Exploring Autoimmune Features in Type 2 Diabetes: A study on Autoantibody Prevalence

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Abstract –

Type 2 diabetes mellitus (T2DM) has been associated with metabolic and immunological dysregulation, yet the role of incretin autoantibodies, such as those against glucagon-like peptide-1 (GLP-1), remains poorly investigated and defined. The aim of this study was to explore relative GLP-1 autoantibody levels in individuals with T2DM in comparison with healthy controls.

To carry this out, blood samples from 85 individuals were analysed utilising an indirect enzyme-linked immunosorbent assay (ELISA). In order to reduce baseline differences, propensity score matching done using a 1:1 nearest-neighbour design was applied based on age, sex, and different comorbidities. Group comparisons upon acquiring of results were then performed using Welch's t-tests in Graphpad Prism (version 10).

Results showed that mean GLP-1 autoantibody absorbance was lower in the T2DM group compared with controls (2.07 vs 2.74), but this difference was not found to be statistically significant ($t(71) = 1.17$, $p = 0.25$). Confidence intervals crossed zero (-0.47 to 1.8), and the effect size was found to be small ($\eta^2 = 0.019$). Propensity score matching further improved covariate balance across groups however this did not alter the non-significant result. Power justification analysis indicated that with 40 samples per group, this study had adequate power (80%) to detect only moderate-to-large effect size, however insufficient power to reliably detect subtle differences in antibody levels.

These findings suggest that GLP-1 autoantibodies are not significantly elevated in type 2 diabetes mellitus compared with healthy controls. However, it is not possible to exclude small effects which this study did not have the power to detect. Larger studies are thus required to clarify and define the role of incretin-targeting autoimmunity in T2DM pathophysiology.

Introduction –

Diabetes mellitus is one of the largest killers of the 21st century and numbers have only been seen to rise over the past decade. Currently in 2025 it is approximated that there are 539 million individuals living with the disease and this number is projected to rise substantially, reaching values close to 1.3 billion in 2050 (International Diabetes Federation, 2023; Lin et al., 2023). Of these numbers 90 – 95% is accounted for by type 2 diabetes mellitus (T2DM). These numbers show just how necessary research into this field has become in the modern day and yet focus has been majorly on treatment of this issue instead of the cause. The condition is currently defined as a result of two major dysfunctions, the first being decreased and abnormal secretion of insulin from the pancreatic beta-cells, and the latter being the decreased sensitivity of receptors upon insulin-sensitive tissues. This thus presents with the characteristic traits of high blood glucose witness in those with T2DM due to the inability of the required body cells to take up glucose from the insulin signal, leading to an array of other issues.

A variety of hypotheses have been proposed to explain the issue of abnormal secretion of insulin described, with some of these including genetic predispositions, lifestyle, and more recently inflammatory factors. Due to the following of the former two hypotheses and the emphasis on type 2 diabetes mellitus as a metabolic disease, previous full-scale research investigating the presence of immunological responses against major proteins in insulin production and processing have not been carried out. At the moment, no previous suggestions of a major HLA association in T2DM in comparison to the well-known HLA-DR/DQ/B/C in T1DM has been seen. Previous GWAS studies have suggested that beta cell function (TCF7L2, KCNJ11), insulin signalling, adipogenesis and other inflammatory areas are involved in the development of T2DM (Voight et al., 2019; Mahajan et al., 2018). The commonality in symptomatic appearance of conditions such as type B insulin resistance, an autoimmune condition in which autoantibodies against the insulin receptor prevent insulin functionality (Flier et al., 1975), also suggests the possibility of autoimmune factors playing a role in the severity of T2DM. Further, studies showing that macrophage-specific MHC-II knockout in obese mice led to better glucose tolerance, enhanced insulin sensitivity and reduced VAT T-cell infiltration in comparison to wild type (Winer et al., 2009). This highlights the large possibility of MHC II involvement and an immunological roll in T2DM. Similarly, it was shown that in CD8 expansions seen as a result of obesity in early murine studies, depleting CD8 cells in these obese mice showed a reduction in inflammation and increased glucose metabolism (Nishimura et al., 2009). All these pieces of evidence point in the direction of the immune system playing a much larger role than recognised in the development and severity of the disease type 2 diabetes.

