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Male Health

ORIGINAL ARTICLE

Serum sex hormone and growth arrest-specific protein 6 levels in male patients with coronary heart disease

Rui Zhao, Yan Li, Wen Dai

Epidemiological studies have shown a high prevalence of low serum testosterone levels in men with cardiovascular disease. Moreover, the tyrosine kinase receptor Axl, the ligand of which is growth arrest-specific protein 6 (GAS6), is expressed in the vasculature, and serum GAS6 levels are associated with endothelial dysfunction and cardiovascular events. Testosterone regulates *GAS*6 gene transcription directly, which inhibits calcification of vascular smooth muscle cells and provides a mechanistic insight into the cardioprotective action of androgens. This study was designed to determine the correlation between serum GAS6 and testosterone levels in male patients with coronary heart disease (CHD). We recruited 225 patients with CHD and 102 apparently healthy controls. Serum concentrations of GAS6 and soluble Axl were quantified by an enzyme-linked immunosorbent assay. Levels of high-sensitivity C-reactive protein, testosterone, estradiol, and other routine biochemical markers were also measured. Testosterone decreased from 432.69 ± 14.40 to 300.76 ± 6.23 ng dl^{-1} ($P < 0.001$) and GAS6 decreased from 16.20 ± 0.31 to 12.51 ± 0.19 ng ml^{-1} ($P < 0.001$) in patients with CHD, compared with control subjects. Multiple linear regression analysis showed that serum testosterone and GAS6 levels were positively associated in male patients with CHD. Alterations in GAS6 levels may influence the development of CHD. Downregulation of GAS6/Axl signaling in the presence of low sex hormone levels during disease progression is a potential mechanism by which GAS6 affects CHD. This study provides novel results regarding the influence of sex hormones on serum GAS6 levels in patients with CHD.

INTRODUCTION

Coronary heart disease (CHD) is a consequence of atherosclerotic disease that causes serious damage to the coronary arteries and represents a major cause of mortality worldwide. Most epidemiological studies have found a high prevalence of low testosterone levels in men with CHD, and this association exists regardless of the patients' age.[1] Evidence also suggests that low testosterone levels should be considered as an independent cardiovascular risk factor.[1–3]

GAS6 was identified in 1988 and was further characterized in mouse embryonic NIH 3T3 fibroblasts in 1993.[4,5] GAS6 is involved in processes related to proliferation, differentiation, and inflammation in adipocytes, endothelial cells, vascular smooth muscle cells (VSMCs), and bone marrow cells and in tissues including ovary, heart, and kidney.[5–7] GAS6 is a secreted Vitamin K-dependent protein in serum-starved fibroblasts[4] and shares its domain organization and ~44% amino acid identity with the anticoagulant protein S.[8] GAS6 interacts with receptor tyrosine kinases of the TAM (Tyro-3, Axl, Mer) family via its C-terminal sex hormone binding globulin-like domain.[5] GAS6 and the soluble receptor form of Axl (sAxl) are present in mouse and human circulatory systems.[9] The vasculature is an important target of the GAS6/Axl system. Vascular endothelial cells, VSMCs, and fibroblasts in the vascular system synthesize and express GAS6 and Axl.[10,11] The GAS6/Axl system has been implicated in different cellular behaviors including survival and proliferation, cell adhesion and migration, homeostasis, and inflammatory cytokine release.[5,12] Studies have reported that serum concentrations of sAxl and GAS6 are elevated in patients with heart failure and acute coronary syndrome.[13,14] However, others found that plasma levels of GAS6 are decreased in patients undergoing coronary artery bypass grafting or with acute coronary syndrome.[15,16] These results indicate that GAS6 reflects the presence of common cardiovascular risk factors and can predict cardiovascular events.

