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ORIGINAL ARTICLE

Gastroenterology: Celiac Disease

Impact of placental and peripheral blood DNA methylation on celiac disease susceptibility

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Abstract

Objectives: Several studies suggest that the first immunogenic insult in celiac disease (CeD) could occur during fetal development. The placenta is a key organ that could link the environment with the genome and future outcomes, including CeD. Our objective is to determine the involvement of placental DNA methylation (DNAm) as potential mediator of the genetic susceptibility to CeD.

Methods: We used Summary-data-based Mendelian Randomization to infer what part of the susceptibility to CeD acts through DNAm in placenta or peripheral blood. We interrogated whether DNAm of the CpGs identified correlated with the expression of adjacent genes in the same tissues, and repeated the procedure only in cases and controls carrying the HLA-DQ2 risk haplotype.

Results: We identified 248 and 215 CpGs associated with CeD in placenta and blood, respectively. Among the former, the DNAm of seven CpGs correlated with the placental expression of *ZFP57*. In contrast, in the latter group, the most represented gene was *RNF5*, with DNAm of 11 CpGs correlating with its expression in blood. In HLA-DQ2 positive individuals, we observed a decrease of placental CpGs associated with CeD, with a remarkable exception in chromosome 2, close to *AHSA2*. In blood, we identified 44 CpGs associated with CeD in the HLA region, with *HLA-DPA1* showing the largest number of DNAm-expression associations.

Conclusions: Our results suggest that placenta does not seem to be a crucial effector in CeD, and show potentially causal relationships between blood

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Jose Ramon Bilbao and Nora Fernandez-Jimenez jointly directed this study.

DNAm and CeD, with independent signals in the HLA, and particularly in the *HLA-DPA1* gene.

KEYWORDS
complex diseases, gene expression, genetic predisposition, HLA region

1 | INTRODUCTION

Celiac disease (CeD) is a chronic autoimmune enteropathy triggered by gluten with an estimated prevalence around 1.4%.[1,2] Genetics plays a key role in the development of CeD, as virtually all patients carry the human leukocyte antigen (HLA)-DQ2 or HLA-DQ8 haplotypes in the HLA region.[1] *HLA* genes are key factors in the pathogenesis of CeD, since they are involved in both the adaptive and innate immune responses and they encode the HLA complexes of the antigen-presenting cells.[2] Additionally, several non-*HLA* genes are also involved in CeD, despite having a much more modest effect, accounting for 15% of the genetic risk.[1] Overall, all the identified genetic variants to date account for 50% of the genetic variance, indicating that additional hereditary factors remain uncovered.[1] Environmental factors also take part in CeD, with immunotoxic gluten peptides being necessary for disease development.[2] However, other potential contributing environmental factors such as microbiota, reovirus or enterovirus infections, among others, and early life exposure to gluten have been extensively explored but remain controversial.[1–4]

It is well known that the intrauterine environment can make an impact on development and later health.[5] In turn, the prenatal origin of CeD is a controversial topic. On the one hand, there are pieces of evidence supporting an association between elective cesarean and the future development of CeD.[6] Moreover, both low birthweight for gestational age and neonatal infection have been described to be associated with an increased risk of developing CeD.[7] On the other hand, a study by Mårild et al. did not find significant associations between immunologic biomarkers in pregnancy and childhood CeD, and therefore refuted the hypothesis that the prenatal systemic immune response influences its debut.[8] In parallel, other authors have reported that there was no association between exposure to antibiotics during pregnancy and the development of CeD in the offspring.[9] Recently, Moreno et al. have described fetal exposure to gluten immunogenic peptides (GIPs) present in amniotic fluid, establishing a positive correlation with maternal gluten intake.[10] Therefore, the mother's intake would influence the intensity of exposure of the fetal immune system to GIP, potentially directing it towards protection or susceptibility of CeD.[10]

