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Title: Application of machine learning to assess interindividual variability in rapid-acting insulin responses following subcutaneous injection in people with type 1 diabetes.

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ABSTRACT

Introduction: Circulating insulin concentrations mediate vascular-inflammatory and prothrombotic factors. However, whether interindividual differences in circulating insulin levels are associated with different inflammatory and prothrombotic profiles in type 1 diabetes (T1D) is unknown. We applied an unsupervised, machine-learning approach to assess whether interindividual differences in rapid-acting insulin levels associate with parameters of vascular health in T1D patients.

Methods: We reanalysed baseline pre-treatment meal-tolerance test data from two randomised control trials in which 32 patients consumed a mixed-macronutrient meal and self-administered a single dose of rapid-acting insulin individualised by carbohydrate-counting. Postprandial serum insulin, tumour necrosis factor alpha (TNFα), plasma fibrinogen, human tissue factor (HTF activity) and plasminogen-activator inhibitor-1 (PAI-1) were measured. Two-step clustering categorised individuals based on shared clinical characteristics. For analyses, insulin pharmacokinetic summary statistics were normalised, allowing standardised intra-individual comparisons.

Results: Despite standardisation of insulin dose, individuals exhibited marked interpersonal variability in peak insulin concentrations (48.63%), time to peak (64.95%), and insulin incremental area under the curve (60.34%). Two clusters were computed: cluster 1 (n=14) representing increased serum insulin concentrations; cluster 2 (n=18) representing reduced serum insulin concentrations (cluster 1: 389.50±177.10 vs. cluster 2: 164.29±;41.91 pmol/L.IU.hr^{-1}; P<0.001). Cluster 2 was characterised by increased fibrinogen, PAI-1, TNFα levels, higher HTF activity, higher HbA1c, BMI, and lower eGDR (increased insulin resistance), older age, and longer diabetes duration (P<0.05 for all analyses).

Conclusions: Reduced serum insulin concentrations are associated with insulin resistance and a prothrombotic milieu in individuals with T1D, and may, therefore, be a marker of adverse vascular outcome.

INTRODUCTION

Basal-bolus insulin therapy, consisting of modern insulin analogues delivered through multiple daily injections or by continuous subcutaneous insulin infusion, is the first-line choice for achieving good glycaemic control in patients with type 1 diabetes (T1D)[1]. The pharmacokinetics and pharmacodynamics of the insulin analogues aspart and lispro have been well-characterised[2] and their relatively rapid and short action-time profiles make these preparations suitable mealtime insulin replacements[3]. Despite their small degree of within-subject variability, both analogues display similarly large between-subject variability in insulin kinetics following subcutaneous injection[2, 4]. For example, even under controlled experimental conditions in diabetes-naïve individuals, the interindividual coefficient of variation (CV) of certain pharmacokinetic summary measures has been reported to range between 20-45% for different preperations[4]. Further, work from our group has previously shown the interindividual CV of subcutaneously injected rapid-acting insulin analogues to be ~55% in well-controlled patients with T1D, even when dose is individualised to a standardised meal using the carbohydrate-counting method and when patients achieve 2-hour post-meal target glucose[5, 6].

Insulin has potent effects on vascular tone and endothelial inflammation, and temporal differences in the appearance of circulating insulin following subcutaneous injection are known to influence vascular-inflammatory and prothrombotic factors[7]. For example, insulin exhibits a class effect with different preparations, differing by pharmacokinetics and pharmacodynamics, eliciting divergent responses in proinflammatory cytokines, adhesion molecules, and thrombin formation[7]. However, whether interindividual differences in circulating insulin concentrations, resulting from the subcutaneous injection of comparable insulin preparations, is associated with different inflammatory and prothrombotic profiles is unknown.

To the best our knowledge, no study has aimed to assess whether individual patients can be classified on the basis of similar mealtime rapid-acting insulin responses, and whether and how such classifications cluster with parameters of vascular health. The classification of patients on the basis of their mealtime rapid-acting insulin response and shared clinical characteristics may enable better individualised treatment regimens to reduce the unpredictability associated with current insulin dosing strategies and heterogeneity in the risk of developing vascular complications. With this aim, we applied

an unsupervised, machine learning approach to data from two RCTs[5, 8] characterising individual mealtime insulin responses following subcutaneous injection of rapid-acting insulin in patients with T1D treated on multiple daily injections. We used this approach to establish a novel classification of patients on the basis of rapid-acting insulin responses and assessed whether differences in response were associated with parameters of vascular health.

METHODS

We used data from two previously published RCTs[5, 8] (Clinical trial registration: clinicaltrials.gov NCT02595658; ISRCTN registration ISRCTN40811115). Both studies received ethical approval from local National Health Service Research Ethics Committees (REC reference 14/NE/1183; REC reference 17/NE/0244) and all participants gave written informed consent.

Detailed information regarding each study has been published previously[5, 8] but summarised here. In the original study by Campbell et al[5] patients were exposed to four experimental conditions consisting of the consumption of mixed-meal tolerance tests differing in macronutrient composition and rapid-acting insulin bolus dose and timing. We used data from a single arm in this RCT featuring a meal-tolerance test with self-administration of a single dose of rapid-acting insulin individualised to each patients' carbohydrate-counting requirements. In the original study by O'Mahoney et al[8] participants were randomised to a dietary supplementation intervention or placebo control for 6-months, with meal tolerance tests administered at baseline, 3-months, 6-months, and 9-months. In response to the meal tolerance test, participants self-administered rapid-acting insulin individualised to their carbohydrate-counting requirements. We used across both esarms from this RCT. In both studies, the carbohydrate content of each meal tolerance test was standardised and individualised to 1 g of carbohydrate per kg of body mass. Similarly, rapid-acting insulin was administered immediately before consumption of each meal tolerance test with the site of injection standardised between participants using prominent anatomical landmarks (equidistant from the most medial portion of the iliac crest and navel) with dose based on individual carbohydrate-counting requirements (dose per 10g: 0.9±0.3 IU); no patient received any further corrective dose of insulin during the postprandial observation window. All testing procedures were conducted during a single morning-time laboratory visit. Prior to each visit, patients adopted an

overnight fast (>10-hours), and avoided strenuous physical activity, caffeine, and alcohol in the 48-hours prior to arrival.

