

The retrospective data were obtained from a hospital's internal Health Level Seven International (HL7) Fast Healthcare Interoperability Resources (FHIR) (https://www.hl7.org/fhir) server. The server is a data repository that stores information generated during the clinical routine at all hospital departments. It was queried for clinical and demographic information such as patient stay, laboratory testing, conditions, transfusions, medications, procedures, gender, and age. In addition, manual spot checks were performed to ensure data validity and quality between source systems and the extracted data set. At first, the completeness of extracted data be-

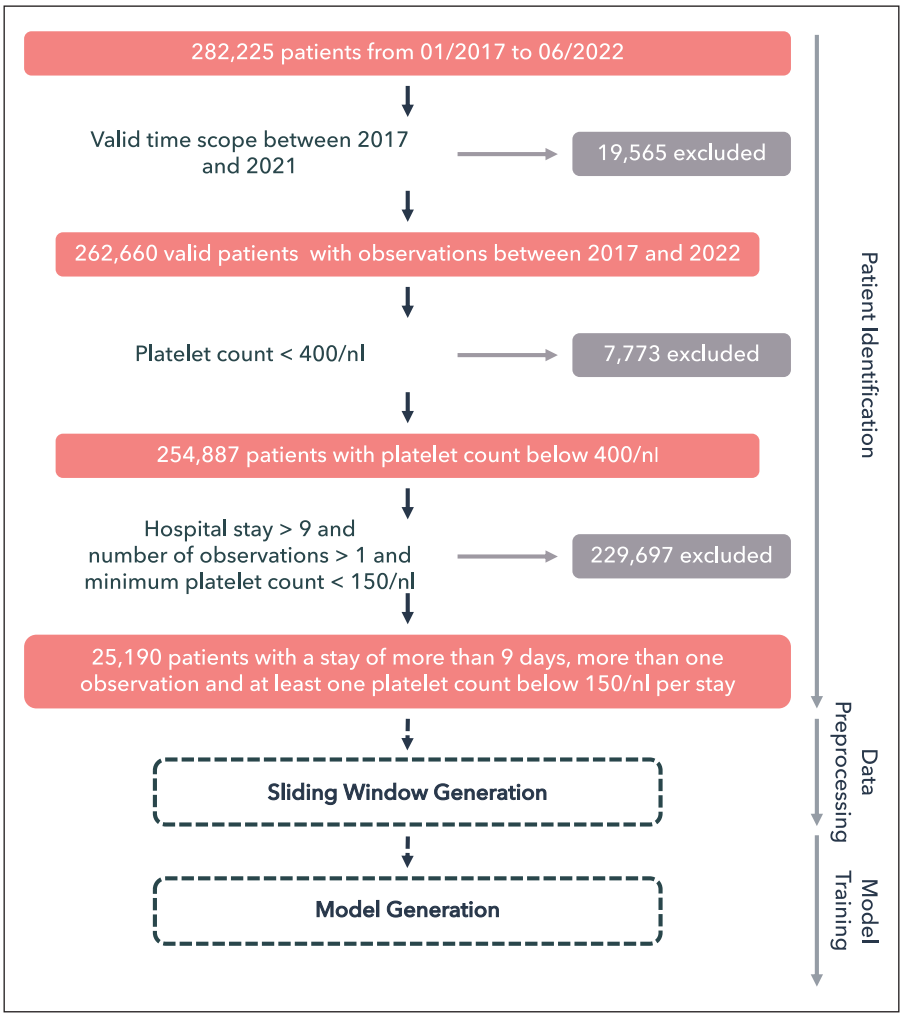

Fig. 2. Cohort identification process, which can be interpreted as a top-down flow diagram within the patient identification process. The entire cohort began with a total of 282,225 patients based on in-house platelet count observations between January 2017 and June 2022. The patient identification process can be divided into three filtering steps. The first filters the time scope of 2017–2022. The second filter excludes patients with platelet counts exclusively above 400 platelet/nL. The final filter ensures that a patient's hospital stay is greater than 9 days, the number of blood observations is greater than one, and at least one platelet count observation needs to be below 150 platelet/nL. After the above-described filter, 25,190 patients are left for the scope of this study.

tween the FHIR server and training data was ensured by comparing the total number of extracted elements for each resource. Second, each FHIR resource's data importers were checked for data completeness. Finally, to ensure end-to-end completeness, data mappings, as seen in Figure 3, were generated on a set of patients. Figure 1 exemplifies the data pipeline applied to extract the patient cohort. The following two subsections explain the data source in detail.

Subjects Identification and Preprocessing

The need for a PC donation is determined by the platelet count in the patient's blood and specified in the hemotherapeutic guidelines [10, 11]. Our study included all patients, independent of the main or first diagnosis, who had platelet count observation between January 2017 and June 2022 and are in total 282,225 patients. A flow diagram visualizing the patient selection process in a schematic overview is represented in Figure 2.

First, a time scope filter was applied as only patients with observations between 2017 and 2021 were considered. While the normal laboratory range for PCs may vary slightly, the usual reference range is between 150 platelet/nL and 400 platelet/nL [12]. Therefore, patients with thrombocyte counts exclusively above the maximum reference range of 400 platelet/nL were excluded. Because each hospitalization had to pass another set of requirements to be included in the analysis, this stage of the identification process reduced the cohort size to 254,887 patients.

First, the hospital stay had to last at least 10 days. Patients with a shorter stay were considered out of scope as these patients had a very low upfront probability of needing a PC and typically had only very few data points. We found the probability for a patient to receive a PC during a stay between 0 and 9 days to be 0.34%. Second, patients with a single observation were considered out of scope because, according to transfusion guidelines, the platelet count should be measured before and after a patient receives a donation [11]. Finally, the minimum platelet count for one observation during a stationary stay had to be less than or equal to 150 platelet/nL, in accordance with the reference range for PC transfusions [12]. This filtering step yielded a final cohort size of 25,190 patients with valid hospitalizations to include in this study. For patients in the final cohort, we collected additional features such as procedures that may impact platelet function (e.g., extracorporeal circulation, hemodialysis), medications that frequently cause thrombocytopenia (e.g., chemotherapeutics such as cytarabine and gemcitabine), conditions that are known to cause thrombocytopenia either directly or through drug-based interference (e.g., leukemic and lymphoid malignancies), the number of received PC transfusions, and time scope of hospital stays. Figure 3 exemplifies the data map for one of the 25,190 patients, with all the occurring features from different resources. A detailed overview of the selected features from medications, procedures, and conditions can be found in Appendix A, available at www.karger.com/doi/10.1159/000528428.

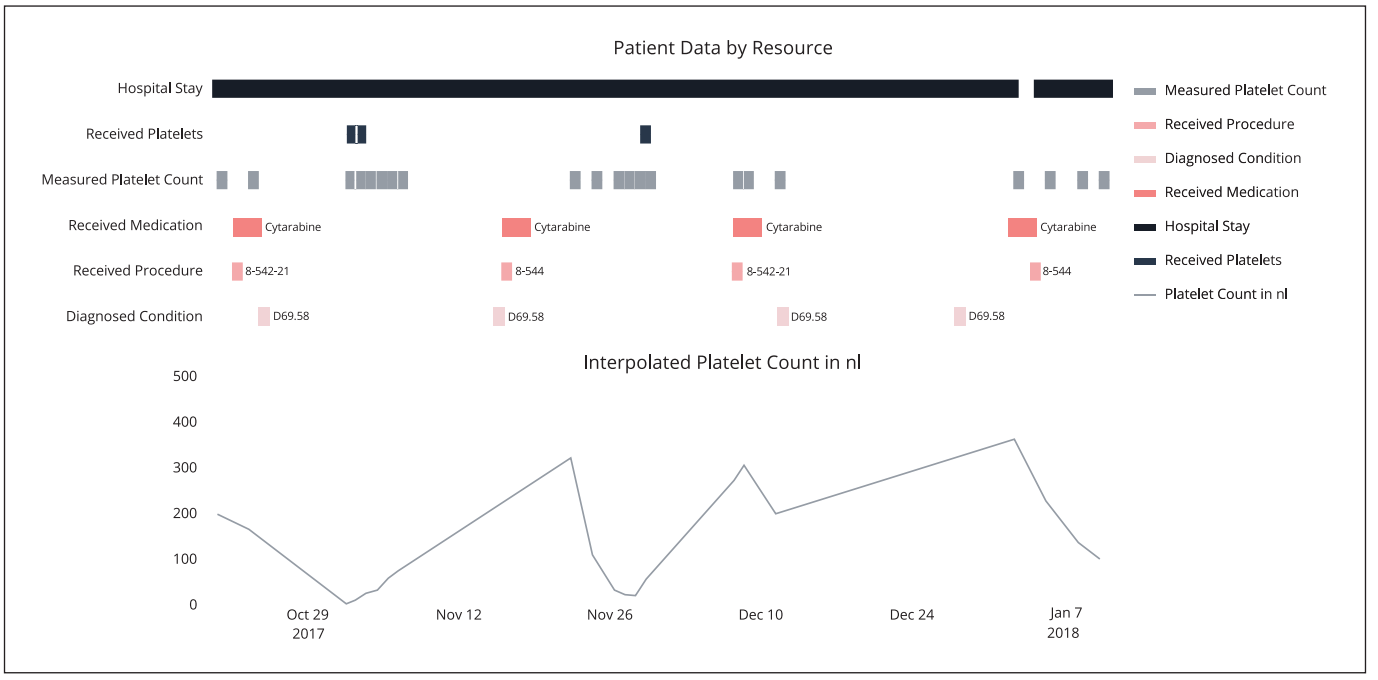

Fig. 3. Exemplary data map of a cohort patient suffering from mantle cell lymphoma, which is represented on multiple occasions as a "diagnosed condition" with the ICD code "C83.1." The patient was therefore subjected to a chemotherapeutic treatment plan with cytarabine (represented in four instances both as "received medication" and "received procedure" with the respective OPS codes "8-542-21" and "8-544"). This led to two occurrences of secondary thrombocytopenia ("D69.58") with a minimal platelet count of 6/nL (November 1, 2017) and 24/nL (November 28, 2017) that were each treated with a PC transfusion ("received platelets").

Sliding Window Sampling

The data needed to be transformed into equally dimensional samples to attain equally shaped data for machine learning models. Figure 4 shows a schematic overview of how each training sample's sliding window was generated. The following criteria define a valid training sample: One platelet count observation needs to be present, and the minimum platelet count needs to be below 150 platelets/nL within the 7-day window.

Each input vector had a time scope of 7 days and a frequency of 12 h, thus 14 time slots. The window sampling was based on time logs of the patient encounters. Consequently, features for the respective time scope were collected using an aggregation function for each valid patient stay at the hospital.

The aggregation function mapped each feature data event within each valid patient stay to one of the 14 time slots. Medications, conditions, and procedures were saved in a list per time slot. The most recent platelet count observation within a 12-h window was preserved, and subsequent empty spaces were linearly interpolated to the next platelet count observation. Additionally, it was recorded whether a platelet count value was interpolated if needed. Finally, each PC unit within a time slot was transformed into the sum of units within a day.

Time slot-independent metadata, such as age and gender, were stored for each patient. Finally, each target vector had a time scope of 1 day and a frequency of 24 h, which contained a list of consumed PC units 3 days into the future. Once preprocessing steps for one input and target vector were calculated, the sliding window algorithm stepped 1 day ahead, and the preprocessing restarted.

Platelet Binary Classification

Classification algorithms were used as the decision of whether a patient should receive a PC can be translated into a binary problem. The first class (0) represented the case of no transfusion, and the second class (1) represented one or more transfusions. The data set was split on the patient level into training and test data using an 80/20 ratio. The data were highly zero-inflated as most samples did not receive any PC transfusion. To address the class imbalance (3.42%), all models were also trained using Gaussian noise up-sampling [13] and the kmeans_SMOTE (synthetic minority over-sampling technique) [14] using default parameters. As the objective was to predict the third day, a single-output classifier was sufficient. For the model training and optimization, scikit-learn [15] [https://scikit-learn.org], eXtreme Gradient Boosting (XGBoost) [16] [https://xgboost.readthedocs.io], Random Forest [17], Dummy Classifier [18], and Optuna [19] [https://optuna.org] were used. Optuna was configured to run 1,000 trials for each model using a tree-structured Parzen estimator sampler [20] for hyperparameter sampling. Random sampling was used on the first 50 trials of each model as warm-up iterations for the tree-structured Parzen estimator algorithm. Samplers decrease the search time, and calculation cost of finding the best-performing hyperparameters for the chosen algorithm compared to a full grid search.

A median pruner (24) was used on top of the iterative process to stop the training process once the improvement plateaued. Pruning was disabled for the first 100 trials of each model run. All models were trained using fivefold stratified-group cross-validation, to ensure that cohorts within a split are not overlapping and the percentage of samples for each class is almost equal. An F2-score was used as an optimization metric to weigh sensitivity/recall

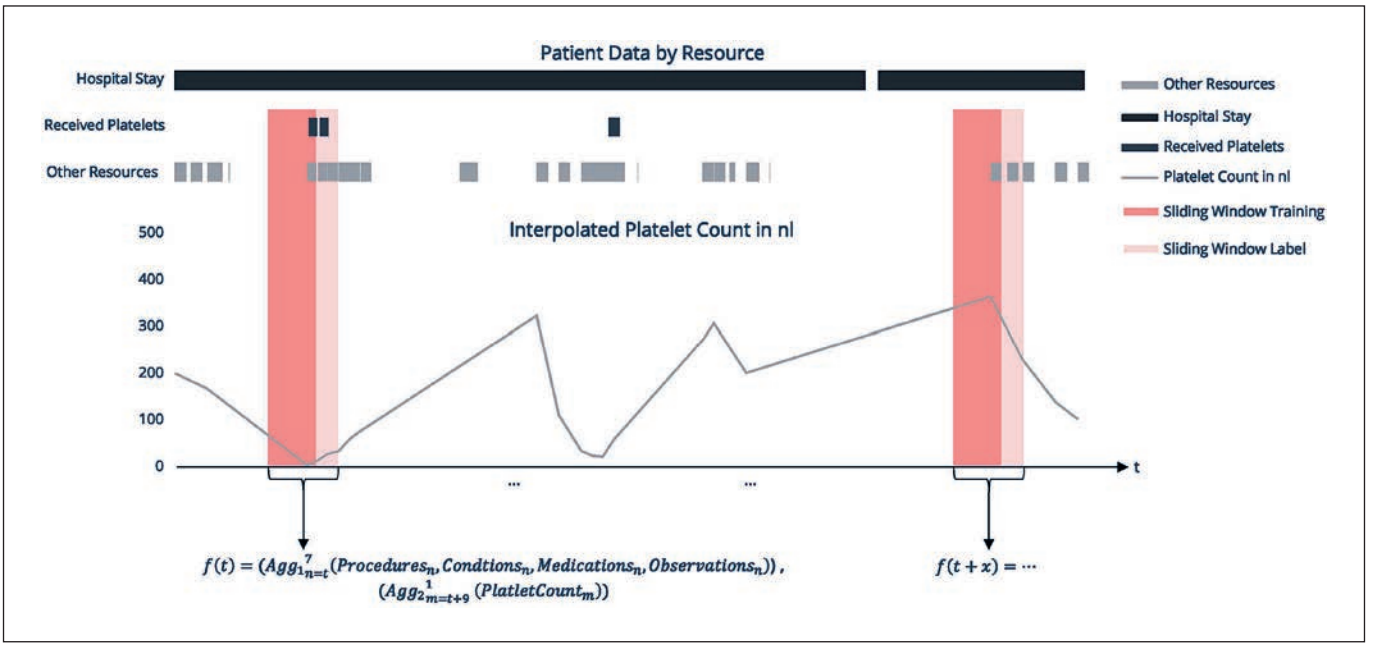

Fig. 4. Generation of the sliding window from raw data for training and labeling schematically. The sliding window frequency of x is one (day) with a frequency of 12 h and 7 days for each training sample and a frequency of 24 h and 3 days for each labeled sample.

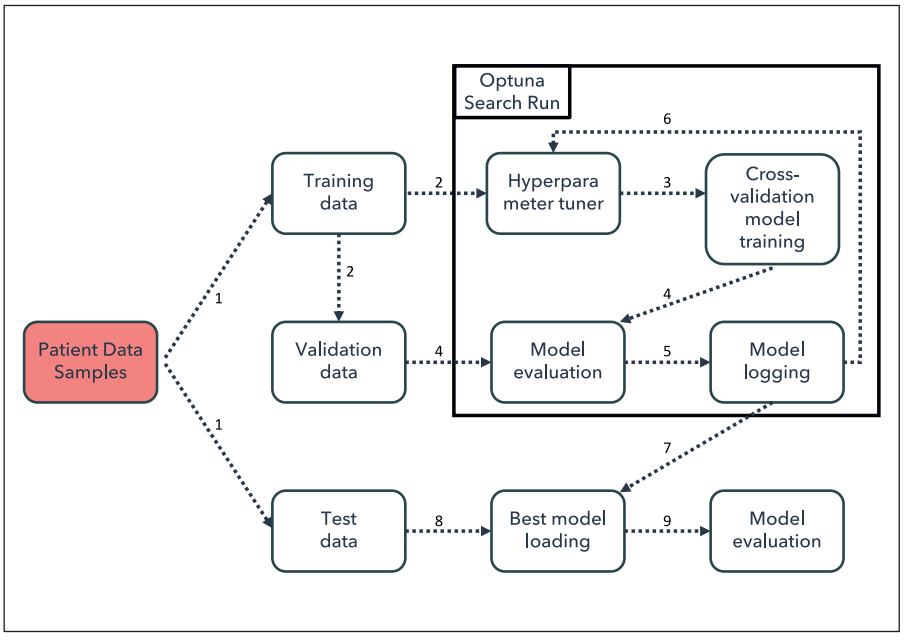

Fig. 5. ML workflow using Optuna. First, the patient data samples were split into training and test data (1). The Optuna run was then initialized (2), which initialized the first algorithm and prepared the data for a cross-validated run (3). In cross-validation, the data were further split into the desired amount of data sets (which consist of training and validation data sets) to further prevent overfitting and selection bias. Each set was then trained, and overall folds were averaged to calculate the model's predictive performance on the validation set (4). The model was then logged with its performance scores and hyperparameter (5). If the pruner decided to train another model, the process would start again at the hyperparameter tuning within the given search space (6). Once the evaluation was finished, the parameters of the best-performing model could be loaded and evaluated on the test data (7–9).

higher than precision. Figure 5 depicts the training process for the machine learning models in a schematic overview.

A dummy classifier with a stratified strategy was used as a baseline model to compare against the above-described classifiers. Dummy classifiers disregard the input features and solely depend on the output values. The stratified method considered the a priori probability and was, therefore, a valid baseline classifier. Subsequently, an XGBoost and a Random Forest model were trained excessively with the hyperparameter search spaces seen in Table 1 using the ML workflow described in Figure 5.

Results

Patient Characteristics

After the processing steps seen in Figures 2 and 4, the cohort consisted of 25,190 patients with a hospital stay of at least 10 days between 2017 and 2021. The cohort data were randomly split on the patient level into 80/20 subsets for training and test data sets. In the training set, the patient's ages ranged from 0 to 101 years, and the mean pa-

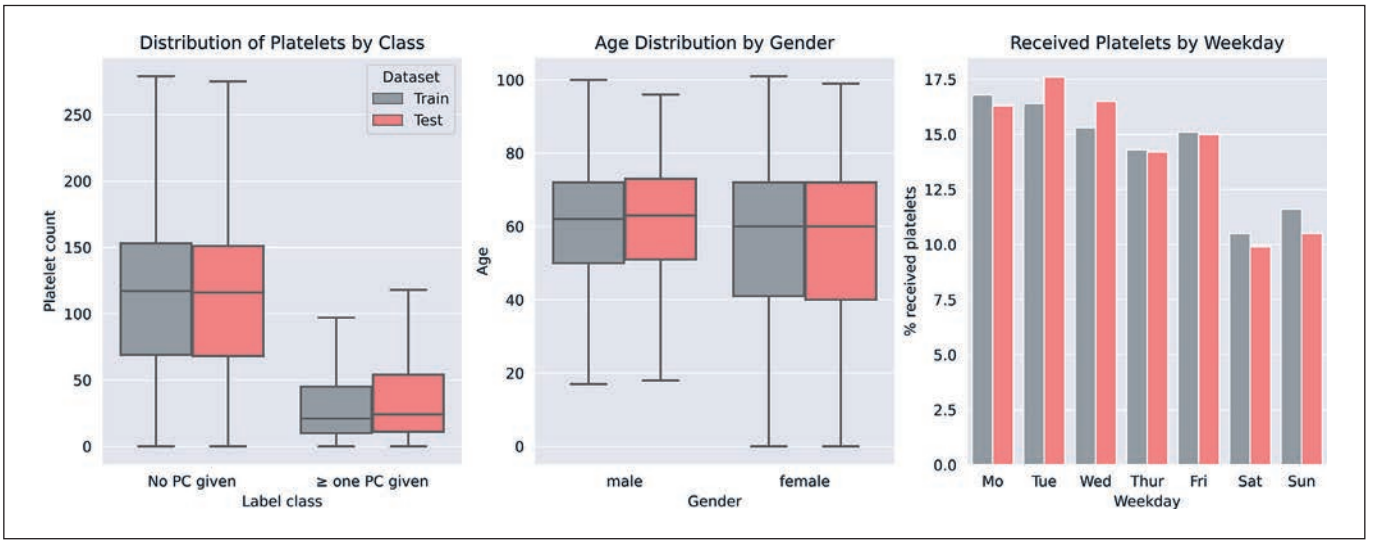

Fig. 6. Patient characteristics by training and test sets. The figure on the left-hand side shows the distribution of platelets by data class. The left-hand figure indicates that patients in the first label class have, in most cases, a platelet count below 50/nL. The center figure shows the age distribution by gender. At the lower end of the distribution, the boxplot has an outlier cutoff for the male cohort below 19 years. However, this does not mean that there are no male patients younger than 19 years. The figure on the right-hand side illustrates the relative received platelets by weekday. It can be observed that most patients receive transfusions between Monday and Friday.

Table 1. Hyperparameter spaces for XGBoost and Random Forest

Parameter	Search space
XGBoost with gbtree booster	
Objective	Binary: logistic
Eval_metric	AUCPR[a]
Lambda	10^{-8}–1.0
Alpha	10^{-8}–1.0
Max_depth	10–30
Eta	10^{-8}–1.0
Gamma	10^{-8}–1.0
Grow_policy	Depthwise, lossguide
XGBoost with gbtree dart	
Objective	Binary: logistic
Eval_metric	AUCPR[a]
Lambda	10^{-8}–1.0
Alpha	10^{-8}–1.0
Max_depth	10–30
Eta	10^{-8}–1.0
Gamma	10^{-8}–1.0
Grow_policy	Depthwise, lossguide
Sample_type	Uniform, weighted
Normalize_type	Tree, forest
Rate_drop	10^{-8}–1.0
Skip_drop	10^{-8}–1.0
Criterion	Gini, entropy
Random Forest	
N_estimators	100–1,000
Splitter	Best, random
Max_depth	10–30
Max_depth	10–30

[a] AUCPR, area under the precision-recall curve.

tient age was 56.5 ± 21.8 years. 42% of the patients were female, and 58% were male. In the test set, the patient age ranged from 0 to 99 years, and the mean patient age was 56.9 ± 22 years. The gender distribution was the same as in the training set. Figure 6 illustrates the data set distribution from different perspectives.

Model Performance

During the observed period of 5 years, 25,190 patients were transfused, with a total of 54,473 PC units. Optuna was configured to run for 1,000 trials for the XGBoost and Random Forest models. The performance of the best-performing classification models on the training data is shown in Table 2. All metrics (area under the precision-recall curve [AUCPR] score, F1 score, MCC score, precision, specificity, and sensitivity) could be improved compared to the dummy classifier. However, all models lack sensitivity.

Figure 7 shows the AUCPR curve across all trained models. The Dummy Classifier is displayed as an almost horizontal line with a score of 0.03, suggesting that the model has no discriminative ability to diagnose patients with and without needing a PC transfusion. The slope of the AUCPR curve on the Random Forest and XGBoost models indicates that the model performs better than the Dummy Classifier.

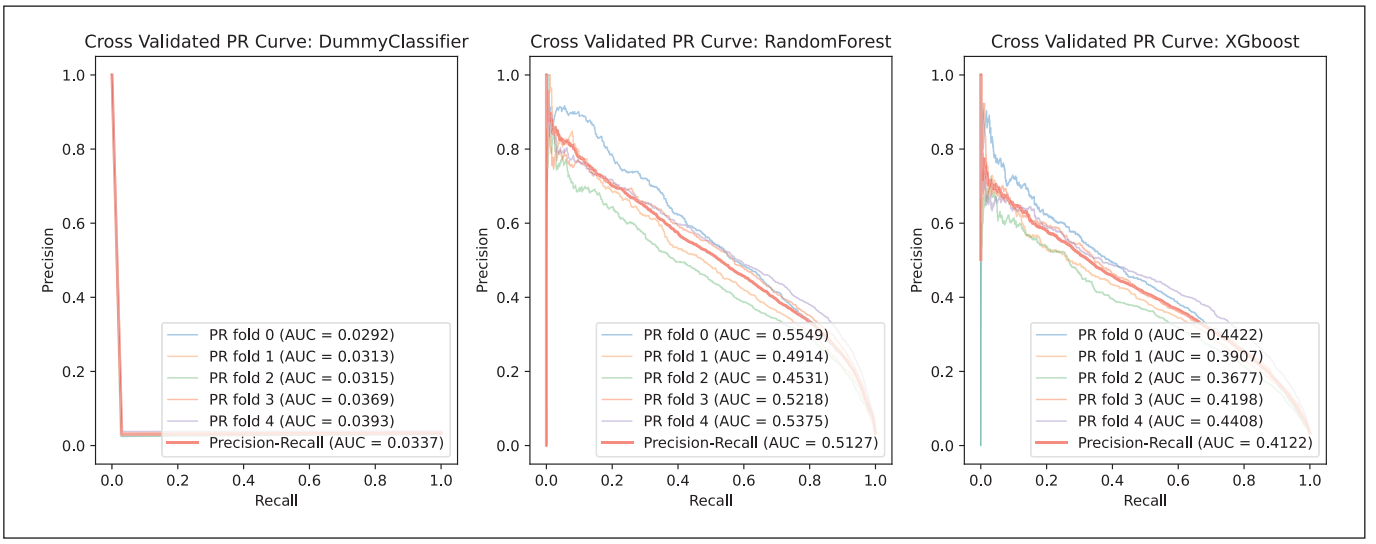

Fig. 7. Area under the precision-recall curve (AUCPR) with cross-validation for each of the five folds. The steepness and roundness toward the right upper corner represent good performance as it is the ideal maximum true-positive-to-false-negative ratio. Furthermore, it clearly shows that the AUCPR scores are almost equal as the data are evenly distributed across every data set created from K-fold cross-validation.

Table 2. Models performance on cross-validation and test data for the Dummy Classifier, XGBoost, and Random Forest

	Model					
	AUCPR	MCC	F1-score	Precision	Specificity	Sensitivity
Cross-validation						
Dummy Classifier	0.0339	0.0006	0.0352	0.0354	0.9671	0.0351
XGBoost	0.4100	0.3939	0.4044	0.4906	0.9875	0.3441
XGBoost with GNUS	0.2618	0.3507	0.3688	0.4083	0.9830	0.3364
XGBoost with SMOTE	0.4435	0.4036	0.4067	0.5348	0.99	0.3284
Random Forest	0.5131	0.3968	0.3596	0.6809	0.9960	0.2445
Random Forest with GNUS	0.51	0.3947	0.3558	0.6843	0.9961	0.241
Random Forest with SMOTE	0.5166	0.3966	0.3565	0.6908	0.9963	0.2407
Test set						
Dummy Classifier	0.0344	0.0018	0.0422	0.0433	0.9662	0.0413
XGBoost	0.4497	0.4247	0.4335	0.5342	0.9882	0.3648
XGBoost with GNUS	0.2916	0.3762	0.3939	0.4407	0.9833	0.3563
XGBoost with SMOTE	0.4786	0.4252	0.4261	0.5715	0.9905	0.3397
Random Forest	0.5467	0.4281	0.3890	0.7267	0.9963	0.2656
Random Forest with GNUS	0.5499	0.4223	0.3824	0.7228	0.9963	0.2600
Random Forest with SMOTE	0.5497	0.4232	0.3824	0.7270	0.9964	0.2594

GNUS, Gaussian noise up-sampling; SMOTE, synthetic minority over-sampling technique.

Discussion/Conclusion

In the present study, we evaluated multimodal data from multiple source systems in a hospital to form a prediction of PC transfusions in 3 days on a per-patient level. To the best of our knowledge, this is the first study to extract individual patient data to decide whether a patient needs a PC transfusion in 3 days for a cohort of more than 25,000 patients on a per-patient level.

XGBoost was the best-performing model with the highest score. However, the recall was too low to integrate the model into a live system as many true positives were misclassified. Yet, specific detection of true negatives was achieved. Therefore, clinical implementation of the trained models could be considered a pretest. The model could run in the background of the clinical routine and filter out all patients who do not need a PC transfusion.

Other studies have investigated the prediction of the blood product requirements for clinic-wide consumption. These studies have already examined the impact of machine learning models on hospital-wide platelet demand [21–24] and have found that it can be predicted with high accuracy [21, 23, 24]. Schilling et al. [21] found that their models could reduce both the shortage and waste of PCs within their institution at RWTH Aachen University Hospital. One limitation of previous studies in this field was the insufficient sensitivity to outliers. Outliers are days of extremely high or low demand for PCs. A patient-specific model, on the other hand, could identify days with outliers of PC demand and react prospectively toward days with a PC shortage.

The current high expiration rates of platelets are the driving factor behind the potential use in clinical routine. Usually, PCs are bought from a third party, supplied by an in-house institution, or both. However, PC demand is determined by the transfusing physicians and often – as is the case at our institution – not reported back to the provider. Digitalization of hospital infrastructure is a potential resource investment in patient care. Concerning PC demand, a system for predicting patient PC transfusion needs could lead to reduced PC expiry, and a doctor-independent decision process could consequently result in widespread economic benefits.

Furthermore, a model that predicts demand at the individual patient level would be an essential step toward improving patient blood management. Patient blood management is a central hemotherapeutic concept for improving patient safety that aims at reducing the need for blood transfusions and takes hospital-wide blood product management into account [25]. By improving the prediction of individual PC supply through our model, potential PC shortages could be prevented.

The most significant limitation of our model is its low sensitivity, which might partly be the result of missing data, particularly regarding future events such as crucial planned medical interventions (e.g., stem cell transplantation). Furthermore, data on medical appliances booked for surgery, especially heart and lung support devices, are currently not stored digitally in our institution. Thus, a new framework for integrating these data into our system will have to be constructed. In addition, the machine learning models used in our study could be considered relatively trivial from a technical perspective. However, a deep learning-based model may improve the model's performance and should be tested in further studies. A recurrent neural network or transformer network with a multi-input, multi-output architecture will be designed. Furthermore, diagnoses and procedures are not permanently recorded for each patient in a timely manner and also only for accounting purposes, so the integration in a live scenario of the data extraction process has to be adapted to using an identifier that merely extracts data of future events in FHIR.

In this study, we built the first model to predict patient individual PC demand. We have shown that an individual patient prediction of patients' PC transfusion needs can be ascertained using various data sources within the FHIR ecosystem and by applying traditional classification algorithms. However, further studies should apply this methodological data approach and adapt the machine learning models to more sophisticated methods, like deep neural networks, to improve sensitivity scores. In particular, however, the validity of a machine learning model depends on the scope and quality of the available data used as input.

In the future, great improvements in the predictive validity of corresponding models can be expected through a consistent, timely, and structured collection of all relevant healthcare data in the clinical routine. Once implemented, predictions for clinic-wide consumption may be derived, laying the groundwork for more efficient, ethical, and economical PC management.

Statement of Ethics

This study was conducted in compliance with the Ethics Committee of the Medical Faculty of the University Duisburg-Essen, approval number 20-9386-BO. Due to the retrospective nature of the study, the requirement of written informed consent was waived by the Ethics Committee.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Merlin Engelke and Vicky Parmar acquired the data. Felix Nensa, Peter Alexander Horn, Merlin Engelke, Vicky Parmar, Rene Hosch, and Sven Koitka designed the study, analyzed the data, and co-wrote the manuscript. Cynthia Sabrina Schmidt, Nils Flaschel, Christian Martin Brieske, and Anisa Kureishi contributed to the study design and critically revised the manuscript. All the authors approved its final content.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

References

1 Henseler O. Bericht zur Meldung nach § 21 TFG für das Jahr 2020; 2021. [cited 2022 Jul 8]. Available from: https://www.pei.de/SharedDocs/Downloads/DE/regulation/meldung/21-tfg/21-tfg-berichte/2020-tfg-21-bericht.pdf?__blob=publicationFile&v=4.

2 Greinacher A, Fendrich K, Hoffmann W. Demographic changes: the impact for safe blood supply. Transfus Med Hemother. 2010;37(3):141–8.

3 Greinacher A, Weitmann K, Schönborn L, Alpen U, Gloger D, Stangenberg W, et al. A population-based longitudinal study on the implication of demographic changes on blood donation and transfusion demand. Blood Adv. 2017 Jun;1(14):867–74.

4 University of Economicsin Katowice, Faculty of Management, Twaróg S, Szołtysek J, University of Economics in Katowice, Faculty of Management, Majewska J, University of Economics in Katowice, Faculty of Informatics and Communication, et al. Influence of demographic change on the blood services in Poland: logistics as a remedy for the future. Gospod Mater Logistyka. 2019 Mar;2019(3):2–11.

5 Festlegung der Haltbarkeitsfrist von Thrombozytenkonzentraten mit dem Ziel der Reduktion lebensbedrohlicher septischer Transfusionsreaktionen durch bakterielle Kontamination. Votum Des Arbeitskreises Blut. 2008 Dec;51(12):1484.

6 Hamada SR, Garrigue D, Nougue H, Meyer A, Boutonnet M, Meaudre E, et al. Impact of platelet transfusion on outcomes in trauma patients. Crit Care. 2022 Dec;26(1):49.

7 Yanagawa B, Ribeiro R, Lee J, Mazer CD, Cheng D, Martin J, et al. Platelet transfusion in cardiac surgery: a systematic review and meta-analysis. Ann Thorac Surg. 2021 Feb;111(2):607–14.

8 Wandt H, Schäfer-Eckart K, Greinacher A. Platelet transfusion in hematology, oncology and surgery. Dtsch Arztebl Int. 2014 Nov;111(48):809–15.

9 DRK-BSD West. Preisliste des DRK-BSD West vom 1.1; 2019.

10 Kaufman RM, Djulbegovic B, Gernsheimer T, Kleinman S, Tinmouth AT, Capocelli KE, et al. Platelet transfusion: a clinical practice guideline from the aabb. Ann Intern Med. 2015 Feb;162(3):205–13.

11 Querschnitts-Leitlinien Hämotherapie; 2020:287.

12 Daly ME. Determinants of platelet count in humans. Haematologica. 2011 Jan;96(1):10–3.

13 Branco P, Torgo L, Ribeiro RP. A survey of predictive modeling on imbalanced domains. ACM Comput Surv. 2016 Nov;49(2):1–50.

14 Douzas G, Bacao F, Last F. Improving imbalanced learning through a heuristic oversampling method based on k-means and SMOTE. Inf Sci. 2018 Oct;465:1–20.

15 Pedregosa F, Varoquaux G, Gramfort A, Michel V, Thirion B, Grisel O, et al. Scikit-learn: machine learning in Python. J Mach Learn Res. 2011;12:2825–30.

16 Chen T, He T, Benesty M, Khotilovich V, Tang Y, Cho H, et al. Xgboost: extreme gradient boosting. R Package Version. 2015;1(4):1–4.

17 sklearn.ensemble.RandomForestClassifier [Internet]. Scikit-Learn. [cited 2022 Jul 19]. Available from: https://scikit-learn.org/stable/modules/generated/sklearn.ensemble.RandomForestClassifier.html.

18 sklearn.dummy.DummyClassifier [Internet]. Scikit-Learn. [cited 2022 Jul 19]. Available from: https://scikit-learn.org/stable/modules/generated/sklearn.dummy.DummyClassifier.html.

19 Akiba T, Sano S, Yanase T, Ohta T, Koyama M. Optuna: a next-generation hyperparameter optimization framework proceedings of the 25th ACM SIGKDD international conference on knowledge discovery & data mining; 2019:2623–31.

20 optuna.pruners.MedianPruner — Optuna 2.10.1 documentation [Internet]. [cited 2022 Jul 19]. Available from: https://optuna.readthedocs.io/en/stable/reference/generated/optuna.pruners.MedianPruner.html.

21 Schilling M, Rickmann L, Hutschenreuter G, Spreckelsen C. Reduction of platelet outdating and shortage by forecasting demand with statistical learning and deep neural networks: modeling study. JMIR Med Inform. 2022 Feb;10(2):e29978.

22 Perelman I, Fergusson D, Lampron J, Mack J, Rubens F, Giulivi A, et al. Exploring peaks in hospital blood component utilization: a 10-year retrospective study at a large multisite academic centre. Transfus Med Rev. 2021 Jan;35(1):37–45.

23 Motamedi M, Li N, Down DG, Heddle NM. Demand forecasting for platelet usage: from univariate time series to multivariate models; 2021 Jan. [cited 2022 Mar 7]. Available from: https://arxiv.org/abs/2101.02305v1.

24 Fanoodi B, Malmir B, Jahantigh FF. Reducing demand uncertainty in the platelet supply chain through artificial neural networks and ARIMA models. Comput Biol Med. 2019 Oct;113:103415.

25 Leahy MF, Hofmann A, Towler S, Trentino KM, Burrows SA, Swain SG, et al. Improved outcomes and reduced costs associated with a health-system-wide patient blood management program: a retrospective observational study in four major adult tertiary-care hospitals. Transfusion. 2017;57(6):1347–58.

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Pilot Study to Gain First Indications for the Impact of a 3-Month's Oral Intake of a Sucrosomial Iron Supplement on Hemoglobin in Iron-Deficient Blood Donors

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Keywords
Sucrosomial iron · Blood donors · Iron deficiency · Oral iron intake

Abstract
Introduction: Regular whole blood donors often suffer from iron deficiency (ID) or iron deficiency anemia due to the loss of 200–300 mg of iron with each donation. Hemoglobin (Hb) as donor eligibility criterion reflects iron stores only poorly. ID in blood donors is typically prevented or treated with orally administered ferrous salts, which frequently cause gastrointestinal side effects. A high daily oral iron dose is counterproductive due to hepcidin upregulation. Oral sucrosomial iron ($_{sucr}$iron) is encapsulated ferric pyrophosphate that may be an option for blood donors due to its supposed high bioavailability and good tolerability. ***Methods:*** This monocentric single-cohort pilot study included fifty whole blood donors (divided into premenopausal women, postmenopausal women, and men) who did not meet Hb donation criteria. Participants aged 18–65 years with ferritin <30 ng/mL and venous Hb <12.5 g/dL in women and Hb <13.5 g/dL in men received oral $_{sucr}$iron (30 mg iron) for 90–120 days. Primary endpoints were the increase of Hb and ferritin. ***Results:*** Forty-seven participants completed the study. With the limitation that no control group was included, there was a substantial overall median increase of 0.94 g/dL Hb and 4.97 ng/mL ferritin (standardized on 90 days of iron intake). These value improvements were likewise observed in each of the subgroups. $_{sucr}$iron was very well tolerated, with almost no gastrointestinal side effects identified. ***Conclusion:*** A clear increase of Hb and ferritin was observed after the intake of $_{sucr}$iron, so it may be a reasonable and useful alternative to traditional oral iron therapy. The ease of administration, pleasant taste, dietary supplement status, and, most importantly, good tolerability highlight the value of $_{sucr}$iron supplementation.

Introduction

Human blood is a crucial element of public health. A sufficient supply of blood products is intrinsically connected to the viability of numerous progressively complex medical and surgical procedures. Therefore, blood and its derivates are included in the World Health Organization's model list of essential medicines [1]. Donation centers have a bidirectional responsibility to maintain adequate blood supply for patients while also caring for the health of donors. As a result of this responsibility, there is a continuous requirement to safeguard blood donors' long-term health. A whole blood donation of 450–500 mL results in a loss of 200–300 mg iron whereas daily average iron resorption is 1–2 mg [2, 3]. Therefore, iron deficiency (ID) and iron deficiency anemia (IDA) are commonly reported in regular blood donors, with a rate of up to 50% in male and up to 75% in female donors [4]. Kiss et al. [5]

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showed that within 168 days after a whole blood donation, 67% of untreated iron-deficient donors were unable to recover iron. Female gender, low body weight, and a high donation frequency are aggravating factors in the failure to restore iron balance [6, 7]. In Styria, donor eligibility criteria include a point-of-care test (POCT) for hemoglobin (Hb) using capillary blood from a fingerstick sample. Lower Hb donation thresholds are 12.5 g/dL for women and 13.5 g/dL for men. However, Hb levels do not accurately reflect body iron stores because IDA is a late consequence of ID [8]. As a result, even if their Hb levels meet donation guidelines, donors may be iron deficient and are at danger of further decreasing their iron stores. ID occurs when any loss of iron cannot be compensated for by diet [9] and is associated with symptoms such as chronic fatigue syndrome, decreased cognitive and physical performance, sleeping disorders, and restless legs syndrome (RLS) [10, 11]. Iron has been substituted orally for decades as ferrous sulfates, citrates, and fumarates. When ferrous salts are used, daily doses are limited due to the upregulation of hepcidin and associated inhibition of iron absorption [12, 13]. Consequently, conventional iron substitution has recently been recommended in either low doses or every other day [13]. Patients' adherence is low due to the long duration of therapy and intake modalities, as well as often-reported gastrointestinal side effects, resulting in therapy cessation and insufficient iron recovery. A promising new oral sucrosomial iron ($_{sucr}$iron), consisting of ferric pyrophosphate encapsulated in sucrosomes through a phospholipid bilayer membrane, is stated to be highly bioavailable, well tolerated, and easy to use. Preclinical data have shown that $_{sucr}$iron preparations pass through the stomach unmodified, avoiding direct irritation of the gastric mucosa, which is frequent with other oral iron formulations. Iron is subsequently released directly in duodenum, allowing iron ions to be transported across the intestinal epithelium without the assistance of divalent-metal transporter 1 [14, 15]. Microfold cells of Peyer's patches in the small intestine allow absorption where macrophages transport iron into the lymphatic system [15]. $_{sucr}$iron supplementation has successfully been tested on patients with chronic renal failure [16], pregnant women without anemia [17], anemic cancer patients [18], patients who are intolerant and/or refractory to iron sulfate [19], and celiac patients [20]. In Austria, $_{sucr}$iron has the status of a food supplement. In this descriptive explorative pilot study, we aimed to obtain first data on the effects of $_{sucr}$iron on Hb and ferritin concentrations in iron-deficient whole blood donors. Additionally, we investigated the acceptability and tolerability of a three-monthly oral intake of the study product and gathered data on symptoms that may be related with ID.

Materials and Methods

Study Design

This monocentric single cohort study in an interventional setting is classified as food study. Recruitment began in November 2019 and ended in June 2020. The study included 50 whole blood donors who had been rejected from blood donation due to low capillary POCT Hb levels (Hb <12.5 g/dL in women and Hb <13.5 g/dL in men). At the blood drive, these donors are routinely offered the opportunity to have their Hb and ferritin levels analyzed at the Department of Blood Group Serology and Transfusion Medicine. These results were taken as the study's baseline values. Further requirements were ferritin levels less than 30 ng/mL and a time period of more than 2 months between the previous whole blood donation and current Hb values. For both men and women, the minimum inter-donation interval for two whole blood donations is 8 weeks. Except for the Hb value, donors had to meet Austrian blood donation requirements and range in age from 18 to 65 years. Donors who met the inclusion criteria for Hb and ferritin were queried for exclusion criteria and invited to participate by phone. Exclusion criteria included pregnancy and lactation, chronic diarrhea, fructose intolerance or any other incompatibilities with the product's contents, as well as iron supplementation during the previous 3 months. The first visit (V1) on day 0 was scheduled within 21 days following blood collection and included the recording of relevant pre-existing medical conditions and/or therapies, as well as the initial completion of the prepared questionnaires. At V1, the participants were given four boxes $_{sucr}$iron, each containing 30 sachets. The last assessment (V2) and conclusion of the participation was scheduled 90 days after V1, on day 90, with a tolerance of 30 days, resulting in an observational period of 90–120 days. Participants had to be subjectively healthy for V2 to avoid infection-associated falsely high ferritin readings; therefore, at V1, they were instructed to postpone V2 if they became ill. At V2, they were queried regarding clinical symptoms of illness. Adverse events (AEs) associated with or unrelated to the test product, accompanying diseases, relevant medication, and potential dropout criteria, were investigated. Dropout would occur if the following conditions were met during the study period: taking fewer than 75% of the prescribed sachets, improper consumption on more than 25% of the days, administration of medication or other dietary supplements that might affect the iron balance, noncompliance with the 30 days tolerance for V2 or omission of V2, trauma or medical interventions involving blood loss or blood donation, diarrhea on more than 25% of the days, pregnancy or withdrawal of consent to study participation. Finally, blood was drawn for Hb and ferritin testing. Included primary endpoints are the increase of Hb and ferritin levels after oral intake of $_{sucr}$iron. Secondary endpoints include assessing the treatment's feasibility, as poor compliance is a major issue with conventional oral iron, as well as the tolerability and safety of the study product by assessing the occurrence of any symptoms related or possibly related to the study product's intake (AEs and serious adverse events). Following V2, a written report with the results and any recommendations was sent to each participant.

Questionnaires

At V1, participants were asked to fill out a questionnaire including any relevant preexisting medical conditions, information on previous iron intake and related AEs, as well as dietary habits. At V2, AEs associated with or unrelated to the test product, accompanying diseases, relevant medication, and potential dropout criteria, were investigated. They were also asked to offer details and score their experience with the $_{sucr}$iron. At each visit, possible clinical symptoms of ID were assessed using validated questionnaires

Table 1. Characteristics of participants consuming at least 3 months of iron supplementation (per protocol) at V1

Characteristic	PrW	PoW	Men	Overall *p* value
n (%), total 47	23 (48.9)	10 (21.3)	14 (29.8)	–
Age, mean (SD), years	36.99 (11.21)	48.95 (11.38)	47.98 (12.91)	0.006[a]
Height, mean (SD), cm	167.13 (5.93)	163.20 (7.27)	178.50 (8.54)	<0.001[b]
Weight, mean (SD), kg	64.57 (8.91)	65.1 (13.82)	80.57 (17.9)	0.003[c]
BMI, mean (SD), kg/m^2	23.16 (3.37)	24.42 (4.52)	25.12 (4.28)	0.202
Vegetarian/vegan, yes/no	3/20	0/10	0/14	0.242
Dietary habits, *n* (%)				Sum
Meat 5-6x per week	4 (20)	1 (10)	6 (42.9)	11
Meat 3-4x per week	9 (45)	2 (20)	6 (42.9)	17
Meat 1-2x per week	7 (35)	7 (70)	2 (14.3)	16
Preference in meat type				
None, *n* (%)	14 (70)	6 (60)	10 (71.4)	30
White meat, *n* (%)	3 (15)	2 (20)	2 (14.3)	7
Red meat, *n* (%)t	3 (15)	1 (10)	2 (14.3)	6
History of anemia	15 (65.2)	5 (50)	11 (78.6)	31
History of oral iron intake	12 (52.2)	3 (30)	5 (35.7)	20

The table contains information on dietary habits, including the frequency of meat consumption per week and meat type preferences, as well as on historical data on anemia and previous conventional oral iron intake. BMI, body mass index; SD, standard deviation. [a] $p < 0.05$ for PrW versus PoW and PrW versus men. [b] $p < 0.05$ for PrW versus men and PoW versus men and [c] $p < 0.05$ for PrW versus men and PoW versus men.

for RLS [21], fatigue [22, 23], sleep [24], and quality of life [25]. For more detailed information, see online supplementary 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000527577).

Laboratory Tests

Laboratory tests were performed at the time of blood donation deferral and at V2. Venous Hb was measured from EDTA blood by cyan methemoglobin method (Advia® 2120/120, Siemens Healthcare Diagnostics, Siemens AG Vienna, Austria), and ferritin was measured from serum using a chemiluminescence immunoassay (Laison® Ferritin, Liaison® XL; DiaSorin S.p.A., Saluggia, Italy), both according to manufacturers' instructions.

Treatment

At V1, donors were given oral $_{sucr}$iron (OLEOvital® EISEN FORTE; PharmaNutra S.p.A., Pisa, Italy, licensed by Fresenius Kabi Austria GmbH, Graz, Austria), containing 30 mg of iron and 70 mg vitamin C. The study product had to be taken daily, regardless of daytime or concurrent use of medication, food, or beverages, for 90–120 days equating to 2,700–3,600 mg iron. The dosage prescribed was in accordance with the manufacturer's recommendations for use.

Statistical Analysis

Before comparing premenopausal women (PrW) versus postmenopausal women (PoW) versus men, all data of continuous variables were checked for normal distribution (test of normality: Kolmogorov-Smirnov with Lilliefors significance correction, type I error = 10%) and for heteroscedasticity (Levene test, type I error = 5%). As none of the variables presented in this paper showed data sets with both normal distribution and homoscedasticity, all comparisons were performed by a nonparametric analysis of variance (Kruskal-Wallis test, followed by Nemenyi's multiple comparisons). Data from the single presented categorical variable (vegetarian + vegan) were compared by the exact χ^2 test.

Pre-post-comparisons of continuous variables with normally distributed data were performed by the paired *t* test; otherwise, the exact Wilcoxon test was used. Dichotomous variables were compared by the exact McNemar test. Multiple regression analyses were used to investigate the influence of the following variables on delta hemoglobinV2-V2/90d and on delta ferritinV2-V2/90d: subgroup membership (PrW vs. PoW vs. men); Hb or ferritin, weight, age, BMI (all at baseline); RLS at baseline and at the end of iron supplementation; ratio iron prescription/iron intake; history of anemia or ID; diarrhea during iron supplementation.

Since the type I error was not adjusted for multiple testing, the results of inferential statistics are descriptive only and the use of the term "significant" in the description of the study results always reflects only a local $p < 0.05$ but no error probability below 5%. Statistical analysis was performed using the open-source R statistical software package, version 4.0.5 (the R Foundation for Statistical Computing, Vienna, Austria).

Results

Between November 2019 and June 2020, 92 eligible whole blood donors were invited to take part in the study of which 50 were enrolled. After the target number of participants was reached, recruitment was discontinued. Three study candidates had to be excluded from the statistical analysis due to protocol violations. One participant donated whole blood (PrW), one took less than 75% of the prescribed sachets (PrW) due to forgetfulness, and one man did not complete the final examination due to an active SARS-CoV-2 infection during the study interval. The remaining participants were as follows: 23 PrW,

Table 2. Results for laboratory parameters at V1 and V2 including 47 participants

Parameter	Reference range	Visit	PrW	PoW	Men	PoW and men
Hb, median (IQR), g/dL	≥12.5/≥13.5[a]	V1	12.0 (11.7, 12.2)	12.0 (11.5, 12.1)	12.5 (12.1, 13.0)	12.1 (11.6, 12.6)
		V2	12.6 (12.3, 13.5)	13.1 (12.7, 13.2)	13.6 (12.7, 14.6)	13.1 (12.7, 14.2)
p value			**<0.001**	**0.002**	**0.001**	**<0.001**
Ferritin, median (IQR), ng/mL	30–150	V1	8.3 (6.8, 10.3)	10.3 (6.3, 16.2)	10.1 (8.2, 13.8)	10.3 (7.3, 15.0)
		V2	14.5 (11.1, 17.1)	18.4 (12.5, 24.5)	16.0 (8.2, 18.8)	16.9 (12.3, 22.6)
p value			**<0.001**	**0.005**	**0.035**	**<0.001**

Hb, hemoglobin; IQR, interquartile range (25th, 75th percentile). [a] Hb values refer to eligibility criteria for donation for women and men.

10 PoW, and 14 men. Overall, the median iron sachet consumption was 97 (interquartile range: 92, 102), equivalent to 2.910 mg $_{sucr}$iron, and the 90-day ratio of prescribed sachets to sachets taken was 1.01 (interquartile range: 1.0, 1.04).

For practical reasons, the period between the first and the second examination and thus the duration of iron intake varied from 90 to 120 days. Therefore, as a result, all outcomes were standardized to 90 days by interpolation.

Characteristics of Participants

Participants' characteristics at baseline and history of ID are summarized in Table 1. A significant difference was found in age, height, and weight. This is due to the participation of both women and men, as well as the distinction between PrW and PoW. The significant difference in baseline Hb between the subgroups at V1 (p = 0.023; for values, see Table 2) is due to the inclusion of men and women, as well as the different cutoff values for admittance to blood donation.

There were only three vegetarians among the participants. Nobody reported daily meat consummation. Eleven participants eat meat 5-6 times per week, 17 3-4 times, and 16 once or twice per week. The majority of participants (30; 68.%) have no preference in meat type, while six prefer red meat and seven prefer white meat (see also Table 1).

History of anemia (for subgroups, see Table 1): thirty-one participants reported that they had previously been diagnosed with anemia. Of these, 20 had already taken conventional iron supplements in the past, of which nine had suffered from side effects (45%). The most common gastrointestinal side effects reported were constipation (3), diarrhea (2), flatulence (2), nausea (2), and gastric distention/cramps (3). The following other side effects were each named once: fatigue, headache, skin rash, itching.

Adverse Events

There were three recorded AEs. One participant had trouble falling asleep at first, another had loose stools on occasion, and one had pain in his fingers and both wrists 2 weeks after starting the iron supplements. There were no further documented side effects or AEs. The latter two incidents were reported to the manufacturer, who did not classify them as possibly caused by $_{sucr}$iron. Overall, the participants rated the $_{sucr}$iron very highly. All of them would also take it again, with the easy handling during intake, the nice flavor, and the perceived favorable effect as key reasons.

