

2019 C-Peptide Standardization Manufacturer Meeting

Minutes

Wednesday August 7 8:00 AM – 10:00 AM
Hilton Anaheim, Anaheim, CA

Participants:

C-peptide Standardization Committee Members

Randie Little—University of Missouri
W. Greg Miller—Virginia Commonwealth University
Daniel Stein--Albert Einstein College of Med (by phone)

Committee members not present

Carla Greenbaum—Benaroya Research Institute
Jerry Palmer—University of Washington
Kenneth Polonsky—Washington University
Lisa Spain—NIDDK

Manufacturer Representatives

Bethany Bell—Alpco Diagnostics
Ken Hood—Tosoh Bioscience
Carissa Jones--Mercodia
Kevin Ley—Beckman Coulter
Stefaan Marivoet—Tosoh Bioscience
Noor Mohammed—Tosoh Bioscience
Shanti Narayanan—Tosoh Bioscience
Yuka Ono—Tosoh Bioscience
Maria-Magdalena Patru—Ortho Clin Diagnostics
Robert Somogyi—Alpco Diagnostics
Koichi Suga—Tosoh Bioscience
Masashi Yamada—Tosoh Bioscience
Jian Yang—Penn State University

Guests

Valerie Arends—University of Minnesota
Christa Cobbaert—IFCC, Leiden Univ, The Netherlands
Shawn Connolly—University of Missouri
Daniel Holmes—St. Paul's Hospital, Vancouver
Andy Hoofnagle—University of Washington
Kuanysh Kabytaev—University of Missouri
Michael McPhaul—Quest Diagnostics
Benjamin Owusu—University of Washington
Curt Rohlfing—University of Missouri
Jesse Seegmiller—University of Minnesota
Michael Steffes—University of Minnesota
Kwaku Tawiah—University of Missouri
Gwen Wark—UKNEQAS/IFCC
Zengru Wu—Quest Diagnostics

By Phone:

Beena Akolkar—NIDDK
Carole Dauscher—Siemens
John Eckfeldt—Univ of Minnesota
Santica Marcovina—University of Washington
Omar Quraishi—Siemens

1) **Welcome and Introduction—Randie Little**

R. Little welcomed those in attendance.

2) **Clinical Update: Type 1 Diabetes—Randie Little**

- Background: C-peptide
 - 1) Pro-insulin is synthesized in the pancreatic beta cells and packaged into granules
 - 2) Pro-insulin is cleaved into Insulin and C-peptide
 - 3) Insulin and C-peptide are secreted in a 1:1 molar ratio.
 - 4) Insulin (but not C-peptide) is cleared by the liver; C-peptide remains in the circulation longer than insulin.
 - 5) C-peptide is a better marker of insulin secretion than insulin.
- Why preserve beta cell function?
 - 1) Many people with Type 1 diabetes still have residual insulin secretion many years after diagnosis.
 - 2) Among DCCT subjects in the intensive treatment group, higher C-peptide was related to:
 - Lower risk of hypoglycemia
 - Better glycemic control
 - 3) Reduction of microvascular complications
 - 4) Treating the underlying disease process, even if not curative, holds the promise of significant clinically relevant benefits.
- Staging for Type 1 Diabetes
 - 1) Genetic predisposition (if have a relative with T1D, 15x greater risk)
 - 2) Immune activation
 - 3) Immune response: development of a single autoantibody

