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Early loss and progressive deterioration of glucagon responses to hypoglycemia in children with type 1 diabetes

Antoine Harvengt,[1] Maude Beckers,[1,2] Ilektra Iakovidou,[1] Anaïs Maure,[1] Zain Syed,[1] Laure Boutsen,[2,3] Dominique Beckers,[3] Philippe A Lysy [1,2]

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[1]Pôle EDIN, UCLouvain, Woluwé Saint Lambert, Belgium
[2]Specialized Pediatrics Service, Cliniques universitaires Saint-Luc, Brussels, Belgium
[3]Pediatric Service, CHU UCL Namur, Yvoir, Walloon Region, Belgium

Correspondence to
Professor Philippe A Lysy; philippe.lysy@uclouvain.be

ABSTRACT

Introduction Impaired glucagon counterregulation is a major contributor to hypoglycemia risk in type 1 diabetes (T1D). While well described in adults, the timing, trajectory, and determinants of glucagon dysfunction in children remain poorly defined. Our objective was to characterize the evolution of glucagon secretion during insulin-induced hypoglycemia in children with newly diagnosed T1D and to identify biomarkers of counterregulatory failure.

Research design and methods GLUcagon REsponse to hypoglycemia in children and adolescents with new-onset type 1 DIAbetes (GLUREDIA) - Work Package (WP) 1 is a prospective, multicenter longitudinal study including 22 children and adolescents (aged 2–17 years) with newly diagnosed T1D and eight healthy first-degree relatives. Participants underwent standardized insulin-induced hypoglycemia tests (IIHTs). In patients with T1D, IIHTs were performed at diagnosis and at 6, 12, and 18 months. Plasma glucagon and counterregulatory hormones were measured at baseline and during hypoglycemia. Continuous glucose monitoring (CGM) metrics and circulating biomarkers were analyzed using longitudinal mixed models and regression analyses.

Results In healthy participants, IIHT elicited a robust physiological increase in glucagon during hypoglycemia ($p<0.001$). In contrast, children with T1D showed no significant glucagon response despite deeper hypoglycemia. Longitudinally, glucagon responsiveness was absent at diagnosis, transiently improved at 6 months ($p=0.04$), absent at 12 months, and paradoxically suppressed at 18 months ($p=0.02$). Basal glucagon levels remained stable over time. Glucagon responses correlated positively with CGM time-in-range (70–180 mg/dL) and negatively with glycemic variability and hypoglycemia frequency. A CGM-based logistic model integrating these metrics predicted impaired glucagon responses with good discrimination (area under the curve 0.79). Circulating glucose-dependent insulinotropic polypeptide, glucagon-like peptide-1, tumor necrosis factor-α, and interferon-γ correlated positively with glucagon secretion, whereas partial remission status did not.

Conclusions Glucagon counterregulation is profoundly impaired early in pediatric T1D and deteriorates dynamically within the first 18 months after diagnosis. Glycemic instability and recurrent hypoglycemia are key determinants of α-cell dysfunction. CGM-derived metrics may serve as non-invasive surrogates of impaired counterregulation and support early risk stratification for hypoglycemia in children with T1D.

Trial registration number NCT06770621.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Impaired glucagon-mediated counterregulation is a major determinant of severe hypoglycemia in type 1 diabetes.

⇒ Although this defect has been extensively described in adults, its onset, longitudinal evolution, and determinants in children and adolescents remain poorly characterized.

WHAT THIS STUDY ADDS

⇒ This prospective longitudinal study demonstrates that glucagon responses to hypoglycemia are already impaired at diagnosis in pediatric type 1 diabetes and deteriorate during the first 18 months after diagnosis.

⇒ Glycemic instability and recurrent hypoglycemia are associated with impaired glucagon secretion, and continuous glucose monitoring (CGM)-derived metrics predict defective counterregulation with good discriminatory performance.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ These findings support the use of CGM-derived digital biomarkers to identify children at increased risk of impaired counterregulation and severe hypoglycemia.

⇒ They also provide a rationale for prospective validation of glucagon-based biomarkers and precision risk-stratification strategies in pediatric type 1 diabetes.

INTRODUCTION

The role of glucagon in glucose homeostasis is well established: secreted by pancreatic α cells, it provides the main defense against falling glycemia by stimulating hepatic glycogenolysis and gluconeogenesis.[1] In type 1 diabetes mellitus (T1DM), this counterregulatory mechanism becomes profoundly impaired early in the disease, leading to a

blunted glucagon response to hypoglycemia and an increased risk of severe hypoglycemia (SH).[2 3] Paradoxically, patients also show inappropriate postprandial hyperglucagonemia, reflecting broader α-cell dysregulation.[2] While these abnormalities are well described in adults, their evolution in children remains less clearly defined.

Importantly, several pediatric studies demonstrate that α-cell dysfunction emerges early, although systematic longitudinal data are lacking. A 24-month prospective study in 21 youth with new-onset T1DM documented progressive abnormalities in mixed-meal glucagon responses during the first 2 years after diagnosis.[4] Cross-sectional hypoglycemia-induction studies similarly show that adolescents may already exhibit a markedly reduced glucagon response within the early disease phase.[5] Long-term follow-up data further support the persistence of dysregulated postprandial secretion: in a 5-year cohort of 40 children, Fredheim *et al* reported an increase in postmeal glucagon over time, associated with higher glycemic excursions and declining C-peptide.[6] Together, these studies highlight that altered glucagon dynamics are present in children, yet they provide only fragmented insight into the timing, rate, and determinants of deterioration of the counterregulatory response.

Mechanistically, the selective loss of the hypoglycemia-induced glucagon response, despite partial preservation of responses to amino acids, exercise, or insulin withdrawal,[2] remains incompletely explained. Proposed contributors include disruption of intra-islet paracrine signaling due to β-cell loss,[7–9] somatostatin hypersecretion from δ cells,[10 11] or altered autonomic regulation,[12] but these hypotheses are derived largely from adult or animal data and may not generalize to developing islets in children.[13]

Children and adolescents with T1DM represent a particularly vulnerable population, given their elevated risk of SH and the potential cumulative impact on neurocognitive development.[14] However, comprehensive longitudinal studies assessing the evolution of glucagon secretion in pediatric T1DM remain limited by small sample sizes, inconsistent hypoglycemia-induction methodologies, and insufficient long-term follow-up.