Hb and Ferritin Levels

When compared to the initial results, there is a marked increase in Hb and ferritin values, with an overall median increase of 0.94 g/dL for Hb and 4.97 ng/mL for ferritin. Men and women have similar median Hb increases (0.84, 0.99, and 0.87 g/dL for PrW, PoW, and men, respectively). The median ferritin increase within the subgroups is 5.08, 7.42, and 2.7 ng/mL for PrW, PoW, and men. Despite the fact that men had the smallest increase in ferritin, the improvement in Hb and ferritin levels was statistically conspicuous in all subgroups (Table 2; Fig. 1).

Multiple regression analyses showed that none of the examined variables had a significant effect on the increase of Hb ($\Delta_{Hb\ (V2\text{-}V2/90d)}$). However, the increase of ferritin ($\Delta_{ferritin\ (V2\text{-}V2/90d)}$) was favored by iron intake compliance (ratio iron prescription/iron intake; p = 0.022) and by RLS at baseline (p = 0.036).

ID-Associated Symptoms at V1 and V2

Table 3 shows the overall results for symptoms associated with ID. PrW experienced the most symptoms including brittle nails (V1, V2: 9 [39.1%], 6 [26.1%]), increased hair loss (V1, V2: 9 [39.1%], 5 [21.7%]), shortness of breath/palpitations (V1, V2: 8 [34.8%], 7 [30.4%]), and dizziness (V1, V2: 7 [30.4%], 6 [26.1%]). There were no reports of men experiencing increased hair loss, load-dependent headaches, or dizziness. A noticeable improvement in brittle fingernails was reported by 54.5% (95% confidence intervals [CI], 23.4–83.3) of participants, in hair loss 75.0% (95% CI, 34.9–96.8), and in shortness of breath/palpitations during exercise 55.6% (95% CI, 21.2–

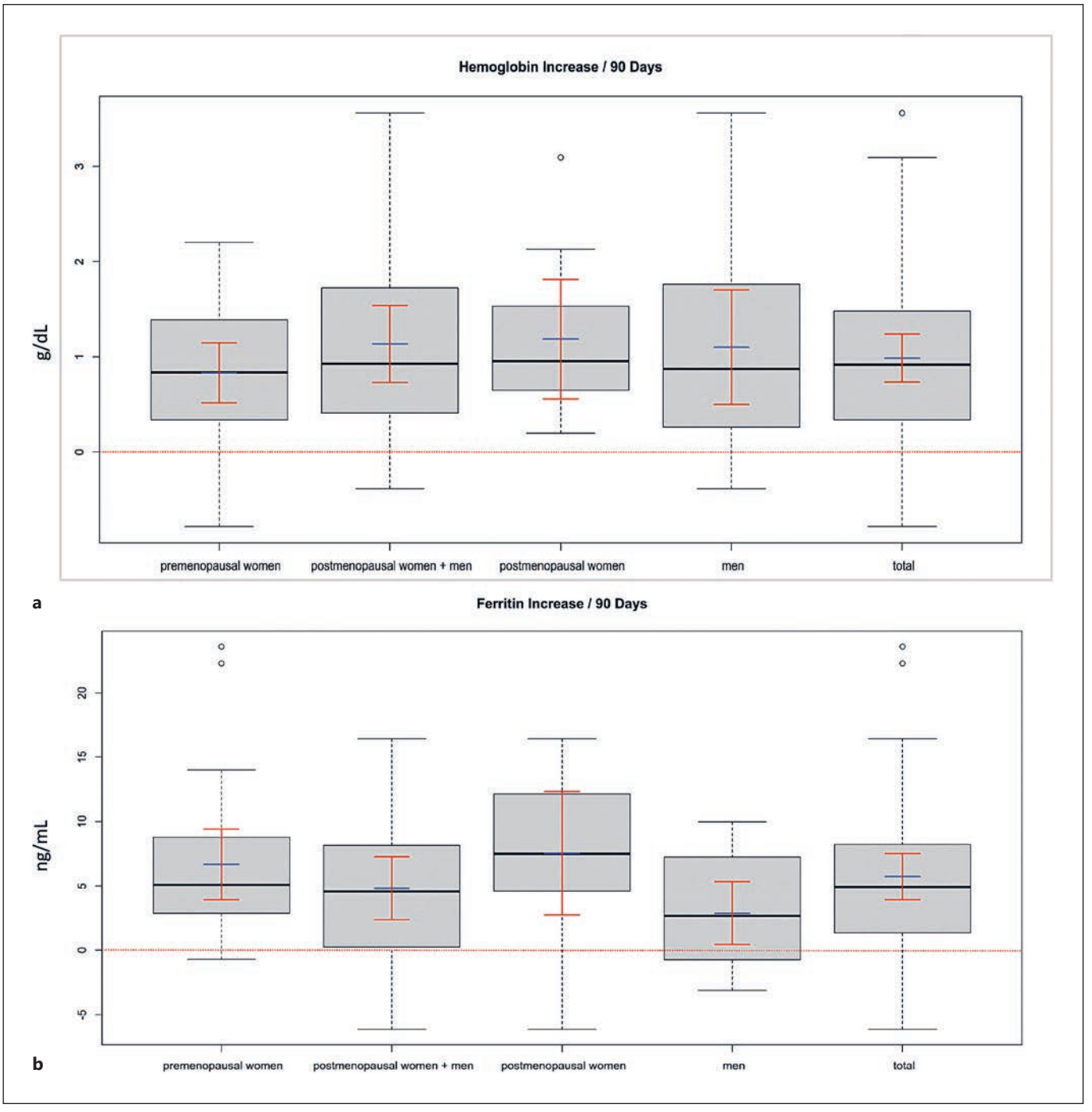

Fig. 1. Increase of Hb and ferritin in subgroups over 90 days. **a** Hb. Boxplot with black line, box, whiskers, and circles (median, interquartile range [IQR], "minimum"/"maximum," and outliers); blue line in boxes, mean; red whiskers in boxes, lower and upper limit of 95% confidence intervals (CI) for mean; red dotted line at y = 0 indicates no change. **b** Ferritin. Boxplot with black line, box, whiskers, and circles (median, IQR, "minimum"/"maximum," and outliers), blue line in boxes, mean; whiskers in boxes, lower and upper limit of 95% CI for mean; dotted line at y = 0 indicates no change.

86.3). However, the improvements were not statistically significant. Online supplementary 1 contains the findings on RLS, fatigue, sleep, and quality of life.

Discussion

In this pilot study, we found that after oral intake of $_{sucr}$iron over 90 days there was a marked increase in both, Hb and ferritin. Overall, the median increases of Hb and

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Table 3. Comparison of symptoms of all participants to ID-associated symptoms at V1 and V2

Symptoms of ID	V1, *n* (%)	V2, *n* (%)	*p* value
Brittle nails	17 (36.2)	11 (23.4)	0.109
Increased hair loss	13 (27.7)	8 (17)	0.063
Load-dependant headache	5 (10.6)	6 (12.8)	>0.999
Shortness of breath/palpitations during exercise	13 (27.7)	9 (19.1)	0.219
Dizziness	7 (14.9)	6 (12.8)	>0.999
Painful or "slippery" tongue	1 (2.1)	0	>0.999
Unexplained food/nonfood cravings (Pica)	0	1 (2.1)	>0.999

ferritin are 0.94 g/dL and 4.97 ng/mL, respectively. In a comparable study, oral administration of 30 mg of conventional iron for 3 months resulted in a mean increase in ferritin of 5.9 ng/mL in PrW with no change in mean Hb [26]. Men benefit less than women do from $_{sucr}$iron intake in terms of ferritin levels (median increase of 2.7 vs. 5.08 and 7.42 ng/mL, men, PrW, and PoW, respectively) despite comparable consumption of sachets and having approximately the same baseline values as PoW. This could be due to men having a higher erythrocyte mass (men 2,087.5 mL vs. 1,405.9 mL in women [27]), or/and skeletal muscle mass (30.6 vs. 38.4% relative to body mass of men [28]), where missing iron could have been utilized to a greater extent, leaving less storage iron. PoW appear to benefit more from iron supplementation than PrW, which seems plausible because PrW lose blood (and thus iron) through menstruation. Nevertheless, a desired ferritin threshold (30 ng/mL) was not reached, which could be resolved by increasing the dose (e.g., 60 mg) or extending the duration of $_{sucr}$iron intake.

Encouragingly, participants experienced remarkably few AEs and/or side effects associated with $_{sucr}$iron intake, although some reported complaints associated with conventional oral iron therapy in the past. It seems reasonable to attribute this to the product's favorable biochemical properties and to the low daily iron intake of only 30 mg in this trial, which is well below the commonly prescribed dosage of 100 mg of elemental iron per day. However, even at substantially lower doses of traditional iron preparations, more side effects are reported [29]. Except from one case of occasionally loose stools in the study period, no gastrointestinal AEs were documented, implying that gastrointestinal tolerance of $_{sucr}$iron appears to be excellent. This reflects the fact that all participants would be willing to take this food supplement again if ever needed. Concerning the presumed AE of pain in the joints and fingers, there is no direct or obvious link between the product's consumption and the complaints. According to manufacturer's comments, similar problems about $_{sucr}$iron supplementation had not been documented in Italy or other countries. Furthermore, only remarkably few participants had to be excluded from the study (n = 3), none of them due to AEs.

Further questions were addressed using validated questionnaires on ID symptoms: RLS, fatigue, sleep, and perception of quality of life. They are discussed in online supplementary 1.

The administration of supplements to otherwise healthy blood donors raises the question, whether such an approach is ethically justifiable. One argument against supplementation might be that treating healthy people with ID is solely masking laboratory values with unnecessary medication and possible side effects. However, we are convinced that iron administration as a preventive measure for donors with ID as a result of a voluntary medical intervention provides both immediate and long-term health benefits and may delay or avoid IDA in future blood donations. In a study with 2-year follow-up, regular blood donors with ID not receiving iron substitution (placebo group and group without any information on iron status) had a mean ferritin increase of 0.8 and 0.3 ng/mL and a mean change in venous Hb of −0.07 and −0.13 g/dL at the final visit, respectively [30]. This underscores the fact, that without additional iron consumption, recovery of iron stores in regular blood donors is poor [5]. If a greater proportion of repeat donors remain healthy, the necessary blood demand can be fulfilled by a greater number of donors, and the donation frequency per donor decreases. This in turn helps to maintain individual iron stores in this population. At this point, $_{sucr}$iron as a food supplement would be a good alternative for blood donors with practically no side effects and flexible modalities for intake (with or without meals, other medications, and at any time of day) making this supplement ideal for donors who are not accustomed to taking medication. A disadvantage is the comparatively high cost of $_{sucr}$iron, which is about ten times higher than conventional oral iron preparations and, due to its classification as a food supplement it is, at least in Austria, not generally covered by health insurances. In addition to oral iron therapy, intravenous iron substitution may also be an effective strategy to replenish iron stores. However, it is substantially more expensive, requiring a venous access as well as monitoring during infusion due to rare hypersensitivity reactions.

With our research, we would like to address the iron stores in blood donors in particular. It was interesting to

see that almost two-thirds of our participants had previously been diagnosed with IDA, and almost 65% of them had also taken iron in the past. The remaining, however, had not. The latter could imply that donors underestimate or are even unaware of the iron loss during blood donation and that they rarely feel the need to take medication without a certain level of suffering. In addition, the symptoms of ID can be ambiguous and elusive, owing in part to the gradual onset of symptoms, which is why donors believe they are healthy enough to donate despite ID. There are numerous approaches to dealing with iron storage in whole blood donors. They include Hb- or ferritin-targeted donation intervals as well as iron supplementation [31]. Nevertheless, current Austrian blood donor regulations do not explicitly address this issue [32] although an adaptation of the regulation is planned. It would be preferable to implement safeguards, such as algorithms that consider Hb, ferritin, and an individual donation frequency, to ensure that blood donors to not develop ID in the first place.

Limitations

Here, we describe the results of a single-arm pilot study. Lacking a control group, spontaneous increase of Hb levels due to naturally occurring auto-regeneration over time could have influenced the results of the study. Mujica-Coopman et al. [26] found a mean increase of 0.6 ng/mL for ferritin and even a slight decrease of −0.3 g/dL for Hb in PrW (one-third with ID of varying severity) after 3 months of placebo intake. In another study, placebo use for 3 months showed a mean change for Hb of −0.05 g/dL and for ferritin of 0.2 ng/mL in PrW with ID [33]. We therefore assume that the improvement in Hb and ferritin levels in our participants may well be attributable to the $_{sucr}$iron consumption, although at least 2,700 mg of $_{sucr}$iron were insufficient to compensate for the iron loss of a whole blood donation. In addition, the small sample size may limit the significance of our findings. $_{Sucr}$iron, like other standard oral iron treatments, contains ascorbic acid. It is unclear whether it enhanced iron bioavailability during the study interval. Although ascorbic acid is acknowledged as an iron uptake enhancer, there are also investigations that have found little effect [34]. The total benefit of vitamin C may be less pronounced because our study participants were not instructed to avoid inhibitors of iron absorption, such as tea, coffee, and dairy items; on the contrary, they were encouraged to take the sachets along with their meals. In terms of clinical symptoms, a placebo effect caused by awareness of an effective supplement could have influenced the study's results.

Overall, the observed increases in outcomes can be understand as trends and indicators of therapeutic success. However, we believe that these findings, along with those from other studies on the use of $_{sucr}$iron administration that included participants with diseases or other physical circumstances, are compelling enough to merit follow-up research. A confirmatory study would have to be conducted with a reasonable number of participants based on sample size estimation, and would have to be double blinded and placebo controlled, using an iron dose of at least 60 mg per day or a comparison of 30 mg per day with 60 mg per day. A comparison group could also receive ascorbic acid. Other iron characteristics that should be examined include transferrin saturation, soluble transferrin receptor, and hepcidin as a critical regulator of iron homeostasis [35].

Strengths of the Study

To our knowledge, this is the first study of $_{sucr}$iron administration in healthy blood donors with ID or IDA who do not have any other major health concerns.

Conclusion

ID and IDA can result in the loss of blood donors who cannot be readmitted to blood donation unless iron supplements are taken. $_{sucr}$iron might be a good alternative to conventional iron therapy, especially for a population that takes little medicine, such as blood donors. The ease of use, the pleasant taste, the status of a food supplement in Austria, and, most importantly, the low occurrence of side effect are all facts in its favor. Overall, it appears to be effective; however, this would need to be confirmed in a detailed follow-up study with higher dosage or an expansion of the administration time.

Statement of Ethics

The Ethical Committee of the Medical University of Graz approved the study (31-435 ex 18/19). It was carried out under GCP guidelines and the Declaration of Helsinki. Furthermore, it was registered at ClinicalTrials.gov (NCT04250298). At the initial visit (V1), participants gave written informed consent to participate in the study.

Conflict of Interest Statement

The authors declare that they have no conflicts of interest relevant to the manuscript.

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Author Contributions

Camilla Drexler-Helmberg, Patrick Paul Torreiter, Peter Schlenke, Petra Krakowitzky, Wolfgang Schimetta, and Wolfgang Helmberg were involved in the design of the study. Patrick Paul Torreiter, Camilla Drexler-Helmberg, and Petra Krakowitzky have conducted clinical visits. Patrick Paul Torreiter, Camilla Drexler-Helmberg, Peter Schlenke, Wolfgang Schimetta, and Wolfgang Helmberg wrote the draft version of the paper. Wolfgang Schimetta contributed statistical analysis and data visualization.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its supplementary files. Further inquiries can be directed to the corresponding author.

References

1 World Health Organization. World Health Organization model list of essential medicines: 21st list 2019. Geneva: World Health Organization; 2019.
2 Cable RG, Brambilla D, Glynn SA, Kleinman S, Mast AE, Spencer BR, et al. Effect of iron supplementation on iron stores and total body iron after whole blood donation. Transfusion. 2016 Aug;56(8):2005–12.
3 Nielsen P. Diagnostik und Therapie von Eisenmangel mit und ohne Anämie. Bremen; 2016.
4 Bialkowski W, Bryant BJ, Schlumpf KS, Wright DJ, Birch R, Kiss JE, et al. The strategies to reduce iron deficiency in blood donors randomized trial: design, enrolment and early retention. Vox Sang. 2015 Feb;108(2):178–85.
5 Kiss JE, Brambilla D, Glynn SA, Mast AE, Spencer BR, Stone M, et al. Oral iron supplementation after blood donation: a randomized clinical trial. JAMA. 2015 Feb 10;313(6):575–83.
6 Cable RG, Glynn SA, Kiss JE, Mast AE, Steele WR, Murphy EL, et al. Iron deficiency in blood donors: analysis of enrollment data from the REDS-II Donor Iron Status Evaluation (RISE) study. Transfusion. 2011 Mar;51(3):511–22.
7 Rigas AS, Sørensen CJ, Pedersen OB, Petersen MS, Thørner LW, Kotzé S, et al. Predictors of iron levels in 14,737 Danish blood donors: results from the Danish Blood Donor Study. Transfusion. 2014 Mar;54(3 Pt 2):789–96.
8 Radtke H, Meyer T, Kalus U, Röcker L, Salama A, Kiesewetter H, et al. Rapid identification of iron deficiency in blood donors with red cell indexes provided by Advia 120. Transfusion. 2005 Jan;45(1):5–10.
9 Zimmermann MB, Hurrell RF. Nutritional iron deficiency. Lancet. 2007 Aug 11; 370(9586):511–20.
10 Carter RC, Jacobson JL, Burden MJ, Armony-Sivan R, Dodge NC, Angelilli ML, et al. Iron deficiency anemia and cognitive function in infancy. Pediatrics. 2010;126(2):e427–34.
11 Macher S, Herster C, Holter M, Moritz M, Matzhold EM, Stojakovic T, et al. The effect of parenteral or oral iron supplementation on fatigue, sleep, quality of life and restless legs syndrome in iron-deficient blood donors: a secondary analysis of the IronWoMan RCT. Nutrients. 2020 May 5;12(5):1313.
12 Moretti D, Goede JS, Zeder C, Jiskra M, Chatzinakou V, Tjalsma H, et al. Oral iron supplements increase hepcidin and decrease iron absorption from daily or twice-daily doses in iron-depleted young women. Blood. 2015 Oct 22;126(17):1981–9.
13 Stoffel NU, Cercamondi CI, Brittenham G, Zeder C, Geurts-Moespot AJ, Swinkels DW, et al. Iron absorption from oral iron supplements given on consecutive versus alternate days and as single morning doses versus twice-daily split dosing in iron-depleted women: two open-label, randomised controlled trials. Lancet Haematol. 2017 Nov;4(11):e524–33.
14 Fabiano A, Brilli E, Fogli S, Beconcini D, Carpi S, Tarantino G, et al. Sucrosomial® iron absorption studied by in vitro and ex-vivo models. Eur J Pharm Sci. 2018 Jan 1;111:425–31.
15 Gómez-Ramírez S, Brilli E, Tarantino G, Muñoz M. Sucrosomial(®) iron: a new generation iron for improving oral supplementation. Pharmaceuticals. 2018 Oct 4;11(4):97.
16 Pergola PE, Fishbane S, Ganz T. Novel oral iron therapies for iron deficiency anemia in chronic kidney disease. Adv Chronic Kidney Dis. 2019 Jul;26(4):272–91.
17 Parisi F, Berti C, Mandò C, Martinelli A, Mazzali C, Cetin I. Effects of different regimens of iron prophylaxis on maternal iron status and pregnancy outcome: a randomized control trial. J Matern Fetal Neonatal Med. 2017 Aug; 30(15):1787–92.
18 Mafodda A, Giuffrida D, Prestifilippo A, Azzarello D, Giannicola R, Mare M, et al. Oral sucrosomial iron versus intravenous iron in anemic cancer patients without iron deficiency receiving darbepoetin alfa: a pilot study. Support Care Cancer. 2017 Sep;25(9):2779–86.
19 Giordano G, Napolitano M, Di Battista V, Lucchesi A. Oral high-dose sucrosomial iron vs intravenous iron in sideropenic anemia patients intolerant/refractory to iron sulfate: a multicentric randomized study. Ann Hematol. 2021 Sep;100(9):2173–9.
20 Elli L, Ferretti F, Branchi F, Tomba C, Lombardo V, Scricciolo A, et al. Sucrosomial iron supplementation in anemic patients with celiac disease not tolerating oral ferrous sulfate: a prospective study. Nutrients. 2018 Mar 9;10(3):330.
21 Allen RP, Picchietti D, Hening WA, Trenkwalder C, Walters AS, Montplaisi J. Restless legs syndrome: diagnostic criteria, special considerations, and epidemiology: a report from the restless legs syndrome diagnosis and epidemiology workshop at the National Institutes of Health. Sleep Med. 2003;4(2):101–19.
22 Glaus A, Müller S. Measuring fatigue of cancer patients in the German-speaking region: development of the Fatigue Assessment Questionnaire. Pflege. 2001;14(3):161–70.
23 Beutel M, Hinz A, Albani C, Brähler E. Fatigue assessment questionnaire: standardization of a cancer-specific instrument based on the general population. Oncology. 2006; 70(5):351–7.
24 Crönlein T, Langguth B, Popp R, Lukesch H, Pieh C, Hajak G, et al. Regensburg insomnia scale (RIS): a new short rating scale for the assessment of psychological symptoms and sleep in insomnia; study design: development and validation of a new short self-rating scale in a sample of 218 patients suffering from insomnia and 94 healthy controls. Health Qual life Outcomes. 2013;11(1):65.
25 Development of the World health organization WHOQOL-BREF quality of life assessment. The WHOQOL Group. Psychol Med. 1998;28(3):551–8.
26 Mujica-Coopman MF, Borja A, Pizarro F, Olivares M. Effect of daily supplementation with iron and zinc on iron status of childbearing age women. Biol Trace Elem Res. 2015;165(1):10–7.
27 Frenkel EP, McCall MS, Reisch JS, Minton PD. An analysis of methods for the prediction of normal erythrocyte mass. Am J Clin Pathol. 1972;58(3):260–71.
28 Janssen I, Heymsfield SB, Wang ZM, Ross R. Skeletal muscle mass and distribution in 468 men and women aged 18–88 yr. J Appl Physiol. 2000;89(1):81–8.
29 Rimon E, Kagansky N, Kagansky M, Mechnick L, Mashiah T, Namir M, et al. Are we giving too much iron? Low-dose iron therapy is effective in octogenarians. Am J Med. 2005;118(10):1142–7.
30 Mast AE, Bialkowski W, Bryant BJ, Wright DJ, Birch R, Kiss JE, et al. A randomized, blinded, placebo-controlled trial of education and iron supplementation for mitigation of iron deficiency in regular blood donors. Transfusion. 2016 Jun;56(6 Pt 2):1588–97.
31 Mast AE. Putting donor health first in strategies to mitigate donor iron deficiency. Transfusion. 2017 Mar;57(3):495–8.
32 Bundesministerium für Arbeit Gesundheit und Soziales. Gesundheitsschutz von Spendern und die Qualitätssicherung von Blut und Blutbestandteilen BGBL II Nr 100/1999 idgF. 1999.
33 Vaucher P, Druais P-L, Waldvogel S, Favrat B. Effect of iron supplementation on fatigue in nonanemic menstruating women with low ferritin: a randomized controlled trial. CMAJ. 2012;184(11):1247–54.
34 Li N, Zhao G, Wu W, Zhang M, Liu W, Chen Q, et al. The efficacy and safety of vitamin C for iron supplementation in adult patients with iron deficiency anemia: a randomized clinical trial. JAMA Netw Open. 2020;3(11):e2023644.
35 Stoffel NU, Zeder C, Fort E, Swinkels DW, Zimmermann MB, Moretti D. Prediction of human iron bioavailability using rapid c-ELISAs for human plasma hepcidin. Clin Chem Lab Med. 2017 Jul 26;55(8):1186–92.

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Immunoglobulin Class Profiles of ABO Antibodies in Saliva and Serum of Healthy Individuals

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Abstract
Introduction: The coronavirus disease (COVID-19) pandemic gave rise to studies investigating the association of ABO blood group with COVID-19 susceptibility. It is hypothesized that ABO antibodies might play a role in neutralizing SARS-CoV-2. However, ABO antibodies were exclusively analyzed in blood samples. Investigation of ABO antibodies in saliva, an easy-to-obtain surrogate for respiratory secretions, may provide novel insights into mucosal immunity crucial in early defense against respiratory pathogens. ***Methods:*** In this study, saliva and serum samples from healthy individuals with known blood groups were investigated using a flow cytometric method for separate anti-A/anti-B IgA, IgM, and IgG class antibody detection. Saliva samples were additionally tested using hemagglutination-based neutral and indirect anti-human globulin test gel cards. This method comparison was complemented by dilution experiments with a high-titer anti-A/anti-B WHO standard. ***Results:*** In saliva, IgA was the most abundant ABO antibody class, followed by IgM; IgG was detected only in low levels in all non-AB blood types. In serum, IgM was the predominant ABO antibody class in all non-AB blood types, followed by IgA and IgG, the latter mainly detected in group O individuals. Saliva and serum samples of group O individuals yielded the highest variability of ABO-specific antibody levels. Regardless of sample material and blood type, major interindividual differences in ABO antibody reactivities were recorded. Antibody levels correlated moderately between these two body fluids. There were no significant sex and age-group differences in ABO antibody levels in both serum and saliva. WHO standard dilution experiments yielded technique-specific limits of detection, illustrating the inherent differences of immunofluorescence versus agglutination. ***Conclusion:*** For the first time, salivary ABO antibodies were investigated by separate detection of the three most relevant antibody classes IgA, IgM, and IgG in a healthy cohort. This study opens new perspectives regarding mucosal ABO antibody class profiles and their potential influence on respiratory infections.

Introduction

The coronavirus disease (COVID-19) pandemic gave rise to studies investigating the association between COVID-19 susceptibility and ABO blood groups [1–3]. Specifically, among infected individuals, blood group O was underrepresented compared to non-group O-types. It was hypothesized that natural ABO antibodies may be a main factor of protection, particularly anti-A of the IgG class, as this was detected preferably in samples of group O individuals [4, 5].

So far, ABO antibodies were exclusively analyzed in blood samples. However, mucosal immunity, particularly secretory IgA, is reported to play a more important role in early defense against respiratory pathogens [6]. Saliva

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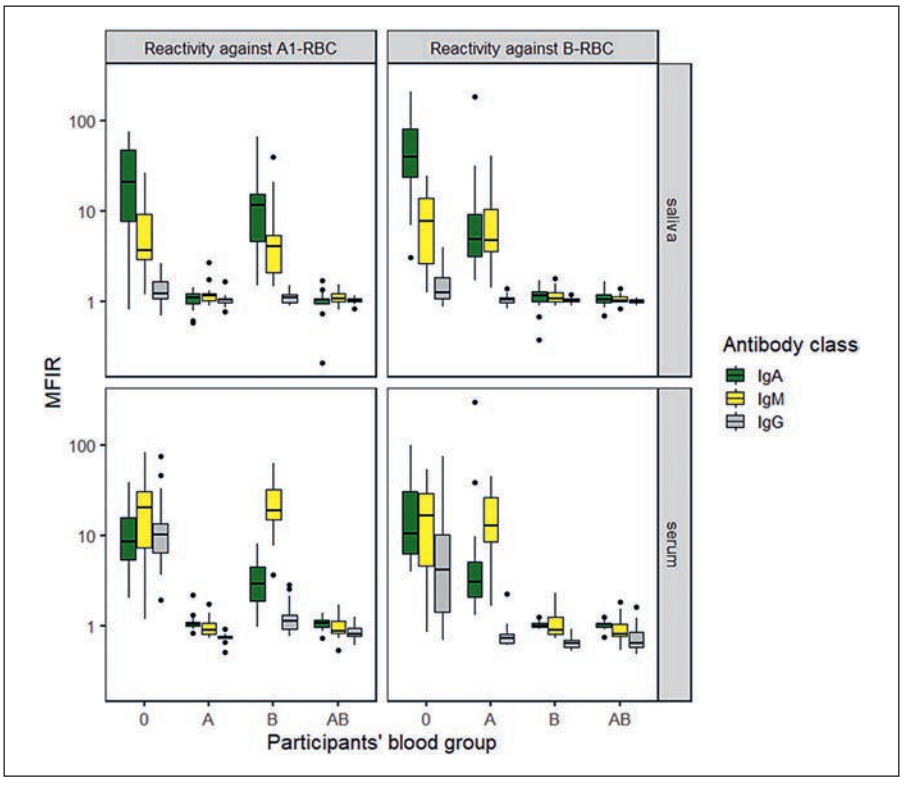

Fig. 1. Boxplots representing MFIR of flow cytometric ABO antibody detection in saliva and serum. Values on the y-axis are displayed on a log(10) scale.

may represent an easy-to-obtain surrogate for respiratory secretions and was insufficiently studied regarding ABO antibodies [7–9]. No data are available on salivary ABO antibodies regarding their immunoglobulin (Ig) classes and corresponding serum levels. Reasons might be challenges in saliva processing caused by high proteolytic activity, viscosity, and composition [10].

In this study, a flow cytometric method was established to differentially detect IgA, IgM, and IgG ABO antibodies in saliva. ABO antibody reactivities in the saliva of healthy individuals were put in relation to serum levels. Aside from providing novel insights into ABO-dependent physiological human salivary composition, this method can be applied to investigate the dynamics of salivary ABO antibody levels in pathological conditions such as COVID-19 [11].

Materials and Methods

Whole blood and saliva samples were collected from apparently healthy Austrian donors with known ABO blood group. Saliva was donated spontaneously after abstaining from food, liquid, or chewing gum for at least 1 h. Saliva specimens were immediately centrifuged (10 min with 15,000 *g* at 4°C) to remove cells and debris. Sera and salivary supernatants were frozen at −20°C until analysis.

Flow cytometric analysis of ABO antibodies in saliva was prepared by incubating 50 µL of saliva containing 0.5 µL protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA) with 50 µL of 0.05% of red blood cells (RBCs; A1, B, and O, diluted in PBS buffer, all serologically typed C-D-E−, K-). After incubation at 20°C for 30 min and washing, 25 µL of saturating secondary antibodies (Alexa Fluor™ 488 F(ab')$_2$ goat anti-human IgA α chain-specific 1:100, R-PE fab fragment goat anti-human IgM $Fc_{5\mu}$-specific 1:50, Alexa Fluor™ 647 fab fragment goat anti-human IgG Fc_{γ}-specific 1:50; Jackson Immuno Research, West Grove, PA, USA) were added. After incubation (30 min at 4°C), washing, and resuspension, RBCs were aspirated twice through a 25-gauge needle to disaggregate potential RBC agglutinates. Detection of ABO antibodies in serum was prepared by the incubation of 25 µL of serum and 25 µL of 0.1% RBCs (previously treated with Karnovsky's Fixative) [12] under otherwise the same conditions as saliva samples. Flow cytometry was performed on a FACS Canto II using FACS Diva software (BD Biosciences, Heidelberg, Germany). Single RBCs were gated by FSC-A versus FSC-H. Median fluorescence intensities (MFIs) were measured in the FITC, PE, and APC channels. Per batch, one group O and one group AB sample served as positive and negative controls, respectively. MFI ratios (MFIR) were calculated by MFI sample of interest/MFI of an AB-sample. Based on the MFIR of all samples, the cut-off for positive results was set at the highest MFIR for each antibody class obtained with O RBCs (online Suppl. Table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000527233).

Serum and saliva samples (the latter equally pretreated with protease inhibitor cocktail, as mentioned above) were additionally tested using hemagglutination-based neutral (NaCl) and indirect anti-human globulin test (IAT) gel cards (MicroTyping system, Bio-Rad, Vienna, Austria) at 20°C and 37°C, respectively, according to manufacturer instructions for serum testing. Results were expressed as agglutination scores (negative, borderline +/−, 1+ to 4+).

Performance of flow cytometry was assessed using a high-titer anti-A/anti-B WHO reference reagent (National Institute for Biological Standards and Control, Hertfordshire, England, UK) in a 1-in-2 dilution series (diluted with AB-serum and AB-saliva pools

Table 1. Number of saliva and serum samples according to their ABO-specific reactivities by flow cytometry

Sample material	Participant's blood group	Samples with ABO type-congruent reactivity in any Ig class (%)	Samples with ABO type-incongruent reactivity in any Ig class (%)[a]	Ig class	anti-A		anti-B		Total
					neg (%)	pos (%)	neg (%)	pos (%)	
Saliva	0	21 (100) anti-A or -B	0 (0)	IgA	1 (5)	20 (95)	0 (0)	21 (100)	21
				IgM	5 (24)	16 (76)	6 (29)	15 (71)	
				IgG	10 (48)	11 (52)	8 (38)	13 (62)	
	A	25 (100) anti-B	1 (4) anti-A	IgA	25 (100)	0 (0)	0 (0)	25 (100)	25
				IgM	25 (100)	0 (0)	4 (16)	21 (84)	
				IgG	24 (96)	1 (4)	22 (88)	3 (12)	
	B	19 (100) anti-A	2 (10) anti-B	IgA	1 (5)	18 (95)	18 (95)	1 (5)	19
				IgM	8 (42)	11 (58)	19 (100)	0 (0)	
				IgG	14 (74)	5 (26)	18 (95)	1 (5)	
	AB	13 (93) negative	1 (7) anti-A/-B	IgA	13 (93)	1 (7)	13 (93)	1 (7)	14
				IgM	14 (100)	0 (0)	14 (100)	0 (0)	
				IgG	14 (100)	0 (0)	14 (100)	0 (0)	
Serum	0	21 (100) anti-A or -B	0 (0)	IgA	2 (10)	19 (90)	0 (0)	21 (100)	21
				IgM	3 (14)	18 (86)	3 (14)	18 (86)	
				IgG	0 (0)	21 (100)	6 (29)	15 (71)	
	A	24 (100) anti-B	0 (0) anti-A	IgA	24 (100)	0 (0)	7 (29)	17 (71)	24
				IgM	24 (100)	0 (0)	2 (8)	22 (92)	
				IgG	24 (100)	0 (0)	23 (96)	1 (4)	
	B	17 (100) anti-A	0 (0) anti-B	IgA	7 (41)	10 (59)	17 (100)	0 (0)	17
				IgM	0 (0)	17 (100)	17 (100)	0 (0)	
				IgG	14 (82)	3 (18)	17 (100)	0 (0)	
	AB	16 (0) negative	0 (0)	IgA	16 (100)	0 (0)	16 (100)	0 (0)	16
				IgM	16 (100)	0 (0)	16 (100)	0 (0)	
				IgG	16 (100)	0 (0)	16 (100)	0 (0)	

[a] All four saliva samples with incongruent results were negative in the respective routine gel card assays.

as well as PBS; 5 replicates each). For these experiments, only saliva from non-secretors was employed to ensure the absence of soluble blood group substances; non-secretor status was ascertained using the Wiener agglutination inhibition test [13]. Precision, dynamic range, and limit of detection were defined as described elsewhere [14, 15]. Data management and statistical analyses were performed using R Version 4.1.0.

Results

Samples were obtained from 88 participants (44 females and 44 males; group O, $n = 21$; A, $n = 25$; B, $n = 19$; AB, $n = 23$). The median age was 46 years, ranging between 19 and 69 years. Blood for serum testing was not available from one A and two B participants. Of 23 AB participants, 7 donated both saliva and serum; further, 7 and 9 donated only saliva and serum, respectively.

Results of flow cytometry are presented in Figure 1; Table 1, as well as online Supplementary Figure 1. All 65 non-AB-saliva samples had detectable ABO antibodies of any Ig class. The predominant ABO antibody class was IgA, detected in all O-, A-, and 18/19 (95%) B-saliva samples, followed by IgM, which was consistently detectable in O-, A-, and in a lower proportion in B-saliva samples. In comparison, the variability of IgG in saliva was very limited in all blood types (see online Suppl. Table 2). Of 62 non-AB sera, 61 (98%) showed positive reactions in any Ig class. All antibody classes were clearly detected in the sera of group O participants. IgA and IgM were consistently detected in A and B sera, with a higher proportion of positive IgM versus IgA (group A/anti-B: 22/24, 92% positive IgM vs. 17/24, 71% positive IgA; group B/anti-A: 17/17, 100% positive IgM vs. 10/17, 59% positive IgA). The proportions of positive IgM versus IgA differed significantly in group B (anti-A, $p = 0.007$) but not in group A (anti-B, $p = 0.137$). IgG was rarely observed in A and B sera. All AB-serum and 13/14 AB-saliva samples showed no anti-ABO reactivity. Samples of group O individuals yielded the highest variability of ABO-specific antibody levels.

The Spearman correlation revealed an association between IgA ABO antibodies in the saliva and serum of A and B donors ($\rho = 0.76$, $p < 0.001$ for anti-B in group A; $\rho = 0.56$, $p = 0.02$ for anti-A in group B, respectively, see online Suppl. Table 3). There was a significant association between IgM anti-B ($\rho = 0.51$, $p = 0.019$) and IgG anti-A

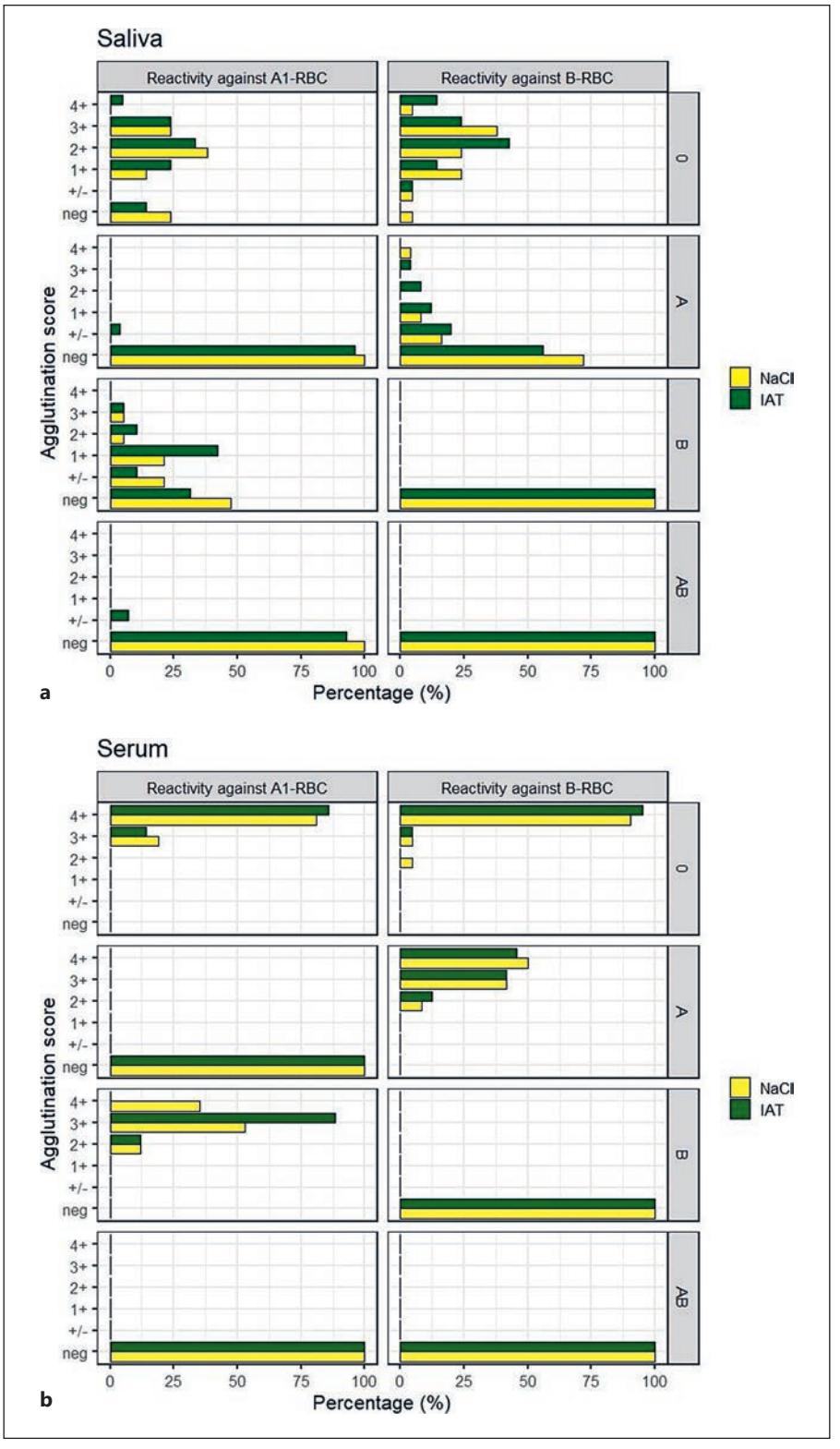

Fig. 2. a ABO-specific reactivities of saliva samples determined by gel cards. One A- and one AB-saliva sample showed borderline reactivities with A1 RBCs, most probably due to mucin clots. Both were negative in flow cytometry. **b** ABO-specific reactivities of serum samples determined by gel cards.

($\rho = 0.62$, $p = 0.003$) in the saliva and serum of O donors. There were no significant sex and age-group differences in anti-A/anti-B MFIRs in both serum and saliva.

Saliva and serum test results in gel cards (NaCl and IAT) are shown in Figure 2a, b and online Supplementary Table 4. In brief, all group O-saliva samples were reactive with A and/or B RBCs, whereas only 6/19 (32%; NaCl) and 11/19 (58%; IAT) group B-saliva samples had detectable anti-A. Positivity rates for group A were even lower (anti-B in NaCl: 3/25, 12%; IAT: 6/25, 24%). No saliva sample reacted with O RBCs, whereas one A and one AB saliva appeared borderline reactive with A RBCs,

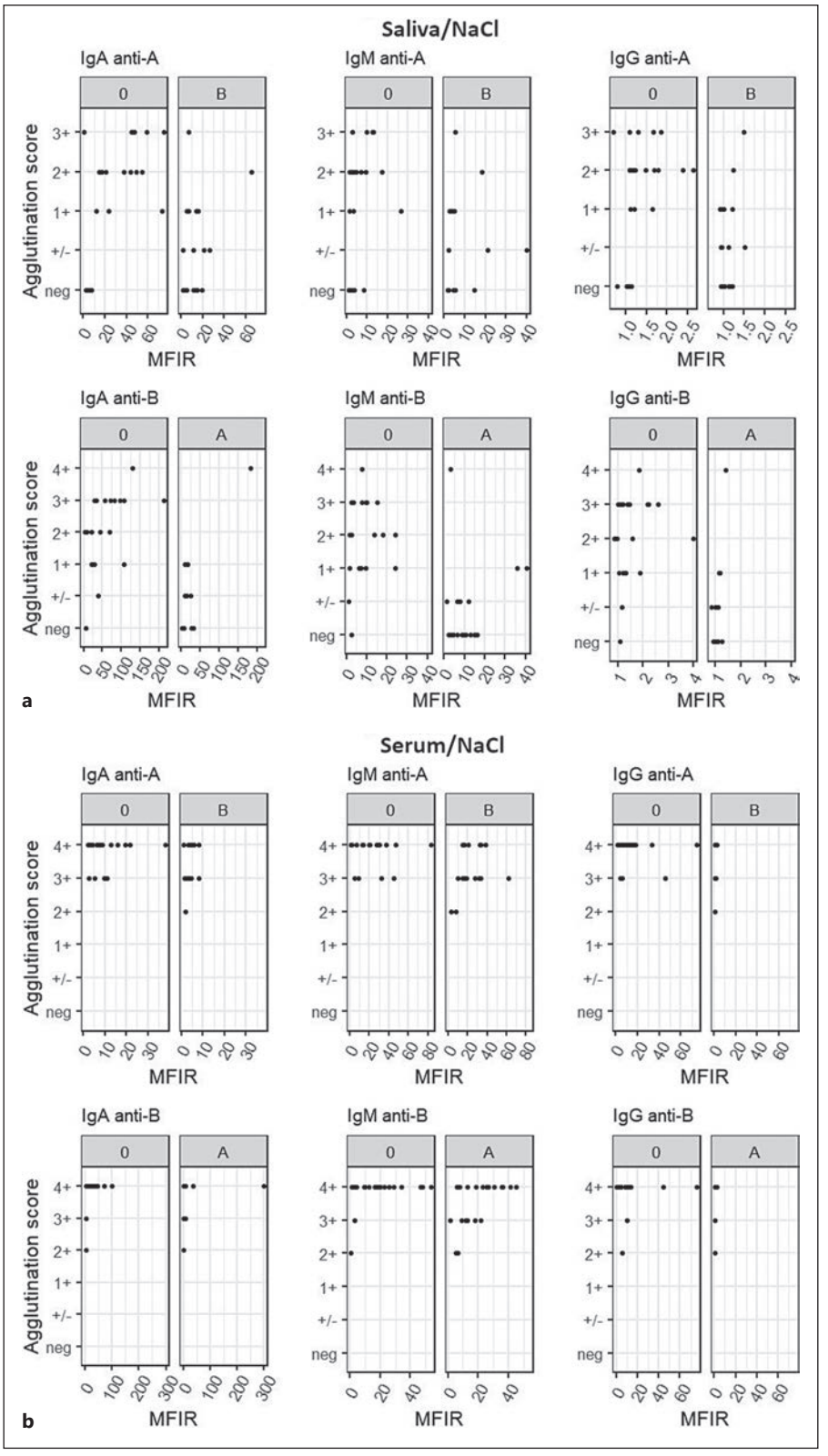

Fig. 3. a Relation between results of NaCl gel cards (y-axis) and anti-A and anti-B MFIR in IgA, IgM, and IgG (x-axis) in saliva. Plots are displayed separately by relevant blood types. **b** Relation between results of NaCl gel cards (y-axis) and anti-A and anti-B MFIR in IgA, IgM, and IgG (x-axis) in serum. Plots are displayed separately by relevant blood types.

most probably due to mucin clots. All sera (100%) reacted according to their blood group in both NaCl and IAT, with the majority of non-AB sera being 4+ or 3+ reactive. The agglutination scores were not consistently associated with MFIR of flow cytometry, reflecting the fact that agglutination was based on combined ABO reactivity of different Ig classes (see Fig. 3, 4a, b).

The high-titer anti-A/anti-B WHO reference reagent showed strong IgM (MFIR anti-A = 95.4, anti-B = 92.1) and moderate IgG levels (MFIR anti-A = 20.3, anti-B =

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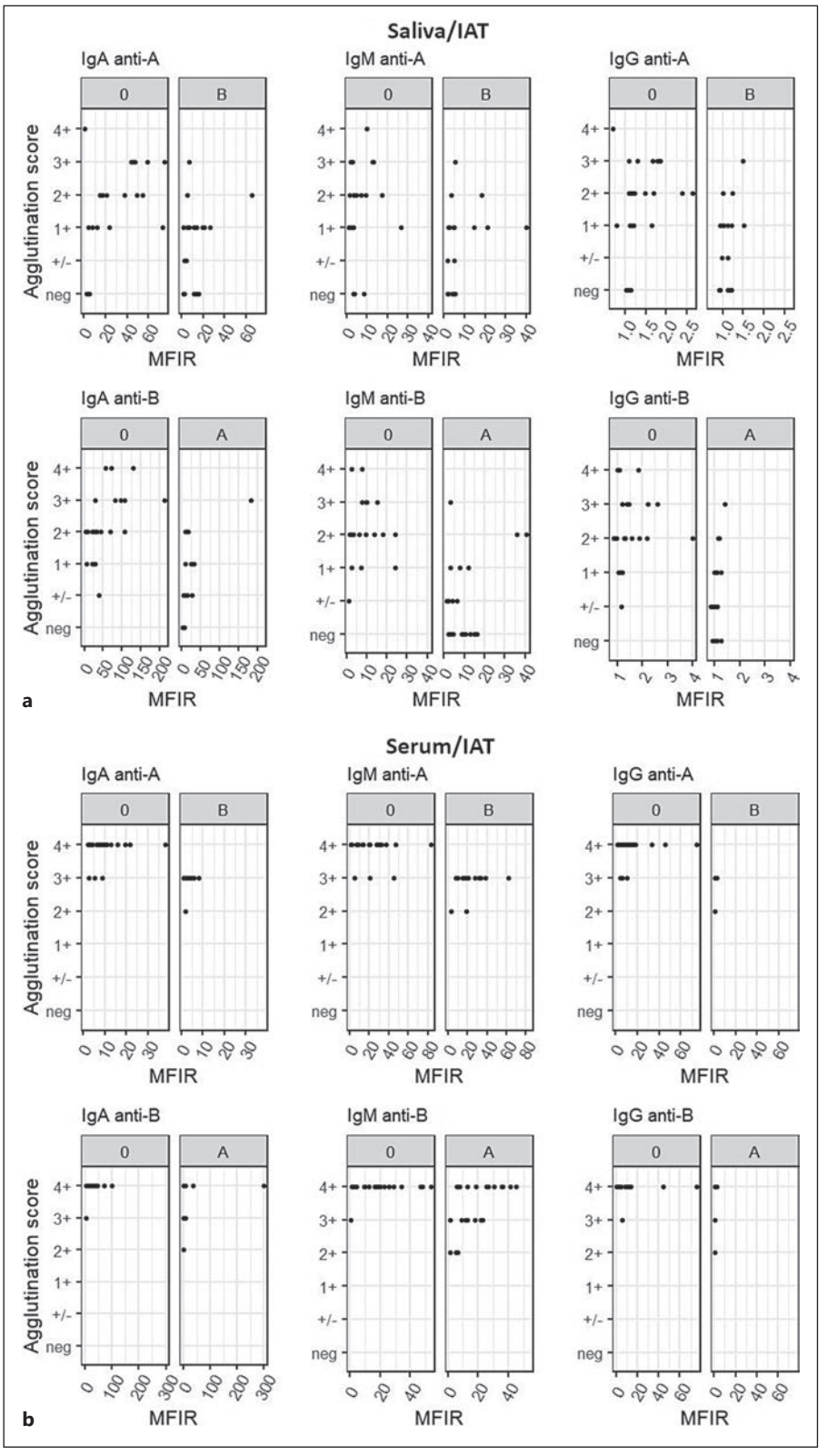

Fig. 4. a Relation between results of IAT gel cards (y-axis) and anti-A and anti-B MFIR in IgA, IgM, and IgG (x-axis) in saliva. Plots are displayed separately by relevant blood types. **b** Relation between results of IAT gel cards (y-axis) and anti-A and anti-B MFIR in IgA, IgM, and IgG (x-axis) in serum. Plots are displayed separately by relevant blood types.

8.4) in flow cytometry. In addition, considerable amounts of IgA (MFIR anti-A = 15.4, anti-B = 41.7) were also detected. Titration experiments using three different diluents (AB-serum and -saliva pools, as well as PBS) revealed that the AB-serum pool had an inhibitory effect on anti-A and -B reactivities in both flow cytometry and hemagglutination as compared to the AB-saliva pool and PBS. Precision defined as relative standard deviation (% RSD) over the 5 replicates and averaged across dilution steps varied between 1.6 and

Table 2. Flow cytometric performance assessment determined by dilution of high titer anti-A/anti-B WHO standard with three different diluents in 5 replicates

Diluent	Ig class	A1 RBC					B RBC				
		flow cytometry				gel card titer NaCl/IAT	flow cytometry				gel card titer NaCl/IAT
		precision (mean % RSD)	DR	LOB	LOD (titer)		precision (mean % RSD)	DR	LOB	LOD (titer)	
AB-Serum Pool	IgA	2.8	15.4	94.9	111.5 (16)	16/128	2.2	41.7	111.1	114.3 (64)	16/128
	IgM	7.4	95.4	93.6	128.7 (64)		4.6	92.1	89.8	92.0 (128)	
	IgG	8.9	20.3	56.4	66.9 (64)		5.2	8.4	103.3	106.9 (2)	
PBS	IgA	2.8	23.4	71.8	74.0 (256)	128/256	2.1	59.0	86.6	90.4 (512)	128/256
	IgM	7.9	144.8	63.8	70.5 (512)		5.5	133.8	63.0	69.5 (512)	
	IgG	6.5	100.9	22.2	24.0 (512)		4.1	44.0	27.3	29.6 (512)	
AB-Saliva Pool	IgA	1.6	15.4	93.3	94.7 (256)	64/256	1.9	41.6	112.3	114.3 (128)	32/128
	IgM	3.8	113.8	60.6	62.5 (1,024)		3.6	108.0	63.3	64.8 (1,024)	
	IgG	6.1	69.0	18.5	20.0 (1,024)		5.4	29.4	28.1	29.0 (512)	

Parameters were defined according to Armbruster 2008 [13] and Wood 2013 [14]. RSD, relative standard deviation; DR, dynamic range (ratio between largest (non-diluted)/smallest (blank) fluorescent signal); LOB, limit of blank (Mean blank + 1.645*SD); LOD, limit of detection (=LOB + 1.645*SD highest dilution).

8.9%. The broadest and narrowest dynamic ranges were obtained by dilution with PBS (up to ratio 144.8 for A1-specific IgM) and AB-serum pool, respectively (Table 2). Regression analyses (online Suppl. Fig. 2AB) revealed a better linear fit (R^2 = 0.89–1.0) for A1 RBCs than for B RBCs (R^2 = 0.66–1.0).

Using gel cards, the WHO reference reagent showed anti-A/anti-B reactivity according to the manufacturer's instructions when diluted with PBS (1:128 in NaCl, 1:256 in IAT). Diluted with the AB-serum pool, anti-A/anti-B titers were 1:16 and 1:128 in NaCl and IAT, respectively. Dilution in AB-saliva pool revealed an anti-A titer of 1:64 and 1:256 and an anti-B titer of 1:32 and 1:128 in NaCl and IAT (Table 2).

