

Genetic association of zinc transporter 8 (ZnT8) autoantibodies in type 1 diabetes cases

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Abstract

Aims/hypothesis Autoantibodies to zinc transporter 8 (ZnT8A) are associated with risk of type 1 diabetes. Apart from the *SLC30A8* gene itself, little is known about the genetic basis of ZnT8A. We hypothesise that other loci in addition to *SLC30A8* are associated with ZnT8A.

Methods The levels of ZnT8A were measured in 2,239 British type 1 diabetic individuals diagnosed before age 17 years, with a median duration of diabetes of 4 years. Cases were tested at over 775,000 loci genome wide (including 53 type 1 diabetes associated regions) for association with positivity for

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ZnT8A. ZnT8A were also measured in an independent dataset of 855 family members with type 1 diabetes.

Results Only *FCRL3* on chromosome 1q23.1 and the HLA class I region were associated with positivity for ZnT8A. rs7522061T>C was the most associated single nucleotide polymorphism (SNP) in the *FCRL3* region ($p=1.13 \times 10^{-16}$). The association was confirmed in the family dataset ($p \leq 9.20 \times 10^{-4}$). rs9258750A>G was the most associated variant in the HLA region ($p=2.06 \times 10^{-9}$ and $p=0.0014$ in family cases). The presence of ZnT8A was not associated with *HLA-DRB1*, *HLA-DQB1*, *HLA-A*, *HLA-B* or *HLA-C* ($p>0.05$). Unexpectedly, the two loci associated with the presence of ZnT8A did not alter risk of having type 1 diabetes, and the 53 type 1 diabetes risk loci did not influence positivity for ZnT8A, despite them being disease specific.

Conclusions/interpretation ZnT8A are not primary pathogenic factors in type 1 diabetes. Nevertheless, ZnT8A testing in combination with other autoantibodies facilitates disease prediction, despite the biomarker not being under the same genetic control as the disease.

Keywords Autoantibody · *FCRL3* · HLA · MHC · *SLC30A8* · Type 1 diabetes · Zinc transporter 8

Abbreviations

GWAS	Genome-wide association study
IA-2A	Autoantibodies to insulinoma-associated antigen-2
IAA	Autoantibodies to insulin
ICA	Islet cell autoantibodies
LD	Linkage disequilibrium
SNP	Single nucleotide polymorphism
T1DGC	Type 1 diabetes genetics consortium
WTCCC	Wellcome Trust Case Control Consortium
ZnT8A	Autoantibodies to zinc transporter 8

Introduction

Autoantibodies to glutamic acid decarboxylase (GADA), insulinoma-associated antigen-2 (IA-2A) and insulin (IAA) and islet cell autoantibodies (ICA) have long been established as associated with the development of type 1 diabetes, with over 90% of newly diagnosed cases positive for at least one of these autoantibodies [1–4]. Most recently, autoantibodies to the pancreatic beta cell-specific protein, zinc transporter 8 (ZnT8A), have also been shown to be associated with type 1 diabetes [5]. Over 60% of newly diagnosed cases are positive for ZnT8A, and 4% of cases are positive for ZnT8A only [5–7], thus suggesting their utility as a predictive and diagnostic marker in type 1 diabetes [8–10]. The percentage of newly diagnosed cases positive for ZnT8A (as well as the magnitude of the ZnT8A titre) has been positively correlated with an older age at diagnosis of type 1 diabetes in children [5, 11] and has been shown to decline following diagnosis of type 1 diabetes [12, 13]. The same correlations have also been found with IA-2A and GADA [14].

The ZnT8 protein is encoded by the *SLC30A8* gene, which is convincingly associated with type 2 diabetes risk at the single nucleotide polymorphism (SNP) rs13266634C>T, an amino acid substitution R325W [15–17]. Although this amino acid variant determines the epitope specificity of ZnT8A in type 1 diabetes, either ZnT8RA or ZnT8WA [8, 11], its association with risk of the disease itself is less clear [18–20].

