Genetic basis of rare blood group variants

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Chapter 9

General discussion
For a safe transfusion practice it is important to transfuse compatible donor red blood cells to an immunized patient. Blood group antigen status in blood donors and blood recipients is most often determined via serological typing. Nevertheless, serological typing is not always possible, for instance, in patients who recently received a red blood cell transfusion or in patients with auto-antibodies. Furthermore, serological typing can be cumbersome if commercial reagents are not available or when an antigen can have low expression levels. Prediction of blood group antigen expression via genotyping can overcome situations in which serology is impossible or impractical. At the moment some blood centers have implemented genotyping assays to accurately predict the blood group status of blood donors and recipients and blood group genotyping has also been used to screen blood donors for rare blood types in a high-throughput fashion. Nevertheless, most blood group genotyping assays are still used as an add-on tool to supplement serological typing, instead as a stand alone assay used to predict antigen expression.

In this thesis we focused on the identification of the genetic basis of rare blood group variants, characterization of the effect of variant alleles on antigen expression and used this knowledge for the development of genetic assays to predict blood group antigen status. The results of our studies and the feasibility of blood group genotyping in general are discussed below.

For most blood group systems prediction of antigens via genotyping is relatively simple, because only a single nucleotide variation is responsible for most antigenic differences. However, the reality of blood group genotyping is not as straightforward as just described. Almost all blood group systems have next to the alleles coding for the antithetic antigens so called variant alleles. These variant alleles have next to the antigenic variation (an) additional mutation(s) that can cause weakened expression of the antigen, can alter antigen expression or can cause the complete absence of the antigen. It is clinically relevant to detect variant alleles, because recipients who have altered antigen expression or lack the complete antigen are at risk of immunization when they come in contact with antigen positive red blood cells. Furthermore, red blood cells from individuals with weakened or altered antigen expression, can immunize individuals who are negative for the antigen. Hence, blood group genotyping assays should also target variant alleles, next to the wild-type alleles that code for the antithetic antigens.

A general advantage of genotyping tests above standard serology is that in one single test multiple targets (antigens) can be detected. However, most available blood group genotyping assays only target a small number of blood group systems. These methods can not cope with the extensive multiplexing that is needed to target all clinically relevant blood group systems. Another disadvantage is that these methods have high start-up and reagents costs.
In chapter 2 we developed and validated a novel genotyping assay based on the Multiplex Ligation-dependent probe amplification [MLPA] technique, the blood-MLPA assay can predict the presence or absence of 48 blood group alleles and 112 variant alleles of the MNS, Rh, Lutheran, Kell, Lewis, Duffy, Kidd, Diego, Yt, Scianna, Dombrock, Colton, Landsteiner-Wiener, Gerbich, Cromer, Knops, Indian and Ok blood group systems. We determined that the blood-MLPA can reliably predict the presence and absence of virtually all clinically relevant blood group antigens, except the ABO antigens. The phenotype prediction of the blood-MLPA is at least as accurate as the determination of blood group antigens by serological typing. Moreover, the blood-MLPA assay has not yet reached its maximum typing capacity and it is still possible to add 29 new "targets", for instance mutations that cause the Jr(a-), Lan- or Vel- phenotypes, of which the genetic basis was detected after we validated the blood-MLPA assay. Because only standard laboratory equipment is needed to perform the assay, the blood-MLPA makes genotyping of blood donors and recipients more feasible.

