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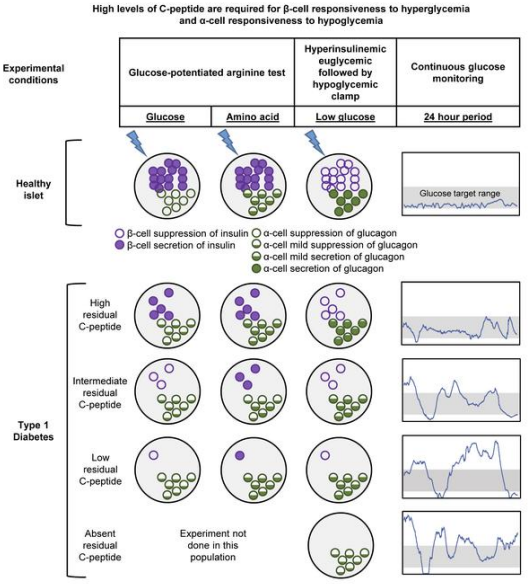

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High residual C-peptide likely contributes to glycemic control in type 1 diabetes

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Abstract

BACKGROUND. Residual C-peptide is detected in many people for years following the diagnosis of type 1 diabetes; however, the physiologic significance of low levels of detectable C-peptide is not known.

METHODS. We studied sixty-three adults with type 1 diabetes classified by peak mixed-meal tolerance test (MMTT) C-peptide as negative (<0.007; *n* =15), low (0.017–0.200; *n* =16), intermediate (>0.200–0.400; *n* =15), or high (>0.400 pmol/mL; *n* =17). We compared the groups' glycemia from continuous glucose monitoring (CGM), β-cell secretory responses from a glucose-potentiated arginine (GPA) test, insulin sensitivity from a hyperinsulinemia euglycemic (EU) clamp, and glucose counterregulatory responses from a subsequent hypoglycemic (HYPO) clamp.

RESULTS. Low and intermediate MMTT C-peptide groups did not exhibit β-cell secretory responses to hyperglycemia, whereas the high C-peptide group showed increases in both C-peptide and proinsulin ($P \leq 0.01$). All groups with detectable MMTT C-peptide demonstrated acute C-peptide and proinsulin responses to arginine that were positively correlated with peak MMTT C-peptide ($P < 0.0001$ for both analytes). During the EU-HYPO clamp, C-peptide levels were proportionately suppressed in the low, intermediate, and high C-peptide compared to the negative group ($P \leq 0.0001$), whereas glucagon increased from EU to HYPO only in the high C-peptide group compared to negative ($P = 0.01$). CGM demonstrated lower mean glucose and more time-in-range for the high C-peptide group.

CONCLUSION. These results indicate that in adults with type 1 diabetes, β-cell responsiveness to hyperglycemia and α-cell responsiveness to hypoglycemia are only observed at high levels of residual C-peptide that likely contribute to glycemic control.

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Introduction

Type 1 diabetes (T1D) results from autoimmune destruction of the insulin-producing β-cells in the endocrine pancreatic islets of Langerhans. After a subclinical period of months to years of autoimmune destruction, the clinical diagnosis of diabetes occurs when the functional capacity for insulin secretion has been markedly reduced (1, 2), and corresponds to a considerable reduction in β-cell volume (3, 4). Interestingly, there is heterogeneity of the pathology affecting the islets of individuals with T1D, with some pancreatic lobules containing islets without β-cells and others containing islets with a near-normal complement of β-cells (3, 4). With increasing time from diabetes diagnosis it becomes harder to find islets containing β-cells (5), although scattered insulin-positive cells can be found in most individuals with T1D (6). Even amongst those with long standing disease, some individuals may have pancreatic lobules with islet containing β-cells (7). Thus, a portion of individuals with T1D appears to maintain a population of β-cells capable of evading immune detection (8).

Consistent with the pathologic description of residual islet containing β-cells, many individuals with T1D will maintain clinically meaningful endogenous insulin secretion, as estimated from levels of mixed-meal stimulated C-peptide >0.200 pmoL/mL (>0.60 ng/mL), for up to 5 years from diagnosis (9). In the Diabetes Control and Complications Trial (DCCT), such residual β-cell function measured within 5 years of disease diagnosis was associated with reduced incidence of retinopathy and nephropathy and a decreased prevalence of severe hypoglycemia (10). Conversely, DCCT participants who had “undetectable” C-peptide at enrollment were at the greatest risk for severe hypoglycemia regardless of treatment intensity (11). While the lower limit of detection for the C-peptide assay used in the DCCT was 0.03 pmol/mL (0.09 ng/mL) (9, 12), most assays performed poorly at this low concentration. Thus,

"negative" has been used across a range from <0.03–0.17 pmol/mL (0.10–0.50 ng/mL), and stimulated C-peptide levels >0.200 pmol/mL have been considered clinically meaningful. However, a more recent analysis of the DCCT (13) suggests that any level of measurable C-peptide may be associated with better clinical outcomes.

With the development and increasing use of improved C-peptide assays, it is now possible to detect residual C-peptide production in the majority of people with T1D during the first 10 years of diabetes, and in a substantial minority of people in their second and third decades with the disease (14-16). These observations have recently been extended to the Joslin Medalist cohort that includes individuals with more than 50 years disease duration (7). However, whether detection of low levels of residual C-peptide has any physiologic significance for affecting the secretory responses of other islet hormones such as glucagon or contributing to glucose control or counterregulation is not known. Moreover, one postulated mechanism for insulin resistance in T1D is peripheral administration of exogenous insulin vs. portal delivery of endogenous insulin that is important for hepatic metabolism (17). Thus, people with intact C-peptide secretion were hypothesized to have higher insulin sensitivity.

The present study was designed to investigate the significance of varying levels of residual C-peptide production for evidencing persistent β-cell function as well as α-cell function that is dysregulated in T1D. Additionally, we sought to determine if a minimum threshold of C-peptide was physiologically important based on a comprehensive evaluation of islet cell responsivity. To accomplish this, residual C-peptide defined by the peak during a mixed meal tolerance test (MMTT) was related to β- and α-cell responsivity to glucose and arginine derived from glucose-potentiated arginine testing, insulin sensitivity measured during a stable glucose isotope-labeled hyperinsulinemic euglycemic clamp, β- and α-cell responsivity to hypoglycemia

and counterregulatory hormone, symptom and endogenous glucose production (EGP) measured during a stable glucose isotope-labeled hyperinsulinemic hypoglycemic clamp, and to glycemic control derived from continuous glucose monitoring. Participants were grouped by their peak C-peptide during the MMTT as negative (<0.007 pmol/mL [<0.02 ng/mL]), low (0.017–0.200 pmol/mL [0.05–0.60 ng/mL]), intermediate (>0.200–0.400 pmol/mL [>0.60–1.20 ng/mL]), or high (>0.400 pmol/mL [>1.20 ng/mL]) based on previously reported distribution of residual C-peptide production in T1D (16). While these were protocol-specified categories, the relationships between physiologic measures were also evaluated by peak C-peptide as a continuous variable.

Results

Participant characteristics

Between June 2016 and February 2017, 63 participants completed the study protocol (Fig. 1). The participants were balanced across groups of C-peptide production for sex, age, and BMI; however, T1D duration was longer (P <0.001) and insulin requirements were greater (P =0.01) for those in the negative C-peptide group (Table 1).

Islet and incretin responses during the mixed-meal tolerance test (MMTT)

As expected from the study design, C-peptide responses during the MMTT increased significantly from no response in the negative group to incrementally greater responses in the low, intermediate, and high C-peptide groups (P <0.0001; Fig. 2A). This relationship was also strongly apparent when evaluated as a continuous variable (r =0.99; P <0.0001; Fig. 2B). There was no relationship between glucagon responses and either categorical (Fig. 2C) or continuous

(not shown) C-peptide responses during the MMTT. No differences in GLP-1 responses were seen across the groups or when C-peptide was assessed as a continuous variable (data not shown). In contrast, there was a relationship between peak C-peptide and the GIP response both as a categorical (P <0.01; Fig. 2D) and continuous (r =0.48; P =0.0001) variable.

