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Predicted Indirectly Recognizable HLA Epitopes Presented by HLA-DRB1 Are Related to HLA Antibody Formation During Pregnancy

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Pregnancy can prime maternal immune responses against inherited paternal HLA of the fetus, leading to the production of child-specific HLA antibodies. We previously demonstrated that donor-specific HLA antibody formation after kidney transplantation is associated with donor-derived HLA epitopes presented by recipient HLA class II (predicted indirectly recognizable HLA epitopes presented by HLA class II [PIRCHE-II]). In the present study, we evaluated the role of PIRCHE-II in child-specific HLA antibody formation during pregnancy. A total of 229 mother–child pairs were HLA typed. For all mismatched HLA class I molecules of the child, we subsequently predicted the number of HLA epitopes that could be presented by maternal HLA class II molecules. Child-specific antigens were classified as either immunogenic or nonimmunogenic HLA based on the presence of specific antibodies and correlated to PIRCHE-II numbers. Immunogenic HLA contained higher PIRCHE-II numbers than nonimmunogenic HLA. Moreover, the probability of antibody production during pregnancy increased with the number of PIRCHE-II. In conclusion, our data suggest that the number of PIRCHE-II is related to the formation of child-specific HLA antibodies during pregnancy. Present confirmation of the role of PIRCHE-II in antibody formation outside the transplantation setting suggests the PIRCHE-II concept is universal.

Abbreviations: IPA, inherited paternal HLA antigens; MFI, mean fluorescence intensity; PIRCHE-II, predicted indirectly recognizable HLA epitopes presented by HLA class II; SAB, single HLA antigen beads

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Introduction

The presence of donor-specific HLA antibodies before transplantation is strongly associated with impaired graft outcome in both solid organ transplantation and allogeneic hematopoietic stem cell transplantation (1,2). These HLA-specific antibodies can be detected in patients who have been exposed to allogeneic HLA after transplantation and blood transfusion. In addition, pregnancy is a major HLA immunizing event (3,4).

During pregnancy, the maternal immune system can be primed toward inherited paternal HLA antigens (IPA) of the fetus (5–7). This IPA-induced priming leads to the production of child-specific HLA antibodies in 10–30% of all pregnant women (4,6–8). The frequency of HLA-specific antibody formation increases with the number of full-term pregnancies (6,9) and depends on the maternal and fetal HLA phenotype (10). Although the clinical relevance of these HLA-specific antibodies in pregnancy outcome has been studied extensively in recent years, no consistent conclusions can be drawn so far from these studies (11). Nevertheless, better understanding of the immunogenesis of humoral immune responses during pregnancy is of great interest for transplant recipients.

The ability of maternal B cells to produce HLA-specific antibodies depends highly on help from CD4+ T cells (12). B cells initially acquire and process the mismatched paternal HLA and present the processed HLA epitopes to T helper cells via HLA class II molecules (12). Subsequently, antigen-specific T cell recognition drives proliferation and differentiation of naive B cells into memory B cells and plasma cells and facilitates IgM-to-IgG isotype switching (12,13).

The risk for developing HLA-specific antibodies during pregnancy is strongly correlated with the number of amino acid sequence mismatches (triplets) between the mother's

HLA and IPA of the fetus, as determined by HLAMatchmaker (14). These triplets are potential linear conformational antibody epitopes that are not shared with the mother's HLA (14). Currently, a redefined version of HLAMatchmaker identifies three-dimensional polymorphic amino acid patches located at the molecular surface of HLA molecules, designated as eplets (15). In addition, the number of mismatched eplets is correlated with HLA sensitization (8). Although considerable research has been devoted to predicting HLA-specific antibody formation, less research has been devoted to predicting the T cell reactivity involved in HLA antibody formation. The production of HLA-specific antibodies against the Bw4 epitope depends on the recipient's class II HLA-DR phenotype; an HLA-DRB1*01 or HLA-DRB1*03 background is associated with production of Bw4 antibodies (16). Moreover, Bw4 peptides can bind to cells positive for HLA-DRB1*01 and HLA-DRB1*03 *in vitro* (17). These observations suggest that T cells may react to processed HLA fragments presented on HLA class II. Inspired by these findings, we developed an *in silico* model that predicts HLA-derived T helper epitopes. This model identifies allopeptides presented by HLA class II molecules, designated as predicted indirectly recognizable HLA epitopes presented by HLA class II (PIRCHE-II) (18–21).

We demonstrated previously that the number of PIRCHE-II is related to *de novo* formation of donor-specific HLA antibodies after kidney transplantation (18). The objective of the present study is to evaluate the role of PIRCHE-II in the formation of child-specific HLA antibodies during a successful pregnancy. We investigated the relationship between PIRCHE-II and the formation of child-specific HLA antibodies in two different populations: primigravidae (women who had their first pregnancy) and multigravidae without prior miscarriage (women who had multiple pregnancies without any prior miscarriage). To exclusively ascribe the results to a successful pregnancy, pregnancies that were preceded by one prior miscarriage or more were excluded from analysis.

Materials and Methods

Population and sample collection

We included 301 women who gave birth at the University Hospital Basel, Switzerland, between September 2009 and April 2011. All women either had their first full-term pregnancy or had previous children who had the same father as the newborn child. Previous blood transfusions and miscarriages were documented. Women who had received prior blood transfusions ($n = 3$) and/or had prior miscarriage ($n = 64$) were excluded from further analysis. Three mother–child pairs were fully matched at all HLA class I loci and were excluded from further analysis. Four children were homozygous for a mismatched locus of the mother. Because no functional HLA class I IPA was present in these children, we excluded these mother–child pairs from analysis, leaving a cohort size of 229.

Peripheral blood samples from the mothers were obtained 1–4 days after delivery. These postdelivery blood samples were used for both HLA typing and HLA antibody analyses. For HLA typing of the newborn child, cord blood samples were obtained immediately after delivery. The study protocol was approved by the local ethics committee, and written informed consent was obtained from all participating women.