The main advantage of the blood-MLPA and of blood group genotyping in general over serological typing is the direct recognition of variant alleles that cause weakened or altered antigen expression. These variant alleles have been described for the ABO, MNS, Rh, Lutheran, Kell, Duffy, Kidd, Dombrock, Colton, Landsteiner-Wiener, I, RHAG, JR, LAN and VEL blood group systems. Standard serology has insufficient sensitivity to detect blood donors with very weak antigen expression. Consequently, donors with very weak antigen expression are incorrectly determined antigen negative, while their red blood cells are able to induce immunization and/or a hemolytic transfusion reaction in a recipient that is negative for the antigen. The D antigen is notorious for the frequent occurrence of variant expression and more than 200 RHD variant alleles have been described. Serology can easily determine normal D+ and D- antigen expression, however, only by extensive serology, sometimes requiring rare patient sera, specific D variants can be recognized. Nowadays blood centers have implemented genetic assays as an additional tool to specify the RHD variant alleles. In chapter 3 we determine that a subset of the blood-MLPA assay, the RH-MLPA that entirely focuses on the detection of RHD and RHCE variant alleles, can reliably determine the majority of RHD variant alleles, while serology could not determined the specific variant. Correct determination of RHD variant alleles is important, because the presence of an RHD variant allele in an individual influences blood transfusion practices. The best way to define RHD variant alleles to efficiently facilitate blood transfusion practices is still being debated. Classically, variant RhD expression is divided into weak D expression and partial D expression, in which weak D individuals are assumed to be incapable of D-immunization, because they express all D epitopes. While partial D individuals are prone to be immunized to the D antigen, because they lack one or more D-epitopes. The partial D and weak D terms are however misleading for transfusion practices. Carriers of some weak D variants can become immunized to the D antigen. On the other hand for some partial D an immunization event to D antigen has never been described, for instance
for the \textit{RHD*05.05}, \textit{RHD*16} and \textit{RHD*18} variant alleles\(^{11}\). Recently, Daniels proposed to replace the terms weak D and partial D with a single collective term: D variant.\(^{12}\) In donor typing the collective term D variant can be implemented, because it is irrelevant whether a blood donor carries a weak D or partial D variant; both are able to immunize a D- recipient. However, I believe that in blood recipient typing the collective term D variant is impractical, because it is of clinical relevance (carriers of many \textit{RHD} variant alleles can become immunized to the D antigen) and of practical relevance (to minimize the unnecessary use of relatively scarce D- red blood cells) to distinguish between D variants. To keep the amount of D immunization in blood recipients with an RhD variant as low as possible and the use of D- red blood cells acceptable, blood recipients with the frequently occurring weak D type 1, 2 and 3 variants (as proposed by Daniels) and also blood recipients with the weak D type 5 variant that all have no to a very low risk of immunization to the D antigen, should receive D+ red blood cells. While individuals with all other D variants should receive D- blood. To be able to follow these criteria RhD typing should be supplemented with a genotyping assay, because serology can not recognize the weak D type 1, 2, 3 or 5 variants. Before we can discuss whether D antigen prediction via genotyping can even completely replace serological D typing, the main drawback of genotyping assays must be discussed.

The main drawback of genotyping assays is the presence of null alleles. Null alleles are variant alleles that have mutations which cause the complete lack of antigen expression. If not tested for these mutations, blood recipients carrying a null allele might be incorrectly typed as antigen positive. In most blood group systems the frequency of null alleles is very low, for instance for the Lutheran, Kidd, Diego, Dombrock, Colton, Cromer and Vel blood group systems. Furthermore, for most blood group systems only a small number of null alleles have been described that can easily be targeted in a genotyping assay, for instance in the MNS, Duffy, Diego, Dombrock, Cromer, RHAG and Vel blood group systems. The D antigen is one of the few antigens in which null alleles frequently occur. Especially in African Blacks the frequency of D- null alleles is high, in 81% of D-negative individuals the \textit{RHD*Ψ} or \textit{RHD*03N.01} null allele is present.\(^{13}\) Moreover, because these null alleles are most often next to a “deleted” allele, carriers of D- null alleles are at high risk of immunization. In several studies among D- Caucasian blood donors over 50 different D- null alleles have been described.\(^{14}\) The blood-MLPA targets twelve of the frequently occurring D- null alleles, including the \textit{RHD*Ψ} and \textit{RHD*03N.01} alleles. As was demonstrated in chapter 3, we failed to detect only one D- null variant allele, the novel \textit{RHD*443G} allele, in a selected set of DNA samples. To determine the true false positive rate of the blood-MLPA in Dutch D- individuals, an assessment of a cohort of extensively typed D- individuals needs to be performed. Moreover, the analysis of the missed D- null alleles might help us to improve the blood-MLPA. Yearly ~25.000 D- Dutch pregnant women are tested using a quantitative fetal \textit{RHD} genotyping assay. This gave us the opportunity to investigate the presence of \textit{RHD} variant alleles in a
cohort of 37,764 D- pregnant woman. In this quantitative fetal \textit{RHD} genotyping assay all variants carrying \textit{RHD} exon 5 and/or exon 7 will be detected, which is the far majority of the variant alleles and only \textit{RHD}*14.01, \textit{RHD}*14.02 and \textit{RHD}*03N.01 are missed. In chapter 4 we show that in 0.96% (95% CI 0.86% - 1.05%) of the Dutch D- pregnant women an \textit{RHD} variant allele is present, which disturbs the quantitative fetal \textit{RHD} genotyping assay. In 53% of the women with a variant allele a D- null allele is present. In 84% of the women with a D- null allele the \textit{RHD}*Ψ allele was present, in 4% of the women one of five different D- null alleles, that were detected by the blood-MLPA, was present. In the remaining 12%, whom carried five known and eight novel D- null alleles, the blood-MLPA gave normal wild type \textit{RHD} results. Hence, to increase the specificity of the blood-MLPA for the Dutch population, the assay should be extended with probes targeting the known \textit{RHD}*922C>T and novel \textit{RHD}*1074-1G>A D- null alleles, that both were detected in three cases of this study. It is impractical to extend the blood-MLPA to target the other eleven D- null alleles that were present in this study, but for which the MLPA has no detecting probes. These alleles are very rare, they were only detected in one or two individuals of this study, respectively. The variant alleles that were not detected by the blood-MLPA assay were all, except one allele, linked with the \textit{RHCE}*02 (RhCe; n = 9) or \textit{RHCE}*03 allele (RhcE; n = 3). A total of 45 different \textit{RHD} variant alleles, including 14 novel variant alleles, were detected and the variant alleles caused in 53% of the woman the D- phenotype, in 32% a partial D phenotype and in 15% a weak D phenotype. Taken the complete cohort into account, we conclude that in 0.26% (CI 0.21% - 0.31%) of the pregnant women the blood-MLPA demonstrated that serology was incorrect, these cases were actually positive for a variant allele that causes weak D expression, while 0.05% (95% CI 0.03% - 0.08%) of the D- women would be incorrectly determined D+ when the phenotype was determined solely on the blood-MLPA assay. Indicating that the blood-MLPA assay can more accurately determine the D- phenotype compared to serological typing.