- 4) Eventually progresses from normal glucose tolerance with ≥ 2 autoantibodies to abnormal glucose tolerance, then to clinical diagnosis of diabetes
- Selection of Subjects for Clinical Trials
 - 1) Approximately 5% of individuals with relatives with T1D have one or more autoantibodies against:
 - islet antigens (GAD65, GADA),
 - insulin [micro-insulin autoantibody (mIAA),
 - insulinoma-associated protein 2 (IA-2A),
 - zinc transporter 8 (ZnT8A),
 - islet cell antibodies (ICA)
 - 2) Those with positive autoantibodies are eligible for regular follow-up in TrialNet
 - 3) 35% of multiple autoantibody-positive relatives with normal glucose tolerance and 65% of those with abnormal glucose tolerance develop clinical type 1 diabetes within 5 years.
 - 4) There is a steep drop in insulin release 6-12 months prior to clinical onset; the “cliff edge”.
 - 5) Attempts to preserve beta cell function should start before the “cliff edge” is reached.
 - 6) Age is also important! Clinical trials (abatacept, rituximab trials) demonstrated that the effectiveness of therapy was most pronounced in children.
- Previous Trials
 - 1) Rituximab (anti-CD20):
 - Slowed decline in stimulated C-peptide in recent onset T1D
 - Was associated with lower insulin requirements and lower HbA1c over 12 months
 - 2) Abatacept (CTLA4-Ig) : In recent onset Type 1, there was 59% more C-peptide secretion which remained 1 year after treatment.
 - 3) Oral insulin: Did not prevent or delay T1D in the primary cohort but there was a significant delay in onset in a secondary cohort of individuals with low first-phase insulin release.
 - 4) From: Bingley, et al. Diabetes Care 2018; 41:653-61
- Clinical Update: 2019
 - TrialNet Anti-CD3 Antibody (Teplizumab)
 - 1) Teplizumab works by reducing the actions of CD8+T lymphocytes on β -cell targets
 - 2) 76 patients (55 children and 21 adults) with a family history of type 1 diabetes, abnormal glucose tolerance and at least two diabetes-related autoantibodies (“the cliff edge”).
 - 3) Patients were randomly assigned to a treatment group (single 14-day course of teplizumab) or placebo group.
 - 4) Treatment slowed the development of the disease by nearly 2 years; this delay is clinically important.
 - 5) After 5 years, 43% of patients were diagnosed compared to the 72% in those not receiving the drug.
 - 6) This is the first time a study has shown that immune therapy can delay progression to Type 1 diabetes!
 - 7) Questions remain
 - This trial included only those with 1st degree relatives with T1D. Most people presenting with T1D do not have 1st degree relatives with T1D. Should we be thinking about a much broader screening strategy?
 - Can Teplizumab actually prevent diabetes in some patients?
 - Distinguishing Different Types of Diabetes
 - 1) The measurement of insulin secretion using C-peptide may be increasingly relevant in clinical practice, with the rise in prevalence of Type 2 diabetes in younger patients and the discovery of monogenic subtypes of diabetes requiring specific management.
 - 2) Some people diagnosed with Type 1 diabetes have the wrong diagnosis!
 - 3) Of 801 patients diagnosed with T1D in Edinburgh UK, 10 new cases of monogenic diabetes and 28 with Type 2 diabetes were found. (Wise et al. BMJ 2019;365:14352)
 - 4) Authors recommended that C-peptide testing be implemented for all people diagnosed with T1D.
 - 5) The correct diagnosis ensures that people get the right treatment early.
- Summary
 - C-peptide is the best marker of insulin secretion.
 - Preserving insulin secretion in Type 1 diabetes can lead to significant clinical benefit.

- Attempts to preserve beta cell function should start prior to diagnosis.
- A recent trial with Anti-CD3 Antibody (Teplizumab) showed that immune therapy can delay progression to Type 1 diabetes.
- Many people diagnosed with Type 1 diabetes and treated with insulin may actually have Monogenic or Type 2 diabetes.
- Screening with C-peptide may be helpful in making the correct diagnosis to ensure the people get the right treatment.

3) A High-throughput Mass Spectrometry Multiplexed Assay to Measure Insulin and C-peptide—Michael McPhaul

