EMERGING TECHNOLOGIES AND THERAPEUTICS

# Evaluation of Long-Term Treatment Effect in a Type 1 Diabetes Intervention Trial: Differences After Stimulation With Glucagon or a Mixed Meal

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## OBJECTIVE

Endogenous insulin secretion, measured by C-peptide area under the curve (AUC), can be tested using both the glucagon stimulation test (GST) and the mixed-meal tolerance test (MMTT). This study compares these two stimulation methods using long-term data from patients newly diagnosed with type 1 diabetes or with latent autoimmune diabetes.

## RESEARCH DESIGN AND METHODS

A recently completed phase 3 intervention study with DiaPep277 demonstrated improved glycemic control and a significant treatment effect of glucagon-stimulated C-peptide secretion. Unexpectedly, MMTT failed to detect differences between the treated and control groups. Data from 343 patients in two balanced-randomized, double-blind, placebo-controlled, parallel-group trials of DiaPep277 were used to compare and correlate between GST- and MMTT-derived C-peptide AUC. Pearson's correlations were calculated for absolute C-peptide AUC at baseline and 12 and 24 months and for long-term changes in AUC ($\triangle$AUC).

## RESULTS

The absolute AUC values obtained at any single time point by the two tests were well correlated in both data sets ($r = 0.74$–0.9). However, the correlations between the $\triangle$AUC were much weaker ($r = 0.39$–0.58). GST-stimulated C-peptide secretion was stable over the fasting glucose range permitted for the test (4–11.1 mmol/L), but MMTT-stimulated C-peptide secretion decreased over the same range, implying differences in sensitivity to glucose.

## CONCLUSIONS

Measurement of long-term changes in stimulated C-peptide, reflecting endogenous insulin secretion, during the course of intervention trials may be affected by the method of stimulation, possibly reflecting different sensitivities to the physiological status of the tested subject.

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The hallmark of type 1 diabetes is deterioration of endogenous insulin secretion. Since insulin and C-peptide are cosecreted by β-cells on an equimolar basis, stimulated secretion of C-peptide is widely accepted as a measure of β-cell function (1–4). Moreover, the Diabetes Control and Complications Trial demonstrated that levels of stimulated C-peptide as low as 0.2 nmol/L are clinically meaningful and could prevent or delay long-term diabetes complications (5,6). C-peptide secretion is commonly stimulated by the intravenous administration of glucagon in the glucagon stimulation test (GST) or by the ingestion of a mixed-meal formula in the mixed-meal tolerance test (MMTT) (7,8). Rapid infusion of glucagon induces insulin secretion immediately by directly stimulating β-cells (9). By contrast, the mixed-meal stimulates insulin secretion from β-cells indirectly after ingestion of carbohydrates, fats, and proteins (10).

The use of the MMTT was recommended for the purpose of standardization between intervention studies involving patients with newly diagnosed type 1 diabetes. Investigators who have chosen to use the GST have been advised to perform also the MMTT at least at baseline and annually in order to obtain comparative data (8). A comparison between the MMTT and the GST conducted by the Type 1 Diabetes TrialNet Research Group and the European C-Peptide Trial Study Group (11) found that both procedures were valid for the determination of β-cell function. Although the MMTT was reported to be more sensitive and reproducible, both tests were demonstrated to be highly reproducible and well correlated when performed within a month. However, the above study did not investigate the use of the two procedures to measure long-term changes in β-cell function.

In this report, we compare and correlate the absolute values for the C-peptide area under the curve (AUC) and the long-term changes from baseline in AUC (ΔAUC) at specific time points obtained using the GST and MMTT procedures. The data are from individual patients with autoimmune diabetes participating in the DIA-AID 1 or LADA intervention trials. These two trials were conducted to evaluate the efficacy of DiaPep277, as a treatment modality for the preservation of β-cell function. The end point was defined as the change from baseline to study end in stimulated C-peptide AUC secretion and was measured by both the GST and MMTT.

