2016 C-Peptide Standardization Manufacturer Meeting
Minutes

Wednesday August 3 7:00 AM – 9:00 AM
Sheraton Philadelphia Downtown, Philadelphia, PA

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
Philip Bryan—Ortho Clin Diagnostics
Sean Conley—Alpco Diagnostics
Carole Dauscher—Siemens
Holly Groth—Ortho Clin Diagnostics
Robert Gunnarsson—Mercodia
Carissa Jones--Mercodia
Iris Kutschera--Diasorin
Qian Ding—Ortho Clin Diagnostics
Stefaan Marivoet—Tosoh Bioscience
Shanti Narayanan—Tosoh Bioscience
Kouichi Saga—Tosoh Bioscience
Chris Wisherd—Alpco Diagnostics

Guests
Valerie Arends—University of Minnesota
Nigel Clarke—Quest Diagnostics
Shawn Connolly—University of Missouri
Linde De Grande—University of Ghent
John Eckfeldt—University of Minnesota
Daniel Holmes—Univ. of British Columbia
Kuanysh Kabytaev—University of Missouri
Michael McPhaul—Quest Diagnostics
Violeta Raneva—ReCCS Japan
Curt Rohlfing—University of Missouri
Amy Saenger—University of Minnesota
Michael Steffes—University of Minnesota
Hirohito Umemoto—ReCCS Japan
Gwen Wark—UKNEQAS/IFCC

1) **Welcome and Introduction—Randie Little**
R. Little welcomed those in attendance, those present introduced themselves. The 2015 meeting minutes were approved.

2) **Clinical Update on Diabetes—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.
  - DM1 is a predictable disease with different phases.
  - Preventing future, 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
  - Over the years can lose ~90% of beta cells before developing glucose intolerance.
  - Continue to lose beta cell mass over time.

- 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 post-prandial 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 (sCP) w/ insulin, but NO change in cut off for significant 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.
- Increasing C-peptide correlates with more insulin+ cells (Keenan et al., Diabetes 2010)
  - Pancreases of people with diabetes for >50 years post-autopsy
  - Saw positive C-peptide responses even after 50 years
- 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
- 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).
  - 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.
- Low levels of C-peptide have clinical significance for established Type 1 diabetes (Kuhtreiber et al, Diabetic Medicine 32:10, 1346-1353, 16 Aug 2015)
  - Use ultrasensitive assay LOQ 2.5 pmol/L
  - Found that low levels of C-peptide were indicative of greater risk of having poorly-controlled diabetes
  - Even very low levels of C-peptide can have prognostic significance
- Conclusions
  - C-peptide production persists for decades after disease onset and remains functionally responsive
  - Very low levels (but detectable by ultrasensitive assays!) of C-peptide are predictive of diabetic complications
  - Patients with advanced disease may benefit from interventions to preserve β-cell function or to prevent complications
- Insulin sensitivity and resistance in-vivo
  - Measurement of insulin and glucose
  - Various ways of calculating response (HOMA, QUICKI, 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) Must have 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)

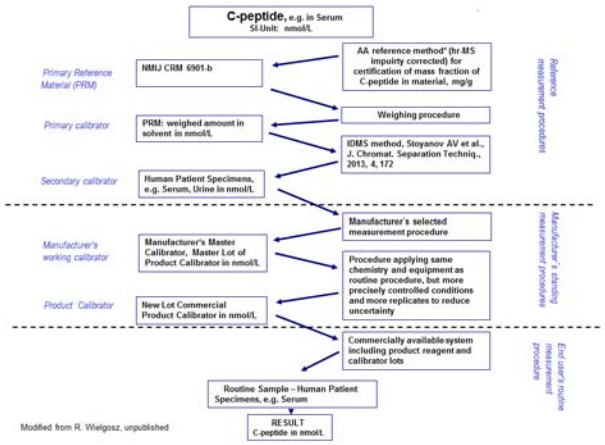
Discussion:

