Towards HLA epitope matching in clinical transplantation

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TOWARDS THE DEFINITION OF THE MOST IMMUNOGENIC HLA CLASS II AMINO ACID MISMATCHES USING HLA-EMMA

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ABSTRACT

In kidney transplantation, polymorphic amino acid configurations on mismatched donor HLA molecules can lead to the development of *de novo* donor-specific antibodies (*dn*DSA). These *dn*DSA are mainly directed against HLA class II, and especially HLA-DQ, and are associated with graft loss. Defining which polymorphic residues are able to induce an antibody response and which not, is pivotal for the development of strategies to prevent *dn*DSA formation. Therefore, the aim of this study was to define the most immunogenic HLA-DQ polymorphic amino acid mismatches in a cohort of kidney transplant recipients.

From multiple Dutch transplant centres we selected non-immunised male recipients that received their first kidney transplant with at least one HLA class II antigen mismatch and subsequently lost their graft due to immunological failure (n=79). Donor and recipient HLA typing was performed at high resolution using next generation sequencing (NGS) and the number of solvent accessible amino acid mismatches was established with HLA-EMMA. Formation of *dn*DSA was determined by screening recipients' sera collected upon graft failure with Luminex single antigen bead assays.

HLA-DQ-specific *dn*DSA were most prevalent, occurring in 35% of the 79 recipients. Increasing numbers of solvent accessible amino acid mismatches resulted in a higher frequency of HLA-DQB1 or HLA-DQA1 *dn*DSA. However, we also observed that a single solvent accessible amino acid mismatch on HLA-DQB1 or HLA-DQA1 was sufficient for the induction of *dn*DSA. In this pilot study with a limited number of cases, it was not yet possible to identify specific amino acid positions or types, which were significantly more immunogenic than others.

Overall, we showed that within this cohort the chance of HLA-DQ-specific *dn*DSA increased with more solvent accessible amino acid mismatches, although on individual level a single amino acid mismatch can be sufficient to trigger an antibody response. This latter observation supports the importance to define the most immunogenic residues, which requires a much larger and diverse cohort.

INTRODUCTION

Human leukocyte antigen (HLA) matching can prevent the occurrence of an alloimmune response. However, most grafts are transplanted with one or more HLA antigen mismatches, which can result in immunisation of the recipient as reflected by the formation of donor specific HLA antibodies. These *de novo* donor-specific antibodies (*dn*DSA) are strongly associated with graft loss,[1-3] and reduce the chance of repeat transplantation.[4] Therefore, it is of utmost importance to prevent the development of *dn*DSA after transplantation.

Induction of *dn*DSA can be triggered by polymorphic amino acid configurations on mismatched HLA antigens and are mainly directed against HLA class II, and more specifically HLA-DQ.[1,2,5] There are several ways of defining and analysing these polymorphic residues. One of these is used by the HLAMatchmaker algorithm, which defines patches of (dis)continuous polymorphic amino acids, called eplets, that theoretically can induce an antibody response.[6,7] Indeed the number of eplet mismatches between donor and recipient have been associated with *dn*DSA formation [8-11] as well as transplant glomerulopathy.[12] In addition, the immunogenicity of HLA mismatches on the population level have also been assessed based on amino acid mismatches and/or physiochemical disparity scores.[13,14] While these approaches provide mismatch scores that are good predictors of sensitisation risk on population level, it remains to be determined which mismatched amino acids triggered the formation of *dn*DSA in an individual patient.

It has been shown that not every eplet mismatch is equally immunogenic[15] and *dn*DSA are observed even when only a low number of triplet, predecessor of eplet, mismatches are present on the mismatched donor HLA.[16] In addition, as mentioned, eplets are theoretically defined and require experimental verification to determine if an antibody can actually bind to these polymorphic residues.[17,18] While data of a recent study showed that both HLA class I and class II antibody-verified eplets were good indicators for risk of graft loss for individual patients, especially for HLA class II additional verification of eplets is required to further improve this risk stratification.[11] Thus, the current list of potential immunogenic polymorphic amino acid configurations is still incomplete.

