Received: 9 June 2017 | Accepted: 24 November 2017

DOI: 10.1111/aji.12802

ORIGINAL ARTICLE

WILEY AJRI American Journal of Reproductive Immunology

Plasma immunological markers in pregnancy and cord blood: A possible link between macrophage chemo-attractants and risk of childhood type 1 diabetes

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Funding information
Norwegian Ministry of Health and Care Services; Norges Forskningsråd, Grant/Award Number: 2210909/F20; Norwegian Ministry of Education and Research; NIH/NINDS, Grant/Award Number: UO1 NS 047537-01 and UO1 NS 047537-06A1; NIH/NIEHS, Grant/Award Number: N01-ES-75558; South-Eastern Norway Regional Health Authority; Oak Foundation; European Research Council; K.G. Jebsen Foundation

Problem: Previous studies have suggested that immune perturbations during pregnancy can affect offspring type 1 diabetes (T1D) risk. We aimed to identify immunological markers that could predict offspring T1D or that were linked to T1D risk factors.

Method of study: We quantified selected circulating immunological markers in mid-pregnancy (interleukin [IL]-1β, IL-1ra, IL-2Rα, IL-2, -4, -5, -6, -10, -12p70, 13, -17A, GM-CSF, IFN-γ, CXCL10, CCL 2, CCL3, CCL4, TNF) and cord blood plasma (neopterin and kynurenine/tryptophan ratio) in a case-control study with 175 mother/child T1D cases (median age 5.8, range 0.7-13.0 years) and 552 controls.

Results: Pre-pregnancy obesity was positively associated with CCL4, CXCL10, kynurenine/tryptophan ratio and neopterin ($P < .01$). The established T1D SNPs rs1159465 (near *IL2RA*) and rs75352297 (near *CCR2* and *CCR3*) were positively associated with IL-2Rα and CCL4, respectively ($P < .01$). There was a borderline association of CCL4 and

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offspring T1D risk, independent of maternal obesity and genotype. When grouping the immunological markers, there was a borderline association (P = .05) with M1 phenotype and no association between M2-, Th1-, Th2- or Th17 phenotypes and offspring T1D risk. **Conclusion**: Increased mid-pregnancy CCL4 levels showed borderline associations with increased offspring T1D risk, which may indicate a link between environmental factors in pregnancy and offspring T1D risk.

KEYWORDS
cord blood, cytokine, human, inflammatory markers, Norwegian Mother and Child Cohort Study, pregnancy, type 1 diabetes

1 | INTRODUCTION

Type 1 diabetes (T1D) is one of the most prevalent chronic conditions in children and is associated with increased morbidity and mortality.[1] Mechanisms driving the rising incidence in most countries over past decades are unknown. The insulin-producing pancreatic β-cells are destroyed in an autoimmune process, triggered or driven by environmental and genetic factors increasing T1D susceptibility,[2] which may operate already in foetal life.[3]

Cytokines are small proteins secreted by nearly all cell types to induce or modulate immune responses and play important roles in a wide spectrum of pathologies. Cytokines are pleiotropic, and combinations of cytokines can dictate T-helper (Th) lymphocyte differentiation, or activate macrophages to pro- and anti-inflammatory states,[4,5] immune cell phenotypes that secrete distinct cytokine repertoires. Several T1D susceptibility genetic markers are in or near genes associated with cytokines or cytokine signalling.[6] Cytokine-driven immune responses are likely factors leading to β-cell destruction,[7] and evidence is accumulating that circulating cytokines and related markers may predict T1D.[8,9]

Cytokines are also involved in regulating normal pregnancy.[10] Pregnant women with an inflammatory state involving cytokines such as infections, obesity[11] and higher age have a higher risk of offspring T1D.[3,12] Maternal cytokine secretion may affect the foetus, for instance, by inducing inflammation in, or crossing over, the placenta. Although some cytokines such as IL-6 can cross the placenta,[13] most cytokines and inflammatory markers do not.[13,14] However, macrophage accumulation[15] and expression of toll-like receptors in the placenta may amplify maternal inflammatory signals,[16] and thus contribute to foetal disease risk.[15] Previous studies have reported associations between maternal inflammatory markers and later health outcomes such as asthma and allergies,[17] supporting that maternal inflammatory cytokines affect the offspring disease susceptibility later in life. To the best of our knowledge, there is only one small study published on maternal cytokines and offspring T1D risk. This study reported an association between increased early-pregnancy serum levels of IFN-γ and IL-1β and higher risk of offspring T1D;[18] however, such associations need to be confirmed in larger studies.

To address the knowledge gap regarding antenatal immune perturbations associated with offspring T1D risk, we aimed to study circulating immunological markers in maternal mid-pregnancy plasma (interleukin [IL]-1β, IL-1 receptor antagonist [IL-1Ra], IL-2 receptor α [IL-2Ra], IL-2, -4, -5, -6, -10, -12p70, -13, -17A, granulocyte-macrophage colony-stimulating factor [GM-CSF], interferon [IFN]-γ, C-X-C motif chemokine 10 [CXCL10, interferon gamma-induced protein 10], C-C motif chemokine ligand [CCL] 2 [monocyte chemo-attractant protein 1], CCL3 (macrophage inflammatory protein [MIP] 1α), CCL4 [MIP-1β], tumour necrosis factor [TNF]) and markers of IFN-γ activation in the child's cord blood plasma (neopterin and the kynurenine/tryptophan ratio [KTR]), in a large case-control study of offspring T1D, nested within one of the largest pregnancy cohorts in the world. The immune markers measured in maternal plasma were carefully selected, either due to prior association with T1D or coeliac disease, to ensure representation of several types (M1 and M2 macrophage phenotypes, and Th1, Th2,

