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GENETIC SIMILARITIES BETWEEN LADA, TYPE 1 AND TYPE 2 DIABETES

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Running Title: Genetics of LADA

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ABSTRACT

Aim: LADA is often considered a slowly progressing subtype of type 1 diabetes, although the clinical picture resembles more type 2 diabetes. One way to improve classification is to study whether LADA shares genetic features with type 1 and/or type 2 diabetes.

Methods/Results: To accomplish this, we studied whether LADA shares variation in the HLA locus, *INS* VNTR, *PTPN22* genes with type 1 or the *TCF7L2* gene with type 2 diabetes, in 361 LADA, 718 type 1, 1676 type 2 diabetic patients and 1704 healthy control subjects from Sweden and Finland. LADA showed, compared to type 2 diabetic patients, increased frequency of risk HLA-DQB1 *0201/*0302 genotype (27% vs. 6.9%; $p<1x10^{-6}$), with similar frequency as to type 1 diabetes (36%). In addition, LADA showed higher frequencies of protective HLA-DQB1 *0602(3)/X than type 1 diabetic patients (8.1% vs. 3.2%, p=0.003). The AA-genotype of rs689, referring to the class I allele in the *INS* VNTR, as well as the CT/TT-genotypes of rs2476601 in the *PTPN22* gene were increased both in T1D ($p=3x10^{-14}$ and $p=1x10^{-10}$, respectively) and LADA (p=0.001 and p=0.002), as compared to controls. Notably, the frequency of the type 2 diabetes-associated CT/TT-genotypes of rs7903146 in the *TCF7L2* were increased in LADA (52.8%; p=0.03), to the same extent as in type 2 diabetes (54.1%, $p=3x10^{-7}$), as compared with controls (44.8%) and type 1 diabetes (43.3%).

Conclusions: LADA shares genetic features with both type 1 (HLA, *INS* VNTR and *PTPN22*) and type 2 (*TCF7L2*) diabetes, which justifies considering LADA as an admixture of the two major types of diabetes.

KEYWORDS. LADA, genetics, diabetes, TCF7L2, HLA, GAD, SNP, transcription factor.

ABBREVIATIONS. LADA: Latent Autoimmune Diabetes of Adults, SNP: single nucleotide polymorphism, OGTT: oral glucose tolerance test, BMI: body mass index, I/G30: insulinogenic index, GADA: glutamic acid decarboxylase antibodies, OR: odds ratio, CI: confidence interval,

Latent Autoimmune Diabetes of Adults (LADA) has in the WHO classification been considered as a subgroup of type 1 diabetes; i.e. slowly progressing type 1 diabetes. Although LADA patients often present with a clinical picture similar to type 2 diabetes, with an adult age at onset and insulin independence at diagnosis, they are characterized by circulating islet autoantibodies similar to those found in type 1 diabetes [1, 2]. One way to shed light on the classification of LADA would be to determine to what extent LADA shares genetic similarities with type 1 diabetes and type 2 diabetes.

There is some support for the view that LADA shares susceptibility genes with type 1 diabetes, but there are only a limited number of reports, which have been large enough to address this issue [3-6]. The HLA locus on the short arm of chromosome 6 that confers most of the genetic susceptibility to type 1 diabetes [7] has shown similar associations with LADA [3, 5], but also distinct differences, e.g. DQB1 *0201/*0302 is a more common genotype in type 1 than in LADA, whereas the protective genotypes *0602/X and 0603/X are more common in LADA than in type 1 diabetes [6]. The insulin gene variable number of tandem repeats (*INS* VNTR) on chromosome 11 falls into two general classes, class I (26-63 repeats) and class III (141-209 repeats), and the short class I have shown strong susceptibility to type 1 diabetes [8] as well as to LADA, as shown in the UKPDS study [4]. However, no association was seen in the Finnish Botnia study [6]. Other loci contributing to the genetic risk of type 1 diabetes include the protein tyrosine phosphatase non-receptor type 22 (PTPN22) gene on chromosome 1 [9].

