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An Increased Diagnostic Sensitivity of Truncated GAD65 Autoantibodies in Type 1 Diabetes May Be Related to HLA-DQ8

Diabetes 2017;66:735–740 | DOI: 10.2337/db16-0891

N-terminally truncated (96–585) GAD65 (tGAD65) autoantibodies may better delineate type 1 diabetes than full-length GAD65 (fGAD65) autoantibodies. We aimed to compare the diagnostic sensitivity and specificity between fGAD65 and tGAD65 autoantibodies for type 1 diabetes in relation to HLA-DQ. Sera from children and adolescents with newly diagnosed type 1 diabetes (*n* = 654) and healthy control subjects (*n* = 605) were analyzed in radiobinding assays for fGAD65 (fGADA), tGAD65 (tGADA), and commercial ^{125}I-GAD65 (RSRGADA) autoantibodies. The diagnostic sensitivity and specificity in the receiver operating characteristic curve did not differ between fGADA and tGADA. At the optimal cutoff, the diagnostic sensitivity for fGADA was lower than tGADA at similar diagnostic specificities. In 619 patients, 64% were positive for RSRGADA compared with 68% for fGADA and 74% for tGADA. Using non-DQ2/non-DQ8 patients as reference, the risk of being diagnosed with fGADA and tGADA was increased in patients with DQ2/2 and DQ2/8. Notably, logistic regression analysis suggested that DQ8/8 patients had an increased risk to be diagnosed with tGADA (*P* = 0.003) compared with fGADA (*P* = 0.09). tGADA had a higher diagnostic sensitivity for type 1 diabetes than both fGADA and RSRGADA. As DQ8/8 patients represent 10–11% of patients with newly diagnosed type 1 diabetes <18 years of age, tGADA analysis should prove useful for disease classification.

GAD (Mr 65 K) autoantibodies (GADA) are strongly associated with type 1 diabetes (1) and are common at clinical onset (2). GADA may be used to classify the type of diabetes, whether autoimmune or nonautoimmune (3). In our previous study of patients with newly diagnosed type 1 diabetes in 1996–2005 in the region Skåne of Sweden, we reported that 5.4% of the patients with type 1 diabetes were negative for seven different islet autoantibodies (2). It may be of value to measure GADA at a higher diagnostic sensitivity and specificity to exclude misclassification (4). Furthermore, patients with HLA-DQ8 have an increased risk of being diagnosed with insulin autoantibodies (IAA), insulinoma-associated protein 2 autoantibodies (IA-2A), or both, whereas patients with HLA-DQ2 have an increased risk of being diagnosed with GADA but a reduced risk of being diagnosed with IA-2A (5). The risk of being diagnosed with Zinc transporter 8 autoantibodies (ZnT8A) was associated with HLA-DQ6.4 (2,6). It may therefore be necessary to include HLA-DQ typing to improve the diagnostic sensitivity and specificity for islet autoantibodies.

It was recently reported that autoantibodies against N-terminally truncated GAD65 (96–585) (tGAD65) had a better chance to detect individuals who progressed to type 1 diabetes compared with full-length GAD65 (1–585) (fGAD65) in some (7,8), but not all, studies (9). The possibility that autoantibodies against tGAD65 (tGADA) may improve the diagnostic sensitivity and specificity of islet autoantibodies prompted the current study as there are

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Received 21 July 2016 and accepted 20 December 2016.

This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db16-0891/-/DC1.

still islet autoantibody-negative patients classified with type 1 diabetes on clinical grounds (2,10). The aim of our study was therefore to compare the diagnostic sensitivity and specificity between fGAD65 and tGAD65 autoantibodies for type 1 diabetes and their possible relation to the patient HLA-DQ genotype (5).

RESEARCH DESIGN AND METHODS

Serum samples at the time of clinical diagnosis were obtained from 654 children consecutively diagnosed with type 1 diabetes (median age 10 years [range 1–19]; 352 [54%] males) (2,11,12). The patients were diagnosed according to the recommendations of the American Diabetes Association (13) between September 1996 and April 2005 (Supplementary Table 1). The control subjects represented a total of 605 serum samples from healthy blood donors (median age 44 years [range 19–81]; 374 [62%] males) in Skåne obtained during 2004 and 2008 (Supplementary Table 1). Informed consent was obtained from all participants. The study was approved by the Human Research Ethics Committee of the Faculty of Medicine, Lund University.

