

**MALONDIALDEHYDE A MARKER OF
OXIDATIVE STRESS IN DIABETES
MELLITUS WITH AND WITHOUT
CORONARY HEART DISEASE**



By

MUHAMMAD AHMAD

**INSTITUTE OF CHEMICAL SCIENCES,
UNIVERSITY OF PESHAWAR
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**A thesis submitted to the University of Peshawar in partial
fulfillment of the requirements for the degree of**

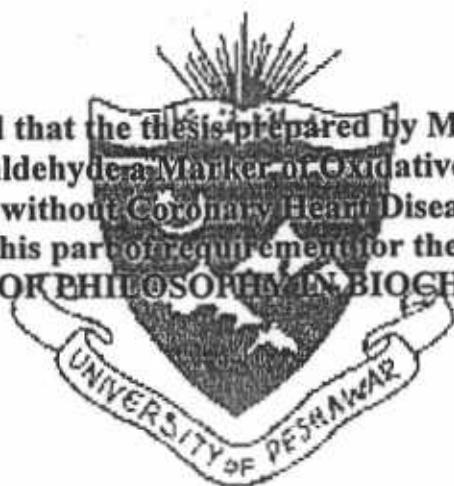
**DOCTOR OF PHILOSOPHY
IN
BIOCHEMISTRY**

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It is recommended that the thesis prepared by Muhammad Ahmad entitled "Malondialdehyde as a Marker of Oxidative Stress in Diabetes Mellitus with and without Coronary Heart Disease" be accepted as fulfilling this part of requirement for the Degree of
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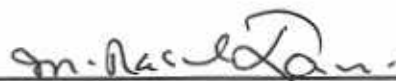
(Dr. Abdus Sattar Khan)
Research Supervisor
Institute of Chemical Sciences,
University of Peshawar,
Peshawar.



(Dr. Anila Jaleel)
External Examiner
Department of Biochemistry,
Ziauddin Medical University,
Karachi.



(Dr. Mudassir Ahmad Khan)
Research Co-Supervisor
Department of Biochemistry,
Khyber Medical College,
Peshawar.



(Dr. Mohammad Rasul Jan)
Director
Institute of Chemical Sciences,
University of Peshawar
Peshawar.

DEDICATED

To

My Parents

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SUMMARY

Diabetes mellitus is an endocrine disorder, which comprises of a large number of complications. Most of the available experimental and clinical evidence suggests that the complications of diabetes result from metabolic derangements mainly hyperglycemia. Diabetes is associated with increased mortality and morbidity due to vascular complications. Diabetic individuals have an increased risk for developing atherosclerotic disease. This is in part a consequence of the association of diabetes mellitus with other atherosclerosis risk factors. Atherosclerosis occurs earlier in diabetics than non-diabetics, its severity is often greater, and its distribution is more diffuse.

Coronary heart disease is frequently associated with diabetes mellitus. Mortality due to cardiovascular disorders is more common in Type 2 diabetes than in Type 1 diabetes mellitus. Oxidative stress has been demonstrated to be present in animal models as well as in patients with diabetes and has been suggested as a possible contributor to the accelerated atherosclerosis seen in diabetics. The generation of reactive oxygen species in diabetics occur via several mechanisms and is initiated not only by glucose, but also by other substances that are found at elevated levels in diabetic patients.

Free iron in the circulation can also produce free radicals, which, in turn cause oxidative stress in diabetes that may promote coronary atherosclerotic disease. The purpose of the study was designed to evaluate and compare the magnitude of serum malondialdehyde (an oxidative stress marker), and biochemical markers of body iron stores (serum ferritin and transferrin saturation)

in control healthy subjects, diabetes mellitus (Type 1 and Type 2) patients, and in patients suffering from coronary heart disease. Further, to compare the serum levels of these parameters in diabetic (Type 2) patients with and those without coronary heart disease.

A total of 410 patients were investigated, they included patients suffering from diabetes mellitus, coronary heart disease and those having diabetes as well as coronary heart disease. One hundred and ninety diabetic patients were enrolled in the study, out of which 90 were Type 1 and 100 were Type 2 diabetics. Moreover 140 patients with coronary heart disease and 80 Type 2 diabetics with coronary heart disease were also part of this study. The control group comprised of one hundred healthy individuals having no history of diabetes mellitus, coronary heart disease, hypertension or any other disease.

Level of serum glucose, triglyceride, total cholesterol, low-density lipoprotein cholesterol, very low density lipoprotein cholesterol and malondialdehyde were found to be significantly higher, whereas levels of high density lipoprotein cholesterol were found significantly lower in all the group of patients in contrast to control subjects. The iron indices i.e. serum iron, total iron binding capacity, transferrin saturation and ferritin are elevated significantly in coronary heart disease, and diabetes mellitus with and without coronary heart disease patients as compared to control subjects. Both type 1 and type 2 diabetic patients when compared with control group showed an increase in malondialdehyde and the iron parameters. The type 2 diabetic patients were having

significantly low concentrations of all the iron indices except for serum ferritin level and an oxidative stress marker malondialdehyde which were found to be significantly high in comparison to that in Type 1 diabetic patients.

It is therefore concluded that diabetes mellitus and coronary heart disease are associated with greater than normal lipid peroxidation. It is also observed that diabetes mellitus and coronary heart disease can alter iron status. Elevated serum malondialdehyde, iron concentration and body iron stores (serum ferritin and transferrin saturation) in diabetic patients reveal a possible role of iron indices in the development of coronary atherosclerosis. Therefore, it is suggested by this study that levels of malondialdehyde and biochemical markers of body iron stores (serum ferritin and transferrin saturation) can be used as an early investigative tool for atherosclerotic coronary artery disease especially in patients with diabetes mellitus.

INTRODUCTION

1.1 FREE RADICALS

Free radicals are highly reactive chemical species that possess an unpaired electron in their outermost shell. They can also be considered as a fragment of a molecule [1]. Free radicals are normally generated in many metabolic pathways [2].

Free radical reactions are critical for the normal operation of a wide spectrum of biologic process. The catalytic action of many cellular enzymes and electron transport processes involve one-electron transfers, which yield free radical intermediates. Because of the ubiquity of molecular oxygen in aerobic organisms and its ability to readily accept electrons, oxygen-centered free radicals are often mediators of cellular free reactions. A wide variety of environmental agents including photochemical air pollutants, hyperoxia, pesticides, tobacco smoke, solvents, anaesthetics, and the general class of aromatic hydrocarbons also cause free radical damage to cells. Certain cellular metabolic states such as hyperoxia, ischaemia, or antibiotic therapy can favor free radical production in excess of basal rates [3].

Free radical species derived from oxygen are often subsumed under reactive oxygen species (ROS). Every living cell with aerobic metabolism exposed to molecular oxygen generates ROS. The ROS may be either unintentionally derived from "accidents of chemistry", i.e., redox reactions of a variety of small molecules (e.g., auto-oxidation of catecholamines) or "leaks" of the mitochondrial

electron transport chain, but more important from a variety of enzymatic reactions [4]. They are superoxide anion radical ($O_2^{\cdot-}$), Hydroperoxyl radical ($HO_2^{\cdot}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), peroxide radical ($ROO^{\cdot}$ Where $R = \text{lipid}$), and singlet oxygen ($^1O_2$) [5]. The superoxide anion is central to ROS chemistry, because it may be converted into other physiologically relevant ROS by enzymatic or nonenzymatic reactions. For example, superoxide anion can react rapidly with nitric oxide ($NO^{\cdot}$) to form peroxynitrite ($ONOO^-$). It can also be converted to H_2O_2 by superoxide dismutase (SOD). Theoretically, both SOD and $NO^{\cdot}$ compete for superoxide anion scavenging. Thus, it is worth mentioning that the rate of superoxide anion conversion to H_2O_2 catalyzed by SOD is only one-third of the reaction rate of superoxide anion with $NO^{\cdot}$. H_2O_2 also serves as a substrate for the production of further detrimental ROS, such as hypochlorous acid ($HOCl$), generated by enzymatic conversion through neutrophil myeloperoxidase, and plasma levels of myeloperoxidase possess predictive value for coronary thrombotic events. Aside from enzymatic conversion, H_2O_2 reacts with ferrous iron (Fe^{2+}), generating ferric iron (Fe^{3+}) and the $OH^{\cdot}$ ("Fenton reaction") [6]. Oxides of nitrogen like $NO^{\cdot}$ and nitrogen dioxide ($NO_2^{\cdot}$) contain odd number of electrons and are free radicals [7]. Nitrogen dioxide is a powerful initiator of lipid peroxidation [8]. $NO^{\cdot}$ can also promote lipid peroxidation. In the presence of superoxide, $NO^{\cdot}$ forms peroxynitrite, a powerful oxidant capable of initiating lipid peroxidation and oxidizing lipid soluble antioxidants [9].

1.1.1 FREE RADICAL REACTIONS

Free radicals can act as both oxidants and reducing agents. When a free radical reacts with a non-radical compound, another free radical can be formed. This enables free radicals to induce chain reactions that may be thousands of events long, for example lipid peroxidation of polyunsaturated fatty acids [5] leading to cellular membrane destruction and consequent tissue damage. This happens only when the reactive oxygen species are formed at an excessive rate and overcome the cellular antioxidant defense mechanisms [10].

Radicals can react with other molecules in several ways. When two free radicals meet, their unpaired electrons can form a shared electron pair in a covalent bond and both radicals are lost. When a radical gives one electron to, takes one electron from or simply adds on to a non-radical, that non-radical becomes a radical. Since most molecules present in living organisms are non-radicals, any free radical produced in the body will most likely react with a non-radical and generate a new radical. Hence, free radical reactions in vivo tend to proceed as chain reactions [8, 11, 12, 13].

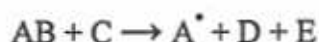
Chemically, free radicals are characterized for sustaining free radical chain reactions, a self propagating kind of reactions in which a free radical reactant yields a product that is also a free radical and which reacts producing another free radical. These feed-forward chemical processes are known as

propagation reactions and are the core of the free radical chain reactions.

Classically, free radical reactions are divided into:

- a) initiation reactions,
- b) propagation reactions, and
- c) termination reactions.

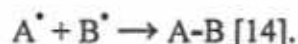
In the initiation reactions a free radical is formed from stable non-free radical chemical species



The propagation reactions involve the reaction of a free radical, also called the reaction center with a stable molecule giving another free radical or reaction center as product.



Whereas, in the termination reactions, two free radicals cancel out their unpaired electrons forming a stable product



1.2 OXIDATIVE STRESS

Oxidative damage inflicted by reactive oxygen species is referred to as "oxidative stress" [15, 16]. Oxidant stress is known to increase the production of free radicals. In the presence of metals, especially iron, these radicals are converted into more damaging species [17, 18].

1.2.1 IRON AND OXIDATIVE STRESS

Iron is an essential element for mammalian cell growth. It is a required constituent of numerous enzymes, including iron-sulphur and haem proteins of the respiratory chain, as well as ribonucleotide reductase, which catalyses the rate-limiting step in DNA synthesis [19].

Iron plays an important role in biology. Body growth may be impaired and mental retardation may occur if the iron supply is inadequate before the late teens. Iron demand after adolescence differs from a minimum for males to a maximum for pregnant females. In males, iron balance should be zero where iron absorbed in the intestine merely compensates for the iron loss from the gut mucosa and skin sequestration. Though iron absorption is regulated in the intestine, most males have a positive balance so that iron begins to accumulate in the liver as storage iron [20]. Iron ions circulate bound to plasma transferrin, and accumulate within cells in the form of ferritin. Under normal circumstances, only trace amounts of iron exist outside these physiologic sinks. In the healthy state there is never an appreciable concentration of 'free iron' (or iron chelated by low molecular weight compounds) [21]. Transferrin does not release its iron at a normal pH, while at a low pH, which may be the case in arterial wall, iron can be released from transferrin and induce oxidation of low density lipoprotein [22]. Serum ferritin levels are affected by age and sex. In normal individuals, ferritin levels are slightly higher at birth and decrease during childhood until puberty. After puberty, body iron storage in males increases progressively with a

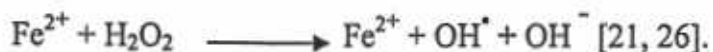
proportional rise in serum ferritin, whereas serum ferritin levels are lower and more stable in females during reproductive period. Ferritin levels only increase after the menopause [23].

The transition property of iron from ferrous ion to ferric ion and vice versa is essential for energy generation in mitochondria. Because the free radicals generated by transition element iron have high potential, all cell organelles are protected by a variety of anti-oxidative systems. Under pathological conditions, however, potential iron, even though small in amount, generates free radicals, resulting in peroxidation of membranous organelles known as oxidative stress. Iron-induced oxidative stress may be negligible in healthy subjects, but can be a trigger of organ damage in some sensitive hosts [20].

Iron catalyzes the formation of reactive oxygen species through the Fenton and Haber-Weiss reactions [24]. The generally accepted view is that the main biological actor in damaged tissues and the one causing oxidative damage to all kinds of molecules, for example, in lipid peroxidation, is mainly caused by the highly toxic $\text{OH}^\bullet$ generated by the transition metal-catalyzed Haber-Weiss reaction [18, 25].



Superoxide anions role in the iron or copper catalyzed Haber-Weiss reaction is the superoxide-assisted Fenton reaction:



The $\text{OH}^\bullet$ is capable of abstracting a hydrogen atom from polyunsaturated fatty acids (LH) to initiate lipid peroxidation.



Once lipid hydroperoxides (LOOH) accumulate, free iron may directly initiate additional lipid peroxidation.



The resulting accumulation of lipid hydroperoxides destroys membrane structure and function. That explains the threat of 'free iron' or 'labile iron' or 'chelatable iron' during life. It can produce an oxidative stress, involved in many pathological manifestations (arteriosclerosis, diabetes, ageing, etc.) [21].

Excess iron and iron overload can facilitate the occurrence of a variety of diseases, including cirrhosis, congestive heart failure, diabetes, and cancer. Pathologic iron accumulation affects many organs, such as the liver, pancreas, and heart, among others. Hereditary iron overload is characterized by excessive intestinal absorption and cellular deposition of iron and is found in several conditions, including hemochromatosis, African iron overload, and iron-loading anemias [27].

1.3 LIPID PEROXIDATION

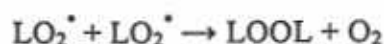
Free radical attacks all of the major class of biomolecules, but lipids are probably the most susceptible. Cell membranes are a rich source of poly-unsaturated fatty acids. They are readily attacked by oxidizing radicals. The oxidative destruction of poly-unsaturated fatty acids is known as lipid peroxidation. Lipid peroxidation then proceeds as a self perpetuating chain reaction, as it provides a continuous supply of free radicals that initiate further peroxidation [1]. Free radicals also cause oxidation of sulfhydryl containing enzymes leading to their inactivation, polysaccharide depolymerization, and base hydroxylation, nicking, cross linkage, scission of deoxyribonucleic acid (DNA) strands causing mutation and inhibition of protein, nucleotide and fatty acid synthesis [5].

Biological lipid peroxidation can be divided into two types:

- a) Non-enzymic lipid peroxidation, and
- b) Enzymic lipid peroxidation [7].

In non-enzymic lipid peroxidation, any free radical ($R^\bullet$) with sufficient energy to abstract a hydrogen atom from a methylene carbon of an unsaturated fatty acid can initiate a chain reaction in bulk lipid. The free-radical

chain reaction of lipid peroxidation propagates until two free radicals annihilate each other to terminate the chain reaction.



or



Whereas, enzymic lipid peroxidation refers only to generation of lipid peroxides at the active center of an enzyme. The hydroperoxides and endoperoxides produced are stereospecific and have important biological functions. Cyclooxygenase and lipoxygenase fulfill this definition. Free radicals are probably important intermediates in the reaction but are localized to the active sites of the proteins. During formation of endoperoxides by cyclooxygenase, a powerful oxidant is generated that is amenable to scavenging by some antioxidants [7].

1.3.1 LIPID PEROXIDATION PRODUCTS

The net amount of lipid peroxidation in a tissue reflects the balance between production and catabolism of the peroxides [28, 29]. Products of lipid peroxidation include hydroperoxides, conjugated dienes, malonaldehyde/malondialdehyde, and fluorochromic proteins or amino-containing phospholipids that develop intra- or intermolecular cross-links with malonaldehyde or malondialdehyde [30].

1.3.2 LIPID PEROXIDATION AND DISEASE

The balance between the increased creation of free radicals in various pathological states, or unfavourable conditions in the environment, and natural antioxidants of a low-molecular (vitamin C, E, glutathione etc.) or enzyme character (superoxide dysmutase, glutathione peroxidase, glutathione reductase, catalase, etc.), plays the chief role in damage which is the cause of many diseases and aging [18].

Free radical damages can accumulate over time and may thereby contribute to cell injury and development of human diseases. Free radicals have been implicated in the development of several diseases including atherosclerosis, diabetes, cancer, chronic inflammatory diseases and neurodegenerative diseases as well as in the process of aging [31, 32, 33, 34, 35, 36, 37].

1.3.3 LIPID PEROXIDATION AND BENEFIT

Free radicals are not only produced as an unwanted product; they are also formed deliberately in the body for useful purposes and have important physiological functions [8, 11, 13, 38]. A well defined role for free radicals is when activated phagocytic cells (neutrophils, monocytes, macrophages and eosinophils) produce superoxide anion radicals and hydrogen peroxide as one mechanism to kill bacteria and fungi and to inactivate viruses [39].

1.3.4 MEASUREMENT OF LIPID PEROXIDATION

Oxidative activity of oxygen radicals in biologic systems can be determined by measuring their oxidative products [40]. Malondialdehyde (MDA) and organic hydroperoxide (OHP), as lipid peroxidation intermediates, are measured to assess the status of oxygen free radicals. Changes in malondialdehyde and organic hydroperoxide concentration indirectly reflect alterations in the level of oxygen free radicals. Malondialdehyde is a volatile product formed from free radical mediated peroxidation of poly-unsaturated fatty acids in cell membranes. Organic hydroperoxide is formed during peroxidation of phosphatidylinositol and arachidonic acid, which also occur in biological membranes [41.Wang and Rogers, 1997]. However, malondialdehyde (MDA) can also be generated as a byproduct of prostaglandin biosynthesis [3.Freeman and Crapo, 1982; 30.Ward et al. 1985; Valenzuela, 1991]. Therefore, one of the most frequently used biomarkers providing an indication of the overall lipid peroxidation is the plasma concentration of malondialdehyde [40, 41, 42, 43].

1.4 ANTIOXIDANTS

The biological efficacy of oxidants is based on a highly regulated equilibrium between the production of oxygen radicals, the contracting defense mechanisms of antioxidant scavenging systems and repair enzymes for the elimination of the degraded bioproducts. Imbalance of this finely tuned, sophisticated equilibrium can result in oxidative stress unleashing a cascade of

pathological processes [44]. To control and reduce lipid peroxidation both in humans and in nature has invoked the use of antioxidants [45]. Antioxidants are compounds protecting other components of the organism (proteins, nucleic acids, lipids, lipoproteins, etc.) against pathogenic oxidations [46]. Propylgallate, butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT) are antioxidants used as food additives. Naturally occurring antioxidants include vitamin E (tocopherol, which is lipid soluble, and urate and vitamin C, which are water-soluble. β -carotene is an antioxidant at low partial pressure of oxygen [45]. Antioxidants fall into two classes:

- a) Preventive antioxidants, which reduce the rate of chain initiation, and
- b) Chain-breaking antioxidants, which interfere with chain propagation [45].

They exist in both the aqueous and membrane compartments of cells and can be enzymes or non-enzymes [1]. Preventive antioxidants include catalase and other peroxidases that react with lipid hydroperoxides, and chelators of metal ions such as diethylenetriamine penta-acetate (DTPA) and ethylenediaminetetraacetate (EDTA). Chain-breaking antioxidants are often phenols or aromatic amines. In vivo, the principal chain-breaking antioxidants are superoxide dismutases (SOD) which act in aqueous phase to trap superoxide free radicals; perhaps urate, and vitamin E, which act in liquid phase to trap lipid hydroperoxide radicals [45].

1.5 DIABETES MELLITUS

Diabetes mellitus is an ancient disease. Symptoms of diabetes mellitus have been found described on an Egyptian papyrus, called the Ebers papyrus, which was dated 1500 B.C. It is a combination of two words, diabetes and mellitus. The former is from a Greek word meaning "siphon" or "to pass through", and the latter is from a Latin word which means, "honey". The word mellitus was added to diabetes because of the sweet nature of the urine in this disease [47].

Diabetes diagnoses are increasing at an alarming rate worldwide [48]. It is one of the most common non-communicable diseases in the world. Diabetes affected about 135 million people in 1995 and the incidence is expected to rise to 300 million in the year 2025 [49]. Diabetes mellitus is a complex metabolic syndrome characterized by chronic hyperglycaemia and associated with alterations in carbohydrate, lipid, protein [50] and mineral metabolism [51]. The World Health Organization (WHO) has defined diabetes mellitus on the basis of laboratory findings as:

- ♦ a fasting plasma venous glucose concentration greater than 7.8 mmol/litre (140mg/dl) or
- ♦ plasma glucose concentration of 11.1 mmol/litre (200 mg/dl) or more two hours after a carbohydrate diet or two hours after oral ingestion of equivalent of 75g of glucose, even if the fasting glucose concentration is normal [52].

Diabetes mellitus is divided into two broad classes as:

- 1- Primary (idiopathic) Diabetes Mellitus, and
- 2- Secondary Diabetes Mellitus [53].

1.5.1 PRIMARY (IDIOPATHIC) DIABETES MELLITUS:

Primary diabetes mellitus has no defined cause [50]. This class is further subdivided into:

- a) Type 1 diabetes mellitus, and
- b) Type 2 diabetes mellitus [50, 53].