ZnT8RA have been reported as associated with HLA-DQB1*0302 genotypes [7] and both ZnT8RA and ZnT8WA have been correlated with HLA-DQB1*0604 haplotypes [8]. No other genetic associations have been reported with ZnT8A to date. Given the association of ZnT8A with type 1 diabetes, the 52 non-HLA regions of the genome associated with type 1 diabetes (www.t1dbase.org, accessed 13 December 2011), have a high prior probability of also being associated with ZnT8A positivity. Therefore, we measured ZnT8A (R and W epitopes) in 2,239 British individuals with type 1 diabetes and tested the known type 1 diabetes regions for association with presence of ZnT8A. A genome-wide association study (GWAS) was also performed to further test for genetic determinants of ZnT8A. Finally, we re-examined the association of rs13266634 in *SLC30A8* with ZnT8A and type 1 diabetes.

Methods

Samples A total of 2,239 British type 1 diabetic individuals diagnosed before the age of 17 years were included in the study (Table 1). These constitute a randomly selected subset of the full collection of 8,000 cases that have been described elsewhere [14]. An independent dataset of 855 family members within 2 years of diagnosis of type 1 diabetes

(average age at diagnosis of 12.0 years) was taken from the type 1 diabetes genetics consortium (T1DGC) affected sib-pair families and have been described previously [21]. All samples were of white European ancestry.

Autoantibody measurement ZnT8A were measured in serum by protein A radiobinding assays as previously described [20] using COOH-terminal (aa268-369) constructs of the ZnT8 R325 (ZnT8RA) and W325 (ZnT8WA) variants, respectively. cDNAs for the preparation of radioligands by in vitro transcription–translation were kind gifts of V. Lampasona (San Raffaele Scientific Institute, Milan, Italy). Samples were considered ZnT8A positive if antibodies to at least one of the ZnT8 variants (ZnT8RA and/or ZnT8WA) were found. Performance in the Diabetes Antibody Standardization Program 2009 workshop is shown as ‘laboratory 121’ in a published report [22]. Details of the GADA and IA-2A assays, and the ZnT8A assay in T1DGC families, have been reported previously [14, 21, 22].

Genotyping The *HLA-DRB1*, *HLA-DQB1*, *HLA-A*, *HLA-B* and *HLA-C* genes were typed at four digit resolution using Dynal RELI SSO assays (Invitrogen, Paisley, UK) as detailed elsewhere [23]. A subset of cases was genotyped at *HLA-DRB1* and *HLA-DQB1* using Roche Molecular Systems SSO reverse dot blot technology (Roche Molecular Systems, Pleasanton, CA, USA). The T1DGC GWAS genotyped 487,592 SNPs that passed quality control checks (2,420 within the extended MHC) using the Illumina 550K Infinium platform [24] (Illumina, San Diego, CA, USA); and 362,838 SNPs (1,687 within the extended MHC) study were genotyped using the Affymetrix GeneChip Human Mapping 500K Array set (Affymetrix, Santa Clara, CA, USA) for the Wellcome Trust Case Control Consortium (WTCCC) GWAS [25]; 75,181 were common to both platforms. Follow-up SNPs were genotyped blind to ZnT8A status using the TaqMan 5′ nuclease assay (Applied Biosystems, Warrington, UK) according to the manufacturer's protocols. All genotyping in the family samples used TaqMan. Two operators scored genotypes blind to disease status.

Statistics All statistical analyses were performed in R (www.r-project.org, accessed 13 December 2011) [26] or STATA (www.stata.com, accessed 13 December 2011). Association with ZnT8A (including R and W epitopes) was assessed using logistic regression models, with autoantibody positivity as the outcome variable and genotype as the predictor. Covariates, age at diagnosis and duration of diabetes were included in the regression models to account for the decrease in positivity for ZnT8A with duration of diabetes and the increase in the number of cases positive for ZnT8A with increasing age at diagnosis. For family analyses, robust variance estimates were used to account for non-

Table 1 Phenotypes of the type 1 diabetic cases by positivity for ZnT8A status

Variable	Cases overall	ZnT8A		ZnT8WA	ZnT8RA
		Positive	Negative	Positive	Positive
<i>n</i> , all samples	2,239	752	1,487	531	642
Age at diagnosis					
Mean (SD)/year	7.57 (3.91)	8.99 (3.36)	6.86 (3.98)	9.14 (3.37)	9.08 (3.31)
Median/year	7	9	7	10	9.5
Duration of diabetes					
Mean (SD)/year	6.61 (7.53)	3.45 (3.83)	8.27 (8.54)	3.38 (3.84)	3.34 (3.60)
Median/year	4	2	6	2	2
Age					
Mean (SD)/year	14.18 (8.05)	12.43 (4.75)	15.13 (9.29)	12.52 (4.70)	12.41 (4.53)
Median/year	13	12	13	13	12
Sex					
Male	1,119 (0.50)	368 (0.49)	751 (0.51)	270 (0.51)	319 (0.50)
Female	1,119 (0.50)	383 (0.51)	736 (0.49)	261 (0.49)	322 (0.50)

