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Maternal microchimerism in cord blood and risk of childhood-onset type 1 diabetes

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Abstract

Background: Maternal microchimerism (MMc), the transmission of small quantities of maternal cells to the fetus, is relatively common and persistent. MMc has been detected with increased frequency in the circulation and pancreas of type 1 diabetes (T1D) patients. We investigated for the first time whether MMc levels at birth predict futureT1D risk. We also tested whether cord blood MMc predicted MMc in plasma at T1D diagnosis.

Methods: Participants in the Norwegian Mother and Child Cohort study were HLA class II typed to determine non-inherited, non-shared maternal alleles (NIMA). Droplet digital (dd) PCR assays specific for common HLA class II NIMA (HLADQB1*03:01, *04:02 and *06:02/3*)* were developed and validated. MMc was estimated as maternal DNA quantity in the fetal circulation, by NIMA specific ddPCR, measured in cord blood plasma from 71 children who later developed T1D and 126 controls within the cohort.

Results: We found detectable quantities of MMc in 34/71 future T1D cases (48%) and 53/126 controls (42%) (OR 1.27, 95%CI 0.71-2.27), and no significant difference in ranks of MMc quantities between cases and controls (Mann-Whitney P=0.46). There was a possible association in the NIMA HLA *DQB1*03:01* subgroup, where presence of MMc was associated with T1D after adjustment (aOR 3.89, 95%CI 1.05-14.4). MMc in cord blood was not significantly associated with MMc at T1D diagnosis.

Conclusions: Our findings did not support the hypothesis that higher levels of MMc in cord blood predict T1D risk. The potential subgroup association with T1D risk should be replicated in a larger cohort.

Keywords: Microchimerism, HLA, Type 1 diabetes, Pregnancy, Childhood

Introduction

Type 1 diabetes (T1D) is an autoimmune disease where insulin-producing beta cells become targeted by the immune system. The main known genetic risk factor for T1D is HLA genotype, and many weaker genetic risk factors exist [1]. Environmental factors are not well characterized, but it has been hypothesized that prenatal and early life factors influence T1D risk [2,3].

Maternal transmission of cells to the fetus during pregnancy, known as maternal microchimerism (MMc), is believed to protect against fetal loss [4], and promote regulatory T cell mediated tolerance against non-inherited maternal antigens [5]. MMc has been shown to persist into adult life [6], is relatively common [7], and has been associated with both protective and adverse effects [8,9]. MMc has been postulated to play a role in autoimmune diseases, where it could be particularly relevant due to the strong association between certain HLA genotypes and autoimmune diseases [8]. MMc can detected or quantified by targeting non-inherited, non-shared maternal allele (NIMA) DNA originating from maternal cells.

MMc has been detected in several tissues, amongst them islet beta cells [10] in the pancreas, with increased frequency of MMc in T1D patients [11]. Higher levels of MMc in the circulation have also been shown for patients with T1D compared with healthy controls, using a quantitative PCR approach to detect non-inherited HLA alleles [10]. This increase could be due to MMc being present before development of T1D, or expansion of existing cells as a result of disease development. No studies to date however have investigated whether those who later develop disease have higher MMc levels prior to disease onset. Although the biological functions of MMc are largely unknown, we hypothesized that higher levels of pre-diagnostic MMc would contribute to lower risk of T1D.

Since MMC likely results from transplacental transfer via cord blood, we developed sensitive allele specific ddPCR assays to test for the first time whether cord blood MMc have any predictive value for childhood-onset T1D. Secondly, we investigated whether cord blood MMC predicted circulating MMc at diagnosis of T1D.

Research Design and Methods

Participants

We designed a nested case-control study in the Norwegian Mother and Child Cohort Study (MoBa) [12], which recruited around 114,000 children and their mothers (41% of eligible mothers participated) from all over Norway during 1999-2008. The current study uses data from repeated questionnaires, maternal postpartum and offspring cord blood samples [13]. All study participants gave written informed consent. The Norwegian Data Protection Authority and The Regional Committee for Medical Research Ethics approved MoBa and the current study. Characteristics of the participants are shown in Table 1. Baseline characteristics for those with available blood samples were largely similar to the whole MoBa cohort, except a lower proportion of caesarean section and premature birth (see [14]).

