Serum alanine transaminase is predictive of fasting and postprandial insulin and glucagon concentrations in type 2 diabetes

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ABSTRACT

The liver plays a key role in glucose homeostasis. Serum liver enzyme levels, including alanine transaminase (ALT), aspartate transaminase (AST) and gamma-glutamyl transferase (GGT), are reportedly predictive of the risk of type 2 diabetes (T2D). However, the link between the liver enzyme profile and metabolic derangements in T2D, particularly the secretion of both insulin and glucagon, is not clear. This study evaluated its relationships with glycemia, insulin and glucagon both during fasting and after an oral glucose load or a mixed meal in T2D. 15 healthy and 43 T2D subjects ingested a 75 g glucose drink. 86 T2D subjects consumed a mixed meal. Venous blood was sampled for measurements of blood glucose and plasma insulin, C-peptide and glucagon. Blood glucose, plasma insulin, C-peptide and glucagon concentrations, both fasting and after oral glucose, correlated directly with ALT, while fewer and weaker correlations were observed with GGT or AST. Subgroup analysis in T2D subjects ascertained that plasma insulin, C-peptide and glucagon concentrations after oral glucose were higher with increasing ALT. Similar findings were observed in the T2D subjects who received a mixed meal. In conclusion, serum liver enzyme profile, particularly ALT, reflects dysregulated fasting and nutrient-stimulated plasma insulin and glucagon concentrations in T2D.

1. Introduction

Hepatic glucose production and disposal are key metabolic processes in glucose homeostasis [1,2]. During fasting, the liver releases glucose via both the breakdown of glycogen (glycogenolysis) and glucose synthesis from precursors including alanine and lactate (gluconeogenesis) [3]. During the postprandial phase, the liver is responsible for the disposal of about a third of ingested glucose [4]. Hepatic regulation of blood glucose is coordinated predominantly by insulin and glucagon released from the pancreatic islets; the secretion of insulin increases glucose uptake in the liver [5], while glucagon augments hepatic glucose production [6,7]. Impairments in insulin secretion and hepatic insulin sensitivity, as well as an augmentation of both fasting and postprandial glucagon secretion, underpin the elevation of blood glucose in type 2 diabetes (T2D) [8,9]. Accordingly, characterization of the relationship between the secretion of insulin and glucagon and liver function is

potentially of major relevance to the understanding of the pathophysiology and rational management of T2D.

In clinical practice, a serum panel of liver enzymes, including gamma-glutamyl transferase (GGT), alanine transaminase (ALT), alkaline phosphatase (ALP) and aspartate transaminase (AST), are assessed usually under 'liver function tests' [10]. Liver enzyme levels are typically elevated in hepatic steatosis (particularly GGT and ALT), hepatocellular injury (ALT and AST), or cholestasis (ALP and GGT) [11]. ALT is released mainly by hepatocytes [12], but other 'liver' enzymes (particularly AST and ALP) are also produced by extra-hepatic tissues, so their elevations are not specific to the liver [13,14]. There is increasing evidence that the liver enzyme profile is predictive of the risk of metabolic disorders, including T2D [14]. For example, a cross-sectional analysis revealed that plasma glucose at 2 h after a 30 g oral glucose load was associated directly with serum GGT in non-diabetic middle-aged men (N = 2419) [15]. In a subsequent cohort study of middle-aged men without

Table 1
Characteristics of healthy and type 2 diabetes (T2D) participants in Study 1.

Parameters	Healthy (N = 15)	T2D (N = 43)				
		Overall	T1 (N = 15)	T2 (N = 15)	T3 (N = 13)	P
Gender (M/F)	9/6	29/14	13/2	9/6	7/6	0.2
Age (year)	67.2 ± 2.3	65.7 ± 1.1	66.4 ± 2.1	64.9 ± 1.3	65.9 ± 1.4	0.9
BMI (kg/m^2)	30.3 ± 0.7	31.5 ± 0.7	29.6 ± 1.0	31.7 ± 1.1	33.5 ± 1.2	0.08
HbA1c (%)		7.1 ± 0.1	6.9 ± 0.2	7.2 ± 0.2	7.2 ± 0.2	0.3
Medication (Diet/Metformin)		4/39	3/12	1/14	0/13	0.17
Total Bili (μmol/L)	10.9 ± 1.4	11.7 ± 0.8	12.9 ± 1.3	10.3 ± 1.0	12.0 ± 1.3	0.4
GGT (U/L)	23.8 ± 3.2	43.3 ± 6.8	23.0 ± 4.2	37.5 ± 10.6	73.2 ± 13.1	0.008
ALP (U/L)	74.7 ± 4.1	76.8 ± 3.5	66.1 ± 2.8	80.5 ± 7.6	84.8 ± 4.7	0.07
ALT (U/L)	24.4 ± 1.7	36.7 ± 2.9 ****	20.8 ± 1.1	33.3 ± 0.8	59.0 ± 4.6	<0.0001
AST (U/L)	23.0 ± 1.2	28.6 ± 2.0	20.5 ± 1.1	25.3 ± 1.0	41.6 ± 3.9	<0.0001
Fasting glucose (mmol/L)	5.6 ± 0.1	7.9 ± 0.2 ****	7.7 ± 0.3	7.9 ± 0.3	8.1 ± 0.5	0.7
Fasting insulin (mU/L)	3.0 ± 0.6	6.7 ± 0.6 ****	4.7 ± 0.5	6.9 ± 1.3	8.6 ± 0.8	0.03
Fasting C-peptide (pmol/L)	346.1 ± 34.3	698.7 ± 42.9 ****	557.5 ± 49.4	694.1 ± 82.3	866.8 ± 68.4	0.003
Insulin clearance	11.8 ± 1.5	12.6 ± 0.7	15.3 ± 1.3	11.2 ± 1.1	11.1 ± 1.2	0.027
Fasting glucagon (pg/mL)	57.2 ± 3.7	66.2 ± 3.8	57.1 ± 3.6	64.8 ± 7.2	78.2 ± 7.8	0.08
HOMA-IR (dL/mg*L/mU)	0.8 ± 0.2	2.3 ± 0.2 * *** *	1.6 ± 0.2	2.4 ± 0.5	3.1 ± 0.3	0.02

Data are means $\pm$ SEM. The Mann-Whitney test (or unpaired student t-test for normally distributed data) was used to evaluate differences between the healthy and T2D groups, except that the distribution of gender and medication were compared using a Chi-square analysis. *P < 0.05, **P < 0.001, ***P < 0.0005, ****P < 0.0001 for healthy vs. T2D (overall). T2D subjects were stratified into three subgroups, including T1 (ALT $\leq$ 29 U/L, N = 15) T2 (ALT = 30–40 U/L, N = 15) and T3 (ALT > 40, N = 13). One-way ANOVA was used to determine statistical differences between the three subgroups, except that the distribution of gender and medication were compared using Chi-square analysis.

diabetes (N = 7458), a relative or absolute increase in serum GGT was found to be an independent risk factor for the development of T2D [16]. Similarly, ALT and AST have been reported as potential markers of diabetes risk in individuals without hepatitis [17–22]. These observations necessitate a deeper understanding of the mechanistic links between metabolic derangement and liver enzymes.