In this context, our project (called GLUcagon REsponse to hypoglycemia in children and adolescents with new-onset type 1 DIAbetes (GLUREDIA) - Work Package (WP) 1) aimed to prospectively characterize the trajectory of the glucagon response to controlled hypoglycemia in newly diagnosed children and adolescents with T1DM. By inducing standardized hypoglycemia at predefined intervals after diagnosis, we sought to define the kinetics of loss of counterregulatory function and identify clinical, metabolic, and hormonal predictors of its deterioration. This study leverages rigorous hormonal profiling, detailed metabolic assessments, and longitudinal analytics to address a critical gap in the understanding of α-cell dysfunction in pediatric T1DM.

MATERIALS AND METHODS

Study design

The GLUREDIA-WP1 study is a prospective, longitudinal, multicenter clinical study designed to characterize specific clinical and biological reactions to hypoglycemia in both children and adolescents with T1DM and healthy first-degree relatives (ClinicalTrials.gov identifier: NCT06770621). The study was conducted at the Cliniques universitaires Saint Luc (CUSL, Brussels, Belgium) and the Centre Hospitalier Universitaire UCL Namur, including both the Sainte-Elisabeth site (Namur) and the Mont-Godinne site (Yvoir, Belgium). Patient enrollment began in June 2022 and was completed in June 2024.

Participants

The study (called GLUREDIA-WP1) included two distinct cohorts: a cohort of children and adolescents with T1DM, and a cohort of healthy first-degree relatives of patients with T1DM.

Inclusion criteria for the diabetic cohort were patients aged between 2 and 17 years, with a diagnosis of T1DM according to the American Diabetes Association criteria, defined as: fasting blood glucose ≥126 mg/dL and/or blood glucose ≥200 mg/dL at 120 minute during an oral glucose tolerance test and/or glycated hemoglobin (HbA_{1c}) ≥6.5% and/or symptoms of hyperglycemia/hyperglycemic crisis with random glucose ≥200 mg/dL, in the presence of at least one positive anti-islet autoantibody (ie, anti-insulin, anti-IA2, anti-GAD65, anti-ZnT8).[15]

Inclusion criteria for the healthy cohort were adults aged over 18 years, first-degree relatives of patients with T1DM, and absence of diabetes markers (ie, negative for anti-islet autoantibodies, HbA_{1c} <5.7%, C-peptide >0.18 nmol/L, fasting glucose <100 mg/dL, and random glucose <200 mg/dL). Both male and female participants were eligible.

Exclusion criteria for both cohorts included use of medications interfering with insulin secretion or sensitivity (eg, sulfonylureas, diazoxide, somatostatin, methylxanthine derivatives, corticosteroids, biguanides, incretins); recent diagnosis (within 1 month) of celiac disease confirmed by pathological duodenal biopsy; presence, at inclusion, of autoimmune or autoinflammatory disease or active malignancy; morbid obesity (body mass index (BMI) Z-score >+3 SD)[16]; hepatic, renal, or adrenal insufficiency; history of bone marrow transplantation; diabetes secondary to hemolytic-uremic syndrome; ischemic cardiomyopathy; epilepsy; pregnancy at the time of inclusion; or participation in another study in the previous 3 months involving blood derivatives or immunomodulatory treatments.

Study procedure

The GLUREDIA prospective study was designed to evaluate the counterregulatory response, particularly glucagon secretion, during insulin-induced hypoglycemia in children and adolescents with newly diagnosed

T1DM, as well as in healthy participants. The study was conducted in three successive phases.

In the first phase, an insulin-induced hypoglycemia test (IIHT) was performed in healthy participants to assess the reliability and safety of the procedure to induce hypoglycemia and to observe a significant physiological increase in glucagon secretion. In the second phase, the same IIHT protocol was applied to children and adolescents newly diagnosed with T1DM. The objective was to evaluate and compare glucagon responses between diabetic patients and healthy controls. The third phase consisted of a longitudinal follow-up of patients with newly diagnosed type 1 diabetes. IIHTs were repeated at 0, 6, 12, and 18 months postdiagnosis to assess the evolution of glucagon secretion over time.

Throughout the study, clinical, glycemic, and biological parameters were collected and analyzed to comprehensively characterize glucagon production dynamics during hypoglycemia.

Insulin-induced hypoglycemia test protocol

This section describes the conditions and standardized procedure for the IIHT used in our study. The day before the test, participants fasted for at least 12 hours and followed a standardized diet. On the day of the test, they rested in a quiet environment for at least 30 min before the test began. A baseline venous blood sample was collected before insulin administration. A catheter was inserted into a superficial vein in the forearm to allow blood sampling. Subcutaneous insulin was administered at an initial dose of 0.1 U/kg, adjusted according to fasting blood glucose (±1 U per 100 mg/dL), with a final dose between 0.05 and 0.15 U/kg. Capillary blood glucose was monitored every 15 min. When capillary blood glucose fell below 54 mg/dL, a second venous blood sample was taken to analyze the hormonal and metabolic response to hypoglycemia. If this value was not reached, the sample was taken at the time of the glycemic plateau, if blood glucose was below 70 mg/dL. The test was contraindicated in cases of SH (<54 mg/dL or neuroglycopenic symptoms) or intense physical activity in the previous 48 hours.

Biochemical analyses

Blood samples collected during the IIHT were used for dynamic quantification of multiple hormonal and metabolic biomarkers. Glucagon levels were measured using a specific ELISA kit (Mercodia) in our research laboratory. Growth hormone, cortisol, catecholamines (epinephrine and norepinephrine), insulin, C-peptide, and arginine-vasopressin were all analyzed within the clinical biochemical platform of CUSL. Cortisol and catecholamines were quantified using liquid chromatography-tandem mass spectrometry at the Clinical Laboratory of CUSL. Growth hormone was measured using a chemiluminescent immunoassay (Liaison XL hGH, DiaSorin). Additional biomarkers, including glucagon-like peptide-1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), somatostatin, glicentin, copeptin, and other peptide hormones, were analyzed using multiplex immunoassays (MSD, Meso Scale Discovery; U-Plex and R-Plex platforms), allowing simultaneous high-sensitivity detection of multiple analytes.