According to the developmental origins of health and disease hypothesis, perinatal and early life environments can impact an individual's health, extending to all life stages.[5] Most organs and tissues are formed and developed during the fetal stage, and therefore, this period is characterized by a great plasticity.[11] It has been proven that modifications of the intrauterine environment can increase the chances of developing pathologies in adulthood.[11] Fetal programming induces changes in gene expression through epigenetic modifications such as DNA methylation (DNAm), which could be the basis for the maintenance of long-term effects.[11] Moreover, methylation quantitative trait loci (mQTL) have proven useful to map the etiology of complex disorders to different organs, tissues, and cell types, and particularly in the case of placenta, also to prenatal stages.[5] In this context, the placenta plays a key role in pregnancy, as it is involved in the transport of nutrients, the immune response, the synthesis of hormones, and the detoxification of substances.[11] Thus, it is an excellent organ for evaluating the prenatal exposome.[5]

Therefore, the purpose of this study was to identify the CeD genetic risk mediated by DNAm of placenta during pregnancy, as a surrogate of the relevance of this stage in etiopathogenesis. It is worth mentioning

What is Known

- The first immunogenic insult of celiac disease (CeD) could occur prenatally, and this is important regarding the gluten consumption of mothers with celiac relatives.
- Placental DNA methylation (DNAm) connects fetal genetics and environment, and could have an impact in later health.
- Understanding whether part of the genetic susceptibility to CeD acts through placental DNAm is relevant to ascertain if the prenatal exposure to gluten could affect disease predisposition.

What is New

- Placental DNAm has a good resolution but does not seem to have a large effect on CeD susceptibility.
- DNAm close to *AHSA2* and *ZFP57* could be an exception and mediate disease predisposition prenatally, in placenta.
- *HLA-DPA1* expression in peripheral blood could operate in CeD susceptibility in a HLA-DQ2 independent manner.

that situating the genetic risk in fetal development could help understand the importance of maternal gluten consumption in the context of pregnancies of women with first-degree relatives diagnosed with CeD. To achieve this aim, we used imputed genotype data from the Immunochip,[12] to perform two association analyses, one of them exclusively on individuals with high-risk genetic profiles. Using a Mendelian Randomization approach, we integrated the genetic association data with placental mQTLs to identify CpG sites potentially associated with CeD. Next, we assessed whether the DNAm of candidate CpG sites correlated with the gene expression of nearby genes (expression quantitative trait methylations, eQTMs) to identify potential gene-based biomarkers. A similar protocol was applied using peripheral blood mQTLs and eQTMs to evaluate the specificity and functional relevance of placenta in CeD.

2 | METHODS

2.1 | Ethics statement

All cohorts of the placental mQTL database obtained ethics approval and informed consent from participants before data collection through their Institutional Ethics Boards, in accordance with the criteria set by the Declaration of Helsinki. This data is already public. The present study did not involve the acquisition of new data and therefore, did not require additional approval by the Ethics Board. It was carried out between April 2024 and December 2024. We have defined all genetic concepts and terms in Table 1 to make this study clearer for the broad readership of the *Journal of Pediatric Gastroenterology and Nutrition*.

2.2 | CeD Immunochip association data

The quality control (QC) of the genotype data from the Immunochip CeD study[12] was performed using the following criteria: single nucleotide polymorphisms (SNPs) with a minor allele frequency (MAF) > 1%, a call rate >95%, and a *p* value for the Hardy–Weinberg equilibrium test $>1 \times 10^{-6}$ were retained. Samples with a call rate >97%, heterozygosity within 4 standard deviations (±4 SDs) of the mean, and no sex discrepancies were included. These QC-ed genotypes were imputed using the Michigan Imputation Server[13] with the HRC r1.1 (GRCh37/hg19) reference panel,[14]

TABLE 1 Glossary of the genetic terms used across the article.

