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# Frequency and Determinants of Pregnancy-Induced Child-Specific Sensitization

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**The aim of this study was to define the frequency and determinants of pregnancy-induced child-specific sensitization shortly after full-term delivery using sensitive single HLA-antigen beads (SAB) and high resolution HLA-typing of the mothers and their children (n = 301). A positive SAB result was defined by a background normalized ratio >1 or a mean fluorescence intensity (MFI) >300, >500 and >1000, respectively. The overall frequency of pregnancy-induced sensitization determined by SAB shortly after full-term delivery was between 45% (MFI > 1000 cut-off) and 76% (ratio cut-off). The rate of child-specific sensitization at the HLA-A/B/C/DRB1 loci was between 28% (MFI > 1000 cut-off) and 38% (ratio cut-off). The number of live birth was associated with a higher frequency of sensitization, which was driven by child-specific, but not third party HLA-antibodies. There was a clear hierarchy of sensitization among the investigated loci (B-locus: 31%; A-locus: 26%; DRB1-locus: 20%; C-locus: 15%; $p < 0.0001$). Some mismatched paternal HLA-antigens led to a significantly higher rate of sensitization than the average (e.g. HLA-A2, HLA-B49, HLA-B51, HLA-C*15). Furthermore, the mother's own HLA-phenotype—especially HLA-A/B homozygosity—was associated with a higher rate and broadness of sensitization. The number of mismatched HLA-A/B/C eplets strongly correlated with the rate of child-specific class I sensitization.**

**Key words: Allo-sensitization, HLA-antibodies, pregnancy, risk assessment, transplantation**

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## Introduction

Preformed donor-specific HLA-antibodies are a major risk factor for antibody-mediated rejection and inferior allograft survival in renal transplantation (1,2). Therefore, pretransplant evaluation of renal allograft recipients includes a sensitization history and a thorough analysis of HLA-antibodies in current and remote sera (1). Beside transplantation and blood transfusion, pregnancy can induce an immune response to mismatched HLA-antigens (3).

Most women had their pregnancies many years before subsequent organ transplantation, and sera dating back to these sensitizing events are usually not available for analysis. It is also well described, that pregnancy-induced HLA-antibodies can diminish over time and, thus, sensitization can be undetectable at the time of evaluation for transplantation (4–6). Therefore, knowledge on the frequency and determinants of child-specific HLA-antibody production soon after delivery could be very helpful to better assess the likelihood of pregnancy-induced sensitization even in the absence of HLA-antibodies in current sera.

Pregnancy-induced sensitization has been reported in the range of 10–40% using CDC-methodology or solid-phase assays (6–10). However, these studies had some limitations, which can under- or overestimate the frequency of sensitization. Indeed, the use of less sensitive CDC-methodology or analysis in sera obtained many years after pregnancy might miss occurred sensitization (5–9). By contrast, describing the overall rate of HLA-antibody positivity after pregnancy without assessing the child-specificity might overestimate the frequency of pregnancy-induced sensitization (6,8–10). It is well known that the rate of pregnancy-induced sensitization is related to the number of pregnancies, while other influencing factors are largely unknown and have not been investigated (6–9). The aim of this study was to define the frequency and determinants of pregnancy-induced child-specific sensitization shortly after full-term delivery using sensitive single HLA-antigen beads (SAB) and high resolution HLA-typing of the mothers and their children.

## Methods

### *Population and sample collection*

This study was approved by the local ethics committee. After obtaining written informed consent, 301 women giving birth at the University Hospital Basel between September 2009 and April 2011 were enrolled in the study. All women had either their first full-term pregnancy or had previous children only from the same partner as the current live birth. A blood sample was drawn from the mother between day 1 and 4 after delivery for HLA-typing and HLA-antibody analysis. Cord blood of the child was obtained immediately after delivery for HLA-typing.

### *HLA-typing*

High resolution HLA-A/B/C/DRB1 typing of the mothers and their children was performed by either SSO DNA-typing (LABType HD; OneLambda, Canoga Park, CA, USA) or sequencing-based typing (www.histogenetics.com).

### *HLA-antibody analysis and assignment as child-specific*

HLA-antibody analysis was performed by single HLA-antigen beads (SAB) for class I (iBeads Lot 1; OneLambda) and class II (LABScreen SA II Lot 9; OneLambda). Ten negative control sera were used to calculate the baseline normalized mean fluorescence intensity (MFI) and the signal-to-noise ratio for every individual bead as follows: Baseline normalized MFI = (MFI bead mother – MFI negative control bead [NCB]) – mean (MFI bead negative control$_{1-10}$ – MFI NCB negative control$_{1-10}$); Ratio = (MFI bead mother / MFI NCB) / 2*(mean+3SD [MFI bead negative control$_{1-10}$ / MFI NCB negative control$_{1-10}$]).

A ratio>1 or a baseline normalized MFI > 300 was regarded as a positive result, respectively. To assess the validity of this definition, the ratio and baseline normalized MFI of the mothers own HLA-alleles were analyzed. The own HLA-alleles had median ratio of 0.24 (IQR 0.16–0.32; mean ± SD: 0.29 ± 0.31) and median baseline normalized MFI of 1 (IQR 1–12; mean ± SD: 26 ± 84), respectively.

