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

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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 immunoenzymometeric 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 Eurpoean 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**	**MMTT**	**GST**
Subjects	**143**	**135**	**116**	**117**
Tests	**278**	**271**	**174**	**174**
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[mean log(x)], SDF =exp(SE of mean log(x)*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
TrialNet, MMTT	Fasting C-peptide (0.013, 1.72 pmol/mL) [†]	165.06 %	146.38, 185.16	< 0.01
	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
European Study, MMTT	Fasting C-peptide (0.010, 0.68 pmol/mL) [†]	170.77 %	150.66, 192.49	< 0.01
	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
TrialNet, GST	Fasting C-peptide (0.013, 1.36 pmol/mL) [†]	179.21 %	163.88, 195.43	< 0.01
	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
European Study, GST	Fasting C-peptide (0.010, 0.58 pmol/mL) [†]	197.90 %	181.76, 214.97	< 0.01
	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.

	TrialNet		European Study	
	MMTT	**GST**	**MMTT**	**GST**
Subjects with duplicate tests	**132**	**121**	**57**	**56**
Basal	0.909 (0.879, 0.940)	0.889 (0.853, 0.927)	0.918 (0.877, 0.959)	0.876 (0.817, 0.931)
Peak stimulated	0.966* (0.955, 0.978)	0.915 (0.886, 0.944)	0.979† (0.969, 0.990)	NA
6 Minute Stimulated Value	NA	0.925 (0.900, 0.951)	NA	0.880 (0.822, 0.941)
AUC-mean	0.978* (0.970, 0.986)	0.932 (0.909, 0.956)	0.980 (0.970, 0.990)	NA

* $P < 0.001$ 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.

	TrialNet		European Study	
	MMTT	GST	MMTT	GST
Subjects	**143**	**135**	**116**	**117**
Tests	**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".

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.
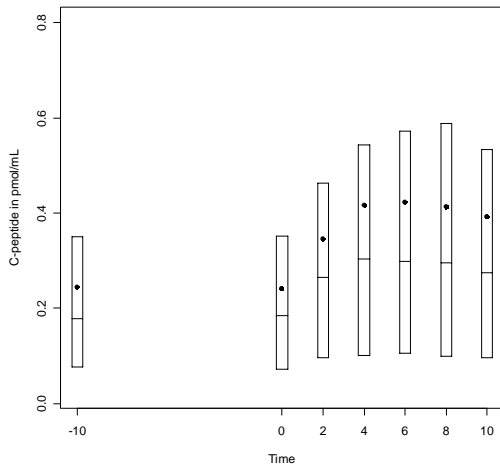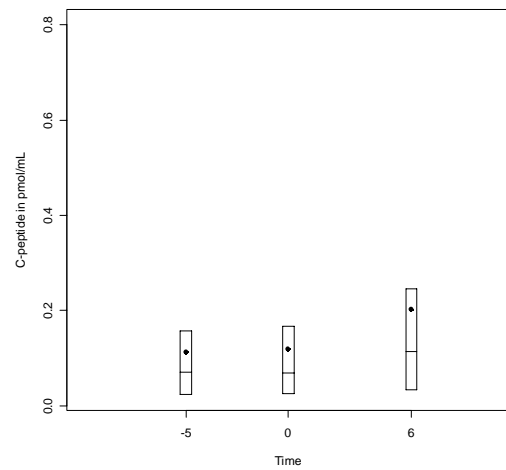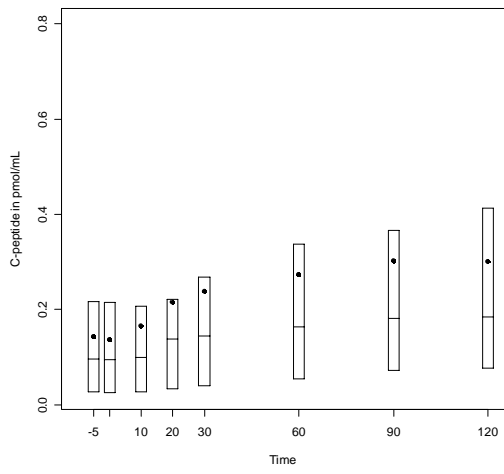
Figure A1

