Journal of Diabetes and Its Complications 33 (2019) 46–52

Contents lists available at ScienceDirect

Journal of Diabetes and Its Complications

journal homepage: WWW.JDCJOURNAL.COM

Autoantibodies against ZnT8 are rare in Central-European LADA patients and absent in MODY patients, including those positive for other autoantibodies☆

Petr Heneberg *, Daniela Šimčíková, Marie Čecháková, Blanka Rypáčková, Petr Kučera, Michal Anděl

Charles University, Third Faculty of Medicine, Prague, Czech Republic

ARTICLE INFO

Article history:
Received 14 May 2018
Received in revised form 10 August 2018
Accepted 7 October 2018
Available online 13 October 2018

Keywords:
Adult-onset autoimmune diabetes
BMI
LYP
LADA
PTPRN
Zinc transporter ZnT8

ABSTRACT

Background: Testing for autoantibodies against the zinc transporter ZnT8 (ZnTA) is becoming routine in pediatric diabetes. However, available data are inconclusive when focusing on adult-onset diabetes, including autoimmune diabetes, which does not require insulin at diagnosis (LADA).
Basic procedures: We examined the ZnTA prevalence and titers and matched them with the clinical phenotype and *PTPN22* genotypes of Czech LADA patients who were positive for GADA and/or IA2A and had a fasting C-peptide level >200 pmol/L at diagnosis as well as *HNF4A*-, *GCK*- or *HNF1A*-MODY patients and healthy controls.
Main findings: Most LADA patients were negative for ZnTA, and the sensitivity of the assay was only 18–20% for patients with LADA-like progression to insulinotherapy compared to healthy controls. In LADA patients, there was no association between the ZnTA and *PTPN22* risk genotypes. LADA patients positive for ZnTA had a lower BMI than those positive for other autoantibodies alone. Importantly, MODY patients were completely negative for ZnTA, and the levels of ZnTA in MODY patients were similar to those in healthy controls.
Conclusions: ZnTA quantification did not improve LADA diagnosis. However, positivity for ZnTA can be used as a negative MODY pre-diagnostic criterion even in the region of Central and East Europe, where other islet cell autoantibodies are common in MODY patients.

1. Introduction

Autoimmune diabetes results from progressive loss of pancreatic β cells through autoimmunity targeted at antigens that are present at secretory granules. Diabetes-associated autoimmunity was initially diagnosed as islet cytoplasmic autoantibodies (ICA). Later, autoantibodies against the four major humoral autoantigens of autoimmune diabetes were distinguished as autoantibodies against insulin (IAA), the 65-kDa form of glutamate decarboxylase (GADA), inactive protein tyrosine phosphatase IA-2 (IA2A) and zinc transporter ZnT8 (ZnTA).[1] More recently, autoantibodies against dozens of other proteins have been demonstrated in autoimmune diabetes, but their prevalence was low or differed dramatically among the studies. The prevalence of these additional autoantibodies was usually below 1% of patients with type 1 diabetes (T1DM), and their prevalence did not consistently exceed 10% when the published studies were compared to each other,[1] except for the most recently reported antibodies against EEF1A1, UBE2L3[2] and tetraspanin 7.[3] Therefore, the clinical diagnosis of (pre-) diabetes-associated autoimmunity still relies on the four auto-antigens that were discovered over a decade ago.

ZnTA were discovered a decade ago by the group of John C. Hutton as highly prevalent in new-onset T1DM (60–80%) and in patients with other autoimmune disorders (Addison's disease 9%, celiac disease 31%, but systemic lupus erythematosus 0% and rheumatoid arthritis 0%) while they are nearly absent in healthy controls (<2%) and low in type 2 diabetes (T2DM) patients (<6%).[4] A similar prevalence of ZnTA (9%) in Addison's disease was recently corroborated by Marta Fichna et al., who also noticed that approximately a half of the ZnTA-positive Addison's disease patients were also T1DM-positive.[5] Several cases positive for ZnTA were also reported among patients with cystic fibrosis (3% prevalence)[6] and Hashimoto's thyroiditis (20–21% prevalence, but there was also a 6% prevalence in healthy controls).[7] In T1DM patients, the positivity for ZnTA is associated with an increased risk of Hashimoto's thyroiditis.[8] In addition, the prevalence of ZnTA increased during and after flu vaccination of pediatric T1DM patients.[9] Importantly, ZnTA may occur in non-autoimmune diabetes. In this regard, the study of Estonian T2DM patients reported 6% of study subjects were positive for ZnTA (and 22% of the analyzed T2DM subjects were positive for at least one of the antibodies against pancreatic β cell antigens).[10] In another study, 2% of T2DM patients in Singapore were positive for ZnTA (and 9% for any antibodies against pancreatic β cell

☆ Conflict of interest: The authors declare no conflict of interest.

* Corresponding author at: Charles University, Third Faculty of Medicine, Ruská 87, CZ-100 00 Prague, Czech Republic.
E-mail address: petr.heneberg@lf3.cuni.cz (P. Heneberg).

https://doi.org/10.1016/j.jdiacomp.2018.10.004
1056-8727/

antigens).[11] In the US-based ACCORD study, the positivity of T2DM patients for ZnTA was associated with severe hypoglycemia accompanied by an inability to achieve an HbA_{1c} level of 42 mmol/mol.[12] Gestational diabetes patients may also be positive for ZnTA; a 5% prevalence of ZnTA was reported for Australia[13] and a 3% prevalence of ZnTA was reported for Sweden.[14] The ZnTA is considered to be absent in patients with autosomal dominant forms of diabetes, known as maturity-onset diabetes of the young (MODY) by the British and U.S. authors,[15,16] but seropositivity of a fraction of patients with MODY was reported from Sweden.[17] In fact, the true prevalence of ZnTA in MODY is difficult to predict, as the absence of antibodies is usually part of the selection strategy for identifying patients for MODY genetic screening, which may lead to an underestimation of the true prevalence of antibodies in MODY.[18]

Testing for ZnTA is becoming routine in pediatric diabetes. However, available data are inconclusive when focusing on adult-onset diabetes, including the autoimmune type of diabetes that does not require insulin for at least 6–12 months from diagnosis, which is also named latent autoimmune diabetes of the adult (LADA) as long as the patients are over 30–35 years old at diagnosis (Table S1; note that the two above-mentioned diagnostic criteria are still unsettled, which reflects the fact that there is likely a continuum of phenotypes ranging from classical T1DM to LADA). The LADA patients were initially thought to undergo the same disease process as T1DM patients in terms of the HLA-mediated genetic susceptibility, spectrum of autoantibodies, low insulin secretion, and higher rate of progression to insulin dependence,[19] but clinical and pharmacological features of the disease distinguish these patients from those with T1DM and T2DM.[20–23] As a result, they clearly need to be analyzed separately irrespective of the problems associated with the disease terminology and classification.

