

**INVOLVEMENT OF OXIDATIVE STRESS IN ISCHEMIC HEART DISEASE (IHD) IN PATIENTS ADMITTED IN A TERTIARY CARE HOSPITAL, WEST BENGAL, INDIA****SANTANU DUTTA<sup>3</sup>, DEBASRI MUKHERJEE<sup>1§</sup>, ELINA MITRA<sup>1</sup>, ARNAB K. GHOSH<sup>1</sup>, MOUSUMI DUTTA<sup>2</sup>, ANJALI BASU<sup>1</sup>, ARUP DAS BISWAS<sup>5</sup>, ARUN BANDYOPADHYAY<sup>4</sup> AND DEBASISH BANDYOPADHYAY<sup>1\*#</sup>**

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*Received: 20 April 2013, Revised and Accepted: 20 May 2013***ABSTRACT**

**Objectives:** The present study is aimed at investigating the involvement of oxidative stress in ischemic heart disease (IHD) of patients in tertiary care hospital in Kolkata. **Methods:** Serum parameters of myocardial injury, oxidative stress biomarkers and antioxidant enzymes were measured. These data were supported by the data of electrocardiography and echocardiography. **Results:** The current studies indicate involvement of oxidative stress in Indian IHD patients, and assessing these biomarkers may be useful in diagnosis of patients with ischemic hypertrophic cardiomyopathy. **Conclusion:** Cardiovascular diseases are becoming one of the leading health problems in India. The majority of cardiovascular diseases and related complications occur due to imbalance of oxidants and pro-oxidants in an individual. Thus, oxidative stress is one of the main factors in ischemic heart disease (IHD).

**Keywords:** Ischemic Heart Disease, Oxidative stress, Hypertrophic cardiomyopathy, Ischemia/Reperfusion ratio (I/R).**INTRODUCTION**

Heart disease is one of the major health problems of developed as well as developing countries of the world. Extensive research through the last decade has shown beyond doubt that free radicals, particularly, reactive oxygen species play a cardinal role in the pathogenesis of oxidative myocardial damage with consequential cardiac malfunction [1]. For much of the past 50 years, ischemia has been an underappreciated (or unrecognized) component of the cardiac disease process, particularly compared with the better-known pathophysiologic mechanisms of LV outflow obstruction [2] and diastolic dysfunction [3].

Coronary artery occlusion resulting from atherosclerotic plaques or vasospasm can result in a reduction in myocardial blood flow that is sufficiently prolonged or severe to produce myocardial injury and necrosis, ultimately leading to diminished (and consequently fatal) cardiac function [4]. Recently, generation of free radicals particularly reactive oxygen species have been implicated in various cardiovascular disorders including ischemia/reperfusion (I/R), atherosclerosis, hypertension, cardiotoxicity induced by drugs etc [5]. The cellular mechanisms involved in the pathogenesis of myocardial ischemia/reperfusion (I/R) injury are complex and involve the interaction of a number of cell types, including coronary endothelial cells, circulating blood cells (e.g., leukocytes, platelets), and cardiac myocytes, most of which are capable of generating reactive oxygen species (ROS). These ROS have the potential to injure vascular cells and cardiac myocytes directly, and can initiate a series of local chemical reactions and genetic alterations that ultimately result in an amplification of the initial ROS-mediated cardiomyocyte dysfunction and/or cytotoxicity.

It has been recently shown that in animal model, isoproterenol-induced myocardial ischemia involves generation of free hydroxyl radicals and serious alterations of enzymatic and non-enzymatic cardiac antioxidant systems leading one to conclude that apart from other factors oxidative stress is involved in the oxidative damage of some critical cellular proteins [6,7,8]. But there are no such evidences about the involvement of oxidative stress mediated IHD in patients of Eastern India and particularly in Kolkata and surrounding areas. The current studies reveal the changes in the

biochemical and functional status of the myocardium due to ischemic injury with or without cardiac hypertrophy among the patients of a leading government hospital of eastern India. An attempt has been made to analyze the oxidative stress status in these patients with the view to correlate the changes brought about by myocardial ischemia with an increased generation of ROS which may indicate a possible target for future therapeutic intervention for IHD. The objective of our study was to generate a database of this disease in areas of eastern India especially West Bengal. The results of our present study provide evidences oxidative stress is one of the causative factors for IHD in Indian patients as well and may have future relevance in developing new therapeutic strategies.

