Study of dyslipidemia, glucose homeostasis and nitrosative stress in diabetes mellitus patients with and without coronary artery disease

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By

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I hereby declare that the thesis entitled **"Study of dyslipidemia, glucose homeostasis and nitrosative stress in diabetes mellitus patients with and without coronary artery disease"** is a bonafide and genuine research work carried out by me under the guidance of **Dr. J. G. Ambekar**, Professor of Biochemistry, BLDE (Deemed to be University)'s Shri B. M. Patil Medical college, Hospital and Research Centre, Vijayapura, Karnataka. No part of this thesis has been formed the bases for the award of any degree or fellowship previously. Shall have the rights to preserve, use and disseminate this dissertation/thesis in print or electronic format for academic/research purpose.

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INDEX

List of tables

List of figures

LIST OF ABBREVIATIONS

ACS – acute coronary syndrome

ACC – American college of cardiology

ADA – american diabetic association

ADMA – Asymmetric dimethyl arginine

AGE – advanced glycation end product

AHA –american heart association

Ang II - angiotensin II

AR - aldose reductase

ApoB - apolipoprotein B

Apo(a) – apolipoprotein (a)

ATP – adenosine triphosphate

ATP-III –adult treatment panel

BMI – body mass index

CAD – coronary artery disease

CDC – centre for disease control

CHD – coronary heart disease

Cm - centimeter

CVD – cardiovascular disease

cGMP - cyclic guanosine monophosphate

DAG - diacylglycerol

DM – diabetes mellitus

ED – endothelial damage

EDGF – endothelial derived growth factor

EDTA – ethylene diamine tetra acetic acid

FFA – free fatty acid

HbA1c – glycosylated hemoglobin

hsCRP – high sensitivity C reactive protein

HDL-C – high density lipoprotein cholesterol

IDDM –insulin dependent diabetes mellitus

IL - Interleukin

KIV2 – kringle IV type-2

LDLR - low density lipoprotein receptor

LPA – apolipiprotein(a) gene

Lp(a) – lipoprotein(a)

LDL-C- low density lipoprotein cholesterol

mg/dL – milligram per decilitre

μIU/L – micro International units per litre

mL – milli liter

mm of Hg – milli metre of mercury

mRNA – messenger ribonucleic acid

n = number of sample

NADP – nicotinamide adenine dinucleotide phosphate

NCEP – national cholesterol education programme

NF-κB – Nuclear factor kappa B

NK cell – Natural killer cell

NO – nitric oxide

NOS – nitric oxide synthase

NIDDM – non insulin dependent diabetes mellitus

Ox Lp(a) – oxidised lipoprotein (a)

OD - optical density

PAI - plasminogen activator inhibitor

PKC – protein kinase C

Plg – plasminogen

ROS –reactive oxygen species

SD – standard deviation

SDMA – Symmetric dimethyl arginine

SPSS – statistical package social system

SMC – smooth muscle cell

SNP – single nucleotide polymorphism

TC – total cholesterol

TG – triglyceride

TNF – tumor necrosis factor

TGF – transforming growth factor

tPA – tissue plasmionogen activator

TF – transcription factor

VLDL – very low density lipoprotein

VSMC – vascular smooth muscle cell

WHO – world health organisation

ABSTRACT

Aims and Objectives: The risk of coronary artery disease (CAD) in diabetic subjects is increased two to four folds over age matched non-diabetic subjects. Conventional risk factors have failed to explain the increasing burden of CAD thus it is necessitating the need to search for other newer risk factors like Lipoprotein (a), homocysteine and inflammatry marker like hsCRP. Lp(a) variant of LDL is considered as one of the risk marker of coronary artery disease (CAD). Lp(a) levels shown wide ethnic variation among human population throughout the world. Endothelial dysfunction associated with diabetes has been attributed to lack of bioavailablity of nitric oxide (NO) due to reduced synthesis of NO from arginine.

Material and Methods: This was hospital based case control study conducted in the department of Biochemistry. The study comprises total 195 participants, and 65 individuals in each groups has healthy controls, diabetes mellitus without CAD and diabetes mellitus with CAD patients. Detail history was taken and general physical examination was done. Fasting venous blood from the participants was used to estimate fasting blood glucose, glycated hemoglobin, total cholesterol, triglyceride, high density lipoprotein, lipoprotein (a), high sensitive C-reactive protein and nitric oxide.

Observations: Serum FBG, HbA1c, TGs, VLDL, Lp(a) and hsCRP were significantly increased in DM without CAD patients and DM with CAD patients as compared to normal healthy controls. HDL-C and NO significantly decreased in DM without CAD patients and DM with CAD patients as compared to healthy controls. Raised serum Lp(a) level is associated with increased risk of CAD in DM patients. Cut-off value above 18 mg/dL in DM without CAD and above 21.6 mg/dL in DM with CAD patients and AUROC more than 0.8, this suggests that Lp(a)

can be used to evaluate risk of CAD in DM patients in north Karnataka population. Lp(a) was found with positive effect and NO with negative effect on the chance of developing CAD in DM patients. With unit increase in Lp(a) level, the chance of CAD was 1.3 times higher in DM without CAD, while in the DM with CAD patients it was almost three times higher compared to healthy controls. Decreased NO levels may be a potential contributor to the pathogenesis of early vascular changes in DM and CAD patients. NO had negative effect on the chance of developing CAD in diabetic patients.

Conclusion: To conclude, serum Lp(a) and hsCRP levels are elevated and NO level reduced in type-2 DM and CAD patients when compared to healthy controls. Thus estimation of Lp(a), NO and hsCRP serve as markers of CAD in type-2 DM patients where lipid profile was within normal range. Thus these tests should be included in panel of investigations for early diagnosis of CAD in DM patients and also to reduce morbidity and mortality associated with it.

Key words: Lipoprotein (a); Nitric oxide; Diabetes mellitus; Coronary artery disease.

Introduction

Cardiovascular disease (CVD) is the most common cause of death among non-communicable diseases (NCD). It accounts for 27% of all deaths due to NCD[1]. Cardiovascular disorders are the disorders that affect blood vessels, in case of CAD it involves coronary vessels which supply blood to the heart. According to global burden disease study, India is expected to have greatest burden of coronary artery disease (CAD)[2]. CVD cause three million deaths per year in India[3]. CAD and diabetes mellitus (DM), together rank first as a cause of death among non-communicable diseases. Morbidity and mortality is increased in DM patients due to cardiovascular complications. The risk of CAD is higher in DM patients.

Risk factors are responsible for initiation and acceleration of the course of CAD. The modifiable risk factors of CAD are dyslipidemia, DM, hypertension, smoking and un-modifiable risk factors include age, gender, genetic factors and family history. These conventional risk factors of CAD have failed to explain the increasing burden of CAD. Inspite of the usage of newer drugs to lower the lipid concentration, CVD continues to be the main cause of death[4], thus necessitating the need to search for newer risk factors like lipoprotein (a) [Lp(a)], homocysteine, inflammatory markers like high sensitivity C-reactive protein (hsCRP), endothelin and thrombogenic factors etc.

Lp(a) variant of low density lipoprotein cholesterol (LDL-C) was first identified by Berg in 1963[5]. Lp(a) consists of apolipoprotein(a) which is covalently bound to apo B through single disulphide bond. Lp(a) is a structural analogue and competitive inhibitor of plasminogen and hence may lead to impaired fibrinolysis. Lp(a) accumulates in the vessel wall, inhibits binding of plasminogen to cell surface. This inhibition also promotes proliferation of smooth muscle cells. Hence Lp(a) has atherogenic and thrombotic properties [6]. Different ethnic populations worldwide showed variations in Lp(a) levels and

different levels of Lp(a) were associated with CAD[7]. Data on Lp(a) levels associated with CAD in diabetes mellitus patients from north Karnataka population are sparse and lacking.

