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Pro/Anti-Inflammatory Cytokines in the Pathogenesis of Premature Coronary Artery Disease

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Proinflammatory interleukin-18 (IL-18), high-sensitivity C-reactive protein (hS-CRP), tumor necrosis factor-alpha (TNF-alpha), and anti-inflammatory IL-10 are involved in the pathogenesis of atherosclerosis. The aim of this study was to determine the imbalance between pro- and anti-inflammatory cytokines in premature coronary artery disease (PCAD) and their association with the degree of angiographic atherosclerotic blockade. A case–control study was carried out at the National Institute of Heart Diseases, Rawalpindi, Pakistan. Three hundred eighty-five stable coronary artery disease patients aged <45 years were screened. A total of 172 subjects participated in this study, comprising 98 PCAD patients and 74 angionegative controls. Serum IL-10, IL-18, and TNF-alpha were measured using enzyme-linked immunosorbent assay and hS-CRP was analyzed using Immulite 1000. The mean age of the patients was 40 ± 4.23 years (69 men and 9 women). Serum IL-18, TNF-alpha, hS-CRP, and IL-18/IL-10 ratio were significantly raised in PCAD patients ($P < 0.01$), whereas IL-10 was moderately increased ($P < 0.05$) when compared with controls. Serum IL-18 and hS-CRP were significantly raised in patients with 86%–100% stenosis ($n = 57$) when compared with patients with 71%–85% stenosis ($n = 41$) ($P < 0.05$). Serum IL-18, TNF-alpha, IL-18/IL-10 ratio, and hS-CRP significantly correlated ($P < 0.01$) with the degree of angiographic blockade. Pro/anti-inflammatory cytokines play a vital role in the pathogenesis of PCAD and have potential to identify the degree of atherosclerosis.

Introduction

Premature coronary artery disease (PCAD) generally occurs in people aged <45 years (Egred and others 2005). Myocardial infarction (MI) under the age of 45 years accounts for about 10% in the United States (Egred and others 2005). Asian population is more prone to develop PCAD (Anand and others 2000), with significant increase being observed in the incidence of cardiovascular disease in the young Pakistani population (Jafary and others 2007).

Multiple proinflammatory and anti-inflammatory cytokines are involved in the pathogenesis of CAD causing overlapping antagonistic and synergistic effects. The inflammatory cascade has counterbalancing factors that maintain a delicate balance of pro- and anti-inflammatory molecules that regulate vascular homeostasis and maintain integrity of the vessel wall (Mills and others 2004). Interleukin-18 (IL-18) and tumor necrosis factor-alpha (TNF-alpha) are proinflammatory cytokines, whereas IL-10 is an anti-inflammatory cytokine. Samnegard and others (2009) demonstrated that the systemic concentrations of IL-18 and TNF-alpha were higher in postmyocardial patients when compared with controls. IL-10 is an anti-inflammatory cytokine that is associated with a humoral immune response that acts by limiting the local inflammatory response and providing stability to the atherosclerotic lesion (Fernandez and others 2002).

Our focus in this study is mainly on the imbalance between the proinflammatory cytokines (IL-18 and TNF-alpha) and the anti-inflammatory cytokine (IL-10) in the pathogenesis of PCAD. Chalikias and others (2005) demonstrated that an imbalance between the pro- and anti-inflammatory forces leads to plaque disruption and recurrent cardiovascular accidents, making IL-18/IL-10 an independent predictor of adverse events in hospitalized coronary syndrome patients. Whether imbalance of proinflammatory (IL-18 and TNF-alpha) and anti-inflammatory cytokines (IL-10) exists in PCAD patients is yet to be established.