M. Steffes asked if anyone has actually looked in the literature to try to extract a standardized approach to the use of C-peptide to measure beta cell function or insulin to measure insulin resistance. D. Stein said no, this is one of the reasons why we are here, standardization of the assays is necessary. This will allow comparison of results across studies and cohorts. It is also important for assessing the many ways of treating diabetes, especially type 2 diabetes, that are now available. When assessing insulin sensitivity and resistance, no one currently looks at the achieved insulin levels, they measure how much insulin is given from the bottle since it is well-characterized. It would be good to have standardized measurements of insulin, and for that matter C-peptide, so that the results could have predictive value. Right now if a clinician orders an insulin level, and it is high, all it tells you is that it's high. C-peptide arguably may be a little better standardized, but the results are still highly variable. S. Narayanan said that a lot of immunoassays for insulin may not detect some insulin analogues. D. Stein agreed, noting that the analogues all have different sequences, it is an issue. The most challenging one in terms of MS measurement is Lispro since the molecular weight is identical to human insulin, it can be detected by either immunoassay or advanced chromatography. All of the other analogues have different molecular weights so they can be detected by MS.

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.
- C-peptide Standardization
  - For the early studies, laboratories analyzed the samples and calibrators, later manufacturers performed the analyses
  - Found that the WHO standard was ineffective in harmonizing results.
  - 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.
- C-peptide Reference Method/Laboratory Comparison
  - In order for manufacturers to re-calibrate their C-peptide assays to the reference method we were told that we must have it listed with the JCTLM. This required a comparison between two reference laboratories.
  - The reference method at D. Stein's lab in New York was replicated in our lab at the University of Missouri.
  - We 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; the method is now listed with JCTLM.
  - A later 2014 comparison between the two reference laboratories showed a better correlation (r^2=0.9921).
- New samples available 2016
  - 7 levels of pooled sera ranging from 0 (undetectable) to 4.00 nmol/L c-peptide (Reference Method assigned values).
  - 80 single donor samples ranging from 0.31 to 5.23 nmol/L c-peptide (Tosoh AIA900).

- Current Issues
  - C-peptide Reference Materials: There was much confusion at last year's C-peptide manufacturer meeting over the different reference materials.
    1) BIPM and NIM China
       - Produced for use in BIPM key comparison (CCQM-K115)
       - Participation was open to all NMIs active in CCQM activities
       - Value assignment is being finalized based on the CCQM results
       - Not high purity, purpose was to test metrology institutes
       - The material will be submitted to the JCTLM
    2) NIBSC
       - Produced by NIBSC for the WHO to replace the previous lot (which was depleted) as an international standard
       - Value assigned by amino acid analysis and HPLC
       - Was compared to previous WHO material
       - A commutability study was performed
       - Is not listed in the JCTLM database
    3) NMIJ CRM (CRM 6901-b)
       - A lyophilized synthetic peptide with high purity
       - Concentration determined by two independent amino acid analyses using liquid and gas phase hydrolyses.
       - Is listed in the JCTLM database
       - We spiked a zero C-peptide sample with the native NMIJ material at different levels and analyzed these samples on LC-MS; results were very close to the theoretical results based on the package insert, with a very tight correlation (r=0.996).
  - Comparison Between NMIJ and DDL Reference Methods
    1) 25% difference in results, still unexplained
    2) NMIJ used a different sample prep
    3) NMIJ uses a derivatization procedure
  - Proposal for Standardization of C-peptide
    1) Primary Reference Material: NMIJ CRM 6901-b
    2) LC/MS method (Stein, et al; Stoyanov, et al)
    3) Secondary Reference Material: pooled and single-donor serum
    4) Proposed reference scheme



- 2015-2016 Goals for Manufacturers: Begin the Process of re-calibration (A letter from ADA is enclosed in your packet)

Discussion:

New reference materials, reference methods
R. Little said the main reason the NIBSC made a new standard was because the old WHO standard was depleted. It is not clear how useful it may be to harmonize routine assays, as any processed material is subject to matrix effects.

