2023 C-Peptide Standardization Manufacturer Meeting
Minutes

Wednesday August 7 8:00 AM – 9:30 AM
Anaheim Marriott, Anaheim, CA

Participants:

C-peptide Standardization Committee Members
Randie Little—Chair, University of Missouri (Virtual)
Kuanysh Kabytaev--Co-chair; University of Missouri
William Hagopian – University of Washington (Virtual)
Andy Hoofnagle – University of Washington (Virtual)
Robert Wielgosz - BIPM, France (Virtual)
Beena Akolkar—NIDDK (Liaison, Virtual)
Salvatore Secchi—NIDDK (Liaison, Virtual)

Committee members not present
Paulo Pozzilli - Campus Bio-Medico Univ. of Rome, Italy

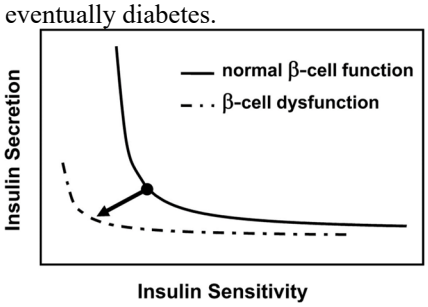
Manufacturer Representatives
Yukie Arizono—Fujirebio (Virtual)
Aya Asagoshi—Tosoh Bioscience (Virtual)
Jose Caciedo—Alpco
Satoshi Kojima—Fujirebio (Virtual)
Holger Lehmann—Roche Diagnostics (Virtual)
Isabelle Lang-Zwosta—Tosoh Bioscience
Shanti Narayanan—Tosoh Bioscience
Godwin Ogbonna—Ortho Clinical
Magnus Simonsson--Mercodia
Mie Wakabayashi—Fujirebio (Virtual)
Chris Wisherd—Alpco

Guests
Shawn Connolly—University of Missouri
Daniel Holmes—St. Paul's Hospital, Vancouver
Steven Kahn—University of Washington (Virtual)
David Leslie—St. Bartholomew's Hosp UK (Virtual)
Violeta Raneva—ReCCS Japan
Curt Rohlfing—University of Missouri
David Sacks—NIH
Gwen Wark—UKNEQAS/IFCC (Virtual)

1) **Welcome and Introduction—Randie Little**
   R. Little welcomed those in attendance. Participants introduced themselves and the 2021 meeting minutes were approved.
2) **C-peptide and Insulin Standardization Update—Randie Little**
   - NIDDK C-peptide Standardization Committee
     - Randie Little – Chair; University of Missouri
     - Kuanysh Kabytaev – Co-chair; University of Missouri
     - Andy Hoofnagle – University of Washington
     - Paulo Pozzilli - Campus Bio-Medico University of Rome, Italy
     - William Hagopian – University of Washington
     - Robert Wielgosz - Bureau International des Poids et Mesures, , Sèvres Cedex, France
     - NIH/NIDDK Liaisons
       1) Beena Akolkar
       2) Salvatore Secchi
   - IFCC-WG on Standardization of Insulin Assay (WG-SIA)
     - R. Little - co-chair US
     - K. Kabytaev - co-chair US
     - D. Holmes CA
     - M. McPhaul US
     - D. Sacks US
     - G. Wark UK
     - S. Kahn US
     - A. Hoofnagle US
     - B. Akolkar - consultant/NIH US
     - K. Van Uytfanghe – consultant BE

3) Clinical Update—Diabetes: Is it worth estimating Insulin and C-peptide?—David Leslie

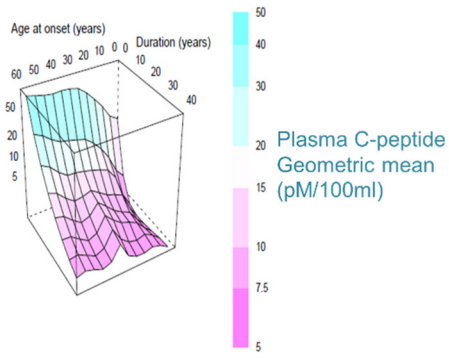
- Spectrum of Diabetes
  - Pro-insulin is synthesized in the pancreas.
  - Pro-insulin is cleaved into Insulin and C-peptide
  - All three are secreted into the bloodstream.
  - C-peptide and insulin are secreted in equimolar amounts.
  - C-peptide is used as a proxy for insulin.
    1) Longer half-life than insulin (not cleared by the liver)
    2) Does not interact with injected insulin
  - Insulin secretion and sensitivity: As the curve moves down and to the left, dysglycemia develops then eventually diabetes.

