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MNSs genotyping by MALDI-TOF MS shows high concordance with serology, allows gene copy number testing and reveals new St(a) alleles

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Results of genotyping with true high-throughput capability for MNSs antigens are underrepresented, probably because of technical issues, due to the high level of nucleotide sequence homology of the paralogous genes *GYPA*, *GYPB* and *GYPE*. Eight MNSs-specific single nucleotide polymorphisms (SNP) were detected using matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry (MALDI-TOF MS) in 5800 serologically M/N and S/s pre-typed Swiss blood donors and 50 individuals of known or presumptive black African ethnicity. Comparison of serotype with genotype delivered concordance rates of 99·70% and 99·90% and accuracy of genotyping alone of 99·88% and 99·95%, for M/N and S/s, respectively. The area under the curve of peak signals was measured in intron 1 of the two highly homologous genes *GYPB and GYPE* and allowed for gene copy number variation estimates in all individuals investigated. Elevated *GYPB*: *GYPE* ratios accumulated in several carriers of two newly observed *GYP*401* variants, termed type G and H, both encoding for the low incidence antigen St(a). In black Africans, reduced *GYPB* gene contents were proven in pre-typed S-s-U- phenotypes and could be reproduced in unknown specimens. Quantitative gene copy number estimates represented a highly attractive supplement to conventional genotyping, solely based on MNSs SNPs.

Keywords: blood group genotyping, MNSs antigens, MNSs alleles, *GYPA*, *GYPB*, MALDI-TOF MS.

Most blood transfusion services routinely test for the major blood group antigens ABO and D only, with determination of other Rh antigens and K in selected cases, whereas minor blood group antigens, such as k, Jk^a, Jk^b, Fy^a, Fy^b, M, N, S and s, usually remain unconsidered. However, transfusion of alloimmunized patients and prevention of alloimmunization may optimally be addressed by enhancing the matching of red cell components based on an extended blood group phenotype. Given that the procurement of extensively phenotyped blood components by agglutination-based assays is costly and laborious, blood group genotyping may offer an alternative approach for this purpose. Genotyping of blood

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donors may be the most appropriate application due to time-insensitive typing needs and availability of suitable high-throughput technologies.

Blood group genotyping is gaining widespread adoption in blood centres and transfusion services(Denomme *et al*, 2011; Denomme, 2013; Flegel *et al*, 2015). A variety of methods have been described for the molecular analysis of *KEL*, *SLC14A1*, *DARC*, *GYPA,* and *GYPB* and the detection of their allelic variants encoding clinically relevant blood group antigens, such as those belonging to the K, Jk, Fy, and MNSs systems (Hashmi *et al*, 2005; Montpetit *et al*, 2006; Polin *et al*, 2008; Hopp *et al*, 2010; Jungbauer *et al*, 2012; Haer-Wigman *et al*, 2013; McBean *et al*, 2014; Meyer *et al*, 2014). Many of these techniques promise high-throughput processing. However, there is no universally accepted definition of this notion.

Matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry (MALDI-TOF MS) based blood group genotyping delivered impressive concordance rates between blood group genotypes and phenotypes, ranging between 99·96% and 99·31% for the antithetic blood group antigens K/k, $Jk^{a/b}$, $Fy^{a/b}$, M/N, and S/s (Table I). Regularly, typing errors were more prevalent in serotyping than in genotyping and there are far fewer data published for M/N and S/s than for K, Jk and Fy (Table I).

The blood group antigens M/N and S/s are encoded on *GYPA* and *GYPB*, respectively (Daniels, 2002; Reid *et al*, 2012). Single nucleotide substitutions, genetic hybrids and gene duplication events (potentially involving a third, highly homologous *GYPE* gene) underlie variant MNSs antigens, ranking the MNSs blood group system second in complexity to the Rh blood group system (Reid, 2009). Glycophorin C and glycophorin D - an abridged version of glycophorin C - are both predetermined by *GYPC* (Le Van Kim *et al*, 1987; el-Maliki *et al*, 1989). *GYPC* encodes for antigens of the Ge (Gerbich) blood group system, but does not share any sequence homology with *GYPA*, *GYPB*, or *GYPE* (Le Van Kim *et al*, 1987). Analysis of nucleotide sequence in non-human primates has actually confirmed that the MNSs genes are classical representatives of paralogous genes or 'paralogs', and has identified *GYPA* as the ancestral gene from which *GYPB* and *GYPE*, but not *GYPC,* arose by sequential gene duplication (Vignal *et al*, 1990; Rearden *et al*, 1993).

Due to excessive nucleotide sequence homology of the paralogs *GYPA*, *GYPB* and *GYPE*, the molecular prediction of antigens in the MNSs system is technically challenging (Kudo & Fukuda, 1989; Huang *et al*, 1991). Thus, primers designed to target single nucleotide polymorphisms (SNPs) of *GYPA* may simultaneously amplify sequences of *GYPB* and *GYPE* and thereby deliver misleading amplicons. The panoply of known and unknown hybrid genes of *GYPA, GYPB and GYPE* further impedes molecular approaches to dissect MNSs alleles. Table II gives a simplified overview of MNS alleles officially recognized by the International Society of Blood Transfusion (ISBT) and addressed in this manuscript (http://www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology/).

To address these issues, we used MALDI-TOF MS-based blood group genotyping. This technology has already shown persuasive characteristics in performance, data quality and reproducibility in the genotyping of the K, Jk and Fy blood groups, true high throughput capability and results that were highly concordant with serotyping (Gassner *et al*, 2013; Meyer *et al*, 2014, 2015; Lopez *et al*, 2015).

Materials and methods

Samples

All 5800 samples were sequentially taken from repetitive red blood cell (RBC) donors, and had historic phenotype data for MN and Ss. Donor samples were collected at the seven regional blood transfusion services headed in Basel [total *n* (tn) = 390, dropout *n* (dn) = 12, valid *n* (vn) = 378], Geneva (tn = 24, dn = 1, vn = 23), Lucerne (tn = 226, dn = 0, vn = 226), Lugano (tn = 381, dn = 5, vn = 376), Neuchatel (tn = 141, dn = 1, vn = 140), St. Gallen (tn = 168, dn = 1, vn = 167) and Zurich (tn = 4472, dn = 37, vn = 4435).

According to the ethical committee of the Canton of Zurich, an ethical approval of the study was not mandatory. However, all donors explicitly permitted genetic laboratory investigations by written consent.

A panel of 50 DNA samples of known, or presumptive black African ancestry was included in this study (Meyer *et al*, 2014). Due to sample depletion, two of the previously used 48 samples were excluded from the present study. Additionally, two S-s-U-/*GYPB*01N* homozygous and two $U+^{W}$/*GYPB*01N*/*GYPB*03N.04* heterozygous samples, were provided by Marion E. Reid (NY Blood Center, NY, USA) for this study.