In this study, the aim was to investigate the presence of autoantibodies against the important incretin GLP-1 and compare between populations of type 2 diabetics and controls. The choice of GLP-1 is due to its importance in insulin production, with it and glucose-dependent insulinotropic polypeptide (GIP) being responsible for the production of 70% of total insulin in the human body, the likelihood of its dysfunctionality playing a role in the abnormal insulin response seen in diabetics are high. Its mimicry in the drug Semaglutide, a very effective drug in the treatment of T2DM further emphasises this hormones importance in regulation of the insulin and glucose system. Despite this, GLP-1 has had limited research regarding the possibility of its malfunction or abnormality in diabetic mellitus.

Thus, following from the previous discussion of immunological factors likely taking effect within the condition of type 2 diabetes, a hypothesis regarding the possible abnormal blocking or clearance of GLP-1 through antibody targeted responses was formed.

Methods –

To answer the stated aim, a laboratory-based case-control investigating GLP-1 autoantibody levels was carried out. The sample (n = 95) consisted of 40 blood samples from individuals who had not been diagnosed with any form of diabetes at the point of bleed, and a further 45 blood samples from

individuals who were diagnosed with type 2 diabetes (T2DM) were used. To select these samples, subcategorization of the blood samples of a previous study was done, allowing for selection of those who were older than 50 and subsequent organisation into diagnosed or undiagnosed categories. This thus meant that ethical approval had been carried out prior and there was no need to obtain new participants or permission.

Power Justification ~

Power calculations were carried out using Excel to identify the minimum detectable effect size at varying sample sizes for GLP-1 autoantibody measurements. Calculations assumed continuous outcomes and parameters were based on the observed pool standard deviation from ELISA absorbance values. A power of 80% and significance level of 0.05 were specified. Both continuous and binary outcome models were formed and investigated into; however, continuous modelling was considered to be most representative of the absorbance data. The representative power justification graph can be seen in figure 1.

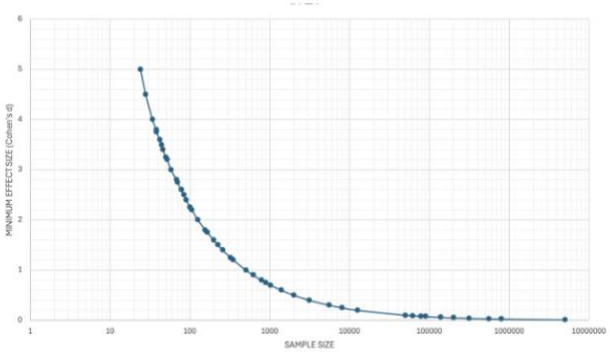


Figure 1 – Power Justification Plot for SD 3

Propensity Matching ~

To reduce confounding factors between the groups and their subsequent effects upon results, propensity score matching was run through the MatchIt package in R (version 4.5.1). This allowed for variables such as age, sex, and other comorbidities, for example, arthritis, hypertension and cancer to be included in the algorithm and matched for. Each diabetes patient was then matched 1:1 without replacement with a calliper set to 0.2 to prevent poor-quality matches and reduce bias. For visualisation and validity checking of balance diagnostics, Love plots, density plots, eQQ and eCDF plots were produced and assessed. These can be seen in figures 2 - 6.