Endothelial dysfunction is common in DM, and is an early indicator of vascular disease. There is a time lag between the initial endothelial injury to development of clinically established CAD cases. Thus, there is a need to detect these vascular changes as early as possible. So that preventive measures can be initiated either by life style modification or pharmacological interventions to reduce morbidity and mortality. This endothelial cell damage can be indirectly measured by nitric oxide (NO) estimation.

Vascular endothelium secretes a gaseous molecule called nitric oxide (NO). It is synthesised by nitric oxide synthase (NOS) enzyme. It acts as a prime signalling messenger in the cardiovascular system[8]. Sufficient levels of NO are required to maintain normal vascular physiology. In DM patients endothelial dysfunction and increased oxidative stress occurs due to uncoupling of endothelial nitric oxide synthase enzyme thus there will be decreased bioavailability of NO[9]. In literature there is limited and controversial data regarding the NO levels in DM and CAD patients.

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Aims and Objectives

Aim of the study: 'To Assess the role of lipid profile, lipoprotein (a), nitric oxide (NO) and high sensitivity C-reactive protein (hsCRP) as markers for early detection of coronary artery disease in type-2 diabetes mellitus patients in and around Bagalkot population'.

Objectives

1. To estimate and compare serum lipid profile, lipoprotein (a), nitric oxide and high sensitive C-reactive protein levels in diabetes mellitus patients without CAD, diabetes mellitus with CAD patients and normal healthy controls.
2. To find the correlation of lipoprotein (a) and nitric oxide with lipid parameters, glycated hemoglobin and high sensitive C-reactive protein.
3. To find the best cut-off value of Lipoprotein (a) in diabetes mellitus without CAD patients and diabetes mellitus with CAD patients to predict risk of developing CAD in and around Bagalkot population.

Hypothesis

Null hypothesis:

'Estimation of serum lipoprotein (a), nitric oxide and high sensitivity C-reactive protein are not effective in the early detection of coronary artery disease (CAD) in type-2 diabetes mellitus patients in and around Bagalkot population'.

Alternate hypothesis:

'Estimation of serum lipoprotein (a), nitric oxide and high sensitivity C-reactive protein are effective in the early detection of coronary artery disease (CAD) in type-2 diabetes mellitus patients in and around Bagalkot population'.

3.1-Diabetes mellitus (DM)

Diabetes mellitus (DM) is derived from the Greek word 'dia' meaning 'through' and 'bainein' meaning 'to go' and diabetes literally means 'pass through'. Greek word 'mellitus' means 'sweet' and that is some sugar is passing in the urine. Diabetes mellitus represents group of metabolic disorders characterized by hyperglycemia due to insulin defect or lack of its action or combination of both[1].

3.1.1 - Epidemiology:

Global scenario – Global prevalence of DM has risen from 4.7% in 1980 to 8.5% in 2014. As per WHO 2016 reports, there were 422 million DM patients worldwide[2].

Prevalence in India - Overall prevalence of DM in India was 7.3%. Higher prevalence of DM was seen in urban areas i.e 11.2% compared to rural area i.e 5.2%[2]. Among the Indian states, maximum prevalence was in Punjab with 10.0% and minimum in Bihar 4.3%. Mortality due to DM was found to be around 2%[3].

3.1.2 - Classification of DM[4]:

I Type-1diabetes mellitus – due to β cell destruction leading to insulin deficiency

- Immune mediated
- Idiopathic

II Type-2 diabetes mellitus – due to insulin resistance with relative insulin deficiency

III Other specific types of diabetes mellitus

I. Genetic defects of β cell development or function characterized by mutations in

- Hepatocyte nuclear transcription factor
- Glucokinase

- HNF-1α
- Insulin promoter factor-1
- HNF-1β
- Mitochondrial DNA
- Subunits of ATP-sensitive potassium channel
- Proinsulin or insulin

II. Genetic defects in insulin action

- Type A insulin resistance
- Leprechaunism
- Rabson-Mendenhall syndrome
- Lipodystrophy syndrome

III. Diseases of exocrine pancreas like pancreatitis, pancreatectomy, neoplasia, cystic fibrosis, hemochromatosis, mutations in carboxyl ester lipase.

IV. Endocrinopathies like acromegaly, Cushing's syndrome, glucaganoma, pheochromocytoma, hyperthyroidism, aldosteronoma.

V. Drug or chemical induced -

Drugs - glucocorticoids, thiazides, hydantoins, antipsychotics, epinephrine, protease inhibitors, diazoxide

Chemicals - rodenticide, nicotinic acid

VI. Infections - congenital rubella, cytomegalovirus, coxsackievirus

VII. Uncommon forms of immune-mediated diabetes mellitus - anti-insulin receptor antibodies

VIII. Other genetic syndromes sometimes associated with diabetes mellitus like Down's syndrome, Turner's syndrome, Klinefelter's syndrome, Friedreich's ataxia, Huntington's chorea, myotonic dystrophy, porphyria.

IX. Gestational DM when diabetes mellitus begins or is detected for first time during pregnancy

3.1.3 - Criteria for the diagnosis of DM[5]:

Symptoms of hyperglycemia and random blood glucose concentration ≥ 200 mg/dL or

Fasting plasma glucose (FPG) ≥ 126 mg/dL or

Glycosylated hemoglobin ≥ 6.5% or

2-hour plasma glucose ≥ 200 mg/dL

3.1.4 - Complications of DM[4]:

I Acute complications

- Diabetic ketoacidosis
- Hyperglycemic hyperosmolar coma

II Chronic complications

- Macrovascular
- Coronary artery disease (CAD)
- Peripheral vascular disease
- Cerebrovascular disease

- Microvascular

- Retinopathy
- Neuropathy
- Nephropathy

3.2 - Coronary artery disease (CAD)

Among the macrovascular complications of DM, coronary artery disease (CAD) is one of the most common and devastating. CAD is "associated with chronic inflammation of the vessel wall which leads to narrowing and remodeling of the coronary arteries that supply the oxygenated blood to the heart. This narrowing of coronary vessels leads to an imbalance between the supply and demand of the heart" [6]. CAD usually manifests as stable angina, acute coronary syndrome (ACS) and sudden death.

3.2.1 - Epidemiology:

As per the WHO reports in India in 2016, 63% of the estimated deaths were due to non-communicable diseases. Out of this, 27% deaths were due to cardiovascular diseases (CVD) [7]. Prevalence of CAD was 6.4% in urban areas and 2.5% in rural areas. It was 6.1% in males and 6.7% in females[8]. By the end of 2025, mortality due to cardiovascular disease will surpass the other diseases like infection, cancer and trauma[9].

3.2.2 - Risk factors [10]:

Modifiable risk factors

1. Diabetes mellitus
2. Hypertension
3. Hyperlipidemia
4. Smoking

Non-modifiable risk factors

1. Age
2. Gender
3. Genetic factors
4. Family history

Others

1. Elevated Lp(a)
2. hsCRP
3. Reduced NO
4. Elevated homocystein

Modifiable risk factors:

1. Diabetes mellitus: As per national cholesterol education programme (NCEP) guidelines, DM is considered as cardiovascular risk equivalent. Type-2 DM patients have two to four fold increased risk of future cardiovascular events and these events will occur two to three decades earlier compared to non-diabetic individuals [11].
2. Hypertension: Contribution of both systolic as well as diastolic blood pressure increases the risk of CAD around 60%. Increased consumption of salt in the diet increases risk of heart disease.
3. Hyperlipidemia: Hypercholesterolemia is one of the major risk factor for atherosclerosis. Increased LDL cholesterol, increased TG and decreased HDL cholesterol are major components of the lipids that predict the risk of future cardiovascular events. Intake of diet rich in saturated fats raises the plasma

cholesterol level. Smoking and obesity lowers HDL, whereas exercise and moderate consumption of ethanol raises HDL levels. Statins like atorvastatin, simvastatin and pravastatin inhibits hydroxylmethylglutaryl coenzyme A (HMG-CoA) reductase which is the key enzyme in cholesterol biosynthesis [12].

