Online-Only Appendix

Randomized Comparisons of the Mixed Meal Tolerance Test versus the Glucagon Stimulation Test for the Assessment of Beta Cell Function in Type 1 Diabetes

Carla J Greenbaum MD, Thomas Mandrup-Poulsen MD PhD, Paula Friedenberg McGee MS, Tadej Battelino MD PhD, Burkhard Haastert PhD, Johnny Ludvigsson MD PhD, Paolo Pozzilli MD PhD, John M. Lachin ScD, Hubert Kolb, MD PhD, and The Type 1 Diabetes TrialNet Research Group and The European C-peptide Trial Study Group.

Participating Investigators

Type 1 Diabetes TrialNet:

Clinical Investigators:
- Dorothy Becker MD, Children’s Hospital of Pittsburgh, Pittsburgh, PA
- Penelope Bingley MD, University of Bristol, Bristol, UK
- Emanuele Bosi MD, San Raffaele Hospital, Milan, IT
- Bruce Buckingham MD, Stanford University, San Francisco, CA
- H. Peter Chase MD, Barbara Davis Center for Childhood Diabetes, Denver, CO
- Peter Colman MD, Walter and Eliza Hall Institute, Melbourne, AU
- Mark Daniels MD, Children’s Hospital of Orange County, CA
- Stephen Gitelman MD, University of California San Francisco, San Francisco, CA
- Robin Goland MD, Columbia University, NYC, NY
- Peter Gottlieb MD, Barbara Davis Center for Childhood Diabetes, Denver, CO
- Leonard Harrison MBBS, MD, DSc; Walter and Eliza Hall Institute/Royal Melbourne Hospital, Melbourne, AU
- Kevan Herold MD, Yale University School of Medicine, New Haven, CT
- Francine Kaufman MD, Children’s Hospital Los Angeles, Los Angeles, CA
- Jennifer Marks MD, University of Miami, Miami, FL
- Toni Moran MD, University of Minnesota, Minneapolis, MN
- Tihamer Orban MD, Joslin Diabetes Center, Boston MA
- Philip Raskin MD, University of Texas, Houston, TX
- Henry Rodriguez MD, Indiana University, Indianapolis, IN
- Desmond Schatz MD, University of Florida, Gainseville, FL
- Diane Wherrett; Hospital for Sick Children; Toronto, Canada
- Darrell Wilson MD, Stanford University, San Francisco, CA

Central Laboratories:
- Jerry Palmer MD, The Beta Cell Function Laboratory, University of Washington, Seattle, WA; William Winter MD, The Central ICA Laboratory, University of Florida, Gainesville, FL; George Eisenbarth MD, PhD, The Central Autoantibody Laboratory, Barbara Davis Diabetes Center, University of Colorado, Denver, CO.

Coordinating Center:
- John M. Lachin ScD, Heidi Krause-Steinrauf MS, Paula Friedenberg McGee MS, The George Washington University Biostatistics Center, Rockville, MD.

Chairman’s Office:
- Jay Skyler MD; University of Miami, Miami, FL;
Carla Greenbaum MD: Benaroya Research Institute, Seattle, WA

TrialNet Study Group: www.Diabetestrialnet.org

European C-Peptide Trial:

Clinical Investigators:
- Magdalena Avbelj MD, Dept. of Pediatric Endocrinology, University Children’s Hospital, Ljubljana, Slovenia
- Natasa Ursic Bratina MD, Dept. of Pediatric Endocrinology, University Children’s Hospital, Ljubljana, Slovenia
- Katerina Dvoráková MD, Institute of Endocrinology, Prague, Czech Republic
- Guido Giani PhD, German Diabetes Center at the Heinrich-Heine University, Düsseldorf, Germany
- Nebošja Lalic MD, PhD, Institute for Endocrinology, Diabetes and Metabolic Diseases, Belgrade, Serbia
- Maria Teresa Martinez Larrad MD, PhD, Department of Internal Medicine II, Hospital Clínico San Carlos, Madrid, Spain
- Claus Morten Larsen MD, Steno Diabetes Center, Gentofte, Denmark
- Roger Lehmann MD, PhD, Division of Endocrinology, Diabetes and Clinical Nutrition, University Hospital, Zürich, Switzerland
- Didac Mauricio MD, PhD, Servicio de Endocrinología y Nutrición Hospital de Sant Pau, Barcelona, Spain
- Moshe Phillip MD, PhD, Schneider Children's Medical Center of Israel, Petah Tikva, Israel
- Antonio Picardi MD, University Campus Bio-Medico, Rome, Italy
- Manuel Serrano Rios, MD, PhD, Department of Internal Medicine II, Hospital Clínico San Carlos, Madrid, Spain
- Olga Perez-Rodriguez MD, Department of Pediatrics Internal Medicine II, Hospital Clínico San Carlos, Madrid, Spain
- Jose Luis Ruibal MD, Department of Pediatrics Internal Medicine II, Hospital Clínico San Carlos, Madrid, Spain
- Nanette Schloot MD, PhD, Institute for Clinical Diabetes Research at the German Diabetes Centre, Leibniz-Center for Diabetes Research at the Heinrich-Heine University, Düsseldorf, Germany
- Edith Schober MD, PhD, University Children's Hospital, Vienna, Austria
- Giatgen A. Spinas MD, PhD, Department of Endocrinology, Diabetes and Clinical Nutrition, University Hospital, Zürich, Switzerland
- Karel Vondra MD, PhD, Institute of Endocrinology, Prague, Czech Republic

Central Laboratory:
- Thomas Mandrup-Poulsen MD PhD; Steno Diabetes Center, Gentofte

Coordinating Centre: Tadej Battelino MD PhD; Dept. of Pediatric Endocrinology, University Children’s Hospital, Ljubljana, Slovenia

Steering committee:
- Thomas Mandrup-Poulsen MD PhD; Steno Diabetes Center, Gentofte, and Dept. of Biomedical Sciences, University of Copenhagen, Denmark
- Tadej Battelino MD PhD, Dept. of Pediatric Endocrinology, University Children’s Hospital, Ljubljana, Slovenia
This Appendix describes methods and additional results for the TrialNet and European studies of the mixed meal tolerance test (MMTT) versus the Glucagon Stimulation Test (GST) to measure β-cell function in type 1 diabetes.

**Methods**

**TrialNet Study.** For the MMTT tests, participants were given 6 ml/kg of Boost up to maximum of 360 ml, to be ingested within 5 minutes. Samples were collected 10 minutes prior to the meal (-10), at the time of ingestion (0), and at 15, 30, 60, 90 and 120 minutes thereafter. For the GST test, 1 mg of glucagon was injected intravenously within 10 seconds. Samples were collected at -10, 0, 2, 4, 6, 8, and 10 minutes. Glucose, C-peptide, and HbA1c were measured at the TN β-cell function laboratory (Seattle, WA). Autoantibodies were measured at TN antibody laboratories (Gainesville, FL and Denver, CO).

C-peptide was measured using a two site immunoenzymometric assay (1) performed on a Tosoh 600 II auto-analyzer (AIA-600 II Analyte Application Manual, Tosoh Bioscience, Inc. South San Francisco, CA). The upper limit of the analytical range of the assay is 30 ng/ml (9.9 pmol/ml). On May 16, 2005, the lower limit of quantification was lowered from 0.2 ng/ml to 0.04 ng/ml (0.066 to 0.013 pmol/ml). The inter-assay and intra-assay coefficients of variation were less than 10%.
**European C-peptide Trial.** MMTT and GST tests were conducted as described for the TN study with the exception that only one post-stimulus sample was obtained after glucagon injection (6 minutes).

Blood glucose measurements were performed by HEMOCUE-meters distributed from the central laboratory at Steno Diabetes Center, Copenhagen. C-peptide, blood glucose, hemoglobin A1c, and serum insulin (ELISA) were all measured in the central laboratory at the Steno Diabetes Center. The C-peptide in a sample was measured using a two-site fluoroimmunometric assay performed using an AutoDELFIA (Perkin Elmer-Wallac) C-peptide kit. The analytical range of the assay is 0.01 – 6.0 pmol/ml, 0.01 pmol/ml being the lower limit of quantification of the assay; the inter-assay coefficient of variation was less than 6%.

