2014 C-Peptide Standardization Manufacturer Meeting
Minutes

Wednesday July 30 8:00 AM – 10:00 AM
Fairmont Chicago, Chicago, IL

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
Martin Blankford—Alpco Diagnostics
Lisa DiMagno—Ortho Clin Diagnostics
Sachiyuki Hasegawa—Tosoh Bioscience
Shanti Narayanan—Tosoh Bioscience
Godwin Ogbonna—Ortho Clin Diagnostics
Judith Ogden—Tosoh Bioscience
Chisato Okamura--Fujirebio
Maria-Magdalena Patru—Ortho Clin Diagnostics
Stefaan Marivoet—Tosoh Bioscience
Hanna Ritzen--Mercodia
Kouichi Saga—Tosoh Bioscience
Sakae Tazoe—Kyowa Medex (Siemens)

Guests
Valerie Arends—University of Minnesota
Shawn Connolly—University of Missouri
Anita Durairaj—University of Missouri
Curt Rohlfing—University of Missouri
Michael Steffes—University of Minnesota
Alexander Stoyanov—University of Missouri
Linda Thienpont—University of Ghent

1) **Welcome and Introduction—Randie Little**
R. Little welcomed those in attendance, those present introduced themselves.

2) **Update on Clinical Trials for Diabetes Prevention—Daniel Stein**
- Main points
  - > 1.4 million with Type 1 diabetes (T1D) in the US; incidence rates rising
  - Type 1 diabetes is (usually) an autoimmune disease.
  - Adolescent (obese) Type 2 Diabetes rapidly increasing.
  - T1D is a predictable disease with different phases.
  - Preventing future T1D, maintaining and/or restoring beta cell function is the goal.
  - C-peptide is the most accurate biomarker of beta cell area and function in beta cell depleted diabetes.
  - Insulin resistance is associated with many "metabolic diseases including obesity, hyperlipidemia, CVD, cancer
  - Insulin is often used as a surrogate marker of insulin resistance
- Natural History of Type 1 Diabetes
  - Genetic predisposition
  - Insulitus/beta cell injury in response to a putative environmental trigger
  - Cellular (T cell) autoimmunity
  - Humoral autoantibodies (ICA, IAA, Anti-GAD65, IA2Ab, etc.)
  - 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
  - Packaged into granules and cleaved to insulin and C-peptide for storage.
  - Insulin and C-peptide are secreted in a 1:1 molar ratio.

- Insulin (but not C-peptide) is cleared by the liver
- C-peptide is the best marker of insulin secretion

- Why preserve beta cell function? Among subjects in the DCCT intensive group:
  - Prevents short-term complications (hypoglycemia)
  - Prevents 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)
- Discriminating Type 1 from Type 2 Insulin Requiring Diabetes (E. J. Besser, A. G. Jones et al Diabet Med. 2012 29:1279-84)
  - MMTT with and without insulin
  - DM1 (56), DM2 (35): all on insulin Rx
  - Results:
    1) 20% reduction peak serum C-peptide w/ insulin, but NO change in cut off for sig endogenous insulin secretion
    2) Fasting sCP α 90 min MMTT (R= 0.97)
    3) Fasting sCP ≥ 0.07 nmol/L (.21 ng/ml): 100% sensitivity, 97% specificity for signficant endog insulin secretion (90 min CP ≥ 0.2 nmol/L)
- Discriminating Pediatric Type 1 from MODY and Type 2 Diabetes (Besser, Shields et al Pediatr Diab 2013, 14:181-8)
  - Subjects

	T1DM	**MODY**	**Ped DM2**
Age onset	0-18	0-18	≥ puberty
Genetics	polygenic HLA +	monogenic HNFs, PDX	polygenic
body type	Lean	Lean	lean/obese
Antibodies	yes	no	no
DKA	yes	no	no
UCPCR	.05	3.51	4.01(nmol/mmol)

  - UCPCR ROC cut offs ≥ 0.7 nmol/mmol
  - 100% sensitive, 97% specific discriminating T1DM from non T1DM (MODY or T2DM)