4. Smoking: Smoking is the most important risk factor for CAD. Prevalence of smoking is increasing among adolescents and women. Risk is directly proportional to number of cigarette consumption. Smoking 20 or more cigarettes daily will increase the risk of CAD by 2-3 folds [13]. It accelerates the atherosclerotic progression, enhances the oxidation of LDL cholesterol and impairs endothelium dependent coronary artery vasodilatation. It enhances platelet aggregation, increases monocyte adhesion etc. There is substantial reduction of risk by cessation of smoking.

Non-modifiable risk factors:

1. Age: Atherosclerosis, the progressive disease, usually manifest in middle or later age. Incidence of MI increases fivefold between the ages 40 and 60 years.
2. Gender: Males and post menopausal women are at higher risk of CAD.
3. Family history: There is an increased risk of premature deaths due to CAD in an individual with positive family history of CAD.

3.2.3 - Pathogenesis [14]:

Atherosclerosis is the main etiopathogenic mechanism in the causation of CAD. Around 90% of the CAD patients have atherosclerosis in the large and medium-sized elastic coronary arteries. Atherosclerosis literally means “hardening of the arteries”. It is a chronic inflammatory and healing response of arterial wall to endothelial injury.

Sequence of pathogenic events in atherosclerosis is as follows [15]:

I. Atherosclerosis initiation

- Endothelial injury
- Accumulation of lipoproteins
- Monocyte adhesion to endothelium
- Foam cell formation

II. Evolution of atheroma

- Platelet adhesion
- Inflammation in atherogenesis
- Smooth muscle cell migration and proliferation
- Extracellular matrix production
- Plaque mineralization

III. Complications of atherosclerosis

- Plaque rupture and thrombosis
- Plaque vulnerability

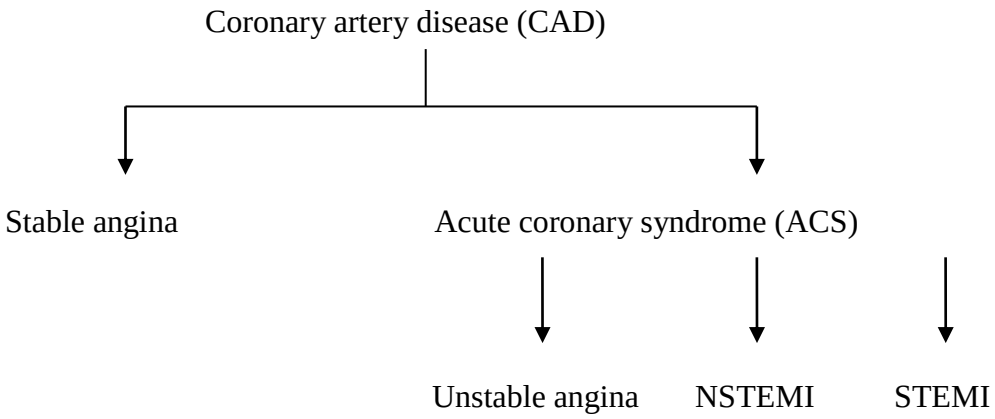
Endothelial injury due to mechanical force, hemodynamic forces, immune complex deposition, chemicals, toxins, irradiation results in intimal thickening. Endothelial dysfunction is an early lesion in the intact endothelium. In DM, dyslipidemia and reduced bioavailability of NO are responsible for initiation of endothelial dysfunction. Thus endothelial injury results in lipoprotein accumulation in the intima. These dysfunctional endothelial cells show increased permeability, endothelial adhesion and altered gene expression. Generally normal endothelial cells resist interaction with leukocytes. Modification of these lipoproteins occurs by processes like oxidation and glycation. These

modified lipoproteins and oxidative stress can induce local cytokine elaboration. Cytokines thus induced increase the expression of leukocyte adhesion molecules and chemo attractant molecules like monocyte chemo attractant protein (MCP-1) and direct them to migrate into intima. The monocytes on entering into intima differentiate themselves into macrophages. Then macrophages bind to modified lipoproteins like oxidized Lp(a) and oxidized LDL via scavenger receptors to become foam cells. These foam cells release mediators like cytokines, interleukins, tumor necrosis factors and molecules like hypochlorous acid, superoxide anion and matrix metalloproteinase's thus resulting in formation of fatty streak.

The different leukocytes like monocytes, macrophages, foam cells and lymphocytes induce cellular as well as humoral immune response and release of inflammatory molecules. Smooth muscle cells (SMC) migrate into the intima from tunica media. After reaching the intima SMC divide and release extracellular matrix, deposits on the growing fatty streak and results in more complex lesion of fibrous cap i.e atherosclerotic plaque. Later, calcification and fibrosis occur. Sometimes, SMC undergo programmed cell death i.e apoptosis.

Integrity of atherosclerotic plaque depends on thickness of fibrous cap. It may be stable or vulnerable plaque. Stable plaques have intact, thick fibrous cap composed of SMC rich in type-I and type-III collagen. Vulnerable plaques have thin fibrous cap mostly composed of inflammatory cells, few SMC and collagen. This type of vulnerable plaques are more prone for rupture and expose the core content of the plaque to circulating proteins leading to thrombosis and occlusion of the arteries. If the occlusion is partial, it is unstable angina or non-ST elevated myocardial infarction (NSTEMI) and if it is total occlusion leads to ST elevated myocardial infarction (STEMI). These are different clinical manifestations of coronary artery disease (CAD).

3.2.4 - Clinical manifestation of CAD [14]:



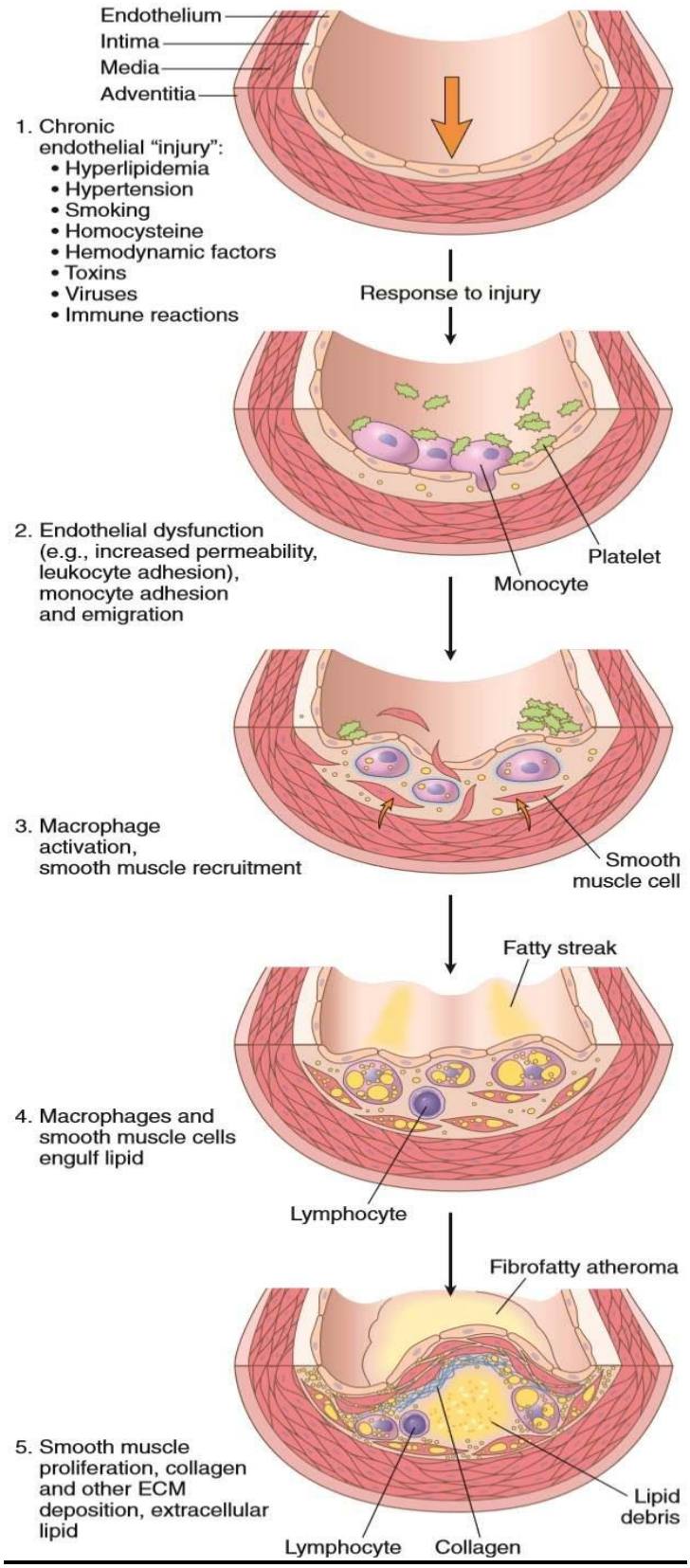


Figure -3.1: Evolution of arterial wall changes in Atherosclerosis
Source: Robbins and Cotran Pathologic Basis of Disease, 2015, 9th edition. Elsevier publishers.

3.3 - Link between DM and CAD

Diabetes mellitus (DM) and CAD are the most common non-communicable diseases. DM is one of the pandemic diseases in India [16]. DM patients are known to have two to four times increased risk of CAD. CAD occurs two to three decades earlier in DM patients compared to non-diabetic individuals [17]. DM patients known to have eight years of reduced life expectancy. CAD constitutes 80% of all deaths in DM patients [18]. Fibrous plaques are more vulnerable to rupture among DM patients [19]. As per national cholesterol education programme (NCEP) guidelines, DM is considered as cardiovascular risk equivalent[11]. Risk factors for DM and CAD almost contribute independently and may cluster each other to increase the risk.

3.3.1 - Biochemical mechanism contributing to CAD in DM [20]

Multiple biochemical mechanisms that lead to vascular changes contributing to CAD are as follows:

1. Sorbital pathway via aldolase reductase activity
2. Increase in fructose-6-phosphate via hexosamine pathway
3. Activation of protein kinase-C via diacyl glycerol
4. Formation of advanced glycation end products (AGEs)
5. Oxidative stress
6. Endothelial dysfunction

Biochemical alterations in various metabolic pathways in DM patients contribute to CAD [20]

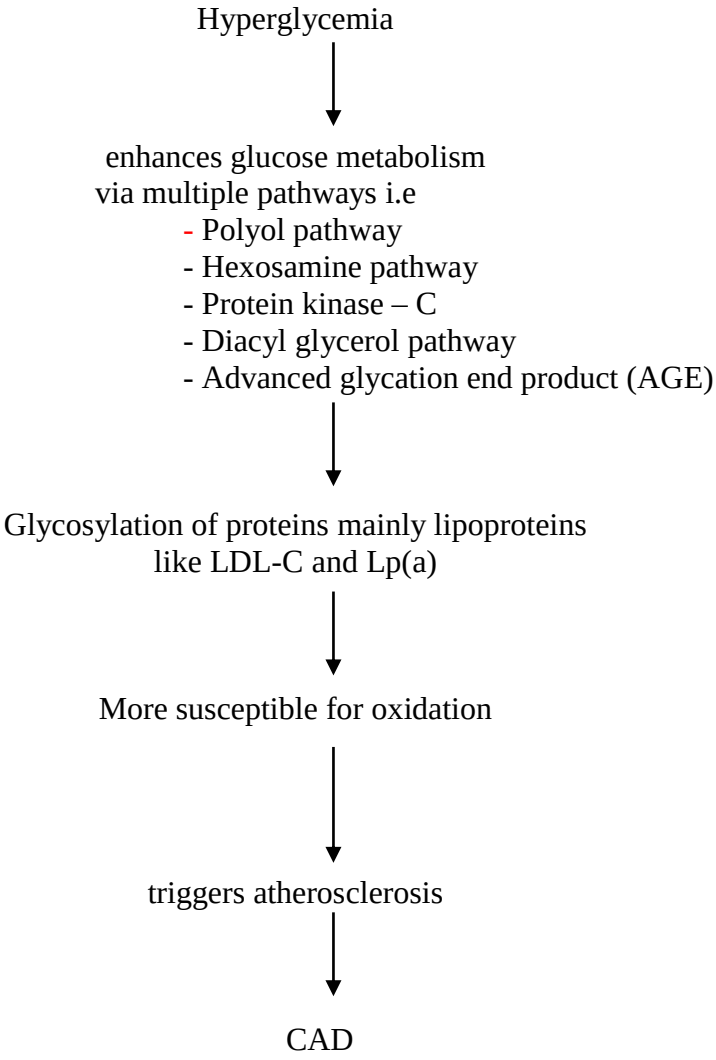


Figure-3.2: Flow chart showing biochemical alterations due to high blood glucose level

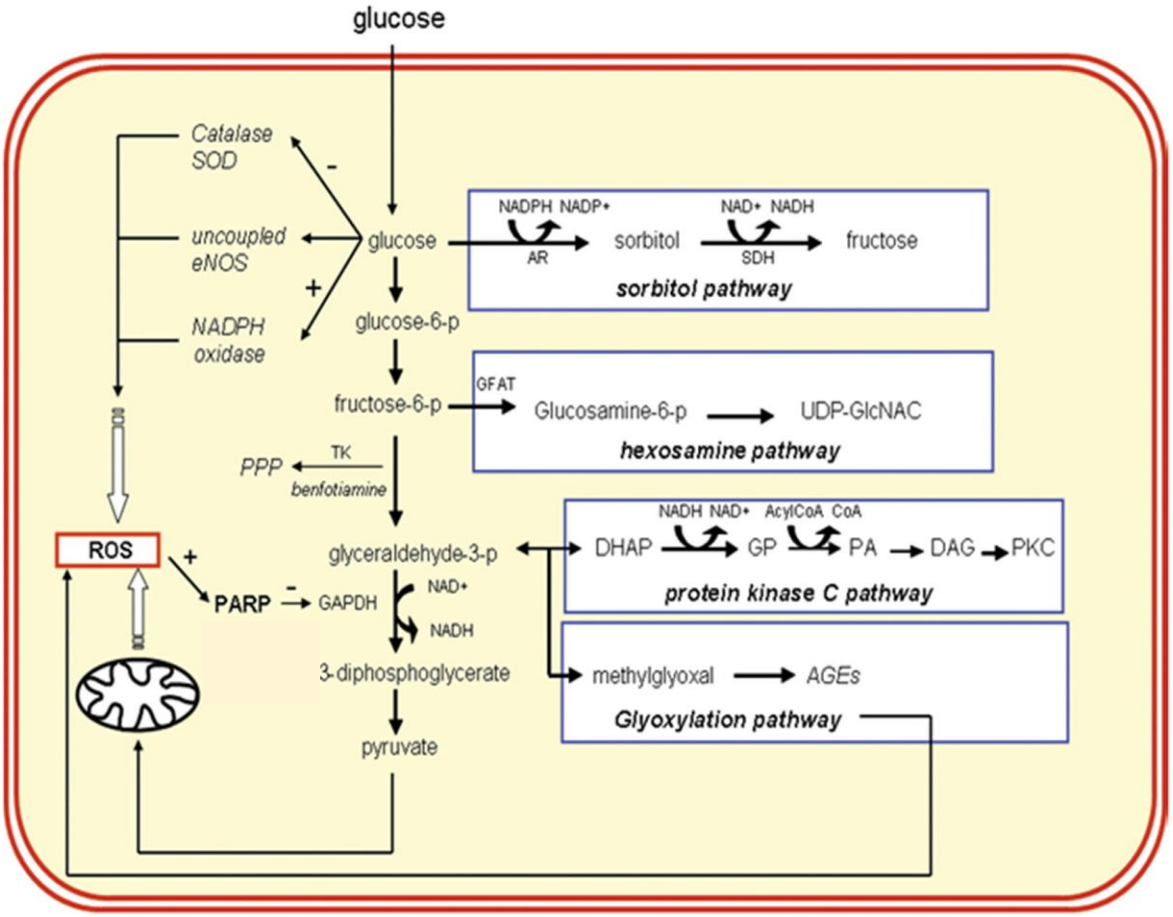


Figure-3.3: Biochemical mechanisms responsible for vascular changes in DM

Source: Schalkwijik CG, Stehouwer CDA. Vascular complications in diabetes mellitus: the role of endothelial dysfunction. Clin Sci 2005;109:143-59.

