# Proinsulin Secretion Is a Persistent Feature of Type 1 Diabetes

*Diabetes Care 2019;42:258–264 | https://doi.org/10.2337/dc17-2625*

*Emily K. Sims,[1,2] Henry T. Bahnson,[3] Julius Nyalwidhe,[4] Leena Haataja,[5] Asa K. Davis,[3] Cate Speake,[3] Linda A. DiMeglio,[1,2] Janice Blum,[6] Margaret A. Morris,[7] Raghavendra G. Mirmira,[1,2,8,9,10] Jerry Nadler,[7] Teresa L. Mastracci,[10,11] Santica Marcovina,[12] Wei-Jun Qian,[13] Lian Yi,[13] Adam C. Swensen,[13] Michele Yip-Schneider,[14] C. Max Schmidt,[14] Robert V. Considine,[9] Peter Arvan,[5] Carla J. Greenbaum,[3] Carmella Evans-Molina,[2,8,9,10,15] and the T1D Exchange Residual C-peptide Study Group**


## OBJECTIVE

**Abnormally elevated proinsulin secretion has been reported in type 2 and early type 1 diabetes when significant C-peptide is present. We questioned whether individuals with long-standing type 1 diabetes and low or absent C-peptide secretory capacity retained the ability to make proinsulin.**

## RESEARCH DESIGN AND METHODS

**C-peptide and proinsulin were measured in fasting and stimulated sera from 319 subjects with long-standing type 1 diabetes (≥3 years) and 12 control subjects without diabetes. We considered three categories of stimulated C-peptide: *1*) C-peptide positive, with high stimulated values ≥0.2 nmol/L; *2*) C-peptide positive, with low stimulated values ≥0.017 but <0.2 nmol/L; and *3*) C-peptide <0.017 nmol/L. Longitudinal samples were analyzed from C-peptide–positive subjects with diabetes after 1, 2, and 4 years.**

## RESULTS

**Of individuals with long-standing type 1 diabetes, 95.9% had detectable serum proinsulin (>3.1 pmol/L), while 89.9% of participants with stimulated C-peptide values below the limit of detection (<0.017 nmol/L; *n* = 99) had measurable proinsulin. Proinsulin levels remained stable over 4 years of follow-up, while C-peptide decreased slowly during longitudinal analysis. Correlations between proinsulin with C-peptide and mixed-meal stimulation of proinsulin were found only in subjects with high stimulated C-peptide values (≥0.2 nmol/L). Specifically, increases in proinsulin with mixed-meal stimulation were present only in the group with high stimulated C-peptide values, with no increases observed among subjects with low or undetectable (<0.017 nmol/L) residual C-peptide.**

## CONCLUSIONS

**In individuals with long-duration type 1 diabetes, the ability to secrete proinsulin persists, even in those with undetectable serum C-peptide.**


Type 1 diabetes results from autoimmune-mediated destruction of the pancreatic β-cell, resulting in the need for exogenous insulin treatment (1). The classic paradigm that type 1 diabetes leads to a complete loss of β-cell mass and absolute insulin deficiency has been challenged by recent data (1). Analysis of pancreatic sections from organ donors with diabetes indicates the presence of residual insulin-containing islets many years after disease onset (2). In addition, multiple groups have reported detectable levels of serum C-peptide in cohorts of individuals with long-duration T1D (3–9). These studies have included highly selected populations, such as the Joslin

[1]*Department of Pediatrics, Indiana University School of Medicine, Indianapolis, IN*
[2]*Center for Diabetes and Metabolic Diseases, Indiana University School of Medicine, Indianapolis, IN*
[3]*Diabetes Clinical Research Program, Benaroya Research Institute, Seattle, WA*
[4]*Department of Microbiology and Molecular Cell Biology, Eastern Virginia Medical School, Norfolk, VA*
[5]*Division of Metabolism, Endocrinology & Diabetes, University of Michigan Medical Center, Ann Arbor, MI*
[6]*Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, IN*
[7]*Department of Internal Medicine, Eastern Virginia Medical School, Norfolk, VA*
[8]*Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, IN*
[9]*Department of Medicine, Indiana University School of Medicine, Indianapolis, IN*
[10]*Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN*
[11]*Indiana Biosciences Research Institute, Indianapolis, IN*
[12]*Northwest Lipid Metabolism and Diabetes Research Laboratories, University of Washington, Seattle, WA*
[13]*Biological Sciences Division, Pacific Northwest National Laboratory, Richland, WA*
[14]*Department of Surgery, Indiana University School of Medicine, Indianapolis, IN*
[15]*Richard L. Roudebush VA Medical Center, Indianapolis, IN*

Medalists, who were identified on the basis of long-term survival, as well as groups more reflective of general diabetes populations, with estimates that up to 80% of individuals with type 1 diabetes retain the ability to secrete small amounts of stimulated C-peptide (3–7).

