2013 C-Peptide Standardization Manufacturer Meeting
Minutes
Wednesday July 31 8:00 AM – 10:00 AM
Hyatt Regency Houston, Houston, TX

Participants:

C-peptide Standardization Committee Members
Randie Little—University of Missouri
Daniel Stein—Albert Einstein College of Medicine

Committee members not present
Judith Fradkin—NIDDK
Carla Greenbaum—Benaroya Research Institute
W. Greg Miller—Virginia Commonweath University
Gary Myers—AACC
Jerry Palmer—University of Washington
Kenneth Polonsky—Washington University
Lisa Spain—NIDDK

Manufacturer Representatives
Corinth Auld--Mercodia
Sean Conley—Alpco Diagnostics
Nadis Corocher—Diasorin
Carissa Jones--Mercodia
Yasunobu Masuda—Kyowa Medex (Siemens)
Beth Schodin—Abbott
Sakae Tazoe—Kyowa Medex (Siemens)
Masashi Tsuura—Tosoh Bioscience

Guests
Shawn Connolly—University of Missouri
Edward Rogatsky—Albert Einstein College of Medicine
Curt Rohlfing—University of Missouri
Michael Steffes—University of Minnesota

Violeta Raneva—ReCCS Japan
Alexander Stoyanov—University of Missouri
Linda Thienpont—University of Ghent
Katleen Van Uytfanghe—University of Ghent

1) **Welcome and Introduction—Randie Little**
   R. Little welcomed those in attendance, those present introduced themselves. The minutes of the 2012 meeting were approved.

2) **Update on Clinical Trials for Diabetes Prevention—Daniel Stein**
   - Main points
     o > 1.4 million with Type 1 diabetes (T1D) in the US; incidence rates rising
     o Type 1 diabetes is (usually) an autoimmune disease.
     o Adolescent (obese) Type 2 Diabetes rapidly increasing.
     o T1D is a predictable disease with different phases.
     o Preventing future T1D, maintaining and/or restoring beta cell function is the goal.
     o C-peptide is the most accurate biomarker of beta cell area and function in beta cell depleted diabetes.
     o Insulin resistance is associated with many "metabolic diseases including obesity, hyperlipidemia, CVD, cancer
     o Insulin is often used as a surrogate marker of insulin resistance
   - Natural History of Type 1 Diabetes
     o Genetic predisposition
     o Insulitus/beta cell injury in response to a putative environmental trigger
     o Cellular (T cell) autoimmunity
     o Humoral autoantibodies (ICA, IAA, Anti-GAD65, IA2Ab, etc.)
     o After 80-90% loss in beta cell mass, enter a pre-diabetes phase followed by clinical onset of diabetes (fasting and post-prandial hyperglycemia).
   - Pro-insulin is synthesized in the pancreatic beta cells
     o Packaged into granules and cleaved to insulin and C-peptide for storage.
     o Insulin and C-peptide are secreted in a 1:1 molar ratio.
     o Insulin (but not C-peptide) is cleared by the liver
     o C-peptide is the best marker of insulin secretion