Continuous glucose monitoring metrics

Raw continuous glucose monitoring data from a 90-day interval were extracted at each outpatient clinical visit from various continuous glucose monitoring devices (ie, FreeStyle Libre, Abbott; Dexcom, Dexcom; Enlite, Medtronic MiniMed).

Partial remission

Partial remission in T1DM is a transient period following diagnosis during which exogenous insulin requirements decrease significantly while maintaining good glycemic control due to a temporary recovery of endogenous insulin secretion. In our study, we defined partial remission by insulin dose-adjusted A1c=HbA_{1c}+(4×total daily insulin dose), where a score below or equal to 9 defines remitters and a score above 9 defines non-remitters.[17]

Statistical analysis

All statistical analyses were performed using R (R Core Team, 2021. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria (https://www.R-project.org/)). The statistical significance level used for all analyses was 0.05. Demographic and clinical data were reported as mean±SD for continuous variables and as numbers and proportions for categorical variables. Normality and homogeneity of variance assumptions were systematically assessed before statistical analyses. Depending on the results of these preliminary tests, group comparisons were performed using Student's t-test, ANOVA, and χ^2 test (or their non-parametric equivalents, the Mann-Whitney U and Kruskal-Wallis test, where appropriate. Linear regression (or Spearman's rank correlation) was used to assess continuous associations. P values were adjusted for multiple testing with the Bonferroni procedure.[18] Marginal R^2 (coefficient of determination) was calculated using generalized linear mixed models,[19] with R packages lme4[20] and lmerTest[21] to consider multiple measurements from the same patient.

RESULTS

The first phase of the study involved performing the IIHT in a cohort of eight healthy adult participants, all first-degree relatives of children or adolescents with T1DM enrolled in the diabetic arm of the study (*healthy cohort*). The cohort had a mean age of 45.5±5.5 years and included a majority of female participants (five out of eight). All individuals were free from metabolic or endocrine disorders (including stage 1 or stage 2 T1DM). HbA_{1c} levels were below 5.7% in all participants and anthropometric parameters (weight, height, and BMI) were within normal reference ranges. Following subcutaneous

insulin administration, all participants developed grade 1 asymptomatic hypoglycemia (blood glucose <70 mg/dL). No case of grade 2 hypoglycemia (blood glucose <54 mg/dL) was observed. Notably, all individuals spontaneously recovered from hypoglycemia without the need for exogenous glucose or glucagon administration.

Paired analysis of glycemic and hormonal parameters demonstrated a decrease in glycemia between baseline (T0) and the hypoglycemic nadir (84.9±7.9 vs 65.1±2.2 mg/dL; p<0.001), an increase in plasma glucagon concentrations (3.7±2.2 vs 5.6±2.3 pM; p<0.001), and a reduction in insulin levels (54.4±48.4 vs 23.6±28.7 pM; p=0.04). Detailed results of the IIHT are presented in table 1. These findings confirm the ability of the IIHT protocol to safely and effectively induce hypoglycemia and elicit a measurable and appropriate glucagon response.

The second phase of the study involved conducting IIHTs in a cohort of 22 children and adolescents newly diagnosed with T1DM (*T1DM cohort,* table 2). Each participant underwent the IIHT at four time points: at diagnosis (month 0), and at 6, 12, and 18 months postdiagnosis. In over 95% of the tests performed, grade 2 hypoglycemia (ie, blood glucose <54 mg/dL) was successfully induced. Only two tests were prematurely interrupted due to the onset of symptomatic grade 1 hypoglycemia (blood glucose between 55 and 57 mg/dL) with patients exhibiting significant autonomic symptoms (notably severe vomiting and intense tremors), necessitating early termination of the procedure.

Paired analysis of glycemic profiles consistently demonstrated a reduction in glycemia between baseline and hypoglycemia (111.9±20.9 vs 50.2±4.6 mg/dL; p<0.001). Moreover, we did not observe any variation in insulin levels (76.5±89.5 vs 79.4±84.7 pM; p=0.96). However, unlike in healthy controls, no increase in plasma glucagon concentrations was observed across time points in the T1DM cohort (4.2±2.0 vs 4.7±2.7 pM; p=0.68). Detailed results of the IIHT are presented in table 3. Finally, we assessed whether partial remission influenced glucagon secretion. In both remitters (baseline vs hypoglycemia: 4.6±2.0 vs 5.4±2.2 pM; p=0.65) and non-remitters (4.1±2.0 vs 4.4±2.8 pM; p=0.21), plasma glucagon concentrations remained unchanged during hypoglycemia. Likewise, the magnitude of glucagon change did not differ between groups (0.3±3.0 vs 0.9±2.1 pM; p=0.52).

In the next phase of the analysis, we compared the biological responses during IIHT between the healthy cohort and the T1DM cohort. For each participant, we calculated the delta variation between baseline and the hypoglycemic time point for all measured biochemical parameters, and these variations were compared between cohorts. Glycemia showed a significantly greater reduction in the T1DM cohort compared with the healthy cohort during hypoglycemia (−61.7±19.5 vs −19.7±7.7 mg/dL; p<0.001). Analysis of counterregulatory hormones revealed distinct patterns between groups. The increase in plasma glucagon concentration was significantly greater in healthy participants compared with patients with T1DM (1.9±1.1 vs 0.4±2.6 pM; p=0.02) while there was no significant difference in basal glucagonemia. Conversely, plasma epinephrine levels exhibited a stronger rise in the T1DM cohort (52.4±136.4 vs 14.1±34.4 ng/L; p=0.02). The detailed results of these comparative analyses are summarized in table 4.