**METHODS AND MATERIALS****Human subjects**

The study cohort included 60 unrelated patients (age below 40 to above 60 years; 80% male). The diagnosis was based on 2-dimensional echocardiographic evidence of a hypertrophied LV (maximal wall thickness 15 mm). Patients were enrolled from a large population followed at our institution, among the 325 patients. Children age below 15 years and potentially child-bearing female patients were excluded. Although representing approximately 25% of potentially eligible patients, the study group is considered representative of the whole cohort, based on age, sex, and clinical status. For all patients, informed consent was obtained, and the study protocol was approved by the Institutional Human Ethics Committee of Dept. of Physiology, University of Calcutta (IHEC/P02/08 dated 26.03.2008).

**Chemicals used**

Thiobarbituric acid (TBA), eosin, 2,2-dithiobis-nitro benzoic acid (DTNB), were obtained from Sigma, St. Louis, MO, USA. Hematoxylin, Trichloro acetic acid (TCA), H<sub>2</sub>O<sub>2</sub> was obtained from Merck Limited, Delhi, India. Sodium pyruvate and bovine serum albumin (BSA) were obtained from Sisco Research Laboratories (SRL), Mumbai, India. Folin-Ciocalteu reagent, sodium carbonates were purchased from E.

Merck Co. (Darmstadt, Germany). All the other chemicals used including the solvents, were of analytical grade.

#### Measurement of activity levels of serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT)

Serum GOT and Serum GPT were measured by standard routine methods as modified by Mukherjee et al. [6]. Values are expressed as IU/L.

#### Measurement of the levels of serum lipid peroxidation and reduced GSH

Lipid peroxides in the serum was determined as thiobarbituric acid reactive substances (TBARS) according to the method of Buege and Aust, 1978 [9] with some modification as adopted by Bandyopadhyay et al. 2004 [10]. Briefly, the serum was mixed with thiobarbituric acid-trichloro acetic acid (TBA-TCA) reagent with thorough shaking and heated for 20 min at 80°C. The samples were then cooled to room temperature. The absorbance of the pink chromogen present in the clear supernatant after centrifugation at 12000 x g for 10 min at room temperature was measured at 532 nm using a UV-VIS spectrophotometer (Bio-Rad, Hercules, CA, USA). Tetraethoxypropane (TEP) was used as the standard. Values were expressed as nmoles of TBARS/mg protein.

Reduced GSH content (as acid soluble sulfhydryl) was estimated by its reaction with DTNB (Ellman's reagent) following the method of Sedlak and Lindsey, 1968 [11] with some modifications as adopted by Bandyopadhyay et al. 2004 [10]. The serum was mixed with Tris-HCl buffer, pH 9.0, followed by DTNB for color development. The absorbance was measured at 412 nm using a UV-VIS spectrophotometer to determine GSH content. Values were expressed as nmoles/mg protein.

#### Assays of superoxide dismutase (SOD) and catalase (CAT)

Copper-zinc superoxide dismutase (SOD1) activity was measured by hematoxylin autooxidation method of Martin et al., 1987 [12]. Inhibition of haematoxylin autooxidation by the serum was measured at 560nm using a UV-VIS spectrophotometer. The enzyme activity was expressed as Units per min per mg protein.

Catalase was assayed by the method of Beers and Seizer, 1952 [13] with some modifications as adopted by Chattopadhyay et al, 2003. The serum was incubated with 0.01ml of absolute ethanol at 4°C for 30 min, after which 10% Triton X-100 was added so as to have a final concentration of 1%. The sample, thus obtained, was used to determine CAT activity by measuring the breakdown of H<sub>2</sub>O<sub>2</sub> spectrophotometrically at 240nm. Values were expressed as  $\mu$ m H<sub>2</sub>O<sub>2</sub>/min/mg protein.

#### Estimation of proteins

Proteins of the different samples were determined by the method of Lowry et al, 1951 [14].

#### Electrocardiography

The first available ECG study for each patient performed at the time of diagnosis. Electrocardiographic analysis was performed using a 12-lead ECG. Abstracted ECG measurements included the PR, QT, ST, and QRS intervals. All QT and PR intervals were confirmed manually. QT intervals were measured using leads II or V5 when possible. In addition, the echocardiographic and ECG analyses were performed independently to ensure a lack of bias [15].