independence within families [27]. Missing genotypes were imputed in the GWAS datasets using a common set of 1,500 controls genotyped on both platforms as reported previously [24]. Imputed genotypes were weighted by their posterior expectation in the regression models. To assess which autoantibody was associated with rs7522061, ZnT8A and IA-2A were used as predictors of rs7522061 genotype in regression models.

Results

ZnT8A was measured in 2,239 British type 1 diabetic individuals. Cases positive for ZnT8A were associated with an older age at diagnosis ($p=3.12 \times 10^{-16}$) and a shorter duration of diabetes ($p=1.10 \times 10^{-57}$), as were both the ZnT8RA and ZnT8WA epitopes ($p < 5 \times 10^{-14}$; Table 1). Whilst the highest positivity for ZnT8A was observed in cases with the shortest duration of disease (≤ 2 years), 10% of cases who

had had diabetes for ≥ 9 years were still positive for ZnT8A (Table 2). Therefore, both age at diagnosis and duration of diabetes were included as covariates in all tests of association with positivity for ZnT8A. This accounts for the decrease in positivity for ZnT8A with duration of disease and earlier age at diagnosis in the statistical association tests, such that all positive associations obtained can be attributed to the genetic variant tested.

A subset of 1,307 cases with ZnT8A measurements had previously been tested for GADA and IA-2A [14, 21]. Of these, just 277 (21%) were negative for all three autoantibodies and 28 were only positive for ZnT8A. There was a strong association of positivity for ZnT8A with both IA-2A and GADA positivity, after accounting for age at diagnosis and duration of disease (OR 4.45 [95% CI 3.32, 5.99] $p=8.46 \times 10^{-26}$ and OR 1.63 [95% CI 1.24, 2.14] $p=5.04 \times 10^{-4}$, respectively); 81% of ZnT8A positives were also positive for IA-2A, whereas 63% were positive for GADA.

Table 2 Positivity for ZnT8A in type 1 diabetes cases by quartiles of the duration of diabetes distribution

Duration	ZnT8A positive <i>n</i> (%)	ZnT8WA positive <i>n</i> (%)	ZnT8RA positive <i>n</i> (%)	ZnT8A negative <i>n</i> (%)
British cases				
≤ 2 years	389 (58)	283 (42)	340 (51)	279 (42)
3–4 years	187 (38)	128 (26)	158 (32)	301 (62)
5–8 years	122 (22)	81 (15)	104 (19)	426 (78)
≥ 9 years	54 (10)	39 (7)	40 (8)	479 (90)
T1DGC families				
<2 years	523 (60)	412 (54)	462 (61)	345 (40)

n number of samples

ZnT8A association in type 1 diabetes regions

Outside of the HLA, no evidence of association with positivity for ZnT8A was obtained in the type 1 diabetes associated regions with the British cases ($p > 0.03$). None of the classical HLA loci, *HLA-DRB1*, *HLA-DQB1*, *HLA-A*, *HLA-B* and *HLA-C*, were associated with ZnT8A ($p > 0.05$; Fig. 1). Evidence of association in the HLA region mapped to the class I region (Fig. 1). The most strongly associated SNP was rs2855812 ($p = 2.25 \times 10^{-5}$) in *MICB* in the T1DGC cases, and rs9258750 ($p = 3.00 \times 10^{-4}$) near *HLA-A* in the WTCCC cases. The genotyping of these SNPs was extended to over 2,100 cases in which ZnT8A was measured (electronic supplementary material [ESM] Table 1). The rs9258750 SNP showed more evidence of association with positivity for ZnT8A ($p = 2.06 \times 10^{-9}$; OR for the major A allele 1.82 [95% CI 1.49, 2.23]) than rs2855812 ($p = 5.80 \times 10^{-5}$; OR for the minor T allele 1.33 [95% CI 1.16, 1.53]). The two SNPs were not in linkage disequilibrium (LD) ($r^2 = 0.02$, $D' = 0.33$) and hence appear to mark independent effects ($p = 7.2 \times 10^{-4}$ for the addition of rs2855812 to rs9258750, and $p = 5.83 \times 10^{-8}$ for the addition of rs9258750 to rs2855812). rs9258750 was in LD with *HLA-A*24* ($r^2 = 0.54$, $D' = 0.98$). *HLA-A*24* was associated with ZnT8A positivity ($p = 2.03 \times 10^{-4}$), but this was due to rs9258750: $p = 0.95$ for the addition of *HLA-A*24* to rs9258750, compared with $p = 0.0015$ for the addition of rs9258750 to *HLA-A*24* in regression models. The association of rs9258750 with positivity for ZnT8A was confirmed in an independent family dataset of white European ancestry ($p = 0.0014$; OR for the major A allele 1.57 [95% CI 1.19, 2.06]) in the 833 affected family members successfully genotyped).

