Background & objectives: Platelet endothelial cell adhesion molecule-1 (PECAM-1) plays a key role in the transendothelial migration of circulating leukocytes (diapedesis) during vascular inflammation. We hypothesized that genetic variation and the level of soluble PECAM-1 could be associated with the development of atherosclerosis and conducted a study on gene polymorphisms of PECAM-1 and soluble PECAM-1 levels in Asian Indian patients with coronary artery disease (CAD) in Singapore.

Methods: Of the 137 angiographically confirmed patients (>70% stenosis) of CAD and 110 controls in Asian Indian population, two single nucleotide polymorphisms (SNPs) of PECAM-1 gene, C+373G (Leu125Val) at exon 3 and G+1688A (Ser563Asn) at exon 8 were analyzed by polymerase chain reaction-restriction fragment-length polymorphism (PCR-RFLP) strategy. In addition, plasma soluble PECAM-1, P-selection and lipid profile were measured. Chi-square test and student t test were adopted to compare categorical and continuous variables, respectively.

Results: A significant decrease in C allele frequency but increase in G allele frequency of the Leu125Val (C/G) polymorphism were observed in CAD patients as compared with controls (0.54/0.46 vs 0.663/0.337 respectively, P=0.008). Alteration in genotype distributions (CC, CG and GG) of the Leu125Val polymorphism between CAD patients and controls (P=0.009) was also significant. A similar trend was observed on the allele frequencies (G/A) and genotype distributions of Ser563Asn (G/A) polymorphism, though the difference did not reach significance. On the other hand, plasma level of soluble PECAM-1 (sPECAM-1) was markedly elevated in CAD patients (P=0.006), and associated with soluble P-selectin and lipid profiles.

Interpretation & conclusion: Our study showed that Leu125Val polymorphism of PECAM-1 gene and elevated soluble PECAM-1 were related to severe coronary artery stenosis in CAD patients of Asian Indian origin in Singapore. Our data also suggest that PECAM-1 plays an important role in the development of atherosclerosis.

Key words Atherosclerosis - coronary artery disease - gene polymorphism - PECAM-1
latter is predominantly mediated by a group of cellular adhesion molecules (CAMs) expressed on the cell surface, such as selectins, intracellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and platelet endothelial cell adhesion molecule-1 (PECAM-1, CD 31)\textsuperscript{2-4}. PECAM-1, a 130-kDa membrane glycoprotein and a member of immunoglobulin (Ig) superfamily, is expressed on the surface of monocytes, some T-lymphocyte subsets, platelets and endothelial cells\textsuperscript{5-7} where it concentrates at cell-cell borders. As a transmembrane glycoprotein, PECAM-1 has 6 Ig-like (homology) extracellular domains (encoded by exon 3 to 8), a short transmembrane domain (encoded by exon 9) and a short cytoplasmic tail (encoded by exon 10-16)\textsuperscript{8,9}. The importance of the first domain of PECAM-1 (encoded by exon 3) is underscored since PECAM-1 forms homophilic binding via its first or the first plus the second extracellular Ig-like domains or heterophilic binding with other molecules to mediate cell-cell adhesion\textsuperscript{10,11}. It has been suggested that PECAM-1 is a multifunctional cell adhesion molecule involved in angiogenesis\textsuperscript{12}, integrin regulation\textsuperscript{13}, apoptosis\textsuperscript{14} and more importantly, transendothelial migration of monocytes(TEM)\textsuperscript{10,15}. Also, PECAM-1 plays an important role in plaque formation and thrombosis\textsuperscript{6,16}.

Earlier studies have shown that genetic variants of PECAM-1 might influence individual susceptibility to coronary artery disease (CAD), association between PECAM-1 gene polymorphism and CAD reported in German\textsuperscript{17} and Japanese\textsuperscript{18} populations. However, there is little information on other ethnic groups. Exon 3 and exon 8 encodes for the 1st and 6th extracellular Ig-like domain of PECAM-1 respectively. The function of 1st Ig-like domain has been emphasized and the function of 6th Ig-like domain is also suggested to be involved in calcium homeostasis\textsuperscript{19}.