- Insulin Assays Have Been Plagued by Considerable Variability
- A New Insulin Assay
 - Characteristics
 - 1) Accurate, reliable quantitation of endogenous intact insulin by LC-MS
 - 2) Simultaneously quantifies intact C-peptide
 - 3) Potential to expand to insulin analogs
 - 4) Potential to identify unusual variants
- Intact Insulin Immunocapture
 - Insulin B chain-specific mouse monoclonal IgG
 - For the multiplexed assay, an anti-C-peptide antibody is included
- Workflow
 - Serum 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
 - 1) MRM on a triple quadrupole MS (Agilent 6490)
 - 2) Internal standards: Bovine (insulin), heavy ID (C-peptide)
- SI Traceability
 - To enable comparisons between laboratories, assays must be standardized (ie, 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 (ie, there is uncertainty in the amount of pure material originally weighed).
 - 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.
- Calibrators and Controls: Peptide Content Measured by Quantitative Amino Acid Analysis (Taylor SW, Clarke NJ, McPhaul MJ. Clin Chem. 2016)
 - Insulin
 - Calibrator: WHO standard (NIBSC code: 83/500)
 - 1) Mean peptide content (SD) =77.9% (2.5%) (Reported 98%)
 - 2) 22.4 IU/mg (Reported 26.0 IU/mg)
 - Control: USP insulin (LOT J0J250)
 - 1) 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) Peptide content variable between vials (56.2% to 76.4%)
 - 4) Reproducible within any given vial, eg, 75.4% to 77.6%
- National Metrology Institute of Japan lyophilized certified C-peptide reference material included in each assay.
- 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 Beckman 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 Siemens 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.
- Progress in Use and Future Steps
 - Application to assign values to ADA pools for insulin and C-peptide - completed
 - Correlate performance with existing C-peptide assays – completed (U of Missouri)
 - Use these assays in clinical studies to explore applications of the assays (next slide set) - ongoing
- Quest comparisons with Univ. of Missouri
 - Both comparisons showed excellent correlation ($r^2 \geq 0.97$) with minimal bias.
 - Difference between assays
 - 1) Quest: Japanese standard material included as calibrators in each assay
 - 2) U of Missouri: Japanese standard material used to assign IS value
- Insulin and C-peptide in Stanford Insulin Resistance Study (F.Abbasi, et. al)
 - Study Population
 - 1) Apparently healthy individuals without history of cardiovascular disease, fasting glucose < 126 mg/dL
 - 2) Insulin resistance (IR) defined as top tertile of steady state plasma glucose (SSPG) in non-diabetic participants
 - 3) 535 participants (36% males, 30% self-described non-Whites) with a complete set of biochemical and anthropometric measurements
 - Results showed that odds ratios for predicting IR greatly increased when insulin and C-peptide were combined vs. each separately
 - Insulin Resistance Risk Score
 - 1) Traceable values of insulin and C-peptide are used to calculate patient's risk score.
 - 2) Report the probability of having clinically significant insulin resistance (defined as top tertile of insulin resistance measured by SSPG), given traceable values of insulin and C-peptide
 - 3) Rather than reporting "positive" or "negative" test result (with cut-point driven sensitivity and specificity) report probability of IR for risk score
 - 4) For example:
 - A patient with 10 μ IU/mL insulin has 53% probability of having IR.
 - This probability goes up to 79% if this patient's C-peptide is 3 ng/mL.
 - Found that both C-peptide and insulin were significant predictors of IR in subjects both with and without metabolic syndrome.
 - Implications
 - 1) Insulin and C-peptide are associated with insulin resistance independently of one another and of traditional risk factors, including fasting glucose.
 - 2) Both Insulin and C-peptide levels are required to adequately model IR as formally assessed using SSPG
 - 3) Insulin and C-peptide can be used to assess probability of insulin resistance in those with and without metabolic syndrome.
 - 4) A risk score combining C-peptide and insulin can be used to provide patients with their probability of having clinically significant insulin resistance.
 - 5) Potential applications:

- Following progress through / response to a DPP
- Quantitative beta cell output in response to stimulus

Discussion:

MS Assay

M. McPhaul noted that the NMIJ material appears to be a very good reference material for C-peptide. Quest still does not have a good reference material for insulin. B. Bell said that although the overall correlation between the Quest and Univ. of Missouri assays was good there were a few samples that showed discrepancies. M. McPhaul said these data are new so they have not had a chance to look into this yet. S. Narayanan asked if the MS results were compared with those of the immunoassay at the Univ. of Missouri lab, M. McPhaul responded that they did not compare with any immunoassay in this study. In the paper describing the MS assay published in CCA they performed a limited number of comparisons with the Siemens assay. S. Narayanan said it would be interesting to see how the new Tosoh C-peptide assay, which uses an antibody specific to the 33-63 sequence, would compare to the MS assay. M. McPhaul agreed and added that they are interested in comparisons with a variety of immunoassays with varying degrees of specificity. R. Little said she would be showing data comparing the Univ. of Missouri Tosoh immunoassay to their MS assay in her later presentation, but it would be good to analyze the outliers from the Quest/UM comparison on the Tosoh as well. Z. Wu said the comparison was only performed once at Quest in their normal clinical setting, so there is some inherent variability in the results. Also this is their ongoing clinical test. They analyze QCs, which are stripped serum spiked with C-peptide, every day, these are used to track the variation of the assay.