Moreover, atherosclerotic plaques contain cells producing interferon-gamma (IFN-γ), IL-18, and TNF-alpha (Buono and others 2003; Branen and others 2004), emphasizing the role of T-helper cells type 1 (Th1) in promoting atherogenesis. IL-18 levels correlated significantly with atherosclerotic plaque area at the carotid and common iliac bifurcations (Yearly and others 2009). T-regulator and T-helper cells type 2 (Th2), on the other hand, are considered to have antiatherogenic effects, with the main anti-inflammatory cytokines being IL-10 and IL-4, respectively (Pinderski and others 2002). Whether

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altered levels of proinflammatory cytokines (IL-18 and TNF-alpha) and anti-inflammatory cytokine (IL-10) are associated with the extent of atherosclerotic stenosis in PCAD patients is yet to be established.

Subjects and Methods

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in the approval by the Institutional Review Committee of Army Medical College, National University of Science and Technology (NUST), Islamabad, Pakistan. The case–control study was conducted at the Clinical Pathology Laboratory (CPL), Army Medical College, Rawalpindi, in collaboration with the National Institute of Heart Diseases (NIHD), Rawalpindi, Pakistan, after obtaining approval. The study duration was 10 months (October 2009 to October 2010).

Subjects

Informed consent was obtained from each patient. A total of 385 stable coronary artery disease (CAD) patients aged <45 years scheduled to undergo coronary angiography were screened over a period of 6 months. Ninety-eight patients who had >70% stenosis in at least 1 coronary vessel on angiography with no history of radiotherapy or chemotherapy were consecutively recruited as PCAD patients. The patients with infectious or autoimmune diseases, familial hyperlipidemia, congenital heart disease, valvular heart disease, rheumatoid arthritis, or life expectance of less than 12 months and those unable to give informed consent were excluded.

Seventy-four subjects who were age and sex matched and angiographically proven to be disease free were recruited as controls after obtaining informed consent. Among the controls, those with acute or chronic illness or those on anti-inflammatory drugs were excluded. Pregnant women and women on contraceptives were also excluded from the study. Demographic characteristics were noted. Medical examination was conducted by a general physician. The patients with a history of hematological, neoplastic, renal, liver, or thyroid disease were excluded.

Coronary angiography was performed by trained cardiologists by Jutkin technique using a quantitative coronary angiographic system. The degree of atherosclerosis was calculated using the Gensini score (Gensini 1983). All coronary angiograms were evaluated by the cardiologist who was unaware of the biochemical analysis results to avoid bias. The patients were divided into 2 subgroups based on the degree of stenosis: 71%–85% stenosis ($n = 44$) and 86%–100% stenosis ($n = 57$) in at least 1 major coronary vessel.

Biochemical analysis

All laboratory investigations were carried out at the Chemical Pathology Laboratory of Army Medical College, NUST, Rawalpindi, Pakistan. Blood samples were taken in the morning on the day of angiography of the respective patients. Five milliliters of blood sample was obtained by venipuncture and transferred to a plain vacutainer tube for serum analysis. Serum was separated by centrifugation at 1500 g for 15 min and stored at -70°C until biochemical analysis.

Sandwich enzyme immunoassay with enzyme linked immunosorbent assay (ELISA) was performed to measure the concentrations of serum IL-10, IL-18, and TNF-alpha using human IL-10, IL-18, and TNF-alpha (Bendermed Systems) commercial kits with monoclonal antibodies. The calculated overall intraassay coefficient of variation (CV) for IL-18, TNF-alpha, and IL-10 was 6.5%, 6.0%, and 3.2%, respectively, whereas the limit of detection was 9, 2.3, and 1.0 pg/mL, respectively.

Serum hS-CRP was analyzed by 2-site sequential chemiluminescent immunometric assay kit (Seimen, LA) on Immulite 1000 (Immulite; Diagnostic Product Corporation). The analytical sensitivity of the method was 0.1 mg/L.

Serum cholesterol was measured by cholesterol oxidase method (CHOD-POD) and serum triglyceride was measured by colorimetric method. These analytes were run on Selectra E (Vital Scientific). The CV of the method was <1%.

