

# Increased serum levels of interleukin-6 and von Willenbrand Factor in early phase of acute coronary syndrome in a young and multiethnic Malaysian population

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## ABSTRACT

**Objective** Interleukin-6 (IL6; proinflammatory marker), von Willebrand Factor (vWF; endothelial dysfunction marker) and P-selectin (platelet activation marker), may play important roles in defining the pathogenesis of vulnerable plaques in acute coronary syndrome (ACS). This study aims to investigate the expression and relationship of these markers in early phases of ACS in a young and multiethnic Malaysian population.

**Design** Peripheral whole blood mRNA, and serum levels of IL6, vWF and P-selectin were measured in 22 patients with ACS, and in 28 controls with angiographically significant coronary artery disease without previous ACS events. Venous blood from ACS patients was obtained within 1 h of hospital admission.

**Results** No significant differences of IL6, vWF and P-selectin mRNA levels between ACS and controls were seen. ACS patients had significantly higher serum levels of IL6 and vWF ( $p < 0.001$ ), compared with controls. P-selectin correlated with IL6 ( $r = 0.697$ ,  $p = 0.003$ ) and vWF ( $r = 0.497$ ,  $p = 0.05$ ) at mRNA levels, indicating a possible association between these three indices of ACS pathogenesis.

**Conclusions** Increased serum levels of IL6 and vWF suggest that inflammation and endothelial dysfunction may play a prominent role in the pathogenesis of the disease during the early phase of ACS.

## INTRODUCTION

Coronary artery disease (CAD), including its manifestation as acute coronary syndrome (ACS) is a well established cause of morbidity and mortality among adults in both developed and developing countries. In recent years, there has been an alarming rise in the incidence of ACS in the Malaysian population, especially in younger age groups.<sup>1</sup> It has been reported that Malaysian ACS patients had a median age of 59 years, which was comparatively younger than the Caucasian population, whose median age was 66 years, as shown by the international multi-centre GRACE Registry.<sup>1,2</sup>

Atherosclerosis is the culprit behind the ACS and stable CAD. The pathophysiology of atherosclerosis includes plaque formation and destabilisation ('atherogenesis'), tendency to thrombus formation ('thrombogenesis'), and the loss of endothelial cell integrity ('endothelial dysfunction') within the coronary arteries, as described by Virchow's vascular triad.<sup>3</sup> These processes are intimately linked, and hence, provide a clue of

diagnosis of eventual ACS as early as in pathogenesis of disease based on composition and vulnerability of plaque.<sup>4</sup>

Currently available cardiac markers for ACS diagnosis, such as troponin-T and creatine kinase-myocardial band (CK-MB) isoenzyme, only detect consequences of myocardial damage after ACS has occurred, thus the information to identify patients at risk of ACS in early phase is not available.<sup>5</sup> Much interest has focused on identifying upstream markers which can detect an individual at risk of atherosclerotic plaque rupture leading to ACS. These include markers of inflammation, interleukin 6 (IL6); endothelial dysfunction, von Willenbrand Factor (vWF), and platelet activation, P-selectin. Although each of these markers has been shown to predict future cardiovascular events in stable CAD patients, as well as recurrent events and death in patients presenting with ACS,<sup>6-8</sup> their association has yet to be studied in a young, multiethnic group of the Malaysian population.

It now supports the concept that all types of blood constituents appear to play a role in plaque formation,<sup>9</sup> and the peripheral blood gene expression may reflect pathophysiology in the vascular wall or the extent of CAD.<sup>10</sup> With increasing awareness of the practical limitations of collecting primary cardiovascular disease tissue, temporal evaluation of blood RNA profiles that are mechanistically associated with disease processes could provide an additional useful tool for screening for disease in an at-risk population. Moreover, studying both transcriptional and translational profiles of markers could lead us to understand the in vivo molecular pathway of atherogenesis, endothelial dysfunction and platelet activation in patients with ACS.

