

## Serum C-reactive protein and C-reactive gene (-717C>T) polymorphism are not associated with periodontitis in Indonesian male patients

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### ABSTRACT

**Background:** Periodontitis is an inflammatory disease caused by periodontal pathogens and influenced by multiple risk factors such as genetics, smoking habit, age and systemic diseases. The inflammatory cascade is characterized by the release of C-reactive protein (CRP). Periodontitis has been reported to have plausible links to increased level of CRP, which in turn has been associated to elevated risk of cardiovascular disease (CVD). **Purpose:** The purpose of this study was to investigate the relationship amongst the severity of periodontitis, CRP level in blood and CRP (-717C>T) gene polymorphism in male Indonesian smokers and non-smokers.

**Method:** The severity of periodontitis was assessed for 97 consenting male Indonesian smokers and non-smokers. The CRP level of the subjects was determined by using immuno-turbidimetric assay performed in PARAHITA Diagnostic Center Laboratory ISO 9001: 2000 Cert No. 15225/2. The rate of CRP (-717C>T) gene polymorphism was determined by using PCR-RFLP in Oral Biology Laboratory, Faculty of Dentistry, Universitas Indonesia. **Result:** The results suggest that the CRP protein level is not significantly associated with the tested CRP gene polymorphism ( $p>0.05$ ). Also, while the severity of periodontitis increased significantly with subject age, the CRP level in blood serum was not significantly related to the severity of periodontitis. The genotypes of the tested polymorphism did not show significant association with the severity of periodontitis either in smokers or in the combined population including smokers and non-smokers. The results naturally do not exclude such associations, but suggest that to discern the differences the sample size must be considerably increased. **Conclusion:** The CRP (-717C>T) gene polymorphism and CRP level in blood serum were not found to be associated with the severity of periodontitis in male smokers or in the combined population of smokers and non-smokers.

**Keywords:** periodontitis; smoking; CRP; polymorphism; CVD

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### INTRODUCTION

In a worldwide population, the National Health and Nutrition Examination Survey III (NHANES 1988-1994) found the prevalence of gingivitis in 50% adult and 40-60% in schoolchild.<sup>1</sup> World Health Organization (WHO) stated that severity level of periodontal disease that being marked by the depth of periodontal pocket ( $\geq 6.0$  mm) was found in 10-15% adult people all over the world<sup>2</sup> and 13.1% in South East Asia.<sup>3</sup>

Particularly in Indonesia, periodontal disease took as the first place (61%) in top ten most complained diseases

according to The Indonesian Health Profile (Profil Kesehatan Indonesia) in 2001.<sup>4</sup> Unfortunately only 29.6% of them had received dental treatment (according to National Basic Health Research/ Riset Dasar Kesehatan Nasional-Riskesdas) in 2007.<sup>5</sup> This epidemiologic data reveals that the prevalence of periodontal disease still high and the ability of patient to be treated is low.

Previous study has proved that individual with periodontal disease has an increasing of C-reactive protein (CRP) level in its blood.<sup>6,7</sup> This protein is an acute protein phase that being controlled as the sign of inflammatory status, and had been identified as main risk factor of

atherosclerosis complication.<sup>7</sup> Another study found relationship between loss of teeth or periodontitis with the risk of atherosclerosis. This sign was immune system fast response toward persistent inflammation.<sup>8</sup> There was also significant increase in adjusted mean levels of CRP in subject with high attachment loss when compared to subjects with healthy periodontium.<sup>9</sup> It could be proposed that patients with periodontitis may have elevated circulating levels of these inflammatory markers like c-reactive protein and hence increase the risk for atherosclerosis.<sup>10</sup>

Periodontitis and atherosclerosis have mutual complex etiologic factors and shared mutual potential bacterial mechanism.<sup>11</sup> The pathway is that periodontal pathogen bacteria and its products will leads to endothelial vascular destruction. Its destruction will indirectly activate the production of platelet. The aggregation of platelets will cause thrombus-embolic formation.<sup>12,13</sup>