Glucose, C-peptide, proinsulin and glucagon during the glucose-potentiated arginine (GPA) test

A GPA test was conducted in the groups with detectable C-peptide as the gold-standard assessment of islet hormone secretion since the β-cell response to arginine is preserved after it is lost to glucose (18, 19), and arginine stimulation allows the α-cell response to be simultaneously quantitated and related (20). Fasting glucose was greater in the low and intermediate than in the high C-peptide group (145±30 vs. 148±31 vs. 115± 0 mg/dL; P =0.02). Across the low, intermediate, and high C-peptide groups, there was increasing fasting C-peptide (0.03±0.02 vs. 0.11±0.03 vs. 0.22±0.13 pmol/mL; P <0.0001; Fig. 3A) and no difference in fasting proinsulin (Fig. 3B). Thus, the fasting proinsulin-to-C-peptide ratio was highest in the low C-peptide group (0.56±0.40 vs. 0.18±0.08 vs. 0.14±0.17; P <0.0001). In response to the ~230 mg/dL hyperglycemic clamp, while the pre-arginine glucose was similar across groups (236±10 vs. 233±9 vs. 239±16 mg/dL), the low and intermediate C-peptide groups did not exhibit a β-cell response to the induction of hyperglycemia, whereas the high C-peptide group showed increases in both C-peptide (P <0.001) and proinsulin (P =0.01). All three groups demonstrated β-cell responses to glucose-potentiated arginine (Fig. 3A, B) with increases across groups in both the acute C-peptide response (0.05±0.03 vs. 0.15±0.04 vs. 0.51±0.26 pmol/mL; P <0.0001) and the acute proinsulin response (0.002±0.001 vs. 0.004±0.002 vs. 0.012±0.012 pmol/mL; P =0.0001).

The proinsulin secretory ratio (PISR), a measure of β-cell stress derived from GPA stimulation (21), was not different across groups. The peak C-peptide during the MMTT was highly correlated with the acute C-peptide response to arginine stimulation (ACR_{arg}: r =0.96; P <0.0001; Fig. 3D) and less so with the acute proinsulin response to arginine (APR_{arg}: r =0.65; P <0.0001; Fig. 3E). While the GPA test was not conducted in the group with undetectable stimulated C-peptide during the MMTT, the y-intercept of the regression line relating ACR_{arg} to the MMTT peak C-peptide equaled zero, supporting that undetectable stimulated C-peptide by one test is predictive for a negative response by the other test. The α-cell response to glucose-potentiated arginine (AGR_{arg}) was not different across groups (Fig. 3C) and there was no relationship between the MMTT peak C-peptide and the acute glucagon response (data not shown).

Insulin sensitivity during the hyperinsulinemic euglycemic (EU) clamp

A hyperinsulinemic euglycemic (EU) clamp was conducted as a gold standard assessment of insulin sensitivity, with infusion of a stable glucose isotope in order to distinguish hepatic from peripheral insulin action using the isotopic dilution method (22). Insulin administration during the clamp resulted in similar levels of plasma insulin during EU across the negative, low, intermediate, and high C-peptide groups (53.0±24.7 vs. 52.4±18.3 vs. 41.3±15.2 vs. 52.3±16.5 μU/mL; Fig. 4A), and there was no difference in plasma glucose during the last 30-min of EU across the groups (93±6 vs. 89±4 vs. 94±6 vs. 90±5 mg/dL; Fig. 4B). No differences were seen across the negative, low, intermediate, and high C-peptide groups for total body insulin sensitivity (S_I, 0.100±0.046 vs. 0.112±0.065 vs. 0.136±0.069 vs. 0.127±0.079 × 10^2 dl·min^{-1}·kg^{-1} per μU/mL), peripheral insulin sensitivity (S_{IP}, 0.052±0.039 vs. 0.060±0.058 vs. 0.073±0.049 vs.

186 0.079±0.048 × 10^2 dl·min^{-1}·kg^{-1} per μU/mL), or hepatic insulin sensitivity (S_{IH}, 0.603±0.172 vs.
187 0.660±0.249 vs. 0.675±0.272 vs. 0.659±0.171).
188
189 **Counterregulatory responses during the hyperinsulinemic hypoglycemic (HYPO) clamp**
190 A hyperinsulinemic hypoglycemic (HYPO) clamp was performed as a gold standard assessment
191 of hormonal and glucose counterregulatory responses to insulin-induced hypoglycemia, with the
192 infusion of stable glucose isotope enabling determination of the endogenous glucose production
193 (EGP) response as the ultimate defense against the development of low blood glucose (23, 24).
194 Plasma levels of insulin were not statistically different during HYPO across the negative, low,
195 intermediate, and high C-peptide groups (52.8±29.1 vs. 47.2±14.3 vs. 35.1±10.5 vs. 38.7±13.6
196 μU/mL; Fig. 4A), and there was no difference in plasma glucose during the last 30-min of
197 HYPO across the groups (52±4 vs. 50±4 vs. 54±6 vs. 52±4 mg/dL; Fig. 4B). Suppression of C-
198 peptide from EU to HYPO was incrementally greater for the groups by increasing C-peptide
199 production (0±0 vs. -0.006±0.005 vs. -0.027±0.008 vs. -0.074±0.042 pmol/mL; P <0.0001; Fig.
200 5A). The increase in glucagon from EU to HYPO was significantly different across the groups
201 by increasing C-peptide production (12.9±7.7 vs. 17.4±16.7 vs. 13.0±14.4 vs. 30.1±16.2 pg/mL;
202 P =0.007; Fig. 5B) with a clearly greater increase in glucagon on average in the high C-peptide
203 group and overlap in the intermediate and low C-peptide groups. The peak C-peptide during the
204 MMTT was highly associated with the suppression of C-peptide during HYPO (r =-0.95; P
205 <0.0001; Fig. 5C) and weakly correlated with the glucagon response to HYPO (r =0.40; P
206 =0.003; Fig. 5D). No differences were seen across the negative, low, intermediate, and high C-
207 peptide groups in the change from EU to HYPO for EGP (0.19±0.71 vs. 0.33±0.69 vs. 0.78±0.56
208 vs. 0.50±0.60 mg·kg^{-1}·min^{-1}; Fig. 6A), FFAs (0.314±0.267 vs. 0.329±0.315 vs. 0.319±0.242 vs.

0.165±0.208 mmol/L; Fig. 6B), epinephrine (380±277 vs. 590±309 vs. 445±203 vs. 451±300 pg/mL; Fig. 6C), or autonomic symptoms (7.62±5.61 vs. 9.02±6.39 vs. 7.69±5.44 vs. 10.04±4.38; Fig. 6D).

Glycemic control as assessed by continuous glucose monitoring (CGM)

Participants from all four groups collected a similar amount of sensor glucose data from the 7-day CGM (Table 2). Mean glucose was lower, time in range 70–180 mg/dL was higher, and time with glucose >180 mg/dL was lower in the group with the highest C-peptide (P <0.05 for all comparisons; Table 2), while statistically significant differences were not seen across groups for glucose CV or time with glucose <70 mg/dL. When evaluated as a continuous relationship with MMTT peak C-peptide, mean glucose was lower (r =-0.356; P =0.005), time in range 70–180 mg/dL was higher (r =0.456; P <0.001), time with glucose >180 mg/dL was lower (r =-0.376; P =0.003), and glucose CV was lower (r =-0.258; P =0.046) with increasing C-peptide, while no relationship was seen for time with glucose <70 mg/dL. No individual with MMTT peak C-peptide >0.400 pmol/mL exhibited less than 50% time in range 70–180 mg/dL (Fig. 7), and so the high C-peptide group appeared protected from experiencing sub-optimal glycemic control (25).

Discussion

Our study is the first to comprehensively assess islet cell responsivity in people with T1D using gold-standard methods across the spectrum of detectable C-peptide production. The group with high peak C-peptide (>0.400 pmol/mL) during a MMTT exhibited lower fasting glucose (111±31 mg/dL), HbA_{1c} (6.8±1.0%) and mean glucose (140±25 mg/dL), and greater CGM-

derived time in target range (72±12%). Given that the high C-peptide group was considerably less often hyperglycemic based on CGM, the lack of difference in peripheral or hepatic insulin sensitivity across the groups supports that insulin resistance in T1D is not strongly related to hyperglycemia as suggested by others (26). The high C-peptide group was the only group who demonstrated β-cell responsivity to glucose during the hyperglycemic clamp conducted prior to the GPA test with measurable increases in C-peptide and proinsulin secretion. Furthermore, this group also demonstrated α-cell responsivity to hypoglycemia with greater increases in glucagon. Evaluation of peak C-peptide as a continuous variable also demonstrated a continuous association with these measures of islet cell responsivity, suggesting that any selected threshold remains somewhat arbitrary. Thus, while the group comparisons suggest that a MMTT peak C-peptide of >0.400 pmol/mL represents a minimum threshold of physiologic importance, the threshold of peak C-peptide >0.200 pmol/mL established by the DCCT as clinically meaningful may be explained by some degree of preserved islet cell responsivity in the intermediate C-peptide group, while lower levels are unlikely to contribute any meaningful benefit for glycemic control in T1D.

