IDENTIFICATION OF AUTOANTIBODY NEGATIVE AUTOIMMUNE TYPE 2 DIABETES PATIENTS

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Running title: Autoantibody negative autoimmune Type 2 diabetes patients.

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Submitted 29 March 2010 and accepted 13 September 2010.

This is an uncopyedited electronic version of an article accepted for publication in Diabetes Care. The American Diabetes Association, publisher of Diabetes Care, is not responsible for any errors or omissions in this version of the manuscript or any version derived from it by third parties. The definitive publisher-authenticated version will be available in a future issue of Diabetes Care in print and online at http://care.diabetesjournals.org.
**Objective:** Islet autoimmunity has long been recognized in the pathogenesis of Type 1 diabetes and is becoming increasingly acknowledged as a component in the pathogenesis of Type 2 diabetes. Islet reactive T cells and autoantibodies (Ab) have been demonstrated in type 1 diabetes. Whereas, islet autoimmunity in type 2 diabetes has been limited to islet autoantibodies. In this study, we investigated whether islet reactive T cells might also be present in type 2 diabetes patients and how islet reactive T cells correlate with beta cell function.

**Research Design and Methods:** Adult phenotypic type 2 diabetes patients (n=36) were screened for islet reactive T cell responses, using cellular immunoblotting, and 5 islet autoantibodies (ICA, GADA, IAA, IA-2A, ZNT8A).

**Results:** We identified 4 subgroups of adult phenotypic type 2 patients based on their immunological status (Ab-T-, Ab+T-, Ab-T+, and Ab+T+). The Ab-T+ type 2 diabetes patients demonstrated T cell responses similar to the Ab+T+ type 2 diabetes patients. Data was adjusted for BMI, insulin resistance and duration of diabetes. Significant differences (p<0.02) were observed between groups for fasting and glucagon-stimulated C-peptide responses. T cell responses to islet proteins were also demonstrated to fluctuate less than autoantibody responses.

**Conclusions:** We have identified a group of adult autoimmune phenotypic type 2 diabetes patients who are Ab-T+ and thus would not be detected using autoantibody testing alone. We conclude that islet autoimmunity may be more prevalent in adult phenotypic type 2 diabetes patients than previously estimated.

Type 1 diabetes results from cell-mediated autoimmune beta cell dysfunction and destruction whereas, type 2 diabetes has been historically considered a metabolic disease (1). However, increasing evidence is pointing toward a relationship between inflammation, insulin resistance, and the subsequent development of type 2 diabetes. In fact, inflammation in the pancreatic islets of type 2 diabetes includes the presence of cytokines (2, 3) and the infiltration of immune cells (3,4).

Despite the fact that the pathological process in autoimmune diabetes involves T-cells, immune markers of autoimmune diabetes have primarily centered on the presence of circulating serum autoantibodies to various islet antigens (5, 6). However, approximately 20% of newly diagnosed autoimmune type 1 diabetes patients are autoantibody negative (7). Recently, it was also found that 9% of autoantibody negative type 1 diabetes patients carry the highest risk HLA genotype DR3-DQ2/DR4-DQ8, strongly suggesting that these patients had autoimmune diabetes but were undetected with autoantibody testing alone (7). Furthermore, Fulminant type 1 diabetes patients, have been reported to be autoantibody negative but demonstrate islet specific T cell responses (8). Therefore, we hypothesized that a group of autoimmune phenotypic type 2 patients may also exist who are autoantibody negative similar to the Ab-type 1 diabetes patients but demonstrate autoimmunity with islet reactive T cells.

Over the years, we have been investigating islet specific T cell responses using cellular immunoblotting in diabetes patients (9-13). Our assay has been validated to have excellent specificity and sensitivity for the detection of islet reactive T cells in
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type 1 diabetes patients (9, 10). Moreover, we have previously observed that T cell reactivity to islet proteins correlates more strongly with impaired β-cell function compared to autoantibody positivity (12). In this study, we utilized our validated T cell assay for the detection of islet reactive T cells to investigate whether autoantibody negative T cell reactive adult phenotypic type 2 patients could be identified. We provide evidence for the existence of this subgroup of autoimmune phenotypic type 2 diabetes patients. We conclude that islet autoimmunity may be more prevalent in adult phenotypic type 2 diabetes patients than previously estimated and assessing autoimmunity through T cells is of importance.

