Plasma levels of mannan-binding lectin-associated serine proteases are increased in type 1 diabetes patients with insulin resistance

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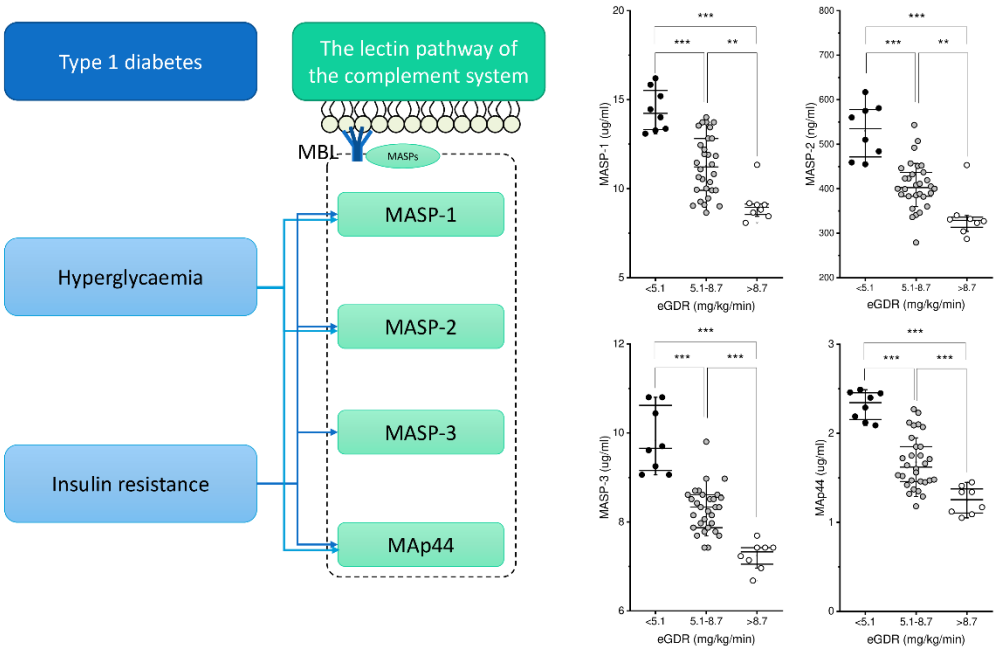
A list of abbreviations

eGDR	estimated glucose disposal rate
IR	insulin resistance
MAps	MBL-associated proteins
MASPs	MBL-associated serine proteases
MBL	mannan-binding lectin
T1D	type 1 diabetes
T2D	type 2 diabetes

ABSTRACT

Activation of the lectin pathway of the complement system, as demonstrated by elevated levels of mannan-binding lectin proteins (MBL), contributes to vascular pathology in type 1 diabetes (T1D). Vascular complications are greatest in T1D individuals with concomitant insulin resistance (IR), however, whether IR amplifies activiation of the lectin pathway in T1D is unknown. We pooled pre-treatment data from two RCTs and performed a cross-sectional analysis on 46 T1D individuals. We employed estimated glucose disposal rate (eGDR), a validated IR surrogate with cut-points of: <5.1, 5.1 – 8.7, and >8.7 mg/kg/min to determine IR status, with lower eGDR values conferring higher degrees of IR. Plasma levels of MBL-associated proteases (MASP-1, MASP-2, MASP-3) and their regulatory protein MAp44 were compared among eGDR classifications. In a subset of 14 individuals, we assessed change in MASPs and MAp44 following improvement in IR. We found that MASP-1, MASP-2, MASP-3, and MAp44 levels increased in a stepwise fashion across eGDR thresholds with elevated MASPs and MAp44 levels conferring greater degrees of IR. In a subset of 14 patients, improvement in IR was associated with significant reductions in MASPs, but not MAp44, levels. In conclusion, IR in T1D amplifies levels of MASP-1/2/3 and their regulator MAp44, and improvement of IR normalises MASP-1/2/3 levels. Given that elevated levels of these proteins contribute to vascular pathology, amplification of the lectin pathway of the complement system may offer mechanistic insight into the relationship between IR and vascular complications in T1D.

KEYWORDS: Type 1 diabetes, Mannan-Binding Lectin-Associated Serine Proteases, Complement, Insulin Resistance



Graphical abstract: Elevated levels of mannan-binding lectin (MBL) is associated with increased vascular complications in individuals with type 1 diabetes (T1D). Hyperglycaemia is one of underlying mechanisms contributing to increased levels of mannan-binding lectin-associated protease-1 and 2 (MASP-1/2) and regulatory protein (MAp44). Insulin resistance in the context of T1D further amplifies MASP-1/2/3 and MAp44 levels.