The GAD2 full-length cDNA gene (14) was previously subcloned from plasmid pcDNAII into the pTNT vector (Promega, Southampton, U.K.) and used in the standard radiobinding assay to detect GADA as previously described in detail (6). Sample duplicates with a coefficient of variation (CV) >20% were reassayed. An in-house standard from an fGAD5 autoantibody (fGADA)–positive patient was diluted to match the World Health Organization (WHO) standard (15). The intra-assay CV for duplicates was 5% and the interassay CV for three positive control sera was 15%. In the Islet Autoantibody Standardization Program (IASP) 2015 workshop, our laboratory had an fGADA workshop sensitivity of 76% and specificity of 95.6%.

The GAD65 (96–585) cDNA corresponding to exon 4–16 or amino acids 96–585 (nucleotide 789–2261 in the mRNA sequence) was purchased in the pJ204 vector (DNA2.0, San Diego, CA) and subcloned into the pTNT vector (Promega).

Coupled in vitro transcription and translation was performed in the TNT SP6 coupled reticulocyte lysate system (Promega) (14) to label the tGAD65 with EasyTag L-(^{35}S)-methionine (PerkinElmer Life Sciences, Shelton, CT). The ^{35}S-tGAD65 was purified and used in the radiobinding assay as previously described in detail (6). All samples were analyzed as duplicates. Sample duplicates with a CV >20% were reassayed. Levels of tGADA were defined as units/mL derived from the WHO standard 97/550 (15). An in-house standard from an fGADA-positive patient was diluted to match the WHO standard (15). The intra-assay CV for duplicates was 5% and the interassay CV for three control sera was 13%. In the IASP 2015 workshop, our laboratory had a tGADA sensitivity of 76% and specificity of 98.9%.

Autoantibodies against GAD65 (GADA) were also analyzed in 619 of 654 patients using a commercially available kit with ^{125}I-GAD65 (RSR Limited, Cardiff, U.K.). The RSR kit was validated in the Diabetes Autoantibody Standardization Program (DASP) with 74% workshop sensitivity and 96% workshop specificity. The intra-assay CV was 8.9% at level 2.0 units/mL and 14.2% at level 44.6 units/mL (2).

IAA, IA-2A, arginine 325 ZnT8A (ZnT8RA), tryptophan 325 ZnT8A (ZnT8WA), glutamine 325 ZnT8A (ZnT8QA), islet cell cytoplasm autoantibodies (ICA), and neuropeptide Y autoantibodies (NPYA) were previously analyzed in the Skåne study as previously described (2,11).

HLA-DQB1 and HLA-DQA1 genotypes were determined using a DELFIA hybridization assay (PerkinElmer). The HLA-DQB1* probes defined the presence of HLA-DQB1*02, 03:02, 03:01, 06:02, 06:03, and 06:04 alleles, and the HLA-DQA1 probes defined the presence of the DQA1*02:01, 03, and 05 alleles (16–18).

Analysis of the data included D'Agostino and Pearson omnibus normality test, nonparametric Spearman rank correlation test, receiver operating characteristic (ROC) curve analysis, Fisher exact test, and McNemar test. All statistical analyses were performed with a two-tailed P value, accepting $P < 0.05$ as significant using either IBM SPSS (version 22) or GraphPad Prism (version 6).

RESULTS

The levels of fGADA and tGADA in the control subjects and patients with newly diagnosed type 1 diabetes (Supplementary Table 1) were not normally distributed ($P < 0.001$) (Fig. 1*A* and *B*). A ROC analysis revealed that the area under the curve for fGADA was 0.971 (95% CI 0.962–0.979) compared with 0.965 (95% CI 0.955–0.975) for tGADA (Fig. 1*C* and *D*). Using 50 units/mL as cutoff, more patients were positive for tGADA (73%; 475 of 654) than for fGADA (68%; 442 of 654; $P < 0.001$), whereas the frequency in control subjects was low and did not differ between fGADA (1.3%; 8 of 605) and tGADA (1.7%; 10 of 605) ($P = 0.727$). The levels of fGADA correlated with the levels of tGADA ($r^2 = 0.984$; $P < 0.001$) (Supplementary Fig. 1*A*). The correlation analysis revealed that among the patients with type 1 diabetes, 0.3% (2 of 654) had fGADA only, 5.4% (35 of 654) had tGADA only ($P < 0.001$), whereas 67.3% (440 of 654) had both fGADA and tGADA and 27% (177 of 654) were double negative (Supplementary Fig. 1*A*).