Diabetes mellitus is a disease characterized by a near-absolute (type 1) or relative (type 2) deficiency in the number of pancreatic beta cells [54], leading to absolute or relative deficiency of insulin [52]. By the year 2025, India alone would have 57 million diabetics mainly of type 2 diabetes constituting 90% of the diabetic population [55].

1.5.1a TYPE 1 DIABETES MELLITUS:

Type 1 diabetes, also called insulin-dependent diabetes mellitus (IDDM) and previously referred to as "juvenile-onset diabetes". This variant accounts for 10% to 20% of all cases of primary diabetes. Type 1 diabetes usually develops in childhood, becoming manifest and severe at puberty [53]. The age of

the patient at the time of diagnosis is usually, but not necessarily under 25 years [56]. Type 1 diabetes mellitus is usually characterized by an abrupt onset of symptoms. This form of diabetes results from a severe, absolute lack of insulin caused by reduction in the beta-cell mass. Patients depend on insulin for survival; hence the term "insulin-dependent diabetes mellitus". Without insulin, they develop serious metabolic complications such as acute ketoacidosis and coma [53, 57].

Type 1 diabetes is associated with a positive family history in less than 10% of the first degree relatives. There is a 50% chance for occurrence of type 1 diabetes in identical twins. Male and female are equally affected by type 1 diabetes. They are usually thin and rarely exhibit loss of weight. [56].

Type 1 (insulin-dependent) diabetes shows a primary association with HLA class II genes on chromosome 6, most notably HLA-DR 3 and DR 4, a determining feature of this type of the disease [58]. There is also prevalence of autoantibodies to pancreatic islet cells in this type of diabetes. Type 1 diabetes is considered as a chronic auto-immune disease, which after progressing through different stages leads to destruction of pancreatic beta-cells, with a gradual decline in insulin secretion [59].

The onset of the disease is associated with some specific infections, which include rubella, mumps, coxsackie virus, infectious hepatitis, infectious mononucleosis and cytomegalovirus. Type 1 insulin-dependent diabetes can also be induced by drugs such as alloxan, streptozotocin and vacor (rodenticide) which selectively destroy the pancreatic beta-cells [57].

In type 1 diabetes there is functional insulinopenia. The level of fasting plasma insulin is low with little or no response to administration of glucose, amino acids, tolbutamide, glucagons or other beta-cell stimulants [50].

1.5.1b TYPE 2 DIABETES MELLITUS:

Type 2 diabetes also called non-insulin-dependent diabetes mellitus (NIDDM) and previously referred to as "adult-onset diabetes". This variant accounts for 80% to 90% of all cases of primary diabetes. Much less is known about the pathogenesis of type 2 diabetes, despite its being by far the most common type [53]. The age of the patient at the time of diagnosis is usually over 40 years. Female are more affected than the men. Type 2 diabetes is associated with a positive family history in more than 20% of the first degree relatives. There is a 90% to 100% chance of occurrence of type 2 diabetes in identical twins [56]. There is no evidence that autoimmune mechanisms are involved in the pathogenesis of type 2 diabetes mellitus. Unlike type 1 diabetes, the disease is not linked to any HLA genes [53].

The two metabolic defects that characterize type II diabetes are a derangement in beta-cell secretion of insulin (beta-cell dysfunction) and an inability of peripheral tissues to respond to insulin (insulin resistance) [53, 60].

Hyperinsulinaemia, which reflects insulin resistance, especially in the presence of normoglycemia is an early abnormality that is characteristic of subjects destined to develop impaired glucose tolerance or Type 2 diabetes mellitus

[61]. Other factors such as obesity, aging [62], and physical inactivity are associated with insulin resistance [63].

Pancreatic beta-cells respond to other insulin secretagogues such as isoprel, arginine, glucagons, and tolbutamide even when the responsiveness to glucose has been lost which demonstrate the persistence of synthesis and storage of insulin [57].

Type 2 diabetics are not prone to ketosis and not dependent on insulin treatment to sustain life. However, they may require insulin for correction of hyperglycemia if this cannot be achieved with diet or oral agents [57]. The deficiency in beta-cell production of insulin and abnormalities in the dynamics of insulin secretion are well-recognized features associated with non-insulin dependent diabetes mellitus [64].

1.5.2 SECONDARY DIABETES MELLITUS:

Secondary diabetes mellitus defines a class of patients in whom hyperglycemia is associated with some other disease, such as endocrine disorders, pancreatic diseases, drugs [50, 53], a variety of genetic syndromes [53] and insulin receptor abnormality [50, 56].

Endocrine disorders leading to secondary diabetes mellitus are acromegaly, Cushing's syndrome, pheochromocytoma [50, 65], primary aldosteronism, and glucagonoma [50].

Pancreatic diseases causing secondary diabetes are chronic pancreatitis, hemochromatosis, pancreatic carcinoma and pancreatectomy [50].

1.5.3 COMPLICATIONS OF DIABETES MELLITUS:

Diabetes mellitus is an endocrine disorder, which comprises of a large number of complications. Most of the available experimental and clinical evidence suggests that the complications of diabetes result from metabolic derangements mainly hyperglycemia [53]. They can be divided into two broad classes:

- a- Acute complications, and
- b- Chronic complications [56].

Acute diabetic complications are diabetic ketoacidosis, hyperosmolar non-ketotic coma, and hypoglycemic coma [56, 66]. Acute complications of diabetes mellitus are life threatening that cause rapid mental and physical deterioration and require prompt treatment [56].

Diabetic ketoacidosis is an important cause of death, particularly in younger type 1 diabetic patients, where other causes of death are low. It can also occur in patients with type 2 diabetes mellitus under conditions of severe stress [67]. Hyperosmolar nonketotic coma is much less common than diabetic ketoacidosis, but it has a much higher mortality rate. It occurs mainly in elderly patients with type 2 diabetes [56]. Hypoglycemic coma is the commonest of the

diabetic emergencies. Hypoglycemia is much commoner in the insulin treated than in the non-insulin treated patients [67].

Chronic complications are associated with long standing diabetes mellitus of either type. They include microvascular disorders, macrovascular diseases, and diabetic neuropathy [56].

Macrovascular disorders include atherosclerotic disease of the medium and large vessels. Atherosclerosis of the vessels may be due to increased blood glucose level. Atherosclerosis of coronary arteries is twice as common in diabetics than in non-diabetics. Atherosclerosis of the coronary arteries leads to the myocardial infarction, which is the most common cause of death in diabetes [68].

1.5.4 METABOLIC DISTURBANCES IN DIABETES MELLITUS

Metabolic derangements in diabetes mellitus are due to absolute or relative lack of insulin, which affects not only carbohydrate metabolism but also lipid and protein metabolism [69].

The plasma glucose level is elevated in diabetes mellitus but if it exceeds 11mmol/litre, the glucose starts appearing in the urine, called glycosuria [52]. The increased urinary glucose concentrations lead to osmotic diuresis and in this way polyuria occurs. Osmotic diuresis causes dehydration which leads to polydipsia, and due to prolonged osmotic diuresis large amounts of urinary electrolytes such as sodium, potassium, magnesium and phosphorus are lost.

Diabetic patients develop polyphagia the pathway of which are not completely defined. This completes the triad of diabetic findings – the polyuria, polydipsia and polyphagia [69].

Increased breakdown of protein starts, which leads to muscle wasting. Proteolysis causes release of amino acids in the blood. Of these amino acids the glucogenic amino acids are taken up by the liver and used in gluconeogenic pathways forming more glucose and worsening the situation [69].

Insulin deficiency also causes lipolysis. The plasma free fatty acid level rises due to enhanced lipolysis. The free fatty acids are taken up by the liver and converted either to acetyl CoA and ketone bodies or they are re-esterified to form endogenous triglycerides and then being incorporated in the very low density lipoprotein (VLDL). Lipoprotein lipase (LPL) hydrolyses chylomicrons and VLDL triacylglycerol and phospholipids and causes release of fatty acids which are then either oxidized or stored, depending upon the requirements of the tissues. The deficiency of insulin causes less synthesis of LPL and this leads to accumulation of chylomicrons and VLDL in the blood. This is the case in type 1 diabetes, whereas in type 2 diabetes there is hypertriglyceridemia along with increase in VLDL and usually not hyperchylomicronemia [70].

In both types of diabetes that is type 1 and 2, the rate of cholesterol synthesis is also increased and along with that an associated increase of low density lipoprotein (LDL) [70].

Lipid disorders are related to the degree of glycaemic control in diabetic persons. The most common lipid abnormalities seen in diabetic persons are elevated VLDL-cholesterol and triglyceride levels and decreased high density lipoprotein cholesterol (HDL-cholesterol) concentrations [71].

1.5.5 DIABETES MELLITUS AND OXIDATIVE STRESS:

Oxidative stress has been demonstrated to be present in animal models as well as in patients with diabetes and has been suggested as a possible contributor to the accelerated atherosclerosis seen in diabetics. The generation of reactive oxygen species in diabetics occur via several mechanisms and is initiated not only by glucose, but also by other substances that are found at elevated levels in diabetic patients [48]. The generation of oxygen free radicals is suggested to be increased in the diabetic state. A local excess of these radicals in the vascular system can induce profound endothelial cell dysfunction, such as vascular permeability via stimulation of platelet activation factor production, abnormal coagulation via inhibition of prostaglandin I_2 synthesis, and cell lysis. These dysfunctions in endothelial cell may be found in diabetes [72]. In diabetics there is increased oxidation of lipids leading to increased levels of lipid peroxides and other end products that have been implicated in the pathogenesis of diabetic complications [73].

1.6 CORONARY HEART DISEASE

Cardiovascular disease is the leading source of morbidity, disability, and mortality in Western industrialized countries, and atherosclerosis is unquestionably the main underlying pathology [74]. The general term for hardening of the arteries is arteriosclerosis. The most prevalent form of arteriosclerosis is characterized by the accumulation of lipid, particularly cholesteryl esters, in the arterial intima. This form of arteriosclerosis is called atherosclerosis. It can lead to myocardial infarction, stroke, aneurysm, or gangrene [75]. Atherosclerosis is derived from the Greek "athera" means gruel or porridge [76, 77], and "sclerosis" stands for hardening [76]. Atherosclerotic coronary artery disease is the focal narrowing of the large and medium sized coronary arteries due to the intimal proliferation of smooth muscle cells and the deposition of lipids [78].

1.6.1 PATHOGENESIS OF ATHEROSCLEROSIS

Atherosclerosis is basically characterized by intimal plaques called "atheromas" that protrude into the lumen, weaken the underlying media, and undergo a series of complications [77]. Lipid deposits initially occur inside arterial smooth muscle cells and macrophages. At this stage the lipid deposits are reversible, and no permanent damage to the vessel wall results. This early lesion is called a fatty streak. Subsequently, lipids begin to accumulate extracellularly in the arterial intima. Ultimately the lipid deposit triggers a fibrotic reaction in the arterial wall, and collagen is deposited to form a fibrous plaque. At this point the process is

no longer completely reversible [75]. This chronic inflammatory reaction results from a sequence of events that begins with the trapping of low density lipoprotein (LDL) in the subendothelial space of the artery wall. The trapped LDL is seeded with oxidative species released by the overlying endothelium, and lipid oxidation is initiated within the LDL particle [79]. This minimally modified LDL induces local vascular cells to produce monocyte chemotactic protein 1 and granulocyte and macrophage colony-stimulating factors, which stimulate monocyte recruitment and differentiation to macrophages in arterial walls. The accumulating monocytes and macrophages stimulate further peroxidation of LDL. The products of this reaction make the protein component of LDL (apolipoprotein B-100) more negatively charged. By virtue of its increased negative charge, this completely oxidized LDL is recognized by scavenger receptors on macrophages and internalized to form so called foam cells. In contrast to the uptake of native (un-oxidized) LDL by LDL (apolipoproteins B and E) receptor on macrophages, the uptake of oxidized LDL by the scavenger-receptor pathway is not subject to negative-feedback regulation and thus results in massive uptake of cholesterol (from oxidized LDL) by the macrophages. In addition to promoting the formation of foam cells, oxidized LDL has direct chemotactic activity for monocytes and stimulates the binding of monocytes to the endothelium. Once monocytes cross the endothelial layer, they become trapped in the subendothelial space, partly because oxidized LDL inhibits their egress from the arterial wall. Oxidized LDL is also cytotoxic to vascular cells, thus promoting the release of lipids and lysosomal enzymes into the intimal extracellular space and enhancing the progression of atherosclerotic lesions [80]. Calcium is deposited within the plaque and the lesion

grows; the result is a decrease in the diameter of the channel through which the blood flows. Subsequently the plaque can degenerate, producing a roughened intimal surface. This, together with the increased turbulence of blood flow resulting from the narrowed arterial lumen, predisposes to clot formation within the blood channel. If the occlusion of the channel persists, the cells that are normally supplied with oxygen by the vessel will become anoxic and ultimately die; the death of tissue is termed an infarction [75].

Atherosclerosis can occur in many different arteries, and the clinical disease that results depends on which vessels are involved. The most frequent location in humans is the coronary arteries, leading to myocardial infarction, which is known commonly as "heart attack" [75].

Myocardial infarction occurs, when for a prolonged period of time, the oxygen requirement of the myocardium exceed the myocardial oxygen supply. This reduced oxygen supply is due severe reduction in coronary blood flow [81]. Coronary heart disease is diagnosed when there is a documented evidence of myocardial infarction (recent or past) or documented evidence of angina with subsequent sudden death or congestive heart failure [82].

1.6.2 RISK FACTORS

The high rate of coronary heart disease in westernized countries is due to the life style factors present in these societies. Autopsy studies, in such populations revealed marked atherosclerosis [83]. The prevalence and severity of

the disease, and therefore the age when it is likely to cause tissue or organ injury, are related to a number of factors, some constitutional and therefore immutable, but others acquired and potentially controllable. The constitutional factors include age, sex, and familial background.

Age: Age is a dominant influence. Although early lesions of atherosclerosis appears in childhood, clinically significant disease, as judged by death rates from ischemic heart disease, rises with each decade, even into advanced age.

Sex: Other factors being equal, males are much more prone to atherosclerosis than females. Females are more or less sheltered from advanced, disease-producing atherosclerosis until menopause, so myocardial infarction is uncommon in premenopausal women unless they are predisposed by diabetes or some unusual (possibly familial) form of hyperlipidemia or have severe hypertension.

Familial Predisposition: There is a well-defined familial predisposition to atherosclerosis and ischemic heart disease. In some instances it relates to familial clustering of other risk factors, such as hypertension and diabetes. In other instances, it involves well-defined hereditary genetic derangements in lipoprotein metabolism that result in excessively high blood lipid levels. The prototype of these conditions is familial hypercholesterolemia, but in addition there are a growing number of familial dyslipoproteinemias, many of which result from mutations that yield defective apolipoproteins [77].

There are four major acquired risk factors that are at least in some part amenable to control: hyperlipidemia, hypertension, cigarette smoking, and diabetes mellitus [77].

Hypertension, hypercholesterolaemia and cigarette smoking are considered as important risk factors in the development of coronary heart disease [84]. Coronary heart disease is frequently associated with diabetes mellitus [85, 86]. Mortality due to cardiovascular disorders is more common in Type 2 diabetes than in Type 1 diabetes mellitus [87]. A number of minor risk factors have also been implicated in the development of coronary atherosclerosis, of which certain trace elements, water hardness, hypercalcemia, hypercoagulability, vasectomy, coffee consumption, hyperuricemia are note worthy. The effect of all these factors in the genesis of coronary atherosclerosis is not completely understood [88]. Oral contraceptives are associated with an increased incidence of myocardial infarction. In smoker women who use oral contraceptives, the increase is more dramatic in incidence of myocardial infarction than in non-smoking women using oral contraceptives. Gout, type A personality, premature arcus senilis, obesity, hypertriglyceridemia, and diagonal ear lobe crease also are associated with an increased risk of atherosclerotic coronary artery disease [78].

1.6.3 CONSEQUENCES OF CORONARY ATHEROSCLEROSIS

Depending on the rate and severity of coronary artery narrowing and the myocardial response, one of four syndromes may develop:

1. Angina pectoris,
2. Acute myocardial infarction,
3. Sudden cardiac death, and
4. Chronic ischemic heart disease with congestive heart failure [89].

More than half of all deaths in the United States are caused by cardiovascular disease, and most of these are attributable to myocardial or cerebral infarction. An infarct is an area of ischemic necrosis caused by occlusion of either the arterial supply or the venous drainage in a particular tissue [90]. Myocardial infarction occurs when myocardial oxygen demand severely exceeds myocardial oxygen supply for a prolonged period of time. This usually is due to a severe reduction in coronary blood flow produced by coronary occlusion [78].

1.6.4 METABOLIC DISTURBANCES IN CORONARY HEART DISEASE

Elevated plasma cholesterol levels are associated with coronary heart disease, resulting in myocardial infarction [91]. The most serious

consequence of an elevation in plasma lipoproteins is atherosclerosis, an arterial disease that causes coronary heart disease and stroke. Increases in low density lipoprotein (LDL), intermediate density lipoprotein (IDL), and very low density lipoprotein (VLDL) lead to this problem. These lipoproteins are rich in cholesterol, giving rise to the view that plasma cholesterol elevations are unhealthy. High density lipoprotein (HDL) also contains cholesterol. High HDL levels afford protection against coronary heart disease, and elevated cholesterol in the form of HDL is beneficial, not harmful. This is the so called "good" cholesterol, as distinct from the harmful cholesterol associated with LDL, IDL, and VLDL [92]. Increased levels of plasma LDL, IDL, and low levels of HDL are found in coronary heart disease [88]. Elevated level of plasma lipoprotein (a) are associated with an increased risk of premature myocardial infarction and in vitro data suggests that lipoprotein (a) may promote thrombosis [93].

1.6.5 CORONARY HEART DISEASE AND OXIDATIVE STRESS:

Oxidative stress is involved in the pathogenesis of atherosclerosis [94]. High blood pressure and increased heart rate can increase consumption of oxygen by the heart, causing increased production of free radicals. If the body's antioxidant system does not respond to quench these increased levels of free radicals, oxidative damage to the heart muscle can result. This is one of the mechanisms whereby obesity can promote lipid peroxidation of the myocardium, the middle layer of the heart composed of heart muscle. Oxidative stress can also impair the ability of the endothelium, the inner layer of cells that line the blood vessels, to expand and dilate in response to blood flow. Based on this scenario, an

accumulation of reactive oxygen species has been linked to "cardiac contractile dysfunction," potentially leading to arrhythmia and heart attack [95]. Lipid peroxidation plays a key role in rendering low density lipoprotein atherogenic [96]. Oxidation, particularly oxidative modification of low density lipoproteins within the artery wall and its subsequent unregulated uptake by macrophages, has been postulated to be an important in disease development [97].

1.7 PURPOSE OF STUDY

Diabetes is a major worldwide health problem predisposing to markedly increased cardiovascular mortality. Elevated blood glucose levels in patients with diabetes mellitus are highly correlated with increased incidence and severity of vascular complications.

To summarize the aforementioned literature it can be said that in diabetes and coronary heart disease there is increased generation of reactive oxygen species. Free iron in the circulation can also produce free radicals, which, in turn cause oxidative stress in diabetes that may promote coronary atherosclerotic disease. So far no study has been reported, at least in Pakistan to ascertain these derangements in the patients suffering from diabetes mellitus, coronary heart disease or in diabetic patients having coronary heart disease.

The present study has been designed to evaluate and compare the magnitude of serum malondialdehyde (an oxidative stress marker), and biochemical markers of body iron stores (serum ferritin and transferrin saturation) in control healthy subjects, diabetes mellitus (Type 1 and Type 2) patients, and in patients suffering from coronary heart disease. Further, to compare the serum levels of these parameters in diabetic (Type 2) patients with and those without coronary heart disease.

**MATERIAL
AND
METHODS**

Patients suffering from diabetes mellitus or coronary heart disease or both were included in the study. Both Type 1 and Type 2 diabetes mellitus patients having a history of at least four years and Coronary heart disease patients who had first attack of myocardial infarction in the last ten days were included in the study. These subjects were selected randomly from amongst the patients attending the outpatient departments, medical and cardiology units of Khyber Teaching Hospital and Hayatabad Medical Complex, Peshawar, Pakistan.

The diabetic patients were diagnosed on the basis of WHO Criteria [52, 98]. Coronary heart disease patients were diagnosed on the basis of electrocardiography (ECG) and laboratory investigations. One hundred and ninety diabetic patients were enrolled in the study, out of which 90 were Type 1 and 100 were Type 2 diabetics. Moreover 140 patients with coronary heart disease and 80 Type 2 diabetics with coronary heart disease were also part of this study. The control group comprised of one hundred healthy individuals having no history of diabetes mellitus, coronary heart disease, hypertension or any other disease. The control subjects were selected from doctors and staff of Khyber Teaching Hospital and Hayatabad Medical Complex, Peshawar, and also from staff of other institutes of Peshawar, Pakistan. All patients suffering from, diabetes mellitus, coronary heart disease, or diabetics having coronary heart disease, and control subjects taking lipid lowering drugs, oral contraceptive pills, and those on multivitamins, especially antioxidant vitamins, were not included.

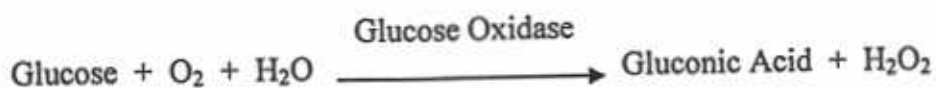
Written informed consent was taken from patients as well as the control healthy subjects. Blood (8 ml) was taken after overnight fast of 12-14 hours, from the antecubital vein of the patients as well as the control subjects. Blood was allowed to clot and serum was separated by centrifugation at 2500 rpm for 15 minutes. Glucose determination was done immediately. Remaining serum was stored at -20° C until analyzed for lipid profile, malondialdehyde, and parameters of iron profile.

