First Genome Wide Association Study of Latent Autoimmune Diabetes in Adults Reveals Novel Insights Linking Immune and Metabolic Diabetes

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INTRODUCTORY PARAGRAPH

Latent autoimmune diabetes in adults (LADA) shares clinical features with both type 1 diabetes (T1D), including the presence of autoimmune autoantibodies, and type 2 diabetes (T2D), with adult age of onset, features of metabolic syndrome and initial insulin independence[1,2]. Despite being at least as prevalent as T1D[4], the genetic basis of LADA remains largely uncharacterized[1,4], except for limited candidate gene analyses suggesting a degree of shared genetic susceptibility with both T1D and T2D[4–14]. We therefore performed the first genome-wide association study of LADA. Together with HLA and gene set enrichment analyses, our data indicate that the leading signals are shared with T1D, but a T2D genetic component is also present. We report a novel signal at *PFKFB3*, encoding a known regulator of glycolysis in energy stressed cells, potentially bridging autoimmunity and metabolism. Furthermore, we observed mutually exclusive genetic co-heritability between LADA and immune traits (associated with T1D) as well as LADA and metabolic/anthropometric traits (associated with T2D), suggesting that the T2D genetic component operates as a modifier of the LADA phenotype.

MAIN TEXT

Although commonly referred to as 'type 1.5 diabetes,' the etiological relationship between LADA and both T1D and T2D is not fully elucidated. In many populations, LADA is at least as prevalent as T1D[3], but is frequently misdiagnosed as T2D[1,4,15,16] given its presentation without need for insulin. As such, LADA subjects could be present in cohort studies for T2D that do not screen out autoantibody-positive cases, potentially resulting in the identification of associations for T2D that are etiologically related to autoimmunity. This challenge is increasingly acute as increasingly larger data sets are assembled to identify additional, common genetic risk factors of smaller effect sizes. Indeed, reflecting this concern, recent genome-wide association study (GWAS) analyses of T2D have reported associations at T1D-associated regions such as *HLA-DQA1* in European ancestry populations[17] and *HLA-B* and *INS-IGF2* in African ancestry populations[18]. While the most recent T2D GWAS[17] claims their signals do not likely represent patients with autoimmune diabetes, they queried only a single T1D SNP for the HLA, although we note that their lead SNP at *HLA-DQA1* (rs9271775) in fact tags the established T1D-associated haplotype HLA-DRB1*15- HLA-DQB1*0602 via proxy SNP rs3135388 (r^2= 0.653). As such, understanding the genetic etiology of LADA will not only aid the much needed characterization of this relatively common form of diabetes, but will also facilitate our understanding of both T1D and T2D.

To date, relatively limited candidate gene studies have been carried out for LADA, but have supported a role for both T1D and T2D risk loci[2,4–13,19]; however, no systematic genome-wide appraisal of LADA has been performed. To address this, we conducted the first GWAS of LADA cases (n = 2,713) versus population-based controls (n = 5,439) of European ancestry in a

discovery meta-analysis setting (**Supplementary Table 1**). Four signals achieved genome-wide significance ($P < 5x10^{-8}$), all at established T1D risk loci (*HLA*, *PTPN22*, *INS*, and *SH2B3*; **Table 1**), despite the adequate power of our study design to detect the strongest T2D-like effects (**Supplementary Table 2**). Pathway analysis with DEPICT[20] for signals at $P < 10^{-5}$ supported a strong immune role in the pathogenesis of LADA (**Supplementary Tables 3-4**), with GSEA implicating 'abnormal cytotoxic T cell physiology' (nominal $P = 6.39 \times 10^{-7}$) as well as the 'mTOR subnetwork' ($P = 6.03 \times 10^{-5}$) and 'cell cycle' ($P = 1.67 \times 10^{-5}$) as also seen in a previous epigenome-wide association study of T1D,[17] and immune system tissue types, including 'natural killer cells' and 'T lymphocytes' (nominal $P = 0.0079$ and 0.0082, respectively). This is consistent with previous reports of these cell types playing a role in the pathogenesis of T1D[21–23] and LADA[24–26].