Our results support the concept that classification of residual C-peptide by peak MMTT response is consistent with the underlying β-cell secretory capacity as demonstrated here using the GPA test. Functional β-cell mass is most accurately determined *in vivo* from the β-cell secretory capacity (27). The β-cell secretory capacity is derived from glucose-potentiation of insulin or C-peptide release in response to injection of a non-glucose insulin secretagogue, such as arginine or glucagon. Glucose-potentiation serves to prime the β-cells by inducing recruitment of secretory granules to a readily releasable pool that is subsequently released in response to membrane depolarization induced by arginine or glucagon (28). Because differences

in glucose concentration affect the priming of β-cells to acute stimulation by arginine, and the repeatability of the measured responses is superior with arginine compared to glucagon (29), we employed a hyperglycemic clamp to create the same degree of glucose-potentiation (~230 mg/dl [12.8 mmol/l]) of arginine-induced insulin secretion in all participants for the most accurate quantification of remaining functional β-cell mass. The MMTT peak C-peptide being highly associated with the acute C-peptide response to GPA (r =0.96; P <0.0001; Fig. 3D) indicates that mixed-meal stimulation may serve as a reasonable correlate to estimate functional β-cell mass in T1D.

An increased proinsulin-to-C-peptide ratio was observed under fasting conditions in the low C-peptide group. This may be explained by greater exposure to hyperglycemia in this group since studies in isolated human islets have shown that hyperglycemia decreases β-cell insulin content and increases β-cell secretion of proinsulin (30). Alternatively, this finding may represent transition within this group to becoming C-peptide negative, where proinsulin secretion may be detected in the absence of C-peptide (31, 32). However, there was no difference in the PISR when hyperglycemia was matched across groups during the hyperglycemic clamp, suggesting that proinsulin processing is not dependent on differences in low residual mass of functioning β-cells. In addition, measures of insulin sensitivity at both the skeletal muscle and liver were not different across groups with residual C-peptide production compared to the negative group, and therefore differences within such low levels of β-cell function do not seem to affect the insulin resistance of T1D (33).

We also show that residual β-cell function does not affect the paradoxical increase in glucagon secretion during meal ingestion in T1D since individuals across all levels of peak C-peptide response had the same post-prandial glucagon levels as those with undetectable C-

peptide, findings that confirm a recent smaller study (34, 35). Consistent with this result, there was also no difference in the acute glucagon response to GPA across groups of increasing residual C-peptide, and prior work demonstrated impaired suppression of glucagon secretion during a MMTT in youth with T1D within the first 2-years of diagnosis (36). Impaired glucagon suppression to hyperglycemia is also seen in individuals with early, asymptomatic T1D manifested by normal fasting but "diabetic" range post-prandial values (1). Moreover, each of these asymptomatic individuals with T1D also had markedly impaired functional β-cell mass, with the acute insulin response to GPA ~25% of normal. In contrast, despite markedly impaired first phase insulin secretion, antibody positive relatives with non-diabetic OGTTs suppress glucagon appropriately in response to IV glucose. We recently reported that multiple antibody positive individuals prior to clinical diagnosis have a wide range of functional β-cell mass (37). Thus, while clearly a continuum, the data to-date suggest a model whereby loss of functional β-cell mass associated with impaired glucagon suppression to hyperglycemia underlies the transition from pre- to post-clinical diagnosis. Then, as currently demonstrated, regardless of residual C-peptide secretion, once a diagnosis of T1D is established, the reduced functional β-cell mass is no longer capable of exerting reciprocal regulation of glucagon secretion as occurs in nondiabetic individuals (38).

Curiously, there was a positive relationship between the peak C-peptide response and the GIP response during the MMTT. While higher levels of GIP would be expected to augment β-cell function and might contribute to the higher C-peptide, the much more robust relationship between the peak C-peptide response and the β-cell secretory capacity evidences that in the low or negative C-peptide group the low/absent C-peptide is a result of β-cell loss, not lack of incretin augmentation. A possible explanation for the correlation of peak C-peptide to the GIP

response during the MMTT is the presence of mild pancreatic exocrine insufficiency in subjects with lower levels of C-peptide that could affect intestinal nutrient sensing and GIP secretion. Endogenous insulin exerts paracrine trophic effect on the exocrine pancreas via an insuloacinar portal circulation (39), and several studies have demonstrated loss of pancreatic exocrine tissue volume in T1D (40, 41). Consistent with this, a positive relationship between residual C-peptide production and pancreatic exocrine function has been reported in T1D (42), although we did not measure pancreatic exocrine function in the present study.

Whereas α-cell responsiveness to nutrient stimulation such as by amino acids remains intact as shown in the present study by MMTT and arginine administration, T1D is associated with the development of a selective defect in α-cell glucagon secretion in response to hypoglycemia (43). This defect in α-cell responsivity to low blood glucose may also be explained by the loss of the reciprocal regulation of glucagon secretion by neighboring β-cells turning off insulin secretion (38). While the glucagon response to hypoglycemia is already markedly impaired at the onset of T1D (44, 45), islets containing β-cells might retain responsiveness of their α-cells to hypoglycemia and contribute to the better glycemic control and avoidance of hypoglycemia associated with increasing amounts of residual C-peptide production. Prior studies examining this relationship have generated conflicting results, some finding a correlation between stimulated C-peptide levels and the glucagon response to insulin-induced hypoglycemia (46-48), and others finding no relationship (49, 50). Our results demonstrate a weak association of peak C-peptide from the MMTT and the glucagon response to insulin-induced hypoglycemia, supporting that a relationship does exist, but again is most significant with high levels of residual C-peptide. We did not, however, see any difference in the EGP response to hypoglycemia across groups of increasing C-peptide when compared to the negative

C-peptide group, a result consistent with other studies reporting no difference in recovery from hypoglycemia in those with or without residual C-peptide (47), while another study did see modestly greater EGP during hypoglycemia in C-peptide positive when compared to negative T1D (48). In this latter study, the epinephrine response was less in the C-peptide negative versus the C-peptide positive group (48), which likely accounts for the lower EGP response that becomes dependent on epinephrine when the glucagon response is impaired (51). Consistent with this premise, the present study identified no difference in the EGP response, while another study identified no difference in the rate of recovery from hypoglycemia (47) when epinephrine responses to hypoglycemia remained intact. Because the epinephrine response is intact at the onset of T1D (44, 45), the maintenance of the EGP response to defend against the development of low blood glucose appears most dependent on preservation of epinephrine and not low levels of glucagon secretion during hypoglycemia.

These results are important to inform the consideration of potential treatment targets for interventions such as immune modulation aimed at preserving or restoring β-cell function in T1D. Studies that evidenced an association between less hypoglycemia and microvascular complications in people with T1D who had mixed-meal stimulated C-peptide in the range of our low C-peptide group (13) involved cohorts with hundreds of people, and the associations, while statistically significant, were very weak. Others have shown that children 3-6 years after diagnosis with stimulated C-peptide >0.040 pmol/mL had significantly less severe hypoglycemic events and lower HbA_{1c} than those with less or no residual secretion (52). Earlier work has shown a benefit of low levels of residual C-peptide in protecting individuals from the development of ketoacidosis in the setting of insulin deprivation when compared to those with negative C-peptide (53). In an analysis of T1D recipients of islet transplantation selected for

experiencing severe hypoglycemia and having undetectable stimulated C-peptide before transplantation, low levels (<0.200 pmol/mL) of mixed-meal stimulated C-peptide following transplantation were associated with poor glycemic control and excessive glucose variability that improved significantly and in a continuous fashion with C-peptide ≥0.200 pmol/mL until insulin-independence was observed with C-peptide >1.000 pmol/mL (54). Consensus guidelines recommend considering β-cell replacement therapy (currently available as islet or pancreas transplantation) in people with either negative or low C-peptide who are experiencing severe episodes of hypoglycemia complicated by hypoglycemia unawareness or marked glycemic lability (55). Such people are clearly not protected by the presence of low levels of residual C-peptide production, and so the goal of intervention is to restore β-cell function with a C-peptide level of at least 0.200 pmol/mL (55). In the present study, while we were unable to pinpoint a threshold level of C-peptide as being physiologically distinct, the low C-peptide group did not behave any differently than the negative group. Clinically, significantly better glycemic control evidenced by CGM was observed in the group with high (>0.400 pmol/mL) mixed-meal stimulated C-peptide. Thus, our data are consistent with the idea that interventions targeting preservation or restoration of β-cell function in T1D should aim for more than "low" levels of C-peptide production.