Recent studies have indicated that sex hormones regulate *GAS*6 gene transcription,[17–19] suggesting a potential role of these hormones in the GAS6/Axl system. The functions of the GAS6/Axl system, and its role in inflammation and vascular diseases may be particularly relevant to CHD in humans. However, little is known about the clinical significance of the GAS6/Axl system and its association with sex hormones, particularly in male patients with CHD. We have addressed this issue by conducting a cross-sectional study to determine whether serum GAS6 levels are associated with sex hormones in male patients with CHD. In this study, we show that GAS6 and testosterone

Department of Clinical Laboratory, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei Province, China.
Correspondence: Dr. Y Li (yanlitf1120@163.com)

concentrations correlate to male patients with CHD. We analyzed the relationship between GAS6 and testosterone with other parameters that are altered in male patients with CHD. The results indicate that decreased testosterone may play a role in downregulating the GAS6/Axl signaling system to induce CHD.

MATERIALS AND METHODS

Subjects

All subjects (225 disease and 102 control cases) were recruited from the Department of Cardiology at the Renmin Hospital of Wuhan University. All cases underwent coronary angiography. Individuals found to have ≥70% occlusion of at least one major coronary artery were defined as having CHD.[20] Control subjects exhibited completely normal coronary arteries.

This study was approved by the Medical Ethics Review Committee of Renmin Hospital, Wuhan University. All study participants were required to provide written informed consent in accordance with policies of the Renmin Hospital of Wuhan University Ethics Committee.

Sample collection

Blood samples were collected from an antecubital vein from all subjects in the morning after fasting for at least 8 h. Blood samples were allowed to clot at 25°C and were later centrifuged at 25°C to obtain serum. The serum was collected, distributed into 2-ml tubes, and kept at −80°C until analysis.

Analytical methods

A commercial enzyme-linked immunosorbent assay kit purchased from Cusabio Inc., (Wuhan, Hubei Province, China) was used to measure human GAS6 and sAxl levels in crude serum samples. All serum samples were also used to determine total bilirubin, alanine aminotransferase, aspartate aminotransferase, blood urea nitrogen, creatinine, uric acid, glucose, lipid profiles, and high-sensitivity C-reactive protein (hs-CRP) by a Siemens Advia 2400 automatic biochemistry analyzer (Siemens, Erlangen, Germany). Testosterone and estradiol levels were measured by a Siemens Advia Centaur CP (Siemens, Erlangen, Germany). White blood cell counts were determined by a SysmexXN-20 (Sysmex, Kobe, Japan).

Statistical analysis

The normality of the distribution variables is expressed as means ± standard error (s.e.). Skewed data are expressed as median and interquartile ranges. Before the statistical analyses, the normality of the distribution and the homogeneity of the variables were evaluated using Levene's test. Differences in age, body mass index, GAS6, triglycerides, and HDL-C, were analyzed using independent *t*-tests. Other analyses used the Mann–Whitney U-test for comparisons of the quantitative variables. Correlations between GAS6 levels and biochemical variables in CHD and control groups were analyzed using Spearman's correlation analyses. Univariate and multivariate logistic regression analyses were performed to evaluate the relationship between the incidence of CHD and the measured parameters. The relationships between GAS6 and cardiovascular risk factors were tested with multiple linear regression analyses. All *P* values reported are two-sided, and those <0.05 were considered statistically significant. The statistical analyses were performed using IBM SPSS version 19.0 statistics software (Chicago, IL, USA).

RESULTS

Characteristics of the study population

The biochemical variables of the control and CHD groups are summarized in **Table 1**. CHD patients had higher triglyceride, LDL-C,

Table 1: Characteristics of biochemical data among control subjects and matched CHD patients