HLA typing

The peripheral blood samples and the cord blood samples were high-resolution typed for the HLA-A, -B, -C and -DRB1 loci using DNA typing methodologies, as described previously (8).

HLA antibody analysis

The presence of child-specific HLA antibodies in postdelivery blood samples was determined, as described previously (8). In short, HLA class I–specific antibodies were analyzed in the maternal sera using single HLA antigen beads (SAB) for HLA class I (iBeads Lot 1, One Lambda; Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions. Mean fluorescence intensity (MFI) values >1000 were regarded as positive. Subsequently, the detected HLA-specific antibodies were assigned as child-specific HLA antibodies by comparing the HLA-antibody specificity of the mother with the HLA typing of the child. Based on the presence or absence of child-specific HLA class I antibodies, the HLA of the child was classified as either immunogenic (i.e. child-specific HLA antibodies were detected in the maternal serum) or nonimmunogenic (i.e. no child-specific HLA antibodies were detected in the maternal serum).

Identification of HLA class I–derived PIRCHE-II

For all mismatched HLA class I molecules of the child, we determined the number of child-derived epitopes that can be presented by maternal HLA class II molecules (PIRCHE-II), as described previously (18,19). Predicting the peptide binding in the HLA class II binding groove is generally challenging. The more open binding groove structure of HLA class II molecules allows binding of peptides with different lengths, and each peptide may bind to the HLA class II molecule with different amino acid positions (22). Consequently, we used the artificial neural network–based NetMHCIIpan-3.0 algorithm (23) to predict at which position a class I–derived peptide may bind to the binding groove of maternal HLA class II molecules. This algorithm subsequently predicts the binding affinity of this predicted nonameric binding core of the peptide to maternal HLA-DRB1 (23). Binding affinities with an IC50 of >1000 nM (24) were defined as HLA class II binders. In our analyses, we included the entire amino acid sequence of the HLA class I proteins, thus including the leader peptide and the transmembrane region. Only child-derived HLA class II binders that are not present in any of the other maternal HLA class I molecules can serve as T helper epitopes; therefore, the predicted nonameric binding cores of child-derived HLA class II binders that differed in at least one amino acid with maternal HLA were considered PIRCHE-II. For each mother–child pair, we calculated the number of PIRCHE-II for all mismatched HLA class I IPA; only unique child-specific epitope–HLA complexes were counted as PIRCHE-II. Both maternal HLA-DRB1 alleles were taken into account as potential PIRCHE-II presenters. Four illustrative examples of different child HLA class I–maternal HLA-DRB1 combinations and their corresponding PIRCHE-II are depicted in Table 1. The PIRCHE algorithm is available online (https://www.pirche.org).

Matchmaker analysis

For all mismatched HLA class I molecules of the child, we determined the number of HLAMatchmaker eplets using HLAMatchmaker version 2.1 (available at http://www.epitopes.net) (25). Fetal eplets that were absent in the mother's HLA-A, -B, -C and -DRB1 locus were counted as mismatched eplets.

Statistical analysis

Statistical analyses were performed with GraphPad Prism software version 6.02 (GraphPad Software, Inc., La Jolla, CA) and SPSS Statistics

Table 1: Examples of PIRCHE-II and eplets for child HLA class I allele–maternal HLA-DRB1 combinations

A		B		C		D	
PIRCHE-II derived from **A*02:01** and presented by **DRB1*11:01**	Eplets present in **A*02:01**	PIRCHE-II derived from **A*02:01** and presented by **DRB1*13:02**	Eplets present in **A*02:01**	PIRCHE-II derived from **A*23:01** and presented by **DRB1*11:01**	Eplets present in **A*23:01**	PIRCHE-II derived from **A*11:01** and presented by **DRB1*03:01**	Eplets present in **A*11:01**
SFYPAEITL	44RME	FLRGYHQYA	62GE	LRIALRYYN	62EE	VGPDGRFLR	62QE
RCWALSFYP	62GE		65RKA	IALRYYNQS	65GKA	AVMAPRTLL	65RNA
LVLLLSGAL	65RKA		107W	DGRFLRGYH	76ERI		70AQS
FGAVITGAV	73TD		142MT	NLRIALRYY	82ALR		113YR
MAAQTTKHK	76VGT		144TKH	YFSTSVSRP			116D
WRFLRGYHQ	107W		207S	AVMWRRNSS			151HA
TLRCWALSF	105S		184A	MAAQITQRK			163R
YIALKEDLR	113YH		193AV				275EL
FLRGYHQYA	127K						
WALSFYPAE	142MT						
HRVDLGTLR	144TKH						
LSFYPAEIT	149AAH						
DWRFLRGYH	151HV						
AVMAPRTLV	207S						
	184A						
	193AV						

For clarity purposes, this table shows PIRCHE-II for only one of the maternal HLA-DRB1 alleles. For our analyses, both maternal HLA-DRB1 alleles were taken into account as presenters.
Only child-derived HLA class-II epitopes that are not present in any of the other maternal HLA class-I molecules were considered a PIRCHE-II and are shown in the table.
A. HLA typing mother: A*01:01, A*01:01, B*07:05, B*35:03, C*04:01, C*15:05, DRB1*01:03, **DRB1*11:01**
HLA typing child: A*01:01, **A*02:01**. B*07:05, B*35:03, C*12:03, C*15:05, DRB1*01:03, DRB1*04:05.
B. Same mismatch as (A), but different HLA-DRB1 as presenter.
HLA typing mother: A*03:01, A*24:02, B*07:02, B*07:02, C*07:02, C*07:02, **DRB1*13:02**, DRB1*15:02
HLA typing child: **A*02:01**, A*24:02, B*07:02, B*51:01, C*07:02, C*14:02, DRB1*11:01, DRB1*15:02.
C. Different mismatch as (A), but same HLA-DRB1 as presenter.
HLA typing mother: A*01:01, A*02:01, B*08:01, B*35:01, C*04:01, C*07:01, DRB1*03:01, **DRB1*11:01**
HLA typing child: A*01:01, **A*23:01**, B*08:01, B*49:01, C*07:01, C*07:01, DRB1*03:01, DRB1*12:01.
D. Different mismatch and different HLA-DRB1 as presenter as (A)
HLA typing mother: A*02:01, A*24:02, B*07:02, B*14:02, C*07:02, C*08:02, DRB1*03:01, **DRB1*15:01**
HLA typing child: **A*11:01**, A*24:02, B*07:02, B*07:02, C*07:02, C*07:02, DRB1*14:01, DRB1*15:01.