Next, we sought to longitudinally assess the glucagon response to insulin-induced hypoglycemia in the T1DM cohort. As previously described, IIHT were conducted at diagnosis, and subsequently at 6, 12, and 18 months postdiagnosis. At each time point, a significant drop in blood glucose levels was consistently observed between baseline and the hypoglycemic phase (p<0.001). Basal glucagon levels remained stable over time, with no differences observed across the four test sessions (p=0.58). At diagnosis, the change in glucagon levels between baseline and hypoglycemia did not reach statistical significance (3.7±1.7 vs 4.5±2.4 pM; p=0.67). Notably, the presence or absence of diabetic ketoacidosis (DKA) at diagnosis did

Table 1 Evaluation of plasma concentrations of various biological parameters during IIHT in healthy first-degree relatives (healthy cohort)

	T_0	T_{Hypo}	Delta	P value
Glycemia (mg/dL)	84.9±7.9	65.1±2.2	−19.8±7.7	<0.001***
Insulinemia (pmol/L)	54.4±48.4	23.6±28.7	−30.8±31.6	0.04*
C-peptide (nmol/L)	0.7±0.4	0.4±0.3	−0.38±0.2	<0.001***
Glucagon (pmol/L)	3.7±2.2	5.6±2.3	1.9±1.1	<0.001***
Epinephrine (ng/L)	25.1±4.3	39.3±32.5	14.1±34.4	0.006**
Norepinephrine (ng/L)	314.7±104.8	403.9±144.3	89.3±75.9	0.02*
GH (ng/mL)	2.3±4.1	3.2±3.1	0.9±4.7	0.63
Cortisol (nmol/L)	406.4±219.1	387.9±207.7	−18.5±148.9	0.73

T_0 corresponds to the baseline (start of the test). T_{Hypo} indicates blood sampling during the hypoglycemic phase. Delta represents the change in biological parameters between T_0 and T_{Hypo} (T_{Hypo}–T_0). The p value, obtained from paired analyses, reflects the significance of variations in glycemic parameters between T_0 and T_{Hypo}.
GH, growth hormone; IIHT, insulin-induced hypoglycemia test.

Table 2 Description of the T1D cohort

	Total (n=22)	Remitters (n=13)	Non-remitters (n=9)	P value
Diagnostic data				
Ketoacidosis—yes	54.5%	61.5%	44.4%	0.43
pH	7.3±0.2	7.2±0.2	7.3±0.2	0.16
Glycemia (mg/dL)	441.7±111.9	444.3±161.7	458.2±113.4	0.34
Bicarbonate (mmol/L)	16.0±6.7	14.3±7.8	15.2±6.0	0.30
GFR (mL/min/1.73 m^2)	83.1±21.8	84.5±17.0	75.33±16.6	0.007**
Positive antibodies—yes	71.4%	75.0%	66.7%	0.68
0 AB	28.6%	25%	33.3%	0.68
1 AB	14.3%	16.6%	11.1%	0.72
2 AB	57.1%	58.3%	55.5%	0.89
3 AB	0%	0%	0%	1
Positive GAD65—yes	47.6%	50%	44.4%	0.80
Positive IA2—yes	42.9%	50%	33.3%	0.45
Positive ZNT8—yes	23.8%	25%	22.2%	0.88
Autoimmune disease—yes	0%	0%	0%	1
T1D in the family—yes	9.5%	8.3%	11.1%	0.83
Clinical follow-up				
Weight (percentile)	56.4±26.6	57.2±20.1	63.5±31.1	0.13
Height (percentile)	46.8±24.9	43.8±20.9	54.1±25.9	0.02*
BMI (percentile)	64.7±24.2	59.3±26.5	68.5±29.9	0.07
TIDD (IU/kg/day)	0.8±0.2	0.6±0.2	0.8±0.2	<0.001***
Treatment				
2I	52.4%	50%	55.6%	0.80
BP	57.1%	50%	66.7%	0.45
Pump	9.5%	8.3%	11.1%	0.83
Glycemic mean (mg/dL)	168.7±27.8	109.2±13.0	188.6±84.5	<0.001***
HBA_{1c} (%)	8.1±1.0	5.9±0.5	8.8±3.1	<0.001***
$TAR_{>180}$ (%)	18.5±10.5	10.6±6.5	27.5±11.7	<0.001***
$TIR_{70–180}$ (%)	74.9±12.0	79.7±10.2	66.1±11.9	<0.001***
$TBR_{<70}$ (%)	6.6±5.2	9.7±7.9	6.4±4.8	0.02*
$IDAA_{1c}$	10.9±1.5	8.3±0.7	11.7±4.0	<0.001***
$GTAA_{1c}$	6.3±1.4	3.5±0.6	7.2±4.1	<0.001***
CGM metrics				
Mean (mg/dL)	135.6±20.2	119.0±10.7	150.4±24.9	<0.001***
Median (mg/dL)	125.8±18.0	110.0±11.2	136.6±26.5	<0.001***
Minimum (mg/dL)	43.6±3.6	45.5±6.2	43.9±5.5	0.10
Maximum (mg/dL)	375.3±66.6	337.4±75.3	399.2±68.1	<0.001***
GMI (%)	6.3±0.7	5.7±0.4	6.8±0.9	<0.001***
CV	0.38±0.07	0.36±0.07	0.42±0.06	<0.001***
$TIR_{70–180}$	0.74±0.13	0.81±0.11	0.66±0.13	<0.001***
$TIR_{70–140}$	0.57±0.16	0.66±0.13	0.46±0.12	<0.001***
$TIR_{60–140}$	0.60±0.16	0.72±0.12	0.50±0.12	<0.001***
$TBR_{<70}$	0.07±0.05	0.09±0.07	0.07±0.05	0.08
$TBR_{<54}$	0.01±0.02	0.01±0.02	0.01±0.02	1
$TAR_{>180}$	0.19±0.11	0.10±0.07	0.27±0.13	<0.001***
$TAR_{>250}$	0.05±0.05	0.02±0.02	0.09±0.09	<0.001***

Continued

Table 2 Continued

	Total (n=22)	Remitters (n=13)	Non-remitters (n=9)	P value
Hypoglycemia frequency (n/day)	0.6±0.3	0.5±0.4	0.6±0.4	0.06

