

# Early Loss of the Glucagon Response to Hypoglycemia in Adolescents With Type 1 Diabetes

ARIS SIAFARIKAS, FRACP, MD<sup>1,2,3</sup>  
ROBERT J. JOHNSTON, MRCP<sup>1</sup>  
MAX K. BULSARA, PHD<sup>3,4</sup>

PETER O'LEARY, PHD<sup>5,6</sup>  
TIMOTHY W. JONES, MBBS, FRACP, MD<sup>1,2,7</sup>  
ELIZABETH A. DAVIS, MBBS, FRACP, PHD<sup>1,2,7</sup>

**OBJECTIVE**—To assess the glucagon response to hypoglycemia and identify influencing factors in patients with type 1 diabetes compared with nondiabetic control subjects.

**RESEARCH DESIGN AND METHODS**—Hyperinsulinemic hypoglycemic clamp studies were performed in all participants. The glucagon response to both hypoglycemia and arginine was measured, as well as epinephrine, cortisol, and growth hormone responses to hypoglycemia. Residual  $\beta$ -cell function was assessed using fasting and stimulated C-peptide.

**RESULTS**—Twenty eight nonobese adolescents with type 1 diabetes (14 female, mean age 14.9 years [range 11.2–19.8]) and 12 healthy control subjects (6 female, 15.3 years [12.8–18.7]) participated in the study. Median duration of type 1 diabetes was 0.66 years (range 0.01–9.9). The glucagon peak to arginine stimulation was similar between groups ( $P = 0.27$ ). In contrast, the glucagon peak to hypoglycemia was reduced in the group with diabetes (95% CI: 68 (62–74) vs. 96 (87–115) pg/mL ( $P < 0.001$ ). This response was greater than 3 SDs from baseline for only 7% of subjects with type 1 diabetes in comparison with 83% of control subjects and was lost at a median duration of diabetes of 8 months and as early as 1 month after diagnosis ( $R = -0.41$ ,  $P < 0.01$ ). There was no correlation in response with height, weight, BMI, and HbA<sub>1c</sub>. Epinephrine, cortisol, and growth hormone responses to hypoglycemia were present in both groups.

**CONCLUSIONS**—The glucagon response to hypoglycemia in adolescents with type 1 diabetes is influenced by the duration of diabetes and can be lost early in the course of the disease.

**H**ypoglycemia is a complication of insulin therapy of type 1 diabetes that can cause significant morbidity and rarely, mortality; as a result, it proves a significant barrier to contemporary targets for glycemic control (1). Young people with type 1 diabetes are especially prone to hypoglycemia due to the nonphysiological nature of insulin therapy, as well as defective counterregulation (1–4). Impairment of the glucagon response to hypoglycemia is well documented in adult patients with long-standing diabetes (2,5); however,

the natural history and underlying pathophysiology have not been well characterized in children and adolescents. Studies with small sample numbers suggested that the glucagon response to hypoglycemia is lost during the first months after diagnosis of type 1 diabetes (6,7), but this remains to be studied further.

The purpose of this study was to 1) assess the glucagon response to both hypoglycemia and arginine as an independent stimulus in adolescents with type 1 diabetes with a range of diabetes duration, as

well as in healthy control subjects, and 2) identify clinical and demographic factors that predict the glucagon response to hypoglycemia.

## RESEARCH DESIGN AND METHODS

Adolescents with type 1 diabetes were identified from the Western Australian Children's Diabetes Database. Eligible patients aged 12–16 years attending the Diabetes Service at Princess Margaret Hospital for Children were approached to participate in the study. Exclusion criteria included a history of seizures not related to hypoglycemia, any episode of hypoglycemia (blood glucose level  $<3.5$  mmol/L) in the 24 h preceding the study, and a history of severe hypoglycemia within the preceding 3 months. Clinical and demographic factors are prospectively recorded from diagnosis and recorded in the Western Australian Children's Diabetes Database. These data were used to determine the factors included in the multivariate analysis. The control subjects were siblings of children with type 1 diabetes recruited from the community. The cross-sectional study design involved admission for a hypoglycemic hyperinsulinemic clamp study followed by an arginine stimulation test. The protocol was approved by the local ethics committee and signed informed consent was obtained for all subjects.