Characteristics	Controls (n=102)	CHD (n=225)	P
Age (year)	58.57±0.84	60.22±0.63	0.133
BMI	24.70±0.29	24.97±0.15	0.364
Smoking (%)	27.45	36.44	0.363
Diabetes (%)	26.47	34.22	0.431
GAS6 (ng ml⁻¹)	16.20±0.31	12.51±0.19	<0.001
sAxl (pg ml⁻¹)	252.03, 190.00–310.66	186.27, 146.06–236.92	<0.001
ALT (U l⁻¹)	24, 15–32	23, 15–35	0.603
AST (U l⁻¹)	24, 18–28	23, 19–34	0.276
T-bil (μmol l⁻¹)	11.95, 8.97–14.32	11.30, 8.20–14.80	0.314
BUN (mmol l⁻¹)	5.80, 4.89–6.86	5.85, 4.63–7.08	0.966
Cr (μmol l⁻¹)	72.1, 62.1–79.8	74.4, 60.5–89.3	0.194
UA (μmol l⁻¹)	352, 308–386	328, 279–397	0.127
Glu (mmol l⁻¹)	5.04, 4.58–5.82	5.14, 4.53–6.32	0.243
TC (mmol l⁻¹)	4.00±0.09	4.11±0.07	0.369
TG (mmol l⁻¹)	1.41, 1.08–1.98	1.66, 1.26–2.29	0.003
HDL-C (mmol l⁻¹)	1.00±0.02	0.97±0.01	0.160
LDL-C (mmol l⁻¹)	2.39, 2.08–2.78	2.52, 2.17–3.14	0.039
T (ng dl⁻¹)	432.69±14.40	300.76±6.23	<0.001
E2(pg ml⁻¹)	31.76, 26.55–36.22	29.78, 25.33–34.70	0.078
T/E2 ratio	12.96, 9.96–17.64	9.83, 7.94–11.97	0.001
WBC	5.67, 4.99–6.90	6.50, 5.30–8.18	0.001
hs-CRP (mg l⁻¹)	1.36, 0.56–3.02	4.31, 1.16–10.09	<0.001
Treatment			
Statin use (%)	21.57	29.33	0.178
Aspirin use (%)	18.63	24.44	0.258
Antihypertension therapy (%)	27.45	30.67	0.603

CHD: coronary heart disease; BMI: body mass index; GAS6: growth arrest-specific protein 6; sAxl: soluble Axl; ALT: alanine aminotransferase; AST: aspartate aminotransferase; T-bill: total bilirubin; BUN: blood urine nitrogen; Cr: creatinine; UA: uric acid; Glu: glucose; TC: total cholesterol; TG: triglyceride; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; T: testosterone; E2: estradiol; WBC: white blood cell; hs-CRP: high-sensitivity C-reactive protein

and white blood cell levels than did the control subjects. **Table 1** and **Figure 1** show that serum testosterone, GAS6, sAxl levels, and the testosterone/estradiol ratio, were lower in the CHD than the control group (*P* < 0.001 and *P* = 0.001, respectively). In contrast, hs-CRP levels were higher (*P* < 0.001) and estradiol levels were unchanged in CHD patients compared to the control subjects.

Correlation between GAS6 levels and biochemical variables in CHD and control groups

Correlational analyses were performed to evaluate whether other commonly used biochemical markers correlated with GAS6 in CHD patients. In both the control and CHD groups, after adjustment for age, serum GAS6 levels were positively correlated with testosterone and sAxl levels (**Table 2** and **Figure 2**). The results showed that CHD patients with low serum GAS6 levels appeared to have lower serum testosterone levels.