#### Echocardiography

Comprehensive 2-dimensional and Doppler echocardiographic studies were performed in each patient using commercially available instruments. Left ventricular hypertrophy was assessed by 2-dimensional echocardiography, and the site and extent of maximal wall thickness and any regional wall motion abnormality were identified [15]. Peak instantaneous LV outflow gradient assessment done to rule out mitral valve systolic anterior motion was estimated with continuous wave Doppler under basal conditions [15].

#### Statistical evaluation

Data were analyzed using with Student's t test for paired with a probability value of less than 0.05 considered significant. All group data are reported as mean + SEM.

#### RESULTS AND DISCUSSION

Understanding of the demographic basis, clinical course, and treatment of IHD has increased substantially in the last decade. In particular, there has been a growing awareness of the clinical and molecular heterogeneity characteristic of this disorder and the many patient subgroups that inevitably influence considerations for treatment. Within the broad disease spectrum, there are little data currently available to definitively guide therapy especially in a developing country like India. From the results as we can see that IHD patients are not only from low income groups but also from high income areas, and number is quiet significant among the total cardiac patients during that part. Whether the cause of the disease is lifestyle, food habits or occupational and other kinds of stress, to know this that further studies are needed.

Chest pain symptoms are a frequent complaint among 25% to 50% of IHD patients. Episodes of chest pain in patients with IHD can be prolonged and atypical in character, occurring frequently under resting conditions, but also may be consistent with classic angina pectoris provoked with exertion and after meals [16]. However, the relationship among the various types of chest pain encountered in IHD and active myocardial ischemia is at present unresolved. In those IHD patients with typical angina in whom suspicion of underlying epicardial coronary artery disease is increased based on more advanced age, assessment of traditional coronary risk factors and pertinent noninvasive testing, coronary angiography (or alternatively cardiac computed tomographic angiography) should be considered to exclude obstructive atherosclerotic coronary artery disease [17] as well as other causes of chest pain (e.g., myocardial bridging or congenital coronary artery anomalies). (Table 1)

**Table 1: The demographic characteristics of control and IHD patients.**

Parameter	Control Subjects	Patients
Age (Mean $\pm$ S.D) years	43 $\pm$ 12	45 $\pm$ 18 60 % middle age 20% below 20, 20% above 60
Sex (Males %)	4:1 (Male : Female)	4:1 (Male : Female)
Religion	All	About Hindu patients (60%)
Geographical area	Kolkata and surroundings	Along gangetic plain - Malda (2 %), West Dinajpur (4 %), Mursidabad (3 %), Nadia (4 %), North 24 Pargana (23%), South 24 Pargana (26%), Howrah (15%), Hooghly (12%) Bardhaman (4%) and Midnapur (7%)
Socio economic	All types	55% from low economic background (Farmer, labourer, etc)
Body weight	Normal	Obesity is one of the risk factor, hypertension, dislipidemia, diabetic
Body mass index (Mean $\pm$ SD), kg/m <sup>2</sup>	24.5 $\pm$ 1.9	29.7 $\pm$ 3.2 (P<0.05) *32 patients had high body mass index
Systolic blood pressure (mm of Hg)	111 $\pm$ 9 mm of Hg	137 $\pm$ 18 mm of Hg** 60% hypertention history
Diastolic blood pressure (mm of Hg)	70 $\pm$ 11	85 $\pm$ 12*

Risk Factors, %		Obesity, hypertension, dislipidemia, diabetes
<b>Smoking Status</b>		
Current smoker	6.5 % of all subjects	80% of the patients**
Ex-smoker	2	15**
Non-smoker	2	7**
Physical activity	Sedentary	Sedentary lifestyle
Hereditary factors	21 % of Normal subjects	25 % of subjects
<b>Dietary Habits</b>		
Fast foods	22%	75%**
Unhealthy diet	40%	80% **
Most common complains/ symptoms	None	Chest pain (mild, frequent during rest period also)

Values are given as mean ± S.E. from 60 subjects in each group; IHD patients compared to control subjects. (\*\*p<0.01, \*p<0.05)

Cardiomyopathy patients showed elevated levels of activity of SGPT (2.04 folds compared to control, \*P<0.001) and the activity of cardiac specific SGOT (2.01 folds compared to control, \*P<0.001) (Fig. 1A and 1B). The basis for clinical diagnosis of pathological symptoms depends on comparing the levels of a number of cytoplasmic enzymes, which are released following cell membrane damage. These enzymes are thought to be released as a result of

physical-chemical alterations in tissue [18]. In our study, increase in the levels of activity of SGPT and SGOT marker enzymes indicates that there has been cellular leakage in the organ and also a loss of functional integrity of membrane architecture. Changes in the concentration of plasma lipids including cholesterol are complications frequently observed in patients with IHD and certainly contribute to the development of Cardiomyopathy.