We feel confident in our results based on the data we obtained from the NMIJ spiking experiment, we are working with the NMIJ lab to resolve the method bias issue. One problem is the difficulty in getting the samples into Japan, there are strict rules. At the recent JCTLM meeting there was some discussion of the fact that when more than one reference method is listed for a given analyte, and these methods run the same samples, the results obtained do not always match. JCTLM and BIPM seem to be taking a harder look at this. We need to make sure that if manufacturers standardize their assays that those will be the numbers and there will not be other references with different numbers. We are working on a paper along with people from the various institutions involved with C-peptide standards and reference methods (BIPM, NIBSC, JCTLM, etc.) to describe these efforts and present the issues we have encountered. There was much confusion regarding the different standards and it is very difficult to get them all working together, the funding streams are all different and they do not routinely meet with each other. We are trying to find better ways of doing standardization studies where there is better communication among all the organizations so that we don't end up with different reference methods that produce different results and different reference materials. D. Stein said that since we asking manufacturers to re-calibrate their assays, we need to state which reference material we will be using to re-calibrate. R. Little responded that manufacturers will use serum samples with values assigned by the reference method to re-calibrate. There is a publication describing the certified reference material (NMIJ). The NIBSC material is not as pure or well-characterized, and is not value-assigned. The BIPM NIM China material is not available yet, and also will not be as pure. N. Clarke said that he suspects the NMIJ reference method is reading lower due to their derivatization causing a high calibration curve. Their results are likely wrong and the D. Stein/Univ. of MO results are likely right. M. McPhaul said that their method (Clin Chim Acta. 2016;455:202-208) should be included in a comparison with the U.S. and NMIJ labs, R. Little agreed. D. Holmes asked whether the use of serum calibrators eliminates the bias between the reference methods, do we know? R. Little thought so, it is difficult to get many measurements from Japan, when we sent samples to Japan they wanted to know the approximate values. K. Kabytaev noted that this is because their method uses double-isotope dilution and briefly described the methodology, noting that there are unanswered questions regarding this method. R. Little said it is good to have another reference method that is different from ours to compare to, but the NMIJ method is very time consuming and it is doubtful that we would be able to send 40 serum samples to them and get results back in a reasonable length of time. M. McPhaul said their method is not time-consuming, it involves a simple immunoprecipitation with no other prep involved. D. Stein said at the recent NIST meeting there were discussions of the BIPM material among members of a lot of serious metrology institutes using the latest technologies to measure this material. They are using C-peptide as a test for standardization of peptides. Using the best MS methods available they found the material was ~88-90% pure. There are salts, water, surfactants, etc. so amino acid analysis needs to be performed to determine peptide content. When it comes to the immunoassays, they are probably picking up some synthetic alterations, post-translational modifications that will likely not be picked up by MS. Also some de-amidation of the standard and samples can occur over time, this needs to be taken into account although for immunoassays it probably does not matter. R. Little said that is why we do not send out pure material for immunoassay analysis. D. Stein said spiking the pure material into serum that is stripped of C-peptide should theoretically make a good calibrator. D. Holmes said in his experience stripped serum is watery and is void of lipids and other things, it is not commutable. N. Clarke agreed, adding that it might work on one method but not another. Unadulterated serum samples with assigned values are the best. D. Stein said he is not sure of the purity of the NIBSC material, his standard and the NMIJ material are probably as pure as one can make them although they are not perfect; the BIPM material is not as pure. S. Connolly added that the BIPM material was deliberately made so as not to be as pure, as its purpose was to test the NMIs. R. Little said it was an unfortunate coincidence that all of the organizations were looking at C-peptide at the same time, the BIPM material was only to be used by metrology institutes.

CAP

R. Little said CAP is planning on a C-peptide survey that will include actual serum samples so that we can get a snapshot of how methods are performing and how well they compare out in the field. Hopefully this will be ongoing so that we can monitor progress over time. S. Narayanan asked about ongoing clinical studies, what should they do if manufacturers perform re-calibration in the middle of these studies? R. Little said there should be a clear-cut relationship between the values before and after so they should be able to relate the two.

Re-calibration of Assays

R. Little asked manufacturers to begin the process of re-calibrating assays once the new serum samples are value-assigned. N. Clarke said based on the results it would seem to be a good idea, even if the results are not 100% accurate at least everyone would be on the same scale. R. Little said the package insert for the NMIJ material is

