Brooks-Worrell B, et al. “Islet autoimmunity is highly prevalent and associated with diminished β-cell function in patients with type 2 diabetes in the GRADE Study”

Supplement

Statistical Analysis

Power and Sample Size: Sample size for the ancillary study was determined to detect differences in β-cell function among T cell positive and negative patients in the GRADE longitudinal study over 3 years. Sample size and power calculations used data from two previous studies [1,2] with similar duration of diabetes and expected trajectory of β-cell function in patients that suggested an effect size of d = 0.37 with standard error of se = 2.37. Using the R-package 'longpower' [3] (https://CRAN.R-project.org/package=longpower) for a linear longitudinal analysis with 3 time points, and allowing for 10% dropout rate, n=102 samples from each treatment arm of GRADE (n=408 total) were found necessary to achieve 90% power for detecting this difference at a significance level of α=0.05.

To calculate the power of our tests for detecting differences in baseline β-cell functions among T cell positive and negative patients, a retrospective analysis was performed based on multivariable linear regressions on multiply imputed data. Given the use of Holm's procedure for multiple comparison adjustment,[4] we calculated the smallest effect size based on Cohen's formula ($f^2 = (R^2_{A,B} - R^2_A)/ (1 - R^2_{A,B})$) [5] for the association between two measures of β-cell function (AUC-CG and C-peptide index) and T cell status, adjusting for either 1/fasting C-peptide or C-peptide based HOMA2-S and other covariates described in the main paper. Here, $R^2_{A,B}$ is the proportion of variance in β-cell function explained by the full model and R^2_A is the proportion of the variance explained by the reduced model excluding T cell status. Using the 'wp.regression' function from 'WebPower' package [6] (https://CRAN.R-project.org/package=WebPower), for n=322

samples with non-missing T cell status, our multiple regression models have >99% power to detect the average minimum effect size of $f^2 = 0.14$ observed across the multiply imputed regressions.

Analyses for primary and secondary hypotheses: To test the primary hypothesis of the study, namely whether islet cell autoimmunity is associated with greater β-cell dysfunction at baseline among T2D patients in GRADE, two measures of β-cell function, namely, incremental AUC-CG and C-peptide index, were considered. Analysis of 273 participant samples with both insulin and C-peptide levels measured in the OGTT samples indicated a high correlation between AUC-based calculations using C-peptide values and those using insulin values (Spearman rho=0.91).

The association between the two measures of β-cell function, i.e., incremental AUC-CG and C-peptide index, with T cell positivity were assessed using linear models with one of the β-cell function measures as the outcome. Both measures are ratios, which can cause issues when used as outcome in linear regression [7]. Moreover, as shown in **Fig S4**, both measures have skewed distributions. To address these issues, the log transformed β-cell measures, i.e. log(incremental AUC-CG) and log(C-peptide index), were used as the outcomes in the multivariable regressions. The effect of this log transformation is to include the denominator of the ratio as an offset term in linear regression, which mitigates the potential issues rising from the use of ratios [7]. However, log transformation of the outcome can also cause issues in the presence of heteroscedasticity [8].

Examining variances of regression outcomes, i.e. log(incremental AUC-CG) and log(C-peptide index), indicates that heteroscedasticity is not a major concern. In particular, the 95% CI for ratio of variances obtained using the 'var.test' function in R is (0.6,1.1), which indicates that variances of log(incremental AUC-CG) in T cell-positive and -negative patients (0.29 and 0.35, respectively) are not statistically different. Similarly, while the variances of log(C-peptide index) in T cell-positive and -negative groups (0.32 and 0.44, respectively) are slightly more distinct, they are not statistically different either, as indicated by the 95% CI for ratio (0.53, 1.02).

We also examined the heteroscedasticity of the regression outcomes, log(incremental AUC-CG) and log(C-peptide index) across the quantitative T cell reactivity measure using the tools in the 'ggfortify' R package [9]. In particular, the residuals-versus-fitted plots and the QQ-lots for normality (**Fig S5**) in regressing models with either log(incremental AUC-CG) or log(C-peptide index) as outcome and T cell reactivity as independent variable indicate no significant trends, or evidence of non-normality. The log transformation and the lack of significant heteroscedasticity justifies the use of the above linear models. To further guard against effects of potential misspecification on validity of inference results, inferences were carried using robust (sandwich) standard errors for regressions with multiply imputed data. As a result, the study findings are valid in terms of projections to the space of covariates, even in the presence of misspecification [10,11].