In conclusion, classification of residual C-peptide production by the peak value obtained during the MMTT is consistent with the underlying β-cell secretory capacity. While a MMTT peak C-peptide >0.4 pmol/mL may indicate a threshold of physiologic importance for β-cell responsivity to hyperglycemia and α-cell responsivity to hypoglycemia, no amount of residual C-peptide in T1D tested in this study is associated with appropriate suppression of glucagon secretion during hyperglycemia. Importantly, even individuals with no residual C-peptide are

capable of maintaining glucose counterregulation in defense against the development of low blood glucose as long as the epinephrine response to hypoglycemia is intact. Because our study was cross sectional, we cannot determine whether the duration of sustained residual C-peptide production may affect these results. We are not able to comment on the mechanisms by which residual insulin secretion contributed to islet cell and counterregulatory responsiveness in the maintenance of glycemic control. Notwithstanding these limitations, the continuous relationship of MMTT peak C-peptide with measures of β- and α-cell function reported here preclude specification of a discrete level warranting further consideration as a potential requirement or treatment target for interventions aimed at preserving or restoring β-cell function in T1D.

Methods

Participants. Participants were recruited at seven sites in the T1D Exchange Clinic Network. Eligible participants were age 18–65 years, had been diagnosed with T1D between 6 months and 46 years and had a disease duration of at least 2 years. Additional inclusion and exclusion criteria are provided in the Supplementary Material.

Design. Participants were grouped by C-peptide such that negative (<0.007 pmol/mL [<0.02 ng/mL]) would have undetectable stimulated C-peptide by the most sensitive assay available and low (0.017–0.200 pmol/mL [0.05–0.60 ng/mL]) would have detectable C-peptide by current standard assays but below the cut-off deemed clinically meaningful by the Diabetes Control and Complications Trial (DCCT) (10). The intermediate (>0.200–0.400 pmol/mL [>0.60–1.20 ng/mL]) and high (>0.400 pmol/mL [>1.20 ng/mL]) groups were assigned to understand the relevance of stimulated C-peptide above the DCCT threshold of 0.200 pmol/ml, and twice that

level, respectively, to assure that we sampled across the distribution of C-peptide values based on approximately 5% of individuals with similar disease duration having random C-peptide levels >0.400 pmol/mL (16). Because the non-fasting C-peptide is predictive of the peak C-peptide during the MMTT (16), participants were selected to proceed with the MMTT based on their screening level of non-fasting C-peptide with the goal of enrolling ~16 participants per group. Participant grouping for analysis was ultimately determined by the peak C-peptide during the MMTT. All metabolic testing was completed within a 30-day period.

Mixed-meal tolerance test (MMTT). Following a 10-hour overnight fast, an antecubital or forearm vein catheter was placed for blood sampling. After baseline blood sampling at t =-10 and -1 min, at t =0, a standardized liquid meal (Boost High Protein, 6 mL/kg up to 360 mL) was consumed over a 5 min period. Additional blood samples were taken at 30, 60, 90, and 120 min from the start of the meal (16).

Glucose-potentiated arginine (GPA) test. Following a 10-hour overnight fast, one catheter was placed in an antecubital vein for infusions, and another catheter was placed in a distal forearm or hand vein for blood sampling, with the hand placed in a heating pad to promote arterialization of the venous blood. After baseline blood sampling at t =-5 and -1 min, at t =0, a hyperglycemic clamp (56) using a variable rate infusion of 20% dextrose was performed to achieve a plasma glucose concentration of ~230 mg/dL. Blood samples were taken every 5 min, centrifuged, and measured at bedside with an automated glucose analyzer (YSI 2300; Yellow Springs Instruments, Yellow Springs, OH) to adjust the infusion rate and achieve the desired plasma glucose concentration. At t =40 and 44 min, blood samples were collected prior to the bolus

infusion of 5 g of 10% arginine over 1-min starting at t =45. Additional blood samples were collected at t =47, 48, 49, and 50 min (corresponding to 2, 3, 4, and 5 min after the infusion of arginine). Participants who were C-peptide negative did not undergo GPA testing.

Hyperinsulinemic euglycemic-hypoglycemic clamp. Participants either spent the night or arrived early in the morning following a 10-hour overnight fast to the clinical research center. One catheter was placed in an antecubital vein for infusions, and another catheter was placed in a distal forearm or hand vein for blood sampling, with the hand placed in a heating pad to promote arterialization of the venous blood. Participants were converted from subcutaneous insulin to a low-dose intravenous insulin infusion protocol to target a blood glucose of 81–115 mg/dL prior to testing. A baseline blood sample was collected for determination of the background enrichment of 6,6-$^2H_2$-glucose. At t =-120 min, a primed (5 mg/kg · fasting plasma glucose in mg/dL/90 given over 5 min) continuous (0.05 mg·kg^{-1}·min^{-1} for 355 min) infusion of 6,6-$^2H_2$-glucose (99% enriched; Cambridge Isotopes Laboratories, Andover, MA) was administered to assess EGP before and during the induction of hyperinsulinemia. After blood sampling at t =-15 and -1 min, at t =0 min a primed (1.6 mU·kg^{-1}·min^{-1} given over 10 min) continuous (0.8 mU·kg^{-1}·min^{-1} for 230 min) infusion of insulin was administered to produce hyperinsulinemia (57). Subsequently, a variable rate infusion of 20% glucose enriched to ~2.0% with 6,6-$^2H_2$-glucose was administered according to the glycemic clamp technique to achieve a plasma glucose ~90 mg/dL by ~60 min and maintained until ~120 min, after which the plasma glucose was allowed to fall to ~50 mg/dL by ~180 min and maintained until 240 min. Blood samples were taken every 5 min, centrifuged, and measured at the bedside with an automated glucose analyzer (YSI 2300) to adjust the glucose infusion rate and achieve the desired plasma glucose concentration.

Additional blood samples were collected at t = 30, 60, 90, 105, 120, 150, 180, 210, 225, and 240 min for biochemical analysis. A questionnaire was administered every 30 min during the study to quantitate autonomic symptoms as the sum of scores ranging from 0 (none) to 5 (severe) for each of the following symptoms: anxiety, palpitations, sweating, tremor, hunger, and tingling (58).

Biochemical analysis. Blood samples were collected into serum separator tubes (for glucose, free fatty acids, insulin, C-peptide, and proinsulin) and on ice into EDTA containing tubes (for 6,6-$^2H_2$-glucose and epinephrine) with protease inhibitor cocktail containing dipeptidyl peptidase 4 inhibitor (for glucagon, glucagon-like peptide-1 [GLP-1], and glucose-dependent insulinotropic polypeptide [GIP]), centrifuged at 4 °C, separated, and frozen at -80 °C for subsequent analysis. Glucose was determined by the hexokinase enzymatic method and free fatty acids by enzymatic colorimetrics (Roche Modular P auto-analyzer; Roche Diagnostics, Indianapolis, IN). Insulin and C-peptide levels were measured by two-site immuno-enzymometric assays (Tosoh 2000 auto-analyzer; Tosoh Bioscience, San Francisco, CA). The C-peptide assay has a sensitivity level of detection at 0.007 pmol/mL (0.02 ng/mL), and the inter-assay coefficient of variation for low-level C-peptide controls is 3.2%. Proinsulin and glucagon were determined by double-antibody radioimmunoassays (Millipore, Billerica, MA). Total GLP-1 and total GIP were measured by enzyme-linked immunosorbent assays (Millipore). Plasma epinephrine was measured by high-performance liquid chromatography with electrochemical detection. Enrichment of 6,6-$^2H_2$-glucose was measured by gas chromatography-mass spectrometry.