Values are expressed as mean ±SD, and percentages may not sum to 100 because of rounding. Percentages were calculated based on the number of available cases for each variable, as some variables contained missing data. Differences between the two cohorts (Remitters vs Non-Remitters) were considered significant when p<0.05, with the following levels of significance: *p<0.05, **p<0.01, and ***p<0.001. The cohorts included the entire T1D population (Total), patients in partial remission (Remitters), and patients not in partial remission (Non-remitters). The following abbreviations are used: AB, autoantibody; TIDD, total insulin daily dose; 2I, insulin regimen with two daily injections (morning and evening); BP, basal–prandial insulin regimen; HbA_{1c}, glycated hemoglobin (%); $TAR_{>180}$, proportion of time spent in hyperglycemia (>180 mg/dL); $TIR_{70–180}$, time in range (70–180 mg/dL); $TBR_{<70}$, proportion of time spent in hypoglycemia (<70 mg/dL); $IDAA_{1c}$, insulin-dose adjusted A1c; $GTAA_{1c}$, glycemic target-adjusted HbA_{1c}; GMI, Glucose Management Indicator; CV, coefficient of glycemic variation; $TIR_{70–140}$, time in range (70–140 mg/dL); $TIR_{60–140}$, time in range (60–140 mg/dL); $TBR_{<54}$, time spent in hypoglycemia (<54 mg/dL); and $TAR_{>250}$, time spent in hyperglycemia (>250 mg/dL).
AB, autoantibody; BP, basal-prandial insulin regimen; CGM, continuous glucose monitoring; CV, coefficient of glycemic variation; GMI, glucose management indicator; $GTAA_{1c}$, glycemic target-adjusted HbA1c; HbA_{1c}, glycated hemoglobin; 2I, insulin regimen with two daily injections (morning and evening); $IDAA_{1c}$, insulin-dose-adjusted A1c; $TAR_{>180}$, proportion of time spent in hyperglycemia (>180 mg/dL); $TBR_{<54}$, time spent in hypoglycemia (<54 mg/dL); $TBR_{<70}$, proportion of time spent in hypoglycemia (<70 mg/dL); T1D, type 1 diabetes; TIDD, total insulin daily dose; $TIR_{60–140}$, time in range (60–140 mg/dL); $TIR_{70–140}$, time in range (70–140 mg/dL); $TIR_{70–180}$, time in range (70–180 mg/dL).

not influence this response (all p>0.05): no difference was found in glucagon variation between patients with and without DKA (1.1±3.3 vs 0.3±2.2 pM; p=0.59). At 6 months postdiagnosis, an increase in glucagon secretion during hypoglycemia was observed (4.3±1.5 vs 6.3±3.4; p=0.04). However, at 12 months, glucagon levels showed no change during hypoglycemia (4.5±2.0 vs 4.7±2.5; p=0.98). Strikingly, at 18 months, a paradoxical response emerged, characterized by a blunted or even inappropriate glucagon secretion during hypoglycemia (4.9±3.0 vs 3.3±2.0; p=0.02) (figure 1).

In the study, we also examined the influence of glycemic control on the physiological counterregulatory response to hypoglycemia, focusing on glucagon secretion. A positive correlation was observed between time in range ($TIR_{70–180}$) and glucagon secretion during hypoglycemia (R=0.57; p=0.03). Conversely, both the frequency of hypoglycemic episodes (R=−0.52; p=0.04) and glycemic variability (coefficient of variation (CV); R=−0.52; p=0.04) were negatively correlated with glucagon response (figure 2A). Finally, patients were categorized as stable (n=7) or unstable (n=8) based on TIR variation between 6 and 18 months, with thresholds arbitrarily set at <10% and >15%, respectively. In stable patients, glucagon variation during hypoglycemia tended to decrease over time, but this trend did not reach statistical significance (0.18±1.44 vs −1.43±1.71; p=0.07). By contrast, unstable patients showed a reduction in glucagon variation (0.80±2.04 vs −1.04±1.15; p=0.03).

To further investigate the relationship between glycemic metrics and the counterregulatory glucagon response, we developed a logistic regression model predicting glucagon response during hypoglycemia based on parameters that showed significant correlation with glucagon secretion: glycemic variability, $TIR_{70–180}$, and frequency of hypoglycemic episodes. The probability of a normal glucagon response (1)

Table 3 Evaluation of plasma concentrations of various biological parameters during IIHT in T1D cohort

	T_0	T_{Hypo}	Delta	P value
Glycemia (mg/dL)	111.9±20.9	50.2±4.6	−61.7±19.5	<0.001***
Insulinemia (pmol/L)	76.5±89.5	79.4±84.7	1.1±48.9	0.96
C-peptide (nmol/L)	0.2±0.2	0.1±0.1	−0.07±0.1	<0.001***
Glucagon (pmol/L)	4.2±2.0	4.7±2.7	0.4±2.6	0.68
Epinephrine (ng/L)	60.9±50.4	113.3±154.6	53.4±136.4	<0.001***
Norepinephrine (ng/L)	215.4±106.5	252.3±102.1	36.9±83.9	0.004**
GH (ng/mL)	1.2±1.7	2.1±2.9	0.8±2.5	0.05
Cortisol (nmol/L)	322.9±136.9	234.2±105.5	−88.1±113.8	<0.001***

Table 3 summarises the longitudinal changes in the different biological parameters assessed during hypoglycemic testing in patients with T1D, including baseline values, measurements obtained during the hypoglycemic phase, and the corresponding within-subject changes. T_0 corresponds to baseline (start of the test), while T_{Hypo} indicates blood sampling during the hypoglycemic phase. Delta represents the change in biological parameters between T_0 and T_{Hypo} (T_{Hypo}–T_0). Due to missing data for certain patients, the mean delta (\Delta) value may slightly differ from the direct difference between the means at T_{Hypo} and T_0. The p-value, obtained from paired analyses, reflects the statistical significance of changes in biological parameters between T_0 and T_{Hypo}, with the following levels of significance:*p<0.05, **p<0.01, and ***p<0.001
GH, growth hormone; IIHT, insulin-induced hypoglycemia test; T1D, type 1 diabetes.