To assess the association between the measures of β-cell function and T cell status, key variables known to affect insulin secretion were pre-determined and included as covariates in the models. The covariates include antibody positivity, age, sex, BMI, duration of diabetes, medications other than metformin and a measure of insulin sensitivity. Two parameters were considered as measure of insulin sensitivity: 1) the homeostasis model assessment-2 for sensitivity based on fasting C-peptide and 2) 1/fasting C-peptide. HOMA2-S of steady state insulin sensitivity was calculated using the HOMA-2 Calculator version 2.2.3 (Diabetes Trials Unit, University of Oxford, Oxford, UK). The HOMA computer module (HOMA2 using insulin values) is correlated with "gold standard" measures of insulin sensitivity (e.g., hyperinsulinemic euglycemic clamp, rapid sampling IVGTT) with (Pearson) correlations in the 0.6–0.7 range [1,2,12,13]. HOMA can be seen as a surrogate for the gold standard measures, which can be interpreted as a covariate measured with error in the regression model. While HOMA is not the exposure of interest in our analyses, measurement error in HOMA can lead to incomplete adjustment for insulin sensitivity or reduced power [14]. Based on data from 3637 GRADE participants with both insulin and C-peptide measurements at baseline, HOMA2-S calculated using C-peptide levels correlates well with that calculated using insulin levels (Spearman rho=0.86). Data from 302 participants with both measurements in our ancillary study showed a similar correlation (Spearman rho=0.88).

Similar models and inferences were also used for assessing the association between β-cell function and T cell reactivity (number of islet blots) and autoantibody positivity. Plots were generated using the 'ggpubr' package in the R-programming language (version

3.6.1). Spearman correlation coefficient was used as a robust (rank-based) alternative to the Pearson correlation coefficient. Exact tests for proportions [5] using the R-function 'binom.test' were carried out to test the null hypotheses about the proportion of autoantibody positive or T cell positive patients. Confidence intervals for proportions were also obtained from the same function, using the method of Clopper and Pearson [15], which guarantees exact coverage.

Missing data considerations: Nine covariates had more than 10% missingness, mainly for the variable of T cell reactivity. Principal reasons for missing T cell reactivity data were severe hemolysis noted upon receipt of the overnight shipped samples, or PBMCs observed by microscopy to be of poor quality or insufficient quantity to proceed with the T cell assay. In some cases, PBMCs that underwent the assay were unresponsive to the Con A control standard (i.e., SI $\leq$ 20 despite the PBMCs appearing structurally intact at the start of the assay), and these samples were also included in the missing category. The occurrence of missing data was likely to have occurred randomly and independent of any measures or unmeasured variables (i.e., data is missing completely at random, MCAR), since if only cases with available values for T cell reactivity were utilized, none of the other variables had significant missing values, while their overall pattern of missed data remained the same. Hence, for the present analysis, only data of participants with available T cell reactivity values were utilized whenever the analysis involved T cell reactivity, and the missing values in the other covariates were multiply imputed to mitigate bias and loss of efficiency. Missing values for other covariates were considered to be missing at random (MAR) and were therefore multiply imputed based

on values of observed covariates. In addition to missing values in data from the parent GRADE study, negative values in C-peptide Index (14 cases) and incremental AUC-CG (1 case) were also set to be missing prior to multiple imputation, as negative values for these measures are biologically implausible and likely occurred due to assay errors.

Imputation methods: Multivariate Imputation by Chained Equations (MICE) implemented in R-package 'mice' [16] was used to impute the missing values. In this approach, multiple imputations are performed using fully conditional specification, providing more flexible imputation for covariates with different distributions. Linear models for assessing the relationships between β-cell functions (log-transformed incremental AUC-CG and log transformed C-peptide Index) as dependent variable, and T cell positivity, T cell reactivity and antibody positivity (adjusting for a measure of insulin sensitivity, age, sex, antibody positivity, BMI, duration of diabetes and medications other than metformin) were fit for each of n = 10 multiply imputed data sets, and results were aggregated using Rubin's rules for multiple imputation [19] implemented in the 'mitools' package in R.