2.1 DETERMINATION OF SERUM GLUCOSE

Serum glucose was estimated by enzymatic colorimetric method, using kit cat. No. GL 1001 supplied by Randox Laboratories Ltd. Ardmore, United Kingdom.

2.1.1 PRINCIPLE

Glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed reacts, under catalysis of peroxidase, with phenol and 4-aminophenazone to form a red-violet quinoneimine dye as indicator.



2.1.2 REAGENTS

1. GOD-PAP Reagent.

Phosphate Buffer	0.1 mol/l, pH 7.0
Phenol	11 mmol/l.
4-aminophenazone	0.77 mmol/l.
Glucose Oxidase	≥ 1.5 kU/l.
Peroxidase	≥ 1.5 kU/l.

2. Standard

Glucose

100 mg/dl.

2.1.3 PREPARATION OF SOLUTIONS

GOD-PAP Reagent. Contents ready for use. Stable up to the expiry date when stored at +2 to +8 °C.

2.1.4 PROCEDURE

1. The blank, standard and serum sample tubes were prepared as follows:

	BLANK	STANDARD (ST)	SAMPLE (S)
GLUCOSE STANDARD	-----	20 µl.	-----
SERUM SAMPLE	-----	-----	20 µl.
REAGENT	2000 µl.	2000 µl.	2000 µl.

2. The contents of the above tubes were thoroughly mixed and incubated at 15-25 °C for 25 minutes.
3. The absorbance (A) of the standard and the sample were measured against the blank within 60 minutes in Spectrophotometer R&M, S-200 at 500 nm.

2.1.5 CALCULATIONS

$$\text{Glucose (mg/dl)} = \frac{A_s}{A_{ST}} \times 100$$

2.1.6 PREPARATION OF CALIBRATION CURVE

A calibration curve was prepared by using the glucose standards containing 100, 200, 300, 400, and 500 mg/dl. Absorbance of the above standards was plotted against their concentrations as in figure-1.

2.2 DETERMINATION OF SERUM TRIGLYCERIDES

Serum triglycerides were estimated by the enzymatic colorimetric method, using kit cat. No. TR 212 supplied by Randox Laboratories Ltd. Ardmore, United Kingdom.

2.2.1 PRINCIPLE

Triglycerides are determined after enzymatic hydrolysis with lipases. The indicator is a quinoneimine formed from hydrogen peroxide, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase.

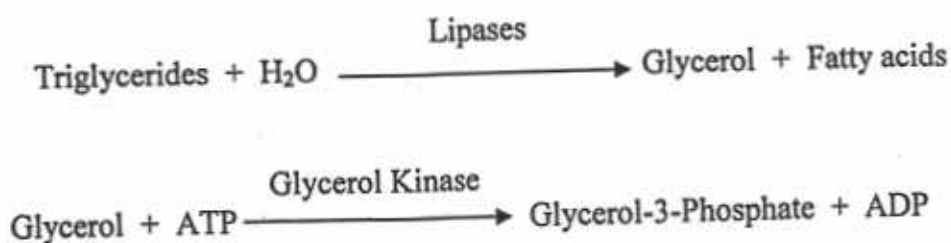
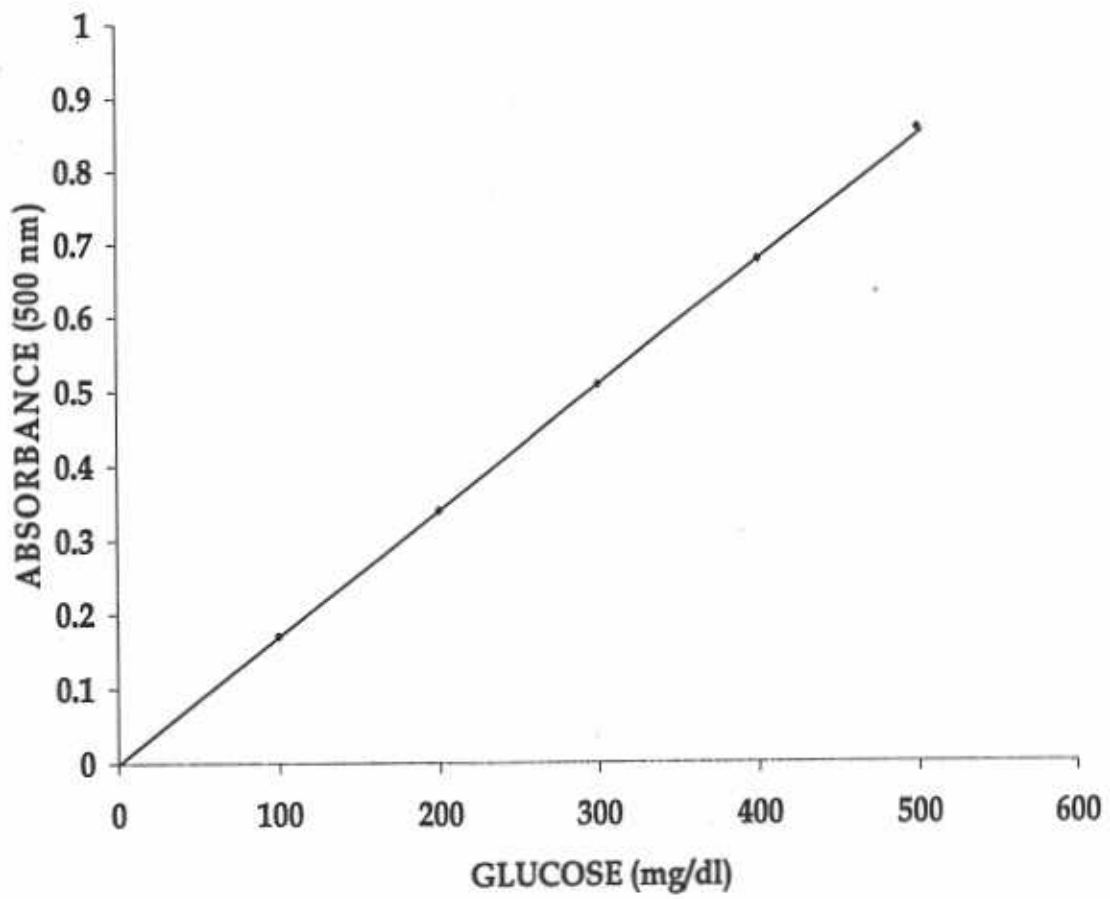
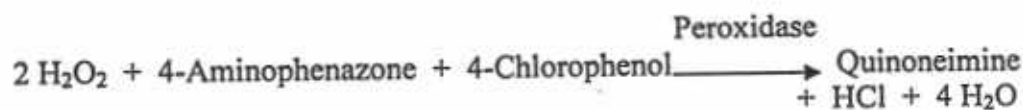
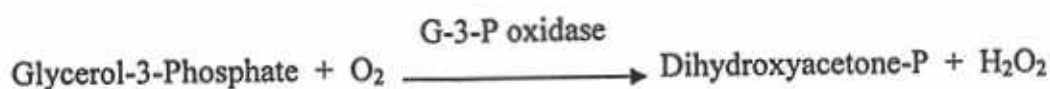


FIGURE-1
CALIBRATION CURVE FOR SERUM
GLUCOSE





2.2.2 REAGENTS

1. Buffer

Pipes Buffer	40 mmol/l, pH 7.6
4-Chlorophenol	5.5 mmol/l.
Magnesium ions	17.5 mmol/l.

2. Enzyme Reagent

4-Aminophenazone	0.5 mmol/l.
ATP	1.0 mmol/l.
Lipases	≥ 150 U/ml
Glycerol Kinase	≥ 0.4 U/l.
Glycerol-3-phosphate oxidase	≥ 1.5 U/ml
Peroxidase	≥ 0.5 U/ml

3. Standard

Glycerol equivalent to 200 mg/dl triolein.

2.2.3 PREPATION OF SOLUTION

1. Buffer.

Contents ready for use. Stable up to the expiry date when stored at + 2 to + 8 °C.

2. Enzyme Reagent.

Reconstitute one vial of Enzyme Reagent 2 with a portion of Buffer 1 and then transfer entire contents to bottle 1, rinsing vial 2 several times. Stable for 21 days at + 2 to + 8 °C, stored protected from light.

2.2.4 PROCEDURE

1. The blank, standard and serum sample tubes were prepared as follows:

	BLANK	STANDARD (ST)	SAMPLE (S)
TRIGLYCERIDE STANDARD	-----	10 µl.	-----
SERUM SAMPLE	-----	-----	10 µl.
WORKING REAGENT	1000 µl.	1000 µl.	1000 µl.

2. The contents of the tubes were mixed and incubated at 20-25 °C for 10 minutes.

3. The absorbance (A) of the standard and the sample were measured against the blank within 60 minutes in Spectrophotometer R&M, S-200 at 500 nm.

2.2.5 CALCULATIONS

$$\text{Triglycerides (mg/dl)} = \frac{A_s}{A_{ST}} \times 200$$

2.2.6 PREPARATION OF CALIBRATION CURVE

Glycerol standard containing 100, 200, 300, 400, and 500 mg/dl, was treated as described for serum sample. The absorbance obtained were plotted against their corresponding glycerol concentrations as in figure-2.

2.3 DETERMINATION OF SERUM TOTAL CHOLESTEROL

Serum cholesterol was estimated by enzymatic colorimetric method, using kit cat. No. CH 201 supplied by Randox Laboratories Ltd. Ardmore, United Kingdom.

2.3.1 PRINCIPLE

The cholesterol is determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantipyrine in the presence of phenol and peroxidase.

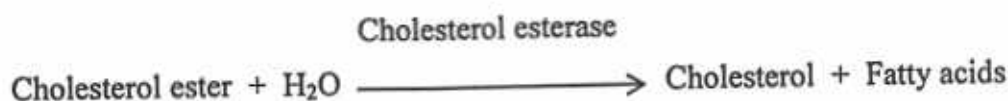
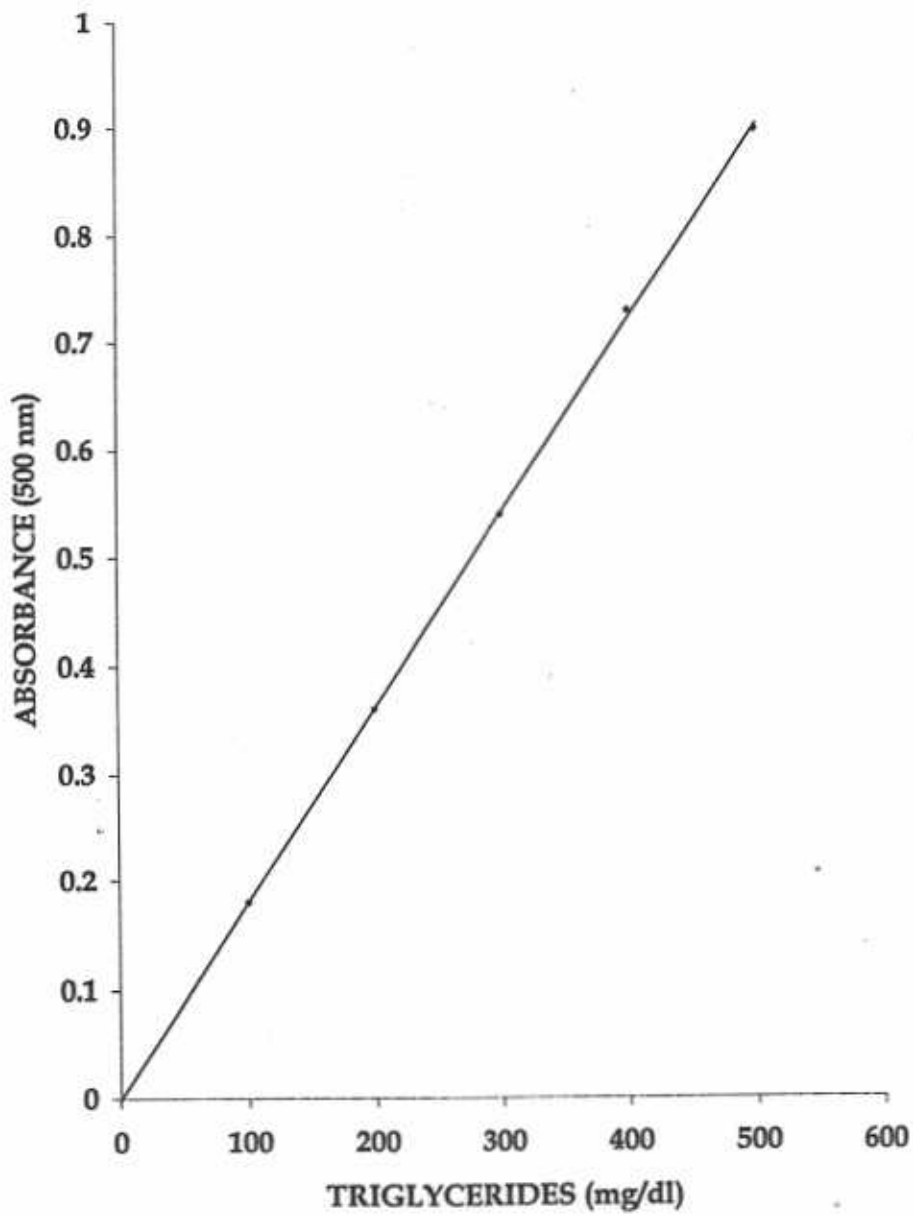
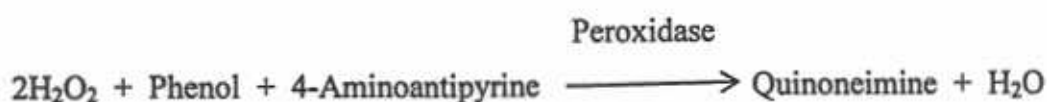
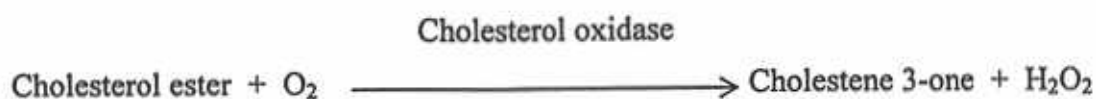


FIGURE-2
CALIBRATION CURVE FOR SERUM
TRIGLYCERIDES





2.3.2 REAGENTS

Reagent Contents

4-Aminoantipyrine	0.30 mmol/l.
Phenol	6 mmol/l.
Peroxidase	> 0.5 U/ml.
Cholesterol esterase	> 0.15 U/ml.
Cholesterol oxidase	> 0.1 U/ml.
Pipes Buffer	80 mmol/l ; pH 6.8

Standard

Cholesterol	200 mg/dl.
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2.3.3 PREPARATION OF SOLUTION

Contents ready for use. The reagent is stable up to the expiry date, when stored at + 2 to + 8 °C, protected from light.

2.3.4 PROCEDURE

1. The blank, standard and serum sample tubes were prepared as follows:

	BLANK	STANDARD (s _T)	SAMPLE (s)
CHOLESTEROL STANDARD	-----	10 µl.	-----
SERUM SAMPLE	-----	-----	10 µl.
REAGENT	1000 µl.	1000 µl.	1000 µl.

2. The contents of the above tubes were mixed thoroughly and incubated at + 20 to + 25°C for 10 minutes.
3. The absorbance (A) of the standard and the sample were measured against the blank within 60 minutes in Spectrophotometer R&M, S-200 at 500 nm.

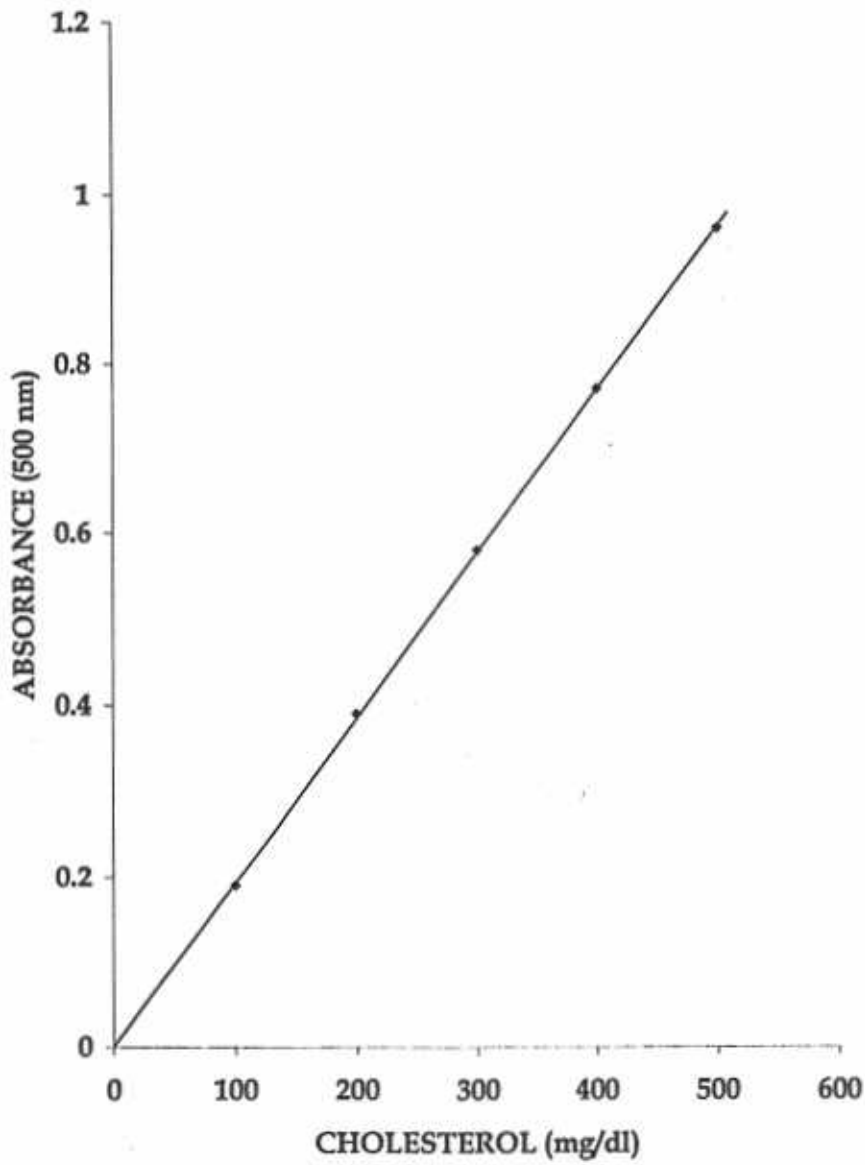
2.3.5 CALCULATIONS

$$\text{Cholesterol (mg/dl)} = \frac{A_s}{A_{ST}} \times 200$$

2.3.6 PREPARATION OF CALIBRATION CURVE

Cholesterol standard solution containing 100, 200, 300, 400, and 500 mg/dl were used in the preparation of calibration curve as in figure-3.

FIGURE-3
CALIBRATION CURVE FOR SERUM TOTAL
CHOLESTEROL



2.4 DETERMINATION OF SERUM

HDL-CHOLESTEROL

Serum HDL-cholesterol was precipitated by using kit cat. No. CH 204 supplied by Randox Laboratories Ltd. Ardmore, United Kingdom.

2.4.1 PRINCIPLE

Low density lipoproteins (VLDL and LDL) and chylomicron fractions are precipitated quantitatively by the addition of phosphotungstic acid in the presence of magnesium ions. After centrifugation, the cholesterol concentration in the HDL (High density lipoprotein) fraction, which remains in the supernatant, is determined.

2.4.2 REAGENTS

Reagent Contents

Phosphotungstic acid	0.55 mmol/l.
Magnesium Chloride	25 mmol/l.

2.4.3 PREPARATION OF SOLUTION

Macro Assay: Contents ready for use undiluted. Stable up to the expiry date specified when stored at + 15 to + 25°C.

2.4.4 ADDITIONAL REAGENTS REQUIRED

Reagent Solution for Cholesterol CHOD-PAP Assay Cat. No. CH 201 supplied by Randox Laboratories Ltd. Ardmore, United Kingdom.

Reagent Contents

4-Aminoantipyrine	0.30 mmol/l.
Phenol	6 mmol/l.
Peroxidase	> 0.5 U/ml.
Cholesterol esterase	> 0.15 U/ml.
Cholesterol oxidase	> 0.1 U/ml.
Pipes Buffer	80 mmol/l ; pH 6.8

Standard

HDL-Cholesterol	40 mg/dl.
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2.4.5 PREPARATION OF ADDITIONAL SOLUTION

Contents ready for use. The reagent is stable up to the expiry date, when stored at + 2 to + 8 °C, protected from light.

2.4.6 PROCEDURE

A. Precipitation:

- 1) 500 µl. of serum sample was added to 1000 µl. of the precipitant reagent in a centrifuge tube.
- 2) The contents of the tube were mixed on vortex shaker and allowed to stand for 10 minutes at room temperature.
- 3) Tubes were then centrifuged at 4000 r.p.m. for 10 minutes.
- 4) Clear supernatant was separated immediately.

B. Cholesterol CHOD-PAP Assay:

1. The blank, standard and serum sample tubes were prepared as follows:

	BLANK	STANDARD (ST)	SAMPLE (S)
HDL-CHOLESTEROL STANDARD	-----	10 µl.	-----
SERUM SAMPLE	-----	-----	10 µl.
REAGENT	1000 µl.	1000 µl.	1000 µl.

2. The contents of the above tubes were mixed thoroughly and incubated at + 20 to + 25° C for 10 minutes.
3. The absorbance (A) of the standard and the sample were measured against the blank within 60 minutes in Spectrophotometer R&M, S-200 at 500 nm.

2.4.7 CALCULATIONS

$$\text{HDL-cholesterol (mg/dl)} = \frac{A_S}{A_{ST}} \times 40 \times 2 \text{ (dilution)}$$

2.5 CALCULATION OF LDL-CHOLESTEROL

LDL-cholesterol was calculated according to formula by Friedewald et al. 1972. [99].

$$\text{LDL-cholesterol (mg/dl)} = \text{Total cholesterol} - \frac{\text{TG}}{5} - \text{HDL-cholesterol}$$

TG = triglyceride.

