GAD autoantibody affinity in schoolchildren from the general population

Christine Bender · Michael Schlosser · Urs Christen ·
Anette G. Ziegler · Peter Achenbach

Received: 10 January 2014 / Accepted: 16 May 2014 / Published online: 18 June 2014
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Abstract

Aims/hypothesis Subtyping GAD autoantibody (GADA) responses using affinity measurement allows the identification of GADA-positive children with a family history of type 1 diabetes who are at risk of developing diabetes. Here, we asked whether GADA affinity is a useful marker to stratify the risk of type 1 diabetes in GADA-positive schoolchildren from the general population.

Methods GADA affinity was measured by competitive binding experiments with [125 I]-labelled and unlabelled human 65 kDa isoform of GAD (GAD65) in sera from 97 GADA-positive children identified in the Karlsburg Type 1 Diabetes Risk Study of a general schoolchild population in north-eastern Germany. GADA epitope specificity was determined using radiobinding assays with [35 S]-labelled GAD65/67 kDa isoform of GAD (GAD67) chimeric proteins.

Results GADA affinity was high, $\geq 10^{10}$ l/mol, in 33 of 35 multiple islet autoantibody-positive children. In contrast, the affinity ranged widely among 62 single GADA-positive

children (median 3.1×10^9 l/mol; range 5.6×10^6 to $>4.0 \times 10^{11}$ l/mol; $p < 0.0001$). High-affinity GADA were associated with HLA-DRB1*03 ($p = 0.02$) and predominantly directed against the C-terminal and/or middle part of the GAD65 protein. At follow-up, the affinity remained relatively constant. Five of the single GADA-positive children developed additional islet autoantibodies and had high-affinity GADA. Twenty-six children progressed to type 1 diabetes; among them, 23 had GADA affinities of $\geq 10^{10}$ l/mol before disease onset.

Conclusions/interpretation Schoolchildren from the general population may develop heterogeneous GADA responses, and a high affinity can identify those GADA-positive children who are more likely to progress to type 1 diabetes.

Keywords Affinity · GAD autoantibodies · General population · Schoolchildren · Type 1 diabetes risk

Abbreviations

GAD65	65 kDa isoform of GAD
GAD67	67 kDa isoform of GAD
GADA	Autoantibodies to GAD
GAD-MID	Middle section of GAD
IA-2A	Autoantibodies to insulinoma-associated antigen-2
IAA	Autoantibodies to insulin
IQR	Interquartile range
ZnT8A	Autoantibodies to zinc transporter-8

Introduction

Type 1 diabetes is one of the most common autoimmune diseases in children and adolescents and is caused by a selective destruction of the insulin-producing beta cells of the

C. Bender (✉) · U. Christen
Pharmazentrum Frankfurt/ZAFES, Goethe University Hospital
Frankfurt, Theodor-Stern Kai 7, 60590 Frankfurt am Main, Germany
e-mail: bender@med.uni-frankfurt.de

M. Schlosser
Department of Medical Biochemistry and Molecular Biology,
Research Group of Predictive Diagnostics, and Institute of
Pathophysiology, Research Group of Predictive Diagnostics,
University Medical Center Greifswald, Karlsburg, Germany

A. G. Ziegler · P. Achenbach (✉)
Institute of Diabetes Research, Helmholtz Zentrum München,
Ingolstaedter Landstrasse 1, 85764 Neuherberg, Germany
e-mail: peter.achenbach@helmholtz-muenchen.de

A. G. Ziegler · P. Achenbach
Forscherguppe Diabetes, Klinikum rechts der Isar, Technische
Universität München, Munich, Germany

pancreatic islets of Langerhans [1, 2]. A characteristic feature of this disease is the occurrence of autoantibodies against beta cell antigens such as autoantibodies to GAD (GADA), insulin (IAA), insulinoma-associated antigen-2 (IA-2A) and zinc transporter-8 (ZnT8A) [3, 4]. The presence of such autoantibodies in the peripheral blood is the first detectable marker of ongoing beta cell destruction. They may appear early in life and predict the development of type 1 diabetes [5–8]. The risk of diabetes increases with the number of autoantibody markers [9, 10]. In contrast, children who do not develop autoantibodies against multiple beta cell antigens rarely progress to clinical type 1 diabetes during childhood. GADA often appear in older age groups and are preferentially associated with HLA-DRB1*03 haplotypes [11]. The GADA level alone does not predict progression to diabetes, and controversial reports exist with respect to the relevance of GADA epitope reactivity to diabetes progression [12–17].

