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ARTICLE

Time dynamics of autoantibodies are coupled to phenotypes and add to the heterogeneity of autoimmune diabetes in adults: the HUNT study, Norway

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Abstract
Aims The aetiology of latent autoimmune diabetes in adults (LADA), assessed by autoimmune markers, is insufficiently clarified. We cross-sectionally investigated the prevalence and prospectively the prediabetic and postdiabetic presence of antibodies to glutamic acid decarboxylase (GADA), insulinoma-associated protein 2 and zinc transporter 8 in LADA and in type 1 diabetes.
Methods We included 208 'classic' type 1, 161 LADA and 302 type 2 diabetic cases from the second (HUNT2: 1995–1997) and third (HUNT3: 2006–2008) Nord-Trøndelag health surveys. Prospective data were available for 59 type 1, 44 LADA and 302 type 2 diabetic cases followed from HUNT2 to HUNT3. From HUNT3, 24 type 1 diabetic and 31 LADA incident cases were available.
Results Cross-sectionally, 90% of LADA cases were positive for only one antibody (10% multiple-antibody-positive). Prospectively, 59% of GADA-positive LADA patients in HUNT2 were no longer positive in HUNT3. LADA patients who became negative possessed less frequently risk HLA haplotypes and were phenotypically more akin to those with type 2 diabetes than to those who stayed positive. Still, those losing positivity differed from those with type 2 diabetes by lower C-peptide levels (p= 0.009). Of incident LADA cases in HUNT3, 64% were already antibody-positive in HUNT2, i.e. before diabetes diagnosis. These incident LADA cases were phenotypically more akin to type 1 diabetes than were those who did not display positivity in HUNT2.
Conclusion/interpretation The pattern of antibodies, the postdiabetic loss or persistence as well as the prediabetic absence or presence of antibodies influence LADA phenotypes. Time-dependent presence or absence of antibodies adds new modalities to the heterogeneity of LADA.

Keywords Autoantibody · Autoimmune diabetes · Diabetes mellitus · GADA · IA-2A · LADA · Type 1 diabetes · ZnT8A

Abbreviations

ai	Antibody index
DASP	Diabetes Autoantibody Standardization Program
GADA	Glutamic acid decarboxylase antibody
HUNT study	Nord-Trøndelag Health Study
IA-2A	Insulinoma-associated protein 2 antibody
ICA	Islet cell antibody

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LADA	Latent autoimmune diabetes in adults
SNP	Single nucleotide polymorphism
ZnT8A	Zinc transporter 8 antibody

Introduction

Type 1 diabetes is identified biochemically by the presence of circulating antibodies. Antibodies include those to glutamic acid decarboxylase (GADA) and insulinoma-associated protein 2 (IA-2A), as well as to insulin and cytoplasmic islet cells (ICA). GADA is the diabetes-associated antibody with the highest penetration, being present in 70–80% at onset of the disease [1, 2].

About 10% of patients with a type 2 diabetes-like phenotype (i.e. not insulin-dependent at the time of diagnosis) are GADA- and/or ICA-positive [3–5]. These patients are commonly classified as having latent autoimmune diabetes in adults (LADA). The presence of autoimmune markers in LADA and their relationship to phenotype, as well as to the similarity to 'classic' type 1 diabetes with onset in adulthood, have not been investigated in detail. In particular, the time course of autoimmunity (e.g. presence before overt diabetes and persistence of positivity after onset) in LADA is only partly elucidated. We reasoned that data collected from the Nord-Trøndelag Health Study in Norway (the HUNT Study) should be appropriate to gain information on these issues.

The main aim of this study was to investigate prospectively the prediabetic appearance of GADA, IA-2A and zinc transporter 8 antibodies (ZnT8A) and the persistence of these antibodies in prevalent LADA after a period of 10–13 years. A second aim was to compare cross-sectionally the presence of the same antibodies in LADA and adult-onset 'classic' type 1 diabetes in relation to diabetes onset and other phenotypic characteristics. We included ZnT8A since this antibody has recently been identified as a new target antigen in autoimmune diabetes [6, 7].