1. Sorbitol (polyol) pathway [21]:

Aldose reductase catalyses the NADPH dependant reduction of glucose to sorbitol. Aldose reductase has very high Km for glucose. At normal glucose concentrations found in non-diabetics, metabolism of glucose by this pathway is a very small percentage of total glucose use. In hyperglycemic state increased intracellular glucose results in increased enzymatic conversion of glucose to sorbitol by this pathway.

2. Hexosamine pathway [21]:

In hexosamine pathway, fructose 6-phosphate is converted into fructosamine 6-phosphate by the enzyme glutamine fructose 6-phosphate amidotransferase and finally converted into N-acetyl glucosamine (GlcNAc).

3. Diacylglycerol (DAG) and protein kinase-C [21]:

Hyperglycemia activates DAG, protein kinase-C. These will induce oxidative stress in mitochondria. Raised DAG and PKC activity inturn affects the blood vessels.

4. Advanced glycation end products (AGEs) [22,23]:

Glucose reacts with most of the tissue proteins by combining with free amino group of lysine residues to form Schiff's base, which further undergoes a series of changes to yield stable fluorescent products termed as maillard products or advanced glycation end products (AGEs). Increased plasma glucose level increases glycosylation of proteins mainly lipoproteins like LDL and Lp(a). AGEs are associated with tissue damage. Glycation of proteins in DM increases the rate of free radical production by fifty fold. These free radicals increase oxidation of lipoproteins thus triggering the atherosclerosis progression.

5. Oxidative stress [24,25]:

Oxidative stress is the disequilibrium between pro-oxidants and antioxidants. Hyperglycemia enhances production of reactive oxygen species (ROS) by activating NADPH oxidase and xanthine oxidase. It also leads to inactivation and reduced expression of the antioxidant enzymes i.e catalase and superoxide dismutase. Uncoupling of mitochondrial oxidative phosphorylation and endothelial nitric oxide synthase (eNOS) also contributes for increased generation of free radicals. Diabetic

endothelium produces an increase in superoxide and hydrogen peroxide (H_2O_2) thus increasing production of hydroxyl radicals. At an injured and inflamed endothelial site there will be increased generation of ROS. ROS at high concentration can cause cell injury and death. Oxidative stress increases endothelial permeability and enhances leukocyte and platelet adhesion, alters endothelial signal transduction and redox-regulated transcription factors. In DM uncoupling of eNOS activity, and activation of vascular NADPH oxidase leads to endothelial damages (ED). Increased production of superoxide, hydrogen peroxide by diabetic endothelium leads to ED.

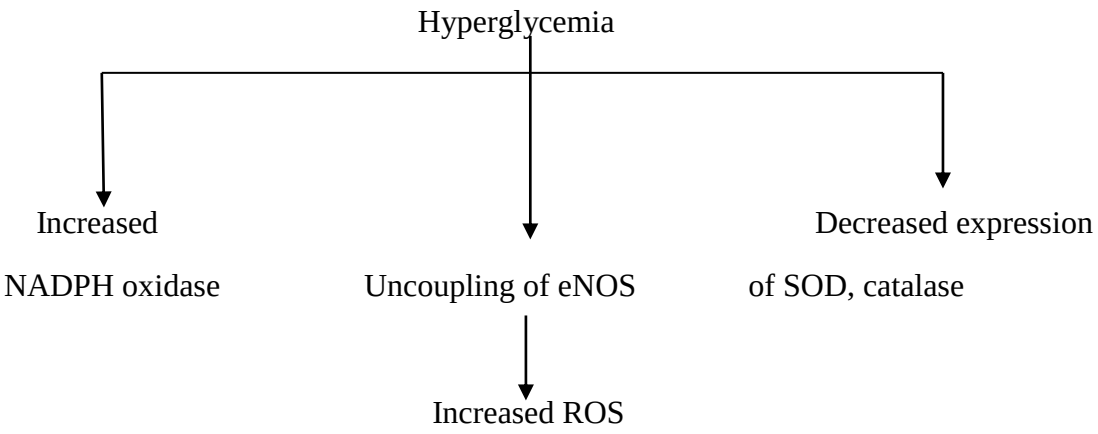


Figure-3.4: Flow chart showing hyperglycemia induced oxidative stress in DM

6. Endothelial dysfunction (ED)[26,27]:

Endothelium is the inner layer of the blood vessels, which maintains the vascular homeostasis. It maintains the vascular homeostasis by releasing certain vasoactive factors that regulate blood flow, vascular tone, coagulation, delivery of nutrients, vascular smooth muscle cell (VSMC) proliferation and migration, inflammation, platelet and leukocyte

adherence[26]. Important molecules produced by endothelium to maintain these functions are nitric oxide (NO), endothelin-1 (ET-1), angiotensin-II, prostacyclin and endothelial derived hyperpolarizing factors (EDRF)[27]. Nitric oxide (NO) can be used to measure endothelial cell damage.

Imbalance in the release of endothelial-derived relaxing factor and contracting factors leads to endothelial dysfunction (ED). ED is common in type-2 DM and independently predicts cardiovascular risk[28].

