

Research Article

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Gilbert's syndrome: protective effect on endothelial dysfunction

Gilbert Sendromu: Endotel Disfonksiyonu Üzerinde Koruyucu Etkisi

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Abstract

Objective: Gilbert's syndrome (GS), is a benign condition characterized by unconjugated hyperbilirubinemia related to a decreased hepatic glucuronidating activity without symptoms and signs of liver disease or overt hemolysis. In the present study, we aimed to assess circulating levels of asymmetric dimethylarginine (ADMA), pentraxin-3 (PTX-3) and high sensitivity C-reactive protein (hs-CRP) between patients with GS and controls and determine the correlation of unconjugated bilirubin (UCB) levels with these molecules as prognostic factors for vascular risk stratification and endothelial dysfunction.

Methods: Forty two patients with GS and 37 age and sex matched control subjects were enrolled in this study. The diagnosis of GS was made by unconjugated hyperbilirubinemia (1 mg/dL or >17.1 µmol/L) on at least two occasions with normal values of other liver function tests, normal hepatic imaging, and absence of hemolysis.

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Results: Serum ADMA, PTX-3 and hs-CRP levels were significantly lower in GS than the healthy controls ($p=0.037$, $p=0.025$ and $p=0.040$, respectively). In correlation analysis, UCB was negatively correlated with ADMA, PTX-3 and hs-CRP ($r=-0.239$, $p=0.034$; $r=-0.280$, $p=0.012$ and $r=-0.224$, $p=0.047$, respectively).

Discussion and conclusion: The present study showed for the first time that decreased levels of ADMA, PTX-3 and hs-CRP may prove the protective effects of hyperbilirubinemia on the endothelial dysfunction.

Keywords: Gilbert's syndrome; endothelial dysfunction; asymmetric dimethylarginine; pentraxin-3; high sensitivity C-reactive protein.

Özet

Amaç: Gilbert Sendromu (GS), karaciğer hastalığı veya aşikar hemoliz belirti ve bulguları olmaksızın, azalmış hepatic glukuronidasyon aktivitesi ile ilişkili, indirekt hiperbilirubinemi ile karakterize benign bir durumdur. Bu çalışmada, GS olan hastalar ile kontrol grubundaki hastaların dolaşımdaki asimetrik dimetilarginin (ADMA), pentraxin-3 (PTX-3) ve yüksek duyarlılık C-reaktif protein (hs-CRP) düzeylerini değerlendirmek ve vasküler risk sınıflandırması ve endotel disfonksiyonu için prognostik faktörler olarak bu moleküller ile indirekt bilirubin (İB) düzeyleri arasındaki korelasyonunu belirlemek amaçlanmıştır.

Yöntem ve Gereçler: GS olan 42 hasta ve yaş ve cinsiyet eş değer olan 37 kontrol hastası çalışmaya dahil edilmiştir. GS tanısı, diğer karaciğer fonksiyon testlerinin normal değerlerde tespit edildiği, karaciğer görüntülemesinin normal olduğu ve hemoliz yokluğunda gerçekleştirilen en az iki farklı ölçümde indirekt hiperbilirubinemi (1 mg/dL veya >17.1 µmol/L) varlığı ile konulmuştur.

Bulgular: Serum ADMA, PTX-3 ve hs-CRP düzeyleri GS hastalarında sağlıklı kontrollere göre anlamlı derecede düşük bulunmuştur (sırasıyla; $p=0.037$; $p=0.025$ ve $p=0.040$). Korelasyon analizinde, İB'nin ADMA, PTX-3 ve hs-CRP ile negatif yönde korele olduğu tespit edilmiştir (sırasıyla; $r=-0.239$, $p=0.034$; $r=-0.280$, $p=0.012$ ve $r=-0.224$, $p=0.047$).

Tartışma ve Sonuç: Bu çalışmada ilk kez ADMA, PTX-3 ve hs-CRP düzeylerindeki azalma ile hiperbilirubineminin endotel disfonksiyonu üzerine olan koruyucu etkileri ortaya konulmuştur.

Anahtar kelimeler: Gilbert Sendromu; endotel disfonksiyonu; asimetrik dimetilarginin; pentraksin-3; yüksek duyarlıklı C-reaktif protein.

