

Jk_{null} alleles in two patients with anti-Jk3

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Background - As of publication, a total of 41 null alleles have been acknowledged by the International Society of Blood Transfusion (ISBT) to cause the rare Jk_{null} phenotype, but none have been discovered in Austria thus far.

Materials and methods - Two patients with anti-Jk3 were serologically identified by a positive antibody screening and typed as Jk(a-b-). The initial genotyping using an SSP-PCR method for the common 838A/G polymorphism indicated a JK*02/02, or JK*01/02 genotype, respectively. To find the disruptive mutations, Sanger sequencing was performed and results were compared to the reference sequence. The patient's antibodies were characterized with a monocyte monolayer assay (MMA) for their potential clinical significance.

Results - Three novel null-mutations of the *SLC14A1* gene were found in two patients. Patient 1 was homozygous for a 10bp deletion in exon 4 (c.157_166del on JK*02). Testing of her family members revealed Mendelian inheritance of the deletional allele. The other patient was compound heterozygous for two mutations: one allele carrying a single base deletion in exon 4 (c.267delC on JK*01) and the other a splice site mutation in intron 3 (c.152-1g>a on JK*02). The MMA results suggest high clinical significance of the anti-Jk3 in both patients.

Discussion - The detected mutations led to Jk_{null} phenotypes and are the first description of Jk_{null} alleles in the Austrian population.

Keywords: immunohaematology, blood group genotyping, HDFN.

INTRODUCTION

The base of the Kidd blood group system are polymorphic sites distributed on a 43 kb multi-pass membrane glycoprotein that acts as an urea transporter on erythrocytes as well as the capillary endothelium of the kidney¹. Its 389 amino acids are encoded by the gene *SLC14A1*, which spans 30 kb of chromosome 18 and is organized in 10 exons, with exons 3 to 10, previously numbered as 4-11, encoding the mature protein².

The system consists of the three antigens Jk^a, Jk^b and Jk³, and was named JK after the initials of a child who suffered from haemolytic disease of the fetus and new-born (HDFN) caused by the antibody now known as anti-Jk^a in 1951³. The antithetical antigen Jk^b was discovered in 1953 and the Jk(a-b-) phenotype, also named Jk_{null} was reported in 1959⁴.

Jk^a is expressed in around 77% of the Caucasian population and Jk^b in around 74%^{1,5}. The high frequency antigen Jk³ is present on all red blood cells (RBCs) that express either

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Jk^a, Jk^b or both antigens and is absent only from very rare Jk(a-b-) RBCs¹. The corresponding antibody anti-Jk3 is clinically highly significant and can cause intermediate or delayed haemolytic transfusion reactions. Additionally severe HDFN was reported to be caused by anti-Jk3, however most cases are mild^{1,6-8}. RBCs that exhibit a Jk_{null} phenotype lack a functional urea transporter, which results in resistance to lysis of the red cells to 2M urea solutions. Although this trait provides a simple screening method for samples of questionable Jk phenotype^{9,10}, the underlying genetic background is heterogeneous. The Asp280Asn polymorphism encoded by c.838G/A in exon 8 distinguishes between the JK*01 and JK*02 genotype, or the Jk^a and Jk^b phenotype, respectively¹¹. Null mutations can be found on both genetic backgrounds, and either homozygosity or compound heterozygosity can lead to a Jk(a-b-) phenotype. Additionally a dominant inhibition as well as a transient loss of Jk antigens were reported¹.

With the exception of Polynesia, where 0.27% of the population exhibit a Jk_{null} phenotype based on a specific mutation of a JK*02 allele, the Jk_{null} phenotype is extremely rare¹². In the Finnish population another variant of the JK*02 allele was found in about 0.03%¹³. A more diverse genetic base for the very rare Jk_{null} phenotypes was described for most other population like Chinese, French, Australian, Swiss, English and Tunisian individuals^{1,14-17}. The distribution of Jk_{null} alleles in many populations is still unknown.

Here we describe two patients, who both presented with a Jk(a-b-) phenotype and consequently anti-Jk3. Patient 1, a 48-year-old female with anti-Jk3, anti-Fy^a and historically known anti-S. Anti-Jk3 developed during her first pregnancy. The first child was not affected and did not show any signs of HDFN. Her second child born three years later developed moderate HDFN and was in need of postnatal blue light therapy and erythropoietin.