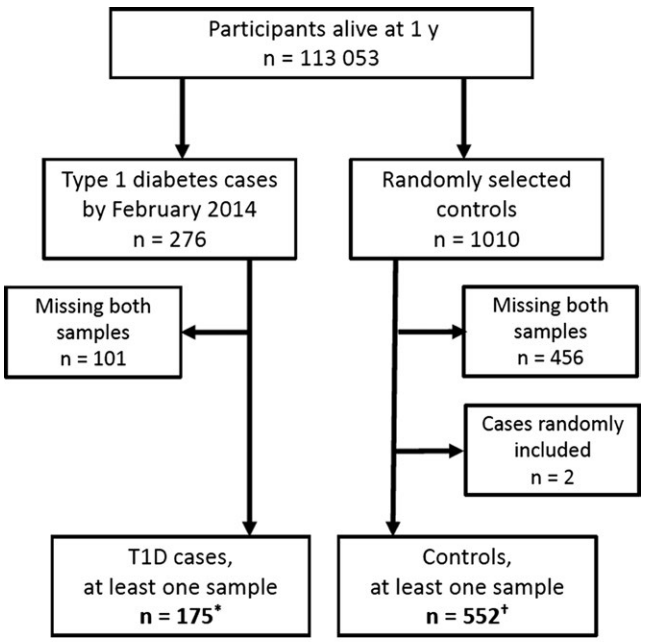


FIGURE 1 Flow chart of the participants

Th17 and Treg) of immune responses, and believed to give reliable measurements given our sample handling. As cord blood plasma were not immediately separated and more frequently are affected by some degree of haemolysis, we measured only neopterin and KTR in cord blood, two inflammatory markers that have been shown to be relatively stable at the sample handling and storage conditions used in this study.[19] A secondary aim was to assess associations of other suggested risk factors for T1D, such as maternal obesity or genotype, and these circulating immunological markers.

2 | METHODS

2.1 | Subjects and design

We designed a nested case-control study in the Norwegian Mother and Child Cohort Study (MoBa).[20] MoBa recruited around 114 000 pregnancies from all over Norway during 1999-2008, and children who developed T1D by 5 February 2014 were identified with a high degree of ascertainment by register linkage to the Norwegian Childhood Diabetes Registry.[21] In all, 175 mother/child pairs were included as T1D cases, and 552 mother/child pairs from a random sample of the cohort with available blood samples were used as controls (Figure 1).The randomly selected controls were largely similar to the whole MoBa cohort, with the exception of Caesarean section and premature birth (15.2% vs 10.3% for Caesarean section, and 6.2% vs 3.4% for premature birth, in the whole MoBa cohort vs picked random controls),[22] conditions associated with missing blood samples in the MoBa Cohort. Characteristics of the study participants are given in Table 1. The establishment and data collection in MoBa have obtained a licence from the Norwegian Data Inspectorate and approval from The Regional Committee for Medical Research Ethics, and the study was approved by The Regional Committee for Medical Research Ethics in South-Eastern Norway. Informed consent was obtained from all study participants.

2.2 | Sample handling

Venous maternal blood was collected in EDTA tubes around pregnancy weeks 17-19, centrifuged and shipped overnight as plasma in ambient temperature to the Norwegian Institute of Public Health for storage at −80°C. At delivery, umbilical cord vein blood was collected in EDTA tubes and shipped overnight in ambient temperature before centrifugation and storage at −80°C until analysis. Cord blood plasma samples were visually assessed for degree of haemolysis and graded according to a four-level colour code.[23] Maternal and cord blood samples were treated separately but equally, with personnel blinded to case-control status at all stages of sample handling and analysis.

2.3 | Laboratory assays

We selected a limited set of cytokines based on prior association with either coeliac disease or T1D, and to ensure representation of macrophage M1 and M2 phenotypes, and Th1, Th2, Th17 and Treg immune responses in the adaptive immune system. We avoided cytokines known or suspected to be present in undetectable concentrations in most healthy individuals, or to give unreliable measurements, for example due to pre-analytical factors. In total, we measured 18 cytokines in maternal mid-pregnancy plasma using Bio-Plex protein array systems (Bio-Rad, Hercules, CA, USA), based on xMAP technology (Luminex, Austin, TX, USA) as previously described[23]—IL-1β, IL-1 receptor antagonist (IL-1Ra), IL-2 receptor α (IL-2Ra), IL-2, -4, -5, -6, -10, -12p70, -13, -17A, GM-CSF, IFN-γ, C-X-C motif chemokine 10 (CXCL10, interferon gamma-induced protein 10), C-C motif chemokine ligand (CCL) 2 (monocyte chemo-attractant protein 1), CCL3 (MIP-1α), CCL4 (MIP-1β), TNF. Concentrations were calculated from a standard curve generated from standards provided by the assay manufacturer, and standard curves showing high sensitivity were selected to obtain similar lower limit of quantification (LLOQ) for all samples. The LLOQ ranged from 0.02 (IL-4) to 1.35 (CCL2) pg/mL and corresponds to the lowest point of the standard curve yielding acceptable recovery. The inter-plate mean coefficient of variation (CV) across all dilutions ranged from 2.2% (CCL4) to 8.0% (IL-4). Further details of limits of quantification, inter- and intra-assay CVs are given in previous publication.[23] Umbilical cord plasma concentrations of cotinine, neopterin, tryptophan and kynurenine were measured using a high-throughput liquid chromatography tandem mass spectrometry at the Bevital laboratory (Bergen, Norway),[24] which have previously demonstrated acceptable pre-analytical stability.[19] The kynurenine/tryptophan ratio (KTR) was calculated by dividing the plasma concentration of kynurenine (nmol/L) by the concentration of tryptophan (μmol/L). A brief summary of the immune markers studied is given in the online repository of Mårild et al.[23]

