High-resolution genotyping of HLA class I loci in children with type 1 diabetes and celiac disease

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Objectives: HLA-DQ2 and DQ8 contribute to the strongest risk haplotypes for type 1 diabetes (T1D) and celiac disease (CD). The variation in genetic risk association is likely linked to different HLA class II loci susceptibility, but association studies of HLA class I alleles are scarce. The aim was to investigate HLA class I A, B, and C alleles polymorphisms in children with only T1D, CD, and a subgroup with both T1D and CD (T1D w/CD).

Materials and methods: HLA class I A, B, and C genes were genotyped using next-generation targeted sequencing. A conditional analysis was performed on 68 children with T1D, 219 children with CD and seven children with T1D w/CD enrolled from a birth cohort study at high genetic risk children from the South of Sweden.

Results: Among 1764 HLA class I allele variants, A*29:02:01 in T1D w/CD was associated with both T1D (OR = 21.42 [1.05, 1322.4], p = 0.0231) and CD (OR = 35 [2.36, 529.12], p = 0.0051) along with C*05:01:01 with both T1D (OR = 5.54 [1.06, 24.8], p = 0.02) and CD (OR = 6.84 [1.46, 26.01], p = 0.0077). No independent effects of HLA-B allele associations were observed in T1D w/CD.

Conclusion: Although the distribution of HLA class I alleles differs between children with T1D and CD, the A*29:02:01 and C*05:01:01 alleles showed shared risk association of both diseases.

KEYWORDS
children, celiac disease, HLA, HLA class I, next-generation sequencing, type 1 diabetes

1 | INTRODUCTION

The human leukocyte antigen (HLA) class II genes, which encode HLA-DR and HLA-DQ proteins, are highly polymorphic and contribute, in combination with many other non-HLA loci across the genome, to the strongest genetic risk for both type 1 diabetes (T1D) and celiac disease (CD).1,3 Although T1D and CD are polygenic disorders of which more than 50 genetic risk loci have been identified,4 both diseases share a strong association with HLA-DR3-DQA1*05:01-DQB1*02:01 (abbreviated DR3-DQ2) and HLA-DR4-DQA1*03:01-DQB1*03:02 (abbreviated DR4-DQ8) haplotypes accounting for 55% of the genetic risk in T1D and 90% in CD.5,6 Children who develop both diseases (T1D w/CD) tend to be younger at T1D onset than those who develop T1D only, of whom 55% were diagnosed with CD within 2 years and 79% within 5 years after T1D onset, respectively.7,8

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We previously demonstrated that children with T1D and CD share three HLA class II loci in DRB3, DRB4, and DRB5 genes using high-resolution genotyping. Although the DRB loci are in linkage disequilibrium (LD) with the major HLA risk loci in the class II regions for both diseases and thereby likely influence the risk associations for HLA class I alleles, extended HLA class I allelic and haplotype diversity in T1D and CD have not been well studied.

Susceptibility of HLA class I genes in T1D have been investigated in previous studies, and been associated with the B*39 and A*24 alleles. The presence of the B*39:06 allele enhances the risk of T1D on distinct HLA-DR-DQ haplotypes (DRB1*08:01-DQB1*04:02 and DRB1*01:01-DQB1*05:01). Since B*39 has been associated with early age at diagnosis, the subtypes B*39:06, A*24:02, and A*29:02 incorporated into a precise genetic risk score (T1D GRS2) to discriminate diabetes subtypes and to predict T1D in newborn screenings. In contrast, B*18 was associated with accelerated progression from autoimmunity to T1D, but only in subjects with DQ2 and A*24 promoted rapid T1D development in the presence of DQ8.

Albeit the majority of CD patients carry DQ2.5 and the remaining either DQ8 or DQ2.2, we previously showed that different HLA-DRB3-DRB1-DQA1-DQB1 haplotypes confer different risk for CD. However, initial reports of HLA class I association with CD pointed to associations with the A*01 and B*08 alleles. Genome-wide association study fine mapping of the MHC region recently explained an additional 18% of CD heritability, independent of the DQ region, and identified B*08:01 and B*39:06 alleles in strong LD with the DR3-DQ2.5 haplotype. Interestingly, A*01:01 and B*08:01 may restrict the adaptive CD8 T cell responses to gluten in patients with CD. In contrast, B*39:06 and A*24:02 conferred increased risk and early T1D disease onset.