Term	Definition
CpG	Short form for cytosine-phosphate-guanine, a dinucleotide where DNAm commonly occurs, influencing gene function.
DNAm	A biochemical process that adds a methyl group to the DNA molecule, generally leading to the formation of 5-methylcytosine. It often affects gene expression and plays a crucial role in epigenetic regulation.
eQTL	Genetic variants that are associated with variation in gene expression levels.
eQTM	A CpG site that is associated with changes in gene expression, suggesting a regulatory role.
HEIDI	A method that seeks to address whether many SNPs in a single region give estimates that are more different from each other than expected by chance, under the assumption that there is a single causal variant and each SNP only exhibits an effect due to LD with the causal SNP.
Horizontal pleiotropy	A phenomenon in which a genetic variant influences multiple traits (methylation, phenotype, etc.) through independent biological pathways. This can complicate causal inference in genetic studies.
Immunochip	A genotyping array specifically designed to study immune-related diseases by targeting genetic variants associated with immune function.
LD	A nonrandom association of alleles at two or more loci, often due to their physical proximity on a chromosome.
mQTL	Genetic variants that are associated with variation in DNAm levels at CpG sites.
MAF	The frequency of the less common allele at a genetic locus within a given population.
PCs	They are the result of PC analyses and capture the most significant variance, allowing for a more efficient representation of the data while preserving essential patterns and structures.
SNP	Variation at a single position in the DNA sequence that may influence complex traits or disease susceptibility.
SMR	A statistical method that integrates genetic and epigenetic data to infer causal relationships between DNAm and gene expression or disease traits.

Abbreviations: DNAm, DNA methylation; eQTL, expression quantitative trait loci; eQTM, expression quantitative trait methylation; HEIDI, HEterogeneity In Dependent Instruments; LD, linkage disequilibrium; mQTL, methylation quantitative trait loci; MAF, minor allele frequency; PC, principal component; SMR, Summary-data-based Mendelian Randomization; SNP, single-nucleotide polymorphism.

focusing on the European population. Finally, SNPs with an imputation accuracy (R^2) <0.9 and MAF < 1% were excluded. The final genotype data set included 21,982 individuals (10,774 cases with CeD and 11,208 controls) and 900,849 SNPs.

Subsequently, we selected those individuals carrying the HLA-DQ2 haplotype, as described in a previous study by our group.[15] Briefly, the DQ haplotypes were imputed using the HIBAG-HLA Genotype Imputation R package (Version 1.32.0).[16] We selected individuals who were either homozygous for HLA-DQB1*02:01 or heterozygous for HLA-DQB1*02:01/DQB1*02:02, resulting in 4264 cases with CeD and 550 controls.

Both association analyses were performed using the score method implemented in SNPTEST (version 2.5.6)[17] under an additive genetic model. The steps for the Immunochip data QC, imputation, and shorting before the association study have been schematically represented in Figure S1.

2.3 | Placental mQTL

We utilized a placental mQTL data set previously described by our group.[5] Briefly, the placental mQTL database was generated by our group between 2020 and 2024 using 368 fetal placental DNA samples from the Infancia y Medio Ambiente cohort. A linear model was implemented using TensorQTL,[18] including genotype, sex, five genotype principal components (PCs), 18 DNAm PCs, and estimated cell types as covariates. The resulting mQTL database, filtered at a p value threshold of $<5.0 \times 10^{-8}$, comprised 9,602,938 SNP-CpG associations, and can be found at the following website: https://irlab.shinyapps.io/shiny_mqtl_placenta/.

2.4 | Peripheral blood mQTL

We used the publicly available whole blood mQTL database reported by Hannon et al.,[19] which includes 1193 individuals (aged >16 years) and 19,288,834 genetic variant-CpG associations at a p value threshold of 7.67×10^{-9}. Briefly, their analysis employed a linear model implemented with MatrixEQTL,[20] incorporating genotype, age, sex, six estimated cell types, two batch variables, and 10 genetic PCs as covariates.

2.5 | Summary-data-based Mendelian Randomization (SMR) analyses

We integrated both the general and the HLA-DQ2-restricted CeD-association summary statistics with the placental and whole blood mQTLs as described above, using the Multi-SNP option of the SMR software[21] to identify CpGs pleiotropically associated with CeD. Multi-SNP SMR employs genetic variants as instrumental variables, DNAm as the exposure, and CeD status as the outcome. In addition to the Multi-SNP SMR test, the software conducts a HEterogeneity In Dependent Instruments (HEIDI) test to distinguish between associations driven by linkage disequilibrium (LD) and horizontal pleiotropy. We adhered to the default settings recommended by the developers and considered significant those results with a Bonferroni-corrected p value for the SMR test ($p_{\text{SMR-multi}}$) < 0.05 and $p_{\text{HEIDI}} > 0.05$.