Material and methods

The HUNT Study The Nord-Trøndelag Health Study consists of three health surveys performed in 1984–1986 (HUNT1), 1995–1997 (HUNT2) and 2006–2008 (HUNT3). In all three surveys the entire adult population (aged ≥20 years) in Nord-Trøndelag county (located in the middle part of Norway) were invited to participate (n=87,259, n=94,187 and n=94,123, respectively). The cases that formed the basis of our analysis were collected from the HUNT2 and HUNT3 surveys. The HUNT2 survey had an overall response rate of 69% (n=65,215). The survey included questionnaires about health and personal habits, a clinical examination and blood sampling. The HUNT2 survey has been described in detail elsewhere [8]. The HUNT3 survey, with a similar design to that of HUNT2, had an overall response rate of 54% (n=50,810). Fifty-seven percent of the participants in HUNT2 also participated in HUNT3 (n=37,059).

Data collection Diabetic cases (n=1,972 in HUNT2 and n=2,189 in HUNT3) were identified from a self-reported answer of 'Yes' to the question 'Do you have or have you had diabetes?'. In HUNT2 and HUNT3, participants declaring diabetes were invited to a follow-up investigation. A total of 1,452 individuals from HUNT2 and 1,168 from HUNT3 took part in these investigations. They completed a more detailed questionnaire concerning diabetes and underwent an interview by a nurse. They furthermore provided a fasting blood sample for measurements of blood glucose, serum C-peptide, GADA and IA-2A (the latter only in HUNT3). Later, additional measurements of antibodies were performed as described below. In addition, GADA was analysed in cases declaring diabetes but not attending the follow-up and having serum available from a non-fasting state (n=432 in HUNT2 and n=984 in HUNT3). The samples were stored at the HUNT Biobank at −70°C prior to analysis.

Classification of diabetes Cases were classified as 'classic' type 1 diabetes (in the following termed type 1 diabetes) if they started insulin treatment within 12 months of diagnosis and were: (1) antibody-positive, or (2) antibody-negative but with fasting C-peptide levels <150 pmol/l. Cases were classified as LADA if they were antibody-positive and had not been treated with insulin within 12 months of diagnosis. No age limit was set for LADA. Cases were classified as having type 2 diabetes if they were GADA-negative and had not been treated with insulin within 12 months of diagnosis. Type 1 diabetic cases were divided into two subgroups based on the median of onset, which was 24 years. The groups were termed young-onset type 1 diabetes and adult-onset type 1 diabetes.

Additional antibody measurements Serum samples from diabetic cases classified as LADA or type 1 diabetes were analysed for IA-2A (if not done already in HUNT3) as well as for ZnT8A. Also serum samples from HUNT2 were used to analyse antibodies in cases classified as LADA and type 1 diabetes in HUNT3 but with no diagnosis of diabetes in HUNT2.

Final study population For LADA and for type 1 diabetic cases we included those with complete data on all three antibodies (120 type 1 and 120 LADA cases from HUNT2 and 147 type 1 and 85 LADA cases from HUNT3) (Fig. 1). For type 2 diabetic cases, we included those who had participated in both HUNT2 and HUNT3 studies (302 cases).

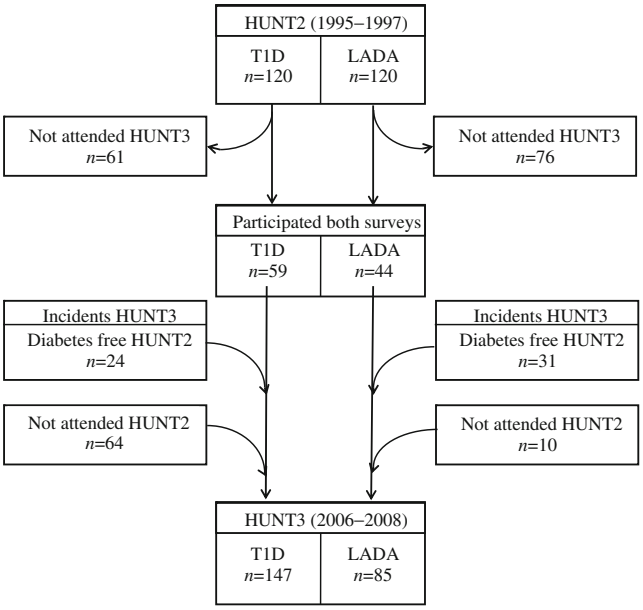

Fig. 1 Flowsheet of included diabetes cases from the HUNT2 and HUNT3 surveys. T1D, type 1 diabetes

We obtained prospective data (HUNT2 to HUNT3; 10–13 years' follow-up) on 44 LADA, 59 type 1 diabetic and 302 type 2 diabetic cases from HUNT2, and 31 LADA and 24 type 1 diabetic incident cases from HUNT3.

Antibody assays GADA, IA-2A and ZnT8A were analysed at the Aker Hormone Laboratory, Oslo University Hospital, Oslo, Norway.