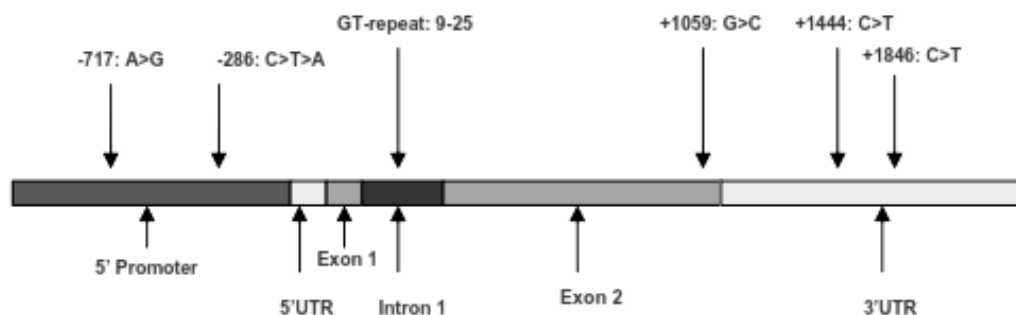
Various studies concerning assessment of etiologic factor of periodontal disease, implied that periodontal disease was multifactorial disease with interacted manifestation between three elements which are bacterial, host and environmental factor. Host immune system is influenced by genetic and epigenetic factor. This study was focused to analyze one of the genetic factors of the periodontal disease's severity which is genetic polymorphism in each individual based on structure difference, diversity of gene expression and function.<sup>14</sup> This study is aimed to investigate the relationship among the severity of periodontitis, CRP level in blood serum and CRP (-717 C>T) gene polymorphism in Indonesian male smokers and non-smokers.

## MATERIALS AND METHODS

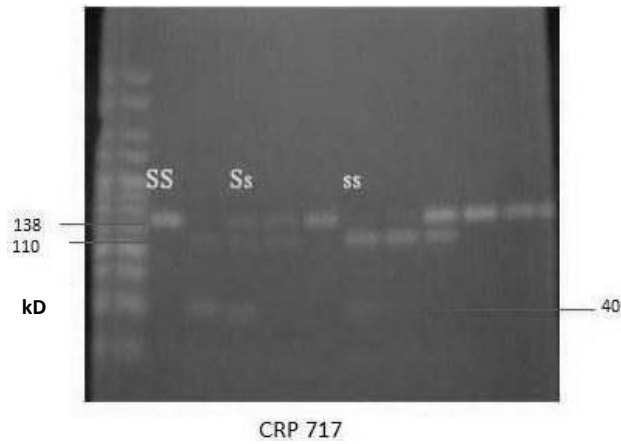
This study is analytic study with laboratory approach. Ninety-seventh male subjects were randomly selected from all patients in Department of Periodontology, Dental Hospital Faculty of Dentistry Universitas Indonesia. Intra oral examination and periodontal health status, attachment loss (AL), probing depth were assessed for all subjects using a standardized procedure at six locations on each tooth. The

sample is stored samples in -20°C (DNA and blood samples) archived in Oral Biology Laboratory, Faculty of Dentistry and Biology Laboratory Faculty of Medicine, Universitas Indonesia. The ethical clearance was approved by the Ethical Committee of the Faculty of Dentistry, Universitas Indonesia and all patients signed written informed consent. Ninety-seventh samples (25-60 years old) were analyzed for its severity of periodontal disease, smoking status (smoker or non-smoker), and CRP level. Systemic disease is excluded for this study. The samples known divided into 59 smokers and 38 non-smokers based on its cigarette consumption habit characteristic. Severity of periodontal disease were divided into mild (CAL<2.0 mm), moderate (CAL 2.0 – 4.0 mm), and severe (>4.0 mm). Levels of CRP were measured from peripheral blood samples by using Immuno-turbidimetric technique assay performed in PARAHITA Diagnostic Center Laboratory ISO 9001: 2000 Cert No. 15225/2. The rate of CRP (-717C>T) gene polymorphism was determined by using PCR-RFLP in Oral Biology Laboratory, Faculty of Dentistry, Universitas Indonesia. Patient who under medication such as antibiotics, corticosteroids, anti-inflammatory drugs and who had a history of periodontal treatment in the past 6 months were excluded from this study.