**Statistical Methods.** The area under the curve (AUC) was computed from all timed collections (including the basal) using the trapezoidal rule. The AUC mean equals the AUC divided by the interval of time, e.g. 120 minutes for a 2 hour MMTT. The AUC could not be computed for the European Study GST that only obtained a 6 minute value.

Analyses were conducted using the log transformation and the results presented as a geometric mean (exp(mean log(x)) and standard deviation factor (SDF = exp(SE of mean log(x)*square root (df))) from which the 95% confidence limits on the geometric mean were computed.

Bland-Altman plots (2) were used to assess homogeneity of error variation over the range of subject values. The ratio of the two values is plotted versus the geometric mean of the two for each subject.
There were no differences between the values of the first and second tests within subjects and thus values from all MMTTs and GSTs were employed with an adjustment for test order and other factors, and allowing for the inter-correlation among repeat measures in the same subject.

The differences in adverse reactions and preferences were compared across groups using the chi-squared test for independence, or Fisher’s exact test, as appropriate.

Results

**Measurable Values:** Table A1 presents the proportion of non-measurable C-peptide values below the lower limit of quantification of the assay.

**C-Peptide Values:** Figure A1 presents the distribution of the C-peptide values from each timed collection during the MMTT and the GST for the TrialNet and the European studies. The box plots present the 25, 50 and 75 percentiles of the distribution, the dot the mean. The strong positive skewness is indicated by the mean being substantially higher than the median (middle bar of each box) and the median being closer to the 25th than the 75th percentile.

The European C-peptide values (panels C and D) appear to be lower than those in TrialNet (A and B). However, the distributions of values in the European study are similar to those in TrialNet when the analysis is restricted to the subjects with the same range of age and duration (panels E and F).

**Inter-correlations:** Table A2 presents the inter-correlations among the various summary measures from the MMTT and GST within the TrialNet and European studies.

**Summary Measures:** Separately for the TrialNet and European studies, Table A3 presents the distribution of the basal (fasting) values, the 90 minute MMTT and 6 minute GST
values, the peak post-stimulus value and the AUC mean. For the European GST, the 6 minute value was used for the peak value. No AUC mean could be computed from the European GST. The quartiles are presented as well as the geometric mean and standard deviation factor (SDF) and the 95% confidence limits. In general, among the stimulated values, the MMTT values are higher than those from the GST.

**Covariate Effects on C-peptide:** Table A4 presents the effects of the log fasting C-peptide, the fasting plasma glucose, age, sex and duration of diabetes on the MMTT and GST peak C-peptide in the TrialNet and European studies.

**Reproducibility and Reliability:** Figure 2 of the main paper presents the scatter plots for the correlation of the AUC C-peptide from the replicate tests of the MMTT in the TrialNet and European studies, and of the AUC from the GST in TrialNet (not computed in the European Study). Figure A2 presents Bland-Altman plots (2) that assess the assumption of homoscedasticity or the extent to which the random errors are scattered evenly over the range of the subject values. These showed that the variation within paired measurements was evenly distributed over the range of values for both MMTT and GST in both studies.

Table A5 presents the intra-class correlations between the duplicate repeat values for the two tests. The differences in the estimated reliabilities from TN versus the ECPT were not statistically significant.

**Adverse Effects:** Table A6 presents the incidence of nausea, vomiting and “other” adverse experiences for each test within each study.

**ADDITIONAL REFERENCES:**

1. Beisher W.: Proinsulin and C-peptide in humans. *Hormones in Normal and Abnormal Human Tissues.* Vol 3, pp. 1-43, 1983.
2. Bland JM and Altman DG: Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet.* 8476:307-10,1986.
Table A1. Proportion of non-measurable C-peptide values below the lower limit of quantification of the assay for each timed collection during the MMTT and the GST within the TrialNet and European studies.