included in the packets, we can obtain more information on the other standards as well if needed. I. Kutschera said that there are countries where they can only buy assays that are validated against WHO. R. Little said we were not aware of this. S. Narayanan clarified that this means a manufacturer is not allowed to bid for the tenders (i.e. large government contracts) if they are not standardized to WHO. R. Little said that is an important issue to bring up with the JCTLM; WHO generally does not list their materials with JCTLM. This needs to be addressed at the higher up level with NIBSC and JCTLM. A. Saenger asked why it has not been addressed by these institutions. R. Little said her impressions are that the issues are political. We may also need to address this in the paper, C-peptide is likely going to be used as an example of issues that arise in harmonization efforts. I. Kutschera said this issue applies to other analytes, not just C-peptide. A different reference material might be more valid, but these countries will not recognize it. There are also regulatory issues with assay re-calibration in some countries, it can require additional submissions which take many months and there can be penalties involved. S. Narayanan agreed but noted that in the U.S. minor re-calibrations are not a major issue, the FDA does not require a whole new submission, since C-peptide is an exempt assay so it just requires a letter although documentation and data also needs to be provided. R. Little said there are many groups that are now focused on standardization and harmonization, this needs to be brought up with JCTLM, we know that the WHO standard for C-peptide is ineffective for harmonizing assays, it probably is for other analytes as well. Regulations need to be brought up-to-date in terms of harmonization efforts. D. Holmes asked if it is possible to have two different calibration sets, S. Narayanan said there are cases such as PSA where there are two different calibrations and the FDA has approved both. I. Kutschera noted that this means all of the work has to be done twice. The best thing for manufacturers would be that, if the NMIJ material is to be the reference, WHO would tie its standard to that standard or use that standard as their material. Manufacturers could then use this material to set the calibrations for their assays. R. Little said NIBSC has been made aware of what is going on; there is probably not a way of making everyones' materials agree, they can assign any value to a material but when manufacturers use them in their assays they produce different results in samples. It's probably the regulatory process that needs to be addressed. I. Kutschera said it is very difficult to get regulatory processes changed.

Samples

S. Narayanan asked how the samples sent out for re-calibration would be used to standardize the low end of the range since different assays have different sensitivities. R. Little said at this point we have not looked closely at the low end, we will need to look at this at some point but right now we are just trying to make results comparable throughout the range. The lowest samples read zero on the reference method, we do not know if they really are zero but we get as low as we can by using subjects with long-term type 1 diabetes. A later goal could be to see how comparable results are in the very low range. S. Conley asked about the volume of the samples, R. Little said they are the same as before (500uL). There is a lot of material, it will take a while to get values for all of the pooled and single-donor samples.

4) IFCC Working Group for Standardization of Insulin Assays (SWG-IA) Update — Amy Saenger, Michael Steffes

- Goal
  - Achieve calibration traceable to an ID-LC-MS/MS reference measurement procedure for all commercially available insulin assays
  - Manufacturers require a reference system listed by JCTLM to enable recalibration of assays
- Current Status of Insulin Assays
  - Commercial methods not harmonized
  - Harmonization by traceability to IDMS was demonstrated to be achievable using single donor sera
  - Calibration algorithms at low concentrations need to be improved for some methods
  - Harmonization using pooled sera was effective over the concentration interval covered by the pools (>50 pmol/L)
  - Use of pure recombinant insulin was not effective in harmonizing results for insulin immunoassays
- IFCC Working Group on Standardization of Insulin Assays (SWG-IA)

Name	Role	Country
A. Saenger	Co-Chair	US
M. Steffes	Co-Chair	US
J. Dekker	Member	NL
D. Holmes	Member	CA
R. Little	Member	US
M. McPhaul	Member	US
G. Miller	Member	US
D. Sacks	Member	US
G. Wark	Member - IFCC	UK

In Collaboration with: American Diabetes Association (ADA), European Association for the Study of Diabetes (EASD), CDC, NIDDK, International Diabetes Federation (IDF)