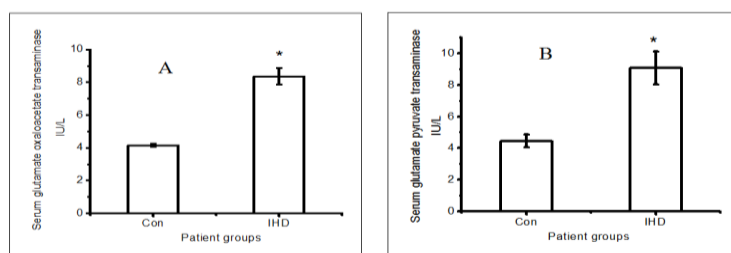


Figure 1: The SGOT [A] and SGPT [B] activities in control and IHD patients.

These parameters are associated with increased IVS thickness (27.77% compared to control, P<0.05) which explains hypertrophy of the myocardium and a decrease in systolic function (EF 17.37% compared to control, P<0.05). Also we found an increase in LVIDD (36.52% compared to control, P<0.01), and a less pronounced

increase in LVIDS (33.82% compared to control, P<0.01) (Table 2). The increase in LVIDD and LVIDS indicate a significant left ventricular dilatation in both diastolic and systolic phases respectively. The representative 2D echocardiography with colour Doppler study of one of our sixty IHD patients is depicted in fig. 2.

Table 2: Various parameters obtained from echocardiography of the control and the IHD patients.

Parameter	CONTROL	IHD
Ejection Fraction (%)	61.6 ± 2.6	50.9 ± 1.6*
IVS thickness (mm)	6.52 ± 0.5	8.33 ± 1.0*
LVIDD (mm)	39.7 ± 2.25	54.2 ± 2.9**
LVIDS (mm)	27.5 ± 1.0	36.8 ± 1.5**

Values are given as mean ± S.E. from 60 subjects in each group. IHD patients compared to control subjects. (\*\*p<0.01, \*p<0.05)

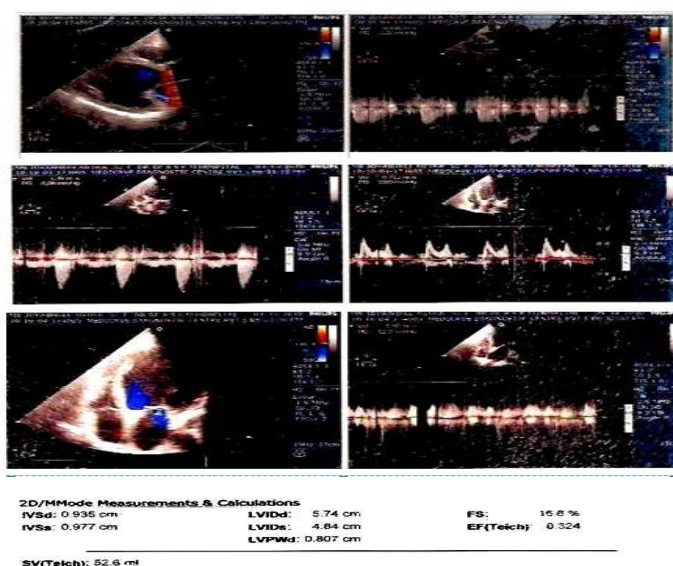
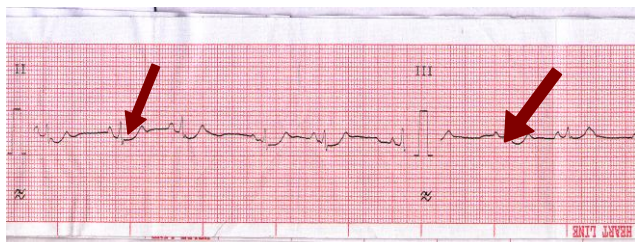
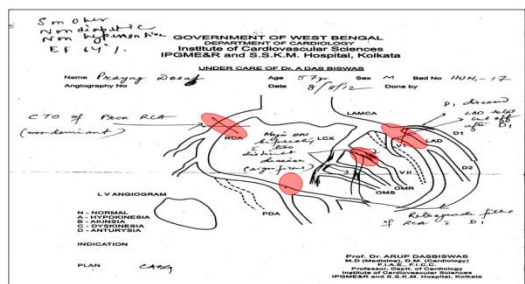