software version 20 (IBM Corp, Armonk, NY). Differences in the PIRCHE-II numbers between immunogenic and nonimmunogenic HLA were analyzed using Mann–Whitney *U* tests. To investigate whether the probability of HLA-specific antibody production during pregnancy is related to the PIRCHE-II numbers in the total cohort, the PIRCHE-II numbers were divided into quintiles (i.e. five equal groups). Gaussian distribution of the number of PIRCHE-II was tested using the D'Agostino-Pearson omnibus normality test to justify the division into quintiles. Pearson's chi-square tests were used to analyze the overall change in immunogenicity over the quintiles. Pearson's chi-square tests with Yates correction were used to analyze differences between two individual PIRCHE-II quintiles. Linear regression analyses were performed to analyze whether the relation between the probability of HLA-specific antibody production and PIRCHE-II was present in primigravidae and multigravidae. For the latter populations, percentage of immunogenic antigens was calculated for PIRCHE-II numbers that were four or more times present in the population. The regression lines of primigravidae and multigravidae were compared using calculations that have been described previously (26) and that are equivalent to the analysis of covariance test. Linear regression analyses were also performed to analyze the relationship between the number of PIRCHE-II and the number of eplets. A p-value <0.05 was considered statistically significant.

Results

Population characteristics

The characteristics of the study cohort have been summarized in Table 2. Of all 229 women, 67.2% had their first full-term pregnancy, 28.4% had their second full-term pregnancy, and 4.4% had their third or more full-term pregnancy. HLA mismatches for three HLA class I alleles

Table 2: Population characteristics

Total number of mother–child pairs	229
Number of pregnancies (%)	
First full-term pregnancy	154 (67.2)
Second full-term pregnancy	65 (28.4)
Third or more full-term pregnancy	10 (4.4)
Number of IPA (HLA-A, -B and -C) (%)	
1 mismatch	17 (7.2)
2 mismatches	66 (28.0)
3 mismatches	146 (61.8)

IPA, inherited paternal HLA antigens.

(i.e. on the HLA-A, -B and -C loci) were found in 63.8% of the mother–child pairs.

The HLA antigens of the child were classified as either immunogenic HLA (specific antibodies detected in maternal serum) or nonimmunogenic HLA (no specific antibodies detected in maternal serum). A total of 99 immunogenic HLAs (38 HLA-A, 45 HLA-B, and 16 HLA-C) and 488 nonimmunogenic HLAs (153 HLA-A, 157 HLA-B, and 178 HLA-C) were identified. For all of these individual mismatched HLA class I antigens, we predicted the number of indirectly recognizable HLA class I epitopes that were derived from the current child and presentable by maternal HLA-DRB1 (PIRCHE-II) (Table 3). The PIRCHE-II numbers ranged from 0 to 72 and did not have Gaussian distribution ($p < 0.0001$) (Figure S1).

Immunogenic HLA contains higher PIRCHE-II numbers

To determine whether the production of anti-HLA IgG antibodies depends on the presence of T helper epitopes, PIRCHE-II numbers of the immunogenic and nonimmunogenic HLA groups were compared. When analyzing the total cohort, immunogenic HLA contained higher PIRCHE-II numbers than nonimmunogenic HLA ($p = 0.0003$) (Figure 1A). The median PIRCHE-II number of the immunogenic HLA group was 1.3-fold increased compared with the nonimmunogenic HLA group.

The total study cohort consisted of primigravidae and multigravidae. Because these groups may have different immunological responses, the cohort was further subdivided into these two groups. For both the primigravidae (Figure 1B) and the multigravidae (Figure 1C), immunogenic HLA contained higher PIRCHE-II numbers than nonimmunogenic HLA ($p = 0.0016$ and $p = 0.1$, respectively). These results indicate that responses of primigravidae and multigravidae are immunologically similar, independent of the number of full-term pregnancies.

The probability of HLA-specific antibody production increases with the number of PIRCHE-II

We subsequently analyzed whether the probability of HLA-specific antibody production during pregnancy is related to the PIRCHE-II numbers. To this end, we divided the number of PIRCHE-II into quintiles, and for each quintile, we determined the percentage of antigens against which the mother develops HLA antibodies. The percentage of immunogenic antigens increased with the number of PIRCHE-II ($p = 0.001$; 0–6 vs. $\geq$25 PIRCHE-II: $p < 0.0001$) (Figure 2A). We repeated this analysis using different group strategies (e.g. PIRCHE-II numbers divided into quartiles or tertiles), and similar results were obtained (data not shown).

To test whether the probability of HLA-specific antibody production was different between primigravidae and multigravidae, we analyzed each group separately. Percentages were calculated for PIRCHE-II numbers with a frequency of four or higher. In both groups, the probability of generating HLA-specific antibodies increased with the number of PIRCHE-II (Figure 2B); however, multigravidae started producing HLA-specific antibodies with fewer PIRCHE-II numbers than primigravidae. Although the slopes of both regression lines were not significantly different ($p = 0.53$), the intercepts of the lines with the *y*-axis differed ($p = 0.01$), indicating that the regression lines of both groups were distinct but parallel. The quintile-division graphs for primigravidae and multigravidae (Figure S2) support this observation.