2.6 CALCULATION OF VLDL-CHOLESTEROL

VLDL-cholesterol was calculated according to the formula proposed by Wilson, cited by Delong et al. 1986. [100].

$$\text{VLDL-cholesterol} = 0.2 \times \text{Triglycerides}$$

2.7 DETERMINATION OF MALONDIALDEHYDE

Malondialdehyde (MDA) was measured as thiobarbituric acid (TBA) activity by using the method recommended by Buege and Aust cited by Valenzuela, 1991. [101].

2.7.1 PRINCIPLE

Malondialdehyde of the serum sample reacts with thiobarbituric acid to form a pink coloured pigment, the absorbance of which is measured at 535 nm.



2.7.2 REAGENTS

- | | | |
|----|----------------------------|-------------|
| 1. | Trichloroacetic acid | 15% |
| 2. | Hydrochloric acid | 0.25 N. |
| 3. | Thiobarbituric acid | 0.375% |
| 4. | Standard | |
| | 1,1,3,3-tetraethoxypropane | 0.997 gm/ml |

2.7.3 WORKING REAGENT

Working reagent of thiobarbituric acid was prepared by mixing 1:1:1 (vol./vol./vol.) solution of 15% trichloroacetic acid, 0.25 N hydrochloric acid and 0.375% thiobarbituric acid.

2.7.4 PROCEDURE

Blank, standard and sample tubes were prepared as under:

	BLANK	STANDARD	SAMPLE
WORKING REAGENT	2 ml	2 ml	2 ml
MDA STANDARD	-----	1 ml	-----
SERUM SAMPLE	-----	-----	1 ml
DISTILLED WATER	1 ml	-----	-----

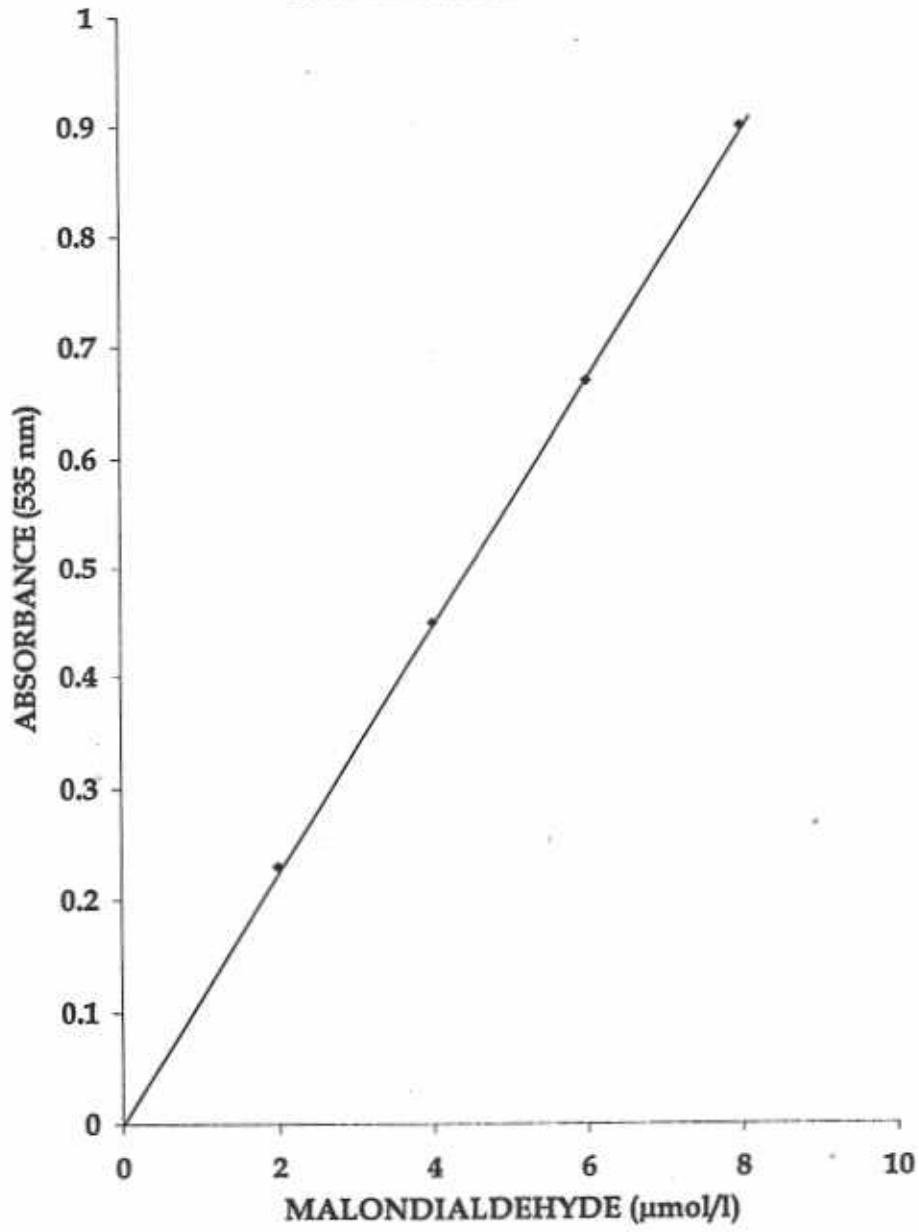
1. The contents of the tubes were mixed and heated in a boiling water bath at 100°C for 25 minutes.
2. The tubes were allowed to cool at room temperature and centrifuged at 1500 g for 5 minutes.

3. The clear, coloured supernatant was separated and its absorbance measured against blank at 535 nm. in Spectrophotometer R&M, S-200.

2.7.5 PREPARATION OF CALIBRATION CURVE

1 ml each of 2, 4, 6 and 8 $\mu\text{mol/l}$. of the diluted standard solution with 2 ml of working reagent was used in the preparation of calibration curve as described in the procedure for test. Absorbance of each of the diluted standard was plotted against the corresponding concentration as shown in figure-4.

FIGURE-4
CALIBRATION CURVE FOR SERUM
MALONDIALDEHYDE



2.8 DETERMINATION OF SERUM IRON

Serum iron was estimated by colorimetric method using kit cat. No. SI 257 supplied by Randox Laboratories Ltd. Ardmore, United Kingdom.

2.8.1 PRINCIPLE

Ferric iron is dissociated from its carrier protein, transferrin, in an acid medium and simultaneously reduced to the ferrous form. The ferrous iron is then complexed with the chromogen, a sensitive iron indicator, to produce a blue chromophore. The absorbance of the coloured complex is determined by a spectrophotometer.

2.8.2 REAGENTS

1. Chromogen

Ferene	22.2 mmol/l..
--------	---------------

2. Reductant

Ascorbic Acid	1.3 mol/l.
---------------	------------

3. Buffer

Acetate Buffer	0.087 mol/l, pH 4.65
----------------	----------------------

Dimethyl Sulphoxide	
---------------------	--

Surfactant	
------------	--

4. Standard

Iron	200 µg/dl.
------	------------

2.8.3 PREPARATION OF REAGENT

Reductant

Dissolve the contents of one vial of Reductant 2 with 15 ml of iron-free deionized water. Stable for 4 weeks at + 4 to + 8 °C.

All other reagents are ready for use as supplied. Stable to expiry date when stored at + 15 to + 25 °C.

2.8.4 PROCEDURE

1. The blank, standard and serum sample tubes were prepared as follows:

	BLANK	STANDARD (ST)	SAMPLE (S)
BUFFER	2 ml.	2 ml.	2 ml.
REDUCTANT	0.1 ml.	0.1 ml.	0.1 ml.
IRON-FREE WATER	0.5 ml.	-----	-----
IRON STANDARD	-----	0.5 ml.	-----
SERUM SAMPLE	-----	-----	0.5 ml.

2. The contents of the above tubes were thoroughly mixed and the initial absorbance of the serum sample and standard was read against the reagent blank at 595 nm. in Spectrophotometer R&M, S-200.
3. Pipette 0.1 ml Chromogen in the blank, standard and serum sample tubes.

4. The contents of the above tubes were mixed thoroughly and incubated at + 20 to + 25°C for 5 minutes.
5. The Final absorbance (A) of the standard and the sample were measured against the reagent blank in Spectrophotometer R&M, S-200 at 595 nm.
6. Subtract initial absorbance from final absorbance to give ΔA for sample and standard.

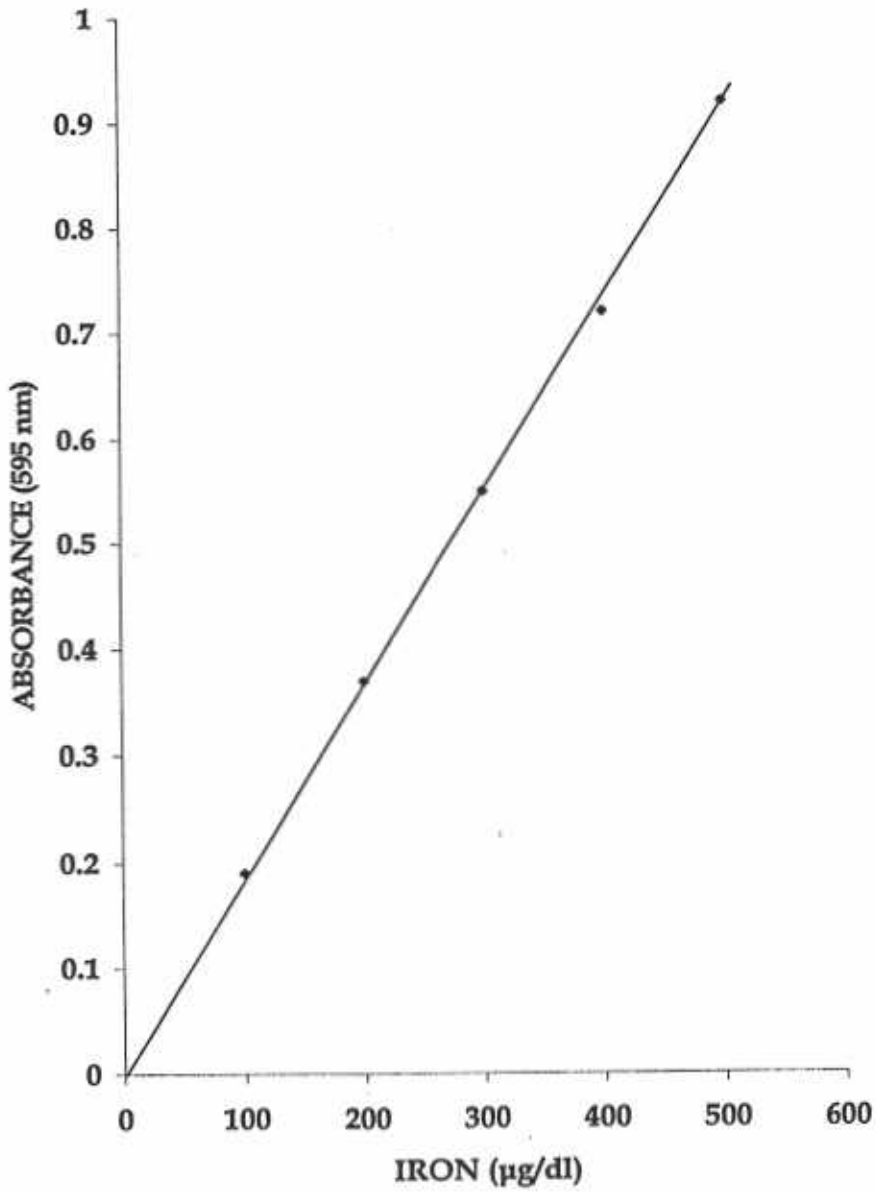
2.8.5 CALCULATIONS

$$\text{Iron } (\mu\text{g/dl}) = \frac{\Delta A \text{ Sample}}{\Delta A \text{ Standard}} \times \text{Standard Concentration } (\mu\text{g/dl})$$

2.8.6 PREPARATION OF CALIBRATION CURVE

A calibration curve was prepared by using the iron standards containing 100, 200, 300, 400, and 500 $\mu\text{g/dl}$. Absorbance of the above standards was plotted against their concentrations as in figure-5.

FIGURE-5
CALIBRATION CURVE FOR SERUM IRON



2.9 DETERMINATION OF SERUM TOTAL IRON BINDING

CAPACITY (TIBC)

Serum total iron binding capacity (TIBC) was estimated by colorimetric method, using kit cat. No. TI 1010 supplied by Randox Laboratories Ltd. Ardmore, United Kingdom.

2.9.1 PRINCIPLE

An excess of iron is added to the serum to saturate the transferring. The unbound iron is precipitated with basic magnesium carbonate. After centrifugation the iron in the supernatant is determined.

2.9.2 REAGENTS

1. Reagent 5

Iron Solution:

Iron 500 µg/dl.

2. Reagent 6

Basic Magnesium Carbonate (powder).

2.9.3 PREPARATION OF SOLUTION

The reagents are ready for use. All reagents are stable up to the date of expiration as specified when stored at + 15 to + 25° C.

2.9.4 PROCEDURE

1. Pipette into 10 ml centrifuge tube:

SERUM SAMPLE	0.5 ml.
REAGENT- 5	1.0 ml.

3. Mix and allow to stand for 5-30 minutes at + 20 to + 25°C.
4. Then add spatula full (180 mg) of basic magnesium carbonate from reagent bottle 6.
5. Let stand for 30-60 minutes at + 20 to + 25°C, mixing frequently during this period.
6. Then centrifuge for 10 minutes at 3000 rpm.
7. Remove the supernatant. The supernatant can be stored for up to one hour.
8. Use 0.5 ml of supernatant for assay with serum iron kit cat. No. SI 257 supplied by Randox Laboratories Ltd. Ardmore, United Kingdom.

2.9.5 REAGENTS

1. Chromogen

Ferene 22.2 mmol/l..

2. Reductant

Ascorbic Acid 1.3 mol/l.

3. Buffer

Acetate Buffer 0.087 mol/l, pH 4.65

Dimethyl Sulphoxide

Surfactant

4. Standard

Iron

200 $\mu\text{g/dl}$.2.9.6 PREPARATION OF REAGENT

Reductant

Dissolve the contents of one vial of Reductant 2 with 15 ml of iron-free deionized water. Stable for 4 weeks at + 4 to + 8°C.

All other reagents are ready for use as supplied. Stable to expiry date when stored at + 15 to + 25°C.

2.9.7 PROCEDURE

1. The blank, standard and serum sample tubes were prepared as follows:

	BLANK	STANDARD (ST)	SAMPLE (S)
BUFFER	2 ml.	2 ml.	2 ml.
REDUCTANT	0.1 ml.	0.1 ml.	0.1 ml.
IRON-FREE WATER	0.5 ml.	-----	-----
IRON STANDARD	-----	0.5 ml.	-----
SERUM SAMPLE	-----	-----	0.5 ml.

2. The contents of the above tubes were thoroughly mixed and the initial absorbance of the serum sample and standard was read against the reagent blank at 595 nm. in Spectrophotometer R&M, S-200.

3. Pipette 0.1 ml Chromogen in the blank, standard and serum sample tubes.
4. The contents of the above tubes were mixed thoroughly and incubated at + 20 to + 25° C for 5 minutes.
5. The Final absorbance (A) of the standard and the sample were measured against the reagent blank in Spectrophotometer R&M, S-200 at 595 nm.
6. Subtract initial absorbance from final absorbance to give ΔA for sample and standard.

2.9.8 CALCULATIONS

$$\text{TIBC } (\mu\text{g/dl}) = \frac{\Delta A \text{ Sample}}{\Delta A \text{ Standard}} \times \text{Standard Concentration } (\mu\text{g/dl})$$

2.10 CALCULATION OF TRANSFERRIN SATURATION

Transferrin Saturation was calculated from the ratio of serum iron concentration to total iron-binding capacity expressed as a percentage [102].

$$\text{TIBC } (\%) = \frac{\text{SERUM IRON}}{\text{TIBC}} \times 100$$

2.11 DETERMINATION OF SERUM FERRITIN

Serum Ferritin was estimated by enzyme linked immunoassay method using kit cat. No. F 31545 supplied by Clinotech[®] Diagnostics & Pharmaceuticals, Inc. Horseshoe Way Richmond, Canada.

2.11.1 PRINCIPLE

The Ferritin is a solid phase direct sandwich enzyme linked immunoassay (ELISA) method. The samples and diluted anti-Ferritin-HRP conjugate are added to the wells coated with MAb to Ferritin. Ferritin in the patients serum binds to anti-Ferritin MAb on the well and the anti-Ferritin-HRP second antibody then binds to Ferritin. Unbound protein and HRP conjugate are washed off by wash buffer. Upon the addition of the substrate, the intensity of color is proportional to the concentration of Ferritin in the samples.

2.11.2 MATERIALS PROVIDED

1. Microwell coated Ferritin MAb (12×8×1 wells). Total 96 wells.
2. Ferritin Standard: 6 vials (0.7 ml each). 0, 35, 80, 150, 250, 500 ng/ml.
3. Enzyme Conjugate: 1 bottle (12 ml).
4. TMB Substrate: 1 bottle (12 ml).
5. Stop Solution: 1 bottle (8 ml).
6. Wash Concentrate: 1 bottle (50 ml, 10×).

2.11.3 REAGENTS PREPARATION

The reagents (Ferritin Standard, Enzyme Conjugate, TMB Substrate, and Stop Solution) are ready for use. All reagents are stable up to the date of expiration as specified when stored at 2 to 8 ° C.

Keep microwells sealed in a dry bag with desiccants.

10× Wash Buffer Concentrate:

To prepare working wash buffer, add the contents of the bottle to 450 ml of distilled water. Store at room temperature.

2.11.4 ASSAY PROCEDURE

Prior to assay, allow reagents to stand at room temperature.

Gently mix all reagents before use.

1. Place the desired number of coated strips into the holder.
2. Pipette 20 µl of Ferritin standards, control and patients sera.
3. Add 100 µl of enzyme conjugate to all wells.
4. Cover the plate and incubate for 60 minutes at room temperature (18-26 ° C).
5. Remove liquid from all wells. Fill wells with wash buffer. Wash three times.

Blot on absorbent paper towels.

6. Add 100 µl of TMB substrate to all wells.
7. Incubate for 10 minutes at room temperature.
8. Add 50 µl of stop solution to all wells. Shake the plate gently to mix the solution.

9. Read absorbance on ELISA Reader at 450 nm within 20 minutes after adding the stopping solution.

2.11.5 CALCULATION OF RESULTS

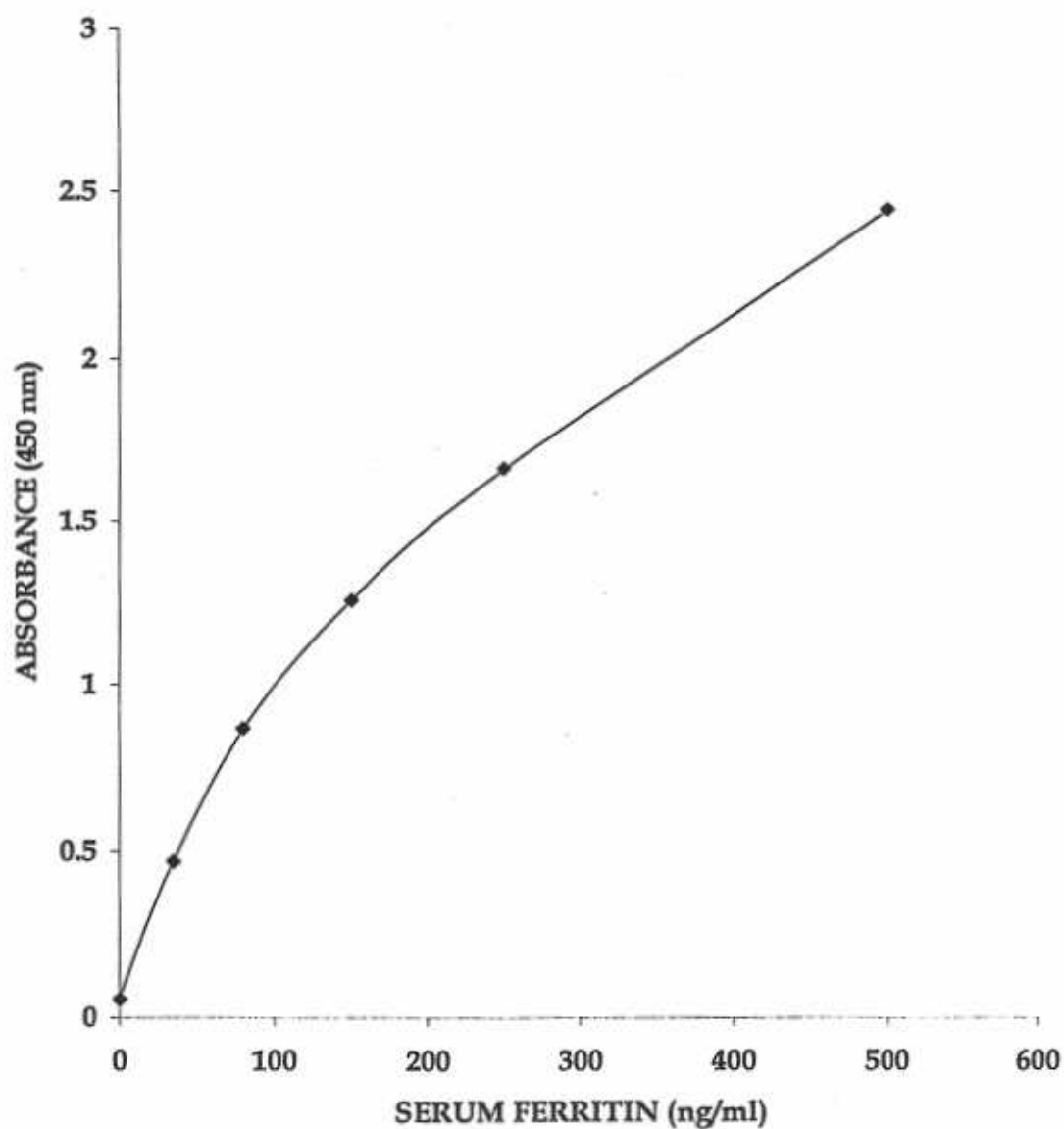
The standard curve is constructed as follows:

1. Check Ferritin standard value on each standard vial.
2. To construct the standard curve, plot the absorbance for the Ferritin standards (vertical axis) versus the Ferritin standard concentrations (horizontal axis) on a linear graph paper. Draw the best curve through the points.
3. Read the absorbance for controls and each unknown sample from the curve. Record the value for each control or unknown sample.