For detection of residual β-cell mass and function, these studies have relied nearly exclusively on the measurement of C-peptide, which is generated from the processing of immature preproinsulin molecules into insulin and C-peptide. Preproinsulin processing begins with cleavage of the N-terminal signal peptide to form proinsulin within the lumen of the β-cell endoplasmic reticulum (ER) (10). Disulfide bond formation and terminal protein folding occurs in the ER and Golgi, and proinsulin is eventually cleaved into mature insulin and C-peptide by the enzymes prohormone convertase 1/3, prohormone convertase 2, and carboxypeptidase E within secretory granules (10). β-Cell dysfunction, such as that caused by inflammatory or ER stress, results in the accumulation and release of incompletely processed proinsulin (11–13). Thus, measurement of C-peptide secretion alone could underestimate the ability of the β-cell to initiate hormone production, while increased proinsulin secretion may provide insight into specific disease pathology.

Studies in human cohorts have suggested that abnormalities in β-cell proinsulin processing have clinical relevance to type 1 diabetes. Levels of circulating proinsulin relative to C-peptide (PI:C ratios) are elevated at the time of type 1 diabetes onset and have been shown to be predictive of type 1 diabetes development in autoantibody-positive individuals (14–16). Similarly, the ratio of proinsulin–to–insulin-positive β-cell area is increased in pancreatic sections from donors with recent-onset type 1 diabetes and in autoantibody-positive donors without diabetes (17). Persistent elevations in islet proinsulin content relative to insulin and C-peptide have also been reported in whole pancreata from donors with long-standing type 1 diabetes (18). However, whether detectable proinsulin secretion is present in individuals with extended-duration type 1 diabetes has not been adequately tested.

To gain insight into the relationships between C-peptide and proinsulin secretion in long-standing type 1 diabetes, we analyzed longitudinal samples from a diverse cohort of subjects with established disease (≥3 years) who were categorized based on the presence or absence of residual stimulated C-peptide secretion. We sought to define patterns of change in C-peptide and proinsulin secretion over 4 years of follow-up and to explore relationships between meal-stimulated C-peptide and proinsulin secretion.

## RESEARCH DESIGN AND METHODS

### Study Approval

Sample collections were performed after institutional review board approval was obtained from T1D Exchange Network sites and Indiana University. Written informed consent was obtained from participants prior to study inclusion.

### Subjects

We previously reported nonfasting serum C-peptide levels in 919 individuals with varying durations of disease (all ≥3 years from disease onset) identified through the T1D Exchange clinic registry who participated in the Residual C-peptide in Type 1 Diabetes Study evaluating residual insulin secretion in those with long-standing type 1 diabetes (3). To be enrolled in this study, an individual must have had a clinical diagnosis of type 1 diabetes made by an endocrinologist on the basis of either positive islet cell antibodies or insulin therapy started around the time of diagnosis and used continually thereafter (with the exception of individuals who received a pancreas or islet transplant) (3). From this group, 319 subjects in whom mixed-meal tolerance tests (MMTTs) were performed were included in the current analysis (3). In brief, this included subjects with detectable random nonfasting C-peptide and 10% of subjects with C-peptide <0.017 nmol/L on a random nonfasting test ($n$ = 99). For subjects with serum C-peptide ≥0.017 nmol/L ($n$ = 220), repeat MMTTs were performed 1, 2, and 4 years after the initial MMTT. In addition, MMTT was performed in a small group of healthy adult control subjects locally at Indiana University School of Medicine ($n$ = 12). Subject characteristics are described in Table 1.

### Measurements and Assays

Standard MMTTs were performed as previously described with blood samples drawn at −10, 0, 30, 60, 90, and 120 min (3,19). Control samples drawn at Indiana University were immediately processed and stored at −80 without freeze/thaw prior to testing. Serum from subjects with diabetes was shipped to Northwest Lipid Metabolism and Diabetes Research Laboratories (NWRL, University of Washington) on cold packs the day of sample collections. Samples were immediately analyzed for C-peptide. Otherwise, serum was stored at −80°C without freeze/thaw until proinsulin measurement. *HLA-DRB1* typing, $HbA_{1c}$, fasting glucose, and autoantibodies against GAD, islet antigen 2 (IA-2), and zinc transporter 8 (ZnT8) were measured as previously described (19).

C-peptide values were measured from each MMTT time point in a blinded fashion by NWRL using the Tosoh two-site immunoenzymometric assay (Tosoh Bioscience), which had a sensitivity of 0.017 nmol/L at study start (3,19). Though the TOSOH assay sensitivity changed to 0.007 nmol/L over the course of the study, for consistency in our longitudinal analyses, the threshold

*Corresponding author: Carmella Evans-Molina, cevansmo@iu.edu, or Emily K. Sims, eksims@iu.edu*

*Received 15 December 2017 and accepted 12 October 2018*

*This article contains Supplementary Data online at http://care.diabetesjournals.org/lookup/suppl/doi:10.2337/dc17-2625/-/DC1.*