## Protocol

**Hyperinsulinemic hypoglycemic clamp study.** A modification of the glucose clamp technique described by Amiel et al. (8) was used. Patients were advised to fast for at least 8 h prior to admission to the research laboratory on the morning of the study. Only short-acting insulin was used in the 12 h preceding the study. Fasting samples were taken for plasma glucose, C-peptide, HbA<sub>1c</sub>, and islet cell/glutamic acid decarboxylase antibodies.

The subjects were given an intravenous infusion of insulin (Humalog; Eli Lilly) at a continuous rate of 80 mU/m<sup>2</sup>/min, with doubling of the rate for the first 5 min. Plasma glucose was measured bedside at 5-min intervals and the glucose infusion

From the <sup>1</sup>Department of Endocrinology and Diabetes, Princess Margaret Hospital for Children, Perth, Western Australia, Australia; the <sup>2</sup>School of Paediatrics and Child Health, The University of Western Australia, Perth, Western Australia, Australia; the <sup>3</sup>Institute of Health and Rehabilitation Research, University of Notre Dame Fremantle, Fremantle, Western Australia, Australia; the <sup>4</sup>School of Population Health, The University of Western Australia, Perth, Western Australia, Australia; the <sup>5</sup>Department of Biochemistry, Princess Margaret Hospital for Children, Perth, Western Australia, Australia; the <sup>6</sup>Centre for Population Health Research, Curtin Health Innovation Research Institute, Curtin University, Perth, Western Australia, Australia; and the <sup>7</sup>Telethon Institute for Child Health Research, Centre for Child Health Research, The University of Western Australia, Perth, Western Australia, Australia.

Corresponding author: Elizabeth A. Davis, elizabeth.davis@health.wa.gov.au.

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rate (20% dextrose in saline) adjusted to achieve the target plasma glucose concentration. Blood samples were taken at 10- to 20-min intervals for measurement of counterregulatory hormones (glucagon, epinephrine, cortisol, and growth hormone) and free insulin levels.

The plasma glucose was initially stabilized between 5.0 and 5.5 mmol/L (90–108 mg/dL) for 60 min (euglycemic phase) and then reduced over a period of 30–40 min to achieve a target nadir of 2.8 mmol/L (50.4 mg/dL). Hypoglycemia was maintained for 40 min (hypoglycemic phase). On completion of the hypoglycemic phase, the insulin infusion was stopped and the plasma glucose was stabilized between 5.0 and 5.5 mmol/L (90–108 mg/dL) for at least 20 min.

### Arginine stimulation test

To assess glucagon release to stimuli other than hypoglycemia and residual insulin reserve, arginine was used as an alternative stimulus (9,10). Baseline samples for plasma glucagon were taken at 5 ( $t = -5$ ) and 2 min ( $t = -2$ ) prior to infusion of 10% arginine solution (5 g in 50 mL 0.9% sodium chloride over 30 s) (11). Stimulated plasma glucagon was measured at 2, 3, 4, and 5 min postarginine (11).