2.11.6 PREPARATION OF CALIBRATION CURVE

A calibration curve was prepared by using the ferritin standards containing 0, 35, 80, 150, 250, and 500 ng/ml. Absorbance of the above standards was plotted against their concentrations as in figure-6.

FIGURE-6
CALIBRATION CURVE FOR SERUM
FERRITIN



2.12 DATA ANALYSIS

Comparisons between control healthy subjects and various groups of patients were analyzed by two-tailed Student's *t* test. Results were expressed as mean $\pm$ Standard Deviation (SD). The level of significance was set at $P < 0.05$. Calculations were performed using the SPSS statistical programme.

RESULTS

A total of 410 patients were investigated, they included patients suffering from diabetes mellitus, coronary heart disease and those having diabetes as well as coronary heart disease, and were placed in three groups as follows:

- Group A - Patients with Diabetes mellitus
- Group B - Patients with Coronary Heart disease
- Group C - Diabetic patients having Coronary heart disease

Table 1 shows the distribution of subjects in different groups, their mean ages and the body mass index. Values are expressed as mean $\pm$ standard deviation (SD). Control group, which consisted of non diabetic healthy subjects, included 50 males and 50 females, with mean ($\pm$ SD) age of 46.04 ± 11.61 years, having body mass index (BMI) of $24.60 \pm 1.63 \text{ kg/m}^2$.

Group A included 190 patients suffering from diabetes mellitus. Out of this 95 were females and 95 were males. The age (mean $\pm$ SD) of this group was 45.43 ± 12.06 years, having a body mass index of $24.27 \pm 1.76 \text{ kg/m}^2$. No significant age and body mass index difference was found on comparing the control group with the Group A patients.

Group B comprised of 140 patients with coronary heart disease, with a male to female ratio of 86:54, the age (mean $\pm$ SD) of 46 ± 7.69 years and body mass index of $26.89 \pm 3.06 \text{ kg/m}^2$. Coronary heart disease patients (Group B) showed a significant difference ($P < 0.001$) in body mass index, whereas a non-

Table - 1
COMPARISON OF SEX, AGE AND BODY MASS INDEX.

Values expressed as mean $\pm$ SD.

GROUPS	TOTAL	SEX		AGE (Years)	BMI (Kg/m ²)
		MALE	FEMALE		
CONTROL	100	50	50	46.04 $\pm$ 11.61	24.60 $\pm$ 1.63
A	190	95	95	45.43 $\pm$ 12.06 §	24.27 $\pm$ 1.76 §
B	140	86	54	46.38 $\pm$ 7.69 § Ψ	26.89 $\pm$ 3.06 ⊕ †
C	80	49	31	49.80 $\pm$ 9.09 ♣ † ϕ	26.40 $\pm$ 2.77 ⊕ † ⤵

♣ P < 0.01 as compared to Control group.

⊕ P < 0.001 as compared to Control group.

† P < 0.001 as compared to Group A.

ϕ P < 0.005 as compared to Group B.

§ Non-Significant as compared to Control group.

Ψ Non-Significant as compared to Group A.

⤵ Non-Significant as compared to Group B.

significant difference was seen in age when compared with control subjects. There were 80 diabetic patients having coronary heart disease in Group C, 49 being male and 31 female, with mean ($\pm$ SD) age of 49.80 ± 9.09 years and body mass index $26.40 \pm 2.77 \text{ kg/m}^2$. Both age and body mass index in Group C patients showed a significant difference as compared to control subjects.

Patients suffering from diabetes mellitus include both types of diabetic patients that is, patients with Type 1 diabetes mellitus and those with Type 2 diabetes mellitus. Table 2 shows comparison of age, sex, and body mass index in both types of diabetes mellitus. Out of 190 diabetic patients 100 had Type 2 diabetes mellitus and 90 had Type 1 diabetes mellitus. Male to female ratio for Type 2 diabetes mellitus and Type 1 diabetes mellitus patients was 49:51 and 46:44 respectively. Mean age of patients suffering from Type 2 diabetes mellitus was 54.12 ± 9.16 years and that of Type 1 diabetes mellitus was 35.78 ± 6.04 years. Age of the Type 1 diabetes mellitus group was significantly lower ($P < 0.001$) when compared with that of Type 2 diabetes mellitus group, while a non-significant difference was seen in the body mass index of both types of diabetic patients.

Table 3 shows the comparison of levels of glucose, total cholesterol and triglycerides between the control subjects and different patient groups. Levels of glucose, total cholesterol and triglycerides were significantly increased in group A, B and C in contrast to the control subjects.

Table - 2

**COMPARISON OF SEX, AGE AND BODY MASS INDEX
BETWEEN TYPE 2 DIABETES MELLITUS AND TYPE 1
DIABETES MELLITUS.**

Values expressed as mean $\pm$ SD.

GROUP-A	TOTAL	SEX		AGE (Years)	BMI (Kg/m ²)
		MALE	FEMALE		
Type 2 DM	100	49	51	54.12 $\pm$ 9.16	24.17 $\pm$ 1.87
Type 1 DM	90	46	44	35.78 $\pm$ 6.04 [†]	24.38 $\pm$ 1.62 [§]

† P < 0.001 as compared to Type 2 DM.

§ Non-Significant as compared to Type 2 DM.

Note: DM = Diabetes Mellitus.

Table - 3

**. FASTING BLOOD GLUCOSE AND BLOOD LIPIDS
IN CONTROL SUBJECTS AND IN VARIOUS GROUPS OF
PATIENTS.**

Values expressed as mean $\pm$ SD.

GROUPS	GLUCOSE (mg/dl)	TRIGLYCERIDES (mg/dl)	CHOLESTEROL (mg/dl)
CONTROL (n = 100)	90.58 $\pm$ 8.06	128.42 $\pm$ 14.63	175.86 $\pm$ 18.61
A (n = 190)	184.87 $\pm$ 40.92 $\clubsuit$	194.84 $\pm$ 27.93 $\clubsuit$	217.82 $\pm$ 30.38 $\clubsuit$
B (n = 140)	93.73 $\pm$ 8.53 ϕ	182.68 $\pm$ 17.37 $\clubsuit$	266.25 $\pm$ 32.98 $\clubsuit$
C (n = 80)	228.30 $\pm$ 46.32 $\clubsuit$	219.81 $\pm$ 47.37 $\clubsuit$	298.67 $\pm$ 38.91 $\clubsuit$

$\clubsuit$ P < 0.001 as compared to Control subjects.

ϕ P < 0.005 as compared to Control subjects.

Comparison of level of lipoproteins in control group and in groups A, B and C patients is shown in Table 4. The levels of high density lipoprotein cholesterol showed a highly significant decrease ($P < 0.001$) in all the groups that is A, B and C, as compared to the control subjects, whereas a highly significant increase ($P < 0.001$) in the levels of low density lipoprotein cholesterol, and very low density lipoprotein cholesterol was observed in patients of all the groups that is A, B, and C as compared to control subjects. The level of blood glucose, lipids and lipoproteins in patient groups as compared to control group are also well evident in figures 7, 8 and 9.

The results of the serum malondialdehyde, iron, total iron binding capacity (TIBC), transferrin saturation and ferritin levels are shown in Table 5 which shows a significant increase in the level of above said parameters in patients from group A, B, and C in contrast to the subjects in the control group. This can be clearly seen in figure 10, 11, 12 and 13.

A highly significant increase ($P < 0.001$) in the levels of serum glucose, total cholesterol and triglycerides was observed (Table 6) in the patients of both the subgroups of diabetic patients that is Type 2 diabetes mellitus and Type 1 diabetes mellitus as compared to the control subjects. Significantly lower levels of serum glucose and total cholesterol were found in patients with Type 1 diabetics when compared to Type 2 diabetic patients, whereas no significant change was seen in the

Table - 4

**COMPARISON OF LIPOPROTEINS IN PATIENT GROUPS
AND CONTROL SUBJECTS.**

Values expressed as mean $\pm$ SD.

GROUPS	HDL-c (mg/dl)	LDL-c (mg/dl)	VLDL-c (mg/dl)
CONTROL (n = 100)	54.28 $\pm$ 7.29	95.93 $\pm$ 17.37	25.65 $\pm$ 2.96
A (n = 190)	39.18 $\pm$ 2.93 ♣	139.65 $\pm$ 30.08 ♣	38.99 $\pm$ 5.57 ♣
B (n = 140)	35.79 $\pm$ 3.85 ♣	193.92 $\pm$ 32.43 ♣	36.56 $\pm$ 3.68 ♣
C (n = 80)	35.20 $\pm$ 5.93 ♣	219.47 $\pm$ 44.48 ♣	44.00 $\pm$ 9.45 ♣

♣ P < 0.001 as compared to Control group.

FIGURE-7
SERUM GLUCOSE, LIPIDS AND
LIPOPROTEINS IN CONTROL
SUBJECTS AND GROUP A PATIENTS.

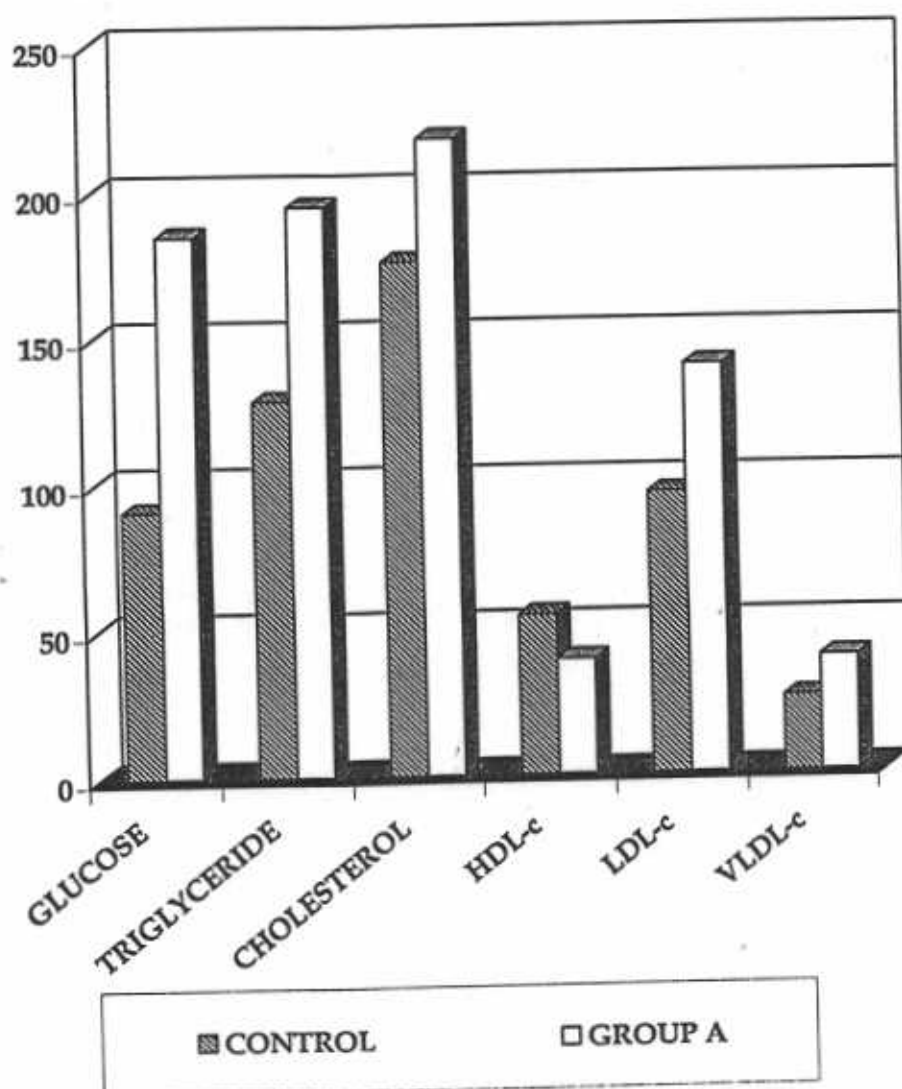


FIGURE-8
SERUM GLUCOSE, LIPIDS AND
LIPOPROTEINS IN CONTROL SUBJECTS
AND GROUP B PATIENTS.

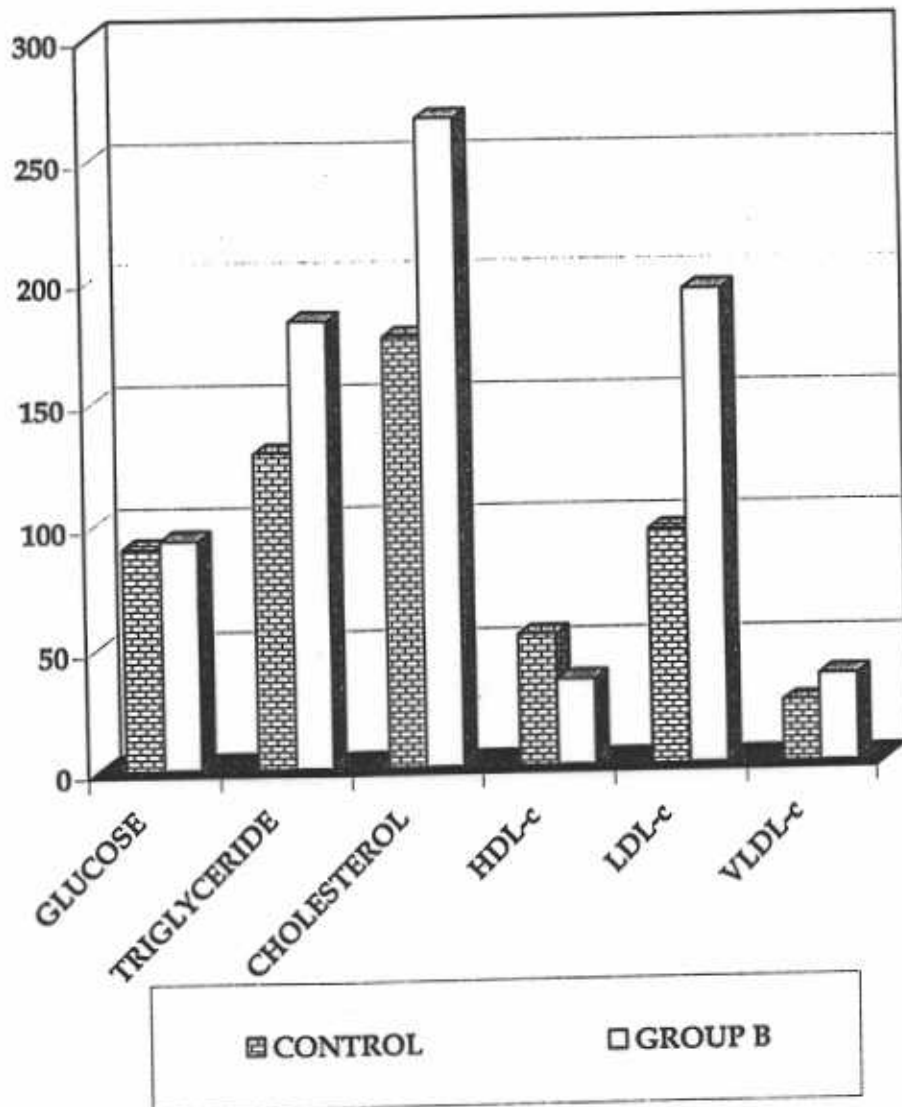


FIGURE-9
SERUM GLUCOSE, LIPIDS AND
LIPOPROTEINS IN CONTROL SUBJECTS
AND GROUP C PATIENTS.

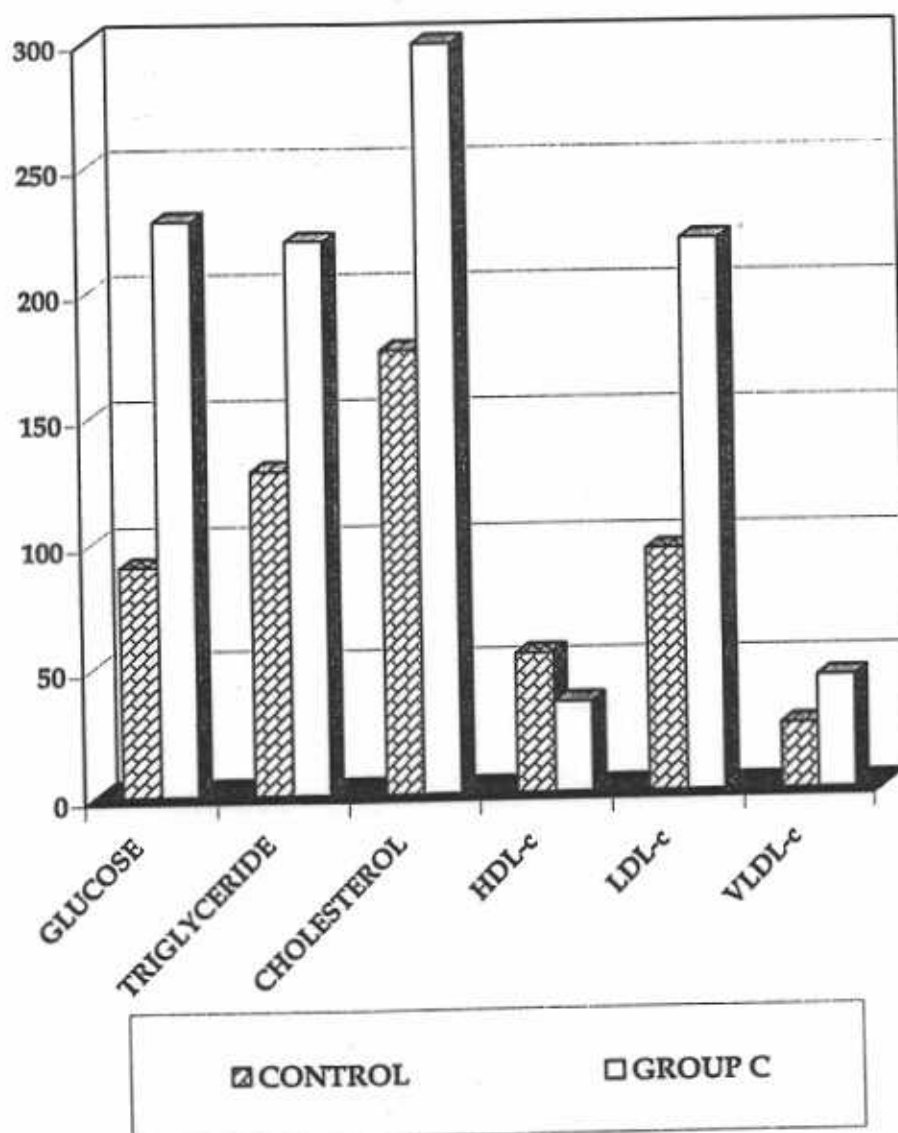


Table - 5

BLOOD LEVELS OF MALONDIALDEHYDE AND IRON INDICES IN PATIENT GROUPS AND CONTROL SUBJECTS.

Values expressed as mean $\pm$ SD.

GROUPS	MDA ($\mu\text{mol/l}$)	IRON ($\mu\text{g/dl}$)	TIBC ($\mu\text{g/dl}$)	TRANSFERRIN SATURATION (%)	FERRITIN (ng/ml)
CONTROL (n = 100)	2.28 $\pm$ 0.47	79.45 $\pm$ 10.86	372.09 $\pm$ 51.43	22.08 $\pm$ 5.78	83.51 $\pm$ 14.97
A (n = 190)	5.30 $\pm$ 0.99 $\clubsuit$	94.56 $\pm$ 11.62 $\clubsuit$	340.28 $\pm$ 37.85 $\clubsuit$	28.38 $\pm$ 6.09 $\clubsuit$	99.07 $\pm$ 12.01 $\clubsuit$
B (n = 140)	3.87 $\pm$ 0.49 $\clubsuit$	85.38 $\pm$ 17.39 $\ddagger$	354.51 $\pm$ 38.10 ϕ	24.91 $\pm$ 7.87 $\clubsuit$	105.26 $\pm$ 11.78 $\clubsuit$
C (n = 80)	4.80 $\pm$ 0.79 $\clubsuit$	83.82 $\pm$ 18.39 $\dagger$	353.10 $\pm$ 38.07 ϕ	24.64 $\pm$ 8.50 $\clubsuit$	110.57 $\pm$ 13.89 $\clubsuit$

$\dagger$ P < 0.05 as compared to Control subjects.

$\ddagger$ P < 0.01 as compared to Control subjects.

ϕ P < 0.005 as compared to Control subjects.

$\clubsuit$ P < 0.001 as compared to Control subjects.

FIGURE-10
SERUM MALONDIALDEHYDE IN
CONTROL SUBJECTS AND IN GROUP A, B
AND C PATIENTS.