**A complete list of the T1D Exchange Residual C-peptide Study Group can be found in Supplementary Data online.*



**See accompanying article, p. 183.**

**Table 1—Characteristics of the study population**

| Variable | C-peptide <0.017 nmol/L (*n* = 99) | C-peptide 0.017–0.2 nmol/L (*n* = 117) | C-peptide ≥0.2 nmol/L (*n* = 103) | Control subjects (*n* = 12) |
|---|---|---|---|---|
| Age at initial MMTT (years)*** | 34.0 (17.0, 56.0) | 29.0 (17.0, 45.0) | 41.0 (28.0, 48.8) | 20.5 (18.0, 25.8) |
| Sex (% male) | 46.5 | 42.7 | 44.7 | 50 |
| BMI (kg/m$^2$) | 25.5 (22.0, 29.0) | 25.4 (22.1, 28.4) | 26.1 (22.7, 29.2) | 24.7 (23.1, 28.4) |
| Race/ethnicity (% non-Hispanic white)* | 93.9 | 87.2 | 86.7 | 66.7 |
| T1D duration at consent (years)*** | 16.0 (7.0, 33.0) | 6.0 (4.0, 11.5) | 7.0 (4.0, 13.0) | n/a |
| Age at diagnosis (years)*** | 15.0 (7.0, 25.0) | 19.0 (11.0, 45.0) | 29.0 (21.5, 38.0) | n/a |
| $HbA_{1c}$ (%)* | 7.8 (5.3, 8.7) | 7.7 (6.8, 9.3) | 7.3 (6.6, 8.1) | n/a |
| GAD positive* | 41.4 | 62.4 | 60 | n/a |
| IA2 positive** | 35.4 | 44.4 | 22.9 | n/a |
| ZnT8 positive*** | 14.1 | 40.17 | 25.7 | n/a |
| ≥1 high risk HLA DRB1 alleles present | 83.2 | 76.2 | 75 | n/a |

Data represent values obtained at initial visit and are median (interquartile range) or percent unless otherwise indicated. AUC, area under the curve; n/a, not applicable. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ for differences among groups using Kruskal-Wallis test for continuous variables and $\chi^2$ test for categorical variables.

of 0.017 nmol/L was applied throughout. Proinsulin levels were assayed at the −10 and 90 min time points in a blinded fashion by NWRL using a radioimmunoassay (catalog number HPI-15 K; Millipore), which detects 100% human intact proinsulin, 95% human Des (31,32) proinsulin, and <0.1% human Des (64,65) proinsulin (20). The reported sensitivity of this assay is 3.1 pmol/L. Intra- and interassay variation determined by NWRL were 1.4–6.0% and 4.84–7.48%, respectively. Cross-reactivity for both human C-peptide and human insulin are <0.1%. Experiments performed to validate assay variation, sensitivity, specificity, and cross-reactivity, including analysis of sera from patients before and after pancreatectomy, analysis of insulin antibody–positive serum, and validation of radioimmunoassay results using targeted mass spectrometry analysis of serum, are included in Supplementary Tables 1–6 and Supplementary Figs. 1–4.

MMTT 90-min values were used to define stimulated C-peptide or proinsulin secretion. For C-peptide–positive subjects, PI:C ratios were calculated as molar ratios × 100.

### Statistics

A Kruskal-Wallis test with Dunn multiple comparisons test was used to compare continuous variables between groups, and a $\chi^2$ test was used to compare proportions. An intraclass correlation coefficient was calculated to quantify the degree of clustering for repeated longitudinal proinsulin values. Analyses were done using SAS, version 9.4, and GraphPad Prism 7.0. For all analyses, two-tailed *P* values of ≤0.05 were considered significant.

## RESULTS

To define patterns of C-peptide and proinsulin secretion in long-standing type 1 diabetes, we analyzed hormone secretion at fasting and in response to mixed-meal stimulation in a cohort of 319 individuals with diabetes of ≥3 years' duration (range 3–81). Demographics of the study cohort are reported in Table 1. MMTTs were performed at baseline and then repeated at 12-month, 2-year, and 4-year follow-up visits. As previously reported, we considered three categories of stimulated C-peptide: *1*) C-peptide positive, with stimulated values ≥0.2 nmol/L; *2*) C-peptide positive, with stimulated values ≥0.017 but <0.2 nmol/L; and *3*) C-peptide <0.017 nmol/L (3). Median fasting and stimulated C-peptide values from the initial visit for each group are shown in Table 2, with individual stimulated values from each visit displayed in Fig. 1*A*. Although most subjects had small reductions in stimulated C-peptide over 4 years of follow-up, most remained within their originally assigned C-peptide category.

To categorize residual proinsulin secretion between the groups, we assayed proinsulin levels from the MMTT fasting

**Table 2—Fasting and stimulated C-peptide and proinsulin values**

| Variable | C-peptide <0.017 nmol/L (*n* = 99) | C-peptide 0.017–0.2 nmol/L (*n* = 117) | C-peptide ≥0.2 nmol/L (*n* = 103) | Control subjects (*n* = 12) |
|---|---|---|---|---|
| Fasting C-peptide (nmol/L) | n/a | 0.023 (<0.017, 0.036)***# | 0.156 (0.095, 0.30)* | 0.601 (0.525, 0.800) |
| Stimulated (90 min) C-peptide (nmol/L) | n/a | 0.079 (0.040, 0.111)***# | 0.513 (0.344, 0.847)* | 1.84 (1.08, 2.47) |
| Fasting proinsulin (pmol/L) | 13.5 (6.2, 23.8) | 10.9 (7.3, 21.5) | 15.2 (9.8, 23.8) | 15.19 (11.8, 17.4) |
| Stimulated (90 min) proinsulin (pmol/L) | 11.2 (4.6, 19.7)***# | 11.3 (7.3, 34.8)***# | 22.5 (17.1, 34.8) | 46.7 (34.3, 74.2) |
| Fasting PI:C ratio | n/a | 37.7 (22.0, 58.5)***# | 9.6 (5.9, 18.1)** | 2.3 (2.0, 3.1) |
| Stimulated PI:C ratio | n/a | 15.4 (8.4, 27.9)***# | 4.9 (2.4, 6.9) | 2.6 (2.4, 3.2) |

Data represent values obtained at initial visit and are expressed as median (interquartile range). n/a, not applicable. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ compared with control subjects and #$P < 0.001$ compared with C-peptide ≥0.2 nmol/L group using Kruskal-Wallis test with Dunn multiple comparisons test.

