

## EVALUATION OF CARDIAC RISK BY OXIDATIVE STRESS AND INFLAMMATORY MARKERS IN DIABETIC PATIENTS

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### ABSTRACT

**Objectives:** To evaluate the diabetic patients for cardiac risk by measuring oxidative stress and inflammatory markers in relation with glycaemic control.

**Methodology:** A total of 140 subjects were included in this case-control study, comprising of 70 diabetic patients with coronary heart disease (CHD) and an equal number, age and sex matched controls. The patients were non-alcoholic and had age >40years, BMI < 30 kg/m<sup>2</sup> and glycosylated hemoglobin (HbA1c) 7-10%. Serum total cholesterol (TC) and gamma glutamyltransferase (GGT) were analyzed on selectra-E auto analyzer. Serum nitrate was measured at 540nm on ELISA. HbA1c on was analyzed by using Human kit. Serum high sensitivity C-reactive protein (hs-CRP) was analyzed on immulite 1000.

**Results:** Patients mean age was 51 (range 40-73) years. Diabetic patients had significantly elevated median of HbA1c (7.9 vs 4.9), hs CRP (6.0 vs 2.12), TC (5.95 vs 4.45), nitrate (19.20vs 10.70) and GGT (29.50 vs 22.50) as compared to controls ( $p < 0.001$ ). HbA1c showed a positive correlation ( $p < 0.001$ ) with hs-CRP ( $r=0.49$ ), TC ( $r=0.69$ ), nitrate ( $r=0.41$ ) and GGT ( $r=0.30$ ).

**Conclusion:** Oxidative stress and inflammatory markers should be used in addition to HbA1c for assessment of increased cardiac risk in un-controlled diabetic patients because of accelerated atherosclerosis due to free radical injury.

**KEY WORD:** Diabetes mellitus, Gamma glutamyltransferase, C-reactive protein, Nitrate, Total cholesterol, Oxidative stress.

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## INTRODUCTION

Diabetes mellitus is commonly associated with both microvascular and macrovascular complications.<sup>1</sup> Increasing evidence supports that atherosclerosis is a co-morbid condition in the diabetic patients.<sup>2</sup> Impairment of vascular endothelial function is an initial step in the development of cardiovascular problems.<sup>3</sup> Recently the important contribution of inflammation and oxidative stress to the pathogenesis of accelerated atherosclerosis in diabetic patients has been emphasized.<sup>4-5</sup>

Hypercholesterolemia causes focal activation of endothelium by infiltration and retention of

LDL -cholesterol in arteries causing inflammatory response and activation of reactive oxygen species (ROS).<sup>6</sup> Modification of LDL, through oxidation and enzymatic activity causes LDL oxidation. OxLDL when recognised by macrophages is converted into foam cells which is a key event in atherogenesis. The central role of dyslipidemia in causing progression of atherosclerosis in adults with diabetes has been elucidated. There are a few researcher who have reported higher levels of total cholesterol, LDL-cholesterol and triglyceride with higher HbA1c concentrations in diabetic patients.<sup>7-8</sup>

Elevated levels of inflammatory markers, including hS-CRP, interleukin-6, tumor necrosis factor- $\alpha$  and GGT have been reported in diabetic patients.<sup>5, 9</sup> Recent research suggests that CRP is etiologically involved in the pathogenesis of diabetes.<sup>10</sup> Hyperglycemia causes glycosylation of proteins and phospholipids. Advanced glycation end products (AGEs) generate reactive oxygen species (ROS) with consequent increased vessel oxidative damage and atherogenesis.<sup>11</sup> Chronic over production of ROS in the diabetic patients leads to redox imbalance leading to increased GGT activity. Among the new emerging markers for evaluation of oxidative stress GGT is receiving increased attention and is an early marker of oxidative stress in humans.<sup>12-13</sup>

Nitric oxide (NO) is another important biomarker of inflammation and oxidative stress. It is a gaseous biological mediator first identified as the endothelium-derived relaxing factor (EDRF). Cytotoxicity attributed to NO is due to peroxynitrite and superoxide anion.<sup>14</sup> Peroxynitrite interacts with lipids, DNA, and proteins via direct oxidative reactions or via indirect, radical-mediated mechanisms.<sup>14</sup> Free radicals, such as superoxide anion, can rapidly react with NO, inactivating it and priming proatherogenic mechanisms.