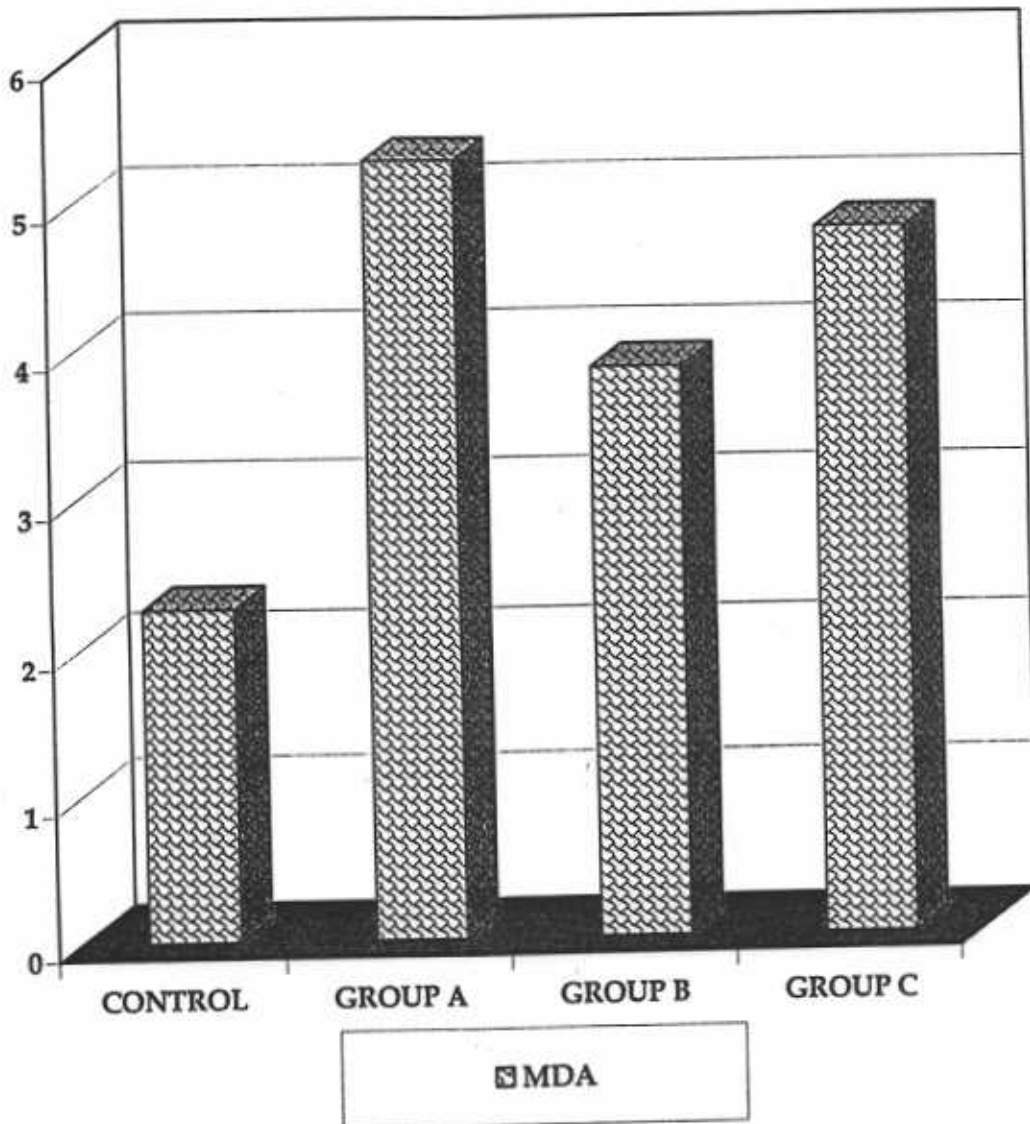


FIGURE-11
SERUM IRON AND TOTAL IRON BINDING
CAPACITY IN CONTROL SUBJECTS AND
GROUP A, B AND C PATIENTS.

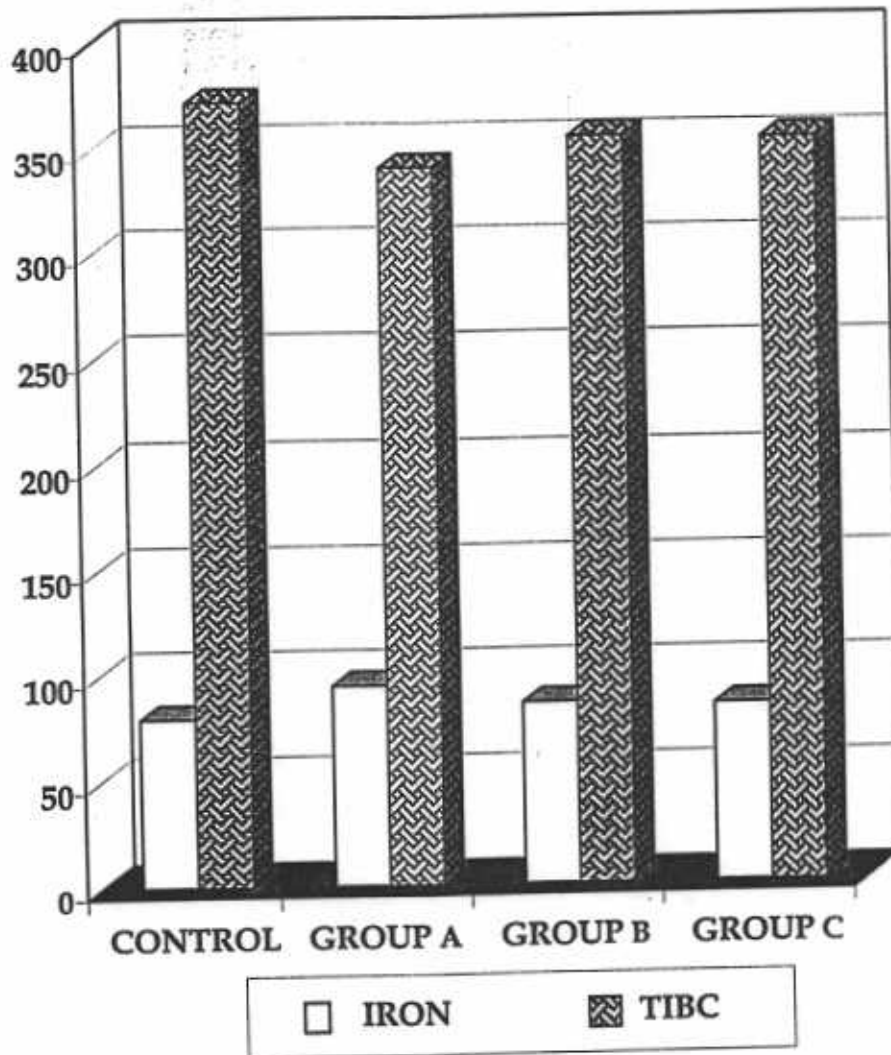


FIGURE-12
TRANSFERRIN SATURATION (%) IN
CONTROL SUBJECTS AND GROUP A, B
AND C PATIENTS.

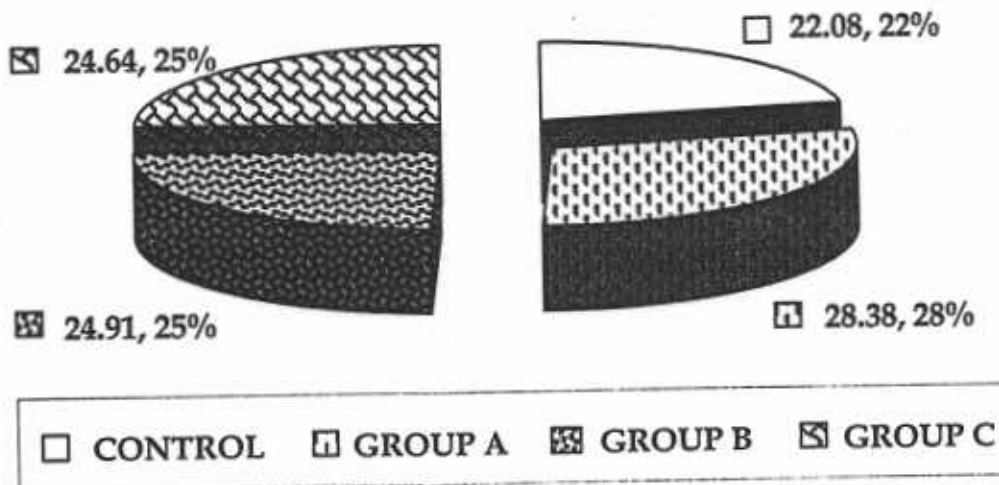


FIGURE-13
SERUM FERRITIN IN CONTROL SUBJECTS
AND GROUP A, B AND C PATIENTS.

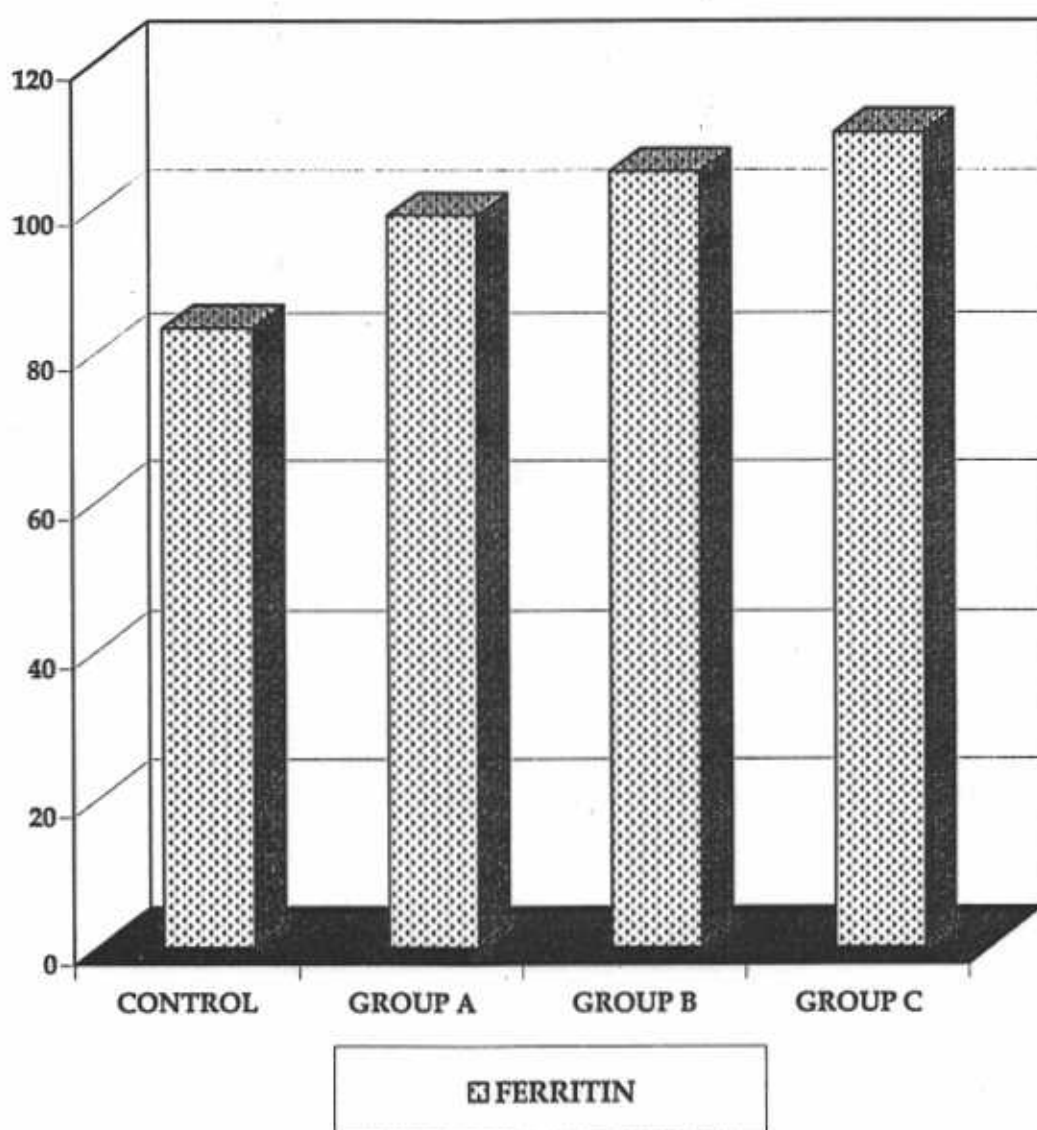


Table - 6

**FASTING BLOOD GLUCOSE AND LIPIDS IN CONTROL
SUBJECTS AND TYPE 2 DIABETES MELLITUS AND TYPE 1
DIABETES MELLITUS.**

Values expressed as mean $\pm$ SD.

GROUPS	GLUCOSE (mg/dl)	TRIGLYCERIDES (mg/dl)	CHOLESTEROL (mg/dl)
CONTROL (n = 100)	90.58 $\pm$ 8.06	128.42 $\pm$ 14.63	175.86 $\pm$ 18.61
Type 2 DM (n = 100)	219.26 $\pm$ 22.98 $\ddagger$	195.19 $\pm$ 34.46 $\ddagger$	237.40 $\pm$ 22.95 $\ddagger$
Type 1 DM (n = 90)	146.67 $\pm$ 12.79 $\ddagger$ $\dagger$	194.45 $\pm$ 18.31 $\ddagger$ $\S$	196.07 $\pm$ 21.55 $\ddagger$ $\dagger$

$\ddagger$ P < 0.001 as compared to Control group.

$\dagger$ P < 0.001 as compared to Type 2 DM.

$\S$ Non-Significant as compared to Type 2 DM.

Note: DM = Diabetes Mellitus.

level of triglycerides on comparison in both Type 2 diabetics and Type 1 diabetic patients.

Table 7 gives comparison of lipoproteins in control subjects, Type 2 diabetics and Type 1 diabetic patients. Both Low and high density lipoprotein cholesterol levels were significantly high ($P < 0.001$) whereas high-density lipoprotein level was found significantly low ($P < 0.001$) in both types of diabetic patients as compared to control group. No significant difference was observed in levels of high and very-low density lipoprotein cholesterol on comparing Type 2 diabetics with Type 1 diabetic patients. The level of blood glucose, lipids and lipoproteins in Type 1 and Type 2 diabetic patient groups as compared to control group are also well evident in figures 14 and 15.

Serum levels of total iron binding capacity was significantly low whereas malondialdehyde, iron, transferrin saturation and ferritin were found to be significantly high when both types of diabetics were compared with control subjects (Table 8). High significant levels of iron profile (except serum ferritin) whereas a significantly low ($P < 0.02$) level of malondialdehyde was observed in Type 1 diabetic group in comparison to Type 2 diabetic group of patients. This can be clearly seen in figure 16, 17, 18 and 19.

Blood glucose, total cholesterol, and triglyceride levels of group A are compared with group B and group C patients in table 9. Blood glucose of patients in group B was significantly lower ($P < 0.001$), whereas of those in

Table - 7

**COMPARISON OF LIPOPROTEINS IN CONTROL SUBJECTS
AND TYPE 2 DIABETES MELLITUS AND TYPE 1 DIABETES
MELLITUS.**

Values expressed as mean $\pm$ SD.

GROUPS	HDL-c (mg/dl)	LDL-c (mg/dl)	VLDL-c (mg/dl)
CONTROL (n = 100)	54.28 $\pm$ 7.29	95.93 $\pm$ 17.37	25.65 $\pm$ 2.96
Type 2 DM (n = 100)	39.30 $\pm$ 2.71 [♣]	159.06 $\pm$ 23.42 [♣]	39.04 $\pm$ 6.87 [♣]
Type 1 DM (n = 90)	39.05 $\pm$ 3.16 ^{♣ §}	118.08 $\pm$ 20.42 ^{♣ ‡}	38.93 $\pm$ 3.65 ^{♣ §}

♣ P < 0.001 as compared to Control group.

‡ P < 0.001 as compared to Type 2 DM.

§ Non-Significant as compared to Type 2 DM.

Note: DM = Diabetes Mellitus.

FIGURE-14
SERUM GLUCOSE, LIPIDS AND
LIPOPROTEINS IN CONTROL SUBJECTS
AND TYPE 1 DIABETES MELLITUS
PATIENTS.

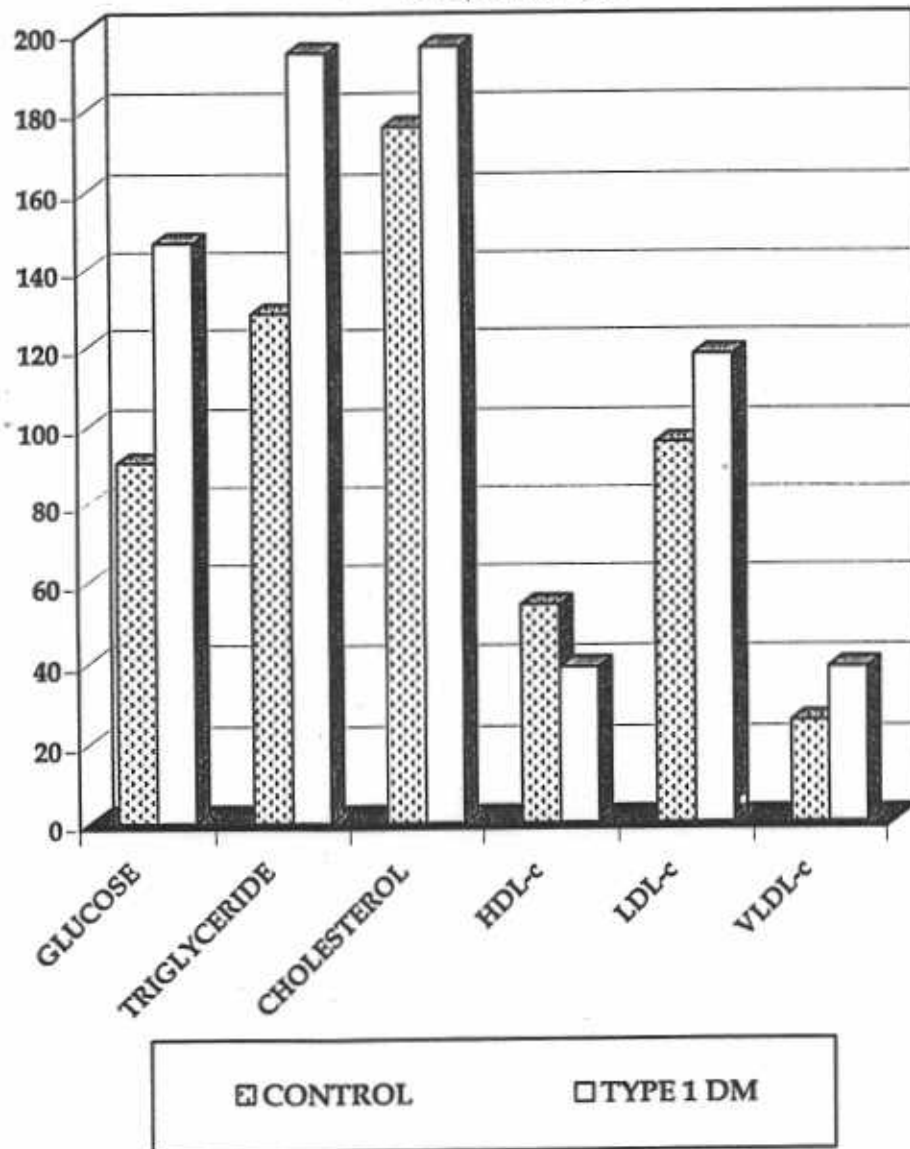


FIGURE-15
SERUM GLUCOSE, LIPIDS AND
LIPOPROTEINS IN CONTROL SUBJECTS
AND TYPE 2 DIABETES MELLITUS
PATIENTS.

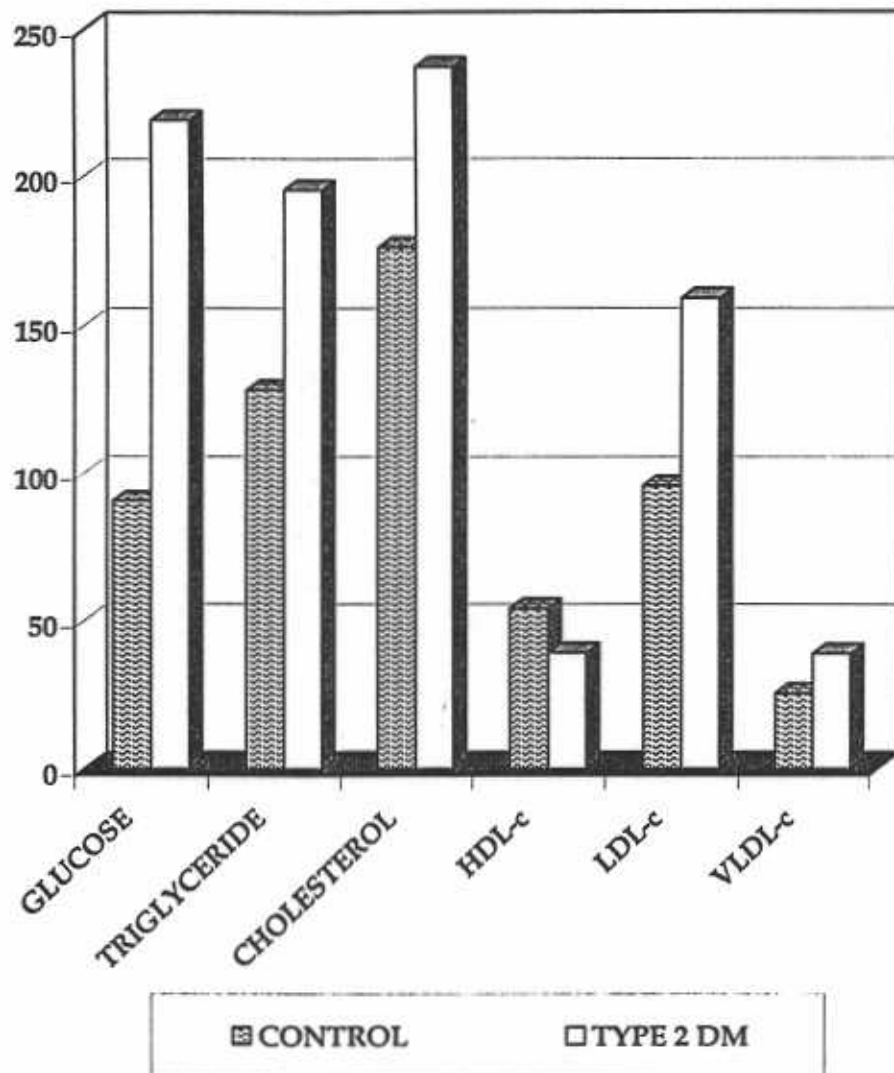


Table - 8

COMPARISON OF MALONDIALDEHYDE AND IRON INDICES IN CONTROL SUBJECTS AND TYPE 2 DIABETES MELLITUS AND TYPE 1 DIABETES MELLITUS.