In subjects with normal glucose tolerance, ALT, AST and GGT have been found to correlate positively with insulin levels during fasting and

Table 2
Characteristics of Study 2 participants in the total group and classified according to serum ALT.

Parameters	Overall	Lower (N = 45)	Higher (N = 41)	P
Gender (M/F)	54/32	26/19	28/13	0.3
Age (year)	64.7 ± 0.7	65.6 ± 0.9	63.7 ± 1.1	0.18
BMI (kg/m^2)	29.9 ± 0.6	29.0 ± 0.8	30.9 ± 0.7	0.04
HbA1c (%)	6.6 ± 0.1	6.5 ± 0.1	6.8 ± 0.1	0.14
Medication (Diet/Metformin)	37/49	19/26	18/23	0.9
Total Bili (μmol/L)	10.1 ± 0.7	9.3 ± 0.6	10.9 ± 1.2	0.63
GGT (U/L)	38 ± 4.9	25.0 ± 2.0	43.4 ± 4.1	<0.0001
ALP(U/L)	74.1 ± 2.0	74.6 ± 2.8	73.6 ± 2.8	0.8
ALT(U/L)	31.4 ± 1.8	20.6 ± 0.8	43.3 ± 2.6	<0.0001
AST(U/L)	26.8 ± 1.0	22.3 ± 0.7	31.5 ± 1.6	<0.0001
Fasting glucose (mmol/L)	8.1 ± 0.2	8.1 ± 0.2	8.2 ± 0.2	0.9
Fasting insulin (mU/L)	6.6 ± 0.6	5.1 ± 0.5	8.3 ± 1.1	0.007
Fasting glucagon (pg/mL)	74.1 ± 2.1	71.2 ± 2.5	77.2 ± 3.3	0.1
Matsuda index (dL/mg*L/mU)	7.3 ± 0.6	8.8 ± 0.9	5.7 ± 0.7	0.0007
HOMA-IR (mmol/L*mU/L)	2.5 ± 0.2	1.8 ± 0.2	3.0 ± 0.4	0.006

Data are means $\pm$ SEM. Participants were divided into two subgroups, with lower ($\leq$ 29 U/L, N = 45) or higher ($\geq$ 30 U/L, N = 41) ALT levels. Mann-Whitney test (or unpaired student t-test for normally distributed data) was used to determine the statistical difference between the Lower and Higher subgroups, except that the distribution of gender and medication were compared using Chi-square analysis.

following a 75 g oral glucose load [23]. Moreover, in healthy men and women, both GGT and ALT correlated positively with hepatic insulin resistance, assessed by the gold standard euglycemic-hyperinsulinemic clamp, with fasting glucagon related positively to ALT [24]. Accordingly, an increase in liver enzymes may represent an early marker of hepatic resistance to insulin and hyperglucagonemia. Although T2D patients with increased liver fat and ALT also displayed greater hepatic insulin resistance than those with normal liver fat and ALT [25], the relationships of the secretion of both insulin and glucagon with serum liver enzyme levels in patients with T2D have not been well characterized.

We have therefore examined the relationships of blood glucose, plasma insulin, C-peptide and glucagon during the fasting state and following a 75 g oral glucose load and a mixed meal with liver enzymes in T2D.

2. Methods

2.1. Participants

T2D patients managed by diet and/or metformin monotherapy, and healthy controls matched for age, gender and body mass index (BMI), were recruited from the community by advertisement for studies to evaluate nutritional and/or pharmacological therapies for T2D in our center (ACTRN12616001059459 and ACTRN1261600055415, ACTRN12612001005842 and ACTRN12614001131640) [26–29]. After excluding subjects who participated in multiple protocols (n = 7) or had missing data (n = 3), a total of 15 healthy older subjects and 129 T2D patients were included in this retrospective analysis. In all subjects, during a screening visit, a fasting blood sample had been collected for serum biochemistry (including renal and liver function), and for HbA1c in the case of T2D, and analysed in a commercial laboratory (SA Pathology, Adelaide, Australia). None of the participants had gastrointestinal symptoms, eGFR of <60 mL/min, or elevation of liver enzymes, including ALT, GGT, AST and ALP, more than two-fold the upper limit of the reference range. The studies were approved by the Royal Adelaide Hospital Human Research Ethics Committee and all participants provided written informed consent.
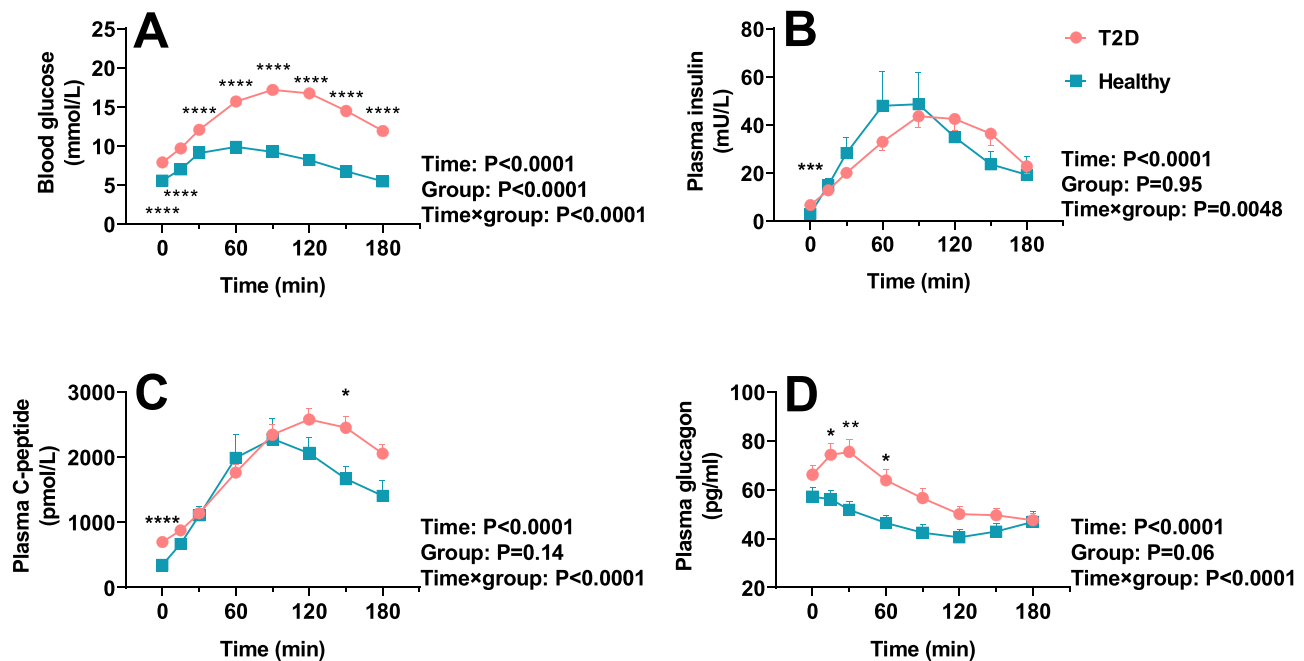
Fig. 1. Blood glucose (A), plasma insulin (B), plasma C-peptide (C) and plasma glucagon (D) responses to a 75 g oral glucose load in healthy subjects (N = 15) and individuals with type 2 diabetes (T2D, N = 43) in Study 1. Repeated-measures two-way ANOVA was used to determine statistical differences. ANOVA results are reported as P values for differences between groups, over time, and due to group by time interactions. *Post hoc* comparisons were adjusted by Bonferroni's correction. Data are means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ for healthy vs. T2D.