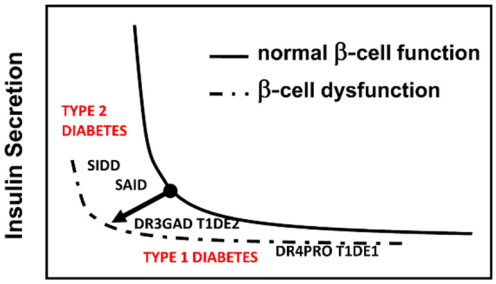


  - Historically, low insulin secretion diabetes was recognized and named insulin dependent diabetes, and was thought of as a disease of childhood.
  - In time it was learned that low insulin secretion diabetes had an immunogenetic association and the disease was named Type 1 diabetes.
  - All other diabetes was labeled non-insulin dependent, and later Type 2, diabetes.
  - We now know that there is a continuum between T1 and T2 diabetes, it is not a binary system.
    1) T1D is predominately a disease of adulthood that often presents itself as non-insulin dependent (T2D) at diagnosis.
    2) This presents a conundrum for the clinician in that if a patient is diagnosed with T2D you may actually be dealing with T1D or T2D.
  - Adult-onset Diabetes: Swedish Cohort (n=14,775): Ahlquist et al Lancet 2018
    1) About 25% of the cases had a high risk of progression to insulin therapy
    2) Cluster 1: 6.4%
      - GADA positive
      - C-peptide low
      - BMI low
      - HbA1c high

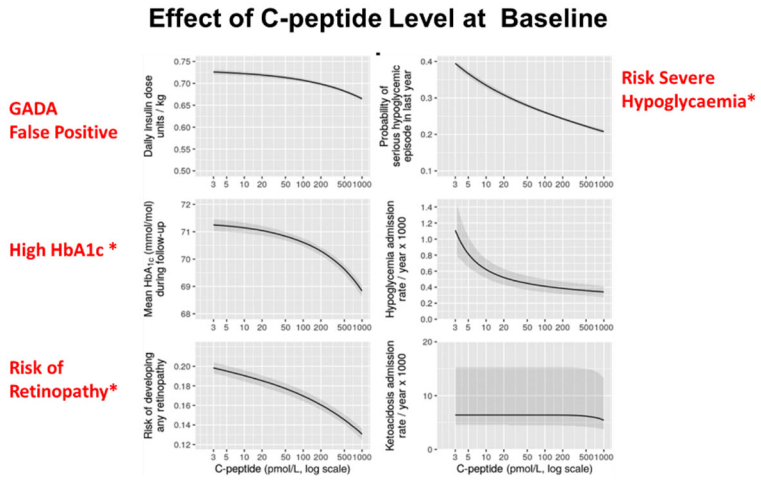
    3) Cluster 2: 17.5%
      - GADA negative
      - C-peptide low
      - BMI low
      - HbA1c high

    4) Cluster 1 appeared to be T1D while cluster 2 appeared to be T2D.
    5) Cluster 1 is sometimes referred to as latent autoimmune diabetes of adults (LADA).
    6) Cluster 2 is sometimes referred to as severe insulin deficient diabetes (subgroup of T2D).
- Spectrum of C-peptide/Insulin
  - C-peptide Falls by Age and T1D Duration (McKeigue, Colhoun et al BMC Medicine 2019, Maddoloni et al DOM 2022)