Routine serological blood group typing and handling of discrepant results

Standard MN and Ss serology was performed as described before (Meyer *et al*, 2014). Serotyping for Vw, Mt(a) and Mg was performed retrospectively on genetically selected individuals with antisera of human origin and provided by Thierry Peyrard [Institut National de la Transfusion Sanguine (INTS), Paris, France]. Samples genetically positive for *GYP*401* and *GYP*201.01*-like were tested for antigens Vw, Mur and Mut with monoclonal antibodies (kindly provided by M. Uchikawa of the Japanese Red Cross, Kanto-Koshinetsu Block Blood Centre, Tokyo, Japan) in/indirect antiglobulin test (ID/IAT)-IgG (BioRad, Cressier, Switzerland), antigens St(a), Hil and Miny with polyclonal antibodies in ID/IAT (BioRad), and M, N, S and s with monoclonal antibodies in tube test (Grifols, Düdingen, Switzerland and Immucore, Rödermark, Germany).

Table I. Selected blood group genotyping approaches.

Reference	Institution location	Technique	*n*	K, k *KEL* Mistyped due to		*n*	Jk a/b *SLC14A1* Mistyped due to		*n*	Fy a/b *DARC* Mistyped due to		*n*	MN *GYPA* Mistyped due to		*n*	Ss *GYPB* Mistyped due to	
				Serology	Genotyping		Serology	Genotyping		Serology	Genotyping		Serology	Genotyping		Serology	Genotyping
Hashmi *et al* (2005)	New Jersey, USA	BeadChip	1468	1	0	866	2	0	1236	5	0	34	0	0	882	7	6 (8)
Montpetit *et al* (2006)	Montréal, Canada	SNPstream	366	0	0	243	6	0	205	6	0	102	2	0	136	2	0
Polin *et al* (2008)	Linz, Austria	RealTime	200	0	0	42	0	0	38	0	0	31	0	0	16	0	0
Hopp *et al* (2010)	Milwaukee, USA	OpenArray	411	0	0	248	2	0	311	1	1	–	–	–	–	–	–
Jungbauer *et al* (2012)	Vienna, Austria	Multiplex PCR-SSP	469	0	0	370	0	0	380	0	0	372	0	0	370	0	0
Haer-Wigman *et al* (2013)	Amsterdam, NL	MLPA	226	2	0	221	0	0	215	1	0	206	1	0	229	2	0
Meyer *et al* (2014)	Zurich, Switzerland	MALDI-TOF MS	4000	0	0	4000	2	1	4000	31	1	–	–	–	–	–	–
McBean *et al* (2014)	Kelvin Grove, Australia	Chip and MALDI	300	0	0	300	0	0	300	2	0	300	0	0	300	0	0
Present study	Zurich, Switzerland	MALDI-TOF MS	–	–	–	–	–	–	–	–	–	5743	8	7	5743	3	3
Total			7440	3	0	6290	12	1	6685	46	2	6788	11	7	7676	14	3
		Mistyped (%)		0·04	0·00		1·91	0·02		6·88	0·03		1·62	0·10		1·82	0·04
		Correctly typed (%)		99·96	100·0		98·09	99·98		93·12	99·97		98·38	99·90		98·18	99·96

Black and grey background represents discrepant findings between serology and genotyping. Black background indicates samples for which the original phenotype was corrected according to genotyping results (mistyped due to serology). Grey background indicates samples for which the genotyping result needed to be corrected according to the original serology (mistyped due to genotyping).

Table II. Simplified overview of MNS alleles officially recognized by the International Society of Blood Transfusion (ISBT) and addressed in this manuscript (http://www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology/).

Gene	Phenotype	Allele name
GYPA	MNS:1 M+	*GYPA*01*
	MNS:2 N+	*GYPA*02*
	MNS:7,9,–40, Vw+	*GYPA*09*
	MNS:-1,-2,11, M^g+	*GYPA*11*
	MNS:14 Mt(a+)	*GYPA*14*
	MNS:7,19,–40, Hut+	*GYPA*19*
GYPB	MNS:4, s+	*GYPB*04*
	MNS :3, S+	*GYPB*03*
	MNS:6, s+, He+	*GYPB*06.01*
	MNS:3,6, S+, He+	*GYPB*06.02*
	MNS:–3,5W, S–U+^{w}	*GYPB*03N.04*
GYP(A-B) hybrid	MNS:-3,4,20,34 S-s+, Hil+, MINY+	*GYP*201.0*
GYP(B-A) hybrid	MNS:15, St(a+)	*GYP*401*
GYP(B-A) hybrid	MNS:–3,4,24, Dantu+	*GYP.402*
GYP(B-A-B) hybrid	MNS:–3,4,8, 20,34,35, S–s+, Mi(a+), Hil+, MUT+, MINY+	*GYP.504*
GYPB deletion	MNS:–3,–4,–5, S–s–U–	*GYPB*01N*

SNPs included in the MALDI-TOF MS MNSs module

The SNPs that were investigated for the common and variant alleles of the *GYPA*, *GYPB* and *GYPE* blood group genes are given in Table III. Assay design, quality control of the primer mixes, and MALDI-TOF MS-based genotyping were done as described before (Meyer *et al*, 2014).

Confirmatory blood group genotyping and sequencing

Confirmatory blood group genotyping and serotyping were performed in case of discrepancy on an independent second sample with two exceptions: In these two cases, original serological results and DNAs were used for further analysis. Discrepant results however, were not counted as 'serological' or 'genotyping' errors. MNSs genotyping was performed after

Table III. Specificities tested by the matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry (MALDI-TOF MS) based MNSs module.