A. TrialNet MMTT
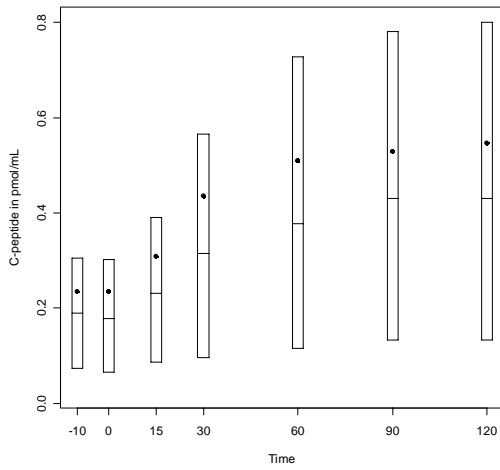
C. European MMTT
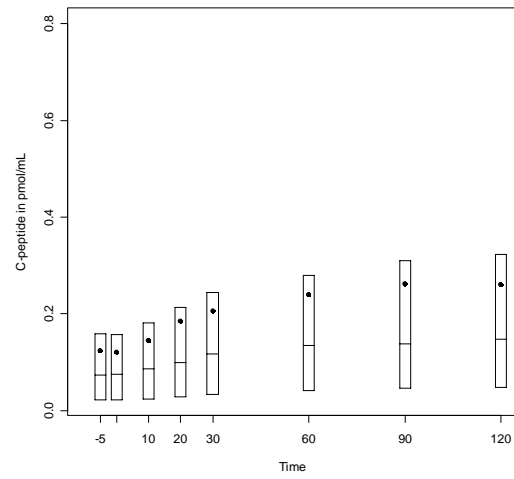
B. TrialNet GST

D. European GST

E. Restricted European MMTT
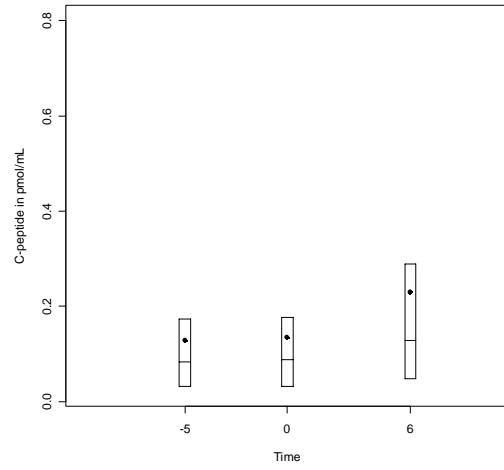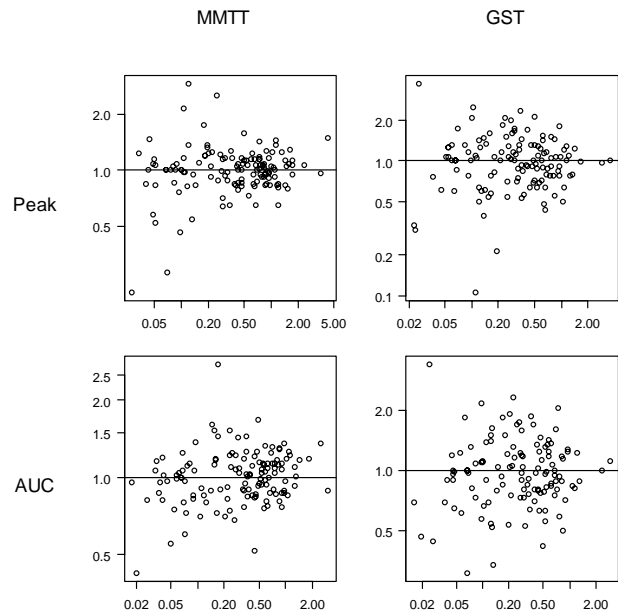

F. Restricted European GST

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