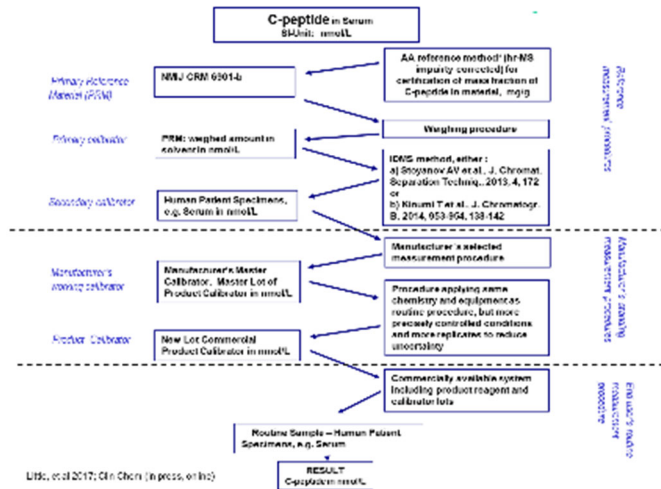
IR study

M. McPhaul said there were some subjects that showed discordances between C-peptide and insulin in the study. They suspect this may be due to the fact that C-peptide is cleared in the kidney while insulin is cleared by the liver. They have been following subjects enrolled in a diabetes prevention program, and generally see that subjects that follow the program in terms of weight management, etc., generally lower their IR risk scores. However, there is subset of subjects that have high IR scores even after following the program. It would be interesting to follow these subjects farther after they have completed the program to see if they eventually develop IR. They are also interested in looking at how reproducible beta cell production is over time.

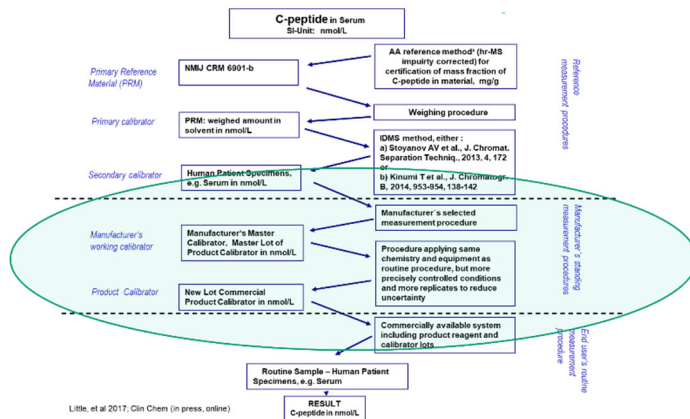
3) C-peptide Standardization Update—Randie Little and Kuanysh Kabytayev

- Background (R. Little)
 - For the early studies analyses were performed by laboratories performing clinical trials.
 - For subsequent studies manufacturers performed the analyses.
 - Manufacturers analyzed individual serum samples and pooled serum calibrators with reference method-assigned values, then obtained results for the individual serum samples using both their usual calibration and calibration using the pooled calibrators.
 - Data showed that use of pooled serum calibrators greatly reduced the variability of results among methods compared to the manufacturers' usual calibration schemes.
 - The original IDMS reference method had been set up in D. Stein's laboratory in New York, a second reference lab was later established at the University of Missouri.
 - Comparisons of results between the two reference labs were published and the method is now listed in the JCTLM database.
 - Dr. Kinumi's group in Japan (NMIJ) also has their reference method listed with JCTLM.
 - C-Peptide Reference Material: NMIJ CRM (CRM 6901-b)
 - 1) A lyophilized synthetic peptide with high purity
 - 2) Concentration determined by two independent amino acid analyses using liquid and gas phase hydrolyses.
 - 3) Is listed in the JCTLM database
 - 4) We evaluated our method with this material by spiking a zero C-peptide sample with the NMIJ Standard. The measured results matched the theoretical values based on the CRM.
 - 2018 comparison between the UM (both previous API-4000 and current API 6500+ MS instruments) and NMIJ methods showed excellent correlation and equivalent results between labs/methods.
 - Published a paper describing the issues and challenges involved with implementing C-peptide standardization (Clin Chem 2017 63:1447-56) along with an accompanying editorial.