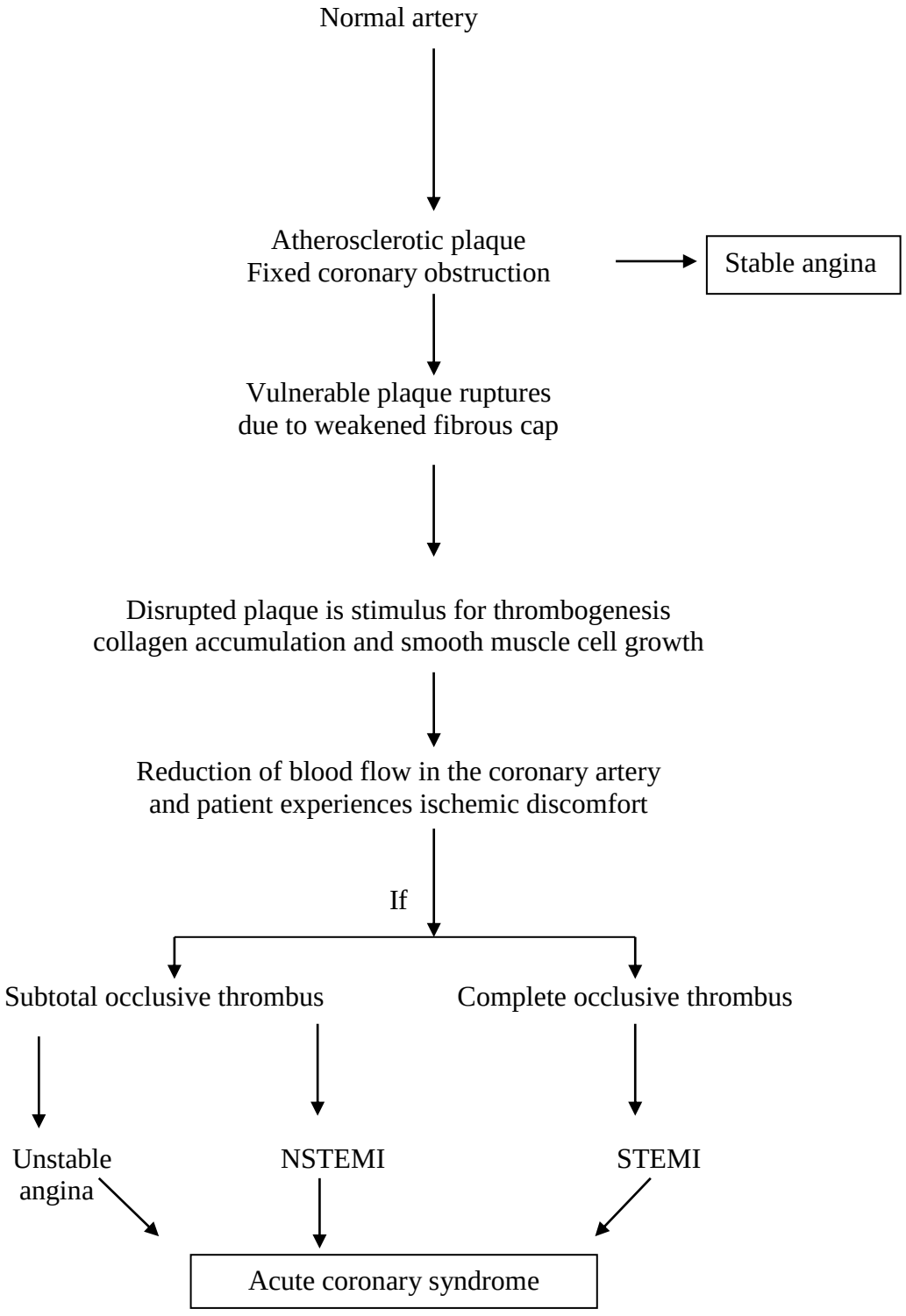


Figure-3.5: Schematic presentation of sequential progression of coronary artery disease

Source: Modified from Libby P: Circulation 2001; 104:365.

3.3.2 - Clinical manifestations of CAD[29]:

Stable angina: It manifests as deep, poorly localized chest discomfort, precipitated on physical exertion and emotional stress and relieved by rest or sublingual nitroglycerin.

Unstable angina: It is angina pectoris or equivalent type of angina pectoris, with atleast one out of three features -

1. Chest pain at rest or on minimal exertion and lasts for more than 20 minutes.
2. Chest pain is severe and usually described as frank pain.
3. Chest pain is severe, prolonged and awakens patient from sleep

STEMI (ST segment elevation myocardial infarction): Evidence of myocardial ischemia, 12-lead ECG shows ST and T wave abnormality.

NSTEMI (Non-ST segment elevation myocardial infarction): Patients with chest pain with no ST elevation in ECG and evidence of myocardial necrosis on the basis of raised biochemical cardiac markers like troponin-T or troponin-I and creatine kinase-MB (CK-MB).

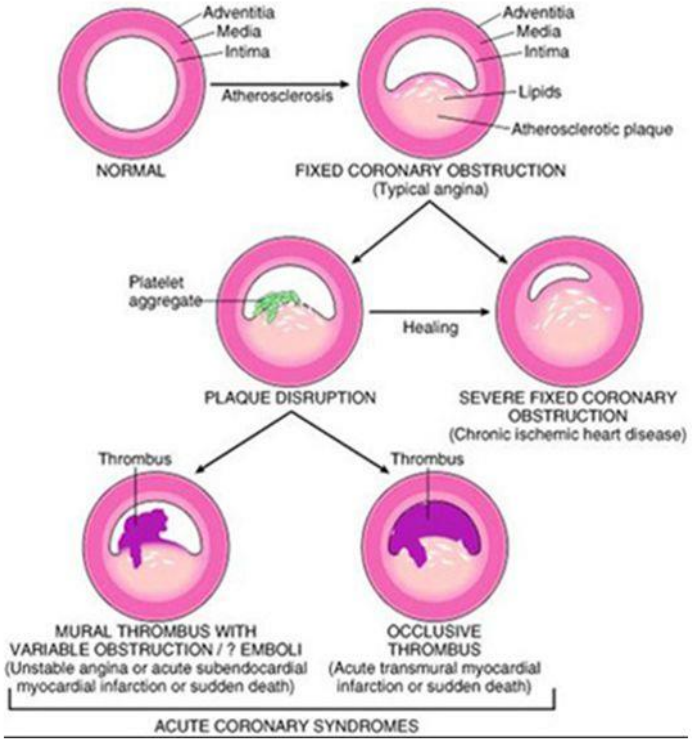


Figure-3.6: Diagram showing different manifestations of acute coronary syndrome

Source: Robbins and Cotran Pathologic Basis of Disease, 2015, 9th edition. Elsevier publishers.

3.3.3 - Clinical features[30]:

Patient complains of chest pain. It is severe, intolerable, prolonged, may last for thirty minutes to few hours. It is usually located in retrosternal region or may radiate down to left ulnar border with tingling sensation. Nausea and vomiting may occur due to stimulation of left ventricular receptors or due to vagal reflex. Other symptoms like palpitation, weakness, dizziness or diarrhea may also be present in these patients.

3.3.4 - Diagnosis[30]:

Coronary angiography - findings showed presence of minimum of 50% stenosis in major coronary arteries like left circumflex artery, left anterior descending artery or right coronary artery.

ECG – Shows ST segment elevation or depression, T wave abnormalities and development of pathologic ‘Q’ wave.

Cardiac markers – Rise in biochemical markers like CK-MB, Troponin-I and Troponin-T.

Imaging – Shows regional motion wall abnormalities.

3.4 - Dyslipidemia

"Alteration in lipid parameters i.e increased levels of triglyceride (TG), low density lipoprotein (LDL-C) and decreased high density lipoproteins (HDL-C) are called dyslipidemia".

Cholesterol:

Cholesterol is a steroid compound, contains 27 carbon units, having a 'cyclopentanoperhydrophenanthrene' ring. It is synthesized in the liver and intestine. Cholesterol acts as a precursor for biologically very important compounds like vitamin D, bile acids and steroid hormones. It is associated with atherosclerosis and heart disease[31]. Increased serum cholesterol is the causative factor in the causation of atherosclerotic diseases. An increased serum cholesterol concentration will markedly increase coronary disease risk[32]. A report by the Cholesterol Lowering Atherosclerosis Study (CLAS) demonstrated the benefit of lowering cholesterol in people with established disease [33].

Triglyceride (TG):

They are esters of glycerol and fatty acids. It act as an important fuel storage reserve. It is synthesized in the liver and adipose tissue and is stored mainly in the adipose tissue in large lipid droplets. It undergoes continuous lipolysis and esterification. The balance between these two determines the free fatty acid pool in adipose tissue and circulating levels in the plasma. Triglycerol is the major lipid in chylomicrons and VLDL. When the TG content in the liver parenchymal cells goes up to 25-30% it is called fatty liver. When the accumulation becomes chronic, fibrotic changes occur in the cells which may finally lead to liver cirrhosis and impairment of liver function. Since it has no charge, it is also called neutral lipid[34].

3.4.1 - Lipoproteins:[35]

Lipoproteins are water soluble macromolecules, composed of lipid and protein that transport insoluble lipids through the blood between different organs and tissues. The protein moiety of lipoprotein is known as an apoprotein or apolipoprotein. These are separated according to their electrophoretic properties. The compositions of lipoproteins are shown in the table.