GADA was measured by immune-precipitation using translation labelled ^{3}H-GAD65 as a labelled reagent (Novo Nordisk, Bagsværd, Denmark). Separation of bound GADA and free labelled antigen was done by means of protein A coupled to Sepharose (procedure developed at the Aker Hormone Laboratory). Antibody levels were expressed as an antibody index (ai) relative to a standard serum. The lower limit of detection was 0.01 ai; no upper limit was defined. An index of ≥0.08 ai was considered positive. This cut-off level of positivity was the one used by the Aker Hormone Laboratory based on data from the Diabetes Autoantibody Standardization Program (DASP) material. Cut-off was set to achieve the highest possible specificity with an acceptable corresponding sensitivity. As calculated from DASP 2003, the assay has a 100% workshop-specificity and 68% workshop-sensitivity. The intra-assay coefficient of variation (CV) was 14% in the lowest (0.11 ai), 8% in the middle (0.22 ai) and 17% in the highest (2.0 ai) range of measurements. The total assay CV was 19% in the lower (0.21 ai) and 23% in the higher (0.66 ai) measurement range.

IA-2A was measured by immune-precipitation using translation labelled ^{3}H-IA-2$_{ic}$ as a labelled reagent. Separation of bound IA-2A and free labelled antigen was done by protein A coupled to Sepharose, using a procedure developed at the Aker Hormone Laboratory. Antibody levels were expressed as an index value relative to a standard serum. A value of ≥0.11 ai was considered positive (method range, 0.01–3.00 ai). The cut-off level was based on the same considerations as for GADA. As calculated from DASP 2003, the assay of IA-2A has 99% workshop-specificity and 70% workshop-sensitivity. Intra-assay CV was 17% in the lowest (0.10 ai), 10% in the middle (0.48 ai) and 7% in the highest (1.96 ai) range of measurements. Total assay CV was 22% in the lower (0.14 ai) and 11% in the higher (3.60 ai) range of measurements.

ZnT8A was measured by immune-precipitation using a translation labelled ^{3}H-ZnT8 C-terminal Arg325 variant fused to a C-terminal Trp325 variant as a labelled reagent (based on a plasmid pJH5.2 SP6, a Dimer human ZnT8 C-terminal Arg325 variant fused to human ZnT8 C-terminal Trp variant from J. Hutton, University of Colorado, Denver, CO, USA). Separation of bound ZnT8A and freely labelled antigen was achieved by protein A plus protein C coupled to Sepharose using a procedure developed at the Aker Hormone Laboratory. Antibody levels were expressed as an index value relative to a standard serum. A value >0.08 ai was considered positive (method range, >0.01 ai). The level of cut-off was based on the same considerations as for GADA and IA-2A. As calculated from DASP 2010, the ZnT8A assay has 100% workshop-specificity and 46% workshop-sensitivity. Intra-assay CV was 7% in the lowest (0.18 ai) and 6% in the highest (0.88 ai) range of measurements. Total assay CV was 20% in the lower (0.18 ai) and 16% in the higher (0.85 ai) range of measurements.

SNP genotyping and HLA haplotyping We had genotyping data available from 44 single nucleotide polymorphisms (SNP) known to be associated with either type 1 or type 2 diabetes. The genotyping was done by using TaqMan or SNPlex Genotyping System (Applied Biosystems, Foster City, CA, USA) as described previously [9]. We had 14 HLA tag SNPs available for use in HLA haplotyping as described previously [9]. The tag SNPs for haplotyping are given in Table 1 of the Electronic supplementary material (ESM).

Statistical analysis PASW statistics software, version 18, was used (www-01.ibm.com/software/analytics/spss/). Categorical data were compared by χ^2 or Fisher's exact test when appropriate. Comparing continuous data between groups was done by a non-parametric analysis of variance test (Mann–Whitney *U* test and Kruskal–Wallis test as appropriate). A Kaplan–Meier log-rank test was performed to assess time to insulin requirement in relation to antibody positivity. Comparison of antibody titre over time (HUNT2 to HUNT3) was performed by a non-parametric Wilcoxon

signed-rank test for two related samples. Phasing HLA haplotypes and testing for association between groups were carried out using PLINK 1.07 software (http://pngu.mgh.harvard.edu/~purcell/plink/) [10]. A p value <0.05 was considered significant.

Ethics All participants gave their written consent. The study was approved by the Regional Committee for Ethics in Medical Research.

