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Evaluation of Tumor necrosis factor-alpha in patients with Coronary Artery Disease

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Abstract :
Background: *Tumor necrosis factor-α (TNF-α) is a multifunctional circulating cytokine derived from endothelial and smooth muscle cells as well as macrophages associated with coronary atheroma. A large number of genetic and epidemiological studies have identified TNF-α as a risk factor for atherosclerotic disease such as Coronary Artery disease and stroke. Based on our knowledge, this is the first study to estimate serum TNF-α concentration in Manipur.*
Aims and objective: *The study was conducted to estimate serum TNF-α in Coronary Artery Disease patients, and its correlation with lipid parameters, blood glucose and anthropometric measurements.*
Materials and Methods: *Study was carried out in 112 CAD patients as cases and 56 healthy subjects as controls. TNF-α was measured by ELISA reader using human TNF-α kit of Krishgen Biosystem.*
Results: *Mean serum TNF-α level in CAD patients group was significantly higher than the healthy control. Serum TNF-α was also significantly higher in males than females in both study groups and control subjects. The difference is statistically significant in ACS and CSA group is evident by P-value<0.05.*
Conclusion:*Our study revealed that coronary artery disease patients have elevated levels of serum TNF-α as compared to healthy subjects. High concentration of serum TNF-α is strongly associated with risk of Coronary Artery Disease.*
Keywords: *Coronary Artery diseas , cytokine, ELISA, lipid parameter,serum TNF-α.*

I. Introduction

Coronary Artery Disease(CAD) or Ischemic Heart Disease (IHD) is a condition in which there is an inadequate supply of blood and oxygen to a portion of the myocardium; it typically occurs when there is an imbalance between myocardial oxygen supply and demand. The most common cause of myocardial ischemia is atherosclerotic disease of an epicardial coronary artery (or arteries) sufficient to cause a regional reduction in myocardial blood flow and inadequate perfusion of the myocardium supplied by the involved coronary artery. Hyperlipidemias and hyperlipoproteinemias are the most important risk factors for atherosclerosis. IHD causes more deaths and disability and incurs greater economic costs than any other illness in the developed world.[1]

Tumor Necrosis Factors (TNF) are cytokines with a molecular weight of 17-70 kDa that exist in either alpha (α) or beta (β) form, they are capable of causing in vivo hemorrhagic necrosis of certain tumor cells, but not affecting normal cells.[2]TNF-α enhances endothelial adhesion and vascular invasion of dendritic cells, the most potent antigen presenting cell type.[3]TNF-α also a pro-inflammatory cytokine which is actively involved in the progression of atherosclerosis.[4] TNF-α is secreted in the vascular wall by endothelial smooth muscle cells and by monocytes or macrophages and is a powerful inducer of local inflammation.[5]TNF-α increase the permeability of the endothelial cell barrier,[6]promotes the expression of leukocyte adhesion molecules via nuclear factor-kappa B[7] and increases the uptake of macrophages in atherosclerotic lesions thus directly promoting atherosclerosis.

Coronary Artery Disease is a serious, chronic and life threatening disease. Till date there is no study done on TNF-α in CAD patients in north east part of India. The present study has been done with an aim to explore this, which in future may enable us to come up with a better approach towards prevention and management of CAD.

II. Aims and Objects

The present study was taken up in the Department of Biochemistry in collaboration with Department of Medicine, Regional Institute of Medical Sciences Hospital ,Imphal to estimate serum level of TNF-α in CAD patients , normal individuals and to compare the findings between them. The study also assess the correlation of TNF-α, lipid parameters ,blood glucose and anthropometric measures in patients of CAD.

III. Materials and Methods

3.1 Study Population

A cross-sectional comparative study was carried out in the Department of Biochemistry, Regional Institute of Medical Science (RIMS), Imphal in collaboration with Department of Medicine, RIMS, Imphal for the duration of two year (September 2013 to August 2015).A minimum of 112 patients above 18 years of age with already diagnosed CAD who attended Cardiac clinic/Medicine OPD/admitted in the medicine ward or ICCU were selected randomly as study group irrespective of sex, religion and socio-economic status. The study group was divided into two subgroups of 56 patients each:

Group 1:Patients with acute coronary syndrome(ACS)

Group2:Patients with chronic CAD presenting as stable angina

For control group, a minimum of age and sex matched 56 normal subjects who were otherwise free from any systemic disease has been selected. The participants were recruited in consideration with possible screening out of exclusion criteria, such as patients with congenital heart disease, severe renal and hepatic impairment, acute and chronic infection, major surgery or trauma within last one month, systemic disease(rheumatoid arthritis)and patients currently receiving NSAID or immunosuppressive therapy.