Of 619 available samples from patients with type 1 diabetes, 64% (393 of 619) were positive for autoantibodies against ^{125}I-GAD65 (RSRGADA) compared with 68% (423 of 619) in the fGADA ($P < 0.001$) and 74% (455 of 619) in the tGADA analysis ($P < 0.001$). The levels of RSRGADA correlated with the levels of both fGADA ($r^2 = 0.938$; $P < 0.001$) and tGADA ($r^2 = 0.939$; $P < 0.001$) (Supplementary Fig. 1*B* and *C*).

Among the 619 patients with type 1 diabetes, 0.3% (2 of 619) had fGADA only, 4% (25 of 619) had tGADA only, and 1% (6 of 619) had RSRGADA only (Supplementary Table 2).

A total of 463 patients were positive for fGADA, tGADA, or RSRGADA, demonstrating in a Venn diagram that 82% (378 of 463) were positive for all three, 43 had both fGADA and tGADA, and 9 had tGADA and RSRGADA, but no patient had both fGADA and RSRGADA (Fig. 2*A*).

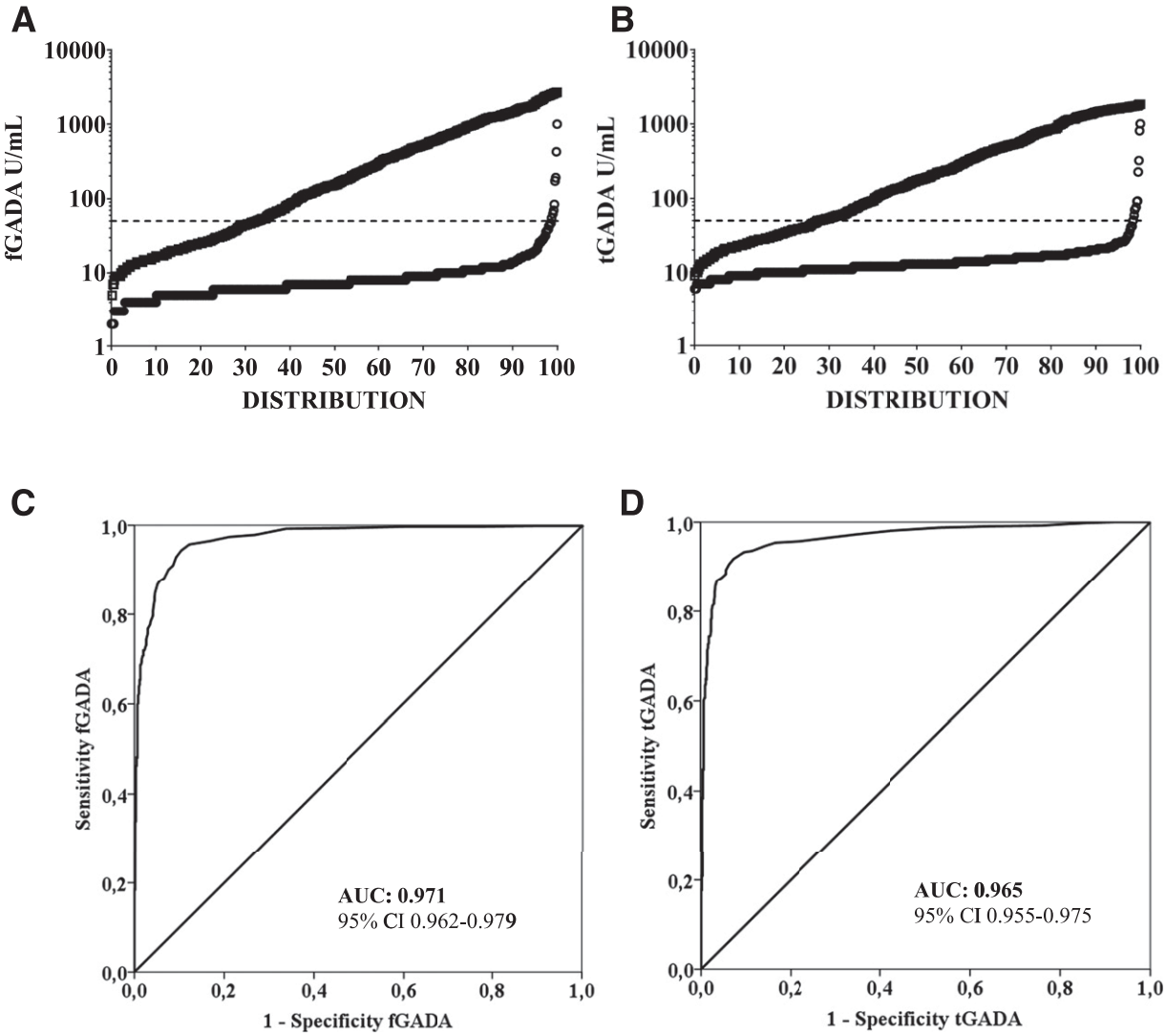

Figure 1—Quantile-quantile plots of patients with newly diagnosed type 1 diabetes (n = 654) and healthy control subjects (n = 605) for fGADA (*A*) and tGADA (*B*), respectively. The ROC curve analyses, including the area under the curve (AUC), are shown for fGADA (*C*) and tGADA (*D*), respectively. The dotted lines in *A* and *B* at 50 units/mL represent the cutoff values determined based on the specificity and sensitivity in ROC analysis.