Results

Age-related occurrence of LADA and type 1 diabetes differ markedly For preliminary analyses we combined all prevalent cases from HUNT2 together with diabetic cases from HUNT3 who did not have diabetes or who did not participate in HUNT2 (n=208 type 1 diabetes and n=161 LADA). The age distribution (Fig. 2) demonstrates decreasing occurrence of type 1 diabetes from the age of 40 years. By contrast, the occurrence of LADA increased markedly from the same age.

Pattern of antibody positivity in LADA influences phenotype Only 17 out of 161 LADA cases were positive for antibodies other than GADA and only 1 of these 17 cases was GADA-negative (ESM Fig. 1a). LADA cases positive for two or three antibodies (10%, n=16) had a higher GADA titre (p<0.001), lower systolic blood pressure (p=0.005) and higher non-fasting blood glucose (p=0.011) than those positive only for one antibody (ESM Table 2).

Phenotypes and antibodies differ in LADA compared with adult-onset type 1 diabetes In comparison with patients with adult-onset type 1 diabetes (age >24 years, n=105), those with LADA were older at onset (p<0.001), had longer diabetes duration (p<0.001), higher BMI (p=0.008), higher systolic blood pressure (p<0.001), lower HDL-cholesterol (p<0.001), lower HbA_{1c} (p=0.041), higher C-peptide levels (p<0.001), and higher GADA titre and lower IA-2A titre (p<0.001 and p=0.002, respectively) (ESM Table 3). LADA cases were also less frequently positive for more than one antibody (p<0.001).

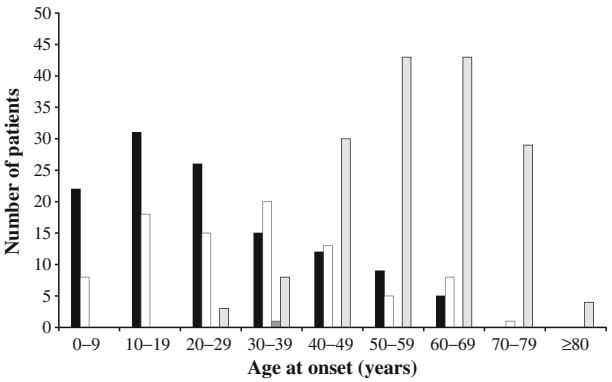

Fig. 2 Number of patients with type 1 diabetes and LADA from HUNT2 and HUNT3, according to GADA negativity/positivity and age at onset. Black bars, type 1 diabetes, GADA-negative; white bars, type 1 diabetes, GADA-positive; dark grey bars, LADA, GADA-negative; light grey bars, LADA, GADA-positive. For diabetes classification, see Materials and methods. Note that individuals with diabetes onset before the age of 20 years did not participate in the HUNT Study before they had reached that age

A majority of diagnosed LADA cases lose antibody positivity Fifty-nine type 1 diabetic, 44 LADA and 302 type 2 diabetic cases in HUNT2 also participated in HUNT3. In HUNT2, LADA cases were antibody-positive by classification. However, after 10–13 years, in HUNT3, a majority of LADA cases (26 of 44, 59%) were now negative for all three antibodies (ESM Fig. 1b). As for type 1 diabetes, 28 cases out of 59 (47%) were already antibody-negative in HUNT2, whereas 31 cases (53%) were antibody-negative in HUNT3. Hence, in contrast to LADA, only three cases (6%) with type 1 diabetes who were positive in HUNT2 had lost positivity in HUNT3.

LADA patients who became antibody-negative during follow-up differed in several aspects from those with persisting positivity. The former were older at onset (p=0.001), more obese (higher BMI and waist circumference; p=0.013 and p=0.009, respectively), had lower HDL-cholesterol (p=0.017) and higher triacylglycerol values (p=0.002) in HUNT2 (Table 1). They also had higher C-peptide levels (p=0.003), lower GADA and IA-2A titre (p<0.001 and p=0.012, respectively) and were less likely to be multiple-antibody-positive (p=0.023). Only 4 of the 44 LADA cases from HUNT2 had multiple antibodies; however, all of these four cases were still positive in HUNT3. Hence, the likelihood of being persistently antibody-positive in HUNT3 after being multiple-antibody-positive in HUNT2 was 100%.

Individuals with persisting GADA positivity did not display any decrease or increase in GADA titre over time (p=0.778, Fig. 3b). However, both IA-2A and ZnT8A displayed a decrease in titre during follow-up (p=0.051 and p=0.014, respectively; data not shown).