- Why preserve beta cell mass?
  - Prevent short-term complications (hypoglycemia)
  - Prevent long term complications (retinopathy, nephropathy, neuropathy, etc.)
- Strategies and Goals for Prevention of T1D
  - Major goal: Prevent T1D before it starts
  - Settling for slowing progression: 5 year delay could make an enormous impact on the challenge of adolescence.
  - Pre-diabetes, new-onset diabetes, versus established diabetes: More dangerous treatments easier to justify once diabetes established.
- Cure Equivalent for Type 1 Diabetes
  - Prevent onset by blocking autoimmunity
  - If T1D established, restore beta cell deficiency with transplantation or regeneration and block autoimmunity
  - Challenges of Islet Transplantation
    1) Supply of insulin-producing cells
    2) Protection from transplant rejection and autoimmunity
- Which tests to measure beta cell function in clinical trials?
  - Glucagon Stimulation Test (GST) or Mixed-meal Tolerance Test (MMTT): MMTT is more physiological and better stimulates C-peptide (Greenbaum et. al 2008).
  - Standard for most clinical trials looking at T1D is MMTT, integrate C-peptide concentration (area under the curve) over two hours
- Urine C-peptide creatinine ratio (UCPCR) is a noninvasive alternative to the mixed-meal tolerance test in children and adults with T1D (Besser RE et al., Diab Care. 2011 Mar;34(3):607-9).
  - Patients with T1D (n=51; 0.2 – 66 yr post diagnosis)
  - Fasting void; MMTT; p2hr MMTT void
  - Serum C-pep at 0, 90, 120 min; cutoff 0.2 nM as diagnostic for T1D
  - 90 min MMTT C-Peptide correlation with 2hr UCPCR (R=0.87)
  - 2hr UCPCR 90 min vs. MMTT C-peptide to detect stimulated C-peptide < 0.2 nM (95% sens, 100% specific)
  - Correlated highly with home pp 120min UCPCR (R=0.8)
- Variable Rates of Beta Cell Killing
  - In pre-T1D beta cell destruction can take years.
  - Transplant of pancreas between identical twins (with and without T1D) – beta cells killed within weeks. What is the role of memory cells?
  - In pancreases of people with T1D for over 50 years, beta cells are virtually always present.
  - Number of beta cells is correlated with level of C-peptide.
- Faustman and colleagues measured C-peptide levels in patients with T1D using a new ultrasensitive immunoassay developed by Mercodia (Wang, C et al. Diab Care 2012;36:599-604).
  - Study Objective: To examine persistence of C-peptide production by ultrasensitive assay years after onset of T1D and factors associated with preserving β-cell function
  - Serum C-peptide levels measured in human subjects (n = 182) by ultrasensitive assay (lower detection limit 1.5 pmol/L), as was β-cell functioning
  - Disease duration, age at onset, age, sex, and autoantibody titers were analyzed by regression analysis to determine their relationship to C-peptide production
  - Another group of four patients was serially studied for up to 20 weeks to examine C-peptide levels and functioning.
  - Results showed that a significant number of subjects that had no detectable C-peptide with the standard assay had measurable C-peptide with the ultrasensitive assay.
  - The number of subjects with measureable C-peptide decreased with duration of diabetes.
- Faustman and colleagues: Pilot study to look at the effects of BCG (immunostimulant) administration on beta cell function in patients with T1D who had undetectable C-peptide with the standard assay that was measurable with the ultrasensitive assay.
  - Subjects administered BCG showed transient increases in C-peptide while the placebo subjects did not.

- o One subject that developed a EBV infection (which provokes a immunostimulatory response similar to BCG) also showed a transient increase in C-peptide.
- Conclusions
  - o C-peptide production persists for decades after disease onset and remains functionally responsive
  - o Patients with advanced disease may benefit from interventions to preserve β-cell function or to prevent complications
- Insulin
  - o Gold standard for measuring insulin: Hyperinsulinemic Euglycemic Glucose Clamp
    1) Give research volunteers a standard dose of insulin which stimulates glucose metabolism and storage (indicates insulin sensitivity)
    2) Difficult to perform
  - o Other ways to use insulin as a biomarker
    1) 1/Fasting Insulin: Reciprocal of fasting plasma insulin concentration, μU/ml
    2) Insulin/Glucose ratio: Ratio of fasting plasma glucose (mg/dl) and insulin (μU/ml) concentration
    3) HOMA: HOMA-IR = {[fasting insulin (μU/ml)] × [fasting glucose (mmol/l)]}/22.5
    4) QUCKI: QUICKI = 1/[Log (fasting insulin, μU/ml) + Log (fasting glucose, mg/dl)]
    5) Matsuda Index: ISI(Matsuda) =10,000/√[(Gfasting (mg/dl) × Ifasting (mU/l)) x (Gmean × Imean)]
    6) Others include Gutt, Avignon, Stumvoll indexes
- Problem for insulin and C-peptide is that validated assays around the world can give 2-3 fold differences, harmonization is needed.
- Unresolved Questions
  - o What is the reproducibility of ultrasensitive C-peptide assays in the same T1D individual over time?
  - o Are very low levels of C-peptide biologically significant?
  - o Are very low levels of C-peptide CLINICALLY significant?
  - o Does this translate to lower rates of complications (hypoglycemia; improved glycemic control)
  - o Does this translate into a positive susceptibility for beta cell regeneration therapies?
- Acknowledgements
  - o Peter Gottlieb
  - o Carla Greenbaum
  - o Barbara Davis Diabetes Center
  - o Mark Pescovitz (ADA Web)
  - o Type 1 Diabetes Trial Net (Jay Skyler)
  - o Immune Tolerance Network (Gordon Weir)