In the present study, we aimed to address the ZnTA prevalence in patients with diabetes mellitus with LADA-like progression to insulinotherapy, who were either positive for GADA and/or IA2A (LADA) or negative for these autoantibodies and with high C-peptide (ID-T2DM). We aimed to determine, whether the variability in ZnTA titers is associated with differences in the clinical phenotype of LADA. We hypothesized that ZnTA could be a prognostic marker for differentiating between autoimmune diabetes and MODY in Central and East Europe, where approximately a quarter of MODY patients are GADA- and/or IA2A-positive.[24,25] We also hypothesized that the ZnTA prevalence and titers are regulated by functional polymorphisms in the Lyp phosphatase, as suggested in T1DM patients.[26]

2. Material and methods

2.1. Study design and populations

We recruited four groups of research subjects to test autoantibodies. We applied the following absolute inclusion criteria:

1. LADA patients (n = 59):
   a. Diabetes onset at 35 years of age or later, and
   b. Treatment of diabetes for six months or longer without the need of insulin
   c. Fasting C-peptide >200 pmol/L, or
   d. Positivity for at least one of the following auto-antibodies:
      i. GADA >50 ng/mL (measurements prior 2002) or > 5 U/mL
      ii. IA2A (autoantibodies against islet antigen-2) >0.9 U/mL
2. T2DM patients with LADA-like progression to insulinotherapy (termed ID-T2DM patients; n = 18):
   a. Diabetes onset at 35 years of age or later, and
   b. Treatment of diabetes for six months or longer without the need of insulin but later conversion to insulinotherapy
   c. Fasting C-peptide >200 pmol/L, or
   d. Negativity for GADA and IA2A
3. MODY patients (n = 63; comprising of 14 *HNF4A*-MODY, 18 *GCK*-MODY and 31 *HNF1A*-MODY):
   a. Confirmed genetic diagnosis of *HNF4A*-MODY, *GCK*-MODY or *HNF1A*-MODY, and
   b. Treatment of diabetes for six months or longer without the need for insulin (often only after the genetic diagnosis), and
   c. Three-generation family history of diabetes
4. Healthy controls (n = 70):
   a. No evidence of diabetes, and
   b. No evidence of autoimmune endocrine diseases

All patients were of European origin and of the Czech nationality. The study cohorts overlapped somewhat with those used in our previous studies focusing on the role of *PTPN22* in the onset and progression of LADA and MODY.[18,27] Some of the supporting measurements were obtained in course of these studies.

2.2. Autoantibodies and other clinical features

We detected the islet cell autoantibodies in serum that was not treated with $CaCl_2$ or thrombin or in plasma. To check for the ZnTA levels, we used the ZnTA ELISA kit ZnT8/96 (RSR, Cardiff, UK) according to the manufacturer's instructions. For each plate, we determined the calibration curve based on the ZnTA-negative sample and samples with 10, 20, 75, 500 and 2000 U/mL of ZnTA (provided by the manufacturer). We measured all samples in duplicate at the wavelengths of 405 nm (A405) and 450 nm (A450). The lower detection limit was 1.2 U/mL. The arbitrary cut-off of ZnTA positivity, suggested by the manufacturer, was 15 U/mL. The assay sensitivity and specificity were 72–76% and 97–99%, respectively, according to the IASP 2012, 2013 and 2015 studies (Zinc Transporter 8 (ZnT8) Autoantibody ELISA Kit - Instructions for use. RSR/38 Rev. 12, 16-Jul-2015 and RSR technical information Zinc transporter 8 (ZnT8) autoantibody ELISA kit ElisaRSR™ ZnT8 Ab™. Undated, valid as of Oct-2016). As the previously reported cut-off values were determined for T1DM patients, we calculated receiver operating characteristic (ROC) curves based on the newly examined LADA and MODY patients and compared them to the healthy controls.

For the analyses of autoantibodies against GAD_{65} (GADA) and IA-2 (IA2A), we used the enzyme immunoassays by Medipan (Dahlewitz, Germany) that were calibrated to the reference material NIBSC 97/550. The analytical sensitivities were 0.8 IU/mL (GADA) and 0.5 IU/mL (IA2A). The arbitrary cut-offs were 5 IU/mL (GADA) and 10 IU/mL (IA2A). We published the ROC curves of the method previously[28]; some of the GADA and IA2A measurements were obtained in course of a previous study of LADA patients.[29]

At the time of autoantibody measurement, we measured the fasting C-peptide, HbA_{1c}, height and weight as well as calculated the body mass index (BMI) and recorded the treatments. We determined the HbA_{1c} by high-performance liquid chromatography on the basis of the reference method of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC).[30] We determined the fasting serum C-peptide level using a chemiluminescent immunoassay Immulite 2000 (Diagnostic Products Corporation, Los Angeles, CA). For patients who were previously examined,[29,31] we retrieved the information on the *PTPN22* polymorphisms that have previously been associated with autoimmune diseases. These included rs2476601 (exon 14, c.1858C>T), rs2488457 (promoter, c.-1123G>C), rs33996649 (exon 10, c.788G>A) and rs1310182 (intron 16, c.1970-852T>C).

