

## Research Article

# Plasma Gamma-Glutamyltransferase Is Strongly Determined by Acylation Stimulating Protein Levels Independent of Insulin Resistance in Patients with Acute Coronary Syndrome

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**Background.** Steatosis is a manifestation of the metabolic syndrome often associated with release of liver enzymes and inflammatory adipocytokines linked to cardiovascular risk. Gamma-glutamyltransferase (GGT) is one sensitive liver marker recently identified as an independent cardiovascular risk factor. Mechanisms involved in enhanced hepatic lipogenesis causing steatosis are not yet identified and are usually linked to insulin resistance (IR). Acylation stimulating protein (ASP), a potent lipogenic factor, was recently shown to increase in patients with steatosis and was implicated in its pathogenesis. **Aim.** To investigate the association of plasma ASP levels with liver and metabolic risk markers in acute coronary syndrome (ACS) patients. **Methods.** 28 patients and 30 healthy controls were recruited. Their anthropometrics, lipid profile, liver markers, insulin, and ASP levels were measured. **Results.** In the patients, ASP, liver, and metabolic risk markers were markedly higher than in the controls. ASP strongly predicted GGT levels ( $B = 0.75$ ,  $P < 0.0001$ ), followed by triglycerides ( $B = 0.403$ ,  $P = 0.017$ ), together determining 57.6% variation in GGT levels. Insulin and IR correlated with metabolic risk components but not with liver enzymes. **Conclusion.** The strong association of ASP with GGT in ACS patients suggests that ASP, independent of IR, may contribute to a vicious cycle of hepatic lipogenic stimulation and GGT release promoting atherogenesis.

## 1. Introduction

Steatosis is the liver manifestation of the metabolic syndrome [1, 2] often associated with release of liver enzymes, inflammatory adipocytokines, interleukins, and complement factors, many of which are linked to cardiovascular risk [3–6]. Mild elevations in liver enzymes are a common feature of the metabolic syndrome reflecting the progression of liver steatosis. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and gamma glutamyltransferase (GGT) are the main liver enzymes used as surrogate markers of liver steatosis after ruling out infectious hepatitis, alcohol abuse, and damage to extrahepatic tissues [3, 7, 8]. GGT, in particular, is a very sensitive enzyme

marker of steatotic liver injury [9]. Importantly also, GGT has recently sparked major interest as an independent cardiovascular risk factor [10, 11].

Mechanisms promoting liver steatosis and subsequent release of liver function markers and inflammatory mediators are rather complex and not fully understood [12]. In general enhanced liver lipogenesis is attributed to the dyslipidemic profile associated with insulin resistance [13, 14]. Several factors including PPAR- $\gamma$  [15] and adipokines such as leptin, adiponectin, tumor necrosis factor- $\alpha$ , and interleukin-6 have been suggested to play a role in fatty liver progression through complex and interactive paracrine and endocrine mechanisms [13]. One adipokine worth highlighting in this regard is acylation stimulating protein (ASP). ASP exhibits potent fat

storage effects, which are independent and additive to insulin effects [16, 17]. Contrary to insulin, ASP stimulates lipogenesis in the tissue microenvironment in a paracrine manner, by activating the rate limiting enzyme in the triglyceride (TG) synthesis pathway [16] and by increasing glucose transport [18]. The ASP receptor was recently identified as C5L2, a 7-transmembrane serpentine receptor highly expressed in adipose tissue depots, skeletal muscle, and liver [19]. ASP levels were shown to increase in various metabolic disorders, including obesity, hyperlipidemia, nephrotic syndrome, and coronary artery disease [16, 20–23], and coordinated with enhanced TG clearance [24, 25]. Genetic linkage studies showed a genetic correlation of ASP with cardiovascular risk factors [26]. Overall, accumulating evidence suggests that ASP may contribute to the clustering of metabolic syndrome components and associated cardiovascular risk [16]. Interestingly, recent findings showed that patients with fatty liver had higher ASP levels than patients with infectious hepatitis and controls [27]. Also, ASP precursors in the liver increase during the progression of steatosis [4, 5]. Reviews by Copaci et al. and Lonardo et al. reflect a role for ASP in fatty liver progression recommending further studies on ASP mediated hepatic lipogenesis [4, 5].

