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Urine α-Glutathione S-Transferase, Systemic Inflammation and Arterial Function in Juvenile Type1 Diabetes

Running head:

Proximal tubular function and arterial function in juvenile diabetes

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Abstract

**Background:** Despite marked improvement in therapy and monitoring of patients with insulin-dependent (type1) diabetes, diabetic nephropathy remains a serious complication, with subsequent end-stage renal disease in about 20% of cases.

**Objective:** To investigate in young patients with type1 diabetes whether urine $\alpha$-Glutathione S-transferase to creatinine ratio ($\alpha$-GST:crea) relates to markers of systemic inflammation and subclinical vasculopathy.

**Design:** Children and adolescents (median age and diabetes duration 14 and 6 years, respectively) with type 1 diabetes screened in a previous study for proximal tubular (urine $\alpha$-GST:crea ratio) and renal (plasma creatinine, cystatin C glomerular filtration rate (GFR), timed urine albumin excretion rate (AER)) function were, within the same timeframe, also investigated for vascular (blood pressure, carotid artery intima-media thickness (IMT) and compliance (CAC), brachial flow-mediated dilatation (FMD) and plasma cyclic guanosine monophosphate (cGMP) and inflammatory (C-reactive protein (CRP), and tumor necrosis factor-alpha (TNF-$\alpha$)) profiles. Exposure to environmental tobacco smoke (ETS) was assessed through questionnaire (n=67 respondents).

**Results:** None of the patients (n=69) had overt renal insufficiency. AER correlated with age (p=0.01, r=0.3), diabetes duration (p=0.02, r=0.3), FMD (p=0.04, r=0.3, n=52), CAC (p=0.03, r=0.3, n=62) and cGMP (p=0.01, r=0.3, n=59). $\alpha$-GST:crea was lower (p=0.03) in patients than in controls. $\alpha$-GST:crea appeared to be particularly lower in older patients (p=0.004, r=0.34 vs age), in those with worse diabetic control (p=0.03, r=0.26 vs HbA1c), and in those with lower carotid artery elasticity (p=0.017, r=0.3 vs CAC). Although ETS had no direct significant impact on $\alpha$-GST:crea, $\alpha$-GST:crea correlated with FMD only in patients with ETS (r=0.5, p=0.009, n=13). Instead, $\alpha$-GST:crea showed positive association with TNF-$\alpha$ (p=0.01, r=0.3).
Conclusion: In children and adolescents with type 1 diabetes, lower levels of urine excretion of \( \alpha \)-GST:crea appear to be associated with decreasing elasticity and endothelial vasomotor function of peripheral arteries, especially in patients with ETS. In contrast, higher levels of \( \alpha \)-GST:crea are more common in patients with elevated markers of systemic inflammation. Large scale prospective studies are needed to clarify the meaning and mechanisms of this association.
Introduction

Previous studies have demonstrated that abnormalities in arterial function and structure occur early in life in patients with type 1 diabetes [1-3]. These changes may be detected non-invasively (e.g. via high-resolution ultrasound), and are seemingly at least in part responsible for the excessive burden of cardiovascular disease in adults with risk factors [3]. For instance, in patients with end-stage renal disease undergoing haemodialysis, decrease in carotid elasticity appeared to be a strong independent predictor of cardiovascular mortality [4]. It has been suggested that decreased arterial elasticity may also independently predict progression to chronic kidney disease but the precise mechanisms remain elusive [5]. Also abnormalities in arterial endothelial function have been documented in patients with renal failure [6].

Already early in the life of patients with type 1 diabetes, accelerated development of adverse changes in the vascular system appears to be accompanied by subclinical renal dysfunction, such as microalbuminuria [7,8]. Hyperglycemia, systemic inflammation, and changes in lipid profile along with increased oxidative stress have been suggested as putative mechanisms [9].

The current diagnostic screening of nephropathy in type 1 diabetes includes assessment of urine albumin excretion (AER) and glomerular filtration rate (GFR). Microalbuminuria has long been considered an acceptable standard in the early diagnosis of nephropathy. Although it has been found to be a predictor of cardiovascular risk [10], microalbuminuria has low sensitivity and specificity for nephropathy given its transient nature especially in young patients with type 1 diabetes.