- Importance of testing C-peptide/Insulin
  - Prediction
    1) Stages of T1D
       - Genetic risk
       - Immune activation
       - Immune response
       - Stage 1: Start of T1D, autoantibodies present
       - Stages 2 and 3: Beta cell decline
    2) After stage 1, can either progress to multiple autoantibodies or maintain a single autoantibody
    3) Caveat: For people with a low risk of autoimmune diabetes, the single autoantibody poses an issue. Even though the specificity of GADA is very high, a positive result in these individuals is a false positive in 40% of cases.
    4) Requires assessment of other biomarkers to determine if there will be progression to T1D.
    5) This is becoming more important as therapies are being developed to delay progression to T1D in at-risk individuals.
  - Categorisation
    1) Options
       - Autoantibodies
       - C-peptide/Glucose
       - Gene Risk Score (not yet available)
    2) Categories include
       - T1D
       - T2D
       - SAID/LADA
       - Gestational Diabetes
       - Ketosis-Prone Diabetes
       - Drug-Induced Diabetes
       - Type 3 Diabetes
       - Monogenic Diabetes
  - Prognosis: Anita Jeyam et al. Dia Care 2021;44:390-398



1) As C-peptide goes down, risks of high HbA1c, retinopathy and severe hypoglycemia all go up.
2) C-peptide measurement allows for identification of those patients at risk for poor glucose control, hypoglycemia and diabetes complications.

Discussion:

D. Holmes noted that although standards are available for GADA measurements, the test is currently not standardized among manufacturer assay methods. D. Leslie acknowledged this, adding that HbA1c standardization took a lot of time and effort and expressed concern that there are tests being utilized by physicians that are not standardized. For example, he sees CGM results from patients that do not always make sense, probably because CGM is not standardized. D. Sacks asked D. Leslie his opinion on how accurate C-peptide results need to be. D. Leslie responded that currently high accuracy is not needed. There are ongoing arguments about what C-peptide levels can be used to define insulin dependence and levels above which insulin therapy is not needed. The curves show that risks associated with C-peptide represent a continuum, there are no sharply defined diagnostic cutoff levels. At the same time, however, he feels it would be a mistake to just accept that the test may sometimes lead in the wrong direction. C-peptide standardization is needed.

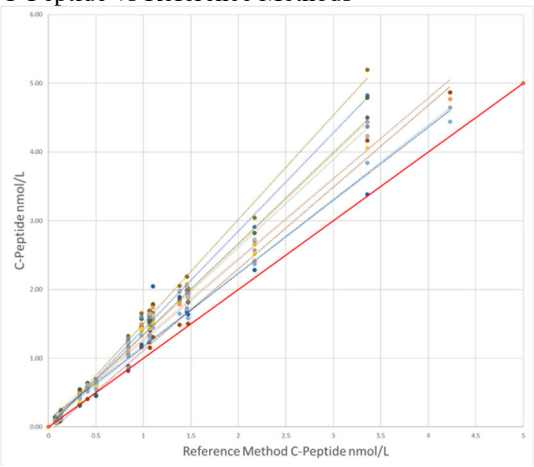
4) Proposal for Standardization of C-peptide & Insulin. Randie Little

- What do we need?
  - Certified Reference Material (CRM)
  - LC/MS Reference Method
  - Commutable Secondary Reference Material
  - Traceability chain
  - Proficiency testing
  - Website
- C-peptide: All six elements are in place.
  - C-peptide Reference Material: NMIJ CRM (CRM 6901-b)
    1) A lyophilized synthetic peptide with high purity
    2) Concentration determined by two independent amino acid analyses using liquid and gas phase hydrolyses.
    3) Is listed in the JCTLM database
    4) Backup C-Peptide Reference Material: Sigma – Cerilliant CRM
  - LC/MS Reference Method: C-peptide reference method is listed in the JCTLM database.
  - Secondary Reference Materials
    1) 40 single donor samples ranging from 0.07 to 4.23 nmol/L c-peptide
    2) All samples have Reference Method assigned values.
  - Traceability chain has been published.
    1) Our studies have shown reduced variability among methods when assays are standardized to reference method values using serum calibrators.