Previous studies were based on lower resolution (1st and 2nd field) HLA typing of selected HLA loci in individuals who share both diseases, providing partial insight into the HLA diversity. The next generation target sequencing (NGTS) technology enables the elucidation of full-length HLA gene sequences, permitting an in-depth characterization of population HLA diversity. NGTS meets the limitations of traditional typing techniques, thereby sustaining optimal HLA and a better understanding of the genetic diversity in T1D and CD.

The aim of the present study was thus to further investigate extended allelic and haplotype diversity to HLA class I A, B, and C loci at high-resolution, 3rd field level, using high-throughput NGTS technique for HLA typing in children with T1D and CD and a subgroup diagnosed with both diseases (T1D w/CD). The aim was not to evaluate the risk of HLA class I allele by comparing to a healthy control population, but precisely ask whether HLA class I alleles differ between the two diseases and if a specific class I alleles contribute to disease predisposition in children affected by both T1D and CD.

## 2 | MATERIALS AND METHODS

### 2.1 | Study population

The study included 68 patients (39 females, 29 males) diagnosed with T1D at median 5.5 years of age (range 0.9–11.3) according to the American Diabetes Association criteria, 219 patients (137 females, 82 males) diagnosed with CD at median 4.5 years of age (range 1.1–11.0) according to the ESPGHAN criteria and a subgroup of seven patients (five females, two males) diagnosed with both diseases (T1D w/CD) (Table 1). All patients were selected among 2525 Swedish participants followed from birth with eligible HLA risk genotypes for T1D and CD in the Environmental Determinants of Diabetes in the Young (TEDDY) study between 2004 and 2010 at the Department of Clinical Sciences, Lund University, Malmö, Sweden as described elsewhere. Written informed consent was collected separately for all participants from a parent or primary caretaker for genetic screening and prospective follow-up participation. Local institutional review boards approved the study.

### 2.2 | HLA class I high-resolution sequencing

DNA was purified from either a dried blood spot punch or a small volume of whole blood lysate specimens from

| TABLE 1 | Demographic characteristics of the study population |
|----------|---------------------------------------------------|
|          | Type 1 diabetes-only (T1D only) (N = 68) | Celiac disease-only (CD only) (N = 219) | Type 1 diabetes with celiac disease (T1D w/CD) (N = 7) |
| Median (IQR) age at diagnosis | 5.5 (0.9, 11.3) | 4.5 (1.1, 11.0) | 4.3 (2.6, 7.2) |
| Female n (%) | 39 (57.3) | 137 (62.5) | 5 (71.4) |
| Male n (%) | 29 (42.6) | 82 (37.4) | 2 (28.5) |
the study subjects. All HLA class I A, B, C loci genotyping followed the manufacturer’s specifications (Scisco Genetics Inc., Seattle WA). Concisely, the laboratory workflow comprises consecutive PCR reactions using long-range amplicon-based 2-stage PCR-based amplification of HLA-class I with barcoding incorporated into the amplicon PCRs for individual DNA sample tracking, amplicon pooling, enzymatic clean-up, barcode pooling, magnetic bead-based size selection, and quantification. Illumina MiSeq paired-end sequencing-by-synthesis was applied. Complete exons are sequenced for HLA genotyping using Scisco HLA v4 typing configuration kit on MiSeq v2 PE500. After assay-specific amplification, samples were pooled together and applied to the MiSeq device, where they were amplified as individual clusters and ordinarily sequenced using universal sequencing primers. Subsequently, sequences were analyzed using genetic system software to simultaneously report unambiguous HLA-class I alleles and haplotypes to patients’ samples.