A total of 61% (378 of 619) were triple positive for fGADA, tGADA, and RSRGADA. Of the patients with type 1 diabetes, 17% (102 of 619) were positive for 5 autoantibody assays and 1% (6 of 619) were positive for all 10 autoantibody assays used (fGADA, tGADA, RSRGADA, IAA, IA-2A, ZnT8RA, ZnT8WA, ZnT8QA, NPYA, and ICA).

The odds of identifying fGADA, tGADA, and RSRGADA, respectively, were tested in a regression analysis adjusted for sex, age at diagnosis, positivity for multiple autoantibodies, and six HLA-DQ genotypes (Table 1). The female patients were at an increased risk for fGADA (P = 0.005), tGADA (P = 0.003), and RSRGADA (P = 0.002) (Table 1). In addition, patients diagnosed at 10 years of age or above had an increased risk for fGADA ($P < 0.001$), tGADA (P = 0.002), and RSRGADA ($P < 0.001$). Patients with HLA-DQ2/2 genotype showed the highest risk of having fGADA (P = 0.002), tGADA ($P < 0.001$), or RSRGADA (P = 0.01) (Table 1). Patients with HLA-DQ2/8 had increased risk for both fGADA (P = 0.03) and tGADA (P = 0.01) but not for RSRGADA (P = 0.6). Notably, patients with HLA-DQ8/8 (n = 74) had a significantly increased risk for tGADA (P = 0.003) but not for fGADA (P = 0.09) or for RSRGADA (P = 0.2). tGADA-only was found in 2 of 80 patients with DQX/X and in 6 of 74 patients with DQ8/8 compared with 1 of 80 with DQX/X and 0 of 74 with DQ8/8 for fGADA-only. Of the 4% (25 of 619) who had tGADA alone, HLA-DQ8/8 was present in 24% (6 of 25).

DISCUSSION

The major findings in this study to define the diagnostic sensitivity and specificity of tGADA in comparison with fGADA and RSRGADA were as follows.