- 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.
- Demonstration of Islet Cell function in patients with 50 yrs or longer of Diabetes (Keenan, Berger, Sun, Eisenbarth, Doria, King ADA abstract, 2014 San Francisco, CA)
  - N=211 subjects
  - Age 67 ± 7
  - A1c 7.1% ± 1.3
  - Random C-peptide; in house RIA
  - Results
    1) 24% C-peptide ≥0.3 ng/ml (100pmol/L)
    2) NO difference prevalence complications > 0.3 ng/ml vs < 0.3 ng/ml
  - Subjects received optimal care and were well-controlled, data from the DCCT indicate that with more variable standards of care residual C-peptide production may have beneficial effects.
- 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).
  - 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.
- McDonald and colleagues (Diabetologia 2014 57, 187-191).
  - T1 DM > 5 years (mean 30; 19-41 years); n=74
  - Age at Dx mean 16 (9-23)
  - Measured fasting and MMTT stimulated CP, and UCPCR
  - Roche, and Mercodia ultrasensitive assays (LOQ 3.3 and 1.5 pmol/L)
  - Low CP group (< 30 pmol/L fasting): 80% stimulated during MMTT
- Conclusions
  - C-peptide production persists for decades after disease onset and remains functionally responsive
  - Patients with advanced disease may benefit from interventions to preserve β-cell function or to prevent complications
- Insulin as a marker of insulin resistance
  - There is correlation between insulin resistance and the amount of fat in the liver.
  - 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
  - Other ways to use insulin as a biomarker
    1) Measurement of insulin and glucose
    2) Various ways of calculating response (Insulin/glucose ratio, HOMA, others)
- 20/(fasting C-peptide x fasting plasma glucose) is a simple and effective index of insulin resistance in patients with type 2 diabetes mellitus: a preliminary report (Ohkura, Shiochi et al Cardiovasc Diabetol (2013) 12:21-29)
  - Fasting C-peptide and glucose measured in T2DM
  - Insulin sensitivity measured by hyperinsulinemic euglycemic clamp
  - Results: Glucose Infusion Rate strongly correlated with 20/(f-CPxf-PG), r=0.83 compared with HOMA-IR (r=-0.74), ISI (r=0.66)

- Limitations:
  1) Normal renal function
  2) Initial group 15 expanded to 25 for ADA 2014

- Unresolved Questions
  - What is the reproducibility of ultrasensitive C-peptide assays in the same T1D individual over time?
  - Are very low levels of C-peptide (endogenous beta cell function) biologically significant?
  - Are very low levels of C-peptide (endogenous beta cell function) CLINICALLY significant?
  - E.g. does this translate to lower rates of complications (hypoglycemia; improved glycemic control)
  - Does this translate into a positive susceptibility for beta cell regeneration therapies?
  - Well designed outcomes of prospective trials of beta cell function using standardized testing procedures, and adjusting for multiple clinical and demographic variables are necessary.
- Acknowledgements
  - Peter Gottlieb
  - Carla Greenbaum
  - Barbara Davis Diabetes Center
  - Mark Pescovitz (ADA Web)
  - Type 1 Diabetes Trial Net (Jay Skyler)
  - Immune Tolerance Network (Gordon Weir)

3) C-peptide Standardization Update—Randie Little

- In 2002, the NIDDK organized a C-peptide standardization committee and funded an international comparison study of C-peptide assays.
- Participating manufacturers
  - Alpco
  - DiaSorin
  - Mercodia
  - Millipore
  - Roche
  - Siemens
  - Tosoh
  - Fugirebio
  - Mercodia
- Harmonization studies
  - Early studies: Laboratories analyzed the samples and calibrators, we performed the normalization calculations to compare results before and after normalization.
  - Later studies: Manufacturers analyzed the samples and calibrators and performed the normalization calculations.
  - Showed that normalization using patient samples with values assigned by an LC-MS reference method greatly reduces the variability among methods and laboratories.
  - Pooled serum calibrators with LC-MS assigned values can be used for method re-calibration by the manufacturer.
- Most recent study
  - Pooled serum calibrator range was 0.01--3.2 nmol/L (based on the LC-MS reference method).
  - 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.
  - Manufacturers submitted results for the patient samples using both their standard calibration and calibration using the supplied LC-MS reference values.
  - 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.
  - Use of the pooled serum calibrators greatly reduced variability in results among methods.
- C-peptide Reference Method/Laboratory Comparison

- 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.
- We have published a comparison between the two laboratories in 2012 showed good correlation between them (r^2=0.9647).
- This comparison data was used to submit the method for listing in the JCTLM database.

