2018 C-Peptide Standardization Manufacturer Meeting
Minutes

Wednesday August 1 8:00 AM – 10:00 AM
Marriott Marquis Chicago, Chicago, IL

Participants:

C-peptide Standardization Committee Members
Randie Little—University of Missouri
W. Greg Miller—Virginia Commonweath University
Daniel Stein—Albert Einstein College of Medicine

Committee members not present
Judith Fradkin—NIDDK
Carla Greenbaum—Benaroya Research Institute
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
Carissa Jones--Mercodia
Stefaan Marivoet—Tosoh Bioscience
Shanti Narayanan—Tosoh Bioscience
Chisato Okamura—Fujirebio
Maria-Magdalena Patru—Ortho Clin Diagnostics
Hanna Ritzen—Mercodia
Chris Wisherd—Alpco Diagnostics
Paul Wynveen—Beckman

Guests
Valerie Arends—University of Minnesota
Shawn Connolly—University of Missouri
Daniel Holmes—St. Paul's Hospital, Vancouver
Kuanysh Kabytaev—University of Missouri
Santica Marcovina—University of Washington
Curt Rohlfing—University of Missouri
Violeta Raneva—ReCCS, Japan
Amy Saenger—University of Minnesota
Jesse Seegmiller—University of Minnesota
Michael Steffes—University of Minnesota
Hirohito Umemoto—ReCCS, Japan
Gwen Wark—UKNEQAS/IFCC

By Phone:
Beena Akolkar NIDDK

1) **Welcome and Introduction—Randie Little**
   R. Little welcomed those in attendance, the 2017 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.
     - T1D 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 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
     - Immune response
       1) Cellular (T-Cell) Autoimmunity
       2) Humoral Autoantibodies ((ICA, IAA, Anti-GAD65, IA2Ab, etc.)
     - Lose ~90% of beta cells before hyperglycemia begins
     - Eventually lose almost all β cell function
   - 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): The two are not equivalent
  - 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
  - T1D (56), T2D (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 was proportional to 90 min MMTT (R= 0.97)
    3) Fasting sCP ≥ 0.07 nmol/L (.21 ng/ml): 100% sensitivity, 97% specificity for significant endogenous 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, insulin positive beta cells are virtually always present. (Keenan et al., Diabetes 2010)
  - Number of insulin positive 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% had C-peptide ≥0.3 ng/ml (100pmol/L)
    2) NO difference in the prevalence of complications > 0.3 ng/ml vs < 0.3 ng/ml
- Oram et. al. Most People With Long-Duration Type 1 Diabetes in a Large Population-Based Study Are Insulin Microsecretors (Diabetes Care, Volume 38: 323-328 February, 2015)
  - Study Design and Methods
    1) 924 patients were recruited from primary and secondary care in two U.K. centers
    2) Patients had a clinical diagnosis of T1D, were under 30 years of age when they received a diagnosis, and had a diabetes duration of >5 years
    3) Median age at diagnosis was 11 years (6-17)
    4) Duration of diabetes was 19 years (11-27)
    5) All provided a home postmeal UCPCR, measured using a Roche electrochemiluminescence assay
  - Results
    1) 80% of patients had detectable endogenous C-peptide levels
    2) Most patients had historically very low undetectable levels
    3) 8% of patients had a UCPCR ≥0.2 nmol/mmol, equivalent to serum levels associated with reduced complications and hypoglycemia
    4) Absolute UCPCR levels fell with duration of disease
    5) Age at diagnosis and duration of disease were independent predictors of C-peptide level in multivariate modeling
- 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
- Long-term reduction in hyperglycemia in advanced type 1 diabetes: the value of induced aerobic glycolysis with BCG vaccinations. *NPJ Vaccines. 2018 Jun 21;3:23*. Kuhtreiber et al.
  - Randomized 8-yr prospective study T1DM received 2 doses of the BCG vaccine
  - Well controlled subjects (HbA1c ~7%)
  - Averaged 18% reduction in HbA1c
  - C-peptide levels measured with an ultrasensitive assay rose from ~2 to ~2.5 pmol/L
  - The BCG impact on blood sugars appeared to be driven by a novel systemic and blood sugar lowering mechanism in diabetes
  - BCG via epigenetics also resets six central T-regulatory genes for genetic re-programming
  - Metabolic changes: Systemic shift in glucose metabolism from oxidative-phosphorylation to aerobic glycolysis, a state of high glucose utilization.
  - Conclusion: These findings set the stage for further testing of a known safe vaccine therapy for improved blood sugar control through changes in metabolism and durability with epigenetic changes even in advanced Type 1 diabetes
- Insulin sensitivity and resistance in-vivo
  - Hyperinsulinemic euglycemic insulin clamp is the gold standard

