The Mixed Meal Tolerance Test Versus The Glucagon Stimulation Test For 
The Assessment Of Beta-Cell Function In Therapeutic Trials In Type 1 Diabetes

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Additional information for this article can be found in an online appendix at http://care.diabetesjournals.org.

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**Objective:** Beta-cell function in type 1 diabetes (T1D) clinical trials is commonly measured by C-peptide response to a secretagogue in either a Mixed Meal Stimulation Test (MMTT) or a Glucagon Stimulation Test (GST). The Type 1 Diabetes TrialNet and the European C-peptide Trial (ECPT) Group conducted parallel randomized studies to compare the sensitivity, reproducibility and tolerability of these procedures.

**Research Design and Methods:** In randomized sequences, 148 TrialNet subjects completed 549 tests with up to two MMTT and two GST tests on separate days; and 118 ECPT subjects completed 348 tests (up to 3 each) with either two MMTTs or two GSTs.

**Results:** Among individuals with up to 4 years duration of T1D, over 85% had measurable stimulated C-peptide values. The MMTT stimulus produced significantly higher concentrations of C-peptide than the GST. Whereas both tests were highly reproducible, the MMTT was significantly more so ($R^2=0.96$ for peak C-peptide response). Overall, the majority of subjects preferred the MMTT and there were few adverse events. Some older subjects preferred the shorter duration of the GST. Nausea was reported in the majority of GST studies, particularly in the young age group.

**Conclusions:** The MMTT is preferred for the assessment of β-cell function in therapeutic trials in type 1 diabetes.

**ClinicalTrials.gov registration:** TrialNet, NCT00105352
The measurement of C-peptide in response to a stimulus provides a direct measure of β-cell function. While type 1 diabetes (T1D) results from an immune mediated loss of pancreatic β-cells, higher residual β-cell function early in the disease has strong long-term beneficial effects (1). The international diabetes research community has highlighted the need to establish the validity of C-peptide, and other assessments, used in clinical trials in recent onset T1D (2); weighing the scientific properties of a test against the burden imposed on a subject.

The recently-issued a draft guidance recognizes preservation of β-cell function, as measured by C-peptide, as an appropriate outcome for therapeutic trials in early T1D (3). Results from trials using therapies such as immunosuppression, T-cell modulation, B-cell modulation, co-stimulation blockade, antigen specific therapy, and metabolic control are likely to change the standard of care for individuals with T1D.

Clinical trials generally employ one of two methods for stimulating C-peptide response. In the Mixed Meal Tolerance Test (MMTT), commonly used in the US, a liquid meal (Sustacal/Boost) is ingested in the fasting state with timed measurements of C-peptide over the subsequent 2-4 hours. In the Glucagon Stimulation Test (GST), glucagon is injected intravenously with timed measurements of C-peptide over the subsequent 10 minutes. Two parallel studies were conducted simultaneously by the Type 1 Diabetes TrialNet (TrialNet) and the European C-peptide Trial (ECPT) study group to evaluate the properties and tolerability of these tests.

**RESEARCH DESIGN AND METHODS**

Details about study design, laboratory and statistical methods are provided in the Online Appendix (available at http://care.diabetesjournals.org).

**Subjects.** TrialNet enrolled 148 subjects between September, 2004 and December, 2005 at 14 North American clinical centers, 1 in Europe and 1 in Australia. The ECPT enrolled 118 subjects between April, 2004 and December, 2004 in 12 European centers. Each site obtained institutional or ethics board approval, and each subject or parent, as appropriate, provided informed consent and/or assent. Subjects were diagnosed with T1D by American Diabetes Association criteria within the past 1 month to 3 years (TrialNet) or by the WHO 1999 criteria within 4 years (ECPT). Patients being treated with drugs that influence β-cell function or influence insulin sensitivity were excluded.

**Methods.** TrialNet randomly allocated subjects 8-35 years of age to receive either two MMTTs followed by two GSTs or the opposite sequence 3 to 10 days apart. ECPT randomized subjects to receive two MMTTs or GSTs followed by one GST or MMTT. Subjects were stratified by age (8-17 versus 18-35 years) and duration of diabetes (1 year ± 3 months, 2 years ± 6 months, 4 years ± 12 months) with approximately equal numbers within each stratum. The latter two duration subgroups were randomized together.