- Initially there was a significant discrepancy between results from the UM and NMIJ methods; this was resolved and the results published in Clin Chem (2017 63:1904-5).
- Proposal for Standardization of C-peptide
 - 1) Primary Reference Material: NMIJ CRM 6901-b
 - 2) LC/MS method (DDL-MO, Japan)
 - 3) Secondary Reference Material: pooled and single-donor serum
 - 4) Traceability Chain



- Recent Studies (K. Kabytaev)
 - Long-term Reproducibility: Studies have shown excellent reproducibility over the period of 2016 to 2019 based on four levels of samples run as QC and comparisons with the Tosoh AIA immunoassay over this time period.
 - Commutability
 - 1) Both patient serum and spiked artificial BSA matrix samples sent to us by a manufacturer were analyzed on the IDMS reference method.
 - 2) The BSA materials showed a wide interference peak that can affect quantitation.
 - 3) We must be careful when analyzing specimens with synthetic components.
 - Comparison of WHO vs NMIJ by LC-MS
 - 1) Workflow:
 - Dilution NMIJ CRM
 - Calibration of isotope labeled standard with NMIJ CRM using LC-MS
 - Measurement of WHO concentration using ID LC-MS
 - Analysis in duplicate using two different NMIJ and WHO vials.
 - 2) Results: The amount of WHO c-peptide (13/146) in terms of NMIJ CRM (6901-b) is lower; 82.8% (83.4%, 82.2%) from the specified value.
 - Comparison of WHO vs NMIJ by Immunoassay
 - 1) The WHO material was also compared with NMIJ c-peptide by Tosoh AIA 900 and showed 82.6 % content.
 - 2) The Roche Elecsys assay showed 86.1% content.
- Implementation of C-peptide Standardization (R. Little)
 - All participating manufacturers have been sent data showing how their methods' results compare to those of the reference method.
 - Results for some methods are already close to those of the reference method, but others show significant deviations.
 - Serum Samples Available
 - 1) 7 levels of pooled sera ranging from 0 (undetectable) to 3.77 nmol/L c-peptide.
 - 2) 40 single donor samples ranging from 0.22 to 5.14 nmol/L c-peptide
 - 3) All samples have Reference Method assigned values.
 - Traceability



- New Accuracy-Based CAP Survey (ABGIC-A 2019)
 - 1) Only 18 labs participated at the time of this report with only one method listed.
 - 2) “Accuracy of C-peptide results will have great importance now that a reference method has been identified and standard diagnostic threshold may be promulgated. With bias of the Roche method as high as almost 50% of assigned values, there is clearly a long way to go.” (reported by DE Palmer-Toy and JA Straseski)
 - 3) Results from all other methods grouped together also showed much higher results overall than the reference method.
- We are developing a web site for C-peptide standardization.

Discussion:

C-peptide Reference Method

M. Patru asked why the MS results were compared to the Tosoh immunoassay when assessing reproducibility. K. Kabytaev said the laboratory already had this method, and it is another way to assess reproducibility besides the samples that were analyzed on the MS over time. As the samples age it is possible this could have some affect on MS results, so this is a good secondary way to verify reproducibility. Also, during this time the MS instrument was switched, so it was good to have another method to compare to, even though the actual results do not match we can still look at the relationship. R. Little added that the Tosoh is the clinical assay with tight QC so we know it is stable and reproducible. B. Owusu asked if, based on the results, we should conclude that the immunoassay is good. R. Little responded that in the early studies the performance characteristics of the various commercial assays were evaluated. Many of the immunoassays showed excellent precision, the problem is that the actual results do not match. That is the purpose of standardization, to make the results from different methods match and are accurate in terms of being as close to the true value as possible. B. Owusu asked if they had ever compared BSA material spiked with synthetic C-peptide to human zero C-peptide serum spiked with synthetic C-peptide. K. Kabyteev said yes, R. Little earlier showed a slide where zero C-peptide serum was spiked with the NMIJ material. We have to be careful when analyzing material with a synthetic matrix, they can cause interference as shown earlier. SIM mode, not MRM, mode is used in the MS method, which is more sensitive to potential interferences. D. Holmes asked K. Kabytaev if he has tried a new Sigma matrix called Sigmatrix which is an alternative to BSA, K. Kabytaev replied that they have not. S.Narayanan asked if the Sigmatrix material is more comparable to human serum. D. Holmes did not know, as the ingredients are proprietary. R. Little noted that for their routine immunoassay they have a frozen human serum QC in addition to commercial controls, as commercial controls can always potentially be subject to matrix effects.

