High GADA titer increases the risk of insulin requirement in LADA patients: a 7-year follow-up (NIRAD study 7)

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Abstract

Objective: The aim of this study was to determine whether glutamic acid decarboxylase antibody (GADA) titer and other clinical parameters could define the risk of progression to insulin therapy in latent autoimmune diabetes in adults (LADA) patients during a 7-year follow-up.

Methods: This study involved 220 LADA and 430 type 2 diabetes subjects followed up for 7 years from the time of GADA screening to evaluate their progression toward insulin therapy. Kaplan–Meier curves and multivariate logistic regression analysis were performed to identify the markers capable of influencing this progression.

Results: During the follow-up, the drop out was 4% in both groups. A total of 119 (56.1%) out of 212 LADA patients required insulin during the 7 years of follow-up. The Kaplan–Meier plots showed that 74/104 (71.1%) of high GADA titer required insulin compared with 45/108 (41.6%) of low GADA titer and with 86/412 (20.9%) of type 2 diabetes ($P<0.0001$ for both). A BMI of ≤ 25 kg/m^2 and IA-2$_{IC}$ and zinc transporter 8 (ZnT8) positivity were also shown as the markers of faster progression ($P<0.0001$ for both). The proportion of LADA patients requiring insulin was significantly higher in the group of subjects treated also with sulfonylurea in the first year from diagnosis compared with those treated with diet and/or insulin sensitizers ($P<0.001$). The multivariate analysis confirmed that the presence of high GADA titer was a significant predictor of insulin requirement ($P<0.0001$, OR$=6.95$).

Conclusions: High GADA titer, BMI $\leq$ 25, ZnT8 and IA-2$_{IC}$ positivity and sulfonylurea treatment, in the first year from diagnosis, significantly increase the progression toward insulin requirement in LADA patients.

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Introduction

Latent autoimmune diabetes in adults (LADA) is a form of autoimmune-mediated diabetes, usually diagnosed by the presence of islet autoantibodies, namely glutamic acid decarboxylase antibodies (GADAs), with or without protein tyrosine phosphatase IA-2 antibodies (IA-2As) (1, 2) and/or zinc transporter 8 (ZnT8) (3).

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Although LADA subjects are characterized by the presence of specific antibodies for autoimmune diabetes, they do not require insulin therapy initially (4). During the early phase of disease, individuals with LADA can retain good metabolic control through diet alone or by taking oral hypoglycemic drugs, and this state can last from months to years. However, a percentage of LADA patients progress toward insulin requirement, indicating that LADA involves a slow and progressive loss of β-cells (5), although the factors influencing this progression are not completely known (6). The first evidence, demonstrating that a high GADA titer is a stronger predictor of insulin requirement than a general GADA positivity, was obtained from two studies with a limited number of LADA patients (7, 8). UKPDS 25 reported, in a larger population of LADA patients, that high GADA titer was associated with a significant higher risk of insulin requirement only among subjects older than 55 years at diagnosis (1). Afterwards, conflicting results were reported in the literature; some studies demonstrated that a high titer of GADA was associated with a shorter insulin-free period (9, 10, 11), whereas others did not support this hypothesis (6, 12).

In a previous study, we demonstrated the presence of two different populations among individuals with LADA; analysis of GADA titer showed a bimodal distribution that identified two subgroups of subjects with either a low or a high GADA titer (13).

Assuming that the more prominent features of insulin deficiency, that characterize high GADA titer compared with low GADA titer (13), define a more reduced β-cell function, it may follow that patients with high GADA titer would require insulin therapy earlier, compared with those with low GADA titer.

The aim of the present prospective study was to determine whether GADA titer would affect progression to insulin therapy in LADA patients during a 7-years of follow-up. We also looked for other biochemical and clinical parameters associated with early development of insulin requirement.