Blood Group	Gene (HGNC)	Position	Allele 1	Allele 2	nt. position	nt. 1	nt. 2	Amino acid exchange	Detected antigens	Detected alleles	Detected SNPs	rs #
M/N	*GYPA*	Exon 2	*GYPA*01*	*GYPA*02*	59	C	T	Ser20Leu	2	2	1	rs7682260
M/N	*GYPA*	Exon 2	*GYPA*01*	*GYPA*02*	72	T	G	Gly24Glu	0	0	1	Open
MN/Vw*	*GYPA*	Exon 3	*GYPA*	*GYPA*09*	140	C	T	Thr47Met	1	1	1	Open
MN/Hut*	*GYPA*	Exon 3	*GYPA*	*GYPA*19*	140	C	A	Thr47Lys	1	1	0	Open
MN/Mt(a*)	*GYPA*	Exon 3	*GYPA*	*GYPA*14*	230	C	T	Thr77Ile	1	1	1	Open
S/s	*GYPB*	Exon 3 (4)	*GYPB*03*	*GYPB*04*	143	T	C	Thr48Met	2	2	1	rs7683365
Ss/He	*GYPB (A-like)*	Exon 2	*GYPA*	*GYPB*06*	59	Y	G	Ser/Leu20Thr	1	1	0	n.a.
S, s/U+^{w}	*GYPB*	Intron 4 (5)	*GYPB*	*GYPB*03N(.03/.04)*	i4(i5)+5	g	t	–	1	1	1	Open
S, s/U–	*GYPA/GYPB*	Intron 1	*GYPA*	*GYPB*	i1 + 9k	c	t	–	1	1	1	Open
S, s/U–	*GYPB/GYPE*	Intron 1	*GYPB*	*GYPE*	i1 + 16k	c	g	–	0	0	1	Open
Total									10	10	8	

*Genetic intron 1 locations 9 kbp and 16 kbp are deduced from an alignment including *GYPA*, *B*, and *E*. There is a remarkable variation in the actual positions of these inter-genetic polymorphisms within the three genes. However, genetic variations are given in brackets and are defined unambiguously by the sequences (C/T)TGCCTTGGCCTCCCAAAGCA-3′, and 5′-AACTTTGGGA-TAAAGTTTTT(C/G), for the 9 kbp and 16 kbp specificities, respectively. *GYPA*01* and *GYPB*04* are considered as reference alleles by the International Society of Blood Transfusion (ISBT) terminology committee (http://www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology/). Number of detected antigens, alleles and SNPs allow for a statistical assessment of the MNSs genotyping module. Redundancies were only counted once. Sole gene names indicate any allele of the respective gene, or any, but the one given in the field 'allele 2'. HGNC, HUGO (Human Genome Organization) Gene Nomenclature Committee; nt., nucleotide; SNP, single nucleotide polymorphism.

manual DNA extraction(Gassner *et al*, 2013) and using commercially available genotyping kits (RBC-Ready Gene MNSs, Inno-Train GmbH, Kronberg im Taunus, Germany).

Sequencing was performed on all samples with confirmed phenotype/genotype discrepancies and for reference purposes on one NNSS phenotype sample with the primers listed in Tables SIA and B. Primer concentration was 300 nmol/l and conditions were those previously described for *RHD* sequencing (Gassner *et al*, 2005). Reference sequences were from one phenotype NNSS individual sequenced in this study (accession number LN880516) and from the NCBI database (accession numbers NG_007470.3, NG_007483.2, and NG_009173.1) (http://www.ncbi.nlm.nih.gov/gv/mhc/xslcgi.cgi?cmd=bgmut/home). ISBT allele names for newly observed alleles were requested from the ISBT terminology committee (http://www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology/).

Bi-specific diagnostic polymerase chain reaction sequence-specific primers (PCR-SSPs) were used to specifically detect *GYP*201.01*-like/GP.Hil and *GYP*401*-/St(a) types G and H, and *GYP.402*/Dantu alleles with the primers listed in Tables SIC and D for linkage analysis (Lo *et al*, 1991). Positive amplification control and PCR set-up conditions were as described by Gassner *et al* (1997), cycling and agarose gel electrophoresis was performed as described by Crottet *et al* (2014).

Allele frequency calculation

Allele-frequencies were estimated by allele counting and were adjusted for serologically hidden and genetically undetected new alleles in heterozygous individuals, according to Hardy–Weinberg proportions (Tables SI and SII) (Hardy, 1908).

Materials and methods

Samples

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SNPs included in the MALDI-TOF MS MNSs module

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Results

The MNSs module: a single multiplex reaction with typing capability for 8 SNPs

The MNSs blood group genotyping module is a single multiplex reaction, comprising a total of 3 biallelic and 2 triallelic SNP assays for the simultaneous genetic analysis of M, N, Vw, Hut, Mt(a), S, s, and He. Additionally, a mutated splice site at the 5th nucleotide of intron 5, specific for *GYPB*03N*, with a pronouncedly weakened expression of U was detected (Huang *et al*, 1997; Storry *et al*, 2003). Furthermore, two inter-genetic polymorphisms, approximately located at positions 9 kbp and 16 kbp of intron 1 of *GYPA*, *GYPB* and *GYPE* were interrogated to allow for respective gene copy number variation estimates (Table SI) (Huang *et al*, 1987).

High-throughput MALDI-TOF MS-based genotyping was performed in eight independent typing runs over 8 d, excluding DNA extraction and analysis of data. Calling failures were caused by samples with negative results for all SNPs (total DNA dropouts $n = 41$), single SNP assay failures per sample ($n = 15$), and one sample with suspected DNA cross-contamination ($n = 1$) and were all excluded from the final 5743 donor data set further analysed, resulting in a total calling failure rate of 0·98% (57/5800).

Performance comparison between Zurich and Hamburg

A random set of 760 blinded samples was chosen to assess the reproducibility of MALDI-TOF MS-based MNSs blood group genotyping using mass spectrometers in two independent laboratories. All process steps, from oligonucleotide order to data analysis, were autonomously carried out in Zurich and Hamburg. Assay performance revealed raw data call rates of 94·6% (Zurich) and 94·3% (Hamburg). Additional manual post-processing cluster analysis led to final call rates of 98·8% (Zurich) and 98·6% (Hamburg). Out of a total of 72 non-templated control samples (H_2O) analysed per centre, provided one and five false positive calls in different individual assays, in Zurich and Hamburg, respectively. Final findings of 3412 replicate genotypes showed 100% genotyping concordance between the two sites.

Common and variant GYPA *alleles among Swiss*

Congruent results comprised 1711 MM (including 1 *GYPA*09*/Vw positive), 2807 MN (10 *GYPA*14*/Mt(a) and 4 *GYPA*09*/Vw positives), and 1208 NN (4 *GYPA*14*/Mt(a) and 4 *GYPA*09*/Vw positives), resulting in a phenotype - genotype concordance rate of 99·70% (5726/5743). Phenotypes Vw, Mt(a) and Mg were confirmed serologically on three *GYPA*09*, nine *GYPA*14* and one *GYPA*11* positive genotypes, respectively. Four MM, ten MN and three NN phenotypes were discrepant to the respective genotype (17/5743), with four MM (1 without follow up), three MN (1 without follow up), and three NN that could be explained by serological mistypings. Phenotypes were then corrected according to the corresponding genotype (Fig 1).