The MMTTs and GSTs were initiated before 10 a.m. Subjects on continuous subcutaneous insulin infusion (CSII, insulin pump) could continue to use the usual basal rate. Subjects were instructed to withhold long-acting insulin on the morning of the test. TrialNet allowed use of rapid acting insulin by injection or CSII up to 2 hours prior, and short acting insulin up to 6 hours prior to the test. ECPT subjects withheld rapid or short acting insulin for 6 hours before the test. Tests were rescheduled if the subject had a capillary glucose value >200 mg/dl or < 70 mg/dl.
peptide concentrations are presented as pmol/mL.

**Statistical Methods.** The ECPT GST only obtained a 6 minute value that was treated as the peak. The post-stimulus area under the curve (AUC) was calculated using the trapezoidal rule. The AUC-mean (in pmol/mL) is the AUC divided by the time of the test. Distributions were positively skewed and a log transformation was used.

The covariate-adjusted mean C-peptide for MMTT and GST was estimated, and the difference tested, using normal errors regression models for repeated measures (4). The reliability of repeated MMTT or GST tests in the same subject was assessed by the intraclass correlation coefficient (5). The difference in reliability of the MMTT vs. GST in TrialNet was tested (6) using subjects who completed all 4 visits.

All analyses were conducted using the Statistical Analysis System (SAS).

**RESULTS**

(See Online Appendix at http://care.diabetesjournals.org for additional tables and figures.)

**Subjects.** TrialNet randomized 77 subjects to receive two MMTTs followed by two GSTs, and 71 to receive the opposite sequence. Of these, 123 completed all 4 tests, 15 completed 3 of the 4 tests, and ten completed 1 or 2 of the tests. ECPT randomized 59 subjects to receive two GSTs followed by a single MMTT, and 59 to receive the opposite sequence. Of these, 115 completed three tests and 3 completed only one test. The baseline characteristics were similar within each sequence group within each study, and were comparable across the two studies with the exception of differences by design in duration of T1D (Table 1).

**Measurable Values.** The C-peptide assay can not measure a value below the lower limit of quantification (LOQ). In TrialNet, the LOQ was lowered during the study. Among assays completed with the new assay, C-peptide was measurable in 88% of the basal samples, 87-95% of stimulated MMTT samples and 92-94% of stimulated GST samples. Results in the ECPT were similar. There were no significant differences either between tests (MMTT vs GST) or studies (TrialNet vs ECPT). Specimens with non-measurable C-peptide were assigned the LOQ value at the time of testing.

**C-Peptide Values.** In TrialNet, the MMTT values increased steadily over the first hour, and then more slowly during the second hour, nearly doubling over the fasting concentration. The GST values reached a peak at 6 minutes and then declined. A similar pattern was observed in the ECPT among the 82 subjects in the same range of age (8 – 35 years) and duration (3 months to 3 years) as in TrialNet. The mean time to the peak value (±SE) was 88.4 ± 2.5 minutes for the TrialNet MMTT, 80.3 ± 10.7 min for the ECPT MMTT, and 5.8 ± 0.3 min from the TrialNet GST. Summary measures from each test were highly correlated, that between the MMTT fasting and stimulated peak or AUC being at least 0.92, that for GST being slightly lower.

Table 2 presents fasting and stimulated C-peptide values obtained from the two tests. In each study, the covariate-adjusted stimulated MMTT value was significantly higher than the GST value. The MMTT stimulated values in TrialNet tend to be higher than those from ECPT. However, in a further analysis (data not shown) using the subset of ECPT subjects in the same range of age and duration as in TrialNet, the MMTT stimulated values were equivalent in TrialNet and ECPT, although the TrialNet basal value remained significantly higher than the ECPT.

**Covariate Effects on C-peptide:**

Peak stimulated MMTT and GST values were positively associated with fasting C-peptide. Fasting glucose was inversely associated with peak MMTT but not GST values. Age and
duration were associated with peak C-peptide only in the TrialNet studies. Similar associations were observed after excluding the small numbers of subjects with a fasting glucose outside of 70-200 mg/dl on the day of the test.