2.4 | Genotyping

To account for established T1D susceptibility markers, participants were genotyped using a custom Illumina Golden Gate assay (Illumina, San Diego, CA, USA). DNA extraction, genotyping methods and quality control procedures are described in detail in previous publication.[23] Briefly, tag-SNPs (n = 144) on chromosome 6 were used to impute human leucocyte antigen (HLA) class II genotype,[25] which was subsequently confirmed by classical HLA genotyping using allele-specific PCR. Based on the previously established risk of T1D conferred by HLA genotype, we categorized genotypes into four groups: protective (carrying at least one copy of HLA DQA1*01:02-DQB1*06:02-DRB1*15:01 [DQ6-DR15]), increased risk (at least one copy of HLA DQA1*03-DQB1*03:02-DRB1*04 [DQ8-DR4] or DQA1*05:01-DQB1*02:01 [DQ2-DR3], but not both haplotypes), high risk (heterozygous HLA DQ2-DR3/DQ8-DR4) or neutral (any other genotype). Established non-HLA T1D risk SNPs (n = 51) were used to calculate a weighted additive genetic risk score (GRS, sum of risk alleles, weighted by their odds ratio [OR] for T1D reported in the literature, see Table S1). SNPs possibly associated with T1D and cytokine levels (n = 19, see Table S2) were tested against maternal

TABLE 1 Characteristics of the study participants

	Controls (n = 552)	Cases (n = 175)
Median age (range) at end of follow-up (years)	8.6 (4.7-14.1)	9.8 (5.0-13.3)[a]
Female sex	272 (49.3%)	87 (49.7%)
Maternal type 1 diabetes	0 (0.0%)	7 (4.0%)
Preterm birth	19 (3.4%)	10 (5.7%)
Missing data	1 (0.2%)	3 (1.7%)
Birthweight (in grams)		
<2500 g	8 (1.4%)	7 (4.0%)
2500-3500 g	231 (41.9%)	66 (37.7%)
>3500-4500 g	285 (51.6%)	94 (53.7%)
>4500 g	28 (5.1%)	8 (4.6%)
Parity		
No earlier births	240 (43.5%)	86 (49.1%)
One	203 (36.8%)	53 (30.3%)
Two or more	109 (19.8%)	36 (20.6%)
Maternal age (years)		
<25	70 (12.7%)	16 (9.1%)
25-35	390 (70.6%)	134 (76.6%)
>35	92 (16.7%)	25 (14.3%)
Self-reported maternal infections in pregnancy		
None	191 (34.6%)	62 (35.4%)
One	172 (31.2%)	54 (30.9%)
Two or more	189 (34.2%)	59 (33.7%)
Maternal smoking during pregnancy		
Not during pregnancy	388 (70.3%)	127 (72.6%)
Stopped smoking prior to pregnancy	14 (2.5%)	5 (2.9%)
Smoked at any time during pregnancy	119 (21.6%)	37 (21.1%)
Missing data	31 (5.6%)	6 (3.4%)
Maternal pre-pregnancy BMI (kg/m^2)		
<25	309 (55.6%)	76 (43.4%)
25-30	136 (24.6%)	55 (31.4%)
>30	50 (9.1%)	28 (16.0%)
Missing data	57 (10.3%)	16 (9.1%)
Child's HLA genotype and genetic risk score		
Protective (DQ6)	171 (31.0%)	3 (1.7%)
Baseline (any other HLA not mentioned)	112 (20.3%)	5 (2.9%)
Increased risk (≥1 copy of either DQ8 or DQ2.5)	210 (38.0%)	94 (53.7%)
High risk (DQ8/DQ2.5 heterozygote)	31 (5.6%)	72 (41.1%)
Missing HLA genotype	28 (5.1%)	1 (0.6%)
T1D non-HLA GRS[b] (median, IQR)	61.2, 47.3-75.2	63.2, 51.0-76.7
Number of freeze-thaw cycles (median, range)[c]		
Cord blood samples	3, 1-9	3, 1-7
Mid-pregnancy samples	4, 1-10	4, 1-9
Caesarean section[d]	57 (10.3%)	34 (19.4%)

[a]The median age at diagnosis of T1D cases was 5.8 (range 0.7-13.0) years.
[b]The genetic risk score was calculated as the number of risk alleles of SNPs associated with T1D, weighted by their ORs (see Table S1).
[c]Categorized as 1, 2, 3, 4, 5, 6.3 for cord blood samples and 1, 2, 3, 4, 5, 6, 7.5 for maternal mid-pregnancy samples. To avoid small groups, all freeze-thaw cycles >5 in cord blood and >6 in maternal mid-pregnancy samples were categorized together at their mean value (6.3 and 7.5 for cord blood and maternal mid-pregnancy samples, respectively).
[d]Includes unknown (n = 1), emergency (n = 53) and elective (n = 37) Caesarean section.

immunological markers to assess if there were any specific genetic effects that should be included in the analysis.