Child-specific HLA-antibodies were assigned by comparison of the HLA-antibody specificities of the mother with the HLA-typing of the child. In the vast majority of cases an HLA-allele in the SAB panel corresponded to the HLA-alleles of the children. If an HLA-allele of the child was not included in the SAB panel, the most representative allele of the same serologically defined HLA-molecule was selected as the child-specific one.

### *HLA-matchmaker analysis*

The HLA-matchmaker program was used to calculate the number of mismatched eplets between the mother and the child at the HLA-A/B/C loci (www.matchmaker.net).

### *Statistical analysis*

We used JMP software version 9.0.2 (SAS Institute Inc., Cary, NC, USA) for statistical analysis. For categorical data, Fisher's Exact test or Pearson's chi-square test was used. Parametric continuous data were analyzed by Student's *t*-tests. For nonparametric continuous data, the Wilcoxon rank-sum test was used for analysis. A p-value < 0.05 (two-tailed) was considered to indicate statistical significance.

## Results

### *Population characteristics*

The characteristics of the population are summarized in Table 1. Sixty-two percent of the women had their first live birth, 30% the second live birth and 8% ≥third live birth. The vast majority were Caucasians (95%), and all had a full-term delivery defined by a gestation week ≥37. In 163 of 301 live births (54%) there were mismatches in all four loci (i.e. HLA-A/B/C/DRB1).

**Table 1:** Population characteristics

| | |
|---|---|
| Age, median (IQR) | 31 (28–34) |
| First live birth, n (%) | 187 (62%) |
| Prior miscarriage(s), n (%) | 32/187 (17%) |
| Second live birth, n (%) | 90 (30%) |
| Prior miscarriage(s), n (%) | 24/90 (27%) |
| ≥Third live birth, n (%) | 24 (8%) |
| Prior miscarriage(s), n (%) | 8/24 (33%) |
| Prior blood transfusions, n (%) | 3 (1%) |
| Ethnicity | |
| Caucasoid, n (%) | 285 (95%) |
| Hispanic, n (%) | 8 (3%) |
| Oriental, n (%) | 6 (2%) |
| Unknown, n (%) | 2 |
| Total A/B/C/DRB1 mismatches with current child | |
| zero, n (%) | 6 (2%) |
| 1 mismatch, n (%) | 11 (3%) |
| 2 mismatches, n (%) | 26 (9%) |
| 3 mismatches, n (%) | 95 (32%) |
| 4 mismatches, n (%) | 163 (54%) |
| Mode of delivery | |
| Spontaneous, n (%) | 178 (60%) |
| Caesarian section, n (%) | 58 (19%) |
| Instrumental delivery, n (%) | 61 (20%) |
| Not reported, n (%) | 4 (1%) |
| Gestation weeks at delivery, median (IQR) | 40 (39–41) |

### *Frequency of overall sensitization*

The frequency of overall sensitization at all HLA-loci using different cut-off is summarized in Table 2. For first live birth it was between 70% (ratio cut-off) and 33% (MFI > 1000 cut-off); for second life birth between 84% (ratio cut-off) and 62% (MFI > 1000 cut-off) and for ≥third live birth between 92% (ratio cut-off) and 75% (MFI > 1000 cut-off).

### *Frequency of child-specific and third party sensitization*

The frequency of child-specific allo-sensitization at the HLA-A/B/C/DRB1 loci was 38% (ratio cut-off), 34% (MFI > 300 cut-off), 31% (MFI > 500 cut-off), and 28% (MFI > 1000 cut-off), respectively (Table 3). The observed rate of third party sensitization at the HLA-A/B/C/DRB1 loci was 31% (ratio cut-off), 29% (MFI > 300 cut-off), 21% (MFI > 500 cut-off), and 10% (MFI > 1000 cut-off), respectively (Table 3).

### *Influence of number of pregnancies and miscarriages on child-specific and third party sensitization*

Using the ratio cut-off, child-specific sensitization increased from 34% (first live birth) to 46% (second live birth) and 46% in women having ≥third live birth (p = 0.12). By the more robust MFI > 1000 cut-off, the frequency of

**Table 2:** Overall frequency of sensitization using different cut-off in the single HLA-antigen bead analysis and stratified by the number of live births