Table No-3.1: Different lipoproteins and their characteristic features:-

Characters	**VLDL-C**	**LDL-C**	**HDL-C**	**Chylomicrons**	**Lipoprotein (a)**
Diameter (nm)	30-90	20-25	5-10	90-1000	26-30
Density (g/mL)	0.95-1.006	1.019-1.063	1.063-1.210	< 0.95	1.040-1.130
Lipid-protein ratio (%)	90:10	80:20	50:50	99:1	75:25
Main lipids	Endogenous TAG	Cholesterol	Phospholipids, cholesterol	TAG	Cholesterol, phospholipids
Apolipoproteins	B-100, C, E	B-100	A, C, E	A, B-48, C, E	apo(a), B-100
Source	Liver	Plasma VLDL	Liver, Intestine	Intestine	Liver
Functions	Transport of TG from liver to peripheral tissue	Transport of cholesterol from liver to peripheral tissue	Transport of cholesterol from peripheral tissue to liver	Transport of dietary lipids from intestine to peripheral tissues	Atherogenic

Table No-3.2: Alteration of lipoproteins in type-2 DM [36]

Type of Lipoprotein	Alterations
TG ↑	Increased production of TG and apo B, decreased clearance of TG and apo B, abnormal composition
HDL-C ↓	Increased clearance of apo A, decreased proportion of large HDL-C, TG enrichment, glycation, decreased reverse cholesterol transport
LDL-C ↑/N	Increased production of LDL apo B, decreased receptor mediated clearance, TG enrichment, glycation & oxidation
Lp(a) ↑	Delayed clearance from circulation

3.4.2 - Dyslipidemia in DM without CAD patients:

The dyslipidemia in type 2 DM consists of increased TG and decreased HDL-C [37]. Triad of small dense LDL-C, increased TG and decreased HDL-C can also be seen in impaired glucose tolerance and insulin resistance. Hyperglycemia and dyslipidemia generally coexist in diabetic patients with poor glycemic control. Although they have been shown to be independent significant risk factors for vascular complications, the interaction of hyperglycemia and dyslipidemia increases the risk of macro and microvascular complications[38].

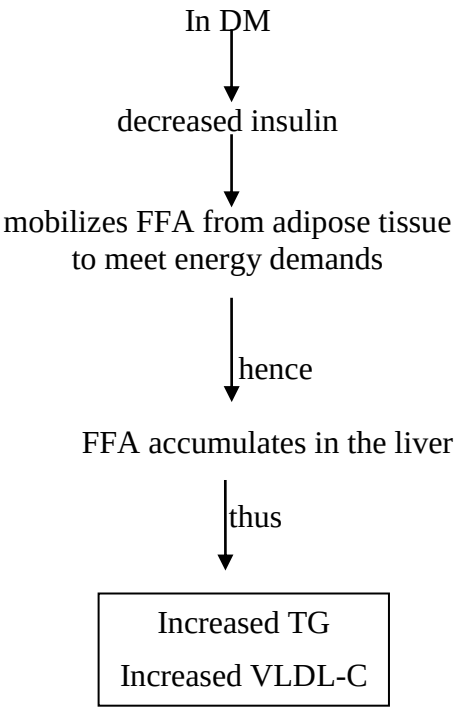


Figure-3.7: Flow chart showing the increased TG and VLDL-C in DM [38]

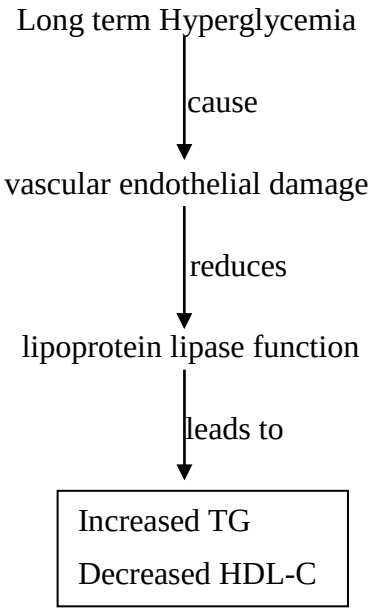


Figure-3.8: Flow chart showing increased TG and decreased HDL-C in DM [38]

A study conducted by Abdulbari Bener et al[39] in 2007, on 214 type-2 DM Arabian patients and 214 healthy controls of the age group between 25 to 65 years showed increased levels of TGs and decreased levels of HDL-C in diabetic patients compared to healthy controls. An animal study documents hypertriglyceridemia and reduced HDL-C due to exchange of VLDL-C triglyceride with cholesteryl esters of HDL-C by cholesteryl ester transfer protein (CETP)[40].

3.4.3 - Dyslipidemia in DM with CAD patients:

Hyperglycemia has been implicated in the pathogenesis of diabetic dyslipidemia. It leads to elevated levels of atherogenic cholesterol-enriched apo-B containing remnant particles by reducing expression of the heparan sulphate proteoglycan perlecan on hepatocytes[41]. Post-prandial hyperglycemia may be more predictive of atherosclerosis than is fasting plasma glucose level or HbA1c[42]. A strong association exists between dyslipidemia and increased risk of microvascular and macrovascular complications such as CAD[43].

A prospective study by United Kingdom Prospective Diabetes study (UKPDS) reported increased CAD was associated with increased TG, LDL-C and decreased HDL-C levels [44]. A study conducted in 2013 by Pant DC et al[45], on 36 newly diagnosed CAD patients, showed high TGs and low HDL-C levels whereas TC and LDL-C were within normal range. Ishfaq A et al[46] and Anthonia OO et al [47] had similar findings. Few studies reported decrease in TC and TG reduced mortality in CAD[48-50].

Thus from above literature survey it is well understood that dyslipidemia plays significant role in development of macrovascula complication like CAD. But studies also reported normal lipid parameters and still the burden of high cardiovascular disease. So thus, this

made an attempt to find the newer risk factors contributing for pathogenesis of CAD. Among the novel parameters we estimated the lipoprotein(a) and its association in DM as well as CAD patients.

3.5 - Lipoprotein(a) Lp(a)

There are many novel risk factors contributing for initiation and progression of atherosclerosis along with good old conventional risk factors. Lipoprotein(a) plays an important contributing risk factor.

3.5.1 – Structure:

Lipoprotein (a) [Lp(a)] is a LDL-C like molecule. It was discovered by Kare Berg in 1963 [51]. Lp(a) consist of a unique apolipoprotein (a) [apo(a)] which is covalently bound to apolipoprotein B by a disulphide bond. Its molecular weight is 2.9 to $3.7x10^6$ daltons, diameter 25 to 30 nm, density is 1.04 to 1.13 g/mL. It is composed of lipid and protein in the ratio of 64:36 and cholesterylester and phospholipids are major lipids. It occupies pre-beta region in the electrophoretic mobility, half-life in the circulation is 3-4 days[52]. In 1987, Richard Lawn and his associates discovered LPA gene[53]. The apo(a) contains variable number of kringle units, the fourth kringle have homology with plasminogen. Hence, it interferes with fibrinolysis[54].

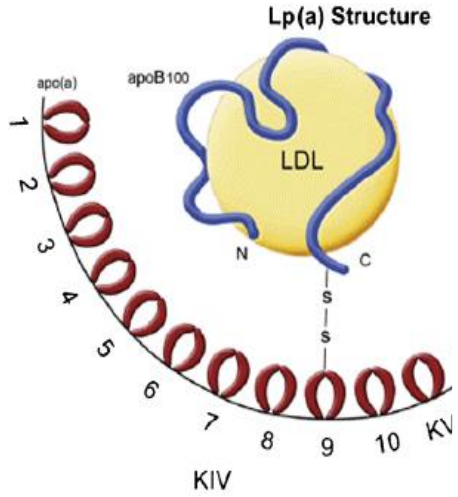


Figure-3.9: Structure of Lipoprotein (a)
Source: Hoover-Plow J, Huang M. Lipoprotein(a) metabolism: Potential sites for therapeutic targets. Metabolism 2013;62(4):479-91.