Association between soluble CAMs released to circulation and CAD has been suggested with soluble form of PECAM-1 (sPECAM-1) detected in both human plasma and in the medium of cultured endothelial cells\textsuperscript{20,21}. While most of studies focus on studying the association between other soluble CAMs and CAD, little is known regarding soluble PECAM-1 in patients of CAD at stable condition. No data are available on the association of PECAM-1 with CAD in Asian Indians. We therefore conducted this study on gene polymorphisms [two most studied single nucleotide polymorphisms (SNPs) located at exon 3 and exon 8 respectively that have potential biological functions] of PECAM-1 and soluble PECAM-1 levels in CAD patients in Asian Indian in Singapore.

**Material & Methods**

**Subjects:** A total of 137 unrelated patients with CAD who were consecutively referred to National University Hospital of Singapore between 2001 and 2003 were included in the study. All CAD patients were angiographically defined (having 1, 2, or 3 major epicardial coronary arteries with \(\geq 70\%\) luminal stenosis). Among CAD patients, 5.7 per cent had single vessel disease, 18.9 per cent had double disease and 75.4 per cent had triple vessel disease. None of the CAD patients recruited in the study had acute myocardial infarction.

110 non CAD controls recruited from the same period were volunteers by advertisement who did not have a history or clinical evidence of CAD. Further, they were confirmed free of CAD by treadmill test.

Most of patients and controls were of south Indian origin and have settled in Singapore for over 3 generations. All participants were interviewed in details, and data on smoking habits, hypertension, and diabetes were recorded. Individuals were defined as hypertensive if their blood pressure was >140/90 mm Hg or if they were receiving any anti-hypertensive treatment. Individuals with a history of diabetes or those receiving any anti-diabetic medication were considered to be diabetic. Smokers included both ex-smokers and active smokers. Both patients and controls with age >70 yr, familial hypercholesterolaemia, or thyroid, kidney or liver disease or autoimmune disease were excluded from the study. The study complied with the declaration of Helsinki and was approved by the Joint Committee for Clinical Investigation (JCCI) of Johns Hopkins University and Hospital as well as the bio-ethics committees from National University Hospital of Singapore, and Singapore General Hospital, which
covers the National Heart Centre of Singapore. Informed consent was obtained from all subjects.

Screening of PECAM-1 gene polymorphisms: Blood (15 ml) was obtained from patients and controls with overnight fasting (12 h). Genomic DNA was isolated from the white blood cell pellets with a protocol modified from Blin and Stafford\(^22\). We have selected two SNPs in the coding sequence, C+373G (Leu125Val) at exon 3 and G+1688A (Ser563Asn) at exon 8 as reported previously\(^17,18,23,24\) in our polymorphism screening. A polymerase chain reaction restriction fragments length polymorphism (PCR-RFLP) procedure was adopted. Based on published sequence of PECAM-1 gene\(^25\), PCR primer pairs were designed to generate two DNA fragments covering these SNPs. A pair of oligonucleotide primers, forward (5’-CTATCGCTGGCCCTGTA-3’) / reverse (5’-TTATGACTGTGCT-3’) with the product size of 504 nucleotides covering the SNP C+373 G (Leu125Val) at exon3; and another pair, forward (5’-CTATCGCTGGCCCTGTA-3’) / reverse (5’-TCTGGAGGCTGTGACT-3’) with the product size of 399 nucleotides covering the SNP of G+1688A (Ser563Asn) at exon 8 were synthesized. The conditions for PCR were: 95°C for 4 min; 95°C for 30 seconds; 62°C for 45 seconds and 72°C for 60 seconds and repeat for 30 cycles; and 72°C for 7 min. PCR product was ethanol precipitated and digested with Pvu II (New England Biolabs, USA, CAG/CTG, from +370 to +375) and Nhe I (New England Biolabs, USA, GCTAG/C, from +1684 to +1689) based on the single nucleotide substitution at C+373G and G+1688A, respectively. Digested PCR products were subjected to agarose gel electrophoresis. Genotyping results from the 15 samples representing 3 genotypes were confirmed by direct sequencing of PCR products using DNA sequencer\(^26,27\).