Continuous glucose monitoring (CGM). CGM was performed blinded as a validated assessment of glycemic control during the month of metabolic study. The CGM device (Dexcom G4 Platinum with 505 software; Dexcom, San Diego, CA) measures interstitial glucose every five minutes from a subcutaneously inserted sensor in the range of 40–400 mg/dL. Participants wore CGMs for up to 7 days, during which they were instructed to monitor their blood glucose at least 3 times daily and calibrate the CGM device at least every 12 hours.

Calculations. Incremental responses from the MMTT for C-peptide, glucagon, GLP-1, and GIP were calculated as peak minus baseline values.

Acute C-peptide, proinsulin, and glucagon responses to arginine during the 230 mg/dL glucose clamp (ACR_{arg}, APR_{arg}, and AGR_{arg}, respectively) were calculated as the peak of the 2-, 3-, 4-, and 5-min values minus the mean of the pre-arginine values (56). The fasting proinsulin-to-C-peptide ratio was calculated as the molar concentration of proinsulin divided by the molar concentration of C-peptide (59). We also examined the proinsulin secretory ratio (PISR) calculated as the molar concentration of the acute proinsulin response to arginine divided by the acute C-peptide response to arginine (59, 60).

The rates of appearance (R_a) and disposal (R_d) of glucose during the hyperinsulinemic euglycemic-hypoglycemia clamp were calculated using Steele's non-steady state equation modified for the use of stable isotopes, as previously described (22). EGP was calculated from the difference between the R_a of glucose in the plasma and the infusion rate of exogenous glucose. Total body (S_I) and peripheral (S_{IP}) insulin sensitivity were calculated from the last 30 min of EU as previously described (61, 62). Hepatic insulin sensitivity was determined from the percent suppression of EGP as $S_{IH} = 1-(EGP_2/EGP_1)$ where EGP_1 and EGP_2 are the endogenous

glucose production at baseline and during the last 30 min, respectively. The magnitude of each hormonal, incremental symptom, and EGP response to hypoglycemia was assessed as the change in values from the last 30 min of euglycemia to the last 30 min of hypoglycemia.

CGM variables were calculated for all participants with a minimum of 72 hours of daytime (0800–2200) and 24 hours of nighttime (2200–0800) data. Interstitial glucose data were summarized to provide mean glucose, glucose standard deviation (SD), coefficient of variation (CV) and percent (%) time with glucose in range 70–180 mg/dL, <70 mg/dL, and >180 mg/dL (63). CV for glucose was calculated from the glucose SD divided by the mean glucose.

Statistical analysis. Data are given as means±SD except where otherwise noted. Comparison of results across the C-peptide groups was performed with the Kruskal Wallis test and when significant differences at $P \leq 0.05$ were found, pairwise comparisons between groups were performed using the Mann-Whitney U test. Linear regression, analysis of covariance, and Spearman's rank correlations were used to evaluate continuous relationships among the different measures of islet function and glucose counterregulation.

Study approval. The institutional review boards of each participating site approved the study, and all participants provided written informed consent to participate.

Author contributions

MRR, CEM and CJG designed the study, researched data, contributed to the statistical analyses, and wrote and edited the manuscript. HTB and AY researched data, contributed to the statistical analyses, and wrote and edited the manuscript. WH, KJN, MAC, JLS, TH, REP, and VNS contributed to the study design, researched data, and reviewed and edited the manuscript. KMM researched data, and reviewed and edited the manuscript. MRR and CJG are the guarantors of this work, and as such, had full access to all the data in the study and take responsibility for the integrity of the data and accuracy of the data analysis.

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Table 1. Participant characteristics

	C-peptide group				
Characteristic	**Negative (*n* = 15)**	**Low (*n* = 16)**	**Intermediate (*n* = 15)**	**High (*n* = 17)**	***P*-value**
Sex (% female)	47	44	60	59	
Age (years)	26 ± 11	29 ± 8	27 ± 9	29 ± 9	0.39
BMI (kg/m^2)	25 ± 3	24 ± 3	24 ± 3	24 ± 3	0.45
T1D duration (years)	13 ± 9	7 ± 4	5 ± 2	5 ± 5	<0.001
Insulin use ($U \cdot kg^{-1} \cdot d^{-1}$)	0.7 ± 0.2	0.6 ± 0.2	0.5 ± 0.2	0.5 ± 0.3	0.01
HbA_{1c} (%)[a]	7.6 ± 0.7	6.9 ± 1.0	7.2 ± 0.8	6.8 ± 1.0	0.08

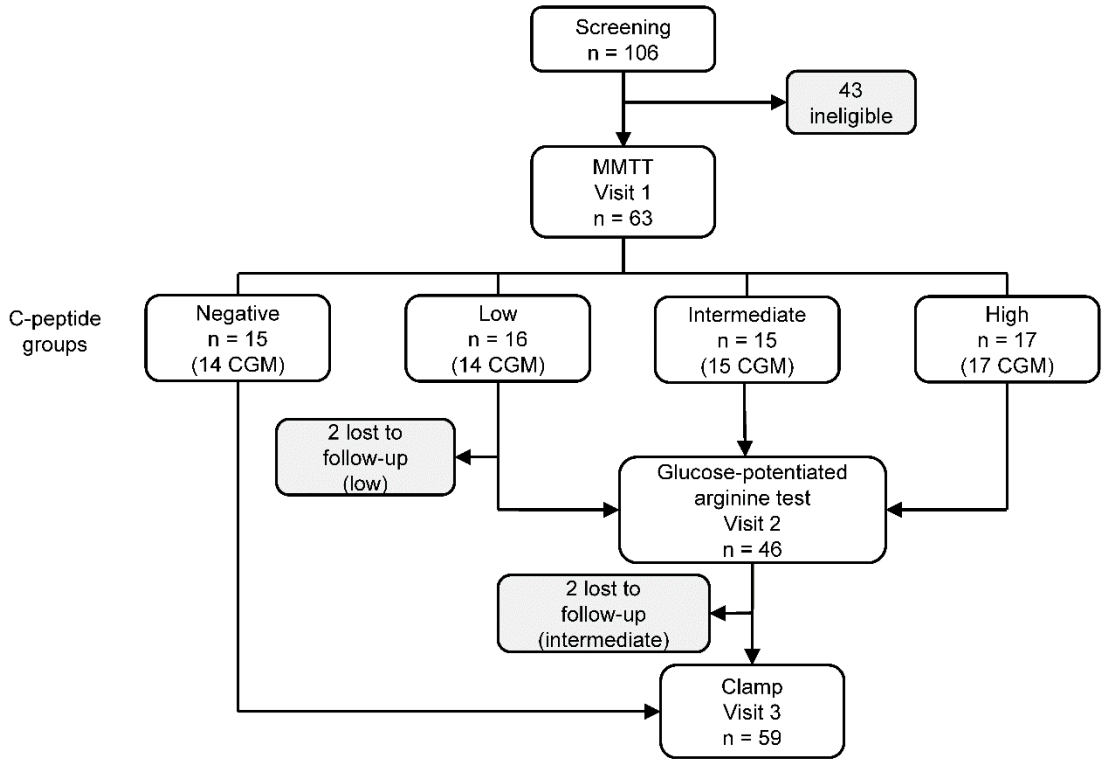
Data are means±SD.

[a]To convert to mmol/mol, multiply by 10.93 and subtract 23.50.

Table 2.Continuous glucose monitoring (CGM)

	C-peptide group				
Variable	**Negative* (*n* = 14)**	**Low* (*n* = 14)**	**Intermediate (*n* = 15)**	**High (*n* = 17)**	***P*-value**
CGM duration (hours)	152 ± 78	165 ± 59	139 ± 26	148 ± 53	0.55
Mean glucose (mg/dL)	161 ± 36	177 ± 29	162 ± 32	140 ± 25	0.02
Time with glucose 70–180 mg/dL (%)	58 ± 15	52 ± 16	59 ± 13	72 ± 12	<0.01
Time with glucose >180 mg/dL (%)	35 ± 18	43 ± 17	34 ± 16	22 ± 13	0.01
Time with glucose <70 mg/dL (%)	7 ± 10	5 ± 4	6 ± 7	7 ± 5	0.79
CV (%)	39 ± 8	41 ± 7	39 ± 7	38 ± 9	0.57

Data are means±SD. CV, coefficient of variation calculated from the glucose SD divided by the mean glucose. *Three participants (1 negative C-peptide, 2 low C-peptide) did not complete continuous glucose monitoring.