Patient 2 is a 91-year-old female with acute ischemia of the left arm, requiring surgical thrombectomy. The aetiology of her anti-Jk3 is unclear.

MATERIALS AND METHODS

RBC serology

Both patient's red cells were typed for Jk antigens using monoclonal anti-Jk^a and anti-Jk^b in a direct agglutination tube method (ImmucorGamma, Norcross, GA, USA) and human polyclonal anti-Jk^a and anti-Jk^b (Biorad, Hercules,

CA, USA) in an indirect agglutination gel column test (ID-Card LISS/Coombs, Biorad). Furthermore, these results were confirmed by attempted adsorption of the polyclonal anti-Jk^a and anti-Jk^b (Biorad), subsequent elution using Gamma ELU-KIT II (Biorad) and investigation of the eluate with Jk(a+) and Jk(b+) cells in an indirect agglutination test.

Antibody titration was performed with ABO-compatible Jk(a+b-) and Jk(a-b+) cells.

The antibody specificity was confirmed by indirect agglutination test with ABO-compatible rare Jk_{null} cells and characterized by using a monocyte monolayer assay (MMA).

Monocyte monolayer assay

Mononuclear cells derived from peripheral blood of randomly selected donors were isolated using lymphocyte separation medium (LSM) (MP Biomedicals, Solon, OH, USA), resuspended in RPMI-1640 Medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% foetal bovine serum (FBS) (Gibco, Dublin, Ireland) and allowed to attach on Lab-Tek chamber slides (Nunc, Thermo Fisher Scientific, Rochester, NY, USA) by incubation at 37 °C to form a monolayer. Human red cells of defined Jk^a and Jk^b phenotypes were sensitized with the patients' sera. After one hour of interaction of the monocyte layer and sensitized erythrocytes and three washing cycles with physiological sodium chloride solution (VWR Chemicals, Leuven, Belgium) the phagocytic activity was quantified by a May-Gruenwald-Giemsa staining (Merck, Darmstadt, Germany) and microscopic enumeration of 600 events (monocytes with bound erythrocytes). A phagocytic rate of <3% is interpreted as clinically irrelevant, while between 3 and 5% the clinical significance is indeterminate. According to literature, a phagocytosis rate over 5% is rated as probably clinically relevant and antibodies with rates >20% will most likely cause a haemolytic transfusion reaction. In contrast to the method described by Arndt&Garratty no fresh complement was added¹⁸.

Urea lysis test

The urea lysis test was modified from Deelert *et al.*¹⁰. A solution of 5-8% erythrocytes diluted with 0.9% saline was incubated with 2M urea and closely inspected for haemolysis at room temperature. As a control, Jk_{null} RBCs of a patient homozygous for a deletion of SLC14A1 exons 3 and 4 were used¹⁴.

Genetic tests

- DNA was purified from EDTA-anticoagulated whole blood samples using the Maxwell 16 device and the Maxwell 16 Blood DNA kit (Promega, Fitchburg, MA, USA) according to the manufacturers' instructions.
 - Routine JK genotyping was performed with an updated version of the multiplex PCR described previously¹⁹.
 - SSP- and sequencing PCRs were carried out in 16 µL reactions using following conditions: 1x Buffer (Promega), 4.1 mM MgCl₂ (Promega), 0.2 mM dNTPs (Thermo Fisher Scientific), 0.04 µM internal control primers, 0.25-0.5 µM gene specific primers, 2 µL genomic DNA.
 - Standard PCR programme for SSP-PCRs and sequencing PCRs: 94 °C 30", 6 cycles (94 °C 30", 67 °C 40" -0.5 °C/cycle, 72 °C 50"), 27 cycles (94 °C 30", 64 °C 40", 72 °C 50"), 72 °C 120".
- PCR for the demonstration of separate alleles in patient 2: the PCR run was performed with the annealing temperature

