Metformin restores prohormone processing enzymes and normalizes aberrations in secretion of proinsulin and insulin in palmitate-exposed human islets

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Abstract

Aim: To elucidate how proinsulin synthesis and insulin was affected by metformin under conditions of nutrient overstimulation.

Materials and Methods: Isolated human pancreatic islets from seven donors were cultured at 5.5 mmol/L glucose and 0.5 mmol/L palmitate for 12, 24 or 72 h. Metformin (25 μmol/L) was introduced after initial 12 h with palmitate. Proinsulin and insulin were measured. Expression of prohormone convertase 1/3 (PC1/3) and carboxypeptidase E (CPE), was determined by western blot. Adolescents with obesity, treated with metformin and with normal glucose tolerance (n = 5), prediabetes (n = 14), or type 2 diabetes (T2DM; n = 7) were included. Fasting proinsulin, insulin, glucose, 2-h glucose and glycated haemoglobin were measured. Proinsulin/insulin ratio (PI/I) was calculated.

Results: In human islets, palmitate treatment for 12 and 24 h increased proinsulin and insulin proportionally. After 72 h, proinsulin but not insulin continued to increase which was coupled with reduced expression of PC1/3 and CPE. Metformin normalized expression of PC1/3 and CPE, and proinsulin and insulin secretion. In adolescents with obesity, before treatment, fasting proinsulin and insulin concentrations were higher in subjects with T2DM than with normal glucose tolerance. PI/I was reduced after metformin treatment in subjects with T2DM as well as in subjects with prediabetes, coupled with reduced 2-h glucose and glycated haemoglobin.

Conclusions: Metformin normalized proinsulin and insulin secretion after prolonged nutrient-overstimulation, coupled with normalization of the converting enzymes, in isolated islets. In adolescents with obesity, metformin treatment was associated with improved PI/I, which was coupled with improved glycaemic control.

Keywords
carboxypeptidase E, free fatty acids, human islets, insulin, metformin, prohormone convertase 1/3, proinsulin, proinsulin to insulin ratio (PI/I)
1 | INTRODUCTION

The prevalence of overweight and obesity among children is rising with almost 200 million children with obesity worldwide. Complications of obesity, including type 2 diabetes mellitus (T2DM), non-alcoholic fatty liver disease and cardiovascular disorders, can develop already during childhood. This development calls for preventative measures including lifestyle-related interventions and, for those who have already developed complications, effective treatment options. Pharmacological agents proven effective in adult patients are important to evaluate for their safety and efficacy in the paediatric population.

Metformin treatment improves glycaemic control in adult subjects with obesity and prediabetes or T2DM. The effects of metformin have been connected with mechanisms including both the insulin-producing beta-cell and enhanced insulin sensitivity of peripheral tissue. However, in adolescent subjects with obesity and prediabetes or T2DM metformin has shown to be less effective. Previously, we and others showed that metformin prevented accentuated glucose-stimulated insulin secretion from isolated human pancreatic islets exposed to nutrients overstimulation by preventing enhanced metabolism and development of endoplasmic reticulum (ER) stress and apoptosis. These positive effects of metformin to alleviate nutrient-induced beta-cell stress in isolated islets depended on the duration of the exposure to elevated palmitate levels and therefore could involve the ability of the beta-cell to process properly the proinsulin to insulin.

Prohormone convertase subtilisin/kexin type 1 (PC1/3) is expressed in different neuroendocrine cells including the pancreatic alpha- and beta-cells. Together with the chaperone protein carboxypeptidase E (CPE), PC1/3 contributes to the processing of proinsulin to insulin in human pancreatic beta-cells. Mutations in PCSK1 coding for PC1/3 and CPE coding for CPE have been connected with obesity and glucose intolerance, along with increased proinsulin levels. However, how proinsulin and insulin secretion from islets are affected under conditions of elevated palmitate levels at normoglycaemia, which is coupled with accentuated glucose-stimulated insulin secretion, and how metformin affects the proinsulin and insulin secretion under these conditions have not been determined. In addition, adult subjects with obesity and prediabetes or T2DM metformin treatment was accompanied by reduced fasting concentration of proinsulin, which was greater than reduction in fasting insulin, and could imply improved processing of proinsulin to insulin. In addition, fasting proinsulin and insulin levels were not determined in adolescent subjects with obesity and prediabetes or T2DM, in whom metformin showed no effect. In the present study, we therefore investigated how metformin treatment affected proinsulin and insulin concentrations from isolated human pancreatic islets treated with palmitate and in adolescents with obesity.