2.2. Protocol

Each subject was evaluated on a single occasion with either a 75 g oral glucose challenge (Study 1), or a standardized mixed meal (Study 2). Prior to their study visit, subjects were instructed to refrain from intensive physical activity for 24 h, and consumed a standardized evening meal (McCain beef lasagne, [592 kcal, McCain Foods, Melbourne, VIC, Australia], together with bread, a non-alcoholic beverage and one piece of fruit) at ~7 pm. Subjects then fasted from all food and nutrient beverages, but were allowed to drink water until midnight, before attending the clinical research laboratory at The University of Adelaide the following morning.

2.2.1. Study 1: oral glucose tolerance test (OGTT)

A total of 15 healthy subjects (9 males and 6 females, age 67.2 ± 2.3 years, BMI 30.3 ± 0.7 kg/m^2) and 43 T2D patients (29 males and 14 females, age 65.7 ± 1.1 years, BMI 31.5 ± 0.7 kg/m^2, HbA1c 7.1 ± 0.1 %) (Table 1) were evaluated with a 75 g oral glucose challenge, during which an intravenous (IV) cannula was inserted in one forearm vein for blood sampling and kept warm for collection of 'arterialized' venous blood. At t = 0 min, a glucose drink (75 g glucose dissolved in water to a final volume of 300 mL) was consumed within 5 min, and subjects remained seated throughout the study. Blood samples were collected immediately before (t = 0 min) and at t = 15, 30, 60, 90, 120, 150 and 180 min after the glucose drink, and were centrifuged at 3200 rpm for 15 min at 4°C within 15 min of collection. Plasma was separated and stored at −80°C until subsequent assays were performed.

2.2.2. Study 2: mixed meal test

86 T2D subjects (54 males and 32 females, age 64.7 ± 0.7 years, BMI 29.9 ± 0.6 kg/m^2, HbA1c 6.6 ± 0.1 %) (Table 2) were evaluated in a similar procedure to Study 1, except that subjects consumed a semisolid meal comprising 65 g powdered potato (Deb Instant Mashed Potato; Continental, Epping, Australia) and 20 g glucose, reconstituted with 200 mL water and one egg yolk (368.5 kcal: 61.4 g carbohydrate, 7.4 g protein and 8.9 g fat) between t = 0–5 min. 'Arterialized' venous blood was sampled before (t = 0 min) and at t = 15, 30, 60, 90, 120, 180 and 240 min after the meal.