**TrialNet**

| GST time point | -10 | 0 | 2 | 4 | 6 | 8 | 10 |
|----------------|-----|---|---|---|---|---|----|
| % Non-measurable | 12.2% | 13.1% | 8.2% | 8.2% | 8.9% | 10.4% | 9.7% |
| N              | 270 | 268 | 269 | 270 | 269 | 270 | 269 |

| MMTT time point | -10 | 0 | 15 | 30 | 60 | 90 | 120 |
|-----------------|-----|---|----|----|----|----|-----|
| % Non-measurable | 12.0% | 13.7% | 13.7% | 11.2% | 9.0% | 6.5% | 8.3% |
| N               | 275 | 277 | 277 | 277 | 277 | 277 | 277 |

**European**

| GST time point | -5 | 0 | 6 |
|----------------|----|---|---|
| Non-measurable (N = 174) | 19.5% | 21.8% | 14.9% |

| MMTT time point | -5 | 0 | 10 | 20 | 30 | 60 | 90 | 120 |
|-----------------|----|---|----|----|----|----|----|-----|
| Non-measurable (N = 169) | 13.2% | 13.2% | 13.8% | 12.1% | 12.6% | 10.3% | 10.9% | 10.9% |
Table A2. Inter-correlations among summary measures on the log scale from TrialNet evaluations (above the diagonal) and from the European Study (below the diagonal). All p<0.001.

|             | MMTT Basal | MMTT peak | MMTT AUC-mean | GST Basal | GST peak | GST AUC-mean | GST 6 min. |
|-------------|------------|-----------|----------------|-----------|----------|---------------|-------------|
| MMTT Basal  | --         | 0.919     | 0.949          | 0.921     | 0.905    | 0.925         | 0.913       |
| MMTT peak   | 0.930      | --        | 0.989          | 0.911     | 0.920    | 0.934         | 0.927       |
| MMTT AUC-mean| 0.956     | 0.992     | --             | 0.929     | 0.928    | 0.945         | 0.936       |
| GST Basal   | 0.872      | 0.886     | 0.896          | --        | 0.948    | 0.969         | 0.953       |
| GST peak    | NA         | NA        | NA             | NA        | --       | 0.990         | 0.979       |
| GST AUC-mean| NA         | NA        | NA             | NA        | NA       | --            | 0.991       |
| GST 6 min.  | 0.875      | 0.907     | 0.914          | 0.966     | NA       | NA            | --          |
Table A3. C-peptide concentration in pmol/mL. Geometric means from a mixed model analysis of the log(C-peptide) adjusted for fasting glucose concentration, age, gender, continuous diabetes duration, test order, sequence group. Analyses of peak value and AUC-mean also adjusted for basal log(C-peptide). Analyses conducted separately for TrialNet and the European study.

|                  | TrialNet | European Study |
|------------------|----------|----------------|
|                  | MMTT     | GST            |
| Subjects         | 143      | 135            |
| Tests            | 278      | 271            |
| Basal Quartiles  | 0.07, 0.18, 0.31 0.08, 0.18, 0.35 0.02, 0.08, 0.16 0.02, 0.07, 0.16 |
| GM */ SDF*       | 0.17 */ 0.85 0.17 */ 0.85 0.07 */ 1.05 0.07 */ 1.05 |
| GM 95% CI        | 0.14, 0.19 0.15, 0.20 0.05, 0.08 0.05, 0.08 |
| Peak Stimulated  |          |                |
| Quartiles        | 0.15, 0.44, 0.88 0.13, 0.34, 0.62 0.06, 0.16, 0.37 NA |
| GM */ SDF        | 0.40 */ 0.35 0.30 */ 0.35‡ 0.13 */ 0.46 NA |
| GM 95% CI        | 0.38, 0.42 0.28, 0.32 0.12, 0.15 NA |
| 90 Minute MMTT/ 6 Minute GST Stimulated Value Quartiles | 0.13, 0.43, 0.78 0.10, 0.30, 0.57 0.05, 0.14, 0.31 0.03, 0.11, 0.25 |
| GM */ SDF        | 0.36 */ 0.36 0.27 */ 0.29‡ 0.12 */ 0.42 0.10 */ 0.31‡ |
| GM 95% CI        | 0.34, 0.38 0.25, 0.28 0.11, 0.13 0.09, 0.10 |
| AUC-mean†        |          |                |
| % measurable     | 276 (99%) 263 (97%) 132 (92%) NA |
| Quartiles        | 0.11, 0.36, 0.65 0.10, 0.28, 0.53 0.04, 0.13, 0.28 |
| GM */ SDF        | 0.31 */ 0.32 0.25 */ 0.33‡ 0.12 */ 0.42 NA |
| GM 95% CI        | 0.29, 0.33 0.24, 0.26 0.11, 0.13 NA |