| | Ratio Ratio | MFI > 300 | MFI > 500 | MFI >1000 |
|---|---|---|---|---|
| All pregnancies (n = 301) | | | | |
| A-locus,% | 40% | 35% | 31% | 25% |
| B-locus,% | 51% | 38% | 32% | 28% |
| C-Locus,% | 21% | 20% | 15% | 13% |
| Class I,% | 61% | 48% | 41% | 34% |
| DRB1-locus,% | 34% | 39% | 28% | 17% |
| DRB3—5-loci,% | 16% | 21% | 16% | 12% |
| DQ-locus,% | 27% | 36% | 27% | 18% |
| DP-locus,% | 21% | 27% | 16% | 10% |
| Class II,% | 50% | 58% | 45% | 28% |
| Overall,% | 76% | 72% | 61% | 45% |
| First live birth (n = 187) | | | | |
| A-locus,% | 33% | 26% | 22% | 19% |
| B-locus,% | 44% | 29% | 24% | 20% |
| C-Locus,% | 17% | 17% | 12% | 10% |
| Class I,% | 55% | 39% | 33% | 26% |
| DRB1-locus,% | 28% | 36% | 24% | 12% |
| DRB3—5-loci,% | 13% | 16% | 10% | 7% |
| DQ-locus,% | 21% | 30% | 20% | 10% |
| DP-locus,% | 19% | 21% | 12% | 6% |
| Class II,% | 43% | 53% | 38% | 20% |
| Overall,% | 70% | 65% | 52% | 33% |
| Second live birth (n = 90) | | | | |
| A-locus,% | 49% | 46% | 41% | 32% |
| B-locus,% | 60% | 50% | 43% | 39% |
| C-Locus,% | 23% | 23% | 18% | 17% |
| Class I,% | 71% | 63% | 53% | 47% |
| DRB1-locus,% | 42% | 42% | 36% | 26% |
| DRB3—5-loci,% | 22% | 30% | 24% | 21% |
| DQ-locus,% | 34% | 43% | 37% | 30% |
| DP-locus,% | 19% | 33% | 19% | 14% |
| Class II,% | 56% | 63% | 52% | 39% |
| Overall,% | 84% | 83% | 74% | 62% |
| ≥Third live birth (n = 24) | | | | |
| A-locus,% | 63% | 58% | 54% | 42% |
| B-locus,% | 63% | 63% | 54% | 46% |
| C-Locus,% | 42% | 38% | 29% | 21% |
| Class I,% | 71% | 67% | 58% | 50% |
| DRB1-locus,% | 46% | 50% | 33% | 29% |
| DRB3—5-loci,% | 17% | 33% | 29% | 17% |
| DQ-locus,% | 50% | 46% | 38% | 33% |
| DP-locus,% | 38% | 50% | 33% | 21% |
| Class II,% | 83% | 79% | 71% | 54% |
| Overall,% | 92% | 88% | 83% | 75% |

sensitization increased from 21% (first live birth) to 37% (second live birth) and 46% (≥third live birth) ($p = 0.003$; Table 3). This increase in sensitization was observed for HLA-A/B/C and HLA-DRB1. Interestingly, the rate of third party sensitization was not different among first, second and ≥third live birth using different cut-off. It ranged from 30–33% (ratio cut-off; $p = 0.93$) to 7–14% (MFI > 1000 cut-off; $p = 0.18$; Table 3).

In women having their first live birth (n = 187), child-specific sensitization was not different if prior miscarriages had occurred or not ($p \geq 0.24$ for all cut-off). The same observation was made in women having their second (n = 90) or ≥third live birth (n = 24) ($p \geq 0.15$ for all cut-off).

### *Child-specific sensitization among different loci*

Overall, there were 1000 HLA-A/B/C/DRB1 mismatches among the 301 pregnancies (234 in the A-locus, 259 in the B-locus, 246 in the C-locus, and 261 in the DRB1-locus). The locus-specific sensitization assessed by the ratio cut-off was highest in the B-locus (31%), followed by the A-locus (26%), the DRB1-locus (20%) and the C-locus (15%; $p < 0.0001$). This locus-specific sensitization hierarchy remained significant using different MFI cut-off ($p \leq 0.009$; Figure 1). This hierarchy was also observed in first, second and ≥third live birth, though it was not statistically significant in ≥third live birth (Figure 1). Furthermore, this hierarchy was present in mothers having simultaneously four HLA-mismatches in the HLA-A/B/C/DRB1 loci (Figure 1). The strength of the immune response against antigens encoded by the different loci assessed by the MFI of child-specific HLA-antibodies was similar among the A-locus (median MFI 6122), the B-locus (median MFI 6638) and the DRB1-locus (median MFI 8608), while much lower in the C-locus antigens (median MFI 1910; $p = 0.005$).

### *Child-specific sensitization related to individual HLA-molecules*

The frequency of child-specific sensitization to individual HLA-mismatches is summarized in Figure 2. There was a clear hierarchy of sensitization to specific HLA-mismatches in the HLA-A, HLA-B, and HLA-C loci, while this was not observed in the HLA-DRB1 locus. An HLA-A2 mismatch led significantly more often to sensitization (48%) than the average HLA-A mismatch (26%; $p < 0.01$). The same observation was made for HLA-B49 (71%) and HLA-B51 (53%), while HLA-B44 (16%) and HLA-B8 (8%) had a lower rate of sensitization than the average HLA-B mismatch (31%). In the HLA-C locus, C*15 demonstrated a higher frequency of sensitization (33%) than the average HLA-C mismatch (14%).

### *Child-specific sensitization related to the HLA background of the mother*

Next, we evaluated whether specific HLA-molecules of the mother are associated with the frequency of sensitization in the respective loci. An HLA-A11 (11%; $p < 0.05$) and an HLA-A2 (19%; $p = 0.06$) background of the mother was associated with a lower frequency of sensitization than other HLA-A molecules (average 26%). HLA-B71 (80%; average B-locus 31%; $p < 0.05$), HLA-C*14 (42%; average C-locus 15%; $p < 0.05$) and HLA-DR13 (34%; average DRB1-locus 20%; $p < 0.01$) of the mother were associated with a higher frequency of sensitization in the corresponding loci.