C-peptide standardization

R. Little said that with HbA1c standardization it was easier to get manufacturers on board because the data were so strong in terms of clinical need. It is clear that we are headed in that direction for C-peptide, as shown earlier. The ADA has issued a letter stressing the importance of C-peptide standardization, the AACC is supportive of these efforts as well. S.Narayanan asked about the correlation between the UM IDMS and Tosoh AIA method; are the results more equivalent to the IDMS with the new Tosoh Cpep II compared to the old assay? R. Little noted that the graph just shows the correlation (X and Y number scales are different), the Tosoh results are still higher than those

of the reference method. S.Narayanan said it would be good to see how the results compare to unity, R. Little agreed and added that Tosoh can always re-analyze the samples on the new assay. M. Patru asked if the comparison showing how all of the manufacturers compare to the reference method was performed recently. R. Little said the data are from 2016, manufacturers would want to re-analyze the 40 samples and obtain current data prior to making their calibration adjustments. Unfortunately we currently cannot see any of the methods other than the Roche method in the accuracy-based CAP survey since all of the other methods' data were combined. R. Little said she would try to get that information from CAP. The low number of participants in the first accuracy-based survey is concerning, one possibility is to include a serum sample in the other survey which would get data from many more participants. J. Eckfeldt confirmed this is being discussed at CAP. The idea is to include a fresh-frozen un-manipulated serum pool in the ING survey which has hundreds of participants. They could alternate fasting and post-prandial pools between surveys to try to get varying C-peptide levels from one survey to the next. This has been proposed at CAP, we will not know for a while if they will decide to implement this. R. Little asked if there will be another accuracy-based survey this year. J. Eckfeldt said it is difficult to get labs to participate and pay hundreds of dollars for an additional survey when the ING survey serves their compliance needs. CAP is not planning to drop the ING survey in favor of an accuracy-based survey, at least for now. S.Narayanan said until C-peptide is standardized an accuracy-based survey does not make sense, as large numbers of labs will fail when compared to the reference values. J. Eckfeldt said the grading could be educational, the data would clearly show which assays were running high or low. R. Little said this has been done with HbA1c as the CAP criteria have been tightened. The labs have been provided an educational grade showing how they performed against the new criteria prior to the implementation of the new criteria. The purpose of the current C-peptide accuracy survey was to show how results look now, which assays are running high, low or close to the target value. As manufacturers start to recalibrate we should see the variability reduced on these surveys in the future. This is why we need accuracy-based surveys; it is the only way to monitor the quality of results in the field.

4) Insulin Analysis Update — Jesse Seegmiller

- Objective
 - Provide overview of approach to measuring markers using LC-MS/MS.
 - Discuss approaches taken.
 - Explain the current state of the assay.
- Formation of C-peptide: Preproinsulin is cleaved into proinsulin, which is then cleaved into C-peptide and insulin
- Why Study Using Mass Spectrometry?
 - Immunoassays analytically have been questionable.
 - Various insulin immunoassays have differing cross-reactivity with insulin analogs. Also may not obtain true insulin value to understand β cell function if someone is taking insulin analogues. A clinical trial we are involved with has glargine treatment branch.
- Insulin analogs include glargine, detemir, lispro, aspart and glulisine.
- Immunoassays: Insulin Analogs
 - Cross-Reactivity between vendors is quite variable for analogs.
 - Reagent lot differences.
- Immunoassays also show considerable variability among assays in patient samples.
- Challenges: Insulin Analysis by LC-MS/MS
 - Solubility: Isoelectric Point = 5.30-5.35 (1996 Merck Index)
 - Sensitivity
 - 1) Protein/peptide size of intact insulin 51 amino acids.
 - 2) Larger protein/peptides present more difficulty in fragmentation.
- Sample Preparation Approach
 - Sample preparation free of antibody based reagents is desirable. Difficult to standardize antibodies.
 - Reports of cation exchange preparation methods were attempted, but produced low recovery of insulin.
 - Current Approach: Mixed Mode Strong Anion Exchange (MAX) with reversed phase properties.
 - Insulin is negatively charged at high pH (≥ 9.0).
- Sensitivity Using this Approach
 - Using 0.5 mL of specimen.
 - Sensitivity with the non-antibody approach is ~ 50 pmol/L.
 - $\sim 10\%$ of the cohort is < 50 pmol/L at baseline.