Discussion:
M. Steffes noted that the ultrasensitive assay for the original study by Faustman and colleagues was performed by the manufacturer (Mercodia) in Sweden, was this true for the later studies as well? The answer was yes, D. Stein said the the ultrasensitive assay involves a modified protocol developed by Mercodia. It is now being offered commercially.

3) Update on Reference Laboratory Comparison Studies—Randie Little
- In 2002, the NIDDK organized a C-peptide standardization committee and funded an international comparison study of C-peptide assays.
- Phase I-IV Studies
  - o Normalization with sample calibrators was effective in reducing variability of C-peptide results.
  - o Normalization using patient samples with values assigned by an LC-MS reference method greatly reduces the variability among methods and laboratories.
  - o Calibrators can be prepared from either single donors or samples pooled from more than one donor

- o Serum is acceptable for the Reference Method and can be used for calibrators
- o Pooled serum calibrators with LC-MS assigned values can be used for method re-calibration by the manufacturer.
- Most recent study
  - o Pooled serum calibrator range was 0.01--3.2 nmol/L (based on the LC-MS reference method).
  - o Patient samples representing a range of C-peptide values were analyzed by the LC-MS reference method and participating manufacturers along with the pooled serum calibrators.
  - o Manufacturers submitted results for the patient samples using both their standard calibration and calibration using the supplied LC-MS reference values.
  - o The fasting and 2-hour samples from one individual in the most recent study had unusual variability in results among methods, it turned out there were mouse antibodies present in this individual donor.
  - o Use of the pooled serum calibrators greatly reduced variability in results among methods.
- C-peptide Reference Method/Laboratory Comparison 2012
  - o In order for manufacturers to re-calibrate their C-peptide assays to the reference method we must have it listed with JCTLM which requires a comparison between two reference laboratories.
  - o The most recent comparison between the two laboratories in 2012 showed good correlation between them (r^2=0.9647).
- Steps for 2012 and 2013
  - o Completed
    - 1) Complete method setup in secondary reference laboratory (MO)
    - 2) Publish comparison
    - 3) Submit Reference method to JCTLM
  - o In process
    - 1) Prepare another set of pooled sera for calibrators (#, levels?)
    - 2) Collect another set of individual test sera
    - 3) Available to ship to manufacturers if needed
- NIDDK C-peptide Standardization Committee:
  - o Judith Fradkin (NIDDK)
  - o Randie Little (Univ. of Missouri)
  - o Greg Miller (Virginia Commonwealth Univ.)
  - o Gary Myers (AACC)
  - o Jerry Palmer (Univ. of Washington)
  - o Kenneth Polonsky (Washington Univ.)
  - o Lisa Spain (NIDDK)
  - o Daniel Stein (Albert Einstein College of Med)

Discussion:
R. Little and C. Rohlfing said the JCTLM committee is supposed to decide if the reference method is approved when they meet in December. If the method is approved manufacturers should be able to begin the process of re-calibrating their assays to the reference method. S. Conley asked if the two reference methods are the same, D. Stein said they are not 100% identical but are very similar.

4) **Insulin Standardization Update—Michael Steffes**
   - WHO has a new insulin standard available that is made from human recombinant insulin. They are willing to provide this for standardization of assays.
   - There is more interest in IDMS assays for insulin as well as C-peptide.
     - o L. Thienpont's group has already developed an IDMS method for insulin.
     - o D. Stein's group is working on developing an insulin method.
     - o Question of whether MS methods are reaching the point where they will be used in clinical laboratories, a recent paper published online in Clin Chem which presents a IDMS method for insulin that is high throughput.
     - o Clinical labs are already using MS assays for other analytes (cortisol, testosterone, etc.), one issue is the availability of internal standards.