Three large studies, performed in Germany\textsuperscript{15}, Poland\textsuperscript{16} and Austria\textsuperscript{17}, on the presence of \textit{RHD} variant alleles in D- donors have been performed. These studies detected a lower percentage of \textit{RHD} variant alleles, 0.6%, 0.2% and 0.4%, respectively. However, these studies were performed on serologically typed D- donors. Serological donor typing has been developed to determine donors who carry the \textit{RHD}*06 allele as D+, in contrast to serological typing of blood recipients and pregnant women, who are determined D-. Hence, these studies did not detect individuals carrying the \textit{RHD}*06 allele. Furthermore, all three studies deliberately did not detect persons positive for the \textit{RHD}*Ψ variant allele. If the frequency of variant alleles in our study is recalculated and the individuals positive for the \textit{RHD}*Ψ or the \textit{RHD}*06 allele are removed, the variant allele frequency of 0.33% (95% CI 0.28% - 0.39%) fits exactly in the frequency determined in the three other studies. In the German, Polish and Austrian studies, the blood-MLPA would not have recognized the presence of a D- null allele in 0.02%, 0.02%, and 0.004% of the donors, respectively. This lower percentage of undetected D- null alleles might be due
to the more variable ethnic background of our Dutch pregnant women, compared to the Caucasian background of the tested blood donors. In agreement with the results of chapter 3, we again demonstrated the superiority of the blood-MLPA over serological typing of blood donors with weak D expression; in 0.07%, 0.09% or 0.21% of the individuals, respectively, the false negative serological results would have been corrected by the blood-MLPA. We can therefore conclude that, at least in West and Central Europe, genotyping of the D- phenotype of blood donors via the blood-MLPA assay is more accurate compared to serological typing. Furthermore, due to the very low false-positive rate of the blood-MLPA, this assay can also be used to type blood recipients or pregnant women for the D phenotype.

Genotyping of the Vel blood group has been a long desire, because serological typing of the Vel antigen is very cumbersome, due the variable antigen expression and due to the lack of commercially available reagents. Genotyping was, however, not possible, since the genetic basis of the Vel antigen was not yet determined. The discovery of the genetic basis of the Vel blood group antigen is described in chapter 5. The Vel blood group antigen is encoded by the SMIM1 gene and the Vel- phenotype is caused by the homozygous presence of a 17-nucleotide frame shift deletion that most likely causes the complete absence of the SMIM1 protein. This is supported by the fact that in an individual heterozygous for the SMIM1*64-80del allele wild-type SMIM1 mRNA is detected, while mRNA with the 17-nucleotide deletion was absent. In chapter 6 we determine that weakened Vel expression is caused by the major allele of rs1175550 small nuclear polymorphism in intron 2 of SMIM1 in combination with a SMIM1*64-80del, SMIM1*152T>A or SMIM1*152T>G allele. Nevertheless, not in all cases with weak Vel expression levels genetic variation in SMIM1 could be hold responsible for the weakened expression and we conclude that genetic factors outside the SMIM1 gene or environmental factors can also be responsible for weak Vel expression. To overcome the difficult serological typing of the Vel antigen we developed and validated a high-throughput genotyping assay to detect blood donors with the Vel- phenotype. In 3,366 screened Caucasian donors we were able to detect two new Vel- blood donors.