Introduction

Gilbert's syndrome (GS), first described in 1901, is a benign condition of the liver characterized by mild, life-long, unconjugated hyperbilirubinemia and intermittent jaundice as a result of decreased hepatic glucuronidating activity (essential for efficient biliary excretion of bilirubin) about 30 percent of normal which is associated with reduced uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) enzyme activity, in the absence of bilirubinuria, symptoms and signs of liver disease or overt hemolysis [1, 2]. GS is the most commonly inherited disorder of bilirubin metabolism, affecting 3%–12% of the general population, occurs predominantly in men with a male to female ratio ranging from 2:1 to 7:1 and the mean bilirubin concentration is significantly higher in males [3, 4]. The hyperbilirubinemia is mild and, by definition, <6 mg/dL ($103 \mu\text{mol/L}$). However, there are many articles published with average bilirubin concentrations lower than 2 mg/dL ($35 \mu\text{mol/L}$) [5–7].

Bilirubin, once considered simply as the major end-product of heme degradation, has now emerged as an important endogenous antioxidant molecule [7]. Previously published several studies have shown that low serum bilirubin predicts future cardiovascular disease (CVD) and increased bilirubin level is associated with protection against oxidative stress-mediated diseases, especially atherosclerotic diseases particularly in men [7–12]. Although elevated serum bilirubin concentrations were argued as protective against endothelial dysfunction, the mechanisms of decreased frequency of atherosclerotic disease in GS are not entirely known but probably multifactorial.

As previously indicated in several studies, elevated levels of asymmetric dimethylarginine (ADMA) may

contribute to the development of endothelial dysfunction and thus identified as an independent risk factor for progression of atherosclerosis-related/cardiovascular mortality by inhibiting endogenous nitric oxide (NO) synthesis [13–15]. These studies were all performed in patients accompanied by different risk factors. Although, an unambiguous inverse relationship between serum bilirubin levels and atherosclerosis was demonstrated many times in previous studies, there is lack of study investigating the circulating levels of ADMA in patients with increased bilirubin levels [15].

Pentraxin-3 (PTX-3) and high sensitivity C-reactive protein (hs-CRP) are the members of the pentraxin superfamily [16]. PTX-3 have a local production in a variety of tissues, innate immunity cells and vascular cells, including monocytes, macrophages, fibroblasts, dendritic cells and epithelial cells exist in wall of cardiovascular system and increase in atherosclerotic conditions in response to proinflammatory signals [17]. Because of its extrahepatic synthesis, in contrast to hs-CRP, the PTX-3 level is believed to be a true independent indicator of disease activity because PTX-3 is produced at sites of inflammation and is intimately linked to endothelial dysfunction in diseases characterized by persistent vascular inflammation, including systemic small vessel vasculitis and chronic heart failure [18]. In addition, as demonstrated in numerous studies previously, acute vascular injuries also cause the overexpression of PTX-3 [18, 19].

Based on the very limited data available, in the present study we aimed to assess circulating levels of ADMA, hs-CRP and PTX-3 between patients with GS and control group and also to determine the correlation of bilirubin levels with these molecules as prognostic factors for endothelial dysfunction and subclinical atherosclerosis.

Methods

The protocol of this study was approved by the Ethics Committee of Kecioren Training and Research Hospital, Ankara, Turkey; which was conducted according to the Helsinki II Declaration and informed consent was obtained from each individual.

Study population

This cross sectional study was conducted on 42 patients with GS and 37 age and sex matched control subjects. The diagnosis of GS was made by unconjugated

hyperbilirubinemia ($>17.1 \mu\text{mol/L}$) on at least two occasions with normal values of other liver function tests, normal hepatic imaging (ultrasonography), and absence of hemolysis based on normal serum lactate dehydrogenase (LDH), haptoglobin levels, reticulocyte count and whole blood analysis [2]. Inclusion criteria were as follows: normal liver enzyme test results [aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and gammaglutamyl transpeptidase (GGT)] and being between the ages of 20–25 years and male sex; while exclusion criteria were: existing chronic diseases (e.g. diabetes mellitus and coronary heart disease), cholelithiasis, a positive test for hepatitis B surface antigen, hepatitis C antibody and other causes of liver disease (autoimmune liver disease, Wilson's disease, haemochromatosis, α 1-antitrypsin deficiency etc.), any acute or chronic inflammatory disease or clinical signs of infection, alcohol consumption ≥ 30 g/day in the last year; exposure to occupational hepatotoxins, known malignancy, drugs and therapy with medications in the last month.

