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Full Length Research Paper

Levels of inflammatory markers (complement C3, Complement C4 and C-reactive protein) in smokers

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The study aimed to discover the relative significance of selected inflammatory markers in smokers. Serum levels of complement C3, C4 and C-reactive protein were determined in 24 male smokers (18 to 73 years). A group of 24 healthy non-smokers was taken as control. C3 and C4 were measured by using radial immunodiffusion technique and CRP was determined by latex agglutination technique. The statistical analysis was done using Student's t-test and Fisher exact test which were used for the comparison of the characteristics of the groups. C3 and C4 were observed to be 33.84 and 45.28% higher in smokers, respectively, but statistically insignificant as (p = 0.122) and (p = 0.078). No significant difference was observed in the case of C-reactive protein levels and the body mass index of smokers and non-smokers (CRP: p = 0.49 and BMI: p = 0.64). These findings repudiated the presence of an acute phase and inflammatory reaction in smokers.

Key words: Smokers, C-reactive protein, body mass index, complement.

INTRODUCTION

Tobacco was first cultivated in North America. The word 'nicotine' is derived from French language after French Ambassador to Portugal Jean Nicot. Nicotine, tar, carbon monoxide, phenols, hydrogen cyanide, benzene, ammonia, formaldehyde and nitrosamine are some of the hazardous compounds in tobacco that are cancer causing and promote the progression of tumours (Adil et al., 2005). Many components of cigarette smoke have an ability to alter the function of immune cells (Sopori, 2002).

The respiratory epithelium of tobacco smokers was observed to contain multifocal premalignant lesions which express the tendency of carcinogens in tobacco smoke to cause mutagenesis (Walser et al., 2008). The occurrence of cancers and upper respiratory tract infections is increased in smokers which reveal their troubled immune system (Moszczynski et al., 2001).

The concentration of cytokines and acute phase proteins (APP) in plasma gets altered to a notable extent due to tissue injury, infection, burn, shock and several types of inflammatory conditions and cancer. Smoking shows a relationship with acute phase proteins like C-reactive protein (CRP) and figrinogen (Yanbaeva et al., 2007). Smokers show increase in levels of circulating leukocyte counts and inflammatory markers including CRP, intercellular adhesion molecule Type-1, interleukin (IL-6), E-selectin and P-selectin (Perlstein and Lee, 2006).

Studies have shown that the biomarker CRP plays a role in the pathogenesis of atherosclerosis (Labarrere and Zaloga, 2004). CRP levels are >10 mg/l and can arrive at >500 mg/l when there is inflammation or infection (Jialal et al., 2004). Factors which increase the risk of adverse cardiovascular events like smoking, hypertension and the use of female synthetic hormones increase the levels of CRP (Life Extension Magazine Report, 2003). Smoking causes an elevation in CRP levels which might be due to the tissue detrimental effect of tobacco smoke (Hoekstra et al., 2001). High levels of CRP aid in the identification of smokers with unusual airway abrasions that are likely to proceed to lung

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Abbreviations: APP, Acute phase proteins; **CVD,** cardiovascular disease; **IL-6,** interleukin-6; **C3,** complement C3; **C4,** complement C4; **CRP,** C-reactive protein; **IgG,** immunoglobulin G; **IgM,** immunoglobulin M; **IgA,** immunoglobulin A.

cancer (Sin et al., 2006).

Complement C3 and C4 are the plasma proteins of vital importance in the immune system complement pathways. The synthesis of these proteins is increased in cases of inflammation and infection but the rate is relatively slower when compared with conventional acute phase proteins (Engstrom et al., 2005). It is assumed that the inhalation of cigarette smoke causes complement activation which is in turn responsible for the production of leukocyte chemoattractants in the lung fluid (Kew et al., 1985). The examination of smoke exposed serum led to the discovery of cleaved properdin factor b and C3 and the presence of C5a. In this way, the reason for incursion of neutrophils and macrophages into the smoker's lungs was found to be complement activation (Robbins et al., 1991).

Long run activation of complement system might have devastating effects. It leads to an increase in the danger of cardiovascular disease (Tungtrongchitr et al., 2002). Preceding studies highlighted the involvement of smoking in alteration of the humoral immunity patterns, thus, causing inflammation and progression to multiple types of diseases. This study aimed to determine which of the markers of inflammation is most strongly associated with cigarette smoke inhalation and discover the alteration in humoral immunity system of smokers.