Recently, we developed a software program, HLA-EMMA, to analyse the compatibility between donor and recipient HLA class I and class II molecules on the amino acid level.[19] The benefits of HLA-EMMA are that large cohorts of donor-recipient pairs from diverse populations can be analysed for HLA class I and class II simultaneously and, in addition, it provides the type and position of the amino acid mismatches. Using this software program, we aim to define

the most immunogenic polymorphic amino acids that ultimately form the basis of specific polymorphic amino acid configurations involved in antibody binding.

As mentioned, the most frequently observed *dn*DSA after transplantation are directed against mismatched HLA-DQ. To prevent HLA-DQ-specific *dn*DSA formation it is essential to determine the immunogenic polymorphic amino acids on the HLA-DQB1 and HLA-DQA1 molecules. To this aim, we performed a pilot study using a kidney transplant cohort study of non-immunised male transplant recipients with at least one HLA class II antigen mismatch, who lost their graft due to immunological failure.

MATERIALS AND METHODS

Study population

From multiple Dutch transplant centres, we selected non-immunised male recipients that received their first renal transplant with at least one HLA class II antigen mismatch, and who subsequently lost their graft due to immunological failure. From Leiden and Rotterdam transplant centre we selected recipients that underwent their first kidney transplantation between 1992 and 2015 (n=40) (Figure 1). Subsequently, the cohort was extended with patients from the PROCARE consortium (PROfiling Consortium on Antibody Repertoire and Effector functions) database with recipients that received their first kidney transplantation between 1995 and 2005 (n=129).

HLA typing

DNA samples were collected from recipients and donors for HLA typing by next-generation sequencing (NGS) of HLA-A, -B, -C, -DRB1, -DRB3/4/5, -DQB1, -DQA1, -DPB1, and -DPA1 loci. NGSgo-AmpX kit (GenDx, Utrecht, the Netherlands) was used for the amplification of HLA genes. Next, library and sequence preparation were performed with NGSgo-LibrX/IndX kit (GenDX) and subsequently sequencing was carried on Illumina MiSeq or MiniSeq (Illumina, San Diego, CA, USA). NGS data was analysed with NGSengine software (GenDx).

Ambiguities at the second field level were still observed for several HLA alleles (n=6 DRB1, n=3 DQB1, n=17 DPB1) after NGS typing. Therefore, we selected the first allele of the ambiguity group.

Definition of donor-specific antibodies

Recipients' sera before transplantation and after graft failure were collected for screening for the presence of HLA antibodies with Lifecodes Lifescreen Deluxe screening kit (LMX,

Immucor Transplant Diagnostics, Stamford, CT, USA). Subsequently, positive serum samples were treated with ethylenediaminetetraacetic acid (6% EDTA) and tested with Lifecodes HLA class I and class II single antigen beads (SAB) (Immucor Transplant Diagnostics). Data was analysed with Match It! Antibody software version 1.3.0 (Immucor Transplant Diagnostics). The screening data were analysed using raw mean fluorescence intensity (MFI) and for the SAB data background corrected MFI (BCM) as provided by software were used. For defining DSA from the SAB data of each individual both the software positive assignments and the BCM of recipient' alleles were considered. For HLA-DRB1, HLA-DRB3, HLA-DRB4, and HLA-DRB5 donor alleles with BCM > 1000 were defined as DSA. For HLA-DQB1 and HLA-DQA1 DSA was assigned if the actual mismatched HLA-DQB or HLA-DQA donor allele present on a bead in combination with respectively the self HLA-DQA or HLA-DQB allele of the recipient was positive, BCM > 1000. If the mismatched allele was not present on a bead in combination with a recipient allele, then DSA was assigned if all beads with mismatched allele were positive and the reactivity was not caused by the other allele on the bead. In case donor HLA allele was not present in SAB assay, no *dn*DSA could be defined (n=3 DRB1, n=1 DRB4, n=1 DQB1, n=1 DQA1) and these cases were excluded from analysis.