Genotyping results of all remaining seven individuals with MM genotypes and MN phenotypes were confirmed to be negative for *GYPA*02*(T59) by commercially available PCR-SSP typing kits and sequencing of *GYPA* exon 2, in all cases. In addition, amplification with primers forward-specific for *GYPB* intron 2 and reverse-specific for *GYPA* intron 3 or intron 4 indicated the presence of *GYPB-A* hybrid-genes for all samples. Sequencing allowed the detection of one *GYPB-A-B*, with high similarity to *GYP*201.01*/GP.Hil and two types of *GYPB-A* hybrid alleles, both with high similarity to *GYP*401*/St(a) and two and four representatives each. For two samples, the break-point was positioned close to exon 3 and in four samples approximately 500 bp after the exon 3/intron 3 border, respectively. For both newly detected *GYP*401*/St(a) cases, antigen positivity for St(a) was confirmed serologically on one sample each. The new *GYP*401* alleles show similarity with previously reported alleles found in St(a) positive samples dubbed St(a) type A-F. Until now, St(a), encoded by *GYPB-A* molecules have been described in detail for St(a) type A in two independent Black Americans (Huang & Blumenfeld, 1991a), type B in two independent Japanese(Huang & Blumenfeld, 1991a), type C in an 'Oriental' family(Huang & Blumenfeld, 1991b), type D in one Polish family(Suchanowska *et al*, 2010) and type E and F in Taiwanese(Chen *et al*, 2014) (Fig 2). Our two new *GYP*401* alleles were genetically different from those described previously, and therefore may be defined as St(a) type G and H. Sequences were deposited under accession numbers LN880514 for *GYP*401* type G, and LN880515 for *GYP*401* type H (Fig 2).

The *GYP*201.01-like*/GP.Hil-like allele showed a complete insertion of the expressed *GYPA* exon 3 at the homologous sequence of *GYPB* (accession number LN880513, Fig 2). In contrast, the originally described *GYP*201.01* was reported to be located on *GYPA* (Fig 3) (Huang & Blumenfeld, 1991b).

GYPA genotypes		MN phenotypes			*GYPB* genotypes		Ss phenotypes		
1st allel	2nd allele	**MM**	**MN**	**NN**	1st allel	2nd allele	**SS**	**Ss**	**ss**
01	***01***	1710	2		***03***	***03***	619	1	
01	***01***		1§						
01	***09***	1							
01	***02***	1	2792	3	***04***	***03***		2415	
01	***GYP*401 type H***		4		***04***	***03(G145A)***		1	
01	***GYP*401 type G***		2		***04***	***06***		1	
01	***GYP*201.01* like**		1						
01	***02***	1§							
01	***11***	2							
02	***14***		10						
01	***09***		4						
01	***GYPB*06***		1						
02	***02***			1200	***04***	***04***		1	2702
02	***09***			4	***04***	***03(N.G218A)***			2
02	***14***			4	***04***	***24***			1
MM, MN and NN samples:		1715	2817	1211	SS, Ss and ss samples:		619	2419	2705
all samples:				5743	all samples:				5743

Fig 1. Comparison of genotypes and phenotypes observed among 5743 Swiss donors. Left and right panel show MN and Ss pheno- and genotyping on *GYPA* and *GYPB*, respectively. The vast majority of samples showed full concordance between phenotype and genotype (no background). Black background indicates samples for which the original phenotype was corrected according to genotyping results ('serological mistypes'). Grey background indicates samples for which the genotyping result needed to be corrected according to the original serology ('genetic mistypes'). Simultaneously, all samples with grey background were sequenced unveiling variant alleles in all cases. *GYPB*06*, *GYP*201* and *GYP*401* are explicitly listed in the left panel to indicate their actual genetic background differing from *GYPA*. § without follow up.

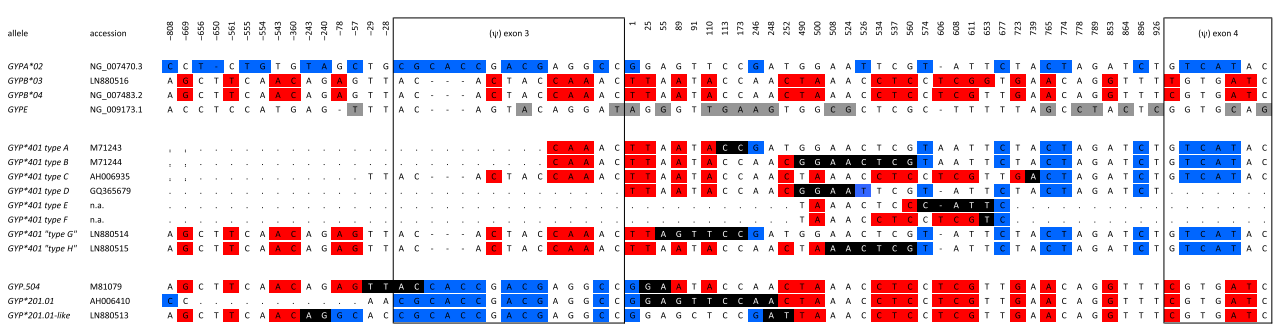

Fig 2. An alignment of informative nucleotide polymorphisms for *GYPA* (blue), *GYPB* (red) and *GYPE* (grey) reference sequences and previously known and new *GYP*401*/St(a) and *GYP*201.01-like* alleles. Reference sequences are shown in the 4 top lines. Exon 3 of *GYPA* and pseudo-exons 3 (ψ) of *GYPB* and *GYPE* (boxed) adjacent intronic sequences, and exon 4 of *GYPA* and *GYPB* and pseudo-exon 4 (ψ) of *GYPE* (boxed) are given. Breakpoint regions between *GYPB* and *GYPA* are indicated by white letters on black background. Deleted nucleotides are given as '-', nucleotides not given are represented by a dot. Centred horizontally, previously published *GYP*401*/St(a) types A to F and *GYP*401*/St(a) types G and H identified in this study are given. For the new *GYP*401*/St(a) alleles, breakpoint region between *GYPB* and *GYPA* could be located between T of i3 + 25 and G of i3 + 246 (type E), and A of i3 + 500 and T of i3 + 574 (type F). All *GYP*401* breakpoint regions are unique. The two sequences displayed at the bottom are the previously published *GYP*201.01*/GP.Hil and the new *GYP*201.01 -like*/GP.Hil-like sequence, observed during this study (Huang *et al*, 1992). Allele *GYP*201.01*-like showed a complete insertion of the expressed *GYPA* exon 3 between *GYPB* specific C of i2-360 and T of i3 + 490. Whereas the two alleles exhibit a completely different molecular background (Fig 3), predicted primary structures only differ by a glutamic acid at position 13 found in *GYP*201.01*, *versus* alanine at the same position in *GYP*201.01-like*. Ignoring the reference sequence of *GYPE*, exact breakpoint regions could be narrowed down further.