Data obtained from the DIA-AID 1 study demonstrated significantly preserved C-peptide secretion in the DiaPep277 treatment arm compared with the placebo arm when measured by GST but not by MMTT (12). This was accompanied by a significant improvement in glycemic control.

Herein, the discrepancy between the GST and MMTT data obtained by long-term follow-up in a clinical setting is further investigated and discussed.

## RESEARCH DESIGN AND METHODS
### Clinical Studies
Data from two trials registered with ClinicalTrials.gov, namely, DIA-AID 1 (protocol 901; trial number NCT00615264) and LADA (protocol 702; trial number NCT00058981) were used. All patients signed informed consent forms. The protocol and consent documents were approved by an independent ethics committee or institutional review board at each participating center (DIA-AID 1, 46 international centers; LADA, 7 U.S. centers).

DIA-AID 1 (Protocol 901) is a phase 3, multinational, balanced-randomized, double-blind, placebo-controlled, parallel-group study that was designed to evaluate the clinical safety and efficacy of DiaPep277 as an additional treatment in newly diagnosed patients with type 1 diabetes who were being treated with insulin. For a full description of this study, see reference (12). Briefly, participants consisted of adolescents and adults (aged 16–45 years) who had been diagnosed with type 1 diabetes, as defined by the American Diabetes Association or the World Health Organization, and who possessed residual β-cell function as evidenced by fasting C-peptide levels ≥0.22 nmol/L at the screening visit. Patients received 1 mg DiaPep277 or placebo by subcutaneous injection at months 0, 1, 3, 6, 9, 12, 15, 18, and 21 of the study, with follow-up continuing for a further 3 months.

The change in stimulated C-peptide secretion from baseline to study end (month 24) was measured as the AUC obtained by the GST (primary end point) and the MMTT (secondary end point). Clinical end points included the proportion of patients who, at study end, maintained HbA$_{1c}$ treat-to-target levels of ≤7% (≤53 mmol/mol), insulin dose at study end, and hypoglycemic event rate (12).

The current study used data from all DIA-AID 1 study patients for whom GST and MMTT data were available both at baseline and at study end (N = 297, designated the subgroup population). Within this subgroup population, 146 patients were treated with DiaPep277 and 151 with placebo.

The LADA study (protocol 702) was a phase 2, randomized, double-blind, placebo-controlled, parallel-group clinical study to investigate the safety and tolerability as well as the immunological and clinical efficacy of multiple subcutaneous doses of DiaPep277 in patients with latent autoimmune diabetes. Participants consisted of adult patients (aged 30–70 years) who had been diagnosed with diabetes for up to 5 years and who were subsequently defined as LADA based on presence of GAD autoantibodies at screening. Patients had fasting C-peptide levels of ≥0.3 nmol/L and received 1 mg DiaPep277 or placebo by subcutaneous injection at months 0, 1, 3, 6, 9, 12, 15, and 18. The study was terminated prematurely following an interim analysis showing that it was not powered to demonstrate statistically significant efficacy, so the majority of patients were only followed up to month 12.

The change from baseline to month 12 in stimulated C-peptide secretion was measured as the AUC obtained by the GST and the MMTT. The current study used data from all LADA study patients for whom GST and MMTT data were available both at baseline and at month 12 (N = 46, 22 in the DiaPep277 group and 24 in the placebo group). Albeit an abbreviated study, long-term changes in C-peptide measured by GST and MMTT in individual patients could be compared.

### GST and MMTT Procedures

In the DIA-AID 1 study, C-peptide levels were measured after stimulation by glucagon at month 1 (GST baseline), month 12, and month 24. MMTT-stimulated C-peptide secretion was measured at month 0 (MMTT baseline) and at months 6, 12, 18, and 24. The GST at months 12 and 24 was performed either 3 weeks before or up to 2 weeks after the MMTT.

In the LADA study, C-peptide levels were measured first by MMTT and two weeks later by GST at baseline and month 12.

Fasting and stimulated C-peptide data from baseline and month 12 of both studies, and from month 24 of the DIA-AID 1 study, were analyzed. The tests were performed in the morning after an overnight fast of 8–10 h only if the fasting plasma glucose level was within the permitted range of 4 to 11.1 mmol/L (72 to 200 mg/dL) (8).