2.6 | Placental eQTM

To investigate the relationship between the placental CpG sites identified and gene expression, we evaluated the correlation between DNAm at these CpG sites and gene expression levels using a placental eQTM data set from the Rhode Island Child Health Study. This data set included 195 samples with available gene expression and DNAm data. The detailed process is described in a previous study conducted by our group.[3] Briefly, eQTMs were calculated using MatrixEQTL,[20] implementing a linear regression model that accounted for sex, five gene expression PCs, and estimated cell types as covariates.

2.7 | Blood eQTM

To determine whether variation in DNAm at CpG sites associated with CeD in blood influences gene expression, we applied the same analytical protocol used for the placental eQTM analysis. We utilized the publicly available blood eQTM data set from the Human Early Life Exposome cohort, which includes data from 832 children as reported by Ruiz-Arenas et al.[22] Briefly, the blood eQTMs were calculated using a linear regression model adjusted for age, sex, cohort, and blood cell type composition. The summary of the whole study and the databases employed are shown in Figure S2.

3 | RESULTS

3.1 | Identification of CpG sites associated with CeD in placenta and peripheral blood

The integration of the placental mQTL data set with the CeD Immunochip summary statistics that included all individuals regardless of their HLA haplotype identified 248 CpGs potentially associated with CeD (Bonferroni corrected $p_{\text{SMR-multi}} < 0.05$ and $p_{\text{HEIDI}} > 0.05$) (Table S1). As expected, 215 of these CpGs were located on chromosome 6, very likely reflecting the strong influence of

HLA genes on the disease. The remaining CpGs were distributed across other chromosomes as follows: 10 on chromosome 15; 7 on chromosome 2; 4 on chromosome 3; 3 on each chromosomes 10, 11, and 16; and 1 each on chromosomes 1, 12, and 14. The CpG with the lowest *p* value was cg23681866, located on chromosome 6 ($p_{SMR\text{-}multi} = 7.34 \times 10^{-29}$, $B_{SMR} = 0.689$).

To determine whether the results obtained using placental mQTLs were tissue-specific, we applied an analogous protocol using peripheral blood mQTLs. The SMR analysis integrating blood mQTLs with the summary statistics of the CeD Immunochip identified 215 CpGs potentially associated with CeD (Table S2). As observed in the placenta, chromosome 6 had the largest number of associated CpGs ($n = 178$), again, very probably due to the critical role of the HLA region in the disease. Interestingly, despite the considerably larger number of blood mQTLs (19,288,834) compared to placental mQTLs (9,602,938), the analysis identified fewer associated CpGs. This result may indicate the higher resolution of the placental methylome, probably due to the trimodal distribution of DNAm in that organ.

3.2 | Identification of CpG sites associated with CeD in HLA-DQ2 positive individuals

When we repeated the analysis using only HLA-DQ2 positive individuals, the number of significant associations decreased substantially for placenta. In fact, only two CpGs remained associated with CeD: cg00362816 on chromosome 2 ($p_{SMR} = 3.00 \times 10^{-7}$, $B_{SMR} = 0.228$) and cg14566696 on chromosome 6 ($p_{SMR\text{-}multi} = 1.10 \times 10^{-6}$, $B_{SMR} = -0.717$) (Table S3). Remarkably, the CpG site on chromosome 2 was located close to *ASHA2*, a pseudogene that has been associated with CeD in previous studies (Figure 1).

On the other hand, the SMR analysis using blood mQTLs in HLA-DQ2 positive individuals also showed a reduction in the number of associated CpGs ($n = 44$)