Ascertainment of case status

Children who developed T1D by February 5, 2014 were identified with a high degree of ascertainment by register linkage to the Norwegian Childhood Diabetes Registry [15] and used as cases. A random set of controls was selected among participants in the MoBa cohort with available blood samples. We later ascertained if the initially selected random controls developed T1D until 1 June 2018. In total, 186 mother/child-pairs where the child developed T1D and 540 mother/child-pairs were used as controls. Of these, 71 T1D cases and 126 controls had an informative HLA DQB1*03:01, *04:02, or *06:02/3 NIMA allele (Figure 1).

Sampling

Briefly, maternal blood samples were collected in EDTA tubes during pregnancy and shortly after birth, and a blood sample was taken at birth from the umbilical cord vein using a syringe [12,13,16]. At diagnosis of T1D, blood samples were collected and shipped to the Norwegian Childhood Diabetes Registry. At the biobank repository, DNA was extracted and stored at -20°C until analysis. For details of sample handling, see [13,16,17].

Genotyping

To account for established T1D susceptibility markers, participants were genotyped using a custom Illumina Golden Gate assay (Illumina, San Diego, CA). DNA extraction, genotyping methods and quality control procedures are described in detail in a previous publication [18]. Briefly, tag-SNPs (n = 144) on chromosome 6 were used to impute human leukocyte antigen (HLA) class II genotype [19], which was subsequently confirmed by classical HLA genotyping using allele specific PCR on all samples [20]. For each informative dyad, we inferred the non-inherited non-shared HLA*DQB1 allele, NIMA (Figure 1, panel A).

Droplet Digital (dd)PCR MMc quantification

MMc were measured by quantifying the amount of NIMA specific DNA in cord blood. We developed allele-specific ddPCR assays for three common NIMAs (DQ01*03:01, DQB01*04:02, DQB01*06:02/*06:03), based on and validated against our previous quantitative PCR assays [10,21], to improve sensitivity for low copy number targets. We used the droplet digital PCR QX200 system (Bio-Rad, California, USA). Final primer and probe concentrations optimised for DQB1*04:02 and DQB1*06:02/3 at 100nM and 300nM, and DQB1*03:01 at 300nM and 100nM respectively. DNA was quantified using the QuantiFluor dsDNA system (Promega, Madison, USA).

Sensitivity of assays determined using serial dilutions (500, 100, 5, 1 and 0.5 genomic equivalent (gEq)) of NIMA positive homozygous DNA in a background DNA quantity of 10,000 genome equivalent negative for NIMA. Specificity for each assay was determined using well-characterised HLA cell lines. Ten wells were run for each sample, loaded at 10,000 gEq equal to 33ng; 100,000 gEq was screened per sample. MMc value was determined by total copy number of HLA allele specific positive cells per 1 000 000 host cells as determined by beta-globin.
A priori, we decided to consider a sample positive only if two or more of the 10 replicates were positive. Samples with only one positive well (n = 62) were set to an MMc value of zero. Figure 1B shows the formation of the analysis sample.

As the measured MMc levels between probes are not directly comparable, we calculated MMc z-scores by subtracting the NIMA specific mean and dividing by the standard deviation (both calculated from control children), all done on log2transformed raw values.

Other covariates

A priori, we chose maternal pre-pregnancy body mass index (BMI) and maternal age as our primary adjusting variables, as these have been associated with T1D [22-24] and could conceivably influence MMc. In a separate model we further included maternal smoking during pregnancy [25] (coded as smoking or non-smoking at end of pregnancy), caesarean section [26] and child`s HLA risk for T1D (coded as a binary variable; protective or neutral vs increased risk) as a sensitivity analysis. Distribution of these covariates are presented in Table 1.

Maternal age at delivery was obtained from the nationwide Medical Birth Registry of Norway (MBRN). Information regarding maternal pre-pregnancy BMI was obtained from mid-pregnancy questionnaires (accessible at www.fhi.no/moba) and MBRN.

Statistical analysis

We tested maternal microchimerism as a dichotomous variable (postive vs negative) and divided into three categories (negative, ≤median of quantifiable MMc values, >median of the quantifiable MMc values), using logistic regression. We also tested whether the ranks of MMc differed between cases and controls using a Mann-Whitney U test. In a secondary analysis, we stratified the above analysis by specific NIMA alleles. The statistical association between MMc in cord blood and at diagnosis of T1D in the same individual cases was estimated using logistic regression (for presence or absence of measurable MMc).