METHODS

Subjects. In this study, we wanted to determine the autoimmune status of recently diagnosed type 2 diabetes patients ages 35-70 years. We screened patients within 5 yrs of diagnosis. However, since diagnosis of type 2 diabetes is often not as definitive of a diagnosis as type 1 diabetes, our inclusion criteria included HbA1c <8.0, no patients on insulin, and patients needed to be able to control glucose levels with only one oral diabetes medication. The inclusion criteria of HbA1c<8.0 was used to ensure all patients were type 2 diabetes patients without “severe” disease thus not requiring insulin treatment. These patients were either obese or had an increased waist-to-hip ratio, had no history of ketonuria or ketoacidosis, and were consecutively chosen from patients meeting the criteria. Thirty-six phenotypic type 2 diabetes patients participated in this study. Patients were evaluated for T cell responses, using cellular immunoblotting, and autoantibody responses to islet proteins (ICA, GADA, IAA, IA-2A, ZnT8A). Written informed consent was obtained from each patient prior to sample collection. Patients were subjected to blood draws on 2 or 3 occasions 3 months apart. Patients were classified as autoimmune if 2/2 or 2/3 tests were positive for any antibody or T cell assay or were classified as non-autoimmune if 2/2 or 2/3 tests were negative.

Autoantibody assays. Assays for autoantibodies (ICA, GADA, IAA, IA-2A) were first performed (screening assays) in Seattle WA and then confirmed in other laboratories (confirmatory assays). All serum samples, both screening and confirmatory, were sent to the laboratories blinded.

Screening Autoantibody assays. Screening autoantibody assays for IAA, IA-2A and GADA were performed at the Northwest Lipid, Metabolism, and Diabetes Research Laboratories at the University of Washington (NWLRL), Seattle, WA. Islet cell autoantibodies (ICA) assays were performed in our laboratory. Zinc transporter autoantibody (ZnT8A) assays were performed by Dr. Liping Yu at the Barbara Davis Center, Denver, CO.

GAD-65 Autoantibody assay (GADA). GADA were measured in a radiobinding immunoassay on coded serum samples as described (14). The levels of GADA were expressed as a relative index (GAD index) using one positive serum (Juvenile Diabetes Foundation (JDF) World Standard for ICA) and three negative standard sera from healthy subjects. GAD index was calculated and a positive was set at >0.085 which is the 99th percentile based on 200 normal controls. Positive and negative controls were run in duplicate in each assay. In the Immunology of Diabetes Society (IDS) sponsored 2007 DASP workshop, the sensitivity of the GAD assay was 78% and specificity was 98% (15).

Insulinoma associated protein-2 autoantibody (IA-2A) Assay. Autoantibodies to IA-2 were measured under identical conditions as described for GADA (14) using the plasmid containing the cDNA coding for the cytoplasmic portion of IA-2. The IA-2A index for each sample was
calculated using the same JDF standard serum and control sera that were used in the GADA assay. An IA-2 index > 0.017, the 99th percentile based on 200 normal controls, was the cut off for positivity. In the IDS sponsored 2007 DASP workshop, the sensitivity of the IA-2A assay was 64% and specificity was 99% (15).

**Insulin autoantibody (IAA) assay.** 125 I-Insulin was incubated with serum and separation was achieved using a 50% Protein-A/8% Protein G-Sepharose mixture. As with the other assays, positivity was set at the 99th percentile of normal controls. In the IDS sponsored 2007 DASP workshop, the sensitivity of the IAA assay was 16% and specificity was 99% (15).

**Zinc Transporter autoantibody assay (ZnT8A).** The presence of the Zinc transporter autoantibody (16) was tested in serum from the patients using a radiobinding assay by Dr. Liping Yu at the Barbara Davis Center, Denver, CO. Cut-off values were set as the mean + 4 SD of the within-assay controls.