Clinical examination

The participants underwent routine medical history, physical examination including anthropometry. Body mass index (BMI, kg/m^2) was calculated as weight (in kilograms) divided by height (in meters) squared and used as an index of body fat.

Sample collection and laboratory measurement

Overnight fasting blood samples were obtained from the antecubital vein, collected in BD Vacutainer® venous blood collection tubes containing clot activator and gel for serum separation and serum samples were separated by centrifugation at 2000 g for 10 min. Subsequently, the analysis of the biochemical parameters were performed without freezing while 2 mL of serum samples were aliquoted and immediately frozen at -80°C for future analyses of ADMA and PTX-3 until examination. All serum samples were protected from light.

Total bilirubin and conjugated bilirubin were both quantified by acid diazo methods with Olympus AU2700 (Beckman Coulter, USA) auto analyzer by using commercially available reagents, while unconjugated bilirubin (UCB) was calculated by subtracting the values of conjugated bilirubin from total bilirubin. Total cholesterol,

triglyceride, high-density lipoprotein cholesterol (HDL-C), ALT, AST, GGT, ALP, LDH, fasting blood glucose (FBG), urea and creatinine levels were also measured by the enzymatic and colorimetric methods with Olympus AU2700 (Beckman Coulter, USA) using reagents from Olympus Diagnostics. Low-density lipoprotein cholesterol (LDL-C) was calculated by Friedewald's formula [20]. Complete blood count (CBC) was performed with an automated blood cell counter (ABX Pentra 120, Horiba, Japan). hs-CRP level was determined in serum by immunoturbidimetric fixed rate method by Olympus AU-2700 autoanalyzer (Beckman Coulter, USA). Intra-assay coefficient of variation (CV) and total CV were 5.73%–5.76%, respectively. The test was linear between 0.08 mg/L and 80 mg/L and the minimum detectable concentration for hs-CRP was 0.07 mg/L.

Serum ADMA (Cat.No: CK-E11310) and PTX-3 (Cat.No: CK-E90303) levels were both measured by using quantitative ELISA kits (Hangzhou Eastbiopharm Co., Ltd, China). Measurements were carried out using ELISA plate reader Bio-Tek Synergy HT (Biotek Instruments Inc., Winooski, VT, USA). Intra-assay CV and inter-assay CV were $<10\%$ and $<12\%$, respectively and assay range was between 200 ng/L and 60000 ng/L with sensitivity 100.21 ng/L for ADMA, while intra-assay CV and inter-assay CV were $<8\%$ and $<10\%$, respectively and assay range was between 0.1 ng/mL and 30 ng/mL with sensitivity 0.05 ng/mL for PTX-3.

Statistical analyses

The Statistical Package for Social Science v 15.0 software (SPSS, Chicago, IL, USA) was used to conduct the statistical analyses. Anthropometric and biochemical features were categorized as categorical variables or continuous variables. All values are expressed as the mean $\pm$ SD or median (25th–75th interquartile range) as appropriate. Comparisons between two groups were assessed for continuous variables with the unpaired t test and Mann-Whitney test, as appropriate. Correlations were performed by the Spearman rank test. All of the reported p-values were two-tailed, and those less than 0.05 were considered to be statistically significant.

Results

The demographics and clinical characteristics of the study population together with the laboratory findings are presented in Tables 1 and 2. The average age of patients with

Table 1: Comparison of anthropometric and laboratory features of patients with Gilbert's syndrome and controls.