Discussion

In this study, a flow cytometric method for separate IgA, IgM, and IgG class-specific ABO antibody detection in saliva was devised. Investigation of a cohort of healthy individuals revealed that IgA was the most abundant ABO antibody class in saliva, followed by IgM and IgG. Total IgA serves as major immunological defense on mucous membranes and is produced locally, similar to IgM. In contrast, as total salivary IgG is mostly serum-derived, it should mirror serum levels, which, however, was not the case for ABO antibodies [6]. All group O and only few A and B individuals had ABO-specific IgG antibodies in serum. In saliva, comparatively fewer group O samples than group B and A samples demonstrated IgG (76%, 26%, and 12%, respectively).

In serum, IgM was the most abundant ABO antibody class, followed by IgA and IgG, the latter mainly detected in group O individuals. Surprisingly, IgA was detected in similar levels to IgG in group O and predominantly to IgG in group A and B individuals. Hence, the traditional division of ABO antibodies into IgM and IgG should be reconsidered in light of the significant presence of IgA. Regardless of sample material, major interindividual differences in ABO antibody reactivities were recorded. With the applied cut-off level strategy, serum testing yielded no unspecific/false-positive reactions at all, and only few unexpected reactivities in saliva. Using O RBCs for the cut-off definition seemed adequate as they do not carry ABO antigens and the respective antibodies were not expected to bind. This cut-off definition leads to a low-level threshold of positivity and should therefore be interpreted with caution.

Saliva represents a poorly studied sample material for ABO antibody detection, as all previous studies were hemagglutination-based [7–9, 16]. Compared to serum, saliva is a heterogenic and unsterile body fluid influenced by several factors like viscosity, composition (water, enzymes, mucins, and antibodies), and microbiome [10]. Standardized sampling circumstances are essential to maintain the integrity of potential biomarkers [17]. A major challenge in saliva processing is its high proteolytic activity that has been overcome by use the of protease inhibitors [10]. Previous experiments had shown that

protease inhibitors exerted only minimal reductions of MFIR (<10%) and no effect on hemagglutination scores in gel cards. Moreover, varying mucin concentrations in saliva specimens may increase the possibility of evaluation errors. ABO antibody levels in saliva might be prone to volatility dependent on fluid balance. Serum levels of ABO antibodies remain stable over time in individuals but demonstrate a large heterogeneity within donor populations [18–20].

Comparison of flow cytometry with the standard hemagglutination technique reflected by gel card testing was additionally performed by dilution experiments using a high-titer anti-A/anti-B WHO reference reagent that has been previously used by Sprogøe et al. [21] to benchmark flow cytometric anti-A IgM and IgG measurements. In that study, flow cytometric results were reported as absolute entities (molecules of equivalent fluorochrome) by relating sample MFI to a standard curve obtained by calibration beads. This methodological setup is quite different from ours, making direct comparison difficult. Our method enables calculating MFIR based on AB-samples serving as negative controls, allowing for evaluating the relative antibody content. This approach has proven efficient for judging different clinical conditions, such as the influence of infections on ABO antibodies or ABO-incompatible transfusion and transplantation [11, 12, 19, 20].

WHO reference reagent titration experiments using three different diluents (AB-serum and -saliva pools as well as PBS) revealed that AB sera had an inhibitory effect on anti-A and anti-B reactivities in both flow cytometry and hemagglutination. We assume that this inhibition was caused by soluble A and B substances in AB sera, which competitively bind anti-A and anti-B of the WHO standard. These blood group substances are common in the serum of both secretors and non-secretors and in other body fluids of secretors [22, 23]. Therefore, care was taken to select exclusively AB-saliva samples of non-secretors for pooling.

Standard serological techniques are based on RBC agglutination and fail to clearly discriminate between Ig classes. It is a common misconception that direct agglutination in neutral gel cards is solely brought about by IgM. In fact, the combined action of different Ig classes (not only IgM but also IgG as well as IgA) will, in concert, lead to direct agglutination. The same holds true for IAT (Coombs card milieu) where not only anti-human globulin-driven agglutination (IgG) but also direct agglutination via IgM and IgA can be observed. For these reasons, a direct comparison of Ig class-specific flow cytometric MFIR and mixed Ig class hemagglutination titers may be misleading (see Fig. 3, 4a, b).

Regarding test performance, flow cytometry supersedes standard semiquantitative hemagglutination techniques by measuring all cell-bound ABO antibodies regardless of their agglutination potential. Preferably, IgM-induced RBC agglutination is prevented by the use of fixed RBCs for serum testing and needle aspiration prior to analysis, both commonly used to obtain single RBCs for flow cytometry without affecting antibody binding [12, 19, 24–26]. Moreover, the use of Ig class-specific secondary antibodies allows for differentiation of ABO antibodies into IgA, IgM, and IgG, including IgG subclasses [19, 20]. Numerical values enable objective and quantitative results. Despite its high-throughput ability, flow cytometry needs more processing time (due to several incubation and washing steps, instrument setting, analysis, and evaluation) compared to standard hemagglutination techniques, which additionally can also be performed and evaluated manually. Both techniques feature comparable costs for reagents and consumables.

Another possibility to quantitatively measure ABO antibodies is by ELISA, which works with the use of synthetic or animal ABO glycans coated onto a microplate surface [27–29]. Anti-A/anti-B antibodies in sera bind these glycans and are detected by enzyme-labeled anti-human IgM or IgG. ELISA techniques are less elaborate than flow cytometry but exhibit an incomplete antigenic structure due to the non-human origin of ABO glycans and the potential conformation loss during the coating procedure. As complete human ABO on intact RBCs could not ideally be reflected by this assay, test sensitivity and specificity has shown to be decreased in comparison to flow cytometry and standard hemagglutination techniques [19].

Detection of salivary ABO antibodies provides previously unknown aspects of mucosal immunity that are possibly important in the current COVID-19 pandemic [11]. So far, no data were available on salivary ABO antibody classes or the association of these antibodies between saliva and serum. Further studies are required to determine the potential mutual influence of infections and mucosal ABO antibody class profiles.

Statement of Ethics

This study was approved by the Ethics Committee of the Medical University of Vienna (EK Nr. 1248/2021). All study participants gave written informed consent.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Marlies Schönbacher established and performed the analyses and wrote the manuscript. Chiara Banfi and Andrea Berghold calculated the statistics. Eva Matzhold and Thomas Wagner discussed the results and contributed to the final version of the manuscript. Wolfgang Mayr provided helpful advice in interpreting the data. Günther F. Körmöczi designed and supervised the study and wrote the manuscript. All the authors critically revised the manuscript.

Data Availability Statement

Further data are available by request after approval granted by the Ethics Committee of the Medical University of Vienna.

References

1 Liu N, Zhang T, Ma L, Zhang H, Wang H, Wei W, et al. The impact of ABO blood group on COVID-19 infection risk and mortality: a systematic review and meta-analysis. Blood Rev. 2021;48:100785.
2 Matzhold EM, Berghold A, Bemelmans MKB, Banfi C, Stelzl E, Kessler HH, et al. Lewis and ABO histo-blood types and the secretor status of patients hospitalized with COVID-19 implicate a role for ABO antibodies in susceptibility to infection with SARS-CoV-2. Transfusion. 2021;61(9):2736–45.
3 Gutiérrez-Valencia M, Leache L, Librero J, Jericó C, Enguita Germán M, García-Erce JA. ABO blood group and risk of COVID-19 infection and complications: a systematic review and meta-analysis. Transfusion. 2022;62(2):493–505.
4 Gérard C, Maggipinto G, Minon JM. COVID-19 and ABO blood group: another viewpoint. Br J Haematol. 2020;190(2):e93–4.
5 Deleers M, Breiman A, Daubie V, Maggetto C, Barreau I, Besse T, et al. Covid-19 and blood groups: ABO antibody levels may also matter. Int J Infect Dis. 2021;104:242–9.
6 Brandtzaeg P. Secretory immunity with special reference to the oral cavity. J Oral Microbiol. 2013;5(1):20401.
7 Bell CE, jr, Fortwengler HP. Salivary anti-A and anti-B activity of group O males. Vox sanguinis. 1971;21(6):493–508.
8 Saneshige Y, Woodfield DG. Detection of ABO isoagglutinins in saliva using toluene. J Forensic Sci Soc. 1984;24(5):489–93.
9 Joshi SR, Vasantha K, Iyer YS, Kulkarni S, Jadhav S. Studies on the ABH-Iso-Agglutinins in serum, saliva and milk from mothers with "Bombay" (Oh) phenotype. Asian J Transfus Sci. 2009;3(1):3–5.
10 Thomadaki K, Helmerhorst EJ, Tian N, Sun X, Siqueira WL, Walt DR, et al. Whole-saliva proteolysis and its impact on salivary diagnostics. J Dent Res. 2011;90(11):1325–30.
11 Matzhold EM, Körmöczi GF, Banfi C, Schönbacher M, Drexler-Helmberg C, Steinmetz I, et al. Lower levels of ABO anti-A and anti-B of IgM, IgG and IgA isotypes in the serum but not the saliva of COVID-19 convalescents. J Clin Med. 2022;11(15):4513.
12 Wahrmann M, Schiemann M, Marinova L, Körmöczi GF, Derfler K, Fehr T, et al. Anti-A/B antibody depletion by semiselective versus ABO blood group-specific immunoadsorption. Nephrol Dial Transplan. 2012;27(5):2122–9.
13 Zhang F, Yin S, Fan Y, Song T, Huang Z, Liang J, et al. Effect of donor and recipient ABH-secretor status on ABO-incompatible living donor kidney transplantation. Front Immunol. 2021:671185.
14 Armbruster DA, Pry T. Limit of blank, limit of detection and limit of quantitation. Clin Biochem Rev. 2008;29(Suppl 1):S49–52.
15 Wood B, Jevremovic D, Béné MC, Yan M, Jacobs P, Litwin V, et al. Validation of cell-based fluorescence assays: practice guidelines from the ICSH and ICCS – part V – assay performance criteria. Cytometry B Clin Cytom. 2013;84(5):315–23.
16 Adinolfi M, Mollison PL, Polley MJ, Rose JM. Gamma-A-blood group antibodies. J Exp Med. 1966;123(5):951–67.
17 Hettegger P, Huber J, Paßecker K, Soldo R, Kegler U, Nöhammer C, et al. High similarity of IgG antibody profiles in blood and saliva opens opportunities for saliva based serology. PloS one. 2019;14(6):e0218456.
18 Sprogøe U, Yazer MH, Rasmussen MH, Antonsen B, Bistrup C, Assing K. Minimal variation in anti-A and -B titers among healthy volunteers over time: implications for the use of out-of-group blood components. J Trauma Acute Care Surg. 2017;82(6S Suppl 1):S87–90.
19 Stussi G, Huggel K, Lutz HU, Schanz U, Rieben R, Seebach JD. Isotype-specific detection of ABO blood group antibodies using a novel flow cytometric method. Br J Haematol. 2005;130(6):954–63.
20 Daga S, Hussain S, Krishnan N, Lowe D, Braitch M, Patel P, et al. Immunoglobulin isotype compositions of ABO specific antibodies are dependent on the individual patient blood group and blood group specificity: results from a healthy donor cohort. J Immunol Methods. 2021;494:113053.
21 Sprogøe U, Assing K, Nielsen C, Rasmussen MH, Yazer MH. Quantification of anti-A of IgM or IgG isotype using three different methodologies. Transfusion. 2021;61(Suppl 1):S214–22.
22 Watkins WM. Blood-group substances. Science. 1966;152(3719):172–81.
23 Høstrup H. A and B blood group substances in the serum of normal subjects. Vox sanguinis. 1962;7:704–21.
24 Flegel WA, Curin-Serbec V, Delamaire M, Donvito B, Ikeda H, Jorgensen J, et al. Section 1B: Rh flow cytometry. Coordinator's report. Rhesus index and antigen density: an analysis of the reproducibility of flow cytometric determination. Transfus Clin Biol. 2002;9(1):33–42.
25 Roback JD, Barclay S, Hillyer CD. An automatable format for accurate immunohematology testing by flow cytometry. Transfusion. 2003;43(7):918–27.
26 Pins MR, Saidman SL, Cosimi AB, Jennings LD, Stowell CP. Accelerated acute rejection of an apparent A2 renal allograft in an O recipient: report of a case with flow cytometric analysis. Transplantation. 1997;63(7):984–8.
27 Buchs JP, Nydegger UE. Development of an ABO-ELISA for the quantitation of human blood group anti-A and anti-B IgM and IgG antibodies. J Immunol methods. 1989;118(1):37–46.
28 Rieben R, Buchs JP, Flückiger E, Nydegger UE. Antibodies to histo-blood group substances A and B: agglutination titers, Ig class, and IgG subclasses in healthy persons of different age categories. Transfusion. 1991;31(7):607–15.
29 Satoh A, Kawagishi N, Minegishi M, Takahashi H, Akamatsu Y, Doi H, et al. Development of a novel ELISA for detection of anti-A and anti-B antibodies in recipients of ABO-incompatible living donor liver grafts. Tohoku J Exp Med. 2007;211(4):359–67.

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Non-Invasive Zinc Protoporphyrin Screening Offers Opportunities for Secondary Prevention of Iron Deficiency in Blood Donors

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Abstract

Background: Frequent blood donors are at high risk of developing iron deficiency. Currently, there is no potent screening during blood donation to detect iron deficient erythropoiesis (IDE) before anemia develops and deferral from donation is inevitable. ***Study Design and Methods:*** In addition to capillary and venous hemoglobin, the iron status of 99 frequent blood donors was assessed by various venous blood parameters and zinc protoporphyrin IX (ZnPP). ZnPP was determined by high-performance liquid chromatography (HPLC) and a new prototype fiber-optic device was employed for non-invasive measurements of ZnPP through the blood collection tubing (NI-tubing) and on lip tissue (NI-lip). We aimed to evaluate the feasibility and diagnostic value of the NI-tubing measurement for early detection of severe iron deficiency in blood donors. ***Results:*** NI-tubing and HPLC reference measurements of ZnPP showed narrow limits of agreement of 12.2 µmol ZnPP/mol heme and very high correlation (Spearman's Rho = 0.938). Using a cutoff of 65 µmol ZnPP/mol heme, NI-tubing measurements ($n = 93$) identified 100% of donors with iron deficiency anemia (IDA) and an additional 38% of donors with IDE. Accordingly, NI-tubing measurements would allow detection and selective protection of particularly vulnerable donors. ***Conclusion:*** NI-tubing measurements are an accurate and simple method to implement ZnPP determination into the routine blood donation process. ZnPP was able to identify the majority of subjects with IDE and IDA and might therefore be a valuable tool to provide qualified information to donors about dietary measures and adjustments of the donation interval and thereby help to prevent IDA and hemoglobin deferral in the future.

Introduction

Blood donation is mostly organized by nonprofit health or social welfare organizations, such as the Red Cross. Worldwide, over one hundred million units of blood are donated each year [1]. Among all blood donors, the vast majority donates blood regularly. For example, the Bavarian Red Cross (BRK) recruits 88% of blood donations from repeat donors. In Germany, male donors are allowed to donate up to 6 times a year, female donors up to 4 times [2]. Each donation draws 500 mL of whole blood, including 200–250 mg of iron. Taking into account that iron stores in men are 500–1,000 mg and 200–400 mg in premenopausal women, each blood donation depletes the iron stores considerably [3], also in comparison to the usual daily loss of about 1 mg [4]. As only a maximum of 7 mg of iron – if ferritin levels are already

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very low – can be absorbed per day from the diet, frequent donors are at a considerable risk of gradually depleting their iron stores [5]. When laboratory tests were made in research studies among regular blood donors, iron deficiency was found in 59% of cases in a US study (log [soluble transferrin receptor/ferritin] >2.07) [6] and in up to 27.4% (men with hepcidin <0.5 nmol/L) in a Danish study [7]. Advanced iron deficiency can lead to symptoms such as headaches, difficulty concentrating, depressive symptoms, and shortness of breath. Persistent iron deficiency eventually leads to iron deficiency anemia (IDA). Only in this case, the hemoglobin (Hb) value drops below predefined eligibility thresholds and donors are deferred from donation on the basis of a pre-donation Hb measurement. The corresponding deferral rates are in the order of a few percent [8]. Rejection from blood donation has been shown to reduce the likelihood of donating again [9, 10]. Thus, early detection and mitigation of iron deficiency is not only relevant for the personal health of the blood donor but could also help prevent on-site donor deferral and the subsequent loss of valuable blood donors. Unfortunately, there is so far no practical, quick and low-cost test, which could indicate the gradual development of iron deficiency at an early stage. All attempts made so far are restricted to study scenarios, and none of the investigated parameters and related devices have found their way into routine use. Blood tests, which indicate iron depletion or – probably more relevant – iron deficient erythropoiesis (IDE) comprise numerous parameters, such as ferritin, transferrin saturation (TSAT), soluble transferrin receptor (sTfR), and erythrocyte indices, which are usually measured and interpreted jointly as an appropriate criterion to conclude about the availability of iron for erythropoiesis. Ferritin is the most frequently studied parameter [11–15] and well accepted as an indicator of iron stores among otherwise healthy people, such as blood donors. However, there is still a lack of consensus with respect to the relevant threshold values used to indicate iron deficiency and to guide iron supplementation recommendations [12, 14]. In addition, possible symptoms induced by low iron stores alone, such as fatigue, restless legs syndrome, or impaired cognitive function, have to be balanced with possible side effects of regular iron supplementation: stomach cramps, constipation, or nausea may occur and may lead to a discontinuation of the iron treatment or – more severe – prolonged iron supplementation may even increase the risk for colon cancer [14, 16]. Hence, iron depletion as defined by low ferritin alone may not be the most relevant condition to look for when aiming to improve blood donor health and availability. It seems reasonable that only individuals with prolonged iron depletion that already impairs heme production during erythropoiesis – as is the case in IDE – will develop significant morbidity and be at risk for future anemia development. These donors should be the target for proactive mitigation measures such as iron supplementation or extension of the deferral period, even when anemia is not yet apparent.

Probably the best-accepted single parameter to indicate IDE is sTfR. The German Society for Hematology and Medical Oncology (DGHO) recommends the use of sTfR for the differential diagnosis of IDE as part of an algorithm for iron deficiency assessment [3]. The diagnostic potential of zinc protoporphyrin IX (ZnPP) for IDE diagnostics is also recognized by this society, with the lack of a leading standard for ZnPP determination cited as an obstacle to routine use [3]. In addition, the most commonly used method for determining ZnPP with a hematofluorometer only yields reliable results when using washed erythrocytes and needs skilled personnel to operate the device reproducibly [17, 18]. It is therefore difficult to integrate ZnPP determination into the blood donation workflow. These reasons may explain why ZnPP is currently not used in blood donor management, despite promising results demonstrating the capability of ZnPP to identify iron-deficient donors in earlier studies [8, 19–23]. The herein proposed approach to measure ZnPP overcomes the limitations of standard hematofluorometer measurements and may be easily integrated into the routine blood donation process.

In earlier work, we have demonstrated that with the use of a high dynamic range spectrometer and two-wavelength excitation, ZnPP fluorescence can be reliably extracted from very large background signals [24, 25]. In this way, ZnPP can be detected directly from whole blood without any prior blood processing. Moreover, the developed innovative device allows for completely non-invasive (NI) measurements of ZnPP through the red vermillion of the lower lip (NI-lip measurement) with a fiber-based applicator, delivers an immediate result and can be used at the point of care [25, 26]. Therefore, it might provide a standard of measurement in the future.

Given the versatility of the device, it appears well suited for integration into the blood donation logistics. To this end, a specific applicator was developed to be clipped onto the transparent hose of the blood collection tubing for ZnPP measurement (NI-tubing measurement). This applicator fixes the optical fiber perpendicularly onto the transparent hose so that light emitted from the fiber can probe the blood flowing through the tubing. The personnel just has to fix the applicator on the tubing (see Fig. 1) and start the measurement at the beginning of blood drawing. The result is available within a few seconds.

Based on the abovementioned medical needs the aims of the study were to:

- validate the NI-tubing measurement against high-performance liquid chromatography (HPLC) reference measurements of ZnPP.

- confirm the accuracy of NI-lip measurements of ZnPP as found in previous studies.
- evaluate the diagnostic value of ZnPP compared to the routine capillary Hb measurement and whether it might offer valuable benefit for blood donors and blood donation services.

Materials and Methods

Study Design

This single-armed, multicenter study was conducted at a series of mobile blood donation events run by the blood donation service of the BRK. Between August and December 2017, 100 frequent blood donors (age ≥18 years) with more than three (women) and four (men) donations in the past 12 months were included at eleven mobile blood donation events organized by the BRK district associations of Munich and Augsburg. Exclusion criteria were blood transfusions in the preceding year (<12 months prior to examination), recent iron replacement therapy (<3 months prior to examination), diagnosis of hemochromatosis and refusal from blood donation due to reasons other than low Hb levels. Written informed consent was obtained from each participant. The study is registered at the German clinical trials register as DRKS00013102 and was approved by the Ethics Committee at the Ludwig Maximilian University of Munich, approval number 17-021.

Blood Sample Collection and Laboratory Analysis

At the mobile blood donation events, each blood donor was routinely screened for low capillary Hb levels. This was done by lancet-puncture of the earlobe (n = 90) or fingertip (n = 10) at the point of care using a Hb reader (CompoLab TM, Fresenius Kabi AG, Bad Homburg, Germany). In addition, venous blood was drawn to assess the iron status. If admitted to blood donation, venous blood samples were collected from the predonation sampling bag of the blood collection system in addition to routine blood sampling by the blood donation service. In case of rejection from blood donation, venous puncture and blood drawing were performed explicitly for the purpose of the study. The venous parameters Hb, serum ferritin, TSAT, sTfR, mean corpuscular Hb, and mean corpuscular volume were determined by the Institute of Laboratory Medicine at the Ludwig Maximilian University of Munich using clinical chemistry analyzers for venous Hb, mean corpuscular Hb and mean corpuscular volume (XN 9000, Sysmex Corporation, Kobe, Japan) and for ferritin and TSAT (AU 5800, Beckman Coulter Corporation, Brea, USA). The sTfR values were determined by BN ProSpec with Dade Behring Assay (Siemens Healthineers AG, Erlangen, Germany). An aliquot of EDTA blood allowed measuring the ZnPP/heme ratio by HPLC as a reference to the executed NI ZnPP measurements. The HPLC measurements were performed by an independent laboratory (MVZ Labor PD Dr. Volkmann und Kollegen GbR, Karlsruhe, Germany) as described in Hennig et al. [25].

NI Measurement of the ZnPP/Heme Ratio

The NI ZnPP measurements were conducted by a team of five authorized examiners with two prototype devices (FID*screen*, FerroSens GmbH, Munich, Germany). The detailed working principle of the prototypes is described elsewhere [25].

In the present study, this measurement method was adapted to application through the tubing of the blood collection system during blood donation. During the study, blood collection systems from Haemonetics (Leukotrap WB, CPD/SAG-M, product code WBT438DCG, Boston, USA) with 70 mL CPD stabilizer for 500 mL blood collection were utilized. The measurement was carried out as follows. After venipuncture, the blood flow through the blood collection tubing enabled the filling of the blood collection bag. To attach the tubing applicator of the measuring device (Fig. 1), the section of the tubing between the puncture site and the blood collection bag was used. To exclude potential interference from environmental contaminants, the measuring site of the tubing was cleaned with alcohol-based disinfectant before insertion into the applicator. The applicator was designed to fix the tubing in a defined depression, so that the blood collection tubing made direct and reliable contact with the glass fiber inside. In order to avoid incorrect measurements due to ambient light, the applicator was magnetically sealed by an opaque plastic lid. The measurement was started after a continuous blood flow into the blood donation bag was ensured and repeated at two further tubing sections for each study participant. The final result was calculated from the mean value of the three measurements. Additionally, a scaling factor was calculated from the comparison of tubing measurements and HPLC results, allowing an offset to correct for disturbances from spectral signatures of the blood collection tubing.

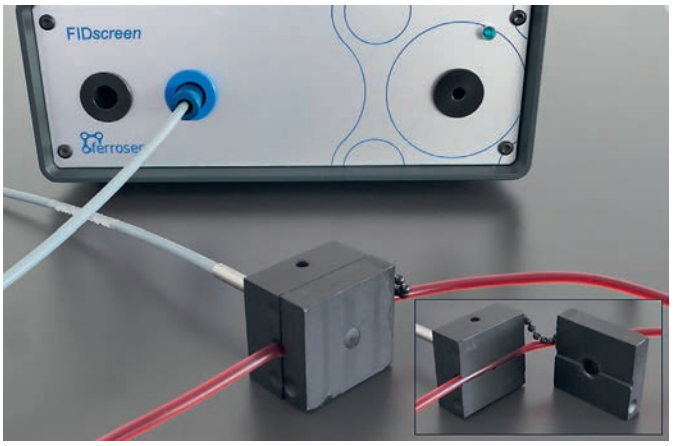

Fig. 1. Applicator for measurement of ZnPP fluorescence through the blood collection tubing. The inserted picture shows how the tubing is clicked into a groove in one part of the applicator, where the measurement fiber then is in contact with the tubing. The two halves of the applicator are fixed to each other by small magnets and shield the tubing from ambient light during measurement.

Study Procedure

If the inclusion and exclusion criteria were satisfied, the implementation of the study started with the NI-lip measurement. This was followed by capillary Hb determination. In case of confirmed eligibility for blood donation based on capillary Hb values of ≥13.5 g/dL (males) and ≥12.5 g/dL (females), the NI-tubing measurement was performed. In addition, venous blood samples were taken from the pre-donation sampling bag of the blood collection system. If the capillary Hb value was too low and the study participant was deferred from blood donation, a venipuncture was performed to obtain venous blood samples. The active study participation ended after the blood donation or after the collection of the venous blood sample.

Clinical Definitions

To enable a statement about the diagnostic performance of the NI measurements, the study population was categorized according

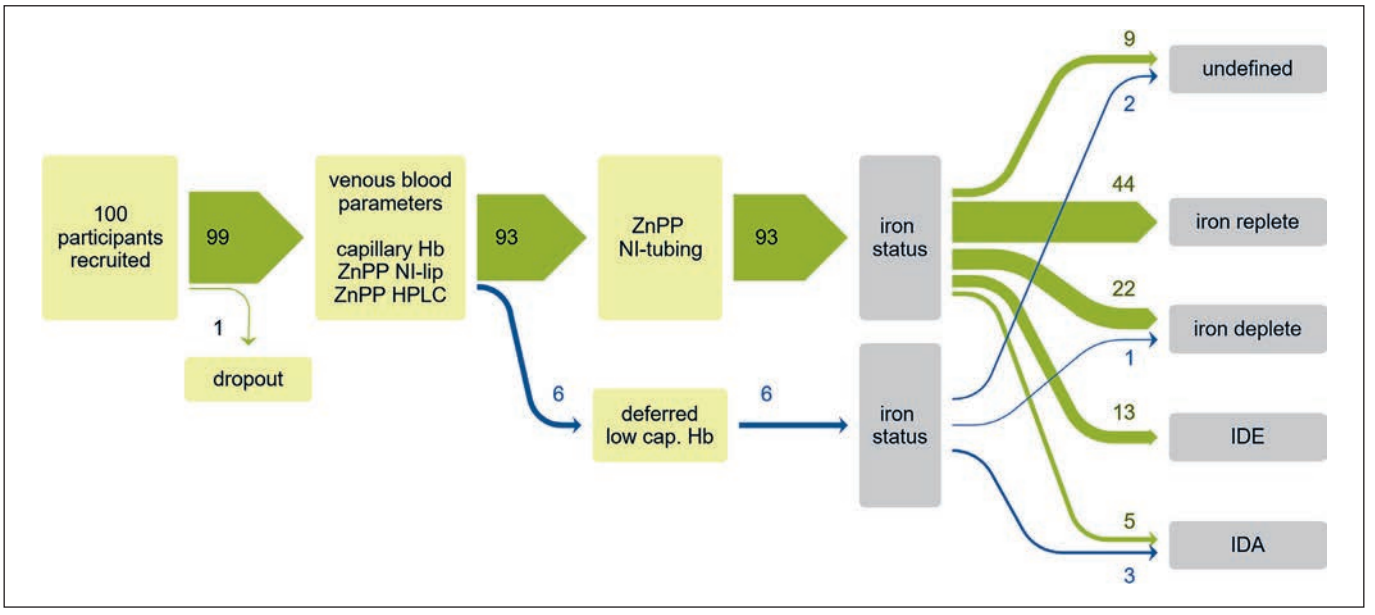

Fig. 2. Flowchart of study. From 100 recruited subjects, 1 dropped out prior to venous blood collection voluntarily. Venous blood parameters as well as capillary Hb and ZnPP measurements on the lip and by HPLC were determined from the remaining 99 subjects. 6 of the remaining 99 subjects were deferred from blood donation due to low capillary Hb and no NI-tubing measurement of ZnPP could be performed. The right side of the chart shows the assignment of the subjects to the applied iron deficiency stages.

to iron deficiency stages. The applied classification is commonly used in literature on iron deficiency diagnosis [27, 28] and is recommended by the DGHO [3]. For the purpose of this study, the iron deficiency stages are defined as follows:

- Iron depletion: reduction of ferritin levels (males <30 µg/L, females <15 µg/L).
- IDE: reduction of ferritin levels and increase of sTfR (>1.760 mg/L).
- IDA: reduction of ferritin levels, increase of sTfR, and drop of venous Hb (males <13 g/dL, females <12 g/dL).

As an alternative to ferritin and sTfR, the measurement of ZnPP also provides a means to detect IDE. According to the reference values of the MVZ Laboratories Volkmann, impairment of erythropoiesis can be ruled out below 40 µmol ZnPP/mol heme. In a previous study, we have identified a "grey zone" between 40 and 65 µmol ZnPP/mol heme with ZnPP levels >65 µmol ZnPP/mol heme being indicative of IDE with a high probability [29]. In the present study, a ZnPP cutoff of 65 µmol ZnPP/mol heme was primarily applied for IDE/IDA detection, but the cutoff of 40 µmol ZnPP/mol heme was tested likewise.

Statistical Analysis

For quantitative comparison of NI-tubing, NI-lip, and HPLC measurements of ZnPP, limits of agreement (LoA) were calculated using the robust τ-estimate of the differences between the results of each two methods according to Bland-Altman analysis [30]. The 95% confidence intervals were calculated by bootstrapping (software R, version 3.2.2, functions scaleTau2, boot, boot.ci). Since none of the data sets was normally distributed, Spearman's Rho correlation coefficient was calculated to assess the quality of the correlation (software IBM SPSS Statistics, version 24).

Results

Study Population

Initially, 100 study participants, who met the inclusion and exclusion criteria, were included in the study. One subject discontinued participation before blood sampling voluntarily. Of the remaining 99 participants, 40 were females and 59 males. The mean age was 46 years for female subjects (range: 18–70 years, median: 51 years) and 51 years for male subjects (range: 20–72 years, median: 52 years).

Prevalence of Iron Deficiency in the Study Population

The enrolled 99 subjects (100%) were classified into the iron deficiency stages mentioned above according to their venous blood values. 11 subjects did not meet the predefined criteria for assignment to one of the iron deficiency stages and were therefore classified under "undefined iron status". Under these conditions, the prevalence of iron deficiency in the study population was 44% of which 23% had only depleted iron stores, 13% had IDE and 8% IDA (see Fig. 2).

Agreement between Fiber-Optic ZnPP Measurements and HPLC Reference

A Spearman Rho correlation coefficient of 0.938 ($p < 0.001$) showed significant correlation between the results of the NI-tubing and the HPLC measurement (Fig. 3a).

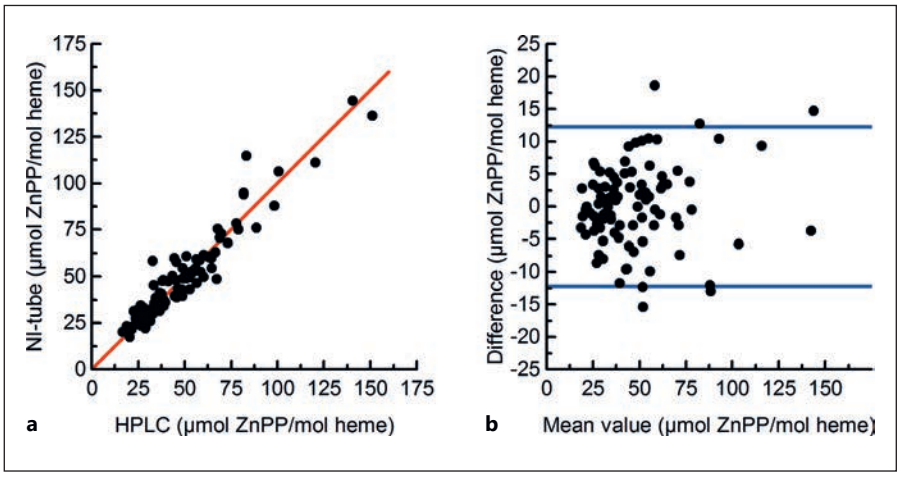

Fig. 3. Comparison of ZnPP measured in venous blood samples by high-performance liquid chromatography (HPLC measurement) and non-invasively through the blood collection tubing during blood donation (NI-tubing measurement). **a** Correlation between the two measurements. The red diagonal line represents the balance line. **b** Bland-Altman plot. The blue horizontal lines indicate the robust limits of agreement of 12.2 µmol ZnPP/mol heme.

Table 1. Comparison between ZnPP measurement methods using Bland-Altman analysis

Compared ZnPP measurement methods	*n*	LoA	CI
HPLC – NI-tubing	93	12.2	9.6–14.5
HPLC – NI-lip	99	20.9	17.6–24.1
NI-tubing – NI-lip	93	17.8	14.4–20.8

The LoA were 12.2 µmol ZnPP/mol heme, with a 95% confidence interval of 9.6–14.5 µmol ZnPP/mol heme (Fig. 3b). Thus, the LoA clearly lie below the ZnPP-difference of 25 µmol ZnPP/mol heme between manifest (>65 µmol ZnPP/mol heme) and excluded IDE (≤40 µmol ZnPP/mol heme). Table 1 additionally shows the results of the Bland-Altman Analysis performed for the comparison between the NI-lip and HPLC measurement as well as the fiber-optic measurements among each other. The best agreement was found between NI-tubing and HPLC measurement.

Diagnostic Value of NI ZnPP Measurements for the Detection of Iron Deficiency Compared to Capillary Hb Measurements

By capillary Hb determination, 3 of 8 subjects with IDA, none of 13 subjects with IDE, 1 of 23 subjects with depleted iron stores, none of 44 iron-replete subjects, and 2 of 11 subjects with undefined iron status were rejected from donation (Fig. 4a). In the cases with low capillary Hb, the NI-tubing measurements could not be conducted, due to the deferral of these subjects.

Of the remaining subjects that were accepted for donation (n = 93), the following number of subjects exceeded a cutoff of 65 µmol ZnPP/mol heme in the respective groups: 5 of 5 donors with IDA, 5 of 13 donors with IDE, 1 of 22 donors with depleted iron stores, 2 of 44 iron-replete donors, and 2 of 9 donors with undefined iron status (Fig. 4b). Using a cutoff of >65 µmol ZnPP/mol heme as indicative of IDE and IDA, 10 of 18 donors with IDE and IDA were correctly identified by this method, while only 3 of 66 donors with either only depleted or replete iron stores also exceeded this threshold.

A cutoff of 40 µmol ZnPP/mol heme was exceeded for the following number of subjects in the respective groups: All 5 of 5 donors with IDA, 12 of 13 donors with IDE, 14 of 22 donors with depleted iron stores, 14 of 44 iron-replete donors and 6 of 9 donors with undefined iron status (Fig. 4c). Using a cutoff of >40 µmol ZnPP/mol heme thus identified 17 of 18 donors with IDE and IDA, while identifying 64% of subjects with only depleted iron stores. However, 32% of iron-replete donors are then classified as iron deficient. Due to the high agreement of the NI-tubing and NI-lip measurement, NI-lip measurement showed similar detection rates when applied in addition to capillary Hb measurement, as shown in Table 2.

Discussion

The primary aim of the present study was to test the feasibility of a new NI approach to measure ZnPP from whole blood as it flows through the transparent blood collection tubing during blood donation. We have previously described a novel spectroscopic method for measuring ZnPP levels, which overcomes most of the limitations of hematofluorometers [24] and is sensitive enough to measure ZnPP non-invasively from the lower lip [25]. With a modification of the light application and sensor probe, the approach proved similar agreement with reference HPLC determination of ZnPP levels as in our previous studies and may offer unique utility in the blood donation setting. These earlier studies demonstrated a good correspondence between NI-lip and reference measurements

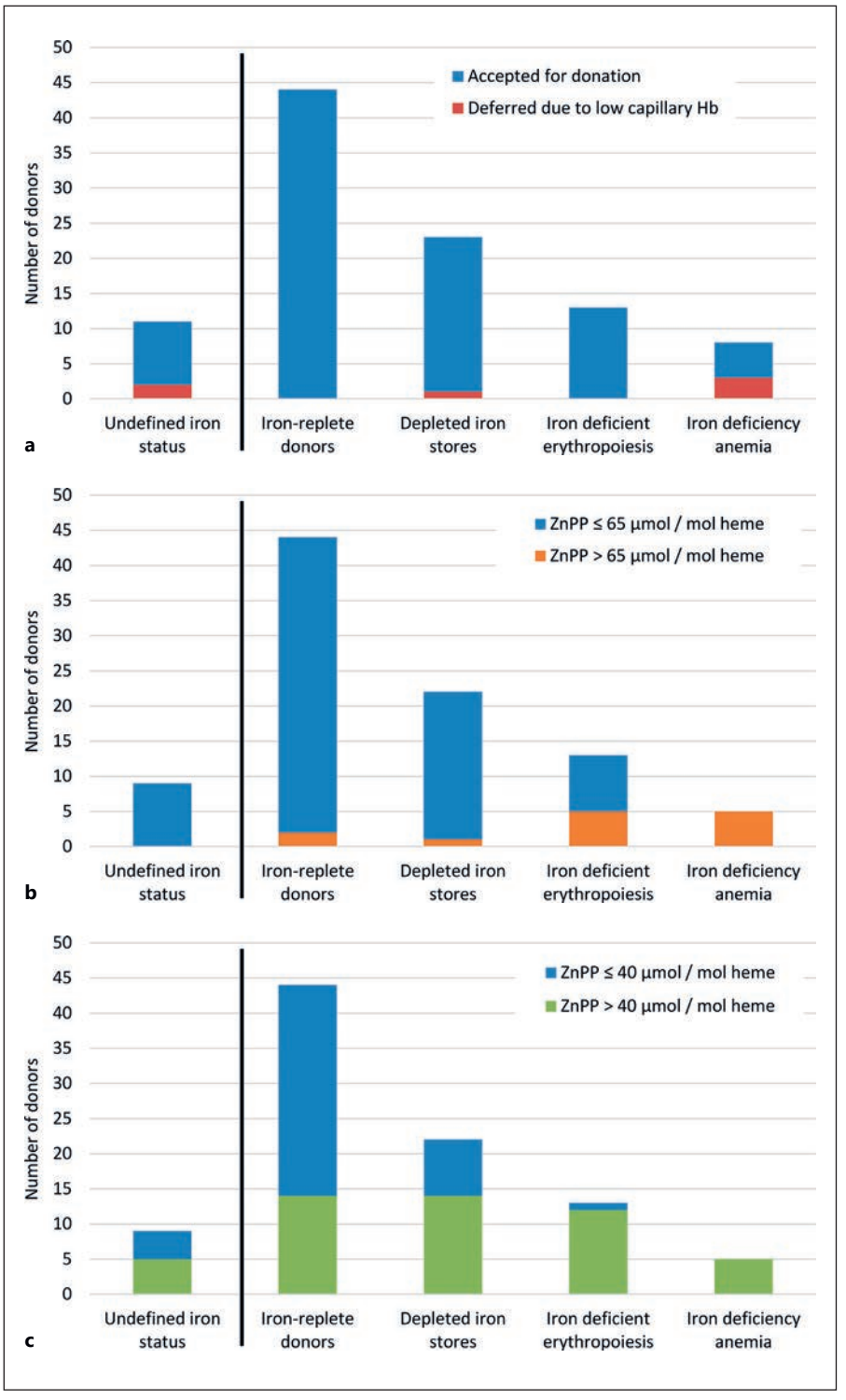

Fig. 4. Bar charts showing the assignment of potential donors to the applied iron deficiency stages. **a** Number of subjects accepted for donation (blue) and number of deferred subjects due to low capillary Hb (red) in each group. **b** Number of accepted donors (without deferred subjects) with NI-tubing measurement of ZnPP below (blue) and above (orange) the cutoff value of 65 µmol ZnPP/mol heme. **c** Number of accepted donors (without deferred subjects) with NI-tubing measurement of ZnPP below (blue) and above (green) the cutoff value of 40 µmol ZnPP/mol heme.

in blood with narrow LoAs between 14 µmol ZnPP/mol heme [31] and 19.7 µmol ZnPP/mol heme [25, 29].

In the current study, LoA between NI-lip measurement and HPLC reference were 20.9 µmol ZnPP/mol heme, comparing well to the previous results and confirming its validity as a method for NI ZnPP determination. Between NI-tubing and HPLC measurements LoA of 12.2 µmol ZnPP/mol heme were found, which demonstrates an excellent accuracy of the NI-tubing measurement. The continuous flow of blood through the tubing alleviates interfering effects of fluorescence photobleaching or sedimentation of erythrocytes, contributing to the high accuracy.

Table 2. Iron deficiency detection through NI-lip measurement

Iron deficiency stage	Detected/total subjects (cutoff at 40 µmol ZnPP/mol heme)	Detected/total subjects (cutoff at 65 µmol ZnPP/mol heme)
IDA	5/5 (100%)	3/5 (60%)
IDE	12/13 (92%)	3/13 (23%)
Depleted iron stores	13/22 (59%)	1/22 (5%)
Iron-replete	23/44 (52%)	2/44 (5%)
Undefined iron status	6/9 (66%)	2/9 (22%)

IDA, iron deficiency anemia; IDE, iron deficient erythropoiesis.

The second important aim of this study was to investigate whether NI measurement of ZnPP can contribute to secondary prevention of IDA in frequent blood donors by recognizing advanced iron deficiency at an earlier stage than routine capillary Hb. To evaluate this, all subjects were classified according to iron status based on ferritin, sTfR, and Hb obtained from venous sample laboratory measurements.

Using a cutoff of 65 µmol ZnPP/mol heme, ZnPP screening by NI-tubing measurements was able to identify all subjects with IDA and additionally 5 of 13 subjects with IDE. In contrast, capillary Hb determination did not identify any of the 13 donors with IDE. More surprisingly, capillary Hb identified only 3 of 8 donors with IDA as defined by venous blood parameters. Of note, NI-tubing measurements were not available for these 3 cases with IDA because of deferral from blood donation. However, both NI-lip and HPLC measurements yielded ZnPP values above 65 µmol ZnPP/mol heme for these subjects (online suppl. Table 2; for all online suppl. material, see www.karger.com/doi/10.1159/000528545). In summary, in the present study, NI measurements of ZnPP clearly outperformed capillary Hb determination with respect to identifying advanced iron deficiency in frequent blood donors.

Different scenarios for improved donor protection through ZnPP screening can be envisioned. A promising approach might be to combine capillary Hb and NI-tubing measurement of ZnPP to decide on future donor management strategy. Assuming donor eligibility continues to be based on capillary Hb, ZnPP measurement on the blood collection tubing could be used to identify donors at risk for future IDA and allow blood centers to propose mitigation measures such as iron supplementation or extension of donation intervals. That strategies involving iron supplementation can improve recovery of donor iron stores and lead to lower deferral rates has already been shown [13, 32].

Another option for blood centers could be to modify their screening strategy for regular donors. For example, if capillary Hb exceeded the threshold for IDA, including a safety margin, AND NI-tubing measurement of ZnPP excluded IDE, one might decide to omit capillary Hb measurements in subsequent donation visits depending on the present capillary Hb and the risk for iron deficiency reflected by the ZnPP value. Some blood establishments have already implemented similar scenarios, for example, by omitting capillary Hb determination before blood donation if the venous Hb from the previous donation exceeded 12.9 g/dL for females or 13.9 g/dL for males [33]. Used in this manner, the proposed ZnPP screening would result in fewer invasive pre-donation screening tests for regular blood donors and would automatically lead to a longitudinal record of ZnPP levels enabling even more precise and appropriate recommendations than based on single ZnPP measurements.

Other approaches to protect donors with respect to iron deficiency are based on ferritin screening. In recent years, a number of blood establishments in Europe and USA have started using routine ferritin testing as an additional measure to better identify and manage donors at risk of developing severe iron deficiency. In the present cohort, ferritin levels were below cutoff for 50% of donors (including 6 donors that were classified with "undefined iron status"). This confirms the findings of other studies [6, 7], but imposing an extended deferral period on all these donors may not be desirable and is likely also not adequate. Therefore, it might make sense to identify only donors with IDE, who are at higher risk of developing anemia than donors with just depleted iron stores. In the present study, by using a ZnPP cutoff of 65 µmol ZnPP/mol, only 19% of the donors would have been considered as needing mitigation. In addition, ferritin testing is not yet available as a point-of-care test and thus requires laboratory assessment. This does not allow for immediate feedback to the donors but instead necessitates post-donation follow-up, which may be logistically complex and not always effective. From a cost perspective, ZnPP measurements also likely offer an advantage over ferritin measurements as the presented approach is purely optical without the need for disposables. Thus, use of ZnPP rather than ferritin to guide targeted interventions allows for

a less restrictive approach, which may be easier to manage by blood centers.

The above considerations are valid for adult donors. For adolescents, the situation is presumably different. Due to their higher demand for iron, iron stores run short more quickly and whole blood donations require longer intervals. In order to determine the appropriate recommended length of intervals between donations, a large study among 30,806 teenagers in the USA has measured ferritin and found that recovery of iron stores took much longer than in adults [34]. In order to protect teenagers from severe iron depletion, ferritin may therefore be the appropriate measure.

The true benefits of a ZnPP-based donor management approach in terms of impact on the donors' iron balance, eligibility, and willingness to donate remain to be fully established in future longitudinal studies. Previous studies found added value of ZnPP measurement in the prediction of iron deficiency in blood donors and suggested that ZnPP might be a helpful parameter in improving donor management but did not yet suggest specific strategies [8, 19–22].

Results from a recent placebo-controlled interventional study [35] involving iron-deficient donors showed that iron repletion following donation dramatically reduced the risk for Hb deferral at the next donation visit, from 58% to 16% in women and from 16% to 0% in men. A recent large-scale study (n = 55,644) in the Netherlands on the consequences of a new policy involving a 6–12 month temporary deferral in case of low ferritin also found that donors managed this way were less likely to unsubscribe permanently from donation than those deferred on site due to low Hb [10]. It seems reasonable that this might also translate to iron deficiency screening based on ZnPP.

The present study has a number of limitations. One relates to the low number of individuals included. In addition, 11 of 99 donors could not be assigned to one of the predefined iron status groups (for an assessment based on additional blood parameters see online Suppl. Table 1, online Suppl. Fig. 1, 2). Furthermore, four assignable donors were deferred due to low capillary Hb, and therefore, no NI-tubing measurement of ZnPP could be performed. Another limitation relates to the ZnPP cutoff values used. The current study focused on very frequent donors as we intended to assess donors with a high-risk profile who could particularly benefit from innovative diagnostics for reliable iron deficiency screening. However, since chronic blood loss can lead to adaptation of erythropoiesis [36], the cutoffs should also be investigated for first-time donors and irregular donors in the future. Similarly, questions may be raised about sTfR thresholds used to define IDE. Although the sTfR limits applied in the study are recommended for diagnosis of suspected IDE, the threshold values may not be adequate for regular blood donors as increased erythropoiesis leads to increased sTfR levels [3, 37]. A careful reconsideration of all threshold values and assignments into iron status groups is therefore indicated for larger studies in the future.

In conclusion, the present study clearly demonstrated the technical and practical feasibility of NI ZnPP measurements both on the lower lip as well as on the blood collection tubing. It also showed that ZnPP screening in addition to capillary Hb screening detects a high percentage of donors with iron deficiency before anemia occurs. Consequently, the NI ZnPP measurement methods may enable selective implementation of secondary preventive measures to protect donors against advanced iron deficiency and IDA. Its routine deployment thus offers an opportunity to improve donor protection and to support blood donation services in retaining their regular blood donors by avoiding future iron deficiency-related Hb deferrals.

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Statement of Ethics

The study is registered at the German clinical trials register as DRKS00013102 and was approved by the Ethics Committee at the Ludwig Maximilian University of Munich, approval number 17-021. All study participants have given their written informed consent.

Conflict of Interest Statement

A.S., A.L., L.H., M.V., and R.S. have disclosed no conflicts of interest. CH is shareholder and managing director of the start-up company FerroSens GmbH (Munich, Germany), which aims at commercializing the technology for NI detection of ZnPP. C.H., G.H., and H.S. are co-inventors of the patent family with application number PCT/EP2016/053389 "Apparatus and method for fluorescence measurements on tissue for the determination of blood fluorophores." F.W. and E.Q. are affiliated to the company "Blutspendedienst des Bayerischen Roten Kreuzes gemeinnützige GmbH" (Munich, Germany).

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Authors Contributions

C.H., G.H., A.L., and H.S. developed the fiber-optic ZnPP measuring device. A.S., C.H., G.H., A.L., R.S., H.S., F.W., and E.Q. developed the concept and designed the study. A.S., C.H., A.L., and H.S. recruited study participants and carried out the fiber-optic measurements. L.H. and M.V. were responsible for the biochemical and hematological laboratory determinations. A.S. and C.H. analyzed the data and performed all statistical analyses. All authors approved the final version of the manuscript. The corresponding author A.S. had full access to all the data in the study and shared responsibility for the decision to submit for publication with C.H. and H.S.

Data Availability Statement

Anonymized source data are available on request from the corresponding author.

HPLC, ZnPP reference measurement by high performance liquid chromatography; NI-lip, non-invasive ZnPP measurement through red vermillion of lower lip; NI-tubing, non-invasive ZnPP measurement through tubing of blood collection system during blood donation; n, number of participants included; LoA, robust limits of agreement according to description under statistical analysis; CI, confidence interval.

References

1 Myers DJ, Collins RA. Blood donation. Treasure island (FL): StatPearls. 2020. Available from: https://www.ncbi.nlm.nih.gov/books/NBK525967/.
2 Bundesärztekammer. (BÄK). Beschluss der Bundesärztekammer über die Richtlinie zur Gewinnung von Blut und Blutbestandteilen und zur Anwendung von Blutprodukten (Richtlinie Hämotherapie) – Gesamtnovelle 2017. Dtsch Arztebl. 2017;114(3132). A-1504/B-272/C-246.
3 Hastka J, Metzgeroth G, Gattermann N. Eisenmangel und Eisenmangelanämie, Empfehlungen der Fachgesellschaft zur Diagnostik und Therapie hämatologischer und onkologischer Erkrankungen. Onkopedia Leitlinien; 2021.
4 Brittenham GM, Klein HG, Kushner JP, Ajioka RS. Preserving the national blood supply. Hematology Am Soc Hematol Educ Program. 2001;422–32.
5 Waldvogel-Abramovski S, Waeber G, Gassner C, Buser A, Frey BM, Favrat B, et al. Iron and transfusion medicine. Blood Rev. 2013 Nov;27(6):289–95.
6 Cable RG, Glynn SA, Kiss JE, Mast AE, Steele WR, Murphy EL, et al. Iron deficiency in blood donors: the REDS-II Donor Iron Status Evaluation (RISE) study. Transfusion. 2012 Apr;52(4):702–11.
7 Baart AM, van Noord PAH, Vergouwe Y, Moons KGM, Swinkels DW, Wiegerinck ET. High prevalence of subclinical iron deficiency in whole blood donors not deferred for low hemoglobin. Transfusion. 2013 Aug;53(8):1670–7.
8 Baart AM, de Kort WL, Moons KGM, Atsma F, Vergouwe Y. Zinc protoporphyrin levels have added value in the prediction of low hemoglobin deferral in whole blood donors. Transfusion. 2013 Aug;53(8):1661–9.
9 Hillgrove T, Moore V, Doherty K, Ryan P. The impact of temporary deferral due to low hemoglobin: future return, time to return, and frequency of subsequent donation. Transfusion. 2011 Mar;51(3):539–47.
10 Spekman MLC, Ramondt S, Sweegers MG. Whole blood donor behavior and availability after deferral: consequences of a new ferritin monitoring policy. Transfusion. 2021 Apr;61(4):1112–21.
11 Finch CA, Cook JD, Labbe RF, Culala M. Effect of blood donation on iron stores as evaluated by serum ferritin. Blood. 1977 Sep;50(3):441–7.
12 Gestsdottir E, Magnusson MK, Lund SH, Johannsdottir V, Jonsson T, Gudmundsson S, et al. Monitoring iron stores in Icelandic blood donors from 1997 through 2019. Transfus Med. 2022 Feb 24;32(2):128–34.
13 Gorlin J, Katz L, Elsmore D, Kirbach K, Erickson Y, Hove A, et al. Prevalence of blood donor iron deficiency and feasibility ferritin-based iron replacement: a blood collection agency-based study. Vox Sang. 2016 Aug;111(2):206–8.
14 Sayers MH. Iron supplementation? Ferritin screening? Why questions persist. Transfusion. 2019 May;59(5):1616–9.
15 Russell WA, Scheinker D, Custer B. Individualized risk trajectories for iron-related adverse outcomes in repeat blood donors. Transfusion. 2022 Jan;62(1):116–24.
16 Chua AC, Klopcic BR, Ho DS, Fu SK, Forrest CH, Croft KD, et al. Dietary iron enhances colonic inflammation and IL-6/IL-11-Stat3 signaling promoting colonic tumor development in mice. PLoS One. 2013;8(11):e78850.
17 Schifman RB, Finley PR. Measurement of near-normal concentrations of erythrocyte protoporphyrin with the hematofluorometer: influence of plasma on "front-surface illumination" assay. Clin Chem. 1981 Jan;27(1):153–6.
18 Hastka J, Lasserre JJ, Schwarzbeck A, Strauch M, Hehlmann R. Washing erythrocytes to remove interferents in measurements of zinc protoporphyrin by front-face hematofluorometry. Clin Chem. 1992 Nov;38(11):2184–9.
19 Schifman RB, Rivers SL. Red blood cell zinc protoporphyrin to evaluate anemia risk in deferred blood donors. Am J Clin Pathol. 1987 Apr;87(4):511–4.
20 Jensen BM, Sandø SH, Grandjean P, Wiggers P, Dalhøj J. Screening with zinc protoporphyrin for iron deficiency in non-anemic female blood donors. Clin Chem. 1990 Jun;36(6):846–8.
21 Harthoorn-Lasthuizen EJ, Lindemans J, Langenhuijsen MM. Zinc protoporphyrin as screening test in female blood donors. Clin Chem. 1998 Apr;44(4):800–4.
22 Schotten N, Zalpuri S, Pasker-de Jong PCM, Swinkels DW, van den Hurk K, de Kort WLAM, et al. Utility of zinc protoporphyrin in management of whole blood donors. Transfusion. 2018 Mar;58(3):692–700.
23 Schifman RB, Rivers SL, Finley PR, Thies C. RBC zinc protoporphyrin to screen blood donors for iron deficiency anemia. JAMA. 1982 Oct 22;248(16):2012–5.
24 Hennig G, Gruber C, Vogeser M, Stepp H, Dittmar S, Sroka R, et al. Dual-wavelength excitation for fluorescence-based quantification of zinc protoporphyrin IX and protoporphyrin IX in whole blood. J Biophotonics. 2014 Jul;7(7):514–24.
25 Hennig G, Homann C, Teksan I, Hasbargen U, Hasmüller S, Holdt LM, et al. Non-invasive detection of iron deficiency by fluorescence measurement of erythrocyte zinc protoporphyrin in the lip. Nat Commun. 2016 Feb 17;7:10776.
26 Heckl C, Eisel M, Lang A, Homann C, Paal M, Vogeser M, et al. Spectroscopic methods to quantify molecules of the heme-biosynthesis pathway: a review of laboratory work and point-of-care approaches. Translational Biophotonics. 2021;3(2):3.
27 Hastka J, Lasserre JJ, Schwarzbeck A, Hehlmann R. Central role of zinc protoporphyrin in staging iron deficiency. Clin Chem. 1994 May;40(5):768–73.
28 Labbe RF, Vreman HJ, Stevenson DK. Zinc protoporphyrin: a metabolite with a mission. Clin Chem. 1999 Dec;45(12):2060–72.
29 Füllenbach C, Stein P, Glaser P, Triphaus C, Lindau S, Choorapoikayil S, et al. Screening for iron deficiency in surgical patients based on noninvasive zinc protoporphyrin measurements. Transfusion. 2020 Jan;60(1):62–72.
30 Bland JM, Altman DG. Measuring agreement in method comparison studies. Stat Methods Med Res. 1999 Jun;8(2):135–60.
31 Homann C, Hennig G, Maier F, Stepp H, Holdt LM, Vogeser M, et al. Non-invasive measurement of erythrocyte zinc protoporphyrin in children. Pediatr Res. 2019 Feb;85(3):349–54.