The number of PIRCHE-II do not correlate with the number of eplets

As reported previously, the frequency of child-specific sensitization toward HLA class I loci is correlated to the number of mismatched eplets (8). Because HLAMatchmaker and our PIRCHE-II model are both based on amino acid dissimilarities between the HLA of the mother and the child, a correlation between the number of PIRCHE-II and eplets is likely. To investigate this potential correlation, we plotted the number of PIRCHE-II against the number of eplets for the primigravidae. For immunogenic HLA, no correlation between the number of PIRCHE-II and the number of eplets was observed ($R^2 = 0.04$; $p = 0.12$) (Figure 3A). For nonimmunogenic HLA, a weak correlation between the number of PIRCHE-II and the number of eplets was observed ($R^2 = 0.30$; $p < 0.0001$) (Figure 3B). The regression lines of both groups are intersecting. Therefore, the amino acid dissimilarities between HLA of the mother and the child that were identified by the HLAMatchmaker model were different than the amino acid dissimilarities identified by our PIRCHE model.

The immunogenicity of individual HLA mismatches cannot be explained by PIRCHE-II numbers

The immunogenicity of individual HLA mismatches is highly diverse; some mismatched HLA molecules lead to sensitization significantly more frequently than other HLA molecules (8,10,27). We investigated whether the immunogenicity of individual HLA molecules could be explained by the number of PIRCHE-II. To this end, we sorted the individual HLA molecules from frequently immunogenic (left) to less frequently immunogenic (right) and calculated

Table 3: Number of PIRCHE-II for individual HLA class I IPA

Total number of HLA class I IPA	587
Number of PIRCHE-II (%)	
0–6	120 (20.4)
7–11	120 (20.4)
12–16	119 (20.3)
17–24	118 (20.1)
≥25	110 (18.7)

IPA, inherited paternal HLA antigens; PIRCHE-II, predicted indirectly recognizable HLA epitopes presented by HLA class II.

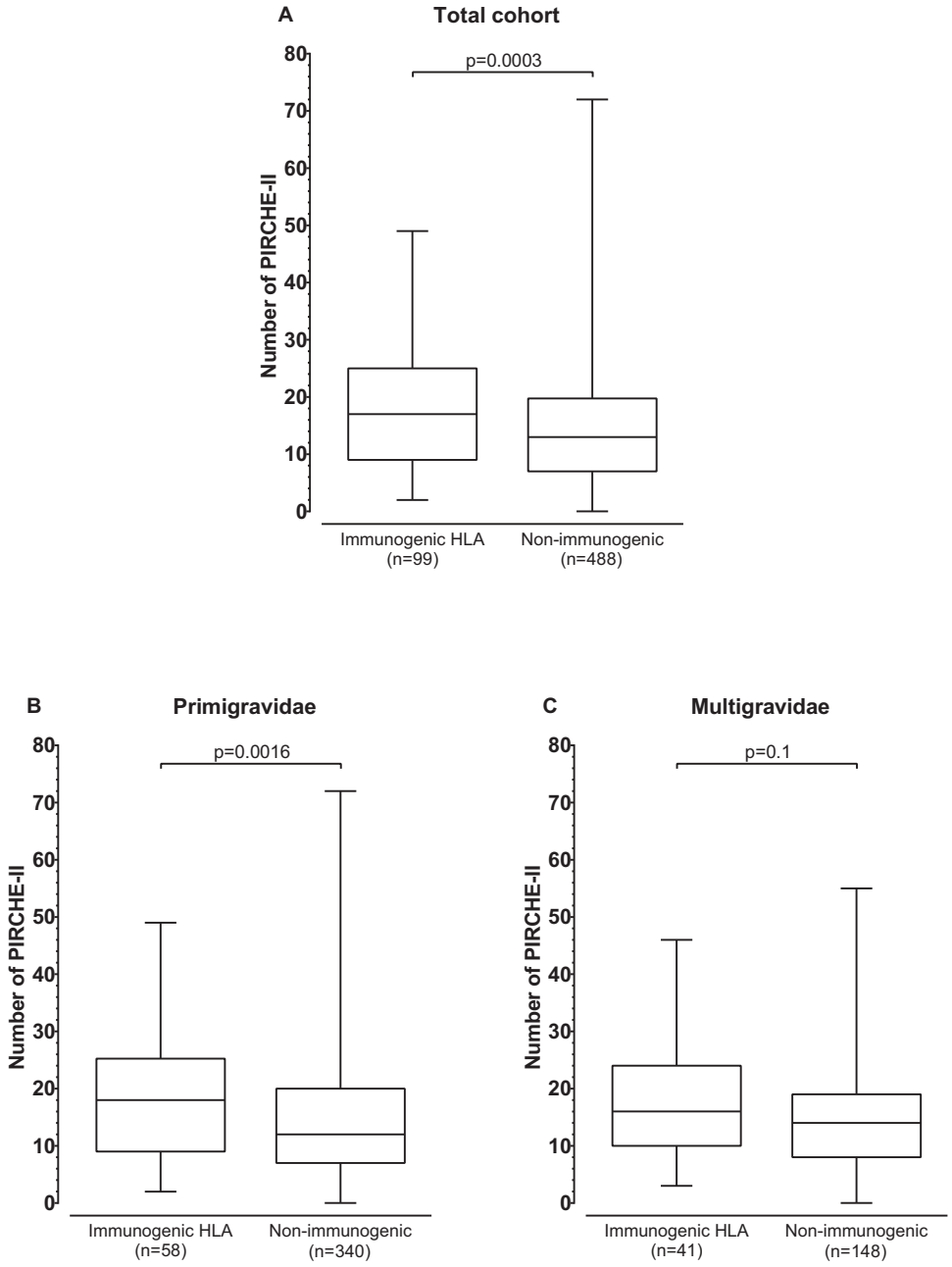

Figure 1: Comparison of the number of PIRCHE-II between immunogenic and nonimmunogenic HLA. Immunogenic HLA contained a higher number of PIRCHE-II than nonimmunogenic HLA in the total cohort (A), in primigravidae (B), and in multigravidae (C). The reported p-values were derived from Mann–Whitney *U* tests. The boxes extend from the 25th to 75th percentiles, and the middle line represents the median. The whiskers are drawn from the lowest to the highest PIRCHE-II value. PIRCHE-II, predicted indirectly recognizable HLA epitopes presented by HLA class II.

the PIRCHE-II numbers for these individual HLA mismatches. The individual HLA molecules that were highly immunogenic in our cohort did not contain higher PIRCHE-II numbers than less immunogenic HLA molecules (Figure 4). We conclude that a difference in the immunogenicity of individual HLA mismatches cannot yet be explained by the number of PIRCHE-II.