Using LADA cases and population samples from an additional two study centers, we attempted replication of 13 signals with suggestive association ($P < 5x10^{-5}$) (**Supplementary Table 5**). We observed a novel signal at 10p15.1 between the two established T1D loci at *IL2RA* and *PRKCQ*, which achieved borderline genome-wide significance (rs1983890-C, OR (95% CI) = 1.22 (1.14-1.32), $P = 5.06 \times 10^{-8}$) (**Fig. 1A-B**). As our LADA signal was in moderate to low LD with established T1D-associated alleles (**Supplementary Table 6**), we conditioned on the T1D SNPs and observed that rs1983890 remained strongly associated with LADA (OR (95% CI) = 1.15 (1.12-1.18), $P = 1.7 \times 10^{-7}$) (**Fig. 1C**). DEPICT gene prioritization analysis[20] identified the gene encoding '6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 3' (*PFKFB3*), the nearest gene to the LADA signal, as the most likely candidate (**Supplementary Table 7**). Previous studies strongly support this gene as a plausible biological candidate, as PFKFB3 plays a key regulatory role in insulin-activated glycolysis in energy stressed cells[27,28]. Furthermore, previous

studies have linked PFKFB3 to both autoimmune diseases (*e.g.*, rheumatoid arthritis, Crohn's disease, and celiac disease[29–32]), as well as T2D biology via insulin resistence[33–37].

Several additional variants with suggestive association to LADA overlapped previously documented T1D associations, including rs11755527 (*BACH2*) and rs941576 (*DLK1*). Taking a candidate approach, we extracted 68 established T1D-associated loci from the LADA vs. population controls meta-analysis, and found that 18 of these 68 loci yielded association with LADA after multiple-test correction ($P < 3.6 \times 10^{-4}$, **Supplementary Table 8**). Taking a similar candidate approach with 70 established T2D loci, none surpassed the significance threshold; however, at the nominal significance level ($P < 0.05$), 12 T1D and 14 T2D variants were associated with LADA, all having the same direction of effect as on T1D and T2D, respectively, except for the T2D locus *CILP2* (rs10401969-T, OR = 0.80 (0.72-0.91), $P = 4.9 \times 10^{-4}$).

In terms of T2D-associated loci, our results differ from previous candidate studies. For instance, our previously reported *HNF1A*[7] locus was not observed in this setting. Furthermore, while previous studies showed an association for the leading T2D risk locus at *TCF7L2* with LADA,[6,12] potentially providing a link between T2D and autoimmune diabetes, our data does not support this finding (**Supplementary Table 9**) (LADA vs. population controls, rs7903146-T: OR (95% CI) = 1.006 (0.924-1.095), $P = 0.896$) despite the adequate power of our study design to detect the leading T2D-like signals (**Supplementary Table 2**). To understand the evidence supporting the previous association, we looked at the allele frequencies of the lead variant in each contributing cohort. This revealed that the difference in risk allele frequency between cases and controls was cohort-specific, and may be explained by cohort differences in LADA inclusion criteria (see **Supplementary Table 1 and Supplementary Note**) or by differences in allele frequencies between ActionLADA cases and our controls. Another

possibility is that inclusion or exclusion of T2D patients from control cohorts would affect the frequency of the risk allele; however, sensitivity analysis with control sets that either excluded or included diabetic patients in Swedish and Danish samples showed the persistence of an association (**Supplementary Table 10**), although not at the genome-wide significance level.