32 Radtke H, Tegtmeier J, Röcker L, Salama A, Kiesewetter H. Daily doses of 20 mg of elemental iron compensate for iron loss in regular blood donors: a randomized, double-blind, placebo-controlled study. Transfusion. 2004 Oct;44(10):1427–32.
33 Ziemann M, Steppat D, Brockmann C, Washington G, Kirchner H, Schlenke P. Selection of whole-blood donors for hemoglobin testing by use of historical hemoglobin values. Transfusion. 2006 Dec;46(12):2176–83.
34 Vassallo RR, Hilton JF, Bravo MD, Vittinghoff E, Custer B, Kamel H. Recovery of iron stores after adolescents donate blood. Pediatrics. 2020 Jul;146(1):e20193316.
35 Hod EA, Brittenham GM, Bitan ZC, Feit Y, Gaelen JI, La Carpia F, et al. A randomized trial of blood donor iron repletion on red cell quality for transfusion and donor cognition and wellbeing. Blood. 2022 Sep 6: blood.2022017288.
36 Fliedner TM, Hoelzer D, Steinbach KH. [Physiological and pathological regulation of erythropoiesis]. Verh Dtsch Ges Inn Med. 1978(84):15–27.
37 Skikne BS. Serum transferrin receptor. Am J Hematol. 2008 Nov;83(11):872–5.

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Identification of Lutheran Blood Groups and Genetic Variants within *KLF1* among Thai Blood Donors

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Keywords

Lutheran blood group system · In(Lu) phenotype · *BCAM* · *KLF1* variants · Thai blood donors

Abstract

Background: Lu[a] and Lu[b] are inherited as codominant allelic characters resulting from a single nucleotide variant (SNV) of the basal cell adhesion molecule (*BCAM*) gene. Red cells of the dominantly inherited suppressor of the Lutheran antigens In(Lu) phenotypically appear as Lu(a–b–) by the haemagglutination test. In(Lu) resulted from heterozygosity for mutations within the erythroid-specific Krüppel-like factor 1 (*KLF1*) gene. This study aimed to determine the frequency of the Lu(a) and Lu(b) phenotypes and genotypes and genetic variants of the distinct In(Lu) among Thai blood donors. ***Material and Methods:*** Samples from 334 Thai donors were phenotyped with anti-Lu[a] and anti-Lu[b]. These DNA samples and an additional 1,370 donor DNA samples with unknown Lu(a)/Lu(b) phenotypes were genotyped using an in-house PCR-SSP. In the case of the three Lu(a–b–) donors, the *BCAM* and *KLF1* genes were analysed by PCR and sequencing. ***Results:*** A total of 331 of the 334 donors were Lu(a–b+), while the other observed phenotype, appearing as Lu(a–b–), was found among three donors. Of those three Lu(a–b–) donors with the *LU*02/02* genotype, we identified *KLF1* variant alleles, consisting of two variants: c.[304T>C, 1001C>G] and c.[304T>C, 519_525dupCGGCGCC], leading to the In(Lu) phenotype, and one homozygous variant (c.304T>C) mutation. Also, only one Thai donor was genotyped as *LU*01/02*, confirmed by serology test and DNA sequencing. ***Conclusion:*** In this study, we identified *KLF1* variants to be included in Lutheran typing analysis in Thai populations. Therefore, the application of genotyping and phenotyping methods has simultaneously been in use to screen and confirm the rare Lu(a+) and In(Lu) phenotypes.

Introduction

The Lutheran (LU) blood group system (International Society of Blood Transfusion, ISBT No. 005) currently consists of 26 antigens [1], which are located on two isoforms of red cell membrane glycoproteins (basal cell adhesion molecule, B-CAM, CD239) belonging to the immunoglobulin superfamily (IgSF) of adhesion/receptor molecules [2, 3]. These antigens are numbered LU1 to LU29: four antithetical pairs – LU1(Lu[a])/LU2(Lu[b]), LU6/LU9, LU8/LU14, and LU18(Au[a])/LU19(Au[b]) – and three antigens (LU10, LU11, and LU15) are declared obsolete [1]. The Lu_{null} or Lu(a–b–) phenotype is inherited as a recessive allelic character in which no Lutheran antigens can be detected in red cells. Thus, individuals possessing this phenotype may generate an antibody against Lu glycoproteins, anti-Lu3, which reacts equally strongly with all red cells except those of the Lu_{null} phenotype [3]. Lutheran antibodies, anti-Lu[a] and anti-Lu[b], are either naturally occurring or immune and are IgM, IgG, or IgA. Clinically significant findings for both antibodies have shown them to be involved in the formation of mild delayed hae-

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molytic transfusion reactions (HTRs) [4–6]. There were some cases of haemolytic disease of the fetus and newborn (HDFN) recorded, and they were not severe enough to require any treatment beyond phototherapy, despite detecting either raised bilirubin or a positive direct antiglobulin test (DAT) [5].

The LU (now *BCAM*) gene is located on chromosome 19 at 19q13.32 and is organised into 15 exons distributed over approximately 12.5 kb. The Lu^a and Lu^b antigens have codominant allelic relationships and represent a single nucleotide variant (SNV), rs28399653, c.230A>G (p. His77Arg) in exon 3 of the *BCAM* gene [7, 8]. The true Lu(a–b–) phenotype is the recessive type and results from either homozygous or double heterozygous mutations responsible for inactivating the *BCAM* gene [9].

In addition to the recessive Lu_{null} phenotype, the rare X-linked suppressor (XS2) and In(Lu) phenotypes usually manifest as the Lu(a–b–) phenotype detected by haemagglutination tests due to the very low levels of Lutheran antigens presented in their red cells. Hence, individuals with the types of XS2 and In(Lu) do not produce anti-Lu3. The X-linked type found in a large Australian family was shown to be caused by a hemizygous mutation in the erythroid transcription factor *GATA1* [10, 11]. In another type governed by the erythroid transcription factor KLF1 (erythroid-specific Krüppel-like factor 1), the In(Lu) phenotype results from heterozygous inheritance of loss-of-function mutations in the dominant suppressor gene, *KLF1* [12], and In(Lu) is also characterized by the diminished expression of the high-prevalence antigen, AnWj, and the antigens for the Indian, P1PK, Landsteiner-Wiener, Knops, OK, RAPH, and I blood group systems [3, 12, 13]. The human *KLF1* gene is located on chromosome 19, encompassing three exons and two introns [14]. KLF1 contains two transactivation domains at the N-terminal end of the protein and three highly conserved zinc fingers at the C-terminal, which regulates the activity of several erythroid genes [14, 15].

Lu^b is a high-prevalence antigen that is present in more than 99.9% of Southeast Asians. Its antithetical antigen, Lu^a, typically occurs very rarely or is absent from Asians [3, 16, 17], although it is widely distributed in certain populations (e.g., it is found in approximately 6% of Europeans (non-Finnish), 5% of Africans/African Americans, and 3% of Latino/admixed Americans) [3, 17]. The recessive Lu(a–b–) phenotype is extremely rare and is observed in all populations [3]. In three Thai reports, in which donor red cells were tested with anti-Lu^a and anti-Lu^b, the following results were revealed: in 1967, 455 were tested, 452 were Lu(a–b+), one was Lu(a+b+), and two were Lu(a–b–) [18]; in 2001, 200 were tested and all were Lu(a–b+) [19]; in 2002, 500 were tested, 498 were Lu(a–b+) and two were Lu(a–b–) [20]. Two predominant Thai studies found two samples of blood that were apparently Lu(a–b–), but it is currently unproven whether these samples are either inherited Lu_{null} or XS2/In(Lu) phenotypes, as identified by the suppressor gene mutations [18, 20]. In addition to considering the inheritance of these antigens, no study among Thai blood donors has ever been genetically investigated. This study aimed to determine the frequency of the Lu(a) and Lu(b) phenotypes and their genotypes and genetic variants of the In(Lu) phenotype among the Thai blood donors.

Materials and Methods

Sample Collection and Preparation

Ethylenediaminetetraacetic acid-anticoagulated samples were collected from the whole blood of 334 healthy Thai blood donors at the Blood Bank at Thammasat University Hospital (TUH) in Pathumtani, Central Thailand, between January and April 2022. Genomic DNA from these samples was extracted using the DNeasy Blood & Tissue Kit according to the manufacturer's instructions (QIAGEN, Valencia, CA, USA), then stored at −20°C until genotyping. An additional 1,370 DNA samples from unrelated healthy Thai blood donors obtained from our inventory of DNA banks (TUH) were used for *LU*01/LU*02* genotype screening by testing with in-house polymerase chain reaction sequence-specific primers (PCR-SSP).

Lu^a and Lu^b Phenotyping

A total of 334 blood samples were typed using the conventional tube technique (CTT). The reaction was tested by adding one drop of human anti-Lu^a and anti-Lu^b (CE-Immundiagnostika GmbH, Eschelbronn, Germany), one drop of 22% bovine albumin (National Blood Centre, Thai Red Cross Society, Bangkok, Thailand), and one drop of 2–3% red cell suspension in 0.9% normal saline to each tube before incubation at 37°C for 30 min. The mixtures were washed three times with normal saline, and two drops of the anti-human globulin (CE-Immundiagnostika GmbH) were added. After centrifugation, the reactions were read macroscopically, and the agglutination reactions were graded as 4+, 3+, 2+, 1+, and w+. After reading the negative reaction under a microscope, IgG-coated RBCs were added to check the validity of the antiglobulin test. Also, a DAT was performed for all samples that gave positive results to rule out false positive results, if any. In the case of apparent Lu(a–b–), column agglutination technology (CAT) – ID card antigen profile-I (P1, Le^a, Le^b, Lu^a, Lu^b) – was additionally used for phenotyping as per the manufacturer's instructions (Bio-Rad, Hercules, CA, USA).

DNA Analysis

The *KLF1* exons 1–3 and *BCAM* exons 3–4 were PCR-amplified using the oligonucleotide primers shown in Table 1. For each PCR reaction, 3 µL of genomic DNA (50 ng/µL) was amplified in a total volume of 40 µL using 0.75 µM forward primer and 0.75 µM reverse primer for each reaction. The PCR was performed with 20 µL of 2× PCR reaction mixture (Phusion High-Fidelity PCR Master Mix, New England BioLabs, MA, USA) and 11 µL of sterile distiled water in a T100 Thermal Cycler (Bio-Rad). PCR conditions were as follows: for *BCAM*, 98°C for 30 s, (98°C for 30 s, 63°C for 40 s, 72°C for 30 s) × 35 cycles, 72°C for 5 min, producing a 1,039-bp amplicon; for *KLF1*, 98°C for 30 s, (98°C for 10 s, 69°C for 60 s) × 10 cycles, (98°C for 10 s, 65°C for 50 s, 72°C for 90 s) × 25 cycles, 72°C for 5 min, producing a 2,692-bp amplicon. PCR products were separated on a 1.5% agarose gel containing SYBR

Table 1. Oligonucleotide primers used for PCR procedures and sequencing

Name of primer	Sequence of primer (5′ to 3′)	Gene target	PCR product size, bp
SEQ-BCAM-F	TCAAGAACTGATAGGGAATGGGG	Exons 3–4, *BCAM*	1,039
SEQ-BCAM-R	TCCTAGGACACAGGAGTCTTCCC		
SEQ-KLF1-F	CCTTCTTTGGAGACCCAATGTC	Exons 1–3, *KLF1*	2,692
SEQ-KLF1-R	AGAGGGTCCATTCGTGGGA		
LU-F	AACTGATAGGGAATGGGGGC	Exon 3, rs28399653, c.230A	214
LU-A-R	CTCAGCCGAGGCTAGGT		
LU-F	AACTGATAGGGAATGGGGGC	Exon 3, rs28399653, c.230 G	214
LU-B-R	CTCAGCCGAGGCTAGGC		
HGH-F	TGCCTTCCCAACCATTCCCTTA	*HGH* gene (internal control)	434
HGH-R	CCACTCACGGATTTCTGTTGTGTTTC		

Bp, base pair; HGH, human growth hormone.

Safe DNA Gel Stain (Invitrogen, Paisley, UK), electrophoresed in 1× TBE buffer at 100 V, and visualised under a blue light transilluminator. Thereafter, amplicons were purified using a gel extraction kit (GeneJET Gel Extraction Kit; Thermo Scientific, Waltham, MA, USA), and eluted fragments were then sequenced by next-generation sequencing-based technology on the Illumina Platform (BTSeq™ Services; Celemics Inc., Seoul, Korea). Regarding P^1/P^2 genotype determination, a noncoding region of *A4GALT* targeting SNPs rs5751348 was analysed according to the method described by Thinley and colleagues [21].

*LU*01 and LU*02 Genotyping*

Additional donor DNA samples were analysed using the in-house PCR-SSP method to screen the *LU*01*/*LU*02* genotypes. The primer pair sequences for each amplicon are shown in Table 1. Two tube reactions were performed, one for the *LU*01* allele (c.230A) and the other for the *LU*02* allele (c.230G). The constituents of the PCR mixtures (10 μL volume per reaction) were as follows: 1 μL (50 ng/μL) of genomic DNA, 1 μM common forward primer (LU-F), 1 μM reverse primer (LU-A-R or LU-B-R), 0.3 μM*HGH* primers (HGH-F and HGH-R), and 5 μL of the 2× PCR reaction mixture (GoTaq® Hot Start Colorless Master Mix; Promega, Madison, WI, USA). The genomic regions of interest were PCR-amplified from DNA template 95°C for 5 min, (95°C for 30 s, 69°C for 60 s) × 10 cycles, (95°C for 10 s, 62°C for 50 s, 72°C for 30 s) × 25 cycles, 72°C for 5 min, producing a 214-bp amplicon (*LU*01* or *LU*02* allele) and a 434-bp amplicon (HGH-internal control). The amplicon separation and visualisation were performed as described above. To validate our in-house PCR-SSP technique for *LU*01*/*LU*02* genotyping, random DNA samples from 170 genotyped blood donors were independently sequenced.

Statistical Analysis

The prevalence of the observed phenotypes was described using descriptive statistics and expressed in percentages and 95% confidence intervals (CI). The genotype and allele frequencies were obtained by direct counting. The Hardy-Weinberg equilibrium (HWE) was calculated by comparing expected and observed genotype frequencies using the Chi-square (χ^2) statistic. All statistical analyses were conducted using SPSS, Version 16.0 (SPSS Inc., Chicago, IL, USA), and p values ≤0.05 were considered statistically significant.

Results

Red Cell Phenotyping by CTT and CAT

A total of 334 blood donor samples were typed for Lu^a and Lu^b antigens. The Lu(a−b+) phenotype was commonly observed in 99.10% of donors (n = 331/334, 95% CI, 0.9740–0.9981), while the other phenotype, Lu(a−b−), was found in 0.90% of donors (n = 3/334, 95% CI, 0.0019–0.0260). Among the three Lu(a−b−) donors, P1, Le^a, Le^b, Lu^a, and Lu^b were additionally tested by CAT, and the prevalence of those phenotypes detected is shown in Table 2. All three donors were Lu(a−b−) and Le(a−b+) phenotypes, whereas only one donor showed a weak reaction for P1 antigen.

Mutation Analysis by DNA Sequencing

A total of three Lu(a−b−) donor samples were selected by the partial *A4GALT* and *BCAM* and the whole *KLF1* gene analysis. Table 2 shows the genomic DNA sequencing results of these donors. The molecular basis for a serologically weakened P1(+) phenotype of BZL286 donor was determined to be the heterozygous genotype at rs5751348 (G/T), representing the P^1/P^2 genotype, while the others (BZL008 and BZL116) had the P^2/P^2 genotype. Moreover, all donors had the *LU*02/02* genotype (c.230G/G). DNA sequencing revealed that one donor (BZL286) was found to have a single mutation (homozygous) for the mutant *KLF1* allele, with a missense c.304T>C (p.Ser102Pro) mutation. The remaining two donors were found to carry additional mutations in *KLF1*. One donor (BZL008) had a homozygous missense c.304T>C (p.Ser102Pro) and a heterozygous missense c.1001C>G (p.Thr334Arg) mutation, while the other donor (BZL116) had a heterozygous missense c.304T>C (p.Ser102Pro) and a homozygous duplication of c.519_525dupCGGCGCC (p.Gly176ArgfsX179). The variant alleles defined by these polymorphisms are c.[304T>C, 1001C>G] and c.[304T>C, 519_525dupCG-

Table 2. Phenotypes, alleles, and genotypes for three In(Lu) donors

Donor	Phenotypes			Genotypes		*KLF1* mutations			
	Lutheran	Lewis	P1PK	*A4GALT*	*BCAM*	Nt change	Aa change	exon	rs number
BZL008	Lu(a–b–)	Le(a–b+)	P1(–)	*P2/P2*	*LU*02/02*	c.304T>C	p.Ser102Pro	2	rs2072597
						c.1001C>G	p.Thr334Arg	3	rs483352841
BZL116	Lu(a–b–)	Le(a–b+)	P1(–)	*P2/P2*	*LU*02/02*	c.304T>C	p.Ser102Pro	2	rs2072597
						c.519_525dupCGGCGCC	p.Gly176ArgfsX179	2	rs483352838
BZL286	Lu(a–b–)	Le(a–b+)	P1(+)	*P1/P2*	*LU*02/02*	c.304T>C	p.Ser102Pro	2	rs2072597

Aa, amino acid; Nt, nuclotide.

Table 3. The genotype, allele and MAF, and the HWE for the *BCAM* SNP rs28399653

Population (N)	Allele (*N*)	Frequency	Genotype (*N*)	Frequency	MAF	HWE	
						χ^2	*p* value
Total (1,704)	*LU*01* (1)	0.0003	*LU*01/01* (0)	0.0000	0.0003	0.0006	0.9997
	*LU*02* (3,407)	0.9997	*LU*01/02* (1)	0.0006			
			*LU*02/02* (1,703)	0.9994			
Known Lu(a) and Lu(b) (334)	*LU*01* (0)	0.0000	*LU*01/01* (0)	0.0000	0.0000	0.0000	1.0000
	*LU*02* (668)	1.0000	*LU*01/02* (0)	0.0000			
			*LU*02/02* (334)	1.0000			
Unknown Lu(a) and Lu(b) (1,370)	*LU*01* (1)	0.0004	*LU*01/01* (0)	0.0000	0.0004	0.0086	0.9957
	*LU*02* (2,739)	0.9996	*LU*01/02* (1)	0.0007			
			*LU*02/02* (1.369)	0.9993			

HWE, Hardy-Weinberg equilibrium; *N*, number; MAF, minor allele frequency.

GCGCC], respectively. The occurrence of electropherogram peaks and the positions of these mutations are shown in Figure 1.

*LU*01 and LU*02 Genotyping by PCR-SSP*

An in-house PCR-SSP method was designed, and the genotype *LU*01/LU*02* alleles displayed by agarose gel electrophoresis are shown in Figure 2. A total of 1,704 DNA samples, containing 334 known and 1,370 unknown Lu(a) and Lu(b) phenotypes, were genotyped for the *BCAM* c.230A>G. The allele and genotype frequencies of these samples are shown in Table 3. The observed genotype frequencies in the overall population studied were found to be conformed to the HWE test ($p > 0.05$) and have a very low minor allele frequency (MAF), *LU*01* as < 1%. Overall, there were only two genotypes, with frequencies of 0.0006 and 0.9994 for *LU*01/02* and *LU*02/02*, respectively. The count of the *LU*01* allele was 1 and the *LU*02* allele was 1,703. A rare genotype of *LU*01/01* was not observed in this study. In the 334 known Lu(a) and Lu(b) population, all DNA samples were *LU*02/02* genotype. One hundred and seventy such random samples were subjected to and confirmed by independent DNA sequencing. These verifications of the PCR-SSP results remained constant across the genotyping results obtained by DNA sequencing. In addition to one donor possessing the *LU*01/02* genotype, the donor's red cells were selected for Lu^a/Lu^b phenotyping, and the results were confirmed as Lu(a+b+), corresponding with the genotype.

Discussion

Blood group phenotype distribution varies globally across different populations and ethnic groups, but limited data exist for ethnic distributions of Lu(a) and Lu(b) phenotype, allele, and genotype frequencies in Thailand. Here, we investigated the frequency of Lu(a) and Lu(b) phenotypes among blood donors in the Central region of Thailand using CTT. The study demonstrated that the frequencies of these phenotypes were related to their published frequencies in Thai cohorts [18–20], supporting a very rare or absent Lu^a antigen frequency in Thais. In accordance with Asian ancestry, this frequency is similar to those in Saudi Arabian, Indian, and Chinese populations [22–24]. The In(Lu) phenotype was found in three of 334

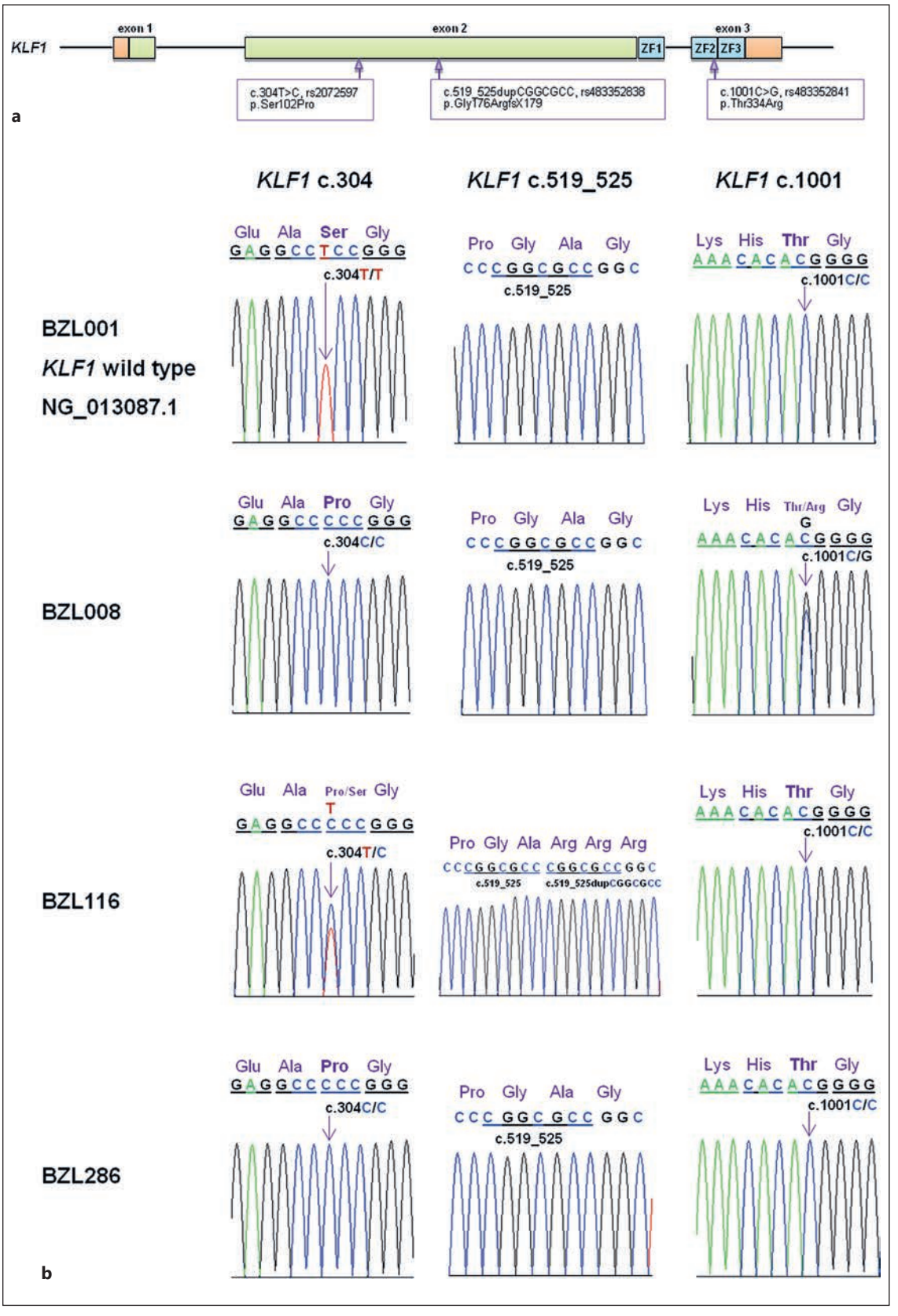

Fig. 1. The *KLF1* gene analysis. **a** Schematic representation of the *KLF1* gene with the location of the mutation variants indicated with an arrow. Exons: orange = non-coding regions; green = coding regions; blue = zinc fingers (ZF1, ZF2, ZF3). **b** Comparison of the partial *KLF1* sequence between the reference (NG_013087.1) and the three apparent Lu(a–b–) phenotypes.

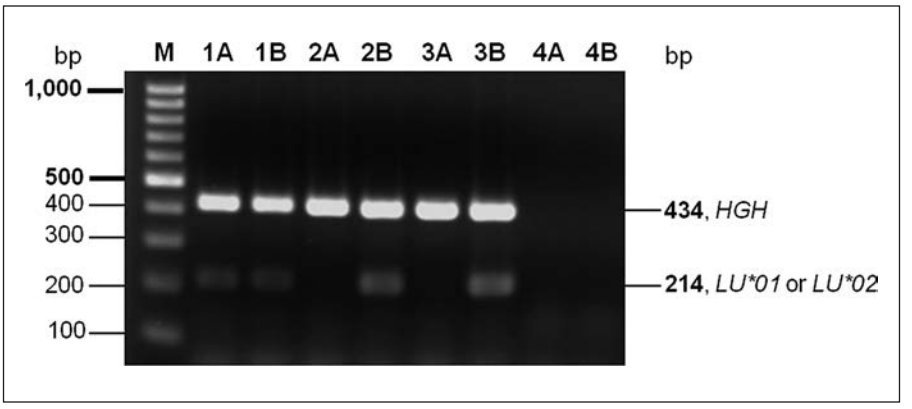

Fig. 2. Representative gel electrophoresis of the PCR-SSP products of the samples 1, 2, and 3 known with the *LU*01/LU*02* genotyping. A 434-bp fragment of *HGH* was present in all lanes. *LU*01* and *LU*02* were deduced from the presence or absence of 214-bp amplicons. From left to right: lane M: 100 bp DNA molecular weight marker. Lanes 1A and 1B correspond to *LU*01/02* genotype, 2A and 2B and 3A and 3B correspond to *LU*02/02* genotype, and lanes 4A and 4B are non-template controls.

donors, or about 0.9%, similar to two published Thai studies [18, 20]. However, additional genetic relationships have not yet been directly elucidated in Thai donors. The absence of genotype information is probably one of the reasons In(Lu) individuals present phenotype-genotype discrepancy, so the application of molecular methods in blood typing can identify *KLF1* alleles through discrepancy resolution [25].

In the present study, we determined the genotype frequencies for the *LU*01* and *LU*02* alleles. Three apparent Lu(a–b–) donors detected by two serological techniques – CTT and CAT – were *LU*02/02* genotype; thus, full-gene *KLF1* sequencing has been required for those donors. Subsequently, we identified three known variants [12] – c.304C>T (*KLF1*BGM12*), c.1001C>G (*KLF1*BGM67*) and c.519_525dupCGGCGCC (*KLF1*BGM34*) – which were responsible for an amino acid substitution, p.Ser102Pro (rs2072597), p.Thr334Arg (rs483352841), and a reading frameshift creating a premature stop codon, p.Gly176ArgfsX179 (rs483352838), respectively. The *KLF1*BGM34* allele has been described by three separate groups as associated with In(Lu) phenotype [26–28]. Vrignaud et al. [29] described the *KLF1*BGM67* allele, and it is noted that another allele associated with the In(Lu) phenotype, *KLF1*BGM48*, involves the same triallelic SNV (rs483352841), but involves c.1001C>T (p.Thr334Met) [30]. Interestingly, the *KLF1* variant alleles found in two donors are characterised by combinations of the above mentioned polymorphisms in *cis*, as c.[304T>C, 1001C>G] and c.[304T>C, 519_525dupCGGCGCC]. Although the effects of missense variant c.304T>C have been reported to cause the In(Lu) phenotype in the early publication, this variant has been shown by three groups to have no impact on Lu^a and Lu^b expression [26, 31, 32]. Helias et al. [26] showed c.304T>C is relatively frequent in general population and thus could not be responsible for the rare In(Lu) phenotype. The potential bias was tested, and no association was found between the mutation and In(Lu) phenotype. Moreover, the study by Keller et al. [31] has reported that this c.304T>C is not, by itself, associated with reduced expression of Lutheran antigens or the In(Lu) phenotype. Eernstman et al. [32] recorded that the c.304T>C distribution was in concordance with the frequencies observed in European populations, showing no correlation with the expression of CD44, band 3, Lu^a, Lu^b, HbF, and HbA2. In the current finding, we identified the c.304T>C variant with missense and frameshift variants, as mentioned above, which is in consonance with a previously published study of one Thai donor, c.[304T>C, 484insC] [25]. In contrast, homozygosity of the c.304T>C variant identified in the remaining donor did not seem to be a significant cause for the In(Lu) phenotype in this cohort. The haemagglutination test for this donor showed weakened expression (w+) of the P1 antigen by sensitive CAT, despite being the P^1/P^2 zygosity. Even though *KLF1* variants affect the expression of the P1 antigen [30], the strength of P1 reactivity could vary individually [3]. Currently, the variant c.304T>C is marked as obsolete in the table for Transcription Factor *KLF1* Alleles that caused the In(Lu) phenotype by the ISBT working party [12]. However, this study has not ruled out the XS2 phenotype in the donor that could be caused by mutations in the *GATA-1* gene [11]. The additional testing for the *GATA*BGM01* allele resulted in suggesting a further study.

The distribution of *LU*01/LU*02* genotype frequencies between known and unknown Lu(a) and Lu(b) phenotypes' populations in this cohort was like those expected in Asians [3] and those found in other Thai populations [18–20]. Both populations were in HWE and had a low MAF of the observed *LU*01* allele (<1%). Based on their MAF in this collection, the *LU*01* allele was confirmed as a rare variant among the Thai population. Even though the research on human genetic variation typically recommends a common MAF threshold at 5% to detect small effects [33], which would require substantially greater sample size of more than 1.1 billion Thai donors [1/(0.0003 × 0.0003)]. Only one donor was screened with the heterozygous *LU*01/02* genotype using the in-house PCR-SSP, which was confirmed by additional serology

test and DNA sequencing. Moreover, robustness showed that the PCR-SSP and DNA sequencing displayed 100% concordance for *LU*01*/*LU*02* genotyping with rounded 10% samples of all donors. Therefore, this in-house assay was successfully validated based on its robustness, good concordance, and genotyping performance compared to the gold-standard DNA sequencing method. Concerning the unknown Lu(a) and Lu(b) population, it would be possible to identify In(Lu) individuals possessing the *LU*02/02* genotype, Lu(a–b+), predicted using PCR-SSP. Therefore, this study recommended that Lu^a/Lu^b antigen typing and In(Lu) status of Thai donors should be determined using combined serological and molecular techniques.

In Thailand, the Lu(a)/Lu(b) phenotyping is not routinely performed, nor have those antigens been included in the screening panels. An identification of Lu^a/Lu^b antibodies has not been reported in transfusion-dependent Thai patients [34], which seems to be in concordance with the rarity of the Lu^a antigen in the Thai population but also because of the use of unspecified Lu^a/Lu^b antigens in panel cells. Less frequently, mild and delayed HTRs can be caused by Lu^a/Lu^b antibodies [4–6]. Nevertheless, the risk of Lu^a and Lu^b alloimmunization in Thais could be low if it almost never takes place. Severe HTRs caused by anti-AnWj, if it occurs, could be transfused with In(Lu) red cell units without any clinical signs or symptoms of haemolysis [35, 36]. In this case, donors exist in whom the In(Lu) is greater in estimated detection probability than the AnWj-negative phenotype in Thai populations. Hence, our In(Lu) detection approach will possess several advantages in terms of reducing the risk of complications and avoiding transfusion risks to effectively transfuse the most appropriate blood components.

Conclusions

This is the first report of Lu^a and Lu^b phenotype, allele, and genotype frequencies in Thai populations and confirms that a high frequency of the *LU*02* allele (Lu^b). We found two distinct characteristics of *KLF1* variant alleles, consisting of two alleles (c.[304T>C, 1001C>G] and c.[304T>C, 519_525dupCGGCGCC]) in Thai donors with serologically apparent Lu(a–b–) that lead to the In(Lu) phenotype, which is important in blood group profiling of donors. However, the limitations of this study are a lack of *GATA-1* investigation and AnWj antigen typing that might have been helpful in defining the phenotype of the apparent Lu(a–b–) samples studied. With well-established blood group genotyping, the application of genotyping and phenotyping methods has simultaneously been in use, at least to screen and confirm the rare Lu(a+) and In(Lu) phenotypes.

Statement of Ethics

Ethics approval for this study was approved by the Committee on Human Rights Related to Research Involving Human Subjects, Thammasat University, Pathumtani, Thailand (COE No. 010/2565).

Conflict of Interest Statement

All authors declare no conflict of interest.

Funding Sources

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Author Contributions

O.N. and K.I. are responsible for experiment design and development. P.K. is responsible for sample collection. K.I. is responsible for carrying out and analysing the phenotype and molecular detections. O.N. and K.I. are responsible for writing and editing the manuscript. All the authors have reviewed and approved the submitted version of the manuscript; the manuscript has neither been published nor is it under consideration for publication elsewhere.

Data Availability Statement

All data generated or analysed during this study are included in this article. Further enquiries can be directed to the corresponding author.

References

1 International Society of Blood Transfusion. Red cell immunogenetics and blood group terminology ISBT working party. 2022. Available from: https://www.isbtweb.org/isbt-working-parties/rcibgt.html.
2 Daniels G. Lutheran. Immunohematology. 2009;25(4):152–9.
3 Daniels G. Human blood groups. 3rd ed. Oxford: Blackwell Scientific; 2013.
4 Greenwalt TJ, Sasaki T. The Lutheran blood groups: a second example of anti-Lub and three further examples of anti-Lua. Blood. 1957 Nov;12(11):998–1003.
5 Klein HG, Anstee DJ. Blood transfusion in clinical medicine. 4th ed. Oxford: Blackwell Publishing; 2005.
6 Molthan L, Crawford MN. Three examples of anti-Lub and related data. Transfusion. 1966 Nov–Dec;6(6):584–9.

7 Parsons SF, Mallinson G, Daniels GL, Green CA, Smythe JS, Anstee DJ. Use of domain-deletion mutants to locate Lutheran blood group antigens to each of the five immunoglobulin superfamily domains of the Lutheran glycoprotein: elucidation of the molecular basis of the Lu(a)/Lu(b) and the Au(a)/Au(b) polymorphisms. Blood. 1997 Jun 1;89(11):4219–25.
8 El Nemer W, Rahuel C, Colin Y, Gane P, Cartron JP, Le Van Kim C. Organization of the human LU gene and molecular basis of the Lu(a)/Lu(b) blood group polymorphism. Blood. 1997 Jun 15;89(12):4608–16.
9 International Society of Blood Transfusion. Names for LU (ISBT005) blood group alleles. 2022. Available from: https://www.isbtweb.org/resource/005lu.html.
10 Norman PC, Tippett P, Beal RW. An Lu(a–b–) phenotype caused by an X-linked recessive gene. Vox Sang. 1986;51(1):49–52.
11 Singleton BK, Roxby DJ, Stirling JW, Spring FA, Wilson C, Poole J, et al. A novel GATA1 mutation (Stop414Arg) in a family with the rare X-linked blood group Lu(a–b–) phenotype and mild macrothrombocytic thrombocytopenia. Br J Haematol. 2013 Apr;161(1):139–42.
12 International Society of Blood Transfusion. Names for transcription factor KLF1 alleles. 2022. Available from: https://www.isbtweb.org/resource/klf1.html.
13 Fraser NS, Knauth CM, Moussa A, Dean MM, Hyland CA, Perkins AC, et al. Genetic variants within the erythroid transcription factor, KLF1, and reduction of the expression of lutheran and other blood group antigens: review of the In(Lu) phenotype. Transfus Med Rev. 2019 Apr;33(2):111–7.
14 Casper J, Zweig AS, Villarreal C, Tyner C, Speir ML, Rosenbloom KR, et al. The UCSC genome browser database: 2018 update. Nucleic Acids Res. 2018 Jan 4;46(D1):D762–9.
15 Tallack MR, Magor GW, Dartigues B, Sun L, Huang S, Fittock JM, et al. Novel roles for KLF1 in erythropoiesis revealed by mRNA-seq. Genome Res. 2012 Dec;22(12):2385–98.
16 Yung CH, Chow MP, Hu HY, Mou LL, Lyou JY, Lee TD. Blood group phenotypes in Taiwan. Transfusion. 1989 Mar–Apr;29(3):233–5.
17 Karczewski KJ, Francioli LC, Tiao G, Cummings BB, Alföldi J, Wang Q, et al. The mutational constraint spectrum quantified from variation in 141, 456 humans. Nature. 2020 May;581(7809):434–43.
18 Chandanayingyong D, Sasaki TT, Greenwalt TJ. Blood groups of the Thais. Transfusion. 1967 Jul–Aug;7(4):269–76.
19 Nathalang O, Kuvanont S, Punyaprasiddhi P, Tasaniyanonda C, Sriphaisal T. A preliminary study of the distribution of blood group systems in Thai blood donors determined by the gel test. Southeast Asian J Trop Med Public Health. 2001 Mar;32(1):204–7.
20 Bejrachandra S, Nathalang O, Saipin J, Kuvanont S, Wichitchinda K, Vongpattranon A. Distribution of blood group systems in Thai blood donors determined by gel test. Siriraj Hosp Gaz. 2002 July;54(7):403–9.
21 Thinley J, Nathalang O, Chidtrakoon S, Intharanut K. Blood group P1 prediction using multiplex PCR genotyping of A4GALT among Thai blood donors. Transfus Med. 2021 Feb;31(1):48–54.
22 Owaidah AY, Naffaa NM, Alumran A, Alzahrani F. Phenotype frequencies of major blood group systems (Rh, kell, kidd, duffy, MNS, P, lewis, and lutheran) among blood donors in the eastern region of Saudi arabia. J Blood Med. 2020 Feb 13;11:59–65.
23 Thakral B, Saluja K, Sharma RR, Marwaha N. Phenotype frequencies of blood group systems (Rh, Kell, Kidd, Duffy, MNS, P, Lewis, and Lutheran) in north Indian blood donors. Transfus Apher Sci. 2010 Aug;43(1):17–22.
24 Yu Y, Ma C, Sun X, Guan X, Zhang X, Saldanha J, et al. Frequencies of red blood cell major blood group antigens and phenotypes in the Chinese Han population from Mainland China. Int J Immunogenet. 2016 Aug;43(4):226–35.
25 Garcia-Sanchez F, Pardi C, Kupatawintu P, Thornton N, Rodriguez MA, Lucea I, et al. Identification of new KLF1 and LU alleles during the resolution of Lutheran typing discrepancies. Transfusion. 2016 Jun;56(6):1413–8.
26 Helias V, Saison C, Peyrard T, Vera E, Prehu C, Cartron JP, et al. Molecular analysis of the rare in(Lu) blood type: toward decoding the phenotypic outcome of haploinsufficiency for the transcription factor KLF1. Hum Mutat. 2013 Jan;34(1):221–8.
27 Wang Z, Luo G, Ji Y. A novel 519_525dup mutation of KLF1 gene identified in a Chinese blood donor with Lu(a–b–) phenotype. Transfusion. 2013 Jul;53(7):1619–20.
28 Yang M, Kuo Y, Hung Y, Hsieh C, Hung C, Lin K. An insertion in EKLF/KLF1 gene causes in (Lu) blood group. Transfusion. 2012 Sep;52(Suppl 3):161A–2A.
29 Vrignaud C, Ramelet S, Lecam S, Calloix M, Hennion M, Azouzi S, et al. Characterization of 7 novel KLF1 alleles responsible for a rare In(Lu) phenotype in French blood donors. Transfusion. 2020 Sep;60(Suppl 5):13A–4A.
30 Kawai M, Obara K, Onodera T, Enomoto T, Ogasawara K, Tsuneyama H, et al. Mutations of the KLF1 gene detected in Japanese with the In(Lu) phenotype. Transfusion. 2017 Apr;57(4):1072–7.
31 Keller J, Vege S, Horn T, Keller MA, Leger RM, Aeschlimann J, et al. Novel mutations in KLF1 encoding the In(Lu) phenotype reflect a diversity of clinical presentations. Transfusion. 2018 Jan;58(1):196–9.
32 Eernstman J, Veldhuisen B, Ligthart P, von Lindern M, van der Schoot CE, van den Akker E. Novel variants in Krueppel like factor 1 that cause persistence of fetal hemoglobin in In(Lu) individuals. Sci Rep. 2021 Sep 17;11(1):18557.
33 Hirschhorn JN, Daly MJ. Genome-wide association studies for common diseases and complex traits. Nat Rev Genet. 2005 Feb;6(2):95–108.
34 Intharanut K, Bejrachandra S, Nathalang S, Leetrakool N, Nathalang O. Red cell genotyping by multiplex PCR identifies antigen-matched blood units for transfusion-dependent Thai patients. Transfus Med Hemother. 2017 Sep;44(5):358–64.
35 de Man AJ, van Dijk BA, Daniels GL. An example of anti-AnWj causing haemolytic transfusion reaction. Vox Sang. 1992;63(3):238.
36 Xu Z, Duffett L, Tokessy M, Cote J, Goldman M, Saidenberg E. Anti-AnWj causing acute hemolytic transfusion reactions in a patient with aplastic anemia. Transfusion. 2012 Jul;52(7):1476–81.

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Circulating Iron in Patients with Sickle Cell Disease Mediates the Release of Neutrophil Extracellular Traps

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Keywords

Sickle cell disease · Hemolysis · Iron · Neutrophils · Neutrophil extracellular traps

Abstract

Introduction: Neutrophils promote chronic inflammation and release neutrophil extracellular traps (NETs) that can drive inflammatory responses. Inflammation influences progression of sickle cell disease (SCD), and a role for NETs has been suggested in the onset of vaso-occlusive crisis (VOC). We aimed to identify factors in the circulation of these patients that provoke NET release, with a focus on triggers associated with hemolysis. ***Methods:*** Paired serum and plasma samples during VOC and steady state of 18 SCD patients (HbSS/HbSβ^0-thal and HbSC/HbSβ$^+$-thal) were collected. Cell-free heme, hemopexin, and labile plasma iron have been measured in the plasma samples of the SCD patients. NETs formation by human neutrophils from healthy donors induced by serum of SCD patients was studied using confocal microscopy and staining for extracellular DNA using Sytox, followed by quantification of surface coverage using ImageJ. ***Results:*** Eighteen patients paired samples obtained during VOC and steady state were available (11 HbSS/HbSβ^0-thal and 7 HbSC/HbSβ$^+$-thal). We observed high levels of systemic heme and iron, concomitant with low levels of the heme-scavenger hemopexin in sera of patients with SCD, both during VOC and in steady state. In our in vitro experiments, neutrophils released NETs when exposed to sera from SCD patients. The release of NETs was associated with high levels of circulating iron in these sera. Although hemin triggered NET formation in vitro, addition of hemopexin to scavenge heme did not suppress NET release in SCD sera. By contrast, the iron scavengers deferoxamine and apotransferrin attenuated NET formation in a significant proportion of SCD sera. ***Discussion:*** Our results suggest that redox-active iron in the circulation of non-transfusion-dependent SCD patients activates neutrophils to release NETs, and hence, exerts a direct pro-inflammatory effect. Thus, we propose that chelation of iron requires further investigation as a therapeutic strategy in SCD.

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Introduction

Sickle cell disease (SCD) is a monogenetic disease wherein a single point mutation in β-globin gives rise to sickle hemoglobin (HbS), affecting millions of people worldwide [1]. Deoxygenation results in intracellular hemoglobin (Hb) polymerization inducing the characteristic cellular shape of sickle erythrocytes. Vaso-occlusion and chronic hemolysis are predominating vascular events that contribute to the pathogenesis of SCD [2]. Vaso-occlusion is thought to trigger acute systemic painful vaso-occlusive crises (VOCs), which is the most common complication of SCD and the leading cause of hospitalization of SCD patients [1, 3]. A role for neutrophils in promoting vaso-occlusion in SCD mice was first demonstrated by Turhan et al. [4]. Later, activated neutrophils were shown to interact with the inflamed vessel wall in vivo, and to adhere to erythrocytes promoting VOC in SCD mice [5]. Also, circulating neutrophils were shown to be primed or constitutively activated in SCD patient [6].

Experimental evidence suggests that hemolysis in SCD mice triggers neutrophil activation and the formation of neutrophil extracellular traps (NETs) within the pulmonary microcirculation, which led to acute lung injury and death of these mice [7]. Earlier, we found levels of neutrophil elastase-α-1-antitrypsin complexes and nucleosomes to be elevated in plasma of SCD patients during VOC, suggesting that neutrophils release NETs in SCD patients [8]. More recently, circulating NET fragments have been shown to contribute to acute chest syndrome in SCD mice [9]. Remarkably, therapeutic neutralization of heme attenuated pulmonary NET release, prevented NET-associated hypothermia and rescued SCD mice from acute chest syndrome and death [7, 10]. In turn, SCD mice receiving heme developed acute chest syndrome, further supporting a central role of heme in systemic vaso-occlusion [10]. Altogether, these findings suggest that therapeutic administration of the heme-scavenging plasma protein hemopexin (Hpx) would protect SCD patients from vaso-occlusion. The therapeutic benefit of Hpx administration to improve the outcome of VOC has been shown in SCD mice [10, 11]. Yet, whether heme instigates clinically high-risk VOC, its cellular mediators herein, and hence, its full therapeutic value is still not clear. Here, we hypothesize a role for iron in SCD patients that had not previously been considered. Using therapeutic blockade by chelation of iron, we show that depletion of iron, and not heme, prevents the release of NETs in SCD patient's blood.

Materials and Methods

Patients

A full description of the patients in our study has been published previously [12]. In brief, consecutive adult patients with SCD admitted for VOC at the Academic Medical Center Amsterdam or the Slotervaart Hospital, Amsterdam, The Netherlands, were approached for inclusion. Patients diagnosed with sickle cell anemia or a compound heterozygous state HbSβ^0-thalassemia, HbSβ$^+$-thalassemia, or HbSC was eligible. Patients were excluded when on hydroxyurea therapy. The study protocol was approved by the Medical Ethical Committee of the participating centers and conducted in agreement with the Helsinki declaration. Written consent was obtained from each participant or their legal guardian. Serum samples and neutrophils of healthy donors (controls) have been obtained from Sanquin. Blood samples were taken by venipuncture. Blood serum tubes of SCD patients were centrifuged for 15 min at 3,000×g at 4°C to obtain serum and stored in aliquots at −80°C until further analysis.

Laboratory Analysis

Total serum heme was measured with a colorimetric method according to the manufacturer's instruction (Quantichrom heme assay kit, Fischer Scientific). Serum Hpx (purified from human plasma with high-performance liquid chromatography) and IgM were determined by ELISA (in-house, see online suppl. Data; see www.karger.com/doi/10.1159/000526760 for all online suppl. material). Purified Hpx was used as standard for the measurement of serum Hpx. Labile plasma iron (LPI) was measured by dihydrorhodamine (Sigma Aldrich) fluorescence as described previously (see online suppl. Data). The differences in the rate of dihydrorhodamine oxidation with and without the iron-chelator deferoxamine (DFO; Sigma Aldrich) represent the fraction of plasma non-transferrin-bound-iron that is redox active. Neutrophil isolation, stimulation, and inhibition of NET formation, analysis of NET formation by neutrophils, immunostaining of NET components, and determination of reactive oxygen species (ROS) production are described in detail in the online supplementary data [13, 14].

Statistical Analysis

Statistical analysis was performed with GraphPad Prism software (version 6.0). Data are presented as mean ± SD of independent experiments. Paired student's *t* test or Wilcoxon matched-pairs signed rank test was used to compare two groups. For comparing more than two groups, one-way ANOVA with Tukey's multiple comparisons post hoc test or Kruskal-Wallis with Dunn's post hoc testing was used where appropriate. Spearman correlation coefficients were calculated to correlate two variables. A *p* value of 0.05 or less was considered statistically significant.