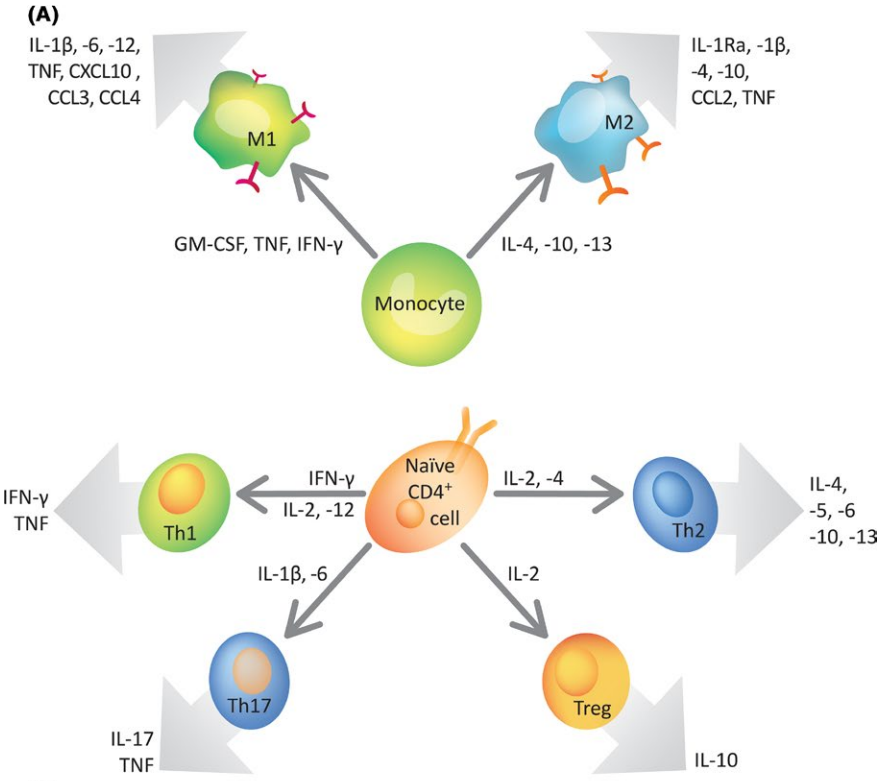
2.5 | Other variables

Predefined variables potentially associated with the selected immunological markers and/or T1D were assessed as predictors for the immunological markers and included as covariates in the analysis of association between cytokines and T1D. Maternal smoking was assessed by self-reported questionnaires and concentration of umbilical cord plasma cotinine[24] (a biomarker for recent nicotine exposure, with children having ≥30 nmol/L assumed to be exposed to cigarette smoke during pregnancy irrespective of maternal self-reported data or missing information). Maternal infection frequency was assessed from self-reported questionnaires.[23] Maternal age at delivery, parity, mode of delivery, child's sex, birth weight, gestational age, month and calendar year of birth were collected from the Medical Birth Registry of Norway. Pre-pregnancy body mass index (BMI, calculated as weight in kilograms divided by height in metres squared) was collected from both questionnaires and birth records, and obesity defined as BMI ≥30. Maternal T1D was collected from birth records, self-reported questionnaires and ICD-10 codes E10.0-E10.9 in the Norwegian Patient Registry. The distribution and categorization of these variables are shown in Table 1. The number of freeze-thaw cycles (median 4, range 1-10 for maternal samples and median 3, range 1-9 for cord blood samples) had a relatively small, but statistically significant association with several cytokine concentrations, while degree of haemolysis in cord blood had a similarly small, but significant effect on neopterin.[23] Maternal samples were spun down at the hospital then shipped overnight (median 1 day in transit, interquartile range [IQR] 1-2 days), while cord blood samples were shipped overnight then spun down at arrival (median 2 days in transit, IQR 1-3 days). Time in transit had a small, but significant effect on neopterin (fold difference 1.04, $P < .01$), but we did not adjust for time in transit as the effect on the reported neopterin estimates was negligible.

2.6 | Statistical analysis

A priori, we decided to base our primary analysis on a logistic regression model with T1D as the dependent variable and $\log_2$-transformed immunological markers as the explanatory variables. In a secondary analysis, we assessed possible deviation from linearity by testing the significance of a squared term of the explanatory variables and by categorical analysis. Based on the distribution of the immunological marker among controls, observations were categorized into tertiles. For markers where >33% of samples were below detection limit (IL-1β, IL-5, IL-12p70, IL-13, IFN-γ, CCL2, IL-2Rα, IL-17A), all the values below the detection limit were put in the lowest category and remaining levels were split in two groups at their median. Associations between predictors (maternal BMI, etc.) and immunological markers in pregnancy and cord blood were assessed using linear regression with the $\log_2$-transformed immunological markers as the dependent variable. As 20 immunological markers with some a priori evidence for association with T1D were tested, and cytokines tend to be positively correlated, Bonferroni correction for multiple testing would be too conservative.[26] We decided a priori to use 0.01 as our significance level in the analyses of each single immunological marker.