By specifically choosing different aspects of the pathophysiology of atherosclerotic plaque rupture, the present study sought to investigate whether IL6, vWF and P-selectin can be used as markers to differentiate patients who had developed ACS, compared with patients with stable CAD during an early phase of hospitalisation.

## MATERIALS AND METHODS

### Study participants

Twenty-two ACS patients, who had transient ST-segment or T-wave changes on a standard 12-lead electrocardiogram, or raised troponin-T levels occurring with their typical symptom onset, were recruited into this study between

March 2009 and December 2010. Each ACS patients was recruited within 1 h of hospital admission in the emergency department of Sarawak General Hospital. Twenty-eight patients with angiographically documented  $\leq 50\%$  coronary stenosis, who had no previous history of an ACS event were recruited as controls. These controls were screened between March 2009 and March 2010 from an original group that had conventional coronary angiography, or coronary angiography by multislice CT of the coronary arteries. History of smoking, diabetes, hypertension, hyperlipidaemia and family history of CAD prior to the index date were ascertained by interview with patients and control subjects, and retrieved from medical records or discharge letters. Written, informed consent was obtained from all subjects. This study was approved by the medical research ethics committee of the Ministry of Health, Malaysia.

### Measurement of serum levels of CRP, IL6, vWF and P-selectin

Baseline serum samples were obtained from 5 ml of whole blood stored in serum-separator tubes after clotting for 30 min, and centrifuged at  $1500 \times g$  for 10 min at room temperature. Samples were analysed by commercial kits of ELISA for high-sensitivity C-reactive protein (CRP), (American Diagnostica, Stamford, Connecticut, USA), IL6 (Invitrogen, Camarillo, California, USA), vWF (AssayPro, St Charles, Missouri, USA), and soluble P-selectin (R&D Systems, Minneapolis, Minnesota, USA). The lower limit of detection by ELISA for each marker was 2.5 ng/ml, 2 pg/ml, 5 ng/ml and 0.5 ng/ml, respectively. Intra-assay and interassay coefficient variations were less than 8% and 10%, respectively. The final results were presented as  $\mu\text{g/ml}$  (CRP and vWF), pg/ml (IL6) and ng/ml (P-selectin).

### RNA isolation, DNases treatment and cDNA synthesis

Total RNA from 5 ml of whole blood was extracted by using TRI-reagent RT-blood reagent (Molecular Research Centre Inc, Cincinnati, Ohio, USA) and further cleaned up using RNeasy mini spin column kit (Qiagen, Hilden, Germany) as per manufacturer's instructions, with slight modification. Following RNA isolation, DNases treatment was performed (RQ1 DNases I, Promega, Madison, Wisconsin, USA) to eliminate contamination of genomic DNA in RNA samples, and then reverse-transcribed to cDNA by using Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Promega, Madison, Wisconsin, USA).

### Real-time PCR

Gene expressions of IL6, vWF and P-selectin at mRNA level in whole blood samples were quantified by delta-delta  $C_T$  ( $\Delta\Delta C_T$ ) relative quantification method using the Rotor-Gene 6000 thermal cycler (Corbett Research, Mortlake, NSW, Australia). Human  $\beta$ -actin primer sets were used as reference gene. All primer sequences were retrieved from online PrimerBank database<sup>11</sup> (IL6 and P-selectin) or qPrimerDepot database<sup>12</sup> (vWF and  $\beta$ -actin), respectively, as listed in table 1.

**Table 1** Forward and reverse primer sequences used in this study

Gene	Forward sequence (5'–3')	Reverse sequence (5'–3')
IL6	CAATCTGGATTCAATGAGGAGAC	CTCTGGCTTGTCTCACTACTC
von Willebrand Factor	TTTCCCAGAGGAGATGTTG	TCGGACCCTTATGACTTTGC
P-selectin	CTGCTGCAAGGCGTTCTACT	GGACAGGTTCCCATGTTGG
$\beta$ -Actin	CCTTGCACATGCCGGAG	GCACAGAGCCTCGCCTT