We previously demonstrated that children with a familial predisposition who developed type 1 diabetes had high-affinity GADA before the onset of their disease, whereas those with low-affinity GADA showed no progression of islet autoimmunity [14]. Nevertheless, approximately 90% of children diagnosed with type 1 diabetes have no first-degree relatives with the disease [18–20]. The intent of the present study was therefore to extend our previous findings in relatives to schoolchildren from the general population. In particular, we asked whether a relationship exists between GADA affinity and other characteristics of GADA such as level and epitope specificity, as well as HLA genotype and the status of autoantibodies to other beta cell antigens.

Methods

Study cohort and samples Samples were obtained from the Karlsburg Type 1 Diabetes Risk Study, in which a total of 11,840 children of a general school population in the north-eastern part of Germany were screened for GADA, IAA, IA-2A and islet cell antibodies [21]. Autoantibody-positive children were invited for re-examination to confirm the autoantibody measurements and for HLA genotyping [22]. The study was authorised by the state government of Mecklenburg-Vorpommern (No. VII 233 A-3201-01) and approved by the ethics committee of the University of Greifswald. All participants gave written informed consent to participate in the study. Investigations were carried out in accordance with the principles of the Declaration of Helsinki, as revised in 2008.

For the present study, the sample volume of 97 GADA-positive schoolchildren at re-examination was large enough to perform measurements of GADA affinity and epitopes. This sample was defined as the initial sample in our study. The children had a median age of 11.2 years (interquartile range [IQR] 9.6–13.5 years), 44 were male, and none had a first-

degree relative with type 1 diabetes. In addition, a follow-up sample was available for analysis from 95 children (median time since the initial sample 4.9 years, IQR 3.3–6.6 years), and a second follow-up sample from 13 children (median time since the first follow-up sample 3.7 years, IQR 2.1–4.4 years). The children were contacted on follow-up to obtain information on diabetes status (median time from the initial sample to last contact 8.6 years, IQR 5.2–10.9 years).

Islet autoantibody measurements Levels of GADA, IA-2A and IAA were measured by radiobinding assays using [125 I]-labelled recombinant human 65 kDa isoform of GAD (GAD65), IA-2 and insulin as previously described [20, 21]. A cut-off level at or above the 98th percentile was used for all autoantibodies [21]. In the 4th Diabetes Antibody Standardization Program 2005 workshop, the GADA and IA-2A assays achieved a diagnostic sensitivity and specificity of 82% and 96% (GADA) and 66% and 100% (IA-2A), respectively [23], and the IAA assay achieved 58% sensitivity and 97% specificity [24].

GADA epitope measurements GADA epitope specificity was determined using radiobinding assays on [35 S]-methionine-labelled GAD65/67 kDa isoform of GAD (GAD67) chimeric proteins as previously described [17]. Thresholds for positivity were defined as the upper limit in the sera of 50 control participants. The chimeric proteins used were GAD65_{1–95}/GAD67_{101–593} (GAD N-terminal; GAD65 amino acid 1–95), GAD67_{1–243}/GAD65_{235–444}/GAD67_{453–593} (GAD-MID, the middle section of GAD; GAD65 amino acid 235–444) and GAD67_{1–452}/GAD65_{445–585} (GAD C-terminal; GAD65 amino acid 445–585). GADA were classified as A (reactive to middle and/or C-terminal GAD65 epitopes), B (reactive to N-terminal GAD65 epitopes only) or C (reactive to none of the GAD65 epitopes represented in the chimeric proteins).

GADA affinity measurements GADA affinity was measured by competitive binding experiments as previously described [14]. Briefly, serum (2 μ l) was incubated in duplicate for 48 h at 4°C in TBST buffer (50 mol/l Tris, 150 mmol/l NaCl, 1% Tween 20, pH 7.4) in the presence of [125 I]-recombinant human GAD65 (0.05 nmol/l) and five increasing quantities of unlabelled human GAD65 (3.3×10^{-11} , 3.3×10^{-10} , 3.3×10^{-9} , 3.3×10^{-8} and 3.3×10^{-7} mol/l) (RSR Ltd., Cardiff, UK) or TBST buffer only in a final volume of 47 μ l. Immune complexes were precipitated for 60 min with 1.5 mg Protein A Sepharose (GE Healthcare, Chalfont St Giles, UK) pre-swelled in 50 μ l TBST, subsequently washed five times with 1.8 ml of ice-cold TBST buffer and measured using a gamma counter (Packard Instruments Co., Meriden, CT, USA). The results were expressed as cpm.