Previously it has been difficult to demonstrate genetic similarities between LADA and type 2 diabetes, as no genes have been consistently associated with type 2 diabetes. However recently, variation in the transcription factor 7-like 2 (*TCF7L2*) gene showed strong association with type 2 diabetes [10, 11], making this gene a good candidate for genetic comparisons between LADA and type 2 diabetes. The *TCF7L2* gene showed no association with type 1 diabetes [12], however a role in type 1 diabetes was suggested for another TCF gene, the *TCF7* [13]. Mutations in yet other TCFs cause monogenic forms of diabetes, e.g. *TCF1* (*HNF1*α; MODY3), *TCF2* (*HNF1*β; MODY5), *TCF14* (*HNF4*α; MODY1).

The aim of this study was to elucidate whether LADA shares genetic similarities with type 1 and type 2 diabetes, by comparing genetic variation within the HLA locus and the *INS* VNTR, *PTPN22* and *TCF7L2* genes between patients with type 1 diabetes, LADA, type 2 diabetes and healthy controls.

RESEARCH DESIGN AND METHODS

Subjects Four groups of individuals from Sweden were included: 164 LADA patients (age at onset>35, GADA positive), 1000 type 2 diabetic patients (age at onset>35, GADA negative), 718 T1D patients (age at onset<35) and 1000 non-diabetic control individuals (age at visit>40) (Table 1). The patients were recruited from the local diabetes registry [14] and diagnosis of diabetes was based upon WHO criteria [1]. The controls were selected from the Malmö Preventive Project [15] without a family history of diabetes or treatment of hypertension. Type 2 diabetes patients and controls were matched for sex, BMI and age, with the controls being at least 5 years older. Three groups of individuals from Finland, participating in the Botnia study [16], were also included: 197 LADA patients (defined by; age at onset>35 and GADA positive), 676 unrelated GADA negative type 2 diabetes patients (age at onset >35) and 704 unrelated control individuals (age at visit >35, no first- or second degree relatives with diabetes) (Table 1).GAD autoantibody (GADA) levels >32 IU/ml or 5 RU were considered

positive (see below for details). The prevalence of GADA positive (LADA) patients among type 2 diabetes patients in the Botnia Study was 9% [6]. All subjects gave their informed consent to the study, which was approved by the local ethics committee.

Measurements and assays. Plasma glucose was measured with a glucose oxidase method using a Beckman Glucose Analyzer II (Beckman Instruments, Fullerton, CA) and insulin was measured with ELISA (DAKO, Cambridgeshire, United Kingdom) with an interassay CV of 8.9 %. Fasting serum C-peptide concentrations were determined by a radioimmunoassay with an interassay CV of 9% (Human C-peptide RIA; Linco, St. Charles, MO, USA). GADA were determined by a radiobinding assay using ^{35}S-labelled recombinant human GAD65 produced by coupled in-vitro transcription-translation as described [17]. Levels exceeding 32 international units/ml (IU/ml) or 5 relative units (RU) were considered positive (RU=(sample cpm-mean cpm of three negative controls)/(cpm of a positive internal reference-mean cpm of three negative controls)x100) and according to the standardised international units, 5 RU equals 32 IU/ml. In the first DASP (2000) our GADA assay showed a sensitivity of 80% and a specificity of 96%; in the second (2002) a sensitivity of 88% and a specificity of 87%; and in the third DASP (2003) a sensitivity of 82% and specificity of 93%.

SNP genotyping. Genotyping of rs689 (*INS* VNTR), rs3842755 (*INS* VNTR), rs2476601 (*PTPN22*), rs7903146 (*TCF7L2*) and rs12255372 (*TCF7L2*) was performed either by allelic discrimination on Applied Biosystems 7900HT system (Applied Biosystems, Foster City, CA, USA) (Swedish cohort: rs689, rs3842755, rs2476601 and Finnish cohort: rs7903146 and rs12255372), or by using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry with the resulting mass spectra analysed by the SpectroTYPER RT 2.0 software (Sequenome Inc, San Diego, CA, USA). An error-rate of <1% was determined with 5-10% re-genotyping. Primer sequences and additional genotyping details are available from the authors.