Variable	Gilbert's syndrome (n=42)	Controls (n=37)	p-Value
Age (years)	23 (21–24)	22 (21–23)	0.094
BMI (kg/m ²)	22.9±1.9	22.8±2.2	0.950
WBC (10 ³ /μL)	6.4±1.5	6.6±1.5	0.571
Hemoglobin (g/L)	159±11	155±10	0.092
Platelet (10 ³ /μL)	229±27	244±52	0.108
FBG (mmol/L)	5.07±0.80	4.97±0.47	0.548
Urea (mmol/L)	4.94±1.68	5±1.21	0.862
Creatinine (μmol/L)	89±10	86±12	0.299
AST (U/L)	22 (19–27)	23 (19–26)	0.794
ALT (U/L)	17 (10–17)	17 (14–21)	0.961
LDH (U/L)	93±20	98±14	0.229
ALP (U/L)	61±21	69±13	0.061
GGT (U/L)	11 (9–13)	15 (13–19)	0.082
T. Cholesterol (mmol/L)	3.70±1.24	4.69±1.01	0.573
Triglyceride (mmol/L)	0.93 (0.70–1.41)	1.04 (0.78–1.50)	0.381
HDL-C (mmol/L)	1.04±0.34	1.22±0.26	0.372
LDL-C (mmol/L)	2.18±1.01	2.95±0.85	0.731
Haptoglobin (mg/dL)	107±57	105±46	0.430
T. Bilirubin (μmol/L)	34.4 (26.7–44.4)	11.5 (9.1–13.7)	<0.001
U. Bilirubin (μmol/L)	27.4 (20.6–35.8)	9.1 (7.6–10.7)	<0.001

Data are expressed as the mean ± SD or median (25th–75th interquartile range) as appropriate. p-Values were calculated using independent-sample t test or Mann–Whitney U test. BMI, body mass index; WBC, white blood cells; FPG, fasting blood glucose; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase; T. Cholesterol, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; T. Bilirubin, total bilirubin; U. Bilirubin, unconjugated bilirubin. Bold values denotes significance at $p < 0.05$.

Table 2: Values of ADMA, Pentraxin-3, and hs-CRP levels in the groups investigated.

Variable	Gilbert's Syndrome (n=42)	Controls (n=37)	p-Value
ADMA (ng/L)	17295 (13907–40322)	26470 (15905–43470)	0.037
Pentraxin-3 (ng/mL)	6.94 (5.92–10.14)	8.74 (6.5–15.19)	0.025
hs-CRP (mg/L)	0.65 (0.24–1.74)	0.94 (0.44–2.31)	0.040

ADMA, asymmetric dimethylarginine; hs-CRP, high sensitivity C-reactive protein. Bold values denotes significance at $p < 0.05$.

GS and controls were 23 (21–24) and 22 (21–23), respectively and all of them were male. There was no statistically significant difference between groups in terms of BMI, white blood cells (WBC) and platelet counts, hemoglobin, total cholesterol, triglyceride, HDL-C, LDL-C, FBG, urea, creatinine, AST, ALT, LDH, ALP, haptoglobin and GGT levels while total bilirubin and UCB levels were significantly higher in patients with GS than controls, as expected ($p < 0.001$ for both). In addition, serum ADMA, PTX-3 and hs-CRP levels were significantly lower in GS than the healthy controls ($p = 0.037$, $p = 0.025$ and $p = 0.040$, respectively) (Figure 1).

In correlation analysis, UCB were negatively correlated with ADMA, PTX-3 and hs-CRP ($r = -0.239$, $p = 0.034$;

$r = -0.280$, $p = 0.012$ and $r = -0.224$, $p = 0.047$, respectively) (Figure 2).

Discussion and conclusion

To the best of our knowledge, the present study showed for the first time that subjects with GS have slightly decreased ADMA, PTX-3 and hs-CRP values compared to healthy controls. In addition, correlation results suggest that there is a negative correlation between UCB and ADMA, PTX-3 and hs-CRP values. These results may prove the beneficial effects of hyperbilirubinemia on the vasculature by contributing to reduced prevalence of vascular complications