Statistical analysis

Data were analyzed using standard SPSS software version-16 (SPSS, Inc.). Kolmogorov–Smirnov test was applied on data, which revealed non-Gaussian distribution for all the variables ($P < 0.05$). Mean, SD, median, and interquartile range (IQR) were calculated for descriptive statistics. Serum cytokine levels among the PCAD patients and controls were compared using Mann–Whitney U test. Intergroup comparison among the subgroups of PCAD patients was done using Mann–Whitney U test. Spearman's rank correlation coefficients were calculated to assess correlation between IL levels, degree of angiographic atherosclerotic blockade, and the established cardiac risk factors. The cardiac risk factors were entered into multivariate logistic regression analysis, taking significant premature atherosclerosis as the dependent variable. Odds ratios (ORs) were calculated using logistic regression. A P value of <0.05 was considered significant.

Results

A total of 172 subjects participated in this study, comprising 98 PCAD patients and 74 controls. The mean age of the patients was 40 ± 4.23 years. The baseline characteristic data of the study groups are shown in Table 1. Significant correlation was seen between IL-18, body mass index, triglycerides, total cholesterol, low-density lipoprotein, high-density lipoprotein, and cigarettes smoked per day ($P < 0.05$), whereas TNF-alpha showed significant correlation with body mass index and cigarettes smoked per day ($P < 0.05$). IL-18/IL-10 ratio showed significant correlation with cigarettes smoked per day and high-density lipoprotein ($P < 0.05$).

As shown in Table 2, the PCAD patients had significantly higher median (IQR) levels of serum IL-18 of 290 pg/mL (237–392 pg/mL) and TNF-alpha of 6.0 pg/mL (3.0–7.6 pg/mL) when compared with controls ($P < 0.05$). IL-18/IL-10 ratio and serum IL-10 level were also significantly raised in patients when compared with controls ($P < 0.05$) (Table 2).

The patients having more than 85% stenosis had significantly ($P < 0.01$) higher serum IL-18 and hS-CRP when compared with those with 71%–85% stenosis (Fig. 1), but TNF-alpha, IL-10, and IL-18/IL-10 ratio were not significantly different between the 2 subgroups (Table 3).

Serum IL-18 and hS-CRP showed a strong significant ($P < 0.01$) positive correlation of 0.658 ($P < 0.01$) and 0.733 ($P < 0.01$), respectively, with the degree of stenosis (Table 4).

Table 1. Demographic Characteristics of Patients and Controls

Parameters	*PCAD patients (n = 98) Mean ± SD*	*Controls (n = 74) Mean ± SD*
Age (years)	40 ± 4.23	35 ± 7.55
Gender (M/F)	89/9	65/9
Weight (kg)	74.1 ± 11.7**	67.7 ± 10.1
Height (m)	1.68 ± 0.06	1.69 ± 0.08
BMI (kg/m^2)	26.32 ± 3.7**	23.6 ± 3.5
Marital status		
Married, *n* (%)	97 (99)	66 (89)
Unmarried, *n* (%)	01 (1)	8 (11)
Ethnicity		
Punjabi, *n* (%)	80 (88)	57 (77)
Pathan, *n* (%)	18 (12)	17 (23)
Smoking		
Smokers, *n* (%)	63 (65)**	20 (27)
Nonsmokers, *n* (%)	35 (35)	44 (73)
Cigarettes/day	10.9 ± 9.23**	2 ± 1.76
Positive diabetes, *n* (%)	36 (37)**	3 (4)
Positive PCAD family history, *n* (%)	43 (44)**	2 (3)
Positive DM family history, *n* (%)	36 (37)**	11 (15)

PCAD, premature coronary artery disease; BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; DM, diabetes mellitus; SD, standard deviation.
**$P < 0.01$.

Similarly, TNF-alpha and imbalance between pro- and anti-inflammatory cytokines as indicated by IL-18/IL-10 ratio showed moderately significant correlation with the degree of stenosis.