IC_{50} and K_d values were calculated by non-linear regression analysis using GraphPad Prism3 (GraphPad Software,

San Diego, CA, USA), and the mean GADA affinity of each sample was expressed as the reciprocal K_d value (l/mol) (Fig. 1). Samples with very high GADA levels that showed binding to [125 I]-GAD65 of >50% of the total [125 I]-GAD65 activity added to the test were diluted. For such samples, GADA affinity was calculated from the competitive binding curve obtained with the diluted sample. The reproducibility of GADA affinity measurements was determined from replicates of a GADA-positive serum that was included in each experiment. The CV of GADA affinity measurements was 3% [14]. The calculation of K_d values was limited to samples with IC_{50} values greater than the concentration of labelled GAD65 (0.05 nmol/l). For samples with an $IC_{50} < 0.05$ nmol/l, the mean GADA affinity of the sample was set at $>4.0 \times 10^{11}$ l/mol.

HLA typing HLA-DRB1 genotyping was performed using PCR-amplified genomic DNA and nonradioactive oligonucleotide probes as described elsewhere [22].

Statistical analysis The Mann–Whitney U test was used to compare GADA affinities between groups (epitope specificity, autoantibody status and HLA genotype). Spearman's correlation was used to determine the correlation between variables. The Wilcoxon logrank test was used to compare GADA affinity over time. For all analysis, a two-tailed p value of 0.05 was considered significant. All statistical analyses were performed using GraphPad Prism software (Version 3.01; GraphPad Software).

Results

Schoolchildren from the general population exhibit large variations in GADA affinity The affinity of GADA varied between the 97 GADA-positive schoolchildren and ranged from 5.6×10^6 to $>4.0 \times 10^{11}$ l/mol. Competitive GADA binding curves to [125 I]-GAD65 were consistent with a one-site binding model in the samples from all the children, suggesting that GADA were of relatively homogeneous affinities within each sample (Fig. 1). The affinity of GADA correlated with the GADA level ($r=0.57$; $p<0.0001$) but was not associated with age ($p=0.2$). Children carrying the HLA-DRB1*03 allele showed higher GADA affinities ($n=28$; median $>4.0 \times 10^{11}$ l/mol, IQR 1.3×10^{10} to $>4.0 \times 10^{11}$ l/mol) compared with those with other HLA alleles ($n=69$; median 1.4×10^{10} l/mol, IQR 8.2×10^8 to $>4.0 \times 10^{11}$ l/mol; $p=0.02$) (Fig. 2a). GADA affinity was particularly higher in children with coexisting IAA and/or IA-2A ($n=35$; median $>4.0 \times 10^{11}$ l/mol, IQR 3.9×10^{11} to $>4.0 \times 10^{11}$ l/mol) compared with those with GADA alone ($n=62$; median 3.1×10^9 l/mol, IQR 6.2×10^8 to 1.8×10^{11} l/mol; $p<0.0001$) (Fig. 2b).

Fig. 1 Sample competitive binding curves. GADA binding to human [125 I]-GAD65 (y-axis) at increasing concentrations of competitive unlabelled human GAD65 (x-axis; log₁₀ transformed values) is shown for a serum with low-affinity GADA of 5.7×10^8 l/mol (sample 1) and a serum with high-affinity GADA of 1.3×10^{11} l/mol (sample 2). Both curves are consistent with a one-site binding model