- Various other models are used to estimate insulin sensitivity (1/Fasting Insulin, HOMA, QUICKI, others)

- Insulin Concentration in Vials Randomly Purchased in Pharmacies in the United States: Considerable Loss in the Cold Supply Chain. J Diab Sci Technol 2018 Jul;12(4):839-841. Carter, Heinemann.
  - Eighteen 10 ml vials from two manufacturers (M1 and M2) were randomly acquired.
  - Used an advanced triple-quadrupole MS TOF method
  - The intact insulin concentration ranged from 13.9 to 94.2 U/ml, mean 40.2 U/ml. No vial met the minimum standard of 95 U/ml.
  - Very controversial, has caused much concern.
  - Concentrations of Intact Insulin Concurs With FDA and EMA Standards When Measured by HPLC in Different Parts of the Distribution Cold Chain. J Diab Sci Technol 2018 Jun 1:1932296818783783. Moses et al. (Published in response to Carter and Heinemann). The data demonstrated that without exception:
    1) Insulin quality based on stability data was maintained, even in scenarios that stressed the normal recommendations for temperature storage conditions,
    2) Insulin content from the last three years of samples returned to Novo Nordisk from patients in the United States (233 vials) was within USP requirements recognized by FDA
    3) Ten years of independent EMA sampling of products obtained at wholesalers and pharmacies across the EU confirmed compliance (n = 43).
  - There is a current grant proposal to further investigate by measuring insulin in vials using various methods.

- 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?
  - Is the cold supply chain for pharmaceutical insulin reliable?
  - Well designed outcomes of prospective trials of beta cell function using standardized testing procedures, and adjusting for multiple clinical and demographic variables are necessary.

Discussion:

Clinical studies
MM Patru asked if the subjects in the BCG study were adults or children. D. Stein responded that they were adults that were children at onset. D. Holmes noted that the Carter/Heinemann insulin results could be an issue with the method, e.g. when making insulin calibrators the insulin sticks to everything. D. Stein said the method was not well-described in the paper, for one thing it referenced a paper where the method was performed differently. Also, they did not implement controls such as measuring insulin in vials obtained directly from the manufacturers. In this case the scientific review process seems to have been inadequate; S. Marcovina agreed. D. Holmes noted that these studies have been performed many times by manufacturers to assess cross-reactivity in their immunoassays. If the levels were really as bad as what is stated in the paper, manufacturers would have noticed this. D. Stein says the lesson of this is that those of us that are dedicated to measuring these analytes properly need to have our validation processes down, make sure we have proper controls in place and have our standards well-defined.

Reference ranges and cutoffs
MM Patru asked about reference ranges in children, what are the reference ranges for C-peptide, insulin and even HbA1c? There are published data but they are very scarce, and there is some variability among the studies. D. Stein said that the same diagnostic cutoffs are generally used for children as in adults, it is important to note that children who present with diabetes generally show other classic symptoms as well, it is not subtle. Where insulin is concerned we use it to measure insulin resistance, not to diagnose diabetes, mainly because of the variability of insulin clearance in the liver. C-peptide is a much better indicator of beta cell function; insulin should not be used to diagnose diabetes. D. Holmes said they have a big problem with wellness clinics using insulin to assess insulin resistance in the ambulatory population that have no symptoms, and they are not even fasting. D. Stein noted that insulin reference ranges vary widely since it is currently not standardized. R. Little said the TEDDY study is following children at risk for T1 diabetes. They have found that when they get to the point where they are positive