On each visit, venous blood samples were obtained via an indwelling catheter placed into the antecubital vein of the non-dominate arm. Blood samples were retrospectively analysed for serum insulin (Invitron insulin; Invitron, Monmouth, UK), tumour necrosis factor alpha (TNFα) (Human TNF-α Quantikine ELISA; R&D Systems, Roche Diagnostics, UK), plasma fibrinogen (ab108842, Fibrinogen Human ELISA Kit; Abcam, Japan), human tissue factor activity (HTF; HTF activity ab108906; Abcam, UK) and plasminogen-activator inhibitor-1 (PAI-1; Human PAI-1/serpin ELISA Kit DSE100; R&D systems, UK) using methods previously described[9]; the intra-assay coefficient of variation was <10% for all biochemical analysis. Further blood samples were obtained at 30-minute intervals for a total duration of 6-hours post-meal and analysed for serum insulin. In addition, we obtained the following physiological characteristics (age, T1D duration, HbA1c, BMI, and blood pressure). Blood pressure was assessed via an automated oscillometric device (Intellisense HEM-907XL, Omron, Japan) with participants categorised as normotensive (<140/90mmHG) or hypertensive (≥140/90mmHG)[10]. Estimated glucose disposal rate (eGDR) was calculated using a composite of BMI, HbA1c and hypertensive status using the following formulae: eGDR = 19.02 – (0.22 X BMI [kg/m^2) – (3.26 X HTN) – (0.61 X Hba1c [%]), whereby HTN is hypertension (1 = yes, 0 = no). Participants were classified as having increased insulin resistance if was eGDR ≤8[11].

In the present analysis, we included participants meeting the following inclusion criteria: aged 18-50 years; diagnosed with T1D for a minimum of 5-years on enrolment; treated with a stable (>12-months) basal-bolus insulin regimen consisting of rapid-acting insulin analogues lispro or aspart and basal insulin glargine delivered through multiple daily injections; familiar with carbohydrate-counting; and free of diabetes-related complications except for early background retinopathy.

Statistical analysis

Data were analysed using SPSS (IBM SPSS Statistics 25, IBM Corporation, USA). Descriptive characteristics of the study population are presented as mean±SD for continuous variables and as frequency (%) for categorical variables. A one-way ANOVA was performed on time-series data to

assess temporal changes in serum insulin concentrations, with multiple comparisons adjusted using Bonferroni correction. The trapezoidal rule was used to calculate individual incremental areas under the serum insulin concentration curve (iAUC). In order to account for differences in rapid-acting insulin dose, iAUC was normalised by dose of insulin administered (IU) allowing standardised comparisons between patients (iAUC/IU). To assess the association between clinical parameters and serum insulin responses, a Pearson correlation coefficient matrix was employed and interpreted as $r > 0.70$ = strong association, $r = 0.50 – 0.70$ = moderate association, $r = 0.30 – 0.49$ = weak association, and $r < 0.30$ = negligible association. To categorise and group individuals based on shared clinical and biochemical characteristics, we utilised two-step clustering with complete data available for continuous variables. In this unsupervised approach the first step estimates the optimal number of clusters on the basis of silhouette width and the second step is based on Bayesian hierarchical clustering. In this application, the method uses agglomerative clustering to partition clinical characteristics based on their abundance/magnitude in the individuals, and partitions individuals based on the abundance/magnitude of their characteristics. We use standardised Z scores of variables and log-likelihood as a distance measure and Schwarz's Bayesian Criterion (BIC) for clustering. BIC is based on the likelihood function and attempts to resolve overfitting to data models; using this approach 2 clusters were deemed to be optimal. Only continuous variables were included as the k-means method does not accommodate binary categorical variables. Cluster labels were assigned by examining cluster variable means. Differences between dichotomised variables were assessed with independent *t*-tests. Statistical significance was set at $P<0.05$ for all analyses.

RESULTS

Baseline characteristics of patients included in the present reanalysis are shown in Table 1. In summary, the 32 T1D patients (male=21) had a mean age of 31±7 years, HbA1c of 58±9 mmol/mol [7.5±0.8%] and rapid-acting bolus dose requirement of 9±3 IU. On average, rapid-acting insulin peaked at 120-minutes post injection at a concentration of 254±124 pmol/L ($P<0.001$), before returning to baseline concentrations at 180-minutes ($P=0.063$) (Figure 1). Individuals exhibited marked interpersonal variability in serum insulin concentrations as determined by summary statistics (Figure 1A-C), including variable peak serum insulin concentrations (CV%: 48.63%; Range: 79 – 546 pmol/L), time to peak

(CV%: 64.95%; Range 30 – 300-minutes), and serum insulin iAUC following adjustment for individual rapid-acting insulin dose (iAUC/IU; CV% 60.34% Range: 69 – 934 pmol/L.IU.hr^{-1}).

Figure 2 shows individual patient clinical profiles ranked by their iAUC/IU. To identify which, and whether clinical parameters associated with individual serum insulin responses, we applied a Pearson correlation coefficient matrix across variables (Figure 3). Serum insulin iAUC/IU was negatively correlated with fibrinogen (r = -0.57; P=0.012), HTF activity (r = -0.45; P=0.010), PAI-1 (r = -0.46; P=0.008), HbA1c (r = -0.64; P<0.007), age (r = -0.63; P<0.005), diabetes duration (r = -0.63; P<0.008), BMI (r = -0.62; P<0.012), and positively correlated with eGDR (r = -0.57; P=0.021). Notably, eGDR was negatively correlated with fibrinogen (r = -0.57; P<0.014), HTF activity (r = -0.41; P=0.020), and PAI-1 (r = -0.49; P=0.005), but not TNFα (r = -0.03; P=0.881). Only weak associations were observed between peak insulin concentrations and fibrinogen, PAI-1, HbA1c, diabetes duration, BMI, and eGDR (P<0.005, Supplementary Figure 1), whereas time to peak was not associated with any clinical parameters (P>0.005, Supplementary Figure 2).