Next, we compared LADA with T2D at the genome-wide level. Similar to the results of LADA vs. population controls, LADA (n = 2,858) vs. T2D (n = 10,444) yielded genome-wide significance for the same four T1D risk loci, as well as for the T1D risk *BACH2* locus (rs6908626)[38] (**Table 1**). As such, comparing LADA cases with both the general population and with T2D cases has implied that LADA is genetically more similar to T1D, comparable to the findings of previous reports[7,39]. We went on to perform a GWAS of LADA (n = 2,533) vs. T1D (n = 971) to assess whether any differences could be detected. Our results showed that only the HLA region was significantly different, representing a relative depletion of the lead signal among LADA cases when compared to T1D cases (rs9273368-A, OR (95% CI) = 0.340 (0.289-0.399), $P = 8.69 \times 10^{-40}$; **Table 1**). To further investigate differences in the HLA region between LADA and T1D cases, we imputed this region using SNP2HLA[40] in 2,159 ActionLADA + CHOP + Swedish LADA cases and 1,990 T1D cases (WTCCC[41]) and compared the frequencies of the leading T1D-associated HLA haplotypes (**Supplementary Table 11**). After removing haplotypes with less than 1% frequency, fifteen known T1D-associated HLA haplotypes were tested for association in LADA compared to T1D. Eleven T1D haplotypes were significantly different in frequency between LADA and T1D cases after correction for multiple testing ($P < 0.003$), with all but four being protective against T1D[42]. The four T1D susceptibility haplotypes, HLA-DRB1*0301-DQA1*0501-DQB1*0201, HLA-DRB1*0401-DQA1*0301-DQB1*0302, HLA-DRB1*0404-DQA1*0301-DQB1*0302, and HLA-DRB1*0405-DQA1*0301-

DQB1*0302[42], had significantly less impact in LADA. This could be partly explained by the established age gradient in HLA frequencies seen in T1D patients[43]; however, the HLA risk genotype frequency has been shown to differ also between LADA patients and T1D patients with age at onset >35 yrs[13,44]. Future studies of the differences in HLA risk haplotypes between T1D and LADA are therefore warranted.

Taken collectively, GWAS and HLA haplotype analysis based on established associations, along with GSEA analyses, supports the hypothesis that the strongest genetic risk loci for LADA are shared with T1D, but that established T2D alleles also appear to play a role, albeit to a lesser degree. To further evaluate this hypothesis beyond established sites by leveraging data from the entire genome, we estimated genetic correlation among LADA, T1D, T2D, and related traits using LD score regression (LDSC) (leveraging the LDSC v.1.0.0 python package[45] or the LD-hub website[46,47], http://ldsc.broadinstitute.org). First, we observed that T1D is, as expected, genetically correlated with autoimmune traits such as rheumatoid arthritis (rg (SE) = 0.452 (0.162), $P = 0.005$) and systemic lupus erythematosus (rg (SE) = 0.364 (0.134), $P = 0.007$) (**Fig. 2; Supplementary Table 12**). Additionally, T2D is strongly correlated with metabolic, glycemic, and anthropometric traits such as waist circumference (rg (SE) = 0.401 (0.04), $P = 3.73 \times 10^{-23}$) and fasting insulin (rg (SE) = 0.483 (0.095), $P = 3.90 \times 10^{-7}$). However, strikingly, T1D and T2D were negatively genetically correlated (rg (SE) = -0.273 (0.092), $P = 0.003$), consistent with previous reports of opposite effects at established sites (e.g., *CTRB1*, at which the T1D risk allele (rs7202877-T) is protective for T2D and vice versa[48]), although a genome-wide negative correlation has not been previously described to our knowledge. It remains unclear whether this negative correlation is due to underlying biology or the mutual exclusion of T1D patients from T2D studies and vice-versa. Notably, LADA was positively genetically correlated

with both T1D (r_g (SE) = 0.379 (0.182), P = 0.037) and autoimmune traits, as well as T2D (r_g (SE) = 0.309 (0.105), P = 0.003) and metabolic/anthropometric traits. Thus, our LADA sample shares genetic etiology with both a T1D-like autoimmune component and a T2D-like metabolic/anthropometric component that are mutually exclusive. GADA assays have a specificity of 95–98%, so by implication, some GADA-positive cases can be incorrectly classified as T2D cases; these should represent only a very small minority of cases and as such will not largely bias our results. Conversely, the small percentage of T2D cases misclassified as LADA patients could affect the estimate of genetic correlation between LADA and T2D to a small degree; however, this impact must be negligible given that we do not observe a positive correlation between T1D and T2D. Nevertheless, our findings lead to the hypothesis that the polygenic component that contributes susceptibility to T2D acts as a modifier to T1D risk, either as a 'second hit' in individuals who have moderate underlying autoimmune susceptibility that is insufficient to trigger childhood T1D but greater than that of the general population, or as a component that delays diabetes onset by protecting against autoimmune beta cell destruction earlier in life.