**Table 3:** Frequency of child-specific and 3rd party sensitization using different cut-offs in the single HLA-antigen bead analysis. The first column indicates the overall frequency of child-specific and 3rd party sensitization. In the following columns, child-specific and 3rd party sensitization is stratified by the number of live birth

| | All (n = 301) | First live birth (n = 187) | Second live birth (n = 90) | ≥Third live birth (n = 24) | p-level* |
|---|---|---|---|---|---|
| A/B/C CSA[1]** | | | | | |
| Ratio, n (%) | 105 (35%) | 56 (30%) | 39 (43%) | 10 (42%) | 0.07 |
| MFI >300, n (%) | 87 (29%) | 44 (24%) | 34 (38%) | 9 (38%) | 0.03 |
| MFI >500, n (%) | 81 (27%) | 39 (21%) | 33 (37%) | 9 (38%) | 0.01 |
| MFI >1000, n (%) | 73 (24%) | 35 (19%) | 29 (32%) | 9 (38%) | 0.01 |
| 3rd party A/B/C | | | | | |
| Ratio, n (%) | 78 (26%) | 46 (25%) | 25 (28%) | 7 (29%) | 0.79 |
| MFI >300, n (%) | 58 (19%) | 28 (15%) | 23 (26%) | 7 (29%) | 0.05 |
| MFI >500, n (%) | 42 (14%) | 22 (12%) | 15 (17%) | 5 (21%) | 0.33 |
| MFI >1000, n (%) | 29 (10%) | 13 (7%) | 13 (14%) | 3 (13%) | 0.12 |
| DRB1 CSA | | | | | |
| Ratio, n (%) | 50 (17%) | 22 (12%) | 22 (24%) | 6 (25%) | 0.02 |
| MFI >300, n (%) | 54 (18%) | 24 (13%) | 23 (26%) | 7 (29%) | 0.01 |
| MFI >500, n (%) | 46 (15%) | 20 (11%) | 20 (22%) | 6 (25%) | 0.02 |
| MFI >1000, n (%) | 41 (14%) | 16 (9%) | 19 (21%) | 6 (25%) | 0.004 |
| 3rd party DRB1 | | | | | |
| Ratio, n (%) | 51 (17%) | 30 (16%) | 16 (18%) | 5 (21%) | 0.81 |
| MFI >300, n (%) | 64 (21%) | 44 (24%) | 15 (17%) | 5 (21%) | 0.42 |
| MFI >500, n (%) | 39 (13%) | 25 (13%) | 12 (13%) | 2 (8%) | 0.78 |
| MFI >1000, n (%) | 11 (4%) | 6 (3%) | 4 (4%) | 1 (4%) | 0.87 |
| A/B/C/DRB1 CSA | | | | | |
| Ratio, n (%) | 115 (38%) | 63 (34%) | 41 (46%) | 11 (46%) | 0.12 |
| MFI >300, n (%) | 103 (34%) | 53 (28%) | 39 (43%) | 11 (46%) | 0.02 |
| MFI >500, n (%) | 94 (31%) | 46 (25%) | 37 (41%) | 11 (46%) | 0.006 |
| MFI >1000, n (%) | 83 (28%) | 39 (21%) | 33 (37%) | 11 (46%) | 0.003 |
| 3rd party A/B/C/DRB1 | | | | | |
| Ratio, n (%) | 94 (31%) | 57 (30%) | 29 (32%) | 8 (33%) | 0.93 |
| MFI >300, n (%) | 87 (29%) | 53 (28%) | 26 (29%) | 8 (33%) | 0.88 |
| MFI >500, n (%) | 63 (21%) | 37 (20%) | 21 (23%) | 5 (21%) | 0.79 |
| MFI >1000, n (%) | 30 (10%) | 14 (7%) | 13 (14%) | 3 (13%) | 0.18 |

*p-levels for comparison of first, second and ≥third live birth.

[1]**CSA, child-specific antibodies.



### *Homozygosity of the mother and the risk of sensitization*

To investigate whether homozygosity of the mother is a risk factor of the rate and broadness of sensitization, we analyzed mothers with a first life birth and a mismatched HLA-molecule in a given specific locus (Table 4). HLA-A homozygous mothers (n = 17) developed significantly more often child-specific antibodies than heterozygous mothers (n = 134) (47% vs. 19%; p = 0.01). In addition, the broadness of sensitization defined by calculated panel reactive antibodies (cPRA) in the HLA-A locus was higher in homozygous mother (69% vs. 59%; p = 0.05). HLA-B homozygous mothers (n = 9) did not develop more often child-specific antibodies than heterozygous mothers (n = 156; 22% vs. 25%; p = 1.0), but the broadness of sensitization was significantly higher (95% vs. 66%; p = 0.002). HLA-C and HLA-DRB1 homozygosity of the mother was associated with a numerically higher frequency of sensitization, but the results were statistically not significant.

### *Mismatched HLA-A/B/C eplets and child-specific sensitization*

Finally, we evaluated the correlation of the number of mismatched HLA-A/B/C eplets and the rate of child-specific sensitization. The median number of mismatched eplets was 11 (range 0–32), which was not different among first, second and ≥third live birth (p = 0.45). Although almost no child-specific HLA-antibodies were observed with <5 mismatched eplets, the rate of sensitization increased stepwise to around 50% with ≥20 mismatched eplets (Figure 3).

## Discussion

To the best of our knowledge, this is the first study evaluating the frequency and determinants of pregnancy-induced child-specific sensitization shortly after full-term delivery using sensitive SAB technology and high resolution HLA-typing.