Given the associations between physiological and clinical parameters with serum insulin levels, next we applied machine learning techniques to classify patients into diabetes subgroups based on their individual serum insulin response and physiological/clinical parameters. We used a TwoStep clustering method with complete data available for continuous clustering variables; Figure 4 shows the cluster characteristics for cluster 1 and 2, with cluster 1 representing increased serum insulin concentrations and cluster 2 representing reduced serum insulin concentrations; (cluster 1: 389.50±177.10 vs. cluster 2: 164.29±;41.91 pmol/L.IU.hr^{-1}; P<0.001). Cluster 2, including 56% (n=18) of the patients, was characterised by increased levels of vascular inflammatory proteins: fibrinogen, HTF activity, PAI-1, and their mediator TNFα, a higher HbA1c, older age, a greater duration of diabetes, and increased BMI, and increased insulin resistance (lower eGDR), in comparison to cluster 1 which included 44% (n=14) of the patients. These data imply that patients with low levels of serum insulin following subcutaneous injection may be associated with increased insulin resistance, age, and diabetes duration, and concomitantly express raised levels of biomarkers associated with increased thrombosis and adverse vascular health. To test this hypothesis, we stratified patients according to their cluster allocation and performed independent t-tests on serum insulin responses and individual clinical parameters (Figure

5). Notably, cluster 2 elicited significantly higher mean fibrinogen (cluster 1: 1559±689 vs. cluster 2: 3073±1283 µg/mL; *P*<0.009), HTF activity (cluster 1: 83.01±39.20 vs. cluster 2: 142.24±48.62 pmol/mL; *P*<0.007), and PAI-1 (cluster 1: 0.86±0.55 vs. cluster 2: 1.71±0.71 ng/mL; *P*<0.005), HbA1c concentrations (cluster 1: 51.76±5.72 vs. cluster 2: 65.96±4.68 mmol/mol; *P*<0.021). Further, cluster 2, elicited a significantly higher mean age (cluster 1: 28±5 vs. cluster 2: 35±7 years; *P*<0.006), a greater mean duration of diabetes (cluster 1: 10±5 vs. cluster 2: 25±7 years; *P*<0.014), a higher mean BMI (cluster 1: 22.68±1.51 vs. cluster 2: 30.34±4.12 kg/m^2; *P*<0.009), a lower mean eGDR (cluster 1: 9.47±1.16 vs. cluster 2: 5.49±2.23; *P*<0.003).

CONCLUSIONS

The results in this paper characterise the interaction between interindividual responses in subcutaneously injected rapid-acting insulin with parameters of vascular health in patients with T1D treated with multiple daily injections. We applied an unsupervised machine learning approach to establish a novel classification of patients on the basis of pharmacokinetic summary statistics that characterise rapid-acting insulin responses and found that an attenuated temporal insulin response characterised by a lower iAUC, peak concentration, and protracted time to peak, clusters with an elevated proinflammatory and prothrombotic biomarker profile.

Our data demonstrate marked interpersonal variability in circulating insulin concentrations following subcutaneous injection assessed using a range of parameters including peak concentration, time to peak, and iAUC adjusted for individualised rapid-acting insulin dose. As such, time to peak ranged from 30- to 300-minutes in our sample with a 13-fold difference in insulin exposure between individuals with the lowest and highest iAUC following adjustment for individualised insulin dose requirements. Research assessing variability in insulin absorption and action so far have focused on variability between injections of different insulin preparations and injection sites within individuals, and physiological (i.e., age and gender, adiposity and anthropometry, skin temperature and subcutaneous blood flow, and presence of complications) and behavioural (smoking, physical activity) factors between individuals[12]. Our data extends these findings by showing that the variability in circulating rapid-acting insulin concentrations is associated with a range of clinical characteristics. The Pearson coefficients calculated herein show that there are significant associations between circulating administered insulin

and clinical characteristics (age, diabetes duration, HbA1c, BMI, and eGDR) as well as prothrombotic vascular biomarkers (fibrinogen, HTF activity, and PAI-1). To explore the strength of these associations in our study further, we used a data-driven cluster analysis to classify patients into two distinct clusters. Cluster 1 (increased serum insulin concentrations) was associated with lower levels of thrombotic parameters, compared to cluster 2 (reduced serum insulin concentrations) which was ubiquitously associated with a raised prothrombotic milieu. Within our study, more pronounced circulating insulin levels may represent a heightened level of insulin sensitivity, which, may have acted to better counteract the increase in post-prandial inflammation and prothrombosis, potentially via increased vasodilation[13], and the suppression of vascular adhesion molecules[13, 14] and prothrombotic-profibrinolytic factors [13, 15-18] such as PAI-1 and human tissue factor, by, at least in part, suppressing monocyte chemoattractant protein-1 (MCP-1) and nuclear factor kappa B(NK-kB)[16]. Given that the higher-risk cluster was also characterised by lower eGDR (indicative of increased insulin resistance), it supports the concept that insulin resistance in T1D is a major driving force in increased vascular risk[19]. For example, in patients with type 2 diabetes, as well as non-diabetes individuals with obesity, the impact of insulin on vasodilation is blunted[20, 21], and we have recently shown that in individuals with T1D, insulin resistance (as measured by eGDR) is associated with an increased risk of vascular complications[19]. However, whether decrements in vasodilation and the subsequent downstream inflammatory and/or thrombotic events is due to insulin resistance or whether insulin resistance is a by-product of reduced skeletal muscle perfusion and downregulated glucose uptake is not known.

Irrespective of the mechanisms at play, the results from this preliminary exploratory study demonstrate a need for more targeted and personalised approaches that seek to address the unpredictability associated with current insulin dosing strategies seen in routine clinic, which, would seem to interact with the heterogeneity in vascular risk[11]. Existing treatment guidelines focus on individualising insulin dose, adjusting as necessary according to meal composition and the results of regular glucose monitoring[1]. However, we suggest that current recommendations are limited in that they do not account for individual physiological and clinical characteristics which are a known source of heterogeneity in response to insulin therapy[22]. It was not our intention to establish distinct subtypes of T1D representative of different aetiologies, and we do not claim that the clustering applied here is in anyway an optimal classification for T1D vascular risk. Additionally, whether patients can move between clusters

needs to be shown in future prospective studies and the exact overlap of weaker association signals warrant investigation in larger cohorts. Indeed, it may be possible to refine the stratification further through inclusion of additional cluster variables, such as genotypes, or genetic risk scores.