In conclusion, in this first GWAS of LADA, we show that although the leading genome-wide significant signals point towards LADA as being a late-onset form of T1D, there is both a reduced potency of key T1D-associated HLA haplotypes and the presence of a T2D-like genetic component. Further in-depth studies are necessary to address how LADA develops, as well as a need for functional studies to investigate how the glycolytic regulator PFKFB3 is situated at the intersection of autoimmune and metabolic diabetes. Furthermore, our LADA dataset should act as a resource to help mitigate the unaccounted presence of autoimmune diabetic patients in T2D GWAS going forward.

ONLINE METHODS

Study subjects

LADA cases were included from cohorts of European ancestry (**Supplementary Table 1**), including 'ActionLada-Plus,' All New Diabetics In Scania (ANDIS), the Botnia Study, Copenhagen LADA (including samples from Danish Centre for strategic Research in Type 2 Diabetes (DD2), Vejle Biobank, Odense University Hospital (OUH), Copenhagen Insulin and Metformin Therapy trial (CIMT), Inter99, and Steno Diabetes Center (SDC)), Diabetes Registry Vasa (DIREVA), GoDARTS, Nord-Trøndelag Health Study (HUNT), and Scania Diabetes Registry (SDR). Controls were either population-based (including samples from the Bone Mineral Density in Childhood Study (BMDCS), Copenhagen controls (with samples from the 1936 Birth Cohort and ADDITION-PRO), GoDARTS, HUNT, and the Malmö Diet and Cancer study) or contained T1D or T2D cases (including samples from GoDARTs, DIREVA, HUNT, and SDR).

Inclusion and exclusion criteria for LADA, T1D, T2D, and population controls varied by cohort (see **Supplementary Table 1 and Supplementary Note** for details). In general, LADA was defined by an age at diagnosis older than 20, 30 or 35 years, with some cohorts restricting the upper age limit to 70 years; the presence of diabetes-associated autoimmune autoantibodies, in particular GADA-positivity; and the lack of insulin requirement for 6 months or 1 year after diagnosis. In some cases, C-peptide level was also used as a filter.

Genotyping and imputation

Each respective cohort performed genome-wide genotyping on the Illumina CoreExome chip, the Illumina OmniExpressExome chip, or the Affymetrix 6 chip. Cases and controls from each study center were matched on the same genotyping chip to reduce batch effects. Standard post-genotyping quality control was performed, including sample exclusions for ambiguous gender, call rate < 95%, and any duplicate or related individuals (pi_hat ≥ 0.2), and SNP exclusions for monomorphic SNPs, SNPs with MAF < 0.05, and SNPs with missingness rate > 0.05. The Haplotype Reference Consortium (HRC) imputation service (URL) was utilized to perform imputation for autosomal SNPs.

Genome-wide association and meta-analysis: LADACTRL, LADAT1D, and LADAT2D

SNPtest [49] was used by each respective cohort to perform case-control GWAS of LADA (n = 2,713) vs. population controls (n = 5,439), LADA (n = 2,533) vs. T1D cases (n = 971), and LADA (n = 2,858) vs. T2D cases (n = 10,444), including sex and the first principal components as covariates (see **Supplementary Table 1** for cohort-specific covariates).

After GWAS, filtering was performed centrally to include only SNPs with a MAF > 0.05, INFO quality score > 0.4, and a Hardy-Weinberg equilibrium $P > 1x10^{-7}$. Meta-analysis was then performed for LADA vs. population controls, LADA vs. T1D, and LADA vs. T2D with GWAMA[50] with two rounds of genomic control (**Supplementary Table 13**).