lowered by 1 °C. PCR products were separated on 1.5% agarose gel (Biozym, Hessisch Oldendorf, Germany) in TBE, electrophoresis 4.5V/cm for 45 min. For sequencing, bands were excised and purified using Promega Wizard SV Gel and PCR clean-up kit. Purified PCR products and primers were shipped to Microsynth AG Austria (Vienna, Austria) for Sanger sequencing. Primers used are listed in **Table I**. Sequencing data were analysed using Chromas 2.6.6 Software (Technelysium Pty Ltd, South Brisbane QLD, Australia), NCBI databases²⁰ and ExPASy Bioinformatics Resource portal²¹. Long-range PCR was carried out in 50µL reactions using GoTaq Long PCR Master Mix (Promega) 50 ng of genomic DNA and 0.4 µM forward and reverse primers. PCR conditions for long-PCR: 95 °C 120", 33 cycles (94 °C 15", 61 °C 20", 65 °C 540"), 72 °C 600". PCR products were separated on 1.5% agarose gel (Biozym) in TBE, electrophoresis 4.5 V/cm for 120 min.

Table I - PCR Primer used

	Sense primer	Nucleotide sequence 5' to 3'	Antisense primer	Nucleotide sequence 5' to 3'	Target
A	JK1-838G-f1	GTCTTTCAGCCCCATTTGcGG	JK-i9-r1	CTTTTCTATCCTCCCAACATCC	c.838G (JK*01)
A	JK2-838A-f2	AGTCTTTCAGCCCCATTTGcGA	JK-i9-r1	CTTTTCTATCCTCCCAACATCC	c.838A (JK*02)
B	JK-x3-seqF	TAGAGCTAGGGCACACGTCA	JK-x3-seqR	TCTGCCCGAAAAGATACAC	Exon2
B	JK-x4-Seq-F	GGGCTCTGCCTTAGGGATAC	JK-x4-Seq-R	TGAAGCCAGGTGCCTTCTAA	Exon3
B	JK-x5-seq-F2	GAGGCTTTCAGGGATCTGGG	JK-x5-seq-R2	GTAAGTGGTCAGCCCCTGTG	Exon4
B	JK-x6-seq-F	TGTGCAAGTGCAACCAAGC	JK-x6-seq-R	CATTCCCTCCTTGCCATGT	Exon5
B	JK-x7-seq-F	TGTGTGAGCTGCTTTGTCA	JK-x7-seq-R	GGCCATTGCGTATCTTGACAG	Exon6
B	JK-x8-seq-F	AGGAGTTTGTGGGTGCCTG	JK-x8-seq-R	CATCCCGGAACTCCCATTTG	Exon7
B	JK-x9-seq-F	CAGAACATCCTGCCTTTAGTCC	JK-x9-seq-R	GTAGTCATGAGCAGCCCTCC	Exon8
B	JK-x10-seq-F	GTAATCAGGGCACTGTGCATTC	JK-x10-seq-R	TGGACTTCAGGAGCATTCC	Exon9
B	JK-x11-F	AGTAGAGGGGGATGCCTTGT	JK-x11-R	AGCAGTTTGCTGAGACCTCG	Exon10
C	JK-x5-ndel-F	AGACAAACCGTGGTGCT	JK-i5-R	TCTACTGCCCTGGTGAGAGT	wildtype
C	JK-x5-del-F	CCAGACAAATCCAGTTCATTGAC	JK-i5-R	TCTACTGCCCTGGTGAGAGT	10bp deletion
D	JK-i4-G-f2	GCTTTACCTCATCCCTTcAG			wildtype
D	JK-i4-A-f2	GCTTTACCTCATCCCTTgCAA			splice site mutation
D			JK-x5-267C-R1	GTGAGAGCCCACCAGGaGT	wildtype
D			JK-x5-267delC-R1	AGTGAGAGCCCACCAGGaGT	single base deletion
E	JK-i4-A-f4	TGCTTTACCTCATCCCTTcAA			splice site mutation
E	JK-i4-G-f4	TGCTTTACCTCATCCCTTcAG			wildtype
E			JK1-838G-r5	CCCAGAGTCCAAAGTAGATaTC	c.838G (JK*01)
E			JK2-838A-r9	CCCAGAGTCCAAAGTAGAaGTT	c.838A (JK*02)
F	GH1-x2-f1	CACCAGCTGGCCTTTGACAC	GH1-x5-r1	TGAAGCAGTAGAGCAGCCCG	1kb control (GH1)
F	GH1-x1-f1	GGATCCCAAGGCCAACTCC	GH1-3'-r1	CATGGCCAGGTAGCCTATGC	2 kb control (GH1)

A: Primers for detection of SNP 838G/838A; B: Primers for Sanger sequencing; C: Primers for detection of the 10bp deletion in Patient 1; D: Primers for detection of mutations in Patient 2; E: Primers for long-range PCR; F: Internal control primers; lower case letters indicate mismatches introduced to increase primer specificity.