In conclusion, this preliminary and exploratory study characterises the interaction between interindividual responses in subcutaneously injected rapid-acting insulin with parameters of vascular health in patients with T1D treated with multiple daily injections. Using an unsupervised machine learning approach to establish a novel classification of patients on the basis of pharmacokinetic summary statistics that characterise rapid-acting insulin responses, we found that an attenuated temporal insulin response characterised by a lower iAUC, peak concentration, and protracted time to peak, clusters with an elevated prothrombotic profile. Future work is planned to interrogate further the mechanisms underpinning interpersonal variation in insulin concentrations and relationship with adverse vascular risk profile.

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FIGURES

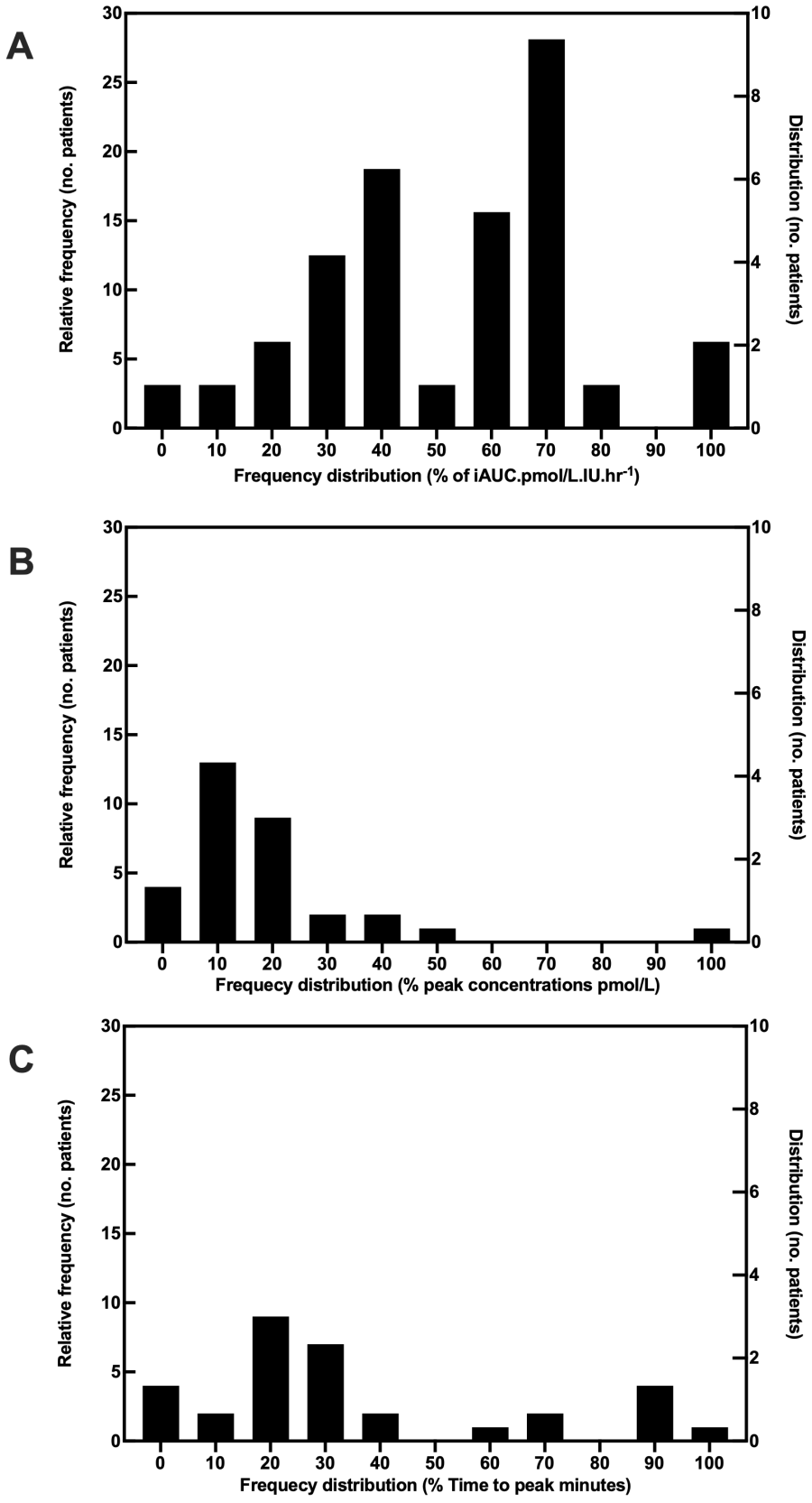


Figure 1. Interindividual responses in serum insulin summary statistics. The distribution of standardised responses following subcutaneous administration of rapid-acting insulin presented as (**A**) iAUC following adjustment for individualised rapid-acting insulin dose requirements, (**B**) %peak concentrations, and (**C**) Time to peak insulin concentrations.