Signals in the secondary tier ($P = 1 \times 10^{-6} – 5 \times 10^{-8}$) for the LADA vs. population controls analysis were followed up in the GODARTS and HUNT cohorts (LADA, n = 345; controls, n = 1,664) and meta-analyzed with the discovery set (total LADA, n = 3,058; controls, n = 7,103) to assess whether any novel signals would reach genome-wide significance.

Conditional analysis

Approximate conditional analysis for known T1D-associated loci was carried out for the LADACTRL summary statistics results for the 10p15.1 locus using Genome-wide Complex Trait Analysis (GCTA)[51,52]. For this locus, LADACTRL + HUNT summary statistics were conditioned on the following T1D-associated SNPs: rs61839660[53], rs10795791[53], rs7090530[54], rs12251307[55], rs41295121[53], and rs11258747[55]. For 12q24.3, two of the T1D-associated SNPs (rs3184504[55] and rs653178[53] were in high LD (r2 > 0.9) with our lead SNP, and the MHC, *PTPN22*, and *INS* loci were not conditioned as the top signals were identified as T1D-associated SNPs.

LD Score Regression

To test for genetic correlations genome-wide between LADA, T1D, T2D, and health-related outcomes, we used LD score (LDSC) regression either through the LDSC v.1.0.0 python package[45] or the LD-hub website[46,47] (http://ldsc.broadinstitute.org/#). Health-related outcomes that were genetically correlated with LADA at $P < 0.05$ were queried for their correlations to T1D[54] and T2D[48]. Details on the GWAS that contributed summary statistics for these comparisons can be found on the LDSC website.

Pathway analysis

DEPICT pathway analysis[20] was used to perform gene set enrichment, tissue enrichment, and gene prioritization analyses.

HLA imputation/analysis

The HLA imputation software SNP2HLA[40] was used to impute chromosome 6 in ActionLADA-Plus (n = 1,365), Swedish LADA cases (n = 794), BMDCS (n = 1,056) and WTCCC T1D cases (n = 1,990). HLA alleles with 4-digit resolution were imputed. The R package 'BIGDAWG' (https://cran.r-project.org/web/packages/BIGDAWG)[56] was used to test for allele frequency differences for established T1D-associated HLA haplotypes between LADA versus T1D, as well as LADA versus BMDCS. Haplotypes with frequencies less than 1% across LADA, T1D, and BMDCS were removed from the analysis given that rare haplotypes can result in unstable variance estimates and unreliable test statistics.

LD between T2D HLA-DQB1 lead SNP and T1D-associated haplotypes

rs9271775 was used to check for evidence of linkage disequilibrium between the top signal near *HLA-DQA1* reported to be associated with T2D in a recent reported analysis[17] and T1D-associated HLA haplotypes (**Table 2**). Tag SNPs for HLA alleles were obtained from de Bakker *et al.*[57]. LADA (n = 1,210) and BMDCS (n = 1,056) chromosome 6 data (imputed as described above by SNP2HLA[40]) was leveraged to calculate pairwise LD between rs9271775 and tag SNPs using PLINK[58]. Information for eight tag SNPs of T1D-associated haplotypes (**Table 2**) were available for testing.