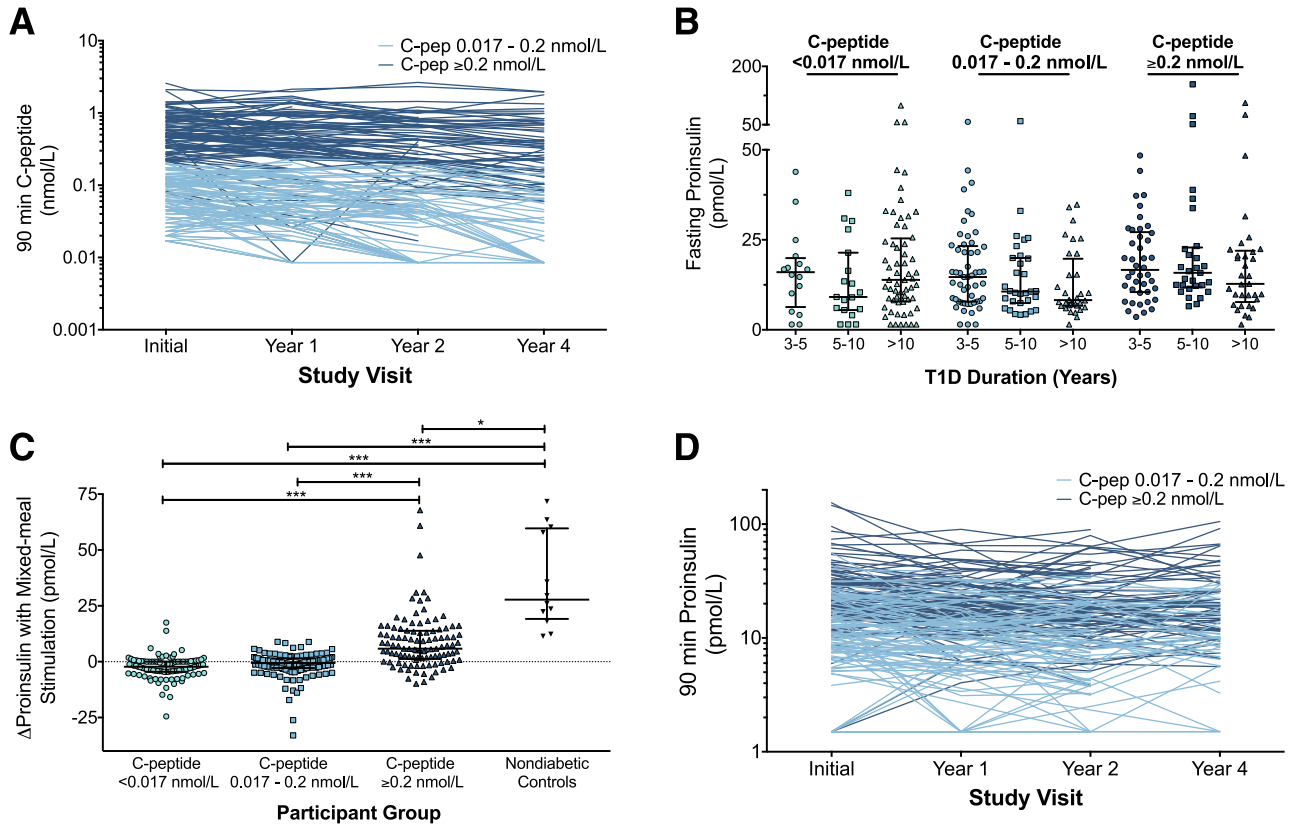


**Figure 1**—Longitudinal C-peptide and proinsulin values in subjects with long-standing type 1 diabetes. Values are color coded based on stimulated C-peptide values (<0.017 nmol/L [teal], ≥0.017–0.2 nmol/L [light blue], or ≥0.2 nmol/L [royal blue]). *A*: Individual stimulated (90 min) serum C-peptide (C-pep) levels over 4 years of follow-up. *B*: Fasting proinsulin values during the initial MMTT, grouped by C-peptide category and type 1 diabetes (T1D) duration. *C*: Scatterplot of individual proinsulin increments from fasting to stimulated MMTT time points. *D*: Individual longitudinal proinsulin values for participants with stimulated C-peptide ≥0.017–0.2 nmol/L or ≥0.2 nmol/L. Subjects with undetectable stimulated C-peptide were not invited for longitudinal MMTTs per study protocol. For graphical depiction, values below the assay limit of detection were plotted as one-half the assay limit of detection. Bars in scatterplot graphs represent medians and interquartile range values. *$P < 0.05$; ***$P < 0.001$.