Subjects and methods

Study design and populations

In the non-insulin requiring autoimmune diabetes (NIRAD) study, we screened 5330 type 2 diabetes patients from 83 diabetes centers equally distributed in the entire mainland and island of Italian territory. A total of 250 subjects of 5330 type 2 diabetes patients were positive for GADA (4.5%) and thus we defined these patients as having LADA. Out of 250 LADA patients, 30 patients did not give their consent to be followed-up in the NIRAD study but only to participate in the initial screening. A total of 220 LADA patients were followed up for 7 years in this study: 430 out of 5080 type 2 diabetes patients, matched for gender and duration of disease with LADA patients, were selected as control group. A total of 220 LADA patients, mean age at onset 50.6±13.1 years, and 430 GADA negative type 2 diabetes subjects, matched for age and gender, mean age at onset 51.2±10.5 years, from the NIRAD study cohort of 5330 consecutive cases of type 2 diabetes subjects, recruited between February 2001 and January 2006 (13, 14), were evaluated in this study.

Inclusion criteria for the NIRAD study were as follows: i) diagnosis of diabetes according to the American Diabetes Association (15); ii) no insulin requirement and no evidence of ketosis from diagnosis to screening time; and iii) disease duration between 6 months and 5 years. The exclusion criteria included prior insulin therapy, pregnancy, and the presence of any other severe disease.

Sixty per cent of LADA subjects identified in the NIRAD study had a disease duration <2 years at the time of antibody screening for GAD.

Antibodies measurement

At the time of GADA screening, the clinical and biochemical characteristics and antibodies (IA-2_{IC}) were evaluated (13, 14).

GADAs and IA-2As were measured centrally at San Raffaele Hospital in Milan and at 'Sapienza' University in Rome, respectively, using a radiobinding assay with *in vitro*-translated [35S] methionine-labeled GAD65 and IA-2_{IC} (amino acids 605–979) (13, 16). All samples were tested in duplicate.

The following results were obtained for these GADA and IA-2A assays at the Diabetes Antibody Standardization Program (DASP) between 2002 and 2005: GADA sensitivity 84, 86, and 88%; GADA specificity 97, 97, and 92% and IA-2A sensitivity 60, 62, and 70% and IA-2A specificity 100, 99, and 99% (13).

The results for GADAs were converted into arbitrary units by extrapolation from a standard curve with a local standard designated 100 arbitrary units. The thresholds for positivity were determined from the 99th centile of control subjects and corresponded to three arbitrary units for GADAs.

The distribution of GADA titer in patients with LADA was independent of diabetes duration and showed a bimodal distribution. Consistently with this

observation, patients with LADA (GADA titer >3 arbitrary units) were divided into subgroups representing the two distributions, namely low (being ≤32 units) and high (being >32 units) GADA titers (13). Based on DASP as a reference (17) the threshold of 32 units was equivalent to 300 WHO units (13).

The samples with low GADA titer were validated for glutamic acid decarboxylase (GAD)-specific binding by competition assay with excess of cold GAD (13).

The sera of LADA patients were re-analyzed and confirmed as GADA positive in a second Italian reference laboratory (at 'Sapienza' University in Rome) also for those participating in the DASP program.

In all patients, ZnT8 antibodies were measured as previously described using RIA (Medipan, Berlin, Germany) at San Raffaele Hospital in Milan (18).

Genotyping

The HLA-DRB1* and DQB1* loci typing was carried out by PCR followed by a reverse line blotting assay using an array of immobilized sequence-specific oligonucleotide probes (19). HLA genotypes were classified in three HLA risk categories based on the absolute risk values obtained in Italian population (20): i) high-risk genotypes: DRB1*03-DQB1*0201/DRB1*04-DQB1*0302 (DRB1*04 different from 0403); ii) moderate-risk genotypes: DRB1*04-DQB1*0302/DRB1*04-DQB1*0302, DRB1*03-DQB1*0201/DRB1*03-DQB1*0201, DRB1*04-DQB1*0302/X, and DRB1*03/X (X different from DRB1*03, DRB1*04-DQB1*0302 or DQB1*0602/03); and iii) low-risk genotypes: all other genotypes.

Follow-up study

The drop out was 4% both in LADA and T2DM patients ($n=8$ GADA positive and $n=18$ type 2 diabetes patients). The reasons of drop out were death, moved out of city, or adverse event (cancer). Therefore, 212 LADA patients and 412 type 2 diabetes were followed up for 7 years from the time of GADA screening to evaluate their progression toward insulin therapy.

In a subgroup of patients ($n=16$ LADA and $n=20$ type 2 diabetes), GADA was re-tested at the time of starting insulin in order to evaluate the stability of positivity and titer.