**Islet Cell autoantibody (ICA) assay.** This assay was performed as previously described (17). All sera with detectable ICA were end-point titered. The lower detection limit of our assay was 1 JDF and the 95th percentile positivity threshold was established at 6 JDF units based on approximately 4,000 normal school children (17). Our laboratory had participated in a total of 8 Immunology of Diabetes Society Workshops (IDW) and IDS-sponsored proficiency programs for ICA with an average sensitivity of 80% and specificity of 100%. In the IDS sponsored Combined Antibody Workshop, our ICA assay had a specificity of 98% and a sensitivity of 76%. Our ICA assay had been validated in a serum exchange with the Diabetes Prevention Trial – Type 1 Diabetes (DPT-1) ICA core lab. In this exchange, the sensitivity of our assay was 85% with a specificity of 100%.

**Confirmatory autoantibody testing.** For confirmation of autoantibody results, autoantibody assays were performed for ICA in the laboratory of Dr. William Winter at the University of Florida, Gainsville (18). Assays for GADA, IAA, and IA-2A were performed in the laboratory of Drs. Liping Yu and George Eisenbarth at the Barbara Davis Center, Denver, CO. (19).

**ICA measurement.** Confirmatory ICA was measured by indirect immunofluorescence method using cryostat-cut frozen sections of human blood type O pancreas. The results were expressed in JDF units, and a value equal to or more than 10 JDF units was set as positive (18).

**GADA and IA-2A assays.** Confirmatory GADA and IA-2A were measured by a combined radiobinding assay as previously described (19). The interassay coefficients of variation are 10 and 5% for GADA and IA-2A, respectively. The upper limits of normal, nondiabetic sera were established as the 99th percentile of 198 healthy controls. In the 2005 DASP workshop, the sensitivity and specificity were 76 and 99% for GADA and 64 and 100% for IA-2A, respectively.

**Insulin autoantibodies (IAA).** IAA was measured by a micro-radiobinding assay as previously described (20). The interassay coefficient of variation was 20% at low positive levels. In the 2005 DASP workshop, the sensitivity and specificity for mIAA were 58 and 99%, respectively.

**HLA haplotyping.** HLA haplotyping was performed at the University of Pittsburgh by Dr. Massimo Trucco's laboratory (21).

**T cell assay: Cellular Immunoblotting.** Cellular immunoblotting was performed on freshly isolated peripheral blood mononuclear cells to test for the presence of islet reactive T cells (9-13). Briefly, normal human islet cell preparations were subjected to preparative one-dimensional 10% SDS-PAGE, and electroblotted onto nitrocellulose. The nitrocellulose particles containing islet
proteins were used to stimulate PBMCs from patients. Positive responses were determined to be T cell proliferative responses to > 4 blot sections. Human pancreatic islets were obtained from the NIH supported Islet Cell Research Centers (ICR-ABCC). The specificity of the T cell responses from diabetes patients to islet proteins has been demonstrated previously (11). We have participated in 2 distinct NIH supported T cell validation workshops designed to test the ability of several different assays, including cellular immunoblotting, to distinguish T-cell responses to islet proteins of type 1 diabetes patients from controls (9, 10). In both workshops, using masked specimens, cellular immunoblotting distinguished type 1 diabetes patients from controls with high sensitivity and specificity, in the ITN workshop 94% and 83% and in the Trialnet workshop 74% and 88%, respectively (9, 10). To control for inter-assay variation of the islet antigen preparations, the quantity and quality of islets are held constant among preparations, and new antigen preparations are compared to and run alongside of older preparations.

**Confirmatory T cell responses.** PBMCs were analyzed for reactivity to 15 test antigens as described previously (10). A response was considered positive if reactivity was positive to 4 or more antigens. Split samples were available to be sent to Dr. Dosch’s laboratory (Hospital for Sick Children, Toronto Canada) from 11 patients (3 in each patient group except Ab+T-where only 2 patient samples were available). The T cell proliferation assay performed by Dr. Dosch’s laboratory has been validated in the ITN supported study (9) with a sensitivity of 58% and a specificity of 94%. Responses obtained from Dr Dosch’s laboratory confirmed our data (results not shown).

**C-Peptide assays.** Fasting and glucagon-stimulated C-peptide responses were used as a measure of endogenous beta cell function in all patients. Stimulated C-peptide was measured 6 minutes after the IV injection of 1 mg glucagon. The C-peptide assay is a two site immunoenzymometric assay, performed using a Tosoh 600 II auto-analyzer (Tosoh Bioscience Inc., South San Francisco, CA) at NWLRL, Seattle, WA (12). The inter-assay and intra-assay precision analysis showed a coefficient of variation (CV) less than 10%. The assay has a sensitivity level of 0.04 ng/ml.