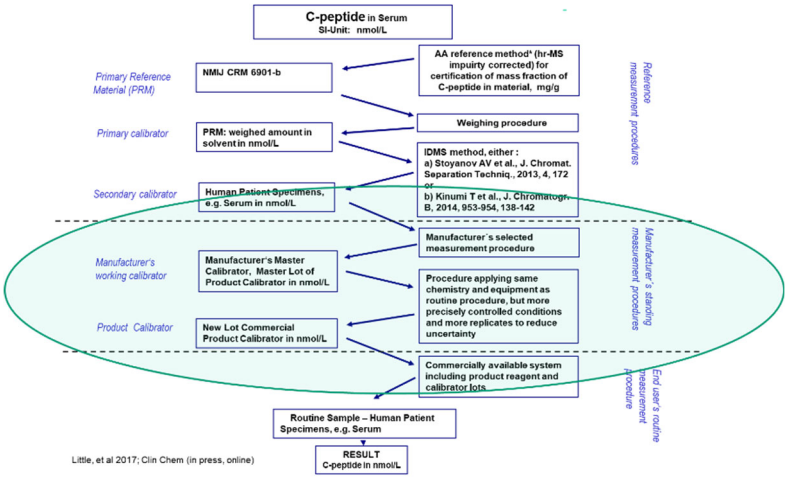
for two or more antibodies they can use a cutoff for HbA1c that allows them to identify subjects that have developed T1D before they experience DKA. This is where HbA1c can fit into the early detection of T1D. Also, reference ranges are not always the same as clinical cutoffs. MM Patru said the manufacturers are often asked about insulin reference ranges in children because the clinicians are requesting this information. Insulin testing volumes are much higher than for C-peptide in both adults and children; S. Naraynan agreed. D. Stein asked if they are using insulin to diagnose diabetes; MM Patru and S. Naraynan said they do not know how the results are being used, but it is requested more than C-peptide. D. Stein suspected that the insulin is being used to try to get an idea of the degree of insulin resistance. Clinically with overweight/obese T2 patients with diabetes, we would measure C-peptide and look at tryglycerides and HDL to determine whether to treat with insulin. The problem with insulin is that we do not have good validation across the spectrum of assays and we do not have cutpoints.

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.
- Determined that there was a need for standardization of C-peptide due to lack of comparability of results among assay methods
- C-peptide Standardization: Comparison Studies
  - WHO material was ineffective in improving the comparability of C-peptide results among methods/laboratories.
  - Use of pooled serum calibrators greatly reduced variability of results.
  - Showed that pooled serum calibrators with LC-MS assigned values can be used for method re-calibration by the manufacturer.
  - Between-lab/manufacturer CVs were much lower after calibration using pooled serum calibrators.
- C-peptide Reference Method/Laboratory Comparison
  - We began working with D. Stein's laboratory which had developed a C-peptide reference method.
  - In order for manufacturers to re-calibrate their C-peptide assays to the reference method we were told that we must have the reference method listed with the JCTLM. This required a comparison between two reference laboratories.
  - The reference method at D. Stein's lab in New York was also set up in our lab at the University of Missouri.
  - We published a comparison between the two laboratories in 2012.
  - 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 continued excellent correlation (r^2=0.9921) and comparability of results.
- C-peptide Reference Material: NMIJ CRM (CRM 6901-b)
  - Produced by the National Metrology Institute of Japan
  - 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
  - Evaluation
    1) Serum with zero C-peptide was spiked with native NMIJ reference material and analyzed by LC/MS as a routine sample.
    2) Results closely matched the theoretical values.
- NMIJ also published a reference method in the JCTLM that is different from the D. Stein/DDL method.
  - Comparison between the reference methods showed that results from the NMIJ method were ~25% lower compared to D. Stein/DDL method.
    1) NMIJ used a different sample prep
    2) NMIJ uses a derivatization procedure
    3) NMIJ and DDL use different internal standard (IS)
  - Another set of samples (12 single-donor, 9 pooled) were sent to NMIJ along with the D. Stein IS.
  - The second comparison showed that when both labs used the D. Stein IS, results from serum samples were equivalent between the labs.
  - Concluded that there was an issue with the NMIJ IS.

- We now have a new more sensitive MS at the DDL (QTRAP 6500+) and have validated that the results are equivalent to those from our previous MS (API-4000) and the NMIJ method.

- Recent publication: Implementing a Reference Measurement System for C-Peptide: Successes and Lessons Learned. Clin Chem 63:9, 1447-1456 (2017).
  - Coauthored with members of JCTLM and other international participants in the standardization efforts.
  - Describes the problems and roadblocks encountered in the process of trying to implement standardization, including lack of communication among the groups involved.
  - Accompanying editorial by GL Myers and WG Miller (Clin Chem 63:9, 1429-1430 (2017).
- After we resolved the discrepancy between the two reference labs, we published a letter to the editor in Clin Chem (63:12, 2017) at the suggestion of WG Miller.
- Proposal for Standardization of C-peptide
  - Primary Reference Material: NMIJ CRM 6901-b
  - LC/MS method (Stoyanov, et al, Kinumi, et al)
  - Secondary Reference Material: pooled and single-donor serum.
  - Traceability chain
- Recent Studies: Internal Standards (K. Kabytaev)
  - Available isotope-labeled C-peptide Internal Standards
    1) We started out using a commercial standard made by Bachem
    2) We subsequently used the D. Stein IS, we have now also synthesized our own IS.
    3) We have also evaluated a commercial standard made by Sigma
  - Sample comparisons show that the D. Stein, DDL and Sigma internal standards produce comparable results in samples.
- Implementation of C-peptide Standardization (R. Little)
  - Traceability Chain