Table 4 Comparison of plasma concentrations of various biological parameters during IIHT between T1D cohort and healthy cohort

	T1D	Healthy	P value
Glycemia (mg/dL)	−61.7±19.5	−19.7±7.7	<0.001***
Insulinemia (pmol/L)	1.1±48.9	−30.8±31.6	<0.001***
C-peptide (nmol/L)	−0.07±0.1	−0.38±0.2	<0.001***
Glucagon (pmol/L)	0.4±2.6	1.9±1.1	0.02*
Epinephrine (ng/L)	52.4±136.4	14.1±34.4	0.02*
Norepinephrine (ng/L)	36.9±83.9	89.3±75.9	0.10
GH (ng/mL)	0.8±2.5	0.9±4.7	0.46
Cortisol (nmol/L)	−88.1±113.8	−18.5±148.9	0.12

Delta represents the change in biological parameters between T_0 and T_{Hypo} (T_{Hypo}–T_0). The p-value, obtained from paired analyses, reflects the significance of variations in glycemic parameters between T_0 and T_{Hypo}, with the following levels of significance: *p<0.05, **p<0.01, and ***p<0.001.
GH, growth hormone; IIHT, insulin-induced hypoglycemia test; T1D, type 1 diabetes.

vs an abnormal response (0) was calculated using the following formula: $logit(p)=[-6.109+4.071\times CV+6.868\times TIR_{70-180}-1.106\times hypoglycemia\ frequency]$ with $p=1/(1+e^{-logit(p)})$. Model performance was assessed using a receiver operating characteristic curve, yielding an area under the curve of 0.79, indicative of good discriminative ability. At a threshold of 0.5, the model demonstrated a sensitivity of 71% and a specificity of 78%, suggesting that the combined assessment of glycemic variability, TIR, and hypoglycemia frequency provides a more effective evaluation of glucagon secretion during hypoglycemia than any single parameter alone.

In parallel, we investigated potential correlations between various circulating biomarkers and glucagon responses (Online supplemental file 1). Biomarker levels were measured at baseline during each IIHT session. Positive correlations were identified between glucagon secretion and several circulating factors, including GIP (R=0.71; p=0.02), GLP-1 (R=0.67; p=0.03), tumor necrosis factor-alpha (TNF-α; R=0.70;

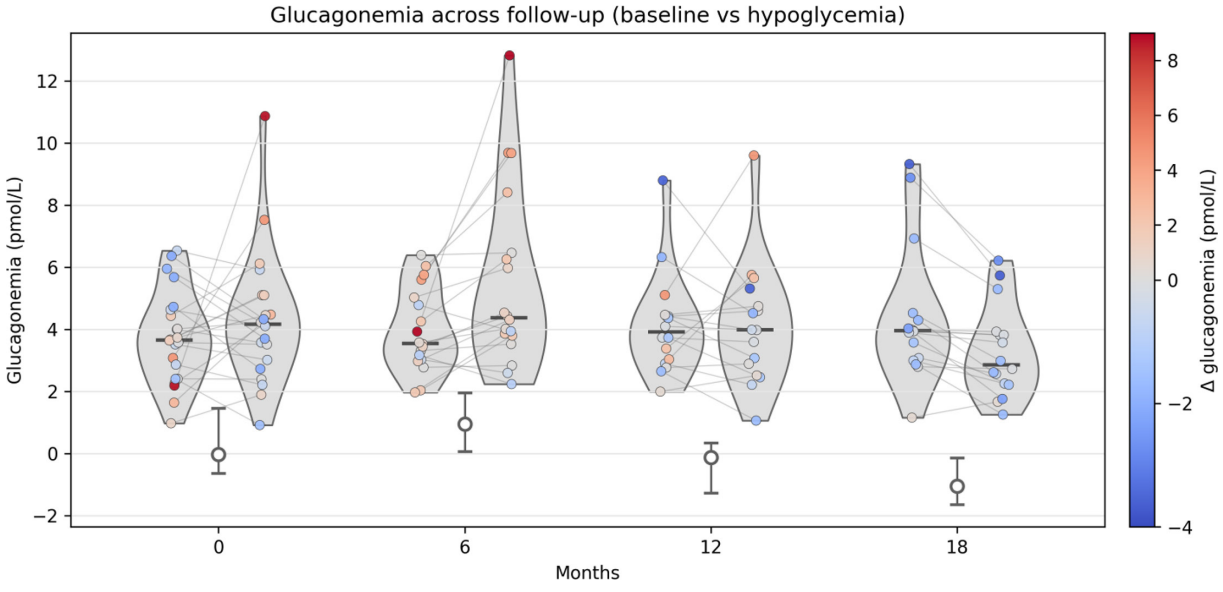

Figure 1 Longitudinal variation in glucagon levels during hypoglycemia in the type 1 diabetes cohort. This figure illustrates the longitudinal changes in plasma glucagon concentrations measured during insulin-induced hypoglycemia tests at different follow-up time points. The x-axis represents the test time points (months). The left y-axis indicates plasma glucagon concentration (pmol/L), while the right y-axis represents the change in plasma glucagon concentration between baseline (T_0) and the hypoglycemic time point (T_{Hypo}), expressed in pmol/L (Δ glucagon). For each test time point, the left violin plot corresponds to values measured at T_0, and the right violin plot corresponds to values measured at the T_{Hypo}. Each dot represents an individual blood sample. Lines connecting the dots indicate the intra-individual change in glucagon levels between T_0 and T_{Hypo} for each patient. The overlaid bar plots represent the change in glucagon levels (Δ glucagon) between T_0 and T_{Hypo} at each test time point. Sample sizes may vary across time points because some participants were unable to complete all scheduled tests.