The rationale for studying the association of ASP with markers of fatty liver is enhanced by the fact that all factors needed for ASP production are present in the liver [5]. The aim of this study is to investigate the association of the major lipogenic hormones ASP and insulin with liver enzymes, as surrogate markers of liver steatosis, and other metabolic syndrome components and metabolic risk factors in patients who developed acute coronary syndrome compared to healthy controls.

## 2. Methods

**2.1. Subjects.** Our main target was patients attending the cardiology clinic at Sultan Qaboos University Hospital diagnosed with acute coronary syndrome (ACS). The study involved 28 consecutive patients who agreed to participate in the study (18 males and 10 females) (mean age  $\pm$  SD: 51.9  $\pm$  10.9 years, range = 25–67 years) and 30 apparently healthy controls selected from the general population (17 males and 13 females), (mean age  $\pm$  SD: 41.1  $\pm$  9.5 years, range = 27–66 years). *Inclusion criteria* included patients diagnosed with ACS based on the clinical presentation identified by a specialized cardiologist. The clinical presentation included severe chest pain in association with dynamic ECG changes and elevations in troponin levels according to the American Heart Association guidelines [28]. Blood samples for troponin measurements were collected at admission, 3, 6, and 12 hours. Blood samples for further biochemical analysis were collected within 24 hours after the initial diagnosis. The patients had at least 3 metabolic syndrome components [29]. *Exclusion criteria* were as follows: subjects with infectious diseases or liver pathologies, not related to the metabolic syndrome, alcohol drinkers, and patients receiving insulin therapy were not included in the study. Two patients were previous smokers and quit more than 3 years before the ACS episode. One patient was still smoking at the time of evaluation. All subjects

fasted overnight before blood samples were collected. The study was approved by Sultan Qaboos University ethics committee under project IG/MED/BIOC/06/04. Informed consent forms were filled out by all subjects participating in the study.

**2.2. Analysis.** Anthropometric measures were recorded for all subjects including BMI and waist circumference. Fasting blood samples were collected in plain tubes with no anticoagulant for lipid and LDL size measurements and EDTA tubes for ASP measurements as described previously [24]. The collection was performed in the morning, and samples were put on ice and immediately centrifuged. The serum and plasma were stored at  $-80^{\circ}\text{C}$  until analysis. All samples were analyzed for ASP, insulin, and metabolic parameters including the lipid profile: TG, total cholesterol (total-C), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), apolipoproteins (apoB and apoA1), and LDL size. The liver markers measured were ALT, AST, GGT, ALP, and bilirubin. Analysis was performed using an automated clinical chemistry analyzer CX 7 Super Clinical System (SYNCHRON) based on enzymatic colorimetric assays for lipid parameters: TG, total-C, HDL-C, and LDL-C. Analysis of samples for apoA and apoB was based on immunoturbidimetry of antigen antibody reactions with the sample apoprotein detected as antigen by IMAGE Immunochemistry Systems. Both analyzers are products of Beckman Coulter, Inc. (Fullerton, CA, USA). Insulin was measured by using Beckman Coulter Access (Fullerton, CA, USA) based on immunoenzymatic assays. The liver enzymes including ALT, AST, GGT and ALP, and bilirubin were measured using COBAS c 111 from Roche Diagnostics, Deutschland. LDL size was measured by polyacrylamide gradient gel electrophoresis (PAGE) for all subjects (patients and controls) as described elsewhere [30]. The homeostatic model assessment (HOMA) was used to quantify insulin resistance (HOMA-IR) from fasting glucose (mmol/L) and insulin levels (mIU/L). HOMA was calculated by the following equation according to Matthews et al. [31]:

$$\text{HOMA - IR} = \frac{\text{Glucose} \times \text{insulin}}{22.5}. \quad (1)$$

A sandwich enzyme-linked immunosorbent assay (ELISA) was used for ASP measurements using a monoclonal antibody as capture antibody and a polyclonal antibody as detecting antibody, as described in detail previously [24].