1 Introduction

Animal and clinical studies have recently confirmed a prominent role of the complement system in the pathogenesis of type 1 diabetes (T1D) by augmenting underlying organ-specific autoimmune processes.[1] Furthermore, the complement system has been implicated in the progression of microvascular and macrovascular complications.[1] Indeed, patients with T1D express elevated circulating levels of several complement system proteins,[2] including the central component C3,[3, 4] and, present with tissue deposits of complement activation products [5, 6] which have been causally associated with vascular-thrombotic complications.[1]

There is now clear evidence for the specific involvement of the lectin pathway of the complement system in the development of vascular complications. Activation of the lectin pathway is mediated by mannan-binding lectin (MBL) pattern recognition molecules via MBL-associated serine proteases (MASPs) and regulatory MBL-associated proteins (MAps), although the role and action of some of these components are yet to be fully elucidated.[7] We have previously shown that initiating the lectin pathway, namely complement activating MASP-1 and MASP-2, to be elevated in patients with T1D [2] and experimental studies demonstrate both MASP-1 and MASP-2 to exhibit thrombin-like activity thus inducing clot formation.[8-10] Further, MBL levels have been reported to be increased in those with overt diabetic nephropathy,[11] and increased MBL levels are associated with progression to end-stage renal disease.[12]

The complement system also plays a role in the development of insulin resistance (IR) and progression to type 2 diabetes (T2D).[13, 14] For example, hepatic- and adipose-derived complement proteins are associated with IR,[15] and *in vitro* and animal work shows upregulation of complement protein synthesis in obese mice and cultured adipose tissue from insulin-resistant humans.[16] This is further supported through the demonstration of adipose tissue and global weight loss in complement protein knockout, and complement protein receptor knockout mice whilst under IR-inducing diet conditions.[17, 18]

Whereas IR is often discussed within the context of T2D, our group [19] and others [20-22] have recently shown IR to be a prevalent feature of T1D and a strong predictor of vascular complications in this population. The evidence for the role of MBL in T1D, vascular complications, and IR, raises the intriguing question of whether IR within the context of T1D further amplifies complement activation, which, if demonstrated could play a pathological role in the increased rate of vascular complications in this population. Therefore, the aim of our present study was to assess plasma concentrations of MBL-associated serine proteases (MASP-1/2/3) and -associated proteins (MAp44) in relation to IR, and, assess whether improvement in IR modifies MASP-1/2/3 and Map44 levels.

2 METHODS & MATERIALS

2.1 Study design and population

We performed a cross-sectional analysis on pooled data from two studies (NCT05231642; ISRCTN13641847) which had previously received ethical approval from local National Health Service Research Ethics Committees. All participants gave written informed consent in accordance with the Declaration of Helsinki.

In the present analysis, we included 46 participants meeting the following inclusion criteria: classical presentation of T1D (including primary osmotic symptoms, weight loss, hyperglycaemia, ketosis, insulin initiation at diagnosis); aged 18-50 years; diagnosed with T1D for a minimum of 5-years on enrolment; treated on a stable (>12 months) basal-bolus insulin regimen consisting rapid-acting insulin analogues lispro or aspart and basal insulin glargine delivered through multiple daily injections or continuous subcutaneous insulin infusion; and free of diabetes-related complications except for background retinopathy.

For our main analysis, we used baseline pre-treatment data across both studies. Data collection occurred during a morning-time laboratory visit, with patients adopting an overnight fast

(> 10 hours). We obtained venous blood samples from which citrated plasma was separated within 2-hours of the collection and stored in aliquots for retrospective analysis. During this visit, we obtained the following clinical data (age, duration of diabetes, HbA1c, insulin requirements, BMI, blood pressure, and eGDR). Blood pressure was assessed via an automated oscillometric device (Intellisense HEM-907XL, Omron, Japan); participants were categorised as hypertensive if ≥140/90mmHG, pre-existing physicians' diagnosis, or antihypertensive use [23]. Insulin resistance was assessed by calculating the estimated glucose disposal rate (eGDR) 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).[24] In a subset of patients, we collected repeat blood samples during routine clinic follow-up at ~6 months following a standardised intervention that aimed at improving IR through achieving weight loss, adjusting insulin doses, and regular patient contact; due to a loss of follow-up or missed appointments repeat blood samples were obtained from 14 patients only.