**HOMA-IR.** To estimate insulin resistance, Homeostasis Model Assessment (HOMA-IR) was calculated from fasting insulin (microunits/ml) and fasting glucose (mg/dl) concentrations using the formula (insulin x glucose)/405 according to Matthews et al. (22).

**Statistics.** The non-parametric Mann-Whitney U test was performed to determine statistical significance between the patient groups. Multiple linear regression analysis was used to estimate associations between measures of C-peptide and Ab and T cell status while adjusting for BMI, HOMA-IR, and duration of diabetes. Adjusted means were computed from these models using Stata 10.1 (College Station, TX). Bonferroni correction was used to account for multiple testing. A p value of <0.02 was considered significant for data adjusted for BMI, HOMA-IR and duration of diabetes.

**RESULTS**

**Subjects.** Patients (n=36) were assayed for their T cell and autoantibody responses and subsequently divided into groups based on their islet autoimmune status. The Ab+T+ patients were all male Caucasian patients whereas, the other patient groups were distributed between different ethnicities. No females were found to be Ab+T+ whereas the other groups were closely split between males and female. The only significant difference (p<0.05) in diabetes duration among the groups was between the Ab-T+ group (2.7 + 1.6 years) compared to the Ab+T- (1.0 + 0.7
years). Therefore, we corrected the C-peptide data for disease duration along with BMI and insulin resistance. We screened patients 2-3 times and were able to classify 75% (27/36) of the patients using 2 blood draws. Only nine patients required a 3rd blood draw for classification. Of the patients requiring a 3rd blood draw, 6/9 demonstrated fluctuating autoantibody responses whereas 3 patients initially negative for T cells demonstrated T cell responses to islet antigens during the 2nd and 3rd blood draws. Low titer ICA (10 JDF units) and IA-2 were the autoantibodies demonstrated to fluctuate. The patient initially identified as Ab-T- but who developed Ab+ and T+ was observed to be positive for IA-2 at 2nd blood draw and GAD at the 3rd blood draw. After screening, ten of the patients were determined to be negative for both islet autoantibodies and islet reactive T cells (Ab-T-), 15 were Ab-T+, 7 were Ab+T+, and 4 were Ab+T-.

Confirmatory Autoantibody responses. All Ab-T- and Ab-T+ patients were confirmed to be autoantibody negative for ICA, IAA, GAD, IA-2 and ZnT8A. The Ab+T- patients were limited to either ICA alone (1 patient) or IA-2 alone (2 patients) or IA-2 with ICA (1 patient). Three of the Ab+T+ patients were positive for both GADA and ICA, two patients were positive for ICA alone, 1 patient was positive for GAD alone and 1 patient was positive for IA-2 alone. Confirmatory autoantibody results were consistent with screening results for samples tested.

T cell reactivity to islet proteins. The number of blots stimulatory to T cells from the patients in each of the 4 groups is shown in Figure 1. There was no significant difference in the number of blots stimulatory to the T cells between the Ab-T+ and Ab+T+ groups. T cell responses to islet proteins from a subset of patients in each group were confirmed by Dr. Dosch’s laboratory in Toronto, Canada (data not shown).

Beta cell function. There were no significant differences between the patient groups in BMI, HbA1c, or age of onset (data not shown). Mean data for C-peptide responses (fasting, glucagon-stimulated, and C-peptide difference) were adjusted for BMI, HOMA-IR, and duration of diabetes. Fasting C-peptide was not calculated for the data adjusted for HOMA-IR as the outcome of fasting C-peptide and HOMA-IR are going to be highly correlated as both calculations reflect insulin resistance. Results are shown in Table 1. For fasting C-peptide, both the Ab+T+ and Ab-T+ patients demonstrated a significantly lower response (p<0.02) compared to the Ab+T- patients when the data was adjusted for BMI and diabetes duration. The Ab+T+ patients demonstrated a significantly lower fasting C-peptide when compared to the Ab-T- patients when the data was adjusted for disease duration but not BMI. For glucagon-stimulated C-peptide, the Ab+T+ and Ab-T+ groups demonstrated significantly lower responses (p<0.02) compared to both the Ab-T- and Ab+T- groups when the data was adjusted for all three variables. There were no differences observed in fasting C-peptide or stimulated C-peptide between the T- (Ab-T- and Ab+T-) or T+ (Ab-T+ and Ab+T+) groups.