  - The upper portion of the traceability chain is now complete, the manufacturer portion needs to be addressed.
  - Manufacturers have a letter from the ADA stating the importance of C-peptide standardization.
  - All manufacturers have received the data showing the relationship between their current results and those of the reference method.
  - Some methods will require small adjustments, others will need to be larger.
  - Once we have standardized results throughout the reported range, we can focus more on results in the very low range.
  - 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.
    4) We have a large supply of these samples.
    5) We will be collecting more samples in the future.
  - What kind of samples would you prefer in the future?

1) Desired levels
2) Desired volume
3) Pooled or single-donor?

Discussion:

C-peptide Reference Method

S. Marcovina noted that the results from the two reference labs are very close at the low end of the C-peptide range but there is more scatter at the upper end, do we know why? R. Little said we do not know, it is possible that if another comparison is done it will look different, in any case we are mainly interested in the lower part of the range. D. Holmes asked about the initial discrepancy between the two reference labs, do we know what the issue was with the NMIJ internal standard? If the IS is not added in concentrations relevant to what is being measured in samples, this can result in discrepancies, you want it to be about in the middle of the range. K. Kabytaev said that based on communications with the lab in Japan, there was a co-eluting interference in their standard, not an issue of the concentration of the standard. J. Seegmiller noted that if the interference was something in the samples, we would expect to see a more scattered relationship, this appears to be a bias due to calibration. K. Kabytaev gave the example that a water adduct will add +18 daltons, if the standard is +16 this can result in interference.

Standardization

R. Little noted that some manufacturers are in the process of re-calibration, looking at the language to be used with customers. Some customers are requesting calibration to WHO; it is time for manufacturers to educate their customers regarding the new reference system for standardization. It would not be good to provide customers with both the old WHO and new calibrations, this is bound to cause confusion. Manufacturers can also help educate clinicians regarding the use of C-peptide and insulin. We can help by providing CAP data throughout the process to assess the impact of the standardization, we can also collect more samples, do further comparisons, etc. WG Miller said there seems to be a barrier in terms of the WHO traceability. One strategy to overcome this would be to publish a paper describing the relationship between the WHO traceability scheme and the new one, clearly pointing out the superiority of the latter. Then customers will ask for it. R. Little said that there is some discussion of this in the recent paper. WG. Miller suggested putting together materials that manufacturers can use in their marketing materials that would be aimed at the customers. R. Little said one way to address this would be through the CAP survey reports. WG Miller agreed but noted that many customers do not read materials included in the CAP reports. S Marivoet asked if the WHO material was ever measured by the reference method, R. Little said no. S. Marivoet suggested doing so, then manufacturers could participate in a project where samples are analyzed and calibrated to the WHO and new systems. They could then make this data available to customers and the national bodies to show them how the new system is better. Right now many customers do not understand the importance of standardization, they just want to be able to produce numbers. In terms of regulation the U.S. just has the FDA, in Europe the situation is more complex. H. Ritzen agreed and added that customers do not understand the issue of commutability. It would help if we could work with WHO to address the issue of commutability for their materials. WG Miller said WHO has made a commitment to try to address the commutability of their materials, but the materials are made in 10-15 year cycles, we cannot wait that long. R. Little said WHO has sent out their new materials to manufacturers to assess commutability, the new C-peptide material may be better but it is still not serum and there is still an issue with commutability. They are calling the material a standard, which makes it even more difficult to get past the problem of insistence on WHO traceability. WG Miller said it is an educational problem, the technology is there to achieve standardization but we need to educate customers to understand that standardization is in the best interests of the patients. MM Patru said that educating the customers is not impossible, it would be helpful to have a publications showing that the WHO standard is not commutable, is this available? R. Little said it is discussed in the recent paper. S. Narayanan asked if the resulting shifts in reference ranges and cutoffs that will occur due to the standardization have been considered. If there are papers published establishing cutoffs, e.g. at 90 minutes, and the data are aligned with the reference method it then makes sense for methods to align to the reference so that clinicians can use these cutoffs. R. Little asked if manufacturers are required to provide reference ranges and cutoffs, MM Patru said they provide reference ranges but do not currently provide cutoffs. D. Stein said that the cutoffs have been based on data from Trialnet, S. Marcovina's laboratory (NWLRML) runs the C-peptide for those studies. The cutoffs for these studies could be adjusted based on a correction factor. R. Little noted that the DDL and NWLRML do regular comparisons, both run the Tosoh assay, so we know how those results relate to the reference method. D. Stein suggested talking to the people at Trialnet, perhaps we could publish an update. S. Narayanan said that would be helpful, some of these trials are going on for many years, changing reference ranges and cutoffs in the middle of these studies can impact the studies. S. Marcovina agreed. R. Little said for long trials