Multiple Comparison Adjustment: Our analysis involves testing the association of two measures of β-cell functions (c-peptide index and iAUC-CG) with T cell status (similarly, antibody status and T cell reactivity). Each of these β-cell response measures were also adjusted for insulin sensitivity using one of two parameters (1/fasting C-peptide and C-peptide-based HOMA2-S). Therefore, for each of T cell status, antibody status and T cell reactivity, our analysis involves four hypotheses (correspondingly, confidence

intervals) that need to be adjusted for multiple comparisons. To account for multiplicity, confidence intervals for the estimates of effect size in each of these 4 models and the corresponding p-values were adjusted for multiple comparisons using Holm's procedure for controlling family-wise error rate (FWER) [17,18]. Holm's procedure for p-values is implemented in the p.adjust() function in R (method = "holm"). Given that existing R functions do not facilitate adjustment of confidence intervals for multiple comparisons, we use an in-house R program that implements the Holm's adjustment. More specifically, Holm's adjustment involves multiplying the significance level for each hypothesis (and the corresponding confidence interval) by a multiplier given by $m - r_k + 1$, where m is the total number of hypotheses (here, 4) and r_k is the rank of the p-value for the kth hypothesis (k = 1, ... , m). To obtain adjusted confidence intervals for transformed parameters, (e.g., relative or absolute differences), we first adjust the confidence intervals for the original parameters (i.e., regression coefficients) and then transform the adjusted confidence intervals in the same manner as the unadjusted confidence intervals – this requires exponentiating the confidence interval endpoints for relative differences and using the delta method [20] for absolute differences. The inference results along with multiply adjusted q-values and confidence intervals for original regression parameters, relative differences and absolute differences are summarized in Supplementary Table 1.

Figure S1. Correlation between beta-cell function values estimated by Inc(AUC-CG) and C-peptide index.