For the GST, 1 mg glucagon (GlucaGen HypoKit, Novo Nordisk) was rapidly administered intravenously at time 0. Blood samples for the determination of C-peptide were drawn at −5 min, at time 0 (immediately before glucagon administration), and at 2, 6, 10, and 20 min postadministration.

For the MMTT, each patient ingested a mixed-meal (Ensure, Abbott, for the DIA AID 1 study and Boost, Nestle, for the LADA study) whose volume was calculated as 6 mL/kg body weight with a maximum dose of 360 mL. Blood samples for the determination of C-peptide were drawn at −10 min, at time 0 (immediately before mixed-meal ingestion), and at 10, 20, 30, 60, 90, and 120 min postingestion.

Fasting C-peptide was defined as the average of the concentrations measured at time −5 min and time 0 (for the GST) or at −10 min and time 0 (for the MMTT). Where a blood sample was collected at only one of these time points, that value was considered time 0.

### Laboratory Tests

All laboratory tests were performed in central laboratories. Samples for C-peptide analysis were shipped frozen from the clinical sites to the LKF GmbH clinical laboratory (Schwentinental, Germany) for the DIA-AID 1 study and to the Northwest Lipid Research Laboratories (Seattle, WA) for the LADA study. C-Peptide was measured using radioimmunoassay kits (Human C-peptide RIA kit, LINCO). The analytical range of the assay is 0.0331–1.655 nmol/L.

### Data Analyses

C-peptide AUC was calculated using the trapezoidal rule. In order to compare the areas under the GST- and MMTT-stimulated C-peptide curves, each AUC was normalized by dividing it by the time interval (20 min for GST or 120 min for MMTT), and thus all data are reported in units of nanomoles per liter per minute.

Changes in C-peptide AUC (ΔAUC) were calculated from baseline to month 12 and, for data from the DIA-AID 1 study, also from baseline to month 24 and from month 12 to month 24, using the mixed-effects model for repeated measurements (13,14). Relative treatment effect was defined as the ratio between the ΔAUC values of the DiaPep277-treated group and the placebo group and is expressed as a percentage. Pearson's correlation coefficients ($r$) were calculated in Microsoft Excel 2007.

Since two different valid methods for measuring a single parameter are being compared, a strong correlation of at least $r = 0.8$ would be expected.

To determine the impact of fasting glucose on the C-peptide response to stimulation, patients were grouped according to their fasting glucose concentration as measured at the start of each test. A stimulation index was calculated for each patient at each visit as $C_{max}$/fasting C-peptide (where $C_{max}$ is the maximum stimulated C-peptide concentration measured post-test administration) to obtain a measure of stimulation in relation to the absolute values of fasting C-peptide.

## RESULTS

### Stimulated C-Peptide and the DiaPep277 Treatment Effect

The DIA-AID 1 clinical study has demonstrated that DiaPep277 treatment significantly improved glycemic control while using similar or even lower insulin dose, indicating preservation of β-cell function. This was accompanied by significant differences in the change of C-peptide levels between the DiaPep277-treated group and the placebo group as measured by GST but not by MMTT (12).

In an attempt to explain the discrepancy between the results of the two stimulation methods, we studied the correlation between long-term changes in stimulated C-peptide AUC obtained by GST and MMTT. Data from 297 patients of the subgroup population who had C-peptide levels measured by both stimulation methods at baseline and at study end were analyzed.

The demographic and baseline parameters of the subgroup population were similar to those of the patients who were analyzed in the modified intent-to-treat (mITT) population of the DIA-AID 1 study (12) (Table 1).

The treatment effect observed in the subgroup population was similar to that of the mITT population when C-peptide was stimulated by glucagon. Likewise, there was no treatment effect when C-peptide was stimulated by the mixed meal.