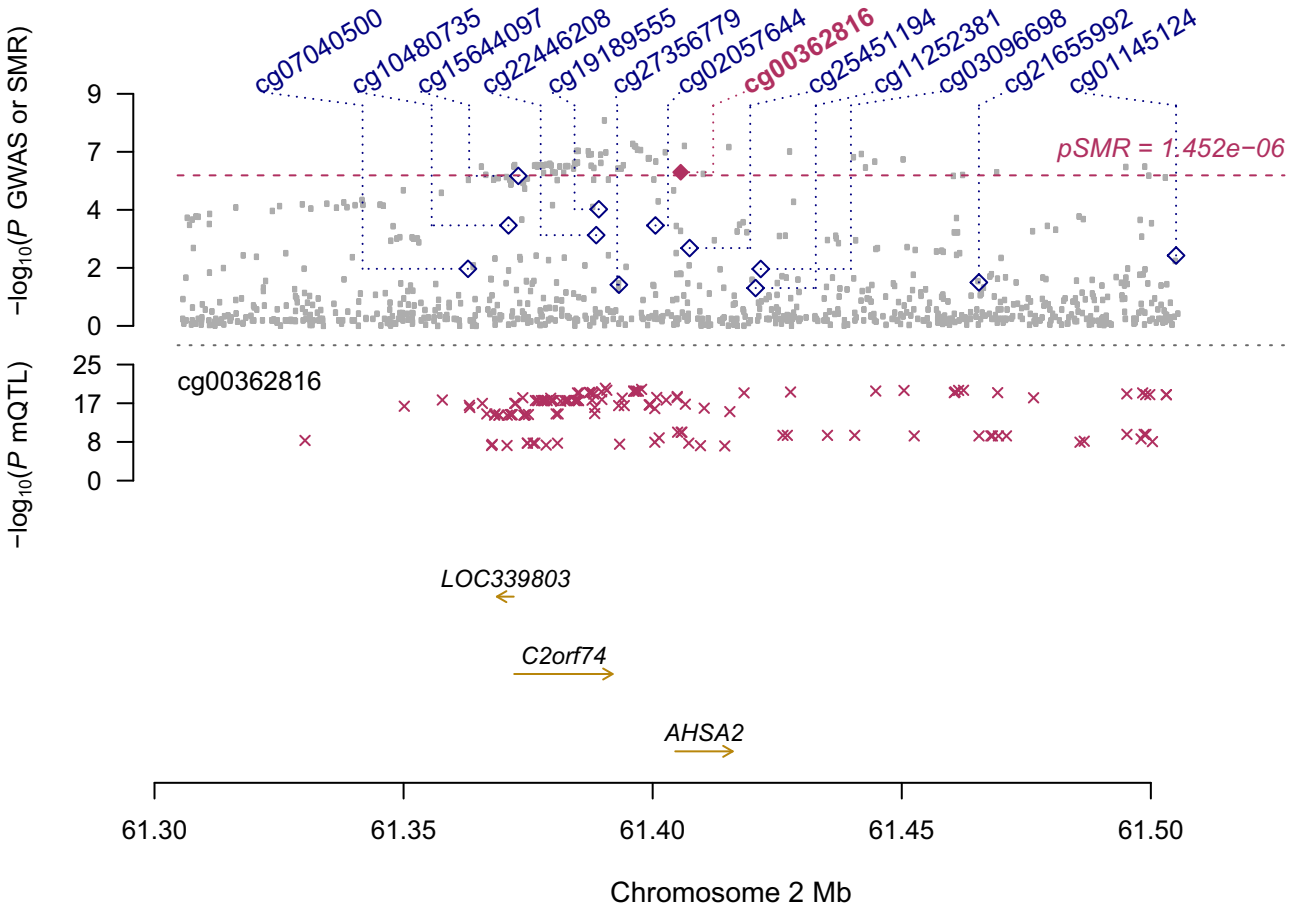

FIGURE 1 SMR locus plot showing the SMR results of cg00362816. In the upper section, gray dots represent $-\log_{10}$ (*p* values) for HLA-DQ2 CeD Immunochip study. Diamonds represent $-\log_{10}$ (*p* values) for CpGs from the SMR analysis, and filled diamonds show those that pass the HEIDI test. In the middle panel, the red crosses represent $-\log_{10}$ (*p* values) for CpGs in the mQTL analysis. In the bottom panel, the location of the CpGs on chromosome 2 is shown. CeD, celiac disease; HLA, human leukocyte antigen; mQTL, methylation quantitative trait loci; SMR, Summary-data-based Mendelian Randomization.