3.2 Data Collection and Procedure

A predesigned, pretested, semi-structured interview schedule was used. Subjects were interviewed after taking informed consent and the prospects of this study for improving the understanding of CAD were explained to the participant. A detailed history including the patient's name, age, sex, socio-demographic data, duration of disease, other undercurrent illness, presence of hypertension, chest pain, personal history of smoking, consumption of alcohol, presence or absence of obesity, history of diabetes mellitus has been obtained.

3.3 Baseline Examination And Measurement

After obtaining history, standardized protocols were used to measure body weight, height, waist and hip circumference with appropriate validation and quality control procedure. Body mass index(BMI) was calculated as weight(kg) divided by the square of the height(m^2).Waist circumference was measured at a level midway between the lower rib margin and iliac crest. Hip circumference was measured as the maximal circumference over the buttocks. Waist hip ratio was calculated by dividing the waist circumference measured by the hip circumference measurement. Blood pressure (BP) was measured with a standard mercury sphygmomanometer and defined as the average of the last two measurements taken at intervals longer than 2 minutes after the participants had been sitting for at least 30 minutes according to the American Heart Association guidelines. BP was measured with the patient's elbow flexed at the heart level and the cuff was applied to upper arm with the lower border not less than 2.5 cm from cubital fossa. Baseline investigations data like ECG, chest X-ray etc available with the patient was taken for reference.

3.4 Collection of Sample

About 5 ml of venous blood was collected by venipuncture from antecubtal vein after an overnight fast.1 ml of blood was collected in fluoride vial for estimation of blood glucose and about 4 ml was collected in plain vial for the estimation of TNF-α was stored immediately at <-20 deg C.All the lipid tests were carried out on the same day. Blood glucose estimation was done on the plasma separated from fluoride vial on the same day.

3.5 Analytical Methods

Serum tumour necrosis factor-alpha were measured by enzyme linked immunosorbant assay(ELISA)using ELISA reader. Total cholesterol was measured by CHOD PAP method, HDL cholesterol by precipitation technique using HUMAN cholesterol liquicolor test kit. Serum triglyceride was measured by GPO-PAP method with colorimetric determinations. Blood glucose was measured by Glucose Oxidase (GOD-PAP) method using Glucose liquicolor kit manufactured by HUMAN, Germany.All the chemicals or reagents used in this study are of analytical grade.

TNF-α was estimated by human TNF-α kit manufactured by Krishgen Biosystem, Mumbai. It provides an accurate method for the quantitative determination of human TNF-α in serum, plasma or other body fluid and cell culture supernatant.

3.6 Principle of The Procedure

The Krishgen Biosystems human TNF-α ELISA employs an antibody specific for human TNF-α and coated on a 96-well plate. During incubation, TNF-α in the sample reacts with the immobilized antibody bound to the microtitration well, After washing, diluted detection antibody solution is added to each well and incubated. This is followed by a second wash after which diluted Avidin horse-radish peroxidase is added and

incubated. After washing away the unbounded enzyme labelled antibody, the bound conjugate is detected by adding freshly prepared tetramethylbenzidine (TMB) substrate. The reaction is stopped by adding stop solution and absorbance is read at 450 nm within 30 minutes. The values are then plotted in reference to the standard curve.

3.7 Data Entry and Analysis

According to the National Cholesterol Education Programme Adult Treatment Panel III[8] ,the lipid profile values of the subjects have been classified from low to high group. According to Joint National Committee on Detection, Evaluation and Treatment for High Blood Pressure (JNC VII) criteria,[9]blood pressure was also categorized According to the obesity criteria established in 2000 of Asia area, BMI has also been classified.