Discussion

In this study, we evaluated the role of child-derived T helper epitopes in the formation of child-specific HLA antibodies during pregnancy. We used a computational approach to predict the binding of HLA-derived epitopes to maternal HLA class II (19–21,23).

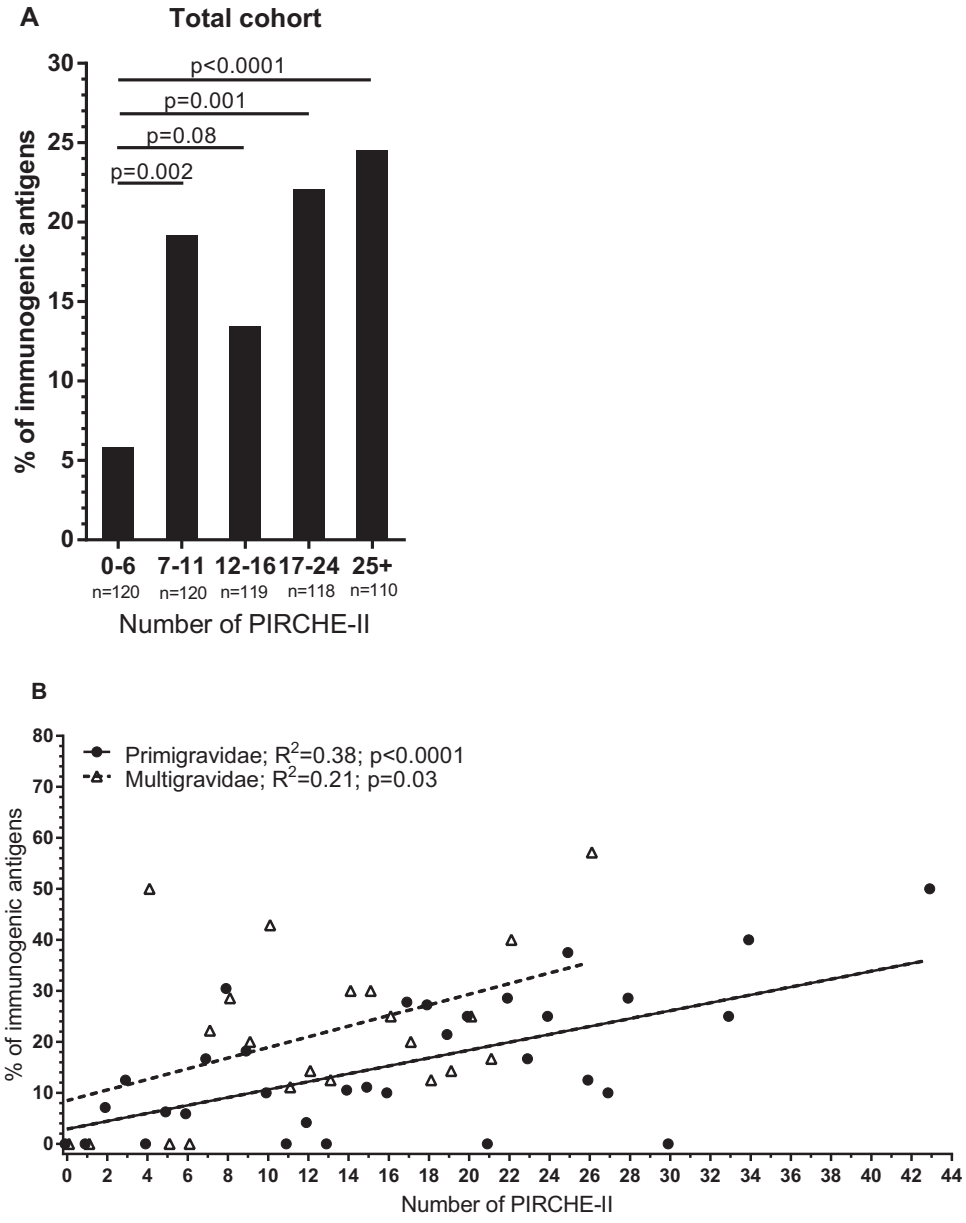

Figure 2: Relationship between the percentage of immunogenic antigens and the number of PIRCHE-II. The probability of HLA antibody formation increases with the number of PIRCHE-II in the total cohort (A) and in primigravidae and multigravidae (B). For panel (A), the number of PIRCHE-II were divided into quintiles (0–6, 7–11, 12–16, 17–24, and ≥25 PIRCHE-II). The reported p-values in panel (A) were derived from chi-square tests with Yates correction. For panel (B), we calculated the percentage of immunogenic antigens for individual PIRCHE-II numbers. Percentages were calculated for PIRCHE-II numbers that were four or more times present. Percentages corresponding to primigravidae are depicted as black dots, and percentages corresponding to primigravidae are depicted as open triangles. Significance of the regression line of primigravidae: $p < 0.0001$; significance of the regression line of multigravidae: $p = 0.03$. PIRCHE-II, predicted indirectly recognizable HLA epitopes presented by HLA class II.