2.2 Laboratory measurements

We measured levels of MASP-1, MASP-2, MASP-3, and MAp44 from citrated samples which had been stored in aliquots at -80°C. MASP-1 was determined with a competition enzyme-linked immunosorbent assay (ELISA) using a MASP-1-specific antibody, as described earlier.[25] Plasma levels of MASP-2 and MASP-3 were measured with commercial ELISA kits (Hycult Biotech, Uden, the Netherlands). MAp44 was determined with a time-resolved immunofluorometric assay (TRIFMA) using a catching antibody and a biotinylated detecting antibody in a sandwich-type assay, as described previously.[26] Intraassay coefficients of variance of all assays were <10%. Routine parameters, namely HbA1c, were determined using local hospital laboratories.

2.3 Statistical analysis

Data were analysed using SPSS Statistics version 25 (IBM SPSS Statistics 25, IBM Corporation, USA). Descriptive characteristics of the study population are presented as mean±SD or median [interquartile range] for continuous variables and as frequency (%) for categorical variables; 95% confidence intervals (CIs) and β coefficients are presented where relevant; statistical significance was accepted at $P<0.05$. For descriptive purposes, we categorised individuals based on IR status, corresponding to eGDR cut-points of: <5.1, 5.1 – 8.7, and >8.7 mg/kg/min, with lower eGDR values conferring higher degrees of IR. We established three eGDR thresholds as derived from previously published work.[27] One-way ANOVA with post-hoc Bonferroni or Kruskal-Wallis test was applied to compare differences in clinical parameters between eGDR categories. Bivariate correlations of parameters were analysed using Pearson's correlation coefficients. We applied unadjusted and adjusted generalised linear regression analyses to examine the relationship between eGDR with MASPs and MAp44, with age, sex, and diabetes duration as potential confounders. As HbA1c is a component for eGDR calculation, the mediation effect of HbA1c, therefore, was tested with the Mediation model using PROCESS v4.0 macro for SPSS.[28] Differences between baseline and 6-month time points were assessed using paired samples t-tests, with the magnitude of change presented as a scattered plot.

3 RESULTS

3.1 Characterisation of diabetes patients

Our study population comprised n=46 patients with T1D. In line with our previously published work,[27] we stratified this cohort by IR status, with IR cut points corresponding to an eGDR of <5.1, 5.1 – 8.7, and >8.7 mg/kg/min, with lower eGDR values conferring higher degrees of IR; baseline demographic and clinical characteristics are presented in Table 1.

3.2 Insulin resistance increases plasma levels of MASPs and MAp44 in T1D patients

MASP-1, MASP-2, MASP-3, and MAp44 levels increased in a stepwise manner across eGDR thresholds with MASPs and MAp44 levels highest in patients with greater degrees of IR (Table 1, Figure 1). Table 2 shows the unadjusted and adjusted associations of eGDR with MASPs and MAp44. In unadjusted regression analyses, eGDR was inversely associated with MASP-1, MASP-2, MASP-3, and MAp44; these findings remained robust following adjustment for confounders (age, sex, and diabetes duration). Additionally, the Mediation model also demonstrated that the effect of eGDR on MASPs and Map44 was not mediated by HbA1c suggesting the effect was driven by other components of eGDR such as BMI or hypertension (Supplementary Figure 1).

3.3 Reducing insulin resistance improves MASPs and MAp44 in T1D patients

In a subgroup (n=14) of patients, we measured levels of MASPs and MAp44 at baseline and 26±1 weeks after improving IR (Supplementary Figure 2). Overall, a small (-0.41±0.19 mg/kg/min [%-6.85±3.24]) but statistically significant increase in eGDR was associated with significant reductions in MASPs, but not MAp44, levels (Figure 2). Reductions in MASP-1, MASP-2, and MASP-3 were statistically significant at a threshold improvement of ≥7% in eGDR (MASP-1: <7%eGDR p=0.180 vs. ≥7%eGDR p=0.042; MASP-2: <7%eGDR p=0.0496 vs. ≥7%eGDR p=0.038; MASP-3: <7%eGDR p=0.146 vs. ≥7%eGDR p=0.021). Collectively, these data indicate that IR may represent an important mediator of MASP levels in T1D.