Twenty-one out of the 44 followed-up LADA cases did not use insulin in HUNT2. In HUNT3 we had data on diabetes treatment from 19 of these 21 cases, of whom ten had started on insulin treatment. The persistent positivity of antibodies in HUNT3 (ESM Fig. 2) was associated with early requirement of insulin therapy by Kaplan–Meier analysis (p=0.001).

We also compared LADA patients who became antibody-negative during follow-up with those with type 2 diabetes. We found that these LADA patients had less preserved C-peptide levels compared with those with type 2 diabetes

Table 1 Comparison of clinical characteristics in HUNT2 for LADA patients who participated both in HUNT2 and HUNT3 and who became either antibody-negative or stayed antibody-positive at HUNT3

Clinical characteristics, HUNT2	LADA		
	Antibody-negative, HUNT3	Antibody-positive, HUNT3	*p* value[a]
N	26	18	
Sex (male), % (*n*)	46.2 (12)	55.6 (10)	0.540
Age at onset (years)	53.5 (42–75)	44.5 (21–60)	0.001
Diabetes duration (years)	7.5 (1–20)	8.0 (1–43)	0.526
Waist circumference (cm)	96 (78–122)	88.5 (71–103)	0.009
BMI (kg/m^2)	27.9 (24.6–44.8)	25.7 (21.9–36.9)	0.013
Systolic blood pressure (mmHg)	158.5 (119–187)	142.5 (102–188)	0.346
Diastolic blood pressure (mmHg)	82 (60–105)	79.5 (65–106)	0.914
Glucose, non-fasting (mmol/l)	7.9 (2.8–14)	8.95 (3.9–22.3)	0.193
Cholesterol (mmol/l)	5.6 (4.1–10.7)	5.45 (4.5–7.9)	0.774
HDL-cholesterol (mmol/l)	1.1 (0.6–2.4)	1.75 (0.7–3.3)	0.017
Triacylglycerol (mmol/l)	1.6 (0.86–4.42)	1.12 (0.44–4.48)	0.002
HbA_{1c} (%)	7.5 (5.5–12.49)	8.2 (0–16.7)	0.114
HbA_{1c} (mmol/mol)	58.5 (36.6–113)	66.1 (0–159)	0.114
C-peptide (pmol/l)	492 (30–1,384)	118.5 (30–588)	0.003
Glucose, fasting (mmol/l)	7.55 (2.9–11.5)	9.0 (3.9–16.9)	0.111
GADA titre (ai)	0.11 (0.08–0.46)	0.51 (0.07–2.43)	<0.001
IA-2A titre (ai)	<0.01 (<0.01–0.07)	0.01 (<0.01–3.0)	0.012
ZnT8A titre (ai)	0.01 (<0.01–0.04)	0.01 (<0.01–0.93)	0.166
Insulin treatment	42.3% (11)	66.7% (12)	0.112
Years without insulin treatment	5.0 (2–10)	3.0 (1–13)	0.111
Number of positive antibodies			0.023
1 (*n*=40)	65% (26)	35% (14)	
2–3 (*n*=4)	–	100% (4)	

Data are presented as percentages and actual numbers (*n*) or median (min–max values)

Antibody-negative, antibody-negative for all three antibodies measured

Antibody-positive, antibody-positive for GADA and/or IA-2A and/or ZnT8A

[a]Unadjusted *p* value calculated by χ^2 test or Fisher's exact test (if number is less than 5) for categorical data and by Mann–Whitney *U* test for continuous data

(median [min–max]: 492 [30–1,354] vs 700.5 [30–2,059]; $p=0.009$). Other parameters were not significantly different between the two groups (data not shown).

HLA haplotypes in LADA cases differ among persisters and losers of antibody positivity SNP data were available for 43 of the 44 LADA patients who participated in both HUNT2 and HUNT3. The HLA haplotypes previously shown to be associated with higher risk in type 1 diabetes [9] were mainly associated with higher risk in LADA antibody persisters compared with losers of antibody positivity (ESM Table 4). The persisters also displayed significantly lower frequency of protective HLA haplotypes.

We did not find any differences in other type 1 diabetic risk loci (such as *INS*, *CTLA4*, *PTPN22*) or type 2 diabetic risk loci (such as *TCF7L2*, *FTO*, *SLC30A8*) (data not shown).

We also tested for differences in HLA haplotype frequency between losers of antibody positivity and those with type 2 diabetes. Haplotyping data were available for 288 of the 302 type 2 diabetic cases. There was only a non-significant higher frequency in losers of positivity compared with type 2 diabetes of the HLA haplotype AACTG for DRB1*0402-DQA1*0301-DQB1*0302 (0.412 vs 0.276; $p=0.063$).