MATERIALS AND METHODS

Subjects

This study included 48 persons separated into 2 groups. Group 1 included 24 current smokers, mean age of smokers at the entry of study was 30.20 ± 7.81 years and the mean number of cigarettes smoked per day is 13.04 ± 2.36. All of them were males.10 smokers were light smokers (1 to 9 cig/day), 10 were moderate smokers (20 to 19 cig/day), 3 smoking individuals were heavy smokers (20 to 39 cig/day) and 1 smoker smoked more than 40 cig/day. Most of the smokers were basically healthy, while 6 smokers suffered from diseases of chest infection, hypertension, heart disease, diabetes and asthma. The conditions experienced by majority of the smokers include pain or tightness in the chest, trouble breathing or shortness of breath, tiredness, leg pain and coughing. Subjects with smoking history of ≥1 cigarette per day and smoking period of at least 6 months were included. Subjects suffering from viral and autoimmune diseases were excluded from the study. The age and sex matched non-smokers chosen in the study were healthy and showed no signs and symptoms of autoimmune diseases.

Body mass index determination

The subjects' height and weight were determined when they were barefooted and were wearing light clothes. Body mass index was calculated with the following formula:

Body mass index (BMI) = Weight (kg)/ Height (m^2)

Sample collection

Blood sampling was performed randomly. Informed consent was acquired from all persons. The study design was accepted by the board of Advance studies and Research and Ethical Committee, University of Punjab Lahore. Smoking history and general physical health was determined with the help of a questionnaire. Blood samples were obtained by performing the venipuncture technique. Blood samples obtained were allowed to clot at room temperature for half an hour, then the serum tubes were centrifuged at 2500 rpm for 15 min. Serum was removed, aliquoted and stored at -70°C.

C-reactive protein

C-reactive protein was measured with the latex agglutination method as described by the manufacturer (Egyptian Company for Biotechnology). The CRP latex reagent was mixed with the serum sample and presence or absence of agglutination was detected. In the semi-quantitative test, serial dilutions of serum were made in saline water. The semi-quantitative test gives the measure of CRP quantity in serum that is higher than normal.

Complement C3 and C4 determination

C3 and C4 were measured by using immunodiffusion plates as described by the manufacturer (Far via Fermi 12-Italy). Serum was pipetted into the wells of the kit and incubated from 48 to 72 h. The sample precipitation diameter was measured and the concentration protein value was read on the reference table. Normal value of C3 is between 50 and 120 mg/dl and for C4 it is between 20 and 50 mg/dl.

Statistical analysis

An assessment of the characteristics of the two groups were made using student's t-test and Fisher exact test. The computing analysis was fulfilled by statistical package for social sciences programme (SPSS).

RESULTS

This cross-sectional study was performed to discover the effects of tobacco usage on the immune system complement pathway. Smoking is injurious to health and has a negative impact on the general heath and immune system. The BMI of smokers was also determined in order to discover the relation between smoking and weight loss. BMI of smokers and controls was observed to be 23.01± 5.07 and 23.53 ± 0.70 kg/m^2. No statistical difference was found between the BMI of smokers and non-smokers ($p > 0.05$) (Table 1).

C3 and C4 were measured in smokers and controls by using radial immunodiffusion technique. C3 and C4 concentrations were normal in 5 (20.83%) smokers, normal C4 and low C4 concentrations were observed in 1 (4.16%) smoker, high C4 and normal C3 concentrations were noted in 5 (20.83%) smokers, high C4 and low C3 concentrations were present in 2 (8.33%) smokers, high C3 and low C4 concentration were seen in 3 (12.5%) smokers, high C3 and normal C4 concentrations were found in 3 (12.5%), smokers, high C3 and high C4 concentrations were present in 2 (8.33%) smokers and low C3 and low C4 concentrations were detected In 3

Table 1. Baseline characteristics of study population.

Paremeter	Smoker (n=24)	Non-smoker (n=24)
Age(yrs)	30.20 ± 7.81	--
Cigarettes used	13.04 ± 2.36	--
Height(m)	1.74 ± 0.09	1.71 ± 0.05
Weight(kg)	52.67 ±18.35	63.28 ± 12.24
Waist circumference(cm)	50.25 ± 26.25	35.4 ± 8.44
BMI	23.01± 5.07	23.53 ± 0.70*

*p > 0.05 indicating no significant difference between the BMI of smokers and non-smokers.
All data are presented as means ± standard deviation.

(12.5%) smokers (Figures 1 and 2).

In the case of non- smokers, C3 and C4 concentrations were normal in 11(45.83%) non-smokers, normal C3 and low C4 concentrations were found in 4 persons (16.66%), high C3 and normal C4 concentrations were present in 1(4.16%) non- smoker, low C3 and normal C4 concentrations were observed in 3 (12.5%) individuals , low C3 and high C4 concentrations were detected in 2 (8.33%) non-smokers, low C3 and low C4 concentrations were present in 1 (4.16%) person, high C3 and high C4 concentrations were seen in 2 (8.33%) healthy persons, while high C3 and low C4 concentrations were noticed in none of the non-smokers (Figures 1 and 2).