2.3. Statistical analyses

For the ZnTA measurements, we calculated the standard curves for both the A405 and A450 values and then calculated the arbitrary units per mL of serum or plasma accordingly. We characterized the obtained data by descriptive statistics (mean ± SE, median, range). We calculated the intra-assay coefficients of variability of the ZnTA assay for values

Table 1
Levels of ZnTA measured in the cohorts of LADA, ID-T2DM, MODY and healthy human subjects.

	[U/mL] based on A405				[U/mL] based on A450				N
	Mean ± SE	Median	Min	Max	Mean ± SE	Median	Min	Max	
LADA	103.2 ± 290.2	7.3	0.0	1350.4	111.4 ± 334.0	7.2	0	1692.3	59
ID-T2DM	5.0 ± 2.4	5.0	1.3	9.2	3.8 ± 2.9	3.4	0.0	8.2	18
HNF4A-MODY	3.0 ± 0.9	2.0	0.0	9.3	2.9 ± 0.9	0.8	0.0	10.6	14
GCK-MODY	3.0 ± 0.5	2.8	0.0	7.0	2.7 ± 0.7	1.8	0.0	8.3	18
HNF1A-MODY	2.8 ± 0.4	2.5	0.0	9.3	2.5 ± 0.4	2.2	0.0	10.2	31
Healthy	3.1 ± 0.4	2.7	0.0	24.0	3.4 ± 0.4	2.4	0.0	25.9	70

over the detection limit (1.2 U/mL). We calculated the inter-assay variability of the ZnTA assay for the absorbance values of 20 U/mL and 500 U/mL standards measured at each plate used. We calculated ROC curve areas with paired analyses of LADA vs. healthy controls or MODY patients. We evaluated the ROC curve area ± SE, 95% CI, *P* value and ROC curve area comparisons between A405 and A450 using the χ^2 test as well as provided the ROC curve area difference ± SE and 95% CI. We calculated the sensitivity and selectivity of the LADA diagnosis based on the ROC curves compared to the healthy controls or MODY patients. We calculated these values (and 95% CI) at the arbitrary cut-off suggested by the manufacturer (15 U/mL); we also calculated the cut-off values and sensitivity (with 95% CI) at ≥95% and ≥99% specificity. We calculated descriptive statistics of the clinical features of patients positive for ZnTA, positive for either GADA or IA2A (but not ZnTA) and negative for all three autoantibodies. We tested these data by the Shapiro-Wilk normality test. We next tested the significance of the data that passed the normality test using one-way ANOVA, and we tested the other data with Kruskal-Wallis one-way ANOVA of ranks. We also checked the power of each test performed and indicated cases for which the desired power was not reached. We similarly tested patients who were stratified according to their *PTPN22* genotypes. We performed post-hoc power analyses of the power of the present study to detect differences between LADA patients and other analyzed groups. We performed the calculations and plotted the figures in PAST 2.14 and SigmaPlot 12.0, except the post-hoc power analyses, which were performed in ClinCalc.

2.4. Compliance with ethical standards

The study was approved by the Ethics Committee of Third Faculty of Medicine at Charles University. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional Ethical Committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. We obtained written informed consent from all individual participants included in the study.

3. Results

3.1. ZnTA as a diagnostic marker of autoimmune diabetes

ZnTA levels measured in LADA, MODY and healthy subjects were generally low; the median values were 2.7 U/mL (healthy controls), 2.0–2.8 U/mL (MODY), 5.0 U/mL (ID-T2DM) and 7.3 U/mL (LADA), while the arbitrary cut-off limit of ZnTA positivity suggested by the manufacturer based on the examination of T1DM patients was set to 15 U/mL. One patient in the cohort of healthy controls exceeded the cut-off limit. All 63 MODY patients were well below the cut-off limit (maximum 9.3 U/mL); this included also all the MODY patients positive for GADA and/or IA2A. In the LADA cohort, 14 patients (24%) exceeded the 15 U/mL cut-off (Table 1). The entire range of values measured was within the linear range at A450; both A405 and A450 provided similar outcomes, but the A450 values were more sensitive for differences in the low concentrations, which allowed for visualization of the difference between healthy controls and MODY patients (Fig. 1). The post-hoc power analysis revealed 97.5% power of the present study to detect a difference between LADA patients and healthy controls at $\alpha = 0.05$ and 98.7% power to detect a difference between LADA and MODY patients at $\alpha = 0.05$.

The ZnTA assay had good reproducibility. Intra-assay coefficients of variability calculated for ZnTA values over the detection limit (1.2 U/mL) reached 15% (for A405) and 10% (for A450). The inter-assay variability calculated for the absorbance of standards measured at each plate