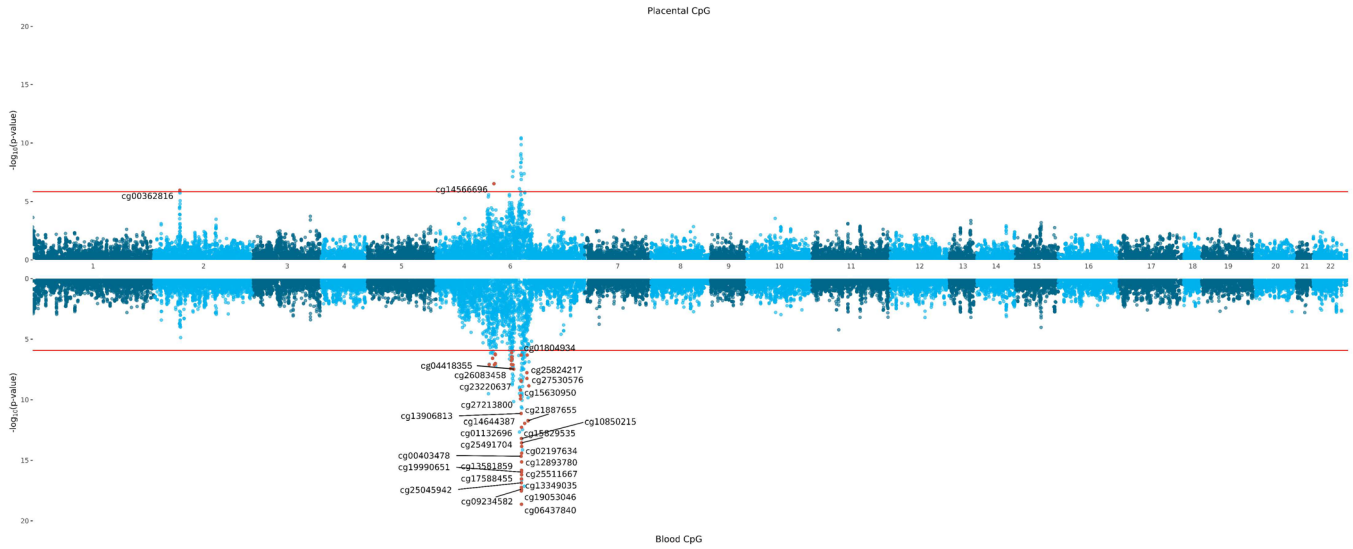

FIGURE 2 Manhattan plot of the SMR analysis results in HLA-DQ2 carriers. The top section represents the SMR results from the placental mQTL analysis. The bottom section shows the SMR results from the blood mQTL analysis. CpGs that are statistically significant for Bonferroni-adjusted $p_{SMR\text{-}multi} < 0.05$ and $p_{HEIDI} > 0.05$ test are colored orange. The horizontal red line represents the Bonferroni corrected p value threshold of 0.05. HLA, human leukocyte antigen; mQTL, methylation quantitative trait loci; SMR, Summary-data-based Mendelian Randomization.

(Table S4 and Figure 2). This decrease was not as pronounced as in placenta, and potentially highlights a more remarkable functional implication of blood cells in CeD compared to placenta, despite the higher resolution of the latter. Notably, all 44 CpGs were located on chromosome 6, underscoring the central importance of this chromosome in CeD, beyond the role of HLA genes.

3.3 | From DNAm to gene expression: eQTM analyses

After identifying CpGs potentially associated with CeD in both placenta and peripheral blood, we analyzed whether changes in DNAm at these sites could affect the expression of nearby genes. This analysis was conducted to shed light on the functional role of the CpGs identified.