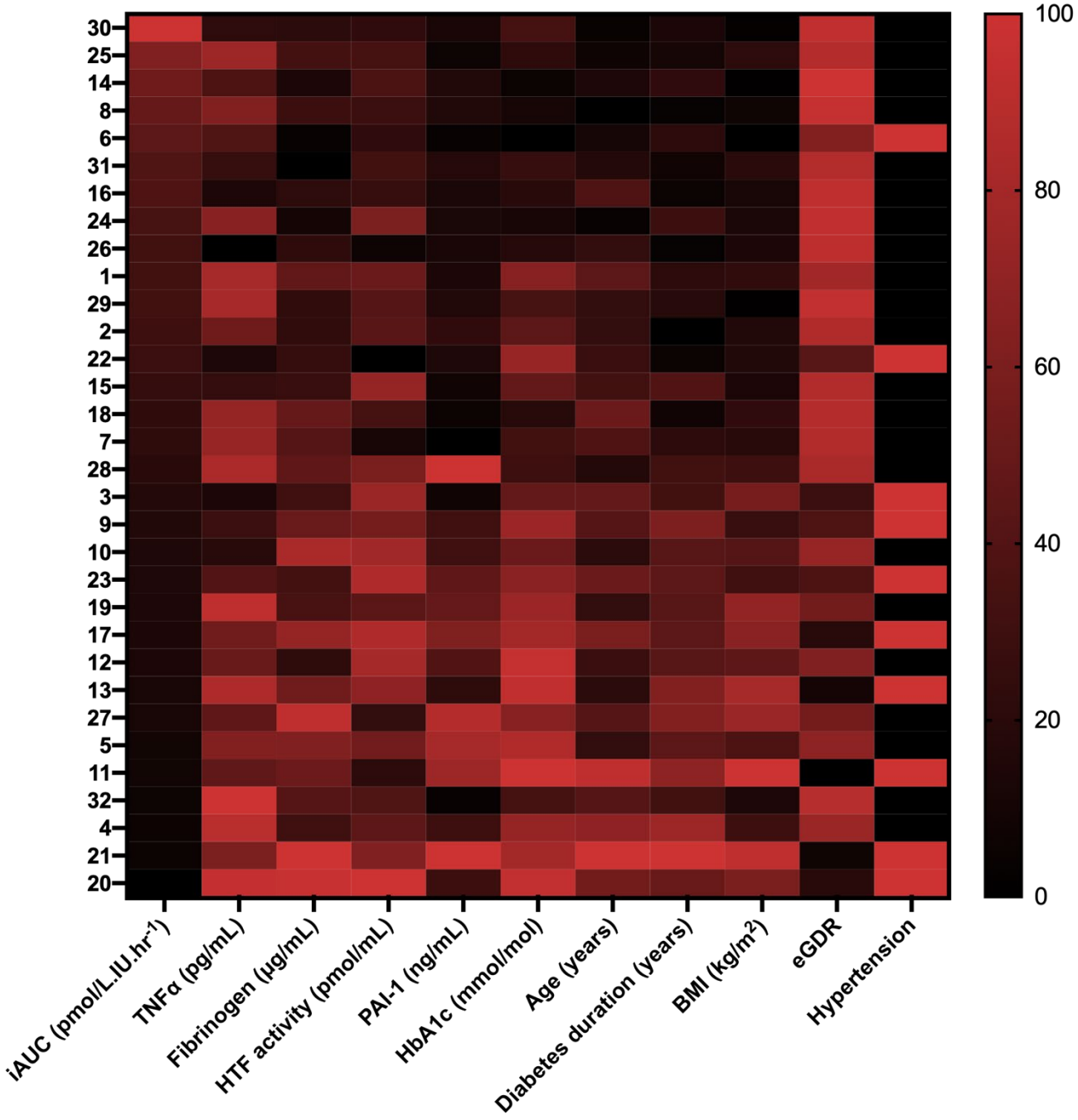


Figure 2. Individual patient clinical profiles (y axis) ranked by iAUC responses adjusted for individualised rapid-acting insulin dose requirements (normalised data). eGDR, Estimated Glucose Disposal Rate; HTF activity, Human Tissue Factor activity; PAI-1, Plasminogen Activator Inhibitor-1; TNFα, Tumour Necrosis Factor alpha.

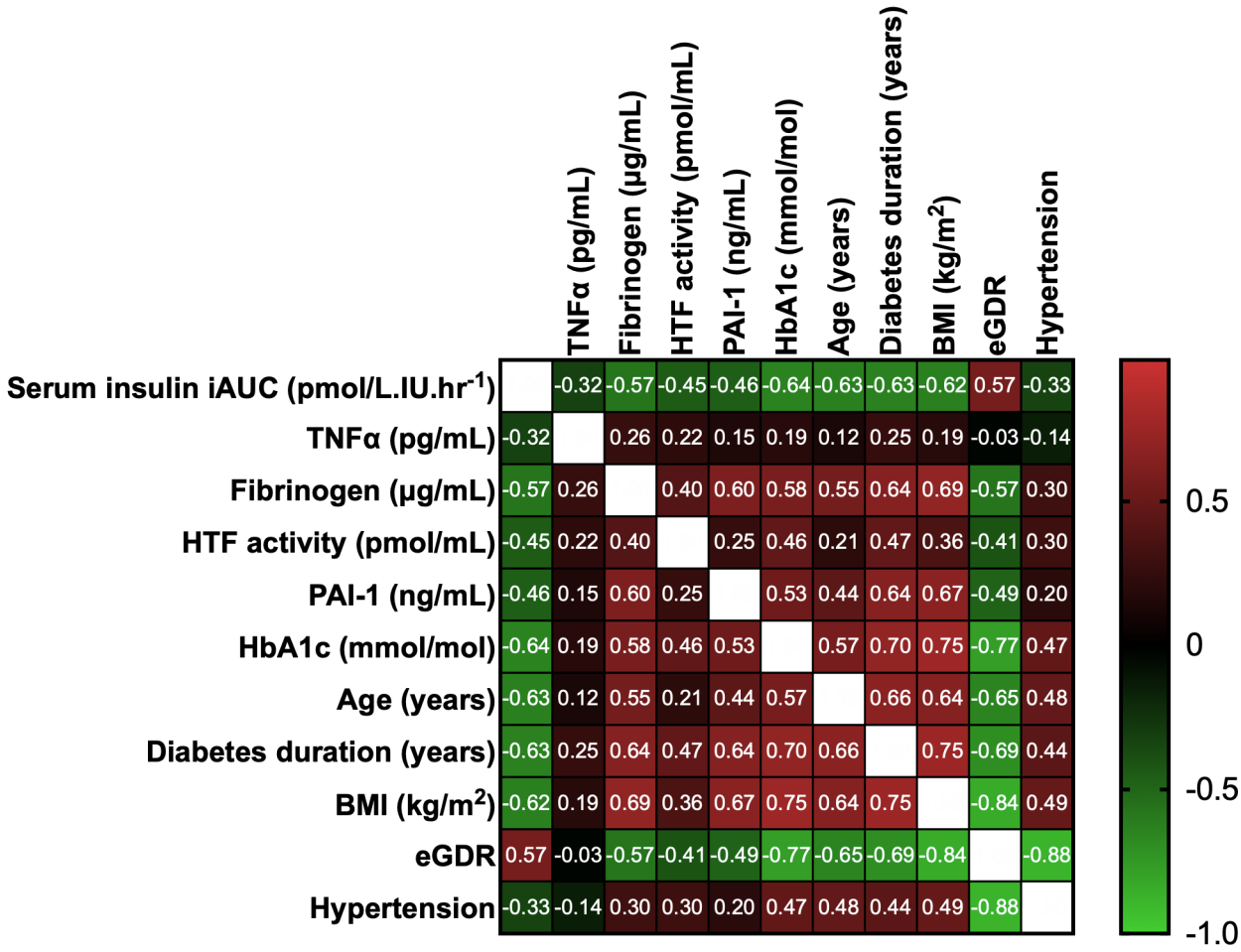


Figure 3. Pearson correlation coefficient matrix illustrating the association between baseline patient characteristics and serum insulin iAUC adjusted for individualised rapid-acting insulin dose requirements. Pearson correlation coefficients (*r*) are highlighted in white text. $r \geq 0.70$ = strong association; $r = 0.50 – 0.70$ = moderate association; $r = 0.30 – 0.50$ = weak association; $r \leq 0.30$ = negligible association. eGDR, Estimated Glucose Disposal Rate; Human Tissue Factor activity; PAI-1, Plasminogen Activator Inhibitor-1; TNFα, Tumour Necrosis Factor alpha; HTF activity.