Initiation of insulin therapy was part of a protocol treatment and the participating centers were blinded to the GADA results. Insulin requirement was defined as the clinical need to start insulin therapy in patients whose glycemic control, at each scheduled visit, became unacceptable (HbA1c ≥58 mmol/mol or ≥7.5%) despite maximally tolerated combination of insulin-sensitizers (metformin and/or glitazones) and sulfonylurea therapy.

Statistical analyses

All analyses were performed using SAS, version 9.3. The frequencies were compared using the χ^2 test, with Yates' continuity correction or Fisher's exact test. The Shapiro–Wilk test was used to test the normality of the distributions of continuous variables. For continuous variables, the difference between the mean value of the two groups was tested by the unpaired Student' *t*-test for normally distributed variables and the Mann–Whitney *U* test for those which were not normally distributed. Kaplan–Meier curves were plotted and the log-rank test was performed to identify possible markers able to influence the progression to insulin requirement. The event time (insulin requirement) in the Kaplan–Meier curve was rounded to the nearest year interval. Cox regression analysis was performed to analyze the most important factors for future insulin requirement.

Multivariate logistic regression was performed to identify the possible predictors of the requirement of insulin. The investigated variables for the two models were as follows: GADA titer, number of antibodies, age at diagnosis, BMI, waist circumference, total cholesterol, HDL cholesterol, HbA1c, fasting glucose, and HLA DRB1*0301-DQB1*0201 haplotype positivity and diabetes treatment. A P value <0.05 was considered statistically significant.

The study was approved by all local ethics committees (rif.491), and written informed consent was obtained from all patients.

Results

During the 7 years of follow-up, 119 (56.1%) of 212 LADA patients and 86 (20.9%) of 412 type 2 diabetes subjects required insulin therapy respectively.

In Table 1, we have given the clinical and biochemical characteristics of LADA patients subdivided according to their progression toward insulin requirement. LADA patients who progressed to insulin requirement were significantly younger, had a lower BMI and waist circumference and a higher HbA1c compared with those who did not progress; the median GADA titer was significantly higher in the group of subjects who progressed to insulin requirement. There was no significant

Table 1 Clinical and biochemical characteristics of LADA patients at the time of GADA screening, subdivided according to insulin requirement during the 7 years of follow-up.

	Non-insulin requirement	Insulin requirement	
	Mean ± S.D.	Mean ± S.D.	*P*
n (male/female)	48/45	65/54	
Age at diagnosis (years)	54.5 ± 10.8	44.5 ± 12.3	<0.0001
Duration of disease (months)	30.7 ± 20.2	27.2 ± 22.6	
BMI (kg/m^2)	27.9 ± 5.1	25.7 ± 4.2	0.02
Waist circumference (cm)	96.9 ± 11.3	90.6 ± 10.8	0.02
Fasting glucose (mg/dl)	159.7 ± 58.0	170.0 ± 53.9	
HbA1c (mmol/mol)	55.2 ± 21.4	62.8 ± 23.5	0.03
HbA1c (%)	7.2 ± 1.9	7.9 ± 2.14	0.03
Total cholesterol (mg/dl)	215 ± 49.2	196.0 ± 46.0	
HDL cholesterol (mg/dl)	48.5 ± 13.6	50.3 ± 14.3	
Triglycerides (mg/dl)	170.2 ± 152.1	135.2 ± 77.6	
Uric acid (mg/dl)	5.2 ± 1.4	4.1 ± 1.3	
GADA titer[a]	11 (7–72.4)	88.9 (7.3–100)	0.01
DRB1*0301-DQB1*0201[b]	27 (29)	57 (47.9)	0.008

[a]Data are expressed as median (interquartile range).
[b]Data are expressed as number and (%).

difference in HLA-DRB1 and HLA-DQB1 genotype distribution (high, moderate, or low) in the two groups of subjects; only the HLA DRB1*0301-DQB1*0201 haplotype frequency was significantly higher in the group of subjects who required insulin therapy earlier compared to the other group (47.9 vs 29% respectively, $P=0.008$).