HLA genotyping. The DQB1 and DRB1 genotypes of the patients were categorized as either "Risk-Associated" if they had HLA genotypes commonly associated with development of type 1 diabetes (0201/0301, 0302/04, or 0502/1601), "Protective" if they had HLA genotypes commonly associated with protection from type 1 diabetes (0602/1501, 0303/0701) or as "Other" (0501/X, 503/1401, 0301/0401). There were no significant differences in the presence of HLA genotypes between the groups of patients (data not shown).

DISCUSSION
In 2006-2007, our group was investigating the relevance of screening for autoantibodies versus islet reactive T cells to identify type 2 patients with varying degrees of beta cell function. We observed the potential existence of a group of phenotypic type 2 patients negative for autoantibodies but positive for islet reactive T cells (12). At that time it was decided that a study to investigate the potential existence of this new subgroup of type 2 diabetes patients (Ab-T+) along with their demographic and beta cell functional status was needed. Thus, the research presented in this manuscript was initiated. We recruited other laboratories recognized for their expertise in assaying either autoantibodies or T cells in diabetes patients for confirmation of both our positive and negative results. We observed that 11/36 (31%) of the type 2 patients were Ab+ with only 4/11 (36%) of the Ab+ patients positive for GADA. If we had defined autoreactivity in this study as GADA+ alone, we would have missed 22/26 (85%) of the autoimmune patients since 0/4 Ab+T- patients were GADA+ and only 4/7 (57%) Ab+T+ were GADA+. Our data demonstrates that autoantibody reactivity may also be more prevalent in type 2 patients than reported previously if multiple autoantibodies are analyzed. One important issue to keep in mind is that the islet autoantibodies we screened for in our study were autoantibodies that have been identified as being important in type 1 patients. The autoantibody negative T cell positive patients may have autoantibodies that are as yet undefined which could raise the percentage of type 2 diabetes patients exhibiting islet autoimmunity even further. Therefore, based on our results, we propose that a sub-group of autoantibody negative islet reactive T cell positive adult phenotypic type 2 diabetes patients (Ab-T+) exists that have diminished beta cell function similar to Ab+T+ phenotypic type 2 diabetes patients.
the age at onset among the groups or the time from diagnosis as all patients are within 5 years of diagnosis. We observed that the autoantibody responses appear to fluctuate more commonly than the islet reactive T cell responses. We also observed the development of islet autoimmunity (development of autoantibodies and/or T cells) in a number of patients screened 2 or 3 times within a short period of time (3 months). This may also add to the misclassification of patients in studies if studies are initiated using only one screening result to classify patients.

Comparing the C-peptide responses between the patient groups, we observed that fasting C-peptide was only different between the Ab+T+ and Ab-T- groups if the data was adjusted for diabetes duration but not BMI. In contrast, both Ab+T+ and Ab-T+ had a significantly lower fasting C-peptide when compared to the Ab+T- group. For the stimulated C-peptide, a significantly lower response was observed in both T+ subgroups compared to the T- groups when adjusted for BMI, insulin resistance and diabetes duration. These data suggest there are most likely differences between the different subgroups, which could have an influence on outcomes of clinical trials if the patients are misclassified. Unfortunately, this study was initiated to only screen newly diagnosed type 2 diabetes patients to determine if the Ab-T+ patient population existed as a substantial subpopulation of type 2 diabetes patients. The lack of longitudinal follow-up in this study precluded us from determining if the presence of the islet reactive T cells correlated with a decline in beta cell function, or if the patients that were singularly positive for either autoantibodies or T cells would become positive for both with long term follow-up. Though the sample size in our manuscript was sufficient to identify the 4 subgroups of T2D patients, future studies to determine the prevalence of these subgroups should incorporate much larger patient sample size.

Based on results published by Greenbaum et al (25), the mixed meal tolerance test (MMTT) has been demonstrated to be superior to the glucagon-stimulation test and thus should replace the glucagon-stimulation test for future studies. However, the study outlined in this manuscript was underway before the results of the comparison of MMTT and glucagon-stimulation tests were available (25).