In our cohort of 229 mother–child pairs, PIRCHE-II numbers were higher in HLA mismatches that elicited child-specific HLA antibodies than in HLA mismatches that did not elicit child-specific HLA antibodies (Figure 1). This result is in agreement with our previous findings that suggested that donor-derived PIRCHE-II is involved in the *de novo* formation of HLA IgG antibodies after kidney transplantation (18). Our data indicate that HLA-specific antibody formation is generally associated with our PIRCHE-II model.

Our cohort consisted of women who did not develop child-specific HLA antibodies at all (nonresponders), women who

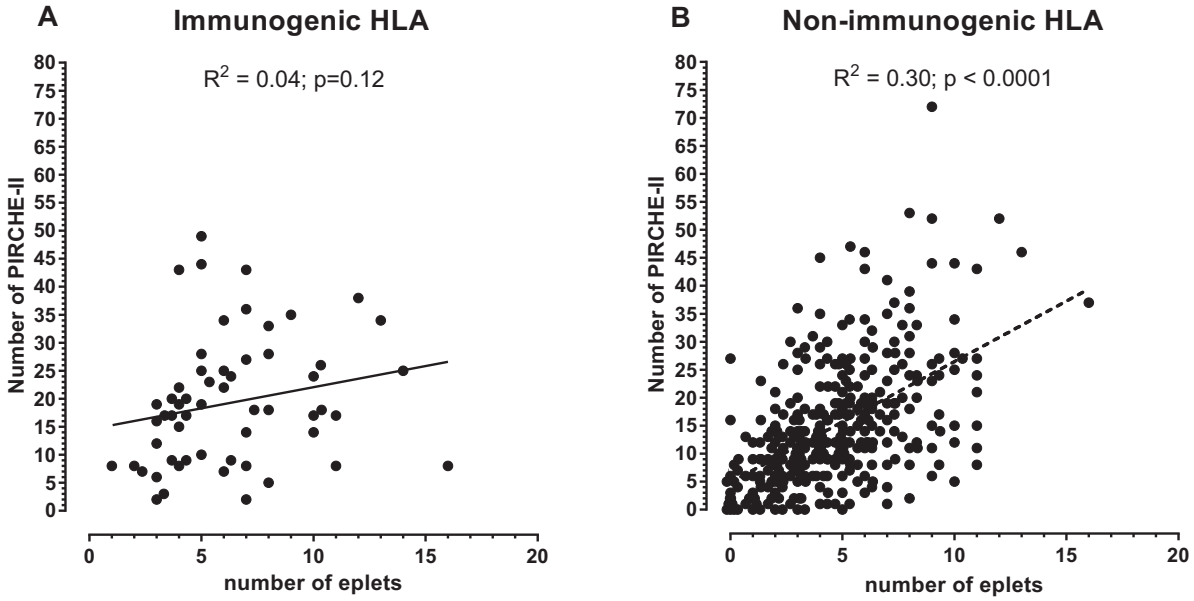

Figure 3: Correlation between the number of PIRCHE-II and the number of eplets. Immunogenic HLAs (A) are depicted as dots, and the corresponding linear regression line is a solid line. Nonimmunogenic HLAs (B) are depicted as triangles, and the corresponding linear regression line is a dotted line. Significance of the regression line is $p = 0.12$ for immunogenic HLA and $p < 0.0001$ for nonimmunogenic HLA. For visualization, overlapping data have been shifted 0.1 unit. PIRCHE-II, predicted indirectly recognizable HLA epitopes presented by HLA class II.

developed child-specific HLA antibodies against all HLA mismatches of the child (full IPA responders), and women who had at least one immunogenic HLA and at least one nonimmunogenic HLA (partial IPA responders). To investigate whether the partial immunogenicity in the latter group corresponded to differences in PIRCHE-II numbers, we analyzed the partial IPA responders separately (data not shown). A Wilcoxon matched pairs analysis showed that the PIRCHE-II numbers within this group differed significantly between immunogenic and nonimmunogenic HLA ($p = 0.0002$), excluding the possibility that our results were biased by data from full IPA responders and nonresponders.

As reported previously (8,9,28,29), the sensitization frequency increases with the number of full-term pregnancies. The increased sensitization in multigravidae may be the result of the increased maternal exposure duration toward (different) IPA (29). Our data suggest that for multigravidae, lower PIRCHE-II numbers are required to obtain the same probability of generating HLA-specific antibodies as primigravidae (Figure 2). Multigravidae seem more sensitive to PIRCHE-II than primigravidae in terms of HLA antibody formation. This increased sensitivity to PIRCHE-II in multigravidae may be explained by the re-exposure toward IPA and, consequently, toward PIRCHE-II during multiple pregnancies.

The number of mismatched HLA antibody–epitopes (eplets), as determined by HLAMatchmaker, is strongly correlated with the HLA-specific antibody response during pregnancy (8,14). Our PIRCHE-II model and HLAMatchmaker are based on the same biochemical principle: Amino acid dissimilarities between the HLA of mother and child invoke an antibody response. Despite this similar basic biochemical principle, HLAMatchmaker and our PIRCHE-II model are based on a different immunological principle: HLAMatchmaker is a predictor of B cell epitopes, whereas the PIRCHE-II model is a predictor of T helper epitopes. In a kidney transplantation setting, the number of eplets correlated only moderately with PIRCHE-II numbers and eplets (18). The lack of correlation may have been due to the relatively small number of recipients in this latter study ($n = 21$). In the current primigravidae population ($n = 154$), we found no correlation between the number of eplets as defined by HLAMatchmaker and the number of PIRCHE-II for immunogenic HLA (Figure 3A) and a weak correlation for nonimmunogenic HLA (Figure 3B). These observations in pregnancy confirm that the PIRCHE-II model and the HLAMatchmaker model are two independent predictors for HLA-specific antibody formation. This independence may be explained by the different epitopes that are predicted by the two models. Indeed, the majority of the HLA class I–derived PIRCHE-II were not part of an eplet (18); therefore, although both models are related to HLA antibody formation, the two algorithms define different polymorphic epitopes (as visualized in Table 1). Further research in larger cohorts is required to investigate whether the two algorithms interact statistically regarding the predictability of HLA antibody formation.