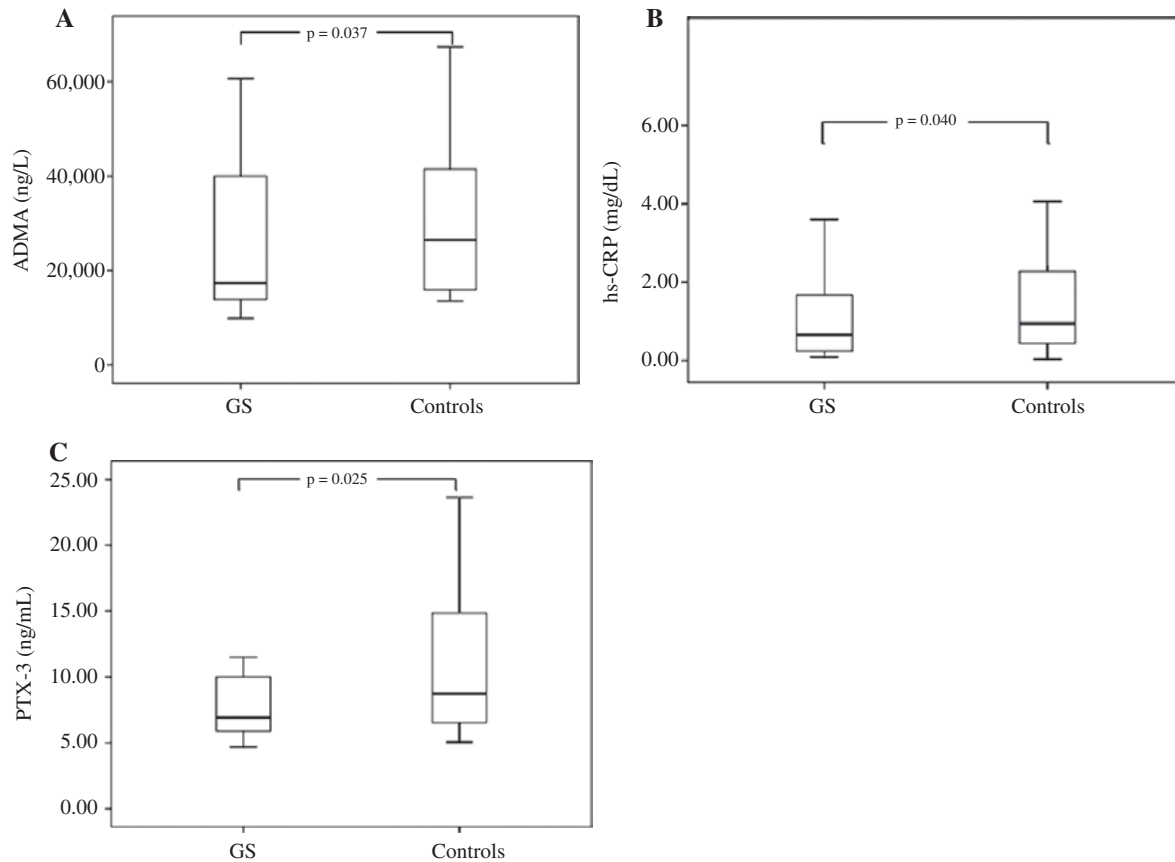


Figure 1: Box plot showing the comparison of (A) ADMA, (B) hs-CRP and (C) PTX-3 levels in controls and patients with GS.

in atherosclerotic patients with GS compared with that in patients without GS [21].

Endothelial dysfunction is the initial step in the pathogenesis of atherosclerosis, leading to cardiovascular complications [22]. There are many clinical studies indicating an inverse correlation between serum concentrations of bilirubin and risk of CVD and peripheral arterial disease [7–12]. Since endothelial dysfunction is a complex, multi-step mechanism where reduced NO levels have been reported as a marker, majority of these studies have typically focused on the potent endogenous antioxidant effect of bilirubin and accordingly beneficial effects on inhibition of the development of atherosclerosis. In a study by Maruhashi et al. bilirubin-induced decrease in oxidative stress was found to augment endothelium-dependent vasodilation in healthy young male patients with GS, suggesting that reactive oxygen species, even under normal conditions, are a predictor of endothelial function [21]. In addition, Mazzone et al. demonstrated that bilirubin may be protective in the development of atherosclerotic diseases by blunting the expression of E-selectin, vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 through a regulation of the

nuclear factor-kappa B (NF- κ B) pathway [23, 24]. Also, as is known, ADMA which is an endogenous inhibitor of NO synthesis impairs effective coronary collateral vessel development. Increased plasma ADMA levels are closely related to poor coronary collateral development [25, 26]. In a study, Gullu et al. showed that elevated concentrations of bilirubin may serve as a protective factor in the development of coronary flow reserve (CFR) impairment, coronary microvascular dysfunction, and possibly in the development of coronary atherosclerosis. They concluded that bilirubin shows the beneficial effects independent of the known coronary risk factors. They suggested that serum bilirubin concentrations in the upper portion of the reference interval for young adult population provide protection against coronary microvascular dysfunction and CFR impairment by improving endothelium-dependent coronary vasodilator function which is related with NO [11]. We found that serum ADMA levels were decreased in our patient group compared to controls accompanied by a negative correlation between UCB and serum ADMA levels. We believe this decreased serum ADMA levels was caused by reduction in dimethylarginine dimethylaminohydrolase activity as indicated many times in previous