For real-time PCR reaction, the following reaction components were prepared: 12.5  $\mu\text{l}$  Rotor-Gene SYBR Green I master-mix (Qiagen, Hilden, Germany), 2.5  $\mu\text{l}$  forward primer (10  $\mu\text{M}$ ), 2.5  $\mu\text{l}$  reverse primer (10  $\mu\text{M}$ ), 6.5  $\mu\text{l}$  nuclease-free water and 1  $\mu\text{l}$  cDNA template to make up total reaction of 25  $\mu\text{l}$ . The following PCR-run protocol was performed: denaturation programme (95°C, 5 min), amplification and quantification programme repeated 40 times (95°C for 5 s, 60°C for 10 s), followed by melting dissociation curve programme. First-choice Human Breast Total RNA (Ambion, Austin, Texas, USA) was used as calibrator, and all the samples were run in duplicate. The final mRNA expression levels were presented as the normalised mean value of mRNA fold change relative to calibrator.

### Statistical analysis

Statistical analyses were carried out using the statistical programme SPSS 16.0 for Windows (SPSS, Chicago, Illinois, USA). Parametric results are expressed as mean with SD, and differences between groups were compared by using one-way analysis of variance (ANOVA). Non-categorical data were compared by the  $\chi^2$  test. Non-parametric results are expressed as medians with IQR, and were compared using Mann-Whitney test. Correlations were examined using Spearman's rank correlation. All comparisons were considered significant at  $p < 0.05$ .

### RESULTS

The demographic and baseline biochemical data from all study subjects are shown in table 2. Among 22 ACS patients, 17 had ST-segment elevation myocardial infarction, four had non-ST-segment elevation myocardial infarction and one had unstable angina. The median duration of chest pain for ACS patients was 61.32 (52.25) min. Among all the study subjects, the prevalence of those with at least one of the five conventional cardiovascular risk factors was 95.4% and 100% in ACS and controls, respectively. The majority of ACS patients had risk factors of smoking and higher admission diastolic blood pressure, heart rate and total white blood cells than the controls ( $p < 0.005$ ).

**Table 2** Baseline demographic and biochemical data in ACS and control subjects

Baseline characteristics	ACS (n=22)	Controls (n=28)	p Value
Age (years)	55.2 $\pm$ 10.4	53.5 $\pm$ 8.4	0.539
Male, n (%)	18 (81.8%)	24 (85.7)	0.709
Risk factor, n (%)			
Current smoking	11 (50)	6 (21.4)	0.017
Hypertension	16 (72.7)	17 (60.7)	0.373
Dyslipidaemia	9 (40.9)	26 (92.9)	<0.001
Diabetes	9 (40.9)	8 (28.6)	0.361
Family history of CAD	11 (50)	16 (57.1)	0.615
Systolic blood pressure (mm Hg)	155.00 (41.75)	130.50 (29.50)	0.091
Diastolic blood pressure (mm Hg)	93.00 (23.25)	81.00 (8.50)	0.004
Heart rate (bpm)	87.00 (26.50)	70.50 (22.25)	0.001
Total white blood cells ( $10^3/\mu\text{l}$ )	11.35 (5.03)	6.29 (2.04)	<0.001
Haemoglobin (g/dl)	14.84 $\pm$ 1.37	14.58 $\pm$ 1.23	0.481
Platelet count ( $10^3/\mu\text{l}$ )	250.82 $\pm$ 77.49	240.43 $\pm$ 41.94	0.547
Creatinine ( $\mu\text{mol/l}$ )	98.14 $\pm$ 21.96	89.07 $\pm$ 17.97	0.115
Total cholesterol (mmol/l)	5.37 (1.58)	4.62 (1.86)	0.190
Triglycerides (mmol/l)	1.57 (0.67)	1.57 (1.26)	0.822
Low density lipoprotein (mmol/l)	1.12 (0.25)	1.30 (0.47)	0.137
High density lipoprotein (mmol/l)	3.19 (1.11)	2.66 (1.31)	0.091

ACS, acute coronary syndrome; CAD, coronary artery disease.