- IFCC SWG-IA (Plans/goals)
  - Establish insulin RMP in 2 laboratories, generate documentation for JCTLM submission
  - Provide manufacturers a panel of frozen aliquots of single donor sera, and/or a set of frozen serum pools, for use to develop internal calibration algorithms. Refine performance of methods, establish recalibration factors for traceability to IDMS values.
  - Serum samples will be value assigned by IDMS for insulin for development of recalibration protocols
  - Evaluate the effectiveness of serum pools in the concentration interval 10-600 pmol/L as secondary reference materials for calibration of insulin assays
  - WHO insulin RM will become available
  - Publish RMP and secondary RM (frozen serum pools) to support JCTLM listing
  - Submit RMP, reference laboratories, and secondary reference material for JCTLM review
  - Get WHO insulin RM JCTLM listed.
  - Develop performance criteria and certification process (if needed).
  - Get RMP listed with JCTLM
  - Serum panel used for surveillance (e.g. EQAS or certification program) of traceability
  - Recalibrated methods available for sale
- Insulin LC-MS/MS Methods
  - Reference Method Procedure: ID-LC-MS/MS University of Ghent (Van Uyfanghe K, Rodriguez-Cabaleiro D, Stocki D, Theinpont LM. Rapid Commun Mass Spec 2007;21:819-21.)
  - Quest/Nichols: will be discussed by Dr. Michael McPhaul
  - University of Minnesota (M. Steffes, A. Saenger): Method is in development on Sciex QTRAP 6500
  - Albert Einstein (D. Stein)
- Development of Patient Pools: Specimens collected and processed according to CLSI C37A
- Patient Pools: Storage of Aliquots
  - Five levels of reference-method value assigned pools (49.8, 217.3, 236.6, 512.9, 676.1 pmol/L)
  - Individual donors (45), donor pools (5)
  - Total aliquots stored at the University of Minnesota: 16,591 aliquots/vials (281 boxes)
- CAP Proficiency Testing (pilot this year will include two serum sample)
  - Insulin, C-peptide
  - Consent, collection, processing, shipping, storage conducted at the University of Minnesota
  - Up to 10 individuals will be consented
  - Donors will be fasting; specimens will be collected when donors are in fasting and non-fasting state
- What are the appropriate target C-peptide concentrations for these two pools
  - What sort of donors and what situation would be used to collect serum to prepare pools with appropriate C-peptide targets (e.g., normal volunteers fasting and non-fasting)
  - Other considerations in making the pools (e.g., need for blood borne virus testing, etc.)

Discussion:

Insulin reference material and methods

A. Saenger said it will be some time before a new WHO insulin standard is available. D. Stein noted the insulin method in NY is on hold at the moment.

CAP survey—use of serum

D. Holmes asked if the sera will be shipped frozen, A. Saenger said yes. Extra aliquots will also be sent to the Univ. of MO and Quest for reference method analysis.

Reference intervals
D. Holmes noted that in the process of migrating from Immulite to Roche for insulin and C-peptide, their lab discovered that the reference intervals are different if people with a high BMI were excluded. Reference interval studies have shown that excluding subjects with BMI>25 results in C-peptide and insulin reference intervals that are about half of what they are if all subjects are included. Even if results are harmonized, we may not be any further ahead if everyone's reference intervals are different. A. Saenger agreed, noting that the reference intervals in different manufacturers' package inserts vary a lot, we need to look at data from epidemiological studies to see how BMI affects reference intervals and determine the best way to define them. R. Little noted that her laboratory uses a cutoff for BMI when defining reference intervals, the parameters just need to be defined and consistent. M. McPhaul noted that a BMI cutoff of 25 results in a lot of exclusions in the U.S., and that there is a lot of variation in insulin results even in the normal range.

5) MS Assay for Insulin—Michael McPhaul

- Insulin Assays Have Been Plagued by Considerable Variability
  - 150 patient samples
  - Assays varied by a factor of 2 for the median and also high insulin values determined in the group
  - > 20 fold difference observed in the low insulin samples
  - Even between two immunoassay platforms, very large differences with some samples (e.g. >300µU/ml vs. 6.1µU/mL, 91µU/ml vs. 6.4µU/ml)
- A New Insulin Assay: Characteristics
  - Accurate, reliable quantitation of endogenous intact insulin by LC-MS
  - Simultaneously quantifies intact C-peptide
  - Potential to expand to insulin analogs
  - Potential to identify unusual variants
- Intact Insulin Immunocapture
  - Insulin Analogs contain different sequences at the last three N terminal AA of B Chain – these are recognized as well - misses Lantus
  - Ab to C-peptide included in the IP step as well
- Workflow
  - Delipidation
  - Immunocapture: Anti-insulin B chain and Anti-C-peptide IgG's coupled to magnetic beads
  - Wash and Elute
    1) Extensive washing with NaCl, PBS, water
    2) Elute with 30% acetonitrile, 0.1% formic acid in water
    3) Stabilize with Trizma base
  - LC
    1) 2 dimensional LC
    2) HLB trapping column
    3) CSH™ (charge surface hybrid) reversed phase eluting column
    4) Water, formic acid acetonitrile gradients
  - MS: MRM on a triple quadrupole MS (Agilent 6490)
- Automated Calibrator and Sample Preparation, Bead Deposition, Immunocapture, Washing, and Elution
  - 150 µL of sample is processed
  - Sample preparation is mostly automated
  - Manual steps involve initial dilution of working stocks, centrifugation, and bead preparation
- Intact Insulin Fragmentation
  - It is well known that insulin does not fragment very well, limiting choices
  - Our assay quantitates using the PK ion
  - We examine 2 qualifier ion fragments to ensure that the measured signal is insulin – both qualifiers ions are derived from the B chain: insulin Tyrosine immonium and y3 ion (last three amino acids of the B chain (=PKT)).
- C-peptide Fragmentation