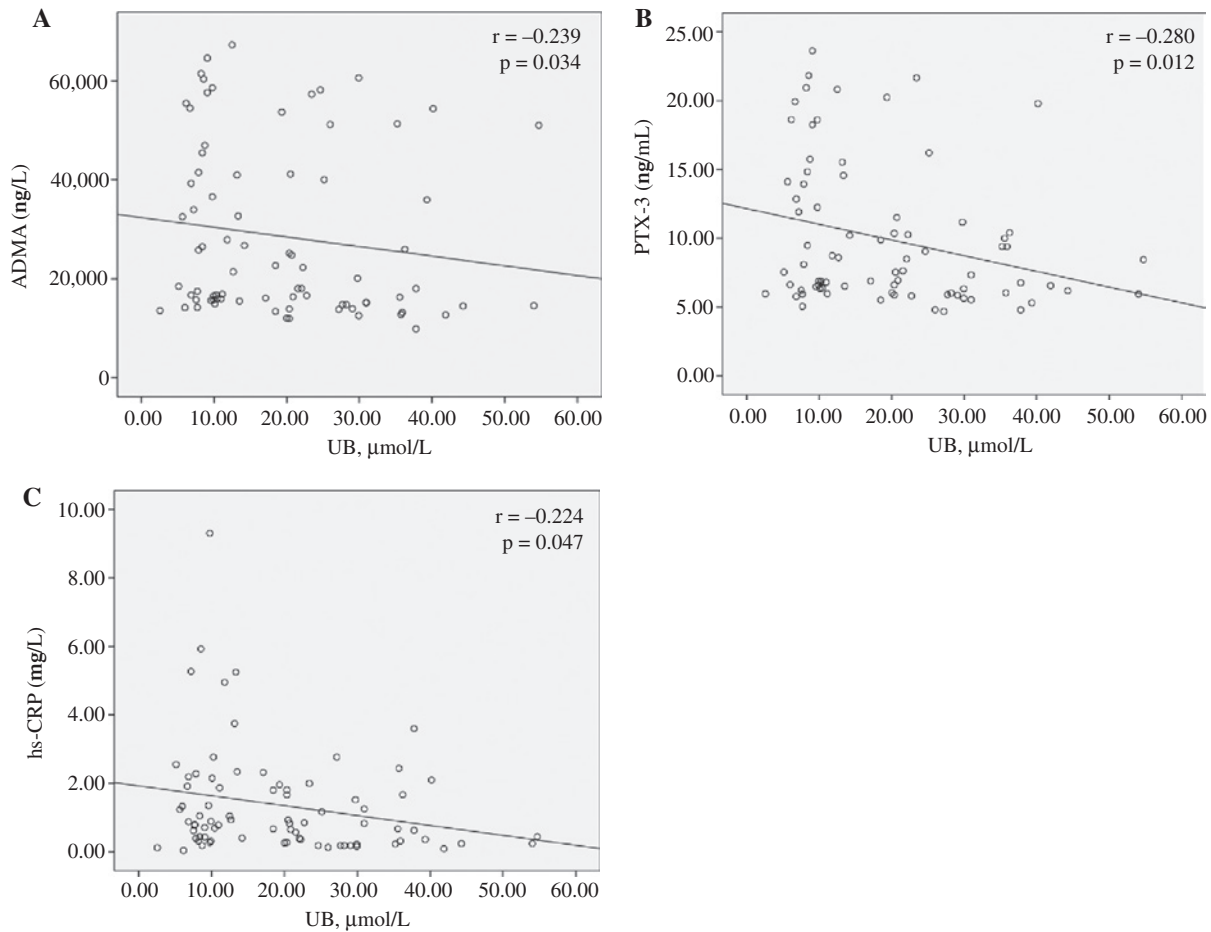


Figure 2: Correlation graphics between the UB values and (A) ADMA, (B) PTX-3 and (C) hs-CRP.

studies on CVD. And, we think that there might be another link between GS and endothelial dysfunction in atherosclerosis prevention due to decreased serum ADMA levels induced by UCB.