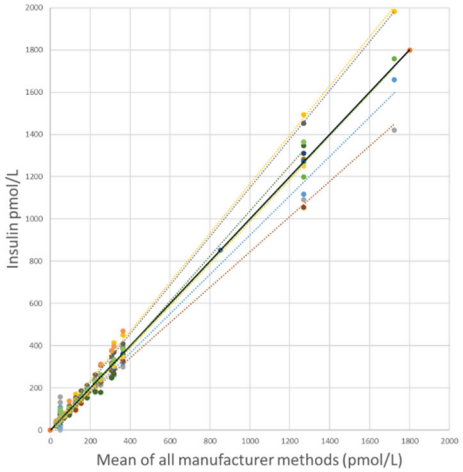
2) Special report published in Clinical Chemistry in 2017 (Little et al. 63:9, 2017, 1447-1456)
3) Editorial published in the same issue (Myers GL, Miller WG. 63:9, 2017, 1429-1430)

- Accuracy-Based CAP Survey: ABGIC
- Web site: www.cpeptide.org

- Insulin
  - Insulin reference material: NMIJ CRM 6209-a
  - LC/MS Reference Method: In progress
  - Secondary Reference Materials: 40 single donor samples ranging from 28.39 to 1724.05 pmol/L
  - Traceability chain has not been established
  - Accuracy-Based CAP Survey: ABGIC
  - Web site has not been developed
- Current Insulin/C-Peptide Standardization Study
  - A new set of single-donor serum samples were collected with a range of insulin and c-peptide levels
  - 20 of these samples (Set A) were sent to 10 manufacturers for analysis of insulin and c-peptide by 13 different methods using routine calibration
  - Each c-peptide method's results were compared to those of the C-peptide Reference Method
  - Each insulin method's results were compared to the mean of all manufacturer method results (temporarily since no Reference Method is available yet).
  - C-Peptide and Insulin Sample Ranges
    1) C-peptide Range: 0.07 to 4.23 nmol/L
    2) Insulin Range: 28.39 to 1724.05 pmol/L
  - C-Peptide vs Reference Methods



  - Insulin vs. Mean of All Manufacturer Methods (3 samples containing exogenous insulin were excluded)



  - Manufacturers were then sent 20 additional samples (set B)

- Data included with set B:
  1) Set A C-peptide Reference Method results
  2) Set A mean of all manufacturer results for insulin
- All manufacturers were requested to:
  1) Analyze all 20 samples in set B using current calibration
  2) Then re-analyze set B samples after adjusting / recalibrating their methods to report results comparable to Reference Method results for C-peptide and "all method means" for insulin
  3) This reanalysis should be based on the data sent for set A
- At the end of this study:
  1) We will have all methods' C-peptide results using current calibrations compared to the C-peptide Reference Method for 40 samples
  2) We will have all methods' insulin results using current calibration compared to the mean of all manufacturer method's results
  3) Insulin results will later be compared to Reference Method results as soon as available
  4) We hope to show once again that adjusted/recalibrated results for C-peptide are comparable to Reference Method results
  5) We hope to show that adjusted/recalibrated results for Insulin are comparable to the mean of all methods and later to Reference Method results
- Next steps
  1) University of Missouri
     - Complete current study and provide each manufacturer with their comparisons from set A and B combined.
     - Complete development of insulin Reference Method
  2) Manufacturers: Provide a white paper for their customers with their c-peptide relationship with the Reference Method

Discussion:

R. Little said a problem with the ABGIC survey is that it is separate from the regular non-accuracy based PT survey and very few labs participate. The plan is to eventually include a serum sample with a reference method-assigned value in the regular survey.

5) Joint Committee for Traceability in Laboratory Medicine (www.jctlm.org) —Robert Weilgosz

- Regulations and Metrological Traceability for IVDs: Essential Requirements of the IVD Directive
  - 98/79/EC of 27 October 1998 on in vitro diagnostic medical devices
  - "The traceability of values assigned to calibrators and/or control materials must be assured through available reference measurement procedures and/or available reference materials of a higher order.. "
- ISO standards for compliance with Traceability Requirements
  - ISO 17511: 2020 In vitro diagnostic medical devices - Requirements for establishing metrological traceability of values assigned to calibrators, trueness control materials and human samples
  - ISO 15193:2009 Requirements for content and presentation of reference measurement procedures (under revision)
  - ISO 15194:2009 Requirements for certified reference materials and the content of supporting (under revision)
  - ISO 15195: 2018 Laboratory medicine — Requirements for the competence of calibration laboratories using reference measurement procedures
- C-peptide entries in the JCTLM Database (www.jctlmdb.org): Assessed to meet ISO standard requirements via independent expert review
  - UMC DDL reference method for serum C-peptide (JCTLM DB Identifier C10RMP12_C-peptide)
  - NMIJ Reference method for serum C-peptide (JCTLM DB Identifier C11RMP1)
  - C-peptide in lyophilized phosphate buffer
    1) National Metrology Institute of Japan (NMIJ)
    2) NMIJ CRM 6901-b, C-peptide
- New Proposal from JCTLM Strategy Task Group. Initiative 2: Acknowledgement by Regulators