**2.3. Statistical Analysis.** The means were compared by independent sample Student's *t*-test. Levene's test was used to test equality of variances. All results in the tables are expressed as mean  $\pm$  standard error. The one-sample Kolmogorov-Smirnov (K-S) test was performed on all measured parameters to test for normality. Bivariate correlations between ASP and insulin with liver enzymes were examined. Pearson's correlation coefficients were used for parameters with normal distributions. Spearman's correlation coefficients were used for parameters with skewed distributions. Stepwise multiple linear regression analysis was performed to determine factors

that significantly explain variations in plasma GGT levels. Analysis was computer-assisted using the SPSS/PC statistical program (version 13.0 for Windows; SPSS, Inc., Chicago, IL, USA).

### 3. Results

**3.1. Metabolic and Anthropometric Measures in Patients and Controls.** Figure 1 shows that all liver enzyme levels were higher in the ACS patient group compared to the controls. The percentage differences between patients and controls for liver markers GGT, ALT, AST, and ALP were (95.6%, 39.4%, 34.2%, 0.72%), respectively, showing the highest increase in GGT levels. All differences were significant except for ALP. Figure 2 shows differences in ASP levels between ACS patients and control subjects. ACS subjects had a 76.4% increase in plasma ASP levels ( $P < 0.001$ ) compared to the controls. Table 1 shows differences in anthropometric and metabolic parameter measures between the patients and controls. These include metabolic syndrome component measures (BMI, WC, TG, HDL-C, insulin and insulin resistance (HOMA IR), and apoA1 glucose) and T-Chol, LDL-C, apoB, and bilirubin levels for all subjects. All lipid profile and metabolic syndrome component measures were higher in the patients compared to the controls except for LDL-C and apoB levels, typical of metabolic syndrome [29]. In contrast, bilirubin plasma levels were 38.6% lower in the patients. All measured parameters for the control subjects were within the normal range [33].

**3.2. Correlation of Liver Markers and MS Components with ASP and Insulin.** Bivariate correlations for liver enzymes with plasma ASP and insulin levels in the patients and the controls are shown in Table 2. In the patients, ASP had the strongest correlation with GGT levels but did not show significant correlations with other liver enzymes. In contrast, insulin did not show significant correlations with any of the liver enzymes. On the other hand, ASP did not show any significant correlations with the metabolic syndrome components, while insulin as expected correlated with most metabolic syndrome components as follows: waist ( $R = 0.49$ ,  $P = 0.007$ ), BMI ( $R = 0.48$ ,  $P = 0.009$ ), TG ( $R = 0.42$ ,  $P = 0.024$ ), and reduced HDL-C ( $R = -0.556$ ,  $P = 0.002$ ). HOMA-IR showed similar correlations with all parameters as with insulin (Table 2).

As for the controls, no correlation was seen between liver enzymes and ASP or insulin although a positive trend was seen for GGT with ASP levels which did not reach significance (Table 2). As for other metabolic syndrome components, ASP showed a significant positive correlation with TG levels ( $R = 0.37$ ,  $P < 0.05$ ), while no correlation was found with other metabolic parameters. On the other hand, insulin significantly correlated with BMI ( $R = 0.57$ ,  $P = 0.001$ ) and WC ( $R = 0.49$ ,  $P = 0.006$ ).