In the previous paragraphs we have showed that for most blood group systems genotyping can accurately predict antigen expression. The most clinical relevant blood group system, the ABO system, has, however, not yet been mentioned. The ABO blood group system is actually the most difficult blood group system to genotype. The ABO gene codes for an enzyme that determines the antigen expression, therefore mutations in ABO have an indirect effect on antigen expression. Furthermore, to correctly predict antigen expression of the ABO blood group system it must be decided if mutations that are almost 2 kilo bases apart are present in cis or in trans. The currently available genotyping assays target specific mutations and none can determine whether mutations are in cis or in trans. The correct ABO (variant) allele can
only be determined when genetic data is supplemented with serological information. Also for other blood group system antigen prediction via genotyping is still impossible or impractical. In chapter 7 we show that the phenotypic prediction of the JR and LAN blood group systems by standard genotyping assays is impractical. Due to the large heterogeneity of mutations that cause the Jr(a-+) or Lan phenotype the developed genotyping assays were not efficient in a Caucasian population. Until suitable reagents are broadly available, serological screening for the Jr(a-) and Lan antigen needs to be combined with genetic confirmation of the Jr(a-) and Lan- phenotype to detect Jr(a-+) and Lan- individuals.

In chapter 8 of this thesis we describe the functional consequences of a rare variation in a gene encoding the Kidd blood group antigen. The genotyping assays we developed to predict the blood group phenotype, were shown to be equally useful to explain a functional phenotype. A duplication of the SLC14A1 gene encoding the Kidd blood group antigen was detected in a family with the rare dominant familial azotemia, of which affected individuals have increased urea concentrations in the blood and decreased urea clearance, while other renal tubular functions are normal. The protein, the UT-B urea transporter, on which the antigens of the Kidd blood group are present, is expressed on red blood cells and also on kidney cells. No serological difference for the Kidd blood group antigens were detected in the affected family members, however, a significantly increased Kidd antigen expression on red blood cells and also a significantly increased UT-B expression in the kidney were detected.

In conclusion, the results presented in this thesis indicate that the prediction of most blood group antigens, including the D antigen, via genotyping is accurate and reliable. Presently available genotyping assay, such as the blood-MLPA, can be used to replace serology for blood group typing of blood recipients, blood donors and pregnant women, when serology is not possible. Genotyping has proven its value in the prediction of antigen expression in blood donors, because unlike genotyping, serology is unable to detect and therefore correctly type very weak expression of an antigen. Furthermore, current genotyping techniques have enough capacity to target the most frequently occurring null alleles in a single assay and can achieve a very low rate of false-positive antigen detection in blood recipients, as we show for D antigen prediction using the blood-MLPA assay.

The continuous improvement of genotyping techniques will ensure that the prediction of blood group antigen expression will become even more accurate and more complete than at present time. For instance, blood group genotyping via next generation sequencing can be used to predict the JR and LAN blood group system, with a large heterogeneity of causative mutations. Because next generation sequencing does not target specific mutations, but can theoretically target entire blood group genes. The first next generation blood group genotyping assay has been developed, however, first test showed that improvement of the
assay is needed. Also for the ABO blood group system next generation might be able to correctly predict antigen expression, when the read lengths become long enough to cover the almost 2 kilo bases to determine whether mutations are in cis or in trans. A fourth generation sequencer in which a nanopore is used to “read” a DNA strand, could easily cover the 2 kilo bases. But despite the very positive views from the companies that develop this technique, nanopore next generation sequencing is still in a developmental stage.

When genetic typing of any antigen will become the golden standard over serological typing, the implementation of genotyping for other blood group antigens will gain momentum, because genotyping assays become relatively cheaper in comparison with serology when more antigens are tested. However, before genotyping assays can replace serology, large population studies need to confirm that the frequency of incorrect antigen prediction, due to variants that are not detected by a genotyping assay, is at a minimum and most importantly lower than the error rate seen with serological typing.

When blood group genotyping assays are implemented in blood centers, the greatest benefit for transfusion practices will arise from comprehensive matching between blood donors and recipients. In the Netherlands, as in most countries, only ABO and D matched red blood cells are transfused in recipients. With the exception of women under 45 and patients with thalassaemia and sickle cell disease, who receive more extensively matched blood, as well as all patients after development of a clinically relevant alloantibody. When genotyping is implemented, all blood donors and recipients will be typed for a large set of blood group antigens in a high-throughput fashion and more comprehensive preventive matching will be possible. Routine preventive matching of for instance the clinically relevant Rh, K, Fy, Jk and Ss antigens, will prevent the occurrence of most immunization reactions and as a result the occurrence of severe immediate or delayed hemolytic transfusion reaction.
References