However, predicting both their primary structures, these two forms only differ by one single amino acid exchange, namely a glutamic acid at position 13 specific for *GYP*201.01*, to alanine at the same position in our *GYP*201.01-like* allele. Serologically, antigenic expression of our *GYP*201.01-like* allele was indistinguishable from the originally reported

*GYP*201.01* and reactions were positive with anti-Hil and anti-Miny, but negative with anti-Mut, anti-Mur and anti-Vw (http://www.isbtweb.org/working-parties/red-cell-immunogenetics-and-blood-group-terminology/).

Frequencies for all common and variant *GYPA* and *GYPB* alleles were calculated and adjusted for serologically hidden and genetically undetected *GYP*201.01*-like and *GYP*401* alleles (Table SII).

Common and variant GYPB *alleles among Swiss*

Genotyping was fully congruent with phenotyping in 619 SS, 2416 Ss and 2702 ss samples, resulting in a concordance-rate of 99·90% (5737/5743). Three discrepancies were due to serological mistypings with subsequent corrections of two Ss to one SS and one ss phenotype (Fig 1). Sequencing of the third serological mistyping with a reported ss phenotype delivered a *GYPB*24* allele (deposited under accession number LN880518) and was serologically positive for a weakly expressed S and 'Mitchell', in accordance with previous reports on *GYPB*24* (Storry *et al*, 2001).

Of the remaining three discrepancies, one sample showed a *GYPB*04* genotype, but had an Ss phenotype. Sequencing revealed a variant *GYPB*03* with a new G145A(Gly49Arg) mutation (accession number LN880519), exactly located in an elongation-primer binding-site of the MALDI-TOF MS MNSs module.

Two other samples showed the same new *GYPB*03* null-allele with a G218A(Gly73Asp) substitution in two S-negative phenotypes (accession number LN880517). One Swiss donor had an apparent MNSs phenotype and showed simultaneous genetic positivity for *GYPA*01*(C59), negativity for *GYPA*02*(T59) and positivity for G59, all together indicating the presence of an allele termed *GYPB*06*, known to express the N-like antigen Henshaw (He) but actually located on *GYPB* (Huang *et al*, 1994). Linkage of G59 to T147(*MNS*03*) could be proven and narrowed the allele designation to *GYPB*06.02*. Frequencies of all *GYPB* alleles were calculated and adjusted for serologically hidden and genetically undetected *GYPB*03 (N.G218A)*, *GYPB*03(G145A)* and *GYPB*24* given in Table SIII.

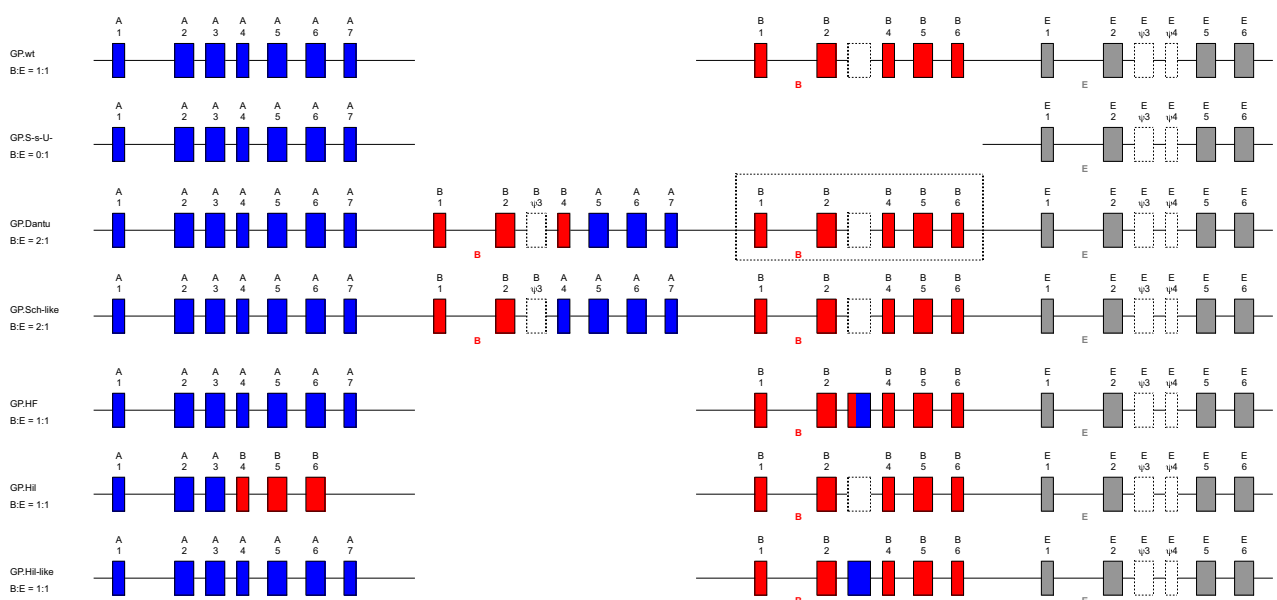

Fig 3. Schematic presentation of genes for *GYPA*, *GYPB* and *GYPE* in different *GYP* haplotypes. A schematic presentation of genes for *GYPA*, *GYPB* and *GYPE* are given in blue, red and grey, respectively. Boxes represent exons. 'ψ' represents pseudo-exons of *GYPB* and *GYPE* with sequence homology to the respective parts of *GYPA*. Correct splicing of dashed ψ -exons into mature mRNA of the respective genes is avoided. GP.wt represents a wild-type *GYP* locus. GP.S-s-U- stands for a hypothetical variation from a previous description with an almost complete deletion of *GYPB*, causative of a S-s-U- phenotype in homozygous status (Rahuel *et al*, 1991). GP.Dantu, encoded by the allele *GYP.402*, is one representative of a haplotype with a duplicated *GYPB-A* hybrid gene, resulting in four *GYP* genes per *GYP*-locus. Appearance and composition of *GYPB* may vary in GP.Dantu haplotypes and is therefore given in a dashed box. GP.Sch, is one representative with positivity for the low frequency antigen St(a), encoded by the allele *GYP*401*. Several *GYP*401* alleles are known and are commonly found on *GYP* haplotypes including *GYPB-A* hybrid gene duplications. In this study two new *GYP*401* alleles, termed type G and H were described, adding to the genetic complexity of previously known *GYP*401* alleles type A to F (Daniels, 2013). GP.HF is encoded by a *GYPB-A-B* hybrid allele, positive for the Mur antigen. GP.Hil is a *GYPA-B* hybrid gene, encoded by *GYP*201.01*. GP.Hil-like, another new observation of this study, is a *GYPB-A-B* hybrid gene, thus with a completely different molecular background to *GYP*201.01* (Huang *et al*, 1992; Daniels, 2013). 'B' and 'E' indicate position 16 kbp of intron 1 used for *GYPB:GYPE* gene copy number ratio detection. Ideally, expected *GYPB:GYPE* ratio values are given below each *GYP* haplotype listed. In physical samples and depending on its type, the second parental *GYP* haplotype will affect respective ratio values accordingly. Based on an illustration in Daniels (2013).