Correlation analyses were performed on the data obtained from patients in each treatment arm of the subgroup population (Supplementary Figs. 1 and 2). A good positive correlation was observed between the absolute C-peptide AUC values obtained by the GST and MMTT methods at specific time points. However, the ΔAUC values obtained by the two stimulation methods were only weakly correlated. This was true in both the placebo arm (Supplementary Fig. 1) and the DiaPep277-treated arm (Supplementary Fig. 2), indicating that the observed discrepancy did not result from the DiaPep277 treatment. Therefore, the data from the two treatment arms were pooled in all subsequent correlation analyses.

The AUC of the stimulated C-peptide secretion levels obtained by the two methods were compared and correlated in each individual patient in the subgroup population (Fig. 1).

The Pearson's correlation coefficients between the absolute C-peptide AUCs obtained by GST and MMTT at individual

**Table 1—Baseline and demographic parameters of the subgroup population compared with the mITT population from the DIA-AID 1 study**

| Parameter | Subgroup population | mITT population from the DIA-AID 1 study |
|---|---|---|
| Number of patients | 297 | 422 |
| Sex (male/female) | 206/91 | 276/146 |
| Age (years) | 27.55 ± 8.00 (16–45) | 26.6 ± 7.74 (16–45) |
| Time from diagnosis (months) | 2.74 ± 1.2 (0.7–7.6) | 2.8 ± 1.2 (0.5–7.6) |
| BMI (kg/m$^2$) | 22.64 ± 2.90 (17.3–33.2) | 22.67 ± 3.0 (17–33.2) |
| Fasting C-peptide (nmol/L) | 0.44 ± 0.21 (0.21–1.84)/[0.28–0.54]* | 0.45 ± 0.23 (0.21–2.28)/[0.3–0.54]* |
| HbA$_{1c}$ (%) | 7.28 ± 1.56 (4.0–14.6) | 7.4 ± 1.7 (4–16.4) |
| HbA$_{1c}$ (mmol/mol) | 56.1 ± 17.01 (20.2–136.1) | 57.5 ± 18.7 (20.2–155.7) |
| Insulin dose (units/kg/day at 3 months) | 0.38 ± 0.19 (0.02–1.21) | 0.38 ± 0.26 (0.02–2.02) |
| Autoantibodies (% positive) | | |
|   IA-2A | 62.6% | 61% |
|   IA | 73.7% | 74% |
|   GADA | 87.5% | 87% |

Data are average ± SD (range). IA-2A, IA-2 protein tyrosine phosphatase; IA, insulin antibody; GADA, glutamic acid decarboxylase. *Interquartile range.

time points were good as expected, $r$ = 0.74, 0.82, and 0.89 at baseline, month 12, and month 24, respectively (Fig. 1A–C). The correlations between the ΔAUC stimulated by GST and MMTT were much weaker over the course of the study, indicating that the change in C-peptide levels measured by one procedure cannot predict the change measured by the other procedure. The correlation coefficients were $r$ = 0.58 across the whole study period (baseline–month 24), $r$ = 0.41 during the first year of the study (baseline–month 12), and $r$ = 0.39 during the second year of the study (months 12–24) (Fig. 1D–F). It should be noted that, already in the first year of the study, the ΔAUC values measured by the two methods were only weakly correlated. The weak correlations for ΔAUC from months 12–24 indicate that the discrepancies between the methods cannot have resulted from measurements at baseline, despite the 1-month difference between the GST and MMTT baselines.

Additional analyses by Bland–Altman plots further support the Pearson's correlation of the test results (see Supplementary Fig. 3).

A similar discrepancy was observed using the smaller data set from the LADA study (protocol 702). While there was good correlation between the absolute values of AUC measured by the MMTT and GST procedures at baseline and at month 12 ($r$ = 0.9 and 0.85, respectively;

Fig. 2A and B), a much weaker correlation ($r$ = 0.48) was found between the ΔAUC values over the study time interval (Fig. 2C).