Results

From 18 patients, paired samples obtained during VOC and steady state were available (11 sickle cell anemia/HbSβ^0-thalassemia and 7 HbSC/HbSβ$^+$-thalassemia). Patients with SCD show reduced levels of the plasma scavenger for heme, Hpx – both in steady state and VOC – compared to healthy donors (shown in Fig. 1a). High systemic levels of heme were found in patients with SCD. Upon comparison of cell-free heme levels in healthy do-

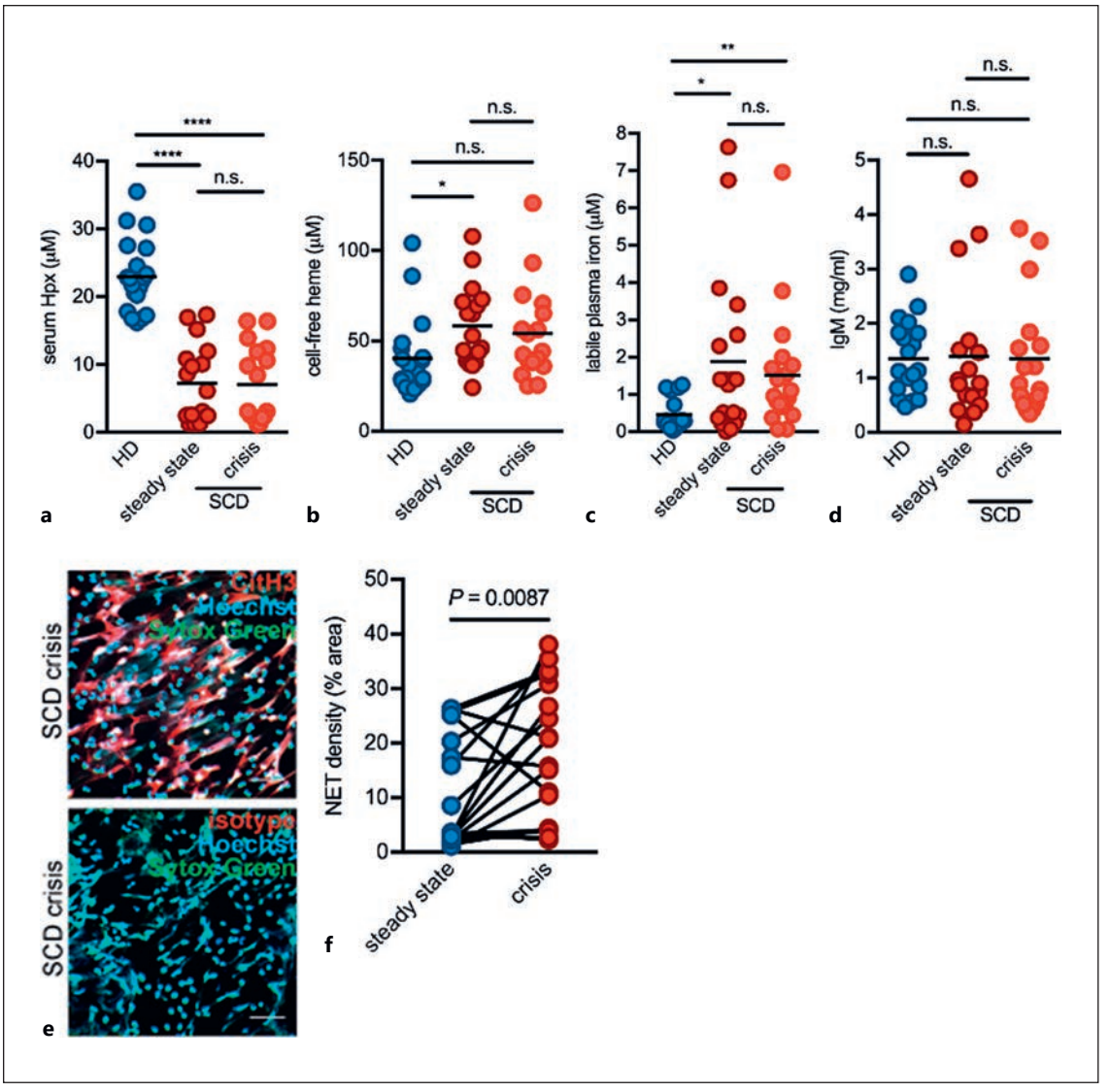

Fig. 1. Neutrophils release more NETs when exposed to sera of patients obtained during VOC compared to the steady state. **a** Serum levels of Hpx in healthy control (HD; $n = 20$) and SCD patients in the steady state and VOC ($n = 17$/group). One-way ANOVA with Tukey's multiple comparisons post hoc test was used. ****$p < 0.0001$. **b** Levels of serum-free heme in HD ($n = 20$) versus patients with SCD in the steady state and VOC ($n = 17$ in each group). A Kruskal-Wallis test with Dunn's post hoc testing was used. *$p < 0.05$. n.s. indicates not significant. **c** Levels of LPI in HD ($n = 19$) versus patients with SCD in the steady state and VOC ($n = 19$ for each group). We used a Kruskal-Wallis test with Dunn's post hoc testing for statistical analysis. *$p < 0.05$, **$p < 0.01$. n.s. indicates not significant. **d** Levels of IgM in serum of HD ($n = 20$) and SCD patients in the steady state and VOC ($n = 17$/group). **e** Immunostaining for citrullinated histone H3 (CitH3) after exposure to SCD serum. Neutrophils from a HD were exposed to serum from a SCD patient in VOC for 180 min. Then, extracellular DNA was stained with Sytox Green (green). Immunostaining was performed on NETs induced by 3 different SCD sera. Representative images are shown. Scale bar, 50 μm. Original magnifications ×40. **f** Quantification of extracellular DNA release in response to sera from SCD patients. A Wilcoxon matched-pairs signed rank test was used for statistical analysis. Incubations of neutrophils with paired SCD sera ($n = 18$) were performed with neutrophils from 3 different HD for each subject group (steady state and crisis). **$p = 0.0087$.

nor plasma and in plasma of SCD patients tested, cell-free heme levels were significantly increased in steady state SCD, though failed to reach significance in VOC as compared to healthy controls (shown in Fig. 1b). In addition, levels of LPI were significantly elevated in samples from SCD patients in steady state and VOC as compared to samples from healthy donors (shown in Fig. 1c), although no significant differences have been observed between LPI levels in VOC and steady state, respectively. Differences in levels of Hpx, heme, and LPI that are caused by hemodilution due to therapeutic hyperhydration during VOC have been excluded by the measurement of IgM levels (shown in Fig. 1d). To determine whether circulating plasma factors in samples obtained from patients with

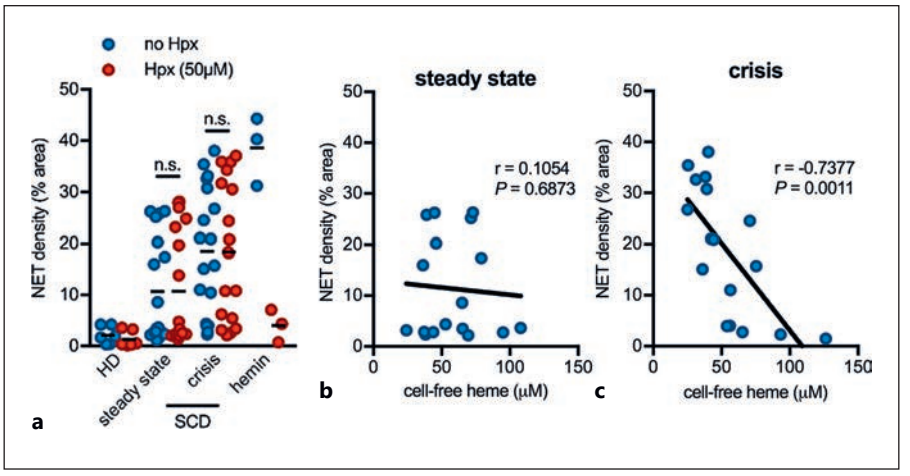

Fig. 2. Plasma-purified hemopexin does not prevent the release of NETs in sera of patients with SCD. **a** Quantification of NET release in response to sera from SCD patients in the presence of plasma-derived Hpx. The densities of extracellular NET-DNA over the image area (i.e., the number of Sytox Green+ pixels divided by the total number of pixels × 100) were determined for paired sera from patients with SCD in the presence or absence of 50-μM plasma-purified Hpx. A Wilcoxon matched-pairs signed rank test was used to compare NET release in response to SCD sera in the presence or absence of Hpx. Incubations of neutrophils with HD (n = 6) or SCD sera (n = 18) were performed with neutrophils from 3 different HD for each subject group. n.s. indicates not significant. **b**, **c** Correlations between levels of circulating heme and NET release in sera from SCD patients (n = 17) in the steady state (**b**) and crisis (**c**). A Spearman test was used to calculate correlation coefficients. p = 0.6873 and $^{**}p$ = 0.0011 in the steady state and crisis, respectively.

SCD induce NET release, we exposed healthy donor neutrophils to sera samples. After 3 h, NETs were detected with a cell-impermeable DNA-binding dye (Sytox Green) and imaged with fluorescence microscopy as described previously [15]. No extracellular DNA was released upon incubation of neutrophils with control serum from healthy donor as evidenced by the absence of Sytox Green staining (shown in online suppl. Fig. S1). By contrast, thread-like NET structures that stained positive for Sytox Green became visible upon incubation of neutrophils with SCD serum (shown in Fig. 1e, suppl. Fig. S3). Here, immunostaining of the NET component citrullinated histone H3 confirmed the identity of NETs. NET release induced by factors in SCD sera was highly reproducible, even when neutrophils from different donors were used (shown in online suppl. Fig. S2). In our hands, priming of neutrophils with TNF-α, as was used by Chen and coworkers [7], was not required to trigger NET formation with SCD serum. The surface area covered by NETs was quantified in paired sera obtained during steady state and VOC (shown in Fig. 1f) as previously described [15, 16]. While NETs were also released in some SCD sera obtained during steady state, NET formation was significantly enhanced in sera obtained during VOC (p = 0.0087).

It was recently suggested that the release of NETs in response to free heme may contribute to vaso-occlusion in a mouse model of SCD [7], and the administration of Hpx improved the outcome of TNF-α induced VOC. To evaluate the effect of Hpx on heme and NET formation in human samples, we purified Hpx from human plasma. Plasma-purified Hpx was functional as it reversed hemin-induced cytotoxicity of HEK-293 cells as determined by standard lactate dehydrogenase release (see suppl. Data and online suppl. Fig. S5a). Similarly, Hpx abolished hemin-induced generation of ROS in neutrophils as measured by the conversion of luminol chemiluminescence (see online suppl. Data, online suppl. Fig. S5b). Then, healthy neutrophils were incubated with hemin, and indeed NETs were formed (shown in online suppl. Fig. S5c). Again, positive immunostaining of the NET components neutrophil elastase and citrullinated histone H3 was found within these NETs (shown in online suppl. Fig. S4). In inhibitor studies, we observed minimal release of extracellular DNA from neutrophils stimulated with hemin when equimolar amounts of plasma-derived Hpx were added (shown in online suppl. Fig. S5c). Albumin also binds to heme, although it binds heme with lower affinity than Hpx [17]. When we added hemin in the presence of 1% normal human serum or 0.5% human serum albumin (HSA), the release of NETs was almost completely impaired (shown in online suppl. Fig. S5b, d). NET release induced by hemin or PMA in the presence or absence of either 1% normal human serum or 0.5% HSA was quantified, and we observed that each negated hemin-induced NET formation, while PMA-induced NET formation was unaffected by the presence of albumin (shown in online suppl. Fig. S5e). Thus, it appears that the presence of albumin is sufficient to prevent the interaction of heme with neutrophils and NET forma-

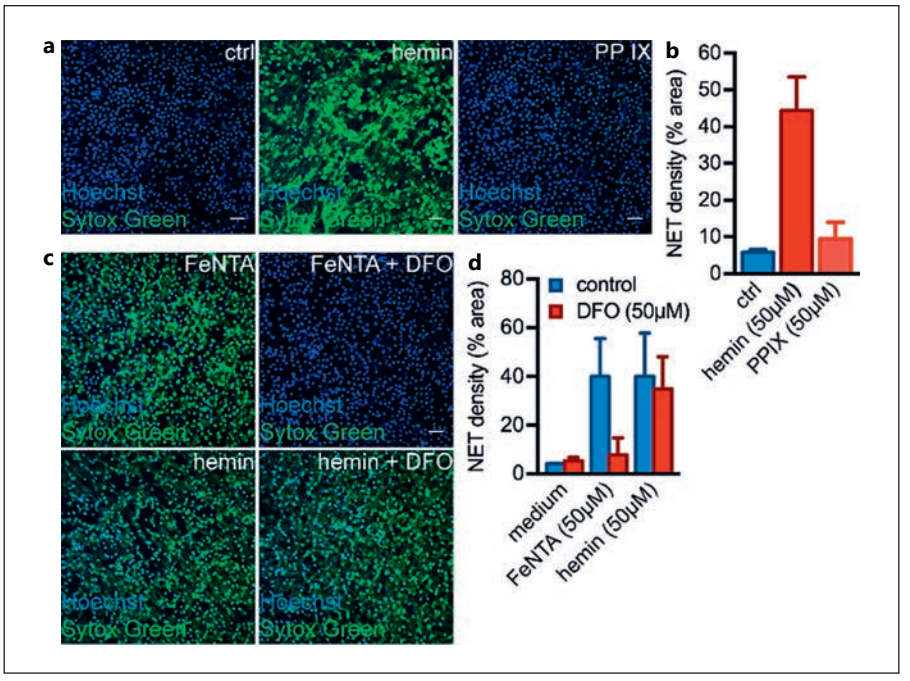

Fig. 3. Iron triggers NET formation, and iron-mediated NET release is blocked by chelation with deferoxamine. **a** Neutrophils isolated from HD were incubated with medium alone (ctrl) or challenged with 50-μM hemin or protoporphyrin IX (PPIX) for 180 minutes. Release of NETs (green in these images) was detected by fluorescence imaging with confocal microscopy using a mixture of 2 DNA-labeling dyes, one cell impermeable (Sytox Green, green) and the other cell permeable (Hoechst 33,342, blue). Depicted are merged images of green and blue fluorescence. All images are representative of 2 independent experiments using neutrophils from different HD. Scale bars, 50 μm. **b** NET formation was quantified after exposure to hemin or PPIX. The densities of extracellular NET-DNA over the image area (i.e., the number of Sytox Green+ pixels divided by the total number of pixels × 100) were determined after the challenge with hemin or PPIX and depicted as mean NET density ± SD in 2 separate experiments. **c** In 2 independent experiments, neutrophils from a HD were exposed to 50-μM FeNTA or hemin in the presence or absence of equimolar amounts of deferoxamine (DFO). After 180 minutes, NETs (green in these images) were visualized with confocal fluorescence microscopy as in panel **a**. Depicted are representative images in which Sytox Green (green) and Hoechst 33,342 (blue) fluorescence are merged. Scale bars, 50 μm. **d** NET release was quantified as in panel b and depicted as mean NET density ± SD (n = 2). Original magnifications ×20 for panels **a**, **c**.

tion. Strikingly, when SCD serum obtained in VOC was preincubated with relatively high levels of Hpx (50 μm) before exposure to neutrophils, NET release was not affected (shown in online suppl. Fig. S5f).

The effect of Hpx supplementation on NET formation was then quantified for all paired SCD sera in our study. Unexpectedly, the addition of high concentrations of Hpx (the normal plasma Hpx concentration is ~ 20 μM) did not alter NET release in the SCD patient sera tested (shown in Fig. 2a). In line with this, high levels of cell-free heme did not positively correlate with the extent of NET formation induced by SCD sera in our cohort (shown in Fig. 2b, c).

In order to investigate the role of iron in the induction of NETs, we compared NET induction of heme to protoporphyrin IX (PPIX) – a porphyrin without iron moiety. Interestingly, PPIX did not induce NET formation suggesting that the iron moiety is required for this process (shown in Fig. 3a, b). We further explored the involvement of iron in NET formation using ferric nitrilotriacetate (Fe^{3+}-NTA). Indeed, incubation of neutrophils with FeNTA triggered NET formation (shown in Fig. 3c, d). In inhibitor studies, FeNTA-induced NET release was prevented with equimolar amounts of iron chelator DFO. Concentrations of 300-μM DFO have previously been shown to provoke NET release by human neutrophils [18]; however, in clinical use, DFO achieves plasma levels of 10 μm in transfusion-dependent patients who receive chelation therapy [19]. At the concentration of 50-μm DFO that we used to supplement sera, we have not observed the formation of NETs. Likewise, DFO had no effect on hemin-induced NET formation since it is not able to remove iron directly from hemin [20, 21] (shown in Fig. 3c, d).

To determine whether iron in the SCD samples in our study may provoke NET release, we tested whether preincubation with DFO or the specific iron-binding protein apotransferrin (apoTf) would affect NET release in SCD sera. We supplemented SCD sera with DFO and apoTf at a concentration of 50 μM, thus, in large excess over the

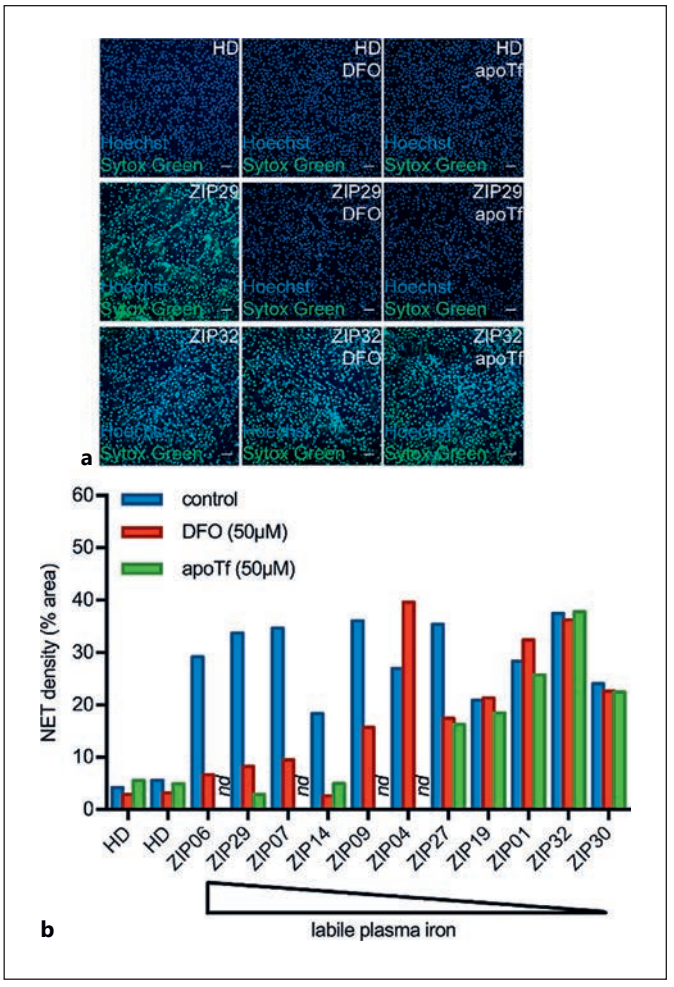

Fig. 4. Iron chelation abrogates NET release in sera from SCD patients. **a** Neutrophils from a HD were exposed to serum from a nonautologous HD or patients with SCD during VOC for 180 minutes in the presence or absence of deferoxamine (DFO, 50 μM) or apotransferrin (apoTf, 50 μM). Release of NETs (green in these images) was visualized with confocal fluorescence microscopy using 2 DNA-labeling dyes, one cell impermeable (Sytox Green), and the other cell permeable (Hoechst 33342). Depicted are merged images of Sytox Green (green) and Hoechst 33342 (blue) fluorescence. All images are representative of experiments performed with sera from 11 different patients. Scale bars, 50 μm. Original magnifications. ×20. **b** Quantification of NET release in response to sera from SCD patients in the presence of iron chelators. The densities of extracellular NET-DNA over the image area (i.e., the number of Sytox Green$^+$ pixels divided by the total number of pixels × 100) were determined for sera from patients with SCD during VOC in the presence or absence of 50-μM DFO or apoTf. Incubations of neutrophils with SCD sera (n = 11) were performed with neutrophils from 3 different HD for each subject group. n.d. indicates not determined.

concentrations of LPI. For these experiments, we focused on SCD patient sera obtained during VOC that had given rise to NETs shown in Figures 1 and 2. The addition of DFO largely abolished (ZIP06, 07, 14, 29) or partly inhibited (ZIP09, 27) the release of NETs in more than half of the sera of SCD patients tested (shown in online suppl. Fig. S5a, b). When DFO was added to the other sera, no effect was observed. Thus, it appears that iron provokes NET release in a significant proportion of SCD patient sera but not all. Indeed, upon ranking the patients according to the level of LPI, it became apparent that DFO addition affected NET release in those sera with high iron (shown in Fig. 4a). As DFO may bind both extra- and intracellular iron, and intracellular iron may directly influence ROS generation and potentially NET formation; we also tested the effect of apoTf on NET formation in SCD sera as it acts on extracellular iron. Unfortunately, we did not have sufficient serum left of all patients in our study to also screen for the effect of apoTf addition. Interestingly, for the samples that have some material left, we observed that apoTf addition appeared to match the effect of DFO on NET release (shown in Fig. 4b). These results indicate that, at least in a subset of patients with SCD, NET release may be induced through the presence of extracellular iron in the blood.

Conclusion

In this study, we reveal a novel role for iron in the circulation of patients with SCD. Our study highlights that the systemic redox-active iron may form an important trigger for neutrophil activation and NET formation in SCD. In our experiments, supplementation of sera from SCD patients with the heme-scavenger Hpx did not prevent the release of NETs from healthy donor neutrophils. By contrast, the addition of DFO or apoTf to scavenge free iron abolished NET release in a significant proportion of SCD sera tested.

In a number of murine models of hemolytic diseases, including SCD and β-thalassemia, heme induced inflammation and tissue damage, an effect that was prevented by injection of heme scavengers [7, 10, 11, 22]. As such, the administration of Hpx has previously shown great potential as novel therapeutic drug. Vinchi et al. [22] showed a beneficial effect of Hpx administration in SCD mice, as it reduced endothelial activation induced by heme. Moreover, in another study, it was shown that hemin injection in SCD mice induced the development of acute chest syndrome and that both TLR4 inhibition and Hpx administration prevented acute chest syndrome development [10]. Of interest, Vinchi et al. [22] have recently shown that hemin-challenged Hpx knockout mice showed signs of heme accumulation in macrophages, and phenotype switching to a pro-inflammatory M1-like phenotype was observed. The latter effect was also found in SCD mice and prevented by Hpx administration [11]. Worth noting, in SCD animal models signs of VOC are often induced by injection of exogenous free hemin. However,

during in vivo hemolysis it is uncertain whether heme, which is a highly hydrophobic molecule, exists as a free form in plasma as it is rapidly sequestered by plasma proteins and lipids [23]. It thus seems plausible that Hpx administration shows greatest benefit in models with administered free hemin where concentrations of free heme are a least transiently increased. By contrast, administration of Hpx to TNF-α-treated SCD mice to lower plasma heme levels prevented pulmonary NET release and ameliorated-associated hypothermia [7]. However, we and others [7] have shown that albumin can efficiently prevent NET formation in response to hemin in vitro, and it remains to be elucidated whether treatment of TNF-α-treated mice with albumin would have similar effects on the phenotype of these mice. Indeed, studies on mice lacking Hpx reinforce the concept of redundancy, overlap, and backup in heme transport provided by Hpx, haptoglobin, and albumin. In the presence of normal albumin levels, Hpx-null mice exhibit a healthy state and a lack of general organ damage [24]. Another important consideration is that heme induces various pro-inflammatory effects other than neutrophil activation and NET formation through interactions with other cells, including effects of heme on macrophages and endothelial cells. Secondary anti-inflammatory effects of Hpx that go beyond the scavenging of heme have also been described [25]. Here, we show that increased levels of circulating heme in patients with SCD do not directly promote NET release but that NET release requires the iron moiety.

The observed role of plasma iron in neutrophil activation is in line with previous in vitro studies, although none of these studies were performed in the context of SCD or investigated LPI in the circulation. Interestingly, Kono et al. [26] have shown that the addition of deferasirox, an iron chelating agent, to neutrophils prevented PMA- or fMLP-mediated ROS production and NET formation in vitro [26, 27]. Saha et al. [27] have shown that this suppressive effect of deferasirox is through chelation of intracellular labile iron that is required for neutrophil oxidative responses and NET release, and can be mimicked by enterobactin, a siderophore expressed by *Escherichia coli* [28]. Previous results had indicated that hemin induces TLR4 signaling to drive pro-inflammatory responses [29, 30]. Recently, however, hemin was shown to provoke NET release in a manner that depends on NADPH oxidase activity and ROS generation but does not require TLR4 signaling [31]. Taken together, these results suggest that heme does not interact with TLR4 to activate neutrophils for NET release but rather that the redox activity of the iron moiety in heme underlies heme-induced NET formation. Indeed, we show that exposure of neutrophils to hemin but not PPIX that triggered the release of NETs, and that the addition of exogenous iron sources, such as FeNTA leads to NET formation.

The recent literature suggests that free, redox-active iron is readily available in the circulation of patients with SCD. Levels of systemic iron were recently found to be elevated in patients with SCD, even in steady state. Consistently, we observed high levels of circulating heme and redox-active iron, which is associated with low Hpx in patients with SCD in steady state. Iron overload is well known to occur in chronically transfused SCD patients who receive prophylactic red-cell transfusions [32–35]. However, patients who have received a blood transfusion in 3 months prior to the development of VOC were excluded from inclusion in our study cohort. Nevertheless, high LPI was found in several SCD patients in our cohort. Thus, it appears that increased LPI levels are caused by continuous hemolysis and thus may also occur in patients who are not on a chronic transfusion scheme [35]. Recently, however, it was shown that chronic hemolysis in SCD mice maintained enhanced iron export and higher levels of circulating iron compared to normal mice [36]. Indeed, excessive release of heme facilitates the export of cellular iron by ferroportin [37–39], and intracellular iron levels are decreased in peripheral blood mononuclear cells from SCD patients [40].

Our results show that the ex vivo addition of the iron scavengers DFO and apoTf limited the NET-inducing effect of labile iron present in a significant proportion of SCD sera tested. The concentration of DFO used was based on a previous report where levels of up to 10-μm DFO were detected in vivo [19]. Clearly, iron chelation did not prevent NET release in all samples from SCD patients, and it is possible that other plasma factors could be involved in NET formation in DFO-insensitive patients. Complement activation, IL-8, and urate crystals are established NET inducers that we hypothesize to play a role in these DFO-insensitive SCD patient samples [15, 41–47].

Our study harbors some limitations that need to be addressed: first, the observation on iron as a trigger of NET formation in SCD is restricted to a small patient group. Second, it also remains unclear why specific iron neutralization only prevented NET formation in a part and not all of the sickle cell patients investigated. This suggests that besides iron other inflammatory mediators (e.g., complement activation products, IL-8, or urate crystals) present in the serum are responsible for NET induction of these patients. Investigating the NET-forming capacity of the serum of sickle cell patients before starting therapeutic iron chelation compared to serum samples when on therapy may give more insights on the clinical relevance of our findings. The effect of iron neutralization on NET formation should further be explored in a mouse model. Further research is required to determine which stimuli induced NET release in patient sera in which targeting iron was less or not effective.

In summary, we show that labile iron plays a role in NET formation in a subset of sera from a cohort of SCD patients and that iron chelation prevents NET formation. Extrapolation to a larger study requires experimental validation. Future studies aimed at validating the therapeutic efficacy of iron chelation therapy as potential novel therapy for VOC in patients with SCD are warranted. As DFO is widely used in patients with chronic iron overload disorders and transfusional iron overload, its use would form a readily available treatment strategy to prevent neutrophil activation, dampen formation of NETs, and possibly the development of VOC.

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Statement of Ethics

The study protocol was approved by the Medical Ethical Committee of the participating centers and conducted in agreement with the Helsinki declaration. Written consent was obtained from each participant or their legal guardian.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Kristof van Avond, Brenda Luken, and Sacha Zeerleder conceived and designed the study, and performed data analysis and interpretation. Kristof van Avond, Ingrid Bulder, and Gerard van Mierlo performed experiments. Marein Schmimmel, Erfan Nur, and Bart Biemond coordinated the collection of patient material. Robin van Bruggen and Sacha Zeerleder obtained grant funding. Kristof van Avond wrote the manuscript. Marein Schimmel, Erfan Nur, Bart Biemond, Brenda Luken, Robin van Bruggen, and Sacha Zeerleder critically reviewed the manuscript. All authors approved the final version of the manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its online supplementary material. Further inquiries can be directed to authors.

References

1 Rees DC, Williams TN, Gladwin MT. Sickle-cell disease. Lancet. 2010 Dec 11;376(9757):2018–31.
2 Zhang D, Xu C, Manwani D, Frenette PS. Neutrophils, platelets, and inflammatory pathways at the nexus of sickle cell disease pathophysiology. Blood. 2016 Feb 18;127(7):801–9.
3 Novelli EM, Gladwin MT. Crises in sickle cell disease. Chest. 2016 Apr;149(4):1082–93.
4 Turhan A, Weiss LA, Mohandas N, Coller BS, Frenette PS. Primary role for adherent leukocytes in sickle cell vascular occlusion: a new paradigm. Proc Natl Acad Sci U S A. 2002 Mar 5;99(5):3047–51.
5 Koehl B, Nivoit P, El Nemer W, Lenoir O, Hermand P, Pereira C, et al. The endothelin B receptor plays a crucial role in the adhesion of neutrophils to the endothelium in sickle cell disease. Haematologica. 2017 Jul;102(7):1161–72.
6 Lum AFH, Wun T, Staunton D, Simon SI. Inflammatory potential of neutrophils detected in sickle cell disease. Am J Hematol. 2004 Jun;76(2):126–33.
7 Chen G, Zhang D, Fuchs TA, Manwani D, Wagner DD, Frenette PS. Heme-induced neutrophil extracellular traps contribute to the pathogenesis of sickle cell disease. Blood. 2014 Jun 12;123(24):3818–27.
8 Schimmel M, Nur E, Biemond BJ, van Mierlo GJ, Solati S, Brandjes DP, et al. Nucleosomes and neutrophil activation in sickle cell disease painful crisis. Haematologica. 2013 Nov 1;98(11):1797–803.
9 Vats R, Tutuncuoglu E, Tejero J, Hillery CA, Gladwin MT, Sundd P. Circulating neutrophil extracellular traps in the pathogenesis of acute chest syndrome of sickle cell disease. Blood. 2019 Nov 13;134(Suppl 1):3556.
10 Ghosh S, Adisa OA, Chappa P, Tan F, Jackson KA, Archer DR, et al. Extracellular hemin crisis triggers acute chest syndrome in sickle mice. J Clin Invest. 2013 Nov 1;123(11):4809–20.
11 Vinchi F, Costa da Silva M, Ingoglia G, Petrillo S, Brinkman N, Zuercher A, et al. Hemopexin therapy reverts heme-induced proinflammatory phenotypic switching of macrophages in a mouse model of sickle cell disease. Blood. 2016 Jan 28;127(4):473–86.
12 Schimmel M, Luken BM, Nur E, van Tuijn CFJ, Sins JW, Brandjes DPM, et al. Inflammatory and endothelial markers during vaso-occlusive crisis and acute chest syndrome in sickle cell disease. Am J Hematol. 2017 Nov;92(11):E634–6.
13 Van Avondt K, Fritsch-Stork R, Derksen RHWM, Meyaard L. Ligation of signal inhibitory receptor on leukocytes-1 suppresses the release of neutrophil extracellular traps in systemic lupus erythematosus. PLoS One. 2013;8(10):e78459.
14 Esposito BP, Breuer W, Sirankapracha P, Pootrakul P, Hershko C, Cabantchik ZI. Labile plasma iron in iron overload: redox activity and susceptibility to chelation. Blood. 2003 Oct 1;102(7):2670–7.
15 Van Avondt K, van der Linden M, Naccache PH, Egan DA, Meyaard L. Signal inhibitory receptor on leukocytes-1 limits the formation of neutrophil extracellular traps, but preserves intracellular bacterial killing. J Immunol. 2016 May 1;196(9):3686–94.
16 McDonald B, Pittman K, Menezes GB, Hirota SA, Slaba I, Waterhouse CCM, et al. Intravascular danger signals guide neutrophils to sites of sterile inflammation. Science. 2010 Oct 15;330(6002):362–6.
17 Satoh T, Satoh H, Iwahara S, Hrkal Z, Peyton DH, Muller-Eberhard U. Roles of heme iron-coordinating histidine residues of human hemopexin expressed in baculovirus-infected insect cells. Proc Natl Acad Sci U S A. 1994 Aug 30;91(18):8423–7.
18 Völlger L, Akong-Moore K, Cox L, Goldmann O, Wang Y, Schäfer ST, et al. Iron-chelating agent desferrioxamine stimulates formation of neutrophil extracellular traps (NETs) in human blood-derived neutrophils. Biosci Rep. 2016 Jun 1;36(3):e00333.
19 Porter JB. Deferoxamine pharmacokinetics. Semin Hematol. 2001 Jan;38(1 Suppl 1):63–8.
20 Keberle H. The biochemistry of desferrioxamine and its relation to iron metabolism. Ann NY Acad Sci. 1964;119(2):758–68.

21 Rouault T, Rao K, Harford J, Mattia E, Klausner RD. Hemin, chelatable iron, and the regulation of transferrin receptor biosynthesis. J Biol Chem. 1985 Nov 25;260(27):14862–6.
22 Vinchi F, De Franceschi L, Ghigo A, Townes T, Cimino J, Silengo L, et al. Hemopexin therapy improves cardiovascular function by preventing heme-induced endothelial toxicity in mouse models of hemolytic diseases. Circulation. 2013 Mar 26;127(12):1317–29.
23 Schaer DJ, Vinchi F, Ingoglia G, Tolosano E, Buehler PW. Haptoglobin, hemopexin, and related defense pathways-basic science, clinical perspectives, and drug development. Front Physiol. 2014 Oct 28;5:415.
24 Tolosano E, Hirsch E, Patrucco E, Camaschella C, Navone R, Silengo L, et al. Defective recovery and severe renal damage after acute hemolysis in hemopexin-deficient mice. Blood. 1999 Dec 1;94(11):3906–14.
25 Lin T, Sammy F, Yang H, Thundivalappil S, Hellman J, Tracey KJ, et al. Identification of hemopexin as an anti-inflammatory factor that inhibits synergy of hemoglobin with HMGB1 in sterile and infectious inflammation. J Immunol. 2012 Aug 15;189(4):2017–22.
26 Kono M, Saigo K, Yamamoto S, Shirai K, Iwamoto S, Uematsu T, et al. Iron-chelating agent, deferasirox, inhibits neutrophil activation and extracellular trap formation. Clin Exp Pharmacol Physiol. 2016 Oct;43(10):915–20.
27 Saigo K, Kono M, Takagi Y, Takenokuchi M, Hiramatsu Y, Tada H, et al. Deferasirox reduces oxidative stress in patients with transfusion dependency. J Clin Med Res. 2013;5(1):57–60.
28 Saha P, Yeoh BS, Olvera RA, Xiao X, Singh V, Awasthi D, et al. Bacterial siderophores hijack neutrophil functions. J Immunol. 2017 Jun 1;198(11):4293–303.
29 Figueiredo RT, Fernandez PL, Mourao-Sa DS, Porto BN, Dutra FF, Alves LS, et al. Characterization of heme as activator of toll-like receptor 4. J Biol Chem. 2007 Jul;282(28):20221–9.
30 Belcher JD, Chen C, Nguyen J, Milbauer L, Abdulla F, Alayash AI, et al. Heme triggers TLR4 signaling leading to endothelial cell activation and vaso-occlusion in murine sickle cell disease. Blood. 2014 Jan 16;123(3):377–90.
31 Ohbuchi A, Kono M, Kitagawa K, Takenokuchi M, Imoto S, Saigo K. Quantitative analysis of hemin-induced neutrophil extracellular trap formation and effects of hydrogen peroxide on this phenomenon. Biochem Biophys Rep. 2017 Sep;11:147–53.
32 Badawy SM, Liem RI, Rigsby CK, Labotka RJ, DeFreitas RA, Thompson AA. Assessing cardiac and liver iron overload in chronically transfused patients with sickle cell disease. Br J Haematol. 2016 Nov;175(4):705–13.
33 Hankins JS, Smeltzer MP, Beth McCarville M, Aygun B, Hillenbrand CM, Ware RE, et al. Patterns of liver iron accumulation in patients with sickle cell disease and thalassemia patients with iron overload. Eur J Haematol. 2010 Mar;85(1):51–7.
34 Ginwalla M, AlMasoud A, Tofovic D, Alin T, Al-Kindi S, Oliveira G, et al. Cardiovascular evaluation and management of iron overload cardiomyopathy in sickle cell disease. Am J Hematol. 2018 Jan;93(1):E7–9.
35 McLeod C, Fleeman N, Kirkham J, Bagust A, Boland A, Chu P, et al. Deferasirox for the treatment of iron overload associated with regular blood transfusions (transfusional haemosiderosis) in patients suffering with chronic anaemia: a systematic review and economic evaluation. Health Technol Assess. 2009 Jan;13(1)1–21.
36 Tangudu NK, Alan B, Vinchi F, Wörle K, Lai D, Vettorazzi S, et al. Scavenging reactive oxygen species production normalizes ferroportin expression and ameliorates cellular and systemic iron disbalances in hemolytic mouse model. Antioxid Redox Signal. 2018 Aug 10;29(5):484–99.
37 Delaby C, Pilard N, Gonçalves AS, Beaumont C, Canonne-Hergaux F. Presence of the iron exporter ferroportin at the plasma membrane of macrophages is enhanced by iron loading and down-regulated by hepcidin. Blood. 2005 Dec 1;106(12):3979–84.
38 Zhang Z, Zhang F, An P, Guo X, Shen Y, Tao Y, et al. Ferroportin1 deficiency in mouse macrophages impairs iron homeostasis and inflammatory responses. Blood. 2011 Aug 18;118(7):1912–22.
39 De Franceschi L, Daraio F, Filippini A, Carturan S, Muchitsch EM, Roetto A, et al. Liver expression of hepcidin and other iron genes in two mouse models of beta-thalassemia. Haematologica. 2006 Oct;91(10):1336–42.
40 Kumari N, Ammosova T, Diaz S, Lin X, Niu X, Ivanov A, et al. Increased iron export by ferroportin induces restriction of HIV-1 infection in sickle cell disease. Blood Adv. 2016 Dec 27;1(3):170–83.
41 Keshari RS, Jyoti A, Dubey M, Kothari N, Kohli M, Bogra J, et al. Cytokines induced neutrophil extracellular traps formation: implication for the inflammatory disease condition. PLoS One. 2012 Oct 26;7(10):e48111.
42 Lanaro C, Franco-Penteado CF, Albuqueque DM, Saad STO, Conran N, Costa FF. Altered levels of cytokines and inflammatory mediators in plasma and leukocytes of sickle cell anemia patients and effects of hydroxyurea therapy. J Leukoc Biol. 2009 Feb 1;85(2):235–42.
43 Cerqueira BAV, Boas WV, Zanette AD, Reis MG, Goncalves MS. Increased concentrations of IL-18 and uric acid in sickle cell anemia: contribution of hemolysis, endothelial activation and the inflammasome. Cytokine. 2011 Nov;56(2):471–6.
44 Arai Y, Nishinaka Y, Arai T, Morita M, Mizugishi K, Adachi S, et al. Uric acid induces NADPH oxidase-independent neutrophil extracellular trap formation. Biochem Biophys Res Commun. 2014 Jan;443(2):556–61.
45 Yousefi S, Mihalache C, Kozlowski E, Schmid I, Simon HU. Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. Cell Death Differ. 2009 Nov;16(11):1438–44.
46 Chudwin DS, Papierniak C, Lint TF, Korenblit AD. Activation of the alternative complement pathway by red blood cells from patients with sickle cell disease. Clin Immunol Immunopathol. 1994 May;71(2):199–202.
47 Sur Chowdhury C, Giaglis S, Walker UA, Buser A, Hahn S, Hasler P. Enhanced neutrophil extracellular trap generation in rheumatoid arthritis: analysis of underlying signal transduction pathways and potential diagnostic utility. Arthritis Res Ther. 2014 Jun 13;16(3):R122.

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Molecular Screening of Blood Donors for *Babesia* in Tyrol, Austria

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Keywords

Babesia · Polymerase chain reaction · Screening · Blood donor · Blood transfusion · Austria

Abstract

Introduction: *Babesia* is a tick-borne intraerythrocytic parasite that is globally ubiquitous, yet understudied. Several species of *Babesia* have been shown to be transfusion-transmissible. *Babesia* has been reported in blood donors, animals, and ticks in the Tyrol (Western Austria), and regional cases of human babesiosis have been described. We sought to characterize the risk of *Babesia* to the local blood supply. ***Methods:*** Prospective molecular testing was performed on blood donors who presented to regional, mobile blood collection drives in the Tyrol, Austria (27 May to October 4, 2021). Testing was conducted using the cobas® Babesia assay (Roche Molecular Systems, Inc.), a commercial PCR assay approved for blood donor screening that is capable of detecting the 4 primary species causing human babesiosis (i.e., *B. microti*, *B. divergens*, *B. duncani*, and *B. venatorum*). A confirmatory algorithm to manage initial PCR-reactive samples was developed, as were procedures for donor and product management. ***Results:*** A total of 7,972 donors were enrolled and screened; 4,311 (54.1%) were male, with a median age of 47 years (IQR = 34–55). No positive cases of *Babesia* were detected, corresponding with an overall prevalence of 0.00% (95% CI: 0.00%, 0.05%). ***Discussion:*** The findings suggest that the prevalence of *Babesia* is low in Austrian blood donors residing in the Tyrol, even during months of peak tick exposure. Although one cannot conclude the absence of *Babesia* in this population given the limited sample size, the findings suggest that the regional risk of transfusion-transmitted babesiosis is low.

Introduction

Babesiosis is the symptomatic infection with any of the members of the *Babesia* genus, a group of tick-borne, intraerythrocytic protozoan parasites [1]. Most cases of human babesiosis are attributed to *Babesia microti*, a species that is known to be transfusion-transmissible [2]. Asymptomatic *Babesia* infection is well described and can persist for months to years in some individuals, thus posing risk of not being detected at the time of blood donation, particularly in the absence of laboratory-based donor testing [3]. Although infection may be mild or subclinical in healthy adults, certain patient groups such as neonates, individuals aged >50 years, the asplenic, and the immunocompromised (HIV, cancer, and immuno-

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suppressant therapy) are at risk of severe or even fatal babesiosis [1]. These same populations are over-represented among transfusion recipients, which likely accounts for the high all-cause mortality (~19%) associated with transfusion-transmitted babesiosis [4].

A 2014 seroprevalence study of Tyrolean blood donors (n = 988) [i.e., in Western Austria] found that 2.1% of donors demonstrated IgG antibodies against the *B. divergens* complex and 0.6% were seropositive against *B. microti* [5]. Regional surveillance and case reporting indicate that *Babesia* is present in humans, ticks, and animal populations in Europe [6–11], although rare, transfusion-transmitted babesiosis has also been described in Europe [8]. We conducted a *Babesia* molecular surveillance study of blood donors in Tyrol, Austria, to characterize the risk of *Babesia* to the local blood supply.

Materials and Methods

Overview of Population and Setting

The study was conducted in the Tyrol region of Western Austria, where both *B. divergens* and *B. microti* have been reported [5].

Sampling and Eligibility

Consecutive sampling was conducted of all community whole blood (WB) donors who presented during the study period (27 May to October 4, 2021) and who consented to participate. Direct-, autologous-, and apheresis platelet/plasma donors were excluded as were individuals with a reported tick bite in the 4 weeks prior to donation. Donor demographics (age, sex, area of residence) were captured at the blood center in Innsbruck, but no personal identifiers were shared with the research personnel, thus protecting donor confidentiality.

Laboratory Processing and Babesia PCR Testing

At the time of the donor visit, trained nurses or technicians collected approximately 1 mL of WB into a Roche WB collection tube specifically for *Babesia* testing. The proprietary collection tube draws up to 1.1 mL of WB into 7.7 mL of a chaotropic reagent that lyses red blood cells and preserves nucleic acid [12].

Babesia PCR

The donors' samples, comprising 850 μL of lysed WB, were tested individually (i.e., single donor specimens) using the cobas® Babesia test (Roche Molecular Systems Inc., Pleasanton, CA). The cobas® Babesia test is a qualitative real-time reverse transcriptase-polymerase chain reaction test capable of detecting four species of *Babesia*: *B. microti*, *B, duncani*, *B. divergens*, and *B. venatorum* [12]. The cobas® Babesia assay is FDA-licensed and CE-marked for blood donor screening. The assay's performance characteristics have previously been described: the reported analytic sensitivity for *B. microti* and *B. divergens* is 6.1 (95% confidence interval [CI]: 5.0, 7.9) and 26.1 (95% CI: 22.3, 31.8), infected red blood cells/mL, respectively. The clinical specificity is 99.999% [12]. The test was run on the cobas® 6800/8800 Systems at the Bavarian Red Cross. The testing was performed in real time, whereby a negative test was required before releasing the associated unit of blood (i.e., from quarantine). Repeat and confirmatory testing was planned using a combination of the cobas® Babesia test, indirect fluorescent antibody testing and sequencing.

Table 1. Characteristics of the study population

	No. of donors (%)[a] (n = 7,972)	No. of donations (%) (n = 8,028)
Study month		
May	1 (0.0)	1 (0.0)
June	686 (8.6)	696 (8.7)
July	2,868 (36.0)	2,912 (36.3)
August	2,128 (26.7)	2,130 (26.5)
September	2,124 (26.6)	2,124 (26.5)
October	165 (2.1)	165 (2.1)
Country		
Austria	7,962 (99.9)	8,018 (99.9)
Germany	8 (0.1)	8 (0.1)
Italy	2 (0.0)	2 (0.0)
Median age (IQR), years	47 (34–55)	47 (34–55)
Age group, years		
18–24	804 (10.1)	812 (10.1)
25–34	1,286 (16.1)	1,297 (16.2)
35–44	1,446 (18.1)	1,456 (18.1)
45–54	2,197 (27.6)	2,214 (27.6)
55–64	1,880 (23.6)	1,887 (23.5)
≥65	359 (4.5)	362 (4.5)
Sex		
Female	3,661 (45.9)	3,688 (45.9)
Male	4,311 (54.1)	4,340 (54.1)
Blood group		
A	3,215 (40.3)	3,240 (40.4)
B	770 (9.7)	774 (9.6)
AB	293 (3.7)	298 (3.7)
O	3,694 (46.3)	3,716 (46.3)

[a] For donor-level data, characteristics were ascertained from the most recent blood donation for donors that presented more than one time during the study period.

Statistical Analysis

Descriptive statistics were used to characterize the study sample of blood donations and blood donors. For donor-level data, characteristics were ascertained from the most recent blood donation for donors that presented more than one time during the study period. The prevalence of *Babesia* and corresponding Clopper-Pearson 95% CIs were estimated at the donor level. Data analysis was conducted in Stata/MP, version 15.1 (StataCorp, College Station, TX).

Human Subjects

The study was approved by the Ethics Committee of the Medical University of Innsbruck (1090/2021) prior to study initiation.

Results

The study sample included 8,028 blood donations collected from 7,972 unique blood donors (56 donors contributed two blood donations) at 71 collection sites (May to October 2021). Of the 11,711 available donors, 7,972 took part in the study, yielding a participation rate of 68.07%. The characteristics of the sampled donor popula-

tion are provided in Table 1. Of the 8,028 blood donations examined, there was no reactive sample for *Babesia*. At the blood donor level (n = 7,972), the overall prevalence of *Babesia* was 0.00% (95% CI: 0.00%, 0.05%).

Discussion

Our study, in which over 8,000 blood donations across the Tyrol were tested for *Babesia* by PCR, found none to be positive for *Babesia*. The findings suggest that the current risk to the local blood supply, if any, is low.

To date, surveillance for *Babesia* outside of the USA has been very limited [2]. The few studies in blood donors that have been conducted outside of the USA have found the prevalence of *Babesia* to be low or even absent in the individual populations that were surveyed [13, 14]. Molecular surveillance has been the exception; one study in Canada found 1 of 50,752 donation samples to be positive [15].

Despite our study's negative finding, tick-borne diseases – including babesiosis – appear to be on the rise [16–18]. Contributing factors include an increase in populations of ticks and associated interaction with their hosts [19–22]. Climate change and global warming have complex, yet unpredictable effects on vectors and vector-borne diseases [23, 24]. There may be changes in how local habitats support tick populations [25]. The birth rates and development of ticks are notably impacted favorably by an increase in temperature [26]. Shorter tick development times due to increased habitat temperatures and increased feeding opportunities for ticks may increase the possibility of human and non-human host infections by *Babesia* [27, 28]. Birds can also carry ticks – including those that are infected – thus introducing tick-borne diseases to areas that have not – historically – been considered to be endemic [29].

This study had limitations. It is still possible that *Babesia* is present but was missed due to the small sample size and/or short observation period. To place the findings in context, in a study of 89,153 blood donations originating in four states in the USA where *B. microti* is highly endemic, 67 donors (i.e., 1 in 1,331) were PCR positive for *Babesia* [30]. Second, serology was not performed; rather, it was planned in the event that PCR-positive cases were detected. While serology offers a better estimate of exposure, molecular methods correlate better with parasitemia and the associated risk of transfusion transmission [30]. Third, testing of Tyrolean *Babesia* samples has not been performed, previously using the cobas® Babesia assay. However, the test has been validated to detect *B. divergens* and *B. microti*, for which evidence of population exposure was found in the previous serosurvey [5]. Molecular testing has advantages over serological testing, thus supporting the rationale for the adoption of molecular screening of blood donors in high-risk areas of the USA. The discordance between our results with those of the previous serosurvey [5] is unsurprising [2]. A high proportion (almost 90%) of infected individuals resolve infection within 1 year of an index positive test (i.e., as reflected by negative molecular testing). By contrast, less than 10% of individuals will serorevert within the same timeframe [30]. There is also the possibility of prior overestimation of the prevalence [5] given challenges surrounding the specificity of antibody-based assays for *Babesia*. Further, donors who reported a tick bite during the 4 weeks prior to donation would have been excluded, diminishing the probability for finding a NAT-positive donation in our study.

In conclusion, no cases of *Babesia* infection – as determined by molecular testing – were found in a limited surveillance study of blood donors in Tyrol, Austria. The findings support vigilance rather than laboratory-based donor screening at this time.

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Statement of Ethics

The study was reviewed and approved by the Ethics Committee of the Medical University of Innsbruck (1090/2021) prior to study initiation. Written informed consent was obtained from all subjects who were involved in the study.

Conflict of Interest Statement

Evan M. Bloch reports personal fees and non-financial support from Terumo BCT, Abbott Laboratories, Tegus, and UpToDate, outside of the submitted work. Steven J. Drews is a paid consultant to Roche and has received research support from Abbott, outside of the submitted work. Harald Schennach reports personal fees from Cerus Corp outside of the submitted work. Anita Siller, Laura Tonnetti, Bryan Spencer, Doris Hedges, Tessa Mergenthal, Marijke Weber-Schehl, Manfred Astl, Eshan Patel, and Manfred Gaber have no conflict of interest to declare.

Evan M. Bloch is a member of the US Food and Drug Administration (FDA) Blood Products Advisory Committee. Any views or opinions that are expressed in this manuscript are those of the author's, based on his own scientific expertise and professional judgment; they do not necessarily represent the views of either the Blood Products Advisory Committee or the formal position of FDA, and do not bind or otherwise obligate or commit either Advisory Committee or the Agency to the views expressed.

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Author Contributions

The following roles were assumed by the individual co-authors: study conception and design (Evan M. Bloch, Anita Siller, Laura Tonnetti, Steven J. Drews, Bryan R. Spencer, and Harald Schennach), subject enrollment and testing (Doris Hedges, Tessa Mergenthal, Marijke Weber-Schehl, and Manfred Gaber), data analysis and interpretation (Eshan U. Patel [lead], Evan M. Bloch, Anita Siller, Laura Tonnetti, Laura Tonnetti, Steven J. Drews, Bryan R. Spencer, Manfred Astl, and Harald Schennach), writing first draft of manuscript (Evan M. Bloch, Anita Siller, and Harald Schennach), and review and editing (all co-authors).

Data Availability Statement

A deidentified dataset will be made available on request. Participant-level data on the blood donor cohort cannot be shared due to regulatory restrictions.

References

1 Vannier E, Krause PJ. Human babesiosis. N Engl J Med. 2012 Jun 21;366(25):2397–407.
2 Bloch EM, Krause PJ, Tonnetti L. Preventing transfusion-transmitted babesiosis. Pathogens. 2021;10(9):1176.
3 Bloch EM, Kumar S, Krause PJ. Persistence of Babesia microti infection in humans. Pathogens. 2019;8(3):102.
4 Herwaldt BL, Linden JV, Bosserman E, Young C, Olkowska D, Wilson M. Transfusion-associated babesiosis in the United States: a description of cases. Ann Intern Med. 2011 Oct 18;155(8):509–19.
5 Sonnleitner ST, Fritz J, Bednarska M, Baumgartner R, Simeoni J, Zelger R, et al. Risk assessment of transfusion-associated babesiosis in Tyrol: appraisal by seroepidemiology and polymerase chain reaction. Transfusion. 2014 Jul;54(7):1725–32.
6 Herwaldt BL, Caccio S, Gherlinzoni F, Aspock H, Slemenda SB, Piccaluga P, et al. Molecular characterization of a non-Babesia divergens organism causing zoonotic babesiosis in Europe. Emerg Infect Dis. 2003 Aug;9(8):942–8.
7 Meer-Scherrer L, Adelson M, Mordechai E, Lottaz B, Tilton R. Babesia microti infection in Europe. Curr Microbiol. 2004 Jun;48(6):435–7.
8 Hildebrandt A, Hunfeld KP, Baier M, Krumbholz A, Sachse S, Lorenzen T, et al. First confirmed autochthonous case of human Babesia microti infection in Europe. Eur J Clin Microbiol Infect Dis. 2007 Aug;26(8):595–601.
9 Ramharter M, Walochnik J, Lagler H, Winkler S, Wernsdorfer WH, Stoiser B, et al. Clinical and molecular characterization of a near fatal case of human babesiosis in Austria. J Trav Med. 2010 Nov-Dec;17(6):416–8.
10 Hildebrandt A, Zintl A, Montero E, Hunfeld K-P, Gray J. Human babesiosis in Europe. Pathogens. 2021;10(9):1165.
11 Bajer A, Beck A, Beck R, Behnke JM, Dwużnik-Szarek D, Eichenberger RM, et al. Babesiosis in southeastern, central and northeastern Europe: an emerging and Re-emerging tick-borne disease of humans and animals. Microorganisms. 2022;10(5):945.
12 Stanley J, Stramer SL, Erickson Y, Cruz J, Gorlin J, Janzen M, et al. Detection of Babesia RNA and DNA in whole blood samples from US blood donations. Transfusion. 2021 Oct;61(10):2969–80.
13 Faddy HM, Rooks KM, Irwin PJ, Viennet E, Paparini A, Seed CR, et al. No evidence for widespread Babesia microti transmission in Australia. Transfusion. 2019 Jul;59(7):2368–74.
14 O'Brien SF, Drews SJ, Yi QL, Bloch EM, Ogden NH, Koffi JK, et al. Risk of transfusion-transmitted Babesia microti in Canada. Transfusion. 2021 Oct;61(10):2958–68.
15 Tonnetti L, O'Brien SF, Grégoire Y, Proctor MC, Drews SJ, Delage G, et al. Prevalence of Babesia in Canadian blood donors: june–october 2018. Transfusion. 2019;59(10):3171–6.
16 Krause PJ, Auwaerter PG, Bannuru RR, Branda JA, Falck-Ytter YT, Lantos PM, et al. Clinical practice guidelines by the infectious diseases society of America (IDSA): 2020 guideline on diagnosis and management of babesiosis. Clin Infect Dis. 2020;72(2):e49–e64.
17 Menis M, Whitaker BI, Wernecke M, Jiao Y, Eder A, Kumar S, et al. Babesiosis occurrence among United States medicare beneficiaries, ages 65 and older, during 2006–2017: overall and by state and county of residence. Open Forum Infect Dis. 2021;8(2):ofaa608.
18 Bloch EM, Day JR, Krause PJ, Kjemtrup A, O'Brien SF, Tobian AAR, et al. Epidemiology of hospitalized patients with babesiosis, United States, 2010-2016. Emerg Infect Dis. 2022 Feb;28(2):354–62.
19 Rodgers SE, Mather TN. Human Babesia microti incidence and Ixodes scapularis distribution, Rhode Island, 1998–2004. Emerg Infect Dis. 2007 Apr;13(4):633–5.
20 Hersh MH, Tibbetts M, Strauss M, Ostfeld RS, Keesing F. Reservoir competence of wildlife host species for Babesia microti. Emerg Infect Dis. 2012 Dec;18(12):1951–7.
21 Vannier EG, Diuk-Wasser MA, Ben Mamoun C, Krause PJ. Infect Dis Clin North Am. 2015 Jun;29(2):357–70.
22 Westblade LF, Simon MS, Mathison BA, Kirkman LA. Babesia microti: from mice to ticks to an increasing number of highly susceptible humans. J Clin Microbiol. 2017 Oct;55(10):2903–12.
23 Medlock JM, Hansford KM, Bormane A, Derdakova M, Estrada-Peña A, George J-C, et al. Driving forces for changes in geographical distribution of Ixodes ricinus ticks in Europe. Parasit Vectors. 2013;6(1):1.
24 Gray JS, Ogden NH. Ticks, human babesiosis and climate change. Pathogens. 2021;10(11):1430.
25 Zając Z, Kulisz J, Woźniak A, Bartosik K, Khan A. Seasonal activity of Dermacentor reticulatus ticks in the era of progressive climate change in eastern Poland. Sci Rep. 2021 Oct 14;11(1):20382.
26 Randolph SE. Evidence that climate change has caused 'emergence' of tick-borne diseases in Europe? Int J Med Microbiol. 2004 Apr;293(Suppl 37):5–15.
27 Gray JS, Kahl O, Lane RS, Levin ML, Tsao JI. Diapause in ticks of the medically important Ixodes ricinus species complex. Ticks Tick Borne Dis. 2016 Jul;7(5):992–1003.
28 Johnson N, Paul Phipps L, McFadzean H, Barlow AM. An outbreak of bovine babesiosis in February, 2019, triggered by above average winter temperatures in southern England and co-infection with Babesia divergens and Anaplasma phagocytophilum. Parasit Vectors. 2020 Jun 12;13(1):305.
29 Hasle G. Transport of ixodid ticks and tick-borne pathogens by migratory birds. Front Cell Infect Microbiol. 2013 2013 September 10;3:48.
30 Moritz ED, Winton CS, Tonnetti L, Townsend RL, Berardi VP, Hewins ME, et al. Screening for Babesia microti in the U.S. Blood supply. N Engl J Med. 2016 Dec 08;375(23):2236–45.