HLA genotyping. HLA genotyping was performed in the Swedish cohort as previously described [18, 19]. Briefly, the second exon of HLA-DQB1 was amplified and hybridized with lanthanide (III) chelate-labelled DNA probes specific for the HLA-DQB1 *0201, *0301, *0302, *0602 and *0603 alleles. Hybridization was evaluated by time-resolved fluorescence (Delfia Research Fluorometer, Wallac OY, Turku, Finland). The symbol X refers to a homozygous allele or any allele other than 0201, 0302 or 0602(3). A Swedish control group was included in the table for comparison [20].

Statistical analysis. Chi-square tests and multivariate logistic regression analyses adjusted for age (controls), age-at-onset (cases), BMI and gender were performed to study association of SNPs with disease. Mantel-Haenszel test were used to test for heterogeneity between the Swedish and Finnish cohorts. In addition, an extensive investigation of potential population stratification between these cohorts has recently been published [21]. The prior hypothesis to be tested was whether LADA shares genetic features with T1D or with T2D. As this has previously been shown for the HLA and INS VNTR loci, we only applied a Bonferroni correction for the two new genes tested, i.e. *PTPN22* and *TCF7L2* by multiplying the p value by 4 (2 genes x 2 cohorts). In pooled analyses of the two cohorts in regression analyses adjustment was made for country-of-origin. A p-value of <0.05 was considered statistically significant. Statistical analyses were performed using Number Cruncher Statistical Systems (NCSS), version 2000 (Kaysville, Utah, USA).

RESULTS

Genotype frequencies of studied SNPs are presented in Table 2 and 3. All genotypes were in Hardy-Weinberg

equilibrium (HWE). A Mantel-Haenzel test revealed no heterogeneity between the two cohorts regarding the tested polymorphisms. Therefore, the Finnish and Swedish cohorts were analyzed together.

HLA-DQB1. The frequencies of the HLA-DQB1 genotypes *0302/X and *0201/*0302 were higher in type 1 diabetes and LADA than in type 2 diabetic patients, whereas the frequencies of the protective genotypes *0602/X and *0603/X were more than twice as high in LADA compared to type 1 diabetic patients (8.1% vs. 3.2%), but markedly lower than in type 2 diabetes (25%) (Table 2).

INS VNTR. The AA-genotype of rs689 (class I) was increased in both type 1 diabetes (73.4%, $p=3x10^{-14}$) and LADA (69.0%, p=0.001) (Table 3). The associations were also observed when using regression analysis adjusting for age, BMI and gender (Table 4).

PTPN22. The frequency of the CT/TT-genotypes of rs2476601 in the *PTPN22* gene were increased in both type 1 diabetes (32.0%, $p=1x10^{-10}$) and LADA (29.7%, p=0.002), compared to controls (17.1%). In addition, the frequency of the CT/TT-genotype was also increased in T2D (23.1%, p=0.008) compared with control subjects (Tables 3 and 4).

TCF7L2. Importantly, the frequency of the CT/TT-genotypes of rs7903146 in *TCF7L2* were increased to the same extent in LADA (52.8%; $p_{pooled}=0.03$) as in type 2 diabetes (54.1%, $p_{pooled}=3x10^{-7}$), compared with controls (44.8%) (Table 3), with no difference between LADA and type 2 diabetes (p=0.59). This was also seen when the data were analyzed using regression analysis adjusted for age, BMI, gender and country of origin (Table 4). The results for SNP rs12255372 were virtually similar as those for rs7903146 (Table 3 and 4). There was no difference in frequency of the CT/TT-genotypes of rs7930146 between type 1 diabetes and controls (43.3% vs 44.8%, p=0.55).