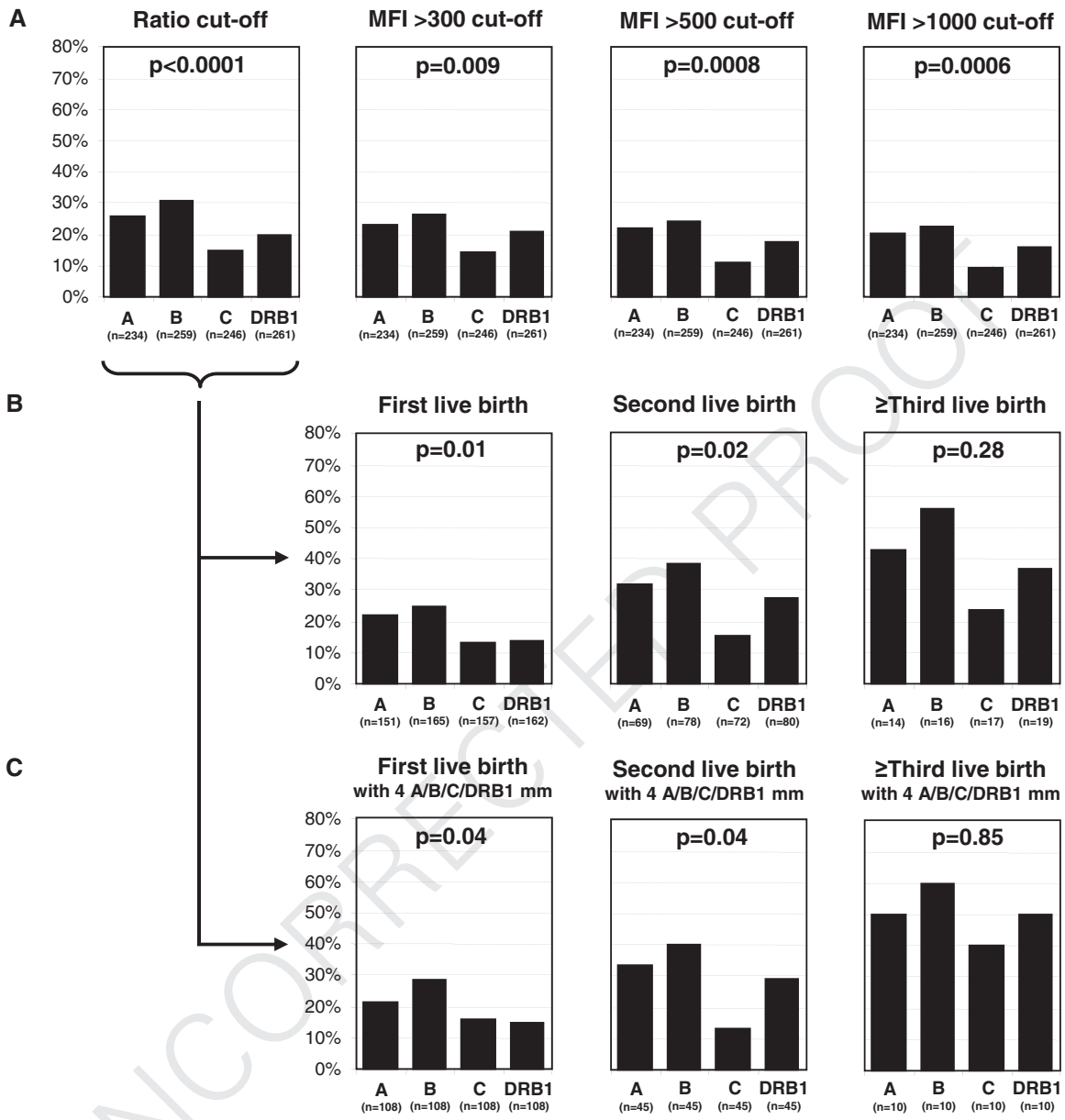


**Figure 1: Child-specific sensitization stratified by the HLA-locus.** (A) Locus-specific rate of sensitization in the whole cohort of 301 pregnancies defined by different cut-off. (B) Locus-specific rate of sensitization stratified by the number of live birth using the ratio cut-off. (C) Locus-specific rate of sensitization among pregnancies with four HLA-A/B/C/DRB1 mismatches stratified by the number of live birth using the ratio cut-off.

The overall frequency of sensitization regarding all HLA-loci was 45% (MFI > 1000 cut-off) to 76% (ratio cut-off), which is expectedly higher than in previous studies using either less sensitive HLA-antibody detection assays or measuring sensitization many years after delivery (6–9). Indeed, pregnancy-induced HLA-antibodies may diminish over time or even disappear completely (4–6). The observed sensitization rate using the very sensitive ratio cut-off is surprisingly high. Currently, there is no widely accepted cut-off to assign a positive result in the SAB analysis. For this reason, we used different cut-off values (i.e. ratio, MFI > 300–1000) and included 10 negative control sera for a more robust determination of background signals. Furthermore, the validity of the most sensitive cut-off (i.e. ratio and MFI > 300) was tested against the signal of the mother's self HLA-antigens, which were clearly below these cut-offs. Therefore, we are confident that signals even just above the used cut-off indicate the presence of antibodies against HLA-molecules. Still, we acknowledge that some positive reactions might be due to antibodies directed against