### Assays

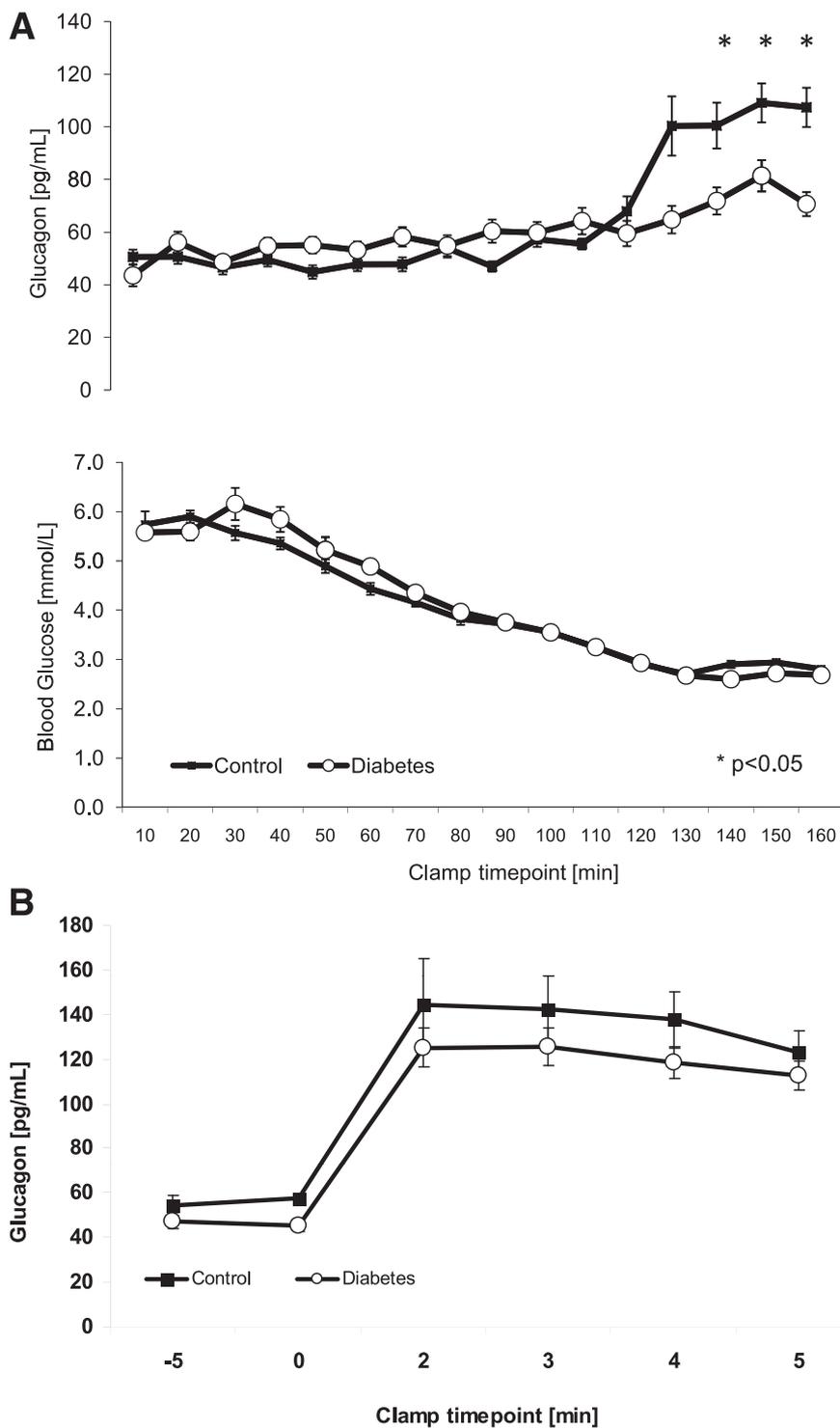
Plasma glucose was assessed at bedside by the glucose oxidase method (YSI analyzer; YSI, Yellow Springs, OH). Blood for plasma glucagon was collected in EDTA with trasylol, immediately stored on ice, separated within 4 h, and stored at  $-70^{\circ}\text{C}$  until assay. Under these conditions, glucagon immunoreactivity is preserved (12). Plasma glucagon was analyzed using a radioimmunoassay (Glucagon RIA; Linco Research, Inc., St. Charles, MO). The coefficient of variation for the glucagon assay was 13.9% at 51.5 pg/mL and 10.2% at 70.1 pg/mL.

Plasma epinephrine levels were measured by ELISA (Diagnostika GmbH, Hamburg, Germany), and samples were analyzed in duplicate according to the manufacturer's instructions (13). Blood was collected into tubes containing EDTA preservative. Samples were stored in an ice bath until they were centrifuged to separate the plasma within 2 h and then stored at  $-70^{\circ}\text{C}$  prior to assay. The inter-assay coefficient of variation (CV) at 10 pmol/L and 5,460 pmol/L were 2 and 5.5%, respectively.

We assessed residual  $\beta$ -cell function based on both fasting- and arginine-stimulated

C-peptide values. Cortisol, growth hormone, and C-peptide were measured by immunoassays (Immulite; Diagnostic Products Corporation, Los Angeles, CA) (14).