Presence of autoantibodies in LADA before diabetes diagnosis associates with insulin deficiency Twenty-one out of 31 (68%) incident cases of LADA in HUNT3 were already antibody-positive 10–13 years earlier in HUNT2 (ESM Fig. 1c). LADA patients who were already antibody-positive before diagnosis of diabetes were diagnosed at a younger age ($p=0.001$) than those who were antibody-negative in HUNT2 (Table 2). The former also had a higher GADA titre ($p=0.002$) and higher fasting blood glucose ($p=0.013$) in HUNT3. Thirteen out of 24 incident type 1 diabetes cases (54%) were already antibody-positive in HUNT2 (ESM Fig. 1c). There were, however, no distinctive differences between antibody-positive and antibody-negative type 1 diabetes either at prediabetes (HUNT2) or at overt diabetes (HUNT3) (data not shown).

Discussion

This study confirms and extends prevalence data and provides novel incidence data on markers of autoimmunity in LADA and adult-onset type 1 diabetes derived from the population-based HUNT Study in Norway. As to prevalence

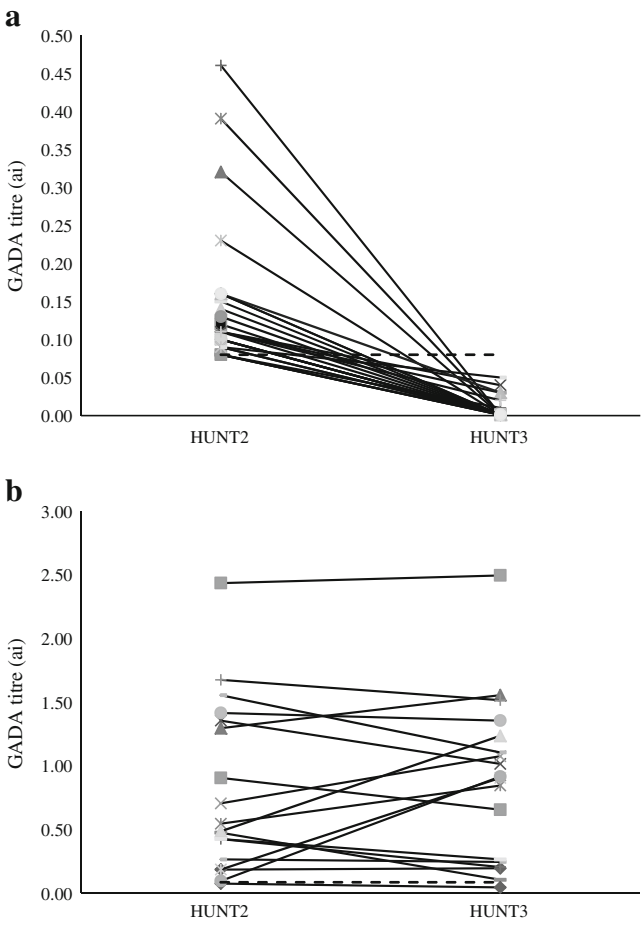

Fig. 3 GADA titre change from HUNT2 to HUNT3 (10–13 years' follow-up) for (**a**) individuals with LADA who became antibody-negative during follow-up (n=26) and (**b**) individuals with LADA who stayed antibody-positive during follow-up (n=18). Dashed line shows the cut-off for antibody positivity

data, this is to the best of our knowledge the first report that details age-related differences in prevalence between LADA and type 1 diabetes. We emphasise that differences in the pattern of antibodies in our study were seen in conjunction with adult-onset type 1 diabetes rather than with all age groups with classic type 1 diabetes. When interpreting our data it should be noted that our classification of LADA cases (no insulin treatment within 12 months of diagnosis) distinguishes LADA more clearly from type 1 diabetes than does the more common definition of LADA (no insulin treatment within 3–6 months of diagnosis) [11]. As to incidence data (the major part of this study), we provide novel observations on the dynamics of GADA and other antibodies. These observations add new modalities of heterogeneity in autoimmune diabetes in adults, in particular in the LADA category.