In TrialNet, but not ECPT, there was a significant interaction between age and test type (MMTT vs. GST) for both the peak and AUC-mean values (p < 0.01 for each) adjusted for other covariates. Figure 1 shows that the mean peak values with MMTT tended to increase with increasing age, especially among those with less than 1 year diabetes duration (Figure 1A), whereas those with GST decrease with increasing age. Thus, the difference between the MMTT and GST peak values also increased with age.

**Reproducibility and Reliability.** Figure 2 shows the relationship between peak and AUC values from the repeat tests of each subject. For each test the relationship is strongly linear with a high $R^2$. The correlation coefficients (reliability) between the duplicate values were high in both studies, although those among the duplicate fasting values were lower (about $r=0.90$) than among the stimulated values. Further, the correlations among stimulated MMTT values were significantly higher than the GST values. The correlations were similar in TrialNet and ECPT and among subgroup categories of gender, age, and duration of diabetes.

**Adverse Effects.** The incidence of adverse effects was substantially higher with GST than with MMTT. With the GST, the incidence of nausea was ~95% in 8-12 year olds falling to ~66% in those over age 18.

**Preference.** In TrialNet, 53% of subjects preferred the MMTT to the GST. The preference depended on age: 86% of those <13 years preferred the MMTT versus 45% of those 13-17 years, and 30% of those ≥18 years (p< 0.01). Males and females did not differ significantly: 61% of females versus 48% of males preferred the MMTT (p=0.17).

Among subjects who preferred the MMTT, 85% cited nausea experienced during the GST as a reason. Among those who preferred the GST, 94% cited the shorter duration of the test as a reason.

**DISCUSSION**

Retention of β-cell function in T1D, as measured by stimulated C-peptide, results in improved glycemic control and reduced incidence of hypoglycemia, retinopathy and nephropathy (3, 7). Thus, intervention studies aimed at preserving β-cell function have usually measured plasma C-peptide following either a liquid mixed meal (MMTT) or an intravenous injection of glucagon (GST). However, the scientific validity of the MMTT versus the GST, and the relative tolerability and practicality, have not been definitively studied.

Two parallel studies have provided clear, concordant results. On repeat testing 3-10 days apart, both tests provided highly reliable (reproducible) measures of stimulated C-peptide responses, the MMTT more so, over a wide range of age and diabetes duration. Thus, changes in these measures over time in clinical trials (and in individual subjects) can be attributed to progression of disease and/or influence of therapy. These data also confirm that almost all individuals with T1D for up to 4 years have measurable stimulated C-peptide, even with an undetectable fasting value, and that most maintain clinically meaningful amounts (> 0.2 pmol/mL) as defined by the DCCT (7).

Both studies also show that the MMTT is a more sensitive test of residual β-cell function, the peak C-peptide response being significantly greater than that in the GST. In the MMTT, the peak response occurred at about 90 minutes as compared to 6 minutes for the GST, thus confirming the selection of this time point for the GST in the ECPT. In multivariate models, longer diabetes duration and younger age were associated
with a lower the C-peptide response to the MMTT in TrialNet, but not the ECPT, perhaps owing to the smaller number of tests and/or the longer allowed duration of diabetes. Further, the difference in peak response with MMTT vs. GST increased with age.

Use of a fixed glucagon dose, rather than dosing based on weight, as was done with the mixed meal, may have resulted in lower peak C-peptide responses in the GST. However, this is unlikely because identical results are obtained after adjusting for weight.

Rather, the greater sensitivity of the MMTT is more likely due to an incretin effect in which an oral glucose stimulus elicits greater insulin secretion due to the actions of GIP and GLP-1 compared to a similar IV stimulus. Although there is a known impairment in the incretin effect early in type 1 diabetes (8), these data suggest that this impairment may be influenced by age. Alternatively, the insulin response to glucagon may be inherently less than the response to the combined stimuli of fat, protein, and carbohydrate, a hypothesis that could be tested in individuals without type 1 diabetes.

Since glucose impacts C-peptide response (9), the MMTT and GST were conducted only if the fasting blood glucose by meter was within 70-200 mg/dl. Within this range, however, the fasting glucose significantly affected the MMTT C-peptide response. Thus, for intervention trials, the distributions of fasting glucose values on the day of the test should be comparable across groups, or controlled for in the statistical analysis.