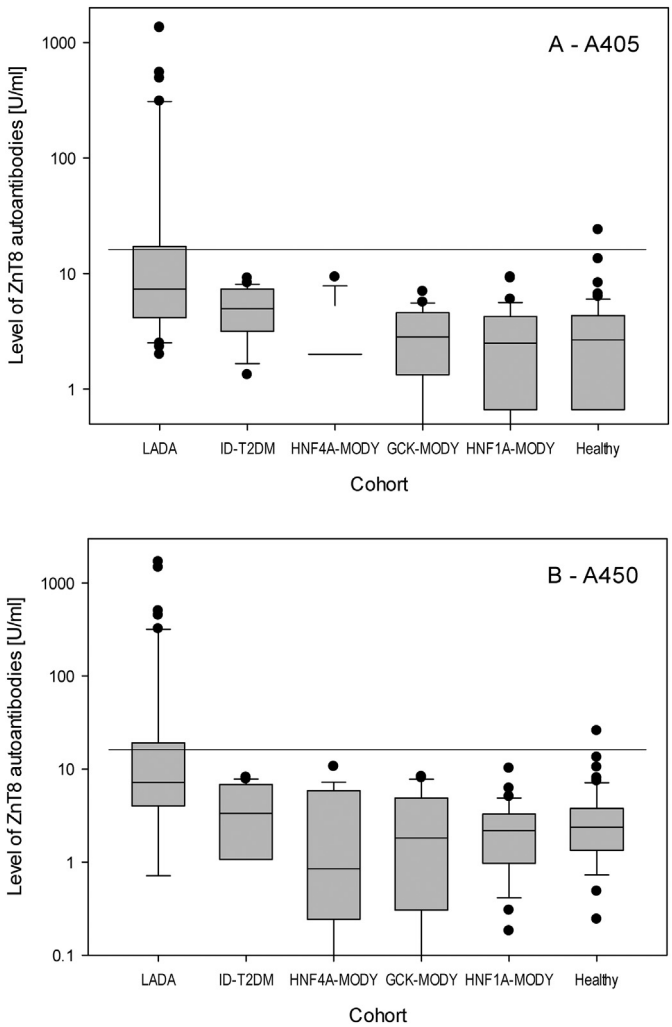

Fig. 1. ZnTA levels in cohorts of LADA, ID-T2DM, MODY and healthy control human subjects. The ZnTA levels were measured at 405 nm (A) and 450 nm (B), and they are shown as Tukey box plots with the median value lines, 25th and 75th percentiles as vertical boxes, 10 and 90th percentiles as error bars, and 5th and 95th percentiles as symbols. Horizontal lines indicate arbitrary cut-off limit (15 U/mL) suggested by the manufacturer of the ZnTA assay used.

reached 5% and 6%, respectively, at 20 U/mL, and 3% and 3% (for A405 and A450), respectively, at 500 U/mL.

As only a limited fraction of LADA patients had a ZnTA level above the cut-off suggested by the manufacturer, we constructed the ROC curves (Fig. 2) to determine what cut-off could be reliably used for differentiating between LADA, healthy controls and MODY patients, and to find out whether the ZnTA levels are altered in MODY compared to healthy controls. The area under the ROC curve (AUC) was 0.7 to 0.8 when comparing the ZnTA levels in patients with LADA-like progression to insulinotherapy with those in healthy controls or in *HNF4A*-, *GCK*- or *HNF1A*-MODY patients (Table S2). However, the AUC did not reach significance when comparing any of the MODY cohorts with healthy

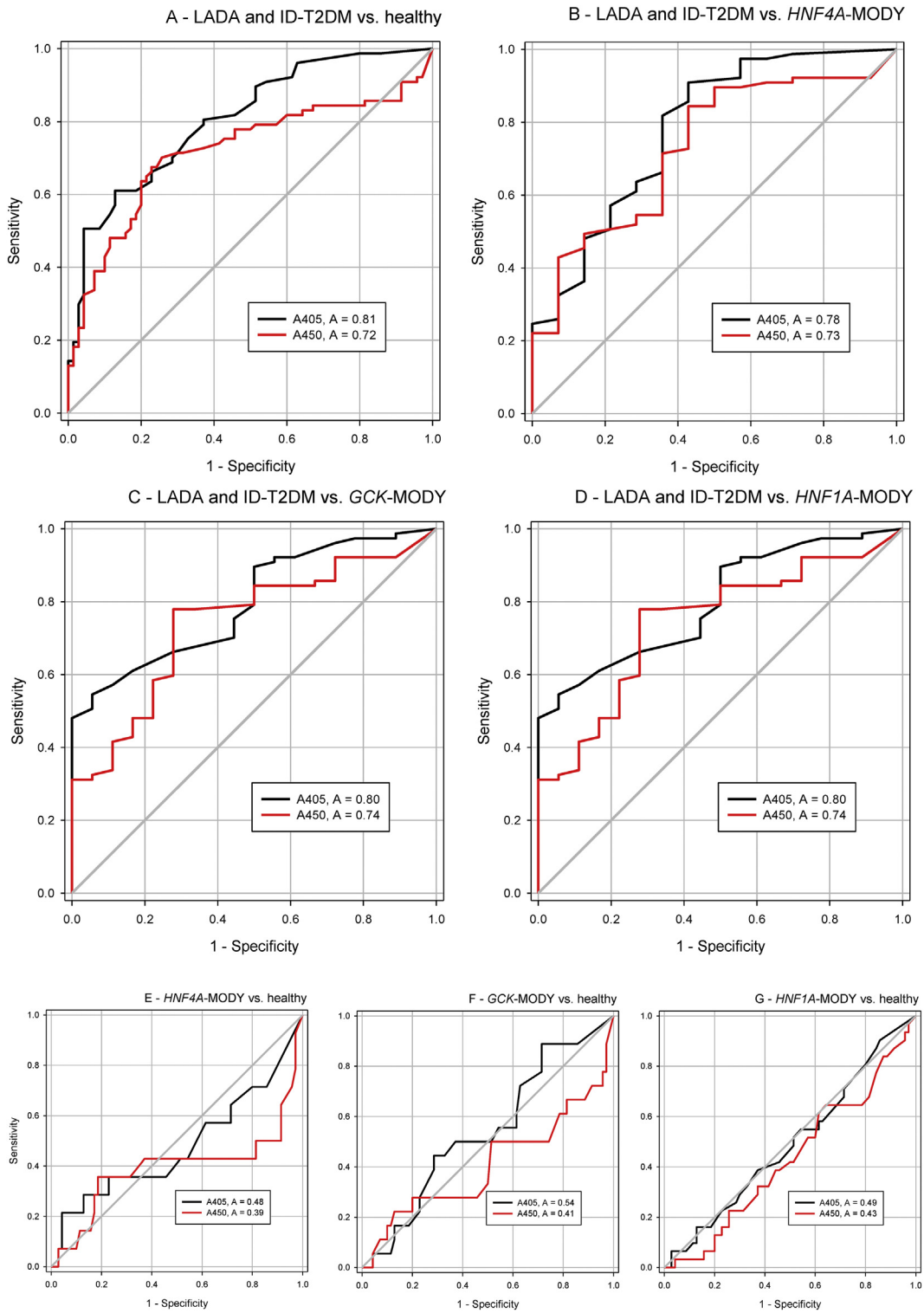

Fig. 2. ROC curves of ZnTA in LADA, MODY and healthy human subjects. The ROC curves were calculated based on A405 and A450 values obtained in course of the ZnTA assay. The following cohorts were compared: (A) LADA and ID-T2DM patients with healthy controls, (B) LADA and ID-T2DM patients with *HNF4A*-MODY, (C) LADA and ID-T2DM patients with *GCK*-MODY, (D) LADA and ID-T2DM patients with *HNF1A*-MODY, (E) *HNF4A*-MODY with healthy controls, (F) *GCK*-MODY with healthy controls, and (G) *HNF1A*-MODY with healthy controls. AUC values are indicated.