To assess the sensitivity of our results towards the cut-off for MMc positivity, we reran the analysis when also calling samples positive if only one of 10 replicates were positive.

All analyses were done in Stata Release 15 (College Station, Tx, USA).

Results

Out of 726 genotyped mother-child dyads, 197 (71 T1D cases and 126 controls) provided data for the analysis (Figure 1). Of the 71 cases, a sample at the time of T1D diagnosis with valid MMc data was available from 60. We measured a positive MMc value in 87/197 samples (44%).

Differences between cases and controls

Having any detectable MMc in cord blood was not significantly associated with later T1D (aOR 1.27, 95% CI 0.68-2.36, p=0.45). Investigating specific NIMAs, MMc tended to be associated with increased risk for later T1D in the group with HLA DQB1*03:01 as the NIMA (Table 2).

Investigating MMc quantity divided into three groups (no detectable NIMA vs. below or at median, and above median) gave similar results, with showed no significant associations with T1D overall, and a suggestive positive association for those with HLA DQB1*03:01 as the NIMA (Table 2).

Comparing the ranks of the levels measured did not show any significant difference between cases and controls when summing all NIMAs (Mann-Whitney P = 0.46), or in specific alleles (Mann-Whitney P = 0.07 and 0.77 for HLA DQB1*03:01 and *06:02/3, respectively).

Association between presence of MMc at birth and diagnosis

There was a tendency for presence of MMc in cord blood to be associated with detectable MMc levels in the circulation at diagnosis of T1D (at an average of 6.1 years later), but this was not statistically significant (OR 2.21, 95%CI 0.76-6.46, p=0.15).

Sensitivity analyses

Including more covariates, or changing the cut-off for positive MMc generally resulted in wider confidence intervals, but odds ratios were largely similar (Table 3).

Discussion

In this first study of its kind, we tested whether cord blood MMc at birth was informative of future T1D risk, but we found no significant overall association.

An important strength of this study was the use of digital droplet PCR, which allowed us to investigate the presence of MMc in a more sensitive and specific manner than earlier studies. We observed more children with detectable MMc than a previous study from Korea (44% in our study vs 23%), but we use ddPCR which is considered a more sensitive method. Earlier studies in T1D have investigated individuals after diagnosis in peripheral blood and pancreatic tissue [10,11]. Our results are not consistent with results from these, which could be due to MMc differences between cord blood at birth, and in the pancreas or circulation, as we found no increased risk associated with the presence, or levels, of total MMc. We found a possible association in the HLA DQB1*03:01 NIMA subgroup with later T1D, which must be interpreted with caution.

While maternal cells are believed to cross into the fetus through the umbilical cord, with increased frequency from mid pregnancy towards term, it is only feasible to measure MMc levels on the day of delivery. In theory, transfer of cells could fluctuate temporally. Our study does not rule out an important role of MMc in T1D, for example in terms of specific cell types or their functions in specific tissues postnatally[10,11]. This is an observational study, and we cannot rule out unknown factors influencing MMc levels and later disease. Although the current analysis emanate from a large undertaking, the number of children with informative and measurable MMc in the final analysis was somewhat limited.

In conclusion, maternal microchimerism measured as amount of maternal DNA in cord blood did not significantly predict the risk of future T1D in children.

Conflicts of interest

No other potential conflict of interest relevant to this article was reported. The authors alone are responsible for the content and writing of the paper.

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Acquisition of childhood incident T1D data: TS, GJ, PRN.
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HLA typing: GM, JY, BG, SCK, KMG.

Development of ddPCR method and experimental procedures: GM, JY, BG, SCK, KMG.
Data cleaning and preparation: GT
Planning statistical analyses: LCS, GT, KS.
Performing statistical analyses: GT.
Interpretation of data: All authors.
Drafting the manuscript: GT
Revising the manuscript critically for important intellectual content: All authors.
Final approval of the version to be published: All authors
Taking responsibility for the integrity of the data and the accuracy of the data analysis: GT, LCS, KMG.
Obtaining funding: LCS, KS, KMG

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Table 1: Characteristics of cases with childhood type 1 diabetes and randomly selected controls in the present study.