### 2.3 Statistical analysis

Allelic frequencies of HLA class I genes were determined using relative predispositional effect (RPE) analysis. Crude odds ratios (ORs) and their associated 95% confidence intervals (95% CI) were calculated, and χ² tests or Fisher’s exact test (if any cell contained fewer than five observations) were used to test whether the frequencies of a given allele/haplotype differed between study cohorts. The RPE method was used to identify the alleles, haplotypes, or genotypes with the strongest predisposing or protective effects at each iteration. The selected alleles were then removed from the dataset, and the analysis was repeated until no risk or protective alleles were identified. Comparisons of HLA-A, -B, and -C allele frequencies were performed for exons 1–7 of chromosome 6p21 by performing pairwise comparisons between all study groups and listed in order of increasing p value, followed by the extended haplotype and genotype frequencies. p values ≤0.05 were considered statistically significant, and alleles with a low frequency (≤1%) were not shown in the analysis. The p values presented are nominal and not adjusted for multiple comparisons. Analyses were performed in R (r-project.org) version 3.6.1 and R package epiDisplay version 3.5.0.1.

### 3 RESULTS

The overall HLA class I allelic frequencies are summarized in Table 2 and presented separately for each of the three study outcomes below.

#### 3.1 HLA class I allelic frequencies in CD

The allelic frequencies of 21 HLA-A, 33 HLA-B, and 22 HLA-C are reported in (Tables S1, S3, S4, S6, S7, and S9). The HLA-A locus analysis uncovered 21 different alleles in CD, none of which with frequency >1%. The most frequent allele was A010101 (43.2%), followed by A020101 (22.1%), A030101 (10.3%), A240201 (4.8%), and A110101 (4.3%), respectively. Together, these allelic variants comprised 85% of the HLA-A allelic diversity. Allelic distribution comparison showed A020101, A030101, and A240201 increased frequency in T1D and T1D w/CD cohorts than CD cohorts. A030101 had the highest positive allelic association between T1D patients and CD patients (OR = 2.47 [1.49, 4.11], p = 0.0003), in contrast to A010101 was the most associated protective allele in T1D compared with CD (OR = 0.28 [0.17, 0.46], p = 7 x 10^-08). In the HLA-B locus, 33 variants were observed. Both HLA-B080101 (53.9%) and B150101 (13.2%) were the major frequent alleles in CD. B150101 showed positive association (OR = 2.83 [1.79, 4.47], p = 5.15 x 10^-06), while B080101 had negative association in T1D compared with CD (OR = 0.36 [0.24, 0.54], p = 6.07 x 10^-07). HLA-C locus genotyping revealed 22 variants, in which the C07 group with four alleles accounted for 60% of allelic variability. C030301, C030401, C060201 and C120301 alleles showed a significant positive association in T1D compared with CD cohort, in contrast to C070101 that showed negative association. A total of 153 different haplotypes were identified of which A010101-B080101-C070101 were found in 25.8% of the children with CD compared with 3.7% in T1D (OR = 0.11 [0.03, 0.27] p = 8.2 x 10^-10) while A020101-B080101-C050101 haplotype presents in 14% of T1D w/CD compared with 0.2% in CD (p = 0.0026) (Tables S10 and S12). A total of 129 different genotypes were identified, of which A010101-B080101-C070101/A010101-B080101-C070101 was the most common in 39 (17.8%) CD children compared with other patient groups. Statistical analysis showed no differences between CD HLA class I genotypes and other study groups.