This study aimed to determine the role of inflammation and oxidative stress markers for assessment of cardiac risk in patients with un-controlled type-II diabetes mellitus. We evaluated status of arteriosclerosis in diabetic patients by measuring oxidative stress, inflam-

matory markers and correlated with HbA1c in our clinical step-up.

## METHODOLOGY

*Subject:* This case-control study was conducted in the clinical pathology laboratory, Army Medical College, Rawalpindi, Pakistan. A total of one hundred thirty subjects, comprising of seventy diagnosed patients of coronary heart disease with type II diabetes mellitus and an equal number, age and sex matched healthy subjects were included after informed consent. The participants were included by purpose sampling technique. The patients had HbA1c between 7 to 10%, BMI < 30 kg/m<sup>2</sup> and were non-alcoholic. Patient suffering from renal failure, hepatitis, acute illness, taking hormonal replacement therapy (HRT), anti-inflammatory drugs or statin were excluded. This study was carried out from April to Oct 2008, after approval of the protocol by institution review committee.

*Methodology:* Blood sample (5ml) was drawn at 7:00-9:00am following an overnight fast. All the assays were performed at Clinical Pathology Laboratory, Army Medical College, Rawalpindi Pakistan following the standard protocols and quality control. Serum hS-CRP was analyzed on Immulite 1000 (Seimen, USA) which is a solid-phase, two-site sequential chemiluminescent immunometric assay method.<sup>15</sup> Two values of serum hS-CRP were obtained optimally two weeks apart, which were then averaged. Serum GGT activity was assessed by a kinetic colorimetric assay at 37°C and expressed as unit per liter. This method was standardized against recommended IFCC method.<sup>16</sup> Serum nitrate was measured by using Griess reagent at 540nm on ELISA strip reader by colorimetric assay kit (Cayman, UK)<sup>17</sup> Analysis for HbA1c was carried out at 415nm on microlab-200 by following the manufacture method.<sup>18</sup> Serum total cholesterol was measured by cholesterol oxidase method (CHOD. POD) on selectra-E auto analyzer.<sup>19</sup> Coefficient of variation was 4-5%.

*Statistical Analysis:* The data was analyzed by using standard SPSS software version-15 (SPSS

Table-I: Comparison of HbA1c, cholesterol, oxidative stress and inflammatory markers in the diabetic patients as compared to healthy controls (n= 140)

Parameters	Diabetic patients Median (IQR)	Controls Median (IQR)	p
HbA1c %	7.90(7.40-9.0)	4.90(4.60-5.30)	0.001
Cholesterol (mmol/L)	5.95(5.60-6.25)	4.45(4.10-4.80)	0.001
Serum CRP (mg/L)	6.0(3.0-10.12)	2.12(1.39-3.45)	0.001
Serum Nitrate(nmol/L)	19.20(12.50-30.25)	10.70(4.07-17.20)	0.001
Gamma glutamyl transferase (U/L)	29 (22-42 )	22 (16.75-32)	0.001

Inc, Chicago) for statistical analysis. Descriptive statistics were applied and data were not following Gaussian distribution. Median and inter quartile range (25-75 percentile) were calculated. Comparison of data of both diabetic patients and control groups were done by applying Mann-Whitney t-test. The correlation between HbA1c, oxidative stress and inflammatory biomarkers was determined by Spearman’s correlation. A p-value <0.05 was considered significant.

### RESULTS

Diabetic patients had mean (SD) age of 51 (6) years and controls 52 (5) years. Male to female ratio was 2:1. The patients had poor glycaemic control and had significantly raised HbA1c as compared to healthy subjects (Fig-1a). The

hs-CRP levels were also markedly elevated in the CHD patients with diabetes as compared to control indicating increased atherosclerosis in these patients (Fig-1b). Serum total cholesterol was also raised in type II diabetics. The patients had increased oxidative stress indicated by elevated GGT activity (Table-I) and high serum NO levels. Correlation of HbA1c levels with CRP in the diabetic patients is exhibit in Fig-2. Our study also revealed a positive correlation of HbA1c with total cholesterol (r=0.69), nitrate (r=0.41) and GGT (r=0.30) in these diabetic patients (Table-II).

### DISCUSSION

Our study has demonstrated that there is an increased inflammatory and oxidative damage of coronary vessels in type II diabetic patients.