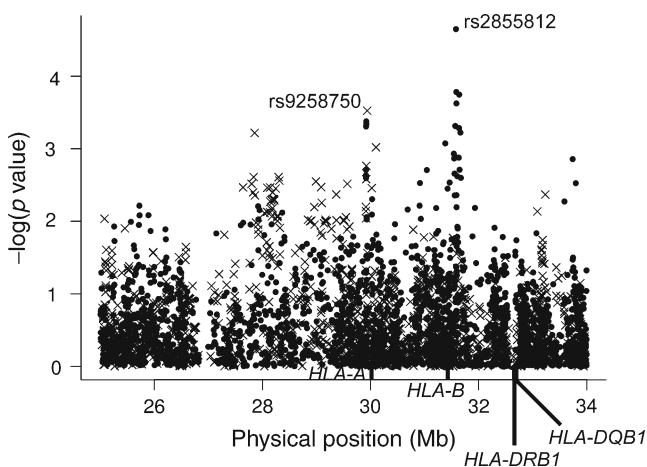


Fig. 1 Association of ZnT8A with SNPs in the MHC region of chromosome 6. SNPs are analysed in up to 1,021 type 1 diabetes cases at the WTCCC SNPs (crosses) and up to 1,123 type 1 diabetes cases at the T1DGC SNPs (dots). $-\log(p$ value for association with ZnT8A positivity) is plotted against physical position in megabases (Mb). The physical locations of the HLA classical loci are also given

Genome-wide association

Having found no evidence of association with positivity for ZnT8A in the type 1 diabetes associated regions, the search was extended genome wide. Up to a maximum of 2,142 cases were analysed at 775,249 SNPs across the genome. Only the *FCRL3* gene region on chromosome 1 attained significance at a GWAS level ($p < 5 \times 10^{-8}$) with all SNPs outside of this region having p values above 1×10^{-6} (Fig. 2 and ESM Fig. 1). The most associated SNP, rs7522061, ($p = 1.13 \times 10^{-16}$) is located in exon 4 of the *FCRL3* gene (Ensembl version 63) and is in LD with rs7528684 ($r^2 = 0.89$ in British controls), which has previously been shown to be associated with IA-2A in type 1 diabetes cases [21, 28]. The region is also associated with Graves' disease and thyroid peroxidase autoantibody positivity at rs11264798 [21, 25].

Neither rs7528684 (the IA-2A associated variant) nor rs11264798 (the Graves' disease associated variant) were genotyped as part of the GWAS, but had been genotyped previously using TaqMan [21]. Both SNPs were associated with positivity for ZnT8A (rs7528684, $p = 1.79 \times 10^{-16}$; OR for the major A allele 1.81 [95% CI 1.56, 2.09] and rs11264798 $p = 4.86 \times 10^{-14}$; OR for the C allele 1.72 [95% CI 1.48, 1.98]). Only the WTCCC samples were genotyped at rs7522061 (with imputation used for the remaining samples in the GWAS), and so this was extended to 2,131 cases by TaqMan genotyping (Table 3). Whilst rs7522061 accounted for the association at rs11264798, both rs7522061 and rs7528684 appear to be marking the same association, as their effects could not be distinguished in the 2,088 cases genotyped at both loci: rs7528684 did not improve a model with rs7522061 ($p = 0.15$) and conversely, rs7522061 did not improve a model with rs7528684 ($p = 0.10$). Given that IA-2A and ZnT8A are correlated, we tested whether the association with positivity for ZnT8A at *FCRL3*