The primary statistical test of the association between immunological markers and the offspring's risk of T1D was a m-1 degree of freedom likelihood ratio test, where m equals the number of immunological markers (testing the complete null hypothesis that none of the markers were associated vs the alternative that at least one marker was associated with offspring T1D risk). Next, we used a likelihood ratio test to assess each of five subsets of immunological markers grouped as M1-, M2-, Th1-, Th2- or Th17-pathway immunological markers (see Figure 2, Panel A for a simplified overview of pathways and their cytokines). We attempted to choose the most characteristic immunological markers for these subsets and tried to avoid including



(B)

Pathway (cell type)	Cytokines	*P*-value
Adaptive immune system		
Th1	IL-12, IL-2rα* (differentiation), IFN-γ and TNF-α (secretion)	.53
Th2	IL-4 (differentiation and secreted by Th1) and IL-2rα* (differentiation), IL-5, IL-13 (secretion)	.44
Th17	IL-6 and IL-1β (differentiation), IL-17 (secretion)	.23
Innate immune system		
M1	GM-CSF and IFN-γ (activation), CXCL10, CCL3 and CCL4 (secretion)	.05
M2	IL-4 and IL-13 (activation), CCL2, IL-1ra and IL-10 (activation and secretion)	.09

FIGURE 2 Simplified overview of the stimulation by, and secretion of, the selected maternal immunological markers. *IL2-Rα chosen as a better marker of IL-2 activity than IL-2 itself

immune markers which overlap between different subsets, thus not all immunological markers shown in the figure are included. Z-scores for each cytokine were calculated and used in the analysis of groups and the model including all cytokines. Further, we tested each single immunological marker as predictors of T1D, adjusted for maternal pre-pregnancy BMI, child's HLA risk category, gestational age at delivery, mode of delivery and year of birth. We also assessed the impact of additional adjustment for maternal smoking during pregnancy, child's non-HLA GRS, maternal T1D, parity, freeze-thaw cycles and maternal age. Finally, we did an exploratory, hypothesis-free analysis of cytokine patterns using k-means cluster analysis with 8 clusters on the $\log_2$-transformed maternal immunological markers. This assigned individuals to clusters (groups) with similar pattern across the 18 maternal immunological markers. We then compared the resulting clusters with respect to risk of offspring T1D in a logistic regression model.

3 | RESULTS

Established risk factors for T1D such as HLA class II and maternal T1D were more common in cases than in controls. Other characteristics were similar in cases and controls, except a higher proportion of maternal BMI above 30 among cases was observed (see Table 1). The child's median age at the end of follow-up was 8.6 (range 4.7-14.1) years in controls (see Table 1), and the median age at diagnosis of T1D cases was 5.8 (range 0.7-13.0) years. The distribution of most immunological markers was approximately symmetrical after $\log_2$-transformation (see Figure S1), although for around half of the markers, a relatively large proportion of the participants had levels below the LLOQ. There were strong positive pairwise correlations between immunological markers measured in mid-pregnancy maternal plasma (IL-1β, -1Ra, -2Ra, -2, -4, -5, -6, -10, -12p70, -13, -17A, GM-CSF, IFN-γ, CXCL10, CCL2, CCL3, CCL4, TNF), but not in markers measured in cord blood plasma (neopterin, KTR) (see Figure S2). Maternal mid-pregnancy IFN-γ was not associated with child's neopterin or KTR.

3.1 | Predictors of immunological markers

There was no significant seasonal variation for the level of any of the immunological markers, which implies seasonal infections do not influence levels of the immunological markers in a systematic fashion (See Figure S3). Maternal pre-pregnancy BMI was positively associated with maternal CCL4 and CXCL10 concentrations, as well as neopterin and KTR in cord blood, and had a borderline (P = .02) positive association with IL-6 (see Table S3 and Figure S4). Notably, maternal BMI significantly predicted cord blood neopterin and KTR also after adjusting for the child's birth weight (or ponderal index), while birthweight did not significantly predict cord blood concentrations of neopterin and KTR when adjusted for mothers BMI. Maternal levels of GM-CSF were lower in preterm deliveries and increased in mothers smoking during pregnancy. Carrying the T1D high-risk HLA genotype (heterozygous DQ2/DQ8) was associated with cord blood KTR (see Table S3 and Figure S4). The T1D non-HLA GRS was not significantly associated with any of the immunological markers (see Table S3). Some maternal SNPs were associated with maternal mid-pregnancy cytokine levels at P < .01, such as *IL2RA* SNP rs11594656[27] associated with soluble IL-2Rα, SNP rs3024505 (flanking *IL10)* associated with IL-10 and SNP rs75352297 (intergenic between C-C chemokine receptor *CCR3* and *CCR2*) associated with CCL4 (see Table S2 and Figure S4).

3.2 | Pregnancy and cord blood immunologic markers and risk of type 1 diabetes

The overall likelihood ratio test of the null hypothesised that none of the 20 immunological markers (IL-1β, -1Ra, -2Ra, IL-2, -4, -5, -6, -10, -12p70, -13, -17A, GM-CSF, IFN-γ, CXCL10, CCL2, CCL3, CCL4, TNF measured in maternal mid-pregnancy plasma, and neopterin and KTR measured in cord blood plasma at birth) were associated with T1D gave P = .17. The likelihood ratio tests of the macrophage M1 (CCL3, -4, CXCL10, IFN-γ, GM-CSF) immunological markers gave P = .046, and the related M2 (CCL2, IL4, IL13, IL1ra, IL10) immunological markers gave P = .09, while tests for the Th1, Th2 and Th17 groups gave $P \geq .20$ (see Figure 2B).

Modelling each single immunological marker for association with T1D revealed no significant associations at the predefined 0.01 level after adjustment for covariates. Maternal mid-pregnancy plasma CCL4 showed a borderline association with T1D per doubling (P = .04 per $\log_2$ increase, see Figure 3). Some immunological markers had an increased estimate and borderline association (ORs between 1.52 and 1.60, P-values between .02 and .05) for the highest tertile category in the unadjusted analysis, such as maternal mid-pregnancy plasma CCL2, IFN-γ, IL-4 and TNF, but these were all not significant after adjustment although their estimates were still increased (data not shown, and see Figure S5). Adjustment for potential confounders had limited influence on most associations (see Figure S6). Likewise, adjusting for genotypes influencing immunological marker levels (eg adjusting for the rs75352297 genotype, which was significantly associated with CCL4 levels, when analysing CCL4) did not influence our results to an appreciable degree (data not shown). Of note, the association between maternal obesity (BMI ≥30) and higher risk of offspring T1D (OR 2.28, 95% CI: 1.35-3.85, compared to BMI <25) remained significant after adjustment for immunological markers CCL4, CXCL10, neopterin and KTR (adjusted OR 1.99, 95% CI: 1.09-3.64).