RESULTS

RBC serology

The anti-Jk3 in both patients was identified by using an indirect agglutination test with ABO compatible Jk(a-b-) cells. Erythrocytes were serologically typed as Jk(a-b-) and confirmed by failure to adsorb and elute polyclonal Jk^a and Jk^b antibodies.

In patient 1's serum anti-Fy^a was detectable and anti-S was historically known but not detectable. The titre of the anti-Jk3 was 1:64 performed with Jk(a+b-) Fy(a-) and S- RBCs in IAT gelcards.

In patient 2 anti-Jk3 with a titre of 1:128 was found as a single specificity with no other antibodies present.

Monocyte monolayer assay

To determine the clinical relevance of the Jk3 antibodies, an MMA was performed with serum from both patients. Each sample was tested with Jk(a+b-) and Jk(a-b+) erythrocytes of blood group O. In total 600 monocytes in each field were counted and the rate of phagocytosis was calculated. For patient 1, whose serum additionally contained anti-Fy^a, two out of four tested cells were Fy(a+). Interestingly the phagocytosis rate was lower when two targeted antigens were present (mean 58.1% with Fy(a-) and 39.6% for Fy(a+) cells with same conditions). For patient 2 phagocytosis rates were even higher with a mean of 70.06%.

There was no major difference whether Jk(a+b-) or Jk(a-b+) RBCs were used in both patients. Antibodies with rates of >20% in MMA are considered as clinically significant and will most likely cause haemolytic transfusion reaction¹⁸.

Urea lysis test

As an additional test to confirm the absence of a functional Kidd-glycoprotein, a urea lysis test was performed. RBCs of patient 1 resisted lysis for 5 min but showed complete lysis after 10 min treatment with 2M urea by tube method. In contrast, RBCs of the patient's family members heterozygous for the c.157_166del mutation and all control samples of Jk(a+) or Jk(b+) individuals showed almost immediate lysis (within a few seconds) when treated with 2M urea. The control Jk_{null} RBC, homozygous for deletion of exon 3 and 4, showed a comparable lysis time (10 min) as the patient 1 cells. Unfortunately, by the time the urea lysis test was performed, fresh RBCs of patient 2 were unavailable for testing.

Genetics

Patient 1

In routine genotyping patient 1 typed as JK*02/02, after the identification of an anti-Jk3. However, in serological testing neither Jk^a nor Jk^b were detected. Sanger sequencing of the coding exons 3 to 10 revealed a 10 bp deletion (c.157_166del) in exon 4 of *SLC14A1*. Homozygosity was confirmed by SSP-PCR with primers specific for the mutation and the corresponding wildtype. The heterozygous 10bp deletion was detected in both parents and the sons of patient 1, but not in her brother. The father of her children could not be tested (see pedigree in **Figure 1**). Additionally, a synonymous variant was detected in exon 6, 588G>A, which was also found in all family members carrying the 10 bp deletion.

This 10 bp deletion (c.157_166del) is submitted as reference SNP rs750167058 with a frequency of 3/121200 (ExAC) and 2/246102 (GnomAD) in the European subgroup in both studies²². Here, we describe the first patient carrying the c.157_166del mutation in a homozygous state and provide evidence that it leads to a Jk_{null} phenotype. In silico translation using the translate tool from ExPASy bioinformatics resource portal²¹ revealed that the frameshift caused by the deletion leads to an altered protein sequence from amino acid 53 and a premature stop codon (p.[Pro53SerfsTer25]). As mRNAs containing premature stop codons are subject to nonsense-mediated mRNA decay it is unlikely that the deletion c.157_166del allele will be transcribed into protein, leaving the erythrocyte membrane without a functional urea transporter²³.