As mentioned above, the prevalence of autoimmunity as demonstrated by testing for multiple autoantibodies and islet reactive T cells among adult phenotypic type 2 diabetes patients is unknown at this time. Future studies investigating the prevalence of autoimmunity in type 2 diabetes patients are needed. Our data suggests that islet autoimmunity may be more common than previously thought and may be an important contributor to the progressive decline in beta cell function observed in phenotypic type 2 diabetes patients. This is the first study identifying a group of phenotypic type 2 diabetes patients demonstrating autoimmunity to islet proteins without the presence of islet autoantibodies.

Author Contribution: Barbara Brooks-Worrell, Jessica Reichow, Amit Goel, Heba Ismail, Jerry Palmer all assisted in researching data, contributing to discussion, writing and editing the manuscript.

ACKNOWLEDGEMENTS
We extend our sincere thanks to Pam Mansfield R.N. (University of Washington) for her assistance in recruitment and scheduling of the study subjects, and Drs. Yu and Eisenbarth (Barbara Davis Center, Denver, CO), and Dr. Winter (University of Florida, Gainsville) for confirmatory autoantibody results. We would also like to thank Dr. Trucco (University of Pittsburgh) for performing HLA typing on our patients.
and Dr. Ed Boyko (University of Washington, Puget Sound Health Care System) and the Clinical Research Core of the Diabetes Endocrinology research group at the University of Washington for helping with statistical analysis. This work was supported (in part) by the Medical Research Service of the Department of Veterans Affairs and GlaxoSmithKline. In addition, the following National Institute of Health grants provided partial support: P01-DK053004 and P30-DK017047.

REFERENCES


FIGURE LEGENDS.

**Figure 1.** Number of blot sections stimulatory to T cells responding for each of the patient categories. The number of positive blot sections are demonstrated on the y-axis. Patient groups are shown on the x-axis. A positive response to 4-18 blot sections is similar to responses of T1D patients (9).

**Table 1.** Mean C-peptide data on 4 subcategories of phenotypic T2D patients adjusted for BMI, HOMA-IR, and Disease Duration.

<table>
<thead>
<tr>
<th>Patient Categories</th>
<th>DATA Adjustment</th>
<th>Ab-T- (n=10)</th>
<th>Ab+T- (n=4)</th>
<th>Ab-T+ (n=15)</th>
<th>Ab+T+ (n=7)</th>
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<td>Mean + SD Fasting C-peptide</td>
<td>3.9 + 1.45</td>
<td>5.8 + 1.38</td>
<td>3.1 + 1.39†</td>
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<tr>
<td>Mean + SD Glucagon-stimulated C-peptide</td>
<td>7.25 + 2.28</td>
<td>10.7 + 2.14</td>
<td>5.61 + 2.13³†</td>
<td>4.06 + 2.17³†</td>
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<tr>
<td>Mean + SD Change in C-peptide</td>
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<td>4.91 + 1.24</td>
<td>2.51 + 1.24³†</td>
<td>1.55 + 1.27³†</td>
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<td>Mean + SD Fasting C-peptide</td>
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<td>Mean + SD Glucagon-stimulated C-peptide</td>
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<td>5.98 + 1.86³†</td>
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<tr>
<td>Mean + SD Change in C-peptide</td>
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<td>4.6 + 1.22</td>
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<tr>
<td>Mean + SD Fasting C-peptide</td>
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<td>3.2 + 1.43³</td>
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<tr>
<td>Mean + SD Glucagon-stimulated C-peptide</td>
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<td>10.3 + 2.32</td>
<td>5.61 + 2.29³†</td>
<td>3.7 + 2.25³†</td>
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<tr>
<td>Mean + SD Change in C-peptide</td>
<td>3.6 + 1.3</td>
<td>4.72 + 1.36</td>
<td>2.48 + 1.36³†</td>
<td>1.35 + 1.30³†</td>
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* p<0.02 compared to Ab-T-
† p<0.02 when compared to Ab+T-

**Figure 1.**
Autoantibody negative autoimmune Type 2 diabetes patients

![Graph showing the number of blots positive for different patient groups.](image)