- Description: By June 2024, develop correspondence with major IVD regulators around the world for their requirements for metrological traceability of IVD MDs and compliance with ISO TC 212 WG2 standards.
- Goal of initiative: To increase awareness amongst regulators of the JCTLM database and its usefulness in supporting regulatory frameworks, and to provide JCTLM with clear links to regulator statements on metrological traceability to increase the perceived value of the Database to other stakeholders.
- Metrics to measure success:
  1) Number of regulators contacted
  2) Number of responses from regulators
  3) Number of statements from regulators that can be referenced in a "support for compliance" with regulations part of JCTLM websites

- JCTLM Stakeholders' Meeting, 4-5 December 2023
  - JCTLM Members' and Stakeholders' meeting and Workshop on 'EQA schemes elucidating the clinical suitability of laboratory results'
  - BIPM, Pavillon de Breteuil, Sèvres, France
  - https://www.bipm.org/en/committees/jc/jctlm/wg/jctlm/2023-12-04

Discussion:

R. Little asked manufacturer representatives present if it is possible to produce white papers showing the relationships between their methods and the reference method for their customer. H. Lehmann said that in general it is doable, but he was not sure if 40 samples would be sufficient to establish the equation. For example, over 100 samples are required to establish a reference range. Also there are some gaps present over the measurement range. FDA for example requires that certain percentages of samples fall within certain parts of the measurement range. R. Little acknowledged that more data may be needed for regulatory compliance, but asked if the 40 sample comparison is adequate for the purposes of a white paper. She will also be looking at the data from the previous 40 sample comparison to see if the relationship has changed, if not then the comparisons could be combined into one set of data. The white paper idea was actually presented by a manufacturer representative at the previous meeting and several others agreed that it was probably doable. She suggested that this is probably the best direction to go for now until the regulatory issues can be sorted out. The progress that has been made by the JCTLM should eventually help to address these issues. The relationships between the methods and reference method is especially important for long-term clinical studies. She will be in contact with individual manufacturers regarding the possibility of white papers. S. Narayanan asked about the content of the white paper, will it be based on the current 40-sample relationship? Also how do we explain the differences among methods? R. Little said she is still receiving data for the latest 40 sample comparison, once she has received it all she will be sending the results out to all of the manufacturers and they can use it for white papers. The current differences among methods are mainly due to the fact that the WHO reference material, which is used by all of the manufacturers, is not commutable. It has already been shown that the use of serum materials with values assigned by the reference method to calibrate assays greatly reduces variability among the methods. There is a new WHO material that appears to be better, but is still not commutable with all methods. There is only one manufacturer that has already switched to the new WHO material and others will follow, but it is still not going to fully standardize methods.

6) Development of LC-MS reference method for insulin quantitation — Kuanysh Kabytaev

- In Search of the Perfect Reference Method
  - Analytical performance:
    1) High sensitivity
    2) High specificity
    3) Adequate CV
    4) High accuracy Long-term assay stability
  - Practical aspects:
    1) Cost efficiency:
    2) "High-throughput"
    3) Ease of implementation
    4) Insensitivity to external factors
    5) Adequate implementation
- Balancing Accuracy, Sensitivity and Specificity: Exploring Different Mass Spectrometry Acquisitions