We found, as have others [12, 13], that the presence of more than one positive antibody (GADA) is far less common in LADA than in type 1 diabetes. Also we confirm previous observations [7, 12] that the presence of more than one antibody in LADA cases is associated with a higher titre of GADA and a tendency towards an increased propensity for insulin treatment. However, we noted that the prevalence of both IA-2A and ZnT8A positivity (8.7% and 4.3%, respectively; ESM Fig. 1a) is less than that found in the Non Insulin Requiring Autoimmune Diabetes (NIRAD) study (20% and 19%, respectively) [7]. That our LADA cases were population-based, whereas those of NIRAD were centre-based, could be of importance for the observation.

As to the prospective part of the study, a major finding was that GADA titre changed from positive to negative in the majority of LADA cases between participation in HUNT2 and the follow-up in HUNT3. Fluctuations in antibody levels such as GADA are common in type 1 diabetes in childhood and adolescence and their disappearance is not closely linked to progression or lack of progression to beta cell failure [14, 15]. Such findings may contrast with the lesser degree of progression seen here in GADA losers vs persisters. The discrepancy highlights aetiological differences between autoimmune diabetes at different ages.

The proportion of LADA cases reverting from GADA-positive to GADA-negative was higher in our study than previously reported [16, 17]. Differences between study populations could cause such a discrepancy. Our population was different from those of previous studies in being whole-population based. Also individuals with LADA were older and phenotypically closer to those with type 2 diabetes (i.e. being more overweight) than the population of the UK Prospective Diabetes Study (UKPDS) [16].

LADA patients who reverted to negative were phenotypically more similar to those with type 2 diabetes. Hence, they were more obese, had higher triacylglycerol, higher C-peptide and higher age at diabetes onset than those who stayed positive. Importantly, however, LADA patients who lost positivity still differed from those with type 2 diabetes by displaying significantly lower levels of C-peptide at baseline. This indicates that even a temporary appearance of the GADA marker of autoimmunity is of clinical importance.

We found that GADA persisters as a group displayed higher levels of GADA than those who lost positivity and were genetically more predisposed to autoimmune diabetes as indicated by HLA haplotyping. These features are known to be associated with a more type 1 diabetes-like phenotype [18]. In our view, the persistence/reversion characteristic should not be regarded as a mere reflection of baseline high and low titres of GADA but as a modality of its own. Hence, we did not find a general decrease in titre with time (which otherwise could have preferentially eliminated positivity in those with lower titres at baseline). Thus, antibody titres in the persisters did not decrease significantly with time. Furthermore, there was some overlap in baseline titres between persisters and reverters. Hence, in LADA cases GADA

Table 2 Clinical characteristics of incident LADA cases from HUNT3 who were either antibody-negative or antibody-positive in HUNT2 (i.e. several years before diagnosis)

Clinical characteristics, HUNT3	LADA		
	Antibody-negative	Antibody-positive	*p* value[a]
N	10	21	
Sex (male), % (*n*)	50% (5)	52.4% (11)	1.00
Age at onset (years)	70 (57–80)	55 (31–79)	0.001
Waist circumference (cm)	101 (93–123)	101 (76–117)	0.597
BMI (kg/m^2)	29.7 (25.7–38.9)	28.3 (23.2–37.7)	0.583
Systolic blood pressure (mmHg)	142 (102–152)	134 (106–169)	0.806
Diastolic blood pressure (mmHg)	74 (55–78)	73 (57–92)	0.888
Cholesterol (mmol/l)	4.95 (4.2–7.8)	4.5 (3.1–6.4)	0.108
HDL cholesterol (mmol/l)	1.20 (0.7–1.7)	1.2 (0.8–1.9)	0.733
Glucose, non-fasting (mmol/l)	6.8 (5.7–12.2)	11.1 (5.0–22.4)	0.033
GADA titre (ai)	0.12 (0.08–1.09)	1.17 (0.1–2.09)	0.002
IA-2A titre (ai)	0.018 (<0.01–0.06)	0.02 (<0.01 to >3.0)	0.227
ZnT8A titre (ai)	<0.01 (<0.01–0.18)	0.01 (<0.01–0.46)	0.790
HbA_{1c} (%)	6.8 (4.7–8.2)	7.4 (5.8–11.9)	0.432
HbA_{1c} (mmol/mol)	50.8 (27.9–66.1)	57.4 (39.9–106)	0.432
C-peptide (pmol/l)	986 (290–2,144)	587 (48–1,496)	0.116
Glucose, fasting (mmol/l)	5.65 (5.2–6.0)	8.0 (5.5–19.6)	0.013
Time to diabetes diagnosis (years)	7.5 (2–11)	4 (0–10)	0.199
Time to insulin treatment (years)	2.0 (1.0–4.0)	2.0 (1.0–7.0)	0.823
Insulin treatment	30% (3)	57% (12)	0.252

Data are presented as percentages and actual numbers (*n*) or median (min–max values)

Antibody-positive, antibody-positive for GADA and/or IA-2A and/or ZnT8A before clinical onset (HUNT2)

Antibody-negative, antibody-negative for all three antibodies measured before clinical onset (at HUNT2)

[a]Unadjusted *p* value calculated by Fisher's exact test for categorical data and by Mann–Whitney *U* test for continuous data

persistence or otherwise could thus be viewed as a novel aspect of pronounced vs less pronounced autoimmune activity.