	Controls (n= 126)	Cases (n = 71)
Median age (range) at end of follow-up* (years)	10.5 (7.0 – 15.9)	11.5 (7.3 – 15.1)
Female sex	64 (50.8%)	34 (47.9%)
Preterm birth	4 (3.2%)	5 (7.0%)
Parity		
No earlier births	60 (47.6%)	35 (49.3%)
One	40 (31.7%)	20 (28.2%)
Two or more	26 (20.6%)	16 (22.5%)
Maternal age (years (median, range))	30 (19 – 40)	30 (22 – 42)
19-24	17 (13.5%)	4 (5.6%)
25-34	86 (68.3%)	55 (77.5%)
35-42	23 (18.3%)	12 (16.9%)
Maternal smoking during pregnancy		
Non-smoker at end of pregnancy‡	104 (82.5%)	61 (85.9%)
Smoked at end of pregnancy	17 (13.5%)	7 (9.9%)
Missing data	5 (4.0%)	3 (4.2%)
Pre-pregnancy BMI (kg/m^2(median, range))	24.1 (18.7 – 37.8)	26.1 (17.3 – 38.6)
<25	67 (53.2%)	28 (39.4%)
25-30	34 (27.0%)	27 (38.0%)
>30	12 (9.5%)	10 (14.1%)
Missing data	13 (10.3%)	6 (8.5%)
Child's HLA genotype§		
Protective (DQ6)	21 (16.7%)	2 (2.8%)
Neutral (any other HLA not mentioned)	29 (23.0%)	3 (4.2%)
Increased risk (≥1 copy of either DQ8 or DQ2.5)	68 (54.0%)	38 (53.5%)
High risk (DQ8/DQ2.5 heterozygote)	8 (6.3%)	28 (39.4%)
Caesarean section‖	15 (11.9%)	7 (9.9%)

* The diagnosis date of the last case included - June 2, 2016

† The median age at diagnosis of T1D cases was 6.3 (range 0.7 – 13.0) years

‡ Including those that quit smoking shortly before or during pregnancy, as the association with T1D has been observed in those that smoked throughout pregnancy [25].

§ Coded as a binary variable in the analysis – protective or neutral (0) vs increased or high risk (1)

|| Includes unknown (n = 1), emergency (n = 9) and elective (n = 13) caesarean section

Table 2: Association between MMC in cord blood and type 1 diabetes

	Cases†	Controls†	OR (95% CI)	aOR (95% CI)*	Adjusted P-value
Presence of detectable MMc					
Any‡ NIMA	32/65	49/113	1.27 (0.71 - 2.27)	1.27 (0.68 - 2.36)	0.45
DQB1*03:01 NIMA	14/20	16/36	2.44 (0.81 - 7.35)	3.89 (1.05 - 14.44)	0.04
DQB1*06:02/3 NIMA	15/33	64/64	1.04 (0.47 - 2.31)	0.96 (0.40 - 2.27)	0.92
Categorical analysis§					
Any‡ NIMA, Undetected	33/65	64/113	Ref.	Ref.	
Any‡ NIMA ≤ median	18/65	26/113	1.52 (0.75 – 3.07)	1.34 (0.64 – 2.81)	0.44
Any‡ NIMA > median	14/65	23/113	1.02 (0.48 – 2.18)	1.20 (0.54 – 2.65)	0.66
Per category increase	65/65	113/113	1.07 (0.74 - 1.53)	1.12 (0.76 - 1.65)	0.56
DQB1*03:01 NIMA, undetected	6/20	20/36	Ref.	Ref.	
DQB1*03:01 NIMA ≤ median	6/20	9/36	2.10 (0.55 – 7.99)	2.74 (0.61 – 12.44)	0.19
DQB1*03:01 NIMA > median	8/20	7/36	2.79 (0.78 – 10.01)	5.77 (1.21 – 27.52)	0.03
Per category increase	20/20	36/36	1.68 (0.89 - 3.17)	2.40 (1.10 - 5.24)	0.03
DQB1*06:02/3 NIMA, undetected	18/33	35/64	Ref.	Ref.	
DQB1*06:02/3 NIMA ≤ median	11/33	15/64	1.65 (0.66 – 4.11)	1.36 (0.52 – 3.62)	0.54
DQB1*06:02/3 NIMA > median	4/33	14/64	0.48 (0.14 – 1.61)	0.51 (0.14 – 1.83)	0.30
Per category increase	33/33	64/64	0.82 (0.49 - 1.36)	0.80 (0.45 - 1.40)	0.43

OR: Odds Ratio; aOR: adjusted OR; CI: Confidence Interval

*: adjusted for maternal age and pre-pregnancy BMI

†: included in the adjusted analysis. There were 71 cases and 126 controls in total in the unadjusted analyses.