- Goals for 2013-2014 (Completed)
  - Prepare another set of pooled and individual sera to ship as needed for re-calibration
    1) 7 levels of pooled sera ranging from 0 (undetectable) to 4.00 nmol/L c-peptide (Reference Method assigned values).
    2) 80 single donor samples ranging from 0.23 to 5.24 mnol/L c-peptide.
  - Obtain JCTLM approval: We have received official notice of approval, the reference method is now listed in the JCTLM database.
  - We need to know what manufacturers need from us in order to harmonize methods.
  - We also need to know what is important in maintaining standardization going forward, how do we monitor the effectiveness of the program?
  - What do we need to consider, e.g. are very low levels critical?

Discussion:

Low C-peptide levels

D. Stein said that the entire range of C-peptide values is important, including very low levels. R. Little said there is interest in low levels and there are now ultrasensitive assays available to measure these levels. M. Steffes said that in their lab they were not able to duplicate the findings of Faustman et. al with the Mercodia ultrasensitive assay in terms of being able to measure very low levels. R. Little asked if there have been improvements to the Mercodia ultrasensitive assay since the Faustman paper. H.Ritzen said they have an updated protocol and in-house results have shown better sensitivity and precision. C. Auld said they have received positive feedback from users, it is important to note that we have the ultrasensitive assay and an alternative protocol to be used with it to obtain the extra sensitivity. This is what the Faustman group has used and has now brought in-house, they and other users report good results. It is extremely important when running this assay to pay very close attention to certain aspects such as wash steps, this is true of any highly sensitive assay for any biomarker. R. Little said that very low levels are mainly a research interest at this point in time. D. Stein noted that there is more than one ultrasensitive assay available.

Monitoring standardization

R. Little said the NGSP has worked with the College of American Pathologists before in establishing the whole blood survey for HbA1c, it would be good to have a matrix-appropriate material (i.e. pooled serum) for the C-peptide survey. There are far fewer participating laboratories compared with HbA1c so it should be doable. M. Steffes said that the samples CAP uses for their surveys are generally processed and show strong matrix effects, they have only used matrix-appropriate materials for a few analytes including HbA1c. R. Little said CAP seems to be moving toward accuracy-based grading which may provide incentive to use matrix-appropriate materials, at least periodically. We will try to get someone from CAP involved. S. Marivoet and S. Narayanan said their new assay is so specific for C-peptide that it will not detect analogs used by CAP and other PT providers to spike samples. R. Little noted that matrix effects are very common with processed materials, they may be useful for comparing within a method group but not for comparing between methods.

Timeline for re-calibration of assays

R. Little asked manufacturers how much time would be required to standardize their assays. S. Narayanan said their new assay is already calibrated to the reference method. The current results match the old Tosoh method but we also tested the value-assigned calibrator samples, so it would just be a matter of changing to the new calibration. H. Ritzen asked if the 80 single-donor samples are newer than what was previously provided, R. Little said they are and there are still some sets of the previous samples. There were storage issues with the most recent set, so in addition a new batch samples will be prepared soon. All of these samples are available to manufacturers upon request. H. Ritzen asked if the raw data for the reference

value assignments could be provided to manufacturers for both the pooled and single-donor samples, R. Little said yes. H. Ritzen said performing the analyses is straightforward, but where the re-calibration is concerned it would depend on the production schedule, we typically make large batches of reagents with a three-year shelf life. R. Little asked if it is possible to re-calibrate existing lots, H. Ritzen said no, this would involve recall and re-validation of lots. It is hard to give an exact timetable because it depends on the production schedule. There is also the regulatory aspect, this could cause us to push the change back further depending upon the degree of change in calibration. J. Ogden and S. Narayanan said it is difficult to give an exact timeframe, for their assay the regulatory issues are not as big of a problem but we still have to do documentation. M. Blankford said they are already looking at this, we have requested another batch of samples and need them soon, especially for our newest assay. The next issue is the low end of the range, we need samples in the 1pg/ml range. R. Little said if additional samples are needed for the low end of the range they can be provided. M. Blankford said there is also the issue of EP-26, serum panels are needed for assessing lot-to-lot variation.