Solvent accessible amino acid mismatches

HLA-EMMA software program version 1.0 was used to define the solvent accessible amino acid mismatches between donor and recipient.[19] With this software program the amino acid sequences of the donor HLA alleles are compared with the recipient' sequences, interlocus for HLA-DRB1, HLA-DRB3, HLA-DRB4, and HLA-DRB5, and intralocus for HLA-DQB1 and HLA-DQA1. HLA-EMMA identified and quantified the position and type of solvent accessible amino acid mismatches of each donor allele.

Visualising solvent accessible amino acid positions

HLA-DQB1*03:02-DQA1*03:02 crystal structure PDB 1JK8 (downloaded from https://www.rcsb.org/ on January 16, 2020) was used to visualise solvent accessible amino acid positions with Swissviewer.[20]

Statistical analysis

SPSS statistics version 25 (IBM, Armonk, NY, USA) was used to perform statistical analysis. Binary logistic regression was used to assess the significance of antigen, allele, or solvent accessible amino acid mismatches as predictors of *dn*DSA formation. P values of <0.05 were considered statistically significant.

Table 1: *dn*DSA formation in study cohort

*dn*DSA	N (missing*)	% of 79
HLA class I	13 (2)	16.5
HLA-DRB1	10 (4)	12.7
HLA-DRB3/4/5	8 (8)	10.1
HLA-DQB1	26 (1)	32.9
HLA-DQA1	22 (1)	27.8

*HLA alleles that are not present in single antigen bead assay

RESULTS

Study cohort

The selection criteria of the study cohort were non-immunised first kidney transplant male recipients with at least one HLA class II antigen mismatch with graft failure due to immunological failure. This resulted in a study cohort consisting of 79 donor-recipient couples for analysis (Figure 1).

Overall within this cohort, 39% of recipients developed HLA class II *dn*DSA (n=31) of which 45% recipients (n=14) also had HLA class I *dn*DSA (Table 1). HLA-DQB1 and/or HLA-DQA1-specific *dn*DSA were most prevalent in the recipients (35% n=28) and 11 of those recipients also developed HLA-DRB1/3/4/5 specific *dn*DSA, while HLA-DRB1/3/4/5 *dn*DSA were only observed in 3 recipients.

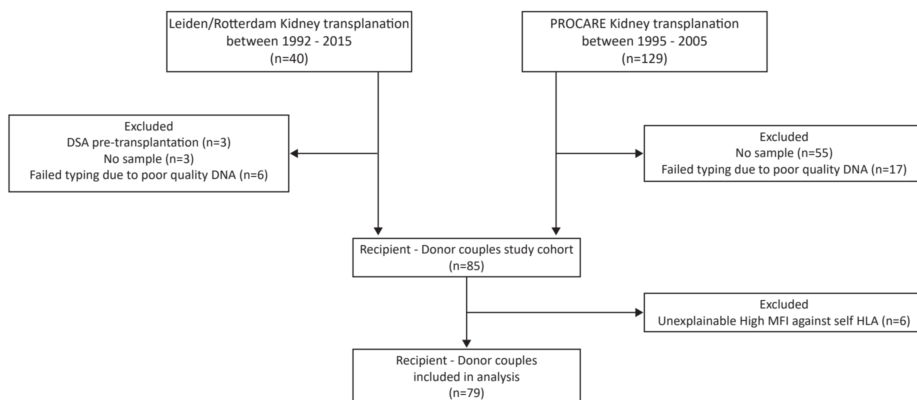


Figure 1: Flow chart of inclusions and exclusions for the study cohort.