2.3. Measurements

Blood glucose concentrations were measured in duplicate at each time point using a glucometer (Optium Xceed; Abbott Laboratories, Lake Bluff, IL, USA), and the mean value was recorded. Plasma insulin concentrations were measured by ELISA (10–1113, Mercodia, Uppsala, Sweden), with a sensitivity of 1.0 mU/L, and intra- and inter-assay coefficients of variation (CVs) of 2.9 % and 6.7 % respectively. Plasma C-peptide concentrations were measured by ELISA (10–1136–01, Mercodia), with a sensitivity if 15 pmol/L and intra- and interassay CVs of 7.7 % and 3.7 % respectively. Plasma glucagon concentrations were determined by radioimmunoassay (RIA, GL-32k; Millipore, Billerica, MA, USA), with a sensitivity of 20 pg/mL, and intra- and inter-assay CVs of 10 % and 3.1 % respectively. We noted that there was a shift in the performance of the Millipore RIA kit after the introduction of a new batch of antibody for glucagon in 2019 [30]. The glucagon data reported here were therefore derived from RIA kits produced before 2019, which we have validated against the highly specific sandwich ELISA kit that detects both the N- and C-terminus of glucagon (i.e. the Mercodia ELISA kit) [30]. Insulin clearance was reflected by the molar ratio of C-peptide AUC$_{0–180\ min}$ to insulin AUC$_{0–180\ min}$ [31]. Whole-body insulin sensitivity and hepatic insulin resistance were assessed by the Matsuda index and homeostatic model assessment for insulin resistance (HOMA-IR), respectively. The Matsuda index was calculated by the following:
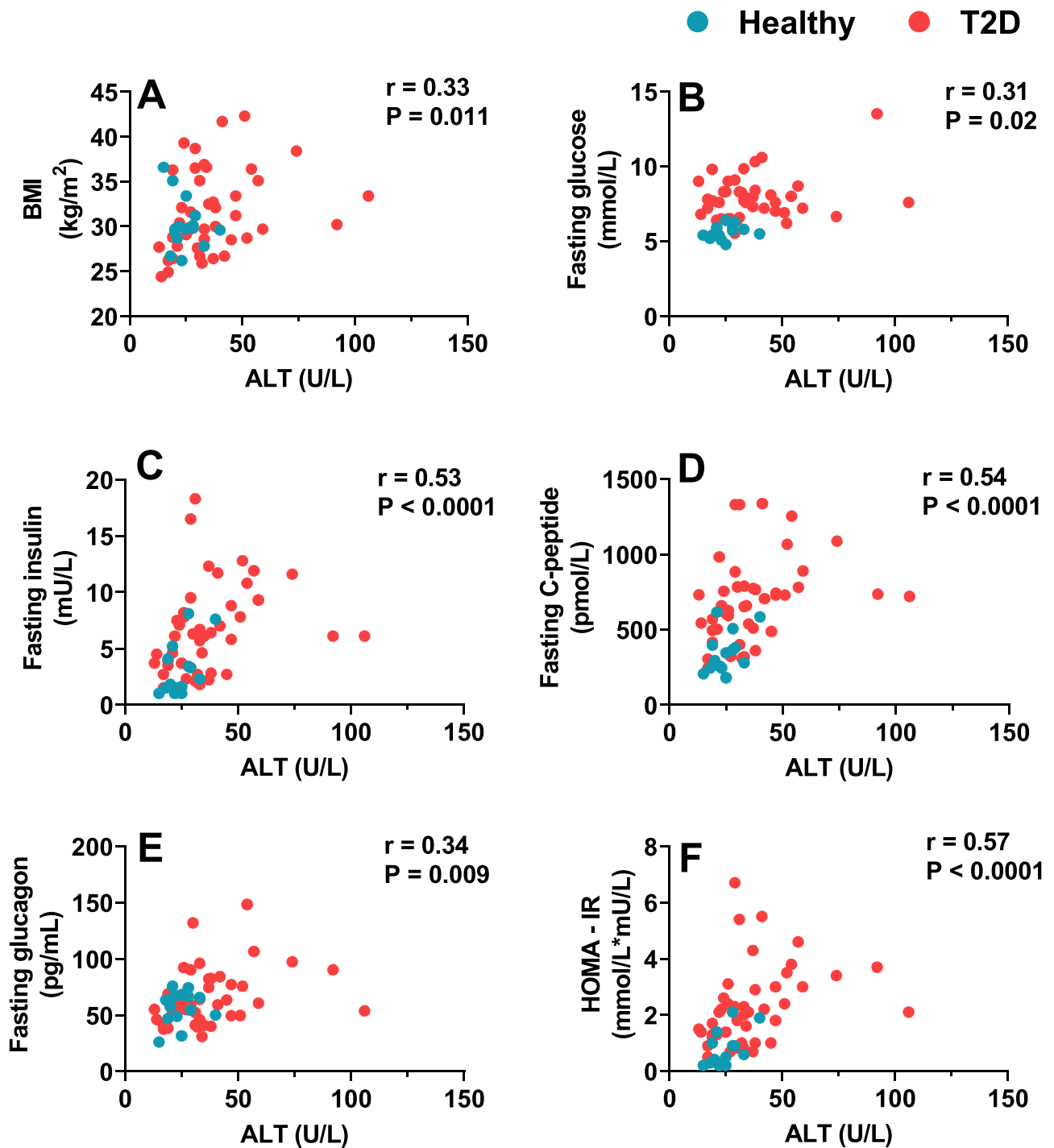
Fig. 2. Relationships of BMI (A), fasting blood glucose (B), plasma insulin (C), plasma C-peptide (D), plasma glucagon (E) and HOMA-IR (F) with alanine transaminase (ALT) in the combined subjects without (N = 15) and with type 2 diabetes (T2D, N = 43) in Study 1. Spearman correlation analysis was employed to examine these relationships.

$$\frac{10000}{\sqrt{G_0 \times 18 \times I_0 \times mean \quad glucose \quad concentration \times 18 \times mean \quad insulin \quad concentration}}$$

where G_0 was the fasting blood glucose (mmol/L), I_0 was the fasting plasma insulin (mU/L) [32]. HOMA-IR was calculated by the following [33]:

$$\frac{G_0 \times I_0}{22.5}$$

Table 3
Relationships between liver enzyme levels and baseline characteristics in healthy and T2D subjects (N = 58 in total) in Study 1.

	GGT		ALP		ALT		AST	
Parameters	r	P	r	P	r	P	r	P
BMI (kg/m^2)	0.29	0.03	0.03	0.8	0.33	0.011	0.15	0.28
HbA1c (%)	0.38	0.01	0.02	0.9	0.28	0.07	0.25	0.11
Fasting glucose (mmol/L)	0.18	0.17	0.04	0.7	0.31	0.02	0.24	0.08
Glucose AUC$_{0-180\ min}$ (mmol/L*min)	0.13	0.34	0.05	0.7	0.27	0.04	0.2	0.13
Fasting insulin (mU/L)	0.41	0.001	0.10	0.4	0.53	<0.0001	0.42	0.001
Insulin AUC$_{0-180\ min}$ (mU/L*min)	0.19	0.15	0.13	0.3	0.35	0.007	0.23	0.07
Fasting C-peptide (pmol/L)	0.36	0.005	0.15	0.27	0.54	<0.0001	0.4	0.002
C-peptide AUC$_{0-180\ min}$ (pmol/L*min)	0.21	0.1	0.24	0.07	0.33	0.01	0.2	0.1
Insulin clearance	-0.14	0.3	-0.02	0.9	-0.35	0.008	-0.2	0.1
Fasting glucagon (pg/mL)	0.01	0.94	-0.02	0.9	0.34	0.009	0.30	0.024
Glucagon AUC$_{0-180\ min}$ (pg/mL*min)	0.1	0.47	0.1	0.4	0.44	0.0006	0.38	0.004
Matsuda index (dL/mg*L/mU)	-0.37	0.004	-0.16	0.2	-0.57	<0.0001	-0.46	0.0004
HOMA-IR (mmol/L*mU/L)	0.43	0.0007	0.12	0.4	0.57	<0.0001	0.44	0.0005

Spearman correlation analysis (or Pearson correlation analysis for normally distributed data) was used to determine the relationship.

2.4. Statistical analysis

Areas under the curves (AUCs) for blood glucose, plasma insulin and plasma glucagon were calculated using the trapezoidal rule. Demographic data were either compared using one-way ANOVA (three subgroups) or unpaired Student's t-tests (designated subgroups) except for gender and medication use, which were compared by Chi-square tests.

In Study 1, the relationships between parameters (including the combined healthy and T2D subjects who received 75 g oral glucose) were examined by either Pearson correlation analyses (when the data were normally distributed) or Spearman correlation analyses (when the data were not normally distributed). Sensitivity analyses, which excluded healthy subjects, were also performed. Given the strong correlation between metabolic parameters and ALT, T2D subjects were also stratified into three subgroups, including T1 (ALT ≤ 29 U/L, N = 15), T2 (ALT = 30–40 U/L, N = 15) and T3 (ALT > 40 U/L, N = 13) (Table 1).

In Study 2, because the majority of the subjects had relatively low ALT values, they were divided into two subgroups, with Lower (≤29 U/L, N = 45) or Higher (≥30 U/L, N = 41) ALT levels (Table 2). Blood glucose, plasma insulin and plasma glucagon curves were compared between the subgroups by two-way repeated measures ANOVA using 'group' and 'time' as factors. If ANOVA revealed significant interactions, post hoc comparisons were performed with Bonferroni's correction.