- Need ~10 fold increase in sensitivity
- Investigated β Chain Analysis
 - Beta chain could offer 2-3x Sensitivity increase.
 - Even with this would not hit ~5-10 pmol/L.
 - This approach offers different challenges and opportunities
- Insulin: α and β Chain: ~20 fold more sensitivity for beta chain than alpha chain
- Glargine Metabolites
 - According to literature studies glargine as injected is not found in circulation when analyzed.
 - Glargine metabolites M1 (major) and M2 (minor).
 - M1 has the same β chain as insulin.
 - Thought was we could get an idea of the major by quantitating the β chain and α chain and subtracting the two.
 - This could be challenging and preliminary data suggest sensitivity would still not hit the desired levels.
- Assay Needs in Clinical Trial
 - Ultimate sensitivity needed: $\sim < 5\text{-}10$ pmol/L
 - Non-antibody SPE approach: ~ 50 pmol/L would miss $\sim 10\%$ of study
 - All sites performing insulin by mass spectrometry detection use an antibody preparation protocol. Some are proprietary; some are commercially available.
- Sample Prep Approach
 - Current:
 - 1) SPE – Mixed Mode
 - 2) Insulin / C-peptide
 - Next Step
 - 1) Immunoaffinity
 - 2) Insulin / analogs only
- Next Step
 - Immunoaffinity – MSIA system: This will allow for a much more pure sample extract, only insulin and analogs.
 - Mass Spectrometry Detection
 - 1) Larger protein/peptide MS/MS detection is an issue. Fragmentation efficiency decreases as >20 amino acids.
 - 2) MS/MS was needed with the less selective SPE approach.
 - 3) Will gain sensitivity running in MS only no fragmentation using accurate mass system.
 - 4) We expect $\sim 20\text{-}50$ fold increase in sensitivity.
 - 5) Process has a liquid handler and is semi-automated.
- Sample Limited
 - Specimen vials are to contain ~ 500 μL .
 - Historically we know many vials will not have this volume. < 500 μL
 - This will present a problem also.
 - With a more sensitive method we can attempt a lower sample load volume, say 400 μL .
- Nominal vs. Accurate Mass Spectrometers
 - Nominal mass allows for Unit resolution.
 - 1) E.g. distinguish m/z 50 from m/z 51 amu.
 - 2) m/z 1000 from m/z 1001 amu.
 - Accurate mass resolution $R = \text{mass}/\Delta\text{mass}$
 - 1) Example $R = 70,000$ at mass 200 amu
 - 2) $\Delta\text{mass} = 200/70,000 = 0.00286$ amu
 - 3) If $R = 140,000$: $\Delta\text{mass} = 0.00142$ amu
- Conclusions
 - Current non-antibody approach SPE protocol will not meet clinical trial sensitivity needs.
 - Next step will be to use a more insulin specific processing step.
 - Immunoaffinity purification is thought to provide a more specific insulin extraction.
 - Accurate mass system would allow for potentially better specificity in MS only analysis.
- Future Work
 - Focus on increasing measurement procedure sensitivity.

- Immunoaffinity
- Potentially use accurate mass
- Will likely increase sensitivity ~20 to 50 fold using MS only and no MS/MS fragmentation.
- Perform validation studies.
- Analyze Samples.
- Acknowledgements: David J. Schmit, Ph.D., University of Minnesota ARDL

Discussion:

New insulin standard commutability study

D. Holmes asked about calibrating for and quantitating the synthetic insulin analogs. J. Seegmiller said they are not going to quantitate the analogs. Glargine is the biggest problem, if we quantitated it we would not quantify the glargine metabolites but would do an external calibration to insulin. D. Holmes and J. Seegmiller noted that the MSIA system should work well for the immunoaffinity purification/extraction. D. Holmes asked R. Little how she felt about a reference method for standardization that's tied to a specific commercial immunopurification product. R. Little said it is better to be independent of specific commercial vendors if possible. K. Kabytaev noted that the C-peptide method is now listed with JCTLM, if we were to make significant changes to it the method might have to be re-submitted. D. Holmes and J. Seegmiller said that the advantage of immunopurification is the gain in sensitivity, it would probably be possible to match what Roche has achieved (5pmol/L). K. Kabytaev agreed that Roche has achieved greater sensitivity than what the MU method has achieved (~20 pmol/L) but wondered if that level of sensitivity is needed. J. Seegmiller said the lower sensitivity is needed mainly for clinical trials. R. Little said that sensitivity is something we need to consider and would like to improve on. However, at this point in time the main concern from a clinical need standpoint is to get the range of clinically relevant results to match.