In this study, we found that frequently immunogenic HLA molecules did not necessarily contain higher PIRCHE-II

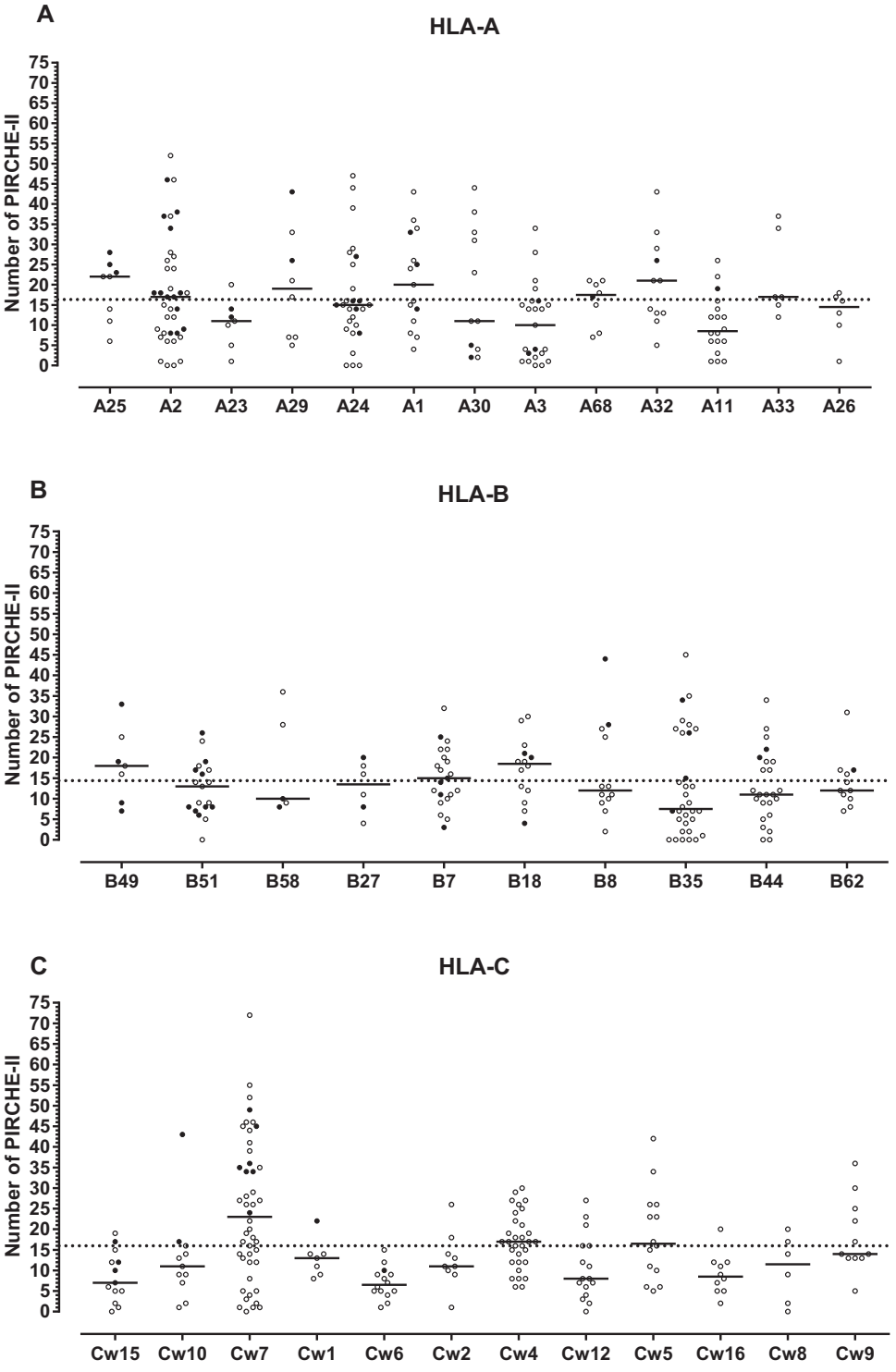

Figure 4: The frequency of sensitization to individual HLA molecules cannot be explained by the number of PIRCHE-II. HLA serotypes (A–C) were derived from high-resolution HLA typing. Individual serotypes were sorted from frequently immunogenic (left) to less frequently immunogenic (right). Immunogenic HLAs are depicted as black dots, and nonimmunogenic HLAs are depicted as open dots. Black lines represent the median. Dashed lines indicate the average number of PIRCHE-II for the individual HLA loci. Analyses were performed on HLA molecules that were at least three times mismatched. HLA serotypes that were excluded from the figure are A31, A66, A69, B13, B35, B37, B38, B39, B41, B45, B47, B50, B52, B55, B56, B57, B60, B61, B65, B72, B75, Cw14, and Cw17. PIRCHE-II, predicted indirectly recognizable HLA epitopes presented by HLA class II.

numbers (Figure 4), indicating that the immunogenicity of individual HLA class I molecules cannot be predicted by the PIRCHE-II algorithm. Several studies, however, showed that the immunogenicity of individual mismatched HLA molecules depends on the HLA phenotype of the responder (16,30). The frequency of antibody production against individual mismatched HLA class I molecules is highly dependent on the HLA-DR background of the responder (30). These observations suggest that the effect of an identical HLA mismatch may differ in responders with a different HLA phenotype and that the immunogenicity of individual HLA class I molecules should be analyzed in the context of the maternal HLA-DR phenotype or might even have to be considered in the context of maternal HLA-DQ and HLA-DP phenotypes. Due to the limited numbers of mismatched HLA in the different maternal HLA-DR backgrounds, we were unable to analyze the individual HLA class I molecules in the context of the maternal HLA-DR phenotype for our cohort.