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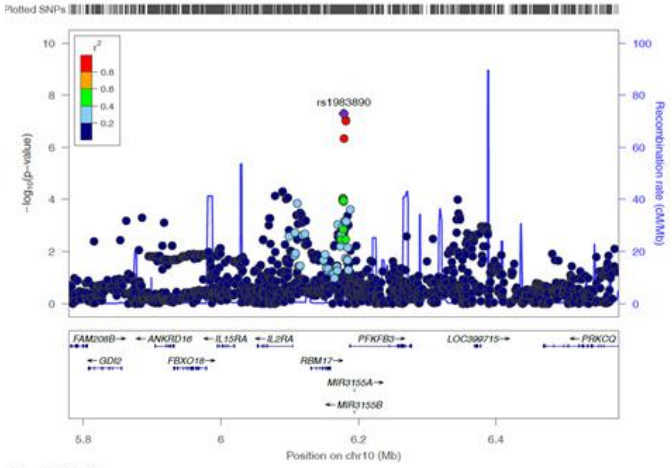
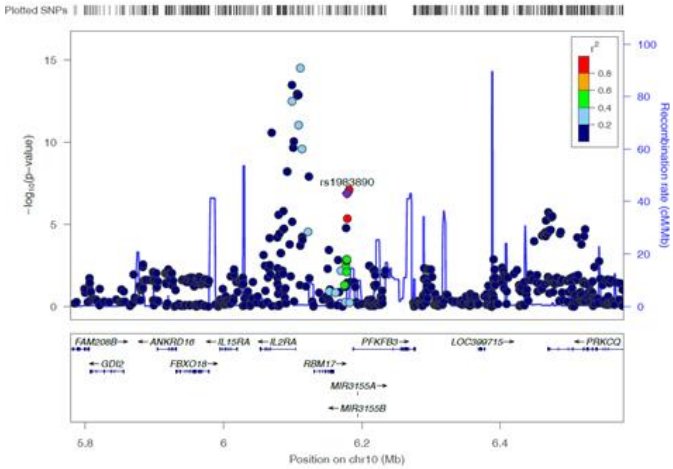
Figure 1. LocusZoom plots for the *PFKFB3* locus. (A) In LADA vs. population controls with the addition of replication samples, rs1983890 reached borderline genome-wide significance. (B) This signal lies in between two T1D-associated loci at 10p15.1 (Bradfield 2011). (C) When we conditioned on the two known T1D loci, the signal in LADA remained. LocusZoom plots were constructed to show the association data of SNPs 400kb upstream and downstream of the lead LADA-associated signal at rs1983890.

A. LADA



B. T1D



C. LADA conditional

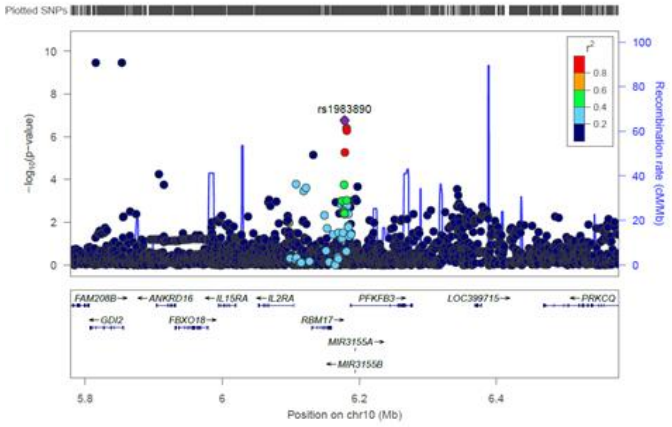


Figure 2. LD score regression (LDSC) analysis. We tested for genome-wide genetic correlations between LADA, T1D, T2D, and health-related outcomes. The MHC region was excluded prior to these analyses. Outcomes that were genetically correlated with LADA at $P < 0.05$ were queried for their correlations to T1D[54] and T2D[48]. Red represents a negative correlation, while blue represents a positive correlation. *, $P = 0.01$-0.05; **, $P < 0.01$; ***, $P < 0.001$.