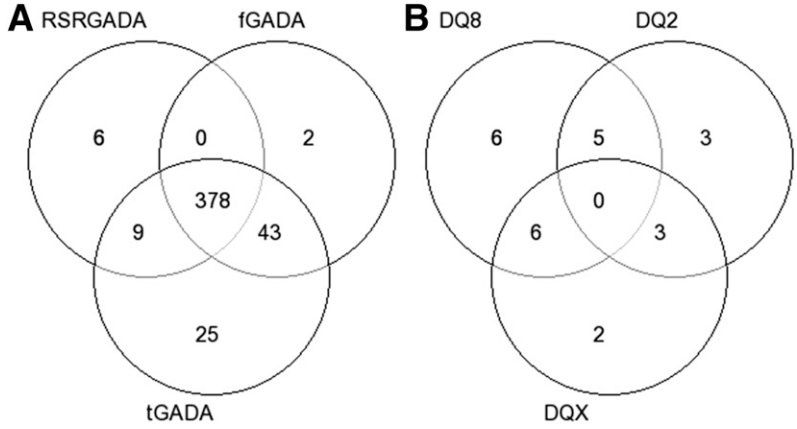

Figure 2—Venn diagram of patients with type 1 diabetes (n = 463) positive for any of the fGADA, tGADA, or RSRGADA variants or in combination (*A*) and of the distribution of the HLA-DQ haplotypes DQ2, DQ8, and DQX (X is any haplotype except for DQ2 or DQ8) in patients with type 1 diabetes positive for tGADA but negative for fGADA and RSRGADA (n = 25) (*B*).

1. Significantly more patients with type 1 diabetes were positive for tGADA than either fGADA or RSRGADA.
2. The optimal cutoff in the ROC curve analysis showed a similar diagnostic specificity for fGADA and tGADA. The optimal cutoff was defined as the point for which (sensitivity + specificity) is maximal. The observation that the diagnostic specificity of tGADA was comparable to fGADA suggests that the difference in diagnostic sensitivity between the two GAD65 variants would not be due to borderline reactive control sera.
3. tGADA and RSRGADA detected autoantibodies with apparent unique epitopes.
4. The increased diagnostic sensitivity seemed to be explained by an unexpected association between tGADA and HLA-DQ8/8. It is noted that the levels of tGADA-only samples were relatively low. However, the tGADA measurements would appear valid as the intra-assay CV for a duplicate determination was only 5%. It is also noted that only two serum samples were fGADA-only reactive (Supplementary Fig. 1*A*).

The present Skåne study precludes any speculation as to the initial autoreactivity against GAD65, i.e., at the time of seroconversion. It is not possible from our data to predict whether some children may have been triggered against tGAD65 only. In order to understand the initial autoantibody reactivity to GAD65, serum or plasma samples from prospective studies carried out from birth in children with increased genetic risk for type 1 diabetes will be needed. Prior epitope mapping of GADA with rFab of GAD65 monoclonal antibodies is consistent with the present data as GAD65 autoantibodies were directed against the middle and COOH-terminal regions of GAD65 (19,20). In genetically predisposed subjects, the autoimmune response may then undergo intramolecular epitope spreading toward epitopes on the N terminus and further epitopes located in the middle (20).

Table 1—The risk for having fGADA, tGADA, and RSRGADA in patients with newly diagnosed type 1 diabetes analyzed with logistic regression adjusted for sex, age at diagnosis, multiple autoantibodies, and the HLA-DQ genotypes

	fGADA (n = 654)			tGADA (n = 654)			RSRGADA (n = 619)		
Patients with type 1 diabetes	OR	95% CI	*P*	OR	95% CI	*P*	OR	95% CI	*P*
Females	1.7	1.2–2.4	0.005	1.8	1.2–2.6	0.003	1.8	1.2–2.5	0.002
Age at diagnosis ≥10 years	2.0	1.4–2.9	<0.001	1.8	1.3–2.7	0.002	2.0	1.4–2.8	<0.001
Positivity for ≥2 autoantibodies*	4.7	2.9–7.4	<0.001	4.5	2.8–7.2	<0.001	5.3	3.2–8.6	<0.001
HLA-DQ genotypes†									
DQX/X (reference)	1.0	—	—	1.0	—	—	1.0	—	—
DQ2/2	4.5	1.7–11.9	0.002	7.5	2.4–22.8	<0.001	3.4	1.3–8.9	0.01
DQ2/8	1.9	1.1–3.3	0.03	2.1	1.2–3.7	0.01	1.7	1.0–3.1	0.6
DQ8/8	1.9	0.9–3.8	0.09	3.3	1.5–7.4	0.003	1.7	0.8–3.4	0.2
DQ2/X	1.4	0.7–2.8	0.3	1.4	0.7–2.9	0.3	1.4	0.7–2.8	0.4
DQ8/X	1.3	0.7–2.4	0.3	1.5	0.8–2.7	0.2	1.1	0.6–2.0	0.7