† Geometric Mean (GM) */ Standard Deviation Factor (SDF). Geometric mean = \(\exp[\text{mean log(x)}]\), SDF =\(\exp(\text{SE of mean log(x)} \times \text{square root (df)})\).
†† AUC-mean = AUC/time period (120 min for MMTT, 10 min for the GST).
‡ p < 0.0001 for the comparison between the TrialNet GST and MMTT.
### Table A4. Covariate effects on the log peak C-peptide in the MMTT and GST for the TrialNet and European Studies.

| Covariate (min,max) | Percent change* | 95% CI         | P-value |
|---------------------|-----------------|----------------|---------|
| Fasting C-peptide (0.013, 1.72 pmol/mL)† | 165.06 % | 146.38, 185.16 | < 0.01 |
| **TrialNet, MMTT** |                 |                |         |
| Fasting glucose (54, 235) mg/dL | -0.49 % | -0.63, -0.35 | < 0.01 |
| Age (8, 35 years) | 1.51 % | 0.50, 2.53 | < 0.01 |
| Sex (Female vs Male) | -5.77 % | -17.21, 7.24 | 0.35   |
| Duration (0.08, 2.93 years) | -9.49 % | -16.49, -1.91 | 0.01   |
| Fasting C-peptide (0.010, 0.68 pmol/mL)† | 170.77 % | 150.66, 192.49 | < 0.01 |
| **European Study, MMTT** |                 |                |         |
| Fasting glucose (63.1, 413.5) mg/dL | -0.33 % | -0.43, -0.22 | < 0.01 |
| Age (8, 40 years) | 0.85 % | -0.32, 2.03 | 0.14   |
| Sex (Female vs Male) | 14.29 % | -3.70, 35.65 | 0.11   |
| Duration (0.75, 4.97 years) | -4.24 % | -11.17, 3.23 | 0.24   |
| Fasting C-peptide (0.013, 1.36 pmol/mL)† | 179.21 % | 163.88, 195.43 | < 0.01 |
| **TrialNet, GST** |                 |                |         |
| Fasting glucose (54.5, 235) mg/dL | -0.05 % | -0.16, 0.06 | 0.38   |
| Age (8, 35 years) | -0.64 % | -1.42, 0.14 | 0.10   |
| Sex (Female vs Male) | -7.05 % | -16.08, 2.96 | 0.15   |
| Duration (0.08, 2.98 years) | -6.49 % | -12.31, -0.28 | 0.04   |
| Fasting C-peptide (0.010, 0.58 pmol/mL)† | 197.90 % | 181.76, 214.97 | < 0.01 |
| **European Study, GST** |                 |                |         |
| Fasting glucose (54.05, 374.77) mg/dL | -0.03% | -0.11, 0.06 | 0.52   |
| Age (8, 40 years) | -0.23 % | -1.03, 0.57 | 0.55   |
| Sex (Female vs Male) | -8.93 % | -19.34, 2.81 | 0.12   |
| Duration (0.75, 4.97 years) | -1.94 % | -6.94, 3.32 | 0.45   |

* Percent change in the geometric mean per 1 unit increase of the covariate
† The model uses the LOG scale for fasting C-peptide, but the range is shown in pmol/mL
Table A5. Intra-class correlation among log C-peptide values (with asymmetric 95% confidence limits) between repeated tests within subjects with duplicate tests, with test of the significance of the difference between correlations for MMTT and GST measures, separately for the TrialNet and European Study.