GYP *gene copy number measurement*

The area under the curve of peak signals was measured for *GYPA versus GYPB* and *GYPB versus GYPE*, by the detection of gene-specific nucleotides approximately located at positions 9 kbp and 16 kbp in intron 1 of the highly homologous genetic regions, respectively. Whereas the assay at position 9 kbp did not deliver any informative results (data not shown) data retrieved from position 16 kbp allowed for *GYPB versus GYPE* gene copy number estimates (Fig 4A).

The two types of outliers were observed in 0·35% of the samples (20/5743), suggesting a reduced *GYPB* gene content (ratio < 0·65), and in 0·71% of the samples (41/5743), suggesting an increased *GYPB* gene content (ratio > 1·35), respectively. In the 20 individuals with an apparently reduced *GYPB* gene content, observation of only seven Ss heterozygous *versus* 13 (presumably) ss homozygous individuals deviated strongly from an expected Hardy–Weinberg disequilibrium (7/13 = 0,5385 vs. 2415/2702 = 0,8938, counts taken from Fig 1), suggesting (partial) deletion(s) of *GYPB* (**03*) in these cases.

In the arbitrarily defined group of 41 individuals with a supposedly increased *GYPB* gene content, four out of six *GYP*401*/St(a) allele carriers were detected, who had been originally identified due to *GYPA*01* homozygosity, but with a discrepant MN heterozygous phenotype. The suggested presence of a fourth, duplicated *GYPB-A* allele in these cases, is in line with previous reports on *GYP*401*/St(a) positive haplotypes (Fig 2) (Huang & Blumenfeld, 1991a,b; Suchanowska *et al*, 2010; Chen *et al*, 2014).

Among other available samples with the highest *GYPB*:*GYPE* ratios, bi-specific diagnostic PCR-SSPs were used to directly detect additional *GYP*401*/St(a) allele-carriers, with one individual found among 21 NN pheno/genotypes, and other five among 25 MN pheno/genotypes. In total, 12 *GYP*401* allele carriers were identified among 5743 Swiss donors. Nine of them accounted for roughly 22% of all 41

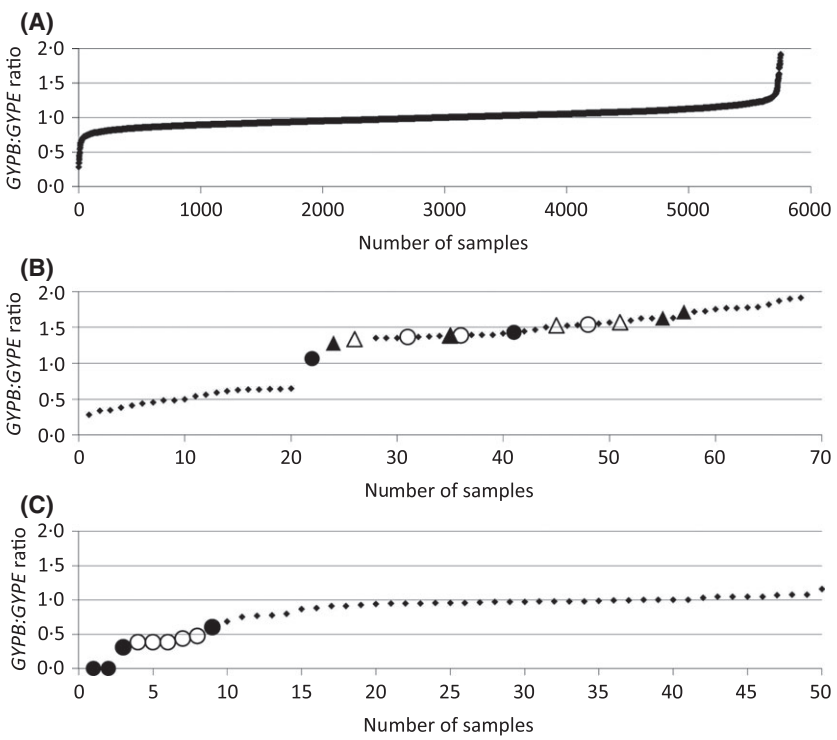

Fig 4. *GYPB*:*GYPE* intron 1, position 16kbp, ratio values. The raw area under the curve of peak signals intensities for *GYPE* were in total 39% higher for all 5743 data points obtained and therefore adjusted to match the theoretically expected *GYPB*:*GYPE* gene copy number ratio of 1:1, as expected for the majority of samples. Ratio is indicated on the *y*-axis, number of samples are given on the *x*-axis. (A) Ratio values for all 5743 Swiss donor samples tested are shown. Ratio values range from 0·28 to 1·92, indicating uneven *GYPB*:*GYPE* gene copy numbers in a minority of samples. (B) Samples with *GYPB:GYPE* ratio values lower than 0·65 (n = 20/5743, 0·35%), and higher than 1·35 are shown (n = 41/5743, 0·71%). In between, three additional samples with ratio values of 1·06 (filled circle), 1·28 (filled triangle) and 1·33 (open triangle) are given. Circles represent samples positive for the allele *GYP*401*/St(a) type G and triangles represent samples positive for the allele *GYP*401*/St(a) type H. Filled symbols represent the first index samples where discrepancy between phenotype MN and genotype MM became apparent (Fig 1, left panel). Open symbols represent additional heterozygous *GYP*401* allele G (circle) and H (triangle) carriers without evident discrepancies between pheno- and genotype. All of them were identified by diagnostic PCR-SSPs among other samples with elevated *GYPB:GYPE* ratios: The most left open triangle was found in an individual with NN phenotype, all other open symbols represent individuals with a MN phenotype. (C) Ratio values for 50 DNA samples of individuals with presumptive Black African ancestry are shown. Previously known U negative and U^{+W} phenotypes with double and haploid *GYPB* deletion are indicated by black circles, respectively. Open circles are samples with *GYPB*:*GYPE* gene ratios in between 0·38 and 0·47, indicative for the deletion *GYPB*, e.g. *GYPB*01N* positivity, on one of the two paternal chromosomes.

individuals with elevated *GYPB* ratios (>1·35, Fig 4B). No attempts were made to test individuals for the presence of additional *GYP*401* allele-carriers among samples with normal *GYPB*:*GYPE* ratios (between 0·65 and 1·35).