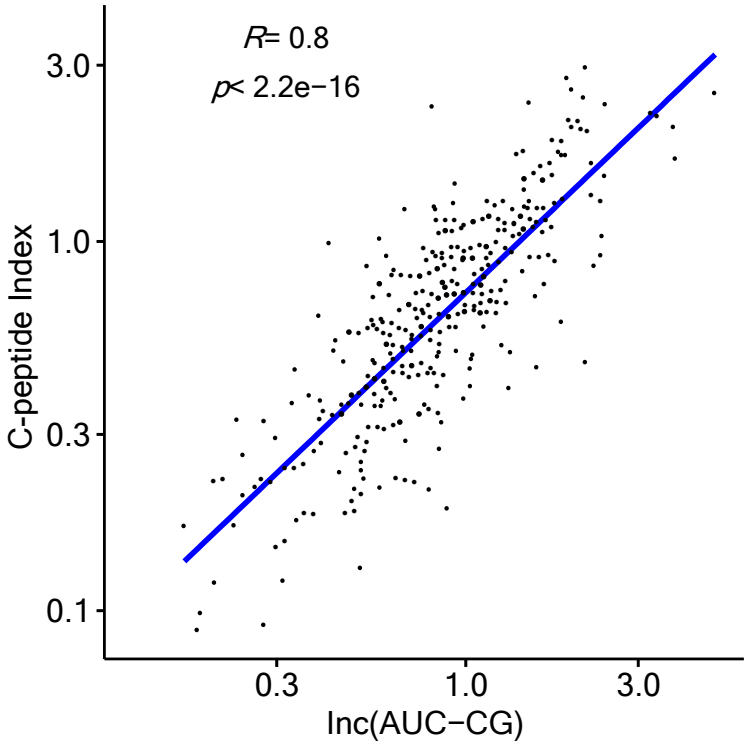
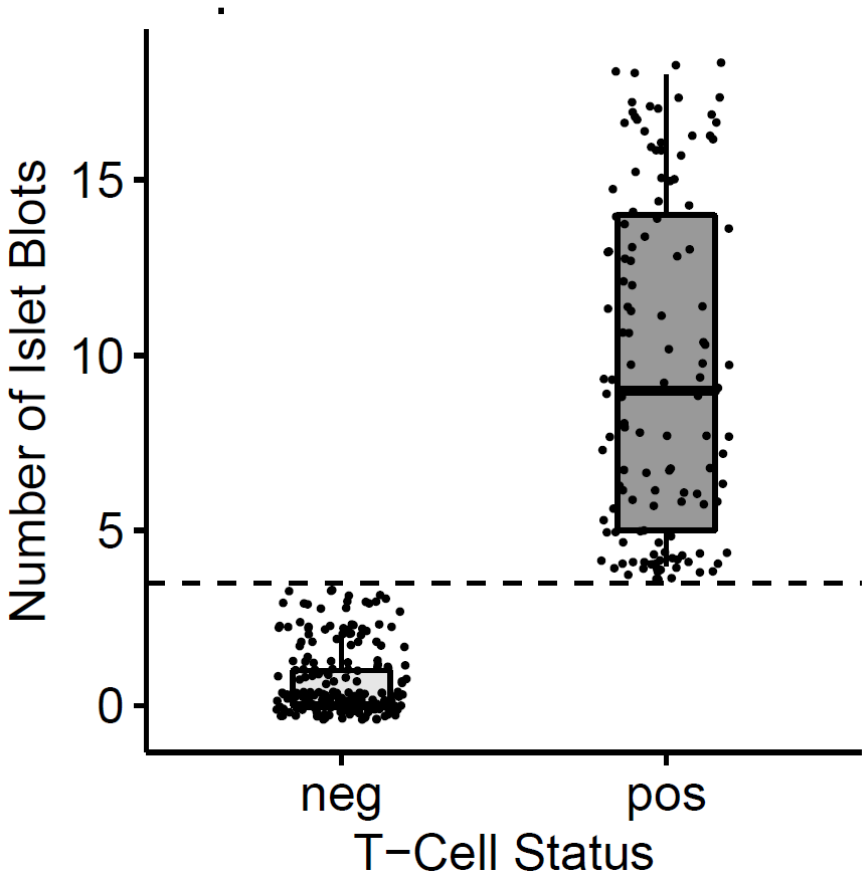
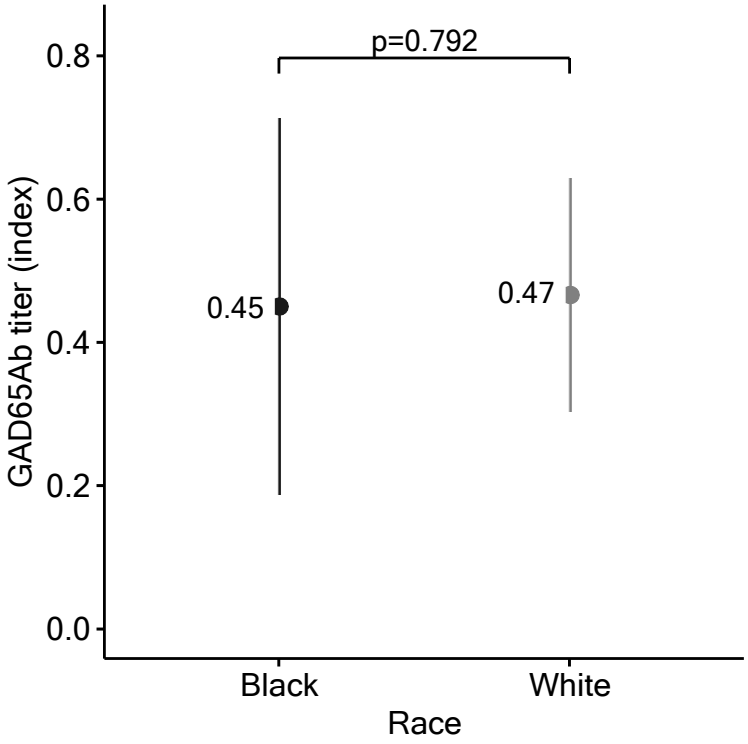


Figure S2. Box-plot of number of reactive blots in T cell-positive (T+) and T cell- negative (T-) groups.



Dashed line indicates cutoff value that distinguishes T cell-negative patients from T cell- positive (T+) patients. T cell-negative (T-) $\leq$ 3 blots; T+ $\geq$ 4 blots. See text for details.

Figure S3. GAD65Ab titers in Black/African American compared with White participants among those who were GAD65Ab-positive.



Note: Black / African American: N=8 GAD65Ab-positive; N=58 GAD65Ab-negative.
White: N=12 GAD65Ab-positive; N=252 GAD65Ab-negative.

Data are mean +/- SE.

Figure S4. Histograms of Inc(AUC-CG) and C-peptide index values.