and 90-min time points. Table 2 displays median baseline fasting and stimulated proinsulin values grouped by C-peptide positivity. As anticipated, nearly all subjects who were C-peptide positive at baseline had detectable fasting or stimulated proinsulin, including 100% in the highest C-peptide group and 98.3% in the group with C-peptide ≥0.017 but <0.2 nmol/L. Unexpectedly, proinsulin was also detectable in 89.9% (89 of 99) of subjects with serum C-peptide levels <0.017 nmol/L. Of note, median fasting proinsulin values among each of the groups with type 1 diabetes (13.5, 10.9, and 15.2 pmol/L) were well above the recommended level of detection for the assay (3.1 pmol/L) and fell in the midpoint of the standard curve for the assay. Fasting proinsulin concentrations were not significantly different between any of the groups, and there was no reduction in presence or level of fasting proinsulin with increasing diabetes duration (Fig. 1*B*). For validation of these findings, targeted mass spectrometry analysis was performed on 10 samples from individuals with undetectable stimulated C-peptide measured by the TOSOH assay (<0.017 nmol/L) but detectable proinsulin measured by the Millipore radioimmunoassay (Supplementary Tables 5 and 6 and Supplementary Figs. 2 and 3). The presence of serum proinsulin in the absence of detectable serum C-peptide was confirmed in all 10 samples. Taken together, these data point to the continued presence of proinsulin production and secretion, even in the presence of minimal stimulated C-peptide secretion.

To define relationships between proinsulin and C-peptide secretion, we examined changes in proinsulin levels in response to MMTT stimulation by comparing fasting and stimulated values of proinsulin among the different C-peptide categories and compared these values with those obtained in a small cohort of young control subjects without diabetes. In adults without diabetes, proinsulin levels increased approximately threefold with mixed-meal stimulation (Table 2, with individual increments during stimulation plotted in Fig. 1*C*). In contrast, among groups with long-standing type 1 diabetes, proinsulin secretion increased with stimulation only in subjects with the highest C-peptide values. In this group, proinsulin secretion increased ~1.5-fold with meal stimulation ($P <$ 0.001 compared with fasting values) (Table 2 and Fig. 1*C*). No significant increase in proinsulin was present with mixed-meal stimulation among the groups with C-peptide <0.017 or C-peptide ≥0.017 but <0.2 nmol/L (Table 2 and Fig. 1*C*). Along these lines, correlation analysis by C-peptide category revealed that stimulated C-peptide and proinsulin values were correlated only in the group with high residual C-peptide secretion (correlation coefficient of 0.54).

Over the course of 4 years of follow-up, stimulated serum proinsulin levels tended to stay relatively stable. Figure 1*D* displays individual stimulated proinsulin values for the groups with C-peptide ≥0.017 but <0.2 nmol/L and C-peptide ≥0.02 nmol/L at each study visit. Among all participants, the intraclass correlation coefficient for repeated proinsulin values was 0.795, confirming a strong clustering of repeated measurements over the duration of the study.

We have previously shown that circulating PI:C ratios are elevated in pediatric subjects with new-onset type 1 diabetes compared with matched healthy control subjects (15). Moreover, elevations in the circulating PI:C ratio were associated with clinical progression to type 1 diabetes in autoantibody-positive individuals, suggesting utility of the PI:C ratio as an indicator of β-cell stress (14). To define whether circulating PI:C ratios differed among the groups with long-standing type 1 diabetes and control subjects, we calculated fasting and stimulated PI:C ratios for subjects with detectable C-peptide and control subjects without diabetes. Similar to findings observed at diabetes onset, fasting PI:C ratios were increased in both groups with type 1 diabetes and detectable C-peptide compared with control subjects without diabetes ($P < 0.001$ for each group compared with control subjects) (Table 2). Consistent with this measure as a reflection of β-cell dysfunction, PI:C ratios were the highest in the group with the lowest residual stimulated C-peptide secretion ($P < 0.001$ compared with group with C-peptide values ≥0.02 nmol/L) (Table 2).

## CONCLUSIONS

Recently, several independent groups have reported that a substantial percentage of individuals retain the ability to secrete C-peptide many years after the diagnosis of type 1 diabetes (3–8). However, less is known about the relationship between proinsulin and C-peptide secretion in long-standing type 1 diabetes. Older analyses of subjects with type 1 diabetes detected circulating proinsulin in subjects with undetectable fasting C-peptide (21–23). In addition, a recent report from a subset of adult subjects from the T1D Exchange registry described detectable circulating proinsulin in 16% of samples from C-peptide–negative subjects with long-standing type 1 diabetes. However, this analysis was performed on randomly collected samples without regard for meal stimulation, and all previous studies were cross-sectional in nature (24). Here, we analyzed longitudinal fasting and stimulated serum samples from subjects with established type 1 diabetes, using a proinsulin radioimmunoassay with negligible cross-reactivity to insulin or C-peptide. In a cohort with a wide distribution of age at diagnosis and duration of disease, we found that almost all individuals tested had detectable serum proinsulin under fasting or meal-stimulated conditions, including 89.9% of subjects with undetectable serum C-peptide (<0.017 nmol/L). Median values for the cohorts were well within the limits of detection for the proinsulin radioimmunoassay used and fell in the midpoint of the standard curve for the assay. In fact, fasting proinsulin values were similar among all groups, irrespective of stimulated C-peptide status. Taken together, these data indicate that the vast majority of subjects with long-standing type 1 diabetes retain the ability to initiate preproinsulin production and secrete proinsulin.