Figure 2: The echocardiograph of an IHD patient



**Figure 3: The ECG report shows ST depression in lead II and III.**

Our studies shows electrocardiographic changes consistent with ischemia such as ST-segment depression (Fig 3) occurs frequently in patients and are most often associated with chest pain. Recent researches showed a clear association between chest pain and microvascular ischemia. However, this has not been established in HCM, suggesting that ischemia often occurs silently [19, 22]. In one study of young asymptomatic HCM patients, reversible thallium perfusion defects were present in about 50% of patients, supporting the concept of clinically silent ischemia [21]. In addition, no consistent relationship has been established between chest pain and LV wall thickness, outflow obstruction, or other HCM disease features [22]. But in our study we found that there is a clear correlation between chest pain and hypertrophy in maximum number of subjects studied.



**Figure 4: The coronary angiogram report shows Triple Vessel Disease.**

**Table 3: Various serum parameters obtained from blood samples drawn from IHD patients**

Parameter	CONTROL	IHD
Cholesterol (mg/dL)	155 ± 2.99	222.75 ± 15.85**
HDL (mg/dL)	52 ± 1.5	31.6 ± 1.0***
LDL (mg/dL)	50.4 ± 3.1	68.1 ± 3.5**
Triglycerides (mg/dL)	79.375 ± 4.8	191.25 ± 16.2***

Values are given as mean ± S.E. from 60 subjects in each group. IHD patients compared with control subjects. (\*\*p<0.01, \*\*\*p<0.001)

Cardiomyopathy patients showed a significant increase in the serum lipid peroxidation level (3.46 folds compared to control, p<0.001) and a decrease in the serum reduced GSH level (40.46 % compared to control, p<0.001) (Fig. 5A and 5B). Lipid peroxidation level is an index of oxidative stress. Lipid peroxidation is a free radical mediated chain reaction and it is self perpetuating [27, 28]. Tissue damage is considered proportional to lipid peroxide contents and thus cell membrane damage is tested by measuring lipid peroxide contents by various ways. Here, we have measured the TBARS content spectrophotometrically. The elevated serum LPO level, shown in the figure, demonstrates that IHD patients suffered from oxidative stress. A reason for increased lipid peroxidation in the serum of patients may be due to a poor enzymatic and non-enzymatic antioxidant defense system. SOD along with catalase, the

enzymatic antioxidants, play a very important role in protection against lipid peroxidation.

Figure 4 depicts a coronary angiogram report showing triple vessel disease. Left anterior descending artery distal to D1 (complete cut off), first diagonal is diffusely diseased, circumflex artery after its branching having significant disease and proximal right coronary artery showing complete cut off. All these indicate the severity and pattern of IHD patients' i.e. multivessel disease which is quite different from western countries where we find mostly involving one or two vessels with discrete lesion [23].

Cardiomyopathy patients showed elevated levels of cholesterol (17.37% compared to control, P<0.01), triglycerides (43.71% compared to control, P<0.001) and LDL (35.12% compared to control, P<0.01) and decrease in HDL level (39.23% compared to control, P<0.001) (Table 3). Cholesterol has been singled out as the primary factor in the development of cardiac diseases. HDL is regarded as one of the most important protective factors against arteriosclerosis. HDL's protective function has been attributed to its active participation in the reverse transport of cholesterol. Numerous cohort studies and clinical trials have confirmed the association between a low HDL and an increased risk of coronary heart disease [24]. The concentration of LDL correlates positively whereas HDL correlates inversely to the development of coronary heart disease. Smokers have significantly higher serum cholesterol, triglyceride, and LDL levels, but HDL is lower in smokers than in non-smokers [25]. Evidence suggests that oxidatively modified LDL contribute to the pathogenesis of heart disease. Increased oxidative stress and the generation of the free oxygen radicals can result in modification of LDL to oxidized LDL that could lead to cardiac diseases [25]. Recent studies demonstrated that disturbed lipid profile is one of the most important and potent risk factors in ischemic heart disease (IHD). According to these researchers, an elevated levels of undesirable pathways including the formation of oxidized LDL (O-LDL) and oxidized cholesterol which encourages cholesterol accumulation in arterial tissues. Here, we mostly find an increase in triglyceride level with a concomitant decrease in HDL level in patients from West Bengal. This lipidemic pattern is quiet different from the western population [26].

enzymatic antioxidants, play a very important role in protection against lipid peroxidation.