Clearly, the high degree of antibody reversion that we detected could cause problems when defining the frequency of autoimmune diabetes in adults. An autoimmune component of diabetes could be missed if measurement of GADA were negative at the time of diabetes diagnosis but possibly positive at an earlier prediabetic stage. Our ongoing prospective study of GADA in non-diabetic individuals in the HUNT Study will address this issue.

Among incident cases of LADA in HUNT3 we found that a majority were already GADA-positive in HUNT2, i.e. several years before diabetes was diagnosed. A recent prospective study reported that GADA positivity in non-diabetic cases markedly increased the risk of future diabetes [19]. We found that those who were antibody-positive both in the prediabetic and diabetic state were phenotypically more similar to individuals with type 1 diabetes compared with those who were positive only after the diagnosis of diabetes. These findings, which confirm the impact of prolonged autoimmunity, seem to be in line with those obtained in the follow-up of LADA cases (preceding paragraphs). Taken together, they indicate that long-term persistence of GADA in LADA cases, whether manifested before or during the course of the disease, is associated with a high impact of autoimmunity on pancreatic beta cells.

An obvious strength in our study is its basis in a population encompassing all adults in a large and geographically defined area, a population fairly representative of Norway [8]. The self-reported diagnosis of diabetes has been shown to have good validity [20]. Participation rates in HUNT3 would ideally have been higher. However, it should be noted that participation was above the overall response rate of 54% of HUNT3 in the majority of the age groups studied (40–79 years old), the mean response rate in these age groups being 67% (data not shown).

The question has been raised whether a distinction between LADA and type 1 diabetes is artificial, since the insulin-free criterion for LADA is open to subjective judgements and different guidelines of treatment [21]. Also the length of insulin-free diabetes that qualifies for a diagnosis of LADA varies in different studies. Still, we would argue that the LADA category is bound to be clinically important. For example, it has been shown that knowledge of LADA from antibody measurements, usually GADA, leads to insulin treatment being instituted earlier in the course of the disease than if antibody positivity were not known [22]. We would also argue that choosing a long insulin-free period (12 months rather than 3 or 6 months) as a LADA criterion

has merits when trying to determine whether aetiological differences exist between LADA and type 1 diabetes. By the criteria used here we are able to detect a marked difference in age of onset of the two forms of autoimmune diabetes (Fig. 2). Such a difference suggests significant differences in aetiology, a notion that is also supported by our HUNT genotyping data [9]. Aetiological differences between LADA and adult-onset type 1 diabetes need, however, to be further tested and documented.

A possible limitation of this study is that long-term storage of serum samples could theoretically affect the results of antibody titre measurements. Few studies have explored the stability of antibodies in long-term storage of frozen serum samples. Männistö et al. reported that the levels of thyroid antibodies in serum stored at −25°C increased with extended storage time, with a marked increase after 14 years [23], and suggested that comparison of samples with different storage times should be carried out cautiously, especially after 14 years of storage. The HUNT samples have been stored at −70°C and have been exposed to limited freeze–thawing cycles, which most likely is better for the stability of antibodies and minimises evaporation compared with storage at −25°C. To our knowledge the long-term effect on levels of diabetes-related antibodies by storing samples at −70°C has not been tested.

Previous studies performed by us [9, 24] and others [5, 12, 25] have shown heterogeneity in phenotype, clinical course and genetic background in LADA cases. Our findings demonstrate that the pattern of antibodies, the postdiabetic loss or persistence as well as the prediabetic absence or presence of antibodies, are all factors that influence LADA phenotypes. The time dynamics of antibodies adds novel modalities to the heterogeneity of LADA.

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Contribution statement EPS analysed the data and wrote the manuscript. FS and KK participated in the interpretation of data and reviewed/edited the manuscript. KM collected the data, participated in the interpretation and reviewed/edited the manuscript. VG designed the study, contributed to the discussion and reviewed/edited the manuscript. All authors gave their final approval of this version to be published.

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