*Autoantibodies included GADA, IA-2A, IAA, ZnT8RA, ZnT8WA, ZnT8QA, NPYA, and ICA; †DQ2/8, DQ A1*05:01-B1*02:01/A1*03:01-B1*03:02; DQ8/X, DQ A1*03:01-B1*03:02/X (X is any haplotype except for DQ2 or DQ8); DQ8/8, DQ A1*03:01-B1*03:02/ A1*03:01-B1*03:02; DQ2/X, DQ A1*05:01-B1*02:01/X (X is any haplotype except for DQ2 or DQ8); DQ2/2, DQ A1*05:01-B1*02:01/A1*05:01-B1*02:01.

The strength of the current study is the large number of patients consecutively diagnosed with type 1 diabetes from a defined geographical region in Sweden. The study may be viewed as population based over 10 years and has made it possible to ask questions about temporal trends (12) in addition to dissecting autoantibody markers of type 1 diabetes at the time of clinical diagnosis to improve classification (2). Also, ROC curve analyses were used to set cutoff values for fGADA and tGADA as the number of patients and control subjects was sufficient.

A limitation of the current study was that the control subjects were not matched to the patients with respect to age. Analysis of age-matched control subjects might generate a different diagnostic specificity as the patients were all <19 years of age. It will therefore be important to solicit serum samples from children and young adults to better define the diagnostic specificity among the young. However, it is unlikely that a possible decrease in diagnostic specificity would change the conclusions of our study as the difference between fGADA and tGADA in diagnostic sensitivity will not change. Another limitation was the use of Protein A Sepharose that does not bind IgG3 (21). The majority of GADA belongs to the lgG1 subclass, but there is a small portion of GADA that belongs to the lgG3 subclass (22). Protein A Sepharose was used in a previous study that measured fGADA and tGADA in parallel (8). Similar to this study, our measurements of fGADA and tGADA were carried out only months apart. Another limitation was the insertion of (^{35}S)-methionine to the N terminus of tGAD65. However, the N-terminal methionine is unlikely to have affected autoantibody binding as it is neither highly charged nor bulky (8).

In conclusion, we have found that removing the N-terminal end of GAD65 reveals an epitope that perhaps is more reactive in HLA-DQ8/8 patients. Further studies of prospective cohorts of children followed from birth, such as the Environmental Determinants of Diabetes in the Young (TEDDY) (23), will be needed to test if DQ8/8 children are able to develop tGADA-only as their first β-cell autoantibody. The increased risk of being positive, albeit at a low titer, for tGADA in DQ8/8 patients suggests that removing the first 95 amino acids in GAD65 may have uncovered a conformation-dependent epitope related to the GAD65 autoimmune response in HLA-DQ8/8 subjects. As DQ8/8 patients represent 10–11% of patients with newly diagnosed type 1 diabetes <18 years of age, tGADA analysis should prove useful to improve not only the diagnostic sensitivity for type 1 diabetes but also the positive predictive values for type 1 diabetes when screening schoolchildren for the risk of type 1 diabetes.

Acknowledgments. The authors thank Ida Jönsson (Lund University Clinical Research Centre, Skåne University Hospital, Malmö, Sweden) for technical assistance.

Funding. A.W. was supported by a summer student stipend from the Lund University Medical Faculty Foundation. This work was also supported by the Swedish Research Council, the Swedish Diabetes Foundation, the Skåne University Hospital Fund, the Skåne County Council for Research and Development, and the Swedish Child Diabetes Foundation.

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. A.W. and H.S. planned and designed the study, executed the analyses, analyzed the data, and drafted the manuscript. A.L. and A.R. executed the analyses. A.C., E.C., B.J., S.A.I., H.E.L., K.L., B.L., and J.N. diagnosed and classified patients and control subjects. M.F. and C.T. recruited the control subjects. Å.L. planned and designed the study, analyzed the data, and drafted the manuscript. All authors revised and approved the final version of the manuscript. Å.L. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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