3.8 Statistical Analysis

Statistical analysis was performed using SPSS software 17 version. Results were reported as mean ± SD (standard deviation) for quantitative variables and number of cases along with the percentages for the categorical/qualitative variables. The group's means were compared by F-test(commonly known as ANOVA) and paired wise comparison was made by Post Hoc test for continuous variables and X^2-test(or Fisher's Exact Test, if necessary)for categorical variables. In order to investigate serum TNF-α level as predictor of coronary artery disease(CAD),sensitivity and specificity of the predictor were calculated for CAD group at various levels of TNF-α by receiver operating characteristic (ROC) curve with 95% confident interval. P-values for<0.05 and <0.01 were used as the cut-off values for significance and highly significance respectively.

IV. Results and Observations

Table 1 shows the distribution of the number of study subjects along with percentage over the three groups considered according to their socio-demographic and behavioural factors. It is seen that number of males is higher than females in all three group studied. However, the sex composition of the one group is almost similar to the sex composition of other group i.e sex is matched (x^2=5.424 P=0.006) In this study sample, the number of urban subjects is more than that of rural subjects. However, no significant difference is observed as evident by P=0.841.Smoking behaviour is classified according to the US Centres for Disease Control and Prevention as[10]. Smoking pattern is quite different(P=<0.001) as the percentage of non smokers is more in the control group while more of current smokers and ex-smokers are present in ACS and CSA groups respectively. Hindus comprises the highest number in each group and there is no significant difference among group as evident by P=0.621.Majority of sedentary workers in all groups. Seventy five percent of the study subjects didn't have the family history of CAD while the remaining twenty five percent had the history. But there is no significant variation as shown by P=0.424.

It is evident from Fig 1 that the majority of study populations are in the age group of >50-60 years. Mean age of the study groups are ACS,(57.39±1145)CSA(58.30±11.39)and control(56.82±10.19).However the difference in age group among the groups is not significant(P=0.991),indicating that all groups are of comparable age.

Table 2 shows the comparison of numerical parameters considered in the present study in terms of their mean and standard deviation(mean±SD)amongst the groups. Here, there is no significant difference in age among groups as P=0.774.The mean weight, waist to hip ratio and BMI show increasing trend from normal group to CSA and to ACS group. The mean systolic as well as diastolic blood pressure level for control group are found to be lowest followed by CSA group and ACS group respectively. The mean fasting blood sugar level for control is 85.78±10.57 mg/dl whereas for the CSA group is 102.55±30.86 mg/dl and for ACS group is 117.44±24.22 mg/dl. The mean triglyceride (TG)level in control, CSA and ACS groups are 111.10±22.70 mg/dl,144.00±24.87 mg/dl and 172.05±55.7 mg/dl respectively. The highest serum total cholesterol (TC)is found in ACS group(197.44±26.78mg/dl)and lowest is in control group(173.75±13.70mg/dl).Again, the mean value for low density lipoprotein(LDL)increases from control to CSA group and then to ACS group are (100.89±14.74mg/dl,118.26±19.65 mg/dl and 130.27±24.99 mg/dl).The differences are statistically significant as evident from P-value(<0.001).Similar trend was also seen in the case of very low density lipoprotein (VLDL).As expected, a reverse finding is observed in cases of HDL-C. The highest mean HDL-C(66.32±10.77mg/dl)is found in control group and the lowest value(41.98±9.29 mg/dl)is seen in ACS group.

The mean TNF-α level is highest in the ACS group (22.41±3.85 pg/ml)followed by CSA group(18.25±3.88 pg/ml) and the lowest value is found in the control group (15.17±2.48 pg/ml).The differences are statistically significant as evident by P-value(<0.001)[as shown in Fig 2]

The mean level of TNF-α is higher in males than females in both the study groups and control subjects. The difference is statistically significant in ACS and CSA group is evident by P-value<0.05[as shown in Fig 3]

Table 3 shows that 30.4% of ACS group and 14.3%of CSA group have BMI≥25 whereas only 3.6% of the control group has BMI≥25.Prevalence of hypertension is highest in the ACS group which is followed by the CSA group. The difference between the groups is statistically significant as evident by P<0.001.