Logistic regression analyses for CHD

To assess the factors predicting the incidence of CHD, univariate and multivariate logistic regression analyses were performed (**Table 3**). Univariate analysis showed that glucose, LDL-C, triglycerides, testosterone, white blood cells, hs-CRP, sAxl, and GAS6 levels were associated with the incidence of CHD. Multivariate analysis showed that testosterone, GAS6, sAxl, and hs-CRP levels were predictors of the incidence of CHD.
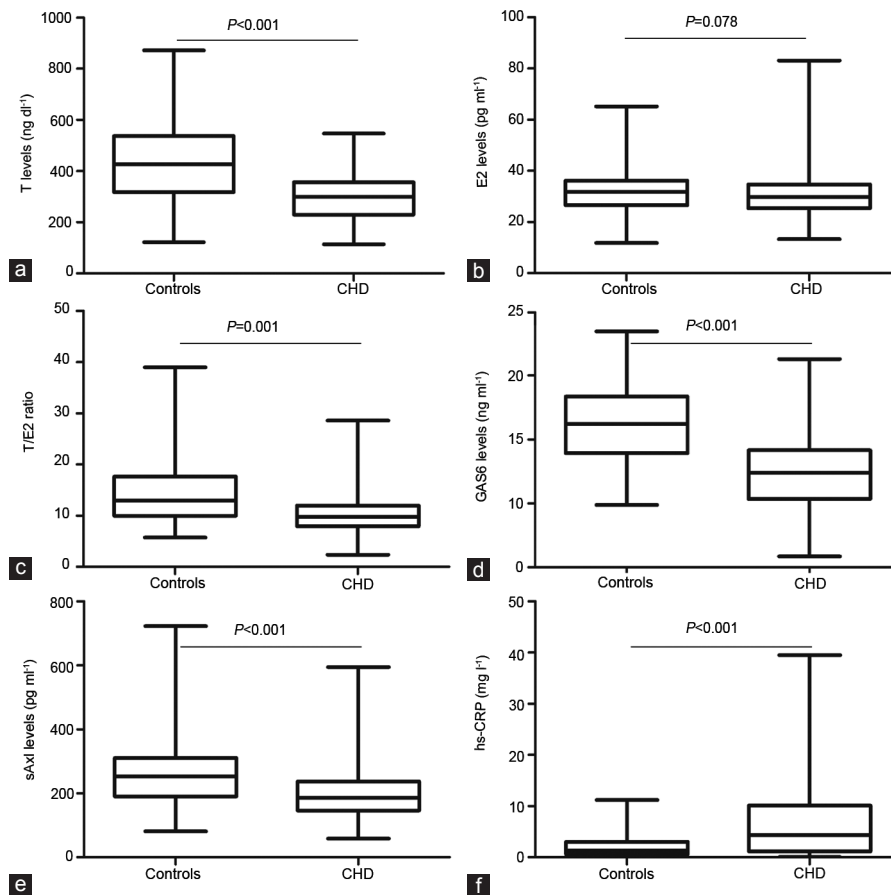
Figure 1: Serum levels of T (**a**), E2 (**b**), T/E2 ratio (**c**), GAS6 (**d**), sAxl (**e**) and hs-CRP (**f**) in control subjects (controls) and patients with coronary heart disease (CHD). Data are presented as medians and interquartile ranges because these values were not distributed normally.

Association between GAS6 and the main cardiovascular risk factors in CHD

The association between GAS6 and cardiovascular risk factors was further assessed by multivariate regression analysis. As shown in **Table 4**, in male patients with CHD, GAS6 had a negative association with triglycerides and a positive association with testosterone.

DISCUSSION

To our knowledge, this is the first study examining the relationship between serum GAS6 and testosterone levels in male patients with CHD. In this study, we showed that serum GAS6 and testosterone levels were much lower in male patients with CHD than in control subjects. Moreover, serum GAS6 concentrations were positively associated with testosterone concentrations. Multiple linear regression analysis also suggested that serum levels of GAS6 and testosterone were closely associated in male patients with CHD.

Testosterone might directly activate androgen-response elements (AREs) of the *GAS6* gene to influence *GAS6* gene expression and protein production.[17] GAS6 is a potential surrogate marker of inflammation and endothelial dysfunction.[21] Therefore, we concluded that the involvement of GAS6 in inflammation and endothelial dysfunction was a significant factor in CHD. Our findings also indicated that decreased testosterone might play a role in downregulating the GAS6/Axl system to induce CHD. Most actions of androgen are mediated by the androgen receptor to activate transcription by binding to AREs in the *GAS6* gene

promoter, which inhibits VSMC calcification.[17] This potential mechanism can explain the upregulation of the *GAS6* gene by testosterone.