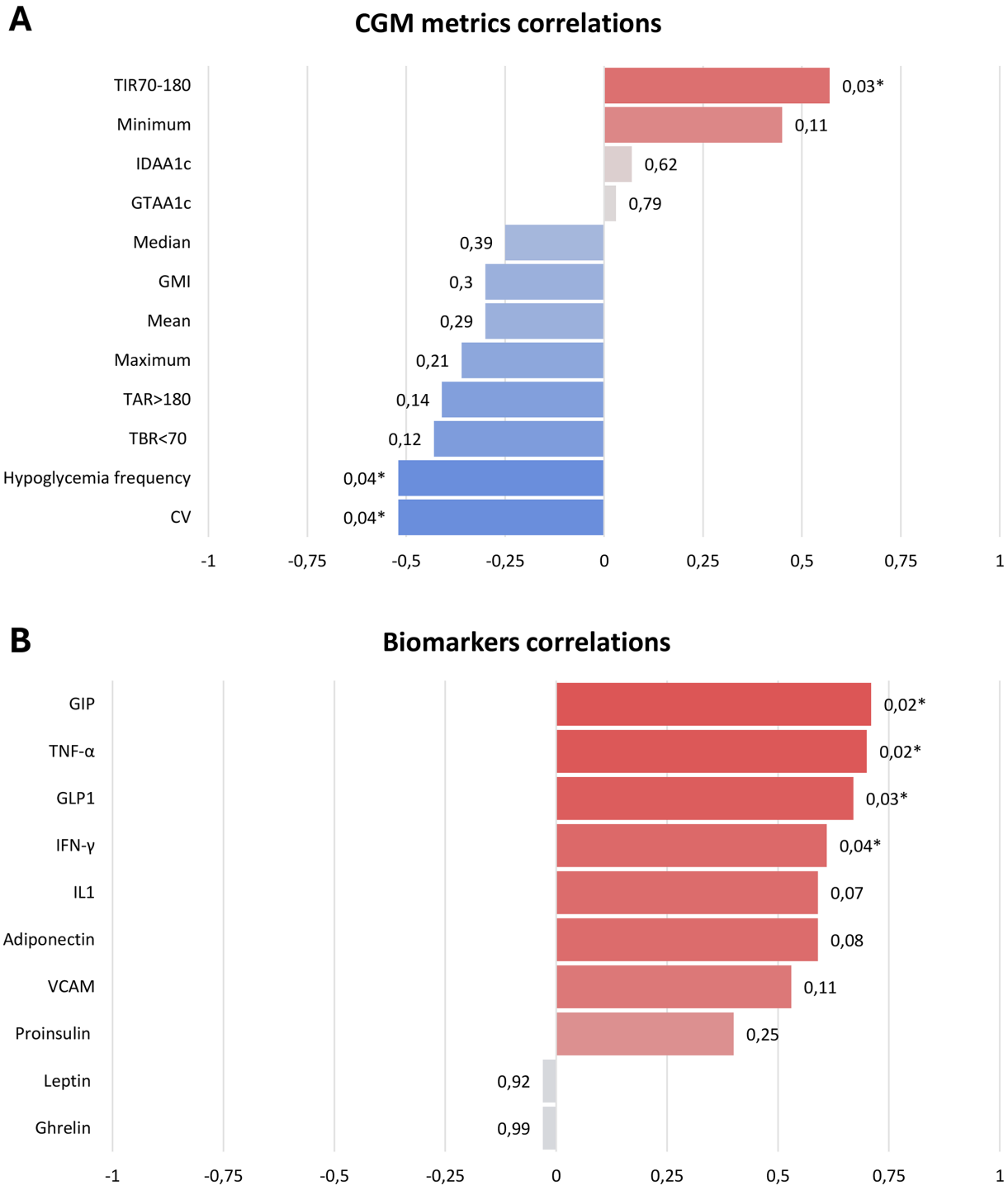

Figure 2 Correlation between glucagon secretion during hypoglycemia and CGM metrics or biomarkers. (A) Correlations between changes in glucagon levels during hypoglycemia and CGM-derived metrics computed from data collected during the month preceding the IIHT. Pearson's correlation coefficients were calculated, with statistical significance defined as $p<0.05$. The value displayed at the end of each bar corresponds to the associated p value. (B) Correlations between changes in glucagon levels during hypoglycemia and circulating biomarkers measured at time 0 of the IIHT using multiplex analysis (Meso Scale Discovery). Pearson's correlation coefficients were calculated, and results were considered statistically significant at $p<0.05$. The value displayed at the end of each bar corresponds to the associated p value. CGM, continuous glucose monitoring; CV, coefficient of variation; GIP, glucose-dependent insulinotropic polypeptide; GMI, glucose management indicator; $GTAA_{1c}$, glycemic target-adjusted glycated hemoglobin; Hypoglycemia freq., frequency of hypoglycemia; $IDAA_{1c}$, insulin-dose-adjusted glycated hemoglobin; IFN, interferon; IIHT, insulin-induced hypoglycemia test; IL, interleukin; Maximum, highest continuous glucose monitoring-measured glucose; Minimum, lowest continuous glucose monitoring-measured glucose; $TAR_{>180}$, percentage of time above 180 mg/dL; $TBR_{<70}$, percentage of time below 70 mg/dL; $TIR_{70–180}$, percentage of time in target range (70–180 mg/dL); TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule.

p=0.02), and interferon-gamma (IFN-γ; R=0.61; p=0.04). In contrast, no associations were found between glucagon secretion and metabolic hormones such as ghrelin (R=−0.03; p=0.99) or leptin (R=−0.03; p=0.92), suggesting these markers may not be correlated with the acute glucagon response under the conditions tested. A detailed summary of these correlations is presented in figure 2B.

DISCUSSION

The aim of the GLUREDIA-WP1 study was to characterize glucagon secretion during hypoglycemia as it occurs in everyday life in T1D. To this end, we implemented an insulin-induced hypoglycemia protocol designed to mimic a typical episode of insulin excess experienced by patients. This protocol, tested in healthy subjects, effectively induced hypoglycemia accompanied by a physiological increase in glucagonemia, validating its relevance for the study of hormonal responses to hypoglycemia.

Many studies have relied on hyperinsulinemic-hypoglycemic clamps to induce controlled hypoglycemia. Although this approach is well standardized and effective, it also presents limitations. Continuous intravenous insulin infusion is not physiological and may alter endogenous glucagon regulatory pathways, and clamp conditions do not fully replicate the clinical scenarios in which hypoglycemia typically occurs in daily life.[22–24] In this context, our insulin-induced hypoglycemia protocol offers a complementary, more practice-oriented method that approximates real-world conditions and may provide additional insights into α-cell function in a clinically relevant setting.

The first major observation of our study is an early and significant alteration in glucagon secretion during hypoglycemia in children and adolescents with T1DM. Patients with T1DM showed no significant variation in glucagon levels during hypoglycemia, in contrast to the physiological increase observed in healthy subjects. Moreover, under normal physiological conditions, plasma glucagon concentrations are expected to rise twofold to threefold during grade 2 hypoglycemic episode,[25] such as that induced in our diabetic patient cohort. This alteration occurs despite basal glucagon levels being comparable between the two groups, even though the glycemic fall is more marked in the T1DM cohort. These data confirm that, despite deeper hypoglycemia, patients with diabetes already exhibit an inadequate α-cell response from diagnosis onwards. This counterregulatory defect, well documented in adults or in animal models, sometimes via hypoglycemic clamp,[3 5 26–28] is observed here from the very first months of the disease, in an experimental context closer to real life. It highlights the increased risk of SH in children, a population already vulnerable due to high insulin sensitivity, variable food intake, and physical activity patterns.