Reporting of results

H. Ritzen asked who will exercise oversight when the manufacturers re-calibrate their assays, how do we monitor the harmonization once the re-calibration processed has occurred? There is the CAP survey but it utlizes processed materials. R. Little said manufacturers can check internally and samples can be provided to manufacturers, but this is a different issue from how it translates down to the clinical labs. We will work with CAP regarding the survey. D. Stein said the manufacturers have their intrinsic raw data values, then they do a correction factor to standardize, can they provide both results to customers? R. Little agreed, users can then see how the old values relate to the standardized ones. S. Marivoet there is risk letting the end-user decide, this could result in confusion on the part of clinicians. R. Little said that with HbA1c some countries presented both IFCC and NGSP numbers before eventually switching over to reporting IFCC, the good thing is that the two sets of numbers could not be confused. With C-peptide manufacturers may decide to report both numbers for a time, there may be ways of reporting them on the instrument that would help avoid confusion. S. Narayanan said that education is important, what are we doing regarding educating clinicians regarding clinical limits, etc., we need guidelines. R. Little agreed and asked what guidelines clinicians follow now. D. Stein said that really low levels are mainly a research question, clinically you are often looking at discriminating between insulin deficiency and sufficiency. This would be around levels 0.5-1 ng/ml. Every lab has their norms. A clinician may use these as the basis for determining whether a patient should go on a pump or injections (T1), or if they are moderately insulin deficient (need insulin) or can just be on oral medication (T2). S. Marivoet said the problem is that physicians may use cutoffs for these determinations that have no relation to the test the lab is using, the lab may change methods while the physician continues to use the same cutoffs. R. Little noted that this is the advantage of standardization. S. Marivoet agreed but said that we can inform the customers of the change in values when we standardize but they may not do anything with this information. The lab may have its reference interval but this is a normal range, this is different from cutoff values that determine what is abnormal. D. Stein said a good clinician will see these results on a spectrum and make decisions based on additional information besides just the C-peptide level. M. Blankford asked if the current bias among the assays is large enough to affect clinical decisionmaking. D. Stein responded that people have experience with their own assay based on this they have cutpoints in mind, sometimes the lab changes assays and physicians complain but they have no control over this. R. Little said for many assays it would not be a large change but it will be for some. D. Stein said harmonization is very important in terms of future clinical practice which will come from the research world. Results from different trials looking at different interventions could be directly compared which will lead to better interventions and treatments. M. Blankford said this is similar to the issues with Troponin. R. Little said there are clinicians on the committee that cannot attend this meeting, they can provide input on what would be useful for clinicians. We also have the labs that did the initial studies, they could also be of help in terms of what would be useful to a research lab.

Reference material

H. Ritzen noted that we do not have a commutable reference preparation, manufacturers have been using the WHO standard which is clearly not commutable. This is why we are harmonizing using serum pools and single-donor sets. Ideally we would like to have a commutable reference preparation. D. Stein said in addition to the WHO standard there is also a new standard from Japan and NIBSC has a new one as well.

The point is that there is analytical variability among the different assays due to different specificities, or some may measure different degradation products, etc. S. Narayanan said that specificity is the issue, noting that some assays show cross-reactivity with proinsulin. D. Stein said you can also have partially processed proinsulins that may react more or less with different assays. S. Narayanan said that as a result of differences in specificity you may see more variability among methods in CAP as opposed to native samples. CAP has indicated that they are working toward a accuracy-based survey using native specimens, this will be a separate survey so the question is how many labs will participate? R. Little said that individual manufacturers have internal controls/calibrators that they know work for their methods, commutability is only an issue when comparing between methods. We will work with CAP to try to get a survey that uses native specimens. Regardless of how quickly that moves along it should not slow down the harmonization process. S. Marivoet that since the reference method is now accepted manufacturers should be able to standardize to the reference method, we do not need the reference material. This should solve the commutability issue unless the serum samples provided are contaminated with antibodies, etc. but that is easy to see. This is the same with Vitamin D and testosterone, the secondary reference material can be used to calibrate the individual assays. This is also true of HbA1c, manufacturers cannot use the primary reference material.