you would not need to change the numbers in the middle of the trial, the issue can be addressed as long as you know the relationship between the numbers. MM Patru asked about FDA, R. Little said they are aware of the standardization efforts. S. Narayanan stated that C-peptide is an exempt assay, so manufacturers do not have to submit a new 510K when re-calibrating to standardize their assays. You have retain the data and categorize the assay for CLIA, a letter needs to be submitted but no data needs to be submitted to FDA. R. Little was asked about the plan for maintaining the standardization, she noted that the plan is to maintain the availability of materials with reference-assigned values. We can send them to manufacturers as needed, and even send them to customers if that is requested.

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

- 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
- 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
K. Van Uytfanghe	Member	BE
G. Wark	Member - IFCC	UK
B. Akolkar	Consultant – NIH/NIDDK	US
V. Arends	Coordinator – UMN	US

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

- IFCC Working Group on Standardization of Insulin Assays (SWG-IA): Corresponding Members

Name	Full and Affiliate Member Societies
Paul Glendenning	Australasian Association of Clinical Biochemists
Syuu Meguro	Japan Society of Clinical Chemistry (JSCC)
Andrejus Coj	Lithuanian Society of Laboratory Medicine
Fayeofori Abbiyesuku	Association of Clinical Chemists Nigeria (ACCN)
Oguzhan Zengi	Turkish Biochemical Society (TBS)
Gary Myers	American Association for Clinical Chemistry (AACC)
Name	**Corporate**
Tanja Dubravcic	Siemens

- C-peptide traceability scheme described by R. Little applies to insulin as well.
- Update on New WHO/NIBSC Insulin Standard
  - WHO 1st IRP 66/304
    1) Human pancreatic insulin in sucrose and dilute acetic acid (lyophilized)
    2) Not suitable for calibration traceability
    3) International Standard of Insulin (24,000 units/g) from 1959. A molecular weight of insulin (5,808 Da) gives 1 unit (1/24,000)/5,8087.174 nmol or 1 U/ml 7.174 pmol/l.
  - Current NIBSC/WHO international standard
    1) Established in the mid-1980's
    2) Potency defined in IU/mg
    3) Human standard: coded 83/500; potency of 26,000 units/g
    4) Correct conversion factor is 1 U/mL = 6.00 pmol/L
  - Value assignment to current insulin standard based on a multi-method collaborative study by in vivo bioassay
  - Need for an updated standard(s) to reflect transition of insulin internationally to a well-characterized, mass-balance assigned molecule
  - New WHO/NIBSC International Standard for Human Insulin (11/212)
    1) 1st International Standard for biosynthetic human insulin
    2) NIBSC code 11/212
    3) Candidate standard consists of highly purified recombinant human insulin, dissolved in ddH2O and acidified with 0.2M HCl to dissolve
    4) Acidified solution was neutralized to a concentration of 10 mg/g

5) Solution dispensed into glass ampoules, lyophilized and sealed

6) Study Protocol – 3 Phases

- Phase 1: Assignment of insulin content to candidate standard (Completed)
  a) Assess suitability to serve as a reference material for insulin immunoassays and therapeutic preparations of biosynthetic insulin
  b) Laboratories used a mass balance approach to determine insulin (plus A21 desamido insulin) content of the bulk active pharmaceutical ingredient
  c) Candidate standard: 9.19 mg/ampoule (uncertainty: 9.14 – 9.24 mg/ampoule)
- Phase 2a: Assess suitability of candidate standard to serve as an International Standard for calibration of diagnostic immunoassays (Protocol in review)
  a) Specific Aims:
  b) Demonstrate suitability of preparation (11/212) to serve as an international standard for human insulin immunoassays by evaluating its "behavior" in currently available immunoassays
  c) Assess relationship between the 1st insulin IRP (66/304) and proposed international standard (11/212) in insulin immunoassays
  d) Evaluate the likely commutability of proposed 1st international standard (11/212) using immunoassays
  e) Demonstrate stability of candidate preparation (11/212) by measuring activity of accelerated thermal degradation samples of candidate standard using immunoassays
  f) Reconstitution/dilution of 66/304 and 11/212 – likely 7 concentrations across the linear range
  g) 15 Serum / 5 Plasma specimens provided to laboratories using various immunoassay platforms
  h) Accelerated thermal degradation: ampoules of candidate international standard incubated at +4∘C, +20 ∘ C, +37 ∘ C, +45 ∘ C for 77 months