All analyses were performed with GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA). Data are presented as means ± SEM. P < 0.05 was considered statistically significant.

3. Results

Both protocols were well tolerated. Demographic and biochemical data of subjects included in Studies 1 and 2 are summarized in Tables 1 and 2, respectively. In Study 1, healthy and T2D subjects were well matched for age, BMI and gender, but ALT was higher, and AST non-significantly higher, in the T2D group (Table 1). As expected, blood glucose concentrations, both fasting (P < 0.0001) and after oral glucose (group effect: P < 0.0001, group by time interaction: P < 0.0001), were substantially higher in T2D than healthy subjects (Fig. 1A). Fasting insulin was higher (P < 0.0001), and the insulin response to oral glucose occurred later (peak at ~ 120 min vs. 60 min) in T2D subjects (P = 0.0048 for group by time interaction, Fig. 1B). Similarly, fasting C-peptide was higher (P < 0.0001), and the C-peptide response to oral glucose occurred later (~120 min vs 90 min) in T2D subjects (group by time interaction: P < 0.0001, Fig. 1C). Fasting glucagon was numerically (P = 0.4) higher in T2D subjects. However, while plasma glucagon was suppressed after oral glucose in healthy subjects, it increased to a peak at t = 30 min in the T2D group before returning to baseline. Accordingly, the glucagon response to oral glucose was greater in T2D

than healthy subjects (group by time interaction: P < 0.0001) (Fig. 1D). In T2D subjects in Study 2, blood glucose, plasma insulin, and plasma glucagon all increased following the mixed meal, with glucose and insulin peaking at around 90 min and glucagon peaking at around 30 min after the meal, as reported previously [28,29,34].

3.1. Relationships between metabolic parameters and liver enzymes in fasting and after an oral glucose load and before and after a mixed meal

In the pooled analysis of both healthy and T2D subjects who received oral glucose (Study 1), BMI (r = 0.33, P = 0.011), fasting blood glucose (r = 0.31, P = 0.02), plasma insulin (r = 0.53, P < 0.0001), C-peptide (r = 0.54, P < 0.0001) and glucagon (r = 0.34, P = 0.009), as well as HOMA-IR (r = 0.57, P < 0.0001), correlated directly with ALT (Fig. 2). The relationship between HbA1c and ALT (r = 0.28, P = 0.07) did not reach significance (Table 3). BMI (r = 0.29, P = 0.03), HbA1c (r = 0.38, P = 0.01), fasting plasma insulin (r = 0.41, P = 0.001), C-peptide (r = 0.36, P = 0.005) and HOMA-IR (r = 0.43, P = 0.0007) correlated directly with GGT, but no significant correlation between fasting blood glucose or plasma glucagon and GGT was observed (Table 3). Likewise, fasting plasma insulin (r = 0.42, P = 0.001), C-peptide (r = 0.4, P = 0.002), glucagon (r = 0.30, P =0.024) and HOMA-IR (r = 0.45, P = 0.0005) correlated directly with AST. The relationship between fasting blood glucose and AST (r = 0.23, P = 0.078) did not reach significance, and there were no significant correlations observed of either BMI or HbA1c with AST (Table 3).

After the 75 g oral glucose load, the AUC$_{0-180\ min}$ for blood glucose (r = 0.27, P = 0.04), plasma insulin (r = 0.35, P = 0.007), C-peptide (r = 0.33, P = 0.01) and glucagon (r = 0.44, P = 0.0006) correlated directly (Fig. 3A-D), whereas insulin clearance (r = −0.35, P = 0.008) and the Matsuda index correlated inversely (r = −0.57, P < 0.0001), with ALT in the pooled analysis (Fig. 3E-F). The Matsuda index also correlated inversely with GGT (r = −0.37, P = 0.004), but there was no significant correlation between the AUC$_{0-180\ min}$ for glucose, insulin, C-peptide or glucagon and GGT (Table 3). There was a direct relationship between the glucagon AUC$_{0-180\ min}$ and AST (r = 0.38, P = 0.004), an inverse relationship between the Matsuda index and AST (r = −0.46, P = 0.0004). However, no significant correlations were observed between the AUC$_{0-180\ min}$ for glucose, insulin or C-peptide and AST, nor between any of the aforementioned parameters and ALP, in the pooled analysis (Table 3).

Sensitivity analysis was also conducted between metabolic parameters and ALT in Study 1 by excluding significant outliers (Fig. S1 and S2), which did not affect the aforementioned relationships, except that the correlation between the AUC$_{0-180\ min}$ for blood glucose and ALT become less apparent (r = 0.25, P = 0.058).