Transfusion Medicine and Hemotherapy

Systematic Review

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Detection of Acute Traumatic Coagulopathy by Viscoelastic Haemostatic Assays Compared to Standard Laboratory Tests: A Systematic Review

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Keywords

Viscoelastic assay · Thromboelastography · Acute traumatic coagulopathy · Wounds and injuries · Blood component transfusion

Abstract

Introduction: The aim of this systematic review was to investigate whether viscoelastic haemostatic assays (VHAs) offer comparative diagnostic ability of acute traumatic coagulopathy (ATC) compared to the standard laboratory coagulation tests (SLCT). ATC is a complication of major trauma characterized by dysfunctional blood clotting, leading to an increased bleeding risk. Additionally, we aimed to analyse the association of VHA with blood product use and health outcomes. ***Methods:*** The search protocol was pre-published and completed on December 2, 2020, assessing manuscripts from 2000 until the present. We searched MEDLINE, Embase, Cochrane Central, BIOSIS, Emcare, CINAHL, and additional online resources and referenced lists. Included were manuscripts that quantitatively reported the detection of ATC using VHAs and SLCTs. A meta-analysis was undertaken including observational studies that reported on patients with injuries to all body regions and results analysed using a random-effects model and reported using pooled odds ratio with 95% confidence intervals (CI). ***Results:*** There were 14 observational studies and one randomized control trial involving 2,715 participants that satisfied inclusion criteria. We observed significant heterogeneity in the definitions of ATC, study design, setting, and patient population. Among observational studies that reported on patients with injuries to all body regions, VHAs were associated with higher odds of diagnosing ATC compared to SLCT (pooled OR 2.4; 95% CI: 1.4–4.1). There was inadequate evidence to suggest VHAs were associated with reduced blood product usage or lower mortality. ***Conclusion:*** VHAs detected more patients with ATC compared to SLCTs. However, the clinical significance and applicability of this finding remains unknown as translation to management was not adequately reported.

Introduction

Trauma is a leading cause of death for young adults worldwide. Despite ongoing medical and public health improvements, the incidence of trauma is increasing in Victoria, Australia and with it, death due to trauma [1]. Approximately 40% of trauma of deaths results from haemorrhage, disproportionately affecting patients within the first 24 h after injury [2]. The management of haemorrhagic shock requires clinically driven trauma protocols and algorithms to address the hypovolaemic status and haemostatic dysfunction. Such damage control resuscitation (DCR) strategies have been developed to help guide clinicians with empirical treatments targeting the conditions that exacerbate haemorrhage [3]. These include a number of surgical, non-invasive and medical interventions.

In the setting of critical bleeding, acute traumatic coagulopathy (ATC) is a common complication after injury

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that leads to significant challenges in management and poor patient outcomes [4]. ATC causes a dysfunction in blood clotting leading to an increased bleeding risk. It occurs early and independently after injury, driven by hypoperfusion and is present in approximately a quarter of trauma patients [5, 6]. It is frequently present in the patients with higher injury severity scores and increasing degrees of hemorrhagic shock. The detection of ATC is difficult due to the acute, dynamic, and complex mechanisms behind it. Accurate diagnosis is important as inappropriate transfusion of blood products has been associated with harm [7]. Furthermore, timely diagnosis is essential to prevent worsening of coagulopathy and clinical deterioration to the point of irreversible physiological derangements.

Traditionally, standard laboratory coagulation tests (SLCT) are used for the detection of ATC, but are slow and only provide a limited snapshot of the clotting time when the sample was taken. Clinical laboratories perform SLCTs in approximately 15–30 min, with additional time to reporting results taking up to an hour from sampling [8]. Point of care devices for the measurement of traditional times to blood clotting have been reported as unreliable in the diagnosis of ATC [9]. By comparison, point of care Viscoelastic Haemostatic Assays (VHAs) have offered the potential for a quicker diagnosis of ATC and ongoing monitoring of transfusion requirements based on coagulation abnormalities [10]. This is a functional, dynamic, and repeatable set of parameters designed to assess clot formation, timing, strength, and dissolution. The two most commonly used VHA devices are Thromboelastography (TEG) (Haemonetics®, Boston, USA) and Rotational Thromboelastometry (ROTEM) (Tem Innovations GmbH, Munich, Germany). The Automated Thromboelastometry (TEM-A) (Framar Biomedica, Rome, Italy) is not currently used.

The use of VHA has been associated with mortality benefit in other areas of medical and surgical care [11, 12]. However, the current published literature has been unable to demonstrate a clear and definitive benefit established for acute trauma resuscitation due to the lack of available and comparable studies [13, 14]. The aim of this systematic review was to determine whether the use of VHA during trauma resuscitation detects a different population of patients with ATC compared to SLCTs. The association of any such difference with patient outcomes was explored as secondary outcome measures.

Methods

Information Sources and Search Technique

A systematic search of databases was conducted on the December 2, 2020 to identify relevant manuscripts. This included MEDLINE, Cochrane Central, Embase, Emcare, BIOSIS, and CINAHL. As a result of ATC first appearing in literature in 2003 and the relatively novel nature of viscoelastic technology being used in trauma, we decided to restrict our search to studies published from 2000 onwards in all databases and secondary searching. Only human trials and full-text available manuscripts were included in the final review.

In addition to the databases listed above, additional searching of bibliographies of included texts, grey literature, websites, and registries including; clinical trials.gov, WHO trials registry, LILACs, Hemonetics, and the transfusion library was conducted. The study protocol was prospectively registered on Prospero [15].

Eligibility Criteria and Study Selection

We aimed to include randomized control trials (RCTs) as well as observational studies. The patient population was adult major trauma patients, as defined by individual manuscripts, excluding children and animal studies. Amongst these manuscripts, those that reported on both VHAs and SLCTs during the trauma resuscitation were selected. Our outcome measures were the proportion of patients with ATC detected on either VHA and SLCT and whether the difference in detection was associated with variation in blood product use in the first 24 h of admission.

Data Abstraction and Analysis

Two reviewers (EF and BM) independently screened the results from the search and collected data according to set of predetermined parameters as per guidance from the study team. The data extracted from each article included author, year, country, sample size, age range, mechanism of injury, injury severity score, VHA model, VHA timing, SLCT tests, and the ATC definition as per the VHAs and SLCTs used. Our primary outcome was reported as the number of patients identified as coagulopathic with VHA and SLCT and the number of those identified as not coagulopathic with VHA and SLCT. Other outcomes were documented as per the authors' reported results in the manuscript.

Two authors (EF and BM) independently assessed the risk of bias from the included studies as per the Newcastle-Ottawa II Scale [16]. Each manuscript was rated to establish the risk of bias from the selection processes, measurement acquisition, result reporting procedures, and loss to follow-up.

Among the included studies that reported on all major trauma patients, we performed a meta-analysis to assess the differential diagnosis of ATC between VHA and SLCTs. Manuscripts that reported on selected subgroups of injured patients, e.g., traumatic brain injury, were excluded from the meta-analysis. Data were analysed using STATA v 15.1 (College Station, TX, USA). A random-effects model was used to account for the assumption that the study effect estimates would show more variance than when drawn from a single population. To accommodate this assumption and minimize the imprecision of the effect estimations, the pooled odds ratios were analysed using the DerSimonian-Laird methods and reported with 95% confidence intervals. Statistical heterogeneity was assessed using the χ^2 test.

Results

The search yielded 2,579 studies after removal of duplicates, of which 47 articles fulfilled criteria for inclusion. The results of the search and respective levels of screening are outlined in the PRISMA flowchart in Figure 1 [17].

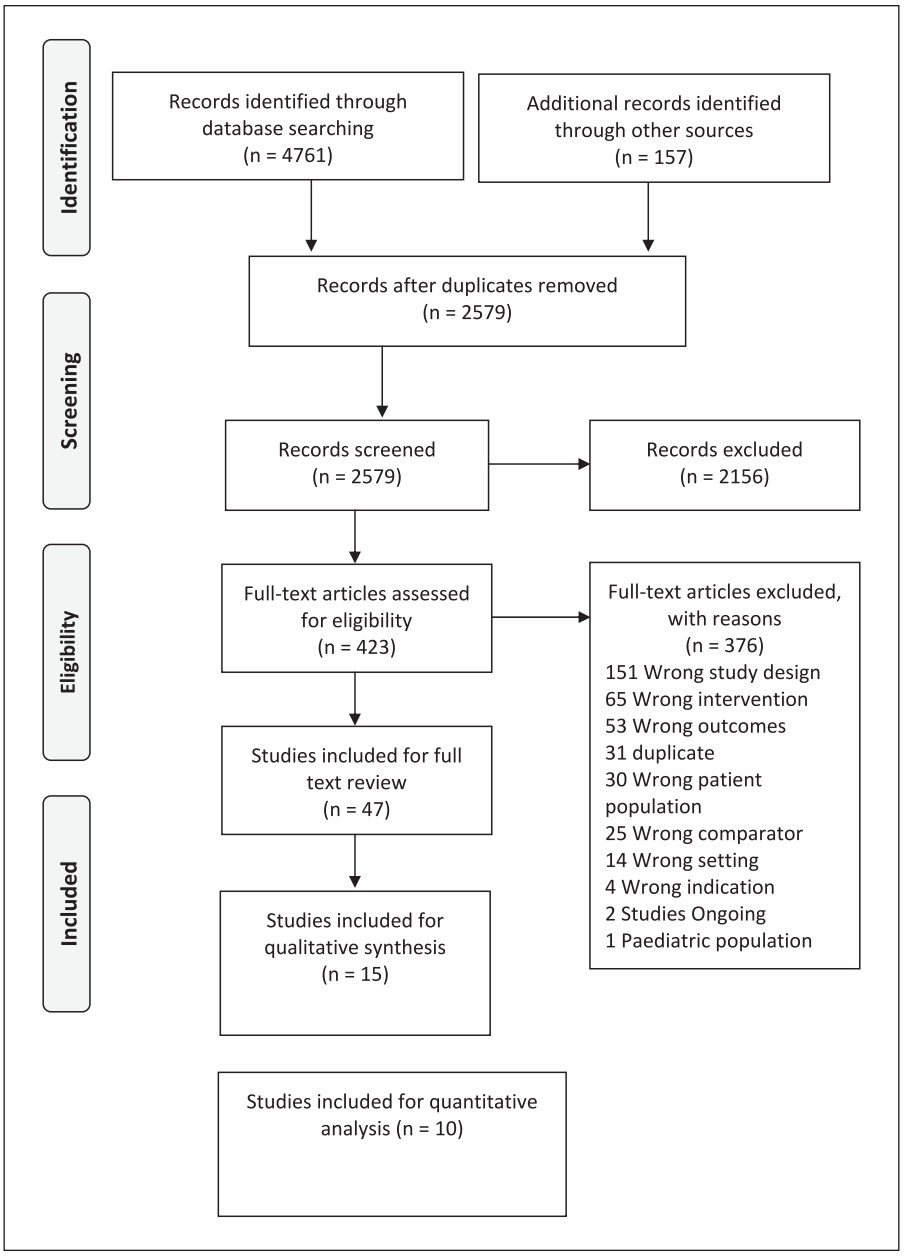

Fig. 1. Selection of studies, PRISMA flow diagram.

Of those 47 articles, there were only 15, comprising of 2,715 patients, which had published data that could be used to quantitatively assess our primary outcome [18–32]. There was one RCT identified and remaining manuscripts were either retrospective or prospective observational studies of moderate methodological quality. Descriptions of the 15 included study characteristics are presented in Table 1. Therefore, the remaining 32 manuscripts were excluded from this analysis as they did not numerically report on the detection rates of ATC between VHA and SLCT [33–64]. The characteristics of these studies are outlined in Table 2.

Five of the studies used VHA-guided resuscitation, incorporating the results into their treatment algorithms. Baksaas-Aasen et al. [32] compared TEG-, ROTEM-, and SLCT-guided resuscitation protocols in the RCT for implementing treatment algorithms for the correction of trauma induced coagulopathy (iTACTIC). Gratz et al. and Bouzat et al. [27, 31] used ROTEM-guided resuscitation with SLCTs performed in conjunction for clinical assessment. Zwinkels et al. [18] applied two different protocols separately to compare ROTEM-guided treatment with SLCT-guided treatment. Tauber et al. [20] used either SLCTs, ROTEM or both to guide clinical practice. Amongst the other studies, treatment was guided by SLCTs and traditional resuscitation protocols, with the VHA results obtained only for research purposes and not provided to the treating doctors [19, 21, 23, 25, 26, 29, 30].

Table 1. Description of included studies for systematic review

Article	Study design	*N*	Population	VHA model	SLCT test
Baksaas-Aasen et al. 2020 UK [32]	Randomized Control Trial	396	Age: 18+ years ISS: 26 (17–36) Blunt/Penetrating (%): 67/33%	ROTEM + TEG (model not specified)	INR, fibrinogen, platelets
Bouzat et al. 2019 Germany, France [31]	Multi-centre Retrospective Cohort	149	Age: 18+years ISS: 28 (20–42) Blunt/penetrating (%): 62/38%	ROTEM Delta + ROTEM Sigma	INR and/or fibrinogen
Cohen et al. 2019 Afghanistan [30]	Prospective Cohort study	40	Age: 16+ years ISS: 22 (14–27) Combat	ROTEM (model not specified)	INR
Doran et al. 2010 UK [29]	Prospective cohort study	31	Age: 18+ years Combat	ROTEM (model not specified)	PT, aPTT
Gozal et al. 2017 USA [28]	Retrospective cohort	190	Age: 18+ years TBI only Anticoagulant use 9%	TEG (model not specified)	Not specified
Gratz et al. 2019 [27]	Multi-centre Prospective Cohort	32	Age: 18+ years ISS: 43 (26–50) TBI only	ROTEM Sigma	INR, aPTT, fibrinogen
Jeger et al. 2009 Switzerland [26]	Prospective Cohort study	20	Age: 16+ years ISS: 29 (16–65) Blunt/penetrating (%): 100/0%	TEG 5000	INR, TT, aPTT, platelet count
Plotkin et al. 2008 USA [24]	Retrospective Cohort	44	Age: unspecified ISS: 21 +/– 9.4 Blunt/penetrating (%): 0/100% Combat	TEG 5000	aPTT, PT, INR
Schochl et al. 2011 Austria [23]	Retrospective cohort	88	Age: 15+ years ISS: 20 (16–26.25) TBI only	ROTEM (model not specified)	PT, aPTT, fibrinogen, platelet count
Subramanian et al. 2014 India [22]	Retrospective cohort	150	Age: 16+ years ISS: 28.4 +/– 11.3	TEM-A	PT and aPTT and/or INR
Sumislawski et al. 2019 USA [21]	Prospective cohort	839	Age: 18+ years ISS: 10 (2–26) Blunt/penetrating (%): 51/49%	TEG 5000	INR, aPTT
Tauber et al. 2011 Austria [20]	Prospective cohort	334	Age: 18+ years ISS: 34 (24–45) Blunt/penetrating (%): 100/0% Polytrauma patients	ROTEM (model not specified)	INR, aPTT, fibrinogen, plaelet count
Tonglet et al. 2018 Belgium [19]	Prospective cohort	50	Age: 14+ years ISS: 13 (9–20)	ROTEM (model not specified)	INR, fibrinogen
TurMartinez et al. 2018 Spain [25]	Retrospective cohort	230	Age: 15+ years ISS: 10 (1–54) Blunt/penetrating (%): 96/4% Polytrauma patients Anticoagulant use 15%	TEG (model not specified)	INR, aPTT, PT

Table 1 (continued)

Article	Study design	*N*	Population	VHA model	SLCT test
Zwinkels et al. 2020 Netherlands [18]	Retrospective cohort	122	Age: 18+ years ISS: 34 (27–48) Blunt/penetrating (%): 55/45% Polytrauma + Massive transfusion patients	ROTEM Delta	INR

VHA, viscoelastic haemostatic assays; SLCT, Standard Laboratory Coagulation Tests; ISS, injury severity score – reported as either median (Interquartile range) or mean +/− standard deviation; TBI, traumatic brain injury, TEG, thromboelastography; ROTEM, rotational thromboelastometry; TEM-A, automated thromboelastometry; aPTT, activated partial thromboplastin time; PT, prothrombin time; INR, international normalized ratio.

The remainder did not report whether the VHA results were available to the treating team or not [22, 24, 28].

To undertake a meta-analysis of observational data exploring the differential diagnosis of ATC, we excluded the manuscripts by Gozal, et al., Gratz, et al., and Schochl et al. [23, 27, 28] as the populations had been restricted to traumatic brain injury (TBI) patients only. Additionally, we excluded the manuscript by Plotkin, et al. [24] as the population only included patients suffering from penetrating injuries [24]. Finally, the RCT by Baksaas-Aasen et al. [32] was excluded from a meta-analysis combining observational studies.

The risk of bias assessment for the observational articles included in the meta-analysis is presented in Table 3 [16]. As per the Newcastle-Ottawa II Scale, the manuscripts were rated as good, fair or unclear, with seven manuscripts rated good [19–22, 25, 26, 31], and three as fair quality [18, 29, 30].

The results of the meta-analysis are shown in Figure 2. There was significant statistical heterogeneity detected among manuscripts ($p < 0.001$), which was adjusted for using random-effects model. The pooled odds ratio for ATC favoured VHA, where viscoelastic testing was associated with higher odds of diagnosing ATC (pooled OR 2.4; 95% CI: 1.4–4.1).

There were four studies included in our analysis that reported a significant benefit in using VHAs to detect the presence of coagulopathy, demonstrating either significant association with the corresponding SLCT parameters or a comparable negative predictive value (NPV) [19, 20, 23, 26]. Sumislawski et al. [21] reported no significant diagnostic benefit and supported the combined use of VHAs and SLCTs, as the SLCTs were able to identify acutely coagulopathic patients with the highest mortality. Subramanian et al. reported that the diagnostic accuracy of ROTEM increased from 46.6% to 66% when using a set of values predetermined by prior research of the specified population, instead of using the manufacturers ranges [22]. The manufacturers' ranges for ROTEM and TEG are presented in Table 4.

Additionally, another six articles reported on the relationship with blood product consumption. Cohen et al. reported the use of ROTEM increased the identification of patients requiring a massive transfusion by 22%, and had a higher sensitivity than using an INR >1.2 [30]. Tonglet et al. [19] determined a normal ROTEM was able to rule out patients at risk of needing >5 units RBC and >3 units plasma in 24 h as well as 30-days mortality with a NPV of 100% and 95.2%, respectively [19]. Plotkin et al. [24] found that the TEG parameters were more accurately able to indicate blood product requirements than SLCTs and the combined use of TEG, platelet count and haematocrit could guide transfusion. Tauber et al. [20] demonstrated that ROTEM MCF was significantly associated with reduced risk for RBC transfusion. Zwinkels et al. [18] compared VHA-guided resuscitation with SLCT-guided resuscitation and discovered that whilst fibrinogen, platelets, and calcium were given more frequently, the rate of plasma transfusions decreased with VHA-guided care. The iTACTIC trial found that patients in the VHA group were more likely to receive a study intervention of blood or blood product than their SLCT group counterparts (VHA 67% vs. SLCT 36%) [32]. It was also reported that the VHA group received those study interventions on average 21 min earlier than the SLCT group. The VHA group received more fibrinogen supplementation. However, at 24 h there was no significant difference in the rate of massive transfusion with an odds ratio of 1.15 and 95% confidence interval of 0.76–1.73. Except in a subgroup analysis of TBI patients, there was an improvement in 28-day mortality in VHA patients [32].

There were 3 studies using ROTEM that reported on early mortality risk or 30-days survival [19, 20, 23]. However, in a comparison of VHA and SLCT-guided resuscitation, Zwinkels et al. [18] demonstrated no significant benefit on 30-days mortality, and ICU or hospital length

Table 2. Description of included studies for full-text review but excluded from analysis

Article	Study Design	*N*	Population	VHA model	SLCT test
Albert et al. 2019 India [33]	Prospective cohort	58	Age: 18+ Isolated severe TBI, GCS <8	TEM-A	PT, aPTT, INR
Baksass-Aasen et al. 2019 UK [34]	Multi-centre prospective cohort	2,287	Age: 18+ ISS: 13 (5–25) Blunt/Penetrating (%): 85/15%	ROTEM Delta, TEG 5000	INR, aPTT, fibrinogen, platelets
Coleman et al. 2018 USA [35]	Prospective cohort	343	Age: 18+ NISS:18 (6–34) Blunt/Penetrating (%): 52/48%	TEG 5000	INR, aPTT, fibrinogen, platelets
Cotton et al. 2011 USA [36]	Prospective cohort study	272	Age: 18+ ISS: 14 (8–25) Blunt/Penetrating (%): 72/28%	TEG 5000	PT, aPTT, INR, platelet
Davenport et al. 2011 UK [37]	Prospective cohort study	300	Age: 15+ ISS: 12 (4–25) Blunt/Penetrating (%): 79/21%	ROTEM (model not specified)	PT, platelets, fibrinogen
David et al. 2016 France [38]	Retrospective cohort study	358	Age: 18+ ISS: 26 (17–34) Blunt/Penetrating (%): 94/6%	ROTEM (model not specified)	INR, aPTT, fibrinogen, platelets
Gonzalez et al. 2016 USA [39]	RCT	111	Age: 18+ ISS: 47.5 (22–59) Blunt/Penetrating (%): 68/32%	TEG (model not specified)	INR, aPTT, fibrinogen, platelets
Guth et al. 2019 France [40]	Retrospective cohort + prospective cohort comparison	372	Age: 18+ ISS: 28 (18–38) Blunt/Penetrating (%): 93/7%	ROTEM Delta	PT, aPTT, fibrinogen
Holocomb et al. 2012 USA [41]	Retrospective cohort study	1,974	Age: 18+ ISS: median 17	TEG 5000	PT, aPTT, INR
Hota et al. 2019 USA [42]	Retrospective cohort	118	Age: 18+ ISS: mean 16 Anticoagulated + TBI patients	TEG (model not specified)	Not specified
Jeger et al. 2012 Switzerland [43]	Prospective cohort study	76	Age: 16+ ISS: mean 18 Blunt/Penetrating (%): 83/17%	TEG 5000	aPTT, INR, TT
Johansson et al. 2009 Denmark [45]	Retrospective cohort study	832	Age: 15+ MT patients	TEG (model not specified)	Platelets, aPTT, INR
Johansson et al. 2013 Denmark [44]	Prospective cohort study	182	Age: 18+ ISS: 17 (9–26) Blunt/Penetrating (%): 92/8%	TEG 5000	INR, aPTT, platelets, fibrinogen
Kashuk et al. 2009 USA [46]	Retrospective cohort	44	Age: 18+ ISS: 29 (23–35) Blunt/Penetrating (%): 80/20%	TEG 5000	INR, fibrinogen, platelet
Kashuk et al. 2012USA [47]	Prospective cohort study	68	Age: 18+ Blunt/Penetrating (%): 68/32% MT patients	TEG 5000	INR, aPTT
Kobayashi et al. 2018 USA [48]	Prospective cohort study	182	Age: 18+ ISS: 9 (4–13) Anticoagulated trauma patients	TEG (model not specified)	aPTT, INR
Lammers et al. 2020 USA [49]	Retrospective cohort study	3,320	Age: 18+ ISS: median 18.8 Blunt/Penetrating (%): 84/16% Combat patients Male: 98%	ROTEM (model not specified)	INR
Leemann et al. 2010 [50]	Retrospective cohort study	53	Age: 18+ ISS: 31.1 +/– 1.7 Blunt/Penetrating (%): 100/0%	ROTEM (model not specified)	INR, aPTT, platelets

Table 2 (continued)

Article	Study Design	*N*	Population	VHA model	SLCT test
Mohamed et al. 2017 USA [51]	Retrospective cohort study	134	Age: 18+ ISS: mean 29 Blunt/Penetrating (%): 63/37% MT patients	TEG 5000	Not specified
Nystrup et al. 2011 Denmark [52]	Retrospective cohort	89	Age: 18+ ISS: 21 (19–23) Blunt/Penetrating (%): 85/15%	TEG (model not specified)	aPTT, INR, platelets
Peng et al. 2019 Canada [53]	Prospective	45	Age: 18+	TEG 5000, ROTEM Delta	INR, PT, aPTT, fibrinogen, platelets
Pezold et al. 2012 USA [54]	Retrospective cohort study	80	Age: 15+ ISS: 29 +/– 1 Blunt/Penetrating (%):38/62%	TEG 5000	INR, aPTT
Prat et al. 2017 Afghanistan [55]	Retrospective Cohort	219	Age: 18+ ISS: 21 (14–29) Blunt/Penetrating (%): 16/84% Combat patients	ROTEM Delta	INR, platelets
Rugeri et al. 2007 France [56]	Prospective cohort	88	Age: 18+ ISS: 22 (12–34)	ROTEM (model not specified)	INR, PT, aPTT, fibrinogen, platelets
Schochl et al. 2010 Austria [57]	Retrospective cohort	131	Age: 18+ ISS: 38 +/– 15 MT patients	ROTEM (model not specified)	Fibrinogen, aPTT, PT
Smith et al. 2020 USA [58]	Retrospective cohort	301	Age: 18+ ISS: >15 Blunt/Penetrating (%): 89/11%	ROTEM (model not specified)	INR
Stettler et al. 2018 USA [59]	Prospective cohort	222	Age: 18+ NISS: 46.5 (38–57)	ROTEM, TEG (model not specified)	INR, aPTT
Tapia et al. 2013 USA [60]	Retrospective cohort	289	Age: 18+ ISS: 23+/– 14 Blunt/Penetrating (%): 34/66% MT patients	TEG (model not specified)	Not specified
Unruh et al. 2019 USA [61]	Retrospective cohort	67	Age: 18+ ISS: 26.7 +/– 14.7 Blunt/Penetrating (%): 76/24% MT patients	TEG 5000	PT, INR, fibrinogen
Van Wessem et al. 2017 Netherlands [62]	Prospective cohort	135	Age: 18+ ISS: 29 (22–38) Blunt/Penetrating (%): 96/4% ICU polytrauma patients	TEG (model not specified)	aPTT, PT, platelets
Walters et al. 2018 Australia [63]	Retrospective cohort	326	Age: 18+ ISS: median 22 ICU trauma patients	ROTEM (model not specified)	Not specified
Yin et al. 2014 China [64]	Retrospective cohort	60	Age: 18+ ISS: 15.2 +/– 6.9 Blunt/Penetrating (%): 83/17% Abdominal trauma	TEG 5000	INR, aPTT

VHA, viscoelastic haemostatic assays; SLCT, standard laboratory coagulation tests; ISS, injury severity score – reported as either median (Interquartile range) or mean +/– standard deviation; NISS, new injury severity score; TBI, traumatic brain injury; TEG, thromboelastography; ROTEM, rotational thromboelastometry; TEM-A, automated thromboelastometry; aPTT, activated partial thromboplastin time; PT, prothrombin time; INR, international normalized ratio; ICU, intensive care unit.

Table 3. Risk of bias assessment – Newcastle-Ottawa quality assessment for cohort studies

Study	Selection				Comparability		Outcome			Rating	
	representativeness of the exposed cohort	selection of non-exposed cohort	ascertainment of exposure	outcome not present at the start	study controls for age and sex	study controls for at least 3 other variables	assessment of outcome	adequate follow-up	loss to follow-up	overall quality score	rating
Bouzat et al. 2019 [31]	*	*	*	*	–	–	*	*	*	7/9	Good
Cohen et al. 2019 [30]	–	*	*	–	*	*	*	*	–	6/9	Fair
Doran et al. 2010 [29]	–	*	*	–	*	*	*	*	–	6/9	Fair
Jeger et al. 2009 [26]	*	*	*	*	*	*	*	*	*	9/9	Good
Subramanian et al. 2014 [22]	*	*	*	*	*	*	*	*	*	9/9	Good
Sumislawski et al. 2019 [21]	*	*	*	*	*	*	*	*	–	8/9	Good
Tabuer et al. 2011 [20]	*	*	*	*	*	*	*	*	*	9/9	Good
Tonglet et al. 2018 [19]	*	*	*	–	*	*	*	*	*	8/9	Good
TurMartinez et al. 2018 [25]	*	*	*	–	*	*	*	*	*	8/9	Good
Zwinkels et al. 2020 [18]	*	*	*	–	–	–	*	*	*	6/9	Fair

Refer to http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp, for explanation of the Newcastle-Ottawa Quality Assessment Scale for cohort studies. The stars (*) represent higher degrees of quality in the respective areas of assessment for each article.

of stay with VHA. The iTACTIC randomized control trial (RCT) found no significant difference between VHA and SLCT groups in the rate of multiple organ failure, number of ventilator-free or ICU-free days, hospital length of stay, quality of life scores, and cause of death profiles [32]. Additionally, they reported that at 24 h, 28 days, and 90 days, there remained no significant differences in the mortality rate.

Discussion

This systematic review demonstrated that the use of VHAs was associated with a higher proportion of patients being diagnosed with ATC when compared to SLCTs. Whilst there was some indication that VHAs may lead to a reduction in blood and blood product use, benefits on patient outcomes were not conclusive and could not be demonstrated from the only RCT on the topic. The clinical significance of early detection of abnormalities on viscoelastic measures of blood clotting is therefore questioned.

The variability of VHA tools including ROTEM Sigma, ROTEM Delta, TEG 5000 versus TEG 6 s and TEM-A added to the heterogeneity of the observations. Additionally, blood was obtained at different times after injury for analysis and this was not always reported on in the included articles. Some authors repeated the assay at either the clinician's discretion or at regular intervals, where others simply obtained the initial result for diagnostic purposes. There was also a variation in whether the VHA results were available to the treating clinicians, research team only or used as the primary method for guiding resuscitation efforts. Those studies that implemented VHA-guided protocols registered an adherence rate of 65–92%, potentially reducing the number of eligible patients from which to draw significant conclusions [18, 27].

Adding to the heterogeneity of VHA measurement tools and timing, there was variability in the definition of ATC by the VHAs and SLCTs. Many studies used a number of arbitrary thresholds to determine the diagnosis of ATC. These introduce significant clinical heterogeneity to this review (online suppl. Material; for all online suppl. material, see www.karger.com/doi/10.1159/000526217). The VHA ranges used were either provided by the manufacturers, a result of author experience, or predetermined for the specific patient population. Subramanian et al. [22] found an improved diagnostic accuracy using values created against a gold-standard SLCT in their laboratory rather than the ROTEM manufacturer reference set. This may impact the comparison of studies between specific trauma populations if the reference ranges also vary in cut-offs and efficacy.

Table 4. VHA manufacturer reference ranges [65, 66]

ROTEM						
parameter	CT (s)	CFT (s)	alpha angle, °	A10, mm	MCF, mm	LI30, *n* (%)
EXTEM	38–79	34–159	63–83	43–65	50–72	94–100
INTEM	100–240	30–110	70–83	44–66	50–71	94–100
HEPTEM	100–240	30–110	70–83	44–66	50–71	94–100
	Comparison with INTEM. A better clot formation in HEPTEM as compared with INTEM indicates the presence of heparin or heparin-like anticoagulants in the sample					
APTEM	38–79	34–159	63–83	43–65	50–72	n/a
	Comparison with EXTEM. A better clot formation in APTEM as compared with EXTEM is a sign of hyperfibrinolysis					
FIBTEM	n/a	n/a	30–70	7–23	9–25	n/a
TEG						
parameter	R value, min	K time, min	alpha angle, °	A10, mm	MA, mm	LY30, n (%)
CK-TEG	4.6–9.1	0.8–2.1	63–78°	n/a	52–69	0–2.6

Citrated Kaolin TEG (CK-TEG), EXTEM, INTEM, HEPTEM, APTEM, and FIBTEM refer to the different reagents used to assess various clot dynamics. CT, clotting time; CFT, clot formation time; MCF, maximum clot firmness; A10, clot amplitude at 10 min; LI30 & LY30, clot lysis at 30 min, *R* value, reaction time; K time, kinetics; MA, maximum amplitude.

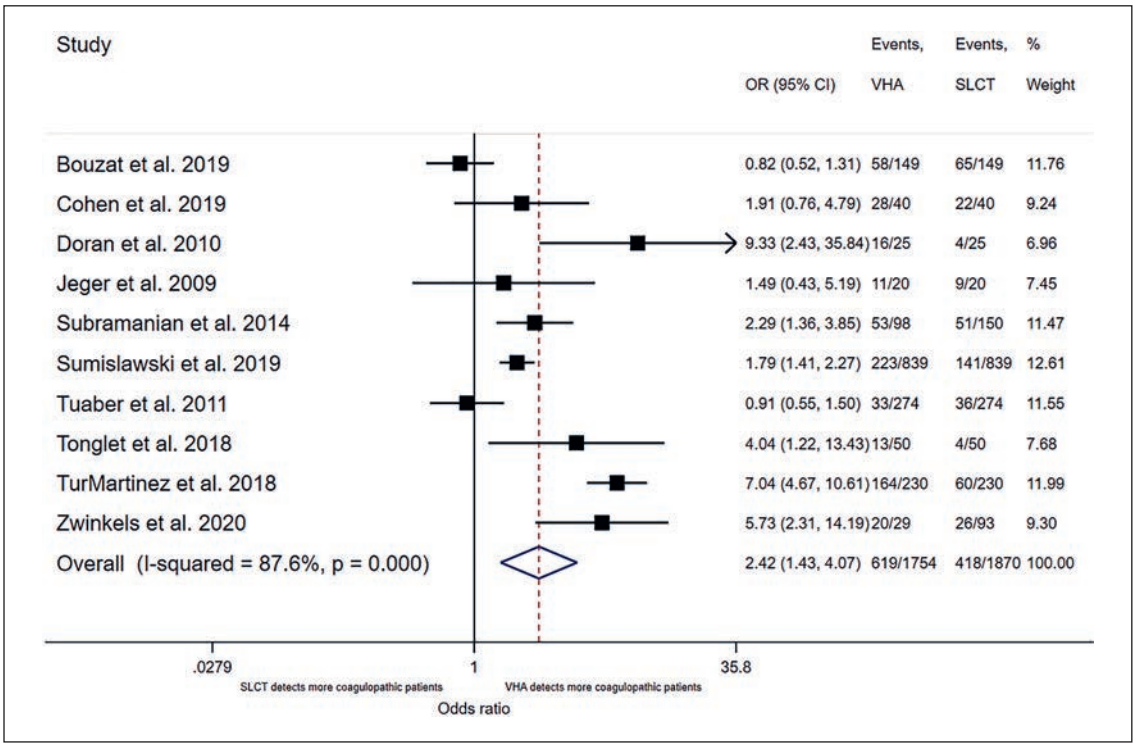

Fig. 2. Meta-analysis of studies assessing general trauma patients.

In recent observational studies, an overall reduction in transfusion rates has been demonstrated using VHA-guided treatment [45, 51, 55, 61, 64]. Some evidence comparing various TEG and ROTEM measurements to SLCTs suggests the ability to forecast outcomes and predict transfusion requirements [19, 20, 24, 35–37, 50, 52, 58, 59]. A systematic review published in 2016, identified that the ROTEM measurements for clot amplitude at 5

min (CA5) and maximum clot firmness (MCF) were able to predict transfusion requirements and mortality [65]. These analyses pose an issue in application of such statistical predictions in a clinical setting that is highly variable, dynamic, and fast-paced, often with unstable patients. It is common practice to identify this correlation using VHA results only obtained for research purposes in patients treated according to their SLCT results. In 2013, Johansson et al. discovered that when they compared TEG parameters obtained during TEG-guided resuscitation, it weakened the statistical signal for prediction of mortality and massive transfusion [44].

Whilst the iTACTIC trial saw a 1.8 times increase in the number of interventions given in the VHA group, they were unable to ascertain whether those additional therapies provided were able to effectively correct the coagulopathy and achieve haemostasis [32]. Furthermore, the iTACTIC trial demonstrated increased fibrinogen supplementation in the VHA treated patients. This may be due to the time delay in SLCTs identifying low fibrinogen levels compared to VHAs, resulting in faster and more frequent detection of low fibrinogen and subsequent supplementation. However, it is important to consider that the iTACTIC trial assessed VHA groups using both TEG and ROTEM devices combined, but research shows that the fibrinogen-based clot integrity levels reported are different between the two devices. This indicates that the potential of VHAs overall to guide fibrinogen supplementation therapy without factoring in this difference may lead to inconsistent and/or inaccurate treatment [66].

In regards to mortality, several articles from 2010 to 2020 showed a trend towards increased survival when using VHA-guided treatment [44, 45, 49, 60], but equally as many studies displayed no significant benefit [18, 40, 42, 47, 51, 62]. A previous study by Mohammed et al. reported only a significant decrease in mortality for patients <30 years old and none overall [51]. They also noted that repeating TEGs had no impact on survival. There are two published RCTs on VHA in trauma, one shows rapid-TEG-guided treatment led to an improved 28-day survival and decreased the rate of early haemorrhage [39], whereas the most recent multi-centre RCT concludes no difference in the mortality and massive transfusion outcomes [32]. A recent publication by Lammers et al. [49], showed a 57% reduction in overall mortality after implementing ROTEM in trauma [49]. In this analysis the reported benefit for 24 h, 30-days and overall mortality with VHA use was inconsistent amongst the studies comparing the VHA- and SLCT-guided resuscitation. Therefore, whilst an identifiable trend is evident in the literature, no further conclusions can be made from this study.

This systematic review was limited in only including one RCT, the wide heterogeneity of repeatable VHA techniques, inconsistent data reporting and lack of comparable definitions for ATC and massive transfusion. Unfortunately, the only other RCT on VHA in trauma by Gonzalez et al. [39] did not have data available on the proportion of patients with and without coagulopathy in each of the two groups, and could not be included in the analysis. Furthermore, there were a handful of studies that focused on certain subgroups of trauma, being TBI [23, 27, 28], penetrating injury [24], anticoagulant usage [25, 28], and combat setting [24, 29, 30]. This is not an accurate reflection of the distribution of patients in a large portion of civilian trauma hospitals. Additionally, there is an evident link between TBI and coagulopathy, although it is not clear if this predisposes to ATC or is a separate phenomenon [67, 68]. The iTACTIC trial reported an unexpected finding of mortality improvement in subgroup analysis of TBI patients in the VHA group. This may indicate an area for future improvement in trauma care as TBI has typically been excluded on the basis that it is unlikely to be affected by changes to haemostatic management due to severity and underlying pathology [32].

One of the more practical considerations in comparisons of VHA- and SLCT-guided resuscitations is the cost of implementing new treatment modalities. Whilst none of our included studies directly reported on the cost effectiveness of VHA in their analysis, a 2015 UK study compared the costs of the TEG and ROTEM devices available at the time and concluded that the cost effectiveness of the VHA devices dominated the SLCTs. The estimated cost savings were £688 for ROTEM and £721 for TEG when compared to the SLCTs. It is, however, worth noting that the authors had minimal access to data on the effectiveness of VHA in trauma and so the results were more "indicative of the potential cost-effectiveness" rather than definitive for trauma patients [69].

The retrospective studies were at risk of selection bias in that they did not assess patients with clinical signs of bleeding and may have missed those who were coagulopathic and not bleeding and vice versa. In regard to the multi-centre articles, Sumislawski et al. [21] noted that they did not have identical transfusion protocols between the two centres for accurate collation and comparison of data. Moreover, there was distinct statistical heterogeneity from variation in sample size between included manuscripts. The marked variation in literature, combined with scarcity of studies, leads to poor clinical applicability and decidedness on the ability of VHA to improve outcomes in trauma.

Conclusion

The current literature on detection of ATC by VHA is predominantly observational and displays variability regarding the utility of VHA. Use of VHAs during trauma resuscitation detected higher rates of ATC, faster time to results, and the potential to anticipate massive transfusions. However, the clinical significance of findings of abnormalities remains unknown. Prospective trials that combine investigations towards ATC with evidence-based therapeutic strategies may further elucidate the benefits of early investigations.

Statement of Ethics

An ethics statement is not applicable because this study is based exclusively on published literature.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

This is an original project with the initial concept proposed by Biswadev Mitra. Ellen Forster was primarily involved in creating and executing the search strategy, data analysis, and manuscript write-up. Biswadev Mitra and Simon Hendel were involved in the editing, draft review, concept construction, and overall direction of the paper. The work presented here is completely original and does not contain any material previously published elsewhere by another author, except where appropriate references have been made.

Data Availability Statement

All data generated or analysed during this study are included in this article and its online supplementary material. Further enquiries can be directed to the corresponding author.

References

1 Victorian State Trauma Registry. State trauma system and registry annual report 2016–2017. Victoria, Australia: Department of Health and Human Services; 2018.
2 Sobrino J, Shafi S, editors. Timing and causes of death after injuries. Baylor University Medical Center ProceedingsTaylor & Francis; 2013.
3 Harris T, Davenport R, Mak M, Brohi K. The evolving science of trauma resuscitation. Emerg Med Clin North Am. 2018;36(1):85–106.
4 Frith D, Davenport R, Brohi K. Acute traumatic coagulopathy. Curr Opin Anaesthesiol. 2012;25(2):229–34.
5 Brohi K, Singh J, Heron M, Coats T. Acute traumatic coagulopathy. J Trauma. 2003; 54(6):1127–30.
6 Maegele M, Lefering R, Yucel N, Tjardes T, Rixen D, Paffrath T, et al. Early coagulopathy in multiple injury: an analysis from the German Trauma Registry on 8,724 patients. Injury. 2007;38(3):298–304.
7 Norfolk D. Handbook of transfusion medicine Sheffield UK: United Kingdom Blood Services 2013 [5th edition]; 2013. Available from: https: //www.transfusionguidelines.org/transfusion-handbook/.
8 Boudaoud L, Divaret G, Marie P, Bezeaud A, editors. Rapid centrifugation for routine coagulation testing. Annales de biologie clinique. 2006;64(4):315–7.
9 Mitra B, O'Reilly G, Collecutt M, Cameron PA, Phillips L, Davis A. Prospective comparison of point-of-care international normalised ratio measurement versus plasma international normalised ratio for acute traumatic coagulopathy. Emerg Med Australas. 2012; 24(4):363–8.
10 Hagemo JS, Christiaans SC, Stanworth SJ, Brohi K, Johansson PI, Goslings JC, et al. Detection of acute traumatic coagulopathy and massive transfusion requirements by means of rotational thromboelastometry: an international prospective validation study. Crit Care. 2015;19(1):97.
11 Goerlinger K, Dirkmann D, Kiss G, Dusse F, Hanke A, Arvieux CC, et al. ROTEM-based management for diagnosis and treatment of acute haemorrhage during liver transplantation: a-322. Eur J Anaesthesiol. 2006;23(Suppl 37):85.
12 Redfern RE, Fleming K, March RL, Bobulski N, Kuehne M, Chen JT, et al. Thrombelastography-directed transfusion in cardiac surgery: impact on postoperative outcomes. Anna Thorac Surg. 2019;107(5):1313–8.
13 Hunt H, Stanworth S, Curry N, Woolley T, Cooper C, Ukoumunne O, et al. Thromboelastography (TEG) and rotational thromboelastometry (ROTEM) for trauma-induced coagulopathy in adult trauma patients with bleeding. Cochrane Database Syst Rev. 2015; 2015(2):CD010438.
14 Wikkelsø A, Wetterslev J, Møller AM, Afshari A. Thromboelastography (TEG) or thromboelastometry (ROTEM) to monitor haemostatic treatment versus usual care in adults or children with bleeding. Cochrane Database Syst Rev. 2016;2018(12):CD007871.
15 Forster E, Hendel S, Mitra B. Detection of acute traumatic coagulopathy by viscoelastic haemostatic assays compared to standard laboratory tests: a systematic review 2020. 2020. Available from: https://www.crd.york.ac.uk/prospero/display_record.php?RecordID=178710.
16 Wells GA, Shea B, O'connell D, Peterson J, Welch V, Losos M, et al. The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomised studies in meta-analyses. 2013.
17 Shamseer L, Moher D, Clarke M, Ghersi D, Liberati A, Petticrew M, et al. Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015: elaboration and explanation. BMJ. 2015;350:g7647.
18 Zwinkels RLJ, Endeman H, Hoeks SE, de Maat MPM, den Hartog D, Stolker RJ. The clinical effect of hemostatic resuscitation in traumatic hemorrhage; a before-after study. J Crit Care. 2020;56:288–93.
19 Tonglet ML, Poplavsky JL, Seidel L, Minon JM, D'Orio V, Ghuysen A. Thromboelastometry in trauma care: a place in the 2018 Belgian health care system? Acta Clin Belg. 2018; 73(4):244–50.
20 Tauber H, Innerhofer P, Breitkopf R, Westermann I, Beer R, El Attal R, et al. Prevalence and impact of abnormal ROTEM(R) assays in severe blunt trauma: results of the "Diagnosis and Treatment of Trauma-Induced Coagulopathy (DIA-TRE-TIC) study". Br J Anaesth. 2011;107(3):378–87.
21 Sumislawski JJ, Christie SA, Kornblith LZ, Stettler GR, Nunns GR, Moore HB, et al. Discrepancies between conventional and viscoelastic assays in identifying trauma-induced coagulopathy. Am J Surg. 2019;217(6):1037–41.
22 Subramanian A, Albert V, Agrawal D, Saxena R, Pandey RM. Evaluation of the utility of thromboelastography in a tertiary trauma care centre. ISRN Hematol. 2014;2014: 8496261.

23 Schöchl H, Solomon C, Traintinger S, Nienaber U, Tacacs-Tolnai A, Windhofer C, et al. Thromboelastometric (ROTEM) findings in patients suffering from isolated severe traumatic brain injury. J Neurotrauma. 2011; 28(10):2033–41.
24 Plotkin AJ, Wade CE, Jenkins DH, Smith KA, Noe JC, Park MS, et al. A reduction in clot formation rate and strength assessed by thrombelastography is indicative of transfusion requirements in patients with penetrating injuries. J Trauma. 2008;64(2 Suppl):S64–8.
25 TurMartínez J, Petrone P, Axelrad A, Marini CP. Comparison between thromboelastography and conventional coagulation test: should we abandon conventional coagulation tests in polytrauma patients? Cir Esp. 2018;96(7): 443–9.
26 Jeger V, Zimmermann H, Exadaktylos AK. Can RapidTEG accelerate the search for coagulopathies in the patient with multiple injuries? J Trauma. 2009;66(4):1253–7.
27 Gratz J, Güting H, Thorn S, Brazinova A, Görlinger K, Schäfer N, et al. Protocolised thromboelastometric-guided haemostatic management in patients with traumatic brain injury: a pilot study. Anaesthesia. 2019;74(7):883–90.
28 Gozal YM, Carroll CP, Krueger BM, Khoury J, Andaluz NO. Point-of-care testing in the acute management of traumatic brain injury: identifying the coagulopathic patient. Surg Neurol Inter. 2017;8:48.
29 Doran CM, Woolley T, Midwinter MJ. Feasibility of using rotational thromboelastometry to assess coagulation status of combat casualties in a deployed setting. J Trauma. 2010; 69(Suppl 1):S40–8.
30 Cohen J, Scorer T, Wright Z, Stewart IJ, Sosnov J, Pidcoke H, et al. A prospective evaluation of thromboelastometry (ROTEM) to identify acute traumatic coagulopathy and predict massive transfusion in military trauma patients in Afghanistan. Transfusion. 2019;59(S2):1601–7.
31 Bouzat P, Guerin R, Boussat B, Nicolas J, Lambert A, Greze J, et al. Diagnostic performance of thromboelastometry in trauma-induced coagulopathy: a comparison between two level I trauma centres using two different devices. Eur J Trauma Emerg Surg. 2019; 47(2):343–51.
32 Baksaas-Aasen K, Gall LS, Stensballe J, Juffermans NP, Curry N, Maegele M, et al. Viscoelastic haemostatic assay augmented protocols for major trauma haemorrhage (ITACTIC): a randomised, controlled trial. Intensive Care Med. 2021;47(1):49–59.
33 Albert V, Subramanian A, Pati HP, Agrawal D, Bhoi SK. Efficacy of thromboelastography (TEG) in predicting acute trauma-induced coagulopathy (ATIC) in isolated severe traumatic brain injury (iSTBI). Indian J Hematol Blood Transfus. 2019;35(2):325–31.
34 Baksaas-Aasen K, Van Dieren S, Balvers K, Juffermans NP, Næss PA, Rourke C, et al. Data-driven development of ROTEM and TEG algorithms for the management of trauma hemorrhage: a prospective observational multicenter study. Ann Surg. 2019;270(6): 1178–85.
35 Coleman JR, Moore EE, Chapman MP, Banerjee A, Silliman CC, Ghasabyan A, et al. Rapid TEG efficiently guides hemostatic resuscitation in trauma patients. Surgery. 2018; 164(3):489–93.
36 Cotton BA, Faz G, Hatch QM, Radwan ZA, Podbielski J, Wade C, et al. Rapid thrombelastography delivers real-time results that predict transfusion within 1 hour of admission. J Trauma. 2011;71(2):407–14; discussion 414–7.
37 Davenport R, Manson J, De'Ath H, Platton S, Coates A, Allard S, et al. Functional definition and characterization of acute traumatic coagulopathy. Crit Care Med. 2011;39(12): 2652–8.
38 David JS, Durand M, Levrat A, Lefevre M, Rugeri L, Geay-Baillat MO, et al. Correlation between laboratory coagulation testing and thromboelastometry is modified during management of trauma patients. J Trauma Acute Care Surg. 2016;81(2):319–27.
39 Gonzalez E, Moore EE, Moore HB, Chapman MP, Chin TL, Ghasabyan A, et al. Goal-directed hemostatic resuscitation of trauma-induced coagulopathy: a pragmatic randomized clinical trial comparing a viscoelastic assay to conventional coagulation assays. Ann Surg. 2016;263(6):1051–9.
40 Guth C, Vassal O, Friggeri A, Wey P-F, Inaba K, Decullier E, et al. Effects of modification of trauma bleeding management: a before and after study. Anaesth Crit Care Pain Med. 2019;38(5):469–76.
41 Holcomb JB, Minei KM, Scerbo ML, Radwan ZA, Wade CE, Kozar RA, et al. Admission rapid thrombelastography can replace conventional coagulation tests in the emergency department: experience with 1974 consecutive trauma patients. Ann Surg. 2012;256(3): 476–86.
42 Hota S, Ng M, Hilliard D, Burgess J. Thromboelastogram-guided resuscitation for patients with traumatic brain injury on novel anticoagulants. Am Surg. 2019;85(8):861–4.
43 Jeger V, Willi S, Liu T, Yeh DD, De Moya M, Zimmermann H, et al. The rapid TEG α-angle may be a sensitive predictor of transfusion in moderately injured blunt trauma patients. Sci World J. 2012;2012:821794.
44 Johansson PI, Sørensen AM, Larsen CF, Windeløv NA, Stensballe J, Perner A, et al. Low hemorrhage-related mortality in trauma patients in a Level I trauma center employing transfusion packages and early thromboelastography-directed hemostatic resuscitation with plasma and platelets. Transfusion. 2013; 53(12):3088–99.
45 Johansson PI, Stensballe J. Effect of haemostatic control resuscitation on mortality in massively bleeding patients: a before and after study. Vox Sang. 2009;96(2):111–8.
46 Kashuk JL, Moore EE, Le T, Lawrence J, Pezold M, Johnson JL, et al. Noncitrated whole blood is optimal for evaluation of postinjury coagulopathy with point-of-care rapid thrombelastography. J Surg Res. 2009;156(1): 133–8.
47 Kashuk JL, Moore EE, Wohlauer M, Johnson JL, Pezold M, Lawrence J, et al. Initial experiences with point-of-care rapid thrombelastography for management of life-threatening postinjury coagulopathy. Transfusion. 2012; 52(1):23–33.
48 Kobayashi LM, Brito A, Barmparas G, Bosarge P, Brown CV, Bukur M, et al. Laboratory measures of coagulation among trauma patients on NOAs: results of the AAST-MIT. Trauma Surg Acute Care Open. 2018;3(1): e000231.
49 Lammers DT, Marenco CW, Morte KR, Bingham JR, Martin MJ, Eckert MJ. Viscoelastic testing in combat resuscitation: is it time for a new standard? J Trauma Acute Care Surg. 2020;89(1):145–52.
50 Leemann H, Lustenberger T, Talving P, Kobayashi L, Bukur M, Brenni M, et al. The role of rotation thromboelastometry in early prediction of massive transfusion. J Trauma. 2010;69(6):1403–8; discussion 1408–9.
51 Mohamed M, Majeske K, Sachwani GR, Kennedy K, Salib M, McCann M. The impact of early thromboelastography directed therapy in trauma resuscitation. Scand J Trauma Resusc Emerg Med. 2017;25(1):99.
52 Nystrup KB, Windeløv NA, Thomsen AB, Johansson PI. Reduced clot strength upon admission, evaluated by thrombelastography (TEG), in trauma patients is independently associated with increased 30-day mortality. Scand J Trauma Resusc Emerg Med. 2011; 19(1):52.
53 Peng HT, Nascimento B, Tien H, Callum J, Rizoli S, Rhind SG, et al. A comparative study of viscoelastic hemostatic assays and conventional coagulation tests in trauma patients receiving fibrinogen concentrate. Clin Chim Acta. 2019;495:253–62.
54 Pezold M, Moore EE, Wohlauer M, Sauaia A, Gonzalez E, Banerjee A, et al. Viscoelastic clot strength predicts coagulation-related mortality within 15 minutes. Surgery. 2012;151(1): 48–54.
55 Prat NJ, Meyer AD, Ingalls NK, Trichereau J, DuBose JJ, Cap AP. Rotational thromboelastometry significantly optimizes transfusion practices for damage control resuscitation in combat casualties. J Trauma Acute Care Surg. 2017;83(3):373–80.
56 Rugeri L, Levrat A, David JS, Delecroix E, Floccard B, Gros A, et al. Diagnosis of early coagulation abnormalities in trauma patients by rotation thrombelastography. J Thromb Haemost. 2007;5(2):289–95.
57 Schöchl H, Nienaber U, Hofer G, Voelckel W, Jambor C, Scharbert G, et al. Goal-directed coagulation management of major trauma patients using thromboelastometry (ROTEM®)-guided administration of fibrinogen concentrate and prothrombin complex concentrate. Crit Care. 2010;14(2):R55.
58 Smith AR, Karim SA, Reif RR, Beck WC, Taylor JR, Davis BL, et al. ROTEM as a predictor of mortality in patients with severe trauma. J Surg Res. 2020;251:107–11.
59 Stettler GR, Moore EE, Nunns GR, Chandler J, Peltz E, Silliman CC, et al. Rotational thromboelastometry thresholds for patients at risk for massive transfusion. J Surg Res. 2018;228:154–9.