To survey the genotype-phenotype variations related to the gene locus polymorphisms of CRP and CRP (-717C>T) gene polymorphism, the polymorphism status of these genes was determined from samples of peripheral blood. DNA fragments amplifications were done using Polymerase Chain Reaction (PCR) method. 12.5 ml top tag master mix 2x (1.25 U top tag DNA polymerase, 1x PR buffer 1.5 mM MgCl<sub>2</sub>, 200 mM dNTP), 2.5 µL coral load, and 4 mL ddH<sub>2</sub>O were used for every 25 ml reactan amplification. Else 5 µL primer forward, 1.5 µL primer reverse, and 5 µL DNA template were used. Solution was mixed with vortex before went into PCR machine. Primer forward 5'-ACTGGACTTTTACTGTCAGGGC-3' and primer reverse 5'-ATCCCATCTATGAGTGAGAACC-3' were used for CRP-717 C>T DNA samples were amplified fp tu 35 cycles with early denaturation in 94° for 5 minutes, then went into cycle consists of annealing and elongation. Time elongation extension 72°C for 7 minutes



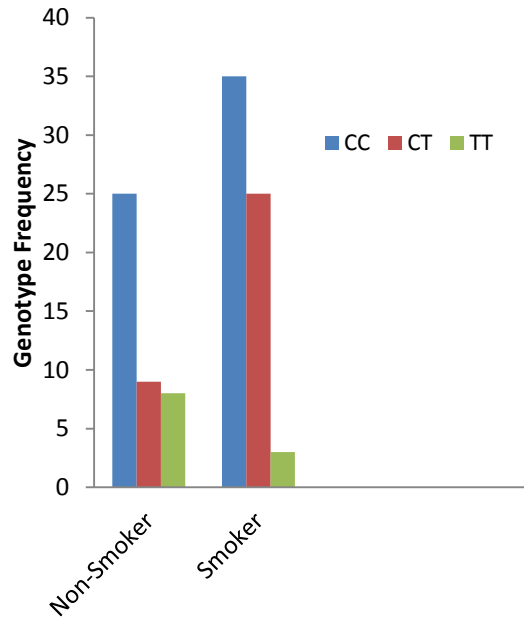
**Figure 1.** CRP polymorphism. CRP polymorphism could take place in promoter (left), UTR (right), exon (right-center), and intron (left-center).<sup>15</sup>



**Figure 2.** Enzyme SacII digest electrophoresis based on molecules weight.  
Note: SS = CT, Ss = CT, ss = TT.

was added at the end of cycle. After DNA amplification process, amplicons were stored at 4°C. Amplification outcomes (PCR) were detached with electrophoresis in agarose gel 2% (Promega) contains 0.1 µL etidium bromide (0.5 mg/mL) inside dapar solution TAE 1X (0.04 M tris-acetate, 0.002 M EDTA ph 8.0). 5 µL DNA PCR yield were mixed with 2 µL tracking dye (0.25% bromopgenol blue, 0.25% xylene xyanole, 25% sucrose), afterwards went into electrophoresis well. The DNA fragment bands were separated with electrophoresis in 80 voltages for 60 minutes. 100 pb DNA ladder were used as marker. The DNA fragment bands outcome of electrophoresis were observed with UV illuminator and photographed using digital camera. There are CRP (-717C>T) polymorphism at various location, it could take place in promoter region, first exon, intron, second exon or in UTR (un-translated regions).<sup>20</sup>

Restriction fragment length polymorphism (RFLP) technique was used to detect the presence of CRP-717 C>T polymorphism. The cutting of PCR yields were done with 20 µL 65°C Sac II enzyme for 4 hours.



**Figure 3.** Genotype frequency distribution in smoker and non-smoker group.

Afterwards, it transferred to 85°C of temperature to inactivation. The outcomes of the cutting were examined using electrophoresis with agarose 3% and buffer TAE. Visualization of the outcome was done using electrophoresis with agarose gel 3% stained by 0.5 mL 0.1% etidium bromide, and poured into dapar TAE 1x solution. Electrophoresis was set in 80 voltages, 400 mA for 60 minutes. For DNA visualization, gel was placed in Gel Doc.

Chi-square testing with SPSS 18.0 was mainly used in the statistical analysis, both for comparing results in the test groups and for assessing the allele and genotype frequencies with predictions with respect to the Hardy-Weinberg equilibrium. Statistical significance was assumed with  $p < 0.05$ .