Emerging novel urine biomarkers like epidermal growth factor, Tamm-Horsfall protein [10], α-1-microglobulin and transforming growth factor-β1 [12], specific for different parts of the kidney, have been suggested to provide additional sensitivity in the early diagnosis of kidney injury, or even to be linked to cardiovascular mortality in adult patients with type 1 diabetes [13]. Cytosolic urine enzyme glutathione S transferase alfa (α-GST) is localized in the cytoplasm and nuclei of cells in the proximal convoluted tubules, medullar tubules and thin loops of Henle [14,15]. It has been suggested that
elevated levels of urine $\alpha$-GST excretion could indicate proximal tubular cell damage [15]. Exposure to nephrotoxic drugs [14] leads to rapidly increased excretion of urine $\alpha$-GST. Cardiac surgery with cardiopulmonary bypass [16], which is known to promote intense system inflammation, is also associated with transient but marked increase in urine $\alpha$-GST, yet with preserved renal function, suggesting that other mechanisms, rather than cell injury, could be implicated as well.

Nevertheless previous study from our center on young patients with type1 diabetes has shown lower levels of urine $\alpha$-GST:crea than in age-matched controls particularly in patients with longer diabetes duration and elevated HbA1c [17]. In alloxan-induced diabetic rats renal GST enzyme expression was decreased but normalized with insulin treatment [18].

To our knowledge, with exception of the afore-mentioned study from our center [17], there are no published clinical pediatric studies linking urine $\alpha$-GST excretion to type1 diabetes. We therefore sought to investigate, in the same population, whether the previously reported decrease in urine $\alpha$-GST:crea could be associated with vascular, metabolic, and inflammatory changes known to appear early in the course of type1 diabetes, and whether extrinsic factors, such as exposure to ETS could have additional impact.

**Subjects**

Of the 184 children and adolescents enrolled in a previous study on urine excretion of GST enzymes, up to 69 patients were included in another study carried out within the same timeframe, in which vascular (blood pressure, carotid artery intima-media thickness and compliance, brachial flow-mediated dilatation and plasma cyclic guanosine monophosphate (cGMP) and inflammatory (high-sensitivity C-reactive protein (CRP), and tumor necrosis factor-alpha (TNF-$\alpha$) markers were investigated. Exposure to environmental tobacco smoke (ETS) was assessed through a confidential questionnaire, and defined as occasional or regular cigarette smoking in the presence of study
participants in or outside home (e.g. private or public transportation, in or around school, playground, other public places). Patients were divided into three groups: 1 = no exposure during the past year; 2 = occasional exposure, i.e. presence in a smoky environment less than once a week; 3 = weekly to daily exposure. In addition the average number of cigarettes smoked per day in or around home by patient’s cohabitants was assessed as well as the number of household smokers. Patients at our department are commonly treated with approximately 50% Insulin Glargin once daily or insulin Detemir twice daily and insulin Aspart with meals. Approximately 10-15% of the diabetic children use an insulin pump with insulin Aspart. HbA1c was measured four times a year upon follow-up visits. Exclusion criteria were age below 6 years, co-presence of familial hypercholesterolemia, active smoking or systemic hypertension. The ethical committee for human research at the Lund University Hospital approved the study. Written and oral consent was obtained from all participants and or parents.

Methods

Urine and blood analyses

The method for urine α-GST analysis is described elsewhere [17]. Timed over night urine was collected at home. A specimen of urine for GST was spared with the addition of a preservative within four hours and provided by the manufacturer (Biotrin International Ltd, Dublin, Ireland). Urine α-GST was measured in 71 patients with a commercially available solid phase sandwich, using an immunosorbent assay from Biotrin International Ltd, Dublin, Ireland. Urine albumin was analyzed by enzyme-linked immunosorbent assay [19]. HbA1c was measured by a high performance linked liquid chromatography (HPLC, Auto-A, Tosoh) with a normal value of 4-5.3%. U-creatinine was measured by an enzymatic calorimetric method (Hitachi Modular-P) with detection limit 0.03 mmol/L (range 0.03-53 mmol/L). Serum cystatin C was determined by an automated particle enhanced turbidimetric method with normal range of 0.55-1.15 mg/L [20]. Plasma creatinine was analysed by a creatininase enzyme-based analyse system (Hitachi Modular-P) with detection limit of 2.7 μmol/L and a normal
value between 5 and 15 years of 25-68, male 60-105, and female 45-90 μmol/L. Plasma high-sensitivity C reactive protein (CRP) was measured by enzyme-linked immunoassay using polyclonal antibodies, reference value 0.05-3.0 mg/L, (DACO Diagnostics, Glostrup, Denmark). Plasma tumor necrosis factor alpha (TNF-α) was detected using chemiluminiscent immunometric assay (Immunite 1000 LKNF1, Siemens Medical Solutions Diagnostics, Llanberis, UK). Plasma cGMP levels were measured using a cGMP EIA Kit (Biomedical Technologies, Stoughton, MA). All children had their height and weight measured. Blood pressure was taken in supine position after 5-minute rest with an arm cuff covering two thirds of the right upper arm.

