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# A quarter of patients with type 1 diabetes have co-existing non-islet autoimmunity: the findings of a UK population-based family study

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## Summary

**Individuals with type 1 diabetes (T1D) are at increased risk of coeliac disease (CD), autoimmune thyroiditis and autoimmune gastritis, but the absolute risks are unclear. The aim of this study was to investigate the prevalence of autoantibodies to tissue transglutaminase (TGA), thyroid peroxidase (TPOA) and gastric $H^+/K^+$-ATPase (ATPA) and their genetic associations in a well-characterized population-based cohort of individuals with T1D from the Bart's–Oxford family study for whom islet autoantibody prevalence data were already available. Autoantibodies in sera from 1072 patients (males/females 604/468; median age 11·8 years, median T1D duration 2·7 months) were measured by radioimmunoassays; *HLA class II* risk genotype was analysed in 973 (91%) using polymerase chain reaction with sequence specific primers (PCR-SSP). The prevalence of TGA (and/or history of CD), TPOA and ATPA in patients was 9·0, 9·6 and 8·2%, respectively; 3·1% had two or more autoantibodies. Females were at higher risk of multiple autoimmunity; TGA/CD were associated with younger age and TPOA with older age. ATPA were uncommon in patients under 5 years, and more common in older patients. Anti-glutamate decarboxylase autoantibodies were predictive of co-existing TPOA/ATPA. TGA/CD were associated with human leucocyte antigen (HLA) DR3-DQ2, with the DR3-DQ2/DR3-DQ2 genotype conferring the highest risk, followed by DR4-DQ8/DR4-DQ8. ATPA were associated with DR3-DQ2, *DRB1*0404* (in males) and the DR3-DQ2/DR4-DQ8 genotype. TPOA were associated with the DR3-DQ2/DR3-DQ2 genotype. Almost one-quarter of patients diagnosed with T1D aged under 21 years have at least one other organ-specific autoantibody. *HLA class II* genetic profiling may be useful in identifying those at risk of multiple autoimmunity.**

**Keywords:** gastric H+/K+-ATPase antibodies, HLA, thyroid peroxidase antibodies, tissue transglutaminase antibodies, type 1 diabetes

## Introduction

Type 1 diabetes (T1D) is one of the major chronic disorders of childhood, with a steady increase in incidence during the last few decades [1–5]. Approximately half the susceptibility to T1D is derived from genetic factors; the rest depends upon environmental triggers [1,6–9]. Autoimmune diseases associated with T1D are often subclinical, and complications are usually recognized retrospectively. Along with the deleterious impact on general health, they can affect glycaemic status negatively in patients with T1D. For example, impaired thyroid function (hypothyroidism) in autoimmune thyroiditis or malabsorption resulting from coeliac disease (CD) lead to frequent hypoglycaemic episodes and poor glycaemic control [10,11]. The long latent stage of autoimmune diseases, when only autoantibodies are present without clinical signs, highlights the opportunity to implement prevention strategies [12].

Analysis of the literature showed large variations in reported frequencies of CD in children with T1D, ranging from 3·3 to 12% [13–15], while in the general population

the prevalence is estimated at 1–2% [16]. Autoimmune gastritis is present in 5–10% of T1D patients, while in the general population the average prevalence is 2% [17]. Autoimmune thyroiditis is one of the most frequent autoimmune diseases associated with T1D. The prevalence of thyroid antibodies varies considerably in different studies; it was estimated to be 5–40% in children [18–22] and 18–30% in adults with T1D [19,20,23] compared with 3–5% and 8–24% in healthy controls, respectively [23,24]. Thyroid hypofunction, which is a sign of progressive thyrocyte damage, was present in 6–10% of patients with T1D, and the prevalence increased after the age of 50 years, eventually reaching 18% in the over-60s [25].

Numerous studies in the last few decades have suggested common pathways and genetic elements involved in the development of autoimmune disorders, but few included patients with multiple autoimmunity (MA). Recent genomewide association studies have underlined the contribution of human leucocyte antigen (HLA) to the development of different autoantibodies in T1D [26], but the genetic determinants critical to the development of MA are not well defined. This study aimed to identify the interplay between genetic determinants and co-existent non-islet autoimmunity in patients with T1D.