60 Tapia NM, Chang A, Norman M, Welsh F, Scott B, Wall MJ Jr, et al. TEG-guided resuscitation is superior to standardized MTP resuscitation in massively transfused penetrating trauma patients. J Trauma Acute Care Surg. 2013;74(2):378–85; discussion 385–6.
61 Unruh M, Reyes J, Helmer SD, Haan JM. An evaluation of blood product utilization rates with massive transfusion protocol: Before and after thromboelastography (TEG) use in trauma. Am J Surg. 2019;218(6):1175–80.
62 van Wessem KJP, Leenen LPH. Thromboelastography does not provide additional information to guide resuscitation in the severely injured. ANZ J Surg. 2018;88(7–8):697–701.
63 Walters K, Wake E, Campbell D, Wullschleger M, Chalasani A, Ho D, et al. Critical evaluation of a targeted point of care ROTEM guided coagulation and haemostasis management programme in severe trauma. Aust Crit Care. 2018;31(2):116.
64 Yin J, Zhao Z, Li Y, Wang J, Yao D, Zhang S, et al. Goal-directed transfusion protocol via thrombelastography in patients with abdominal trauma: a retrospective study. World J Emerg Surg. 2014;9(1):28.
65 Veigas PV, Callum J, Rizoli S, Nascimento B, da Luz LT. A systematic review on the rotational thrombelastometry (ROTEM®) values for the diagnosis of coagulopathy, prediction and guidance of blood transfusion and prediction of mortality in trauma patients. Scand J Trauma Resusc Emerg Med. 2016;24(1):114.
66 Prüller F, Münch A, Preininger A, Raggam RB, Grinschgl Y, Krumnikl J, et al. Comparison of functional fibrinogen (FF/CFF) and FIBTEM in surgical patients: a retrospective study. Clin Chem Lab Med. 2016;54(3):453–8.
67 Castellino FJ, Chapman MP, Donahue DL, Thomas S, Moore EE, Wohlauer MV, et al. Traumatic brain injury causes platelet adenosine diphosphate and arachidonic acid receptor inhibition independent of hemorrhagic shock in humans and rats. J Trauma Acute Care Surg. 2014;76(5):1169–76.
68 Lee TH, Hampton DA, Diggs BS, McCully SP, Kutcher M, Redick BJ, et al. Traumatic brain injury is not associated with coagulopathy out of proportion to injury in other body regions. J Trauma Acute Care Surg. 2014;77(1):67–72; discussion 72.
69 Whiting P, Al M, Westwood M, Ramos IC, Ryder S, Armstrong N, et al. Viscoelastic point-of-care testing to assist with the diagnosis, management and monitoring of haemostasis: a systematic review and cost-effectiveness analysis. Health Technol Assess. 2015;19(58):1–228.

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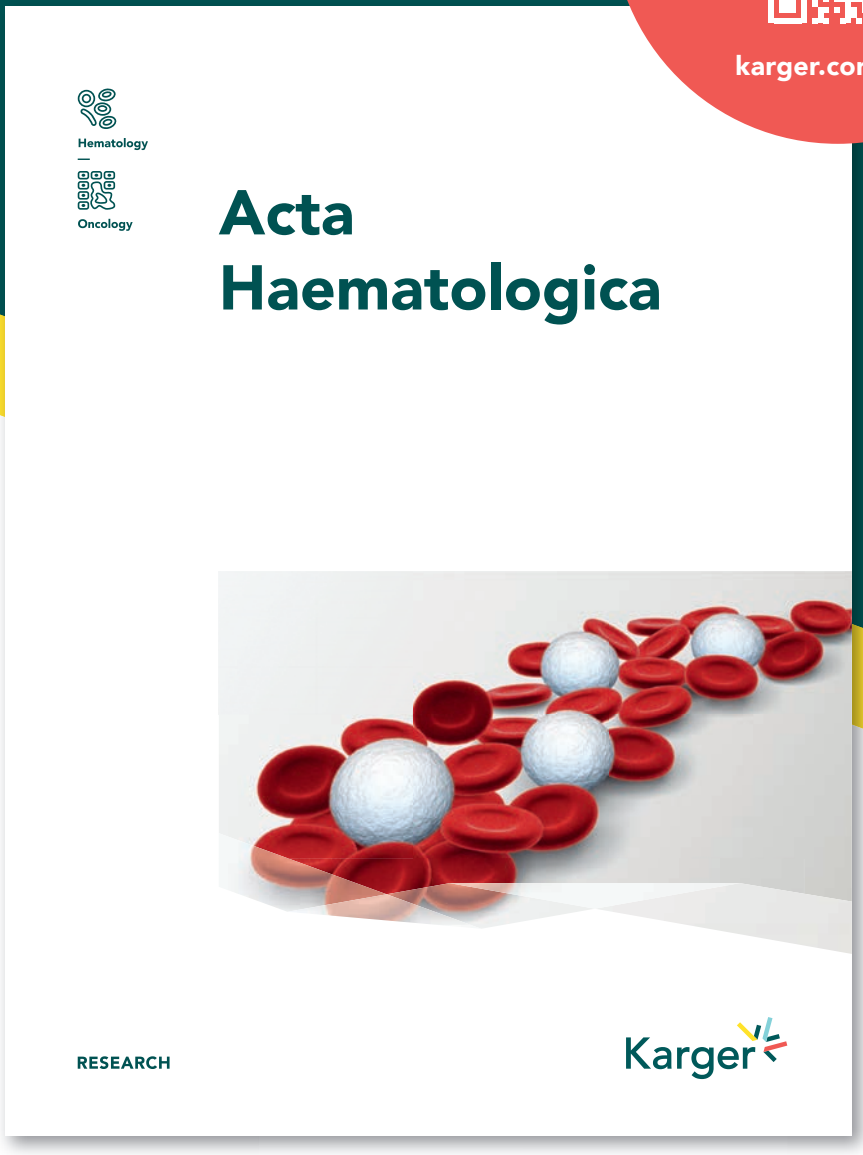

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Platelet-Rich Fibrin in Oral Surgery and Implantology: A Narrative Review

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Keywords

PRF · Oral surgery · Bone augmentation · Third molar extraction · MRONJ

Abstract

Background: The application of blood concentrates has gained popularity in dentistry in recent years. Platelet-rich fibrin (PRF) has been discussed frequently due to a high content of growth factors and the option of chair-side manufacturing in a simple centrifugation process. PRF is free from adjuvants and inexpensive to produce. The number of studies reporting beneficial effects of PRF in various clinical applications such as alveolar ridge preservation, sinus floor elevation, management and prevention of medical-related osteonecrosis of the jaw, third molar extractions, and guided bone regeneration in dentistry has increased recently. However, to date, neither clinical recommendations nor guidelines are available. The present narrative review aims to summarize the level of evidence on the clinical application of PRF within the field of oral surgery and implantology. ***Summary:*** A literature search in Pubmed and Medline has identified 34 articles as a basis for this narrative review. The effectiveness of the clinical application of PRF has been analyzed for five indications within dentistry: medical-related osteonecrosis of the jaw, wisdom tooth extraction, guided bone regeneration, sinus floor elevation, and alveolar ridge preservation. The amount of data for third molar extractions, socket preservation, and guided bone regeneration is extensive. Less data were available for the use of PRF in combination with sinus floor elevations. There is a lack of studies with scientific evidence on PRF and medical-related osteonecrosis of the jaw; however, studies positively impact patient-related outcome measures. Most studies report on beneficial effects when PRF is additionally applied in intrabony defects. There is no evidence of the positive effects of PRF combined with bone graft materials during sinus floor elevation. However, some benefits are reported with PRF as a sole filling material. ***Key Messages:*** Many recently published studies show the positive clinical impact of PRF. Yet, further research is needed to ensure the validity of the evidence.

Introduction

Blood concentrates have a lengthy history in dentistry and started with fibrin glues. The idea of this product is simple. Blood presence and its contents around the wound have always been the key to proper healing. Thus, establishing a blood clot at surgical sites appears to support natural healing processes [1–3].

The first described autologous platelet product used in dentistry was platelet-rich plasma (PRP) [4, 5]. It is produced in two centrifugation steps, depending on the applied protocol [6]. Platelets get activated by adding throm-

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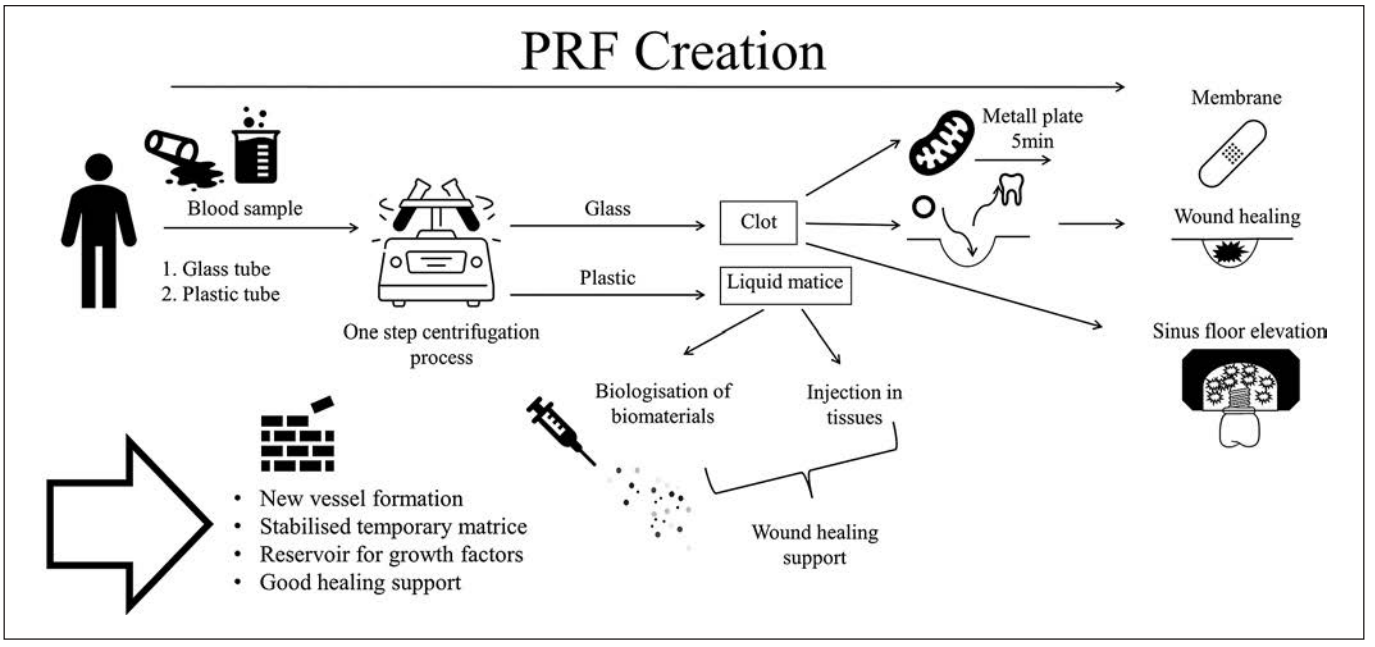

Fig. 1. PRF creation.

bin, usually of bovine descent, as well as calcium chloride or a combination of both. As a result, a liquid substance is fabricated. With certain technologies, its viscosity can be changed [6]. It contains approximately 5 times more platelets than noncentrifuged, natural blood and a variety of key growth factors that accelerate wound healing and tissue remodeling [7]. PRP stimulates certain cells' proliferation and differentiation, such as osteoblasts [4, 8]. It supports healing processes [9] and interacts with physiological processes, especially in the first days after surgeries, to prevent serious complications, like ankylosis [10]. PRP has been considered safe in clinical application with a low-risk of infections and allergies [11]. PRP further allows bleeding management during the treatment of patients with anticoagulant medications [12]. Despite all these positive effects, there are some limitations. In the literature, there are opposing results reported on the effects of PRP, especially on the promotion of bone healing [13–15]. In addition, PRP releases growth factors only once, right when it is applied [2]. Another disadvantage is its preparation, which requires time due to the two-step centrifugation process. As a result, PRP seems rather unlikely to become a routine clinical practice in the future [13].

More than 10 years ago, Choukroun et al. [16] introduced platelet-rich fibrin (PRF). PRF is the first platelet concentrate easily generated in a single-stage centrifugation process free of anticoagulants [17] (see Fig. 1). PRP clots' size is rather small compared to PRF [13]. The matrix of PRF tends to be more penetrable than the one of PRP. This might ease the release of growth factors and other important substances of the wound healing cascade. Also, its permeability for cells simplifies the organization of the wound healing process [1, 3, 18]. There are two consistencies of PRF-solid [19] and liquid [20]. In oral surgery, the solid version of PRF is preferably often and specially used as a membrane, for example, to cover bone substitute in bone augmentation procedures. Membranes can be created by pressing PRF-clots with a plate [21]. With certain centrifugation protocols using glass tubes or silica-coated tubes, PRF forms as a solid clot. Being weighted with a panel PRF turns into a stable membrane used for guided bone regeneration (GBR) procedures in various trials [22, 23]. Further cylindric clots are frequently used for alveolar ridge preservation techniques or as filling material in cyst cavities [23]. Due to the centrifugation process, three fractions are visible: acellular plasma, fibrin, and erythrocytes on the bottom [21]. PRF consists of various blood cells, growth factors, and fibrinogen which play a crucial role in natural healing processes [21, 23]. The cell composition of PRF includes: platelets, lymphocytes, monocytes, neutrophils, and basophiles [24].

The complex process of wound healing can be partitioned into several overlapping phases. After the immediate excess of blood to flush out the wound, the hemostasis and coagulation cascade are initiated. Surrounding vessels contract for a few min and platelets initiate the formation of a clot as a temporary matrix. This active matrix involving fibrin acts as a storage for invading cells. Vessels dilate, leukocytes enter the wound stimulated by activated platelets and their growth factor release. The inflammatory phase is initiated by release of IL-1 and IL-6 [25]. TGF-β1 regulates inflammatory processes and the

synthesis of collagen type III [25–27], and VEGF leads to new blood vessel formation and stabilization. VEGF further supports the proliferation and division of fibroblasts and endothelial cells. New bone formation seems to be enhanced [28, 29]. Neutrophils perform wound cleansing during inflammatory phase by phagocytosis and secretion of proteinases. Monozytes differentiated to macrophages participate in the process of wound debridement and trigger the proliferative phase through the release of TGF-β, PDGF, and VEGF. Additionally, they act as antigen-presenters [25, 30] with the aim of granulation tissue formation; vessels grow in the proliferative phase. Collagen synthesis is aiming to close the wound in the first place and to decrease again as keratinocytes start re-epitheliazation. Finally, collagenases and elastases are released to dissolve intercellular bonds and restore the permeability of the tissue. Dense new formed vascular nets, granulocytes, macrophages, fibroblasts, and collagen bundles are the main components of the granulation tissue developed. During remodeling, collagen type III is exchanged to more robust collagen type I [25].

The PRF-clots head describes the part which is abutted in the red blood cells after the centrifugation process. Along the border, authors describe a high leukocyte content [28].

Complications stimulate the development of new methods. In recent years, the topic of PRF has reached interest in the field of clinical research. The number of published articles in dentistry has grown. Ghanaati et al. have summarized the first 15 years of PRF in a systematic review [31], The quantity of publications and reports on the application of PRF in dental surgery and tissue regeneration has significantly increased and deepened. The number of articles indexed in Pubmed related to the keyword combination "platelet-rich fibrin" AND "oral surgery" has expanded exponentially since 2006 from 8 publications to 125 publications in 2021. However, no clinical recommendations or guidelines for indications and clinical protocols can be extracted. Therefore, the present narrative review aims to give an overview of the current level of evidence on the application of PRF within the field of dentistry and the regeneration of oral tissues.

Main Text

Materials and Methods

Literature search has been conducted via Pubmed and Medline. The following keywords have been used: "PRF" AND "Third molar" OR "SOCKET PRESERVATION" OR "SINUS FLOOR ELEVATION" OR "STICKY BONE" OR "GUIDED BONE REGENERATION" OR "ALVEOLAR RIDGE PRESERVATION" OR "MRONJ."

PICO approach has been applied to verify the current knowledge effect of PRF in oral surgery.

Population

Adult human beings receiving third molar extractions, socket preservation, alveolar ridge preservation, sinus floor elevation, GBR, or a surgical intervention due to osteonecrosis of the jaw.

Intervention

Randomized clinical trial (RCTs)/case series/cohort studies/prospective studies or retrospective studies with additional application of PRF in oral surgery.

Comparison

RCTs/case series/cohort studies/prospective studies or retrospective studies without application of PRF in oral surgery.

Outcome

Healing of soft tissue, healing of hard tissue, the patient-centered outcome such as pain.

Inclusion Criteria

In vivo studies, RCTs, case series, cohort studies, prospective studies, retrospective studies, only studies published between 2018 and 2021. Ghanaati et al. summarized relevant clinical data about PRF in the field of dentistry up to 2017 [31].

Exclusion Criteria

Reviews, animal studies, in vitro studies, studies published in languages other than English (see Fig. 2).

Scientific evidence, according to the US Agency for Healthcare Research and Quality, has been classified (see Table 1).

Third Molar Extractions

The extraction of wisdom teeth is a frequently performed oral surgical intervention potentially causing distress in patients. This may be related to possible postoperative complications such as trismus, pain, and severe swelling, which are usually more frequent than infections, alveolar osteitis, and sensibility disorders [33]. In order to improve patients' postsurgical outcomes in regard to swelling, trismus, and pain, filling the defect with a PRF clot has been described in the literature [34].

Out of 22 articles published since 2018, only eight studies have been selected for this review. This selection is due to the number of patients, follow-up rate, and examination parameters. Studies with at least 20 patients were included. In total, a number of 315 patients were involved in the mentioned articles. Follow-up in the first week to report pain and swelling results up to 6 months to assess bone healing was included. Parameters investigated in the use of PRF for wisdom tooth removals are most likely pain assessment [29, 35–41], level of swelling

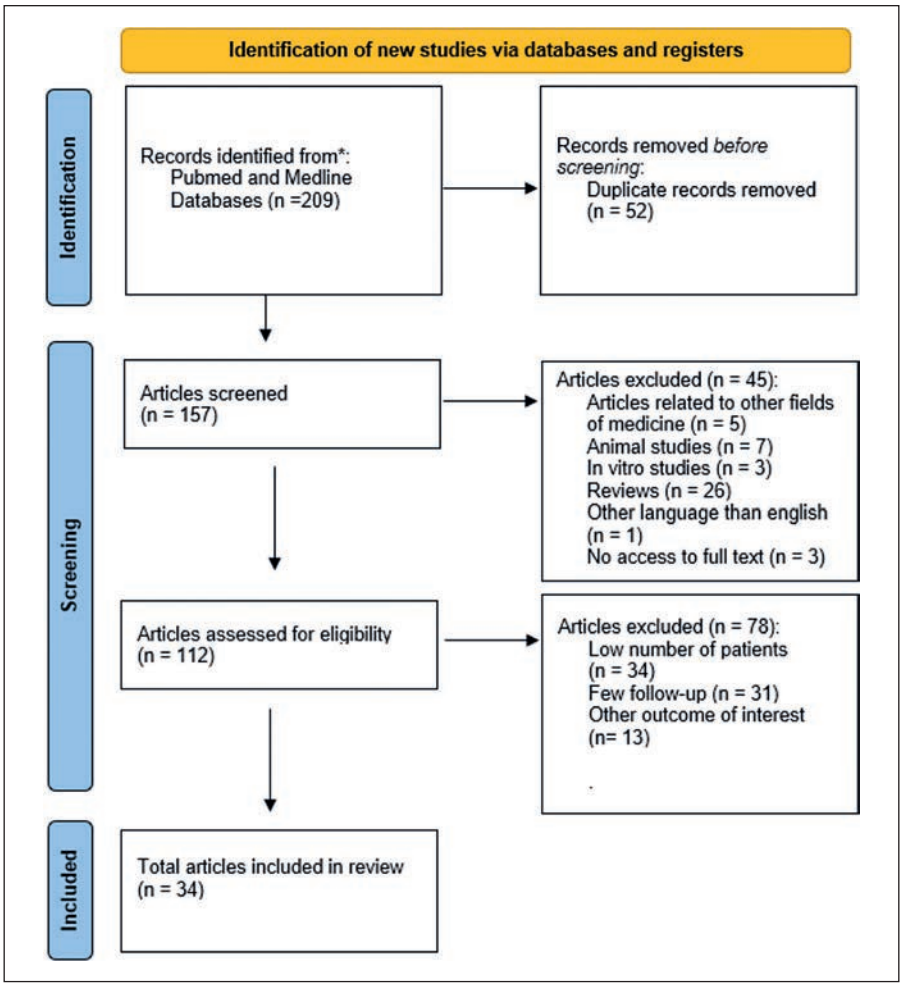

Fig. 2. Adapted after: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021; 372:n71. doi: 10.1136/bmj.n71.

Table 1. Level of scientific evidence adjusted according to the US Agency for Healthcare Research and Quality [32]

Level of scientific evidence adjusted according to the US Agency for Healthcare Research and Quality
Ia…. Systematic reviews/Meta-analyses of RCTs
Ib…. A minimum of one RCT
IIa…. A minimum of one controlled study, well-designed, without randomization
IIb…. A minimum of one other type of quasi-experimental study, well-designed
III…. Descriptive studies such as comparative, correlation or case-control studies, nonexperimental, and well-designed
IV…. Repot/opinions from expert committees

[29, 35–37, 39, 41], effect on trismus [29, 41], the incidence of alveolar osteitis [38], soft tissue healing [29, 35, 39–41], and bone healing [29, 35–37, 39–41]. A follow-up to evaluate pain, swelling, trismus has been reported within the first postsurgical week. Incidence of alveolar osteitis and soft tissue healing has been examined in the first 3 months. Evaluation of bone healing has been performed generally after three to 6 months [29, 35–41]. All included studies are designed as split-mouth studies. Lower impacted third molars have been extracted, and in test groups, the alveolus has been filled with PRF, and control sides have been left for conventional healing. Gupta et al. [29] found significantly better results concerning pain, trismus, swelling, and soft tissue healing at day 3 in the PRF group ($p < 0.05$). Several other groups also observed reduced pain in the test group [29, 36–39, 41]. Multiple research teams examined less swelling in the first postsurgical week [36–39, 41]. Only one group, Ritto et al. [40] notified no statistically significant difference between test and control group related to pain and soft tissue healing. Additionally, Gupta et al. [29] showed that bone healing seemed to be influenced positively in the

PRF group significantly ($p < 0.05$) measured radiographically. Five studies confirm these results with similar results [35, 37, 39–41]. In contrast, Sybil et al. [36] reported no statistically significant influence in bone height between the groups. One of the included studies [38] evaluated the presence or absence of alveolar osteitis showing more infectious events in the control groups.

Six studies with scientific evidence type Ib [29, 36–38, 40, 41] and two studies type IIa [35, 39]were taken in account. The extraction of wisdom teeth is a frequently performed intervention in oral surgery. Distinct advantages regarding patient-related outcome measures emerge from the studies listed above. In most of the studies, PRF appears to be a valuable supplement with clinical effect, especially in soft tissue healing [29, 36–39, 41], There are some limitations. Publications with small case numbers predominate in this field. Studies about simultaneous upper and lower third molar extractions would be closer to practical relevance. Additional data reflecting these frequently occurring interventions are still missing.

Alveolar Ridge Preservation

Alveolar ridge preservation techniques are usually performed by filling the extraction socket with autogenous bone or substitute materials. An autogenous or exogenous barrier membrane can be applied to protect the bone graft particles from soft tissue ingrowth and dislocation [42]. Soft tissue closure is recommended in some cases [43]. To ensure the emergence profile around implants and to maintain a natural appearing gingival margin, the preservation of sufficient soft tissue is mandatory. In this field, PRF is used to support the preservation of soft tissues around extracted teeth. At the same time, these surgical interventions are also associated with postoperative discomforts such as pain, swelling, and the risk of inflammation. These could be reduced by the use of PRF [23]. The application in the included studies was performed either with PRF as a sole filling material [44–49] or PRF in combination with bone graft materials and a collagen plug compared to spontaneous healing of the alveolus [49–51]. Eight out of 32 articles dealing with this topic were included in our analysis. Excluded articles had a low number of participants or little follow-up. A total of 294 patients were treated in the studies included. Follow-up is reported up to 6 months.

For this purpose, studied parameters were defined as dimensions of alveolar bone and its changes [44–51], new bone formation [44, 46, 47, 51], pain [49–51], swelling [51], soft tissue healing [48, 49, 51–53]. Follow-up for soft tissue healing was documented in the first postsurgical week [44, 45, 47, 48] and for hard tissue healing between up to 6 months [44–51]. Statistically, significant less reduction in bone dimension using PRF in combination with bone graft material was described in four articles [46, 47, 50, 51]. In addition, improved soft tissue healing due to PRF application could be detected in three included articles [45, 47, 48]. Ahmed et al. compared PRF as a sole filling material to natural healing as well as to PRF in combination with a collagen plug [45]. They found that PRF combined with a collagen plug was superior to socket preservation with PRF alone. These findings were reflected in the examination of bone dimensions [45]. Histomorphometric analyses were carried out by two groups and showed significantly higher percentages of new bone formation after 3 months using PRF as a sole graft material in clinical studies [46, 47]. In contrast, Aravena et al. [44] could not find significant differences in wound healing or bone formation and bone dimension [$p = 0.78$] between socket preservation using PRF as a sole graft material and natural healing. Pain in the first postsurgical week was slightly lower in the group treated with PRF, according to Kumar et al. [49]. Santhanakrishnan et al. and Yewale et al. [50, 51] reported no statistically significant differences in pain values between the groups. No statistically significant differences were observed in the study of Yewale et al. [51] evaluating patients' swelling.

Six studies at a level of evidence Ib [44–46, 49–51] and two studies at a level IIa were included in the analysis [47, 48]. Results on the application of PRF for the indication of alveolar ridge preservation are controversial. In the absence of evidence, no firm recommendation for the use of PRF can be made in this area.

Guided Bone Regeneration

PRF has been used for GBR to treat defects. With this type of intervention, success is particularly dependent on the healing readiness of the surrounding tissue. Inflammatory tissue is removed as thoroughly as possible and, if feasible, a restitutio ad integrum should be aimed for by creating ideal healing conditions. The application of PRF is intended to additionally support the formation of new tissues, both bone and soft tissue. Seven studies out of 31 have been analyzed in detail. Follow-up after 6 months is reported in each of the included studies. In total, 318 patients received treatment with PRF. Parameters examined in the seven studies were probing depth [52–55], clinical attachment loss [52–55], gingival recession [52, 55], bone fill [52–54, 56], bone height [53, 54, 57], percentage of vital bone [58], intrabony component [54], pain, bleeding [56], wound healing, and tooth mobility [53]. Intrabony defects have been treated with a variety of treatment options. Sun et al. [56] compared PRF and bone powder covered with PRF membranes and a flap curettage combined with GBR. Pain evaluation has been reported during 24 h postsurgical; however, bleeding was reported after 7 days. Both parameters were statistically significantly lower in the PRF group. After 60 days and 120 days, degrees of bone defects and bone density have been reexam-

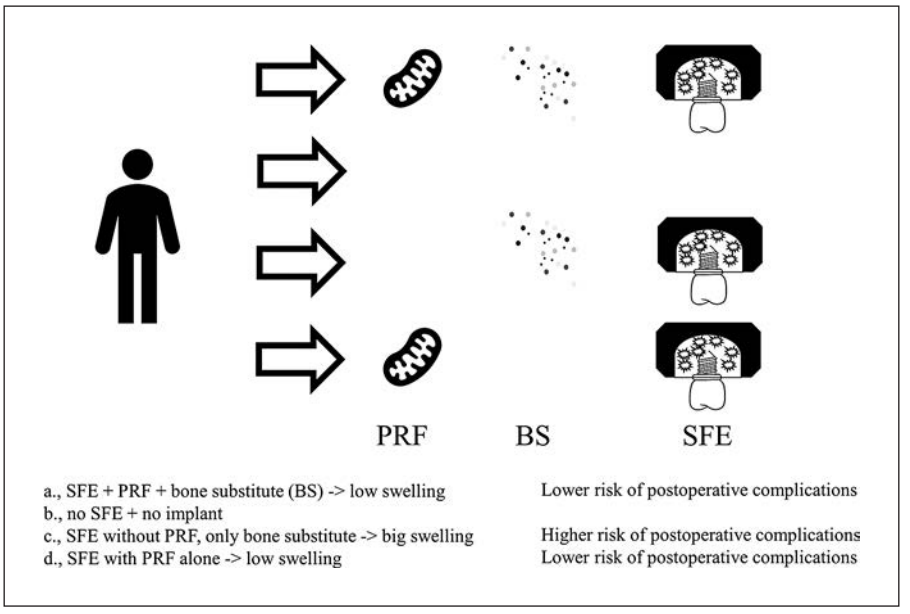

Fig. 3. Sinus floor elevation with and without PRF.

ined. The PRF group had significantly lower values in bone defect measurements ($p < 0.05$) after 60 days. The level of bone density was significantly higher after 60 days as well as after 120 days ($p < 0.001$). The study was carried out in patients with bone defects caused by peri-implantitis [56]. In the split-mouth, designed study of Bodhare et al. [52] bioactive glass has been either combined with PRF or applied as a sole filling material. Three-month follow-up and 6-month follow-up showed a significantly greater clinical attachment level, bone fill, and a reduction in probing depth in the PRF group [52]. Lei et al. [54] have compared three groups. Guided tissue regeneration used PRF, concentrated growth factors [CGF], and a control group treating periodontal intrabony defects. After 6 months, there was no difference in pocket depths, clinical attachment level gain, or bone height between PRF and CGF. However, both PRF and CGF achieved significantly better results concerning intrabony component depth and percentage of bone fill at the defect side than the control group [54]. A histological examination was performed by Hartlev et al. [58] six months after the surgical intervention. The group investigated the percentage of vital respectively nonvital bone and the number of blood vessels.

The test group was treated with autologous bone graft and PRF, the control group with bovine bone and a resorbable collagen membrane. In this article, no statistically significant difference of the examined parameters was found [58]. Similarly, Işik et al. like Hartlev et al. [57] conclude that both bovine bone graft alone and bovine bone graft combined with PRF deliver success used for GBR. Treatment of intrabony defects was compared by Pham et al. trying three different approaches. Open flap debridement combined with PRF, guided tissue regeneration, and open flap debridement alone. Periodontal parameters such as probing depth, clinical attachment level, tooth mobility, and bone defect fill were superior when defects were treated with open flap debridement and PRF, respectively, guided tissue regeneration [53]. Gingival recession defects were handled applying sticky bone, a mixture of bone allograft and liquid PRF, covered with collagen membranes coated with liquid PRF by Kapa et al. [55]. According to the case series, 6 months after the surgical intervention, positive effects in root coverage and increase of gingival thickness were noted [55].

The level of scientific evidence of the studies mentioned was Ib [52, 53, 55, 57, 58] and III [54, 55]. The evidence levels of the cited studies in this field appear to be high. The present studies included indicate potential benefits when using PRF during GBR procedures compared to control groups. However, also for this indication, no clear guideline can be extracted from the literature.

Sinus Floor Elevation

When jawbone loses its function after tooth removal, it recedes. However, a certain height and width of bone is a prerequisite for placing dental implants. Bone height can be reconstructed in the upper jaw using GBR techniques such as sinus floor elevation [59–61]. Sinus floor elevation is usually either performed through a lateral window or transcrestal approach [61, 62]. The cavity between the Schneiderian membrane and the bony sinus floor is usually filled with autogenous bone and or bone substitutes (see Fig. 3). When using a lateral window, the bone substitute is usually covered with a synthetic or xenogenic membrane [63]. In order to facilitate the integration of bone substitute in patients' maxilla, PRF has been used for a biologicalization of bone substitutes.

This review includes only one study in which transcrestal sinus floor elevations were performed [64]. Three other studies with lateral sinus floor elevations were also analyzed [65–67]. One study examined lateral and transalveolar sinus floor elevations [68]. Studies included in this review consider 147 patients in total. Molemans et al. Cho et al. and Barbu et al. [64, 66, 68] used PRF as a sole filling material. Pichotano et al. and Irdem et al. [65, 67] combined PRF with deproteinized freeze-dried bovine bone mineral (DBBM) in the test group, whereas in control groups, SFE was performed with DBBM alone. Cho et al. [64] compared PRF as a sole filling material with saline as a control. The studies of Pichotano et al. Cho et al., and Irdem et al. [65, 67] were designed as split-mouth randomized controlled clinical trials. The case series of Barbu et al. [66] report about large sinus membrane perforation preserved with PRF as a sole filling material and simultaneous implant placement. Molemans et al. [68] did twenty-two transcrestal and six lateral SFE using a prospective, single-cohort study design. Most likely, newly formed bone was measured as a parameter. Histological analysis, radiographs, or Micro-CT were made after three to 12 months [65–68]. Pichotano et al. observed implant survival, as did Cho et al. at a 1-year follow-up [64, 65] and Barbu et al. [66] at a 4-year follow-up. Two groups collected histological samples to detect the amount of vital bone [66, 67], fibrous tissue, and residue from bone graft [69]. PRF as a sole filling material in simultaneous SFE showed clinically integrated implants [68, 70]. A significantly higher intrasinusoidal bone level could be measured using PRF (2.6 ± 1.1 mm) as a sole filling material in comparison to a saline filling (1.7 ± 1.0 mm) ($p < 0.5$) [68]. Pichotano et al. reported statistically significant higher percentages of vital bone in the test group with PRF and DBBM ($p = 0.0087$). The amount of residual graft was higher in the control group with DBBM as a sole filling material ($p = 0.0111$) [71]. Using PRF as a filling material in surgeries with a large perforation of the Schneiderian membrane was tested by Barbu et al. [72]. The case series observed nine complicated sinus floor elevations resulting in successfully osseointegrated implants after 4 years of follow-up [72]. Surprisingly Irdem et al. found no statistically significant difference in none of the investigated parameters. Neither new bone formation was more, nor residual graft amount was lower, in the test group using PRF additionally to DBBM instead of DBBM alone in the control group [69]. The low number of cases may have had an influence on the result.

There are three studies about sinus floor elevation and PRF at a scientific evidence level Ib [68, 69, 71]. One study with scientific evidence level IIa [70] and one study at a level of III were included in this narrative review [72].

The results obtained from the involved studies report controversial data on the application of PRF during sinus floor elevation. However, potential clinical benefit and new pathways in bone augmentation might arise, that have to be confirmed in further investigations.

This applies to both transcrestal and lateral SFE. Especially, the use of PRF combined with bone substitutes and as sole filling material might to be a promising alternative to the use of bone substitute alone [64, 68].

Medication-Related Osteonecrosis of the Jaw

Medication-related osteonecrosis of the jaw is a big challenge in oral surgery. The necrosis usually occurs as a side effect in patients under i.v. or oral antiresorptive therapy to treat osteoporosis and osteoclastic metastasis. This group of patients requires particularly careful treatment. There are great difficulties in mucosal healing. Patients often suffer from dehiscence and exposed bone for weeks after extraction or surgical debridement of necrotic bone. Quality of life can be reduced dramatically, and treatment can be easily accompanied by complications [71]. Since oral surgery may sometimes be necessary during antiresorptive therapy, it is important to support fast and natural healing as much as possible in order to avoid dehiscence and wound healing disorders and necrosis. Covering wounds with PRF membranes might compensate healing disturbances.

To illuminate the effect of PRF used at surgical interventions for the treatment of osteonecrosis of the jaw, six of thirteen studies were reviewed in detail [73–78]. Four studies compared surgical treatment with additional use of PRF and conventional treatment [73–75, 78]. Three studies observed management of preexisting MRONJ using PRF [74, 75, 77]. Overall, 443 patients were observed in the studies mentioned above. Giudice et al. [70] compared a test group adding PRF to the conventional surgical treatment of MRONJ to a control group. Guidice et al. [75] could show that the PRF group had a statistically significant better mucosal integrity, no infection and less pain after 1-month follow-up. However, after 6 months and 1 year, no differences were found. Zelinka et al. concluded a success rate of 85% after 12 months of treating patients with early stages of MRONJ. Early stages are defined as necrosis which could be removed completely [77]. Pain, exposed bone, mucosal closure, and signs of inflammation were defined as outcome parameters. Zelinka et al. [77] assumed additional PRF application in surgical therapy of early stages of MRONJ is effective. Comparing three different treatments, Tenore et al. investigated healing, a transition from higher to lower stage of MRONJ, and persistence of pain and bone exposure. In the test groups, surgery was performed, and antibiotics and PRF were applied. In a second control group, additional photobiomodulation was performed. As a consent of this article, therapy with PRF, surgery, antibiotics, and photobiomodulation via low-level laser therapy (LLLT)

Table 2. Stages of MRONJ after [80]

Stage 0	Non-specific clinical signs such as difference in bone density, tooth mobility without periodontal disease, pain in mandibular, no exposed necrotic bone
Stage 1	Necrotic bone exposed but asymptomatic, no infection
Stage 2	Infected exposed necrotic bone accompanied by symptoms
Stage 3	Infected exposed necrotic bone accompanied by symptoms additionally to severe signs of MROJ such as fracture, extra-oral fistula or extended osteolysis

contribute to the management of MRONJ [74]. Nica et al. described a heterogenous patient cohort consisting of low-risk patients, patients suffering from MRONJ stage zero, and patients suffering MORNJ stage 1–3 for whom an extraction was necessary (see Table 2). Low-risk patients were treated with sutures and LLLT, stage 0 patients took antibiotics, sutures, and LLLT, stage 1–3 patients received a perioperative antibiosis and were treated with piezo-surgery and additional LLLT and PRF were applied at the wound. The study concluded a high rate of success in all groups and a total healing rate of 91.66% in the third group using PRF [73]. Two studies investigated the possible prevention of MRONJ followed by oral surgeries in patients at risk [76, 78]. The study participants received antiresorptive therapy for up to 6 months [73, 74, 78]. Pain [78], exposed bone, mucosal healing, symptoms indicating inflammation were the assessed outcomes [73, 74, 78]. Miranda et al. treated patients under antiresorptive or antiangiogenic agents requiring acute dental extraction. In the control group, extractions were performed carefully and minimally invasive to prevent postoperative complications such as MRONJ. Test group extractions sockets were filled with a PRF plug. In the control group, 19.23% of patients suffered from MRONJ, whereas none of the test group patients developed necrosis [78]. Şahin et al. analyzed 63 dental extractions treated with PRF in 44 patients with a risk of MRONJ. They reported uneventful healing in all cases and success preventing MRONJ after 6 months of follow-up [79].

One study with scientific evidence level Ib was included [75]. One study was at level IIa [77] and four studies at level III [73, 74, 76, 78].

In conclusion, PRF could provide notable improvements for patients suffering from MORNJ. In the presented clinical studies, it seems that the prevention and management of MRONJ could benefit from a PRF application.

Discussion

PRF is created from whole blood samples without additional ingredients [75]. Thus, a low risk of side effects can be assumed. Another advantage is the simplicity of its preparation process [76] and by that the intervention it is not associated with high costs [77]. PRF is applicable as liquid as well as solid matrix [19, 78] and seems to encounter a range of reasonable clinical applications. A variety of different manufacturing protocols have been published in the literature; however, the lack of a standardized protocol creates confusion and difficulty in the comparison of data [31]. Various parameters are required for the definition of a protocol. In the literature, this was often not fully specified, which hindered the reproducibility of study protocols. The different devices and protocols used for PRF-creation seem to have an influence on the quality of the blood product [28]. In particular, the literature suggests that the reduction of centrifugal force has a positive effect on the release of growth factors in vitro [19, 20, 24]. According to the available data on the application of PRF within field of dentistry, the following conclusion seems to be plausible:

A considerable number of RCTs report beneficial outcomes when using PRF in third molar extractions. Focusing on patients' comfort and avoiding complications during postoperative care, supplemental use of PRF could be a valuable treatment option.

Results on the application of PRF for the indication of alveolar ridge preservation are controversial. There is a lack of evidence to make a clear statement for the benefit of PRF in this field of application.

In the prevention and therapeutic management of MRONJ, potential benefits might derive from an application of PRF. However, to date, the degree of scientific evidence is rather low. Larger RCTs are urgently needed to confirm or otherwise reject these preliminary observations since in this area any benefit for the patients seems to be most crucial.

Results on PRF application during sinus floor elevation and GBR are also heterogeneously. PRF might be a promising supplement to bone substitutes or even sole filling material during SFE justifying further clinical investigations. In addition, PRF obviously represents a possibility for managing perforated sinus membranes.

Conclusion

There is a tendency that the available data on PRF in dentistry may have a positive impact on patient-related postoperative outcomes. However, there is a huge heterogeneity of protocols and a lack of guidelines for clinical application for PRF. Further studies are urgently needed to justify the standardized use of PRF within oral surgery.

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Conflict of Interest Statement

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References

1 Fan Y, Perez K, Dym H. Clinical uses of platelet-rich fibrin in oral and maxillofacial surgery. Dent Clin North America. 2020 Apr 1; 64(2):291–303.
2 Chou TM, Chang HP, Wang JC. Autologous platelet concentrates in maxillofacial regenerative therapy. Kaohsiung J Med Sci. 2020 May 1;36(5):305–10.
3 Feigin K, Shope B. Use of platelet-rich plasma and platelet-rich fibrin in dentistry and oral surgery: introduction and review of the literature. J Vet Dent. 2019 Jun 1;36(2):109–23.
4 Lang S, Loibl M, Herrmann M. Platelet-rich plasma in tissue engineering: hype and hope; 2018 [cited 2021 Dec 5]; Available from: www.karger.com/esrwww.karger.com/esr.
5 Whitman DH, Berry RL, Green DM. Platelet gel: an autologous alternative to fibrin glue with applications in oral and maxillofacial surgery. J Oral Maxillofacial Surg. 1997 Nov 1;55(11):1294–9.
6 Dohan Ehrenfest DM, Rasmusson L, Albrektsson T. Classification of platelet concentrates: from pure platelet-rich plasma (P-PRP) to leucocyte-and platelet-rich fibrin (L-PRF). Trends Biotechnol. 2009 Mar;27(3): 158–67.
7 Alsousou J, Thompson M, Hulley P, Noble A, Willett K. The biology of platelet-rich plasma and its application in trauma and orthopaedic surgery: a review of the literature. J Bone Joint Surg Br. 2009 Aug;91-B(8):987–96.
8 Arpornmaeklong P, Kochel M, Depprich R, Kü Bler NR, Wü Rzler KK. Influence of platelet-rich plasma [PRP] on osteogenic differentiation of rat bone marrow stromal cells. An in vitro study. Int J Oral Maxillofac Surg. 2004;33(1):60–70.
9 Naik B, Karunakar P, Jayadev M, Rahul Marshal V. Role of platelet rich fibrin in wound healing: a critical review. J Conser Den. 2013 Jul;16(4):284–93.
10 Wikesjö UME, Sorensen RG, Kinoshita A, Jian Li X, Wozney JM. Periodontal repair in dogs: effect of recombinant human bone morphogenetic protein-12 [rhBMP-12] on regeneration of alveolar bone and periodontal attachment: a pilot study. J Clin Periodontol. 2004 Aug;31(8):662–70.
11 Anitua E, Sánchez M, Orive G. The importance of understanding what is platelet-rich growth factor [PRGF] and what is not. J Shoulder Elb Surg. 2011;1:4–23.
12 Della Valle A, Sammartino G, Marenzi G, Tia M, Lauro A, Ferrari F, et al. Prevention of postoperative bleeding in anticoagulated patients undergoing oral surgery: use of platelet-rich plasma gel. J Oral Maxillofac Surg. 2003; 61(11):1275–8.
13 Simonpieri A, del Corso M, Vervelle A, Jimbo R, Inchingolo F, Sammartino G, et al. Current knowledge and perspectives for the use of platelet-rich plasma [PRP] and platelet-rich fibrin [PRF] in oral and maxillofacial surgery Part 2: bone graft, implant and reconstructive surgery. Curr Pharm Biotechnol. 2012 Jun 12; 13(7):1231–56.
14 Grageda E. Platelet-rich plasma and bone graft materials: A review and a standardized research protocol. Implant Dent. 2004 Dec;13(4):301–9 [cited 2021 Dec 19]. Available from: https://journals.lww.com/implantdent/Fulltext/2004/13040/Platelet_Rich_Plasma_and_Bone_Graft_Materials__A.7.aspx.
15 Jakse N, Tangl S, Gilli R, Berghold A, Lorenzoni M, Eskici A, et al. Influence of PRP on autogenous sinus grafts: an experimental study on sheep. Clin Oral Implants Res. 2003 Oct;14(5):578–83.
16 Dohan DM, Choukroun J, Diss A, Dohan SL, Dohan AJ, Mouhyi J, et al. Platelet-rich fibrin [PRF]: a second-generation platelet concentrate. Part I: technological concepts and evolution. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2006Mar;101(3):e37–44.
17 Dohan DM, Choukroun J, Diss A, Dohan SL, Dohan JAJ, Mouhyi J, et al. Platelet-rich fibrin [PRF]: a second-generation platelet concentrate. Part II: Platelet-related biologic features. Oral Med Oral Pathol Oral Radiol Endod. 2006 Mar;101(3):e45–50.
18 Liu Y, Sun X, Yu J, Wang J, Zhai P, Chen S, et al. Platelet-rich fibrin as a bone graft material in oral and maxillofacial bone regeneration: classification and summary for better application. Biomed Res Int. 2019;2019:3295756.
19 el Bagdadi K, Kubesch A, Yu X, Al-Maawi S, Orlowska A, Dias A, et al. Reduction of relative centrifugal forces increases growth factor release within solid platelet-rich-fibrin [PRF]-based matrices: a proof of concept of LSCC [low speed centrifugation concept]. Eur J Trauma Emerg Surg. 2019 Jun 1;45(3):467–79.
20 Choukroun J, Ghanaati S. Reduction of relative centrifugation force within injectable platelet-rich-fibrin [PRF] concentrates advances patients' own inflammatory cells, platelets and growth factors: the first introduction to the low speed centrifugation concept. Eur J Trauma Emerg Surg. 2018 Feb 1; 44(1):87–95.
21 Dohan Ehrenfest DM, del Corso M, Diss A, Mouhyi J, Charrier JB. Three-dimensional architecture and cell composition of a Choukroun's platelet-rich fibrin clot and membrane. J Periodontol. 2010 Apr;81(4):546–55.
22 Valladão CAA Jr, Monteiro MF, Joly JC. Guided bone regeneration in staged vertical and horizontal bone augmentation using platelet-rich fibrin associated with bone grafts: a retrospective clinical study. Int J of Implant Dent. 2020 Dec;6(1):72.
23 Miron RJ, Zucchelli G, Pikos MA, Salama M, Lee S, Guillemette V, et al. Use of platelet-rich fibrin in regenerative dentistry: a systematic review. Clin Oral Invest. 2017;21(6):1913–27.
24 Wend S, Kubesch A, Orlowska A, Al-Maawi S, Zender N, Dias A, et al. Reduction of the relative centrifugal force influences cell number and growth factor release within injectable PRF-based matrices. J Mater Sci Mater Med. 2017;28(12):188.
25 Reinke JM, Sorg H. Wound repair and regeneration. Eur Surg Res. 2012 Aug;49(10):35–43.
26 Niu Y, Li Q, Ding Y, Dong L, Wang C. Engineered delivery strategies for enhanced control of growth factor activities in wound healing. Adv Drug Deliv Rev. 2019 Jun;146:190–208.