controls (Table S3). At the arbitrary cut-off suggested by the manufacturer (15 U/mL), the ZnTA assay had a sensitivity of only 18.2–19.5% when comparing the ZnTA levels in patients with LADA-like progression to insulinotherapy with those in healthy controls or in *HNF4A*-, *GCK*- or *HNF1A*-MODY patients although the specificity was high, at 97.1–100.0% (Table S4). Decreasing the specificity to just 95% decreased the cut-off to values to between 6.4 and 10.9 U/mL, but it only marginally improved the assay sensitivity. Additionally, at the 95% specificity, the sensitivity reached just 32.5%–50.7% when comparing the patients with LADA-like progression to insulinotherapy and healthy controls, and it was even lower when comparing patients with LADA-like progression to insulinotherapy and MODY patients. For example, the sensitivity of the ZnTA assay at 95% specificity was only 22.1%–24.7% when comparing patients with LADA-like progression to insulinotherapy to the *HNF4A*-MODY cohort (Table S4). Among ID-T2DM patients, we did not identify any single individual, who was positive for ZnTA (Fig. 3). Therefore, the addition of a test for ZnTA to the routinely performed tests for GADA and IA2A had no effect on the identification of islet cell autoimmunity in LADA patients. Instead, it only identified one false positive case in the control cohort.

3.2. Characteristics of ZnTA$^+$ LADA patients

The ZnTA$^+$ LADA patients examined in the present study had a similar age at diagnosis and diabetes duration as the LADA patients who were only positive for other autoantibodies against islet cell antigens (GADA and/or IA2A) and ID-T2DM patients (Table 2). Surprisingly, the three cohorts differed in their weight, height and BMI; ZnTA$^+$ LADA patients were shorter and leaner, while ID-T2DM patients were taller and had a higher BMI. ZnTA positivity was not associated with any gender. The trend towards lower C-peptide and higher HbA_{1c} levels in ZnTA$^+$ LADA patients was not significant (Table 2). The ZnTA titers did not show any association with the *PTPN22* risk genotypes (c.1858CT and c.-1123GC) but there was a trend towards lower ZnTA titers in patients expressing the loss-of-function LYP mutant (genotype c.788GA). However, the c.788A allele is rare in LADA because of its generally protective role against autoimmune diseases; therefore, we were only able to test four patients with the c.788A allele (Table S5).

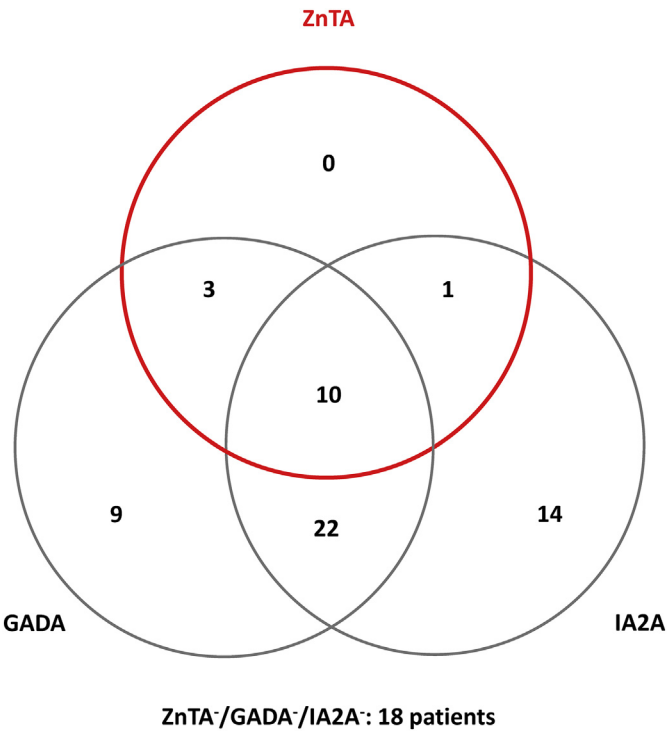

Fig. 3. Distribution of autoantibodies against islet cell antigens (ZnTA, GADA and IA2A) in LADA and ID-T2DM patients examined in the present study, as shown by the Venn diagram.

Table 2
Clinical characteristics of LADA patients positive for ZnTA (ZnTA$^+$), positive for other autoantibodies but negative for ZnTA (ZnTA$^-$) and ID-T2DM patients negative for any tested autoantibody but with C-peptide >200 pmol/L (Ab$^-$). The differences were tested by one-way ANOVA.

Cohort	LADA, ZnTA$^+$	LADA, ZnTA$^-$	ID-T2DM, Ab$^-$	One-way ANOVA[a]
N	12	34	18	
Age at diagnosis [years]	52.5 ± 4.3	52.6 ± 2.1	52.7 ± 2.8	>0.05[a]
Diabetes duration [years]	7.3 ± 3.8	13.4 ± 2.5	10.6 ± 2.1	>0.05[a]
Height [cm]	163.8 ± 3.5	170.3 ± 1.7	173.4 ± 2.5	0.058[b]
Weight [kg]	62.6 ± 3.2	75.3 ± 2.3	84.2 ± 5.3	0.007[a]
BMI [kg/m^2]	23.5 ± 1.4	25.9 ± 0.7	27.8 ± 1.4	0.062[b]
C-peptide [pmol/L]	152.5 ± 33.5	196.4 ± 43.5	346.1 ± 80.0	>0.05[a]
HbA_{1c} [mmol/mol]	101.3 ± 13.1	71.1 ± 5.3	76.0 ± 7.2	>0.05[a]
Sex	4M, 8F	20M, 14F	9M, 9F	>0.05[a]

[a] For data that failed the Shapiro-Wilk normality test, the Kruskal-Wallis one-way ANOVA was used; these are indicated by an asterisk.
[b] The power of these tests was below the desired power of 0.800.