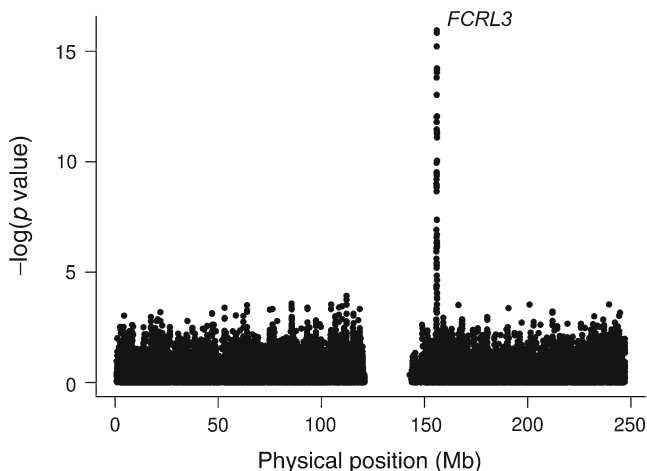


Fig. 2 Association of positivity for ZnT8A with SNPs on chromosome 1 in up to 2,142 type 1 diabetes cases

Table 3 Association of positivity for ZnT8A with rs7522061 T>C in *FCRL3*

rs7522061	T allele		OR [95% CI]	<i>p</i> value
	<i>n</i> (frequency)			
	Positive	Negative		
British cases				
ZnT8A	876 (0.61)	1,395 (0.49)	1.82 [1.57, 2.10]	1.13×10^{-16}
ZnT8WA	630 (0.62)	1,641 (0.51)	1.70 [1.45, 1.98]	8.13×10^{-12}
ZnT8RA	735 (0.61)	1,536 (0.50)	1.65 [1.42, 1.91]	1.74×10^{-11}
Family samples				
ZnT8A	601 (0.59)	322 (0.49)	1.50 [1.22, 1.84]	1.20×10^{-4}

The minor allele is negatively associated with the presence of ZnT8A in both the British cases and the family samples, so ORs and allele frequencies are given for the major T allele. The association of rs7522061 with ZnT8A is consistent across both ZnT8WA and ZnT8RA epitopes

was due to the known association with IA-2A. Using 1,241 cases in a model that included both ZnT8A and IA-2A, positivity for ZnT8A predicted the rs7522061 genotype ($p=5.51 \times 10^{-11}$) and IA-2A positivity did not ($p=0.10$).

The association at *FCRL3* was confirmed in up to 855 affected family members of white European ancestry. Confirmation of association with ZnT8A was obtained at rs7522061, rs7528684 and rs11264798 ($p=1.20 \times 10^{-4}$, 3.14×10^{-4} and 9.20×10^{-4} , respectively) with the same direction of effect as in the British cases (OR for the T allele at rs7522061, 1.50 [95% CI 1.22, 1.84]; Table 3).

Association of ZnT8A with *SLC30A8*

In the GWAS, whilst evidence of association with positivity for ZnT8A was obtained at rs13266634/R325W in *SLC30A8* ($p=5.29 \times 10^{-5}$), it was not at a level required for GWAS

significance. As genotypes were only available for those samples genotyped using the Illumina platform, rs13266634 was genotyped in all samples using TaqMan (as detailed in the Methods). The SNP was associated with positivity for ZnT8A ($p=3.66 \times 10^{-11}$). However, a multiplicative allelic effects model did not fit the data. The minor T/T homozygous genotype was associated with ZnT8A positivity and the heterozygous C/T genotype with absence of ZnT8A (Table 4). Analysis of the two epitopes, ZnT8WA and ZnT8RA, revealed that the T allele at rs13266634 was associated with ZnT8WA positivity ($p=9.26 \times 10^{-27}$) and ZnT8RA negativity ($p=7.20 \times 10^{-17}$; Table 4). This inverse association of the two ZnT8A epitopes with rs13266634 accounted fully for the deviation from a multiplicative allelic effects model with positivity for ZnT8A overall. Importantly, this SNP is not associated with type 1 diabetes overall ($p=0.15$ in 7,680 British type 1 diabetes cases and 7,200 British controls; ESM Table 2).