Patient 2

Patient 2 with a Jk_{null} phenotype was initially typed as JK*01/02 in the routine SSP-PCR genotyping. Sanger sequencing of exon 4 and flanking intron sequences revealed two mutations: a single base deletion, c.267delC in exon 4, and a putative splice site mutation at position -1 in intron 3, c.152 -1g>a (see **Figure 2A and B**). Both mutations and corresponding wildtype were confirmed by SSP-PCR and shown to be located on separate alleles: no PCR product was formed for patient 2 with the wildtype primers. However, a 155bp PCR product was formed with primers for wildtype intron sequence with a 267delC specific primer, as well as for the intron 3 mutation with the wildtype 267C. No PCR product was formed when primers specific for both mutations were used

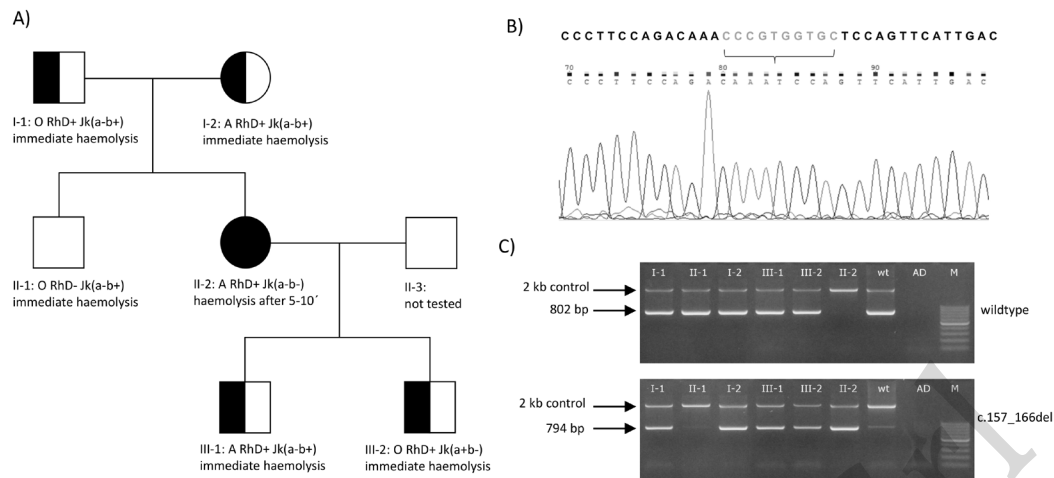


Figure 1 - Mendelian inheritance of a 10bp deletion in patient 1 and family members

(A) Pedigree of patient 1's family indicating the zygosity of the *JK* c.157_166del mutation. ABO, RhD blood group and serological *Jk* phenotype are given and below the result of urea lysis test. The father of patient 1's children was not tested. (B) Partial DNA Sequencing chromatogram of *SLC14A1* exon 4, Patient 1. Top panel shows the wildtype sequence with the 10 bases deleted in Patient 1 shown in grey. (C) SSP-PCR for detection of c.157_166del and corresponding wildtype using primer pairs JK-x5-ndel-F/JK-i5R and JK-x5-del-F/JK-i5-R respectively. Numbers indicate the persons as given in pedigree. wt: Jk(a+b+) blood donor; A.D: water control; M: 100bp marker.