**3.3. Stepwise Multiple Regression Analysis.** As GGT levels showed the highest increase in the patients, a stepwise multiple linear regression model was set to determine components that significantly predicted plasma GGT level variations

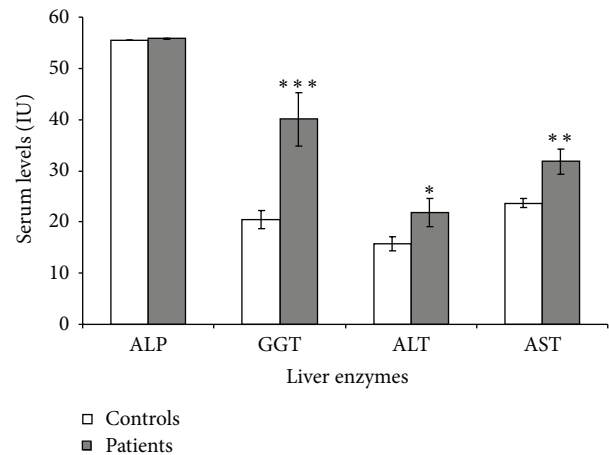


FIGURE 1: Plasma liver enzyme levels in ACS and control subjects. The values are given as mean  $\pm$  standard error of the mean. \*Significant difference: \*\*\*  $P = 0.001$ , \*\*  $P = 0.002$ , and \*  $P = 0.04$ .

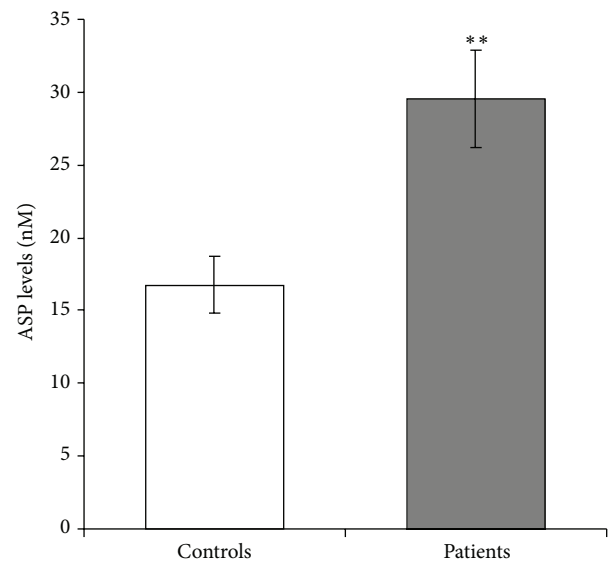


FIGURE 2: Plasma ASP levels in ACS and control subjects. The values are given as mean  $\pm$  standard error of the mean. Significant difference: \*\*  $P = 0.002$ .

in ACS patients (Table 3). GGT was set as the dependent variable in the regression model. ASP and insulin and all measured metabolic and anthropometric parameters in the study were included as independent variables. In the patients, regression analysis showed that ASP was the strongest predictor of GGT levels ( $R = 0.75$ ,  $P < 0.0001$ ), followed by triglycerides ( $R = 0.403$ ,  $P = 0.017$ ). ASP determined 42% of GGT level variation, and together with triglycerides determined 57.6% variation as indicated by  $R^2$  values. Insulin, insulin resistance (HOMA-IR), and other MS and metabolic risk components did not contribute as predictors (based on regression analysis). When other liver markers, ALT, AST, and ALP were set as dependent parameters in separate regression models, they were not predicted by any of the measured parameters in this study.

TABLE 1: Differences in anthropometric and metabolic measures between acute coronary syndrome patients and controls.