- Qualifiers – ratio of three ions (Y5, Y6, and Y9)
- Quantitation – the sum of the same three ions are employed

- Insulin and C-peptide Chromatography
  - Qualifier ions superimposed on the quantifiers.
  - If the ions weren't superimposed, the chromatographic profiles would be different and they would not be from insulin.
  - Thus qualifiers confirm molecular identity.
- Insulin and C-peptide standard curves: Both assays are linear over two orders of magnitude (dynamic range of quantitation)
- SI-traceability
  - To enable comparisons between laboratories, assays must be standardized (i.e., traceable to a common reference material).
  - Ideally, the reference material is traceable to the International System of Units (SI). However, most peptide assays are standardized against World Health Organization (WHO) reference materials that are not SI-traceable (i.e., there is uncertainty in the amount of pure material originally weighed).
  - Furthermore, the reference materials previously used for the standardization of most insulin (NIBSC code: 66/304) and C-peptide assays (NIBSC code: 84/510) are no longer available.
  - Calibrators were used to measure the response of the assay to increasing concentrations of peptide. Controls were used to check the accuracy of the assay; different suppliers provided calibrators and controls.
  - Insulin and C-peptide concentrations in patient samples were determined using the LC-MS/MS assay and immunoassays. Results were compared.
- Calibrators and Controls: Peptide Content Measured by Quantitative Amino Acid Analysis (Taylor SW, Clarke NJ, Chen Z, McPhaul, MJ. Clin Chim Acta. 2016;455:202-208).
  - Insulin
    1) Calibrator: WHO standard (NIBSC code: 83/500)
    2) Found: mean peptide content (SD) =77.9% (2.5%) (Reported 98%)
    3) 22.4 IU/mg* (Reported 26.0 IU/mg)
  - Control: USP insulin (LOT J0J250)
    1) Found: mean peptide content (SD) =76.1% (0.85) (Not reported)
    2) 21.9 IU/mg* (Reported 26.4 IU/mg)
  - C-peptide
    1) Calibrator: Anaspec (0.5 mg/vial)
    2) Control: Bachem (0.5 mg/vial)
    3) Found: peptide content variable between vials (56.2% to 76.4%)
    4) Reproducible within any given vial, eg, 75.4% to 77.6%
- Accuracy: Controls versus Calibrators Adjusted for Peptide Content

	Insulin (μIU/mL)*			C-peptide (ng/mL)		
	QC Low	QC Medium	QC High	QC Low	QC Medium	QC High
Target	13.3	43.1	172.7	0.48	2.07	7.70
Overall Mean	14.1	43.3	173.0	0.51	1.96	7.77
Overall SD	1.5	3.6	12.7	0.05	0.14	0.35
Overall CV	11%	8%	7%	10%	7%	5%
Overall Accuracy	106%	100%	100%	106%	95%	101%

- Correlations with immunoassays
  - Insulin: Good linear correlation with Beckman ICMA, values were comparable
  - C-peptide: Good linear correlation with Centaur ICMA, but Centaur results were ~20% higher.
    1) This may because the immunoassay is standardized to a reference material that has not been corrected for peptide content and/or the higher specificity of the LC-MS/MS method.
    2) Results are consistent with those reported in the literature.
- Conclusions
  - The peptide content based on quantitative amino acid analysis of calibrators and controls differed from the information provided by the commercial suppliers.
  - The LC-MS/MS assay is accurate for insulin and C-peptide. When the controls were run through the assay, their concentrations matched values calculated based on peptide content.