Jiang *et al*.[16] have demonstrated that GAS6 levels are significantly lower in stable angina pectoris and acute coronary syndrome patients than in control patients, and have indicated a significant correlation between the degree of CVD and plasma GAS6 levels. GAS6 is derived from endothelial cells, fibroblasts, and VSMCs, and can be released into the circulation in response to disease.[22,23] GAS6 is also very important for the phagocytosis of apoptotic cells.[24,25] In dendritic cells, GAS6 can modulate the inflammatory response by downregulating TNF-α, IL-6, and interferon.[26] GAS6 and Axl are involved in activating the endothelium in response to inflammation, increasing the extravasation of leukocytes, and the rejection of transplanted tissues.[27]

GAS6 and sAxl are present in normal human serum and plasma.[9] Strong evidence from mouse models has suggested that the GAS6/Axl signaling pathway plays an important role in the vasculature via the PI3K/Akt pathway, resulting in cell survival, proliferation, adhesion, and protection from cellular death.[28] For example, GAS6 promotes the survival and migration of VSMCs,[23,29] and is overexpressed, along with its receptor Axl, in rat arterial neointima formation following balloon injury.[25] Jin *et al*. demonstrated that GAS6 plays a role in cell cycle arrest by promoting the G1/S phase transition and alleviating senescence in VSMCs. Axl plays a key role in this process, which is mediated by the binding of GAS6 thereby activating the PI3K/Akt/FoxO signaling pathway.[30] Axl is

Table 2: Spearman partial correlation coefficients between GAS6 levels and biochemical variables in CHD and control groups

	Controls (n=102)		CHD (n=225)	
	r	P	r	P
BMI	−0.178	0.073	0.094	0.160
ALT	0.001	0.999	0.137	0.040
AST	0.072	0.475	0.093	0.167
T-bil	0.037	0.714	0.015	0.819
BUN	0.148	0.137	0.045	0.500
Cr	0.096	0.337	0.151	0.023
UA	−0.011	0.911	0.112	0.095
Glu	0.024	0.813	−0.033	0.621
TC	−0.012	0.904	0.055	0.412
TG	0.069	0.488	−0.052	0.435
HDL-C	−0.087	0.386	−0.030	0.654
LDL-C	−0.014	0.891	0.103	0.122
T	0.528	<0.001	0.285	<0.001
E2	0.081	0.421	0.077	0.250
T/E2 ratio	0.382	<0.001	0.205	0.002
WBC	−0.020	0.840	0.106	0.114
hs-CRP	−0.064	0.525	0.210	0.002
sAxl	0.217	0.028	0.289	<0.001

CHD: coronary heart disease; BMI: body mass index; GAS6: growth arrest-specific protein 6; ALT: alanine aminotransferase; AST: aspartate aminotransferase; T-bill: total bilirubin; BUN: blood urine nitrogen; Cr: creatinine; UA: uric acid; Glu: glucose; TC: total cholesterol; TG: triglyceride; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; T: testosterone; E2: estradiol; WBC: white blood cell; hs-CRP: high-sensitivity C-reactive protein; sAxl: soluble Axl.