#### 3.2 HLA class I allelic frequencies in T1D

Observed frequencies of a total of 17 HLA-A, 23 HLA-B and 16 HLA-C alleles are reported in (Tables S1, S2, S4, S5, S7, and S8). NGTS genotyping revealed 17 different HLA-A alleles in T1D, of which 11 alleles with frequency >1%. The most frequently allele was A020101 (32.4%),
| HLA allele | CD n (%) (N = 219) | TID n (%) (N = 68) | TID w/CD n (%) (N = 7) | CD versus TID | CD versus TID w/CD | TID versus TID w/CD |
|------------|-------------------|-------------------|------------------------|----------------|-------------------|-------------------|
| A*01:01:01 | 189 (43.2%)       | 24 (17.6%)        | 2 (14.3%)              | 0.28 (0.17, 0.46) | 7 × 10⁻⁸ | 0.22 (0.02, 1.01) | 0.005* |
| A*02:01:01 | 97 (22.1%)        | 44 (32.4%)        | 5 (35.7%)              | 1.68 (1.1, 2.57)  | 0.0157 | 1.95 (0.5, 6.66) | 0.325* |
| A*03:01:01 | 45 (10.3%)        | 30 (22.1%)        | 2 (14.3%)              | 2.47 (1.49, 4.11) | 0.0003 | 1.45 (0.15, 6.85) | 0.648* |
| A*24:02:01 | 21 (4.8%)         | 13 (9.6%)         | 1 (7.1%)               | 2.1 (1.02, 4.31) | 0.0398 | 1.53 (0.03, 11.13) | 0.508* |
| A*29:02:01 | 2 (0.5%)          | 1 (0.7%)          | 2 (0.5%)               | 1.61 (0.03, 31.21) | 0.556* | 35 (2.36, 529.12) | 0.005* |
| A*68:01:02 | 12 (2.7%)         | 2 (1.5%)          | 2 (14.3%)              | 0.53 (0.06, 2.43) | 0.536* | 5.87 (0.58, 31.32) | 0.065* |
| B*15:01:01 | 98 (22.4%)        | 41 (30.1%)        | 4 (28.6%)              | 2.83 (1.79, 4.47) | 6 × 10⁻⁷ | 0.48 (0.12, 1.61) | 0.276* |
| B*18:01:01 | 12 (2.7%)         | 10 (7.4%)         | 1 (7.1%)               | 2.82 (1.19, 6.67) | 0.0144 | 2.72 (0.06, 21.24) | 0.339* |
| B*35:03:01 | 1 (0.2%)          | 5 (3.7%)          | -                      | 16.58 (1.83, 787.18) | 0.00342* | -                 | -     |
| B*44:02:01 | 16 (3.7%)         | 6 (4.4%)          | 2 (14.3%)              | 1.22 (0.47, 3.17) | 0.687 | 4.37 (0.44, 22.33) | 0.103* |
| C*03:03:01 | 20 (4.6%)         | 15 (11%)          | 5 (35.7%)              | 2.59 (1.29, 5.21) | 0.00593 | 1.61 (0.04, 11.76) | 0.491* |
| C*03:04:01 | 77 (17.6%)        | 37 (27.2%)        | 4 (28.6%)              | 1.75 (1.12, 2.75) | 0.014 | 0.78 (0.08, 3.62) | 0.0099* |
| C*05:01:01 | 24 (5.5%)         | 9 (6.6%)          | 4 (28.6%)              | 1.22 (0.55, 2.7)  | 0.618 | 6.84 (1.46, 26.01) | 0.007* |
| C*06:02:01 | 6 (1.4%)          | 6 (4.4%)          | -                      | 3.32 (1.05, 10.48) | 0.0303 | -                 | -     |
| C*07:01:01 | 237 (54.1%)       | 43 (31.6%)        | 5 (35.7%)              | 0.39 (0.26, 0.59) | 4 × 10⁻⁶ | 0.47 (0.12, 1.6)  | 0.187* |
| C*12:03:01 | 3 (0.7%)          | 5 (3.7%)          | 2 (14.3%)              | 5.51 (1.06, 35.97) | 0.0209* | -                 | -     |