Discussion

We have obtained convincing evidence of association between positivity for ZnT8A and the nonsynonymous SNP, rs7522061, in *FCRL3*, and in our datasets this association was indistinguishable from the association with the non-coding SNP, rs7528684. The T allele of rs7522061, which is associated with positivity for ZnT8A, alters the amino acid sequence (Asp to Asn), which could alter protein function and/or RNA stability/splicing. Only in relation to multiple sclerosis have there been reports of this SNP being associated; the same study also reported disease association with rs7528684 [29]. The minor G allele at rs7528684 is associated with risk of rheumatoid arthritis and systemic lupus erythematosus [30], protection from autoimmune Addison disease [31] and multiple sclerosis [29], and has

Table 4 Association of rs13266634 in *SLC30A8* with positivity for ZnT8A in 2,191 type 1 diabetes cases

rs13266634	ZnT8A			ZnT8WA			ZnT8RA		
	<i>n</i> (frequency)		OR [95% CI]	<i>n</i> (frequency)		OR [95% CI]	<i>n</i> (frequency)		OR [95% CI]
	Positive	Negative		Positive	Negative		Positive	Negative	
T	500 (0.34)	928 (0.32)	1.07 [0.93, 1.24]	478 (0.46)	950 (0.28)	2.38 [2.02, 2.80]	310 (0.25)	1,118 (0.36)	0.51 [0.43, 0.60]
C/C	348 (0.47)	651 (0.45)	1.00 (reference)	152 (0.29)	847 (0.51)	1.00 (reference)	348 (0.56)	651 (0.42)	1.00 (reference)
C/T	272 (0.37)	684 (0.47)	0.64 [0.52, 0.78]	254 (0.49)	702 (0.42)	2.01 [1.59, 2.55]	246 (0.39)	710 (0.45)	0.54 [0.44, 0.67]
T/T	114 (0.16)	122 (0.08)	1.90 [1.38, 2.62]	112 (0.22)	124 (0.07)	6.31 [4.48, 8.88]	32 (0.05)	204 (0.13)	0.23 [0.15, 0.35]
<i>p</i> value			3.66×10^{-11} ^a			9.26×10^{-27} ^b			7.20×10^{-17} ^b

n number of alleles or genotypes

^a Note that the multiplicative model did not fit the data, $p=6.44 \times 10^{-12}$, hence the *p* value reported is for a model that does not assume a specific mode of inheritance

^b Note the multiplicative model was an appropriate approximation for the association with ZnT8WA ($p=0.056$) and ZnT8RA ($p=0.38$)

been found to alter the binding affinity of nuclear factor- κ B [30], making it a good functional candidate SNP. Interestingly, *FCRL3* is not associated with type 1 diabetes risk [21], even though it is strongly associated with IA-2A positivity [21, 28]. We have shown here that this association is probably due to a primary association with positivity for ZnT8A and not with IA-2A positivity directly. The association with positivity for ZnT8A was marked equally well by rs7522061 or rs7528684, and consequently, further work is required to unravel the precise nature of the association with the presence of ZnT8A in the *FCRL3* region and the mechanisms involved.

None of the regions known to be associated with type 1 diabetes, outside of the HLA, were associated with positivity for ZnT8A. Interestingly, evidence of association with positivity for ZnT8A was confined to the HLA class I region, but the most associated variant, rs9258750, was not associated with type 1 diabetes ($p=0.13$ in 8,232 British cases and 9,757 British controls; data not shown). The known associations of IA-2A with *HLA-A*24* [14, 32] and of GADA with rs9266722 in HLA class I [14] were independent of the ZnT8A associations described in the present study. In contrast to previous reports in smaller sample sets, no association with *HLA-DQB1*0302*, **0604*, **04* alleles and ZnT8A was obtained ($p>0.15$), neither with positivity for ZnT8A ($p>0.15$) nor ZnT8WA ($p>0.16$).