*Carotid artery intima-media thickness*

A high-resolution ultrasound system (Acuson Sequoia C512, Siemens AG, Germany) equipped with a 15 MHz probe was used. The imaging protocol was described in details previously. In short, longitudinal scans in bi-dimensional mode of the 1-cm-long distal end of the common carotid artery of the nondominant arm (left in all patients) were imaged so that the lumen-intima and media-adventitia interfaces were distinguishable. All images corresponded to the R-wave on electrocardiogram (ECG). Four scans obtained from each individual were recorded on videotape for offline analysis of intima-media thickness (IMT). The ultrasound scans were taken by two sonographers unaware of the infection status. The mean carotid IMT was measured from each scan manually. Mean IMT obtained from all scans from the same subject were averaged and the resulted mean IMT was used for statistical analyses.

*Carotid artery compliance*

Longitudinal scans of the brachial artery (nondominant arm) were imaged several centimetres above the antecubital fossa via a 15-MHz linear ultrasound transducer of an Acuson Sequoia™ C256
(Siemens AG, Germany). The ultrasound beam frequency was set at 8 MHz. Four scans obtained from each individual were recorded on videotape for offline analysis of the carotid artery compliance (CAC), stiffness index (SI), and intima-media thickness (IMT). The ultrasound scans were taken by two sonographers unaware of the infection and smoke exposure status. The mean carotid IMT was measured from each scan manually. Mean IMT obtained from all scans from the same subject were averaged and the resulted mean IMT was used for statistical analyses. CAC and SI were calculated according to the following formulas: 

\[ \text{CAC} = \frac{(D_s - D_d)}{D_d}(P_s - P_d) \]

\[ \text{SI} = \ln \left( \frac{P_s}{P_d} \right) \frac{(D_s - D_d)}{D_d} \]

where \(D_s\) is systolic diameter, \(D_d\) is diastolic diameter, \(P_s\) is systolic blood pressure, and \(P_d\) is diastolic blood pressure.

**Brachial artery endothelial vasomotor function**

The dilatory responses to hyperaemia (endothelium-dependent) and glycerol trinitrate (GTN, endothelium-independent) were obtained in 62 patients. Briefly longitudinal scans of the brachial artery (nondominant arm) were imaged several centimetres above the cubital fossa via a 15-MHz linear ultrasound transducer of an Acuson Sequoia C512 (Siemens, Germany). The ultrasound beam frequency was set at 8 MHz. Once the image was obtained, the transducer was fixed throughout the ultrasound study with aid of a transducer holder manufactured in 2001 at the Great Ormond Street Hospital, London, UK. ECG-gated end-diastolic scans of the artery were recorded at baseline, and a pressure cuff tourniquet placed around the forearm was thereafter inflated to 50 mmHg over the systolic blood pressure for 5 minutes. A new series of frames was taken for 15 seconds before, and for 120 seconds after cuff deflation. Arterial flow velocity was obtained before and during the first 15 seconds after cuff release by pulsed Doppler signal at 70° to the vessel with the range gate in the centre of the artery. Blood flow volume was calculated by multiplying the velocity time integral of the Doppler signal by heart rate and the vessel cross sectional area. Following a ten-minute recovery period, additional frames were taken before and over a four-minute period after sublingual administration of 400 μg GTN spray. Flow-mediated dilatation (FMD) and GTN-induced dilatation of
the brachial artery were expressed as maximum percent dilatation following cuff deflation and GTN administration, respectively. Two experienced sonographers who were unaware of the patients’ clinical characteristics took all scans.