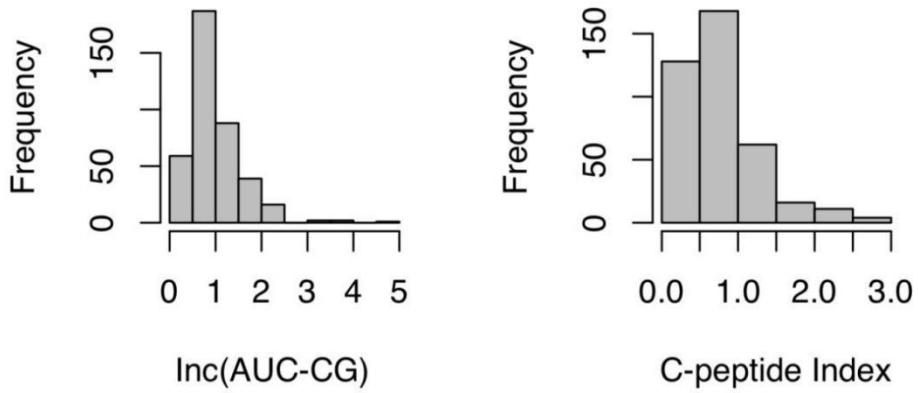
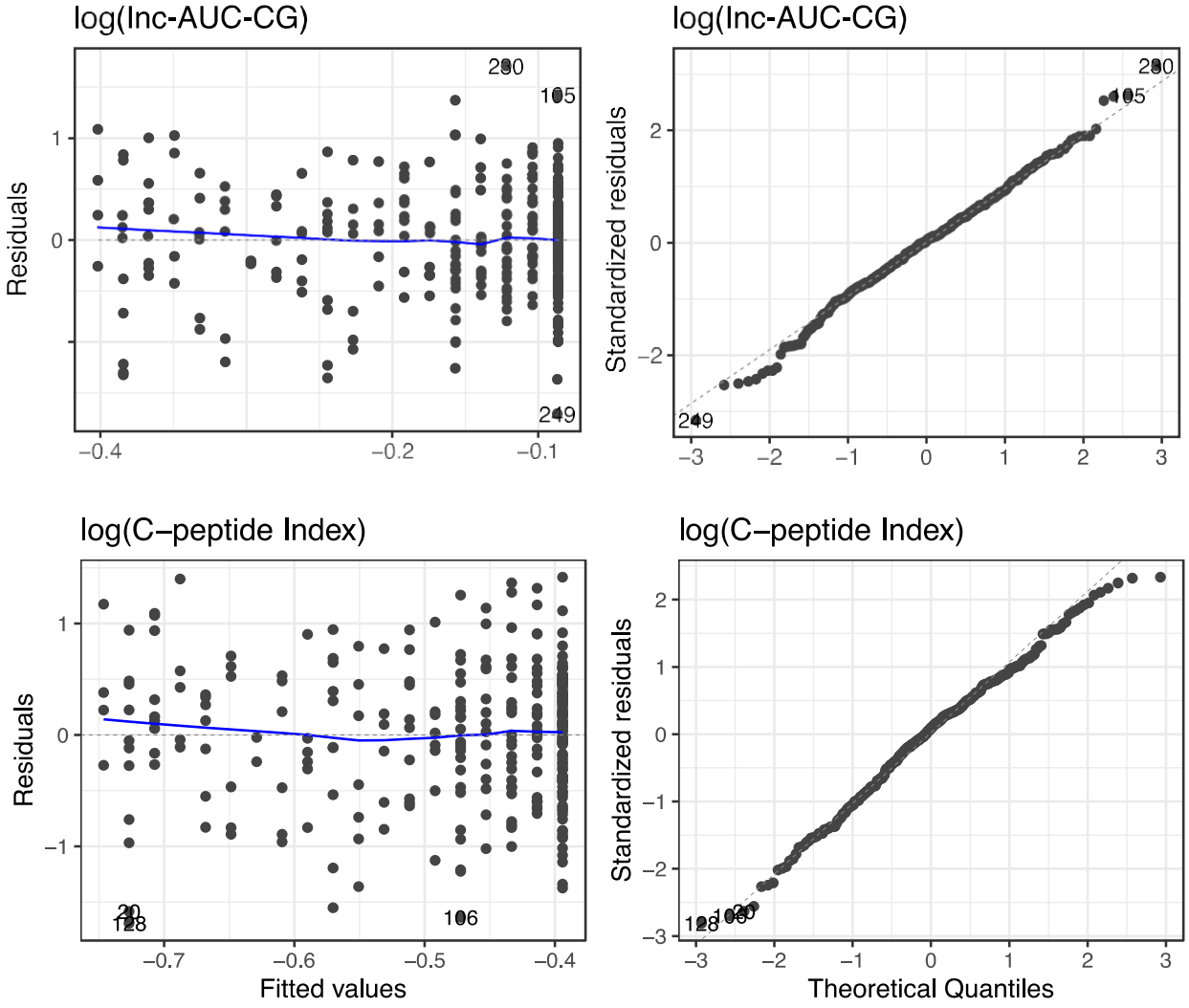


Figure S5. Residual-vs-fitted plots and QQ-plots for assessing the heteroscedasticity of regression outcomes over the range of T cell reactivity values.