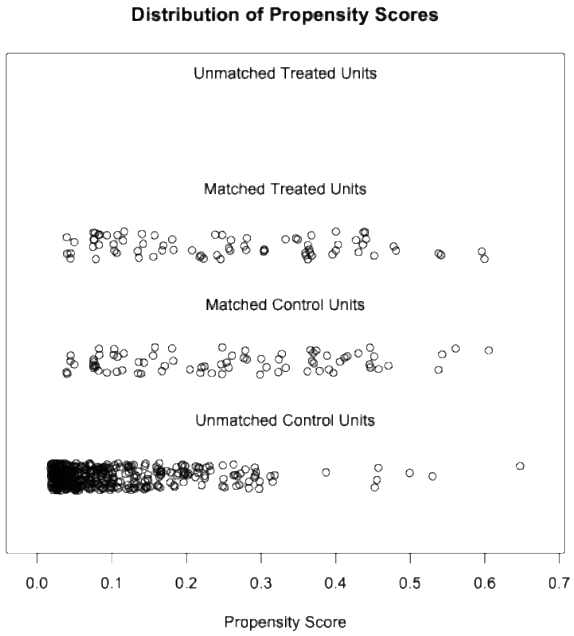


Figure 2 – Propensity Score Distribution

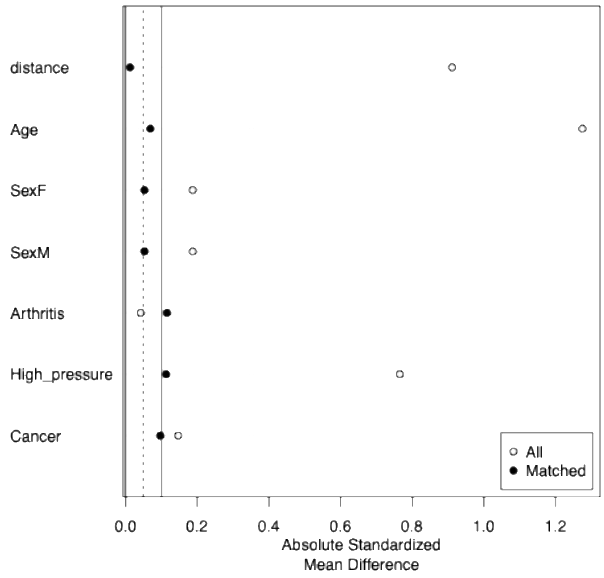


Figure 3 – SD Mean Difference Love Plot

Figure 4 – eCDF Plots

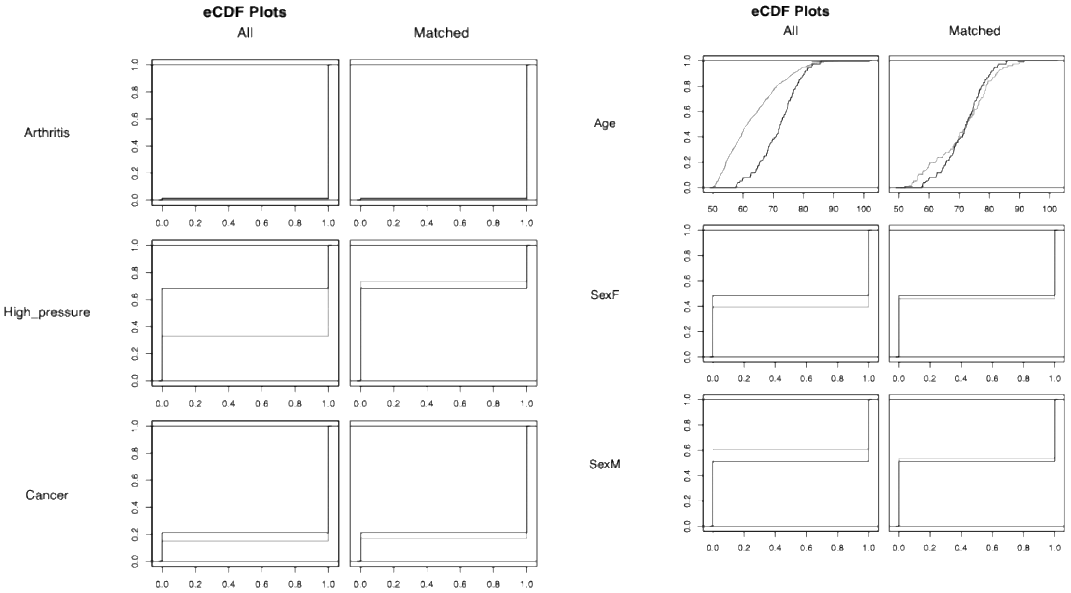


Figure 5 – eQQ Plots

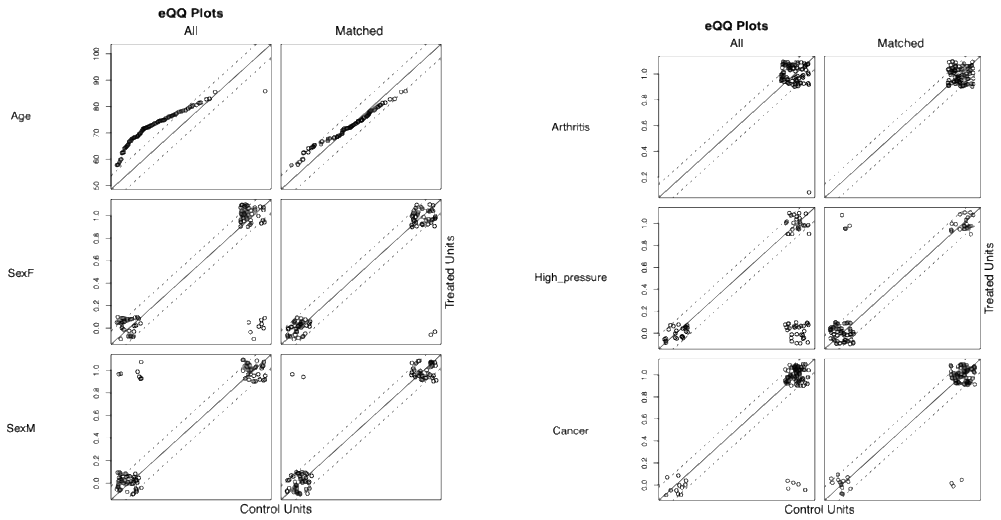
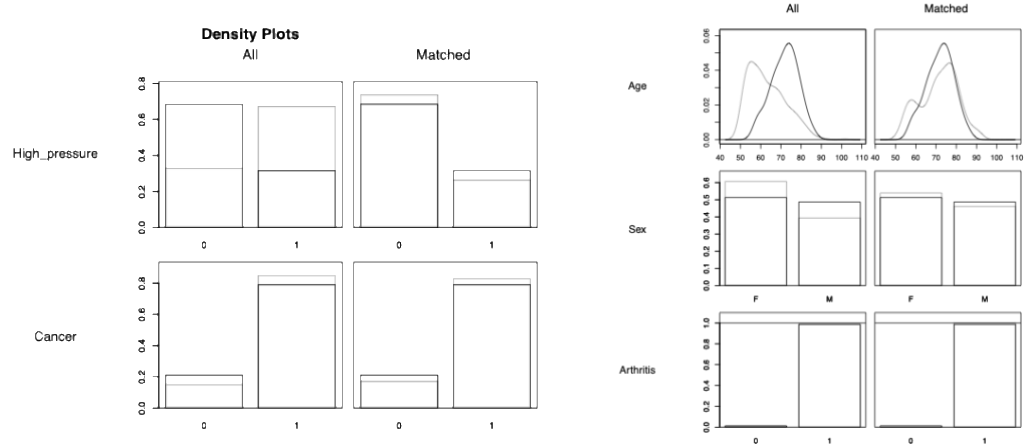


Figure 6 – Density Plots



Enzyme-linked Immunosorbent Assay (ELISA) ~

A verified 96 well ELISA kit produced by the company ThermoFisher[1111] of catalogue number BMS2194 was obtained and utilised. This was chosen due to its usage in previous studies (reference) and recommendations. To prepare the blood samples, these were removed from the freezer and a subaliquot from the original large storage samples formed the day prior to carrying out the ELISA. Following the kit protocol, standards and samples were also diluted and prepared on this day.