Statistics

Results are given as mean±SEM or as median and range for variables with skewed distribution (see below). Analysis of variance (ANOVA) followed when applicable (i.e. significant p value) by Bonferroni posthoc test were used to assess the differences between groups. Simple regression analysis was used to assess the relationship between certain dependent and independent variables. To this aim, variables with skewed distribution (CRP, GFR, α-GST:crea, and AER) and HbA1c, were log-transformed. For statistical purpose, patients with α-GST:crea below the detection limit (n=3) were assigned a value of 0.01 ng/ml. The α-GST:crea data were compared with those from a previously studied healthy material [17] to whom patients were age-matched. Significance was accepted when p ≤0.05. The analyses were performed using the Stat View for Windows as statistical package (USA).

Calculations

Urine excretion rates were calculated in relation to 1.73 m² body surface area (= Weight0.425 x Height0.725 x 71.84/100) to adjust for size and gender. The ratio between the urine component and urine creatinine was used to correct for failures in timed collections. Cystatin C clearance was used to estimate glomerular filtration rate (GFR; ml/min/1.73 m² body surface area) =84.69 x cystatin C (mg/L) and x 1.384 for children <14 years [21].

Urine α-GST:crea ratio below 0.21 or above 2.86 μg/mmol, the lowest and the highest, respectively, in the control material, was considered abnormal [17]. Likewise a urine excretion rate above the highest value for control children for AER >4.15 μg/min was considered abnormal [17].
Results

The main demographic, diabetic, renal, inflammatory, and vascular characteristics are presented in Table 1.

Urine albumin excretion

Urine albumin, expressed as timed Albumin Excretion Rate (AER), cystatin C, and cystatin C clearance (GFR) showed no signs of overt renal disease (Table 1). AER rose with increasing age (p=0.008, r=0.31) and diabetes duration (p=0.017, r=0.29), and correlated inversely with FMD (p=0.049, r=−0.29), cGMP (p=0.008, r=−0.34), and CAC (p=0.047, r=−0.25). No relationship was found between AER and HbA1c (p=0.6) or inflammatory markers (p≥0.2).

Urine α-Glutathione S-transferase to creatinine ratio

Urine α-GST:crea was available in 65 patients. The data were discarded in 4 patients due to measure errors. Microlbuminuria (>20 mg/L) was found in 2 patients, whose data were therefore discarded. As compared with previously studied, age-matched control material [17], α-GST:crea was significantly lower in patients (p=0.03) than in controls. Thirty-one patients (46%) had α-GST:crea levels below the lowest α-GST:crea (ie, 0.21) in the control material. None had α-GST:crea above the highest corresponding value in the control group.

α-GST:crea appeared to be particularly lower in older patients (p=0.004, r=−0.34 vs age, Figure 1/Panel A), in those with worse diabetic control (p=0.03, r=−0.26 vs HbA1c) and higher BMI (p=0.05, r=−0.23), and in those with lower carotid artery elasticity (p=0.017, r=0.3 vs CAC; Figure 1/Panel B). α-GST:crea showed no clear association with AER (p=0.08), and, also, no relationship to carotid IMT (p=0.4). There was a correlation of α-GST:crea with FMD only in patients with ETS (p=0.01, r=0.43;
Figure 2/Panel A); nevertheless, there was no difference in $\alpha$-GST:crea between patients with and without ETS exposure ($p>0.1$).

In contrast, $\alpha$-GST:crea correlated positively with TNF-$\alpha$ ($p=0.01$, $r=0.32$; Figure 2/Panel B). The relationship between $\alpha$-GST:crea and inflammation strengthened in patients with shorter diabetes duration (ie, up to 6 years=median: TNF-$\alpha$: $p=0.001$, $r=0.58$; CRP: $p=0.0.02$, $r=0.39$) but lost significance in the remaining patients ($p>0.2$).

**Discussion**

We have earlier shown [17], and confirmed herein in a smaller segment of the earlier studied cohort without signs of overt renal disease, that young patients with type 1 diabetes with longer diabetes duration and poor diabetes control are predisposed to decreased urine excretion of $\alpha$-GST:crea. We also found that lower levels of $\alpha$-GST:crea were related to decreased carotid artery elasticity. The latter is regarded as an important risk factor for cardiovascular disease, which is far more prevalent in type 1 diabetes than in healthy individuals [4,5]. The decrease in urine $\alpha$-GST:crea appeared to be dampened by systemic inflammation, as suggested by the positive correlation between $\alpha$-GST:crea and inflammatory markers CRP and TNF-$\alpha$.