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Supplementary Table 1. Summary of inferences for hypotheses involving T cell status, T cell reactivity (continuous variable) and antibody status and measures of β-cell function (C-peptide index and Inc(AUC-CG)).

outcome	adjust	variable	beta	reldiff	absdiff	pvalue	qvalue	beta_CI	rdif_CI	adif_CI	beta_adjCI	rdif_adjCI	adif_adjCI
iAUCCG	1/cpep0	tcellpos	-0.121	0.114	-0.091	0.042	0.044	-0.238,-0.004	0.004,0.212	-0.174,-0.008	-0.238,-0.004	0.004,0.212	-0.174,-0.008
cpepIndex	1/cpep0	tcellpos	-0.195	0.177	-0.114	0.006	0.017	-0.333,-0.057	0.055,0.283	-0.188,-0.041	-0.364,-0.026	0.026,0.305	-0.188,-0.041
iAUCCG	HOMA2	tcellpos	-0.141	0.132	-0.104	0.022	0.044	-0.262,-0.02	0.02,0.23	-0.187,-0.021	-0.279,-0.003	0.003,0.244	-0.187,-0.021
cpepIndex	HOMA2	tcellpos	-0.210	0.190	-0.122	0.003	0.013	-0.351,-0.07	0.067,0.296	-0.195,-0.049	-0.389,-0.031	0.031,0.323	-0.195,-0.049
iAUCCG	1/cpep0	tcellcnt	-0.013	0.013	-0.010	0.022	0.025	-0.025,-0.002	0.002,0.024	-0.019,-0.002	-0.025,-0.002	0.002,0.024	-0.019,-0.002
cpepIndex	1/cpep0	tcellcnt	-0.019	0.019	-0.012	0.005	0.015	-0.032,-0.006	0.006,0.032	-0.02,-0.004	-0.035,-0.003	0.003,0.035	-0.02,-0.004
iAUCCG	HOMA2	tcellcnt	-0.015	0.015	-0.012	0.013	0.025	-0.027,-0.003	0.003,0.027	-0.021,-0.003	-0.029,-0.002	0.002,0.028	-0.021,-0.003
cpepIndex	HOMA2	tcellcnt	-0.020	0.020	-0.013	0.003	0.012	-0.034,-0.007	0.007,0.033	-0.021,-0.004	-0.038,-0.003	0.003,0.037	-0.021,-0.004
iAUCCG	1/cpep0	antibody	0.004	0.004	0.003	0.968	1.000	-0.185,0.193	-0.169,0.212	-0.148,0.154	-0.185,0.193	-0.169,0.212	-0.148,0.154
cpepIndex	1/cpep0	antibody	-0.136	0.128	-0.082	0.200	0.800	-0.345,0.072	-0.075,0.292	-0.2,0.035	-0.403,0.13	-0.138,0.331	-0.2,0.035
iAUCCG	HOMA2	antibody	0.010	0.010	0.008	0.917	1.000	-0.182,0.202	-0.166,0.224	-0.145,0.161	-0.209,0.23	-0.189,0.258	-0.145,0.161
cpepIndex	HOMA2	antibody	-0.132	0.124	-0.080	0.221	0.800	-0.344,0.08	-0.083,0.291	-0.199,0.04	-0.391,0.127	-0.135,0.324	-0.199,0.04

"outcome": the outcome of the regression model (applied after log transformation); "adjust": variable used to adjust for insulin sensitivity (1 over fasting C-peptide or c-peptide-based HOMA2-S); "variable": covariate of interest (one of T cell status (tcellpos), T cell reactivity (tcellcnt), or antibody status (antibody)); "beta": coefficient of regression model; "reldiff": relative difference (fractional change in outcome for change in variable); "absdiff": absolute change in outcome for change in covariate for an average male patient (see main text for); "pvalue": p-value for testing beta=0; "qvalue": adjusted p-value for multiple comparison using Holm's procedure; "beta_CI", "rdif_CI" and "adif_CI": confidence intervals for the original parameter (beta), the relative difference and the absolute difference; "beta_adjCI", "rdif_adjCI" and "adif_adjCI": corresponding adjusted confidence intervals using Holm's procedure.

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