Table 4 shows decreased HDL-C is found in 42.9% of ACS and 10.7% of CSA group. Hypertriglyceridemia is found in 35 ACS cases(62.5%)out of which 42.9%have borderline high values and 19.6% have high values.51.8%of CSA cases have borderline high values of triglyceride and 1.8% has high values. Increased LDL-C is found 87.5% of ACS cases and 83.9% of CSA cases. Borderline high values of cholesterol are found in 55.4% of ACS cases and 53.6% of CSA cases. High cholesterol value is found in 12.5% and 1.8% of ACS and CSA cases respectively.

V. Discussion

In the present study, CAD was found to be more predominant in male which constitutes about 73.2% of the ACS group and 69.6% of the CSA group. This is in agreement with published data from Singapore myocardial infarction registry for acute MI cases aged between 20 and 64 years which showed that men were four times more prone to these events than women.[11]Higher number if current smokers and ex-smokers was found in the ACS and CSA group respectively. Cigarette smoking is an established risk factor for MI. The main mechanisms by which cigarette smoking affects CHD are as a chronic promoter of atherosclerotic lesions and an acute risk factor increasing sympathetic stimulation and enhancing clotting.[12]Yathis TR et al[13] also showed that cigarette smoking not only accelerates the early onset of coronary artery disease but also increases the risk of the development of coronary artery disease by more than 80%.The prevalence of dyslipidaemia, hypertension and smoking were significantly higher in the CAD group compared to the control group.This is similar to the findings of Goswami B et al[14] and Biswas et al[15] .Penelva et al[16] found that the variable TC/HDL was associated with the number of vessels affected, and was higher in the two-vessel and multivessel groups when compared to the one vessel group. LDL-C was also found to be increased with the number of vessels affected. In a study comparing the Meiteis and the Aggarwals of Delhi,it was found that SBP and not DBP played a significant role in contributing to the risk of CAD among the Meiteis while among the Aggarwals both SBP and DBP were important contributors, though systolic hypertension had the highest contribution. Height was found to be a contributing factor in the Meiteis while WHR may be considered an environmental factor. Genetic makeup of the Meiteis appears to make them more susceptible to risk of hypertension, while Aggarwals may be predisposed to hypertension due to their dietary habits.[17] CAD cases were found to have higher mean level of TNF-α than the normal subjects. This is supported by the findings in Caucasians and Asian Indians.[18,14] Level of TNF-α was also found to be higher in the ACS group than CSA group. These findings are consistent with the reports given in several studies.[19,20] The study also reported a higher mean level of TNF-α in the normal subjects of this ethnic population compared to whites. However the mean value of TNF-α was almost similar with those reported from northern India (15.17±2.48 vs 15.2±4.23 pg/ml)[14] Peterson et al[21] reported that TNF-α in Asian Indians is found to be higher than whites. TNF-α is found to play an important role in the pathophysiology underlying CVD by its action on the modulation of lipid metabolism, obesity susceptibility, insulin resistance and production of other inflammatory cytokines as well as its effect on myocardial contractility. The mean level of TNF-α was found to be higher in males than females in all the 3 groups. This is similar to a study done in Swiss population where they found male sex, increased BMI, greater age, current age were associated with greater TNF-α LEVELS.[22] Verthelyi D et al[23] found that pre-menopausal women had significantly more cells actively secreting TNF-α than did post menopausal women Sex hormones may modulate cytokine production in vivo and contribute to gender related differences in normal and pathological immune responses. In this study, TNF-α showed significant positive correlation with BMI. This is similar to the findings of Marques-Vidal P et al.[21] Mendall MA et al[24] also found that serum TNF-α level was positively correlated with BMI. The association of BMI with raised serum TNF-α is consistent with recent work showing that the adipocytes of obese subjects synthesize increased amounts of TNF-α mRNA and that returns to normal on losing weight. TNF-α was found to be positively correlated with waist circumstance, fasting blood glucose, blood pressure and triglyceride and inversely correlated with HDL-C.In a similar study. Moon YS et al[25] found that serum TNF-α concentrations were positively correlated with BMI, waist circumference, triglyceride, DBP, and negatively correlated with HDL-C. The present study found that TNF-α have significant inverse correlation with HDL-C. This is similar to previous studies conducted in whites.[24,19] TNF-α level was found to be positively correlated with age. This is similar to findings of previous studies.[22]