High-affinity GADA display a characteristic epitope specificity The GADA response was predominantly directed against the C-terminal (residues 445–585) and/or middle part (residues 235–444) of the GAD65 protein. This epitope reactivity was associated with high GADA affinities ($n=70$; median $>4.0 \times 10^{11}$ l/mol, IQR 4.4×10^9 to $>4.0 \times 10^{11}$ l/mol) compared with GADA responses restricted to the N-terminal part (residues 1–95) of the protein, which were associated with lower affinities ($n=12$; median 9.0×10^8 l/mol, IQR 2.1×10^8 to 2.9×10^9 l/mol; $p<0.0001$) (Fig. 2c). Furthermore, GADA levels correlated well with GADA reactivity to the C-terminal ($r=0.73$; $p<0.0001$) and middle ($r=0.77$; $p<0.0001$) part of GAD65 (Fig. 3a, b), but not with GADA reactivity to the N-terminal region ($r=0.07$; $p=0.5$) (Fig. 3c). GADA of unknown epitope specificity (i.e. GADA that did not bind to the GAD-MID, C-terminal, or N-terminal constructs) showed variable affinities (median 1.5×10^{10} l/mol, IQR 7.7×10^8 to $>4.0 \times 10^{11}$ l/mol) (Fig. 2c).

GADA affinity on follow-up and progression to type 1 diabetes We obtained a first follow-up serum sample from 95 of the 97 GADA-positive schoolchildren, and a second follow-up sample from 13 children. No follow-up sample was available from two multiple islet autoantibody-positive children (GADA affinity 3.1×10^{10} and $>4.0 \times 10^{11}$ l/mol, respectively). A total of 26 children developed type 1 diabetes (median time from initial sample to diagnosis 6.5 years, IQR 2.8–10.6 years).

GADA affinity remained relatively constant on follow-up in the majority of children (Fig. 4). Children with multiple islet autoantibodies ($n=33$) predominantly had GADA affinities of $\geq 10^{10}$ l/mol. Of these, 29 children remained multiple autoantibody-positive on follow-up, and 21 of them developed diabetes. Four children reverted to single GADA, but two of them nevertheless developed diabetes (Fig. 4c, d). In

Fig. 2 Relationship between GADA affinity and HLA genotype, islet autoantibody status or GADA epitope binding. The GADA affinities of 97 schoolchildren are plotted and compared between (a) children with the HLA DRB1*03 allele (HLA-DR3; $n=28$) vs those without this allele (non-HLA-DR3; $n=69$); (b) children with GADA only ($n=62$) vs those with GADA plus IAA and/or IA-2A ($n=35$); and (c) children with GADA binding to epitopes within GAD65 residues 235–585 (MID and/or C-terminal; $n=70$), restricted binding to epitopes within GAD65 residues 1–95 (N-terminal only; $n=12$) or binding to unknown epitopes (MID-, C-, N-terminal negative; $n=15$). The median GADA affinity is indicated for each group. * $p<0.05$, *** $p<0.001$

contrast, children with single GADA ($n=62$) showed a broad spectrum of GADA affinities ranging from 10^6 to 10^{11} I/mol. Among these, 57 children remained single GADA-positive on follow-up, and two developed diabetes. Five children became multiple islet autoantibody-positive on follow-up (GADA affinity, range 1.2×10^{10} to $>4.0 \times 10^{11}$ I/mol), and one developed diabetes (Fig. 4a, b).

GADA affinity, level and epitope specificity define distinct GADA profiles On considering the affinity, level and epitope

reactivity of GADA, as well as islet autoantibody status and HLA genotype, several distinct GADA profiles could be identified to stratify the risk of type 1 diabetes in schoolchildren (Fig. 5).

The first, most frequent GADA profile (profile P1; Fig. 5a, d) was found in 50 (51.5%) of the 97 GADA-positive children. This profile displayed high-affinity GADA with median to high antibody levels and reactivity against the middle and/or C-terminal epitopes of the GAD65 protein. Of the children with this profile, 20 (40%) carried the HLA-DRB1*03 allele, 31 (62%) were multiple islet autoantibody-positive, and 23 (46%) developed type 1 diabetes.

A second GADA profile (profile P2; Fig. 5a) was also associated with reactivity against the middle and/or C-terminal regions of GAD65, but showed low GADA affinity and low antibody levels. This profile was found in 20 (20.6%) of the 97 children who were all single GADA-positive. Only four of them (20%) carried the HLA-DRB1*03 allele, and one (5%) progressed to type 1 diabetes.

The third GADA profile (profile P3; Fig. 5b, e) was characterised by restricted GADA binding against the N-terminal epitopes of GAD65, low GADA affinity, low antibody levels and low frequency of the HLA-DRB1*03 allele. Twelve (12.4%) children displayed this profile, of whom 11 were single GADA-positive and none progressed to type 1 diabetes.