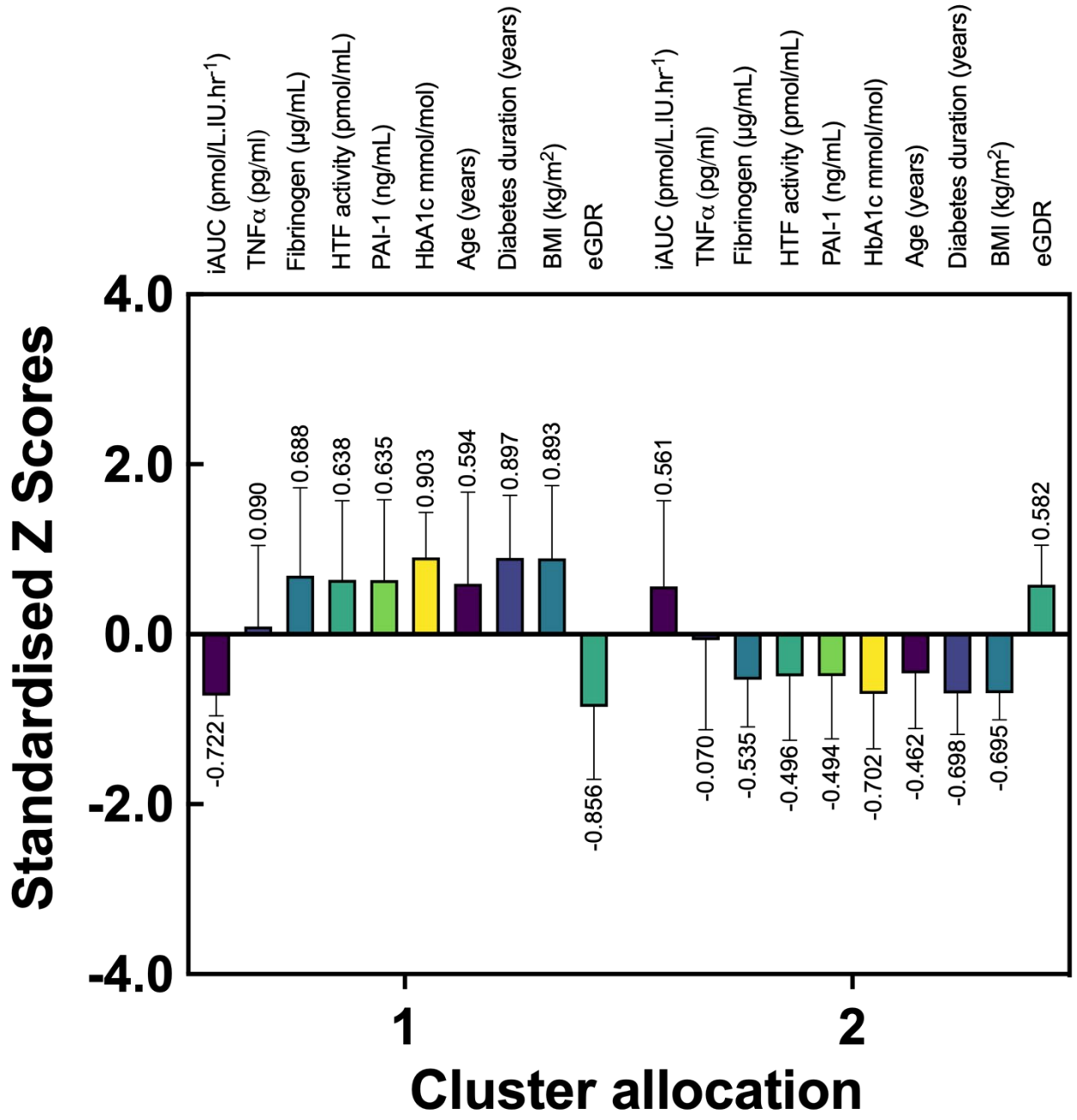


Figure 4. Cluster characteristics. Variables are presented as standardised Z scores. eGDR, Estimated Glucose Disposal Rate; Human Tissue Factor activity; PAI-1, Plasminogen Activator Inhibitor-1; TNFα, Tumour Necrosis Factor alpha; HTF activity.