These data provide an important clinical measure that substantiates recently published findings quantifying elevations in proinsulin at the level of the islet. This includes increases in islet PI:insulin area in euglycemic individuals with positive islet autoantibodies as well as in subjects with recent-onset type 1 diabetes (17). In pancreatic sections from donors with long-standing type 1 diabetes, persistence of islet insulin mRNA and proinsulin protein, despite reduced islet insulin and C-peptide content, was also recently reported (18). Our study is the first to examine longitudinal relationships of circulating C-peptide and proinsulin values in established type 1 diabetes. Our data show that although C-peptide levels decreased gradually, proinsulin levels remained stable over 4 years of follow-up.

In light of these findings, we suggest that detectable proinsulin in subjects with low or undetectable C-peptide levels provides additional information regarding β-cell hormone production over that afforded by measurement of C-peptide alone. Interestingly, our analysis indicates that in long-standing type 1 diabetes, the relationship between meal stimulation and proinsulin secretion is influenced by the level of ambient and residual β-cell function. Only subjects with highly functional β-cells (stimulated C-peptide levels ≥0.2 nmol/L) exhibited increased proinsulin levels with meal stimulation. No significant increase in proinsulin values was observed with stimulation among the groups with undetectable (<0.017 nmol) or low stimulated C-peptide values. Along these lines, stimulated proinsulin and C-peptide values were correlated only in the group with high residual stimulated C-peptide secretion. The etiology of this observation is unclear but will require further testing in human samples to explore underlying mechanisms.

Many individuals in this cohort were diagnosed with type 1 diabetes as adults. Still, 130 of 133 (97.7%) of subjects diagnosed as children had detectable proinsulin, suggesting that applicability of these findings is not limited to individuals with a later age of diagnosis. Because only a small number of individuals exhibited undetectable proinsulin and C-peptide values, we were unable to adequately examine differences in clinical characteristics of this group, but future analyses are warranted to explore factors related to absolute proinsulin and C-peptide deficiency. Additionally, some subjects in the T1D Exchange cohort were diagnosed with type 1 diabetes using clinical parameters, and we cannot exclude the possibility that some individuals may have been misdiagnosed. We calculated PI:C ratios as a potential proxy for β-cell stress, which is the standard method of analysis in the field (14). While our data identified an increase in PI:C ratios in subjects with long-standing type 1 diabetes compared with a small cohort of healthy control subjects without diabetes, we acknowledge that differences between the groups were driven by lower levels of C-peptide among subjects with diabetes.

Finally, our proinsulin and C-peptide assays exhibit different sensitivities. Although more sensitive C-peptide assays are available, the TOSOH assay used here has been most widely used in large clinical networks including the T1D Exchange, TrialNet, nPOD (Network

for Pancreatic Organ Donors With Diabetes), and the Immune Tolerance Network (3,19,25,26). Additionally, standardization of C-peptide assays is not performed at lower levels of C-peptide, such as those observed in many of our subjects. The National Institutes of Health has recently highlighted issues with research rigor and reproducibility, related in large part to specificity of assay reagents. To address this, we performed multiple assay validation experiments, including analysis of sensitivity, intra- and interassay variability, proinsulin recovery, reproducibility with mass spectrometry testing, and testing for specificity, with testing for human insulin, human C-peptide, proinsulin in the context of insulin autoantibodies, and proinsulin in individuals after pancreatectomy requiring exogenous insulin analogs. These results were all reassuring. However, we cannot guarantee with absolute certainty that our proinsulin assay is binding only intact proinsulin or proinsulin split products in sera from our subjects with type 1 diabetes. Analysis of additional cohorts using different proinsulin assays, including those measuring both intact and total proinsulin, should be performed.

Notwithstanding these limitations, our findings demonstrate that persistent circulating proinsulin can be detected in almost all subjects with long-standing type 1 diabetes, including 89.9% of those with low or absent C-peptide. The ability to increase proinsulin secretion under conditions of meal stimulation occurred only in those patients with significant C-peptide levels. Together, these observations suggest a potential hierarchy of β-cell dysfunction, which begins with a healthy β-cell that secretes mostly C-peptide. Early defects are characterized by increased proinsulin secretion with relatively intact C-peptide secretion. Increased progression of β-cell dysfunction is characterized by lower C-peptide secretion but retained ability to increase proinsulin in response to stimulation. Ultimately, as C-peptide levels fall further, proinsulin secretion in response to meal stimulation is blunted. Whether this hierarchy represents distinct stages of disease that are common among all individuals or whether aspects of this framework can be applied to dissect pathophysiological heterogeneity in type 1 diabetes is uncertain. However, the observation of persistent proinsulin production in late-stage disease raises the tantalizing proposition that therapies aimed at β-cell health could have utility in improving insulin secretion in type 1 diabetes.