The following day, the 96 well microstrips were washed twice using the wash buffer supplied in the kit. Sample dilutant was added to all wells in volumes which would allow for a total of 100uL in each respectively once the 50 uL samples were added following completion of the first step. Samples were added to the plate in the layout displayed in figure 7. The wells were then coated with the antigen, GLP-1 peptide, and following this 50uL of diluted biotin-conjugate was added to all wells and after covering of the plate it was placed into incubation at 20 degrees upon a shaker for 2 hours. After this period, the plate was washed 6 times before the immediate addition of streptavidin-HRP to all wells. The plate was then covered again and left for a further 1 hour in the same conditions as the first incubation. Following this, the adhesive covering was removed again, and the plate was washed a further 6 times. 100uL of TMB substrate solution was added to all wells to allow for development of colour and the microwell strips were left at room temperature (18 to 25 degrees Celsius) for 30 minutes in a covered area. The colour development was monitored periodically using the ELISA absorbance reader at 620nm and the solution was stopped through the addition of 100 uL stop solution when standard 1 had reached an OD of around 0.8. Results were then immediately taken through the absorbance reading utilising a spectrophotometer with 450 nm as the primary wavelength. The plate reader was blanked using the blank well allowing for the determination of the absorbance of both the samples and standards.

Figure 7 – ELISA Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	Ctrl1	Ctrl9	Ctrl17	Ctrl25	Ctrl33	C1	C9	C17	C25	C33	C41
B	S2	Ctrl2	Ctrl10	Ctrl18	Ctrl26	Ctrl34	C2	C10	C18	C26	C34	C42
C	S3	Ctrl3	Ctrl11	Ctrl19	Ctrl27	Ctrl35	C3	C11	C19	C27	C35	C43
D	S4	Ctrl4	Ctrl12	Ctrl20	Ctrl28	Ctrl36	C4	C12	C20	C28	C36	C44
E	S5	Ctrl5	Ctrl13	Ctrl21	Ctrl29	Ctrl37	C5	C13	C21	C29	C37	C45
F	S6	Ctrl6	Ctrl14	Ctrl22	Ctrl30	Ctrl38	C6	C14	C22	C30	C38	
G	S7	Ctrl7	Ctrl15	Ctrl23	Ctrl31	Ctrl39	C7	C15	C23	C31	C39	
H	Blank	Ctrl8	Ctrl16	Ctrl24	Ctrl32	Ctrl40	C8	C16	C24	C32	C40	

Ctrl = Non-diabetic control sample

C = Diabetic case sample

S = standard

Due to the limitation on sample numbers and plates available the process was not carried out in duplicate.

Data Processing and Statistical Analysis ~

Standard curves were generated using 4-parameter logistic (4PL) regression in Graphpad Prism (version 10) utilising the data obtained by the standards provided by the kit. Sample concentrations were then interpolated from the standard curve and the removal of negative values in which optical density fell below the measured blank that were present likely because of technical error was carried

out. These values were retained for transparency but were not included in primary analysis to allow for appropriate calculations and graph formation.

All statistical analysis was performed also through usage of Graphpad Prism (version 10). Comparisons between groups were then formed using unpaired t-tests under Welch's correction accounting for potential inequality of variances between groups. Variance homogeneity was then further assessed through the usage of an F-test. Results were then processed and expressed as means ± standard error of the mean, allowing for more informative results in place of raw means. Alongside this, the range where the true difference likely falls was expressed through the confidence interval (95% CI). Calculations were only identified as statistically significant if proven through a two-tailed $p < 0.05$ value.

Results –

A total of 85 serum samples were analysed, comprising 45 from individuals diagnosed with type 2 diabetes mellitus (T2DM) and 40 from healthy controls (n = 85). Groups were broadly comparable in terms of age and sex distribution following propensity score matching.