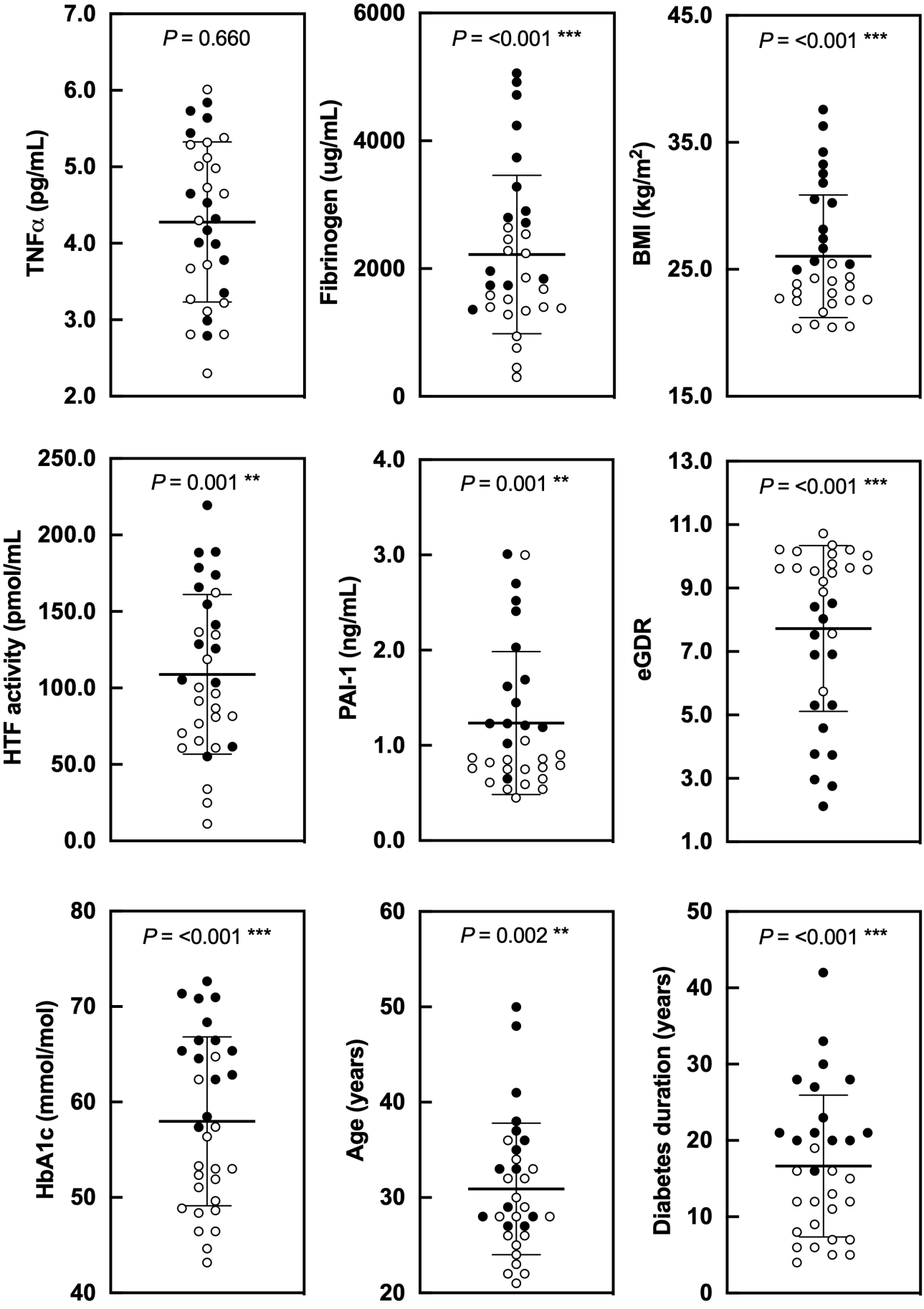


Figure 5. Patient characteristics stratified by cluster allocation. Black circles = cluster 1; white circles = cluster 2. Statistically significant differences between clusters calculated using independent *t*-tests. * denotes *P*<0.05; **

denotes *P*<0.01; *** denotes *P*<0.001. eGDR, Estimated Glucose Disposal Rate; Human Tissue Factor activity; PAI-1, Plasminogen Activator Inhibitor-1; TNFα, Tumour Necrosis Factor alpha; HTF activity.