## Materials and methods

### Patients

A cohort of children and young adults ($n$ = 1072, 468 females, median age = 11·8 years, range = 0·8–28 years; median age at T1D onset = 10·9 years, range = 0·4–21 years; median T1D duration = 2·7 months, range = 0–16·4 years) with T1D from the well-characterized population-based Bart's–Oxford (BOX) family study was investigated. Since 1985, the study has been recruiting individuals under the age of 21 years with newly diagnosed T1D and their first-degree relatives (residents of the area administered formerly by the Oxford Regional Health Authority, UK), 76% of whom are still in regular contact with the study [4]. For the majority (86%) of the cohort, serum samples were available close to diagnosis (within 2 years), while sera from the rest were collected post-diagnosis (within 2–16 years). Thirty-seven (3.4%) patients were diagnosed with CD, as reported by questionnaire. Genetic samples (whole blood or mouth brush) were available from 973 participants (91%) [27], 958 (98%) of whom were tested for all three non-islet autoantibodies.

The BOX study is currently approved by the South Central – Oxford C National Research Ethics Committee. Participants provided informed, written consent and the study was performed according to the principles of the Declaration of Helsinki.

### Methods

*Autoantibody measurement by radioimmunoassay.* Autoantibodies specific for the T1D-specific antigens, insulin (IAA), glutamate decarboxylase (GADA), islet antigen-2 (IA-2A) and zinc transporter 8 (ZnT8A), as well as the CD-specific antigen tissue transglutaminase (TGA IgG/IgA), were measured using fully validated in-house radioimmunoassays (RIAs), as described previously [28,29]. Of the 1072 patients, 1071 (99%) were tested for TGA.

The measurement of autoimmune gastritis-specific anti-$H^+/K^+$-ATPase autoantibodies [ATPA immunoglobulin (Ig)G] was performed in 1055 of the 1072 patients (98%) using a newly established in-house RIA. Plasmid pcDNA3.1 with the *ATP4A* gene encoding a 251 amino acid fragment of the $H^+/K^+$-ATPase 4A polypeptide [30,31] was used for *in-vitro* transcription and translation in the TnT-coupled reticulocyte system (Promega, Madison, WI, USA) to obtain $^{35}S$ methionine-labelled human-$H^+/K^+$-ATPase. Radioactive antigen [20 000 counts per minute (cpm) in 25 µl] bound by serum antibodies (incubated for 19–21 h) was precipitated with protein-A sepharose (GE Healthcare, Uppsala, Sweden) and measured in a beta scintillation counter. Standards were prepared from a serum highly positive for ATPA, serially diluted with seronegative human serum, spanning a range from 0·4 (1/1024) to 100 arbitrary units/ml (1/4). The interassay coefficients of variation for positive control samples were 14% at 24 units/ml and 23% at 79 units/ml. Thyroid autoimmunity was assessed in 1066 of the 1072 patients (99%) by measurement of anti-thyroid peroxidase autoantibodies (TPOA IgG) using a commercial RIA kit (RSR Ltd, Cardiff, UK) following minor modification of the protocol.

*Positivity thresholds for RIAs.* The TGA assay positivity threshold (1·31 units) was set at the 97·5th percentile of 5470 children (median age = 7·5 years, range = 6.9–9·5 years) from the Avon Longitudinal Study of Parents and Children (ALSPAC), a population-based birth cohort study [29]. Sera collected from healthy children attending schools in the Oxford and Windsor regions during 1989–1990 were tested to establish in-house positivity thresholds for TPOA and ATPA. The threshold for the ATPA assay was 21·6 units/ml, set at the 97·5th percentile of 318 schoolchildren tested (median age = 11 years, range = 9–14 years). The threshold for the TPOA assay using the calibrators provided was 8·8 units/ml (kit threshold = 0·3 units/ml), set at the 97·5th percentile of 205 schoolchildren tested (median age = 10 years, range = 9–13 years).

*HLA genotyping.* All DNA samples extracted from whole blood or mouth swab samples were whole genome amplified utilizing a polymerase chain reaction (PCR)-based whole genome amplification protocol (Illustra GenomiPhi V2 DNA amplification kits; GE Healthcare). HLA class II genotype for high-risk DR3-DQ2

(*DRB1*03-DQA1*0501-DQB1*0201*) and DR4-DQ8 (*DRB1*04-DQA1*0301-DQB1*0302*) as well as low-risk DRX (representing all other HLA DRB1 variants) alleles was analysed using PCR with sequence-specific primers as described previously [32]. Genotypes were coded as either DR3-DQ2/DR3-DQ2, DR3-DQ2/DR4-DQ8, DR4-DQ8/DR4-DQ8, DR3-DQ2/DRX, DR4-DQ8/DRX or DRX/DRX. The *DRB1*0404* gene, reported recently to increase the risk of MA [33], was also analysed.