2 | MATERIALS AND METHODS

2.1 | Human pancreatic islets

Islets from brain-dead, metabolically healthy human donors were supplied from either the Nordic Network for Clinical Islets Transplantation, Uppsala University Hospital, Uppsala or Prodo Laboratories (Prodo Laboratories Inc.) (Table S1). The former islets were cultured for 2-7 days in Connaught Medical Research Laboratories (CMRL) 1066 medium (Invitrogen) supplemented with 10% foetal bovine serum (Invitrogen), 1% glutamine (Invitrogen), 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen) at 37°C in humidified air containing 5% CO2 before further culture and treatment. The latter islets were cultured in islet specific medium (Prodo Laboratories Inc.) for 2 days before further culture and treatment.

2.2 | Free fatty acids and metformin preparation

Palmitate was prepared as previously described. Briefly, palmitate (Sigma-Aldrich) was dissolved in 50% ethanol to a concentration of 100 mmol/L. Final concentration of 0.5 mmol/L palmitate solution was obtained by diluting the stock solution in culture medium with 0.5% fatty acid free bovine serum albumin (Sigma-Aldrich) at 37°C for 60 min. Metformin (Sigma-Aldrich) was prepared from stock solution (25 mmol/L) diluted in CMRL culture medium (Invitrogen), as previously described. Final concentration of 25 μmol/L was obtained by diluting the stock solution in culture medium.

2.3 | Islet culture and treatment

Isolated human islets were collected and cultured in CMRL (Invitrogen) supplemented with 5.5 mmol/L glucose and with 0.5 mmol/L palmitate for 12, 24 or 72 h. Metformin was introduced after 12 h. Approximately 30 islets were hand-picked for each culture condition.

2.4 | Proinsulin and insulin measurements

Human islets were transferred 12 h before termination of the culture period to new Eppendorf tubes filled with 200 μl fresh culture medium, which contained the same concentrations of palmitate and metformin as during the initial culture period. At the end of the culture period samples of culture media were collected and proinsulin and insulin concentrations were determined by enzyme-linked immunosorbent assay (ELISA) as described by the manufacturer (Mercodia) and normalized to total protein of islets lysates. A detergent compatible protein assay (Bio-Rad) according to the Lowry method was used to measure total protein in the lysates.

2.5 | Western blot

To determine the levels of proinsulin processing enzymes, western blot was performed on human islets. After culture, islets were washed with Dulbecco’s phosphate-buffered saline (DPBS) (Gibco) and lysed in DPBS containing 1% Triton X100 (Sigma-Aldrich). Islets were
subsequently lysed and reduced with 50 mmol/L dithiothreitol (Sigma-Aldrich) and mixed with loading buffer supplemented with 0.05% bromophenol blue dye (Sigma-Aldrich), 10% sodium dodecyl sulphate (Sigma-Aldrich), 50% glycerol (Sigma-Aldrich) and 0.313 mol/L Tris-HCl (Sigma-Aldrich). Prepared proteins were separated by 8.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to PVDF membrane (Bio-Rad). After blocking, PVDF membrane with proteins were incubated in rabbit anti-PC1/3 (Cell Signaling), rabbit anti-CPE (Invitrogen) and mouse actin antibodies (Invitrogen) overnight at 4°C. Immunoreactive bands were detected by the Fluor-S Multimager MAX (Bio-Rad) and quantified with Quantity One software (Bio-Rad).

2.6 Childhood obesity study

We retrospectively studied the medical charts of participants in the ULSCO childhood obesity cohort. The inclusion criteria were: (a) treatment with metformin for at least 6 months, (b) oral glucose tolerance test conducted before and after metformin treatment, (c) body mass index standard deviation score ≥2. Exclusion criteria were: (a) other endocrine or metabolic disease, (b) poor clinical compliance to treatment, as determined from the medical journals by a physician working clinically with the ULSCO cohort, (c) treatment with other anti-glycaemic, anti-obesity or other medications that could influence glucose homeostasis.