**Fasting C-Peptide and Glucose Levels Prior to C-Peptide Stimulation**

Fasting glucose was shown to significantly affect the C-peptide response to a mixed meal (11,15). Therefore, we assessed the influence of glucose levels on the extent of C-peptide stimulation (regardless of the state of the disease). The levels of C-peptide secretion stimulated by glucagon and by a mixed meal were compared and grouped according to the fasting glucose range within which the tests were performed (4–11.1 mmol/L). We used a stimulation index, defined as $C_{max}$/fasting C-peptide, to compensate for differences in fasting C-peptide levels. The indices obtained by the glucagon procedure (1.6–1.8) were stable over the glucose range permitted for the tests. In contrast, the indices obtained by MMTT were affected by the fasting glucose level: 4.5 at glucose levels of 4–5 mmol/L that decreased to 1.9 at glucose levels of 10–11.1 mmol/L (Fig. 3A).

Fasting C-peptide and glucose were measured prior to applying the C-peptide stimulation procedures at baseline and at study end (Fig. 3B). Similar median fasting C-peptide values were obtained prior to the MMTT and GST at baseline (0.33 and 0.34 nmol/L,

respectively) and at study end (0.175 and 0.177 nmol/L, respectively) in the subgroup population. For fasting glucose, the median values were also similar prior to these procedures at baseline (MMTT, 5.96 mmol/L; GST, 6.13 mmol/L) and at study end (MMTT, 6.91 mmol/L; GST, 7.14 mmol/L). As expected from the natural history of the disease, the median fasting C-peptide levels decreased by month 24 and the median fasting glucose levels increased, reflecting a decline in endogenous insulin secretion (Fig. 3B).

## CONCLUSIONS

Stimulated C-peptide provides a good approximation of endogenous insulin secretion (and thus residual β-cell function). This parameter, while not a direct measure of β-cell mass, is the currently accepted primary efficacy end point parameter in immune intervention studies in type 1 diabetes (3). Clinical benefits are generally evaluated as secondary end points to support the primary efficacy end point.