After the exclusion of healthy subjects, the relationships between the metabolic parameters and liver enzymes were generally unchanged,
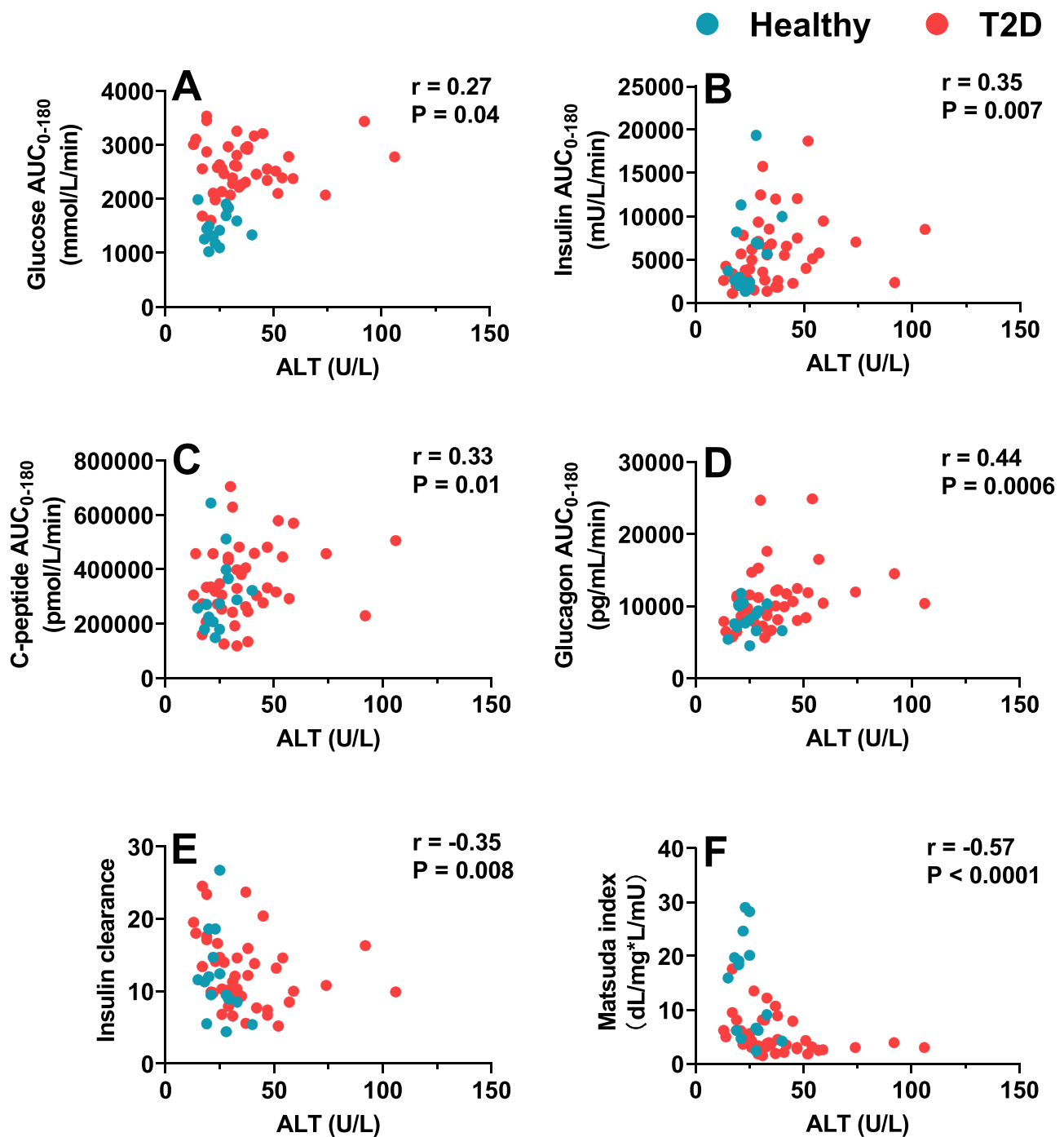
Fig. 3. Relationships of the area under the curve (AUC) for blood glucose (A), plasma insulin (B), plasma C-peptide (C), plasma glucagon (D) between t = 0–180 min, insulin clearance (E) and Matsuda index (F) with alanine transaminase (ALT) in the combined subjects without (N = 15) and with type 2 diabetes (T2D, N = 43) in Study 1. Spearman correlation analysis was employed to examine these relationships.

except that the correlations of liver enzymes with blood glucose were no longer apparent (Table S1).

There were also direct relationships between the plasma insulin concentration after a mixed meal and liver enzymes (GGT and ALT) in Study 2, although no relationships between plasma glucagon concentrations and liver enzymes were evident, most likely due to the small inter-individual variations in liver enzymes (data not shown).

3.2. Blood glucose and plasma insulin, C-peptide and glucagon in T2D subjects classified according to ALT

The demographic data of subjects in each subgroup are summarized in Table 1. GGT (P = 0.008) and AST (P < 0.0001) were higher in subgroups with higher ALT, and BMI and ALP also tended to be higher. However, neither the distribution of gender, age, or HbA1c differed between the three subgroups. While blood glucose concentrations did not differ over 180 min following the 75 g oral glucose challenge between the three subgroups of T2D subjects (Table 1 and Fig. 4A), plasma insulin concentrations were higher in subgroups with higher ALT both
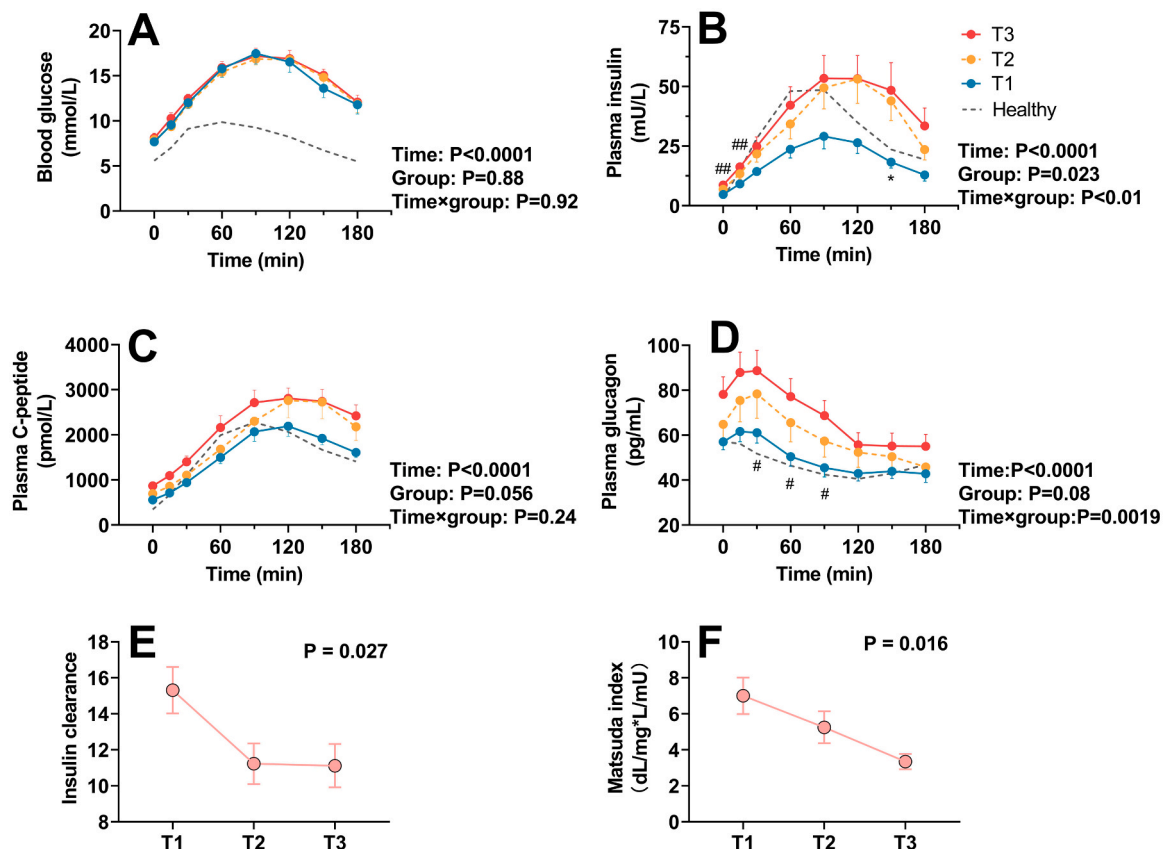
Fig. 4. Blood glucose (A), plasma insulin (B), plasma C-peptide (C) and plasma glucagon (D) in response to a 75 g oral glucose load in subgroups of individuals with type 2 diabetes (T2D), stratified by ALT, i.e. T1 (low ALT, N = 15), T2 (intermediate ALT, N = 15) and T3 (high ALT, N = 13) in Study 1. Relevant curves for healthy participants were added to the figures for reference purposes but were not included in the statistical analysis. Repeated-measures two-way ANOVA was used to determine statistical differences. ANOVA results are reported as P values for differences by groups, the difference over time and differences due to group by time interaction. *Post hoc* comparisons were adjusted by Bonferroni's correction. Data are means ± SEM. *$P < 0.05$ for T1 vs. T2; #$P < 0.05$, ##$P < 0.01$ for T1 vs. T3; (E) and (F) show the insulin clearance and Matsuda index of the three ALT subgroups, respectively. One-way ANOVA was used to determine the statistical differences.