*Statistical analysis.* Data analysis was performed using IBM SPSS version 23 software. Differences in categorical data were investigated by $\chi^2$ or Fisher's exact test. Non-parametric data analysis included Pearson's correlation. Models adjusted for independent factors and covariates (age, gender, HLA DR-DQ) were analysed by logistic regression; a two-tailed *P*-value $< 0{\cdot}05$ was considered statistically significant. The reference genotype for genetic analysis was DRX/DRX.

## Results

### Prevalence of multiple autoimmunity in patients with T1D and non-genetic risk factors

The distribution of non-islet autoantibodies in patients and schoolchildren is shown in Supporting information, Fig. S1. Overall, coeliac autoimmunity (TGA and/or CD) was detected in 9% ($n$ = 97, median age = 9·7 years, median age at T1D onset = 8·7 years) of cases tested and was associated with younger age [odds ratio (OR) = 0.9, 95% confidence interval (CI) = 0·8–0·9, $P < 0{\cdot}0001$]. When those with a medical history of CD alone were not considered, TGA positivity was present in 7·3% ($n$ = 78) of patients. Gastric autoantibodies were detected in 8·2% of patients ($n$ = 87, median age = 12·8 years, median age at T1D onset = 11 years), and were less common in children under 5 years of age ($P < 0{\cdot}05$). Thyroid autoantibodies were detected in 9·6% of patients ($n$ = 102, median age = 13 years, median age at T1D onset = 11·7 years), and were associated with older age (OR = 1·1, 95% CI = 1·04–1·1, $P$ = 0·0001) (Fig. 1). Thirty-three T1D patients (3·1%) had more than one type of non-islet autoantibody, and autoimmunity to TPO and $H^+/K^+$-ATPase was the most common ($n$ = 23) combination of MA in individuals with T1D.

Non-islet autoantibodies were not associated with T1D duration. All non-islet autoantibodies were associated with female gender (OR = 1·9, 95% CI = 1·2–2·9, $P$ = 0·006; OR = 2·1, 95% CI = 1·3–3·3, $P$ = 0·002, OR = 2·7, 95% CI = 1·7–4·2, $P < 0{\cdot}0001$ and OR = 3·4, 95% CI = 1·6–7·2, $P$ = 0·002 for TGA/CD, ATPA, TPOA and multiple non-islet autoantibodies, respectively).

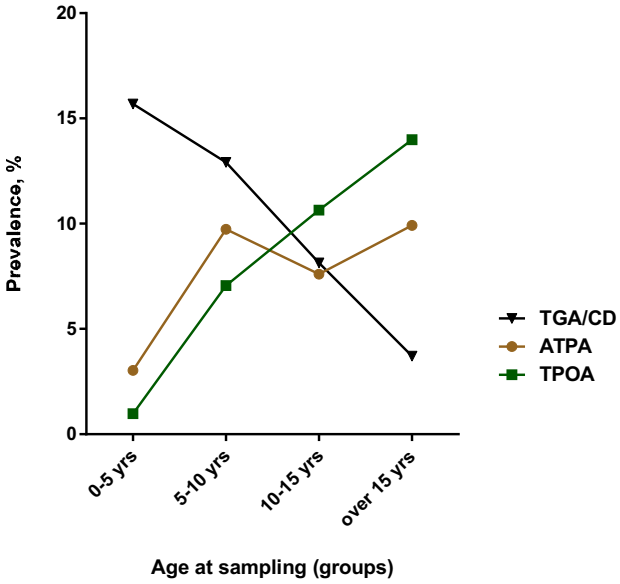


**Fig. 1.** The prevalence of non-islet autoantibodies in children and young adults with type 1 diabetes (T1D) stratified by age group. The prevalence of anti-tissue transglutaminase autoantibodies/coeliac disease (TGA/CD) reduced with age ($P < 0{\cdot}0001$), while anti-$H^+/K^+$-ATPase autoantibodies (ATPA) were uncommon in children under 5 ($P < 0{\cdot}05$), and the prevalence of anti-thyroid peroxidase autoantibodies (TPOA) increased with age ($P$ = 0·0001).