- We should be able to distribute pools and single samples soon.

Discussion:
R. Little asked why it is important to have a MS assay in the clinical lab as long as you have a good MS reference method to calibrate assays. M. Steffes replied that the MS should give a more accurate, though not necessarily more precise, result. The instrumentation has become easier to work with than in the past making it more feasible for MS to be used for a number of different analytes in the clinical lab. D. Stein said this view may be a bit optimistic, especially for analytes like peptides, there are challenges. Compared to immunoassays MS assays tend to be more expensive and more laborious, and more things go wrong. In the case of Vitamin D, MS has become the typical method of analysis; it is cost effective and gets around analytical issues relating to interferences. With regard to insulin and C-peptide I am more skeptical of MS becoming a routine method but it is useful as a reference method to harmonize other assays.

5) **C-peptide and Insulin Reference Methods—Dan Stein**
- C-peptide
  - Ultra-high sensitivity IDA LC/MS candidate reference method for C-peptide established (Rogatsky et al 2006)
  - Harmonization of well validated C-peptide immunoassays successfully completed based upon single and pooled donor specimens
  - Second site for IDA LC/MS C-peptide has completed validation (Missouri)
  - JCTLM Application for reference method submitted 5/13
  - New Ultrasensitive C-peptide ELISA introduced (Mercodia). LOQ 1.25 pmol/L (3.8 pg/ml), Cross reference to Einstein C-peptide LC/MS planned.
  - Preliminary report MSD ECL based ultrasensitive assay (Endocrine Sciences/Labcorp) LLOQ 1.3 pmol/L (4 pg/ml)
  - C-peptide certified reference material now available (Kinumi et al. Anal Bioanal Chem (2012) 404:13-21).
    1) Chemical (FMOC) synthesis, HPLC purification
    2) Metrologically traceable via amino acid analysis via IDMS
    3) Purity by HPLC and MALDI-TOF MS
    4) Minimum stability 6 months at -80C, lyophilized
    5) Certified value of 80.7 ± 5.0 µg/ml intact C-peptide; total 81.7 ± 5.1 µg/ml (98.7% pure C-peptide).
    6) Acceptable agreement with NIBSC 84/510 (86.6 ± 5.4).
  - The New York and Missouri labs show excellent agreement in the latest comparison.
- Insulin
  - Initial IDA LC/MS insulin assay method reported (Rodriguez-Cabaleiro Clin Chem 53:1462-69).
    1) Method
       - Affinity capture (antibody) concentration and purification
       - Reverse phase separation followed by MS/MS
       - Reference material provided by collaborating vendor
    2) Results
       - LOQ 12 pmol/L ; 2µU/ml (72 ng/L)
       - CV 3.2-6.3%; 3.8-10.3% intra; interassay CV, achieved goal <32% error as per ADA guidelines
    3) Limitations:
       1) Individualized disposable affinity columns (expensive)
       2) Slow process, relatively labor intensive
       3) Low throughput
  - Quantitative Insulin Analysis Using Liquid Chromatography–Tandem Mass Spectrometry in a High-Throughput Clinical Laboratory (preliminary publication, Chen et al., Clin Chem,, May 24, 2013, available online)
    1) Method
       - Reduction/alkylation B- and A-chains of insulin
       - Solid phase extraction (SPE) followed by one dimensional RP chromatography

- MS/MS of B-chain only (686.9[m+5]->768.5[m+2], 753.2[m+2]
- Bovine Insulin Internal Standard
- WHO human insulin Std (66/304) from NIBSC

2) Results
- LOQ 3µU/ml (18 pmol/L) [6 pg on column]
- CV's 3-7.9%, 7.1-14.0%; intra- and interassay
- Recovery 94-113%
- Serum and heparin plasma equivalent
- Reference Range (95%) < 13.7µU/ml (82.2 pmol/l)