3.3 | Post hoc analyses

For immunological markers where there was a suggestive threshold association with T1D at the highest category (CCL4, IFN-γ, IL17a, IL2Rα, TNF, neopterin, see Figure S5), we explored associations using the 90th percentile as threshold. CCL4 (OR 2.37, 95% CI 1.10-5.10, P = .03), neopterin (OR 0.40, 95% CI 0.16-0.99, P = .05) and IL17a (OR 2.16, 95% CI 1.03-4.54, P = .04) had suggestive associations with T1D development, but these must be interpreted with caution as the analyses were data-driven.

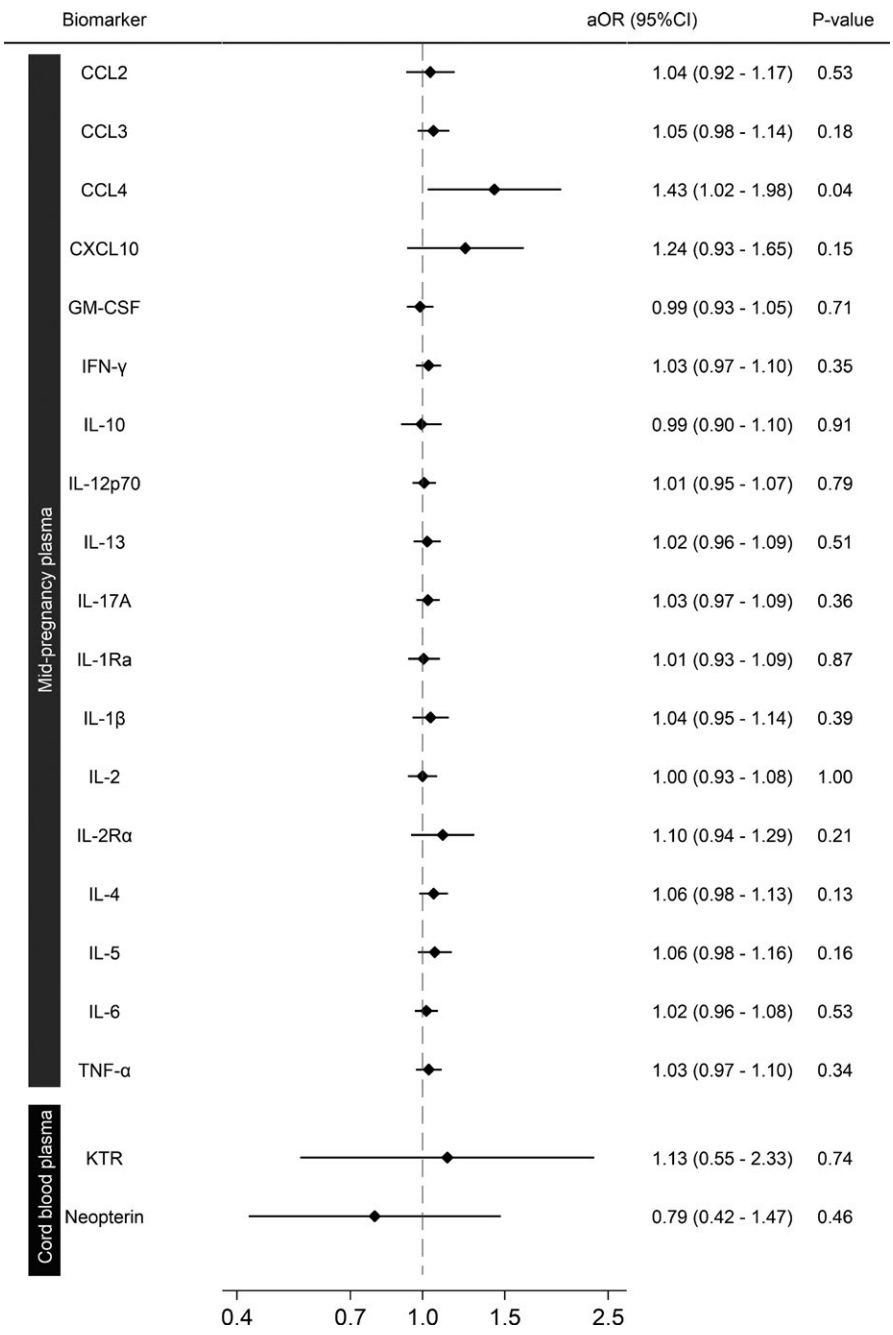


FIGURE 3 Immunological markers and their association with offspring T1D per $\log_2$ increase. A $\log_2$ increase represents a doubling. These results are adjusted for maternal pre-pregnancy BMI, child's T1D HLA risk category, gestational age at delivery, mode of delivery and year of birth. Neopterin is additionally adjusted for degree of haemolysis in cord blood. aOR, adjusted odds ratio; 95% CI, 95% CI confidence interval

As a sensitivity analysis, we adjusted for coeliac disease (present in 21 cases and 5 controls), which only slightly changed our estimates, with the largest change in the adjusted estimate per $\log_2$ increase in CCL4 and T1D risk (OR 1.52, 95% CI 1.08-2.13, P = .02).