4) Insulin Standardization Update—Michael Steffes

- Proinsulin synthesized and then converted in the beta cell to insulin and connecting peptide (c-peptide). Insulin and c-peptide secreted in equimolar amounts.
- NIBSC has been working on a new reference material for insulin, they are coordinating it with insulin manufacturers and are making a large amount of it
- History: Publications
  - Comparison of 11 Insulin Assays: Implications for Clinical Investigation and Research (Manley et. al, Clin Chem 53:5 922-932, 2007)
  - Standardization of Insulin Immunoassays: Report of the American Diabetes Association Workgroup (Marcovina et. al, Clin Chem 53:4 711-716, 2007)
  - Toward Standardization of Immunoassays (Miller et. al, Clin Chem 55:5 1011-1018, 2009)
  - Quantitative Insulin Analysis Using Liquid Chromatography-Tandem Mass Spectrometry in a High-Throughput Clinical Laboratory (Chen et. al, Clin Chem 59:9 1349-1356 2013) (Quest Nichols Institute)
- Insulin Standardization: Current
  - L. Thienpont's group has already developed an IDMS reference method for insulin and shown the feasibility of calibrating routine assays to this method using individual donor sera.
  - Proposed resources
    1) Aliquots from single donors and pools with values-assigned from the 2009 study (Thienpont laboratory) and another laboratory (2nd laboratory is required for JCTLM listing)
    2) Additional aliquots with values-assigned from two laboratories
  - Suggested Process for Standardization
    1) Obtain pure human insulin to use as a calibrator
    2) Once assay is established utilize our aliquots to confirm standardization

Discussion:
R. Little asked if the Quest Nichols lab that developed the high-throughput assay has analyzed any of the 2009 samples, M. Steffes said he is working toward this. He has contacted them several times, they have not yet made the assay available for commercial use, they are working on it.

5) Insulin and C-peptide IDA LC/MS assay Update (July 2014)—Dan Stein

- C-peptide IDA LC/MS assay
  - 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

- JCTLM Application for reference method accepted! 6/14
- New Ultrasensitive C-peptide ELISAs introduced
  1) Mercodia. LOQ 1.5 pmol/L (4.5 pg/ml)
  2) Alpco LOQ 3.0 pmol/L (9.0 pg/ml)
  3) Roche LOQ 3.0 pmol/L (9.0 pg/ml)
  4) Calbiotech LOQ 4.3 pmol/L (13 pg/ml)
  5) Labcorp LLOD 1.3 pmol/L (4 pg/ml) MSD ECL based
- C-peptide certified reference material now available

- Comparison of two Ultrasensitive C-peptide assays (Oram et al Diabetol (2014) 57 :187-191)
  - Subjects with T1 diabetes
  - Compared Roche serum C-peptide electrochemiluminescence assay with Mercodia Ultrasensitive ELISA. Mean difference (standard deviation) was 23 (20) pmol/l
  - Detection of low concentrations of C-peptide by the Roche and Mercodia assay.
    1) In the 67 samples selected for having low or undetectable levels of C-peptide, C-peptide was detectable in 24/67 samples using the ROCHE assay and only 15/67 samples using the Mercodia assay.
    2) All patients detected by the Mercodia assay were also detected by the Roche assay.
    3) This suggests that the Roche assay is able to detect C-peptide at lower concentrations than the Mercodia assay.
  - 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).
  - International Collaborative Study to Establish the 1st WHO International Standard for Human C-peptide
    1) Primary calibrant of recombinant purified human C-peptide
    2) PC01 209 ug/vial
    3) Candidate Standard 13/146
    4) Nominal 8.5 ug human C-peptide
    5) Value assignment
       - Assay by UV-HPLC 214 nm (all participants)
       - Assay by LC/MS (Einstein)
       - Recovery by LC/MS IDA (Einstein)
  - The New York and Missouri labs show excellent agreement in the last comparison (2012).
- 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 (Chen et. al, Clin Chem 59:9 1349-1356 2013).
  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
    - Min vol 0.2 ml
    - RR (95%) < 13.7 uU/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
    - Bias of bovine vs insulin B-chain isotope std, and bias vs ICMA Insulin
- Einstein Biomarker Analytical Core Lab Insulin LC/MS