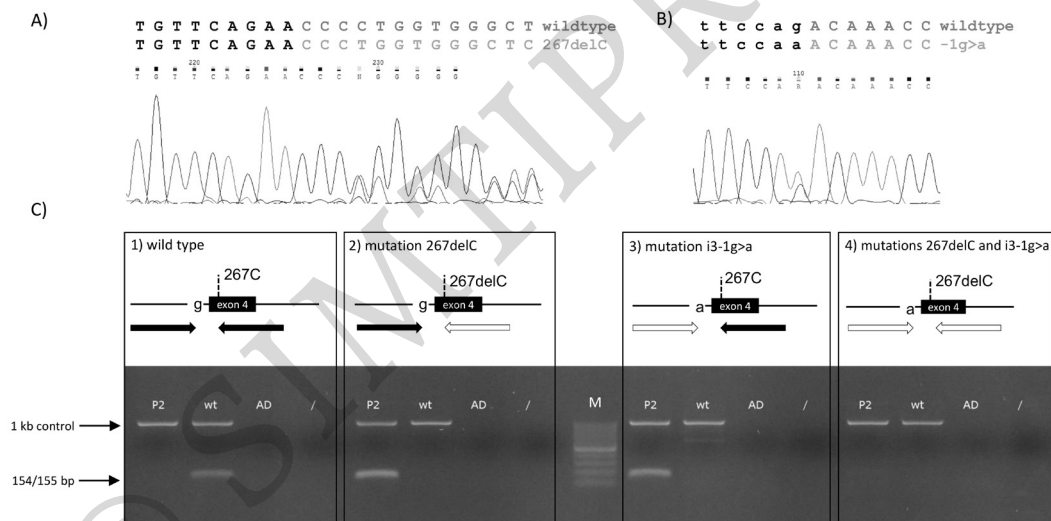


Figure 2 - Detection of two mutations on separate alleles in patient 2

(A) Partial sequencing chromatogram of *SLC14A1* exon 4. Top panels shows wildtype sequence and overlapping deletional allele c.267delC. (B) Partial DNA sequencing chromatogram of *SLC14A1* intron 3 and exon 4. Top panels shows wildtype sequence and overlapping allele with SNP -1g>a in intron 3. Lower case letters are used for intron sequences. (C 1-4) Schematic representation of exon 4 genomic region and SNPs are shown above the gel. Arrows indicate primers used in each assay (black: wildtype, outlined: mutation). Agarose gel is shown below for SSP-PCRs using the primer pairs indicated above. P2: patient 2; wt: Jk(a+b+) blood donor; M: 100bp marker; AD: water control.

(see **Figure 2, C1-4**). Thus, we conclude that two separate alleles are responsible for the *Jk*_{null} phenotype; one carrying the c.267delC mutation and the other carrying the intron 3 splice site mutation. Subsequent long-range PCR using mutation specific forward primers with *JK**01 and *JK**02 specific reverse primers revealed that c. 267delC is located

on the *JK**01 allele, whereas the intron variant c.152 -1g>a is located on the *JK**02 allele.

The deletion (c.267delC in exon 4) is listed as rs766335775 and was found with a frequency of 1/246190 by GnomAD and 1/121398 by ExAC in the European subgroup²². It leads to a frameshift with an altered protein sequence from amino

acid 91 and a premature stop codon (p.[Trp91GlyfsTer15]). The SNP detected in intron 3 (c.152 -1g>a) is submitted as rs373247991 and was found in 1 European sample out of 121146 samples by ExAC²². It is listed as a splice acceptor variant and will likely lead to skipping of exon 4 causing a frameshift and premature stop codon (p.[Asp51GlyfsTer3])

and thus a non-functional urea transporter protein. As mentioned above, the premature stop codons are expected to lead to nonsense-mediated mRNA decay, eventually leading to a Jk_{null} phenotype.

The proposed effects of the mutations on the JK protein are summarised in **Figure 3**.

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Reference:      41 D M K E L A N Q L K D K P V V L Q F I D 60
267delC:       41 D M K E L A N Q L K D K P V V L Q F I D 60
c.157_166del:  41 D M K E L A N Q L K D K S S S L T G F S 60
i3-1g>a:       41 D M K E L A N Q L K G H X

Reference:      61 W I L R G I S Q V V F V N N P V S G I L 80
267delC:       61 W I L R G I S Q V V F V N N P V S G I L 80
c.157_166del:  61 G A Y P K W C S S T T P S V E S X

Reference:      81 I L V G L L V Q N P W W A L T G W L G T 100
267delC:       81 I L V G L L V Q N P G G L S L A G W E Q 100

Reference:     101 V V S T L M A L L L S Q D R S L I A S G 120
267delC:      101 W S P L X

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Figure 3 - Proposed effect of mutated alleles on the urea transporter protein

Protein sequences were derived from reference sequence NM_015865.7 using the translate tool from ExPASy bioinformatics resource portal²¹. Amino acid positions 41 to 120 are shown. For *in silico* translation of c.152 -1g>a, exon 4 was omitted from the reference sequence. Altered amino acids are written bold. Stop is indicated by an X.