4. Discussion

Previous data on the prevalence of ZnTA in LADA are conflicting (Table S1), and the previously reported prevalence in different populations differ by over one order of magnitude, ranging from 4.3% in Norway to 48.3% in Argentina. In type 1 diabetes, the ZnTA detection is generally thought to be associated with high specificity and selectivity. Most previous authors have also used these sensitivity and specificity values for their studies on LADA patients. However, we report here that most of the Central European LADA patients examined in the present study were ZnTA-negative, and the sensitivity of the commercial assay was only approximately 18–20% when calculated for the cohort of Central European patients with LADA-like progression to insulinotherapy versus healthy controls. This suggests that the use of ZnTA quantification for differential diagnosis of diabetic syndrome in adult patients of Central European ancestry may be of limited advantage. In addition, when we analyzed the ID-T2DM patients, which display the same progression towards insulinotherapy as the LADA patients, we found that none was positive for ZnTA (Fig. 3). ZnTA quantification did not improve the diagnosis, instead, its use was associated with increased type I error in the form of occasional positivity within the control cohort. The type I error could be eliminated by requiring positivity for at least two of the three tested autoantibodies (as is already common in some diabetes testing schemes).[32–34] However, as suggested by the data shown in Fig. 3, such a change would dramatically increase the type II error, and the prevalence of false-negative diagnoses would be as high as 30% (the number of single-positive patients within the examined cohort of patients with LADA-like progression to insulinotherapy). Importantly, the limitation of the present study consists of testing the patients at various stages of the disease progression. In T1DM, the ZnTA decrease during first years after the disease onset,[35] and their decline in T1DM follows the kinetics of the C-peptide.[36] There are no such data available from LADA patients. Considering the slow progression of autoimmune β cell destruction in LADA compared to T1DM, we expect the decrease in ZnTA levels in LADA to be slower than that known from T1DM, but a longitudinal study is needed to provide conclusive evidence regarding the kinetics of ZnTA in LADA. Despite the unsatisfactory value of ZnTA identification in the LADA diagnosis, ZnTA was of interest in the pre-screening of MODY among Central European patients. In Central and East Europe, approximately a quarter of MODY patients are positive for autoantibodies, which distinguish them from those examined in the United Kingdom, Japan, or elsewhere. In the Czech Republic, we previously found that 25% of

MODY patients are positive for GADA or IA2A.[24] Similarly, in Germany and Austria, 17% of MODY patients were positive for islet cell autoantibodies.[25] Similar, yet unpublished, data were recently obtained in the European part of Russia. The causes of positivity for islet cell antibodies in Central and East Europeans are unclear. Autoantibody-positive MODY patients from this region have been reported to be negative for *HLA-DRB1*, *HLA-DQB1* and *PTPN22* risk alleles.[24,31] Therefore, positivity for islet cell autoantibodies is a serious problem when pre-screening diabetic patients to refer them for genetic confirmation of the MODY diagnosis according to the widely accepted MODY criteria.[18,27] Here we found that a cohort of Czech MODY patients (including 63 *HNF4A*-, *GCK*- or *HNF1A*-MODY patients; Table 1) was completely negative for ZnTA, and the ZnTA levels in Central European MODY patients were similar to or lower than the levels in healthy controls (Fig. 2E–G). Therefore, positivity for ZnTA can likely be used as a negative MODY prediagnostic criterion even in the region of Central and East Europe, where MODY patients positive for other islet cell autoantibodies are common. We speculate that the ZnTA are more specific than GADA for the autoimmune destruction of β cells. GADA are highly prevalent not only in autoimmune diseases of the endocrine cells and tissues (similarly to ZnTA) but also in neurodegenerative disorders (such as stiff person syndrome), rheumatoid diseases and vasculitis (such as lupus erythematosus) and non-autoimmune forms of diabetes (MODY, T2DM). Despite ZnTA are less prevalent than GADA in both LADA and T1DM, their higher specificity for the autoimmune cause of diabetes may highlight their potential particularly for the above-mentioned pre-screening of diabetic patients referred for genetic confirmation of the MODY diagnosis in the region of Central and East Europe.

Another unresolved issue consists of the association between high levels of islet cell autoantibodies and underlying genetic characteristics of the patients. In the present study, we addressed the link with *PTPN22* polymorphisms. The frequencies of some *PTPN22* alleles are known to be altered in LADA; in particular, the frequency of the protective allele c.788A is low as is the frequency of the c.1970-852C allele. In contrast, the main autoimmunity-associated *PTPN22* risk alleles, c.1858T and c.-1123C, are not increased in LADA.[29] There are contradictory data on the association between the c.1858T risk allele and c.1858TT risk genotype with positivity for islet cell autoantibodies in LADA patients. Our group and Andersen et al. previously reported that they are not associated with any increased risk of positivity for autoantibodies in the Czech LADA cohort,[29,37] which, however, is not in agreement with the NIRAD study.[38] In agreement with our previous data on GADA and IA2A, here we did not find any statistically significant association of the four *PTPN22* SNPs tested with positivity for ZnTA in LADA; instead, there was only a trend towards lower ZnTA levels in c.788GA patients, which are rarely represented among the LADA patients (Table S5).

The issue to be solved is the newly identified association between ZnTA (and other islet cell autoantibodies) and differences in the BMI of the examined Central European LADA patients (Table 2). There are limited data available on this topic. However, Weber et al., who examined a non-overlapping group of Czech LADA patients, previously reported that the BMI was negatively associated with GADA positivity in LADA patients,[39] which is in agreement with the data found in the present study (Table 2). Similarly, Brazilian LADA patients with high GADA titers had a lower BMI than those with low GADA titers or who were GADA negative.[40] The same pattern was also reported in the cross-sectional Action LADA study, which was in combination with lower serum triglycerides, a lower waist circumference and a lower waist-hip ratio.[41] Further research should therefore address the question of whether ZnTA can also be used as a predictor of the diabetes phenotype, as shown repeatedly for GADA.