27 Miron RJ, Fujioka-Kobayashi M, Bishara M, Zhang Y, Hernandez M, Choukroun J. Platelet-rich fibrin and soft tissue wound healing: a systematic review. Tissue Eng Part B Rev. 2017 Feb 1;23(1):83–99.
28 Dohan Ehrenfest DM, Pinto NR, Pereda A, Jiménez P, Corso MD, Kang BS, et al. The impact of the centrifuge characteristics and centrifugation protocols on the cells, growth factors, and fibrin architecture of a leukocyte- and platelet-rich fibrin [L-PRF] clot and membrane. Platelets. 2018;29(2):171–84 [cited 2021 Dec 5]. Available from: https://doi-1org-10013b5kd0d18.han.medunigraz.at/101080/09537104201712938l2.
29 Gupta N, Agarwal S. Advanced: PRF-clinical evaluation in impacted mandibular third molar sockets. J Stomatol Oral Maxillofacial Surg. 2021 Feb 1;122:43–9.
30 Qing C. The molecular biology in wound healing & non-healing wound. Chin J Traumatol. 2017 Aug;20(4):189–193.
31 Ghanaati S, Herrera-Vizcaino C, Al-Maawi S, Lorenz J, Miron RJ, Nelson K, et al. Fifteen years of platelet rich fibrin in dentistry and oromaxillofacial surgery: How high is the level of scientific evidence? J Oral Implantol. 2018;44(6):471–92.
32 Gonzallez-Garcia R. Scientific evidence in surgery for the treatment of temporomandibular joint internal derangement. Stomatological Dis Sci. 2019 May 29:2019.
33 Bailey E, Kashbour W, Shah N, Worthington HV, Renton TF, Coulthard P. Surgical techniques for the removal of mandibular wisdom teeth. Cochrane Database Syst Rev. 2020 Jul; 26;7(7):CD004345.
34 Xiang X, Shi P, Zhang P, Shen J, Kang J. Impact of platelet-rich fibrin on mandibular third molar surgery recovery: a systematic review and meta-analysis. BMC Oral Health. 2019 Jul 25;19(1):163.
35 Das D, Malhotra A, Kapur I, Sharma A, Gupta M, Kumar M. Comparative evaluation of bone regeneration with platelet-rich fibrin in mandibular third molar extraction socket: a randomized split-mouth study. Natl J Maxillofac Surg. 2020;11(2):241.
36 Sybil D, Sawai M, Faisal M, Singh S, Jain V. Platelet-rich fibrin for hard- and soft-tissue healing in mandibular third molar extraction socket. Ann Maxillofac Surg. 2020 Jan 1; 10(1):102–7.
37 Kapse S, Surana S, Satish M, Hussain SE, Vyas S, Thakur D. Autologous platelet-rich fibrin: can it secure a better healing? Oral Surg Oral Med Oral Pathol Oral Radiol. 2019 Jan 1; 127(1):8–18.
38 Unsal H, Erbasar G. Evaluation of the effect of platelet-rich fibrin on the alveolar osteitis incidence and periodontal probing depth after extracting partially erupted mandibular third molars extraction. Niger J Clin Pract. 2018;21: 201–6.
39 Dar MM, Shah AA, Najar A, Younis M, Kapoor M, Dar JI. Healing potential of platelet rich fibrin in impacted mandibular third molar extraction sockets. Ann Maxillofac Surg. 2018;8(2):206.
40 Ritto FG, Pimentel T, Canellas JVS, Junger B, Cruz M, Medeiros PJ. Randomized double-blind clinical trial evaluation of bone healing after third molar surgery with the use of leukocyte- and platelet-rich fibrin. Int J Oral Maxillofac Surg. 2019;48(8):1088–93.
41 Jeyaraj P, Chakranarayan A. Soft tissue healing and bony regeneration of impacted mandibular third molar extraction sockets, following postoperative incorporation of platelet-rich fibrin. Ann Maxillofac Surg. 2018 Jan 1;8:10.
42 Buser D, Dula K, Hirt HP, Schenk RK. Lateral ridge augmentation using autografts and barrier membranes: a clinical study with 40 partially edentulous patients. J Oral Maxillofac Surg. 1996;54(4):420–32; discussion 432-3.
43 Tonetti MS, Jung RE, Avila-Ortiz G, Blanco J, Cosyn J, Fickl S, et al. Management of the extraction socket and timing of implant placement: consensus report and clinical recommendations of group 3 of the XV European Workshop in Periodontology. J Clin Periodontol. 2019 Jun 1;46:183–94.
44 Christian Aravena P, Pilar Sandoval S, Eduardo Pizarro F, Isabel Simpson M, as Castro-Adams N, Eng M, et al. Leukocyte and platelet-rich fibrin have same effect as blood clot in the 3-Dimensional alveolar ridge preservation. A split-mouth randomized clinical trial. J Oral Maxillofac Surg. 2021 Mar;79(3):575–84.
45 Ahmed N, Gopalakrishna V, Nagraj V, Imran M, Kumar P. Efficacy of PRF vs PRF + Biodegradable collagen plug in post-extraction preservation of socket. J Contemp Dent Pract. 2019;20(11):1323–1328.
46 Canellas JVd S, da Costa RC, Breves RC, de Oliveira GP, Figueredo CMd S, Fischer RG, et al. Tomographic and histomorphometric evaluation of socket healing after tooth extraction using leukocyte- and platelet-rich fibrin: a randomized, single-blind, controlled clinical trial. J Cranio-Maxillofac Surg. 2020 Jan 1;48(1):24–32.
47 Zhang Y, Ruan Z, Shen M, Tan L, Huang W, Wang L, et al. Clinical effect of platelet-rich fibrin on the preservation of the alveolar ridge following tooth extraction. Exp Therap Med. 2018 Mar 1;15(3):2277–86 [cited 2021 Dec 12].
48 Srinivas B, Das P, Rana MM, Qureshi AQ, Vaidya KC, Ahmed Raziuddin S. Wound healing and bone regeneration in postextraction sockets with and without platelet-rich fibrin. Ann Maxillofac Surg. 2018 Jan 1;8:28.
49 Girish Kumar N, Chaudhary R, Kumar I, Arora SS, Kumar N, Singh H. To assess the efficacy of socket plug technique using platelet rich fibrin with or without the use of bone substitute in alveolar ridge preservation: a prospective randomised controlled study. Oral Maxillofac Surg. 2018 Jun 1;22(2):135–42.
50 Santhanakrishnan M, Ramesh N, Kamaleeshwari R, Subramanian V. Research Article Variations in Soft and Hard Tissues following Immediate Implant Placement versus Delayed Implant Placement following Socket Preservation in the Maxillary Esthetic Region: A Randomized Controlled Clinical Trial. Biomed Res Int. 2021 Oct 4;2021:5641185.
51 Yewale M, Bhat S, Kamath A, Tamrakar A, Patil V, Algal AS. Advanced platelet-rich fibrin plus and osseous bone graft for socket preservation and ridge augmentation: a randomized control clinical trial. J Oral Biol Craniofac Res. 2021;11(2):225–33.
52 Bodhare GH, Kolte AP, Kolte RA, Shirke PY. Clinical and radiographic evaluation and comparison of bioactive bone alloplast morsels when used alone and in combination with platelet-rich fibrin in the treatment of periodontal intrabony defects—a randomized controlled trial. J Periodontol. 2019;90(6): 584–94.
53 Vu Pham TA, Pham AV. Intrabony defect treatment with platelet-rich fibrin. Guided tissue regeneration and open-flap debridement: a randomized controlled trial. J Evid Based Dent Pract. 2021 Sep;21(3):101545.
54 Lei L, Yu Y, Han J, Shi D, Sun W, Zhang D, et al. Quantification of growth factors in advanced platelet-rich fibrin and concentrated growth factors and their clinical efficacy as adjunctive to the GTR procedure in periodontal intrabony defects. J Periodontol. 2020 Apr 1;91(4):462–72.
55 Kapa BP, Nk S, Gv G, Mehta DS. Coronally advanced flap combined with sticky bone and i PRF coated collagen membrane to treat single maxillary gingival recessions: case series. Clin Adv Periodontics. 2021 May 15;12(3):147–51.
56 Sun G, Cao L, Li H. Effects of platelet-rich fibrin combined with guided bone regeneration in the reconstruction of peri-implantitis bone defect. Am J Transl Res. 2021;13(7): 8397–402.
57 Işık G, Meltem, Yüce Ö, Koçak-Topbaş N, Günbay T. Guided bone regeneration simultaneous with implant placement using bovine-derived xenograft with and without liquid platelet-rich fibrin: a randomized controlled clinical trial. Clin Oral Investig. 2021 Sep;25(9):5563–75.
58 Hartlev J, Erik Nørholt S, Spin-Neto R, Kraft D, Schou S, Isidor F. Histology of augmented autogenous bone covered by a platelet-rich fibrin membrane or deproteinized bovine bone mineral and a collagen membrane: a pilot randomized controlled trial. Clin Oral Implants Res. 2020 Aug 1;31(8):694–704.
59 Tatum H. Maxillary and sinus implant reconstructions. Dent Clin North Am. 1986 Apr; 30(2):207–29.
60 Boyne PJ, James RA. Grafting of the maxillary sinus floor with autogenous marrow and bone. J Oral Surg. 1980;38(8):613–6.
61 Riben C, Thor A. The maxillary sinus membrane elevation procedure: augmentation of bone around dental implants without grafts-A review of a surgical technique. Int J Dentistry. 2012;2012:1–9.
62 Pjetursson BE, Lang NP. Sinus floor elevation utilizing the transalveolar approach. Periodontol. 2000;66(1):59–71.
63 Starch-Jensen T, Jensen JD. Maxillary sinus floor augmentation: a review of selected treatment modalities. J Oral Maxillofac Res. 2017 Sep 30;8(3):e3.
64 Cho YS, Hwang KG, Jun SH, Tallarico M, Kwon AM, Park CJ. Radiologic comparative analysis between saline and platelet-rich fibrin filling after hydraulic transcrestal sinus lifting without adjunctive bone graft: A randomized controlled trial. Clin Oral Implant Res. 2020 Nov 1;31(11):1087–93.

65 Pichotano EC, Molon RS, Souza RV, Austin RS, Marcantonio E, Zandim-Barcelos DL. Evaluation of L-PRF combined with deproteinized bovine bone mineral for early implant placement after maxillary sinus augmentation: a randomized clinical trial. Clin Implant Dent Relat Res. 2019 Apr 1;21(2):253–62.
66 Barbu HM, Iancu SA, Hancu V, Referendaru D, Nissan J, Naishlos S. PRF-Solution in Large Sinus Membrane Perforation with Simultaneous Implant Placement-Micro CT and Histological Analysis. Membranes. 2021;11(6):438.
67 Irdem H, Dolanmaz D, Esen A, Ünlükal N, Şimsek S, Evaluation of the effectiveness of liquid platelet-rich fibrin and deproteinized bovine bone mineral mixture on newly formed bone in maxillary sinus augmentation: a split-mouth histomorphometric study. Niger J Clin Pract. 2021 Sep;24(9):1366–72.
68 Molemans B, Cortellini S, Jacobs R, Teughels W, Pinto N, Quirynen M. Simultaneous sinus floor elevation and implant placement using leukocyte- and platelet-rich fibrin as a sole graft material. Int J Oral Maxillofac Implants. 2019 Sep;34(5):1195–201.
69 Tenore G, Zimbalatti A, Rocchetti F, Graniero F, Gaglioti D, Mohsen A, et al. Management of medication-related osteonecrosis of the jaw [MRONJ] using leukocyte- and platelet-rich fibrin [L-PRF] and photobiomodulation: a retrospective study. J Clin Med. 2020 Nov 1;9(11):3505.
70 Giudice A, Barone S, Giudice C, Bennardo F, Fortunato L. Can platelet-rich fibrin improve healing after surgical treatment of medication-related osteonecrosis of the jaw? A pilot study. Oral Surg Oral Med Oral Pathol Oral Radiol. 2018;126:390–403.
71 Beth-Tasdogan NH, Mayer B, Hussein H, Zolk O. Interventions for managing medication-related osteonecrosis of the jaw. Cochrane Database Syst Rev. 2017 Oct 6;10(10):CD012432.
72 Nica DF, Riviş M, Roi CI, Todea CD, Duma VF, Sinescu C. Complementarity of Photo-Biomodulation, Surgical Treatment, and Antibiotherapy for Medication-Related Osteonecrosis of the Jaws [MRONJ]. Medicina. 2021 Feb 1;57(2):1–14.
73 Zelinka J, Blahak J, Perina V, Pacasova R, Treglerova J, Bulik O. The use of platelet-rich fibrin in the surgical treatment of medication-related osteonecrosis of the jaw: 40 patients prospective study. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2021;165(3):3227.
74 Miranda M, Gianfreda F, Raffone C, Antonacci D, Pistilli V, Bollero P. The Role of Platelet-Rich Fibrin [PRF] in the Prevention of Medication-Related Osteonecrosis of the Jaw [MRONJ]. BioMed Res Int. 2021;2021:4948139.
75 Miron RJ, Fujioka-Kobayashi M, Hernandez M, Kandalam U, Zhang Y, Ghanaati S, et al. Injectable platelet rich fibrin [i-PRF]: opportunities in regenerative dentistry? Clin Oral Investig. 2017 Nov;21(8):2619–27.
76 Zrnc TA, Metzler P, Zemann W, Ghanaati S. Dentistry: a short synopsis about implementation and workflow [in German]. Swiss Dent J. 2018;128:712–3.
77 Karimi K, Rockwell H. The benefits of platelet-rich fibrin. Facial Plast Surg Clin North Am. 2019;27(3):331–40.
78 Al-Maawi S, Herrera-Vizcaíno C, Orlowska A, Willershausen I, Sader R, Miron RJ, et al. Biologization of collagen-based biomaterials using liquid-platelet-rich fibrin: New insights into clinically applicable tissue engineering. Materials. 2019 Dec 1;12(23):3993.
79 Şahin O, Tatar B, Ekmekcioğlu C, Aliyev T, Odabaşi O. Prevention of medication related osteonecrosis of the jaw after dentoalveolar surgery: an institution's experience. J Clin Exp Dent. 2020 Aug 1;12(8):e771–76.
80 Ruggiero SL, Dodson TB, Fantasia J, Goodday R, Aghaloo T, Surgery M. Medication-related osteonecrosis of the Jaw-2014 update special committee on medication-related osteonecrosis of the Jaws. J Oral Maxillofac Surg. 2014 Oct;72(10):1938–56.

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Diagnosis of Bone Marrow Necrosis following Severe Vaso-Occlusive Crisis in Patient with Compound Heterozygous Sickle Cell Disease

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Keywords

Bone marrow necrosis · Sickle cell disease · Leucoerythroblastic reaction · Fat embolism syndrome · Atypical hemolytic uremic syndrome · Microangiopathic anemia

Abstract

Introduction: Bone marrow necrosis is a rare entity that can develop in context of a sickle cell disease vaso-occlusive crisis. Its physiopathology is related to an endothelial dysfunction taking place in bone marrow microvasculature. ***Case Presentation:*** A 30-year-old patient with history of compound heterozygous sickle cell disease was admitted following SARS-CoV-2 infection with fever and diarrhea. After initial favorable evolution, he developed a severe vaso-occlusive crisis with intense hemolysis and multi-organ ischemic complications. Patient then developed high fever and hypoxemia. With the suspicion of acute thoracic syndrome, a red blood cell exchange was performed. Respiratory symptoms ceased but patient persisted febrile with very high levels of acute phase reactants, persistent pancytopenia, and leucoerythroblastic reaction. An infectious cause was ruled out. Afterward, bone marrow aspiration and bone marrow biopsy showed a picture of bone marrow necrosis, which is an extremely rare complication of vaso-occlusive crisis but, paradoxically, more frequent in milder heterozygote cases of sickle cell disease. Ultimately, large deposits of complement membrane attack complex (particles C5b-9) were demonstrated after incubation of laboratory endothelial cells with activated plasma from the patient. ***Discussion:*** The clinical presentation and findings are consistent with a case of bone marrow necrosis. In this setting, the demonstration of complement as a potential cause of the endothelial dysfunction mimics the pattern of atypical hemolytic uremic syndrome and other microangiopathic anemias. This dysregulation may be a potential therapeutic target for new complement activation blockers.

Introduction

Reported cases of bone marrow necrosis (BMN) in literature correspond mostly to acute hematological malignancies with massive infiltration of bone marrow stroma, affecting myeloid precursors, and preserving bone trabeculae structure. Other causes are autoimmune syndromes (e.g., systemic lupus erythematosus and antiphospholipid syndrome) and hemoglobinopathies. The common aspect of these varied pathologies is a dysfunction and lesion of sinusoidal endothelium, leading to a diffuse failure of bone marrow microcirculation [1]. As a result, an edema and infarction of the bone marrow initiate an inflammatory cascade which leads to liberation of fat, marrow cells, and bony spicules to the bloodstream.

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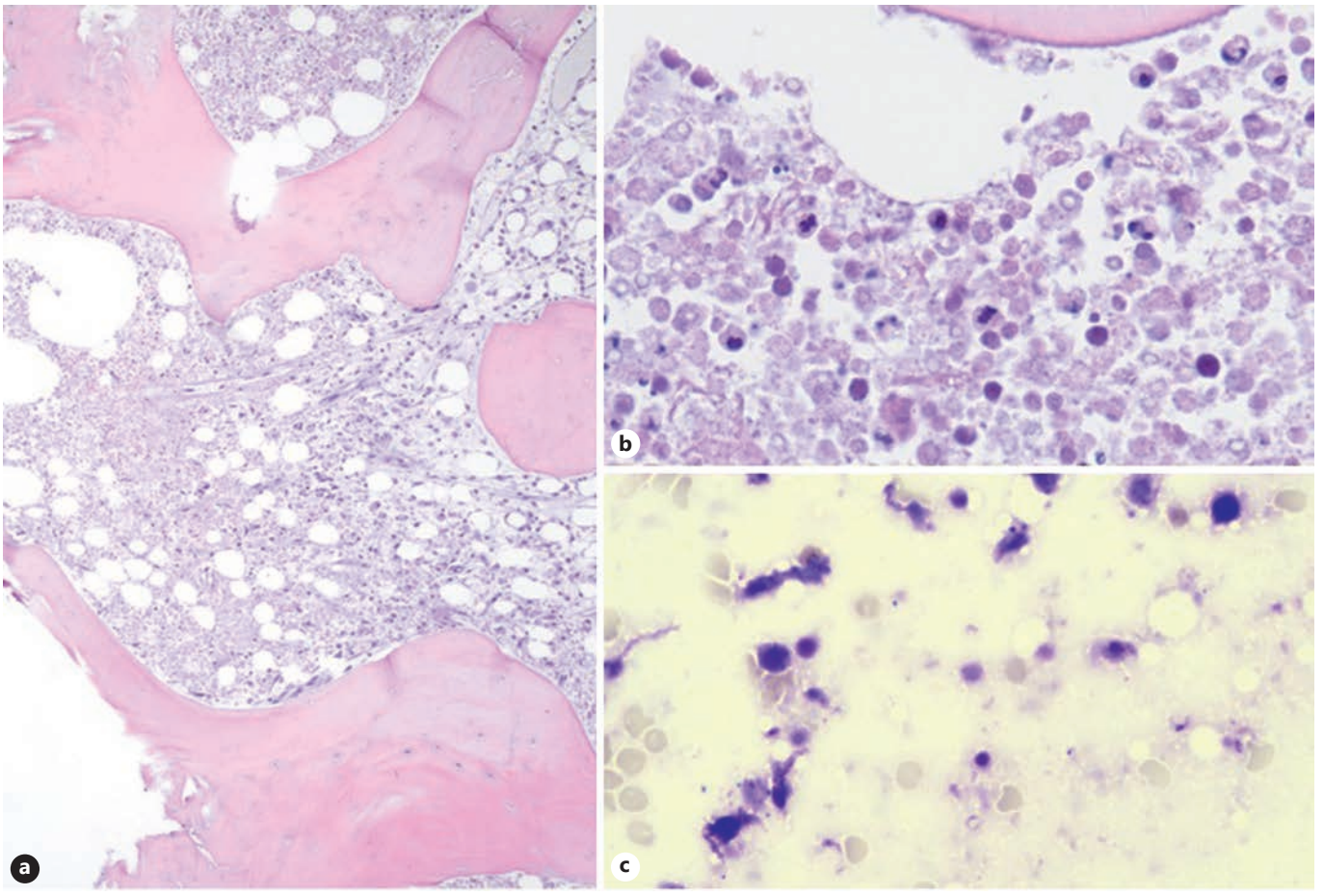

Fig. 1. Bone marrow aspirate and biopsy. **a** (H&E staining, × 100): micrography of bone marrow showing necrotic occupation of medullary spaces with conserved bone structure (no signs of osteonecrosis). **b** (H&E staining. , × 400): micrography of bone marrow showing large area of ghost cells. **c** (May-Grünwald Giemsa staining, × 400): bone marrow aspirate showing scarce viable content with predominance of necrotic material.

These lesions promote a pro-inflammatory state, dysregulation of adhesion molecules, and imbalance between vasodilator and vasoconstrictor agents in endothelium [2], resulting in severe multi-organ complications.

Sickle cell disease (SCD) is a group of inherited hemoglobinopathies affecting â-globin gene alleles. Regarding literature reports of BMN in SCD, it seems that cases of BMN tend to be delayed or confused with other microangiopathic anemias [2, 3], not deserving bone marrow evaluation in most cases. Regarding literature reports of BMN in SCD, it is paradoxically more frequent in those patients affected by less severe heterozygote genotypes. Perhaps because in those cases the total red blood cell (RBC) count tends to be higher and, therefore, blood viscosity increases [4]. The present case exemplifies the clinical presentation and diagnostic challenge of a patient with BMN in context of SCD vaso-occlusive crisis (VOC), stressing the need of better understanding of this entity and its implications toward endothelial dysfunction and interaction with the alternative complement pathway.

Case Presentation

Patient was a 30-year-old male born in Ghana. He had been living in Spain for 10 years. Last trip to his country of origin was 2 years before. In February 2021, was diagnosed with SCD (hemoglobin SC genotype) with a hemoglobin level of 117 g/L. He had never had any complications and he was not taking any medication.

He was admitted with acute diarrhea and fever, oral intolerance, and mild dehydration. Blood tests at admission showed elevated acute phase reactants without anemia. Screening for SARS-CoV-2 resulted positive. Empiric antibiotic and standard COVID-19 treatment were initiated. During the first 5 days of hospitalization, digestive symptoms evolved favorably but patient developed an acute severe VOC. Despite treatment with abundant hydration and rigorous pain control, patient did not improve.

On day +6, he was febrile, tachypneic, and mildly tachycardic, blood pressure was 154/90 mm Hg, oxygen saturation was 89% on room air (improving to 97% with O_2 3 L/min). On physical exam, he appeared uneasy. Cardiopulmonary auscultation was normal and abdomen exploration was soft and non-tender, without hepatosplenomegaly. He seemed somnolent but oriented and without focal neurological deficits. Arterial blood gas analysis at room air confirmed hypoxemia (pO_2 67 mm Hg). Electrocardiogram

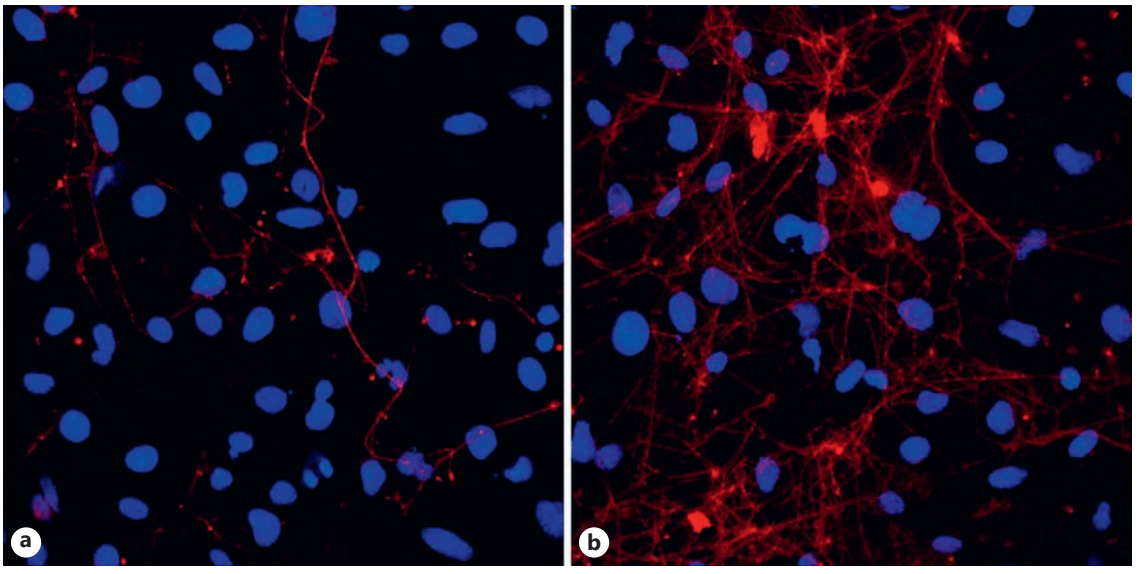

Fig. 2. C5b-9 fluorescence immunostaining imaging showing complement deposit (red) in laboratory endothelial cells (blue, DAPI staining). The human dermal microvascular endothelial cell line (American Type Culture Collection) was seeded on glass coverslip. Cells were washed with test medium (HBSS without calcium or magnesium, 0.5% BSA; Life Technologies) and activated or not with 10 μM ADP (Sigma-Aldrich) (10 min, 37°C). Cells were then incubated (4 h) with activated plasma (citrated plasma mixed with a control sera pool 1:1) diluted with test medium in proportion 1:2. Control sample was obtained by mixing healthy plasma from donors. Cultures were then washed and fixed. For C5b-9 immunostaining, cells were treated with 2% BSA (1 h) and incubated with a rabbit anti-human complement C5b-9 complex (Calbiochem), followed by Alexa594-conjugated goat anti-rabbit secondary antibody (Life Technologies). Micrographs were captured by fluorescent microscopy (Leica DM4000 B) through a video camera (Leica DFC310FX) and analyzed using Fiji (ImageJ). **a** Plasma control. **b** Plasma from patient.

showed sinus tachycardia and a chest X-ray did not show alterations.

Complete blood count showed marked hemolysis (hemoglobin had dropped to 72 g/L with a reticulocyte count of 177 × 10^9/L) with an extremely high LDH (39,000 IU/L), and thrombocytopenia (41 × 10^9/L). Peripheral blood smear reported 3 schistocytes per field, immature granulocytes, and nucleated RBC. No *Plasmodium* spp. was reported on peripheral smear review. Bilirubin level was 2.9 mg/dL, ferritin was 27,469 ng/mL, D-dimer was >35,000 ng/mL (DDU) with normal basic coagulation tests. Patient associated acute kidney injury (GFR 56 mL/min) and the remainder of the tests showed analytic signs of ischemic damage of different organs (pancreas, liver, muscle, and myocardium). Blood, stool, and urine cultures were negative. ADAMTS-13 activity was 57%. A CTA scan excluded acute thromboembolic embolism, COVID-19 signs of lung injury or organizing pneumonia, showing normal pattern of pulmonary parenchyma.

In the light of rapidly progressive anemia and thrombocytopenia, the most likely scenario was a severe VOC drepanocytic crisis triggered by the viral infection, with subsequent bone and multiorgan microinfarctions. Although the respiratory failure did not accomplish diagnostic criteria for acute chest syndrome (ACS), a RBC exchange was performed on day +8. Patient hematocrit at that time was 21% and his total blood volume based on Nadler method was 5,933 mL, corresponding to 1,245 mL of RBC volume. A total of 2.5 RBC volumes of the patient were exchanged in 66 min with the goal of achieving a hematocrit of 30%. RBC exchange was performed with Spectra Optia v12.0 (TerumoBCT, Lakewood, CO) and 10 RBC units (accounting for 2,772 mL of RBC fluid), with an estimated 30% fraction of patient cells remaining after the procedure. Overall, after the procedure, hemoglobin was 115 g/L and levels of S/C hemoglobin dropped from 41%/45% to 9%/8%.

Following RBC exchange, pain improved rapidly and the hypoxemia ceased. Levels of hemoglobin stabilized around 80–100 g/L and a progressive improvement of ischemic alterations was observed. Despite this apparent improvement, from day +9, the patient continued with daily high fever (up to 39°C), progressive thrombocytopenia with a nadir of 29 × 10^9/L, persistent elevation of acute phase reactants (C-reactive protein 17 mg/dL, ferritin 24,667 ng/mL), and a leucoerythroblastic reaction in peripheral blood. On the first hand, the combination of signs and symptoms could be consistent with a hemophagocytic syndrome considering the fever, pancytopenia, and ferritin elevation. Nevertheless, it would not be expected to find a leucoerythroblastic reaction in peripheral blood. On the other hand, a possible concealed infection was considered and multiple serologies (including human immunodeficiency virus, Epstein-Barr virus, Cytomegalovirus, Parvovirus-B19, atypical bacteria, *Toxoplasma*, and *Leishmania*) were performed, obtaining either passed infections or negative results.

Meanwhile, bone marrow aspiration was performed showing scarce material, with complete absence of viable hematopoietic cells and abundance of necrotic material. Consequently, a bone marrow biopsy was performed on day +15: revealing predominant necrotic occupation of medullary spaces (Fig. 1), without histologic signs of osteonecrosis, congruent with a case of BMN following the severe VOC. In view of this event, supportive treatment with transfusions was maintained until spontaneous recovering occurred, with normalization of complete blood count and analytic alterations by day +26. Patient was discharged after a month of hospitalization.

Retrospectively, complement membrane attack complex (C5b-9) deposition after incubation of laboratory endothelial cells with activated plasma from the patient was assessed (Fig. 2). This novel technique provides a semiquantitative estimation of plasmatic in vitro ability to induce complement activation [5], and in our patient, the endothelial reactivity to C5b-9 was reported as intensely positive.

Conclusion

Several cases of SARS-CoV-2 infection and drepanocytic crisis have been reported [6]. In this particular case, respiratory failure was initially interpreted as an ACS. Diagnostic criteria for ACS are based in guiding symptoms plus lung infiltrates on chest imaging, in the context of VOC [7]. The patient had a congruent clinical presentation but CT-scan ruled out the diagnosis. Putting respiratory support aside, the treatment of ACS is the same of a severe VOC: thus, RBC exchange was guaranteed. RBC exchange terminated the VOC had little effect on BMN and hematopoietic function. In fact, platelet count kept dropping. High persistent fever, extremely elevated LDH (× 64 upper limits of normal), and ferritin levels (× 68 upper limits of normal) along with the leucoerythroblastic reaction were cornerstones to raise suspicion of this rare complication [2–4]. It is a genuine example of the frequent underdiagnosis of BMN following VOC. It also stresses the importance of a prompt actuation (e.g., RBC exchange) in case of severe VOC to prevent potential complications.

More precisely, the pathogenesis of the inflammation implicates the secretory form of phospholipase A2, which converts phospholipids of bone marrow fat into free fatty acids, which have a potent inflammatory activity and are prone to generate tissue injury (compared to neutral fats) when unleashed from bone marrow microinfarctions to different organs, notably with pulmonary involvement. This phenomenon is called fat embolism syndrome [8].

Notwithstanding the previous considerations, the novel aspect is the investigation of complement activation in the patient plasma. This finding is consistent with the endothelial dysfunction hypothesis and mimics the pattern of varied pathologies gathered under the "atypical hemolytic uremic syndrome" (aHUS). New evidence suggests that several thrombotic microangiopathies other than complement-mediated aHUS could also be associated with dysregulation of the alternative complement pathway [9]. Particularly, there is an increasing interest regarding VOC in the setting of SCD, which could involve both coagulation and complement pathways in its complex pathophysiology [10, 11]. Further research is needed to elucidate if these findings can act as the primary cause of the inflammatory cascade or whether are another consequence of the vicious circle it creates. Hence, recognition of patients with complement overactivation is a matter of interest because treatment with complement activation blockers (e.g., Eculizumab) may have a role in SCD [9]. Future prospective studies are needed to better understand the role of complement and testing new potential therapeutic targets for SCD.

Statement of Ethics

The case report was written based on our center protocol concerning publication of patient-related information. The patient described in the text has given his written informed consent to publish his case (including publication of images). All data that could lead to identification of the patient has been omitted. Ethical approval was not required for this study in accordance with local/national guidelines.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Marco, Garrote, Seguí, and Bullich took part in the writing of the manuscript. Lozano and Cid supervised and corrected it before submission.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

References

1 Badar T, Shetty A, Bueso-Ramos C, Cortes J, Konopleva M, Borthakur G, et al. Bone marrow necrosis in acute leukemia: clinical characteristic and outcome. Am J Hematol. 2015;90(9):769–73.
2 Gangaraju R, May JE, Williams LA 3rd, Reddy VB, MacLennan P, Marques MB. Fat embolism syndrome due to bone marrow necrosis in patients with hemoglobinopathies: a life-threatening complication mimicking thrombotic thrombocytopenic purpura. Am J Hematol. 2019;94(2):64–6.
3 Gangaraju R, Reddy VVB, Marques MB. Fat embolism syndrome secondary to bone marrow necrosis in patients with hemoglobinopathies. South Med J. 2016;109(9):549–53.

4 Tsitsikas DA, Gallinella G, Patel S, Seligman H, Greaves P, Amos RJ. Bone marrow necrosis and fat embolism syndrome in sickle cell disease: increased susceptibility of patients with non-SS genotypes and a possible association with human parvovirus B19 infection. Blood Rev. 2014;28(1):23–30.
5 Palomo M, Blasco M, Molina P, Lozano M, Praga M, Torramade-Moix S, et al. Complement activation and thrombotic microangiopathies. Clin J Am Soc Nephrol. 2019;14(12):1719–32.
6 Lee JX, Chieng WK, Lau SCD, Tan CE. COVID-19 and hemoglobinopathies: a systematic review of clinical presentations, investigations, and outcomes. Front Med. 2021;8:757510.
7 Howard J, Hart N, Roberts-Harewood M, Cummins M, Awogbade M, BCSH Committee, et al. Guideline on the management of acute chest syndrome in sickle cell disease. Br J Haematol. 2015;169(4):492–505.
8 Dang NC, Johnson C, Eslami-Farsani M, Haywood LJ. Bone marrow embolism in sickle cell disease: a review. Am J Hematol. 2005;79(1):61–7.
9 Cavero T, Rabasco C, López A, Román E, Ávila A, Sevillano Á, et al. Eculizumab in secondary atypical haemolytic uraemic syndrome. Nephrol Dial Transpl. 2017;32(3):466–74.
10 Tampaki A, Gavriilaki E, Varelas C, Anagnostopoulos A, Vlachaki E. Complement in sickle cell disease and targeted therapy: I know one thing, that I know nothing. Blood Rev. 2021;48:100805.
11 Gavriilaki E, Mainou M, Christodoulou I, Koravou EE, Paleta A, Touloumenidou T, et al. In vitro evidence of complement activation in patients with sickle cell disease. Haematologica. 2017;102(12):481–2.

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Immundefekte

Neues Immunglobulin mit guter Verträglichkeit

Für die Therapie von Immundefekten steht mit Yimmugo® seit Dezember 2022 ein neues, intravenöses Immunglobulin (IVIG) aus humanem Blutplasma zur Verfügung. In Deutschland ist es zur Substitutionstherapie bei primären und sekundären Immunmangelsyndromen sowie zur Immunmodulation verschiedener Krankheitsbilder zugelassen [1]. Die gute Verträglichkeit des neuen IVIG, so Dr. Jörg Schüttrumpf von Biotest auf der Launch-Pressekonferenz, erklärt sich unter anderem aus der jahrzehntelangen Erfahrung seiner Firma mit diesen Präparaten.

Primäre Immunmangelsyndrome (PID), fährt er fort, sind eine Klasse genetischer Störungen, die durch einen angeborenen Defekt im menschlichen Immunsystem gekennzeichnet sind. PID betreffen etwa 1–2% der Weltbevölkerung. Sekundäre Immunmangelsyndrome (SID) sind erworbene Funktionsstörungen des Immunsystems und treten häufiger auf als PID. Die Ursachen sind vielfältig und können Konsequenzen aus unterschiedlichen Erkrankungen sein (z.B. HIV-Infektion, Krebs, Autoimmunerkrankungen) sowie aus metabolischen Störungen oder Behandlungen resultieren (Immunsuppressiva, Chemotherapie) [2, 3].
Zur Relevanz von IVIG betont Schüttrumpf: «Menschen mit primären oder sekundären Immundefekten sind auf die Versorgung mit Immunglobulinen angewiesen. Ohne diese Immunglobuline kann ein banaler Infekt lebensgefährlich werden. Yimmugo bietet dazu eine weitere wichtige Behandlungsoption.»

Studienergebnis: Rate schwerer Infektionen auf niedrigem Niveau

Die Wirksamkeit und Verträglichkeit von Yimmugo bei PID-Patienten wurde in einer prospektiven, unkontrollierten, multizentrischen Phase-III-Studie nachgewiesen [4], die Dr. Christiane Staiger von der Biotest AG vorstellte. Dabei erhielten 49 Erwachsene und 18 Kinder im Alter von 2 bis 76 Jahren Dosen zwischen 0,2 und 0,8 g/kg Körpergewicht über einen Zeitraum von etwa 12 Monaten in Abständen von 3 oder 4 Wochen. Der primäre Endpunkt war der Nachweis, dass die mittlere Rate der akuten schweren bakteriellen Infektionen (SBI) pro Patientenjahr unter 1,0 lag. Yimmugo erreichte den primären Endpunkt und die adjustierte SBI-Rate lag bei 0,015 pro Patientenjahr. Nur 1 erwachsene Person aus dem Kollektiv erlebte ein Ereignis, das als SBI eingestuft wurde. Die Ergebnisse der sekundären Endpunkte bestätigten die Wirksamkeit und Sicherheit von Yimmugo in allen Altersgruppen. Insgesamt waren 8% der Infusionen mit ≥ 1 infusionsbedingten unerwünschten Ereignissen verbunden, die innerhalb von 72 Stunden nach der Infusion begannen. Diese infusionsbedingten Nebenwirkungen umfassten vor allem Kopfschmerzen (2,4%), Müdigkeit (0,9%) und Übelkeit (0,5%). Die gute Verträglichkeit von Yimmugo zeigte sich insbesondere bei hohen Infusionsgeschwindigkeiten von > 4,0 ml/kg/h, unterstreicht Staiger. Auch dann traten keine weiteren Nebenwirkungen auf [4].

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Der Studienarzt Prof. Gergely Kriván, Budapest, fasst seine eigenen Praxiserfahrungen folgendermaßen zusammen: «Yimmugo schützt Erwachsene, Jugendliche und Kinder mit Immunmangelsyndromen, egal ob diese angeboren oder erworben sind, vor schweren bakteriellen Infektionen; dabei ist es gut verträglich.»

Yimmugo wird als erstes kommerzielles Präparat in der neuen Produktionsanlage «Biotest Next Level» am Standort Dreieich hergestellt. Die innovative Produktionsanlage repräsentiert einen wichtigen Meilenstein auf dem Weg zu der von Biotest angestrebten vollständigen Klimaneutralität bis zum Jahr 2035.
Schüttrumpf ergänzt: «Mit ‹Biotest Next Level› sind wir in der Lage, in innovativen Prozessschritten aus dem kostbaren Spenderplasma ein Maximum an Plasmaprodukt herzustellen. Um nachhaltig zu produzieren, versuchen wir, die Inanspruchnahme natürlicher Ressourcen, wo immer es uns möglich ist, zu optimieren.»

Reimund Freye, Baden-Baden

Quelle

Launch-Pressekonferenz Yimmugo®, Dreieich, 1. Juni 2023; Veranstalter: Biotest

Literatur

1 Fachinformation Yimmugo® 100 mg/ml Infusionslösung, aktueller Stand.
2 Modell V et al.: Immunol Res 2016;64:736–753.
3 Onkopedia Leitlinien. Immundefekte, sekundär: Schwerpunkt: Therapieinduzierte Immundefekte in der Hämatologie und Onkologie; *www.onkopedia.com/de/onkopedia/guidelines/immundefekte-sekundaer/@@guideline/html/index.html, 2019.*
4 Kriván G et al.: Vox Sang 2022;117:1153–1162.

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Zulassung der Europäischen Kommission für Biosimilar Eculizumab bei PNH

Die Europäische Kommission hat Ende Mai 2023 EPYSQLI™, einem Biosimilar des Referenzproduktes Soliris (Eculizumab) [1, 2], für die Behandlung von Erwachsenen und Kindern mit paroxysmaler nächtlicher Hämoglobinurie (PNH) die Marktzulassung erteilt.
Die Zulassung durch die Europäische Kommission basierte auf der Gesamtheit der Nachweise, einschließlich analytischer nichtklinischer In-vitro-Daten sowie klinischer Daten. Eine randomisierte, doppelblinde, multizentrische Crossover-Phase-III-Studie zu EPYSQLI zeigte durch Auswertung der Laktatdehydrogenase (LDH) bei PNH-Patient*innen eine gleichwertige klinische Wirksamkeit von EPYSQLI und dem Referenzprodukt Eculizumab. Dies deutet auf die Bioäquivalenz von EPYSQLI mit Eculizumab hin [3].
«Die Zulassung von EPYSQLI, dem ersten Biosimilar von Samsung Bioepis für die Hämatologie, spiegelt unsere laufenden Bemühungen wider, mehr Behandlungsmöglichkeiten für PNH-Patient*innen in Europa zu schaffen», so Byoungin Jung, Vice President und Regulatory Affairs Team Leader bei Samsung Bioepis. «Die Zulassung ist ein weiterer Schritt, um das Leben der Patient*innen einschließlich derer mit seltenen Krankheiten durch unseren bahnbrechenden und innovativen Einsatz von Wissenschaft und Technologie zu verbessern», so Byoungin Jung weiter.

Literatur

1 Soliris ist eine eingetragene Marke von Alexion Pharmaceuticals, Inc.
2 Europäische Arzneimittel-Agentur: Soliris Produktinformation, *https://www.ema.europa.eu/en/documents/product information/soliris epar product-information_en.pdf* (letzter Zugriff Mai 2023).
3 Jang JH et al.: EJHaem 2022;4:26–36. DOI: 10.1002/jha2.632.

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CMV-Prophylaxe nach allogener HSZT

Von der Innovation zum Goldstandard: 5 Jahre Letermovir

Letermovir (Prevymis®) ist die erste und einzige [1] zugelassene Cytomegalievirus (CMV) -Prophylaxe für erwachsene CMV-seropositive Patient*innen (R+) nach einer allogenen hämatopoetischen Stammzelltransplantation (HSZT) [2]. Zugelassen im Jahr 2018 [2] wurde Letermovir bereits 2019 in die ECIL7-Leitlinie aufgenommen [3] und erhielt den NUB-Status (NUB = Neue Untersuchungs- und Behandlungsmethoden) [1]. Auch unter Real-World-Bedingungen konnte Letermovir inzwischen sein positives Wirksamkeits- und Verträglichkeitsprofil zeigen [4]. Eine Reaktivierung des CMV gehört zu den häufigsten Komplikationen nach einer allogenen HSZT: Etwa 60–70% der CMV-seropositiven Patient*innen (R+) sind betroffen. Unkontrollierte CMV-Reaktivierungen können zu einer lebensbedrohlichen CMV-Erkrankung führen [5].

Mit Letermovir gibt es seit der EU-Zulassung [2] erstmals eine Option [1] zur CMV-Prophylaxe für erwachsene R+ Patient*innen nach allogener HSZT. Letermovir verfügt über einen einzigartigen Wirkmechanismus [6]: Es hemmt spezifisch den CMV-DNA-Terminase-Komplex, der für die Spaltung und Verpackung viraler Nachkommen-DNA erforderlich ist [2]. Für die Entwicklung des Wirkstoffes wurden Prof. Helga Rübsamen-Schaeff und Dr. Holger Zimmermann von AiCuris 2018 mit dem Deutschen Zukunftspreis ausgezeichnet.

Die Wirksamkeit und Sicherheit von Letermovir wurde in einer multizentrischen, randomisierten, doppelblinden, Placebo-kontrollierten Phase-III-Studie (n = 565) untersucht. Primärer Endpunkt war der Anteil an Patient*innen mit einer klinisch signifikanten CMV-Infektion zu Woche 24 nach der Transplantation unter Patient*innen ohne detektierbare CMV-DNA bei Randomisierung. Der Anteil an Personen, bei denen ein primäres Endpunkt-Ereignis auftrat, war unter Letermovir signifikant geringer als unter Placebo (37,5% vs. 60,6%, Unterschied: –23,5% (95%-Konfidenzintervall (KI) –32,5; –14,6), $p < 0,001$) [7].

Hinsichtlich der Häufigkeit und Schwere von Nebenwirkungen waren die Letermovir- und die Placebo-Gruppe vergleichbar. Zu den unerwünschten Ereignissen gehörten unter anderem Erbrechen (18,5% unter Letermovir vs. 13,5% unter Placebo), Ödeme (14,5% vs. 9,4%), Vorhofflimmern oder Vorhofflattern (4,6% bzw. 1,0%) [7].

Lassen sich die positiven Ergebnisse der oben genannten Phase-III-Studie auch unter Real-World-Bedingungen bestätigen? Mit dieser Frage beschäftigte sich eine monozentrische, retrospektive Vergleichsanalyse von Derigs et al. 2020 [4]. Die Studie ergab, dass mit Letermovir-Prophylaxe bis zu Tag 100 nach allogener HSZT bei CMV-seropositiven Patient*innen signifikant weniger klinisch bedeutsame CMV-Infektionen auftraten als ohne Letermovir-Prophylaxe (Hazard Ratio (HR) 0,29 (95%-KI 0,15–0,57); $p < 0,001$). Darüber hinaus war bei den mit Letermovir behandelten Patient*innen im Vergleich zur historischen Kontrollgruppe seltener eine präemptive Therapie notwendig und weniger Patient*innen mussten hospitalisiert werden. In der Studie wurden keine Nebenwirkungen beobachtet, die auf eine Behandlung mit Letermovir zurückgeführt werden konnten [4]. Mittlerweile ist Prevymis weltweit in mehr als 50 Ländern zugelassen [8]. Von medizinischen Experten, die in den vergangenen Jahren Erfahrungen mit dem Einsatz der Substanz sammeln konnten, wird die Möglichkeit einer CMV-Prophylaxe positiv bewertet: «Die CMV-Prophylaxe mit Letermovir ist fester Bestandteil der klinischen Praxis geworden», äußert Prof. Michael Schmitt, Heidelberg. Und Prof. Matthias Stelljes, Münster, ergänzt: «In den letzten Jahren gab es eine Reihe von Neuentwicklungen im Bereich der allogenen Stammzelltransplantation. Eine, die insbesondere die Therapie von vielen Patient*innen nach Stammzelltransplantation revolutioniert hat, ist Prevymis®.»

Derzeit untersucht MSD den Einsatz von Letermovir zur CMV-Prophylaxe bei Erwachsenen nach Nierentransplantation: Kürzlich wurden Ergebnisse einer klinischen Phase-III-Studie veröffentlicht [9].

Literatur

1 *https://www.dgho.de/publikationen/stellungnahmen/fruehe-nutzenbewertung/copy_of_letermovir/letermovir-dgho-dag-kbt-stellungnahme-20180605.pdf/view* (letzter Zugriff Mai 2023).
2 Fachinformation Prevymis® Filmtabletten, Stand Februar 2023.
3 Ljungman P et al.: Lancet Infect Dis 2019;19:e260–e272.
4 Derigs P et al.: Ann Hematol 2021;100:2087–2093.
5 Einsele H et al.: Blood 2020;135:1619–1629.
6 Shigle TL et al.: Ther Adv Hematol 2020;11:2040620720937150.
7 Marty FM et al.: N Engl J Med 2017;377:2433–2444.
8 Unveröffentlichte Daten bei MSD.
9 Limaye AP et al.: IDWeek 2022;Präsentation P002.

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EBMT-Kongress 2023: Großes Interesse an Treosulfan

Im Rahmen der Tagung der Europäischen Gesellschaft für Blut- und Knochenmarktransplantation (EBMT) fanden das von medac unterstützte medscape Symposium «Optimizing Conditioning Regimens and Advancing Allogeneic Hemopoietic Stem Cell Transplantation Practice», sowie eine zusätzliche Meet-the-Expert-Session zum Thema Treosulfan (Trecondi®) bei pädiatrische Patient*innen [1] statt.

Trecondi® ist das einzige Treosulfan in Europa, das für die allogene hämatopoetische Stammzelltransplantation (alloHSCT) zugelassen ist. Treosulfan in Kombination mit Fludarabin ist als Teil der Konditionierungsbehandlung vor alloHSCT bei erwachsenen sowie bei pädiatrischen Patient*innen, die älter als 1 Monat sind, mit malignen und nicht malignen Erkrankungen indiziert [1].

Der Experte Prof. Dr. Krzysztof aus Kalwak/Polen richtete beim Meet-the-Expert den Blick auf die jüngste Zulassungserweiterung von Trecondi® für pädiatrische Patient*innen mit nicht malignen Erkrankungen. Nach einer Einführung in das pädiatrische klinische Studienprogramm von der medac GmbH, bei der die hervorragenden Ergebnisse in Bezug auf die Wirksamkeit und die sehr niedrige behandlungsbedingte Mortalität hervorgehoben wurden, folgte eine interessante Diskussion, die sich auf weitere Details wie Lebensqualität, Dosierung und die Vielseitigkeit von Trecondi® als Konditionierungsmittel in einer Vielzahl von Indikationen konzentrierte.

Literatur

1 Trecondi® Fachinformation, *www.medac.eu/product-information*, aktueller Stand.

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Hämophilie A und B: Erste positive Daten zu Marstacimab hinsichtlich Wirksamkeit aus zulassungsrelevanter Phase-III-Studie

Marstacimab ist ein neuartiger, in der Erprobung befindlicher Antikörper gegen den Tissue Factor Pathway Inhibitor (Anti-TFPI), der zur Behandlung der Hämophilie A oder B bei Patienten ohne Hemmkörper gegen Faktor VIII (FVIII) oder Faktor IX (FIX) untersucht wird. Ende Mai 2023 hat die zulassungsrelevante Phase-III-Studie BASIS (NCT03938792) zu Marstacimab nun ihre primären Endpunkte erreicht und eine statistisch signifikante und klinisch bedeutsame Wirksamkeit gezeigt. In der Studie wurde Marstacimab wöchentlich in einer fixen Dosierung (körpergewichtsunabhängig) als subkutane 300-mg-Initialdosis, gefolgt von einer 1-mal wöchentlichen 150-mg-Erhaltungsdosis, verabreicht [1, 2].
Die BASIS-Studie zeigte, dass die prophylaktische Behandlung mit Marstacimab bei Menschen mit schwerer Hämophilie A und mittelschwerer bis schwerer Hämophilie B ohne Hemmkörper zu einer statistisch signifikanten und klinisch relevanten Verringerung der annualisierten Blutungsrate (ABR) führte. 116 Hämophilie-Patienten wurden über einen Zeitraum von 12 Monaten mit Marstacimab behandelt, im Vergleich zur vorherigen 6-monatigen Beobachtungsphase, in der sie im Rahmen der üblichen Versorgung eine intravenöse Prophylaxe bzw. Bedarfsbehandlung mit FVIII oder FIX erhielten. In der Patientenkohorte, die in der Beobachtungsphase mit einer bedarfsorientierten Faktorersatztherapie (on demand) behandelt wurde, zeigte Marstacimab eine Überlegenheit ($p < 0{,}0001$) mit einer Verringerung der Blutungen um 92%. Die Ergebnisse zeigten auch eine Überlegenheit ($p = 0{,}0376$) von Marstacimab im Vergleich zur Prophylaxe, mit einer Reduzierung der ABR um 35% [1, 2].
Das Sicherheitsprofil von Marstacimab entsprach den Ergebnissen der Phase-1/2-Studie und die Prophylaxe war im Allgemeinen gut verträglich [1, 2].

Literatur

1 *https://clinicaltrials.gov/ct2/show/NCT03938792?term=NCT03938792&draw=2&rank=1* (letzter Zugriff Juni 2023).
2 Pfizer Pharma: «Pfizer Announces Positive Marstacimab Results from Pivotal Phase 3 Hemophilia A and B Trial», Pfizer Pressemitteilung 30. Mai 2023.

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CAR-T-Zell-Therapie Axicabtagen-Ciloleucel: signifikant längeres OS versus SOC in der Zweitlinie beim r/r LBCL

Bei der diesjährigen Jahrestagung der American Society of Clinical Oncology (ASCO) wurden erstmals detaillierte Daten zum Gesamtüberleben (OS) der Phase-III-Studie ZUMA-7 präsentiert: Sie zeigen, dass Axicabtagen-Ciloleucel (Axi-Cel) (Yescarta®) bei erwachsenen Patient*innen mit einem rezidivierten/refraktären großzelligen B-Zell-Lymphom (r/r LBCL) in der Zweitlinie das Sterberisiko im Vergleich zur bisherigen Standardbehandlung signifikant senken kann [1]. Damit ist Axi-Cel die erste Behandlung in nahezu 30 Jahren, die eine statistisch signifikante Verbesserung des OS im Vergleich zur Standardtherapie in der Zweitlinientherapie des r/r LBCL in einem kurativen Setting demonstrieren konnte [2, 3]. Die Phase-III-Studie ZUMA-7 verglich das Überleben nach einer 1-maligen Infusion Axi-Cel mit der bisherigen Standardbehandlung (SOC)[#] bei erwachsenen LBCL-Patient*innen, deren Erkrankung innerhalb von 12 Monaten nach Abschluss einer Erstlinien-Chemoimmuntherapie rezidiviert oder gegenüber dieser refraktär war (r/r LBCL) [1]. Die Daten wurden jetzt auch vom New England Journal of Medicine publiziert [4]. Die primäre Analyse (intention-to-treat) des OS erfolgte gemäß Protokoll 5 Jahre, nachdem der erste Studienteilnehmende randomisiert wurde; das mediane Follow-up betrug rund 4 Jahre (47,2 Monate) [1]. Die Auswertung der Daten zeigte, dass die Einmalbehandlung mit Axi-Cel in einem signifikant längeren OS resultierte (Hazard Ratio (HR): 0,726; 95%-Konfidenzintervall (KI): 0,540–0,977; 1-seitiger stratifizierter Log-Rank-p-Wert = 0,0168) – entsprechend einer Risikoreduktion um 27,4% gegenüber SOC bzw. einer relativen Verbesserung des OS um 38% [1, 4].

[#]Definiert als 2 bis 3 Zyklen einer Standard-Chemoimmuntherapie (R-ICE, R-DHAP oder R-DHAX, R-ESHAP oder R-GDP) gefolgt von einer Hochdosistherapie und einer allogenen Stammzelltransplantation (HDT-ASZT) bei Patient*innen mit Ansprechen der Erkrankung.

Literatur

1 Westin JR et al.: ASCO 2023;abstr #LBA107.
2 Philip T et al.: N Engl J Med 1995;333:1540–1545.
3 Brown R et al.: Blood 2020;136(suppl 1):38.
4 Westin JR et al.: N Engl J Med 2023, in press. DOI: 10.1056/NEJMoa2301665

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Positive CHMP-Empfehlung für die Zulassung von Nonacoq beta pegol zur Prophylaxe und Behandlung von Blutungen bei Kindern mit Hämophilie B ab 0 Jahren

Der Ausschuss für Humanarzneimittel (CHMP) der Europäischen Arzneimittel-Agentur (EMA) hat am 22. Juni 2023 die Zulassung von Nonacoq beta pegol (Refixia®) nun auch für die Prophylaxe und Behandlung von Blutungen bei Kindern mit Hämophilie B ab 0 Jahren empfohlen [1]. Nonacoq beta pegol ist ein glycopegylierter rekombinanter Faktor IX mit verlängerter Halbwertszeit, der bislang für Erwachsene und Jugendliche ab 12 Jahren zugelassen war [2]. Die Hämophilie B ist eine seltene angeborene Blutgerinnungsstörung, die auf dem Mangel des Gerinnungsfaktors IX (FIX) beruht.
Die positive Stellungname des CHMP basiert auf den Ergebnissen der beiden klinischen Phase-III-Studien paradigm 5 [3, 4] und paradigm 6 [5], die die Wirksamkeit und Sicherheit von Nonacoq beta pegol bei Kindern mit schwerer Hämophilie B (FIX-Aktivität ≤ 2%) gezeigt haben. paradigm 5 untersuchte vorbehandelte Kinder von 0 bis ≤ 12 Jahren [3, 4] und paradigm 6 nicht vorbehandelte Kinder von 0 bis ≤ 6 Jahren [5]. Alle Kinder erhielten 1-mal wöchentlich 40 I.E./kg Körpergewicht Nonacoq beta pegol zur Blutungsprophylaxe; in paradigm 6 war zudem eine individualisierte Dosierung zur Präprophylaxe möglich [3–5].

Literatur

1 European Medicines Agency (EMA), *https://www.ema.europa.eu/en/medicines/human/summaries-opinion/refixia.*
2 Refixia® Fachinformation, Stand Dezember 2022.
3 Carcao M et al.: J Thromb Haemost 2016;14:1521–1529.
4 Carcao M et al.: Thromb Haemost 2020;120:737–746.
5 Chan AK et al.: Res Pract Thromb Haemost 2020;4:1101–1113.

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32. Jahrestagung

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1. Fachinformation Hemlibra®, Stand der Information März 2023.

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