**Table 3** Serum levels of CRP, IL6, vWF and P-selectin in ACS and control subjects

Marker	ACS	Controls	p Value
CRP, µg/ml, median (IQR)	4.90 (10.11)	0.55 (1.09)	<0.001
IL6, pg/ml, median (IQR)	7.95 (22.28)	0.00 (1.23)	<0.001
vWF, µg/ml, median (IQR)	16.85 (14.51)	8.38 (5.54)	<0.001
P-selectin, ng/ml, median (IQR)	79.42 (50.43)	68.63 (45.09)	0.082

ACS, acute coronary syndrome; CRP, C-reactive protein; vWF, von Willebrand Factor.

### Serum protein levels of CRP, IL6, vWF and P-selectin in ACS and control subjects

Serum levels of CRP, IL6, vWF and P-selectin are presented in table 3. All markers were increased in patients with ACS compared with the controls, except for P-selectin. Univariate analysis showed that there was no correlation among the serum levels of all tested markers in the control group. A moderate correlation was found between IL6 and CRP levels in the ACS group ( $r=0.474$ ,  $p=0.026$ ).

### mRNA expression levels of IL6, vWF and P-selectin in ACS and control subjects

In this part of the study, only some samples were selected for mRNA expression analysis for each gene. Samples which showed primer dimer, or had poor RNA quality, were excluded from further analysis. No significant difference of IL6, vWF and P-selectin mRNA levels was found in ACS subjects and controls (table 4). With the limitation of small sample size, and assumption of both ACS and stable CAD which shared similar pathogenesis of atherosclerosis, we combined both groups in order to understand the integral relationship of IL6, vWF and P-selectin at transcriptional level. The correlation test showed that mRNA level of P-selectin was significantly associated with IL6 ( $r=0.697$ ,  $p=0.003$ ) and vWF ( $r=0.497$ ,  $p=0.050$ ).

### Comparison of mRNA and serum protein expression levels

Theoretically, the changes in mRNA level could be reflected in the level of protein expression of one gene. A correlation test was therefore performed to check the relationship between mRNA and serum level of each gene in order to understand their possible molecular pathogenic role in development of atherosclerosis. In the present study, we observed a large discrepancy between mRNA and serum protein expression levels of IL6 and vWF, but not P-selectin in our patients (IL6:  $r=-0.03$ ,  $p=0.891$ ; vWF:  $r=-0.002$ ,  $p=0.991$ ; P-selectin:  $r=0.358$ ,  $p=0.048$ ).

**Table 4** mRNA levels of IL6, vWF and P-selectin in ACS and control subjects

Marker	ACS	Controls	p Value
IL6, median (IQR)*	0.13 (0.24)	0.16 (0.31)	0.536
vWF, median (IQR)†	0.040 (0.01)	0.0025 (0.01)	0.227
P-selectin, median (IQR)‡	388.30 (479.24)	373.34 (403.65)	0.675

Due to consideration of sample qualities, a varying total number of samples to be analysed for relative mRNA fold change in IL6, vWF and P-selectin were taken. Some samples were also excluded for further analysis as only one replicate recorded a positive  $C_T$  and the other was negative (undetected), or showed primer dimer.

\*IL6: 12 ACS versus 7 controls.

†vWF: 17 ACS versus 12 controls.

‡P-selectin: 19 ACS versus 12 controls.

ACS, acute coronary syndrome; vWF, von Willebrand Factor.

### Influence of changes in cardiovascular risk factors

Despite the discrepancy between mRNA and protein level, it is most probably the measurement of protein levels of each gene that is the ideal clinical source of information to differentiate patients at risk of ACS. However, the changes in protein level could be influenced by other confounding factors. We therefore performed additional analyses to assess whether the changes in the serum levels of each marker were associated with changes in the conventional cardiovascular risk factor. Only soluble P-selectin was found correlated with smoking ( $r=-0.438$ ,  $p=0.041$ ) and diabetes mellitus ( $r=0.462$ ,  $p=0.030$ ) in ACS patients.