The DIA-AID 1 study indicated significant preservation of β-cell function supported by clinical benefits when the stimulation of C-peptide secretion was evaluated by GST. However, changes in C-peptide secretion stimulated by MMTT were not significant and did not correlate with the observed clinical benefits (12).
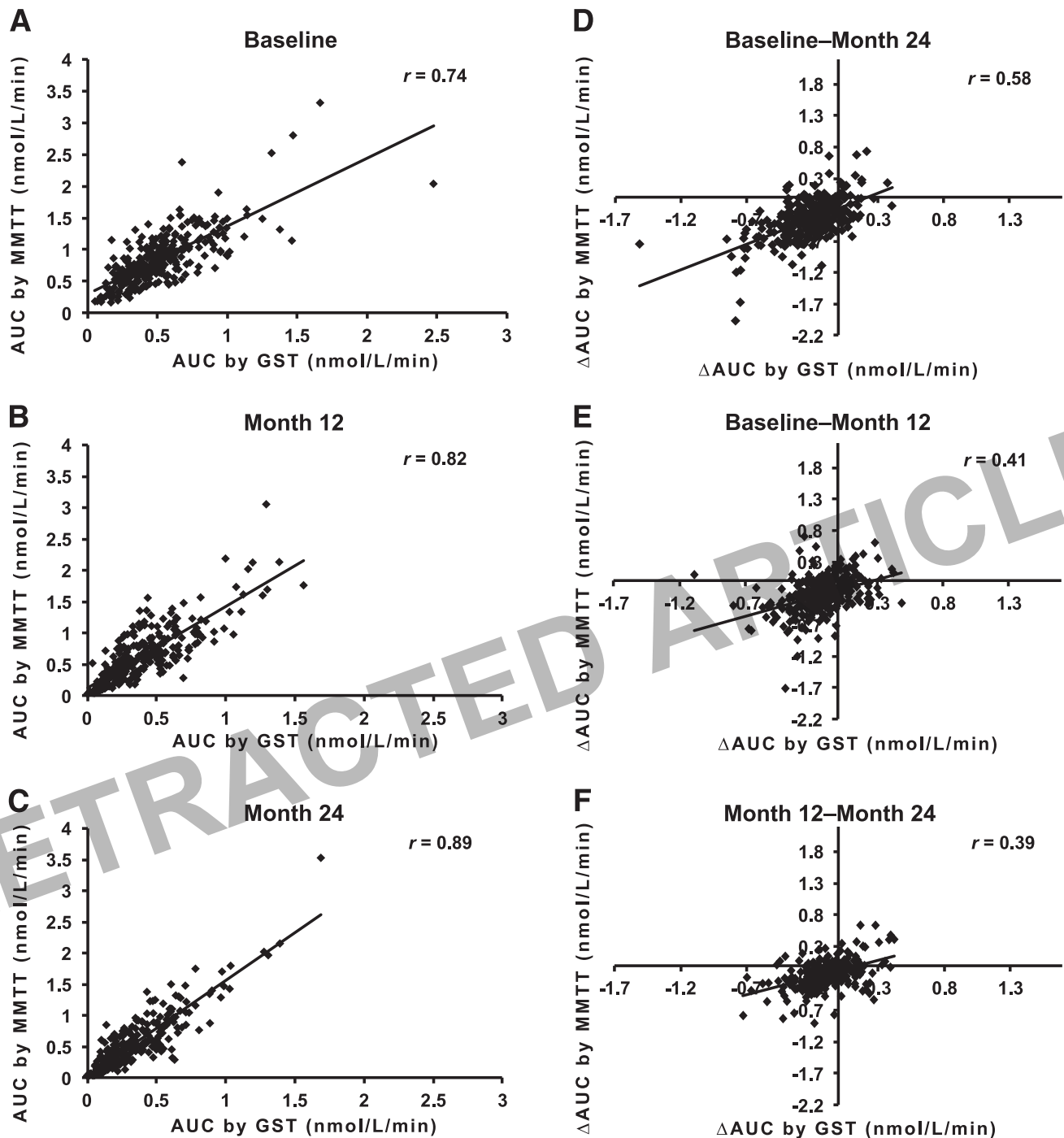
**Figure 1**—Comparison of C-peptide stimulation methods in the DIA-AID 1 study subgroup population (N = 297). The correlations between the AUCs for stimulated C-peptide concentration obtained using the GST and the MMTT at (A) baseline, (B) month 12, and (C) month 24. The correlation between the changes in stimulated C-peptide AUC (ΔAUC) measured by GST and MMTT (D) from baseline to month 24 (whole study period), (E) from baseline to month 12 (first year of the study), and (F) from month 12 to month 24 (second year of the study). Each data point represents a single patient.

The current analysis was undertaken to elucidate this inconsistency using two independent data sets from the DIA-AID 1 and LADA trials. It revealed that the absolute values of stimulated C-peptide measured by GST and MMTT were reproducible and well correlated when evaluated at each individual time point, so confirming the findings of previous reports (7,11,16). However, the ΔAUCs obtained by the two methods over the course of the studies were only weakly correlated. This discrepancy is of particular importance since it is ΔAUCs (rather than absolute AUCs) that indicate change over time from baseline and thus reveal the dynamics of disease progression. As such, it is only ΔAUCs
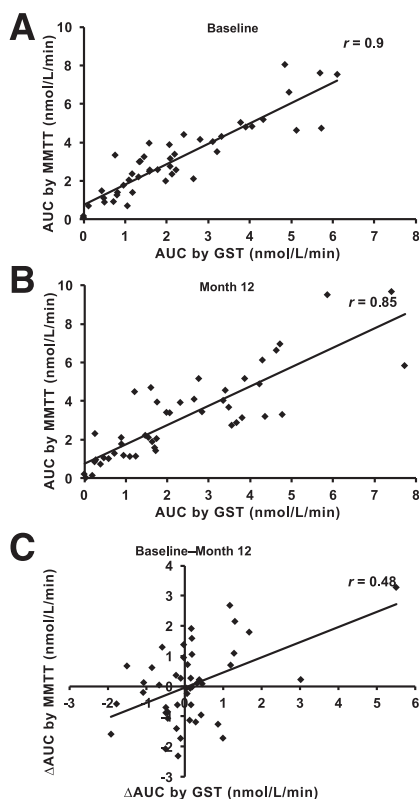
Figure 2—Comparison of C-peptide stimulation methods using data from the LADA study (N = 46). The correlations between the AUCs for stimulated C-peptide measured using the GST and MMTT at (A) baseline and (B) month 12. (C) The correlation between changes in AUC (ΔAUC) measured by GST and MMTT from baseline to month 12. Each data point represents a single patient.