Solvent accessible amino acid mismatches are associated with dnDSA formation

Per individual we determined if a HLA-DQB1 and/or HLA-DQA1 *dn*DSA was formed and observed that for HLA-DQB1 the chance of antibody response was higher for two HLA-DQB1 antigen mismatches, based on serological split typing, compared to one antigen mismatch although this was not significant (Table 2). Interestingly, the chance of *dn*DSA formation was lower for 2 HLA-DQB1 or HLA-DQA1 allele mismatches compared to 1 allele mismatch (Tables 2 and 3), although this was only significant for HLA-DQB1. However, when defining the sum solvent accessible amino acid mismatches for HLA-DQB1 and HLA-DQA1 per individual we found that the chance of *dn*DSA formation was higher for solvent accessible amino acid mismatches above the mean, 12 for HLA-DQB1 and 10 for HLA-DQA1. This was further supported when comparing the number of HLA-DQB1 and HLA-DQA1 solvent accessible amino acid mismatches of one allele mismatch with two allele mismatches (Figure 2). For many double allele mismatches the sum of solvent accessible amino acid mismatches was very low and often lower than the number of solvent accessible amino acid mismatches of several single allele mismatches.

Table 2: HLA-DQB1 mismatches predicting *dn*DSA using logistic regression analysis

	OR (95% C.I.)	p value	No DSA	DSA
DQB1 antigen 1 mm (ref)			33	23
DQB1 antigen 2 mm	2.323 (0.575-9.381)	0.237	10	3
DQB1 allele 1 mm (ref)			30	22
DQB1 allele 2 mm	0.260 (0.078-0.865)	0.028	21	4
DQB1 SA AA mm <=12 (ref)			35	11
DQB1 SA AA mm >12*	2.983 (1.123-7.927)	0.028	16	15

OR = Odds Ratio, C.I. = Confidence Interval

* grouped based on mean

Table 3: HLA-DQA1 mismatches predicting *dn*DSA using logistic regression analysis

	OR (95% C.I.)	p value	No DSA	DSA
DQA1 allele 1 mm (ref)			37	18
DQA1 allele 2 mm	0.514 (0.150-1.762)	0.290	16	4
DQA1 SA AA mm <=10 (ref)			39	8
DQA1 SA AA mm >10*	4.875 (1.686-14.097)	0.003	14	14