| Subjects with duplicate tests | TrialNet | European Study |
|-------------------------------|----------|----------------|
|                               | MMTT     | GST | MMTT | GST |
| Basal                         |          |     |      |     |
|                               | 0.909    | 0.889 | 0.918 | 0.876 |
|                               | (0.879, 0.940) | (0.853, 0.927) | (0.877, 0.959) | (0.817, 0.931) |
| Peak stimulated               |          |     |      |     |
|                               | 0.966*   | 0.915 | 0.979† | NA |
|                               | (0.955, 0.978) | (0.886, 0.944) | (0.969, 0.990) | |
| 6 Minute Stimulated Value     | NA       | 0.925 | NA   | 0.880 |
|                               |          | (0.900, 0.951) |          | (0.822, 0.941) |
| AUC-mean                      |          |     |      |     |
|                               | 0.978*   | 0.932 | 0.980 | NA |
|                               | (0.970, 0.986) | (0.909, 0.956) | (0.970, 0.990) | |

* P < 0.001 between TN MMTT and GST.
† P < 0.001 for MMTT peak stimulated versus GST 6-minute stimulated value for the European Study.
Table A6. Proportion of tests wherein a subject experienced an adverse experience, overall and stratified by age.

| Subjects | TrialNet MMTT | TrialNet GST | European Study MMTT | European Study GST |
|----------|---------------|--------------|---------------------|--------------------|
| Tests    | 143           | 135          | 116                 | 117                |
|          | 272           | 265          | 174                 | 174                |
| Nausea   | 10 (3.6%)     | 221 (81.5%)  | 1 (0.6%)            | 126 (75.0%)        |
| 8 – 12 y | 4 (4.2%)      | 85 (95.5%)   | 0 (0%)              | 34 (94.4%)         |
| 13 – 17 y| 5 (5.6%)      | 76 (86.4%)   | 0 (0%)              | 29 (74.4%)         |
| 18 + y   | 1 (1.1%)      | 60 (63.8%)   | 1 (1.0%)            | 63 (67.7%)         |
| Vomiting | 0 (0%)        | 29 (10.7%)   | 0 (0%)              | 9 (5.4%)           |
| 8 – 12 y | 0 (0%)        | 24 (27.0%)   | 0 (0%)              | 4 (11.1%)          |
| 13 – 17 y| 0 (0%)        | 5 (5.7%)     | 0 (0%)              | 1 (2.6%)           |
| 18 + y   | 0 (0%)        | 0 (0%)       | 0 (0%)              | 4 (4.3%)           |
| Other*   | 17 (6.1%)     | 51 (18.8%)   | 1 (0.6%)            | 14 (7.5%)          |
| 8 – 12 y | 6 (2.2%)      | 16 (18.0%)   | 1 (3.0%)            | 3 (8.1%)           |
| 13 – 17 y| 6 (2.2%)      | 17 (19.3%)   | 0 (0%)              | 2 (5.0%)           |
| 18 + y   | 5 (5.4%)      | 18 (19.2%)   | 0 (0%)              | 9 (9.3%)           |

* “Other” for TrialNet included excessive bleeding (1 for GST), vasovagal/fainting (2 GST, 3 MMTT), headache (14 GST, 4 MMTT), and various “other” conditions. “Other” for the European study was simply defined as “other”.

9/23/2008
Figure A1. Box plots showing the median (-), upper and lower quartiles, and the mean (**)C-peptide at each timed collection during the MMTT and the GST tests from TrialNet (Panels A and B) and the European Study (Panels C and D) using all measures in all subjects. Panels E and F present data from the European Study restricted to subjects with the same duration and age range as in TrialNet.

Figure A1

A. TrialNet MMTT

B. TrialNet GST

C. European MMTT

D. European GST
E. Restricted European MMTT

F. Restricted European GST
Figure A2. Bland-Altman plots of the ratio of the first versus the second measurement of the peak and AUC-mean C-peptide versus the mean of the duplicate measurements in the MMTT and the GST for subjects in TrialNet (A) and the European Study (B).

A. TrialNet

B. European Study