Table 3: Univariate and multivariate logistic regression analysis for CHD

Factor	Univariate			Multivariate		
	OR	95% CI	P	OR	95% CI	P
Age	1.020	0.994–1.046	0.134			
BMI	1.046	0.950–1.151	0.363			
ALT	1.008	0.994–1.023	0.264			
AST	1.012	0.996–1.028	0.145			
T-bil	0.989	0.950–1.029	0.583			
BUN	1.017	0.892–1.160	0.799			
Cr	1.010	0.999–1.021	0.076			
UA	0.999	0.997–1.001	0.446			
Glu	1.169	1.004–1.362	0.045	1.066	0.863–1.316	0.554
TC	1.114	0.880–1.412	0.369			
TG	1.615	1.199–2.176	0.002	1.359	0.899–2.054	0.146
HDL-C	0.456	0.152–1.367	0.161			
LDL-C	1.488	1.076–2.058	0.016	1.316	0.801–2.161	0.279
T	0.990	0.988–0.993	<0.001	0.994	0.990–0.999	0.009
E2	0.992	0.967–1.017	0.517			
T/E2 ratio	0.828	0.780–0.880	<0.001	0.980	0.883–1086	0.698
WBC	1.195	1.057–1.352	0.005	1.014	0.863–1.192	0.867
hs-CRP	1.209	1.125–1.300	<0.001	1.216	1.106–1.336	<0.001
GAS6	0.677	0.615–0.746	<0.001	0.747	0.658–0.847	<0.001
sAxl	0.993	0.990–0.995	<0.001	0.994	0.990–0.998	0.002

OR: odds ratio; CI: confidence interval; CHD: coronary heart disease; BMI: body mass index; ALT: alanine aminotransferase; AST: aspartate aminotransferase; T-bill: total bilirubin; BUN: blood urine nitrogen; Cr: creatinine; UA: uric acid; Glu: glucose; TC: total cholesterol; TG: triglyceride; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; T: testosterone; E2: estradiol; WBC: white blood cell; hs-CRP: high-sensitivity C-reactive protein; GAS6: growth arrest-specific protein 6; sAxl: soluble Axl.

also involved in maintaining the integrity of the vasculature, and its expression is upregulated at sites of vascular injury, suggesting a role for Axl in vascular remodeling.[22] The GAS6/Axl system can mediate potential proinflammatory signals, and this may be a particularly relevant pathway for inflammatory disease conditions such as CHD. Our results demonstrated that serum GAS6 and testosterone levels were significantly lower in male patients with advanced CHD than in control subjects. Testosterone deficiency may, at least in part, be attributed to low GAS6 levels as well as reduced GAS6/Axl signaling in male CHD patients. These findings suggest that GAS6/Axl signaling is related to endothelial dysfunction and that GAS6/Axl signaling may have a crucial role in the pathogenesis of atherosclerosis through testosterone.

Testosterone is a vasoactive hormone that predominantly exerts vasodilatory effects on several vascular beds. Acute and chronic testosterone administration increases coronary artery diameter and flow, improves cardiac ischemia and symptoms in men with chronic stable angina, and reduces peripheral vascular resistance in chronic heart failure. Testosterone can also beneficially enhance biological processes involved in atheroprotection, in particular, lipid deposition and inflammation both within the arterial wall and in the circulation.[31] Testosterone deficiency is highly prevalent in men with CVD and is associated with an increased mortality. Low testosterone also has an adverse effect on several cardiovascular risk factors including insulin resistance, diabetes, dyslipidemia, central adiposity, and endothelial dysfunction.[32] Testosterone may decrease this risk, perhaps through modification and improvement of CVD risk factors, and offer potential therapeutic benefits for patients with CVD.[33]

The effect of testosterone replacement therapy is controversial,[34,35] although testosterone deficiency is associated with CVD in men.[36] Androgens act mainly through the transcriptional control of target genes mediated by the nuclear androgen receptor.[37,38] The receptor-dependent action of androgens protects against angiotensin II-induced vascular remodeling.[39] Some studies showed that apoptosis plays a central role in Pi-induced VSMC calcification through downregulation of the GAS6-mediated survival pathway.[40,41] Androgens prevented VSMC apoptosis and restored GAS6 expression and Akt survival signaling. These inhibitory effects of androgens on apoptosis and calcification were eliminated by flutamide and GAS6 siRNA.[17] A decrease in circulating testosterone levels may, therefore, lead to the downregulation of GAS6 expression, either locally in the aortic tissues or systemically in the vasculature.