Included patients were stratified as having normal glucose tolerance (NGT), prediabetes or T2DM according to plasma levels of fasting and/or 2-h glucose. Adolescents with obesity with impaired fasting glucose and/or impaired glucose tolerance (IGT) were included in the prediabetes group. NGT, impaired fasting glucose, IGT and T2DM were defined according to WHO criteria.

2.7 Blood sampling and analyses

Oral glucose tolerance test was performed and venous blood samples were collected at fasting, and 5, 10, 15, 30, 60, 90 and 120 min after glucose ingestion. Glycated haemoglobin (HbA1c) was measured at Uppsala University Hospital laboratory in accordance with local standard operation procedure. Blood glucose levels were determined by glucose oxidation method (Architect c8000 instrument; Abbott Diagnostics). Fasting proinsulin and insulin were measured by the ELISA according to the manufacturer's instructions (Merodia).

2.8 Ethics

Written informed consent was obtained from all study participants and their legal guardians. The study was performed in accordance with the Declaration of Helsinki. The local regional ethics committee approved the clinical study (EPN 2012/318). Ethical permission to study human islets was obtained from the local regional ethics committee (EPN 2010/006).

2.9 Calculations and statistical analyses

Data were presented as mean ± SEM unless stated otherwise. The material was tested for normal distribution using the Shapiro-Wilk test. The differences between the groups were analysed by one-way ANOVA, with post hoc analyses using Šidák’s multiple comparisons test. Oral disposition index (oDI), an index of beta-cell function adjusted for insulin sensitivity, was calculated as (Insulin_{30} – Insulin_{0})/(Glucose_{30} – Glucose_{0}) × 1/fasting insulin. Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated as fasting insulin (μIU/L) × fasting glucose (mmol/L)/22.5. The proinsulin to insulin ratio (PI/I) was calculated as concentration of proinsulin (pmol/L) divided by the concentrations of insulin (pmol/L) measured in venous blood samples from patients and in accumulated supernatants from islets. The difference before and after metformin treatment was assessed by paired Student’s t-test, when following the Gaussian distribution, or Wilcoxon signed-rank test, when not. Simple and multiple linear regression was used to analyse the correlation either between PI/I and 2 h-glucose or between PI/I and HbA1c. \( p < 0.05 \) was considered statistically significant.

3 RESULTS

3.1 Metformin normalizes proinsulin and insulin secretion from palmitate-exposed human islets

In isolated human pancreatic islets, palmitate treatment at 5.5 mmol/L glucose for 12, 24 and 72 h significantly increased proinsulin concentrations compared with control (Figure 1A). After 72 h of palmitate treatment, proinsulin concentrations were almost three-fold higher compared with control (Figure 1A). During this period of nutrient overstimulation with elevated palmitate concentrations, insulin concentrations almost doubled initially, when culturing with palmitate for up to 24 h (Figure 1B). After prolonged culture for 72 h in the presence of palmitate, insulin did not further increase (Figure 1B). Consequently, PI/I remained stable after 12 and 24 h of palmitate treatment but increased almost two-fold (\( p < 0.05 \)) after 72 h compared with control (Figure 1C). Treatment with metformin for 60 h after an initial 12-h period with palmitate treatment normalized proinsulin (Figure 1A), insulin (Figure 1B) and PI/I (Figure 1C) in isolated islets exposed to palmitate. Metformin alone did not affect proinsulin and insulin concentrations or PI/I (Figure 1A,B,C, respectively).

3.2 Metformin normalizes prohormone convertase 1/3 and carboxypeptidase E expression in palmitate-exposed human islets

To understand the mechanisms by which metformin normalized proinsulin and insulin concentrations, expression levels of PC1/3 and CPE
FIGURE 1  Metformin attenuated palmitate-induced increment of (A) proinsulin, (B) insulin secretion, per islet protein amount, and the (C) PI/I ratio from isolated human islets of Langerhans. Islets were cultured in the absence (C/Control) (black bar) or presence of palmitate alone (white bars) for 12 (P12), 24 (P24), or 72 (P72) h, in combination with metformin (25 μmol/L) (dark grey bars) for 60 h after an initial 12 h of palmitate (P72M60) or cultured in presence of metformin alone (light grey bars) for 60 h (M60) at basal (5.5 mmol/L) glucose. Results are presented as means ± SEM, n = 7 separate experiments, *p < .05 compared with control, #p < .05 compared with P72. PI/I ratio, proinsulin to insulin ratio.