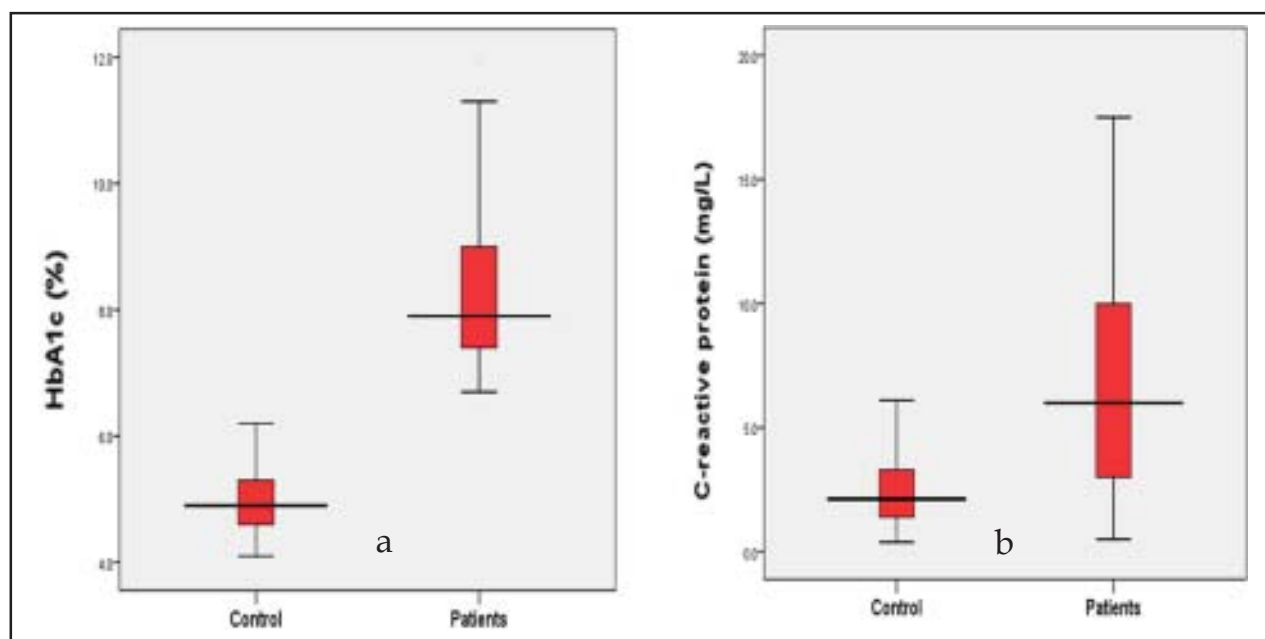


Fig-1: The boxplots of (a) glycated hemoglobin % (b) C-reactive protein in diabetic patients as compared with controls (n= 140).

Table-II: Spearman's correlation between glycated haemoglobin and biomarkers of oxidative stress and inflammatory markers in the diabetic patients.

Parameters	r value	p value
C-reactive Protein (mg/L)	0.49**	0.001
Cholesterol (mmol/L)	.69**	0.001
Nitrate (nmol/L)	.41**	0.001
Gamma Glutamyl transferase (U/L)	.30**	0.001

HbA1c is a marker of long-term glycaemic control and for every one-percent increase in HbA1c, increase the relative risk for cardiovascular events increase.<sup>20</sup> Thus the measurement of HbA1c levels is important not only for monitoring of diabetes but also for assessment of the risk of CHD in diabetics.<sup>21</sup> Cardiovascular morbidity is a major burden in patients with type II diabetes mellitus, with endothelial dysfunction as an early sign of diabetic vascular disease. Atherosclerosis in diabetes mellitus is more progressive than in non diabetic patients. Both type 1 and type 2 diabetes mellitus are associated with abnormalities of lipid metabolism. Several researchers reported similar abnormal lipid metabolism in un-controlled diabetic patients.<sup>7-8</sup>

We found that serum hs-CRP levels were much raised in patients with DM than in control group. Experimental evidence suggests that CRP is a direct participant in the progression of atherosclerosis and is an important cardiovascular risk marker in patients with diabetes mellitus.<sup>22</sup> Inflammatory course of the atherosclerotic process is more severe in diabetic patients than in non-diabetic subjects and our study results strongly support it.<sup>23</sup> The identification of a genetic variant in the human CRP locus associated with a high serum CRP and an increased risk of diabetes support the hypothesis that CRP is also involved in the pathogenesis of diabetes.<sup>10</sup> It is a liver - derived protein secreted under the influence of cytokines such as interleukin - 6 and TNF-  $\alpha$ .<sup>24</sup> Its levels increase with the stage of beta cell dysfunction and insulin resistance. It serves as a chemoattractant