On performing multivariate logistic regression analysis with premature atherosclerosis as the dependent variable, IL-18, TNF-alpha, and CRP remained highly significant ($P < 0.01$) (Table 5). As far as the classical cardiac risk factors were concerned, only smoking remained a significant predictor of premature atherosclerosis in the study group when included in the multivariate regression analyses model.

Discussion

The role of cytokines in atherosclerotic plaque development has gained considerable attention (Schuett and others 2009). Cytokines are important modulators of inflammatory events during all stages of atherogenesis.

We demonstrated that serum IL-18 and TNF-alpha levels are significantly high in the cases of PCAD when compared with disease-free controls. This is in agreement with a study conducted by Samnegard and others (2009), who demonstrated that the systemic concentrations of IL-18 and TNF-alpha were higher in postmyocardial patients when compared with controls. The proposed mechanisms suggest that IL-18 induces the expression of proinflammatory cytokines and chemokines such as IL-6 and IL-8 (Sahar and others 2005). It also mediates the thymus helper cell-type 1 (TH-1) immune response and has been shown to have a role in plaque destabilization (Mallat and others 2001). Similarly, TNF-alpha is also a proinflammatory cytokine that is upregulated in ischemia, inducing the activation of xanthine oxidase and production of O_2^- and leading to coronary endothelial dysfunction (Zhang and others 2006). According to a study, individuals with elevated levels of TNF-alpha were at increased risk for acute myocardial infarction and CAD (Biswas and others 2010).

High-sensitivity C-reactive protein (hs-CRP) is an acute-phase reactant produced predominantly by hepatocytes under the influence of IL-6 and TNF-alpha (Schultz and Arnold,1990). Serum hS-CRP is 2-fold higher in men with documented CAD when compared with controls (Anderson and others 1998). CRP induces adhesion molecule expression and the production of IL-6 and MCP-1 in human endothelial cells, enhancing a local inflammatory response within the atherosclerotic plaque (Pasceri and others 2001). The patients with CAD had higher hS-CRP levels than those without CAD and correlated with the severity of coronary atherosclerosis (Momiyama and others 2010). However, certain studies state that in patients hospitalized with chest pain, there is no association of serum levels of hs-CRP or TNF-alpha with coronary atherosclerotic burden or major cardiac events at 6 months after adjustment for traditional CAD risk factors (Sukhija and others 2007).

As far as IL-10 is concerned, our study shows that it is also increased significantly in the cases when compared with controls. Studies have shown that IL-10 is an anti-inflammatory cytokine that is associated with a humoral immune response that acts by limiting the local inflammatory response and providing stability to the atherosclerotic lesion (Fernandez and others 2002). Kirbis and others (2010) also demonstrated that IL-10 levels were significantly raised in patients with acute coronary syndrome (ACS) probably to counter the effects of proinflammatory markers.

Our focus in this study was also on the imbalance between the proinflammatory cytokines (IL-18 and TNF-alpha) and the anti-inflammatory cytokine (IL-10), as demonstrated by

Table 2. Comparison of Cytokine Levels in Premature Coronary Artery Disease Patients and Controls

Parameters	*PCAD patients (n = 98) Median (IQR)*	*Controls (n = 74) Median (IQR)*	P *value*
IL-18 (pg/mL)	290 (237–392)**	150 (80–200)	0.0001
IL-10 (pg/mL)	1.9 (1.0–3.3)*	1.4 (0.6–3.1)	0.048
TNF-alpha (pg/mL)	6.0 (3.0–7.6)**	4.4 (3.0–6.0)	0.0001
hS-CRP (mg/dL)	6.62 (4.2–16.0)**	0.80 (0.49–2.01)	0.0001
IL-18/IL-10 ratio	176.0 (100–307)**	105 (44.0–208.3)	0.004
TNF-alpha/IL-10 ratio	3.1 (1.86–6.48)	2.5 (1.48–5.4)	0.410

IL, interleukin; TNF-alpha, tumor necrosis factor-alpha; hS-CRP, high-sensitivity C-reactive protein; IQR, interquartile range.
*$P < 0.05$ and **$P < 0.01$ applying Mann–Whitney *U* test.