VI. Conclusion

This is the first study of serum TNF-α level in the Indian Manipuri population. The present study shows a significant difference of TNF-α levels between the groups of CAD and control, and between two groups of CAD i.e acute coronary syndrome and chronic stable angina. The study agrees with previous findings that CAD is associated with high level of TNF-α. Its level in the normal subjects of this population are found to be

similar to the values in Asian Indians(eastern and northern India),but higher than Europeans. Outcomes in ACS are influenced by the speed and accuracy of diagnosis and the timeline of appropriate therapy. Unfortunately, many injury markers are limited in their diagnostic value during the very early stages of ACS. Most markers of myocardial cell injury that reflect the structural consequence of coronary ischemia do not increase until 3 to 4 hours after the onset of pain. Recently, the American College of Cardiology and the American Heart Association guidelines have recommended that risk stratification is one of the most important initial steps in the evaluation and treatment of acute coronary events. Recently, new plasma markers that reflect neuro-hormonal activation and impending plaque rupture have been linked to increased risk in ACS. Markers of plaque inflammation and neuro-hormonal activation make early diagnosis of ACS possible and, as a consequence, help determine the need for and type of aggressive therapeutic interventions. Since higher circulating level of TNF-α was associated with CAD in this ethnic population, identification of high risk subgroups may be useful for anti-inflammatory interventions.

VII. 7. Recommendations

Despite its relatively small sample size, the present study provides evidence of usefulness of estimation of TNF-α level as a convenient and sensitive biomarker for the prediction of coronary artery disease. Prospective and population based studies on a large scale are however required to confirm the associations.

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9. Tables And Figures

Table 1: group-wise distribution of cases with respect to baseline characteristics

Parameters		Group			X2 value	d.f	P-VALUE
		Acute coronary syndrome	Chronic stable angina	Control			
		n(%)	n(%)	n(%)			
Sex	Male	41(73.2)	39(69.6)	30(53.6)	5.424	2	0.066
	Female	15(26.8)	17(30.4)	26(46.4)			
Inhabitancy	Urban	32(57.14)	35(62.5)	33(58.93)	0.346	2	0.841
	Rural	24(42.86)	21(37.5)	23(41.07)			
Occupation	Laborer	7(12.5)	11(19.6)	3(5.4)	6.555	6	0.364
	Household work	19(33.9)	16(28.6)	21(37.5)			
	Sedentary	24(42.9)	26(46.4)	26(46.4)			
	Others	6(10.7)	3(5.4)	6(10.7)			
Smoking	Current	35(62.5)	21(37.5)	8(14.3)	44.000	4	<0.001
	Non-smoker	11(19.6)	12(21.4)	36(64.3)			
	Ex-smoker	10(17.9)	23(41.1)	12(21.4)			
Religion	Hindu	36(64.3)	33(58.9)	38(67.9)	4.414	6	0.621
	Christian	10(17.9)	9(16.1)	11(19.6)			
	Muslim	8(14.3)	9(16.1)	6(10.7)			
	Others	2(3.6)	5(8.9)	1(1.8)			
Family history	Yes	17(30.4)	14(25.0)	11(19.6)	1.714	2	0.424
	No	39(69.6)	42(75.0)	45(80.4)			