### DISCUSSION

The strengths of this study include the availability of whole blood and serum samples at a very early phase of ACS, with a median duration of chest pain of approximately 60 min before acute pharmacotherapy was fully established. Another aspect is the ethnic diversity of the ACS cases and controls. To date, no study has examined the association of whole blood gene expression and serum levels of markers of inflammation—IL6, endothelial dysfunction, vWF and platelet activation—P-selectin in this combination, in a multiethnic Malaysian population during early onset of ACS. The present study affirmed higher serum levels of IL6 and vWF<sup>13 14</sup> but not P-selectin in ACS patients compared with controls.

Evidence, to date, suggests that systemic inflammation originates from local inflammatory processes within the arterial wall after plaque disruption is often an index of further events of ACS.<sup>15</sup> CRP is a valuable marker for underlying systemic inflammation, and its release is largely mediated by cascade response of proinflammatory cytokines including IL6 and TNF- $\alpha$ .<sup>16</sup> Though TNF- $\alpha$  is the main determinate of IL6 synthesis and has been widely studied, it was observed that the IL6 could be a stronger predictor of coronary mortality than TNF- $\alpha$  in unstable angina patients.<sup>17</sup> In the present study, IL6 found closely associated with CRP affirmed that IL6 is a primary stimulant for hepatic production of CRP.<sup>16</sup> Although this may signify an increase of systemic inflammation after vascular injury at the site of the lesion, we lack the prognosis value of IL6 and CRP as a combining marker strategy to predict for future events after an ACS event in our population.

In addition, the increased serum level of vWF indicates the extent of damage in the vascular endothelium, or simply the reaction of acute-phase reactant to various stimuli, such as hypoxia and inflammatory cytokines.<sup>18</sup> A recent report has shown that ACS patients were found to have higher oxidative stress compared with healthy controls, and that oxidation process during haemostasis could induce the release of vWF from the endothelium.<sup>19</sup> As an endothelial dysfunction marker, the causative role of vWF in the process of atherosclerosis process is to support platelet adhesion to the injured vessel walls to promote platelet aggregation and fibrin clot formation.<sup>18</sup> Our data further suggest that vWF can be a strong indicator of existence of any kind of ACS.<sup>19</sup>

The evaluation of gene expression of heterogenous whole blood cell population may better elucidate the true biology of inflammation,<sup>20</sup> however, the difference in serum protein level we observed was not reflected at mRNA level of IL6 and vWF. The poor correlations between mRNA and protein levels of markers may be attributed to the complicated biological processes, such as post-transcriptional splicing, translational modifications, and so on.<sup>21</sup> In a complex inflammatory process, such

as angiogenesis, which is tightly regulated both spatially and temporally, the mRNA content of some specific genes may be 'switched off' after translated into functional protein product.<sup>22</sup> Since IL6 and vWF are involved in proinflammatory or atherogenesis activities, their mRNA content may be terminated quickly or translated into proteins as a consequence of excessive immune response.<sup>23</sup> As a result, the mRNA content will be degraded, and the translated protein product might be released quickly into blood circulation to be detected as markers of disease or as mediators of subsequent inflammatory processes.

There were no significant elevations of P-selectin level in ACS patients in contrast with previous studies,<sup>14 24</sup> P-selectin was found to be correlated with IL6 and vWF at mRNA level in both ACS and control patients. P-selectin may have its enhanced effects in platelet activation only after being upregulated through exposure to other inflammatory mediators, particularly CD40 ligand.<sup>25</sup> It is unclear to us whether P-selectin expression will be upregulated through exposure to IL6 or vWF; however, the correlation between these markers at mRNA levels suggested a possible association between processes of inflammation, endothelial damage and platelet activation—that inflammation and endothelial dysfunction take precedence over platelet activation, and the degree of rise could be indicative of the severity of ACS.