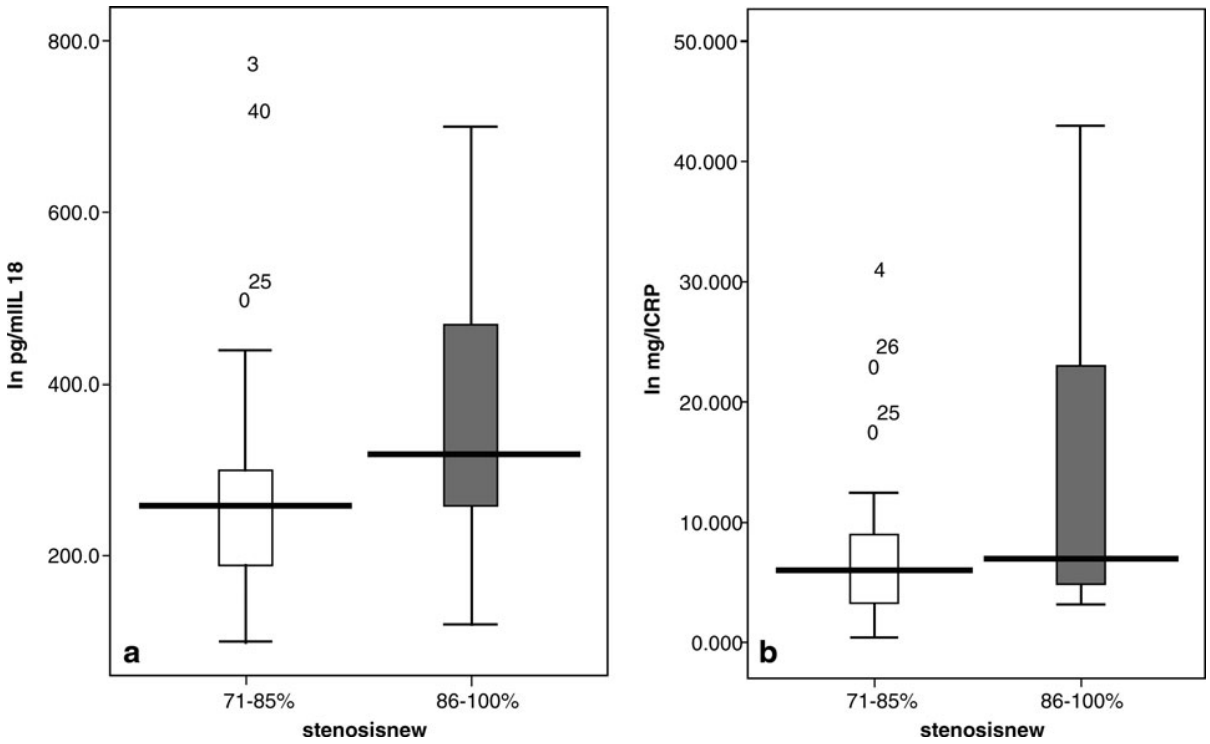

FIG. 1. Box plots showing the increase in serum level of **(a)** interleukin-18 and **(b)** hS-CRP with increasing degree of stenosis.

the IL-18/IL-10 and TNF-alpha/IL-10 ratios. IL-18/IL-10 ratio in PCAD patients was considerably increased in the cases when compared with controls in our study, showing the imbalance between pro/anti-inflammatory cytokines in these patients. Chalikias and others (2005) demonstrated that an imbalance between the pro-and anti-inflammatory forces leads to plaque disruption and recurrent cardiovascular accidents, making IL-18/IL-10 an independent predictor of adverse events in hospitalized coronary syndrome patients. As far as stable coronary artery disease is concerned, Camargo and others (2007) showed that IL-18/IL-10 ratio independently predicts cardiovascular events in patients with stable CAD. A probable reason for this may be that increased IL-18/IL-10 ratio may result in increased macrophage and T-regulator cell expression in the initial plaque (Puranik and others 2009), leading to further release of inflammatory cytokines and accelerated atherogenesis and hence to PCAD.