Changes in urine $\alpha$-GST are thought to merely reflect damage to proximal tubuli [14,22]. Although the role of this enzyme in the redox system is well defined, the precise meaning of its increased or decreased excretion in the urine remains a challenge. Furthermore, since this enzyme lacks in normal vascular endothelial cells [23], any putative association with cardiovascular indices in terms of causality should be interpreted with caution.

Two previous studies have found transitory increased urine excretion of $\alpha$-GST early after cardiac [16] and renal [24] surgery. Open heart surgery is associated with intense complement activation and systemic inflammation. Inflammation is a powerful stimulus of tissue oxidation, which sets in action
redox mechanisms, such as upregulation of antioxidative enzymes. Oxidative stress is an important feature of type1 diabetes. Previous in vitro study showed upregulation of other GST isoenzymes following exposure to inflammatory cytokines [25].

In alloxan-induced diabetic rats renal GST enzyme expression was decreased but normalized with insulin treatment [18]. Although experimental observations cannot always be extrapolated to humans, it is possible that the reduced urinary excretion of α-GST, observed in nearly half of the studied cohort, is due to down-regulation of this enzyme in the proximal tubuli. Whether the observed decline in α-GST:crea with age and diabetes duration, observed in our young material, continues later in life remains speculative. A recent study on adult patients with normo-, micro-, and macro albuminuria in type1 and type2 diabetes found no difference in α-GST:crea in relation to age, glomerular damage, nor as compared to healthy controls [10].

Previous electron microscopy study on streptozotocin-induced diabetic rats found an association between renal microvascular and tubule injury, suggesting possible causal relationship between these two [26]. Impaired microvascular function in systemic diseases, including type1 diabetes, has been documented previously, being shown to be present in the whole body [3,7,8]. These changes are seemingly present in large arteries as well [27]. Renal microvascular dysfunction has been related to changes in carotid artery elasticity [4,5]. In this context, the observed association of decreased urine excretion with lower carotid artery compliance is interesting, and warrants further studies.

Our finding indicating apparently preserved urine excretion of α-GST:crea in patients with elevated levels of CRP and TNF-α could therefore suggest, although do not imply, possible redox compensatory mechanisms involving α-GST pathway. This might explain the significant association between α-GST:crea and systemic inflammation (TNF-α, CRP) in patients with shorter diabetes duration, in whom compensatory redox mechanisms are assumed to be better preserved than in those with longstanding diabetes.
We found significant correlation between higher levels of AER, a marker of glomerular function, and decreased FMD of the brachial artery, which indicates arterial endothelial dysfunction. In the present study, the association between urine α-GST excretion and FMD was only found in those with ETS. This is in agreement with findings from other studies where exposure to smoke was demonstrated to have an impact on artery endothelial dysfunction and also on kidney function [2,28]. Similar to active smoking, ETS seems to adversely influence vascular function [28] even in healthy children [29], and to cause noxious effects on glomerular function [30].

Study limitations: the control cohort includes relatively few individuals; nevertheless, α-GST dispersion was small, in congruence with previous data in adult controls [14, 22]. Although significant, the correlations between α-GST and most of the studied variables are weak, and obtained in a relatively small cohort. In order to mitigate the “outlier” impact, we performed log-transformation for all these variables. The fact that a recent adult study [10] did not show any association of urine α-GST with albuminuria, which often becomes manifest in adult patients with type1 diabetes, raises concern that the putative decrease in α-GST found in our pediatric cohort may not be persistent over time. This important issue needs to be addressed in a future long-term prospective study. Although our findings suggest association of α-GST:crea with adverse changes in arterial function (FMD and CAC), they do not prove any causal relationship. Further experimental studies are warranted.

In conclusion, in children and adolescents with type1 diabetes, lower levels of urine α-GST:crea seem to be associated with decreasing arterial elasticity and endothelial vasomotor function. In contrast, higher levels of α-GST:crea appear to be more common in patients with elevated markers of systemic inflammation. Large scale prospective studies are needed to clarify these associations between endothelium-mediated vascular tone and renal proximal tubule endothelium in type1 diabetes nephropathy.
Acknowledgments

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References


brachial artery: a report of the International Brachial Artery Reactivity Task


Table legend

Table 1:

Descriptive characteristics of the study population.