during fasting (P = 0.03, Table 1) and after the oral glucose load (group by time interaction: P = 0.01, Fig. 4B). Similarly, plasma C-peptide concentrations were higher in subgroups with higher ALT at fasting (P = 0.003, Table 1) and after oral glucose load (group effect: P = 0.056, Fig. 4C). Plasma glucagon concentrations were numerically higher during fasting (P = 0.08, Table 1), but significantly higher after oral glucose (group by time interaction: P < 0.0019, Fig. 4D), in subgroups with higher ALT. In addition, both insulin clearance (P = 0.027, Fig. 4E) and the Matsuda index decreased (P = 0.016, Fig. 4F), while HOMA-IR increased (P = 0.02, Table 1), with increasing ALT.

The demographic data in T2D subjects in Study 2 stratified into lower and higher ALT subgroups are summarized in Table 2. There were no differences in gender distribution, age, HbA1c, ALP or the use of metformin between the two subgroups. As in Study 1, GGT and AST were elevated in the subgroup with higher ALT in Study 2 (Table 2). Blood glucose concentrations did not differ between the two subgroups at baseline or after the meal (Fig. 5A). Both fasting (P = 0.007) and meal-induced insulin concentrations were higher in the subgroup with higher ALT (group by time interaction: P = 0.019, Fig. 5B). While fasting glucagon concentrations were numerically higher, postprandial glucagon concentrations were significantly higher in the subgroup with higher ALT (group by time interaction: P = 0.016, Fig. 5C). In addition, a lower Matsuda index (P = 0.0007) and a higher HOMA-IR (P = 0.006) were observed in the subgroup with higher ALT (Table 2).

4. Discussion

While associations of serum liver enzymes with insulin resistance and future risk of T2D in normoglycemic individuals have been increasingly appreciated, links to specific metabolic derangements in T2D remain poorly characterized. The present study showed that T2D patients with relatively good glycemic control and managed by diet and/ or metformin displayed higher serum liver enzyme levels compared to age- and BMI-matched healthy controls, and most importantly that circulating glucose, insulin, C-peptide and glucagon concentrations, both fasting and after oral glucose, correlated closely with serum liver enzymes, particularly ALT. In T2D subjects, subgroup analysis indicated that higher ALT was predictive of greater insulin resistance, higher insulin and glucagon responses to both an oral glucose load and a mixed meal, even in the absence of differences in glycemia. Together, these findings suggest that serum liver enzymes, particularly ALT, represent biomarkers for diabetogenic changes in insulin and glucagon secretion (and action) in T2D.

The observed relationships between metabolic parameters and liver enzymes in T2D subjects represent a substantial development from prior knowledge in non-diabetic subjects. As reported in the latter [20,23,35], we observed that both insulin resistance (HOMA-IR) and the insulin response to 75 g oral glucose correlated directly with ALT, AST and GGT in the T2D group. A direct relationship between fasting glucagon and some liver enzymes (ALT and GGT) has been observed in healthy individuals [35]. To our knowledge, there is no report on the relationship
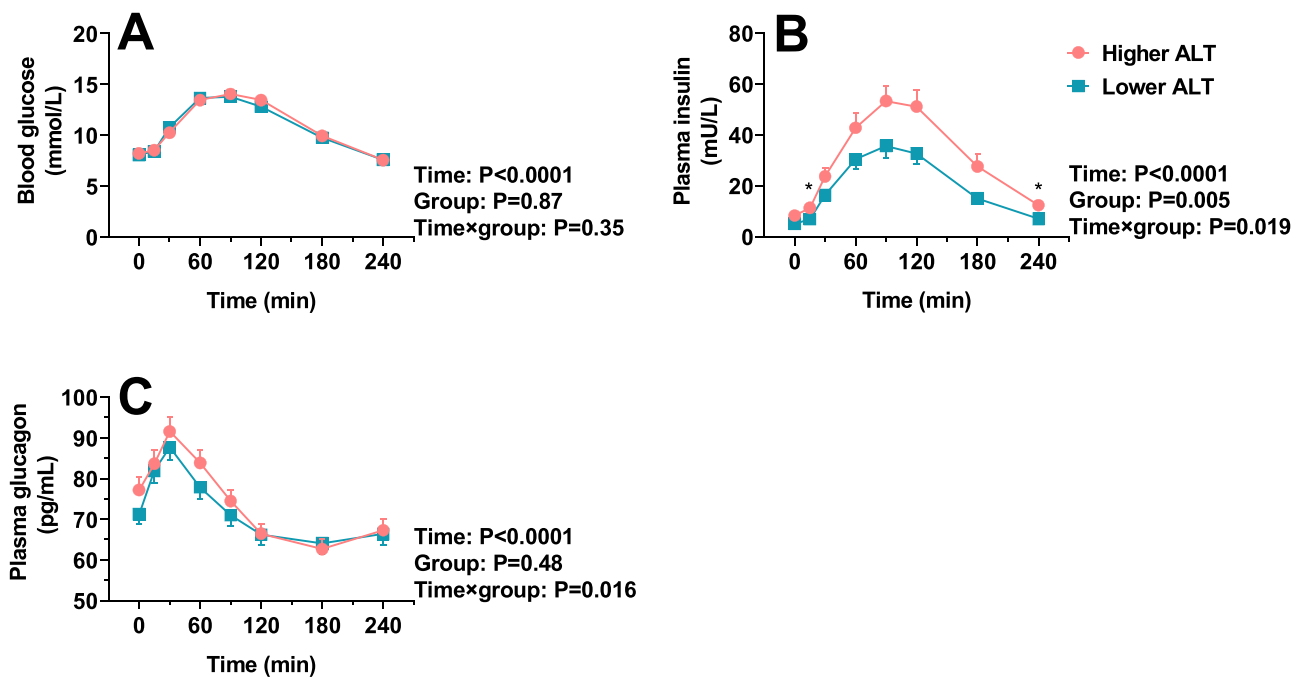
Fig. 5. Blood glucose (A), plasma insulin (B) and plasma glucagon (C) in response to a mixed meal in the two subgroups of individuals with type 2 diabetes in Study 2, i.e. lower ALT group (N = 45) and higher ALT group (N = 41). Repeated-measures two-way ANOVA was used to determine statistical differences. ANOVA results are reported as P values for differences by groups, the difference over time and differences due to group by time interaction. *Post hoc* comparisons were adjusted by Bonferroni's correction. Data are means ± SEM. *$P < 0.05$ for the higher ALT subgroup vs. lower ALT subgroup.