**Acknowledgments.** For this project serum samples were used and subject data were derived from subjects participating in the Residual C-peptide in Type 1 Diabetes Study conducted under the auspices of the T1D Exchange. The authors acknowledge the T1D Exchange Biobank and participating investigators and staff in the T1D Exchange Clinic Network (listed in Supplementary Data) for subject recruitment, evaluation, and follow-up. The authors also acknowledge Anthony Acton (Indiana University) and Wojciech Grzesik (Eastern Virginia Medical School) for technical assistance with proinsulin measurement and laser capture microdissection (performed in association with Eastern Virginia Medical University).

**Funding.** This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases grant K08-DK0103983 to E.K.S.; a Pediatric Endocrine Society Clinical Scholar Award to E.K.S.; National Institutes of Health grants DP3 DK110844 (to W.-J.Q.), R01-DK-093954 (to C.E.-M.), R01-DK-48280 (to P.A.), and UC4-DK-104166 (to C.E.-M. and R.G.M.); U.S. Department of Veterans Affairs Merit Award I01BX001733 (to C.E.-M.); JDRF Strategic Research Agreement 2-SRA-2017-498-M-B to E.K.S.; JDRF Pioneer Award and Strategic Research Agreement (to C.E.-M., L.A.D., and J.B.); JDRF grant 47-2014-299-Q-R (to J.B. and C.E.-M.); and JDRF grant 47-2012-744 (to L.A.D.). Mass spectrometry-based proinsulin work was performed in the Environmental Molecular Sciences Laboratory, a national scientific user facility sponsored by the Department of Energy and located at Pacific Northwest National Laboratory, which is operated by Battelle Memorial Institute for the Department of Energy under Contract DE-AC05-76RL0 1830. Core services for this work were provided by the Diabetes Research Center grant P30-DK-097512 (to Indiana University School of Medicine). T1D Exchange is a program of Unitio, Inc., and supported by the Leona M. and Harry B. Helmsley Charitable Trust.

**Duality of Interest.** No potential conflicts of interest relevant to this article were reported.

**Author Contributions.** E.K.S. planned analyses, evaluated and interpreted data, and wrote the manuscript. H.T.B. performed statistical analysis, evaluated and interpreted data, and edited the manuscript. J.Ny. performed analyses, evaluated data, and edited the manuscript. L.H. performed experiments, interpreted data, and wrote and edited the manuscript. A.K.D. designed and implemented the Residual C-peptide in Type 1 Diabetes Study. C.S. designed supplemental studies to validate proinsulin measurements, interpreted data, and edited the manuscript. L.A.D. interpreted data and edited the manuscript. J.B. interpreted data and edited the manuscript. M.A.M. collected data and edited the manuscript. R.G.M. interpreted data and edited the manuscript. J.Na. interpreted data and edited the manuscript. T.L.M. performed experiments and edited the manuscript. S.M. performed experiments, interpreted data, and edited the manuscript. W.-J.Q. designed experiments, interpreted data, and edited the manuscript. L.Y. performed experiments, interpreted data, and edited the manuscript. A.C.S. performed experiments, interpreted data, and edited the manuscript. M.Y.-S. assisted with sample acquisition, interpreted data, and edited the manuscript. C.M.S. assisted with sample acquisition, interpreted data, and edited the manuscript. R.V.C. designed and performed experiments, interpreted data, and edited the manuscript. P.A. performed analyses, evaluated data, and edited the manuscript. C.J.G. directed the Residual C-peptide in Type 1 Diabetes Study, planned analysis and evaluated data from the clinical study, and edited the manuscript. C.E.-M. planned analyses, interpreted data, and wrote the manuscript. E.K.S., H.T.B., J.Ny., L.H., A.K.D., C.S., L.A.D., J.B., M.A.M., R.G.M., J.Na., T.L.M., S.M., W.-J.Q., L.Y., A.C.S., M.Y.-S., C.M.S., R.V.C., P.A., and C.J.G. approved the final version of the manuscript. E.K.S. and C.E.-M. 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 the accuracy of the data analysis.

**Prior Presentation.** Parts of this study were presented in abstract form at the 76th Scientific Sessions of the American Diabetes Association, New Orleans, LA, 10–14 June 2016; the 2016 HIRN Annual Investigator Meeting, Bethesda, MD, 25–26 May 2016; the 2017 JDRF nPOD Annual Scientific Meeting, Fort Lauderdale, FL, 19–22 February 2017; and the Endocrine Society's 99th Annual Meeting & Expo, Orlando, FL, 1–4 April 2017.