Mean GLP-1 absorbance values were lower in the T2DM group in comparison to the controls (2.07 vs 2.74, respectively; shown in figure 8). However, this difference did not reach statistical significance (Welch-corrected t (71) = 1.17, p = 0.246). The mean difference between groups was 0.67 ± 0.56 absorbance units, with a 95% confidence interval ranging from -0.47 to 1.80.

Test	Value	Result
Mean absorbance (T2DM)	2.07	
Mean absorbance (Control)	2.74	
Mean difference ± SEM	0.67 ± 0.56	
95% CI	−0.47 to 1.80	Not significant
Welch's *t* (df=71)	1.17	*p* = 0.25
F-test for variances	F(39,39) = 1.83	*p* = 0.064
Effect size (η^2)	0.019	Small

Figure 8 – Table of Results and Tests

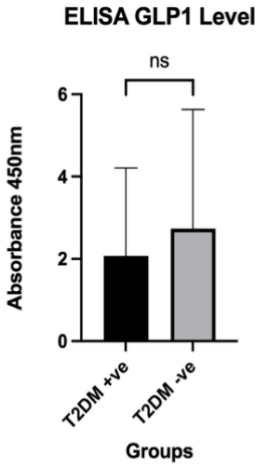


Figure 9 – Bar Graph of Obtained Results

Variance homogeneity was assessed using an F-test. Variability in antibody levels did not differ significantly between the two groups (F (39,39) = 1.83, p = 0.064), thus suggesting that heterogeneity of variance was improbable to be the explanation of the non-significant result as shown in figure 9.

The calculated effect size was small ($\eta^2 = 0.019$), indicating minimal practical difference in GLP-1 autoantibody levels between the T2DM samples and controls.

Power analysis curves were generated alongside the results to assess the sensitivity of the study (figure 1). With around 40-45 participants per group, the study had 80% power to detect effects of sizes moderate-to-large (Cohen's $d \geq 0.6 - 0.7$). This thus corresponds to an approximate minimum

detectable difference of value 1.5 – 1.7 absorbance units, given the observed pooled standard deviation of 2.5. As a result, smaller effect sizes (Cohen's $d < 0.5$) would not be reliably detectable, which may account for the lack of statistical significance in the study.

Discussion –

This study examined levels of GLP-1 autoantibodies in individuals with diagnosed type 2 diabetes mellitus (T2DM) in comparison to matched healthy controls through usage of an ELISA-based absorbance assay. Although mean GLP-1 absorbance among the T2DM group was found to be lower than that among controls (2.07 vs 2.74), the difference did not reach the needed value to suggest statistical significance ($p = 0.246$). Further, the variance between groups was not found to be significantly different, and the effect size was small ($\eta^2 = 0.019$). Power analysis indicated that with 40-45 samples per group, the study was only powered to reliably detect effect sizes following Cohen's $d \geq 0.6 – 0.7$. Together, this data suggests that there is no strong evidence from this sample to state that there is moderate or large elevation or reduction in GLP-1 autoantibody levels in T2DM relative to controls. Due to the limited sample size, subtle differences cannot be denounced and may have been undetected due to limitations in the power or variability.

It has been debated as to whether GLP-1 autoantibodies play a significant role in type 2 diabetes mellitus, or whether their levels differ within T2DM populations resulting in variation of symptom severity. A large majority of the current literature has placed focus upon agonist therapies for the GLP-1 receptor and their effects on things such as glycaemic control, weight loss, and the cardiovascular outcomes. Limited discussion has been had regarding its possible involvement immunologically, more specifically, in association with autoantibodies. A recent trial is an example of this large interest in the former, with the study carried out demonstrating the capability of GLP-1 receptor agonists to reduce HBA1c levels and improve cardiovascular risk markers; all while showing safety in large T2DM populations (Marso et al., 2016).