DISCUSSION

The present study shows that LADA shares genetic features with both type 1 (HLA-DQB1, *INS* VNTR, *PTPN22*) and type 2 diabetes (*TCF7L2*), suggesting that LADA represents an admixture of the two major types of diabetes (figure 1).

The HLA and *INS* VNTR findings are in keeping with previous results. Several studies have shown increased frequencies of type 1 diabetes-associated high-risk HLA genotypes in patients with LADA, thereby concluding that LADA represents a subgroup of type 1 diabetes [3, 4, 22, 23]. It should though be emphasized that LADA differs from type 1 diabetes with a smaller effect size of the associations with HLA, *INS* VNTR and PTPN22, as was previously shown for both HLA [6, 24] and *INS* VNTR [24]. The association of *INS* VNTR with LADA from the UKPDS study [24] was in contrast to the previous report from our laboratory in Finns [6]. We cannot rule out whether this is due to population differences or power issues. The former is unlikely as the frequency of the AA genotype of the *INS* VNTR was similar in the LADA patients from UK as in our Scandinavian sample. The latter is more likely as the current study included almost twice as many LADA patients as the previous Finnish study.

The *PTPN22* gene on chromosome 1 encodes the lymphoid-specific tyrosine phosphatase LYP, involved in the suppression of T cell activation and thereby T-dependent antibody production [25], and has been associated with increased susceptibility to type 1 diabetes [9] and other autoimmune diseases [26]. In this study we provide novel information that LADA also shows increased frequency of the CT/TT-genotypes in the *PTPN22* gene compared with controls, although less than in type 1 diabetes. Somewhat surprisingly, the same genotype(s) were also increased in patients with T2D. Although this could be an interesting support for the role of inflammation in T2D, we should note that this was a secondary analysis which would

require much more stringent corrections for multiple testing. It is though a finding that deserves replication efforts in other cohorts of T2D patients.

Taken together the HLA, *INS* VNTR and *PTPN22* data clearly point at a common genetic background between LADA and type 1 diabetes.

The identification of the *TCF7L2* gene as the strongest candidate gene for type 2 diabetes [10, 11] has now opened for the possibility to test whether LADA also shares genetic features with type 2 diabetes. We provide compelling evidence that this is the case, LADA patients showed the same increased frequency of risk genotypes in the *TCF7L2* gene as type 2 diabetic patients. The association between LADA and TCF7L2 was not affected when the quartile with the lowest GADA levels were excluded, so the finding was not due to inclusion of false-positive type 2 diabetic patients in the LADA group (data not shown). In contrast, and in keeping with a previous report [12], the frequency of these risk genotypes did not differ between type 1 diabetic patients and controls.

There have been a striking increase in both type 1 and type 2 diabetes in Scandinavian countries during the past decades, with a convergence of the previously distinctive phenotypes and it has been proposed that weight gain and insulin resistance could serve as a trigger for diabetes in both type 1 and type 2 diabetes ("accelerator hypothesis") [27]. This was though challenged by the fact that type 1 diabetes does not share a genetic background, like *TCF7L2*, with type 2 diabetes [28]. Our data partially challenge this argument as the late-onset form of autoimmune diabetes, LADA, clearly shares a genetic background with type 2 diabetes.

The mechanism by which variation in the *TCF7L2* gene contributes to diabetes is unclear, however the intestinal proglucagon gene shows binding sites for TCF7L2 [29] and a potential mechanism could involve the incretin axis. We have recently shown that carriers of the risk T-allele show impaired insulin secretion, impaired incretin effect and enhanced expression of the *TCF7L2* gene in human islets [30]. Also in previous studies, the *TCF7L2* variants have been associated with impaired insulin secretion [11, 31]. It is thus likely that similar non-autoimmune mechanisms are operative in islets from both patients with type 2 diabetes and LADA, causing impaired insulin secretion.