7) New WHO/NIBSC International Standard for Human Insulin (11/212): Future Steps

- Phase 2b: Assess suitability of candidate standard to serve as an International Standard for calibration of secondary reference preparations used to assign potency of therapeutic preparations of insulin
- Opportunity to utilize new insulin reference material in calibration of higher order reference method procedures (i.e. LC-MS/MS)
- RMP can be used to confirm/assign values to aliquots in insulin serum biobank

Discussion:

New insulin standard commutability study

R. Little noted that the previous WHO standard showed lack of commutability, why would we expect the new standard to be commutable since it is also a purified material? A. Saenger did not expect it to be commutable. G. Wark noted the evaluation of commutability is just a standard part of the protocol. WG Miller said that the most important thing about the new standard is that it can be used to calibrate the reference method, which in turn can assign values to the existing serum samples which are commutable. It would be good if some MS assays were also included as part of the commutability experiment. G. Wark said she has been talking to NIBSC about doing this and will continue to do so. WG Miller added that it would be good if NIBSC would provide something in their documentation to indicate that the material is not to be used to calibrate routine assays.

5) Update: LC-MS/MS Method Development for Human Insulin— Jesse Seegmiller

- Challenges: Intact Insulin Analysis by LC-MS/MS
- Solubility: Isoelectric Point = 5.30-5.35 (1996 Merck Index)
- Sensitivity
  - Protein/peptide size of intact insulin 51 amino acids.
  - Larger protein/peptides present more difficulty in fragmentation
- Addressing Insulin Solubility: Studies and products suggest low pH is important for both storage and solubility.
  - This appears to be the case for insulin and some analogs.

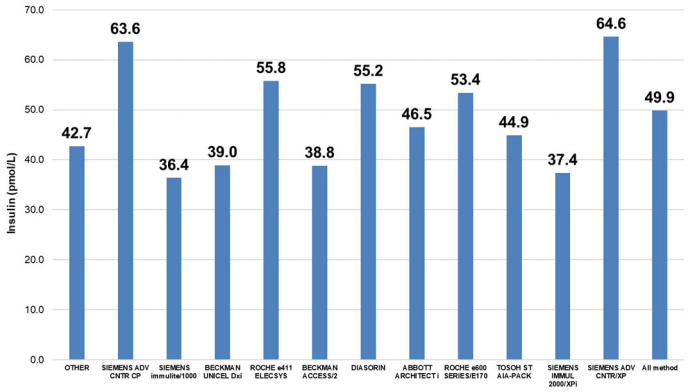
    - Insulin stock solutions made in acidic solutions.
- Sample Preparation Approach
    - Sample preparation free of antibody based reagents is desirable. Difficult to standardize antibodies.
    - Reports of cation exchange preparation methods were attempted, but produced low recovery of insulin.
    - Current Approach: Mixed Mode Strong Anion Exchange (MAX) with reversed phase properties.
    - Insulin is negatively charged at high pH (≥9.0).
    - Negatively charged insulin attracted to positive charge on the solid phase.
- Current Approach to Sample Preparation
    - Protein precipitation using acidic media
    - Vortex, Centrifuge 10 min
    - Elevate extract pH (ammonium hydroxide)
    - Perform solid phase extraction
    - Presently in a MAX plate format
    - Transfer to autosampler vial.
    - Perform injection
- Current LC-MS/MS MRM Transitions Selected for Intact Insulin
    - ESI Positive Mode
    - Multiple Reaction Monitoring (MRM)
        1) 1162.6 – 226.2 Da (Insulin +5H)
        2) 968.8 – 226.2 Da (Insulin +6H)
        3) 1170.2 – 226.2 Da (Insulin +5H Int Std)
        4) 975.3 – 226.2 Da (Insulin +6H Int Std)
    - Insulin Internal Standard: 4-[D10]Leu-Insulin: Provides a 40 Da increased mass difference from human insulin.
- Calibration Curve Example
    - 8 Calibration Levels Used (pmol/L): 8.6, 17.3, 86.3, 172.5, 345.1, 862.7, 1294.1, 1725.4
    - Calibrators made in delipidized serum (Golden West).
- Preliminary quantitative results suggest promising analytical performance for human insulin. Twelve pmol/L (LoQ, 20%CV) is the analytical sensitivity target.
- Conclusions
    - Low pH is important for solubility/stability.
    - Have used Low protein binding materials: Pipet Tips, Tubes, Plates
    - MAX strong anion exchange for sample clean-up and concentration.
    - Preliminary results suggest protocol sensitivity is near the need for a potential measurement procedure.
- Future Work
    - Focus on increasing measurement procedure sensitivity. Further refinement of extraction protocol.
    - Explore 2D-Chromatographic approaches.
        1) Grant funding dependent.
        2) May lead to a less manual sample preparation process.
    - Perform validation studies.
    - Analyze Samples/Pools