    - Intact insulin: SIM and MRM
    - Chain B reduced: SIM and MRM
    - Chain B alkylated: SIM and MRM
    - Glu-C digestion: SIM and MRM
    - Resolutions: Low, Unit and High
- Tested conditions:
    - Insulin reduction/alkylation: TCEP, DTT, iodoacetic acid or iodoacetamide under different temperatures and pHs
    - Insulin IS: porcine, d-labeled, N15-Iabeled
    - Vial materials: LoBind, glass.
    - Presence of detergents: ProteaseMAX, zwittergent
- Insulin Enrichment/Purification: An Antibody-Free Approach
    - Protein precipitation and centrifugation ➡
    - C-18 filtration and buffer addition ➡
    - Ion Exchange column elution
- Commercially available CRM insulin for metrological traceability chain
    - Cerilliant: I-034-0.1ML, Human Insulin in PBS, 100 uL/Ampoule in PBS pH 7.2
    - NMIJ CRM 6209-a, Purified recombinant human insulin in solution, (77.9± 2.3) mg/L
    - WHO, 11/212, 2nd International Standard for human insulin, 9.19 mg per ampoule
- Issues Requiring Deeper Examination:
    - Insulin absorption/aggregation and long-term stability of samples
    - Insulin concentration is very low in some patient samples, so we need to improve LLOQ
    - Which CRM and IS to use?
    - Dilemma: final reference method should be antibody-free but chemical methods usually lack efficiency.
- Plans:
    - Monitor Long-Term Stability of the assay.
    - Collaborate with reputable metrology institutes or laboratory to perform method comparison study.

Discussion:

Insulin Reference Method

K. Kabytaev said that it would be better if the method did not require use of an antibody, but it may end up being necessary. D. Holmes asked K. Kabytaev if he had tried running A. Hoofnagle's Glu-C digestion method, he said yes but not exactly the same way because they did not have the multi-channel robot. A. Hoofnagle noted that they actually are not using a robot for the assay, they are using a handheld multi-pipettor with a Waters 96 well ion-exchange microplate. For their MS they use a Waters instrument but they are currently working on setting up the method on QE and Sciex instruments. They use DMSO in the mobile phase to help with ionization. They have noticed that Glu-C really enhances the detection of insulin. There are two different peptides in the insulin B chain that they can detect, it is a way to improve the sensitivity of the insulin. D. Holmes said it sounded like it should work on at least several different MS instruments, A. Hoofnagle thought so. There are differences, for example the use of DMSO helped with the Waters instrument but that was not the case for the QE. K. Kabytaev said that similarly DMSO did not help with their Sciex system, Glu-C improved the MRM sensitivity but it was still lower than when SIM mode was used. He asked A. Hoofnagle what resolution they use, A. Hoofnagle said unit resolution as they are not using a high-resolution MS. D. Holmes said the method needs to be shown to work on other MS instruments, if it does not it cannot be a reference method. He agreed with K. Kabytaev that the method should not be antibody-dependent if possible, but if it is vendor-dependent that is an even bigger problem. A. Hoofnagle and K. Kabytaev agreed to have further discussions regarding the insulin reference method. K. Kabytaev asked A. Hoofnagle if his lab is still running the methods and if so if they can do further sample comparisons. A. Hoofnagle said yes, they are still measuring both insulin and C-peptide, it is a high-throughput method and they do not claim it is a reference method but they are happy to help. K. Kabytaev then asked about their insulin method, have they tied it to serum materials. A. Hoofnagle said they tried, the CRM has commutability issues and he would like to have well-characterized secondary materials.

CAP ABGIC Survey

A. Hoofnagle, asked the representatives present to please urge their labs to participate in the CAP ABGIC survey. Currently very few labs participate and the data are needed, it is a very important part of the ongoing standardization efforts. CAP is hoping to increase the number of samples in the survey and get wider insulin and C-peptide ranges, as well as to include a wildcard serum in the regular survey. Getting more participants in ABGIC is very important, R. Little asked if the cost for participating could be lower, A. Hoofnagle said they cannot as the processing is expensive. G. Wark said their lab has tried to participate, the problem they have is getting the samples delivered to them. A. Hoofnagle acknowledged that it is difficult to ship the specimens overseas. R. Little agreed, saying the NGSP has been having issues when shipping samples internationally. A. Hoofnagle told G. Wark that he can see about doing some stability studies with CAP to see if they can ship samples to them on blue ice instead of frozen, as it is easier to get them to the destination in a timely manner. G. Wark said it would be great if that could be done, it would help in expanding the number of participants.

R. Little thanked everyone present for their attendance, the meeting was adjourned at 9:40 AM.

Minutes prepared by Curt Rohlfing 10/11/2023. Modified by K. Kabytzev 10/12/2023.