TABLE 2: GROUP-WISE COMPARISON OF MEAN±SD OF CLINICAL AND LABORATORY PARAMETERS

Parameter	ACS(n=56)	CSA(n=56)	Control(n=56)	F-value	P-value
	mean±SD	mean±SD	mean±SD		
Age	57.39±11.45	58.30±11.39	56.82±10.19	0.257	0.774
Weight	62.69±6.32	60.04±7.41	58.08±5.28	7.307	0.001
WC	85.16±7.75	84.19±7.52	81.94±7.45	2.652	0.074
WHR	0.90±0.05	0.88±0.05	0.84±0.05	13.248	<0.001
BMI	23.73±3.40	22.91±2.85	21.19±2.00	11.885	<0.001
SBP	136.82±15.43	131.28±13.48	120.00±8.04	25.475	<0.001
DBP	84.37±7.80	80.00±4.39	78.60±4.74	14.797	<0.001
FBG	117.44±24.22	102.55±30.86	85.78±10.57	25.522	<0.001
TG	172.05±55.71	144.00±24.87	111.10±22.70	36.880	<0.001
TC	197.44±26.78	185.67±25.62	173.75±13.70	15.097	<0.001
LDL-C	130.27±24.99	118.26±19.65	100.89±14.74	29.838	<0.001
VLDL	34.40±11.14	28.80±4.97	22.22±4.54	36.861	<0.001
HDL-C	41.98±9.29	54.10±11.30	66.32±10.77	75.295	<0.001
TNF-α	22.41±3.85	18.25±3.88	15.17±2.48	61.298	<0.001

Table 3: Test For Association Between Groups Among Clinical Parameters

Parameters		Group			X^2 value	df	P-value
		ACS(n=56)	CSA(n=56)	Control(n=56)			
BMI GROUPING	<18	2(3.6%)	3(5.4%)	2(3.6%)	30.582	6	<0.01
	18-22.9	16(28.6%)	24(42.9%)	42(75.0%)			
	23-24.9	21(37.5%)	21(37.5%)	10(17.9%)			
	≥25	17(30.4%)	8(14.3%)	2(3.6%)			
BMI CUT-OFF	<25	39(69.6%)	48(85.7%)	54(96.4%)	15.092	2	0.01
	≥25	17(30.4%)	8(14.3%)	2(3.6%)			
SBP	<120	4(7.1%)	5(8.9%)	31(55.4%)	59.502	6	<0.01
	120-139	23(41.1%)	34(60.7%)	22(39.3%)			
	140-159	23(41.1%)	13(23.2%)	3(5.4%)			
	≥160	6(10.7%)	4(7.1%)	-			
DBP	<80	7(12.5%)	17(30.4%)	25(44.6%)	53.939	6	<0.01
	80-89	28(50.0%)	39(69.6%(	31(55.4%)			
	90-99	20(35.7%)	-	-			
	≥100	1(1.8%)	-	-			

Table 4: Test For Association Between Groups Among Biochemical Parameters

Parameters		Group			X^2 value	df	P value
		ACS(n=56)	CSA(n=56)	Control(n=56)			
HDL-C	<40	24(42.9%)	6(10.7%)	2(3.6%)	69.118	4	<0.001
	40-60	29(51.8%)	35(62.5%)	15(26.8%)			
	>60	3(5.4%)	15(26.8%)	39(69.6%)			
TG	<150	21(37.5%)	26(46.4%)	50(89.3%)	48.247	4	<0.001
	150-199	24(42.9%)	29(51.8%)	6(10.7%)			
	200-499	11(19.6%)	1(1.8%)	-			
LDL-C	<100	7(12.5%)	9(16.1%)	28(50.0%)	55.314	8	<0.001
	100-129	12(21.4%)	25(44.6%)	25(44.6%)			
	130-159	29(51.8%)	20(35.7%)	3(5.4%)			
	160-189	5(8.9%)	2(3.6%)	-			
	≥190	3(5.4%)	-	-			
TC	<200	18(32.1%)	25(44.6%)	52(92.9%)	52.739	4	<0.001
	200-239	31(55.4%)	30(53.6%)	4(7.1%)			
	≥240	7(12.5%)	1(1.8%)	-			