Considering all subjects who required insulin (both LADA and type 2 diabetes patients), LADA subjects showed a significantly lower age at diagnosis, BMI, and waist circumference ($P\leq0.02$ for all comparisons), higher levels of fasting glucose, although not statistically significant, and higher levels of HbA1c and HDL cholesterol ($P=0.04$ and $P=0.03$ respectively) (data not shown) compared with type 2 diabetes patients.

Considering all subjects who did not require insulin, LADA patients showed a significantly higher levels of fasting glucose, HbA1c, and HDL cholesterol compared with type 2 diabetes subjects ($P\leq0.01$ for all comparisons); for the other parameters, there were no differences between the two groups of subjects (data not shown).

As illustrated in the Kaplan–Meier plots (Fig. 1A), patients with a significantly higher number of high GADA titer, 74/104 (71.1%), required insulin during the 7 years of follow-up compared with those with low GADA titer 45/108 (41.6%) and to those with type 2 diabetes 86/412 (20.9%) ($P<0.0001$, for both comparisons). Low GADA titer subjects showed, indeed, a faster progression to insulin requirement than type 2 diabetes subjects ($P<0.01$; Fig. 1A). LADA patients with a BMI ≤25 kg/m^2 had a faster progression to insulin requirement than those with BMI >25 kg/m^2 (62 vs 48% respectively) ($P<0.0001$; Fig. 1B).

We also observed that positivity to both IA-2_{IC} and ZnT8 ($n=26$ subjects) was associated with a faster disease progression than that observed in subjects with both IA-2_{IC} and ZnT8 negativity (66 vs 48% respectively) ($P<0.001$; Fig. 1C). The presence of ZnT8 antibodies showed a slight, but not significant, earlier requirement of insulin therapy ($P=0.06$).

Figure 1D shows the Kaplan–Meier plots for LADA patients subdivided according to the treatment followed in the first year after diagnosis. The proportion of LADA patients who required insulin therapy was significantly higher in the group treated also with sulfonylurea in the first year after diagnosis than in those treated with diet and/or insulin sensitizers alone (either metformin and/or glitazones; $P<0.001$). Approximately 12% of LADA patients treated with diet and/or insulin sensitizers alone progressed to insulin requirement in the first 3 years of follow-up compared with 55% of LADA patients treated also with sulfonylurea during the first year after diagnosis ($P<0.01$; Fig. 1D).

At the time of starting insulin therapy, 13 out of 16 LADA patients maintained the same titer of GADA, whereas in the other three, all with low GADA titer at the first screening, the titer further decreased, although remaining above the limit of positivity; none of the 20 type 2 diabetes patients converted to GADA positivity.

Cox regression analysis showed that the most important factors associated with disease progression were: BMI ≤25 kg/m^2 (HR=2.47; $P<0.006$) and high GADA titer (HR=3.42; $P<0.0003$).

Multivariate logistic regression analysis of insulin requirement confirmed that only the presence of high GADA titer was a significant predictor of insulin requirement ($P<0.0001$, OR=6.95; CI: 2.5–17.7). The presence of multiple antibodies was not a significant predictor ($P=0.12$).

Discussion

This prospective study shows that 56.1% of LADA patients required insulin therapy during 7 years of follow-up compared with 20.9% of type 2 diabetes