Figure 3—C-peptide and glucose levels from the DIA-AID 1 study subgroup population (N = 297). (A) C-peptide stimulation index calculated from the MMTT and GST stimulations and grouped according to fasting glucose levels prior to administration of the test. The numbers below the graph represent the number of observations. (B) Fasting C-peptide (left) and fasting glucose (right) prior to C-peptide stimulation using the MMTT (white circles) or the GST (black circles) at baseline and at study end. The horizontal bars indicate median values.

that should be used to calculate treatment effect and preservation of residual β-cell function in intervention studies. This inconsistency between the GST- and MMTT-stimulated C-peptide results has not been reported in other long-term intervention trials because no other trials have evaluated patients by both procedures. In fact, these are the first long-term intervention trials to use both procedures and so enable such an observation.

Several mechanisms could contribute to the differences in stimulated C-peptide secretion after glucagon or a mixed-meal administration.

Glucagon stimulates the first-phase insulin release, which occurs within <10 min, while a slow rise in blood glucose following an ingested meal will stimulate the second phase of insulin
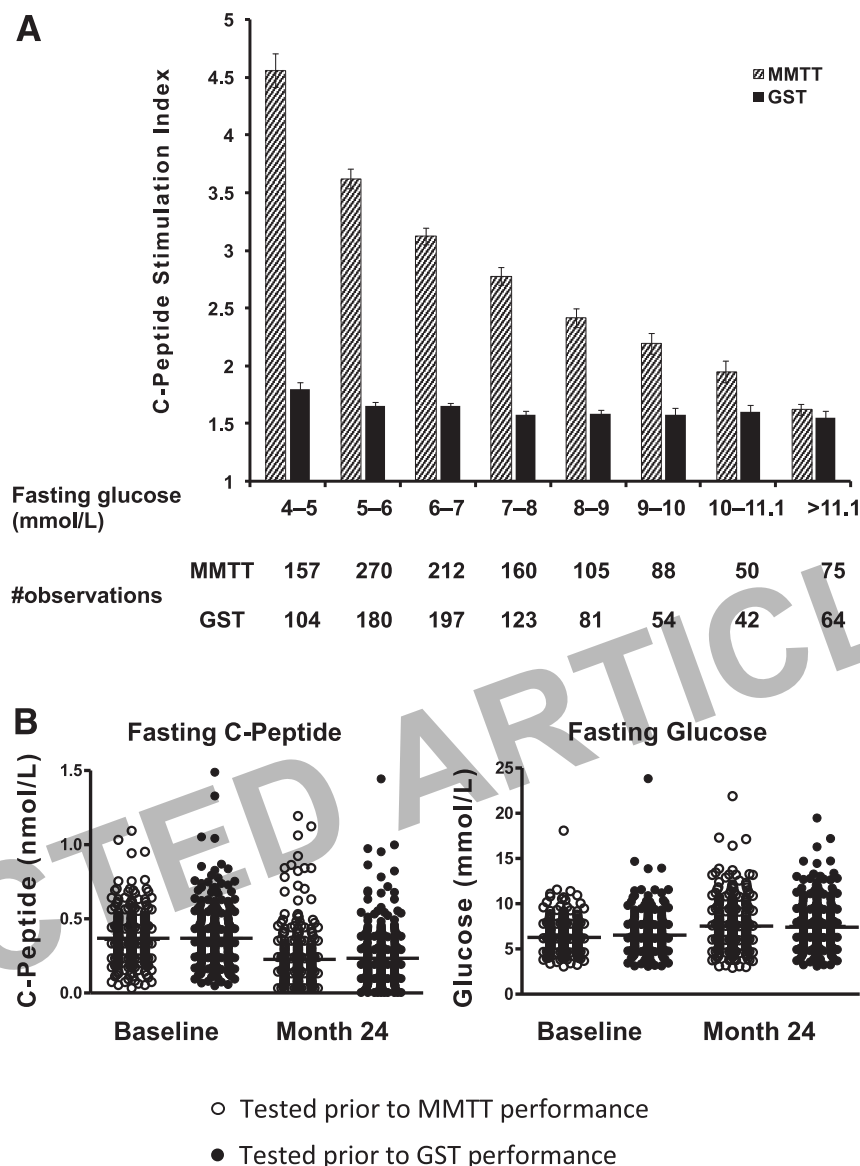
secretion. It is possible that the different storage compartments within the β-cell are differently affected by the progression of diabetes and possibly by the interventional treatment (10,17).

