RESPONSE TO COMMENT ON SIMS ET AL.

Proinsulin Secretion Is a Persistent Feature of Type 1 Diabetes. Diabetes Care 2019;42:258–264

Diabetes Care 2019;42:e85–e86 | https://doi.org/10.2337/dci19-0012

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] and Carmella Evans-Molina[2,8,9,10,15]

In a cohort of 319 individuals with long-duration type 1 diabetes (T1D), we found that 89.9% of participants with undetectable stimulated C-peptide had measurable fasting or stimulated serum proinsulin (1). To ensure the validity of our results, we performed rigorous validation of the Millipore human intact and Des (31,32) proinsulin radioimmunoassay that included spike-in experiments using insulin, C-peptide, and proinsulin; independent calculation of a lower limit of detection; assessment of inter- and intra-assay coefficients of variation; analysis of linearity of dilution; analysis of samples pre- and postpancreatectomy; analysis of effects of increased insulin autoantibody titers; and quantitative validation of our findings using mass spectrometry (1). The letter by Steenkamp et al. (2) in response to our article suggests the Millipore assay may overestimate proinsulin levels compared with the ALPCO STELLUX human total proinsulin ELISA. The authors' concerns are based on their finding that, using the ALPCO assay, proinsulin was detected in only 16% of random samples from a smaller subset of C-peptide–negative individuals from the same T1D Exchange Residual C-peptide Study (3).

Quantitative immunoassay performance differences between different assay platforms are not surprising due to antibody interactions with different epitopes on native antigens and assay calibration differences. Key points in support of our findings are that most detected values were well within the standard curve for the Millipore assay and that we performed specificity analyses (analysis of cross-reactivity, analysis of pancreatectomy samples, analysis of effects of increased insulin autoantibody titers, and quantitative testing using mass spectrometry) to ensure that the measured analyte in our samples was truly proinsulin (1). Because all samples compared in our study were tested using this assay, there is no reason to question the validity of our findings. In their letter, Steenkamp et al. (2) reference independent validation of the ALPCO assay sensitivity to detect proinsulin split products, and spike-in analyses of assay cross-reactivity. However, none of these data are included in the referenced publications (3,4). They also reference unpublished comparisons of the Millipore, Mercodia, and ALPCO assays using the 09/296 proinsulin standard obtained through the National Institute for Biological

[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*

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

Standards and Control. To be able to adequately respond to the authors' statements, data on experimental conditions, number of replicates, and concentrations tested across the standard curve for proinsulin, proinsulin split products, C-peptide, and insulin are required. If available, we suggest the authors submit these important data for critical analysis through peer review.

In addition, it is important to note that the Millipore assay, as well as the Mercodia assay, use proinsulin standards corresponding to the original 84/611 World Health Organization proinsulin standard, while the ALPCO assay is calibrated to the 09/296 standard. The 09/296 proinsulin standard was evaluated in 2014 and was assigned a mass value based on a designation as intact proinsulin (5). However, high-performance liquid chromatography analysis of the standard by several laboratories detected "impurities" that may represent partially processed forms of proinsulin. These "impurities" were not evaluated or adjusted for during assignment of mass content to the standard. Therefore, an obvious etiology of the reported differences in immunoassay performance could be due to the use of the 09/296 standard for calibration of one assay and not in the others, especially when the standards may contain differing amounts of Des (31,32) proinsulin.

Notably, our results are consistent with published work from other groups showing that proinsulin protein is universally present in insulin- and C-peptide–negative islet extracts from individuals with T1D (6). Our results in serum have also recently been reproduced by the group from the University of Exeter in a separate cohort using a TECO intact proinsulin assay, where intact proinsulin was detected in 63% of individuals with undetectable C-peptide (using a more sensitive C-peptide assay than that used in the T1D Exchange study) (7).

We agree that identification and validation of optimal methods to measure proinsulin are needed. This is why we employed multiple approaches to validate results obtained with the assay used in our article. We would welcome the opportunity to participate in future collaborative workshop efforts to test differences among existing assays and compare results in different clinical cohorts, using appropriate standards in which differing amounts of proinsulin split products have been quantified. An essential component of the evaluation and standardization of assays to measure this complex analyte will be the development of a reference method independent of the antigen-antibody reaction, such as targeted mass spectrometry.

Acknowledgments. This project utilized serum samples and subject data derived from subjects participating in the Residual C-peptide in Type 1 Diabetes Study conducted under the auspices of T1D Exchange.

Funding. This manuscript was supported by funding from National Institute of Diabetes and Digestive and Kidney Diseases grant K08DK103983 to E.K.S.; a Pediatric Endocrine Society Clinical Scholar Award to E.K.S.; JDRF grant 2-SRA-2017-498-M-B (to E.K.S.); JDRF grant 47-2012-744 (to L.A.D.); National Institutes of Health grants UC4 DK 104166 (to R.G.M. and C.E.-M.), DP3 DK110844 (to W.-J.Q.), R01 DK48280 (to P.A.), and R01 DK093954 (to C.E.-M.); JDRF Pioneer Award and Strategic Research Agreement (to L.A.D., J.B., and C.E.-M.); JDRF grant 47-2014-299-Q-R (to J.B. and C.E.-M.); and U.S. Department of Veterans Affairs Merit Award I01BX001733 (to C.E.-M.). This work utilized core services provided by the Diabetes Research Center grant P30 DK097512 (to Indiana University School of Medicine). 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 U.S. Department of Energy under Contract DE-AC05-76RL0 1830.

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

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