### Genetic risk factors for multiple autoimmunity in patients with T1D

Haplotype analysis found significant associations of TGA/CD (OR = 2·4, 95% CI = 1·4–3·9, $P$ = 0·001) and ATPA (OR = 2·7, 95% CI = 1·6–4·7, $P$ = 0·0003) with HLA DR3-DQ2. Gender analysis of HLA effects also identified an increased risk of ATPA in males carrying the *DRB1*0404* allele (OR = 2·9, 95% CI = 1·1–7·2, $P$ = 0·025) (Table 1).

Genotype analysis showed that increased risk for TGA/CD was associated with DR3-DQ2/DR3-DQ2, DR4-DQ8/DR4-DQ8, DR3-DQ2/DRX and DR3-DQ2/DR4-DQ8 genotypes compared with the reference genotype DRX/DRX; OR = 6·4 (95% CI = 1·7–24·2, $P$ = 0·006), OR = 6·0 (95% CI = 1·5–22·9, $P$ = 0·009), OR = 4·7 (95% CI = 1·3–16·5, $P$ = 0·016) and OR = 4·2 (95% CI = 1·3–14·1, $P$ = 0·019), respectively. Risk for TPOA was associated with DR3-DQ2/DR3-DQ2 (OR = 2·7, 95% CI = 1·1–7·1, $P$ = 0·036) and risk for ATPA with DR3-DQ2/DR4-DQ8 (OR = 3·6, 95% CI = 1·2–10·3, $P$ = 0·019).

### Individuals with T1D who were seropositive for more than one non-islet autoantibody

Of 1052 individuals tested for all three non-islet autoantibodies, 33 (3·1%) had antibodies of more than one specificity (Supporting information, Table S1). No significant associations between HLA DR risk haplotypes and MA

**Table 1.** Summary of human leucocyte antigen (HLA) DR-DQ effects on the development of multiple autoimmunity in individuals with type 1 diabetes (T1D)

| HLA DR-DQ | TGA/CD ($n = 91$) | ATPA ($n = 79$) | TPOA ($n = 96$) |
|---|---|---|---|
| Haplotype analysis | | | |
| DR3-DQ2 | 2·4, $P = 0·001$ | 2·7, $P = 0·0003$ | 1·3, $P = 0·251$ |
| DR4-DQ8 | 0·9, $P = 0·610$ | 1·2, $P = 0·543$ | 0·8, $P = 0·474$ |
| *DRB1*0404* | 1·3, $P = 0·457$ | 1·6, $P = 0·106$ | 1·6, $P = 0·127$ |
| Genotype analysis | | | |
| DR3-DQ2/DR3-DQ2 | 6·4, $P = 0·006$ | 2·5, $P = 0·153$ | 2·7, $P = 0·036$ |
| DR3-DQ2/DR4-DQ8 | 4·2, $P = 0·019$ | 3·6, $P = 0·019$ | 1·3, $P = 0·498$ |
| DR3-DQ2/DRX | 4·7, $P = 0·016$ | 2·6, $P = 0·104$ | 1·4, $P = 0·506$ |
| DR4-DQ8/DR4-DQ8 | 6·0, $P = 0·009$ | 1·2, $P = 0·797$ | 1·8, $P = 0·280$ |
| DR4-DQ8/DRX | 1·4, $P = 0·621$ | 1·2, $P = 0·769$ | 1·1, $P = 0·832$ |
| Gender analysis | | | |
| DR3-DQ2 Male | n.s. | n.s. | n.s. |
| Female | n.s. | n.s. | n.s. |
| DR4-DQ8 Male | n.s. | n.s. | n.s. |
| Female | n.s. | n.s. | n.s. |
| DRB1*0404 Male | n.s. | 2·9, $P = 0·025$ | n.s. |
| Female | n.s. | n.s. | n.s. |
| DR3-DQ2/DR3-DQ2 Male | n.s. | n.s. | n.s. |
| Female | 8·0, $P = 0·017$ | n.s. | 7·3, $P = 0·007$ |
| DR3-DQ2/DR4-DQ8 Male | n.s. | n.s. | n.s. |
| Female | n.s. | 5·0, $P = 0·033$ | n.s. |
| DR3-DQ2/DRX Male | n.s. | n.s. | n.s. |
| Female | n.s. | n.s. | n.s. |
| DR4-DQ8/DR4-DQ8 Male | n.s. | n.s. | n.s. |
| Female | 7·9, $P = 0·013$ | n.s. | n.s. |
| DR4-DQ8/DRX Male | n.s. | n.s. | n.s. |
| Female | n.s. | n.s. | n.s. |