4 Discussion

It is well established that MBL is elevated in people with insulin resistance, T2D, and T1D, and that increased MBL levels are associated with vascular complications and mortality.[11, 12, 29-31] However, plasma levels of the MBL-associated serine proteases, MASP-1, MASP-2 and MASP-3, and their regulator MA4p44, have not been studied in T1D individuals with concomitant insulin resistance. Here we show for the first time that individuals with T1D with IR express higher levels of MASP-1, MASP-2, MASP-3, and their regulator MAp44 as compared to IR-naive T1D individuals. Specifically, we show that plasma levels of MASPs, and MAp44, increase in a stepwise fashion across eGDR thresholds, and that subsequent improvement in eGDR at a threshold $\geq$ 7% is associated with significant reductions of MASP-1, MASP-2, and MASP-3.

Overactivation of the lectin pathway has been consistently reported in individuals with T1D [2, 31, 32] with previous work suggesting glycaemic control to modulate amplification.[2, 33] For example, in mice, increased MBL-C levels increased as a consequence of increasing plasma glucose concentrations in streptozotocin-induced diabetes in mice,[34] and other studies demonstrate protection from hyperglycaemic complications in MBL knockout or insulin-treated mice.[35] Complement proteins contribute to glucose homeostasis via pleiotropic effects on glucose uptake, storage, and disposal in hepatocytes.[36] Previous work has demonstrated an interaction between MBL and the glycation product fructoselysine resulting in activation of the complement lectin pathway,[33] and consequently amplification of the inflammatory response. In the present study, MASP levels in our patients without IR were elevated to a similar level as previously reported.[2]

However, our data show that MASPs and MAp44 are elevated further in the presence of IR – an effect which we also report is reversed following IR remission. Importantly, our mediation model revealed that this effect was not mediated by HbA1c, which would suggest that IR has an independent and direct role in increasing MBL and its associated serine proteases. Whereas insulin-resistant states typically bolster advanced glycation end products (AGEs) via a hyperglycaemic and

hyperlipidaemic milieu,[37-39] it is possible that mechanisms beyond or independent to AGEs are mechanistically linked to insulin sensitivity, potentially via lipogenic and proinflammatory pathways which directly impair insulin signalling and induce immunologic alterations. Indeed, prior work within the setting of IR-T2D individuals demonstrate that serine proteases are heavily implicated in the development of complications – an association in which IR is likely the main modulator [11, 12, 29-31]. If consistent in T1D, this would support our hypothesis that IR is a fundamental pathological mediator of vascular complications in this population.

Our study is not without limitations. Firstly, because of limited available evidence on the topic of IR in T1D, we chose a pilot study case-control design with a conservative sample size, and we were unable to measure precursors of other complement pathways. Further, eGDR, although a validated surrogate of IR in T1D is not a direct assessment of IR, and therefore we cannot exclude the potential for an interaction between the constituent components of eGDR, namely body weight, hypertension, and hyperglycaemia. Notwithstanding these limitations, this work is the first to report the mediating effect of IR in T1D on MASPs and MAp44 providing a benchmark to launch future larger, prospective, and mechanistic studies investigating the role of the complement system in the development of complications in T1D individuals with IR.

DATA AVAILABILITY STATEMENT: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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ETHICAL APPROVAL STATEMENT: This study was conducted using the pooled data from two studies (NCT05231642; ISRCTN13641847) which had previously received ethical approval from local National Health Service Research Ethics Committees. All participants gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS: GES recruited and characterised patients, performed the laboratory measurements, analysed the data. **NK** analysed data and wrote the manuscript. **MDC** designed the study, analysed the data, and wrote the manuscript. **JB, VS,** and **RA** contributed to the interpretation of data and writing of the manuscript. All authors revised and approved the manuscript.

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6 TABLES

Table 1. Clinical characteristics and MASPs and MAp44 levels of the study population