Levels of sPECAM-1 and soluble P-selectin (sP-selectin) were measured by enzyme-linked immunosorbtent assay (ELISA), according to manufacturer’s instruction. ELISA kits were purchased from Bender MedSystem (MedSystems Diagnostics GmbH Rennweg 95bA-1030 Vienna, Austria). Lipid panel (lipids, cholesterols and lipoproteins) was determined by routine analytical methods at the Pathology department of National University Hospital\(^28\).

Statistical analysis: c\(^2\)-test was used to compare categorical variables. Because of skewed distribution, sP-selectin was expressed as median (25th/75th interquartiles) and compared by Mann-Whitney U test. Other continuous variables were expressed as mean and standard deviation and significance of differences between two groups was assessed by Student’s t test. Hardy-Weinberg equilibrium was analyzed by c\(^2\)-test for the frequencies of the PECAM-1 genotypes\(^29\). Pearson or Spearman correlation coefficients were computed to assess the association between parameters according to the status of distribution. P<0.05 was considered as significant. All computations were performed with Statistical Package for Social Sciences (SPSS,) version 10 (Chicago, IL).

Results

Patients with CAD were older, more likely to be males. The occurrence of diabetes mellitus, smoking, and hypertension were also significantly higher in CAD patients than that in controls (Table I). Moreover, patients with CAD had significantly higher levels of triglyceride (TG), higher ratio of total cholesterol (TC) to HDL-C, but lower levels of HDL-C and apoA1, as well as lower ratio of apolipoprotein A1 (apoAl) to apolipoprotein B (apoB). The levels of TC, LDL-C and apoB were lower in CAD patients compared with controls. There was no difference in lipoprotein (a) [Lp(a)] levels between the two groups (Table II).

Genotyping for C+373G (Leu125Val) and G+1688A (Ser563Asn) polymorphism: The presence of two SNPs, C+373G (Leu125Val) at exon 3 and G+1688A (Ser563Asn) at exon 8 were confirmed in our subjects.

| Table I. Demographic details of patients and controls |
|---------------------------------|-----------------|
|                                | Controls (n=110) | CAD patients (n=137) |
| Age (yr), (mean±SD)            | 52.88±10.08     | 60.28±10.42**        |
| Sex (% of male)                | 41.8            | 81.8**               |
| Diabetes mellitus (%)          | 11.8            | 63.5**               |
| Smoking (%)                    | 7.3             | 38.7*                |
| Hypertension (%)               | 15.5            | 60.6**               |

CAD, coronary artery disease

P*<0.05, **<0.001 compared to controls
The genotype frequencies were in agreement with those predicted by the Hardy-Weinberg equilibrium. We found a significant association between the genotype distributions of C+373G (Leu125Val) polymorphism and CAD (P=0.009), and G allele frequency was also significantly higher in CAD patients than in controls (P=0.008) (Table IIIA). After adjusting for other risk factors for CAD including age, gender, smoking, hypertension, diabetes, the level of TC and HDL-C by multivariate logistic regression test, GG homozygous was significantly associated with CAD compared with CC plus CG genotypes [we assumed a recessive model of inheritance, odds ratio (95% confidence interval): 1.123 (1.060-1.190), P<0.05]. However, the genotype distribution of G+1688A (Ser563Asn) polymorphism did not significantly differ between two groups (P=0.148), and though the frequency of A allele was higher in CAD patients than in control, the difference did not reach significance (P=0.058) (Table III B). The combined effect of two gene polymorphisms was also studied and the results showed that the combination of CG+GG (for Leu125Val) and GA+AA (for Ser563Asn) was significantly increased in patients compared to the controls (67.8 and 51.5% respectively, P=0.014). There was no significant association for Leu125Val or Ser563Asn polymorphisms with the number of affected vessels.

Plasma sPECAM-1 level in Indian CAD patients:
Patients had significantly higher sPECAM-1 level compared with controls (71.92 ± 25.62 ng/ml vs 62.77 ± 25.46 ng/ml, P<0.01). The odds ratio (95%CI) was 1.19 (1.07-1.43). After controlling for other risk factors for CAD including age, gender, smoking, hypertension, diabetes, the level of TC and HDL-C by multivariate logistic regression test.