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773 **Figure 1. Study design.** Eligibility was determined at a screening visit where measurement of
774 non-fasting C-peptide was used to balance recruitment of participants to C-peptide groups. C-
775 peptide group was ultimately determined from the mixed-meal tolerance test (MMTT) peak C-
776 peptide measured at Visit 1. Participants in the detectable (low, intermediate, and high) C-
777 peptide groups underwent a glucose-potentiated arginine test at Visit 2, and participants in the
778 undetectable (negative) and detectable C-peptide groups underwent a hyperinsulinemic
779 euglycemic followed by hypoglycemic clamp at Visit 3, as well as continuous glucose
780 monitoring (CGM).

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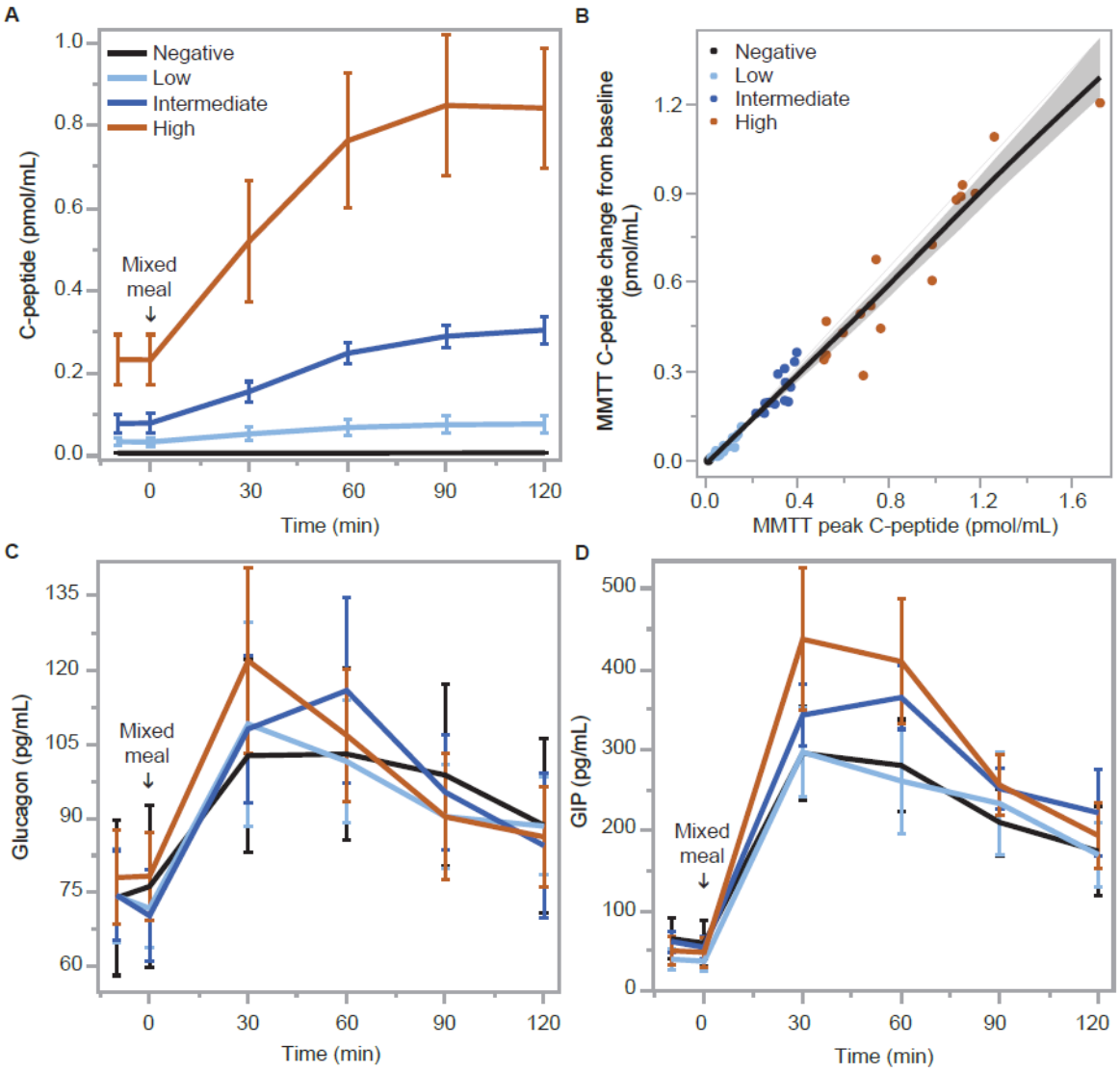


Figure 2. Mixed-meal tolerance test (MMTT). (**A, B**) Serum C-peptide response to ingestion of a standardized liquid meal was different by group based on peak C-peptide level (negative, <0.007 pmol/mL [<0.02 ng/mL]; low, 0.007–0.200 pmol/mL [0.05–0.60 ng/mL]; intermediate, >0.200–0.400 pmol/mL [>0.60–1.20 ng/mL]; high, >0.400 pmol/mL [>1.20 ng/mL]), and by continuous relationship to peak C-peptide. (**C, D**) Plasma glucagon response was not different while plasma glucose-dependent insulinotropic polypeptide (GIP) response was different to meal ingestion by peak C-peptide level group. Data are means with error bars denoting 95% confidence intervals.

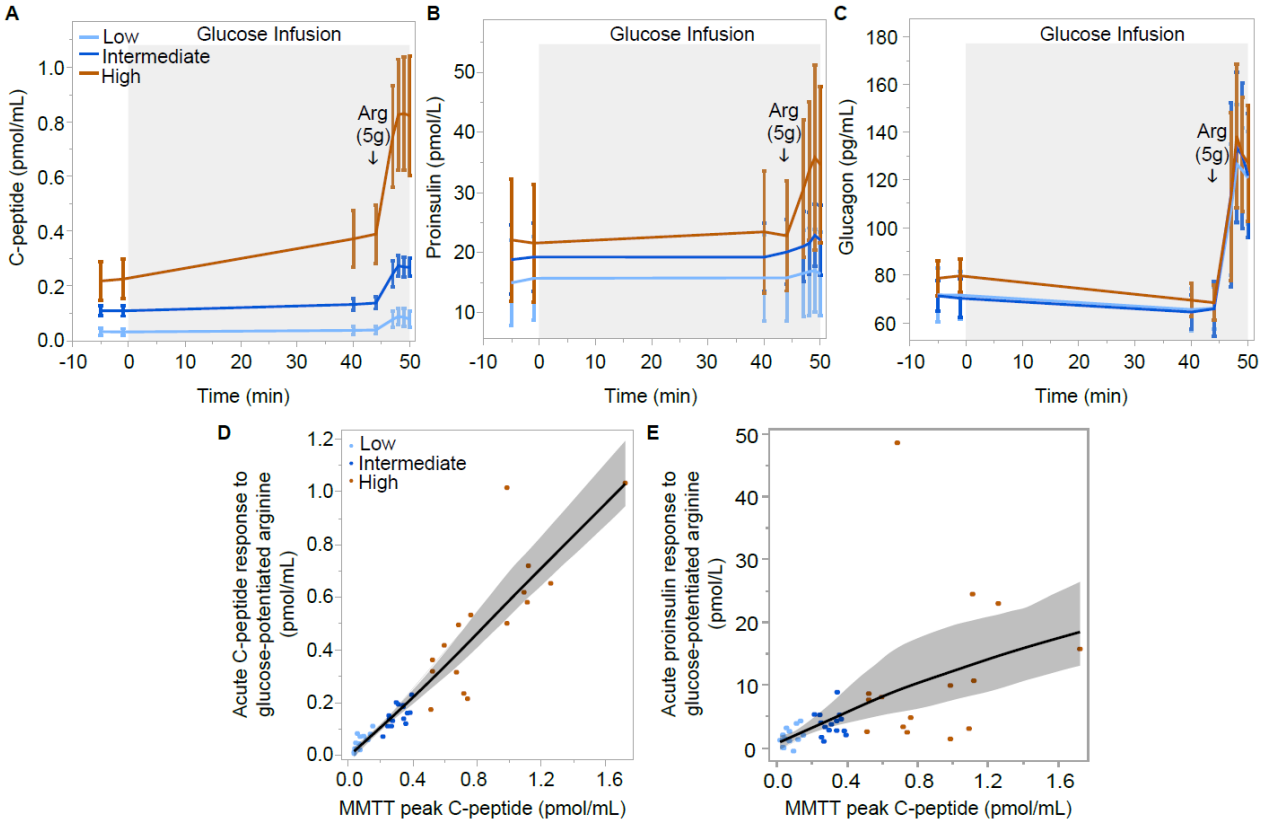
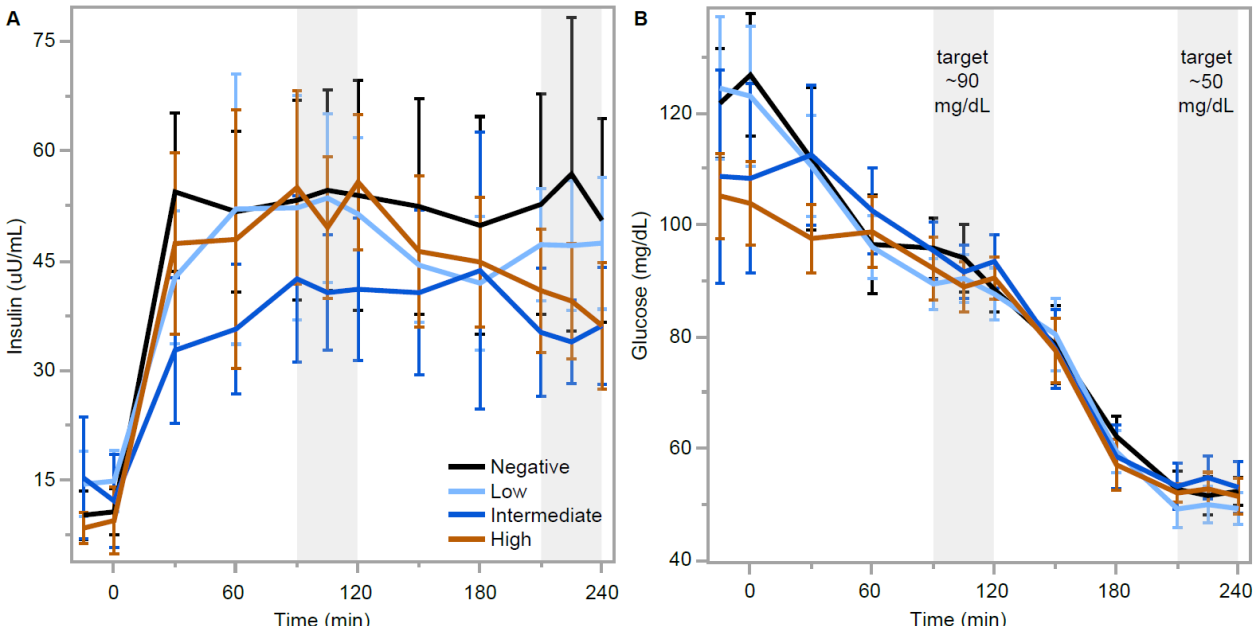