Alternatively, the lack of relationship between the immunogenicity of distinct HLA class I molecules and the number of PIRCHE-II might be due to using HLA antibody response as a readout instead of CD4+ T cell responses; however, data about CD4+ T cell alloimmune responses is generally hard to obtain. Because numerous tetramers and function assays are required, measuring the CD4+ T cell alloimmune response is hardly feasible, as illustrated by the fact that the number of potential T helper epitopes can be up to ≈72 peptides and that such epitopes are only marginally overlapping between different mothers. Furthermore, our observations may also be explained by the poorly characterized immunogenicity of individual PIRCHE-II. Each PIRCHE-II represents a peptide with potential immunogenicity but with an unknown degree of immunogenicity. PIRCHE-II immunogenicity may well differ per peptide due to preferential processing and/or binding to HLA. Defining the immunogenicity of individual PIRCHE-II may give more insight in the clinical relevance of each individual PIRCHE-II.

No clear consensus currently exists regarding the MFI threshold to classify whether HLA-specific antibodies are present or absent; different MFI thresholds for assigning child-specific HLA can be used. Because the MFI threshold determines the classification of child-specific HLA antigens into immunogenic or nonimmunogenic HLA (8), the study outcome might depend on the chosen threshold. Consequently, we repeated the PIRCHE-II analyses using lower and higher MFI thresholds, but the outcome did not change (data not shown).

Although immunogenic HLA contain higher PIRCHE-II numbers than nonimmunogenic HLA in pregnancies without prior miscarriages, maternal antibody responses were also raised against HLA that contained low PIRCHE-II numbers or against HLA for which we did not predict any PIRCHE-II. These immunogenic HLAs might contain epitopes that can bind to maternal HLA class II molecules other than HLA-DRB1, such as HLA-DRB3, -DRB4 or -DRB5 and HLA-DPA1; -DPB1 heterodimers or HLA-DQA1; -DQB1 heterodimers. Our HLA-typing data are restricted to HLA-DRB1. Moreover, HLA-DPB1 and -DQB1 presentation is not implemented in our current PIRCHE model; therefore, we were not able to estimate the number of PIRCHE-II that can bind to maternal HLA-DPA1; -DPB1 heterodimers or HLA-DQA1; -DQB1 heterodimers. The addition of PIRCHE-II presented by HLA-DPA1; -DPB1 heterodimers and HLA-DQA1; -DQB1 heterodimers may more accurately describe the role of PIRCHE-II in the formation of IPA antibodies in our cohort.

In contrast, several HLA containing high PIRCHE-II numbers were nonimmunogenic. This observation could reflect the maternal immunological tolerance toward the fetus during pregnancy (31). IPA-specific T cells can be tolerogenic *in vivo* during pregnancy (32); therefore, PIRCHE-II–specific T cells may be anergized during pregnancy and thus may not be able to provide T cell help to B cells. Alternatively, our PIRCHE-II model predicted presentation of child-derived HLA epitopes by maternal HLA class II but not T cell recognition of these HLA class II–presented epitopes. Some PIRCHE-II, as determined by our model, may not be recognized by CD4+ T cells, thus these HLA containing high PIRCHE-II numbers could be non-immunogenic due to a lack of CD4+ T cell recognition.

In addition to the lack of predicting CD4+ T cell recognition of PIRCHE-II and the unknown degree of immunogenicity of individual PIRCHE-II, our study has several other limitations. As mentioned previously, HLA typing of our study cohort was restricted to HLA-DRB1, whereas PIRCHE-II presentation by HLA-DPA1; -DPB1 and by HLA-DQA1; -DQB1 heterodimers may also occur. Moreover, maternal serum samples were collected 1–4 days after delivery. Because these maternal serum samples were taken shortly after delivery, our observations with respect to PIRCHE-II and HLA antibody formation are most likely to be ascribed to the pregnancy rather than to effects during delivery. After delivery, HLA antibody formation may increase due to fetal exposure during delivery. Because maternal serum samples at later time points are not available for our cohort, we were unable to test this aspect in our cohort. Furthermore, T helper–independent child-specific IgM antibodies may have been formed and may interfere with the detection of child-specific IgG antibodies in our Luminex-based SAB assay (33); however, the presence of child-specific IgM antibodies was not tested in the maternal sera.

In summary, we show that the development of child-specific HLA antibodies during pregnancy is related to higher numbers of PIRCHE-II. The probability of HLA-specific antibody formation increases with the number of PIRCHE-II. No firm conclusions with regard to risk quantification can be drawn based on the current data.

Further studies are required to investigate whether PIRCHE-II can be used in the future as a tool for risk stratification and extrapolation to unacceptable HLA antigens. The present confirmation of the role of PIRCHE-II in antibody formation in a different setting than transplantation suggests that our PIRCHE-II model may be a tool to predict HLA antibody formation in general.

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Disclosure

The authors of this manuscript have conflicts of interests to disclose as described by the *American Journal of Transplantation*. The University Medical Center Utrecht has filed a patent application for the prediction of an alloimmune response against mismatched HLA. E.S. is listed as inventor on this patent. The other authors have no conflicts of interest to disclose.

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Supporting Information

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

Figure S1: Distribution of the number of PIRCHE-II. Dashed lines indicate the quintile group cut-offs.

Figure S2: Relation between the percentage of immunogenic antigens and the number of PIRCHE-II for primigravidae (A) and multigravidae (B). The number of PIRCHE-II were divided into quintiles (0-6 PIRCHE-II, 7-11 PIRCHE-II, 12-16 PIRCHE-II, 17-24 PIRCHE-II, and 25+ PIRCHE-II). The reported p-values are derived from Chi-square tests with Yates' correction.