The levels of sPECAM-1 did not differ among subjects with different genotypes. Also, there was no significant association between sPECAM-1 levels and the number of affected vessels. Soluble PECAM-1 levels were positively correlated with sP-selectin (r=0.314, P=0.005). Also there were weak associations between sPECAM-1 and TG, LDL-C, HDL-C and apoA1 (r=0.134, r=0.173, r= -0.133, and r= -0.144 respectively, P<0.05).

### Table II. Lipid panel of the patients and controls

<table>
<thead>
<tr>
<th></th>
<th>Control (n=110)</th>
<th>CAD patients (n=137)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG (mM)</td>
<td>1.42±0.72</td>
<td>1.73±0.92***</td>
</tr>
<tr>
<td>TC (mM)</td>
<td>5.45±1.22</td>
<td>4.35±1.12***</td>
</tr>
<tr>
<td>HDL-C (mM)</td>
<td>1.28±0.39</td>
<td>0.93±0.28***</td>
</tr>
<tr>
<td>LDL-C (mM)</td>
<td>3.55±0.99</td>
<td>3.21±0.89*</td>
</tr>
<tr>
<td>TC/HDL-C</td>
<td>4.53±1.39</td>
<td>4.98±1.65*</td>
</tr>
<tr>
<td>apoA1 (mg/dl)</td>
<td>145.47±38.86</td>
<td>113.50±28.29***</td>
</tr>
<tr>
<td>apoB (mg/dl)</td>
<td>109.00±36.03</td>
<td>97.27±27.48**</td>
</tr>
<tr>
<td>apoA1/apoB</td>
<td>1.59±1.15</td>
<td>1.30±0.83*</td>
</tr>
<tr>
<td>Lp (a) (mg/dl)</td>
<td>30.67±27.74</td>
<td>30.00±27.11</td>
</tr>
</tbody>
</table>

CAD, coronary artery disease; TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; apoA1, apolipoprotein A1; apoB, apolipoprotein B; Lp(a), lipoprotein(a). Values are mean±SD.

### Table III. Genotypic distributions of the C+373G (Leu125Val) and G+1688A (Ser563Asn) polymorphism in controls and CAD patients

<table>
<thead>
<tr>
<th></th>
<th>A: C+373G (Leu125Val)</th>
<th>B: G+1688A (Ser563Asn)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patients</td>
<td>Controls</td>
</tr>
<tr>
<td>Genotype frequency (%)</td>
<td>n=137</td>
<td>n=110</td>
</tr>
<tr>
<td>CC</td>
<td>23.4</td>
<td>42.7</td>
</tr>
<tr>
<td>CG</td>
<td>60.6</td>
<td>49.1</td>
</tr>
<tr>
<td>GG</td>
<td>16.0†</td>
<td>13.6</td>
</tr>
<tr>
<td>Allele frequency (%)</td>
<td>2n=274</td>
<td>2n=220</td>
</tr>
<tr>
<td>C allele</td>
<td>0.536</td>
<td>0.664</td>
</tr>
<tr>
<td>G allele</td>
<td>0.464††</td>
<td>0.336</td>
</tr>
</tbody>
</table>

P†=0.009, ††=0.008 compared to controls
Plasma sP-selectin level in Indian CAD patients: There was a significant increase in sP-selectin in CAD patients in comparison with controls (median (25th/75th interquartiles): 276.02 (186.19/452.84) ng/ml vs. 166.36 (112.72/228.83) ng/ml respectively, \( P = 0.001 \)). Levels of sP-selectin negatively correlated with HDL-C and apoA1 (\( r = -0.358, \ P = 0.002 \), and \( r = -0.273, \ P = 0.002 \) respectively).

PECAM-1 genotypes and other confounders among CAD group: Among CAD group, the genotypes and allele frequencies of Leu125Val were not significantly associated with gender, smoking, diabetes and hypertension (Table IV). Neither did genotypes of Ser563Asn(data not shown).

### Discussion

Since Behar et al\(^{23}\) reported PECAM-1 was polymorphic and a role of the Leu125Val polymorphism in the 1st (Ig)-like domain in acute graft-versus-host disease in 1996, the association between PECAM-1 gene polymorphism and CAD has been studied sporadically. The two commonly studied polymorphisms are Leu125Val polymorphism and Ser563Asn located at the 1st and 6th (Ig)-like domains, respectively.