The incretin effect plays a role in the MMTT, but not in the GST (11). Indeed, the contribution of the incretin response to the MMTT was recently discussed in type 1 diabetes (18). Moreover, the MMTT elicits a response in insulin secretion because of the combined action of gastric inhibitory

polypeptide and glucagon-like peptide-1 (GLP-1); the GST, by contrast, has a direct effect on the β-cell (19).

Endogenously secreted GLP-1 affects the regulation of postprandial glucose excursions in type 1 diabetes by modulating glucagon levels, gastric emptying rate, and β-cell responsiveness to glucose (18). Approximately 50% of type 1 diabetic patients may have abnormalities in gastric emptying (without correlation with symptoms) that could confound

interpretation of C-peptide concentrations post-MMTT (20–22).

Thus the MMTT may be affected by gastrointestinal mechanisms triggered by an oral nutrient load, which can be variable during the peridiagnosis period of type 1 diabetes (23,24).

A recent study has demonstrated that type 1 diabetic patients in partial remission have higher levels of proinsulin and lower levels of GLP-1 and glucagon and that GLP-1 differed significantly between patients in remission and not in remission (25). It was also shown that high proinsulin and low GLP-1 levels measured close to diagnosis could predict remission after 1 year, indicating that the actions of GLP-1 influence partial remission. These observations are in agreement with a previous study showing that GLP-1 can reverse the antiproliferative effect mediated by inflammatory cytokines such as IL-6, TNF-$\alpha$, and IFN-$\gamma$ in rat islets (26). Thus it could be considered that cytokine inflammation may be less aggressive in patients in remission and that the GLP-1 level as a response to that is lower. Therefore, we may speculate that in patients who have responded to therapy such as those treated with DiaPep277, stimulation with glucagon may elicit a higher response of C-peptide while stimulation with mixed-meal may elicit a lower response, thus masking the treatment effect.

Stimulation of C-peptide is dependent on the blood glucose level (11,15,27), and this is the basis for the requirement that the test be performed only within certain glucose limits (4–11.1 mmol/L). Here, stimulation by the GST was stable over the permitted glucose range; in contrast, stimulation by MMTT showed a marked decrease at higher glucose levels, similar to that shown by Madsbad et al. (27). It could be speculated that MMTT-stimulated C-peptide may be affected by daily fluctuations in fasting glucose levels that are common in patients with type 1 diabetes (28,29). Regardless of any actual change in β-cell function, a subject with increased fasting glucose level could manifest an attenuated C-peptide response by MMTT but not by GST.

Both GST and MMTT are valid and accepted methods for C-peptide stimulation (11). The MMTT is affected by the physiological status of the patients, which is very variable during the first year of type 1 diabetes. On the other hand, the GST directly stimulates the endogenous insulin secretion regardless of the physiological status of the patient.

While further research is required, the analysis presented here suggests that since different stimuli measure different outcomes, both GST and MMTT should be used for evaluating changes in insulin secretion in newly diagnosed type 1 diabetic patients.

This strategy is being implemented in our ongoing second phase 3 intervention study with DiaPep277 (DIA-AID 2).

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