Free and total insulin were measured by radioimmunoassay after precipitation of endogenous antibodies with polyethylene glycol. Analytical recovery of free insulin was 99.3%, of total insulin



**Figure 1**—A and B: Glucagon response to hypoglycemia and arginine. The line diagrams show glucagon levels from clamp studies as mean and SEM during euglycemia and in response to hypoglycemia and arginine infusion, respectively.  $n = 28$  for type 1 diabetes and  $n = 12$  for control subjects. A linear mixed model was used for analysis.

96.4%. For free insulin, assay precision (CV) was 4.0–13.0% (intra-assay) and 7.8–10.7% (interassay); for total insulin, 3.6–9.5 and 6.6–11.7%, respectively (15). Glycated hemoglobin was measured by agglutination inhibition immunoassay (Ames DCA 2000, non-type 1 diabetes reference interval <6.2%).

### Data analysis

Demographic data were expressed as mean and range. Duration of diabetes was also expressed as median and range to allow a more detailed analysis. All other results were described as mean and 95% CIs.

For the calculation of baseline and stimulated values, we used the average of time points 0–60 min for euglycemia and 130–150 min for hypoglycemia. A positive response to hypoglycemia was defined as a stimulated value greater than 3 SDs from the respective baseline during euglycemia. This approach was used by the Diabetes in Children Network Study Group (16) (see Table 2).

The glucagon responses to hypoglycemia and arginine were compared between the groups for each time point of analysis and are illustrated in Fig. 1A and B.

A linear mixed model was used for analysis of the glucagon responses. Stepwise linear regression was used in the subjects with type 1 diabetes to identify influencing factors. Statistical significance was set at  $P < 0.05$  (Stata Statistical Software, release 12; StataCorp, College, Station, TX).

## RESULTS

### Subjects

A total of 28 individuals with type 1 diabetes (14 females and 14 males) and 12 healthy volunteers (6 females and 6 males) were studied. Clinical characteristics are summarized in Table 1. All patients with type 1 diabetes were islet cell and/or glutamic acid decarboxylase antibody positive and on regular insulin therapy: short-acting insulin via insulin pump ( $n = 6$ ) and combinations of long- and short-acting insulin using two ( $n = 7$ ), three ( $n = 6$ ), or four ( $n = 9$ ) injections per day. One subject was taking thyroxine for autoimmune hypothyroidism. Median duration of type 1 diabetes was 0.66 years (range 0.01–9.9).

### Counterregulatory response

The glucagon peak to arginine stimulation was similar between groups ( $P = 0.27$ ) (Fig. 1B). In contrast, the glucagon peak to hypoglycemia was reduced in the group with diabetes (95% CI: 68 (62–74) vs. 96 (87–115) pg/mL ( $P < 0.001$ ) (Fig. 1A). A positive response was defined as greater than 3 SDs from baseline (25 pg/mL for control subjects and 48 pg/mL for the group with type 1 diabetes). Based on these values, only 7% of subjects with type 1 diabetes demonstrated a positive response in comparison with 83% of control subjects (Table 2). The response was lost at a median duration of diabetes of 8 months and as early as 1 month after diagnosis ( $R = -0.43$ ,  $P < 0.01$ ) (Table 3).

Fasting and stimulated C-peptide were higher in the control group (Table 1).

Epinephrine, cortisol, and growth hormone responses to hypoglycemia were present, consistent with the generation of an adequate hypoglycemic stimulus ( $P < 0.05$ ) (Table 2).

### Regression analysis

Stepwise linear regression revealed a significant correlation between glucagon response, duration of diabetes, and weight. There was no significant correlation with HbA<sub>1c</sub>, BMI, fasting and stimulated C-peptide, height, and insulin level during hypoglycemia (Table 3).

**CONCLUSIONS**—This study demonstrates that in adolescents with type 1 diabetes, the glucagon response to an arginine stimulus is similar to healthy control subjects. However, adolescents with type 1 diabetes have a blunted glucagon response after hypoglycemia. Although this finding is not novel, this, to our knowledge, is the first study to show that the glucagon response to hypoglycemia was lost as early as 1 month after diagnosis and at a median duration of diabetes of 8 months. The glucagon response to hypoglycemia decreased with increasing duration of diabetes.