‡: combining DQB1*03:01, *04:02 and *06:02/3. The DQB1*04:02 NIMA subgroup was not analysed separately due to low number of children with measurable quantities in this group.

§: the median value was calculated from controls with detectable levels

Table 3: Sensitivity analyses

	aOR (95% CI)*, Main analysis	aOR (95% CI)†, additionally adjusted	aOR (95% CI)*, including unreplicated positives
Presence of detectable MMc			
Any‡ NIMA	1.27 (0.68 – 2.36)	1.28 (0.65 – 2.52)	1.19 (0.62 – 2.27)
DQB1*03:01 NIMA	3.89 (1.05 – 14.44)	6.33 (0.79 – 50.75)	2.39 (0.56 – 10.26)
DQB1*06:02/3 NIMA	0.96 (0.40 – 2.27)	1.05 (0.42 – 2.65)	1.26 (0.49 – 3.20)
Categorical analysis§			
Any‡ NIMA, Undetected	Ref.	Ref.	Ref.
Any‡ NIMA ≤ median	1.34 (0.64 – 2.81)	1.42 (0.63 – 3.20)	0.75 (0.33 – 1.69)
Any‡ NIMA > median	1.20 (0.54 – 2.65)	1.12 (0.47 – 2.69)	1.35 (0.80 – 3.40)
Per category increase	1.12 (0.76 – 1.65)	1.10 (0.72 – 1.68)	1.29 (0.89 – 1.87)
DQB1*03:01 NIMA, undetected	Ref.	Ref.	Ref.
DQB1*03:01 NIMA ≤ median	2.74 (0.61 – 12.44)	5.99 (0.51 – 70.90)	0.83 (0.13 – 5.14)
DQB1*03:01 NIMA > median	5.77 (1.21 – 27.52)	6.65 (0.61 – 72.76)	4.40 (0.91 – 21.30)
Per category increase	2.40 (1.10 – 5.24)	2.64 (0.81 – 8.68)	2.24 (1.01 – 4.97)
DQB1*06:02/3 NIMA, undetected	Ref.	Ref.	Ref.
DQB1*06:02/3 NIMA ≤ median	1.36 (0.52 – 3.62)	1.64 (0.56 – 4.79)	1.20 (0.41 – 3.53)
DQB1*06:02/3 NIMA > median	0.51 (0.14 – 1.83)	0.50 (0.13 – 1.92)	1.31 (0.46 – 3.77)
Per category increase	0.80 (0.45 – 1.40)	0.82 (0.45 – 1.48)	1.17 (0.70 – 1.97)

aOR: adjusted OR; CI: Confidence Interval.

Main analysis is shown leftmost for comparison, followed by an analysis including more covariates, and an analysis including the measured values of samples which were positive in only one of ten replicates (originally set to zero in the main analysis).

*: adjusted for maternal age and pre-pregnancy BMI

†: adjusted for maternal age, pre-pregnancy BMI, sustained smoking during pregnancy, caesarean section, childs' gender and HLA risk for T1D.

‡: combining DQB1*03:01, *04:02 and *06:02/3. The DQB1*04:02 NIMA subgroup was not analysed separately due to low number of children with measurable quantities in this group.

§: the median value was calculated from controls with detectable levels.