OR = Odds Ratio, C.I. = Confidence Interval

* grouped based on mean

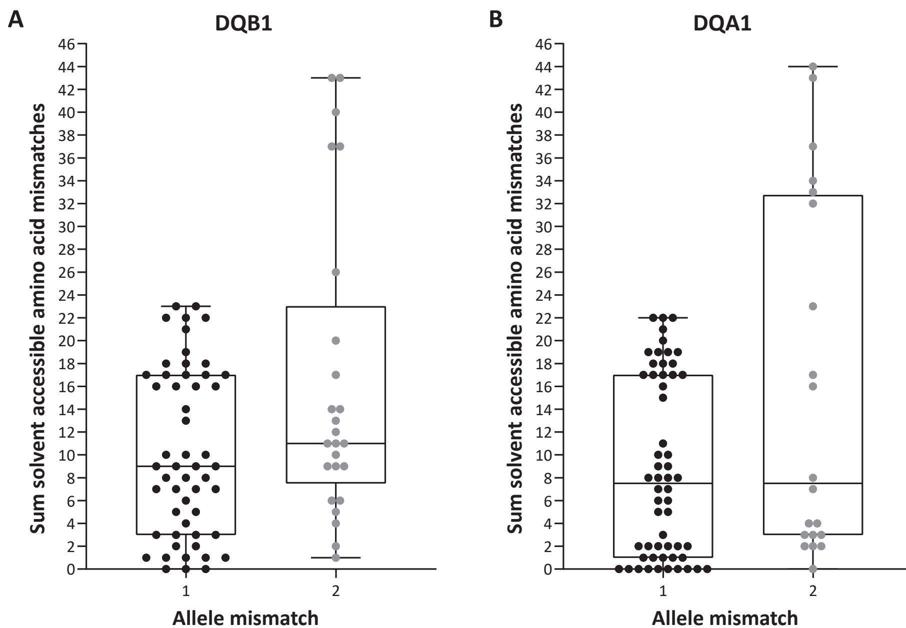


Figure 2: HLA-DQB1 and HLA-DQA1 solvent accessible amino acid mismatches and association with *dn*DSA formation. The number of solvent accessible amino acid mismatches were defined and *dn*DSA per mismatched donor allele for HLA-DQB1 (A) and HLA-DQA1 (B). A higher frequency of *dn*DSA formation is observed with increased number of solvent accessible amino acid mismatches. Groups were equally divided in tertiles and zero amino acid mismatches was set as separate group.

Next, we defined the number of solvent accessible amino acid mismatches for each mismatched donor allele, and we analysed whether an antibody response was formed against that donor allele. Mismatched donor HLA alleles that were not present in SAB assay were not included in the analysis. For HLA-DRB1/3/4/5, HLA-DQB1 and HLA-DQA1 we determined whether a correlation between the number of solvent accessible amino acid mismatches and *dn*DSA existed. For HLA-DR, no association was observed between *dn*DSA formed and number of solvent accessible amino acid mismatches due to low number of pairs (data not shown). For both HLA-DQB1 and HLA-DQA1 a higher frequency of *dn*DSA formation was observed with increasing number solvent accessible amino acid mismatches (Figure 3). The HLA-DQB1 and HLA-DQA1 solvent accessible amino acid mismatched were grouped based on tertiles, and this resulted in odds ratio (OR) 3.788 (1.1912 – 7.504) and OR 3.677 (1.908 – 7.086), respectively. The risk of *dn*DSA formation was also associated with an increased number of solvent accessible amino acid mismatches with OR 1.121 (1.049 – 1.198) for HLA-DQB1 and OR 1.138 (1.064 – 1.217) for HLA-DQA1 if no groups were defined on basis of tertiles.

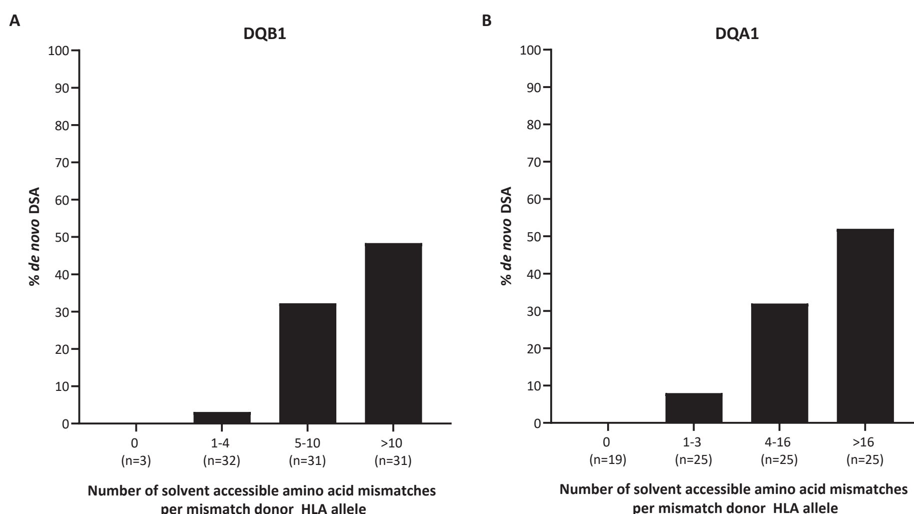
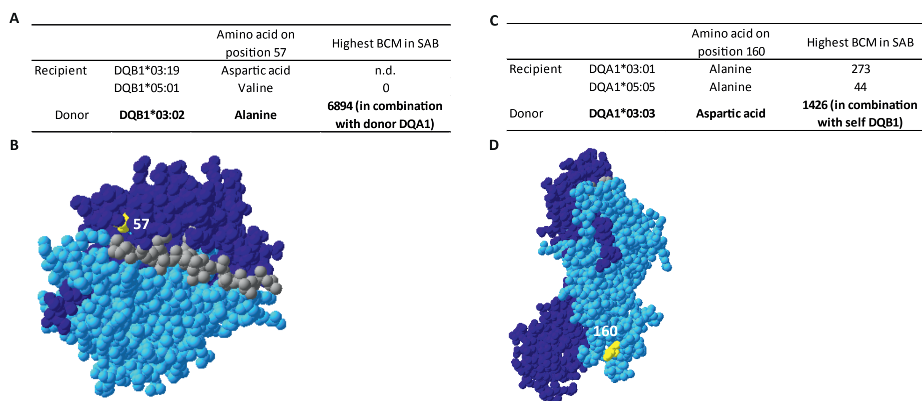