The biological significance of the co-expression of GAS6 and Axl proteins in CHD patients remains to be clarified but may be related to the pathogenesis of advanced CVD in the presence of low testosterone levels. More longitudinal studies are required to elucidate the clinical significance of circulating GAS6 levels in the development of metabolic or cardiovascular diseases in human adults. One of the main limitations of this study was the small sample size resulting in the high variability of the GAS6 levels. Thus, further larger scale studies are required to confirm these results. The results of the current study are the first to show that circulating GAS6 levels are associated with CHD and that GAS6 levels could be used as a nonconventional risk factor in advanced CHD.
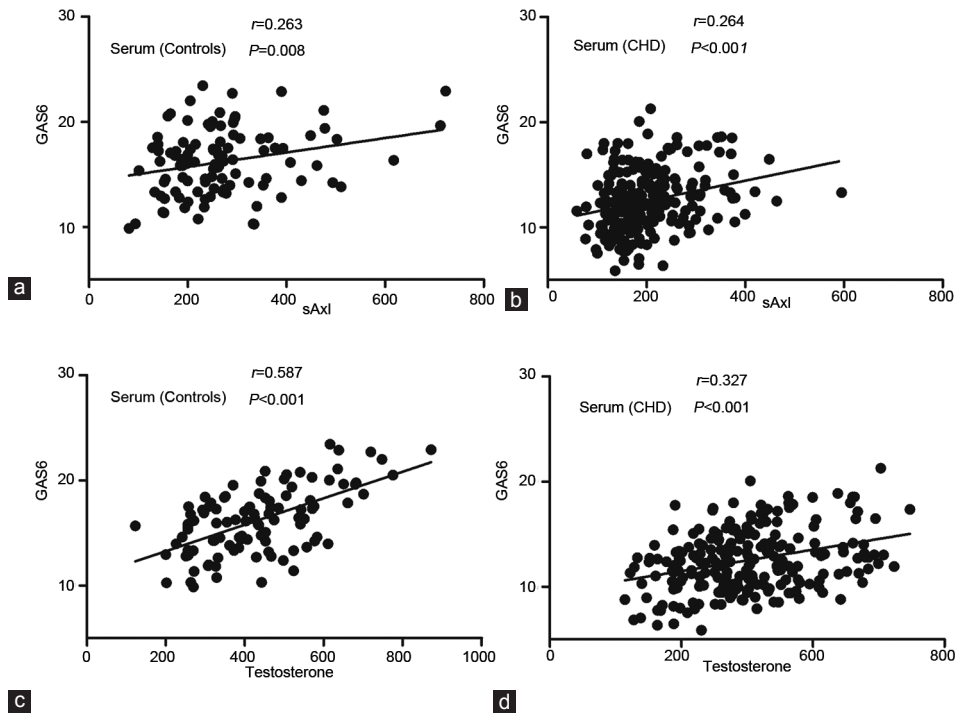
AUTHOR CONTRIBUTIONS

Figure 2: Scatter diagrams illustrating the correlation between serum GAS6 and sAxl (**a**, **b**), and the correlations between GAS6 and testosterone (**c**, **d**), in control subjects (controls) and patients with coronary heart disease (CHD).

Table 4: Multiple regression analysis for GAS6 and cardiovascular risk factors

	TC	TG	HDL-C	LDL-C	Glu	T	T/E2 ratio	WBC	hs-CRP
β-coefficient	−0.013	−0.128	−0.005	0.073	−0.007	0.511	0.101	0.094	0.055
P	0.835	0.010	0.913	0.225	0.883	<0.001	0.139	0.057	0.275

After adjusting for age. TC: total cholesterol; TG: triglyceride; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; Glu: glucose; T: testosterone; E2: estradiol; WBC: white blood cell; hs-CRP: high-sensitivity C-reactive protein; GAS6: growth arrest-specific protein 6

COMPETING INTERESTS

All authors declare no competing interests.

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