We assessed the counterregulatory response to hypoglycemia using clamp studies in adolescents with type 1 diabetes early in the course of the disease. Hoffman et al. (6) and Singer-Granick et al. (7) also demonstrated an impaired glucagon response to hypoglycemia in adolescents with type 1 diabetes. To further analyze and specify the participants' response, Hoffman et al. (17) also included the pancreatic polypeptide release after hypoglycemia and the glucagon response to a mixed-meal stimulus. The interpretation of these studies, however, is limited by the methodology that used an insulin bolus to induce hypoglycemia. Compared with using clamp studies, this does not produce a reproducible hypoglycemic stimulus, resulted in a wide range of insulin levels, and is a technique in which the response to hypoglycemia cannot be compensated. This is why the stimulus may be modified. The Diabetes Research in Children Network Study Group demonstrated that the counterregulatory response can be lost in children aged 4–7 years with a diabetes duration of  $3.3 \pm 1.1$  years (16). Hypoglycemia was induced using a subcutaneous insulin infusion protocol. In contrast to our study, that

**Table 1—Clinical characteristics**

	Control subjects	Type 1 diabetes	P value
Number and sex of subjects	12 (6 female/6 male)	28 (14 female/14 male)	
Age at study date (years)	15.3 (12.8–18.7)	14.9 (11.2–19.8)	0.21
Duration of diabetes (years)	—	1.87 (0.01–9.9) Median: 0.66	—
HbA <sub>1c</sub> (%)	5.0 (4–6)	7.6 (5–11)	<0.001
Fasting C-peptide (nmol/L)	0.37 (0.33–0.41)	0.22 ( $\leq 0.2$ –0.23)	<0.001
Stimulated C-peptide (nmol/L)	0.52 (0.38–0.66)	0.22 ( $\leq 0.2$ –1.5)	0.001
Insulin during euglycemia (mU/L)	118 (18–224)	77 (23–165)	0.05
Insulin during hypoglycemia (mU/L)	91 (57–148)	83 (27–147)	0.54
Height (cm)	1.69 (1.58–1.79)	1.67 (1.5–1.85)	0.37
Weight (kg)	67.1 (48–126)	64.6 (41–90)	0.67
BMI (kg/m <sup>2</sup> )	23.3 (17.7–41)	23.1 (16.2–32)	0.93

Data are presented as mean and range, except duration of diabetes, which is mean and median with range. P values refer to differences between healthy control subjects and patients with type 1 diabetes.

Table 2—Counterregulatory hormone response to hypoglycemia

	Euglycemia	Peak response to hypoglycemia	Comparison eu/hypoglycemia	Response (change from baseline)	Participants with response >3 SDs n/total (percentage)
Glucagon (pg/mL)					
Control subjects	51 (42–59)	96 (87–115)	<0.0001	53 (39–66)	10/12 (83%)
Type 1 diabetes	53 (48–58)	68 (62–74)†	0.027	16 (8–25)	2/28 (7%)
Epinephrine					
Control subjects	32 (13–51)	167 (88–247)	0.03	117 (56–179)	4/5 (80%)
Type 1 diabetes	31 (16–47)	133 (67–199)	0.006	61 (22–99)	4/11 (36%)
Cortisol (mmol/L)					
Control subjects	259 (203–316)	414 (321–508)	0.02	152 (17–286)	0/12
Type 1 diabetes	272 (236–308)	386 (324–447)	0.004	103 (19–188)	3/28 (11%)
Growth hormone (units/L)					
Control subjects	3 (4–9)	32 (5–56)	0.04	29(4–46)	1/12 (8%)
Type 1 diabetes	7 (3–11)	29 (22–36)†	<0.001	19 (3–33)	0/28

Data are presented as mean values with 95% CIs. P values in column 4 refer to differences between euglycemia and hypoglycemia. †Significant difference (P < 0.001) between groups.

group focused on hypoglycemia-associated autonomic failure. Data on the glucagon release suggest a blunted response to hypoglycemia similar to our findings. However, in contrast to our study, this was not compared with a control group of healthy individuals (16).

As has been shown repeatedly in humans (5) and animal models (18), the preservation of the release of glucagon after arginine stimulation in all participants demonstrates that  $\alpha$ -cells of patients with type 1 diabetes maintain their capability to release glucagon.