Our study is the largest of its kind to date, using 2,239 case samples, 70% of whom had had ZnT8A measured, having been diagnosed with type 1 diabetes more than 2 years earlier. Therefore, we have been able to confirm recent reports that showed that positivity for ZnT8A declines after diagnosis of disease [12, 13]. Approximately 40% of cases tested for ZnT8A who have had diabetes for 4 years are expected to test positive compared with 62% at diagnosis [12]. In our samples it was comparable (Table 2). A consequence of this decline in positivity for ZnT8A with duration of diabetes is that the rate of ZnT8A negative samples will be elevated amongst cases with longer disease duration. Consequently, almost half the cases classed as ZnT8A negative who had had diabetes for 9 or more years would probably have tested positive had they been tested at diagnosis. This causes the association signal at some loci to be diluted in these samples, but does not affect the validity of the positive associations detected. The *HLA* and *FCRL3* associations with ZnT8A in cases who have had diabetes for ≤ 2 years are of comparable strength to those who have had diabetes for ≥ 9 years (ESM Tables 3 and 4). All positive associations were confirmed in the cases from the families affected by type 1 diabetes, all of whom were tested within 2 years of diagnosis. The lack of association outside of the HLA and *FCRL3* may be attributable to lack of power, which was exacerbated by the increased frequency of ZnT8A negativity amongst samples with a longer duration

of disease. The inverse correlation of positivity for ZnT8A with duration of diabetes was not known when we designed our study, and hence, the statistical power of future studies into the genetic architecture of presence of ZnT8A would benefit from using cases as close to diagnosis as possible, as we have done for the replication in family samples.

For autoantibodies to be causal for type 1 diabetes, the variants associated with risk of type 1 diabetes would also be expected to be associated with autoantibody positivity. Our results, therefore, indicate that autoantibodies to ZnT8 are a downstream event to the primary genetic pathogenesis of type 1 diabetes. Given their specificity for type 1 diabetes [6], however, this does not detract from their well established value, in combination with other autoantibodies, in assessing risk of the disease [6, 10, 20], but does illustrate how useful biomarkers need not necessarily have associations with genetic markers that confer susceptibility to disease.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement JMMH analysed and interpreted the data and wrote the manuscript. SK, AGZ, EB and PA tested case control samples for ZnT8A positivity. HS managed and prepared DNA samples and interpreted data. DJS performed TaqMan genotyping. JW and JH measured ZnT8A in T1DGC families. EB, JAT, PA and JMMH designed the study. All authors reviewed/edited/approved the manuscript.