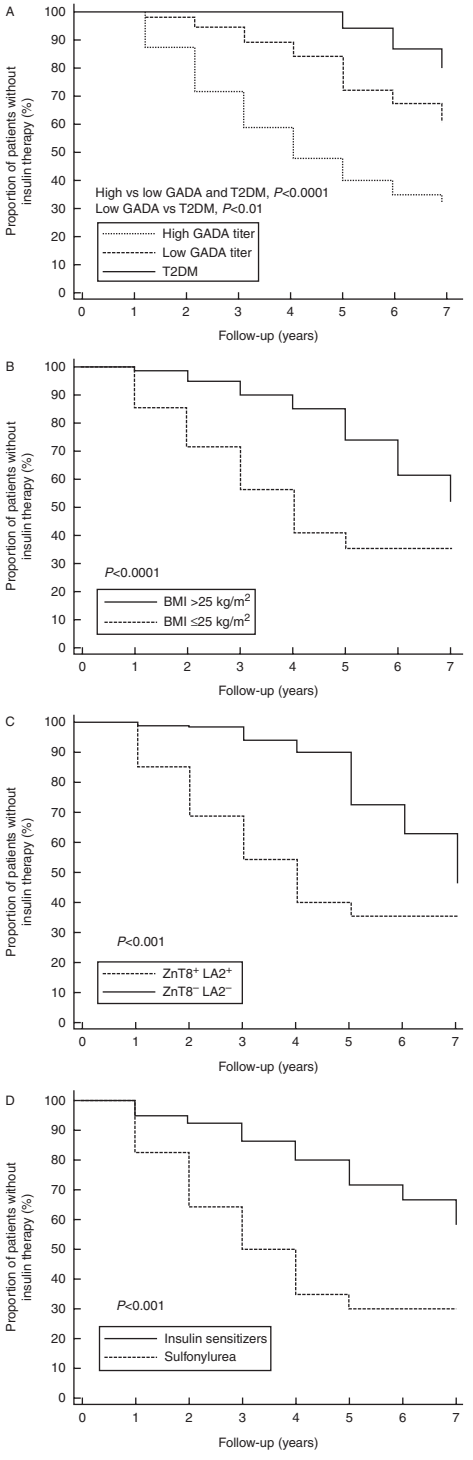


Figure 1

Kaplan–Meier plots of proportion of subjects, requiring insulin during 7-year follow-up, LADA with high or low GADA titer, and type 2 diabetes (A), Kaplan–Meier plots of proportion of LADA subjects subdivided according to BMI (B), according to IA-2IC and ZnT8 positivity (C), according to type of treatment (D).

patients. This finding is in line with previous studies which report that a higher proportion of LADA subjects require insulin within a few years from diagnosis, compared with type 2 diabetes subjects (21, 22).

Brophy *et al*. (23) showed that in countries where the antibodies are assessed at the time of diagnosis, insulin therapy is initiated much earlier in LADA patients and this could be a bias in the comparison of different studies. In our study, the participating centers were not aware of antibodies positivity, and thus their decision to initiate insulin therapy was not influenced by this.

We have observed that the progression to insulin requirement is significantly higher and, above all, occur sooner in high GADA titer subjects compared with those with low GADA titer. These results underlined and reinforced our previous observations, showing that the presence of high GADA titer in LADA patients is associated with a more prominent trait indicative of insulin deficiency and consistent with a more severe profile of autoimmunity.

Following the first reports (1, 7, 8), other studies have investigated whether GADA titer could influence the risk of progression to insulin therapy in LADA patients (6, 11, 12, 21, 22, 24, 25, 26). Some of these studies did not find a positive association between high GADA titer and risk of progression to insulin therapy; for example, differently from the UKPDS 25, the UKPDS 77 (12) found no association between GADA levels (classified according to thirds of the GADA level at 0.5 years) and insulin requirement, implying that GADA level was not associated with aggressiveness. In a recent cross-sectional study, the Action LADA 7 (27) high GADA titer patients were compared with low GADA titer patients in terms of time to insulin requirement, but no statistical significant difference was found. However, in this case, type 1 diabetes and LADA patients were considered as a single group and the LADA group was not evaluated separately. Another consistent group of studies found that high titer of GADA was associated with a shorter insulin-free period (9, 21, 25).

Our data, evaluated by Kaplan–Meier plots, suggest that GADA titer, rather than its presence, could play an important role in determining the risk of progression to insulin requirement. Multiple regression analysis further extends these findings showing that among the factors we investigated, high GADA titer was found to be the best predictor of progression to insulin requirement.

In the light of the previous considerations, our study definitely adds some arguments in favor of the role played by the high GADA titer in increasing the risk of insulin requirement in LADA: different from most of the other studies, which were cross-sectional, this study is a

prospective one and is based on a high sensitivity and specificity assay to detect GADA.

Furthermore, we compared the insulin requirement between the two distinct populations we obtained from a previous evidence of a bimodal distribution of GADA titers (low and high GADA) in LADA patients.