Fig 1: Group-Wise Percentage Comparison With Respect To Age

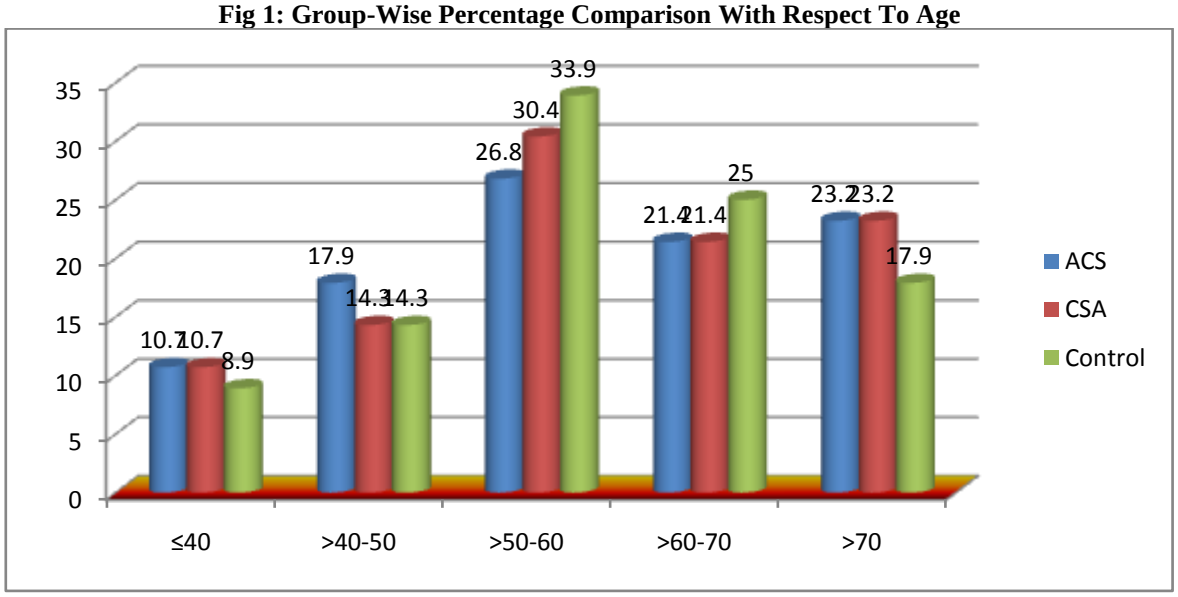

FIG 2: GROUP-WISE DISTRIBUTION OF MEAN TNF-A LEVEL

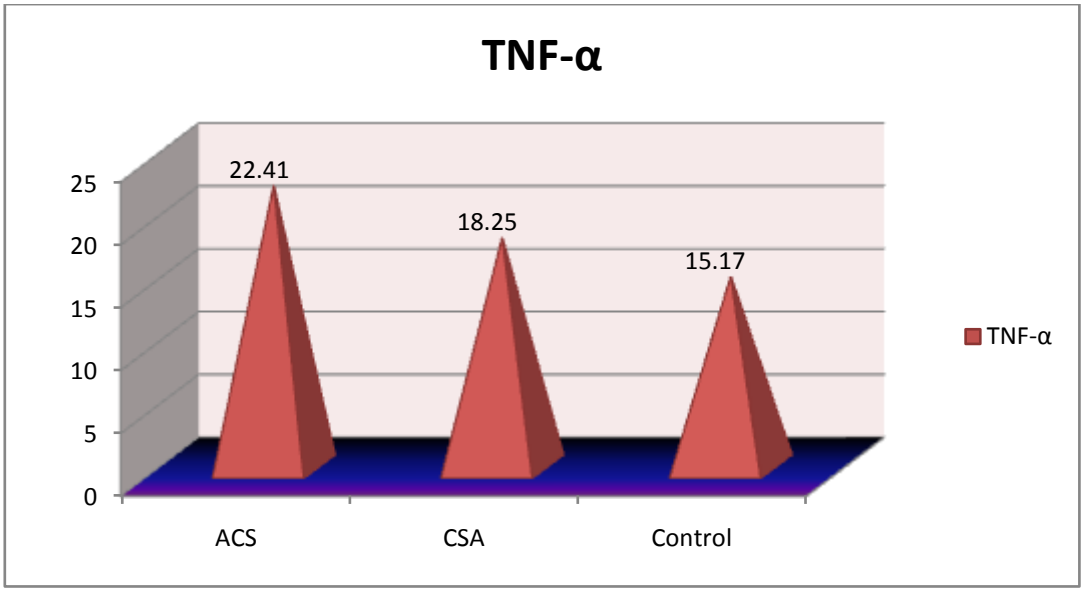

FIG 3: SEX-WISE DISTRIBUTION OF MEAN TNF-A LEVEL