Figure 1: Study overview

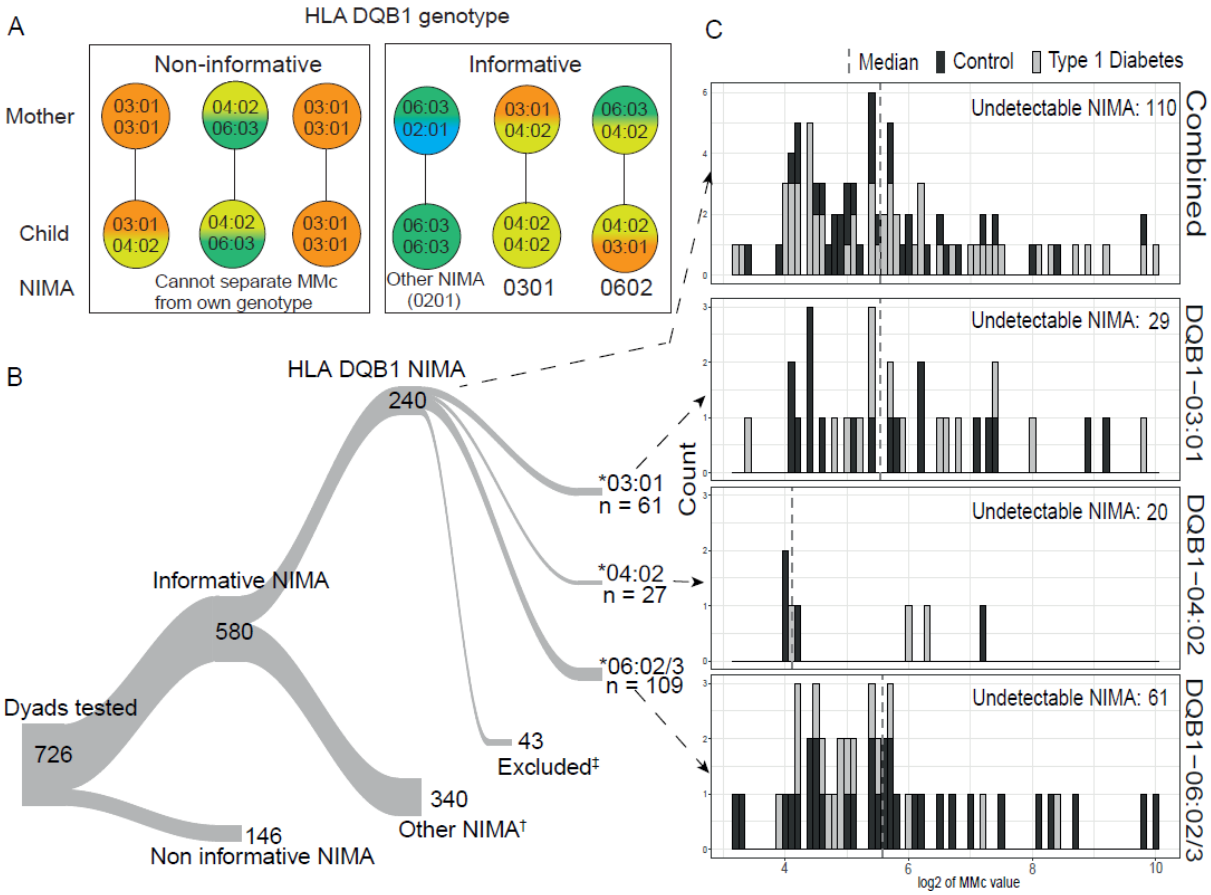

Figure 1 shows how the study design and distribution of participants: Panel A shows example HLA-DQB1 genotypes in mothers and offspring to illustrate whether a NIMA can be measured (informative NIMA), and the resulting NIMA used to measure MMc. Note that as DQB1*06:02 and *06:03 assay are considered one genotype in this study due to the assay used. NIMA was ascertained by comparing maternal and offspring genotypes. If the mother is homozygous, or if mother and child share both alleles, we are unable to measure MMc as we cannot determine NIMA and child's genomic levels of the genotype would mask the maternal signal (non-informative MMc). If the NIMA is informative (child has a NIMA that would not be masked by its own genotype), only the most common NIMA with ddPCR assays available were tested: HLA DQB1*03:01, *04:02 or *06:02/3; others are referred to as "other NIMA". Panel B shows a Sankey diagram to illustrate how many dyads fall into each category in our cohort. Panel C shows a histogram of observed MMc values in cord blood (log2 transformed), in cases and controls, with the median of the positive samples (calculated using values from controls only) marked with a dashed line, and number of negative values listed in the top right corner of each facet. The top facet shows the total of all measured MMc, while the lower panels show per HLA genotype (DQB1*03:01, *04:02 and *06:02/3, in descending order).

†: Includes also dyads without a non-DQB1 NIMA (n = 42).

‡: This group includes samples (n = 26) that were tested with an earlier version of the ddPCR DQB1*03:01 probe which turned out to interact with other alleles, and were excluded. It also includes samples not found or with insufficient DNA (n = 8), samples with genomic levels of MMc (n = 8) potentially caused by contamination from maternal blood and maternal samples included by mistake (n = 1).