TNF-alpha/IL-10 ratio in our study population was not significantly raised but showed a trend toward increase. This is not in agreement with certain studies, for instance, TNF-alpha/IL-10 ratio was increased considerably in CAD patients of northern India when compared with healthy controls (Goswami and others 2009). The patients with CAD and preserved systolic performance of the left ventricle demonstrated a higher value of TNF-alpha/IL-10 ratio when compared with controls (Kosmala and others 2008). The reason why a significant change may not have been seen in our study is the possibility that the study population consisted of stable coronary artery disease patients in whom the acute cardiovascular event had occurred over 2–3 months before the day of their angiography. As the protective response comes into play after the acute event, there is a significant rise in IL-10, which counters the high levels of TNF-alpha, leading to a decrease in the TNF-alpha/IL-10 ratio.

Table 3. Comparison of Cytokine Levels Among Subgroups of Premature Coronary Artery Disease Based on the Degree of Angiographic Stenosis

Parameters	*71%–85% stenosis (n = 44) Median (IQR)*	*86%–100% stenosis (n = 57) Median (IQR)*
IL-18 (pg/mL)	260 (182–300)	320 (258–483)**
IL-10 (pg/mL)	1.7 (1.0–2.2)	2.0 (1.0–3.4)
TNF-alpha (pg/mL)	6.0 (4.9–7.9)	6.4 (5.0–7.5)
hS-CRP (mg/dL)	5.0 (3.1–9.0)	7.0 (5.0–24.0)**
IL-18/IL-10 ratio	175 (100–280)	179 (111–395)

**$P < 0.01$ applying Mann–Whitney *U* test.

Table 4. Spearman's Correlation Between Cytokine Levels and Degree of Coronary Stenosis in Premature Coronary Artery Disease Patients

Parameters	*Degree of stenosis,* r	P *value*
IL-18 (pg/mL)	0.658**	0.0001
IL-10 (pg/mL)	0.121	0.11
TNF-alpha (pg/mL)	0.351**	0.0001
hS-CRP (mg/dL)	0.733**	0.0001
IL-18/IL-10 ratio	0.302**	0.0001

r, Spearman's correlation rank coefficient.
**$P < 0.01$.

Table 5. Multivariate Analyses Showing Odds Ratio for the Prediction of Premature Atherosclerosis

	B	*SE*	*Exp(B) (95% CI)*	*Sig.*
PCAD family history	1.987	1.044	0.137 (0.018–1.06)	0.057
DM	1.588	1.074	0.204 (0.025–1.678)	0.139
Smoking	1.867	0.648	6.470 (1.819–23.0)**	0.004
BMI (kg/m^2)	0.092	0.079	1.097 (0.940–1.280)	0.242
IL-18 (pg/mL)	0.010	0.004	1.010 (1.003–1.018)**	0.006
IL-10 (pg/mL)	0.316	0.287	0.729 (0.415–1.281)	0.272
TNF-alpha (pg/mL)	0.606	0.182	1.832 (1.282–2.618)**	0.001
CRP (mg/dL)	0.495	0.135	1.641 (1.261–2.137)**	0.000
IL-18/IL-10 ratio	0.003	0.003	0.997 (0.992–1.002)	0.270

DM, diabetes mellitus; SE, standard error; Exp(*B*), odds ratio; CI, confidence interval; Sig., significance.
**$P < 0.01$.