Given that elevated *GYPB* gene ratios result from a similar molecular event, as does *GYP*401*/St(a), all samples with elevated *GYPB* gene ratio values were investigated for an assumed presence of *GYP.402*/Dantu (Fig 3) (Huang & Blumenfeld, 1988; Huang *et al*, 1995). No *GYP.402*/Dantu positives were observed. However, these results must be taken cautiously, since no *GYP.402*/Dantu positive samples were available for use as positive controls. Neither potentially reduced *GYPE*, nor actually reduced *GYPB* gene copy numbers were further investigated.

GYP *genotyping of 50 individuals with known or presumptive black African origin*

For a panel of 50 DNA samples with known or presumptive black African ancestry, *GYPB versus GYPE* gene copy number variation was estimated as described above. Two previously defined S-s-U- phenotype samples showed a complete absence of *GYPB* at position 16 kbp in intron 1 (ratio 0·00). Unexpectedly, one of these had a heterozygous 143C/T SNP located in *GYPB* exon 3, usually indicative of an Ss phenotype. This observation could suggest the presence of at least three different haplotypes in these two samples, with either complete or partial *GYPB* deletions, each recessively causative of S-s-U- phenotypes.

Furthermore, two previously defined $U+^{W}$ positive samples were positive for *GYPB*03N.04*, as indicated by the simultaneous presence of G59 and i5 + 5t. The two samples had a *GYPB*:*GYPE* ratio of 0·31 and 0·61, indicating the deletion of *GYPB* on the other paternal haplotype. Two further samples without previous data also showed *GYPB*03N.04* on one paternal haplotype, were both heterozygous for 143C/T and had divergent *GYPB*:*GYPE* ratios of 0·48 and 0·88, respectively. In these two cases, the lower ratio would suggest a heterozygous *GYPB*01N*/*GYPB*03N.04*, the higher a *GYPB*04*/*GYPB*03N.04* genotype, with an expectedly expressed s. However, this assumption could not be proven because no Ss serological determination was available for these samples.

Of the 44 remaining samples, five delivered reduced *GYPB*:*GYPE* ratios between 0·38 and 0·47, indicating the presence of only one *GYPB* gene as detected in intron 1 and a second *GYP* haplotype with a deleted *GYPB* gene termed *GYPB*01N*. All remaining 39 samples showed regular *GYPB*:*GYPE* ratios ranging between 0·68 and 1·16 (Fig 4C).

Linkage analysis

Linkage of *GYPA*09* Vw and *GYPA*14*/Mt(a) positive samples with coding nucleotide 59 on *GYPA*, specific for M or N was directly tested by bi-specific PCR-SSPs. Eight out of nine *GYPA*09* positive samples were in linkage with T59, and one with C59, indicating an 89% (8/9) linkage of Vw with N and only 11% with M (1/9). All *GYPA*14* positive samples showed linkage of coding nucleotide T59 on *GYPA* with T230 of the same allele, indicating an exclusive linkage of Mt (a) with N (data not shown). All these observations were in line with previously published data (Daniels, 2002).

On *GYPB*, all G59 positive samples, including the four black African cases and the single example from the 5743 Swiss cohort, were located on a *GYPB*03* allele (nucleotide T147, exon 3), as tested by bi-specific PCR-SSPs (data not shown). Therefore, expression of He-specific G59 would most likely be expected together with S (nucleotide T147) in most cases. However, the four cases found among black Africans were simultaneously found positive for the i5 + 5t mutation, indicating the presence of *GYPB*03N.04*, with postulated S positivity at a $U+^{W}$ phenotype expression level (Storry *et al*, 2003).

Our *GYP*201.01*-like mutant was linked to *GYPA*01*(M) and had an expressed s, as shown by the sequencing results and deduced from the Ss heterozygous phenotype. The observed haplotype linkages for *GYP*401* alleles were as follows: three of five *GYP*401* type G alleles were unequivocally located on a *GYPA*01*(M)-*GYPB*04*(s) haplotype, whereas two were observed in Ss heterozygous samples and therefore unpredictable; four of seven *GYP*401* type H were found on a *GYPA*01*(M)-*GYPB*04*(s) haplotype, one on *GYPA*01*(M)-*GYPB*03*(S), one on *GYP*02*(N), and the last on *GYPA*01*(M), the latter two have unpredictable further linkage, as both were heterozygous for Ss.

Discussion

General performance of MALDI-TOF MS-based genotyping for MN and Ss detection

The concordance rate of phenotypes and genotypes for MN genotyping on *GYPA* and Ss genotyping on *GYPB* were 99·70% and 99·90%, respectively. Cleared for serological typing errors, our results show a genotyping accuracy of 99·88% (5736/5743) and 99·95% (5740/5743) for MN and Ss, respectively. Looking at the 46344 SNP genotype data sets of this study (8 SNPs per 5743 plus 50 samples), and after exclusion of the calling failures, there was not a single genotyping error due to methodological insufficiency. All observed 'genotyping errors' could be explained by rarely occurring genetic variants of common alleles or by new alleles. Similar to the previously reported module for MALDI-TOF MS-based genotyping of K, Jk, and Fy, throughput capacity and robustness of findings for MNSs genotyping are highly competitive as compared to serotyping and other published high-throughput genotyping methods (Hashmi *et al*, 2005; Montpetit *et al*, 2006; Polin *et al*, 2008; Hopp *et al*, 2010; Jungbauer *et al*, 2012; Haer-Wigman *et al*, 2013; McBean *et al*, 2014; Meyer *et al*, 2014). Consequently, from a

technological point of view, blood donor serotyping for MNSs blood groups can be complemented or, theoretically, even replaced by genotyping.

Improved S-s-U- genotyping employing GYPB *gene copy number estimates*

MALDI-TOF MS has already shown to provide reliable gene number estimates for *RHD* relative to *RHCE* (Gassner *et al*, 2013). In our study, this methodological property of MALDI-TOF MS was applied at the MNSs-locus on chromosome 4q31.21, detecting *GYPB:GYPE* gene copy number ratios for each individual donor. Beside the duplication of *GYP* genes, as known from the *GYP*401* allele family, deletion of *GYPB* in S-s-U-individuals proved to be indicative of the phenotypic variability of antigens of the MNSs blood group system (Fig 4) (Reid, 2009).