Values expressed as mean $\pm$ SD.

GROUPS	MDA (μ mol/l)	IRON (μ g/dl)	TIBC (μ g/dl)	TRANSFERRIN SATURATION (%)	FERRITIN (ng/ml)
CONTROL (n = 100)	2.28 $\pm$ 0.47	79.45 $\pm$ 10.86	372.09 $\pm$ 51.43	22.08 $\pm$ 5.78	83.51 $\pm$ 14.97
Type 2 DM (n = 100)	5.44 $\pm$ 1.06 [♣]	90.55 $\pm$ 11.13 [♣]	334.40 $\pm$ 39.04 [♣]	27.76 $\pm$ 6.41 [♣]	103.17 $\pm$ 10.74 [♣]
Type 1 DM (n = 90)	5.14 $\pm$ 0.90 ^{♣ †}	98.01 $\pm$ 10.53 ^{♣ ‡}	346.81 $\pm$ 35.56 ^{♣ ⊕}	29.08 $\pm$ 5.67 ^{♣ §}	94.53 $\pm$ 11.76 ^{♣ ‡}

♣ P < 0.001 as compared to Control group.

⊕ P < 0.025 as compared to Type 2 DM.

† P < 0.02 as compared to Type 2 DM.

‡ P < 0.001 as compared to Type 2 DM.

§ Non-Significant as compared to Type 2 DM.

Note: DM = Diabetes Mellitus.

FIGURE-16
SERUM MALONDIALDEHYDE IN
CONTROL SUBJECTS AND TYPE 1 AND
TYPE 2 DIABETES MELLITUS PATIENTS.

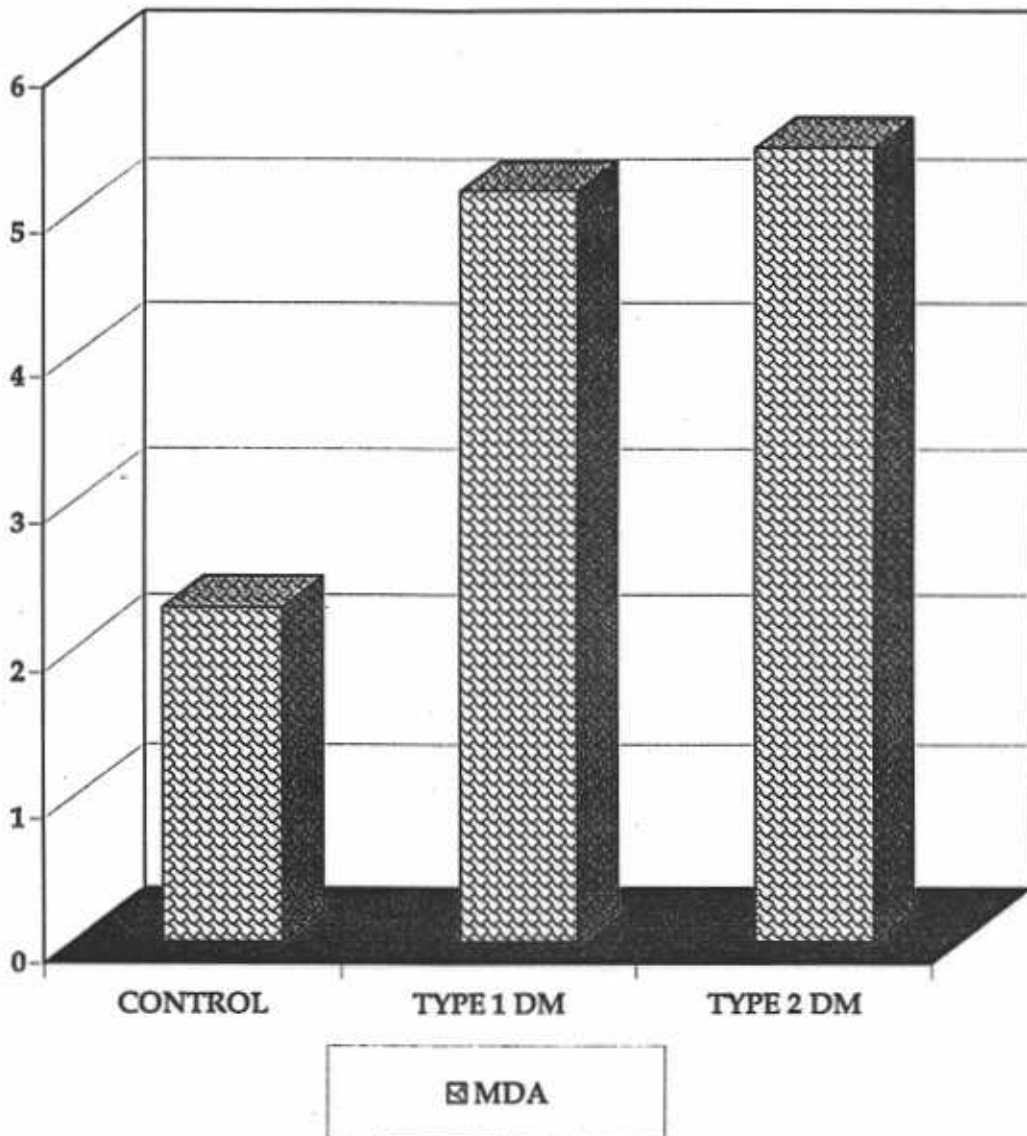


FIGURE-17
SERUM IRON AND TOTAL IRON BINDING CAPACITY
IN CONTROL AND TYPE 1 AND TYPE 2 DIABETES
MELLITUS PATIENTS.

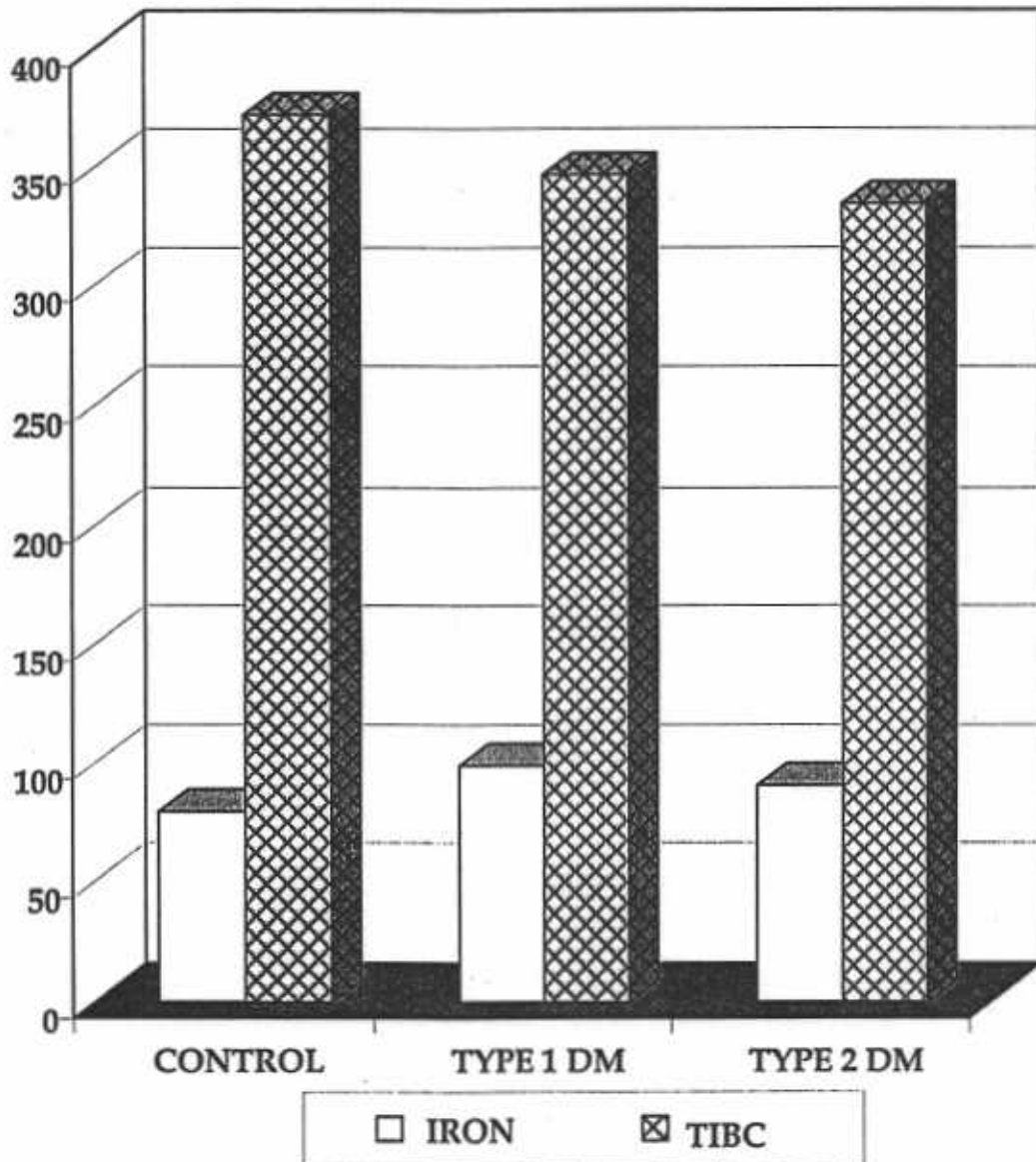


FIGURE-18
TRANSFERRIN SATURATION (%) IN CONTROL
SUBJECTS AND IN TYPE 1 AND TYPE 2 DIABETES
MELLITUS PATIENTS.

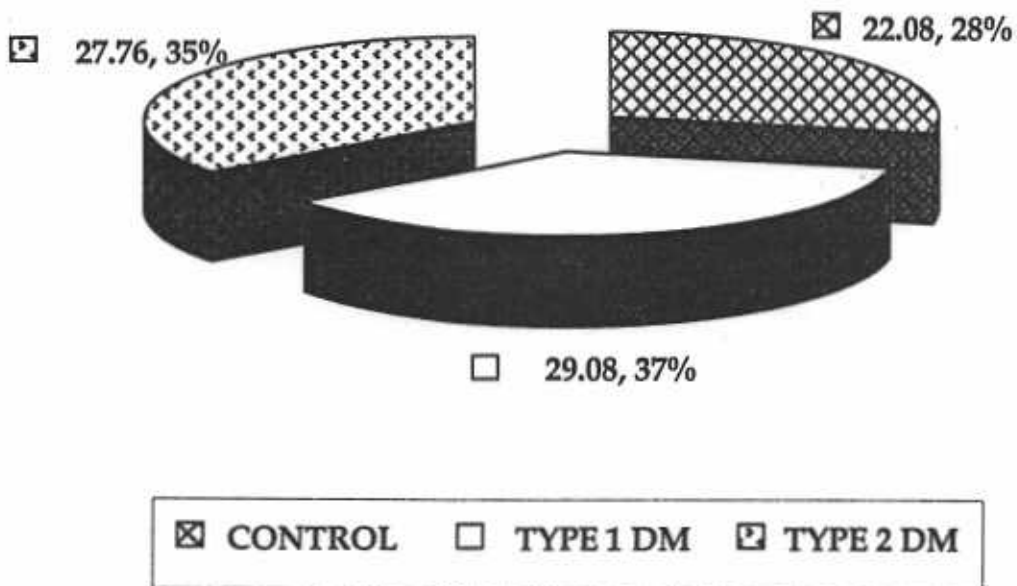


FIGURE-19
SERUM FERRITIN IN CONTROL SUBJECTS
AND TYPE 1 AND TYPE 2 DIABETES
MELLITUS PATIENTS.

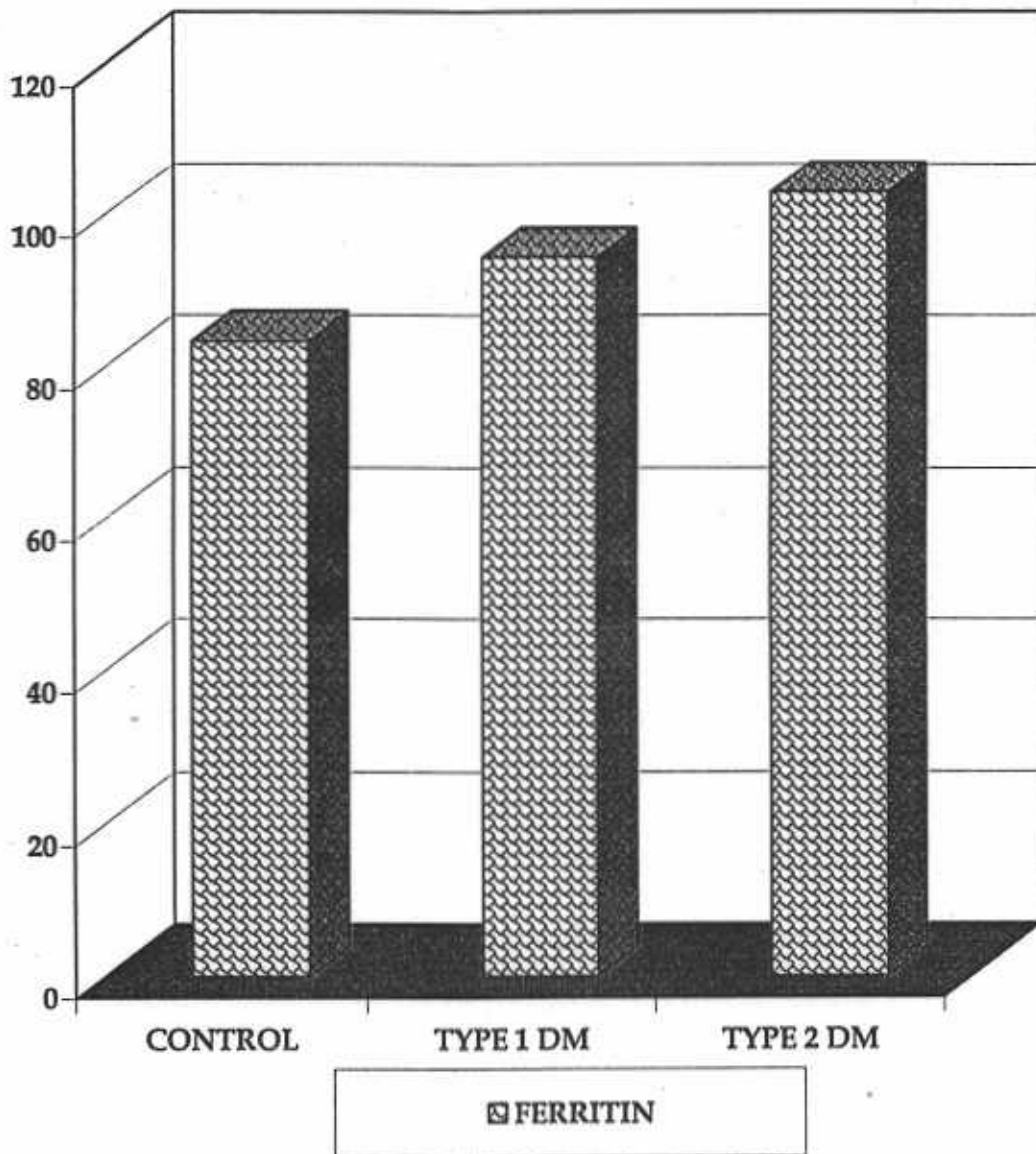


Table - 9

**COMPARISON OF FASTING BLOOD GLUCOSE, AND
BLOOD LIPIDS IN GROUP A, AGAINST GROUP B, AND
GROUP C PATIENTS.**

Values expressed as mean $\pm$ SD.

GROUPS	GLUCOSE (mg/dl)	TRIGLYCERIDES (mg/dl)	CHOLESTEROL (mg/dl)
A (n = 190)	184.87 $\pm$ 40.92	194.84 $\pm$ 27.93	217.82 $\pm$ 30.38
B (n = 140)	93.73 $\pm$ 8.53 †	182.68 $\pm$ 17.37 †	266.25 $\pm$ 32.98 †
C (n = 80)	228.30 $\pm$ 46.32 †	219.81 $\pm$ 47.37 †	298.67 $\pm$ 38.91 †

† P < 0.001 as compared to Group A.

group C it was significantly high ($P < 0.001$) as compared to that of group A patients. Total cholesterol was found markedly increased ($P < 0.001$) in both group B and C patients as compared to those in group A. Triglycerides were observed to be significantly decreased ($P < 0.001$) in group B and significantly increased in group C patients as compared to group A patients.

The levels of lipoprotein cholesterol in group A are compared with group B and group C in table 10. A significant decrease ($P < 0.001$) in the level of high density lipoprotein cholesterol in group B and C patients as compared to the group A patients. Low-density lipoprotein cholesterol in both group B and C, and also very-low-density lipoprotein cholesterol in group C are significantly increased ($P < 0.001$) as compared to group A patients.

The results given in table 11 show a significant decrease ($P < 0.001$) in malondialdehyde, iron, and transferrin saturation whereas significantly high serum total iron binding capacity and ferritin levels were seen in group B and C patients as compared to group A patients.

Table 12 shows the comparison of blood glucose, triglyceride and total cholesterol levels in group B and C patient groups. The results reveal a significant increase ($P < 0.001$) in blood glucose, triglycerides and total cholesterol in group C patients as compared to group B patients. In Table 13 a non-significant decrease was seen in case of high density lipoprotein cholesterol whereas a significant increase

Table - 10

**COMPARISON OF LIPOPROTEINS IN GROUP A, AGAINST
GROUP B, AND GROUP C PATIENTS.**

Values expressed as mean $\pm$ SD.

GROUPS	HDL-c (mg/dl)	LDL-c (mg/dl)	VLDL-c (mg/dl)
A (n = 190)	39.18 $\pm$ 2.93	139.65 $\pm$ 30.08	38.99 $\pm$ 5.57
B (n = 140)	35.79 $\pm$ 3.85 †	193.92 $\pm$ 32.43 †	36.56 $\pm$ 3.68 †
C (n = 80)	35.20 $\pm$ 5.93 †	219.47 $\pm$ 44.48 †	44.00 $\pm$ 9.45 †

† P < 0.001 as compared to Group A.

Table - 11

BLOOD LEVELS OF MALONDIALDEHYDE AND IRON INDICES IN GROUP A, AGAINST GROUP B, AND GROUP C PATIENTS.

Values expressed as mean $\pm$ SD.

GROUPS	MDA (μ mol/l)	IRON (μ g/dl)	TIBC (μ g/dl)	TRANSFERRIN SATURATION (%)	FERRITIN (ng/ml)
A (n = 190)	5.30 $\pm$ 0.99	94.08 $\pm$ 11.45	340.28 $\pm$ 37.85	28.38 $\pm$ 6.09	99.07 $\pm$ 12.01
B (n = 140)	3.87 $\pm$ 0.49 [†]	85.38 $\pm$ 17.39 [†]	354.51 $\pm$ 38.10 [†]	24.91 $\pm$ 7.87 [†]	105.26 $\pm$ 11.78 [†]
C (n = 80)	4.80 $\pm$ 0.79 [†]	83.82 $\pm$ 18.39 [†]	353.10 $\pm$ 38.07 [‡]	24.64 $\pm$ 8.50 [†]	110.57 $\pm$ 13.89 [†]

† P < 0.001 as compared to Group A.

‡ P < 0.01 as compared to Group A.

Table - 12

**COMPARISON OF FASTING BLOOD GLUCOSE, AND
BLOOD LIPIDS BETWEEN GROUP B, AND GROUP C
PATIENTS.**

Values expressed as mean $\pm$ SD.

GROUPS	GLUCOSE (mg/dl)	TRIGLYCERIDES (mg/dl)	CHOLESTEROL (mg/dl)
B (n = 140)	93.73 $\pm$ 8.53	182.68 $\pm$ 17.37	266.25 $\pm$ 32.98
C (n = 80)	228.30 $\pm$ 46.32 [†]	219.81 $\pm$ 47.37 [†]	298.67 $\pm$ 38.91 [†]

[†] P < 0.001 as compared to Group B.

Table - 13

**COMPARISON OF LIPOPROTEINS BETWEEN GROUP B,
AND GROUP C PATIENTS.**

Values expressed as mean $\pm$ SD.

GROUPS	HDL-c (mg/dl)	LDL-c (mg/dl)	VLDL-c (mg/dl)
B (n = 140)	35.79 $\pm$ 3.85	193.92 $\pm$ 32.43	36.56 $\pm$ 3.68
C (n = 80)	35.20 $\pm$ 5.93 §	219.47 $\pm$ 44.48 †	44.00 $\pm$ 9.45 †

† P < 0.001 as compared to Group B.

§ Non-Significant as compared to Group B.

Table - 14

COMPARISON OF MALONDIALDEHYDE AND IRON INDICES BETWEEN GROUP B, AND GROUP C PATIENTS.

Values expressed as mean $\pm$ SD.