Discussion:

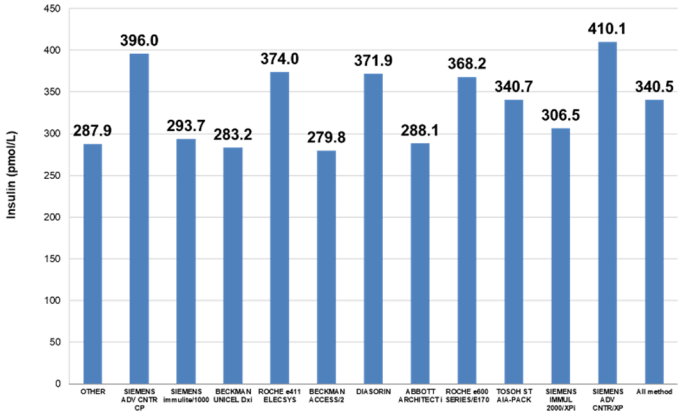
Univ. of MN Insulin Reference Method
D. Holmes asked about when the IS is added and if the method has been tried in patient samples since Golden West serum is clean. J. Seegmiller said at the beginning of the prep process, and they have analyzed patient samples. D. Holmes noted that Quest is routinely operating their MS method and Mayo is working on a method, their approaches use an antibody. K. Kabytaev said the University of Missouri C-peptide prep method is similar to the Univ. of MN insulin but employs ion-exchange. G. Wark and D. Holmes expressed concerns that bias could be introduced depending upon the calibrator matrix, e.g. Golden West serum could induce bias. J. Seegmiller acknowledged that this can happen. D. Stein said that for the NY reference method they used an albumin matrix and utilized a 2-D chromatographic approach to get a cleaner background. A complex matrix is good but in the end you need a clean background. D. Holmes noted that their lab has seen insulin in a serum matrix disappear in a few hours, but they had to use a neutral pH. J. Seegmiller said they have not seen this issue in their lab under refrigerated storage.

6) Update on Timeline for CAP/Proficiency Testing Implementation of Serum Pools— Amy Saenger

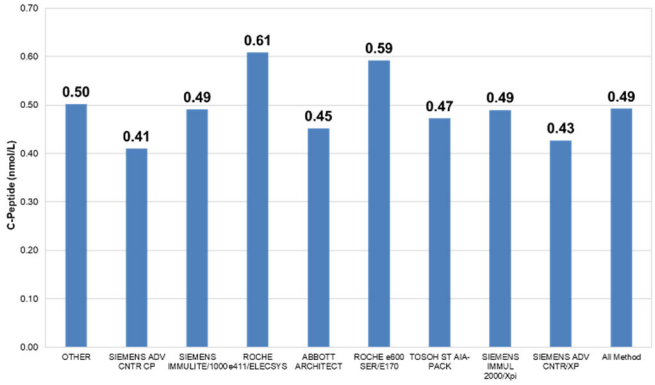
- CAP Proficiency Testing / Wild Card Pools
  - Insulin, C-peptide
  - Consent, collection, processing, shipping, storage conducted at the University of Minnesota
  - Donors were fasting; specimens collected when donors were in fasting and non-fasting state (post-glucose load)
- Results: Insulin Serum Pool (Low)



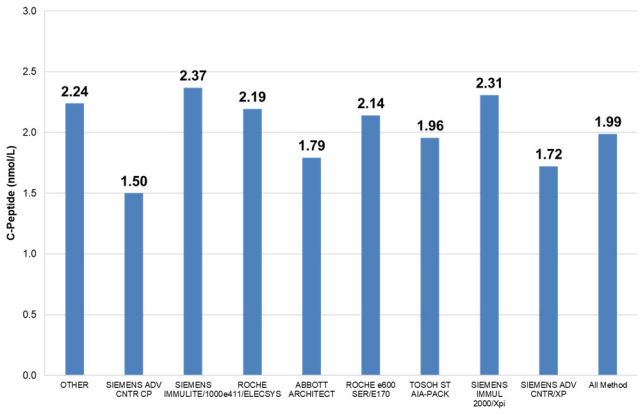
- Results: Insulin Serum Pool (High)