Finally, 15 (15.5%) children had GADA that did not bind to any of the epitope constructs (profile P4; Fig. 5c, f). These GADA were of either low or high affinity, had low antibody levels and were found in children with single GADA or multiple islet autoantibodies. Only two children with this fourth GADA profile progressed to type 1 diabetes (both of whom were multiple autoantibody-positive).

A summary of the main characteristics of all the GADA profiles is shown in Fig. 5g. Among multiple islet autoantibody-positive children, profile P1 was the predominant GADA profile (88.6%; Fig. 5j), whereas single GADA-positive children showed a rather variable distribution into the four profiles (Fig. 5h).

Discussion

Screening for autoantibodies to beta cell antigens is a powerful method to assess the risk of type 1 diabetes [10]. It has recently become evident that besides the autoantibody level, both affinity and epitope specificity may be used to further stratify risk [12, 14, 25, 26]. We were previously able to demonstrate in children who had at least one parent affected by type 1 diabetes that an assessment of GADA affinity and epitope specificity allowed us to define discrete GADA profiles associated with risk of type 1 diabetes [14]. The present study is

Fig. 3 Relationship between GADA levels and epitope binding. The GADA levels of 97 schoolchildren are correlated with the levels of GADA binding to epitopes within (a) GAD65 residues 445–585 (GADA C-terminal) ($r=0.73$, $p<0.0001$), (b) GAD65 residues 235–444 (GADA-

MID) ($r=0.77$, $p<0.0001$), or (c) GAD65 residues 1–95 (GADA N-terminal) ($r=0.07$, $p=0.5$). Thresholds for antibody positivity are indicated by broken lines for each epitope specificity. AU, arbitrary units; KU, Karlsburg units

the first to expand this observation to the general population and to analyse GADA profiles in schoolchildren without a first-degree family history of type 1 diabetes. As in the relatives [14], we found GADA profiles that were characterised by their GADA affinity and epitope specificity. In particular, children with high-affinity GADA that recognised the middle and/or C-terminal part of GAD65 (profile P1) were at risk of progression to clinical diabetes. IAA and/or IA-2A as well as the HLA-DRB1*03 allele were frequently present in GADA-positive children with this profile.

The affinity of GADA varied among the schoolchildren over almost five orders of magnitude and was correlated with GADA levels. Interestingly, GADA binding to the middle and/or C-terminal part of GAD65 could be of high or low affinity, and this was strongly related to the islet autoantibody status. The occurrence of GADA plus IAA and/or IA-2A was predominantly seen in children with high-affinity autoantibodies, confirming previous reports from us and others [25, 27, 28]. In contrast, GADA binding only to the N-terminal part of GAD65 involved predominantly single low-affinity

Fig. 4 GADA affinity over time in 203 follow-up samples from 95 schoolchildren. GADA affinity on follow-up is shown for 62 children with GADA only (a, b) and 33 children with GADA plus IAA and/or IA-2A (c, d) in their first serum samples. Follow-up samples from individual children are connected by lines. Multiple islet autoantibody-positive sera are indicated by filled circles. Children who progressed to type 1 diabetes (T1D) on follow-up ($n=26$) are presented separately (b, d)

Fig. 5 GADA profiles. Sera from 97 schoolchildren are grouped according to islet autoantibody status: GADA only (**a, b, c**), or GADA plus IAA and/or IA-2A (**d, e, f**). GADA epitope reactivity: GAD65 middle and/or C-terminal epitopes (MID and/or C-terminal) (**a, d**); GAD65 N-terminal epitopes only (N-terminal only) (**b, e**); or unknown GAD65 epitope specificity (MID-, C-, N-terminal negative) (**c, f**). Sera with similar GADA profiles are circled (profiles P1–P4). Children with allele HLA-DRB1*03 are indicated by filled triangles (**a–f**). The table summarises the characteristics of the GADA profiles (**g**). The frequency of profiles is shown for children with GADA only (**h**) or GADA plus IA-2A and/or IAA (**j**). KU, Karlsburg units

antibodies and was not related to the development of diabetes. Overall, low-affinity GADA were rarely found in multiple autoantibody-positive children.