Figure 3: HLA-DQB1 and HLA-DQA1 allele mismatch was associated with a wide range of solvent accessible amino acid mismatches. Each data point represents the sum of solvent accessible amino acid mismatches for HLA-DQB1 (A) or HLA-DQA1 (B). The boxplot indicates the median with minimum and maximum values.

A single HLA-DQB1 or HLA-DQA solvent accessible amino acid mismatch can already be sufficient to induce dnDSA

In many publications a specific cut-off of epitopes or eplets is used to stratify patients into low or high risk to develop *dn*DSA. Also in our analysis above, we could identify a cut-off for HLA-DQB1 and HLA-DQA1 that correlated with a high risk of *dn*DSA development. However, low numbers of solvent accessible amino acid mismatches can already result in *dn*DSA formation. Upon in-depth analysis, we found that for one mismatched donor HLA-DQB1 allele and for one mismatched donor HLA-DQA1 allele there was only a single solvent accessible amino acid mismatch leading to *dn*DSA formation. For the HLA-DQB1 allele, DQB1*03:02, the mismatch was alanine on position 57 (Figure 4A), which is located on the top of HLA-DQ molecule (Figure 4B). In contrast, the amino acid aspartic acid on position 160 located near the bottom of the HLA-DQ molecule was the only mismatched amino acid of HLA-DQA1*03:03 but is nonetheless still regarded as solvent accessible (Figure 4C and D).



A

		Amino acid on position 57	Highest BCM in SAB
Recipient	DQB1*03:19	Aspartic acid	n.d.
	DQB1*05:01	Valine	0
Donor	**DQB1*03:02**	**Alanine**	**6894 (in combination with donor DQA1)**

C

		Amino acid on position 160	Highest BCM in SAB
Recipient	DQA1*03:01	Alanine	273
	DQA1*05:05	Alanine	44
Donor	**DQA1*03:03**	**Aspartic acid**	**1426 (in combination with self DQB1)**

Figure 4: A single solvent accessible amino acid mismatch is sufficient to induce *dn*DSA formation. The amino acid alanine on position 57 was the only mismatch between donor HLA-DQB1*03:02 and recipient's HLA-DQB1 alleles (A), but still resulted in *dn*DSA formation. Position 57 is located on the top of the molecule (B). Aspartic acid on position 160 was the only mismatch between donor HLA-DQA1*03:03 and recipient HLA-DQA1 alleles (C), but position 160 is located near the bottom of the molecule (D).

No specific amino acid position is preferentially associated with dnDSA formation

Next, we determined whether in this pilot study it was already possible to define specific amino acid positions or type preferentially resulting in *dn*DSA formation. To this aim, we calculated how often specific position or type occurred as mismatches and how often that specific position or type was mismatched on a mismatched donor HLA-DQ allele that resulted in *dn*DSA formation. So far, no specific amino acid position could be identified for HLA-DQB1 (Figure 5A), nor for HLA-DQA1 (Figure 5B), as the average frequency of *dn*DSA of positions is 39% (range 15-53%) and 40% (range 0-57%), respectively. Interestingly, in the current cohort no mismatches were observed for 116 solvent accessible amino acid positions of HLA-DQB1 and for 17 positions of HLA-DQA1. In addition, when further looking into the type of mismatched amino acids, no specific solvent accessible amino acid type resulting more frequently in *dn*DSA formation could be identified (data not shown).