In placental tissue, when all individuals were considered, 20 of the 248 CeD-associated CpGs showed DNAm levels that correlated with the expression of 13 genes *(ITPR3*, *VARS2*, *TOB2P1*, *MPI*, *ZKSCAN4*, *ZSCAN23*, *ZNF165*, *HCP5*, *RNF39*, *ZFP57*, *TRIM26, HLA-C*, and *C2orf74)*, resulting in a total number of 24 eQTMs or CpG-gene combinations (Table S5). Notably, nearly half of these genes (six) are located in the HLA region on chromosome 6. Among them, *ZFP57* exhibited the highest number of associations, with its expression correlated with the DNAm levels of 7 CpGs. In peripheral blood, although fewer CpGs were identified by SMR (namely, 215), the analysis revealed 137 eQTMs, involving 55 CpGs and the expression of 63 genes, 53 of which were located in the HLA region (able S6, Figure 3). *RNF5* had the highest number of associations, and its expression correlated with the DNAm of 11 CpGs.

The analysis of individuals with the HLA-DQ2 genotype shed different results. In placental tissue, neither of the two CpGs identified were associated with the expression of nearby genes. In contrast, in peripheral blood, 22 of the 44 CeD-associated CpGs influenced the expression of 33 genes, yielding a total number of 65 eQTMs (Table S7, Figure 3). All these eQTMs were located on chromosome 6, and *HLA-DPA1* was the gene showing the largest number of associations, with its expression correlated with the DNAm of 11 CpGs.

4 | DISCUSSION

Our work highlights several genes that could be of special interest as potential mediators of the genetic risk in CeD. In placenta, *ZFP57* is the gene with the largest number of associations between CeD-associated CpG sites and expression. *ZFP57* shows altered expression in CeD and regulates the expression of the insulin-like growth factor 2 (*IGF2*).[23] Additionally, it is a well-known transcription factor involved in the establishment and maintenance of DNAm of imprinted loci, and mutations in this gene can cause transient neonatal diabetes.[24] These findings align with the observed significance of ZFP57 in the placenta, suggesting that it may play a key role during prenatal development.

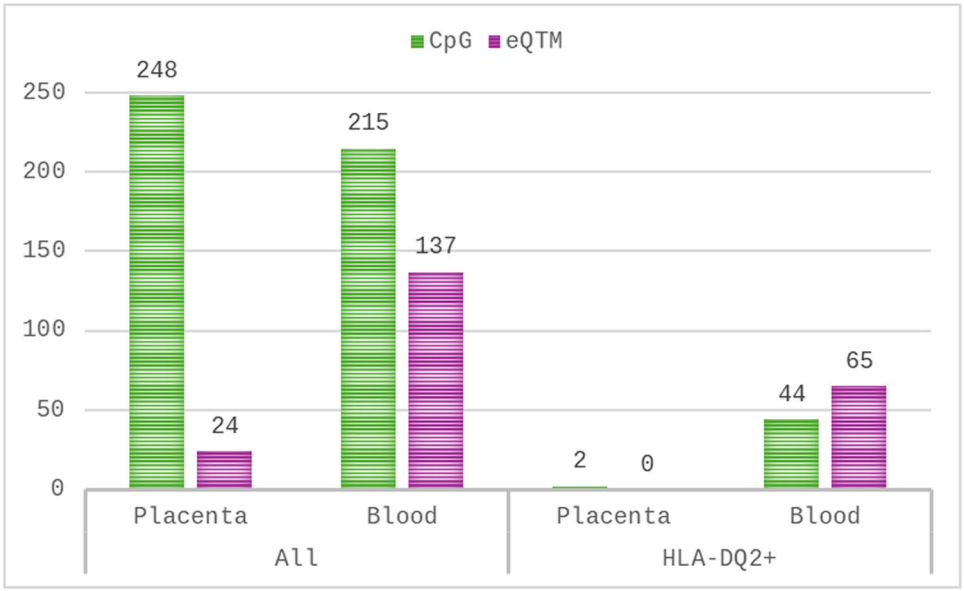

FIGURE 3 Number of SMR-significant CpGs and eQTMs for placenta and peripheral blood in studies considering all individuals (All) and HLA-DQ2 positive individuals (HLA-DQ2+). eQTM, expression quantitative trait methylation; HLA, human leukocyte antigen; SMR, Summary-data-based Mendelian Randomization.

Regarding *RNF5*, its expression in blood is correlated with CeD-associated DNAm at multiple sites, and it has been reported to increase the risk of CeD in another recent work.[25] Furthermore, *RNF5* expression is altered even in the absence of gluten consumption, revealing a potentially constitutive role in CeD.[26] However, the fact that the abovementioned genes are both located in the extended HLA haplotype hinders making definitive conclusions, due to the strong LD across that genomic region.