Figure 3. Glucose-potentiated arginine (GPA) test. (**A, B**) Serum C-peptide and proinsulin responses to an approximately 230 mg/dL hyperglycemic clamp and to the injection of arginine after 45 min of glucose infusion were different by group based on MMTT peak C-peptide level. (**C**) Plasma glucagon responses to glucose-potentiated arginine were not different by group. (**D, E**) Relationship between the acute C-peptide and proinsulin responses to glucose-potentiated arginine and MMTT peak C-peptide. Data are means with error bars denoting 95% confidence intervals.



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808 **Figure 4. Hyperinsulinemic euglycemic (EU) followed by hypoglycemic (HYPO) clamp.** (**A**)

809 Serum insulin levels were not statistically different across groups based on MMTT peak C-

810 peptide levels during the EU or HYPO phases of testing. (**B**) Serum glucose levels were well

811 matched across groups during the EU (~90 mg/dL) and HYPO (~50 mg/dL) phases of testing.

812 Data are means with error bars denoting 95% confidence intervals.

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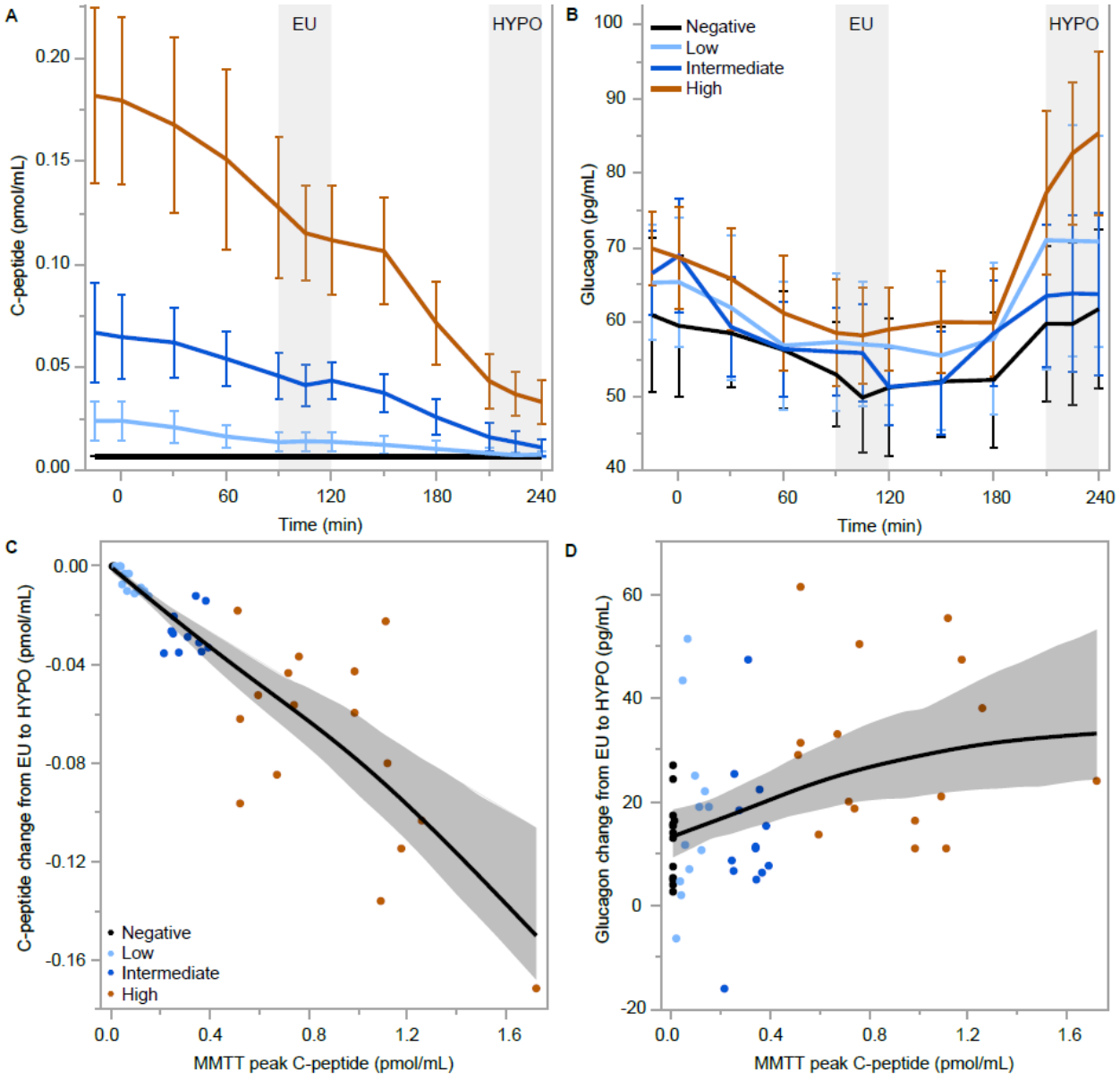


Figure 5. β- and α-cell responses from the euglycemic (EU) to hypoglycemic (HYPO) clamp condition. (**A, B**) Suppression of C-peptide and increase in glucagon were greater by group based on MMTT peak C-peptide level. (**C, D**) Relationships of the change in C-peptide and glucagon levels between the EU and HYPO conditions to MMTT peak C-peptide. Data are means with error bars denoting 95% confidence intervals.