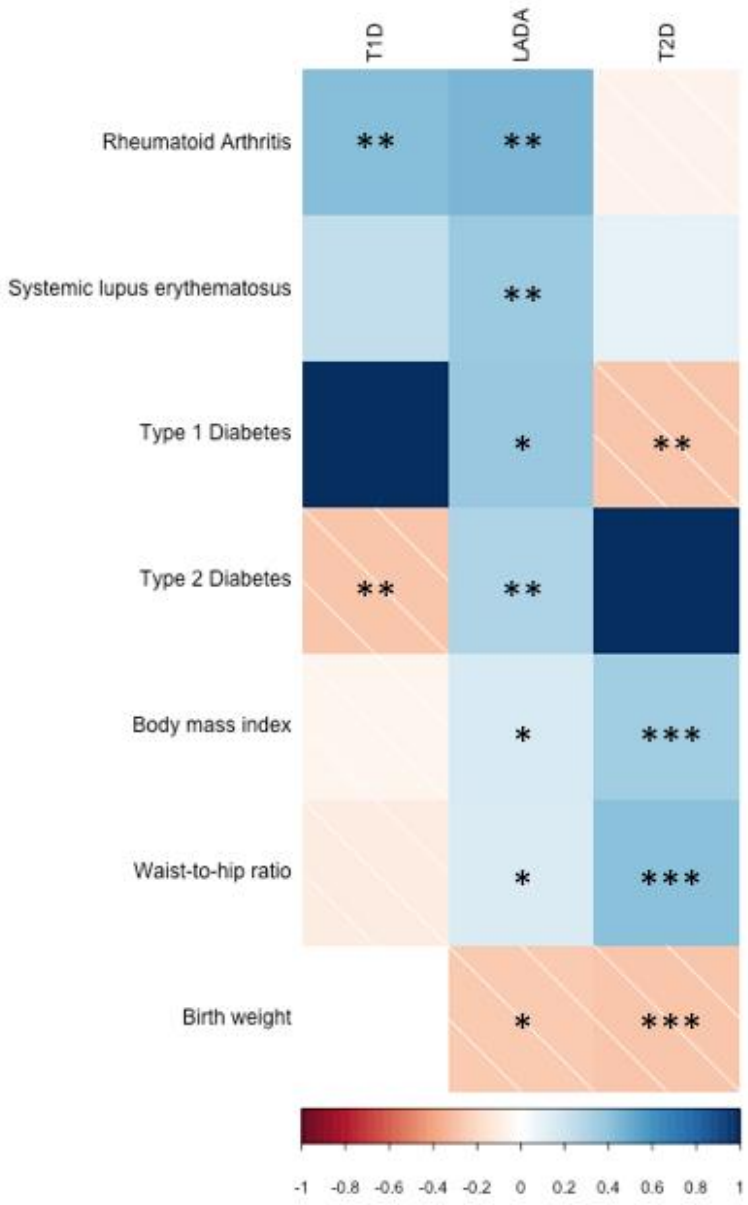


Table 1. Genome-wide significant signals associated with LADA.

SNP	Chr	Position (b37)	Ref/other allele	Effect allele frq (cases/ctrls)	OR	95% CI	*P*	Gene
LADA (n =2,713) vs. population controls (n =5,439)								
rs9273368	6	32626475	A/G	0.499/0.279	3.116	2.859-3.396	2.23×10^{-146}	*HLA-DQB1*
rs2476601	1	114377568	A/G	0.158/0.102	1.711	1.534 -1.908	5.71×10^{-22}	*PTPN22*
rs689	11	2182224	T/A	0.272/0.197	1.480	1.361-1.610	1.01×10^{-19}	*INS*
rs7310615	12	111865049	C/G	0.487/0.459	1.302	1.209 -1.401	2.93×10^{-12}	*SH2B3*
LADA (n = 2,533) vs. T1D cases (n = 971)								
rs9273368	6	32626475	A/G	0.415/0.649	0.340	0.289-0.399	8.69×10^{-40}	*HLA-DQB1*
LADA (n = 2,858) vs. T2D cases (n = 10,444)								
rs9273364	6	32626302	G/T	0.426/0.296	2.447	2.231-2.684	3.46×10^{-80}	*HLA-DQB1*
rs689	11	2182224	T/A	0.783/0.715	1.475	1.355-1.606	2.72×10^{-19}	*INS*
rs2476601	1	114377568	A/G	0.142/0.103	1.590	1.420-1.779	7.41×10^{-16}	*PTPN22*
rs6908626	6	91005743	T/G	0.201/0.164	1.35	1.218-1.491	7.3×10^{-9}	*BACH2*
rs3184504	12	111884608	C/T	0.544/0.520	1.242	1.155-1.339	8.33×10^{-9}	*SH2B3*

We performed three genome-wide association approaches, first for LADA versus population controls (top panel), then for LADA versus type 1 diabetes (T1D, middle panel) and LADA versus type 2 diabetes (T2D, lower panel). Odds ratios (ORs) are given for the LADA risk allele except for rs92773368 in LADA vs. T1D, to illustrate that the T1D risk allele was depleted in LADA.