TGA/CD, ATPA and TPOA = autoantibodies to tissue transglutaminase and/or coeliac disease, autoantibodies to $H^+/K^+$-ATPase and autoantibodies to thyroid peroxidase, respectively. Odds ratios and *P*-values are represented; n.s. = not significant result ($P > 0·05$). Increased risk of TGA/CD was linked to the DR3-DQ2 haplotype, with the highest risk in DR3-DQ2/DR3-DQ2 individuals; increased risk of ATPA was linked to DR3-DQ2, *DRB1*0404* (in males) and DR3-DQ2/DR4-DQ8; increased risk of TPOA was linked to DR3-DQ2/DR3-DQ2. Reference genotype = DRX/DRX.

characterized by more than one non-islet autoantibody were observed. In individuals with thyrogastric autoimmunity, the frequency of the HLA DR3-DQ2 ($n = 17$; 73·9%) and the HLA DR4-DQ8 ($n = 15$; 65·2%) haplotypes was not different from the rest of the cohort ($n = 546$; 57·3%, $P = 0·061$ and $n = 702$; 73·7%, $P = 0·565$, respectively, for DR3-DQ2 and DR4-DQ8).

### Islet-autoantibodies in patients with multiple autoimmunity

Weak correlations of ATPA ($r = 0·184$, $P < 0·0001$) and TPOA ($r = 0·176$, $P < 0·0001$) with GADA positivity were observed, but no associations of non-islet antibodies with IAA, IA-2A or ZnT8A were found.

## Discussion

Although the association between T1D and other autoimmune disorders is well known, and risk factors for each autoimmune disease have been investigated extensively, studies of MA or autoimmune polyglandular syndromes are very limited, especially including genetic analysis [19,30,33]. This study is the first population-based study in the United Kingdom, to our knowledge, to investigate the prevalence of MA in children and young adults with T1D, as well as genetic and non-genetic risk factors.

### Prevalence of multiple autoimmunity in T1D

Multiple autoimmunity was common in patients with T1D diagnosed during childhood and adolescence. The prevalence of MA was higher in females and was influenced strongly by age. It was also associated strongly with HLA genotypes, particularly those including DR3-DQ2 haplotypes. Furthermore, risk of ATPA increased with *DRB1*0404* in males.

The high frequency of TGA in individuals with T1D established in this study is in line with previous reports [20,23,34–41]. The prevalence of ATPA and TPOA was lower than reported previously (18–25%) [19,30,33,41,42], although exceeding that in the general population by up to

three times. Discrepancies could be caused by differences in study design (population-based *versus* hospital-based), use of different testing techniques, assay thresholds or variations in the age of the cohorts tested. For example, our patient cohort comprises children and young adults and we therefore based our threshold on a schoolchild cohort. Using the commercial kit threshold, which is based on healthy adult blood donors, would increase the prevalence of TPOA in our patients to 17·2%.

The detection of non-islet autoimmunity does not necessarily presage the onset of clinical disease, especially in those with levels close to the threshold. Complete clinical data were not available for the patients in this study and therefore we were unable to estimate the prevalence of latent disease or calculate predictive values for the different antibodies. However, it is likely that the majority of antibody-positive patients will develop subclinical disease. While pernicious anaemia was found in only a minority of ATPA positive Belgian T1D patients, more than half of adult patients with parietal cell antibodies had autoimmune gastritis [43]. Thyroid dysfunction was also common in those with TPOA [19]. A Romanian study found that 87% of TPOA-positive, but initially euthyroid, patients with T1D progressed to subclinical hypothyroidism within $5 \pm 3{\cdot}3$ years [44]. The high prevalence of multiple autoimmunity we observed, therefore, even from a relatively young age, supports the case for targeted screening of T1D patients. However, the optimal timing and frequency of autoantibody testing as an aid to cost-effective diagnosis still needs to be determined. Prospective clinical studies of non-islet autoimmunity in T1D patients are needed.

### Risk factors for multiple autoimmunity

The increased risk for females developing most autoimmune diseases is well known [30,33,43,45–48], with sex hormones speculated to be involved [43,49]. Coeliac-specific autoantibodies were more common in younger children [33,50–52], in contrast to ATPA and TPOA [19,20,33,43,53–55]. However, as the majority of individuals in this cohort were tested close to T1D diagnosis, it was difficult to discriminate between the effects of age and age at T1D onset on non-islet autoantibody prevalence.