Importantly, the longitudinal arm of the study reveals that α-cell dysfunction is not static, but evolves dynamically over the first 18 months. Glucagon responsiveness at diagnosis appeared heterogeneous across individuals, with some patients showing preserved responses during hypoglycemia. Overall, we observed a transient improvement in glucagon responsiveness at 6 months, followed by a progressive decline and ultimately a paradoxical suppression of glucagon during hypoglycemia at 18 months. This profile has already been described in vitro or during clinical studies and could contribute to major metabolic imbalances: hyperglucagonemic hyperglycemia on the one hand, or hypoglycemia in the absence of glucagon response on the other.[29–31] While this dysfunction is generally associated with a long-standing course of diabetes, our results show that it sets in early, as observed in certain adult cohorts.[3 5 9] Furthermore, the initial severity of diabetes, in particular the presence or absence of ketoacidosis at diagnosis, does not appear to influence this hormonal response. Unlike fulminant forms, where early alpha-cellular damage has been reported,[32] our cohort shows a homogeneous alteration in glucagon response, whatever the initial clinical presentation.

Beyond documenting α-cell dysfunction, our study suggests that glycemic stability is a key determinant of preserved glucagon secretion during hypoglycemia. Higher time in target ($TIR_{70–180}$) and lower glycemic variability correlated positively with glucagon responsiveness, whereas frequent hypoglycemia and high glycemic coefficient of variation correlated with blunting of the glucagon response. Patients maintaining stable glycemic profiles between 6 and 18 months showed relative preservation of glucagon secretion, while those with worsening TIR showed a significant decline. These findings reinforce the concept that recurrent hypoglycemia and glycemic volatility impair counterregulatory defenses through habituation and desensitization of glucose-sensing networks.[33]

This is consistent with our previous observations showing that SH typically occurs after multiple preceding hypoglycemic episodes, indicating a cumulative effect of recurrent hypoglycemia on counterregulatory failure.[34 35] In those studies, SH events characterized by impaired consciousness occurred at a mean glycemia of 48 mg/dL and were not necessarily preceded by a steep glucose decline. Interestingly, the asymptomatic grade 2 hypoglycemias induced during IIHT occurred at nearly similar glycemic levels, yet were associated with minimal or absent symptoms. Taken together, these data suggest that the frequency and chronic exposure to hypoglycemia, rather than the absolute glucose value, represent the most relevant determinants of both glucagon secretory capacity and symptom severity during hypoglycemia.[34 35] In contrast, partial remission status did not meaningfully influence glucagon secretion, differing from previous reports suggesting more efficient counterregulation in remitters due to preserved intra-islet insulin dynamics.[36]

Building on the strong correlations observed between CGM parameters and glucagon secretion, we developed a logistic regression-based score aimed at predicting

normal versus impaired glucagon responses during hypoglycemia in children and adolescents with T1DM. The objective was to establish a non-invasive, clinically applicable tool relying on routinely collected glycemic metrics, avoiding the need for provocative hypoglycemic testing. The model incorporated CV, $TIR_{70–180}$, and hypoglycemia frequency and demonstrated good discriminative performance. Importantly, the combined assessment of these three CGM parameters provided a more effective evaluation of glucagon secretion during hypoglycemia than any single metric alone, highlighting the added value of integrating multiple aspects of glycemic control. While these initial results are promising, the score requires validation in larger, independent pediatric cohorts to determine its generalizability and potential utility as a non-invasive surrogate marker of counterregulatory α-cell function.

Finally, the correlations observed between glucagon response and circulating biomarkers such as GIP, GLP-1, TNF-α, and IFN-γ provide new insights into the hormonal and immune factors that may influence α-cell function in early T1DM. Integrating these biomarkers into routine monitoring could enhance our understanding of the disease both at the population level and for individual patients. Regular assessment of these parameters may help anticipate potential complications associated with T1DM, such as impaired counterregulatory responses and increased risk of SH. Moreover, incorporating biomarker data into patient follow-up could facilitate more personalized diabetes management, allowing clinicians to tailor preventive and therapeutic strategies to the specific physiological profile of each child or adolescent, which is essential for optimizing long-term outcomes.

In summary, our study provides evidence that glucagon counterregulation is profoundly altered early in the course of pediatric T1DM, evolves dynamically within the first 18 months, and is strongly influenced by glycemic variability. By using a physiologically relevant model of hypoglycemia, we provide a detailed characterization of α-cell dysfunction and identify parameters that could be leveraged into predictive tools for clinical risk stratification. However, these findings should be interpreted in light of certain limitations, including the relatively small sample size and the use of an adult control cohort, which was selected because ethical constraints precluded performing hypoglycemic testing in healthy children. Consequently, potential age-related differences in the development, physiology, and anatomy of the islets of Langerhans, as well as in glucose homeostasis and metabolic regulation, may have influenced the observed responses and should be considered when interpreting our findings. Future work should validate the proposed probability-based approach in larger and more diverse cohorts, extend follow-up to capture later disease stages, and investigate whether preserving glycemic stability can meaningfully alter the trajectory of α-cell decline.

Contributors PAL: conception, planning, data analysis, manuscript drafting and corrections, study supervision. AH and MB: planning, data acquisition and analysis, manuscript drafting and corrections. Il and ZS: data collection and experimental procedures. AM: coordination of ethics and regulatory aspects. LB and DB: data acquisition. All authors reviewed, revised, and approved the final manuscript. PAL is the guarantor.

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Patient consent for publication Not applicable.

Ethics approval This study was approved by the Hospital-Faculty Ethics Committee (Comité d'Éthique Hospitalo-Facultaire) of the CUSL (approval/reference no. 2022/02/FEV/043; Belgian registration no. B403202200013). All procedures were carried out in accordance with the ethical principles set out in the Declaration of Helsinki. Participants gave informed consent to participate in the study before taking part.

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ORCID iD

Philippe A Lysy https://orcid.org/0000-0003-1598-7998

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