Figure Legends

Figure 1:

Association of α-GST:crea (log-transformed) with age (Panel A: $p=0.004$, $r=-0.34$), and with carotid artery compliance (CAC, Panel B: $p=0.017$, $r=0.3$).

Figure 2:

Association of α-GST:crea (log-transformed) with tumor necrosis factor-alpha (TNF-α, Panel A: $p=0.014$, $r=0.32$), and with brachial artery’s flow-mediated dilatation (FMD, %, Panel B: $p=0.01$, $r=0.43$) in patients exposed to environmental tobacco smoke (ETS).
<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Std. Dev.</th>
<th>Count</th>
<th>Skewness</th>
<th>Median</th>
<th>IQR</th>
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<td>Age (years)</td>
<td>14,667</td>
<td>3,526</td>
<td>69</td>
<td>-3.07</td>
<td>14,000</td>
<td>5,000</td>
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<tr>
<td>Diabetes Duration (years)</td>
<td>6,913</td>
<td>4,188</td>
<td>69</td>
<td>4.21</td>
<td>6,000</td>
<td>6,000</td>
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<tr>
<td>HbA1c (%)</td>
<td>6,877</td>
<td>1,342</td>
<td>69</td>
<td>4.88</td>
<td>6,700</td>
<td>1,550</td>
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<tr>
<td>BMI (kg/m2)</td>
<td>20,968</td>
<td>3,175</td>
<td>69</td>
<td>1.92</td>
<td>20,830</td>
<td>4,866</td>
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<tr>
<td>Diastolic BP (mm Hg)</td>
<td>70,565</td>
<td>6,670</td>
<td>69</td>
<td>-0.59</td>
<td>70,000</td>
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<tr>
<td>Systolic BP (mm Hg)</td>
<td>112,783</td>
<td>10,990</td>
<td>69</td>
<td>-4.71</td>
<td>115,000</td>
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<tr>
<td>alpha GST:Crea</td>
<td>0.369</td>
<td>0.552</td>
<td>69</td>
<td>4.098</td>
<td>0.222</td>
<td>0.265</td>
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<tr>
<td>AER (ug/min)</td>
<td>2.898</td>
<td>2.296</td>
<td>69</td>
<td>2.015</td>
<td>2.313</td>
<td>2.260</td>
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<tr>
<td>Cystatin C (mg/L)</td>
<td>0.817</td>
<td>0.119</td>
<td>67</td>
<td>-0.613</td>
<td>0.830</td>
<td>0.160</td>
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<tr>
<td>GFR (ml/min(-1)(1.73m2)(-1))</td>
<td>145,119</td>
<td>47,572</td>
<td>67</td>
<td>2.082</td>
<td>141,000</td>
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<tr>
<td>CRP (mg/L)</td>
<td>1,834</td>
<td>3,213</td>
<td>68</td>
<td>2.266</td>
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<td>1,370</td>
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<tr>
<td>TNF-alpha (pg/ml)</td>
<td>9,600</td>
<td>2,330</td>
<td>55</td>
<td>0.306</td>
<td>10,000</td>
<td>3,000</td>
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<td>IMT (mm)</td>
<td>0.040</td>
<td>0.005</td>
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<td>0.349</td>
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<td>CAC (mm2/mmHg)</td>
<td>2,694</td>
<td>0.908</td>
<td>60</td>
<td>-0.021</td>
<td>2.795</td>
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<td>FMD (%)</td>
<td>10,051</td>
<td>7,201</td>
<td>51</td>
<td>0.325</td>
<td>10,126</td>
<td>10,286</td>
</tr>
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</table>
Figure 1/Panel A

- Log alpha GST:crea
- Age (years)

[Graph showing a scatter plot with age on the x-axis and log alpha GST:crea on the y-axis]
Figure 1/Panel B
Figure 2/Panel A

![Graph showing the relationship between log alpha GST:crea and TNF-alpha (pg/ml). The x-axis represents TNF-alpha (pg/ml) ranging from 4 to 18, and the y-axis represents log alpha GST:crea ranging from -3 to 1. The data points are scattered with a trend line.]
Figure 2/Panel B