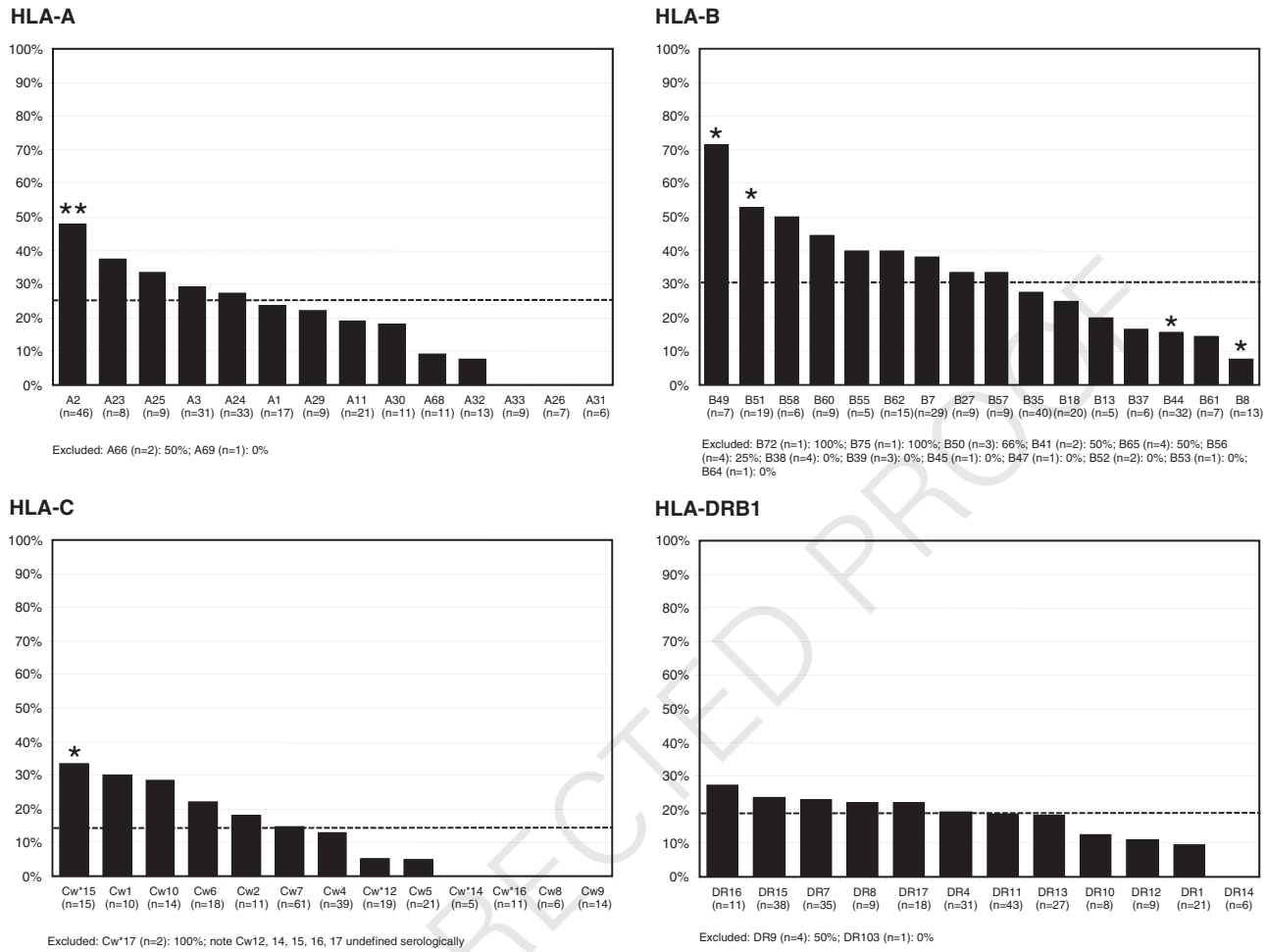


**Figure 2: Frequency of sensitization to specific HLA-molecules.** For this analysis, high-resolution HLA-typing results were converted into the corresponding serotypes. The dashed line indicates the average frequency of sensitization for the individual HLA-loci. The analysis included only HLA-molecules that were at least 5 times mismatched. Excluded HLA-molecules and their frequency of sensitization are indicated below the figures. *$p < 0.05$, **$p < 0.01$.

**Table 4:** Rate and broadness of sensitization related to homozygosity of the mother

| | Homozygous | Heterozygous | p-Level |
|---|---|---|---|
| HLA-A locus, n | 17 | 134 | |
| CSA[1]*,% | 47% | 19% | 0.01 |
| Broadness,% cPRA[2]** | 69% | 59% | 0.05 |
| HLA-B locus, n | 9 | 156 | |
| CSA,% | 22% | 25% | 1 |
| Broadness,% cPRA | 95% | 66% | 0.002 |
| HLA-C locus, n | 12 | 145 | |
| CSA,% | 25% | 12% | 0.2 |
| Broadness,% cPRA | 46% | 46% | 0.99 |
| HLA-DRB1 locus, n | 17 | 145 | |
| CSA,% | 24% | 12% | 0.25 |
| Broadness,% cPRA | 60% | 52% | 0.68 |

[1]*Child-specific antibodies.