The cross-sectional design of this study could not inform us as to the timing of seroconversion and we cannot identify which component of MA could trigger or predict another. However, the highest frequency of coeliac autoimmunity was in children with T1D diagnosed under age 5 years, which may suggest the presence of shared causative factors predisposing to early development of these diseases. Later onset of gastric autoimmunity may suggest that infectious agents such as *Helicobacter pylori* or local gastric inflammation, which are more common in older age, could contribute to the loss of tolerance to ATPase [56,57]. The importance of *H. pylori* however, remains doubtful, as these bacteria were detected in only 16% of autoimmune gastritis cases [58]. An age-dependent increase in TPOA demonstrates the overall tendency of autoimmunity, especially thyroiditis, to develop more often in puberty and beyond [19,43,54,55]. Furthermore, while we have attempted to match the median age of the schoolchild controls to those of the patients, the distribution of ages within the cohorts was different and this should be considered when interpreting these results.

Our genetic findings provide additional evidence of the contribution of the HLA DR3-DQ2 haplotype to the risk of clustering of T1D, coeliac and thyroid autoimmunity [19,33,59–62], as well as novel information concerning its contribution to the clustering of T1D and gastric autoimmunity. The HLA DR4-DQ8/DR4-DQ8 genotype demonstrated an unexpectedly strong effect on risk (second after DR3-DQ2/DR3-DQ2) for developing coeliac autoimmunity in T1D patients, given the fact that the DR4-DQ8 haplotype shows a much weaker association with CD than DR3-DQ2 [61]. The observed risk effect of *DRB1*0404* on the development of ATPA replicates findings of a recent large study [30]. No significant associations between genes and the presence of more than one non-islet autoantibody was observed, probably because of the small number of affected individuals, although an effect of the HLA DR3-DQ2 haplotype on the development of thyrogastric autoimmunity came close to significance. Further work is needed to reveal the precise contribution of HLA and non-HLA genes to the risk of MA.

### Overlap with islet autoantibodies

Our results confirm the overlap between ATPA/TPOA and GADA [15,19,20,30,34,35,45] in spite of correction for HLA DR3-DQ2, which is known to predispose to GADA. This may suggest common disease pathways, given that the GAD65 enzyme is expressed ubiquitously in the pancreas, thyroid gland and stomach [63]. Chance coincidence should also be considered however, as GADA and ATPA/TPOA tend to be more common in older age and in patients with a longer duration of T1D [19].

## Conclusion

In summary, T1D is associated frequently with additional autoimmune conditions such as coeliac, gastric and thyroid disease, which are also characterized by production of organ-specific autoantibodies. Measurement of non-islet autoantibodies in young individuals with T1D is important for early detection of multiple autoimmunity, as this can improve the in-time diagnosis and prognosis. Knowledge of the genetic and non-genetic risk factors predisposing to clustering of autoimmune diseases may allow a deeper understanding of shared pathogenetic mechanisms and inform targeted screening strategies.

## Author contributions

Study design: A. K., A. W., K. C. and K. G.; development of the ATPA assay: A. K., J. W., H. D. and A. W.; testing for autoantibodies: A. K., R. W., C. C., C. W., K. C. and A. W.; HLA class II genotyping: R. A., K. G.; data analysis: A. K., A. L., R. W.; first draft of the manuscript: A. K.; all authors contributed to its completion.

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## Disclosure

None.

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## Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's website:
**Fig. S1.** The distribution of non-islet autoantibodies in healthy children and young patients with type 1 diabetes (T1D). Antibody levels are shown for (a) anti-tissue transglutaminase autoantibodies (TGA), (b) anti-$H^+/K^+$-ATPase autoantibodies (ATPA) and (c) anti-thyroid peroxidase autoantibodies (TPOA). The thresholds indicated by the dotted lines were set at the 97·5th percentile of the 5470 children of the ALSPAC cohort for TGA, 318 schoolchildren for ATPA and 205 schoolchildren for TPOA. Those samples with levels below 1 TGA unit, 5 ATPA units/ml and 2 TPOA units/ml are represented by the numbers in boxes. The distribution of ATPA and TPOA levels in patients appears distinct from those of controls, while that of TGA seems similar in the two cohorts.

**Table S1.** Prevalence of multiple antibodies (MA) to non-islet antigens in 1052 patients with type 1 diabetes tested for all antibodies to anti-tissue transglutaminase autoantibodies (TGA), anti-$H^+/K^+$-ATPase autoantibodies (ATPA) and anti-thyroid peroxidase autoantibodies (TPOA).

  