When we retrospectively evaluated the HbA1c values of patients at baseline, we noted a significant difference between patients who progressed and did not progress to insulin requirement (Table 1). However, in the Kaplan–Meyer analysis, when we subdivided patients according to different variables (GADA titer, BMI, ZnT8/IA-2 positivity, and type of treatment) we did not observe any significant difference at baseline between the groups as far as HbA1c is concern.

β-cell secretion could have been impaired in the early phase of the disease in patients with higher HbA1c at baseline (Table 1). This could have influenced the frequency of insulin treatment during the course of the study. The influence of prior β-cell damage vis-à-vis continued autoimmune destruction of β cells cannot be conclusively determined from our data.

The stability of GADA levels and titers have been investigated in LADA patients (2, 12). Desai *et al.* (12) found that GADA levels varied between 0.5 and 6 years after diagnosis in some patients, but those with higher values at 0.5 years, tended to remain with a high titer at 3 and 6 years, whereas in those with low values at 0.5 years remained low thereafter. Borg *et al.* (28) also found that in LADA patients, GADA titer persisted for up to 12 years. In this study, some samples of LADA patients were retested for GADA at the time of starting insulin therapy: the majority maintained the same titer of GADA. We also observed that none of the type 2 diabetes patients converted to GADA positivity. In view of these results we could hypothesize that GADA is persistent over time.

Some evidence has previously demonstrated that the presence of multiple autoantibodies rather than high GADA titer could predict insulin requirement in LADA subjects: Bottazzo *et al.* (2) found that in type 2 diabetes patients, the presence of IA-2A in addition to GADA increased the likelihood of insulin therapy within 6 years from diagnosis; more recently Maioli *et al.* (6) found that the number of antibodies was more important than high titers of GADA for predicting insulin dependence.

However, we observed that high GADA titer is associated with faster insulin progression during a 7 years of follow-up regardless of the number of antibodies and that the presence of multiple antibodies is not a significant predictor of insulin requirement.

Although according to our multivariate analysis, high GADA titer seems to be the best predictor of progression to insulin dependence, Kaplan–Meier plots demonstrate that positivity for both IA-2_{IC} and ZnT8 increased the risk of progression to insulin therapy. Kawasaki *et al.* (29) found that the presence of ZnT8 and/or IA-2_{IC} increases the progression to insulin requirement in LADA patients. We showed that HLA DRB1*0301-DQB1*0201 haplotype frequency was significantly higher in subjects requiring insulin, which is consistent with the observation that patients with high GADA titer have the highest frequency of DRB1*03-DQB1*0201 (50%) (13). LADA patients with a BMI $\leq 25\ kg/m^2$ have a faster progression to insulin requirement than those with a BMI $>25\ kg/m^2$; this is in line with previous studies (6, 24, 30, 31).

In this study, we show that insulin sensitizers maintained the insulin-free period longer than sulfonylurea. This could be either due to the increased β-cell apoptosis from use of sulfonylurea (32, 33) or due to the protective effect of insulin sensitizers (34).

When we analyzed BMI and HbA1c levels in patients subdivided into two groups depending on prescribed sulfonylureaor insulin sensitizer, we observed that there were no statistical significant differences for these parameters among the two groups ($P<0.05$ for all), thus we would hypothesize that the type of treatment may influence the progression toward insulin requirement.

There are no current guidelines for LADA treatment at present; however some beneficial effects of insulin have been reported. The Tokyo study has showed improved maintenance of the serum Σ-C-peptide over 5 years in the group receiving insulin compared with sulfonylurea (35). The subgroup analysis suggested that patients with high GADA titers and preserved C-peptide at baseline were less likely to progress to the insulin-dependent stage with early administration of small doses of insulin. These observations reinforce the importance of the identification of LADA patients with high GADA titer and the administration of early insulin therapy in order to preserve β-cell function for a longer time (Supplementary Tables 1 and 2, see section on supplementary data given at the end of this article).

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/EJE-14-0342.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

S Zampetti wrote the manuscript; G Campagna and J Osborn performed data analysis; C Tiberti and E Bosi responsible for antibody testing, M Spoletini, M L Arpi, G De Simone, E Cossu, L Cocco, and F Giorgino researched data and reviewed the manuscript; M Spoletini performed the genetic typing and reviewed the manuscript; and R Buzzetti implemented the study design and wrote the manuscript. All authors approved the final version of the manuscript.

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