5) Candidate Insulin Reference Preparation 11/212— Gwen Wark & Melanie Moore

- Collaborative study for candidate preparation 11/212
 - 19 laboratories in 11 countries in two phases
 - 1) Phase 1: value assign the candidate preparation
 - 2) Phase 2:
 - Provide confirmatory data on 11/212 immunoreactivity & suitability to be an IS for calibration of human insulin IA
 - To assess the relationships among the 1st IRR for insulin immunoassay 66/304, local standards and 11/212
 - To assess the commutability of 11/212 in insulin IA
 - To assess the stability of the candidate preparation, 11/212, after accelerated thermal degradation
 - Phase I study for 11/212
 - 1) Pure human insulin preparation
 - 2) Candidate preparation value assigned (SI units)
 - 3) Mass balance insulin content 9.19 mg/ampoule (9.14 – 9.24 mg/ampoule)
 - 4) Confirmatory data by HPLC and total nitrogen analysis
 - HPLC: 9.19 mg/ampoule (9.07 - 9.31 mg/ampoule)
 - Total nitrogen: 9.14 mg/ampoule (8.97 – 9.30 mg/ampoule)
 - Phase II study for 11/212
 - 1) Participants were requested to:
 - Prepare and measure the insulin concentration in a minimum of 7 core dilutions of 66/304 and 11/212 (covered a minimum of 5 dose points in the linear part of their standard curve).
 - 1) Core dilutions were 288, 144, 72, 36, 18, 9 and 4.5 µIU/ml.
 - 2) Initial concentration 576 µIU/ml.
 - Measure, in duplicate or triplicate, the insulin concentration of these dilutions and the 14 serum & 5 plasma samples using the immunoassay(s) normally in use in their lab.
 - 3 independent assays, using fresh ampoules and including all the preparations allocated in each assay.
 - 2) Insulin methods included in the study (15 labs):
 - Abbott Architect & Abbott Alinity I
 - Beckman Access 2 & Beckman Dxl

- Diasorin Liaison & Diasorin Liaison XL
 - Invitron ELISA
 - Roche Elecsys
 - Mercodia ELISA
 - Siemens ADVIA Centaur
 - Ortho Clinical Diagnostics VITROS
 - TOSOH AIA and TOSOH AIA-CL
- 3) Phase II study for 11/212: Immunoreactivity
- Intra-assay variability was acceptable for measurement of both 66/304 and 11/212 with reported CV% values of laboratory estimates < 5% in the majority of assays.
 - Both standards appear to behave in a similar manner in each assay.
 - After taking into account the dilution factor from ampoule reconstitution, laboratory geometric mean estimates are equivalent to 3.18 IU/amp for 66/304 and 267.1 IU/amp for 11/212.
 - Values are in very good agreement with the assigned 3 IU/amp for 66/304 and 9.19 mg/amp for 11/212, which is equivalent to 264.8 IU/amp after application of the activity conversion factor for pure insulin, 1 IU = 0.0347 mg insulin.
- 4) Phase II study for 11/212: Commutability
- 5 plasma & 14 serum patient samples
 - Commutability assessed using a difference in bias approach.
 - 14 labs demonstrated a constant patient sample bias:
 - 1) 11/212 fully commutable with patient samples in 8 labs
 - 2) 6 labs where 11/212 was non-commutable
 - 2 of these used the same methods in which the candidate standard was shown to be commutable by other laboratories.
 - In a further 2 methods the candidate standard had a comparable response to the 1st IRP 66/304.
 - Remaining 2 laboratories (the same method on different platforms) were outside the statistically-defined limits of commutability used in this study.
- 5) Data from HPLC and IA estimates of accelerated thermal degradation samples indicate that the candidate standard is sufficiently stable when stored at -20°C to serve as an IS.
- 6) Therefore it is proposed that the candidate preparation in ampoules coded 11/212 is established as the 1st International Standard for insulin, human, with an assigned content of 9.19 mg/ampoule (expanded uncertainty of 9.14 – 9.24 mg/ampoule; k=2).
- 7) This value can be converted into IU using the internationally recognised specific activity of pure insulin (1IU=0.0347 mg).
- Future steps for 11/212
- 1) Assess suitability of candidate preparation for calibration of secondary reference materials.
 - 2) Opportunity to use the candidate preparation in the calibration of higher order reference methods.

Discussion:

R. Little asked if any of the new standard material has been provided to labs running MS; G. Wark said no but they plan to do so.

R. Little thanked everyone present for their attendance, the meeting was adjourned at 10:05 AM.

Minutes prepared by Curt Rohlfing 11/4/2019. Modified by R. Little 11/5/2019.