Finally, *k*-means cluster analysis on the maternal cytokines reliably clustered individuals in eight groups based on similarity in pattern of cytokine concentrations. Visual inspection of patterns of mean cytokine concentrations across the eight groups did not have any obvious correspondence to any of our a priori pathways (Th1, M1, etc.), and there was no clearly significant association between assigned group and risk of T1D, with the exception of Cluster 8 which had a borderline protective association (OR 0.52, 95% CI 0.29-0.92, P = .03) when compared to Cluster 1. Cluster 1 was characterized by high levels of all cytokines, in particular IFN-γ and IL-17a, while Cluster 8 was characterized by average and slightly higher than average level of all measured maternal immune markers except CXCL10, CCL4 and IL2-Rα, which were slightly lower than average (Figure S7).

4 | DISCUSSION

While none of the immunological markers were significantly associated with childhood-onset T1D according to our predetermined 0.01 level, there were borderline significant associations of increased maternal CCL4 with childhood T1D risk. Another finding was that

maternal pre-pregnancy obesity was associated with higher concentrations of CCL4, CXCL10, neopterin and KTR and remained associated with T1D also after adjustment for these immunological markers.

Interestingly, a common feature of CCL4 and other cytokines with a possible association with T1D risk in the offspring, such as IFN-γ, is activation of pro-inflammatory macrophages. In response to tissue damage and pathogen invasion, $CD4^+$ T-cells are differentiated to Th1, which main product is IFN-γ. IFN-γ stimulates macrophages to become pro-inflammatory,[28] designated M1 macrophages known to secrete chemokines like CCL4,[29] as opposed to M2 macrophages that are anti-inflammatory and a result of stimulation by IL-4 and -10 and with a different secretory repertoire. Pregnancy is dependent on a finely tuned regulation of immune responses, and after the first trimester, the maternal circulating cytokine pattern skews towards a type 2 polarization,[30] while IFN-γ levels are depressed throughout the whole pregnancy.[31] Similarly, decidual macrophages exhibit a mixed M1/M2 phenotype that can secrete CCL4[32] during the first half of the pregnancy, before switching to a M2 phenotype in the latter part of pregnancy.[33] Deviations in maternal immune status in pregnancy have been linked with immune pathologies. Maternal exposure to Th2-inducing environmental irritants enhance offspring allergic responses,[34] and maternal IL-17a promotes autism-like behaviour abnormalities in offspring in mice.[35] In humans, Lindehammer and co-workers found an association between increased IFN-γ in maternal sera and T1D in their children.[18] Our observation of an association between M1 cytokine levels in mothers and offspring T1D risk supports that a skewing towards type 1 immune responses in mothers could influence offspring T1D risk.[36,37] The underlying mechanism behind the potential association of increased maternal plasma levels of M1 cytokines, in particular CCL4, and offspring T1D remains speculative. CCL4 is a CC chemokine that has affinity for the C-C chemokine receptor type 5 (CCR5) and is a chemo-attractant for, and activates, several immune cells.[38] In the pregnant woman, there are several sources for plasma cytokines, including circulating leucocytes and the placenta. Whether the cytokine pattern observed during pregnancy in the maternal circulation reflect the foetal circulation is uncertain, but maternal inflammation has been associated with cord blood inflammation.[39] The crossing of these cytokines over the placenta has not been studied in detail, although CCL4 is expressed at the maternal-foetal interface.[40] CCL4 is found in cord blood, with higher levels in preterm infants,[41] so macrophages with phenotypes similar to M1 macrophages could be present in foetal life. Indeed, macrophages are involved in foetal development, and recent evidence suggests that the majority of tissue macrophage lineages in adults, at least in mice, are established during embryonic development.[42] It is therefore tempting to speculate that the maternal immunological state can program foetal macrophages to a M1 phenotype causing a susceptibility to T1D development in the offspring. Such programming could be a result of maternal IFN-γ crossing the placenta or affecting foetal immune cells at the maternal-foetal interface, or involve a higher level of regulation, such has epigenetic factors, but these possibilities need to be assessed in further studies and are currently unclear.

Our findings are in accordance with studies in T1D patients which demonstrate elevated circulating levels of CCL4,[43,44] CCL2[7,43] and IFN-γ[8]. High-risk individuals without T1D had an elevated level of CCL4[9,45] and IFN-γ[8], and IFN-γ levels were higher in high-risk persons than in patients with recent onset T1D.[46] In experimental studies using mouse models, these cytokines are essential in initiating pancreatic inflammation. Overexpression of IFN-γ in β-cells has been shown to lead to destruction of these cells,[47] and expression of CCL2 by β-cells leads to accumulation of macrophages in the islets.[48] Intriguingly, pancreatic macrophages are thought be essential in T1D pathogenesis,[49,50] being the first cells to infiltrate Langerhans' islets.[51] Thus, previous studies support a mechanistic role for M1-associated cytokines in orchestrating inflammation and injury early T1D development, although experimental studies are contradictory regarding the role of CCL4.[52]