Reduced glutathione (GSH) is an important constituent of the cellular defense mechanism of the body against various exogenous as well as endogenously produced xenobiotics. It plays the role of a sulfhydryl (SH) group provider for direct scavenging reactions. GSH acts both as a substrate in the scavenging reaction catalyzed by glutathione peroxidase (GPx) and as a scavenger of vitamins C and E radicals [29]. In our study we found that there occurred depletion in reduced glutathione level in the IHD patients. This again points out that the cardiac tissue has suffered from ischemic damage due to oxidative imbalance thereby decreasing this biomarker, since GSH is a thiol containing non enzymatic antioxidant of the body.

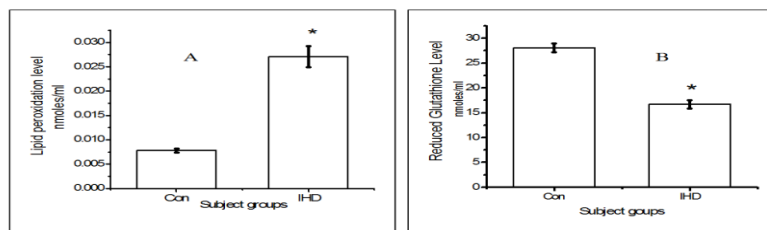


Figure 5: The levels of serum LPO [A] and GSH [B] in control and IHD patients

Cardiomyopathy patients have also show a significant increase in the activity of serum Cu-Zn SOD (2.55 folds compared to control,  $p < 0.001$ ) and in the activity of catalase (40.40% decrease compared to control,  $p < 0.001$ ) (Fig. 6A and 6B). Superoxide dismutase is important antioxidant enzymes and is used as biomarker to indicate the formation of ROS [29, 30]. Free radical-scavenging enzymes such as SOD, CAT and GPx are the first line of cellular defense against oxidative injury, dismutating  $O_2 \cdot$  and decomposing  $H_2O_2$  before

interacting to form the more reactive hydroxyl radical (OH). These enzymes protect the red cells against  $O_2 \cdot$  and  $H_2O_2$ -mediated lipid peroxidation [25]. In our present study, IHD patients showed a significant increase in SOD activity, with a concomitant decrease in the catalase activity indicating a possible accumulation of hydrogen peroxide (a reactive oxygen species) which has the potential to bring about oxidative damage of the bio-macromolecules and the consequential tissue damage.

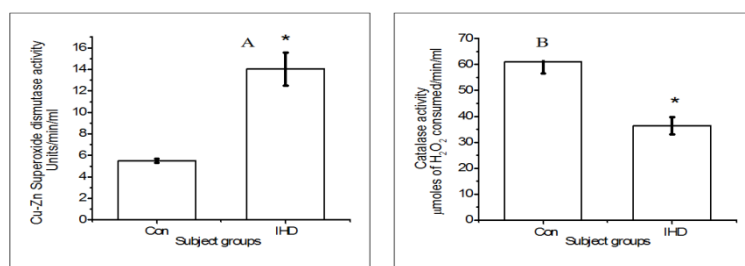


Figure 6: The serum SOD [A] and catalase [B] activities in control and the IHD patients.

## CONCLUSION

We may conclude that our study shows a significant increase in lipid peroxidation and cardiac biomarkers in the circulation of patients with ischemic cardiomyopathy. A significant decrease in the antioxidant status was also observed only in hypertrophic cardiomyopathy patients suffering from IHD. Thus, our study indicates an imbalance between oxidant and antioxidant molecules in these patients. Therefore, assessing these biomarkers of oxidative stress may be useful in diagnosis of patients with ischemic hypertrophic cardiomyopathy. Our work is perhaps a step forward toward creating a database of parameters important in the diagnosis of ischemic heart disease of patients from Kolkata and adjoining areas and the study may also point toward development of efficient management of IHD patients with the knowledge of our own database. Further studies are needed to correlate these associations and accordingly future antioxidative therapy may be beneficial or helpful in IHD patients.

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