3) Limitations
- 4% samples from healthy population below LOQ (< 3µU/ml)
- 3% of results not interpretable (?reason)
- Limit of blank 1.4µU/ml suggesting background contamination of B-chain
- Full data (supplementary data not yet available for evaluation)

o Insulin Einstein Biomarker Analytical Core Lab Insulin LC/MS

1) Method
- **Problem:** Insulin is susceptible to protein microheterogeneity due to deamidation
- **Solution:** Generate stable tryptic peptide (GFFYTPK) after HPLC purification
  a. LOQ persistently above 1 ng/ml (14-30µU/ml)
  b. Recovery inconsistent from non-disposable Perfinity column
  c. Decided an alternative approach was needed.

2) Have developed an excellent C13 labeled internal standard

3) New approach
- SPE (remains to be optimized); should allow 0.5 ml plasma
- 2D RP/RP similar to C-peptide
- Conversion to Agilent 6490 for enhanced sensitivity
- Approach is MS/MS of intact insulin molecule
- Currently able to identify 10 pg on column (1.7 fmol) with SNR 8
- Theoretical sensitivity: LOD 0.3µU/ml; LOQ 0.6µU/ml

o Future Insulin IDA LCMS Goals

1) Purification and quantification of U-13C human insulin internal standard
2) Optimization of RP large plasma volume initial extraction
3) Transfer of ABI Sciex ABI4000 2D RP/RP insulin method to Agilent 6490
4) Optimization of ultra-high sensitivity 2D RP/RP LC/MS detection.
5) Fasting insulin 3-20µU/ml. Occasional levels 1-2µU/ml
6) Goal LOQ < 2µU/ml = 12pmol/L = 72pg/ml
7) Total maximum CV 3% (7% at LOQ), bias limit 5%
8) Cross validation vs. Chen et al LC/MS method and ? other immunoassays w/ single, donor pools 10-1000pM

Discussion:

Stability of Specimens
M. Steffes said that even though L. Thienpont's laboratory no longer runs their insulin method there are frozen samples available that were originally analyzed in her laboratory that could be used for comparison. The only problem is they have been frozen at -80°C for 6-7 years. D. Stein said insulin should be stable under these conditions. R. Little asked if anyone has looked at long-term stability of C-peptide in serum at -80°C. D. Stein did not know, they have found that their clinical research samples collected as plasma with aprotinin and DPP4 inhibitor are very stable at -80C. However, manufacturers use serum and many research samples are also serum, C-peptide is likely not stable at -20°C but I am not sure about -80°C. M. Steffes asked how long their laboratory has kept them before analysis. D. Stein said several years but this is for plasma with aprotinin and DPP4 inhibitor. For serum we have shown stability for at least 6 months. R. Little said a serum control was made several years ago that has been analyzed several times by the NY and MO labs and the results were reproducible, we should consider making controls that would be analyzed in each run and overlapped

every few years. S. Conley asked if the use of plasma with aprotinin and DPP4 inhibitor was done just as a conservative approach; were there any paired comparisons done? D. Stein said the use of aprotinin was based on work of 40-50 years ago with glucagon, this became standard practice. The sample still must be chilled immediately to avoid degradation. We started adding DPP4 inhibitor after a group in Arizona showed evidence of clipping of the last two amino acids of C-peptide which are substrates for DPP4. We have not quantified the actual percentage of this; it is not a large amount. R. Little said stability studies done at MO showed that aprotinin showed no significant difference for samples with vs. without aprotinin. D. Stein reiterated that it is vital to chill the sample immediately in order for aprotinin to be effective, once the proteolytic cascade starts, it does not work. With serum the proteases do not get activated which probably explains why serum works, at least in the short term, we are not sure of long-term stability. C. Rohlfing noted that we have stored samples that can be re-analyzed. L. Thienpont said it would be good for the NY lab to measure the same samples that were measured in the her lab, but suggested also measuring these by one of the very specific assays used in the original method comparison, this way if a discrepancy is also seen in this assay you know that there is a stability problem. D. Stein agreed that this was a good idea.