FIGURE 2  Metformin normalized PC1/3 and CPE expression in palmitate-exposed human islets. (A,C) PC1/3 and (B,D) CPE levels were measured in human islets cultured in the absence (C/Control) (black bar) or presence of palmitate (white bars) for 72 h (P72). Metformin (25 μmol/L) (dark grey bars) was introduced for 60 h after an initial 12 h of palmitate (P72M60) or alone (light grey bars) for 60 h (M60) at basal (5.5 mmol/L) glucose. (A,B) Quantification of Western blots of PC1/3 (n = 6 separate experiments) and CPE (n = 7 separate experiments), respectively, normalized to actin and expressed as fold of control. (C,D) Representative examples of PC1/3 and CPE Western blots, respectively. Results are presented as means ± SEM, *p < .05, **p < .01 compared with control, #p < .05 compared with P72. CPE, carboxypeptidase E; PC1/3, prohormone convertase 1/3.

TABLE 1  Clinical characteristics of adolescents with obesity with varying glucose tolerance

<table>
<thead>
<tr>
<th></th>
<th>NGT (n = 5)</th>
<th>Prediabetes (n = 14)</th>
<th>T2DM (n = 7)</th>
<th>p value</th>
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</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>13.6 ± 1.51</td>
<td>14.1 ± 1.89</td>
<td>14.1 ± 1.84</td>
<td>0.88</td>
</tr>
<tr>
<td>Duration of treatment, years</td>
<td>1.39 ± 0.74</td>
<td>1.25 ± 0.72</td>
<td>1.11 ± 0.64</td>
<td>0.80</td>
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<tr>
<td>Dosage, mg/kg</td>
<td>17.3 ± 5.8</td>
<td>13.8 ± 4.3</td>
<td>15.5 ± 3.0</td>
<td>0.33</td>
</tr>
<tr>
<td>BMI-SDS</td>
<td>3.2 ± 0.3</td>
<td>3.2 ± 0.3</td>
<td>2.9 ± 0.5</td>
<td>0.33</td>
</tr>
<tr>
<td>Sex, female; n (%)</td>
<td>2 (40)</td>
<td>2 (14)</td>
<td>2 (29)</td>
<td>0.48</td>
</tr>
<tr>
<td>Pubertal status, n (%)</td>
<td>2 (40)</td>
<td>3 (21)</td>
<td>1 (14)</td>
<td>0.58</td>
</tr>
</tbody>
</table>

Note: Adolescents with obesity with NGT, prediabetes or T2DM treated with metformin. Data are shown as number of cases in indicated categories, number of cases (proportion %) or as mean ± SD.

Abbreviations: BMI-SDS, body mass index standard deviation score; NGT, normal glucose tolerance; T2DM, type 2 diabetes mellitus.
were measured in human pancreatic islets in the presence or absence of palmitate with or without metformin. Palmitate exposure reduced PC1/3 and CPE expression by approximately 35% and 50%, respectively, compared with control (Figure 2A,B). Introduction of metformin to the culture medium normalized both PC1/3 and CPE proteins levels (Figure 2A,B). Metformin alone did not alter the expression levels of PC1/3 or CPE (Figure 2A,B).

3.3 Adolescents with obesity treated with metformin showed lower fasting proinsulin/insulin ratio and 2-h glucose levels

The patients who had NGT ($n = 5$), prediabetes ($n = 14$) or T2DM ($n = 7$) before metformin treatment were not significantly different regarding age, sex, body mass index standard deviation score, pubertal

![Graphs showing changes in fasting proinsulin, insulin, PI/I, glucose, HbA1c, oDI, and HOMA-IR before and after metformin treatment.](https://dom-pubs.pericles-prod.literatumonline.com/doi/10.1111/dom.15270)