between circulating glucagon concentrations (either during fasting or in response to nutrient loads) and liver enzymes in patients with T2D. Therefore, the current study has added an important new insight into the link of liver enzymes to metabolic derangements in T2D. Together, these findings are indicative of the development of hepatic resistance to both insulin and glucagon signalling in the course of T2D, i.e. insulin and glucagon secretion may be exaggerated to compensate for reduced hepatocyte sensitivity to these hormones [36], which may predispose to the deterioration of glycemic control [37–40]. The mechanism underlying the relationship between liver enzymes and glucagon secretion in T2D is not clear. There is recent evidence that hyperglucagonemia in metabolic diseases, such as non-alcoholic fatty liver disease, may be driven by elevated circulating amino acid levels due to impaired amino acid catabolism in the face of hepatic glucagon resistance [41]. It may also reflect, in part, reduced metabolism of pancreatic hormones due to compromised liver function. In keeping with this concept, hepatic insulin clearance, as assessed by the C-peptide to insulin ratio, was reduced in T2D subjects with elevated ALT. The diminished insulin sensitivity associated with T2D may also be of relevance to hyperglucagonemia, since there is a counter-regulatory relationship between insulin and glucagon [42].

We recognize that serum liver enzymes are often inter-related – for example, individuals with T2D who have higher ALT levels frequently also exhibit raised AST and GGT concentrations. Relative to other liver enzymes, ALT appeared to have a closer relationship to metabolic parameters, probably due to the higher specificity of ALT for reflecting liver dysfunction [10,14]. Subgroup analysis in T2D patients revealed that an increase in ALT was predictive of augmented insulin and glucagon secretion after an oral glucose load. Similar observations were evident in an independent group of T2D patients after a standardized mixed meal. However, the reasons accounting for elevated ALT in T2D were not further examined in the current study. In people with non-alcoholic fatty liver disease, adipose tissue insulin resistance and liver triglyceride content have been shown to be major determinants of plasma ALT levels [43]. It is therefore plausible that the accumulation of liver fat in the context of insulin resistance in T2D may be relevant to

inter-individual variations in ALT. However, we note that liver enzyme levels were, at most, only modestly elevated in the T2D subjects in our study, so the degree of hepatic steatosis may not have been great. Imaging of liver fat content, e.g. by ultrasound, would add insights into this aspect.

Despite substantial differences in the insulin and glucagon responses to oral glucose and a mixed meal between subgroups of T2D subjects with different ALT levels, blood glucose concentrations did not differ between the subgroups. Similarly, the direct relationship between ALT and glycemia after the oral glucose challenge was no longer evident after excluding the healthy subjects. This is likely to reflect the fact that the development of insulin and glucagon resistance occurs in advance of deterioration in blood glucose control, and that the T2D subjects included in the current study were at a relatively 'early' stage of T2D and were able to compensate for hepatic insulin and glucagon resistance. Further studies are, therefore, warranted to evaluate the potential for liver enzymes to both characterize the pathological defects of T2D and inform interventional strategies (e.g. focussing on normalising liver enzymes) to prevent or delay the progression of the disease.

Several limitations should also be acknowledged when interpreting our findings. First, the current observational study involves only a small number of participants, lacks a healthy control in Study 2, and does not provide information about the impact of liver enzymes on future metabolic outcomes, so that a large longitudinal study involving well-matched healthy subjects as a reference in both settings is warranted. Second, the T2D subjects included in the current study were relatively homogenous, with well-controlled glycemia and without large variations in liver enzymes, which limited the capacity to demonstrate relationships with metabolic parameters. Accordingly, evaluations involving a full spectrum of T2D patients with both normal and abnormally elevated liver enzyme levels are needed. Third, the current study has focussed only on the links of insulin and glucagon secretion with liver enzymes. Concurrent measurements of other metabolic targets, such as the incretin hormone glucagon-like pepetide-1 (GLP-1) and insulin-degrading enzyme (IDE), may yield complimentary insights. Finally, given recent evidence that circulating amino acids and fatty

acids are key mediators in communication between the liver and the pancreatic islets, measurements of these may shed light on the observed dysregulated insulin and glucagon concentrations in relation to liver enzymes.

In summary, our study has revealed close relationships between metabolic derangements, including insulin resistance and hyperglucagonemia, and serum liver enzymes in individuals with relatively well-controlled T2D. That ALT was predictive of exaggerated insulin and glucagon responses to an oral glucose load or a mixed meal, in the absence of differences in glycemia, suggests that ALT may represent an 'early' biomarker of metabolic dysfunction during the progression of T2D.

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CRediT authorship contribution statement

WH was involved in data processing and interpretation, statistical analysis and writing of the manuscript; CX, NJWA, MS, SZ, KLJ and MH were involved in the conception of the study, data interpretation and reviewing of the manuscript. CKR and TW were supervisors of this work and were involved in the conceptualization and design of the study, data interpretation, statistical analysis, and reviewing and editing of the manuscript.

Conflict of Interest

NJWA has received speaker fees from Mercodia and MSD, and research support from Mercodia and Novo Nordisk, and acts on advisory boards on Boehringer Ingelheim. KLJ has received research funding from Sanofi Aventis and AstraZeneca and is a share-holder for Glyscend. MH has participated in the advisory boards and/or symposia for Novo Nordisk, Sanofi, Novartis, Eli Lilly, Merck Sharp & Dohme, Boehringer Ingelheim, Inova and AstraZeneca and has received honoraria for this activity, and is a share-holder for both Satiogen and Glyscend. CKR has received research funding from AstraZeneca, Merck Sharp & Dohme, Eli Lilly, Novartis, and Sanofi, and is a share-holder for both Satiogen and Glyscend. TW has received travel support from Novartis and Sanofi and research funding from Novartis and AstraZeneca. None of the other authors has any personal or financial conflict of interest to declare.

Data Availability

Data will be made available on request.

Acknowledgement

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.peptides.2023.171092.

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