Parameter	Controls	Patients	P value
T-Chol (mM)	5.0 ± 0.15	4.9 ± 0.18	0.62
TG (mM)	1.0 ± 0.1	1.96 ± 0.2	< 0.0001
HDL-C (mM)	1.4 ± 0.05	1.15 ± 0.09	0.028
LDL-C (mM)	3.33 ± 0.16	3.22 ± 0.16	0.64
LDL size (nm)	26.4 ± 0.19	24.0 ± 0.74	0.003
ApoA1 g/dL	1.33 ± 0.03	1.15 ± 0.04	0.003
ApoB g/dL	0.93 ± 0.05	1.01 ± 0.05	0.28
Insulin (mM)	5.75 ± 0.62	11.04 ± 2.3	0.02
Glucose (mM)	5.05 ± 0.09	6.6 ± 0.6	0.01
HOMA-IR	1.3 ± 0.15	3.9 ± 1.07	0.02
BMI	28.5 ± 1.03	28.8 ± 0.73	0.02
WC (cm)*			
Males	92.8 ± 3.0	98.8 ± 3.1	0.17
Females	77.8 ± 2.2	88.7 ± 6.5	0.03
Bilirubin (μM)	4.4 ± 0.6	2.7 ± 0.32	0.02

Values are given as average ± standard error of the mean. Differences are significant at  $P < 0.05$ .

\*WC measures identified as a metabolic syndrome component are (males WC > 101.6 cm; females: WC > 88.9 cm) [32].

TABLE 2: Bivariate correlations of ASP and insulin with liver enzymes levels in serum.

Liver enzyme	ASP		Insulin	
	Controls	Patients	Controls	Patients
GGT	$R = 0.318$ $P = 0.087$	$R = 0.517^{**}$ $P = 0.001$	$R = 0.14$ $P = 0.46$	$R = 0.11$ $P = 0.95$
AST	$R = 0.116$ $P = 0.54$	$R = 0.28$ $P = 0.15$	$R = 0.109$ $P = 0.57$	$R = 0.049$ $P = 0.80$
ALT	$R = 0.27$ $P = 0.14$	$R = 0.079$ $P = 0.69$	$R = 0.285$ $P = 0.127$	$R = 0.076$ $P = 0.70$
ALP	$R = -0.195$ $P = 0.4$	$R = -0.246$ $P = 0.31$	$R = 0.287$ $P = 0.21$	$R = -0.195$ $P = 0.41$

\*\* Correlations significant at  $P < 0.05$ .

TABLE 3: Stepwise multiple regression analysis of GGT with measured parameters: ASP, insulin, lipid profile, and anthropometric parameters.

Model	Independent parameter	$R^2$	$\beta$	P value
1	ASP	0.424	0.651	0.001
2	ASP	0.576	0.750	<0.001
	TG		0.403	0.017

Predictors: GGT and TG. Probability to enter the model  $\leq 0.02$ .

Excluded parameters; insulin, insulin resistance (HOMA-IR), age, waist, BMI, T-CHOL, HDL-C, LDL-C, LDL size, apoA1, apoB, glucose and bilirubin.

## 4. Discussion

Mild increases in serum liver enzyme levels within the normal range are used as markers of fatty liver in patients with the metabolic syndrome [3, 7, 33]. Regarding ACS patients in this

study, serum liver enzyme levels increased, within the normal range, compared to the controls. Notably, the highest increase was in plasma GGT levels. These findings are in agreement with recent reports linking increased GGT levels with adverse coronary events in ACS patients [11, 34] and that even mild increases in GGT levels is associated with metabolic risk [32, 35]. Several studies have highlighted GGT as an independent predictor of coronary artery disease, myocardial infarction, stroke, and diabetes [36–39]. GGT was suggested to initiate adverse cardiovascular events by degradation of the key antioxidant glutathione, thereby initiating subclinical inflammatory and oxidative pathways [40–43].

This explanation was further supported by studies demonstrating accumulation of GGT in atherosclerotic plaques [43] and evidence of circulating GGT in the form of distinct protein aggregates that contribute to metabolic risk [44]. In a long-term study including 160,000 Austrian adults, the GGT test was cited as one of the simplest ways to determine the risk of death from cardiovascular disease [45].