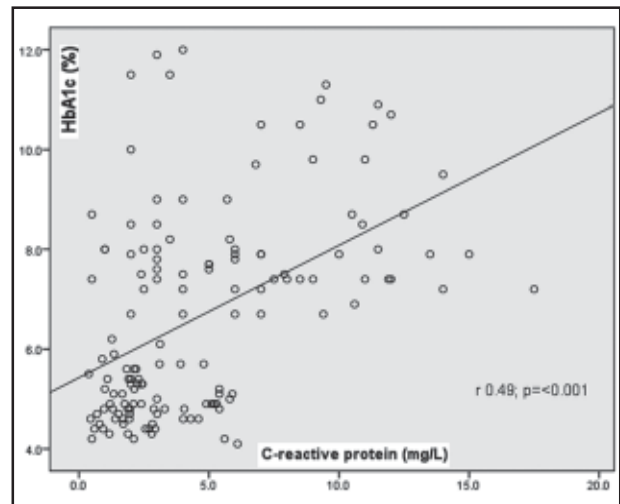


Fig-2: Correlation of HbA1c levels with C-reactive protein in the diabetic patients.

for monocytes. Increasing evidence suggests that CRP has a stronger predictive value for the risk of cardiovascular disease.<sup>25</sup> Patients with CRP concentrations >5 mg/L at the time of hospital admission had a 50% to 330% increase in risk of death with diabetes.<sup>26</sup>

Nitric Oxide plays an important role in homeostatic vasodilatation and the regulation of blood flow. The increased plasma NO levels in patients with type II diabetes may be associated with the pathogenesis of vascular complications.<sup>27-28</sup> Nitric oxide is an important protective molecule in the vasculature. Endothelial nitric oxide synthase (eNOS) is responsible for most of the vascular NO. produced. Uncoupling of the endothelial nitric oxide synthase (eNOS) in blood vessels of diabetic patients leads to excessive superoxide anion ( $O_2^-$ ) production and diminishes NO availability.<sup>29-30</sup> It has also been suggested that CRP effects nitric oxide pathway and elevated serum CRP levels may cause endothelial dysfunction.<sup>31</sup> Raised levels of both serum NO and hs-CRP in our diabetic patient indicate an acceleration in the atherosclerotic process.

We also studied levels of role of GGT for assessment of oxidative processes in relation to heart disease progression in diabetic patients. Baseline serum GGT activity most strongly predicts incident type 2 diabetes.<sup>9</sup> Gamma

glutamyltransferase is a surrogate marker of oxidative stress and a positive correlation is found between serum GGT and CRP.<sup>12, 32</sup> It is the key enzyme that initiates the metabolism and turnover of glutathione (GSH) which is the main antioxidant in mammalian cells. Chronic over production of ROS lead to redox imbalance leading to increased GGT activity and represents early marker of oxidative stress in humans.<sup>13</sup> The serum determination of GGT activity is a low-cost and sensitive laboratory test for assessment of oxidative in our patients. Population-based epidemiological studies showed a strong association of serum GGT activities within the reference interval in the metabolic syndrome.<sup>33</sup> Vasular complications may have a defective cellular antioxidant response against the oxidative stress generated by hyperglycemia. Both oxidative stress and inflammation form a vicious cycle and measuring these markers can help in improvement of health status in diabetic patients.

The limitation of our study was that it mainly included type-2 diabetic patients so it can not be applied on type-I diabetic patients. This was one local study in Rawalpindi and need to be conducted as multicenter study in Pakistan with larger sample size. Future studies can be done by using other markers of inflammation and oxidative stress in the prediction of cardiac risk in type-I and type-II diabetic patients with CHD.

### CONCLUSION

Our study strengthens the role of oxidative stress and inflammation as a common mediator in the pathogenesis of accelerated atherosclerosis in the diabetic patients. Oxidative stress and inflammatory bio-markers should be used in addition to HbA1c for assessment of cardiovascular status in the un-controlled type-2 diabetic patients.

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