Note: The odds ratios (OR) and their associated 95% confidence intervals (95% CI) were estimated using all other alleles as the reference group for each estimate. The p values are based on a chi-squared test, and * indicates those based on Fisher's exact test. %, percentage of genotyped alleles (438 for CD, 136 for TID, 14 for TID w/CD). Abbreviations: 95% CI, 95% confidence interval; CD, celiac disease; n, number of alleles; OR, odds ratio; TID, type 1 diabetes; TID w/CD, type 1 diabetes with celiac disease.
followed by A^*03:01:01 (22.1%), A^*01:01:01 (17.6%), and A^*24:02:01 (9.6%). Together, these allelic variants comprised 81% of HLA-A allelic variability. RPE analysis revealed positive association with A^*29:02:01 (OR = 21.42 [1.05, 1322.4], \( p = 0.0231 \)) and A^*68:01:02 (OR = 10.78 [0.72, 161.44], \( p = 0.0437 \)) in T1D w/CD group compared with the T1D group. Within the HLA-B locus, 23 different allelic variants were identified of which B^*15:01:01 (30%), B^*08:01:01 (29.4%) and B^*40:01:02 (7.4%) were the most frequent alleles. Together with B^*18:01:01 (7.4%) and B^*44:02:01 (4.4%) accounted for 78.4% of HLA-B allelic variability. HLA-C locus genotyping uncovered 16 different allelic variants within 10 allelic families. The most polymorphic was the C^*07 group with four alleles, representing 36% of the HLA-C alleles. C^*07:01:01 (31.6%) followed by C^*03:04:01 (27.2%), C^*03:03:01 (11%) and C^*05:01:01 (6.6%). RPE analysis revealed C^*07:01:01 allele was negatively associated in T1D patients (OR = 0.39 [0.26, 0.59], \( p = 4.56 \times 10^{-6}\)) compared with CD while C^*05:01:01 allele was positively associated with the T1D w/CD patients (OR = 5.54 [1.06, 24.8], \( p = 0.02 \)) compared with the T1D patients. In the T1D, 84 different haplotypes were identified. Of these haplotypes, A^*01:01:01-B^*08:01:01-C^*03:04:01 (8.1%) and A^*02:01:01-B^*08:01:01-C^*05:01:01 haplotypes were positively associated with T1D w/CD (14.3%, OR = 21.42 [1.05, 1322.4], \( p = 0.02 \)) (Table S11). Among the 58 different genotypes identified, A^*01:01:01-B^*08:01:01-C^*03:03:01/A^*02:01:01-B^*15:01:01-C^*07:01:01 was most frequent in T1D children (4.4%).

### 3.3 HLA class I allelic frequencies in T1D w/CD

A total of 8 HLA-A, 6 HLA-B, and 6 HLA-C alleles frequencies are reported in (Tables S2, S3, S5, S6, S8, and S9). Of the 8 HLA-A alleles, the most observed allele was A^*02:01:01 (28.6%), followed by A^*03:01:01, A^*29:02:01, and A^*68:01:02; all of them have similar allele frequencies (14.3%). Similarly, A^*01:01:01, A^*23:01:01, A^*24:02:01 and A^*30:02:01 expressed similar frequencies (7.1%). RPE analysis showed that A^*29:02:01 was positively associated with T1D w/CD and CD (OR = 35 [2.36, 529.12], \( p = 0.0051 \)) and T1D (OR = 21.42 [1.05, 1322.4], \( p = 0.0231 \)), respectively. Notably, A^*68:01:02 was also positively associated with T1D (OR = 10.78 [0.72, 161.44], \( p = 0.0437 \)), while A^*01:01:01 was negatively associated with CD (OR = 0.1 [0, 0.68], \( p = 0.0055 \)). Within the HLA-B locus, six different allelic variants were identified of which B^*08:01:01 (35.7%), B^*15:01:01 (28.6%) and B^*44:02:01 (14%) accounted for 78.6% of HLA-B allelic variability in T1D w/CD group. Similarly, six different allelic variants were identified in the HLA-C locus of which C^*07:01:01 (35.7%), C^*05:01:01 (28.6%), and C^*03:04:01 (14%) were the most frequent alleles. RPE analysis showed that C^*05:01:01 was positively associated with T1D w/CD compared with both T1D (OR = 5.54 [1.06, 24.8], \( p = 0.02 \)) and the CD group (OR = 6.84 [1.46, 26.01], \( p = 0.0077 \)). A^*02:01:01-B^*08:01:01-C^*05:01:01 was the most frequent haplotype (14%) and positively associated compared with T1D (OR = 21.42 [1.05, 1322.4], \( p = 0.02 \)) and with the CD group (OR = 69.16 [3.39, 4146.7], \( p = 0.002 \)) (Tables S11 and S12). All seven children with T1D w/CD carried different HLA class I haplotype combinations in comparison with the T1D and CD groups.