In our study, we found that Leu125Val polymorphism is significantly associated with CAD in Indian patients. We observed significant correlation between genotype distribution of Leu125Val polymorphism and CAD and G allele frequency was significantly higher in CAD patients than in controls.

To the best of our knowledge this is probably the first report on the association of PECAM-1 polymorphisms in Indian CAD patients.

Up to date, only a few studies on PECAM-1 gene polymorphism and CAD have been reported in Caucasians and Japanese. In the German population, Wenzel et al\(^{17}\) reported that in 103 healthy controls and in 98 patients (Caucasians) with more than 50 per cent stenosis, the allele frequencies of the Leu125Val polymorphism were 0.49/0.51 in controls and 0.35/0.65 in patients (\( P < 0.01 \)) and the allele frequencies of the Ser563Asn polymorphism were 0.50/0.50 in controls and 0.37/0.63 in patients (\( P < 0.05 \)). Moreover, the homozygous combination of Leu125Val and Ser563Asn polymorphisms was associated with early severe coronary heart disease. In the Japanese population, Sasaoka et al\(^{18}\) studied the above two polymorphisms (Leu125Val, Ser563Asn) and Arg670Gly polymorphism, and found frequencies of 125Leu, 563Ser, and 670Arg alleles were significantly increased in Japanese patients with myocardial infarction (MI) than controls. However, their results were unexpected since increased frequencies of 125Val, but not 125Leu, and 563Asn, but not 563Ser, in patients were reported in Wenzel’s study. Similarly, our results are in agreement with Wenzel’s finding. In contrast, negative findings have also been reported. In another study in German patients by Gardemann et al\(^{30}\) did not find PECAM-1 C/G (Leu125Val) gene polymorphism to be an independent risk factor of CAD. Leu125Val polymorphism is located at the 3rd exon, which encodes for the 1st extracellular Ig-like domain of the PECAM-1, thus it might play an important role in

<table>
<thead>
<tr>
<th></th>
<th>Male (n=112)</th>
<th>Female (n=25)</th>
<th>Smoker (n=53)</th>
<th>Non Smoker (n=84)</th>
<th>DM (n=87)</th>
<th>Non-DM (n=50)</th>
<th>HP (n=83)</th>
<th>Non-HP (n=54)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC(n)</td>
<td>25</td>
<td>7</td>
<td>13</td>
<td>19</td>
<td>18</td>
<td>14</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td>CG(n)</td>
<td>68</td>
<td>15</td>
<td>27</td>
<td>56</td>
<td>53</td>
<td>30</td>
<td>56</td>
<td>27</td>
</tr>
<tr>
<td>GG(n)</td>
<td>19</td>
<td>3</td>
<td>13</td>
<td>9</td>
<td>16</td>
<td>6</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>C(n)</td>
<td>118</td>
<td>29</td>
<td>53</td>
<td>94</td>
<td>89</td>
<td>58</td>
<td>90</td>
<td>57</td>
</tr>
<tr>
<td>G(n)</td>
<td>106</td>
<td>21</td>
<td>53</td>
<td>74</td>
<td>85</td>
<td>42</td>
<td>76</td>
<td>51</td>
</tr>
</tbody>
</table>

DM, Diabetes mellitus; HP, hypertensive
No significant association found
atherosclerosis. Since the interaction/activation of PECAM-1 is mainly via homophilic binding with its 1st extracellular Ig-like domains\textsuperscript{10,11}, the Leu\textsubscript{125}Val polymorphism might affect the homophilic binding capability, and therefore might influence monocyte/endothelial interaction during the early development of atherosclerotic plaques. On the other hand, the function of the 6th Ig-like domain of PECAM-1 in which Ser\textsubscript{563}Asn is located, is less understood, and it could be implicated in calcium homeostasis\textsuperscript{19} and monocyte passage through extracellular matrix (interstitial migration) prior to TEM (diapedesis)\textsuperscript{31}.