DISCUSSION

Kidd antibodies, especially anti-Jk3, are a known cause for transfusion reactions reaching from mild and delayed haemolytic reactions⁸ to severe and fatal immediate haemolysis²⁴⁻²⁶. Because of the rare phenotype, erythrocyte concentrates from homologous donors are scarcely available worldwide. Thus, both patients were urged to donate blood for cryopreservation if clinical state permits.

Due to the high percentage of phagocytosis observed in the MMA of both patients, we strongly advise to transfuse Jk:-3 blood exclusively.

In general, clinical symptoms of a HDFN caused by anti-Jk3 are described as mild^{6,7}, however in the case of the second child of patient 1 we would classify the HDFN as moderate. He was born at 35 weeks and 6 days gestational age, after medical induction of labour because of maternal hypotonia and an antibody titre of 1:1024 determined with Fy(a-) Jk(a-b+) S- cells by gelcard method. Prenatal sonography showed no signs of fetal anaemia, and haemoglobin after birth was 14.4 g/dL. The child had a 3+ direct agglutination test (DAT) and bilirubin levels reaching up to 17.6 mg/dL. He was diagnosed with icterus praecox and gravis and blue light therapy was performed over 7 days. The

mild anaemia was treated with erythropoietin up to 3 months after birth. There were no details available about immuno-haematological examination by elution and the causative antibody could not be differentiated between Fy^a and Jk3 antigens. Because the MMA does not discriminate between IgG1 and IgG3 it might not be as predictive for HDFN severity as it is for the estimation of haemolytic transfusion reaction risk. Nevertheless, as in this case, it is already useful for the classification of antibodies against high frequency antigens and unknown specificities^{18,27}.

Patient 2 was not transfused and we do not know the *in vivo* properties of her antibody.

Regarding the urea lysis test, we could not observe a prolonged lysis resistance of cells heterozygous for the c.157_166del mutation. Additionally the lysis resistance we observed for the Jk(a-b-) cells of patient 1 and the Jk(a-b-) control was generally shorter than described in literature but significantly longer than in Jk(a+) or Jk(b+) controls. Therefore, the test was considered valid. The urea lysis test is certainly a low-cost test to screen for Jk_{null} individuals. However, it is likely that if Jk phenotypes were questionable, the investigation would proceed to molecular analysis^{10,15}.

The mutations leading to the JK-null phenotype in patient 1 and patient 2 occur at a very low rate and so far were

only detected in a few individuals by large scale genomic sequencing²². None of the mutations were detected in an SSP-PCR screening of 200 randomly selected blood donors at the Vienna Blood Center (data not shown). Additionally, the increasing number of known null-alleles challenges routine SSP-PCR testing. Nevertheless, the common JK*838A/838G polymorphism might still be sufficient to predict the Jk^a/Jk^b phenotype in most routine samples, but not in patients carrying unusual Jk-phenotypes or Jk antibodies⁴. Especially in clinical cases with unexpected allo-antibodies where Jk phenotype cannot be clearly determined by serological methods or where serology and standard SSP-PCR do not match, we suggest enhanced/further molecular testing.

CONCLUSIONS

In a world with increasing possibilities for cost-efficient Next Generation Sequencing, data generated from blood donors and patients will likely lead to the detection of both well-known as well as yet uncharacterised variants. This applies equally to other blood group systems, where alloantibodies are of great clinical significance and compatible blood is rare. Notwithstanding the advances in genetic testing, characterization of the effects of novel mutations at the protein level will remain critical for future investigations.

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AUTHORSHIP CONTRIBUTION

WA and LW contributed equally to this study as first Authors. They conceived the study design and wrote the manuscript. WA carried out genetic tests and data analysis. LW and ML performed RBC serology, urea lysis test and MMA. LW, NL and LG collected patient samples and provided clinical data. ES and CJ supervised the project and critically revised the manuscript. All Authors approved the manuscript's final version and take responsibility for the integrity and accuracy of the data. All Authors have reviewed and approved of the paper.

The Authors declare no conflicts of interest.

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