The consistent pattern in P-selectin level in ACS patients could be due to the uses of serum instead of plasma sample for measurement. It was reported that the difference in P-selectin level between diseased and healthy patients was significantly found only in the plasma sample, as the serum samples may not truly reflect the *in vivo* platelet activation activity of P-selectin.<sup>26</sup> The detection of P-selectin level in serum samples may be due to excess P-selectin released from activated platelets or by the process of coagulation and clot formation.<sup>26</sup> Nonetheless, it was reported that although P-selectin levels are significantly higher in serum compared with plasma, the absolute concentration of P-selectin remain significantly and moderately correlated in both serum and plasma. This suggests that qualitatively similar results are likely to be obtained, irrespective of which sample is used to measure P-selectin level.<sup>27</sup>

In the present study, all ACS patients were enrolled during their early phase of hospitalisation. It is therefore believed that the P-selectin may have a late time frame to be elevated.<sup>24</sup> Experimental evidence suggests that P-selectin is expressed after interaction between CD40 and CD40 ligand to enhance platelet activation activity to facilitate plaque destabilisation and thrombus formation.<sup>26</sup> Moreover, P-selectin levels were associated with diabetes<sup>28</sup> and smoking.<sup>8</sup> Although it can be construed that the measurement of circulating P-selectin level may not be a suitable marker specifically for ACS detection, the unexpected finding of negative correlation between P-selectin and smoking could be due to the impact of small sample size in the present study. Nevertheless, we assume that if an elevated P-selectin level detected during the ACS presentation could indicate significant systemic platelet activation and/or delayed presentation of ACS, this may also carry prognostic value.

The correlation test showed that serum levels of IL6 and vWF could be used as independent markers to study the pathogenesis of ACS independent of conventional cardiovascular risk factors, however, the relevance of this observation needs to be interpreted carefully as the current study only relied on a single baseline sample. The exact time frame of peak elevations of IL6 and vWF and their prognostic values to detect ACS are yet to

be evaluated in this study. Hence, prospective studies are needed to fully evaluate IL6 and vWF as potential mechanisms/markers of specific detection and poor cardiovascular outcome in ACS.

### Study limitations

One of the main goals of this study was to evaluate the potential of blood as surrogate marker of risk of ACS, yet evidence indicates that *in vivo* expression of IL6 and vWF is found abundantly in atherosclerotic plaque.<sup>29 30</sup> Hence, evaluation of peripheral whole blood gene expression alone may have provided reports of results with diminished value since the heterogeneous cell population of whole blood may reflect only one aspect of a complex pathophysiology.<sup>10</sup>

In addition, the assessments of coronary stenosis used as parameters to define controls of this study were evaluated using conventional visual estimation at our centre. However, this type of assessment may underestimate or overestimate the degree of stenosis, and it is difficult to tell whether these coronary plaques are prone to rupture (ie, 'the vulnerable plaque'). It is possible that among these controls that are prone to plaque rupture, they could have an increased level of inflammatory responses, thus, higher the risk of getting ACS in the future. This study also involved a relatively small number of patients which could have compromised the statistical validity of our results. Nevertheless, to our knowledge, this is the first study reporting the relationship of inflammation, endothelial dysfunction, and platelet activation markers in early phase of ACS in a Malaysian population, suggesting the potential use of these markers as diagnosis tools. The current study did not involve the clinical outcomes follow-up of patients, thus it would be interesting to incorporate this consideration into future study to investigate the prognosis value of IL6 and vWF as predictor of the risk of adverse cardiac events, particularly in control patients.

### CONCLUSIONS

Our findings suggest that elevated markers of inflammation and endothelial dysfunction, rather than platelet activation, puts an individual at a higher risk of developing ACS. Hence, IL6 and vWF might be useful markers in the early assessment of the risk of ACS in a young, multiethnic, Malaysia population, as well as to target subjects who may benefit from anti-atherosclerotic or antithrombotic strategies aimed to reduce IL6 and vWF levels. In the future, we may consider large multicentre studies to validate the true relationship between IL6, vWF and P-selectin, as well as their prognostic value, to detect ACS.

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