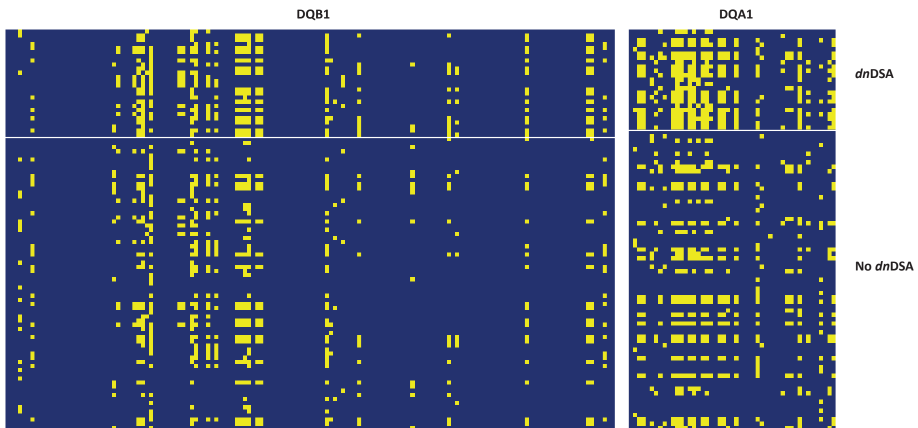


Figure 5: Overview of all mismatched solvent accessible amino acid positions. All solvent accessible amino acid positions of HLA-DQB1 and HLA-DQA1 are depicted in the columns, and each row is a mismatched donor allele. Yellow indicates the positions that were mismatched between donor and recipient and blue are the matched positions. *Dn*DSA was observed against the donor HLA alleles above the line and no *dn*DSA was observed for mismatched donor alleles below the line.

DISCUSSION

The development of *dn*DSA after transplantation is associated with inferior graft survival and reduces the chance of repeat transplantation. Interestingly, since the introduction of the sensitive Luminex SAB assays it is appreciated that *dn*DSA are often directed against HLA-DQ.[1-5] In the current study, we showed that the formation of HLA-DQB1 and HLA-DQA1-specific *dn*DSA is associated with the number of solvent accessible amino acid mismatches on mismatched HLA-DQ antigens. These findings are in accordance with previous studies based on eplet, amino acid, or physiochemical properties mismatches.[8,9,14,21] However, in the present study no association was observed between the number of HLA-DR solvent accessible amino acid mismatches and *dn*DSA formation, which is probably due to exclusion of large number of donor-recipient pairs (n=30) as the mismatched HLA alleles were not present in SAB assay used in this study resulting in low number of pairs and subsequently allele mismatches for analysis.

In addition, *dn*DSA formation, albeit at lower frequency, was observed for low number, or even a single, solvent accessible amino acid mismatches on both HLA-DQB1 allele or HLA-DQA1 alleles, similar as recently observed for HLA-DQ molecule mismatches.[22] One should take this into consideration, both in donor selection and risk estimation, and not only trust on the definition of eplet loads that have been proposed as good indicators for sensitisation

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risk[9,21,23] and have already been applied in kidney allocation to paediatric patients.[24] These data support the notion that with an increasing number of amino acid differences, the chance that an immunogenic disparity is present increases, but that in individual cases, a single amino acid can already be immunogenic.[25]