Studies of endogenous GLP-1 secretion, variability and receptor signalling indicate considerable heterogeneity among individuals with T2DM. Watkins et al. (2023) show this, displaying that GLP-1 secretion in people with T2DM versus non-diabetic controls is inconsistent, with some diabetics showing blunted secretion, and others showing similar levels to their healthy counterparts. This implies that other variables such as comorbidities, age, and BMI, may influence GLP-1 biology.

However, in spite of these studies, there has been no strong precedent for a reliably reproducible increase or decrease in GLP-1 autoantibodies in T2DM. From the investigations into past published assays and trials, it appears that the majority of GLP-1 studies have focused on the functional response to GLP-1 or its actions on receptor agonists. This thus leaves the possibility that if the GLP-1 autoantibodies exist or vary within populations, their effect size may be small or

only apparent under specific conditions, for example patients treated with GLP-1 receptor agonists, or with certain genetic backgrounds.

Evidence regarding this follows beta-cell failure and its biomarkers in a study carried out by Jones et al. (2016) which found that markers of beta-cell failure were able to predict poor glycaemic response to GLP-1 receptor agonist therapy. Following this logic, if autoantibodies were interfering with GLP-1 action, they may be expected to correlate with reduced response to therapy or worse beta cell function; however, this remains speculative in absence of direct autoantibody quantification.

Possible Explanations for Non-significant Findings ~

There are several plausible factors as to why this study did not detect a statistically significant difference.

A major example of this is due to the effect size being too small. Through the carrying out of the power analysis, it can be seen that only moderate to large effect sizes ($d \geq 0.6 - 0.7$) would be detectable with the sample size used ($n = 85$). If in reality the differences in GLP-1 autoantibodies are smaller, these would be unable to be reliably identified in this study.

Another possibility is the high variability in GLP-1 antibody measurements. The observed pooled SD (2.5 absorbance units) is relatively large. Biological variation in antibody levels, low antibody titres near detection limits, or measurement error inherent in ELISA could all contribute to noise. This would in turn reduce the signal-to-noise ratio.

Heterogeneity of the study population could also act as an explanation for these results, as despite matching for age, sex, and basic comorbidities, other unmeasured factors could not be accounted for. Thes include things such as duration of diabetes, glycaemic control, medication use – including previous exposure to exogenous GLP-1 agonists, infection history, immune status, and the possibility of the healthy sample being undiagnosed or pre-diabetic, which could all confound or dilute group differences.

Finally, ELISAs may have limited sensitivity, especially if autoantibodies of low affinity are present at low concentrations. Further, nonspecific binding, cross-reactivity or factors within the blood and serum may have interfered with the detection of these antibodies and subsequently affected results.

Strengths and Limitations ~

The major limitations in this study involve the modest sample size which is unable to detect small effect sizes, and the lack of detailed clinical data. The latter, though some was acquired regarding past medical histories, did not include important factors such as medication use, disease duration, severity, glycaemic control, BMI or obesity. Further, this study was carried out at a single time point and thus temporal variation or causal inference cannot be discussed or addressed.

Despite these limitations, this study had several notable strengths. These include the usage of propensity score matching, which was used to mitigate confounding by age, sex and various other comorbidities. This thus increased comparability between type 2 diabetes mellitus and control groups. The inclusion of a power analysis allows for transparent assessment of what effect sizes the study was able to reliably detect or not detect. Further, use of Welch's correction to accommodate for possible variance heterogeneity adds to the rigour of the results and analysis carried out.

Conclusion -

This study aimed to examine GLP-1 autoantibody presence and levels in individuals with type 2 diabetes in comparison to healthy controls. It was found that mean absorbance values were lower in the diabetes group, the difference was not statistically significant, with a small effect size and wide confidence intervals. Power analysis indicated that the study was only able to detect moderate-to-large differences, suggesting that subtle effects may have gone undetected. Overall, the findings do not support a strong role for GLP-1 autoantibodies in T2DM, but they are unable to state whether there is a smaller effect which may compound with other factors in the condition to produce the disease seen. These results highlight the need for larger, better-powered studies to clarify whether these possible smaller differences may influence disease mechanisms and/or therapeutic responses.

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