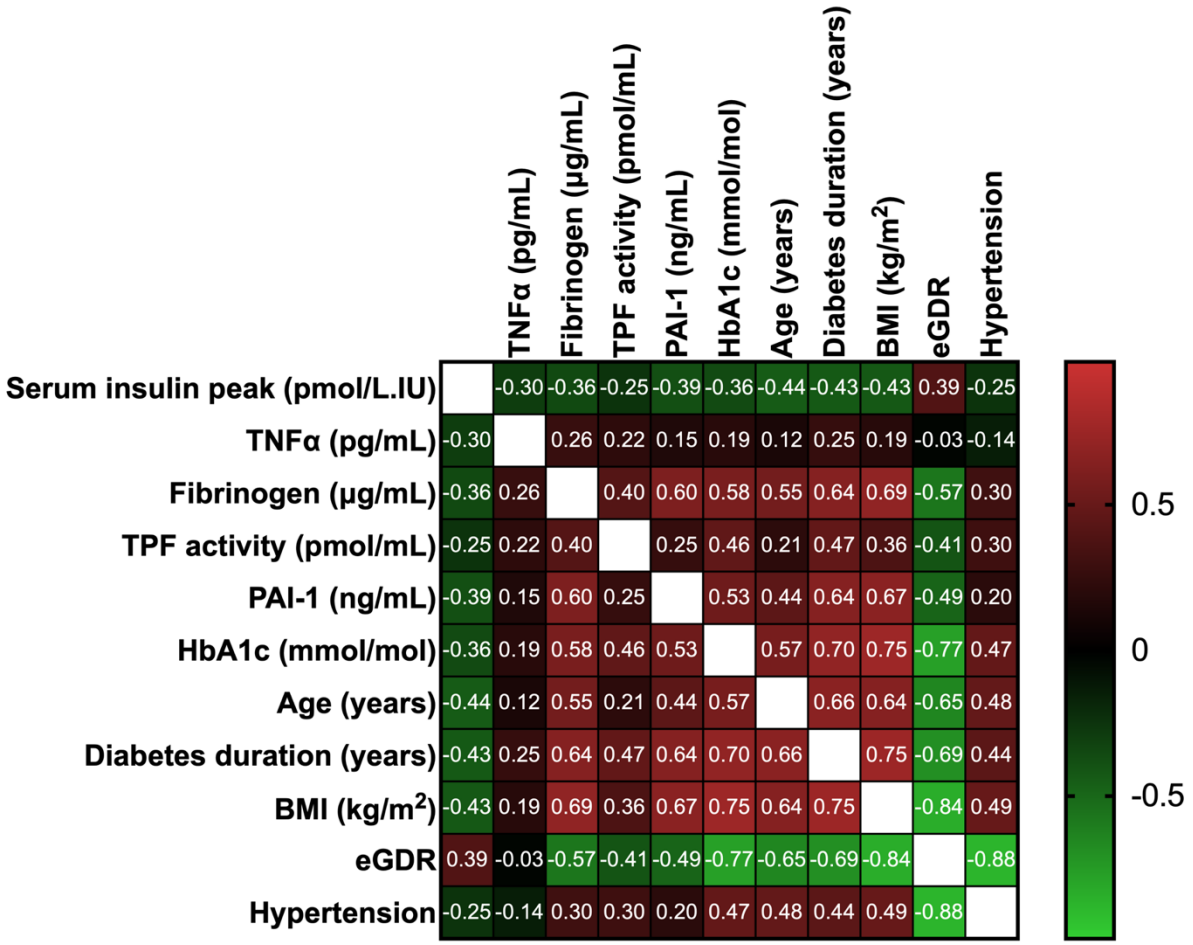
TABLES

Table 1. Baseline characteristics of patients

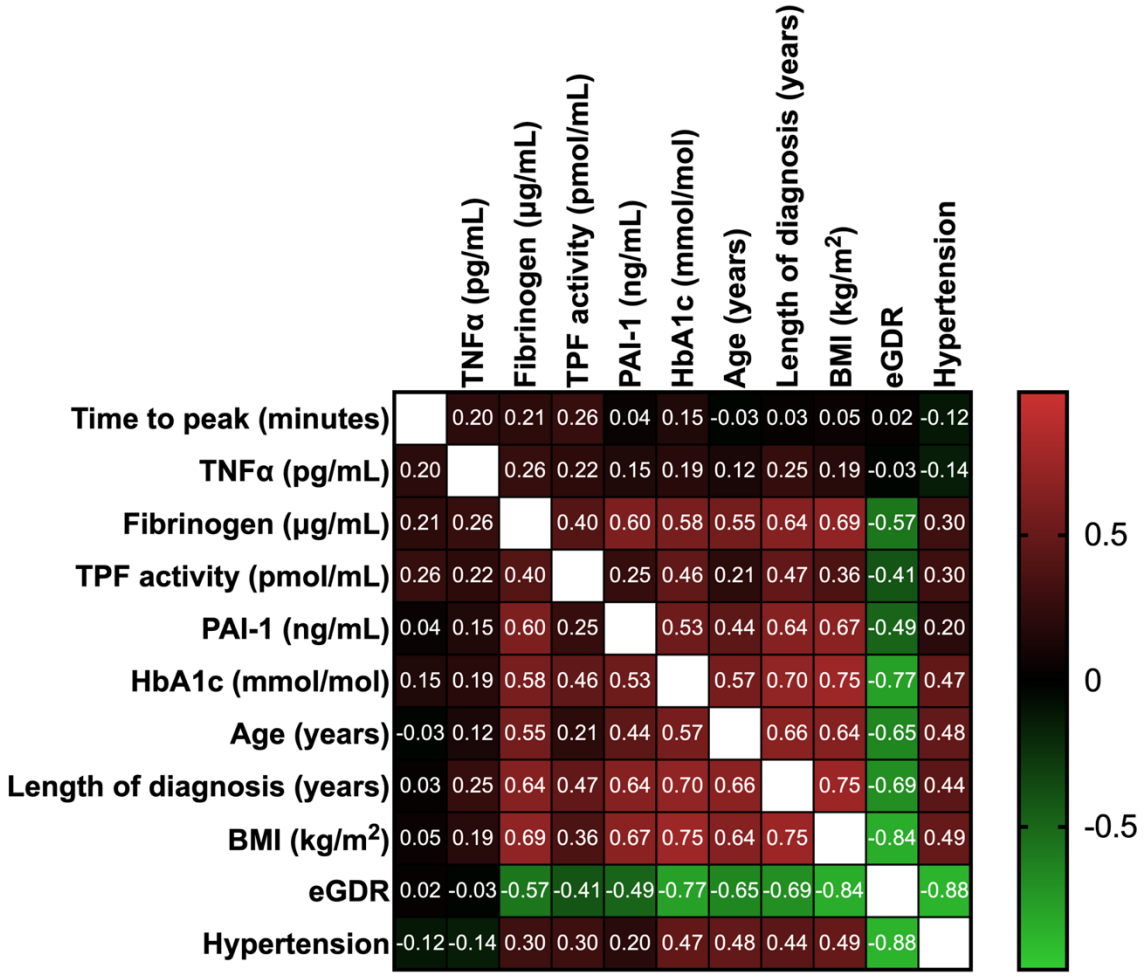
Clinical parameters	
n	32
Age (years)	31±7
Male (%)	66
BMI (kg/m^2)	26.03±4.82
eGDR	7.73±2.61
Hypertension (%)	31
HbA1c (mmol.moL [%])	57.97±8.85 [7.45±0.81]
Diabetes duration (years)	17±9
Rapid-acting insulin dose (IU)	9±3
Insulin apart users (%)	63
Vascular and inflammatory parameters	
TNFα (pg/mL)	4.28±1.05
Fibrinogen (µg/mL)	2221±1238
HTF activity (pmol/mL)	108.93±52.20
PAI-1 (ng/mL)	1.23±0.75

Metric variables presented as mean±SD; categorical data presented as frequency (%). eGDR, Estimated Glucose Disposal Rate; Human Tissue Factor activity; PAI-1, Plasminogen Activator Inhibitor-1; TNFα, Tumour Necrosis Factor alpha; HTF activity.

Supplementary material



Supplementary Figure 1. Pearson correlation coefficient matrix illustrating the association between baseline patient characteristics and serum insulin peak adjusted for individualised rapid-acting insulin dose requirements. Pearson correlation coefficients (*r*) are highlighted in white text. $r \geq 0.70$ = strong association; $r = 0.50 – 0.70$ = moderate association; $r = 0.30 – 0.50$ = weak association; $r \leq 0.30$ = negligible association. eGDR, Estimated Glucose Disposal Rate; Human Tissue Factor activity; PAI-1, Plasminogen Activator Inhibitor-1; TNFα, Tumour Necrosis Factor alpha; HTF activity.



Supplementary Figure 2. Pearson correlation coefficient matrix illustrating the association between baseline patient characteristics and serum insulin time to peak adjusted for individualised rapid-acting insulin dose requirements. Pearson correlation coefficients (*r*) are highlighted in white text. $r \geq 0.70$ = strong association; $r = 0.50 – 0.70$ = moderate association; $r = 0.30 – 0.50$ = weak association; $r \leq 0.30$ = negligible association. eGDR, Estimated Glucose Disposal Rate; Human Tissue Factor activity; PAI-1, Plasminogen Activator Inhibitor-1; TNFα, Tumour Necrosis Factor alpha; HTF activity.