Serum levels of C3 and C4 in the group of smokers were 102.63 ± 14.69 and 43.54 ± 6.29, respectively. Serum levels of C3 and C4 in the control group of non-smokers were 76.68 ± 7.41 and 29.97 ± 4.16, respectively. C3 was observed to be 33.84% higher in smokers when compared with the controls. C4 concentration was 45.28% higher in smokers when compared with the controls. Thus, mean serum levels of C3 and C4 were higher in the smoker subjects but when independent t-test was applied it was found to be statistically insignificant as the p value was > 0.05 (Table 2).

The Fisher exact test applied showed no positive association between the CRP status and smoking (p > 0.05). Positive CRP test results ≥ 6 mg/l (8.33%) were observed only in 2 smokers, while the rest of the 22 smokers showed negative CRP tests < 6 mg/l (92%) (Figure 3). Positive CRP tests showed values of 6 and 24 mg/l. Negative CRP test results (< 6 mg/l) were observed in non-smokers (100%) (Table 3).

DISCUSSION

Smoking has an impact on the immune system of human beings and is found to be involved in inflammation. Smoking induces an inflammatory stimulus resulting in an increase in the expression of regulatory proteins (Airoldi et al., 2005). Extreme complement activation is involved in the pathogenic mechanism of several inflammatory conditions. The products of complement activation are concerned in inflammation in lungs and diseases of the respiratory tract like asthma, pneumonia and damage stimulated by smoke, most probably involve complement activation (Rawal, 2006). In Pakistan, there are about 25 million smokers and the majority of smokers are males as the ratio of male to female is 4:1 (Chaudry et al., 2009). In this study, 100% of the smokers are males.

Complement component C3 indicated persistent vascular inflammation. C3/C4 ratio plays an important role in the prediction of new vascular events as a result of severe coronary disorders (Szeplaki et al., 2004). Few data is available on the association between smoking and the induction of inflammatory pathways. Aral et al. (2006) studies the parameters of humoral immunity in two groups of smokers which were the users of smoke and smokeless tobacco and also the non-smoker group. In this study, the selected indices of humoral immunity, that is, C3 and C4 are considered in a single group of cigarette smokers versus control group. Mean serum levels of C3 and C4 were observed to be higher in the smoker subjects but these were statitiscally insignificant (p > 0.05).

No statistical difference was found between the BMI of smokers and non-smokers which is in agreement with a prevoius study by Saarni et al. (2004). The results of this study are in accordance with the work of Aral et al. (2006) and Moszczynski et al. (2001), which demonstrated no statistical difference in the levels of C3 and C4 in the groups of smokers and controls. However, Moszczynski et al. (2001) also reports decreased levels of IgG, IgM, IgA and lysozyme in smokers.

The levels of complement proteins in serum are linked with the body mass index and the levels of glucose and lipids in serum (Yilmaz et al., 2001). In this study, five subjects who were non-smokers also diplayed high levels of C3 and C4 which can be due to the inflammation induced by tobacco smoke in the environment or pollution.

Most of the persons recruited in the study were healthy smokers. They belonged to the same socioeconomic class and used cigarettes of brands named red and