In conclusion, the present study questioned the value of ZnTA testing for precising the diagnosis of LADA. However, unexpectedly, the present study also raised the possibility of the use of ZnTA in the pre-screening of MODY in Central and East Europe, where many MODY patients are positive for GADA and/or IA2A. It also raised the possibility of using ZnTA as a predictor of the LADA phenotype. Future research should further address the role of ZnTA as a predictor of the LADA diabetes phenotype as examination of a higher number of ZnTA-positive LADA patients is needed to have sufficient power to address the research questions relevant to differences in the LADA phenotype.

Acknowledgement

We would like to thank the patients, their families and the referring physicians for their kind help. We thank Michal Boušek, Marcela Znamirowská and Kamila Riegerová for expert technical assistance.

Funding

This study was supported by the Czech Science Foundation project 15-03834Y and Charles University projects Primus/MED/32 and 260387/SVV/2017. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jdiacomp.2018.10.004.

References

1. Wenzlau JM, Hutton JC. Novel diabetes autoantibodies and prediction of type 1 diabetes. Curr Diab Rep 2013;13:608-15.
2. Koo BK, Chae S, Kim KM, Kang MK, Kim EG, Kwak SH, et al. Identification of novel autoantibodies in type 1 diabetic patients using a high-density protein microarray. Diabetes 2014;63:3022-32.
3. Walther D, Eugster A, Jergens S, Gavrisan A, Weinzierl C, Telieps T, et al. Tetraspanin 7 autoantibodies in type 1 diabetes. Diabetologia 2016;59:1973-6.
4. Wenzlau JM, Juhl K, Yu L, Moua O, Sarkar SA, Gottlieb P, et al. The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes. Proc Natl Acad Sci U S A 2007;104:17040-5.
5. Fichna M, Rogowicz-Frontczak A, Żurawek M, Fichna P, Gryczyńska M, Zozulińska-Ziółkiewicz D, et al. Positive autoantibodies to ZnT8 indicate elevated risk for additional autoimmune conditions in patients with Addison's disease. Endocrine 2016;53:249-57.
6. Bizzarri C, Giannone G, Benevento D, Montemitro E, Alghisi F, Cappa M, et al. ZnT8 antibodies in patients with cystic fibrosis: an expression of secondary beta-cell damage? J Cyst Fibros 2013;12:803-5.
7. Masala S, Cossu D, Palermo M, Sechi LA. Recognition of zinc transporter 8 and MAP3865c homologous epitopes by Hashimoto's thyroiditis subjects from Sardinia: a common target with type 1 diabetes? PLoS ONE 2014;9, e97621.
8. Rogowicz-Frontczak A, Zozulińska-Ziółkiewicz D, Litwinowicz M, Niedźwiecki P, Wyka K, Wierusz-Wysocka B. Are zinc transporter type 8 antibodies a marker of autimmune thyroiditis in non-obese adults with new-onset diabetes? Eur J Endocrinol 2014;170:651-8.
9. Svensson M, Ramelius A, Nilsson AL, Delli AJ, Elding Larsson H, et al. Antibodies to influenza virus A/H1N1 hemagglutinin in type 1 diabetes children diagnosed before, during and after the SWEDISH A(H1N1)pdm09 vaccination campaign 2009–2010. Scand J Immunol 2014;79:137-48.
10. Haller-Kikkatalo K, Pruul K, Kisand K, Nemvalts V, Reimand K, Uibo R. GADA and anti-ZnT8 complicate the outcome of phenotypic type 2 diabetes of adults. Eur J Clin Invest 2015;45:255-62.
11. Ng ML. Prevalence of glutamate acid decarboxylase (GAD65) and zinc-transporter 8 (ZnT8) autoantibodies in adult-onset diabetes patients in Singapore. Diabetes Res Clin Pract 2014;106:S36-7.
12. Chow LS, Chen H, Miller ME, Marcovina SM, Seaquist ER. Biomarkers related to severe hypoglycaemia and lack of good glycaemic control in ACCORD. Diabetologia 2015;58:1160-6.
13. Rudland VL, Pech C, Harding AJ, Tan K, Lee K, Molyneaux L, et al. Zinc transporter 8 autoantibodies: what is their clinical relevance in gestational diabetes? Diabet Med 2015;32:359-66.
14. Dereke J, Palmqvist S, Nilsson C, Landin-Olsson M, Hillman M. The prevalence and predictive value of the SLC30A8 R325W polymorphism and zinc transporter 8 autoantibodies in the development of GDM and postpartum type 1 diabetes. Endocrine 2016;53:740-6.
15. Kleinberger JW, Pollin TI. Undiagnosed MODY: time for action. Curr Diab Rep 2015;15:110.
16. Chambers C, Fouts A, Dong F, Colclough K, Wang Z, Batish SD, et al. Characteristics of maturity onset diabetes of the young in a large diabetes center. Pediatr Diabetes 2016;17:360-7.
17. Vaziri-Sani F, Delli AJ, Elding-Larsson H, Lindblad B, Carlsson A, Forsander G, et al. A novel triple mix radiobinding assay for the three ZnT8 (ZnT8-RWQ) autoantibody