The most interesting finding in this study was that ASP levels were significantly increased and highly predictive of serum GGT levels in ACS patients, which contrasts with the lack of association of insulin levels or insulin resistance with any of the liver enzymes. In spite of significant elevations in the liver enzymes ALT and AST, no correlation of the levels of these enzymes with ASP levels was found. These results complement findings in previous studies showing that complement C3, the main precursor of ASP, was a significant independent covariate of serum GGT levels [46], and Rensen et al. showed widespread activation of the complement system during the progression of fatty liver which was associated with disease severity [47]. Furthermore, activated C3 [48], GGT [43] were found in atherosclerotic plaques. These findings suggest that ASP and GGT may be released as products of an inflammatory response and may contribute to initiation of ASP mediated lipogenesis and subsequent atherogenic events. Overall the findings suggest a potential role for ASP in the pathogenesis of fatty liver, not only as a product of an inflammatory process but also in liver lipogenesis. On the other hand, although insulin levels were elevated and correlated positively with most metabolic syndrome components in the patients, as expected [49], no correlation was found between insulin and any of the liver enzymes [50].

Interestingly, in contrast to insulin, ASP acts in a paracrine manner [51], which supports the hypothesis that increased ASP levels, in the hepatic microenvironment, may act directly to activate lipogenesis and enhance liver fat accumulation. The copious supply of fatty acids available from circulating TG levels due to insulin resistance would further promote cellular fatty acid trapping and hepatic lipogenesis by ASP. As a consequence, further fat accumulation may result in increased production of liver enzymes and inflammatory factors. Importantly, studies have shown that both ALT and GGT correlate with the amount of liver fat present as measured by magnetic resonance imaging (MRI) or ultrasound [52–54]. However, GGT has been mostly linked to cardiovascular events while ALT was weakly associated to these events [55]. Our results demonstrating pronounced

GGT elevation in ACS patients agree with these findings. No correlation was found for GGT or ASP with LDL-C or apoB levels, which is compatible with previous reports [20, 56] and consistent with established features of the metabolic syndrome.

Furthermore, the results showed a significant decrease in bilirubin levels in ACS patients compared to the controls. This finding certainly agrees with studies showing decreased bilirubin levels in patients with cardiovascular disease. Contrary to GGT, bilirubin was found to be a powerful antioxidant *in vitro* and *in vivo* [57]. In the controls, a positive correlation of plasma ASP with TG levels was found as previously reported [20, 58, 59] although an association with liver enzymes was not detected.

Recently, four GGT fractions (big, medium, small, free GGT) were described [60]. Franzini et al. reported that the big GGT (b-GGT) fraction had the highest diagnostic accuracy for NAFLD compared to other fractions and that b-GGT increased in NAFLD but not chronic hepatitis C [60]. These findings are interesting as GGT fraction analysis may contribute to further identification of surrogate markers of NAFLD that may be linked to cardiovascular disease.

In summary, we hypothesize that the significant increase in GGT levels in association with other metabolic derangements may have contributed to the progression of atherosclerosis in ACS patients. The strong predictive value of serum ASP levels on GGT levels suggests that both markers may contribute to a vicious cycle of hepatic lipogenic stimulation by ASP and further release of GGT and atherogenic factors contributing to the progression of cardiovascular disease. Nevertheless, the findings may also reflect increased ASP levels as a result of tissue resistance and together with insulin resistance may exacerbate the dyslipidemic state. The concept of ASP resistance was proposed in several previous studies [16, 58, 59]. In summary, the findings suggest that ASP may play a unique role in the progression of adverse cardiovascular events, independent of insulin. These findings may contribute to further understanding of factors that may contribute to enhanced hepatic lipogenesis and associated cardiovascular risk and provide focus for more risk factors that warrant further investigation.

## Conflict of Interests

The authors declare that they have no conflict of interests.

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