To bypass this problem, we decided to minimize the impact of LD by selecting those participants of the CeD Immunochip study carrying the DQ2 risk haplotype. In placenta, only two CpG sites remained significant, one on chromosome 2, in the promoter region of *ASHA2*. This gene was previously prioritized in another SMR study in CeD conducted by our group.[27] Furthermore, CeD risk polymorphisms have been reported to be associated with *ASHA2* expression levels in the thymus, suggesting implications on T cell development.[28] Finally, eQTL mapping in blood identified different disease-associated genes compared to those previously reported in fine-mapping studies, and prioritized *AHSA2* instead of *PUS10.*[29]

One of the key findings here is that *HLA-DPA1* expression in blood could independently influence the susceptibility of developing CeD in HLA-DQ2 carriers. It is known that certain *HLA-DPB1* alleles (in strong LD with *HLA-DPA1*) may reduce the risk of production of tissue transglutaminase antibodies among children at high risk of CeD, confirming that additional variants in the HLA region may influence the susceptibility to suffer the disease.[30] In yet another study in a Finnish population, the frequency of a given *HLA-DPB1* allele was shown to be higher in CeD patients, but the association appeared secondary to that driven by the HLA-DQ2 haplotype.[31] In summary, the independent implication of molecules other than HLA-DQ2, even in close proximity to the high-risk haplotype, is not novel. However, our study sheds light on the specific gene that could be exerting the effect of those reported secondary signals, specifically by means of its expression in peripheral blood.

Remarkably, our findings indicate a higher resolution of placental mQTLs compared to that observed for peripheral blood mQTLs. This occurs despite the lower number of mQTLs used in the SMR approach in placenta, and very likely arises from the trimodal distribution of DNAm in this ephemeral organ, with a higher number of partially methylated domains and more abundant genomic loci with intermediate DNAm levels compared to any other human tissue or fluid.[32] These intermediate levels will very likely translate into more numerous and more significant associations, with genotype and with anything else, and consequently, in a better resolution in genomic approaches such as the one employed here.

However, the higher statistical power and resolution of placental mQTLs did not translate into more findings at the gene expression level. In fact, the lower number of positive findings in peripheral blood resulted in more associations with gene expression, related to a plausible functional role of that fluid in CeD, and underscoring the importance of validating findings in multiple omics layers. Nevertheless, we cannot rule out the possibility that SMR findings in blood resulted in more

hits at the gene expression level due to the larger sample size and statistical power of the blood eQTM database compared to the placental eQTM data set. In any case, placental mQTL-based SMR could be utilized as a preliminary approach to assess the relevance of DNAm in different disorders, but should be supplemented with additional searches in multi-tissue/fluid expression databases for the evaluation of functionality. Additionally, it is important to note that our approach is purely theoretical, as we are not conducting any in vitro or in vivo validation. Therefore, we cannot draw any definitive conclusions.

Finally, when analyses were restricted to individuals with the HLA-DQ2 genotype, there was a remarkable decline in the number of eQTMs in both tissues. Nevertheless, this decrease was much more pronounced in the case of placenta. Again, this fact reveals an effector role for blood in CeD in opposition to placenta, since stratified and conditional analyses will usually prioritize results with a more prominent functional potential.

5 | CONCLUSIONS

Despite the greater resolution of placental DNAm, its impact on gene expression is more limited than that observed for blood, suggesting a limited effector function of placental DNAm in CeD. Thus, we have little evidence supporting the involvement of prenatal stages in the development of CeD, although other methods and resources could very likely result in different conclusions. Thus, we still do not know whether gluten intake should be controlled during high CeD risk pregnancies, and we cannot make recommendations for this period based on these data. However, some notable exceptions, such as *ZFP57* and *AHSA2*, require further investigation. Finally, we report a potentially causal relationship between CeD risk variants and peripheral blood DNAm. For instance, expression of *HLA-DPA1* in blood seems to be an additional risk factor for HLA-DQ2 carriers.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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