Although an association between the PECAM-1 polymorphisms and CAD follows the same pattern in Indian population as that in other populations, we have observed unique allele frequencies of the above two polymorphisms in Indian population as compared to other populations. In the case of Leu\textsubscript{125}Val, the allele frequencies of C and G are 0.517/0.483 in Chinese controls\textsuperscript{32} in contrast with 0.618/0.382 in Indian controls, and 0.401/0.599 in Chinese CAD\textsuperscript{32} in contrast with 0.529/0.471 in Indian CAD patients. Other populations such as German\textsuperscript{17} and Japanese\textsuperscript{18} have the allele distributions similar to Chinese population. The results suggest the frequency of G allele is much lower in Indian population compared with that in other populations. Similarly a striking lower frequency of A allele for Ser\textsubscript{563}Asn polymorphism was observed in Asian Indian CAD population in general. This finding is interesting, however, in terms of associations of PECAM-1 polymorphisms with CAD, it is in contrast to previous studies that showed that Asian Indians have the higher prevalence of CAD as compared to all other ethnic groups\textsuperscript{33}. We are not clear about how the unique allele distributions of PECAM-1 polymorphism are associated with the prevalence of CAD in Indian population. Nevertheless, our study could add PECAM-1 as one of the risk factors responsible for higher incidence of CAD in Asian Indians. We speculate that PECAM-1 could interact with other risk factors such as adult-onset diabetes, low HDL-C, increased Lp (a) levels as well as low birth weight\textsuperscript{33,34} etc., and collectively contribute to the early onset of CAD in these populations. Our present study is also the first to examine the plasma level of sPECAM-1 and CAD in the Indian population. We found that soluble PECAM-1 was higher in CAD patients than in controls. Similar results were obtained in Chinese CAD patients (Wei \textit{et al}, unpublished data). Up to date, very limited data were reported on the association between sPECAM-1 and CAD. Serebruany \textit{et al}\textsuperscript{20} found a higher sPECAM-1 level in patients with acute MI. However, Blann \textit{et al}\textsuperscript{35} did not find a difference in sPECAM-1 level between patients with frank atherosclerosis and controls. In the present study, since almost all patients had at least two vessels affected (more than 70% stenosis), our results suggested that the sPECAM-1 level increased in severe coronary stenosis in Indian CAD patients. Although the difference in sPECAM-1 between CAD patients and controls is small, it is consistent with the previous studies\textsuperscript{20,32}. Given the roles that PECAM-1 plays in endothelial dysfunction and vascular inflammation, sPECAM-1 level might serve as a useful marker to monitor the individualized pathological changes and evaluate the effect of endothelial protective therapy. Moreover, since sPECAM-1 was found to be positively correlated with sP-selectin, a marker of platelet activation, it suggests that PECAM-1 might be involved in platelet activation and perhaps related to thrombosis.

In the present study weak correlation was found between sPECAM-1 levels and lipid panel. The levels of sPECAM-1 were positively correlated with TG, LDL-C, while it was negatively correlated with HDL-C and apoA1. Similarly, sP-selectin were also negatively correlated with HDL-C and apoA1. Some studies have also reported that HDL-C downregulated\textsuperscript{36} and LDL-C upregulated the expression of cell adhesion molecules\textsuperscript{37,38}. The relationship between lipid and cell adhesion molecules might suggest that serum lipid level may influence cell adhesion molecules expression. However, the association may also be due to their similar effects on CAD which may be independent of each other.

High TG, low HDL-C, as well as high Lp (a) are the typical lipid disorders for Indian CAD patients. As expected, we found higher levels of TG, higher ratio of TC to HDL-C, but lower levels of HDL-C and apoA1, as well as lower ratio of apoA1 to apoB in Indian CAD patients compared with controls. Our study design had some limitations. The major concern was the big difference in other parameters between control and CAD patients, which compromised the interpretation of the results. Nevertheless, our study
still provides some interesting data on PECAM-1 in Asian Indian and foster additional and in depth research in this area.

In summary, we found that the Leu125Val polymorphism of PECAM-1 and the level of sPECAM-1 were correlated with CAD. In addition, a unique pattern of allele frequencies of PECAM-1 polymorphisms was observed in Asian Indian population in Singapore. Our data suggest that PECAM-1 plays an important role in thrombosis and the development of atherosclerosis in Asian Indians.

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