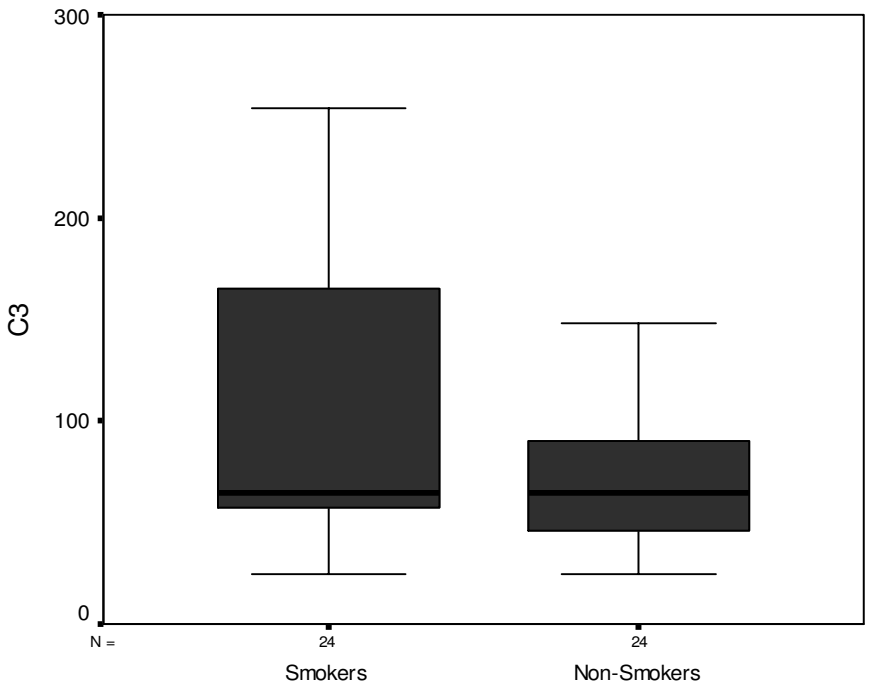

Figure 1. C3 concentration in smokers and non-smokers. The box-plot in Figure 1 indicates a wider box in the case of smokers and and the shift of box towards the lower end indicating that the sample is positively skewed. In the case of non-smokers, the box shows a thinner peak and is positively skewed.

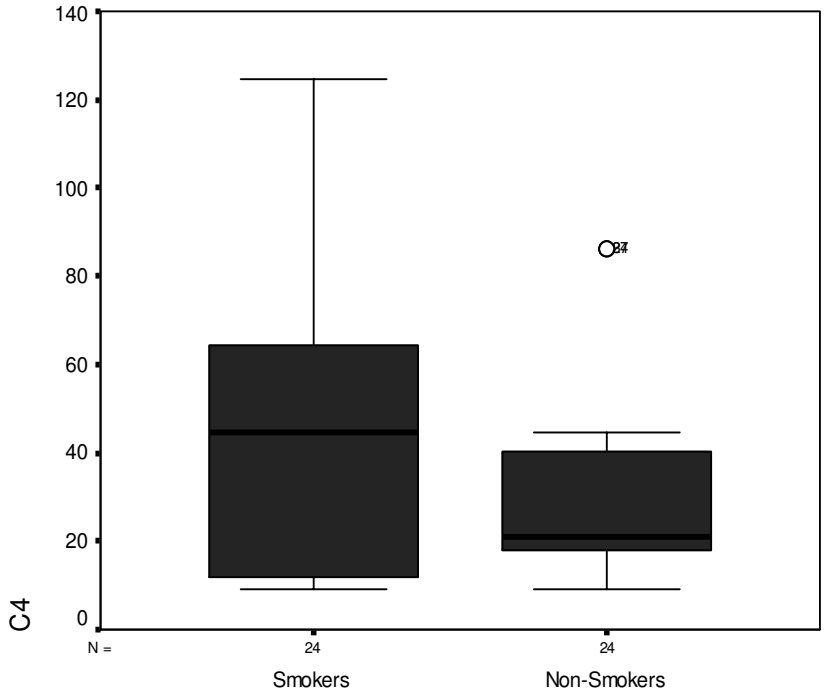

Figure 2. C4 concentration in smokers and non-smokers. The box-plot in figure 2 indicates a wider peak in the case of smokers and shows that the sample is positively skewed. The box-plot shows a wide peak. In non-smokers, the sample is negatively skewed and a thi

Table 2. C3 and C4 levels in smokers and controls. **A.** t-Test; Group statistics.

T-Test ; Group statistic		
	C3	
Parameter	**Smoker**	**Non-smoker**
N	24	24
Mean	102.62	76.68
Standard deviation	71.98	36.29
Standard error mean	14.69	7.41

Table 2B. Independent sample test.

	Parameter	Levene's test for equality of variance		t-test for equality of mean						
		F	Significance	t	df	Significance (2-tailed)	Mean difference	Standard error difference	95% confidence interval of the difference	
									Lower	Upper
C3	Equal variances assumed	15.67	0.000	1.58	46	0.12†	25.9417	16.45	-7.18	59.06
	Equal variances not assumed			1.58	33.98	0.124	25.94	16.45	-7.50	59.38

†p > 0.05 statistically not significant

Table 2C. t-Test; Group statistics.

Parameter	C4	
	Smoker	**Non-smoker**
N	24	24
Mean	43.54	29.97
Standard deviation	30.80	20.38
Standard error mean	6.29	4.16

Table 2D. independent samples test.

	Parameter	Levene's test for equality of variance		t-test for equality of mean						
		F	Significance	t	df	Significance (2-tailed)	Mean difference	Standard error difference	95% confidence interval of the difference	
									Lower	Upper
C4	Equal variances assumed	3.72	0.060	1.80	46	0.078*	13.57	7.54	-1.60	28.75
	Equal variances not assumed			1.80	39.90	0.079	13.57	7.54	-1.67	28.81

*p > 0.05 statistically not significant.