**FIGURE 3** In adolescents with obesity and NGT ($n = 5$), prediabetes ($n = 14$) or T2DM ($n = 7$) plasma levels of (A) fasting proinsulin, (B) fasting insulin, (C) fasting PI/I, (D) fasting glucose, (E) 2-h glucose, (F) HbA1c, (G) oDI and (H) HOMA-IR were measured before (white bars) and after (grey bars) metformin treatment. Results are presented as means ± SEM. * $p < .05$ compared with NGT before metformin treatment, § $p < .05$ compared with prediabetes before metformin treatment, $\Psi p < .05$ compared with before metformin treatment within groups. HbA1c, glycated haemoglobin; HOMA-IR, homeostatic model assessment of insulin resistance; NGT, normal glucose tolerance; oDI, oral disposition index; T2DM, type 2 diabetes mellitus.
status, metformin doses or duration of the treatment (Table 1). Before treatment, fasting proinsulin in adolescents with obesity and T2DM was five times higher ($p < .05$) than the prediabetes group, and eight times higher than the NGT group, although not significant ($p = .08$) (Figure 3A). Similar differences were also observed for fasting insulin among the groups (Figure 3B). Fasting PI/I was not different among the varying glucose tolerance groups (Figure 3C). Fasting glucose (Figure 3D), 2-h glucose (Figure 3E) and HbA1c (Figure 3F) levels were significantly higher in patients with T2DM than patients with prediabetes as well as children with NGT, as expected. In the T2DM group oDI was lower ($p < .01$) (Figure 3G) and HOMA-IR was higher (Figure 3H) than the other groups.

After metformin treatment, fasting PI/I decreased 18% in children with prediabetes and 52% in children with T2DM (Figure 3C). In addition, 2-h glucose was reduced in patients with prediabetes by 1.0 mmol/L and with T2DM by 3.7 mmol/L (Figure 3E). HbA1c decreased significantly in patients with T2DM (Figure 3F). Fasting glucose (Figure 3D), oDI (Figure 3G), and HOMA-IR (Figure 3H) were not changed significantly following treatment.

3.4 | Reduction of proinsulin/insulin ratio correlated with improvement of 2-h glucose and glycated haemoglobin

As PI/I was reduced after metformin treatment in patients with prediabetes and T2DM (Figure 3C), we investigated the correlations between the changes in PI/I ($\Delta$PI/I) and 2-h glucose ($\Delta$2-h glucose) and HbA1c ($\Delta$HbA1c) in these patients. Positive correlations were observed between $\Delta$PI/I and $\Delta$2-h glucose ($r^2 = 0.2$, $p = .03$) (Figure 4A), as well as between $\Delta$PI/I and $\Delta$HbA1c ($r^2 = 0.3$, $p = .01$) (Figure 4B). Correlations persisted when correcting for age and sex between $\Delta$PI/I and $\Delta$2-h glucose ($R^2 = .2$, $\beta = 11.5$, $p = .04$), and $\Delta$PI/I and $\Delta$HbA1c ($R^2 = 0.3$, $\beta = 37.8$, $p = .03$).

4 | DISCUSSION

In the present study, in isolated human pancreatic islets, nutrient overstimulation, in the form of elevated palmitate in normal glucose concentrations, caused an increase in proinsulin and insulin secretion. Prolonged exposure led to continued proinsulin rise without matching insulin increase due to reduced proinsulin-to-insulin processing enzymes. Metformin restored enzyme expression, normalizing proinsulin and insulin. In adolescents with obesity, metformin reduced PI/I, linked to lowered 2-h glucose and HbA1c.

The relation between proinsulin and insulin levels, PI/I, in islet culture medium depends on the performance of the beta-cell to process proinsulin to insulin, as the degradation of proinsulin and insulin is minimal under culture conditions. In this process the prohormone processing enzymes are key components. At normal glucose concentrations, proinsulin concentrations account for 2%-6% of the total secreted insulin plus proinsulin from isolated islet cells observed in this study and in previous studies. Increased proinsulin and insulin secretion from human pancreatic islets in response to elevated palmitate at normal glucose concentrations were previously reported. Free fatty acids (FFAs) are important modulators of insulin secretion from the beta-cell. PC1/3 and CPE are key endoproteolytic processing enzymes of proinsulin to insulin, and are mainly expressed in beta-cells of the human islet. In this study, we found that both CPE and PC1/3 expression were downregulated after prolonged palmitate treatment, which is in accordance with our previous results. Delayed processing of PC1/3 as well as significant reduction of CPE were found in long-term FFA-exposed MIN6 cells and human islets. In addition, FFAs affected proinsulin synthesis and processing to insulin by regulating transcription factors PDX1 and SREBP1, which were also involved in the regulation of PC1/3 and CPE.