[2]**cPRA, calculated panel reactive antibodies. cPRA were calculated using the Eurotransplant cPRA tool available at http://www.etrl.org/etrlpra/webform1.aspx.

denatured HLA-antigens despite the use of the latest version of SAB (i.e. iBeads), which presumably have less such HLA-molecules on the surface (11).

On the other hand, the rate of sensitization might even be higher 1 or 2 months after delivery, because additional exposure to fetal cells can occur during delivery. Unfortunately, we had only serum samples of the mothers collected within the first four days after delivery missing such additional sensitization.

As reported by many studies and confirmed in our cohort, the number of live birth is associated with the frequency of sensitization (6,8,9). This higher rate of sensitization at the HLA-A/B/C/DRB1 loci was driven be child-specific HLA-antibodies, while third party antibodies were equally often detected among first, second and $\geq$third life birth. This suggests that immediately after delivery child-specific antibodies are dominant, and HLA-antibodies

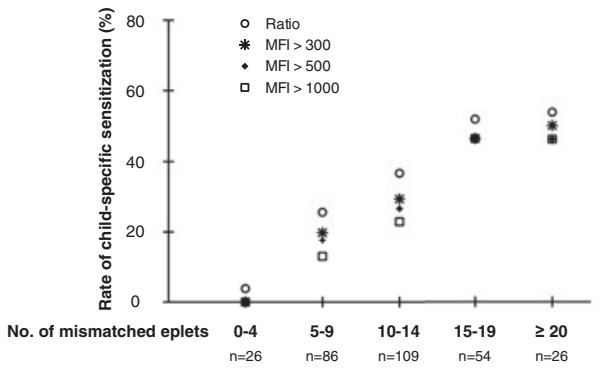


**Figure 3: Correlation of the number of mismatched eplets and the frequency of child-specific sensitization at the HLA-A/B/C loci.** The frequency of child-specific sensitization was assessed using different cut-off (i.e. ratio, MFI > 300, MFI > 500, MFI > 1000). Correlations were significant for all cut-off with $r^2$ between 0.94 and 0.96 ($p < 0.001$).

induced by previous pregnancies (e.g. by a different paternal HLA-haplotype) might be below detectable levels even by the very sensitive SAB assay. To prove this hypothesis, HLA-typing of previous children from women with multiple pregnancies is necessary, but this information is unfortunately not available. Notably, it has been shown that remote sensitization by pregnancies might become apparent after antigenic stimulation by blood transfusions at later time points (5,12). The higher frequency of child-specific sensitization after multiple life births is very likely due to a stronger immune response upon re-challenge with the same paternal HLA-haplotype.

Interestingly, the rate of overall and child-specific sensitization did only slightly increase beyond the second life birth in our cohort. This is consistent with Middelburg et al. who found a "plateau" of sensitization with the third pregnancy (i.e. around 38%; 9). A possible explanation for this observed upper level of sensitization might be that, beyond the second live birth, the mother had contact with and reacted against all immunogenic mismatched paternal HLA-antigens. If no sensitization occurred with the second live birth, the mother might not develop antibodies against the paternal HLA-antigens in subsequent pregnancies. The women included in our study had only children from the same partner, which lends support for this interpretation. Miscarriages, in addition to the number of live birth, did not further increase the frequency of sensitization suggesting that sensitization mainly occurs at a later stage of the pregnancy (i.e. >28 gestation week; Ref. 7).

An interesting observation in our study was that there is a hierarchy of sensitization in different HLA-loci (HLA-B > HLA-A > HLA-DRB1 > HLA-C). This hierarchy was consistent using different cut-off, among different number of live birth, and in mothers having simultaneously four mismatches in the HLA-A/B/C/DRB1 loci. The higher frequency of sensitization in the HLA-B locus might be related to its larger polymorphism compared to the other HLA loci. Furthermore, the lower expression of HLA-C antigens in the tissue might explain the lower rate of sensitization in the HLA-C locus, as well as the quantitatively lower amount of child-specific HLA-C antibodies compared to the HLA-A/B/DRB1 antibodies (i.e. median MFI 1910 vs. >6000; Ref. 13).

The most intriguing result in this study was that individual HLA-molecules induce a different frequency of sensitization. This was most prominent in the HLA-A/B loci, but rather limited in the HLA-DRB1 locus. While some HLA-A/B mismatches led to sensitization in ≥48% of mothers (i.e. A2, B49, B51), others were associated with a sensitization rate below 10% (e.g. A68, A32, A33, A26, A31, B8). These results are consistent with those reported by Dankers et al., who investigated the frequency of pregnancy-induced HLA-antibodies using CDC-methodology (14). Our data further expand the knowledge of pregnancy-sensitization to the HLA-C and HLA-DRB1-locus, and strongly support the concept that individual HLA-antigens have different immunogenicity (14–16).

The HLA background of the mother is also an important factor for the frequency and broadness of sensitization. Indeed, homozygocity in a given HLA-locus implies a limited repertoire of self HLA epitopes, and thus a higher chance that a mismatched HLA-antigen will represent many allo-epitopes. These HLA allo-epitopes might be shared by several other HLA-antigens leading to a broader sensitization. Our data clearly point towards this interpretation, although the results were not statistically significant among all HLA-loci.