## References

1. American Diabetes Association. 2. Classification and diagnosis of diabetes: *Standards of Medical Care in Diabetes—2018*. Diabetes Care 2018;41(Suppl. 1):S13–S27

2. Campbell-Thompson M, Fu A, Kaddis JS, et al. Insulitis and β-cell mass in the natural history of type 1 diabetes. Diabetes 2016;65:719–731

3. Davis AK, DuBose SN, Haller MJ, et al.; T1D Exchange Clinic Network. Prevalence of detectable C-peptide according to age at diagnosis and duration of type 1 diabetes. Diabetes Care 2015; 38:476–481

4. Oram RA, Jones AG, Besser RE, et al. The majority of patients with long-duration type 1 diabetes are insulin microsecretors and have functioning beta cells. Diabetologia 2014;57: 187–191

5. Oram RA, McDonald TJ, Shields BM, et al.; UNITED Team. Most people with long-duration type 1 diabetes in a large population-based study are insulin microsecretors. Diabetes Care 2015; 38:323–328

6. Wang L, Lovejoy NF, Faustman DL. Persistence of prolonged C-peptide production in type 1 diabetes as measured with an ultrasensitive C-peptide assay. Diabetes Care 2012;35: 465–470

7. Keenan HA, Sun JK, Levine J, et al. Residual insulin production and pancreatic β-cell turnover after 50 years of diabetes: Joslin Medalist Study. Diabetes 2010;59:2846–2853
8. McGee P, Steffes M, Nowicki M, et al.; DCCT/EDIC Research Group. Insulin secretion measured by stimulated C-peptide in long-established type 1 diabetes in the Diabetes Control and Complications Trial (DCCT)/Epidemiology of Diabetes Interventions and Complications (EDIC) cohort: a pilot study. Diabet Med 2014;31:1264–1268
9. Greenbaum CJ, Anderson AM, Dolan LM, et al.; SEARCH Study Group. Preservation of β-cell function in autoantibody-positive youth with diabetes. Diabetes Care 2009;32:1839–1844
10. Liu M, Wright J, Guo H, Xiong Y, Arvan P. Proinsulin entry and transit through the endoplasmic reticulum in pancreatic beta cells. Vitam Horm 2014;95:35–62
11. Cnop M, Welsh N, Jonas JC, Jörns A, Lenzen S, Eizirik DL. Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities. Diabetes 2005;54 (Suppl. 2):S97–S107
12. Marhfour I, Lopez XM, Lefkaditis D, et al. Expression of endoplasmic reticulum stress markers in the islets of patients with type 1 diabetes. Diabetologia 2012;55:2417–2420
13. Stull ND, Breite A, McCarthy R, Tersey SA, Mirmira RG. Mouse islet of Langerhans isolation using a combination of purified collagenase and neutral protease. J Vis Exp 2012;67: e4317
14. Sims EK, Chaudhry Z, Watkins R, et al. Elevations in the fasting serum proinsulin–to–C-peptide ratio precede the onset of type 1 diabetes. Diabetes Care 2016;39:1519–1526
15. Watkins RA, Evans-Molina C, Terrell JK, et al. Proinsulin and heat shock protein 90 as biomarkers of beta-cell stress in the early period after onset of type 1 diabetes. Transl Res 2016; 168:96–106.e1
16. Truyen I, De Pauw P, Jørgensen PN, et al.; Belgian Diabetes Registry. Proinsulin levels and the proinsulin:c-peptide ratio complement autoantibody measurement for predicting type 1 diabetes. Diabetologia 2005;48:2322–2329
17. Rodriguez-Calvo T, Zapardiel-Gonzalo J, Amirian N, et al. Increase in pancreatic proinsulin and preservation of β-cell mass in autoantibody-positive donors prior to type 1 diabetes onset. Diabetes 2017;66:1334–1345
18. Wasserfall C, Nick HS, Campbell-Thompson M, et al. Persistence of pancreatic insulin mRNA expression and proinsulin protein in type 1 diabetes pancreata. Cell Metab 2017;26:568–575.e3
19. Beck RW, Tamborlane WV, Bergenstal RM, Miller KM, DuBose SN, Hall CA; T1D Exchange Clinic Network. The T1D Exchange clinic registry. J Clin Endocrinol Metab 2012;97:4383–4389
20. Bowsher RR, Wolny JD, Frank BH. A rapid and sensitive radioimmunoassay for the measurement of proinsulin in human serum. Diabetes 1992;41:1084–1090
21. Heding LG, Ludvigsson J. Human proinsulin in insulin-treated juvenile diabetics. Acta Paediatr Scand Suppl 1977;270:48–52
22. Sobey WJ, Beer SF, Carrington CA, et al. Sensitive and specific two-site immunoradiometric assays for human insulin, proinsulin, 65-66 split and 32-33 split proinsulins. Biochem J 1989;260:535–541
23. Heding LG. Insulin, C-peptide, and proinsulin in nondiabetics and insulin-treated diabetics. Characterization of the proinsulin in insulin-treated diabetics. Diabetes 1978;27(Suppl. 1):178–183
24. Steenkamp DW, Cacicedo JM, Sahin-Efe A, Sullivan C, Sternthal E. Preserved proinsulin secretion in long-standing type 1 diabetes. Endocr Pract 2017;23:1387–1393
25. Greenbaum CJ, Beam CA, Boulware D, et al.; Type 1 Diabetes TrialNet Study Group. Fall in C-peptide during first 2 years from diagnosis: evidence of at least two distinct phases from composite Type 1 Diabetes TrialNet data. Diabetes 2012;61:2066–2073
26. Herold KC, Gitelman SE, Ehlers MR, et al.; AbATE Study Team. Teplizumab (anti-CD3 mAb) treatment preserves C-peptide responses in patients with new-onset type 1 diabetes in a randomized controlled trial: metabolic and immunologic features at baseline identify a subgroup of responders. Diabetes 2013;62: 3766–3774