Some weaknesses of the study should be emphasized. Autoimmune diabetes has been subdivided into a rapidly (type 1 diabetes) and a slowly progressing (LADA) form (WHO). In the diagnosis of LADA lack of insulin treatment during the first 6 months after diagnosis is often used to distinguish LADA from adult-onset type 1 diabetes. However, the decision to initiate insulin therapy is very subjective and ketosis-prone type 1 diabetes in this adult age group very rare (< 10% in our experience). We therefore decided only to use hard criteria like age at onset and presence of GAD antibodies for the diagnosis of LADA. Also the use of two different cohorts (Finnish and Swedish) in the analysis could introduce heterogeneity. However these populations have successfully been used in a whole genome association study for type 2 diabetes with stratification biases excluded using Eigenstrat [21].

In conclusion, the data from this study positions LADA genetically as an admixture of type 1 and type 2 diabetes, rather than as a subgroup of type 1 diabetes.

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FIGURE LEGENDS

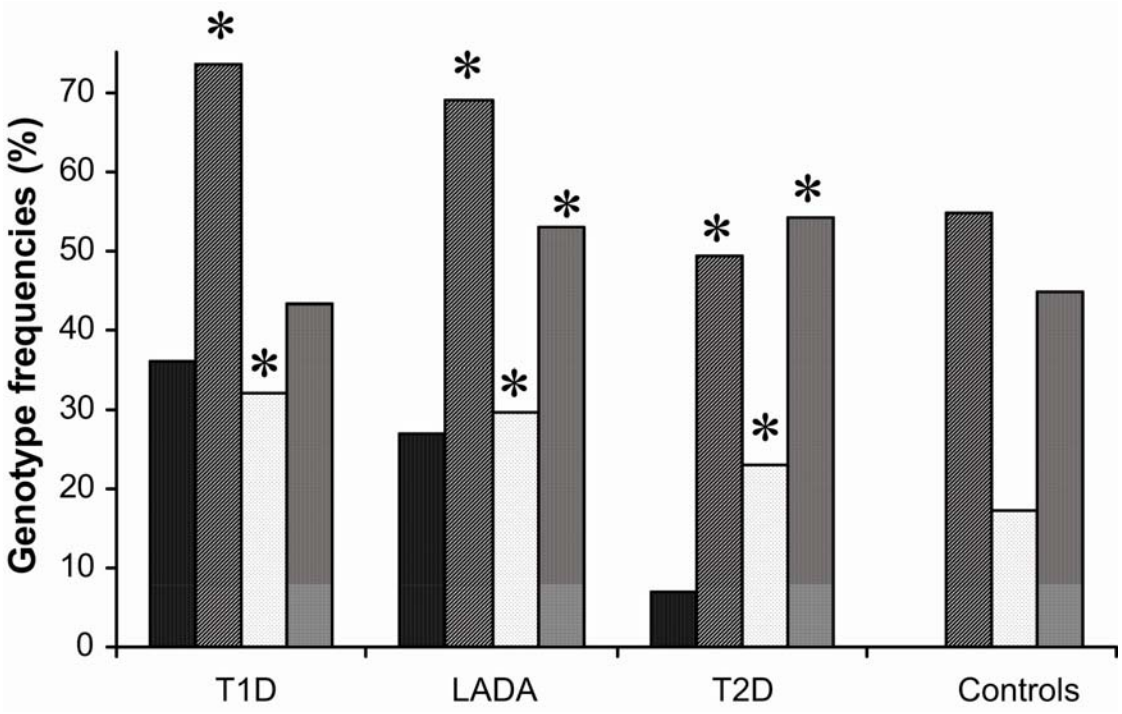
Figure 1. Risk genotype frequencies of the four different susceptibility locus in T1D, LADA and T2D. HLA-DQB1 was not genotyped in controls, * represents a significant association to diabetes ($p<0.05$).

 =HLA-DQB1 (*0201/*0302)

 =INS VNTR-rs689 (AA-genotype)

 =PTPN22-rs2476601 (CT/TT-genotypes)

 =TCF7L2-rs7903146 (CT/TT-genotypes).


Genotype frequencies (%)
0
10
20
30
40
50
60
70
T1D
LADA
T2D
Controls
*