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References

- Borg H, Marcus C, Sjoblad S, Fernlund P, Sundkvist G (2002) Insulin autoantibodies are of less value compared with islet antibodies in the clinical diagnosis of autoimmune type 1 diabetes in children older than 3 yr of age. *Pediatr Diabetes* 3:149–154
- Bilbao JR, Rica I, Vazquez JA, Busturia MA, Castano L (2000) Influence of sex and age at onset on autoantibodies against insulin, GAD65 and IA2 in recent onset type 1 diabetic patients. *Horm Res* 54:181–185
- Verge CF, Gianani R, Kawasaki E et al (1996) Number of autoantibodies (against insulin, GAD or ICA512/IA2) rather than particular autoantibody specificities determines risk of type I diabetes. *J Autoimmun* 9:379–383
- Atkinson MA, Eisenbarth GS (2001) Type 1 diabetes: new perspectives on disease pathogenesis and treatment. *Lancet* 358:221–229
- Wenzlau JM, Juhl K, Yu L et al (2007) The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes. *Proc Natl Acad Sci USA* 104:17040–17045
- Wenzlau JM, Moua O, Sarkar SA et al (2008) Slc30A8 is a major target of humoral autoimmunity in type 1 diabetes and a predictive marker in prediabetes. *Ann N Y Acad Sci* 1150:256–259
- Brorsson C, Vaziri-Sani F, Bergholdt R et al (2011) Correlations between islet autoantibody specificity and the *SLC30A8* genotype with *HLA-DQB1* and metabolic control in new onset type 1 diabetes. *Autoimmunity* 44:107–114
- Andersson C, Larsson K, Vaziri-Sani F et al (2011) The three ZNT8 autoantibody variants together improve the diagnostic sensitivity of childhood and adolescent type 1 diabetes. *Autoimmunity* 44:394–405
- Vermeulen I, Weets I, Asanhanwa M et al (2011) Contribution of antibodies against IA-2beta and zinc transporter 8 to classification of diabetes diagnosed under 40 years of age. *Diabetes Care* 34:1760–1765
- De Grijse J, Asanhanwa M, Nouthe B et al (2010) Predictive power of screening for antibodies against insulinoma-associated protein 2 beta (IA-2beta) and zinc transporter-8 to select first-degree relatives of type 1 diabetic patients with risk of rapid progression to clinical onset of the disease: implications for prevention trials. *Diabetologia* 53:517–524
- Wenzlau JM, Liu Y, Yu L et al (2008) A common nonsynonymous single nucleotide polymorphism in the *SLC30A8* gene determines ZnT8 autoantibody specificity in type 1 diabetes. *Diabetes* 57:2693–2697
- Wenzlau JM, Walter M, Gardner TJ et al (2010) Kinetics of the post-onset decline in zinc transporter 8 autoantibodies in type 1 diabetic human subjects. *J Clin Endocrinol Metab* 95:4712–4719
- Vaziri-Sani F, Oak S, Radtke J et al (2010) ZnT8 autoantibody titers in type 1 diabetes patients decline rapidly after clinical onset. *Autoimmunity* 43:598–606
- Howson JM, Stevens H, Smyth DJ et al (2011) Evidence that HLA class I and II associations with type 1 diabetes, autoantibodies to GAD and autoantibodies to IA-2, are distinct. *Diabetes* 60:2635–2644
- Zeggini E, Weedon MN, Lindgren CM et al (2007) Replication of genome-wide association signals in UK samples reveals risk loci for type 2 diabetes. *Science* 316:1336–1341
- Scott LJ, Mohlke KL, Bonnycastle LL et al (2007) A genome-wide association study of type 2 diabetes in Finns detects multiple susceptibility variants. *Science* 316:1341–1345
- Sladek R, Rocheleau G, Rung J et al (2007) A genome-wide association study identifies novel risk loci for type 2 diabetes. *Nature* 445:881–885
- Raj SM, Howson JM, Walker NM et al (2009) No association of multiple type 2 diabetes loci with type 1 diabetes. *Diabetologia* 52:2109–2116
- Gohlke H, Ferrari U, Koczwara K, Bonifacio E, Illig T, Ziegler AG (2008) *SLC30A8* (ZnT8) polymorphism is associated with young age at type 1 diabetes onset. *Rev Diabet Stud* 5:25–27
- Achenbach P, Lampasona V, Landherr U et al (2009) Autoantibodies to zinc transporter 8 and *SLC30A8* genotype stratify type 1 diabetes risk. *Diabetologia* 52:1881–1888
- Plagnol V, Howson JM, Smyth DJ et al (2011) Genome-wide association analysis of autoantibody positivity in type 1 diabetes cases. *PLoS Genet* 7:e1002216
- Lampasona V, Schlosser M, Mueller PW et al (2011) Diabetes antibody standardization program: first proficiency evaluation of assays for autoantibodies to zinc transporter 8. *Clin Chem* 57:1693–1702
- Nejentsev S, Howson JM, Walker NM et al (2007) Localization of type 1 diabetes susceptibility to the MHC class I genes *HLA-B* and *HLA-A*. *Nature* 450:887–892
- Barrett JC, Clayton DG, Concannon P et al (2009) Genome-wide association study and meta-analysis find that over 40 loci affect risk of type 1 diabetes. *Nat Genet* 41:703–707
- Wellcome Trust Case Control Consortium (2007) Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature* 447:661–678
- R Development Core Team (2011) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna
- Cordell HJ, Clayton DG (2002) A unified stepwise regression procedure for evaluating the relative effects of polymorphisms within a gene using case/control or family data: application to HLA in type 1 diabetes. *Am J Hum Genet* 70:124–141
- Howson JM, Rosinger S, Smyth DJ, Boehm BO, Todd JA (2011) Genetic analysis of adult-onset autoimmune diabetes. *Diabetes* 60:2645–2653
- Matesanz F, Fernandez O, Milne RL et al (2008) The high producer variant of the Fc-receptor like-3 (*FCRL3*) gene is involved in protection against multiple sclerosis. *J Neuroimmunol* 195:146–150
- Kochi Y, Yamada R, Suzuki A et al (2005) A functional variant in *FCRL3*, encoding Fc receptor-like 3, is associated with rheumatoid arthritis and several autoimmunities. *Nat Genet* 37:478–485
- Owen CJ, Kelly H, Eden JA, Merriman ME, Pearce SH, Merriman TR (2007) Analysis of the Fc receptor-like-3 (*FCRL3*) locus in Caucasians with autoimmune disorders suggests a complex pattern of disease association. *J Clin Endocrinol Metab* 92:1106–1111
- Qu HQ, Polychronakos C (2009) The effect of the MHC locus on autoantibodies in type 1 diabetes. *J Med Genet* 46:469–471