The immune system recognizes epitopes on mismatched HLA-antigens (17). The mismatched HLA allo-epitopes are determined by, and must be assessed in the context of the self HLA epitope repertoire. Indeed, the number of mismatched eplets at the HLA-A/B/C loci calculated by the HLA-matchmaker software strongly correlated with the frequency of child-specific class I sensitization, which is in agreement with data reported by Dankers et al. (18). Clearly, the next step will be to determine the HLA allo-epitope specificity of the HLA-antibodies. This would allow investigating the immunogenicity of individual HLA-epitopes (i.e. number of mothers producing an antibody against epitope A divided by the number of mothers exposed to epitope A), which could significantly enhance our understanding of the humoral immune response.

The advantages of this study are the use of sensitive SAB technology and high-resolution HLA-typing to reliably assess the frequency and determinants of pregnancy-induced sensitization. However, the study has also some limitations. As we included mostly individuals of European origin, the results are perhaps not representative and applicable to other ethnic groups. Furthermore, the sample size

is still small for a conclusive statistical analysis regarding the relative immunogenicity of all HLA-molecules. In addition, the analysis of child-specific antibodies was restricted to HLA-A/B/C/DRB1 loci.

In conclusion, the frequency of pregnancy-induced sensitization determined by SAB shortly after full term delivery is between 45% (MFI > 1000 cut-off) and 76% (ratio cut-off). Child-specific sensitization at the HLA-A/B/C/DRB1 loci is between 28 (MFI > 1000 cut-off) and 38% (ratio cut-off), and depends on the number of live birth, the mismatched paternal HLA-molecules and the mother's own HLA-phenotype.

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## Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

## References

1. Gebel HM, Bray RA, Nickerson P. Pre-transplant assessment of donor-reactive, HLA-specific antibodies in renal transplantation: Contraindication vs. risk. Am J Transplant 2003; 3: 1488–1500.
2. Dunn TB, Noreen H, Gillingham K, et al. Revisiting traditional risk factors for rejection and graft loss after kidney transplantation. Am J Transplant 2011; 11: 2132–2143.
3. Scornik JC, Meier-Kriesche HU. Blood transfusions in organ transplant patients: Mechanisms of sensitization and implications for prevention. Am J Transplant 2011; 11: 1785–1791.
4. van Kampen CA, Versteeg-vd Voort Maarschalk MF, Langerak-Langerak J, Roelen DL, Claas FH. Kinetics of the pregnancy-induced humoral and cellular immune response against the paternal HLA class I antigens of the child. Hum Immunol 2002; 63: 452–458.
5. Rebibou JM, Chabod J, Alcalay D, et al. Flow cytometric evaluation of pregnancy-induced anti-HLA immunization and blood transfusion-induced reactivation. Transplantation 2002; 74: 537–540.
6. Densmore TL, Goodnough LT, Ali S, Dynis M, Chaplin H. Prevalence of HLA sensitization in female apheresis donors. Transfusion 1999; 39: 103–106.
7. Regan L, Braude PR, Hill DP. A prospective study of the incidence, time of appearance and significance of anti-paternal lymphocytotoxic antibodies in human pregnancy. Hum Reprod 1991; 6: 294–298.
8. Triulzi DJ, Kleinman S, Kakaiya RM, et al. The effect of previous pregnancy and transfusion on HLA alloimmunization in blood donors: Implications for a transfusion-related acute lung injury risk reduction strategy. Transfusion 2009; 49: 1825–1835.
9. Middelburg RA, Porcelijn L, Lardy N, Briet E, Vrielink H. Prevalence of leucocyte antibodies in the Dutch donor population. Vox Sang 2011; 100: 327–335.
10. Endres RO, Kleinman SH, Carrick DM, et al. Identification of specificities of antibodies against human leukocyte antigens in blood donors. Transfusion 2010; 50: 1749–1760.
11. Morales-Buenrostro LE, Terasaki PI, Marino-Vazquez LA, Lee JH, El-Awar N, Alberu J. "Natural" human leukocyte antigen antibodies found in nonalloimmunized healthy males. Transplantation 2008; 86: 1111–1115.
12. Scornik JC, Ireland JE, Salomon DR, Howard RJ, Fennell RS, III, Pfaff WW. Pretransplant blood transfusions in patients with previous pregnancies. Transplantation 1987; 43: 449–450.
13. McCutcheon JA, Gumperz J, Smith KD, Lutz CT, Parham P. Low HLA-C expression at cell surfaces correlates with increased turnover of heavy chain mRNA. J Exp Med 1995; 181: 2085–2095.
14. Dankers MK, Roelen DL, Korfage N, et al. Differential immunogenicity of paternal HLA Class I antigens in pregnant women. Hum Immunol 2003; 64: 600–606.
15. Dankers MK, Roelen DL, Van Der Meer-Prins EM, et al. Differential immunogenicity of HLA mismatches: HLA-A2 versus HLA-A28. Transplantation 2003; 75: 418–420.
16. Doxiadis II, Smits JM, Schreuder GM, et al. Association between specific HLA combinations and probability of kidney allograft loss: the taboo concept. Lancet 1996; 348: 850–853.
17. Duquesnoy RJ. A structurally based approach to determine HLA compatibility at the humoral immune level. Hum Immunol 2006; 67: 847–862.
18. Dankers MK, Witvliet MD, Roelen DL, et al. The number of amino acid triplet differences between patient and donor is predictive for the antibody reactivity against mismatched human leukocyte antigens. Transplantation 2004; 77: 1236–1239.

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