Importantly, the insulin infusion during clamp studies in itself can negatively impact the response to both hypoglycemia and arginine (19). Liu et al. (20) described that the glucagon response is suppressed by insulin levels of 125 mU/L. In a study by Diamond et al. (21), the glucagon response was negatively affected at

insulin levels >279 mU/L. Mean insulin levels in this study were 91 and 83 mU/L in the control subjects and the type 1 patients with diabetes, respectively, and glucagon responses in the control group were greater after arginine than the hypoglycemic stimulus, suggesting that  $\alpha$ -cell function was not significantly blunted by circulating insulin. Prior to the arginine stimulation, insulin was stopped and normoglycemia established.

The exact mechanisms regulating glucagon secretion in vivo remain to be identified. Several hypotheses have been proposed to explain the blunted response of glucagon release to hypoglycemia in type 1 diabetes patients. Changes to the intraislet insulin concentration as the result of  $\beta$ -cell loss is the most favored concept (22–24). Recent studies focused on the importance of zinc as a cofactor; however, this remains controversial (25,26). Looking at the central detection of hypoglycemia to induce and regulate counterregulatory responses, it appears from rat models that signal transduction in the ventromedial hypothalamus plays an important role. Proposed transmitters include glutamate,  $\gamma$ -aminobutyric acid, and catecholamines (27). They can be directly influenced by local glucose and insulin concentrations (28,29). In addition, the hypothalamus and brainstem might be involved as well (27). Other areas of research include the transcriptional control of pancreatic cell development and of the glucagon gene as well as proglucagon processing (30).

With respect to alternative stimuli of glucagon release, Pörksen et al. (31) conducted a large prospective study on

the effect of a mixed-meal stimulus on glucagon release in adolescents with type 1 diabetes 1, 6, and 12 months after diagnosis. No association with residual  $\beta$ -cell function, age, and sex was noted, but blood glucose and glucagon-like peptide-1 were observed to be influencing factors. Similarly, Brown et al. (32) analyzed the change in glucagon response to a mixed-meal stimulus in young patients on five occasions during the first year after the diagnosis of diabetes. They observed a decline in C-peptide secretion paralleled by an increase in glucagon response over time, also suggesting that the  $\alpha$ -cell secretory reserve had not been affected by the autoimmune process in type 1 diabetes. Neither study included an analysis of the response to hypoglycemia.

Several demographic and clinical factors were identified as predictors of the counterregulatory response to hypoglycemia. Consistent with our findings, the impact of duration of diabetes was reported previously. Most studies focused on adults (5,33,34). Results for children and adolescents vary; a loss of the counterregulatory response was detected as early as at the diagnosis of type 1 diabetes and shortly thereafter (6,35). Singer-Granick et al. (7) demonstrated a positive correlation with age but did not find a correlation between glucagon response and duration of type 1 diabetes in children and adolescents. This may be explained by a significant number of prepubertal participants, indicating a possible effect of puberty that could not be replicated in our group. Unlike Ross et al. (36) and Singer-Granick et al. (7), we did not find a relationship between glycemic control and the preservation

Table 3—Regression analysis

Factor	R	P value (ANOVA)
Duration of diabetes	−0.43	0.008
Weight	0.35	0.029
HbA <sub>1c</sub>	−0.27	0.10
BMI	−0.12	0.47
Fasting C-peptide	−0.12	0.49
Age at study	0.11	0.52
Height	0.09	0.56
Stimulated C-peptide	−0.05	0.78
Insulin level during hypoglycemia	−0.03	0.88

This table includes the detailed regression analysis against the glucagon response to hypoglycemia with R and P values resulting from an ANOVA.

of counterregulation in children and adolescents.

We conclude that adolescents with type 1 diabetes have impaired glucagon responses to hypoglycemia within 12 months of diagnosis. Prospective studies in children and adolescents starting from the onset of type 1 diabetes are needed to further characterize the mechanisms influencing the change over time in the glucagon response to hypoglycemia.

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A.S., T.W.J., and E.A.D. developed the study protocol, researched data, contributed to discussion, and wrote, reviewed, and edited the manuscript. R.J.J. researched data, contributed to discussion, and reviewed and edited the manuscript. M.K.B. contributed to discussion and reviewed and edited the manuscript. P.O. researched data and reviewed and edited the manuscript. E.A.D. 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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