GROUPS	MDA (μ mol/l)	IRON (μ g/dl)	TIBC (μ g/dl)	TRANSFERRIN SATURATION (%)	FERRITIN (ng/ml)
B (n = 140)	3.87 $\pm$ 0.49	85.38 $\pm$ 17.39	354.51 $\pm$ 38.10	24.91 $\pm$ 7.87	105.26 $\pm$ 11.78
C (n = 80)	4.80 $\pm$ 0.79 [†]	83.82 $\pm$ 18.39 [§]	353.10 $\pm$ 38.07 [§]	24.64 $\pm$ 8.50 [§]	110.57 $\pm$ 13.89 [‡]

† P < 0.001 as compared to Group B.

‡ P < 0.01 as compared to Group B.

§ Non-Significant as compared to Group B.

($P < 0.001$) was seen in the level of low density lipoprotein cholesterol and very-low-density lipoprotein cholesterol in group C patients in comparison to group B patients.

Serum malondialdehyde, iron, total iron binding capacity, transferrin saturation and ferritin in group B and C are shown in Table 14. The results of malondialdehyde and ferritin show a highly significant increase in group C patients in comparison to group B patients. The levels of serum iron, total iron binding capacity and transferrin saturation reveal a non-significant difference amongst both group B and group C patients.

DISCUSSION

Diabetes is increasingly common and creates a substantial burden of suffering and cost. A growing body of evidence suggests that earlier detection and treatment of hyperglycaemia and related metabolic abnormalities may be beneficial. The degree and duration of hyperglycaemia are associated with the development of microvascular complications, which can be attenuated by intensive reduction of blood glucose. Hyperglycaemia is also associated with the development of macrovascular disease [103]. Diabetes mellitus is known to be underreported on death certificates and hence underestimated by national mortality statistics [104]. Diabetes is associated with increased mortality and morbidity due to vascular complications [105, 106]. Most of the effects of diabetes mellitus, both type 1 diabetes mellitus and type 2 diabetes mellitus, on the macrovasculature is the result of an acceleration of atherosclerosis and increase in thrombosis [107]. Diabetic individuals have an increased risk for developing atherosclerotic disease. This is in part a consequence of the association of diabetes mellitus with other atherosclerosis risk factors [71, 108]. Atherosclerosis occurs earlier in diabetics than non-diabetics, its severity is often greater, and its distribution is more diffuse [109]. Atherosclerotic complications are the leading cause of death in people with diabetes [110]. Many studies have shown that diabetes mellitus is consistently associated with changes in plasma lipids and lipoproteins and these alterations are of interest because of their possible role in the etiology of the increased cardiovascular disease associated with diabetes [111].

The most common type of lipid abnormalities encountered in a patient with diabetes mellitus are elevated plasma levels of triglycerides,

very low density lipoprotein cholesterol, low density lipoprotein cholesterol and lipoprotein(a), and decreased high density lipoprotein cholesterol concentrations [71, 108, 112].

The results of the present study showed a significant elevation in the level of triglycerides, very low density lipoprotein cholesterol and low density lipoprotein cholesterol, and a significant decrease in high density lipoprotein cholesterol in diabetic patients as compared to the control subjects. These results are in agreement with the work of Schwartz et al. [108].

Alaupovic and co-workers [113] reported significantly high concentrations of triglycerides but normal levels of total cholesterol in diabetic patients in comparison with non-diabetic control subjects. In diabetic patients the concentration of very low density lipoprotein cholesterol was significantly higher and the concentration of high density lipoprotein cholesterol was showing a significant decrease than in control subjects, however, slight but not significant increase was seen in low density lipoprotein cholesterol in diabetic patients. As far as triglycerides, very low density lipoprotein cholesterol, and high density lipoprotein cholesterol, are concerned the results of this study are in line with the aforementioned workers. On the other hand the significant increase in total cholesterol and low density lipoprotein cholesterol in diabetic patients of the present study is in accordance with the observations of Ramirez and his group [71].

Diabetes mellitus is considered to be one of a rank of free radical diseases. The existence of hyperglycaemia produces increased oxidative stress via non-enzymatic glycation, glucose autoxidation, and alterations in polyol pathway activity with subsequent influences on the whole organism [114]. The increased oxidative stress in diabetes mellitus [115, 116] is an acknowledged pathogenic mechanism in diabetic complications. Hyperglycaemia is a widely known cause of enhanced free radical concentration [117, 118]. Diabetes mellitus is accompanied by an increase in serum conjugated diene and malondialdehyde level as well as by an increased malondialdehyde concentration in red blood cells [119]. Increased lipid peroxidation occurs in diabetes mellitus. This is due to an altered intracellular ratio between free radicals and antioxidant systems [120]. Type 2 diabetes mellitus have higher levels of plasma thiobarbituric acid reactive substances than normal individuals. The thiobarbituric acid based assay is the most popular and easiest method used as an indicator of lipid peroxidation and free radical activity in biological samples. The low density lipoprotein has been reported to be more prone to oxidation in diabetes mellitus [121].

Gallou and co-workers [122] determined the malondialdehyde levels in Type 1 diabetes mellitus and Type 2 diabetes mellitus patients, and showed a significant increase in the level of malondialdehyde in both types of diabetic patients as compared to non-diabetic control subjects. However, the malondialdehyde levels between Type 2 diabetes mellitus and Type 1 diabetes mellitus patients were not significantly different. Altomare and his group [120] reported significant increase in Lipid peroxidation end product called

malondialdehyde in poorly controlled diabetic patients as compared to the non-diabetic control subjects. Griesmacher and his group [115] also reported significantly elevated levels of thio-barbituric acid reactive substances in both types of diabetic patients as compared to the control subjects, but in contrast Gallou and co-workers [122], found significantly higher levels of thio-barbituric acid reactive substances in Type 2 diabetes mellitus than in Type 1 diabetes mellitus patients. Soska et al [123] and Roussel et al [124] observed significantly increased levels of malondialdehyde in Type 2 diabetes mellitus patients as compared to the control subjects, whereas on the other hand Seghieri et al. [125] found significantly higher level of the same parameter in Type 1 diabetes mellitus patients on comparison with the control subjects. Pasaoglu and colleagues [126] observed increased levels of malondialdehyde in newly diagnosed type 2 diabetic and those type 2 diabetic patients on oral antidiabetic agents as compared to healthy subjects. Freitas et al. [127] showed significantly increased level of lipid peroxidation in normolipidemic Type 2 diabetes mellitus patients than control subjects. They were unable to show any correlation of malondialdehyde with fasting blood glucose, total cholesterol and triglycerides. Mahboob and colleagues [128] observed a significant elevation of malondialdehyde concentration in both male and female Type 2 diabetic patients in comparison to non-diabetic controls. On comparing both types of diabetic patients Skrha and Hilgertova [129] found significantly higher malondialdehyde concentrations, where as when both types of diabetic patients were compared for malondialdehyde concentrations with the control subjects, significant increase was observed only in Type 2 diabetes mellitus patients. The present results also show significantly increased levels of serum

malondialdehyde in diabetic patients as compared to the non-diabetic control group. Both Type 2 diabetes mellitus and Type 1 diabetes mellitus patients on comparison with control subjects showed significantly increased levels of malondialdehyde, whereas significantly elevated level of the malondialdehyde was found in Type 2 diabetics than the Type 1 diabetic patients as shown in the study of Skrha and Hilgertova [129].

Free iron is capable of stimulating the production of free radicals which cause oxidative damage such as lipid peroxidation [130]. The general effect of catalytic iron is to convert poorly reactive free radicals, such as H_2O_2 , into highly reactive ones, such as the hydroxyl radical. Increased accumulation of iron affects insulin synthesis and secretion in the pancreas and interferes with the insulin-extracting capacity of the liver. Iron deposition in muscle decreases glucose uptake because of muscle damage. Conversely, insulin stimulates cellular uptake through increased transferrin receptor externalization. Thus, insulin and iron can mutually potentiate their effects, leading, after a vicious cycle, to insulin resistance and diabetes [131]. One of the most important mechanism of antioxidant defense is thus the sequestration of iron in a redox-inactive form by transferrin [130]. Iron chelating agents and blood donation can prevent the development of diabetes in transfusional iron overload. Blood letting is commonly used in the treatment of hemochromatosis and has been consistently demonstrated to lower iron stores and to be safe in individuals with diabetes [131].

Epidemiological studies [132, 133], revealed that increased iron stores predicted the development of diabetes, however lower prevalence of diabetes was recorded among frequent blood donors by Facchini [134]. Our results showed significantly increased levels of iron indices in patients with diabetes mellitus than the normal control subjects. Both type 1 and type 2 diabetic patients when compared with control group showed an increase in the iron parameters. The type 2 diabetic patients were having significantly low concentrations of all the iron indices except for serum ferritin levels which were found to be significantly high in comparison to that in Type 1 diabetic patients. Mainous and colleagues [135] discovered non significant association between elevated serum transferrin saturation and the development of diabetes. Al-Saleh and his group [136] found no significant difference in venous blood levels of iron in patients with gestational diabetes and control pregnant women. Thomas and associates [137] showed prevalence of elevated transferrin saturation ($> 35\%$), which was 3-4 fold higher in patients with diabetes, compared with historical prevalence described in the general Australian population. They also reported elevated iron indices including serum ferritin, iron, unsaturated iron binding capacity and transferring saturation in diabetics as compared to control general population. A number of research groups [138, 139, 140, 141] found significantly elevated levels of serum ferritin in Type 2 diabetic patients as compared to healthy control subjects. Canturk and associates [139] also confirmed that serum ferritin was increased in diabetic patients as long as glycemic control is not achieved. Gallou and colleges [142] compared serum ferritin concentrations in poorly controlled Type 1 diabetic patients and in non-diabetic control subjects and found elevated levels of serum ferritin in Type 1

diabetic patients. Oba et al. [143] revealed, mean levels of serum ferritin, glycosylated ferritin, and non-glycosylated ferritin, and percent glycosylated ferritin did not differ significantly between Type 2 diabetic patients without retinopathy and health controls. Taher and co-workers [144] reported elevated concentration of iron and total iron binding capacity in both male and female diabetic patients as compared to controls. Ekmekcioglu et al. [145] compared the level of seven trace elements including iron in whole blood, blood plasma, erythrocytes and lymphocytes of type 2 diabetic patients and control subjects. They discovered significantly higher levels of iron in lymphocytes of diabetic group, whereas in general, no considerable differences could be found when comparing the other fractions between patient group and controls. Ekin and colleagues [146] found higher level of iron in type 2 diabetics than the control group. Experiments on rats by Fujimoto et al [147] showed lower concentration of serum iron and TIBC in diabetic rats than those of control rat sera, on the other hand, Liu and associates [148] reported decreased levels of serum iron in middle-aged and aged people with slight and severe diabetes in comparison to control group.

Cardiovascular disease is a serious threat to life and health, accounting for 44% of the nation's mortality and much of its morbidity [149]. Coronary heart disease is an acute disease caused by the long term deposit of blood lipids in coronary arteries, leading to atherosclerosis and necrosis of the heart tissue [150]. Atherosclerotic cardiovascular disease is a growing problem because of the aging population. Indications are that atherosclerotic cardiovascular disease is a generalized process that involves the heart, brain, and peripheral arteries [149].

The main event in myocardial infarction is the occlusion of coronary artery with a thrombus. In some of these individuals, endogenous thrombolysis occurs within minutes to hours, necrosis is confined to the subendocardium and a non-Q wave infarction occurs, whereas in others the occlusive thrombus persists for hours to days, resulting in a Q-wave infarction [93].

In Western countries, it has been shown that coronary heart disease is related to high serum total cholesterol levels. In less developed continents such as Asia and Africa, serum lipid levels are low and coronary heart disease incidence is much lower as compared with Western countries. With growing urbanization and industrialization in Asia, it has been shown that there is a concomitant rise in the level of serum total cholesterol and with it rise in coronary heart disease. In all the Asian countries, serum total cholesterol levels are also higher in the urban compared with the rural population [151]. Moliterno and co-workers [93] showed no significant change in the plasma concentrations of total cholesterol, triglycerides, very low density lipoprotein cholesterol, low density lipoprotein cholesterol and high density lipoprotein cholesterol in myocardial infarction patients with a patent infarct artery and those with an occluded artery. Hargreaves [152] reported no relation of serum cholesterol to coronary heart disease. He noted no significant difference in the serum cholesterol of subjects with coronary heart disease and those without coronary heart disease whereas, the low density lipoprotein cholesterol concentrations was significantly higher and high density lipoprotein cholesterol concentrations was significantly lower in patients with coronary heart disease as compared to those without coronary heart disease. He

showed that both, a raised low density lipoprotein cholesterol concentration and a low high density lipoprotein cholesterol concentrations are contributory to the development of coronary heart disease.

Wu and associates [150] found no significant difference in total cholesterol, triglycerides and low density lipoprotein cholesterol concentrations in coronary heart disease patients and in control subjects. However, they reported lower high density lipoprotein cholesterol and apolipoprotein A1, and higher levels of apolipoprotein B and lipoprotein (a) in coronary heart disease patients. In the United States, the Framingham Heart Study (cited by Farmer and Gotto) [88] has shown that the incidence of coronary heart disease is positively associated with serum cholesterol levels.

Serum cholesterol has been widely accepted as a risk factor for coronary heart disease, although high concentrations of cholesterol in the low density lipoprotein fraction and decreased amounts of cholesterol in the high density lipoprotein fraction are more predictive of coronary heart disease [153].

Sandkamp and co-workers [154] reported significantly increased concentrations of total cholesterol, low density lipoprotein cholesterol, apolipoprotein B and lipoprotein (a) concentrations, and decreased concentration of high density lipoprotein cholesterol and apolipoprotein A1 in coronary heart disease patients than in control subjects. The results of the present study also show a significant increase in total cholesterol triglyceride, low density lipoprotein

cholesterol, and very low density lipoprotein cholesterol whereas, high density lipoprotein cholesterol was found to be significantly low in patients with coronary heart disease as compared to control subjects. These results are in accordance with the reports of earlier workers.

Myocardial ischaemia can result from a number of causes. Ischaemia can result from atherosclerosis or thromboembolism, or can be produced during surgical interventions such as, percutaneous transluminal coronary angioplasty, coronary artery bypass or transplantation. Whatever is the source of ischaemia, consequences are always the same, lack of oxygen of the myocardium and a lack of suitable substrates for metabolism [155]. An acute coronary occlusion causes severe myocardial ischemia, which might progress to necrosis and scar tissue formation. Thrombolytic therapy, spontaneous reperfusion, or collateral blood flow can modify the evolution of an acute myocardial infarction and may salvage ischemically injured myocardium. Restoration of coronary blood flow may therefore lead to an improvement in segmental contractile function in some patients but prove ineffective in those with completed injury [156]. Oxygen free radicals have been implicated in cardiac ischaemic injury. These free radicals (super oxide anions and hydroxyl radicals) are produced in the body by reduction of oxygen. In normal circumstances they are removed by the different scavenger systems present in blood and tissues. In case of myocardial ischaemia which can lead, to myocardial infarction, excessive free radicals may be generated [157].

Oen and co-workers [158] showed significantly increased levels of lipid peroxides in patients suffering from coronary heart disease as compared to the control subjects. Belch and associates [157] showed evidence of increased free radical activity in patients with myocardial ischaemia than the control subjects. This study also show significantly increased malondialdehyde levels in coronary heart disease patients as compared to the control subjects, which are in agreement with the work of Belch et al. [157].

Iron is essential for many physiological processes; excess iron can lead to tissue by producing the generation of reactive oxygen species. Iron is capable of supporting lipid peroxidation, acceleration of atherosclerosis development has been postulated as a potential mechanism by which iron overload may increase the risk of ischemic cardiovascular events. Iron may also have deleterious effects on vascular function. Locally enhanced vascular production of reactive oxygen species decreases the bioavailability of nitric oxide, impairing vasorelaxation and promoting platelet adhesion and aggregation. Therefore, a dynamic interaction exists between endothelial function and thrombosis, both of which may be influenced by oxidative stress [159].

Stadler and colleagues [97] used the minimally invasive technique of electron paramagnetic resonance (ERP) spectroscopy and inductively coupled plasma mass spectroscopy (ICPMS) to quantify iron in ex vivo healthy human arteries and carotid lesions. They detected statistically elevated levels of iron in the intima of lesions compared with healthy controls. Metwalli et al. [160] showed

serum iron non-significantly decreased in patients with acute myocardial infarction. In a study conducted by Baer and his group [161] failed to show iron deficiency as defined by low transferrin saturation to be protective against acute myocardial infarction. They concluded by saying that their observations do not support the hypothesis that coronary artery disease risk is related to iron stores. Baykan et al. [162] reported after a 45 day follow-up study, serum iron concentration to reach there maximum on the 1st post myocardial infarction day, serum ferritin level started to increase from the admission day of the patients and showed a mean peak value on the 3rd day and decreased to a-minimum level on the 45th day. On the contrary, the transferrin level started to decrease on the 1st day, decreased to minimum level on the 3rd day and continued approximately the same level until the 45th day. Ahmed and associates [163] found significantly increased levels of serum iron, transferrin saturation and serum ferritin in both groups that is smokers having coronary heart disease and in the subjects without any major risk factor. The total iron binding capacity was found to be significantly low in these groups reflecting large volume of iron. The high levels of stored iron found in the subjects of coronary heart disease with smoking risk factor, as compared with controls suggest that there is a cumulative risk of high stored iron in the development of coronary heart disease. They also observed that in the coronary heart disease without any major risk factor, stored iron was also significantly increased than in controls, suggesting that stored iron may be independent risk for coronary heart disease. Both groups Haidari et al. [24] and Bozzini et al. [164] reported significantly increased levels of serum ferritin in coronary heart disease patients as compared to normal control subjects. Haidari and his group [24] also

observed a significantly increased level of serum ferritin in men with coronary heart disease than normal healthy men; on the other hand they could not find a significant difference in serum ferritin concentration when women with coronary heart disease were compared with healthy women with no coronary heart disease. Our results are also in agreement with afore-mentioned workers, showing serum iron and transferrin saturation significantly high, whereas total iron binding capacity was found to be significantly low in coronary heart disease patients as compared to control subjects.

Clinical complications of diabetes mellitus can affect either large or medium arteries leading to coronary heart disease and peripheral artery diseases, or small arteries causing diabetic microangiopathy – retinopathy and nephropathy [165]. Diabetes is associated with accelerated atherosclerosis and subsequent cardiovascular disease. People with diabetes are at two- to fourfold greater risk of developing cardiovascular disease than people without diabetes, and the majority of type 2 diabetic patients die of atherosclerotic disease. The mechanisms underlying diabetes-accelerated atherosclerosis are poorly understood. Diabetics have hyperglycemia, increased levels of reactive oxygen species, production of advanced glycation end products and glycation of lipoproteins, and lipid abnormalities [166]. Increased levels of lipid peroxides have been implicated in the pathogenesis of diabetic complications [167]. Free oxygen radicals are one of the factors which participate via lipoperoxides in the development of atherosclerosis. Atherosclerotic complications affect large proportion of diabetic patients [123]. Free iron serves as a catalyst for lipid and protein oxidation and the formation of

reactive oxygen species [137]. Hyperinduced oxidant stress may have a role in the pathogenesis of diabetes [168].

Barbagallo and his group [169] reported higher total cholesterol, triglycerides, low density lipoprotein cholesterol, apolipoprotein B and lower high density lipoprotein cholesterol and apolipoprotein A1 values in diabetic patients with coronary heart disease as compared to those diabetic patients without coronary heart disease. The present study yielded similar results as that of Barbagallo and his group [169]. The diabetic patient with coronary heart disease showed significant elevation of total cholesterol, triglyceride, low density lipoprotein cholesterol, and very low density lipoprotein cholesterol, and significantly low concentration of high density lipoprotein cholesterol levels in comparison to diabetic patients without coronary heart disease.

Gallou and co-workers [122] reported significantly higher malondialdehyde levels in diabetic patients with macroangiopathy as compared to the diabetic patients with no vascular complications. Abdella and his group [167] also reported increased malondialdehyde levels in diabetic patients with coronary heart disease than those diabetic without coronary heart disease. The results of the present study are also in accordance with the observations of Abdella et al. [167] and Gallou et al [122].

Sulieman and his group [170] demonstrated labile plasma iron elevated in over one-third of all diabetic individuals presenting with acute

myocardial infarction and that it is an independent and powerful predictor of mortality in diabetic individuals presenting with acute myocardial infarction. Mert and colleagues [23] observed a non-significant decrease in the level of serum iron and total iron binding capacity where as a significant increase in serum ferritin levels in diabetic and non-diabetic patients with acute myocardial infarction. Metwalli et al. [160] also reported a non-significant decrease in serum iron concentration in diabetic patients with acute myocardial infarction as compared to control group. Our results showed significant increase in the concentration of serum iron, transferrin saturation and ferritin whereas a significant decrease in total iron binding capacity was seen in diabetics with coronary heart disease as compared to normal control healthy subjects. Patients with acute myocardial infarction are associated with a large flux of serum iron and the intracellular iron stores [162, 171]. Labile plasma iron may be elevated to a greater extent in acute myocardial infarction patients with diabetes due to both a change in the proteins that normally sequester iron and an increase in glycation of serum proteins such as albumin, thereby increasing their affinity for iron [170].

From above mentioned reports it is concluded that diabetes mellitus and coronary heart disease are associated with greater than normal lipid peroxidation. It is also observed that diabetes mellitus and coronary heart disease can alter iron status. Elevated serum malondialdehyde, iron concentration and body iron stores (serum ferritin and transferrin saturation) in diabetic patients reveal a possible role of iron indices in the development of coronary atherosclerosis. Therefore, it is suggested by this study that levels of malondialdehyde and

biochemical markers of body iron stores (serum ferritin and transferrin saturation) can be used as an early investigative tool for atherosclerotic coronary artery disease especially in patients with diabetes mellitus.

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