	All patients	IR Status		
		eGDR <5.1	eGDR 5.1 – 8.7	eGDR >8.7
n	46	8	30	8
Sex (%male)	50	37.5	50.0	62.5
Age (years)	29.6±6.8	29.1±7.5	29.6±6.1	29.8±9.1
BMI	27.2±2.9	29.9±1.7	27.0±3.0*	25.1±1.1*
Hypertension (%)	52.2	100	53‡	0*
HbA1c (%)	7.74±0.96	8.50±0.81	7.81±0.88‡	6.71±0.42*
eGDR (mg/kg/min)	6.61±1.88	4.00±0.74	6.56±1.12*‡	9.39±0.47*
Diabetes duration (years)	17.0 [12.8, 19.3]	16.7 [11.8, 21.3]	17.0 [14.0, 20.0]	12.5 [8.0, 17.5]
Daily insulin dose (U/day)	44.1±6.7	55.9±4.9	42.9±3.2*‡	36.7±1.0*
MASP-1 (µg/ml)	11.35 [9.25, 13.39]	14.24 [13.30]	11.23 [9.91, 12.82]*‡	8.96 [8.50, 9.16]*
MASP-2 (ng/ml)	407 [352, 456]	535 [465, 579]	403 [383, 439]*‡	329 [309, 338]*
MASP-3 (µg/ml)	8.33 [7.69, 8.77]	9.65 [9.11, 10.71]	8.33 [7.87, 8.61]*‡	7.33 [7.01, 7.42]*
MAp44 (µg/ml)	1.62 [1.40, 2.09]	2.34 [2.14, 2.46]	1.62 [1.46, 1.88]*‡	1.25 [1.10, 1.39]*

Note: Metric variables are reported as mean±SD or median [interquartile percentile]; categorical variables are reported as frequency (percentage). Conditional differences were assessed using one-way ANOVA or Kruskal-Wallis test. * = significantly different from eGDR<5.1; ‡ = significantly different from eGDR>8.7; MASP = mannan-binding lectin-associated serine proteases; MAp44 = mannose-binding lectin-associated protein.

Table 2. Linear regression analysis between eGDR and MASPs and MAp44 in patients with T1D

	Model 1		Model 2	
	β (CI)	**P value**	**β (CI)**	**P value**
MASP-1 (µg/ml)	-0.852 (-1.082 to -0.622)	<0.001**	-0.876 (-1.113 to -0.639)	<0.001**
MASP-2 (ng/ml)	-34.78 (-41.72 to -27.84)	<0.001**	-35.42 (-42.53 to -28.33)	<0.001**
MASP-3 (µg/ml)	-0.433 (-0.505 to -0.360)	<0.001**	-0.435 (-0.508 to -0.361)	<0.001**
MAp44 (µg/ml)	-0.197 (-0.225 to -0.169)	<0.001**	-0.203 (-0.231 to -0.176)	<0.001**

Note: Model 1 is unadjusted; Model 2 was fit to estimate associations with adjustment for age, sex, and diabetes duration;
*denotes significant association at P<0.05; ** denotes a significant association at P<0.001. MASP = mannan-binding lectin-associated serine proteases; MAp44 = mannose-binding lectin-associated protein.

7 FIGURE LEGENDS

Figure 1. MASPs and MAp44 levels in T1D patients stratified by IR status. A: MASP-1; B: MASP-2; C: MASP-3; D: MAp44. *denotes significant association at P<0.05; ** denotes a significant association at P<0.001; *** denotes a significant association at P<0.0001; Closed circles = eGDR <5.1, grey circles = eGDR 5.1 – 8.7; open circles = eGDR >8.7; MASP = mannan-binding lectin-associated serine proteases; MAp44 = mannose-binding lectin-associated protein.

Figure 2. %Change from baseline to 6 months in MASPs and MAp44 (Y-axis) following improvement in eGDR (X-axis) in T1D patients. A: MASP-1; B: MASP-2; C: MASP-3; D: MAp44.