On the other hand, not every mismatched donor HLA allele with a high number of solvent accessible amino acid mismatches resulted in *dn*DSA formation, as in this group the *dn*DSA frequency was 48% and 52%, for HLA-DQB1 and HLA-DQA1 respectively. The data suggest that for some allele mismatches the amino acids mismatches that are present are of low immunogenicity.[26] The HLA phenotype of the recipient itself plays a major role in immunogenicity. So is class switching to IgG antibody producing cells dependent on the T cell epitopes presented by the recipients' HLA class II molecules of the B cell.[27,28] However, in current study, we could not find an association between the recipients' HLA-DR phenotype and *dn*DSA HLA-DQ-specific formation could be defined (data not shown), also likely due to limited sample size. Besides T cell dependency for IgG antibody formation, also the type of amino acid substitution plays a role in immunogenicity, because if the size and charge are like those of the recipients than the HLA molecules structure and physiochemical properties can be similar.[29]

Defining either an immunogenic amino acid position or type of the HLA-DQB1 and HLA-DQA1 alleles was not reached. This is probably due to the relatively low number of donor-recipient pairs. While the cohort consist of high number of HLA-DQB1 and HLA-DQA1 allele mismatches and HLA-DQ-specific *dn*DSA formation, the strict selection rules and the requirement of presence of DNA samples for NGS-based HLA typing resulted in a low number of donor-recipient couples. In addition, the numbers for analysis were even more reduced as not all HLA alleles obtained with NGS typing were present in SAB assay used in this study and therefore were excluded from analysis.

Nonetheless, high resolution typing is essential as it provides optimal accuracy in compatibility analysis on the amino acid level, and while for certain populations translation from low resolution to high resolution typing is possible,[30] for individuals with rare alleles this does not apply. High resolution HLA typing was also pivotal for assigning *dn*DSA towards HLA-DQ. In this study, we analysed HLA-DQB1 and HLA-DQA1 separately, and while indeed antibodies can be clearly specific for one chain, it is also possible that an antibody is specifically directed against the whole HLA-DQ molecule.[31,32] This is further complicated by the fact that certain HLA-DQB1 and HLA-DQA1 alleles can not only occur in cis form but also as trans-encoded

heterodimers,[33] and *dn*DSA against trans-encoded heterodimers have been observed.[34] Thus, further studies are required to fully understand what the HLA-DQ antibodies recognise.

To facilitate the analysis of HLA-DQ immunogenicity, it has been proposed that a cohort consisting of patients that have been transplanted with two HLA-DQ mismatches an developing *dn*DSA to only one of the mismatches can be useful, as well as recipients homozygous at the HLA-DQ locus.[22] This will be one of the components of the 18th International Immunogenetics and Histocompatibility Workshop (IHIWS) (https://www.ihiw18.org/). Our study indicates the need to perform larger and more diverse studies, such as the ones proposed in the IHIWS, to identify the most immunogenic HLA-DQ amino acid mismatches. In the current, relative small cohort, we observed that for the HLA-DQB1 locus most of polymorphic solvent accessible amino acid positions were never mismatched, because HLA-EMMA software includes the polymorphic positions for all HLA alleles in IMGT database, even rare alleles. The reason why these positions were never mismatched in the current study may be due to their monomorphic character in our study population, which does not exclude that these positions may be relevant in other study populations with other ethnicities.

Eventually, identification of the immunogenic polymorphic amino acids should lead to defining the relevant amino acid configurations to which an antibody can actual bind, similar as has been done for eplets. These configurations can then be used as parameters for allocation to prevent *dn*DSA formation after transplantation. In addition, amino acid configurations can be used for highly sensitised patients to define acceptable and unacceptable HLA alleles.[26,35]

In summary, in this pilot study we showed that even though the chance of HLA-DQ-specific *dn*DSA is higher with more solvent accessible amino acid mismatches, on individual level a single amino acid mismatch can already be sufficient to trigger an antibody response. Thus, to prevent *dn*DSA formation the most immunogenic residues must be avoided during allocation rather than the HLA alleles with high number of polymorphic amino acid mismatches with relative low immunogenicity. To accomplish this, it is pivotal to define the most immunogenic residues, especially for HLA-DQ, in larger and more diverse cohorts.

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