Metformin has direct effects on pancreatic islets in addition to improving insulin sensitivity in peripheral tissues. The direct effects of metformin include protecting beta-cells from lipotoxicity, nutrient stress and suppressing palmitate-induced ER stress markers and reactive oxygen species levels in an AMPK-independent
manner. In addition to these effects of metformin on the beta-cell, the present study showed that metformin normalized the proinsulin processing enzymes PC1/3 and CPE, which were downregulated in human islets exposed to palmitate. Although mechanisms of metformin’s effects on these two enzymes are yet to be defined, the beneficial effects of metformin on ER homeostasis might be a plausible explanation. In addition, in obese and high-fat diet mice, metformin reduced lipogenesis by suppressing elevated mRNA expression of SREBP1, which regulate CPE expression. Furthermore, activation of the GLP-1 receptor upregulated levels of PC1/3 and PC2 in MIN6 cells via the CAMP-PKA signalling pathway. Metformin enhanced the expression of GLP-1 receptor on mouse islets. Therefore, metformin might indirectly stimulate PC1/3 expression via the GLP-1 receptor although this needs to be studied further.

Hyperproinsulinaemia is commonly observed in adult subjects with obesity with or without glucose intolerance or diabetes. In adults with obesity and T2DM, elevated plasma proinsulin or disproportionate proinsulin to insulin levels were reported as a marker for beta-cell dysfunction. In agreement with our results, elevated proinsulin levels at fasting have been observed and coupled to beta-cell dysfunction in children and adolescents with obesity and reported to be further increased in subjects with IGT and T2DM. To have an indication of how proinsulin to insulin processing is affected, proinsulin levels have to be compared with insulin levels, that is, determining PI/I. In youth with obesity and T2DM proinsulin and insulin were both elevated leaving PI/I unaffected, as were also found in the present study, and suggested that the beta-cell processing of proinsulin to insulin may still be functional.

Interestingly, we observed a decrease in PI/I following metformin treatment in subjects with prediabetes and T2DM, which was coupled with improved glucose tolerance. The improved glucose tolerance particularly in the prediabetes group supports the potential role of metformin to prevent disease progression and further strengthens our hypothesis that decreasing the beta-cell workload is beneficial.

In the clinical study, the main limitation was the limited number of patients available for inclusion in the study, and the retrospective study design. In the islet study, a main limitation was the age of the donors. Whereas the islets used in the study were isolated from adults, the clinical study was conducted in children/adolescents. Although, the in vitro study cannot directly translate into the clinical setting or vice versa, it informs relevant mechanisms. Another limitation of this study was that the ELISA method to measure proinsulin not only detects the intact proinsulin but also detects the split versions of proinsulin. Of note is that elevated levels of both the intact and split forms of proinsulin were associated with T2DM.

In conclusion, our study showed that metformin in isolated human islets normalized palmitate-induced elevated proinsulin and insulin secretion coupled with normalizing PC1/3 and CPE expression. In adolescents with obesity and prediabetes or T2DM, we observed a reduction in fasting PI/I following treatment with metformin, which was accompanied by improved 2-h glucose and HbA1c levels.

**AUTHOR CONTRIBUTIONS**

AIC and QW performed the experiments on human islets. RS and QW collected clinical data from medical journals. PB, AIC, and QW contributed to the first draft of manuscript QW, AIC, BA, MD, RS, AF, and PB took part in editing and approving the final version of the manuscript. QW performed all data analyses and together with PB take responsibility for the accuracy of the data analyses. QW is the guarantor of the study.

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**CONFLICT OF INTEREST STATEMENT**

The authors have no conflicts of interest to declare.

**DATA AVAILABILITY STATEMENT**

The datasets generated during and/or analyzed in the current study are available from the corresponding author upon reasonable request.

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**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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