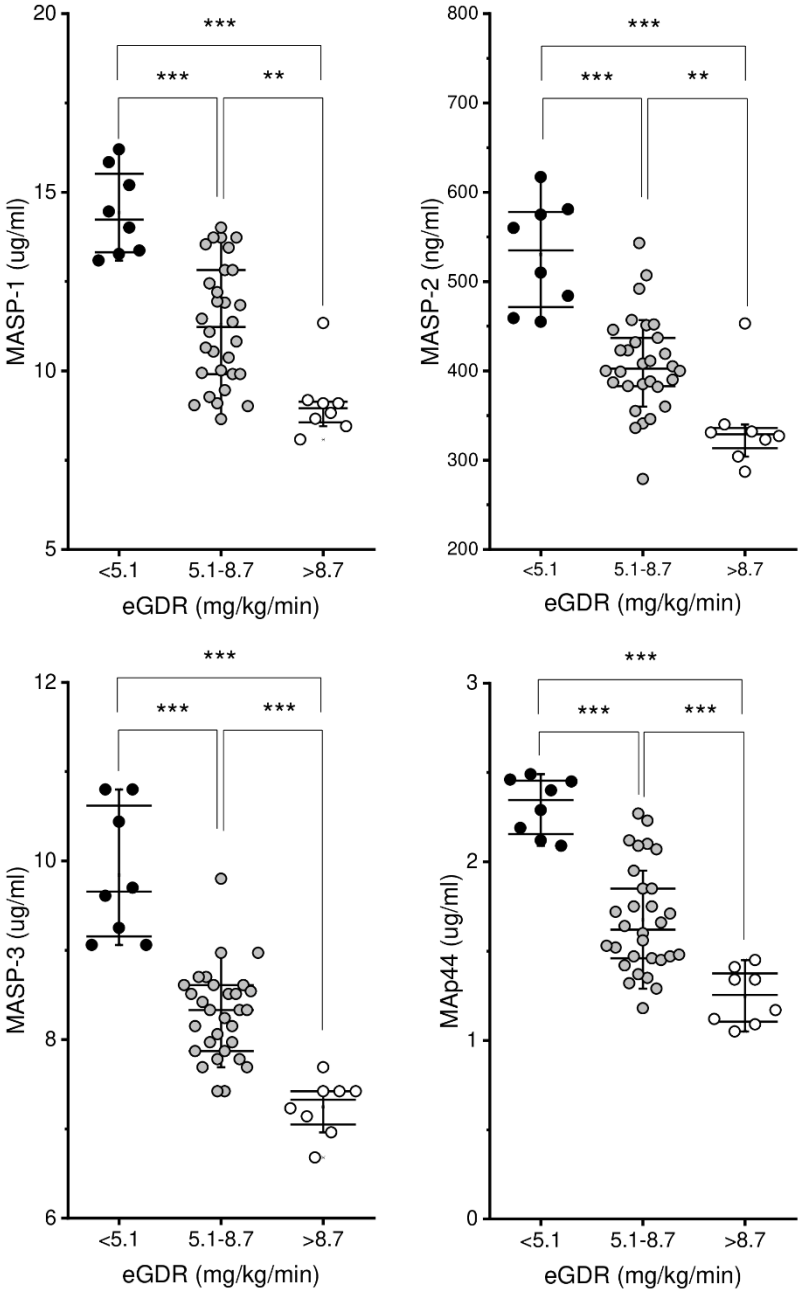

Figure 1. MASPs and MAp44 levels in T1D patients stratified by IR status. A: MASP-1; B: MASP-2; C: MASP-3; D: MAp44. *denotes significant association at P<0.05; ** denotes a significant association at P<0.001; *** denotes a significant association at P<0.0001; Closed circles = eGDR <5.1, grey circles = eGDR 5.1 – 8.7; open circles = eGDR >8.7; MASP = mannan-binding lectin-associated serine proteases; MAp44 = mannose-binding lectin-associated protein.

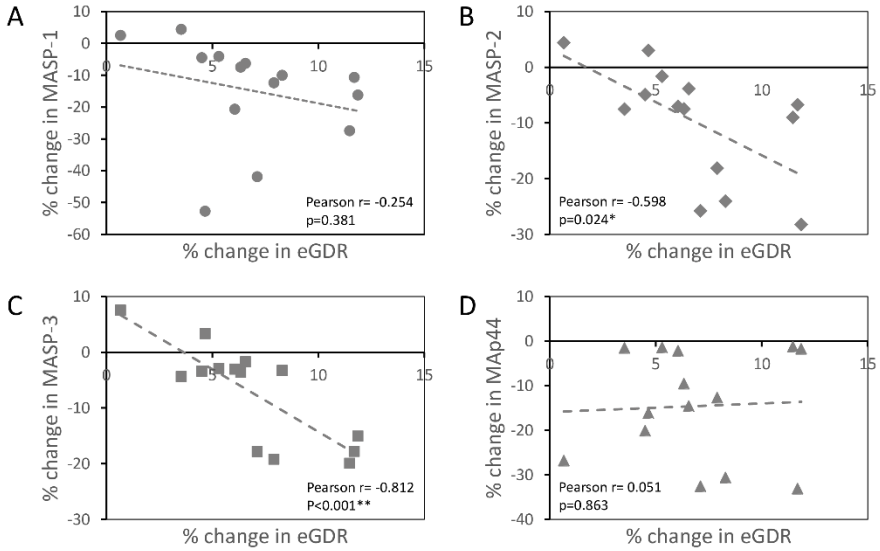

Figure 2. %Change from baseline to 6 months in MASPs and MAp44 (Y-axis) following improvement in eGDR (X-axis) in T1D patients. A: MASP-1; B: MASP-2; C: MASP-3; D: MAp44.