PTX-3 is a protein produced locally by the endothelium and other cells in the area of inflammation and has a close relation with endothelium cell functions [27]. There are studies indicating that PTX-3 has two inverse effect on atherosclerosis. A group of studies postulate that PTX-3 is expressed in atherosclerotic plaques, PTX-3 positive neutrophils can infiltrate into atherosclerotic plaques and finally circulating levels of PTX-3 are increased in different pathophysiological conditions, often affecting the cardiovascular system and contributing to ailments such as vasculitis and acute myocardial infarct [28–31], while another study reveals that PTX-3 may have cardioprotective functions by inhibiting inflammatory responses and macrophage accumulation [32]. In the present study, there are significant differences in serum levels of PTX-3 between patients with GS and controls, suggesting that inflammation is decreased in patients with GS. And,

probably augmentation of endothelial function is due to a decrease in inflammation in patients with GS who may not have cardiovascular risk factors [33]. In addition, as indicated by Deban et al. PTX-3 can act as a negative feedback mediator by dampening excessive neutrophil recruitment and limits inflammation [34]. However, the causalities between PTX-3 levels and cardiovascular outcomes are still unclear and there is still a need for more comprehensive studies on the effects of PTX-3 on endothelial dysfunction.

Chronic inflammation may also contribute to endothelial dysfunction through a decrease in NO production, and/or an increase in NO inactivation [21]. It is known that bilirubin exerts anti-inflammatory effects on the vasculature [35]. In a study performed on healthy subjects, a significant difference in plasma hs-CRP levels were reported between the higher and lower bilirubin groups [36]. Also, a significant decrease in plasma hs-CRP levels with a negative correlation between hs-CRP and UCB levels were determined in studies performed on patients with GS [2, 37]. In accordance with previous studies, we

determined significantly lower concentrations of hs-CRP in GS patients than the healthy controls. In addition, UCB was found to be negatively correlated with hs-CRP in correlation analysis. Eventually, we think that lower levels of hs-CRP may contribute in reducing systematic inflammation and this may be another explanation of decreased CAD prevalence in GS.

In a study by Vitek et al. HDL-C level was significantly higher in subjects with GS than in controls [38]. In addition, Yoshino et al. and Bhuiyan et al. found that the bilirubin level was positively correlated with HDL-C in various patient groups [39, 40]. Unlike these findings, in another study by Tapan et al. it was reported that the level of HDL-C in GS group was lower than control group [2]. On the other hand, as is known hyperglycemia and insulin resistance are associated with endothelial dysfunction. In previous studies FBG level was found to be same in subjects with GS and controls [2, 41]. Moreover, there are studies indicating that serum bilirubin level play a role in glycemic control [42]. In the current study, the GS group's HDL-C level was lower and FBG level was higher than that of the control group, however, there was no statistically significant difference between groups. These correlations should be evaluated further in larger patient groups.

The present study does pose some limitations. Firstly, although a definitive diagnosis of GS can be established by assays of UGT1A1 enzymatic activity or invasive studies, such as liver biopsy (LB), they are rarely necessary [43]. Since it is known that LB is only mandatory to rule out other liver diseases if the diagnosis is in doubt, LB was not performed. Secondly, despite the fact that vitamin B12 deficiency is known to produce unconjugated hyperbilirubinaemia due to the phenomenon of ineffective erythropoiesis, vitamin B12 levels were not evaluated in our study population due to lack of obvious signs of vitamin B12 deficiency such as anemia and peripheral neuropathy and the absence of laboratory results like decreased haptoglobin levels along with an increased serum LDH levels compatible with the ineffective erythropoiesis. Thirdly, though the sample size, the strict inclusion criteria and the study population consisting of young subjects (between the ages of 20 and 25 years), the findings obtained are not representative for all subjects with GS. However, as mentioned above in details, we think that the design of our study was a requirement for the goals to achieve. Fourthly, all participants were men. GS occurs predominantly in men with a male to female ratio ranging from 2:1 to 7:1 [4]. Larger clinical studies will be necessary for confirmation of this relationship. In addition, as of the case-control nature of the design, the association between the bilirubin and

ADMA, PTX-3 and hs-CRP levels do not necessarily indicate causality.

In conclusion, the findings of this study show that circulating levels of ADMA, PTX-3 and hs-CRP are decreased in GS. The results also indicate that UCB plays a complex role in preventing endothelial dysfunction and inflammation in GS. Further studies with larger populations may provide more information regarding the role of ADMA, PTX-3 and hs-CRP preventing endothelial dysfunction and inflammation in GS.

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