  1) Method
    - **Problem:** Insulin is susceptible to protein microheterogeneity due to deamidation (7 potential deamidation sites)
    - **Solution:** Generate stable tryptic peptide (GFFYTPK) after HPLC purification

  2) Have developed an excellent C13 labeled internal standard
- Issue of different types of insulins

  1) At least 6 different types in addition to normal human (Humalog, Lispro, etc.), all except Glargine are modified on the B chain

  2) Lispro mw is identical to intact human insulin

  3) Accurately determining the insulin species can be important

  4) Multidimensional LC/MS-MS Enables Simultaneous Quantification of Intact Human Insulin and Five Recombinant Analogs in Human Plasma (Chambers et. al, Anal. Chem. 2014;86, 694-702)
    - Method
      a. 250 ul plasma
      b. Bovine IS
      c. Protein precipitation
      d. SAX/MM SPE
      e. 2D LC/MS/MS (Waters Xevo TQ-S)
      f. Precursor product pairs max SNR, minimal background
    - Results
      a. LLOQ 50pg/ml (8.3-8.6pmol/L: h-insulin, lispro, glargine, glulisine, others 2-4x more
      b. CV's 2.5, 8.3% intra-, inter-day
      c. Recovery 92-104%
    - Limitations
      a. 1/22 samples below LLOQ (?T1DM)
      b. Effect of bias vs bovine vs authentic IDA standard
      c. Affect of de-amidations on quantification not investigated
- 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
9) Purification and quantification of U-13C h-insulin IDA standard, but-need reference unlabeled insulin std for comparison!
10) Design and optimization of IDA isotope std and plasma reformatting on automated liquid handler system
11) Optimization of on line affinity capture and trypsinization
12) Optimization of ultrahigh sensitivity 2D RP/RP LC/MS detection. Fasting insulin 3-20 µU/ml.
13) Current LOQ 14 uU/ml

Discussion:

Ultrasensitve Assays
C. Auld said that the for the Oram et. al study they ran the Mercodia ultrasensitive assay on an automated system that has been shown to not work well with the assay. She has contacted the laboratory, they were not aware of this when they performed the study. D. Stein said they should be encouraged to publish a correction in the journal, many people are likely not aware of this.

Insulin Methods and Standards
M. Steffes asked about the use of the isotope-labeled human insulin B chain standard vs. the bovine standard, D. Stein said that Chen et. al showed that there is a slight bias between them. If you use the human standard it is only the B chain, have to assume there is complete alkylation/reduction, maybe they achieved this but they do not state this. Chambers et. al used a bovine insulin standard which is a potential limitation, but at least the bovine standard undergoes all of the same processes as the insulins being measured. Chen et. al showed a slight bias between the bovine standard and the human B chain IS, it is unclear whether this is due to incomplete alkylation/reduction or some other factor. It is a good assay, they should be able to answer this question. M. Steffes asked if D. Stein's group will incorporate the Chambers et. al into their assay, D. Stein responded that they will likely try to adopt the Chambers assay, there are less questions about it. The Chen assay should also be acceptable, they are showing good results, I would just like to see a little more data addressing the bias between the two standards. The perfect assay would be using a isotope-labeled intact human insulin. M. Steffes noted that obtaining a labeled human insulin is expensive while bovine insulin is very inexpensive. D. Stein agreed and said he does not know why investigators are not using porcine insulin, it is only one amino acid different from human insulin as opposed to three for bovine insulin.

Reference Laboratories
R. Little asked if either group (Chen, Chambers) would be interested in participating in insulin standardization, at least two reference labs are needed. M. Steffes said he is hoping one or both of them are interested but he does not know at this time. D. Stein said it is good that there are now two different working assays. R. Little asked if the two labs had done any cross-comparisons, D. Stein and M. Steffes did not think so.

RL thanked everyone for their attendance, the meeting was adjourned at 10:00 AM

Minutes prepared by Curt Rohlfing, 8/21/14.