Many of our findings are coherent with the earlier literature and support that obesity and smoking are inflammatory states. We demonstrated an association between increased GM-CSF levels and maternal smoking status, a link supported by in vitro studies.[53] Individuals with obesity in general have been shown to have higher circulating levels of CCL4,[54] CXCL10,[55] neopterin[56] and KTR,[57] in line with our results in pregnant women. We also found an association of maternal IL-6 with BMI (fold difference 1.05, $P = .02$), which has been contradictory in previous studies.[58] Obesity is associated with increased M1 polarization,[59] and maternal obesity is a risk factor for offspring diabetes.[12] Although inflammation could be a potential link between maternal obesity and offspring T1D risk, the fact that this association and offspring T1D were not materially changed after adjustment for immunological markers suggests that the immunological markers measured in our study did not explain the link between these two conditions. Furthermore, we found significant associations between the established T1D susceptibility SNP rs11594656 (near *IL2RA*) and soluble IL-2Rα levels, supporting similar observations in non-pregnant adults,[27,60] and between rs3024505, flanking the *IL10* locus and maternal circulating IL-10, consistent with a previous study.[61] The association between rs75352297 (a SNP near CCR2 and CCR3) and CCL4 levels is somewhat surprising as the main receptor for CCL4 is CCR5. However, rs75352297 is in linkage disequilibrium with rs6808835, which has been reported to influence CCL4 levels in cerebrospinal fluid,[62] and is in the *CCR-Like 2* gene which is close to *CCR5*. Thus, rs75352297 might be a marker for haplotypes which could influence CCL4 levels, although the specific mechanisms are unknown.

Strengths of the study include the prospective design, the large number of samples tested and prospectively collected information on covariates that allowed us to statistically adjust for a number of relevant factors. Weaknesses of the study include suboptimal sample handling. In cord samples, marker concentrations could be influenced by a longer period before separation, and an increase in neopterin levels has been reported in similar conditions.[63] However, because of our prospective design, any influence of suboptimal sample handling should not depend on outcome (T1D case status) and is therefore not likely to create any spurious association. On the other hand, we cannot exclude type II errors. Some samples underwent several freeze-thaw cycles, but our data indicate that the measured concentrations are only

slightly changed by repeated freeze-thaw cycles. We cannot exclude the role of processes taking place in other periods of the pregnancy, local effects by cytokines that are not secreted systemically or effects exerted by cytokines restricted to the foetal circulation. Reports of links between cytokine levels in newborns and T1D risk[64] indicate that the cytokine patterns in the foetal circulation may hide additional cues to the evolvement of T1D predisposition. Although we attempted to limit the number of immunological markers to test, we cannot exclude multiple testing problems.

In conclusion, increased mid-pregnancy maternal levels of cytokines related to activated M1 macrophages, in particular CCL4, might influence offspring T1D risk, although more studies are needed to confirm this association.

ACKNOWLEDGMENTS

The authors thank Siri Beisvåg Rom and Gro Jensen at Department of Medical Biochemistry at Diakonhjemmet Hospital for excellent technical assistance. The Norwegian Mother and Child Cohort Study is supported by the Norwegian Ministry of Health and Care Services and the Ministry of Education and Research, NIH/NIEHS (contract no N01-ES-75558), NIH/NINDS (grant no.1 UO1 NS 047537-01 and grant no.2 UO1 NS 047537-06A1). We are grateful to all the participating families in Norway who take part in this ongoing cohort study. The Norwegian Childhood Diabetes Registry is funded by the South-Eastern Norway Regional Health Authority. Ketil Størdal was supported by an unrestricted grant from the Oak Foundation, Geneva, Switzerland. Costs of all data acquisition, including laboratory assays in MoBa (the sub-study PAGE; Prediction of Autoimmune Diabetes and Coeliac Disease in Childhood by Genes and Perinatal Environment), were supported by grant 2210909/F20 from the Norwegian Research Council (Dr. Lars C. Stene). The study was in part supported by funds from the European Research Council, the K.G. Jebsen Foundation and the Research Council of Norway (to Dr. Pål R: Njølstad).

AUTHOR CONTRIBUTIONS

Conception and design: LCS, MV, GT, KS, KM, JPB. Literature search: MV, GT, LCS. Acquisition of pregnancy cohort data: LCS, KS, PM. Acquisition of childhood incident T1D data: TS, GJ, PRN. Laboratory measurements of immunological markers: MV, GT, ØM, PMU. Genotyping and HLA imputation: KMG, MKV, GT. Data cleaning and preparation: GT, MV, LCS, KS, KM. Planning statistical analyses: LCS, GT, MV, KS, KM. Performing statistical analyses: GT, LCS. Interpretation of data: All authors (MV, GT, KM, ØM, PMU, MKV, PM, JPB, KMG, TS, PRN, GJ, KS, LCS). Drafting the manuscript: MV, GT, LCS. Revising the manuscript critically for important intellectual content: All authors (MV, GT, KM, ØM, PMU, MKV, PM, JPB, KMG, TS, PRN, GJ, KS, LCS). Final approval of the version to be published: All authors (MV, GT, KM, ØM, PMU, MKV, PM, JPB, KMG, TS, PRN, GJ, KS, LCS). Taking responsibility for the integrity of the data and the accuracy of the data analysis: GT, LCS, MV. Obtaining funding: LCS.

CONFLICT OF INTEREST

Dr. Ueland is a member of the steering board of the non-profit Foundation to Promote Research into Functional Vitamin B_{12} Deficiency, which owns Bevital. Dr. Midttun is an employee at Bevital, who carried out the analyses on cord blood. No other potential conflict of interest relevant to this article was reported. The authors alone are responsible for the content and writing of the manuscript.

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SUPPORTING INFORMATION

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How to cite this article: Vistnes M, Tapia G, Mårild K, et al. Plasma immunological markers in pregnancy and cord blood: A possible link between macrophage chemo-attractants and risk of childhood type 1 diabetes. *Am J Reprod Immunol*. 2017;e12802. https://doi.org/10.1111/aji.12802