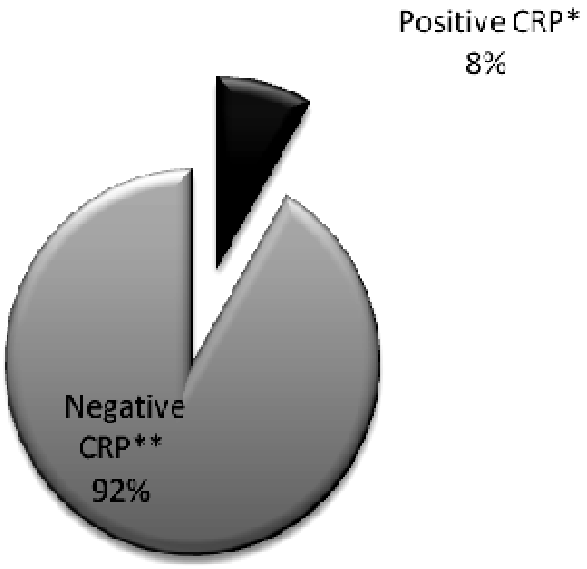

Figure 3. CRP test result in smokers. The figure indicates negative CRP test result in smokers demonstrating absence of inflammation or infection.

Table 3. Results of CRP test in smokers and non-smokers (n= 24).

S/N	Result of CRP	Number of smoker (n = 24)	Number of non-smoker (n = 24)	p-value
1	Positive CRP*	2 (8.33%)	0 (0%)	
2	Negative CRP**	22 (91.66%)	24 (100%)	0.49φ

Positive CRP* ≥ 6 mg/l; Negative CRP** < 6 mg/l; Φ p > 0.1 statistically not significant.

white, gold leaf and K2. They belonged to urban areas and were not exposed to hazardous chemicals. It is complex to make comparisons between the immune system parameters of smokers as they smoke different brands of cigarettes which have different compositions of chemicals and tobacco (Moszczynski et al. 2001). They also differ in age, body mass index, the number of cigarettes smoked per day and their place of residence.

The mean values of C3 and C4 were higher in smokers when compared with non-smokers but overall present in the normal ranges of C3 and C4. Therefore, the results rule out the possibility of involvement of complement components C3 and C4 in the pathogenesis of smoke induced inflammation.

The next marker of inflammation chosen to study in healthy smokers and controls was C-reactive protein which is a major predictor of cardiovascular risk. CRP is deposited in those sites of human coronary arteries that are rich in lipids. It showed an attraction towards monocytes due to chemotactic activity and might also cause complement activation (Ferns, 2001).

Our results are in contrast to numerous other studies which report a positive association between smoking and CRP levels. Loughlin et al. (2008) studied the association between cigarette smoking and CRP in adolescents. A positive relationship between smoking and high levels of CRP was observed and this relationship was observed to be stronger in heavier past-month smokers suggesting a linear association of CRP with the number of cigarettes.

Ohsawa et al. (2005) demonstrated the elevation of CRP levels in smokers but these are unrelated to the quantity of cigarettes and are reduced by long term smoking termination in male smokers. The results showed that the decline in CRP levels might elucidate the reduction in CVD risk caused by smoking termination. In this study, CRP test was negative in all of the smokers except for two smokers, they had CRP levels of 6 and 24 mg/l.

These results suggested that the activation of inflammatory pathways in these smokers might be due to other reasons besides smoking. Both of these smokers were basically healthy and did not suffer from any serious illness. They were moderate smokers who smoked 10 to

12 cigarettes per day. Elevation in CRP levels might also be due to other factors like increased age, reduced general finess and weight.

A study by Pinto-Plata et al. (2006) supported the results of this study. It reported raised level of CRP in chronic obstructive pulmonary disease patients but similar levels of CRP in the control groups of smokers and non-smokers. Thus, it can be deduced from the results that absence of systemic inflammation has been observed in healthy smokers and the non-smokers group. The negative results of CRP test in (92%) smokers and (100%) controls suggested the absence of inflammation, infection or tissue damage.

Conclusions

On the basis of our findings, we can conclude that the C3 and C4 which are the two serum proteins of the complement cascade were present in higher concentration when compared with the controls, but found to be statistically insignificant. No significant difference was found between the CRP levels of smokers and non-smokers. There was no statistical difference between the BMI of smokers and non-smokers. It is most probably because the majority of smokers are younger smokers. The results of this study disclaim presence of cigarette smoke induced inflammation in smoking persons.

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