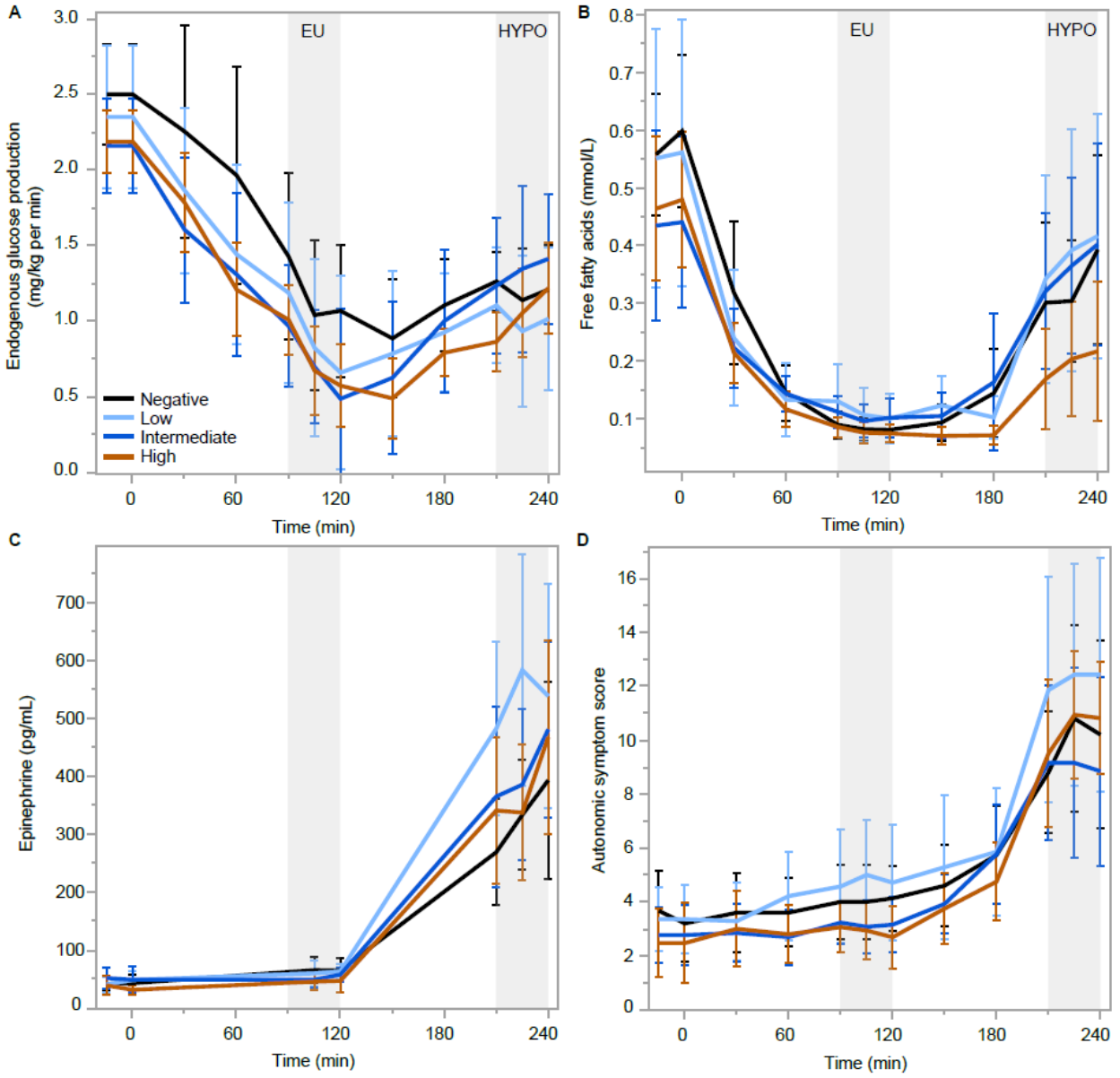
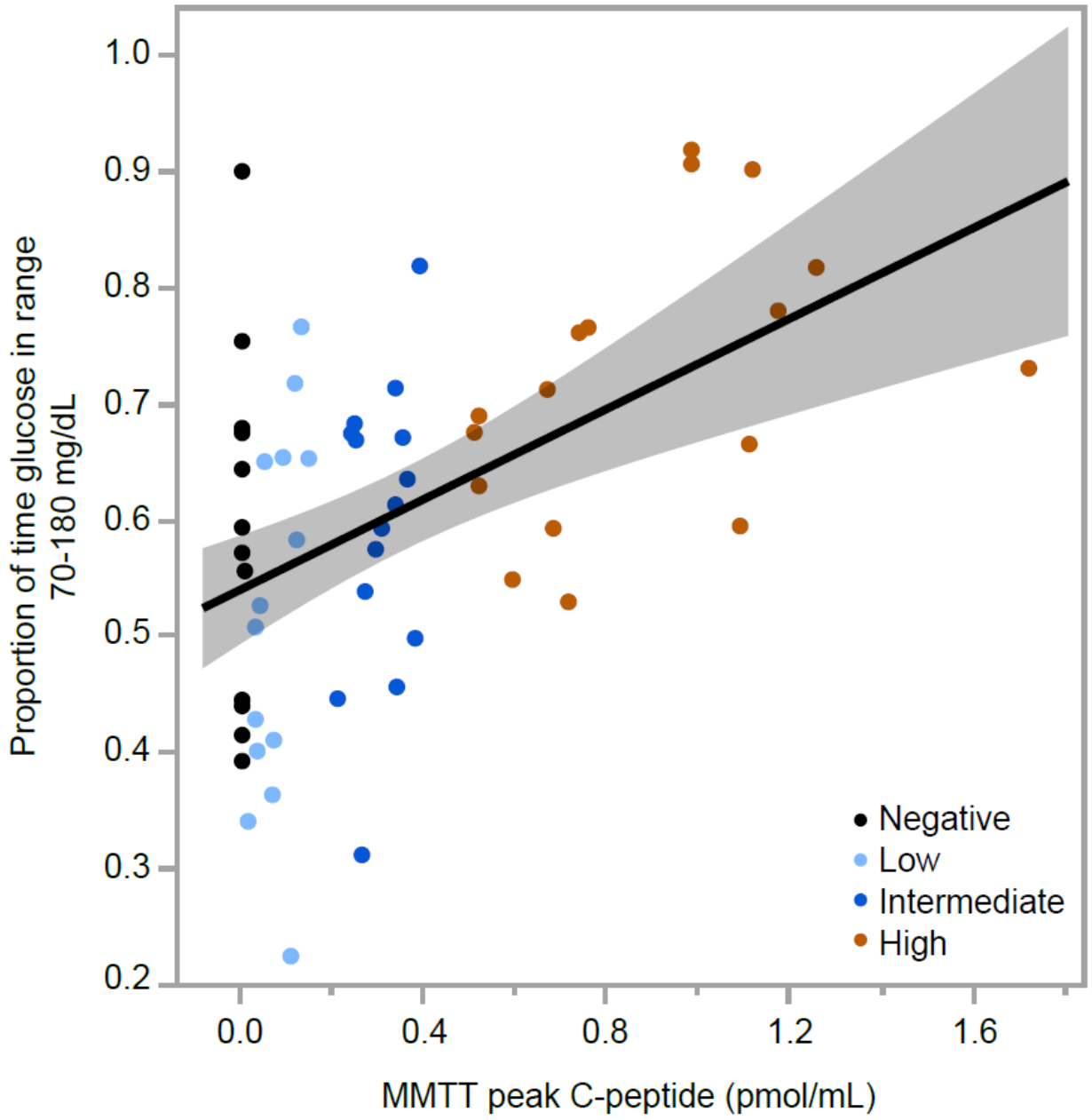


Figure 6. Glucose counterregulatory responses from the euglycemic (EU) to hypoglycemic (HYPO) clamp condition. No differences were seen across groups based on MMTT peak C-peptide level for responses of counterregulatory endogenous glucose production (**A**), serum free fatty acids (**B**), plasma epinephrine (**C**), or autonomic symptom generation (**D**). Data are means with error bars denoting 95% confidence intervals.



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839 **Figure 7. Glucose time in range by MMTT peak C-peptide.** Relationship between proportion
840 of glucose time in range of 70–180 mg/dL by continuous glucose monitoring (CGM) and MMTT
841 peak C-peptide. All individuals in the high C-peptide group (MMTT peak C-peptide >0.400
842 pmol/mL) maintained greater than 50% time in the target glucose range.