In our study, known S-s-U- individuals completely lacked *GYPB*-specific sequences with coinciding *GYPB:GYPE* ratios of 0·0, and samples with known heterozygosity for S-s-U-encoding haplotypes showed reduced *GYPB* contents. As expected, such findings were much more prevalent among Black Africans as compared to the mainly Caucasian Swiss cohort, e.g. 10·87% (5/46) vs. 0·35% (20/5743). Due to migration, some individuals with a deletion of *GYPB* were to be expected among the Swiss donor population, and could offer a plausible explanation for the 20 Swiss individuals with an apparently reduced *GYPB:GYPE* ratio [Federal Department of Justice and Police (FDJP), State Secretariat for Migration (SEM) of the Swiss Confederation, 2015].

However, the molecular cause for S-s-U-, e.g., the precise breakpoint(s) of the *GYPB* deletion(s), is currently unknown and therefore direct positive molecular detection is not possible, though highly desirable. Consequently, all *GYPB* deleted heterozygous and compound heterozygous genotypes remain non-comprehensive in this context and harbour uncertainty in deducing phenotypes from genotyping. Assuming that there are more than one causative haplotype for S-s-U-, e.g., caused by different (partial) deletions of *GYPB*, accurate genotyping of compound heterozygotes would be even more uncertain. In our study, we addressed this issue by estimating *GYPB*:*GYPE* gene copy number and show the relevance of such information in addition to SNP-based typing of MN and Ss.

GYP*401 *type G and H positive haplotypes encode St(a) and N, but genotype negative for* GYP*02

With respect to MN genotyping on *GYPA*, three types of variant alleles became apparent and are of considerable impact: all showed unambiguous expression of N, while being genetically *GYPA*02*(N) negative.

A new *GYPB-A-B* hybrid gene was detected, which appears phenotypically as Hil but has a different molecular background compared to the first reported Hil genotype with a *GYPA-B* hybrid gene, dubbed *GYP*201.01* (Huang & Blumenfeld, 1991b). Additionally, our *GYP*201.01-like* allele shows remarkable similarity to *GYP.504*, one expressing Mur (Fig 3), which is one of the most important RBC phenotypes in Southeast Asia. This allele needs to be considered in Mur genotyping strategies, because carriers of *GYP*201.01-like* would wrongly be detected as Mur positive (Hsu *et al*, 2013).

A far more frequent observation concerned the two newly identified *GYPB-A* hybrid alleles, most frequently found in individuals with elevated *GYPB* gene contents. The observations are in line with previous reports on *GYP* haplotypes encoding the low incidence antigen St(a). St(a) is encoded by *GYPA-B-A*, *GYPA-E-A*, and *GYPB-A-B-A* hybrid genes in a few individuals, but it is usually associated with a duplicated *GYPB-A* molecule located between *GYPA* and GYP*B*, explaining duplicated *GYPB* gene counts when assessed in intron 1 (Figs 3, 4) (Daniels, 2013). St(a) is far more frequent in East Asian populations than in Caucasians [6% in Japan (Madden *et al*, 1964) vs. 0·1% in Europeans (Cleghorn, 1962; Metaxas & Metaxas-Buhler, 1972)], and comprises types A-F encoded by *GYPB-A* (Huang & Blumenfeld, 1991a,b; Suchanowska *et al*, 2010; Chen *et al*, 2014). The St(a) alleles differ in their exact breakpoint positions in intron 3 of the respective *GYPB-A* hybrid genes. We found two new St(a) genotypes, both with new *GYPB-A* intron 3 breakpoints, which point to an antigen frequency of 0·21 (12/5743) in the investigated Swiss-Caucasian population. We propose to term the new *GYP*401* alleles 'type G' and 'type H'.

The relevance of these newly discovered alleles lies not only in their expression of the low incidence antigen St(a) of approximately 0·35% (1/286), but in the simultaneous expression of commonly detectable N despite negativity for the prototypic *GYP*02*(N) SNP. *GYP*01*(M) homozygous individuals may therefore be misinterpreted as M+N- phenotypes if St(a) *GYPB-A* hybrid alleles are present, and this may be expected at a frequency of approximately 0·35% (1/286) among *GYP*01*(M) homozygotes in Switzerland. In contrast to unexpressed, so called 'null-'alleles, which show simultaneous antigen negativity and genetic positivity, these St(a) alleles are an example of the opposite, e.g., presenting antigen positivity with 'genetic negativity'. Therefore, such alleles bear a risk in donor genotyping if exclusively genetic approaches are applied, and thus should be considered in SNP-based *GYPA* and *GYPB* genotyping.

Conclusions

The measurement of the *GYP* gene content by MALDI-TOF MS with estimation of gene ratio, provides a reliable and helpful tool for genotyping the MNS blood group antigens, a system with a complex molecular background and of high clinical relevance, also allowing the detection of variants in a heterozygous state (Daniels, 2002). Measurement of *GYP* gene content, and further extending this potential to two,

three, or even more different genetic locations, would offer an elegant and helpful supplement to the currently existing molecular approaches. Facing the growing ethnic complexity of populations, overcoming the drawbacks of serological typing of MNS blood group antigens would lead to a significant optimization of transfusion strategies, and be most relevant in the clinical management of individual cases.

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Two S-s-U-/*GYPB*01N* homozygous and two U+W/*GYPB*01N/GYPB*03N.04* heterozygous samples, were provided by Marion E. Reid (NY Blood Center, NY, USA) and reanalysed in the course of this study. Serotyping for Vw, Mt (a) and Mg was performed with human antisera provided by Thierry Peyrard [Institut National de la Transfusion Sanguine (INTS), Paris, France] and monoclonal antibodies for antigens Vw, Mur and Mut kindly provided by M. Uchikawa of the Japanese Red Cross, Kanto-Koshinetsu Block Blood Centre, Tokyo, Japan.

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Authorship

S.M, C.V., N.T., S.S., C.P., H.H. and C.G. performed experiments and analysed data. J.G., J.R., A.M., L.I., A. B., SA.D., E.R., D.C., B.W., A.M., A.S., M.B. and J.T. contributed essential material and collected data. S.M., C.V., BM.F. and C.G. discussed the results and commented on the manuscript. S.M., C.V., BM.F. and C.G. designed and supervised the study. C.G. made the figures and wrote the manuscript. All authors edited the manuscript.

Conflict of interest

Christoph Gassner is an employee of the Blood Transfusion Service Zurich, SRC, and acts as a consultant for inno-train GmbH, Kronberg i. T., Germany. Caren Vollmert is employed at Agena Bioscience GmbH, Hamburg, Germany. All other authors do not disclose any competing interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table SI: Primers used.

Table SII: Concordance (CC) and origin of discordance (DC) in *GYPA* genotyping.

Table SIII: Concordance (CC) and origin of discordance (DC) in *GYPB* genotyping.

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