- There was agreement between the LC-MS/MS assay and the immunoassay for insulin; the WHO reference material previously used to standardize the insulin immunoassay may have been SI-traceable.
- There was negative bias between the LC-MS/MS assay and the immunoassay for C-peptide; the WHO reference material previously used to standardize the C-peptide immunoassay may have been of uncertain peptide content and therefore not SI-traceable.

- Next Steps
  - Assignment of values to the sample pools produced for the Insulin and C-peptide Standardization efforts
  - Clinical studies on well characterized patient samples correlating insulin, C-peptide and insulin / C-peptide ratios
  - To development of diabetes
  - To the degree of measured insulin resistance

Discussion:

LC-MS/MS method
A. Saenger asked if they have seen variations among the lots of magnetic beads used for the LC-MS/MS method. N. Clarke said they initially looked at three different lots and did not see significant variation; however, if the step was performed manually by different technicians they saw variations that they do not see when using robotic preparation. D. Holmes asked about insulin analogs, M. McPhaul said they have validated the insulin assay for quantifying the natural insulin sequence but they can detect the different analogs. N. Clarke added that they can also get partial separation of Lispro. D. Holmes said Lispro is problematic for their method since the antibody they use for insulin is directed to the n terminal of the b chain.

Discordance between insulin immunoassays
D. Holmes said that with the Immulite platform heterophile antibody interference was common, and that they have seen sharp increases in C-peptide levels in patients with chronic renal impairment. Patients with GFRs of ~15 that have had islet cell transplants seem to have a lot of C-peptide even though the graft is no longer functioning, is this due to cross-reactivity with the immunoassay? M. McPhaul said they have not looked at this specifically, but they do see cases where there are large discrepancies between methods that are reproducible even over time within a subject. N. Clarke said that it is not an issue where one immunoassay method is always right and the other is always wrong, one method can be correct in one case but the other method might be correct in another case. A. Saenger asked if the interference can be due to the patient being on insulin, M. McPhaul said no, these subjects are not on insulin. D. Holmes asked about the percentage of subjects that show these large discrepancies, M. McPhaul said approximately 2-4%. Also, we have found evidence of excess insulin b chains in some subjects, N. Clarke said they see discordances between intact insulin and b chains on MS. M. McPhaul said they are interested in finding out if this represents a subgroup that has insulin resistance. D. Holmes wanted to know if the signal and capture antibodies used in insulin assays are always targeted at the a and b chains, M. McPhaul said no, they are all over the place. D. Holmes said that the phenomenon of excess b chains in some is a problem for standardization, you can have analytical standardization but you still do not have biological standardization of metabolism. M. McPhaul said this is likely the reason for the discordances we see between immunoassays for these subjects. R. Little said that we can better look at these discrepancies once we achieve standardization of the assays. We would not want to use these types of individuals in our sample panels or reference materials. M. McPhaul noted that these individuals represent a small percentage of people. There is no such thing as free b chain, and there has been no binding proteins or post-translational modifications described for insulin. The only rationale he can think of for what they are seeing is that in solution, the insulin is either self-associating or there is something else that is highly abundant with low affinity that is not being identified. S. Narayanan asked if it is possible that this is an interference from biotin, M. McPhaul did not think so. D. Stein recalled that in the literature they have reported b chain being detected in urine. D. Holmes suggested looking at renal function in these subjects, S. Narayanan said the urine could be tested for b chains. D. Holmes said we will get resistance from manufacturers if we start telling them their antibody selection is not good, but this does represent a barrier to standardization. R. Little said we could run insulin on the samples we have already collected for C-peptide and see what kind of range we have, we might be able to use the same sample panels for both. D. Holmes suggested they could be run on different immunoassay methods to check for discrepancies. M. McPhaul said they are doing further studies, they wanted to come to this meeting to have these kinds of interactions and make sure what they are doing is aligned with other groups that are moving forward.

RL thanked everyone for their attendance and noted that there will be many issues to discuss further once manufacturers have begun the process of re-calibration of their assays. The meeting was adjourned at 9:15 AM.

Minutes prepared by Curt Rohlfing, 8/26/16, modified by R. Little 8/31/16.