**Table 1.** Genotype CRP-717 C>T (N, %) distribution in smoker and non-smoker

Genotype	CC	CT	TT	Total
Smoker	33 (55.93%)	24 (40.68%)	2 (3.39%)	59 (100%)
Non-smoker	24 (63.15%)	8 (21.05%)	6 (15.80%)	38 (100%)
Total	57 (58.76%)	32 (32.99%)	8 (8.25%)	97 (100%)

**Table 2.** Relationship between The Severity of periodontal disease (PP) with CRP Level (mg/L)

PP		Mild(11)	Moderate(47)	Severe(39)	P value
CRP (mg/L)	Median (r)	0.09 (0.01-1.35)	0.10 (0.01-0.94)	0.11 (0.02-2.08)	0.740*
	Mean±SD	0.21±0.39	0.20±0.23	0.22±0.36	

\* Kruskal-Wallis test, post-hoc analysis was using Mann-Whitney to compare mild, moderate, and severity group  $p < 0.05$

**Table 3.** Relationship between severity of periodontal disease (PP) with Age and CRP with genotype in all subject population either smoker or non-smoker; r = data interval

PP		Mild(11)	Moderate(47)	Severe(39)	P value
Age (years)	Median (r)	34 (25-51)	38 (25-56)	45 (25-60)	0.002*
	Mean±SD	35.0±9.6	37.3±9.4	44.2±9.6	
Genotype	CC	10(17.5%)	25(43.9%)	22(38.6%)	0.173**
	CT	0(0.0%)	18(56.3%)	14(43.8%)	
	TT	1(12.5%)	4(50.0%)	3(37.5%)	
Smoking	No	8(21.1%)	20(52.6%)	10(26.3%)	0.005**
	Yes	3(5.15)	27(45.8%)	29(49.2%)	

\* Kruskal-Wallis test, post-hoc analysis was using Mann-Whitney to compare mild, moderate, and severity group

\*\* Chi-square test

p<0.05

**Table 4.** Relationship between genotype with CRP Level (mg/L)

Genotype		CC (57)	CT (32)	TT (8)	P value
CRP (mg/L)	Median (r)	0.09 (0.01-2.08)	0.13 (0.01-0.78)	0.20 (0.03-0.94)	0.181*
	Mean± SD	0.20 ± 0.35	0.20 ± 0.20	0.30 ± 0.31	

\* Kruskal-Wallis test, post-hoc analysis was using Mann-Whitney to compare mild, moderate, and severity group

p<0.05

**Table 5.** Relationship between genotype with Age and CRP and severity of periodontal disease (PP) in all subject population either smoker or non-smoker; r = data interval.

Genotype		CC (57)	CT (32)	TT (8)	P value
Age (years)	Median (r)	38.0 (25-60)	40.5 (25-56)	49.5 (26-55)	0.038*
	Mean± SD	38.4 ± 9.8	40.5 ± 10.2	47.3 ± 9.0	
PP	Mild	10 (90.91%)	0 (0.0%)	1 (9.09%)	0.173**
	Moderate	25 (53.19%)	18 (38.30%)	4 (8.51%)	
	Severe	22 (56.41%)	14 (35.90%)	3 (7.69%)	
Smoking	No	24 (63.15%)	8 (21.05%)	6 (15.80%)	0.027**
	Yes	33 (55.93%)	24 (40.68%)	2 (3.39%)	

\* Kruskal-Wallis test, post-hoc analysis was using Mann-Whitney to compare mild, moderate, and severity group

\*\* Chi-square test

p<0.05

## RESULTS

There were three genotypes from genotype and alypotype distribution enzyme SacII cutting result, that is CC at 138bp band, CT at 138bp, 112bp, 40 bp, and TT at 112bp and 40bp (Figure 2).

All of examined genotype subjects were divided based on wild type or dominant homozygote (CC), mutant heterozygote (CT), and recessive homozygote (TT). Frequency of smoker and non-smoker with those three genotypes were showed in Table 1. There is a significant difference on severe group, so that it could be concluded that there is a differences between the mild-moderate and severe group (Table 5). We could assumed that non-smoker

group (left) and smoker (right) group have much the same of genotype, most of it was CC, the CT and TT was the least (Figure 3).