Performance and Validation of Reference Methods
L. Thienpont said that even with small molecules people assume that if the method is MS it is accurate but it must be validated. We do see discrepancies between MS methods of up to 10 or 20%. D. Stein agreed and said they see this in comparisons of MS assays for Vitamin D; it still comes down to who does the work. Running MS well requires an understanding of the equipment and proper maintenance. R. Little noted that this is why it is important to have at least two labs, with HbA1c we have two whole networks and run constant comparisons to ensure there is no drift. M. Steffes said there can be differences but a good lab can run a MS method well, the availability of internal standards is an issue though. We saw an example with creatinine where there was an issue with the calibration. This shows it is possible to not do even an assay as routine as creatinine well. D. Stein said assay harmonization is extremely important for both clinical and research applications. Being able to translate an insulin value into a measure of insulin resistance would be extremely useful , this is the basis of many diseases that characterize the metabolic syndrome including diabetes, high cholesterol, cancer, etc. S. Conley asked why L. Thienpont's group did the initial affinity purification. D. Stein responded that a sample must be cleaned up prior to analysis. Our initial insulin method used affinity; we are now using a SPE approach with our new method. Affinity works well with disposable columns but we found it did not work well with reusable columns. L. Thienpont said they emphasize sample preparation in their reference method procedures--it is essential. For example, with 25 hydroxy D we initially used SPE and Sephadex prior to the analysis. After the method was published we further optimized the procedure. K. Van Uytfanghe said they changed to using magnetic beads to which an antibody is coupled; only small volumes are required which makes it more cost-effective and efficient. It also made the SPE step redundant. D. Stein said the WHO insulin reference material will be very useful.

JCTLM Approval of Reference Method for C-peptide
R. Little asked L. Thienpont about the timeline for JCTLM approval of the C-peptide reference method. L. Thienpont said the review team deadlines are in October, it will be discussed at the stakeholders and executive meeting in December, approved methods will be added to the database shortly thereafter. R. Little asked what happens if the method is not approved. L. Thienpont said the nominating person is informed of why it is not approved and what additional information/validation is needed. R. Little asked if there is an opportunity to say what additional information is needed prior to the meeting. L. Thienpont thought so.

Recalibration of Manufacturer C-peptide Assays
R. Little asked manufacturers what their timeline would be as far as re-calibrating the assays once the reference method is approved. B. Schodin said for Abbott once there is approved material and protocol it is 12-18 months for it to be made available to customers. There would need to be feasibility work done internally as well. L. Thienpont noted that there can be regulatory issues. B. Schodin said if there is a small change there is little regulatory impact; if the shift is 10% or more it requires verification of package insert claims, determination of impact on customers, etc. L. Thienpont said her group will be visiting Dr. Gutierrez at the FDA to discuss the consequences of standardization of

thyroid hormones. R. Little asked if FDA needs to be approached about each analyte. L. Thienpont said yes. R. Little said hopefully the process is easier than the diagnostic claim for HbA1c. With C-peptide there should be no real impact on patients; it should be less of a safety issue. B. Schodin said that the diagnostic claim for HbA1c involved a new clinical indication, for re-standardization of insulin and C-peptide the intended use of the assay would not change so it should be much easier. L. Thienpont said the reference intervals and decision limits would change and need to be revised. R. Little said we need to be ready to provide manufacturers with what is needed when the time comes; we also need to further investigate sample stability. B. Schodin asked if samples will be collected and tested by the reference method once the method is approved. R. Little said we will be doing so prior to then and we still have samples from previous comparisons as well. L. Thienpont noted that a primary C-peptide material is already available so if the reference measurement procedure is approved, the reference system will be complete. B. Schodin asked if the scheme is to provide the samples to manufacturers so they can determine where they are now and what they need to do to standardize. R. Little said if they participated in the previous comparison they can use that data to look at re-calibration then check using the next set of calibrators and samples.

RL thanked everyone for their attendance, the meeting was adjourned at 9:40 AM
Minutes prepared by Curt Rohlfing, 8/16/13. Modified by Randie Little 8/21/13.