### 4 DISCUSSION

This study performed extended polymorphism of HLA class I genes in children with T1D, CD, and in a subgroup of children having both diseases (T1D w/CD). The goal was to identify genetic diversities or similarities between T1D, CD, or their coexistence. The main finding of importance showed that eight alleles in the HLA-A region and six alleles in both HLA-B and HLA-C regions were shared between T1D, CD, and T1D w/CD, respectively. Among them, only A^*29:02:01 and C^*05:01:01 showed a similar positive association between T1D or CD and those with both T1D and CD. The present study also identified A^*68:01:02 as an additional allele positively associated between T1D and T1D w/CD patients; however, that was not shown between CD and T1D w/CD patients. Moreover, HLA-B alleles were mostly dominated by two alleles, B^*08:01:01 and B^*15:01:01, similarly comparable between T1D and T1D w/CD. Only one genotype, A^*03:01:01-B^*08:01:01-C^*03:04:01/A^*24:02:01-B^*15:01:01-C^*07:01:01, was shared between T1D and T1D w/CD, albeit this genotype was only found in two children.

The positive association of B^*18 and A^*24 with T1D is in line with previous reports.\(^{16,32}\) In contrast to other studies, we found B^*39:06 not to be associated with T1D.\(^{33,34}\) Since B^*39 is relatively rare and found in 0.5%–1.2% of Europeans, the conflicting results between our and those previous studies might relate to regional differences in the study populations.\(^{35}\) Two previous studies have investigated the role of HLA class I and class II DRB1 and DQB1 on the risk of developing T1D, CD, or both diseases (T1D w/CD) and found that individuals with T1D w/CD are genetically more similar to T1D than to CD patients.\(^{36,37}\) In contrast to these previous studies that directly analyzed HLA class II in patients and control subjects, we utilized the NGTS for extended
genotyping of HLA-A, -B, and -C without information on the descent. The sequencing of the coding region of exon 1–7 of HLA class I allowed the high-resolution detection of all associated genes differences in T1D and CD groups and subgroup T1D w/CD cohorts. Applying this analysis showed several alleles in DRB3, DRB4 and DRB5 levels among T1D w/CD seem to have an extended HLA polymorphism more similar to that in children with T1D than that in children with CD.9 The use of RPE analysis provided an estimate of the allele/haplotype association when more than one is associated to a disease, and thus estimating the relative risk for disease between HLA class I alleles/haplotypes and each of the study outcomes giving the influence of a high frequency of certain allele on deviating the frequency of other alleles.

Our objective was to enhance the understanding of how HLA class I genes may contribute to T1D w/CD. This knowledge might contribute to build more precise genetic risk models to identify individuals with either T1D or CD who are at high risk of developing both. Compared with our analysis, the HLA class II loci still confers the strongest association with disease co-existence.38 However, our study shows that the HLA class I alleles associated with T1D w/CD are not the same as those associated with having either T1D or CD.

A limitation of the present study is the low number of children with T1D w/CD and the need for independent verification. Despite this, it was possible through the RPE analysis to identify few HLA class I alleles to be associated with both diseases. Another limitation is not including healthy children from the general population as a control group. Despite this fact, the study population was enrolled from the same geographical site to address whether the allelic variation in the HLA region could be different between the T1D and CD groups. More studies are therefore needed to validate our findings in other cohorts directly genotyped for both HLA class I and II alleles.

In conclusion, although the distribution of HLA class I alleles differ between children with T1D and CD, the A*29:02:01 and C*05:01:01 alleles showed shared risk association of both diseases.

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CONFLICT OF INTEREST
All the authors declare they have no conflicts of interest.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request and approval. The dataset generated in the current study is not publicly available due to Swedish law and GDPR on protecting human subjects. Still, it is available from the corresponding author and guarantor of the study (DA) upon application and approval.

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**SUPPORTING INFORMATION**

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

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