## DISCUSSION

From all subject population consists of smoker and non-smoker group like being showed in Table 2-3, could be grouped based on the degree of periodontal disease severity, mild, moderate and severe. There were 3 people (5%) with mild periodontal disease, while 27 people (46%) with moderate periodontal disease, and 29 people (49%) with severe periodontal disease in smoker group. While

**Table 6.** The significant differences among the severity of periodontitis

Severity	P value
Mild-Moderate	0.409
Mild-Severe	0.011*
Moderate-Severe	0.002*

\* p<0.05 Mann-Whitney test

in non-smoker group, there were 8 people (21%) with mild periodontal disease, 20 people (53%) with moderate periodontal disease and 10 people (26%) with severe periodontal disease. From various studies reported that smoking could affect systemic or local disease especially periodontal disease. Individual with poor oral hygiene would increase the periodontal disease susceptibility, and decline the immune response.<sup>15</sup> Smoking habit as a part of environment factor along with genetic factor that could affect the patient susceptibility toward periodontal disease. In conclusion, from statistical analytic as shown in Table 3, there is a significant difference between the two group, smoker and non-smoker with p=0.005.

In this study, the polymorphisms of CRP (genotype CC, CT, TT) and CRP (-717C>T) genes have been compared to the periodontal disease degree and serum CRP level of 97 Indonesian male subjects. These studies also compare the relationship between the degree of periodontal disease and the smoking status. All samples included relatively few mild periodontitis subjects in comparison with the number of those with moderate and severe groups; this portion seems relevant with the plausible theoretical revealed that oral infection in severe attachment loss of periodontal tissue could spread its toxin systemically through capillary blood vessel and expressed in peripheral blood. The results prove a clear relationship between the severity of periodontitis and smoking habit, so that severe periodontitis was significantly associated with higher lifetime smoking exposure (p=0.005). Moreover there also a confirmation result shown in Table 3, that the severity of periodontitis is related with increasing of age (p=0.002). The genotype of CRP (CT, CT, TT) found significantly correlated with age (p=0.038) and smoking status (p=0.027) as seen in Table 4. In contrast, the genotype of CRP is not related with periodontitis severity.

Periodontal bacteria such as *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia*, *Treponema denticola*, and *Eikenella corrodens*, was found in atherosclerosis plaque.<sup>15</sup> Indeed, *P. gingivalis* has the highest risk because of its ability to regulate adhesion molecules like ICAM-1, VCAM-1, selectins (P and E). These molecules are necessary to bind leucocytes in the endothelium layer at the early stage of atherosclerosis.<sup>16</sup> These bacteria will produce a leucotoxin that induces degranulation and lysis in human neutrophils, caspase-1 activation, and abundant secretion of cytokine from human macrophages, promoting a tissue destruction including loss

of alveolar bone.<sup>17</sup> Therefore, theoretical based revealed that the level of CRP by the induction of bacterial leucotoxin are supposed largely expressed in severe periodontitis group. Contrast with the result of our study that shown insignificant relation between the polymorphisms of CRP (genotype CC, CT, TT) and CRP (-717C>T) genes and periodontitis severity (Table 2-3 and Table 3-4).

Chronic periodontitis patients either with or without atherosclerosis symptoms had increase of CRP level.<sup>18</sup> Chronic periodontitis patients with atherosclerosis symptoms had twice CRP level than patients without atherosclerosis symptoms. Other study by De Freitas *et al.*<sup>19</sup> stated that non-surgical periodontal therapy could reduce CRP level significantly. Blum<sup>20</sup> did research to post-therapy periodontal disease patients and after three months, he found a decreasing of CRP level and a reducing the risk of cardiovascular disease.

However, the reported association between periodontitis, CVD, and individual polymorphisms of CRP (-717C>T) genes is tend to be correlated. If the links between individual genes and their polymorphisms are relatively weak, this means that the individual polymorphisms itself are not predominantly work as influential risk indicators to the disease. Economically the conventional indicators for periodontitis examination such as measurement of pocket depth and alveolar bone loss may remain more useful and effective. While for CVD indicator, the high blood pressure, smoking, obesity, and CRP level is suited. An appropriate treatment of periodontitis can reduce inflammation and circulating CRP levels, which directly reduced the risk of CVD.<sup>17</sup> Furthermore, the potential indicators like CRP (-717C>T) genes polymorphism status did not serve as a complementary factor to confirm the conventional indicators and other research should be made to tested it accurately.

It can be concluded that no relation between polymorphism promoter gene CRP-717C>T with the degree of periodontal disease severity in either smoker or non-smoker male, except the difference between moderate and severe group of the severity degree. There's no association between CRP level in serum with the severity of periodontal disease either in smoker or non-smoker male.

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