GADA profiles were relatively homogenous in schoolchildren with multiple islet autoantibodies and those who developed diabetes, but were highly variable in children who had only GADA. Moreover, children with single low-affinity GADA could be classified into two profiles according to their binding to epitopes within the middle/C-terminal region of GAD65 (profile P2) or within the N-terminal region only (profile P3). Besides the absence of reactivity to multiple islet autoantigens, both profiles were further characterised by relatively low GADA levels and a low frequency of the HLA-DRB1*03 allele. Only one child displaying profile P2, and no child with profile P3, developed type 1 diabetes during the observation time. These findings in the GADA-positive children from the general population are consistent with what we

and others have previously observed for GADA, IAA or IA-2A in first-degree relatives and in IAA-positive children without a familial predisposition [14, 25, 26, 28, 29].

A limitation of our study is that, in contrast to the BABYDIAB study [8], only sera from schoolchildren with established GADA responses have been included, and the age at autoantibody seroconversion in these children was not known. This may explain the low number of changes from single GADA to multiple autoantibody-positive status. Only a few single GADA-positive children developed IAA and/or IA-2A on follow-up. Another limitation of our study is that the schoolchildren were not tested for the presence of ZnT8A. Therefore, we cannot exclude the possibility that some of the schoolchildren who were classified as single GADA-positive may have had ZnT8A and would shift to the multiple islet autoantibody-positive group. ZnT8A have been associated with rapid disease progression in the relatives of patients with

type 1 diabetes [30–32], which we did not observe for schoolchildren with GADA only in the present study. Yet the selected cohort of schoolchildren in our study allowed for an analysis of the development of GADA affinity and epitope specificity over time. Since the children are still being followed prospectively into adulthood, we cannot identify the timeframe within which children with a specific GADA profile will progress to clinical diabetes. Nevertheless, our findings confirm immune profiles found in the relatives of type 1 diabetes patients [14]. Therefore, the data support the hypothesis that findings related to the immune pathogenesis of type 1 diabetes identified in people with a familial predisposition can be transferred to the general population. Recent advances in the reliability and specificity of islet autoantibody measurements, such as electrochemiluminescence-based antibody assays [33], may further improve screening in the general population.

In summary, we conclude that an assessment of GADA affinity and epitope specificity in addition to the GADA level, islet autoantibody status and HLA genotype allows for the determination of several distinct GADA profiles and a more accurate stratification of the risk of type 1 diabetes in the general population. Furthermore, GADA affinity could be a useful diagnostic marker for the recruitment of people from the general population to studies on the prevention of type 1 diabetes.

Acknowledgements The authors thank B. Rees-Smith (RSR, Cardiff, UK) for providing recombinant human GAD65, R. Wassmuth (Department of Quality Management, University Medical Center, University of Duesseldorf, Germany) for HLA genotyping and S. Krause (Forscherguppe Diabetes, Klinikum rechts der Isar, Technische Universität München, Germany) for expert technical assistance.

Funding This study was supported in part by grants from the European Union (EP7-HEALTH-2007, DIAPREPP N202013) and from the German Federal Ministry of Education and Research to the Competence Network for Diabetes mellitus (FKZ 01GI0805) and to the German Center for Diabetes Research (DZD e.V.). PA received support from the JDRF (11-2005-1117).

The Karlsburg Type 1 Diabetes Risk Study was supported by the German Federal Ministry of Research and Technology (BMFT 07NBL02/D4), the government of Mecklenburg-Vorpommern (EMAU16/1995), the Else-Kröner-Fresenius-Stiftung, the Deutsche Diabetes-Stiftung, the Community Medicine Project of the Ernst Moritz Arndt University Greifswald, and the BRAHMS Diagnostica GmbH, Berlin. Additional support was obtained from the BMFT-funded Center for Interdisciplinary Clinical Research at the Friedrich Alexander University Erlangen-Nuernberg (01KS9601), the Deutsche Forschungsgemeinschaft (SFB263), and the Association for Support of Diabetes Research, Karlsburg, Greifswald e.V.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement CB performed all the experiments, researched data and wrote, reviewed and edited the manuscript. MS and UC contributed to the writing of the manuscript. PA designed and supervised the

study, researched data and reviewed and edited the manuscript. All authors (CB, MS, UC, AGZ and PA) contributed to the interpretation of data, critically revised the manuscript for important intellectual content, and approved the final version of the manuscript. PA takes responsibility for the integrity of the work as a whole.

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