Nausea occurred in 75-81% of the GSTs and was more prevalent among younger subjects, perhaps owing to the fixed glucagon dose regardless of body weight. The relatively high frequency is possibly due to directed questioning. Most episodes of nausea were mild, although 5-11% involved vomiting.

Most subjects preferred the MMTT. However, older individuals, more concerned with time and who experienced less nausea, preferred the GST. Nevertheless, compliance with the sequence of tests was high; 83% of TrialNet subjects completing all 4 tests, 93% completing 3 or 4 tests, and 97% of ECPT subjects completed all three tests, all within a 1 month period. Thus, it is unlikely that the choice of MMTT vs GST will impact compliance with testing in a clinical trial.

Recently, C-peptide response during a two-phase glucose-clamp procedure has been used to measure β-cell function (10), which poses a substantial additional burden on the subject and investigator. However, responses to this procedure have not been compared to the GST or MMTT in subjects with type 1 diabetes. Arginine stimulation test (AST) has also been used, particularly in post-transplant studies. The AST, like the GST, is short, but unlike the GST does not cause nausea and thus might be the test with the lightest subject burden. One small study did not find any significant differences in sensitivity between the AST and MMTT (11); however, it did not assess reproducibility or the overall comparability.

Any stimulated test is expensive and time consuming and thus may not provide benefits in clinical practice beyond a fasting or a random C-peptide measurement. As shown herein, a fasting (basal) C-peptide has a high correlation with the preferred MMTT AUC-mean response ($r=0.95$ and 0.93 in the two studies, see Online Appendix) and a random C-peptide has been shown to differentiate subjects with type 1 versus type 2 diabetes [12]. However, the emphasis on stimulated C-peptide levels in type 1 diabetes stems from the DCCT demonstration that stimulated C-peptide response at baseline in the intensive therapy group was directly associated with improved glycemic control,
Reproducibility and Tolerability of MMTT and GST

with lower risks of microvascular complications and less hypoglycemia [1, 7]. The DCCT has not published an analysis of the like associations with the fasting C-peptide levels. Without a formal study using a fasting or random C-peptide in addition to a stimulated test, it is not possible to determine which is more clinically meaningful in an individual subject.

In conclusion, two parallel studies definitively show that the MMTT is superior to the GST when conducted under standardized conditions. The MMTT is more sensitive, providing higher post-stimulus C-peptide responses, is more reproducible, and is better tolerated; and thus is the preferred method to measure residual β-cell function in clinical trials in type 1 diabetes. Clinicians interpreting results from clinical trials to interdict the T1D disease process should be aware that these two commonly used outcome measures are not directly comparable.

ACKNOWLEDGEMENTS

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Hubert Kolb MD PhD; German Diabetes Center, Düsseldorf, Germany
REFERENCES:


Table 1. Baseline Characteristics of the TrialNet and European Trial Participants.
* Restricted to TrialNet and European study subjects with 3 months to 3 years duration, and 8 to 35 years of age