- Results: C-peptide Serum Pool (Low



- Results: C-peptide Serum Pool (High)



- CAP Proficiency Testing: Insulin

Method	Survey Specimen Method CV (%)				
	INGW-98 (Low Serum Pool)	INGW-99 (High Serum Pool)	Y-04	Y-05	Y-06
OTHER	55.5	52.8	35.3	25.5	34.4
SIEMENS ADV CNTR CP	0.0	0	3.0	6.2	2.4
SIEMENS Immulite/1000	6.2	5.4	9.9	9.1	9.1
BECKMAN UNICEL Dxi	6.2	5.6	6.4	6.5	6.1
ROCHE e411 ELECSYS	7.1	5.3	8.2	7.7	6.4
BECKMAN ACCESS/2	4.1	4.3	5.7	5.2	4.9
DIASORIN	14.6	11.5	6.0	5.4	5.9
ABBOTT ARCHITECT i	6.0	3.6	4.5	2.9	3.4
ROCHE e600 SERIES/E170	4.6	3.9	4.8	4.9	7.3
TOSOH ST AIA-PACK	4.4	2.5	3.0	1.9	2.9
SIEMENS IMMUL 2000/XPi	9.2	5.5	18.8	25.9	22.4
SIEMENS ADV CNTR/XP	5.7	5.2	7.3	6.6	6.4
All Method %CV	**22.4**	**16.9**	**27.1**	**31.3**	**27.1**

- CAP Proficiency Testing: C-peptide

Method	Survey Specimen Method CV (%)				
	INGW-98 (Low Serum Pool)	INGW-99 (High Serum Pool)	Y-04	Y-05	Y-06
OTHER	29.0	15.1	19.2	13.5	16.5
SIEMENS ADV CNTR CP	0.0	0.0	0.0	0.0	0.0
SIEMENS Immulite/1000	10.2	9.6	6.1	4.5	2.9
ROCHE e411 ELECSYS	7.2	6.2	4.0	4.7	4.4
ABBOTT ARCHITECT i	8.3	7.7	4.7	4.0	4.3
ROCHE e600 SERIES/E170	4.4	4.9	3.3	3.2	3.2
TOSOH ST AIA-PACK	13.4	10.5	57.2	67.7	50.1
SIEMENS IMMUL 2000/XPi	6.1	4.8	5.2	5.6	4.9
SIEMENS ADV CNTR/XP	8.3	7.5	7.6	6.4	6.7
All Method %CV	**15.6**	**14.0**	**17.2**	**15.3**	**14.6**

- CAP Proficiency Testing / Serum Pools
  - Insulin/C-peptide will be included in CAP surveys on a continuous basis beginning Q1 2019
  - Low and high serum pool
  - Contract pending between CAP/UMN
  - Need confirmation of shipping/handling logistics
  - Reference method required if accuracy based

Discussion:

CAP Survey Materials

R. Little said the main concern for the CAP serum pools is shipping and handling. A. Saenger agreed, they make and freeze the pools at the University of Minnesota but they need to check on the distributor, they last time the samples had stability issues after they left the UM lab. R. Little said we need to know the specifics of how they will be shipped, dry ice would be ideal, also she will again ask manufacturers about the sample stability for their assays. A. Saenger agreed and said that there needs to be a place on the receipt form requesting the temperature upon receipt of the samples. S. Marcovina noted that stability is a significant issue, degradation can result in higher CVs compared to lyophilized materials, and labs do not always test samples immediately upon receipt which can result in

more degradation. S. Narayanan said that unspiked serum pools are better than spiked samples, e.g. the current Tosoh C-peptide only measures intact C-peptide and does not measure the form used to spike the CAP samples. R. Little said CAP will be a critical part of the standardization process.

Standardization Process
R. Little said we need feedback from manufacturers regarding the recalibration process, our materials and any other areas of concern. We did receive a question from a Japanese manufacturer asking if we have or could approach the Japanese societies (e.g. JCCLS, JSCC). We have had contact but we are not sure if they have had any communication with manufacturers. S. Narayanan said that in Japan customers look to their associations, it is important to have them on board but communication with them has been problematic. R. Little said she has received initial feedback that was somewhat vague, but she will dig deeper. S. Narayanan asked R. Little to notify the manufacturers if she gets an e-mail response from Japan, that would be helpful to them.

R. Little thanked everyone present for their attendance, the meeting was adjourned at 10:12 AM.

Minutes prepared by Curt Rohlfing 8/16/2018. Modified by R. Little 817/2018.