variants in children with newly diagnosed diabetes. J Immunol Methods 2011;371:25-37.
18. McDonald TJ, Colclough K, Brown R, Shields B, Shepherd M, Bingley P, et al. Islet autoantibodies can discriminate maturity-onset diabetes of the young (MODY) from Type 1 diabetes. Diabet Med 2011;28:1028-33.
19. Pozzilli P, Di Mario U. Autoimmune diabetes not requiring insulin at diagnosis (latent autoimmune diabetes of the adult) – definition, characterization and potential prevention. Diabetes Care 2001;24:1460-2467.
20. Canivell S, Gomis R. Diagnosis and classification of autoimmune diabetes mellitus. Autoimmun Rev 2014;13:403-7.
21. Brophy S, Davies H, Mannan S, Brunt H, Williams R. Interventions for latent autoimmune diabetes (LADA) in adults. Cochrane Database Syst Rev 2011;9, CD006165.
22. Luo SM, Lin J, Xie Z, Xiang Y, Zheng P, Huang G, et al. HLA genetic discrepancy between latent autoimmune diabetes in adults and type 1 diabetes: LADA China Study No. 6. J Clin Endocrinol Metab 2016;101:1693-700.
23. Xiang Y, Zhou Z, Deng C, Leslie RD. Latent autoimmune diabetes in adults in Asians: similarities and differences between East and West. J Diabetes 2013;5:118-26.
24. Urbanová J, Rypáčková B, Procházková Z, Kučera P, Cerná M, Anděl M, et al. Positivity for islet cell autoantibodies in patients with monogenic diabetes is associated with later diabetes onset and higher HbA_{1c} level. Diabet Med 2014;31:466-71.
25. Schober E, Rami B, Grabert M, Thon A, Kapellen T, Reinehr T, et al. Phenotypical aspects of maturity-onset diabetes of the young (MODY diabetes) in comparison with Type 2 diabetes mellitus (T2DM) in children and adolescents: experience from a large multi-centre database. Diabet Med 2009;26:466-73.
26. Capasso F, Rapini N, Di Matteo G, Testi M, Arcano S, Lidano R, et al. A variable degree of autoimmunity in the pedigree of a patient with type 1 diabetes homozygous for the *PTPN22* 1858T variant. Pediatr Diabetes 2013;14:304-10.
27. Thanabalasingham G, Owen KR. Diagnosis and management of maturity onset diabetes of the young (MODY). BMJ 2011;343:d6044.
28. Urbanová J, Rypáčková B, Kučera P, Anděl M, Heneberg P. Should the negativity for islet cell autoantibodies be used in a prescreening for genetic testing in maturity-onset diabetes of the young? The case of autoimmunity-associated destruction of pancreatic β-cells in a family of *HNF1A*-MODY subjects. Int Arch Allergy Immunol 2013;161:279-84.
29. Heneberg P, Kocková L, Čecháková M, Daňková P, Černá M. Autoimmunity-associated *PTPN22* polymorphisms in latent autoimmune diabetes of the adult differ from those of type 1 diabetes patients. Int Arch Allergy Immunol 2018;177:57-68.
30. Maple-Brown LJ, Ye C, Retnakaran R. Area-under-the-HbA_{1c} curve above the normal range and the prediction of microvascular outcomes: an analysis of data from the diabetes control and complications trial. Diabet Med 2013;30:95-9.
31. Heneberg P, Malá M, Yorifuji T, Gat-Yablonski G, Lebenthal Y, Tajima T, et al. Low frequencies of autoimmunity-associated *PTPN22* polymorphisms in MODY patients, including those transiently expressing islet cell autoantibodies. Int Arch Allergy Immunol 2015;166:189-98.
32. Chmiel R, Giannopoulou EZ, Winkler C, Achenbach P, Ziegler A-G, Bonifacio E. Progression from single to multiple islet autoantibodies often occurs soon after seroconversion: implications for early screening. Diabetologia 2015;58:411-3.
33. Helminen O, Aspholm S, Pokka T, Hautakangas MR, Haatanen N, Lempainen J, et al. HbA_{1c} predicts time to diagnosis of type 1 diabetes in children at risk. Diabetes 2015;64:1719-27.
34. Maclaren N, Lan M, Coutant R, Schatz D, Silverstein J, Muir A, et al. Only multiple autoantibodies to islet cells (ICA), insulin, GAD65, IA-2 and IA-2β predict immune-mediated (type 1) diabetes in relatives. J Autoimmun 1999;12:279-87.
35. Vaziri-Sani F, Oak S, Radtke J, Lernmark K, Lynch K, Agardh CD, et al. ZnT8 autoantibody titers in type 1 diabetes patients decline rapidly after clinical onset. Autoimmunity 2010;43:598-606.
36. Wenzlau JM, Walter M, Gardner TJ, Frisch LM, Yu L, Eisenbarth GS, et al. Kinetics of the post-onset decline in zinc transporter 8 autoantibodies in type 1 diabetic human subjects. J Clin Endocrinol Metab 2010;95:4712-9.
37. Andersen MK, Lundgren V, Turunen JA, Forsblom C, Isomaa B, Groop PH, et al. Latent autoimmune diabetes in adults differs genetically from classical type I diabetes diagnosed after the age of 35 years. Diabetes Care 2010;33:2062-4.
38. Petrone A, Suraci C, Capizzi M, Giaccari A, Bosi E, Tiberti C, et al. The protein tyrosine phosphatase nonreceptor 22 (*PTPN22*) is associated with high GAD antibody titer in latent autoimmune diabetes in adults: Non Insulin Requiring Autoimmune Diabetes (NIRAD) Study 3. Diabetes Care 2008;31:534-8.
39. Weber P, Ambrosova P, Canov P, Weberova D, Kuklinek P, Meluzinova H, et al. GAD antibodies in T1D and LADA—relations to age, BMI, c-peptide, IA-2 and HLA-DRB1*03 and DRB1*04 alleles. Adv Gerontol 2011;24:312-8.
40. Rosário PW, Reis JS, Fagundes TA, Calsolari MR, Amim R, Silva SC, et al. Latent autoimmune diabetes in adults (LADA): usefulness of anti-GAD antibody titers and benefit of early insulinization. Arq Bras Endocrinol Metabol 2007;51:52-8.
41. Hawa MI, Kolb H, Schloot N, Beyan H, Paschou SA, Buzzetti R, et al. Adult-onset autoimmune diabetes in Europe is prevalent with a broad clinical phenotype: action LADA 7. Diabetes Care 2013;36:908-13.