<table>
<thead>
<tr>
<th></th>
<th>TrialNet (n=148)</th>
<th>European (n=118)</th>
<th>Combined data* (n=230)</th>
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<tbody>
<tr>
<td>Male (%)</td>
<td>91 (61%)</td>
<td>70 (59%)</td>
<td>138 (60%)</td>
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<tr>
<td>Age (years)</td>
<td></td>
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<tr>
<td>Median (range)</td>
<td>15 (8-35)</td>
<td>19 (8-40)</td>
<td>16 (8-35)</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>16.2 (6.19)</td>
<td>20.3 (7.54)</td>
<td>17.2 (6.53)</td>
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<tr>
<td>Categories</td>
<td></td>
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<tr>
<td>8 – 12 y (%)</td>
<td>51 (34%)</td>
<td>26 (22%)</td>
<td>73 (32%)</td>
</tr>
<tr>
<td>13 – 17 y (%)</td>
<td>49 (33%)</td>
<td>26 (22%)</td>
<td>66 (29%)</td>
</tr>
<tr>
<td>18 y and older (%)</td>
<td>48 (32%)</td>
<td>66 (56%)</td>
<td>91 (40%)</td>
</tr>
<tr>
<td>Race</td>
<td></td>
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<tr>
<td>White</td>
<td>128 (86%)</td>
<td>116 (98%)</td>
<td>208 (90%)</td>
</tr>
<tr>
<td>African-American</td>
<td>6 (4%)</td>
<td>0 (0%)</td>
<td>6 (3%)</td>
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<tr>
<td>American Indian</td>
<td>3 (1%)</td>
<td>0 (0%)</td>
<td>3 (1%)</td>
</tr>
<tr>
<td>Asian</td>
<td>4 (3%)</td>
<td>1 (0.85%)</td>
<td>5 (2%)</td>
</tr>
<tr>
<td>Other</td>
<td>7 (5%)</td>
<td>1 (0.85%)</td>
<td>8 (3%)</td>
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<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
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<tr>
<td>% Hispanic</td>
<td>10 (7%)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Duration of T1D (years)</td>
<td></td>
<td></td>
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<tr>
<td>Median (range)</td>
<td>1.36 (0.08-2.98)</td>
<td>2.32 (0.75-4.97)</td>
<td>1.49 (0.08-2.98)</td>
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<td>Mean ± SD</td>
<td>1.4 ± 0.88</td>
<td>2.5 ± 1.27</td>
<td>1.54 ± 0.84</td>
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<td>Categories</td>
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<td>0 - &lt;1 years, N (%)</td>
<td>60 (41%)</td>
<td>15 (13%)</td>
<td>75 (33%)</td>
</tr>
<tr>
<td>1 - &lt;2 years, N (%)</td>
<td>39 (26%)</td>
<td>34 (29%)</td>
<td>72 (31%)</td>
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<td>2 - &lt;3 years, N (%)</td>
<td>49 (33%)</td>
<td>34 (29%)</td>
<td>83 (36%)</td>
</tr>
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<td>3 - &lt;4 years, N (%)</td>
<td>N/A</td>
<td>14 (12%)</td>
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<td>4 - &lt;5 years, N (%)</td>
<td>N/A</td>
<td>21 (18%)</td>
<td>N/A</td>
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<tr>
<td>HbA1c (%) Mean ± SD</td>
<td>7.3 ± 1.4</td>
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</table>

Table 2. Fasting and stimulated 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 stimulated values also adjusted for basal log(C-peptide). Analyses conducted separately for TrialNet and the European study.

<table>
<thead>
<tr>
<th></th>
<th>TrialNet</th>
<th>European Study</th>
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<tr>
<td></td>
<td>MMTT</td>
<td>GST</td>
</tr>
<tr>
<td>Subjects</td>
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<td>135</td>
</tr>
<tr>
<td>Tests</td>
<td>278</td>
<td>271</td>
</tr>
<tr>
<td>Fasting GM* (95% CI)</td>
<td>0.17 (0.14, 0.19)</td>
<td>0.17 (0.15, 0.20)</td>
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<tr>
<td>Peak Stimulated GM* (95% CI)</td>
<td>0.40 (0.38, 0.42)</td>
<td>0.30 (0.28, 0.33)</td>
</tr>
<tr>
<td>90 min MMTT/6 minute GST GM* (95% CI)</td>
<td>0.36 (0.34, 0.38)</td>
<td>0.27 (0.25, 0.38)</td>
</tr>
<tr>
<td>AUC Mean GM* (95% CI)</td>
<td>0.31 (0.29, 0.33)</td>
<td>0.25 (0.24, 0.26)</td>
</tr>
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</table>

* Geometric mean = exp[mean log(x)].
† p < 0.0001 for the comparison between the TrialNet GST and MMTT.
Figure Legends:

Figure 1: TrialNet mean peak C-peptide, and 95% confidence bands, from a MMTT and GST with respect to the age of onset in subjects with duration of T1D less than 1 year (a), 1-2 years (b), and 2-3 years (c) obtained from separate models within each duration category with an interaction between test (MMTT vs. GST) and age at onset of T1D, adjusted for fasting C-peptide, fasting glucose, weight, visit number, and randomization group.

Figure 2: Relationship between the duplicate measurements of the peak and AUC mean C-peptide values from the MMTT and the GST. The 6 minute European GST values presented in lieu of the peak values.

Figure 1A
Reproducibility and Tolerability of MMTT and GST

Figure 1B

Figure 1C

C-peptide (pmol/mL) vs Age at Onset

MMTT

GST

Age at Onset
Figure 2

A. Peak Values (with 6 minute European Study GST values)
B. AUC Mean Values