Our present study also shows that serum IL-18 and hS-CRP correlated strongly with the degree of atherosclerotic blockade and were highest in the subgroup of PCAD patients with the maximum degree (86%–100%) of stenosis. This is in agreement with another study in which IL-18 levels correlated significantly with atherosclerotic plaque area at the carotid and common iliac bifurcations (Yearly and others 2009). Similarly, certain other studies have shown positive correlation between carotid intima-media thickness and coronary plaque area (Hulthe and others 2006; Chong and others 2009). This is probably because IL-18 overexpression induces atheroma formation, whereas the endogenous inhibitor of IL-18 slows plaque development (de Nooijer and others 2004).

Similarly, the total plaque area was considerably greater in women with high hS-CRP levels when compared with those with low hS-CRP levels (Prahl and others 2010). In ACS patients, the hS-CRP level correlated well with the coronary artery stenosis (Arroyo-Espliguero and others 2004). CRP is being quoted as the marker of disease activity as well as disease severity in cases of CAD (Arroya-Espliguero and others 2004). This is because in the early stages of atherosclerosis it correlates extremely well with the rate of progression of atherosclerosis (Wiedermann and others 1999). However, some studies deny the relationship of CRP with the extent of coronary atherosclerosis (Veselka and others 2002).

TNF-alpha also correlated moderately with the degree of stenosis in our PCAD patients. According to Branen and others (2004), TNF-alpha is actively involved in the progression of atherosclerosis and its inhibition reduced atherosclerosis in mice. Although still restricted to animal studies, one of the possible reasons for the association of TNF-alpha levels with the degree of stenosis may be the fact that TNF-alpha–converting enzyme is elevated in the atherosclerotic plaques, leading to disease progression and increase in the severity of atherosclerotic burden (Canault and others 2006). Thus, the proinflammatory cytokines initiate the endothelial injury and attract more inflammatory cells at the affected site, which get incorporated into the initial plaque formed. These cells then release yet more cytokines and eventually start a vicious circle leading to increased coronary artery stenosis.

Serum IL-18/IL-10 ratio was also raised significantly in the PCAD patients but correlated only moderately with the degree of coronary stenosis. This may indicate that the imbalance between the pro- and anti-inflammatory cytokines is a better marker of disease pathogenesis than the disease burden.

After adjustment for major cardiac risk factors, multivariate analyses showed that IL-18 ($P < 0.01$), TNF-alpha ($P < 0.01$), and CRP ($P < 0.01$) were significant independent predictors of severity of premature atherosclerosis, making them important indicators of disease burden. Gotsman and others (2008) reported that TNF-alpha and combined levels of TNF-alpha and IL-16 were significant independent predictors of coronary artery disease, whereas IL-6 was independently predictive of the Gensini severity score.

The major strengths of our study are that it is the first study to review the role and effect of imbalance between pro- and anti-inflammatory cytokines in the pathogenesis of PCAD in the Pakistani population. Moreover, we have adequately highlighted the role of immune system in the atherosclerotic process, paving way for new therapeutic modalities. Our study has adequately highlighted the association of cytokine levels with the atherosclerotic disease burden, keeping in view the degree of atherosclerotic blockade as ascertained by the current gold standard coronary angiography.

A limitation of our study is the relatively small sample size of the patients, which may cause discrepancy in the correlations due to other risk factors not taken into account in the statistical analysis. Moreover, our study mainly concentrated on stable PCAD patients and so we cannot generalize these findings on patients with ACS. Future studies can be conducted with a larger sample size and by recruiting PCAD patients with ACS along with stable CAD patients for better evaluation of the disease state and for comparative purposes. Moreover, we have not seen the possible synergism between disease burden and disease activity in our study and so it is hard to ascertain whether raised cytokines indicate causality of the disease or are just reflective of the disease burden.

Conclusion

Pro/anti-inflammatory cytokines play a distinct role in the pathogenesis of PCAD and correlate well with the severity of PCAD.

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Author Disclosure Statement

No competing financial interests exist.

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