3.5.2 - Genetics:

The Lp(a) occurrence was documented by genetic, biochemical and immunological evidence in humans and other primates like baboon, rhesus monkeys and apes. Lp(a) expression of apo(a) has been reported for other tissues like testes, aorta and carotid arteries [55]. Its blood concentrations are controlled by size of apo(a) isoforms gene located on chromosome 6q(26-27)[56]. There is an inverse relation between apo(a) size and Lp(a) levels. Smaller isoform tend to have higher plasma Lp(a) levels thus leads to an increased risk for CVD [57,58]. The apo(a) size polymorphism determines the Lp(a) levels in the blood. Polymorphism of C/T variation in the promoter region of apo(a) gene and variation in pentanucleotide TTTTA repeats differed a cross ethinicity[59]. LPA gene is a single nucleotide (SNP) polymorphism can be associated with higher and lower Lp(a) levels [60,61]. Differences in Lp(a) levels and apo(a) size distribution noted in Asian populations. South Asians have higher mean Lp(a) levels[62]. Around 90% of Lp(a) concentration is under genetic regulation. Eventhough Lp(a) levels are under genetic control, some conditions may effect on Lp(a) levels. They are diabetes mellitus, liver and renal failure, acute-phase response, hormonal homeostasis and defects in the LDL receptor gene which have all been shown to influence Lp(a) metabolism[63].

3.5.3 – Metabolism of Lp(a):

Lp(a) is mainly synthesized in the liver as a precursor with low molecular mass, which is processed and then secreted into circulation as mature form. Around 90% of Lp(a) concentration is under genetic control. Along with it some other factors also may have effect on Lp(a) levels, which are acute phase response, liver and renal conditions, hormonal homeostasis and LDL receptor gene may influence the metabolism[63]. There were conflicting observations regarding the catabolism of Lp(a) and apo(a). It is thought to be catabolised mainly by hepatic and renal pathway. Apo(a) may be degraded to fragments

in plasma, liver and other tissues and few fragments excreted into urine. Apo(a), Lp(a) and its fragments bind to ECM proteins. It may also be catabolised by binding to VLDLR/ megalin and then taken up by hepatocytes, fibroblasts and macrophages. It is postulated that Lp(a)degradation is mediated by LDL receptor as it resembles LDL[64]. But LDL receptor seems to play very minimal role in Lp(a) elimination. Studies showed nonspecific receptor pathway, human skin fibroblast cultures, human monocyte derived macrophages, plasminogen receptor and asialoglycoprotein receptor are also responsible for its elimination[65]. Evidence also suggests that, VLDL receptor in skeletal muscle may be of significance for Lp(a) binding and degradation[66]. Studies also showed role of kidney in Lp(a) catabolism. Circulating Lp(a) interacts with kidney cells and cause cleavage of 2/334 of amino terminal part of apo(a) by collagenase type protease. Part of these apo(a) fragments are found as excretory products of Lp(a) in urine[67].

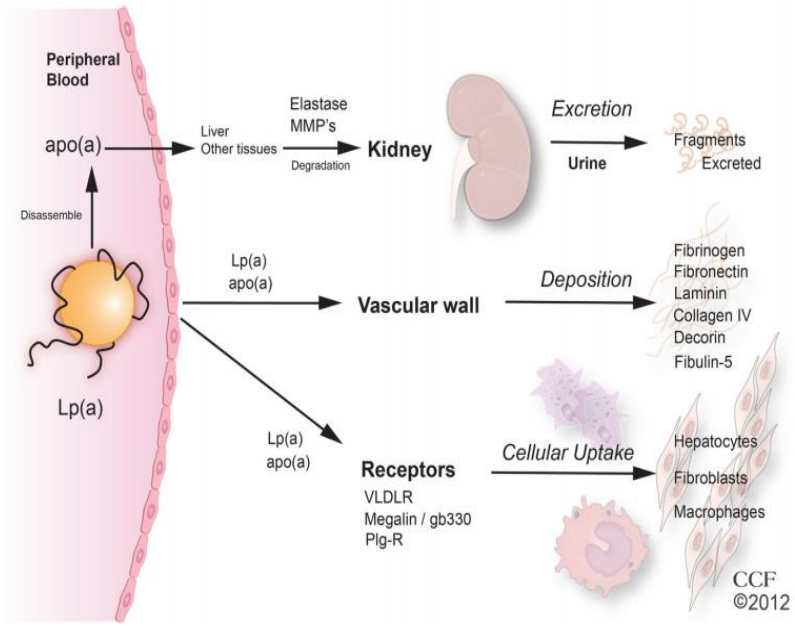


Figure-3.10: Catabolism of Lp(a)
Source: Hoover-Plow J, Huang M. Lipoprotein(a) metabolism: Potential sites for therapeutic targets. Metabolism. 2013;62(4):479-91.

3.5.4 - Atherogenicity of Lp(a):

1. Native form of Lp(a) has tendency of self-aggregation and precipitation. It has been shown to bind to glycosaminoglycans and other vascular structures. It binds to endothelial cells and increases permeability, platelets, macrophages, fibroblasts and sub-endothelial matrix. It promotes vascular smooth muscle cell migration and proliferation[68]. It also enhances foam cell formation[69].
2. Apo(a) component of Lp(a) is structural analogue of plasminogen. It binds to fibrinogen and fibrin and results in inhibition of plasminogen binding. It also compete with plasminogen for its receptors on endothelial cells and leads to decreased plasmin formation. Thereby delays the clot lysis and favouring thrombosis[70,71].
3. Lp(a) induces production of plasminogen activator inhibitor-1 (PAI-1) and inhibits tissue-plasminogen activator[72].
4. Lp(a) promotes generation of free radicals in monocytes[73].
5. Lp(a) via apo(a) stimulates the production of various adhesion molecules in an endothelial cells system like intercellular adhesion molecule, vascular cell adhesion molecule, E-selectin, endothelin-1 and IL-8[74,75].
6. Lp(a) via apo(a) inhibits transforming growth factor thus results in increased vascular smooth muscle proliferation[76].

3.5.5 - Modifications of Lp(a):

Lp(a) molecule can undergo oxidative modification. Oxidation of Lp(a) and other lipoproteins like LDL-C affect the catabolism of lipoproteins. These include receptor recognition, catabolic rate and retention in the vessel wall and thus accelerate the atherosclerosis[77]. A study showed that Lp(a) particles are prone to oxidation, these oxidized lp(a) [Ox-Lp(a)] might attenuate fibrinolytic activity by reducing plasminogen

activation, enhance PAI-1 production in vascular endothelial cells and impair endothelium dependent vasodilatation. Studies demonstrated that CAD patients had increased ox-Lp(a) levels [78,79]. A study of autopsy findings revealed that deposition of Ox-Lp(a) in the vessels of calcified area[80] . Another study showed the mitogenic effect of Lp(a) and Ox-Lp(a) on human vascular smooth muscle cells. Ox-Lp(a) showed stronger stimulatory action on vascular smooth muscle cell (VSMC) compared to native Lp(a)[81].

A study by Seimon TA et al in the year 2010, documented that Lp(a) and oxidized phospholipids (OxPL) on apoB mediate apoptosis in endoplasmic reticulum stressed macrophages by signaling through CD36/TLR2 receptor pathway to activate ERK. Thus ERK stimulates p47 clusture and NADPH oxidase dependent generation of ROS[82]. There is presence of Lp(a) and OxPL in vulnerable human carotid artery thin cap fibroatheroma, which is the precursor of plaque destabilization and rupture [83]. Elevated levels of OxPL on apoB are associated with the presence and progression of CAD and peripheral arterial disease and predict new CVD events. It also provides predictive value in models with established risk factors or Framingham risk score estimates [84].

Glycation of lipoprotein may contribute to the premature atherogenesis in patients with diabetes mellitus. This diverts the lipoproteins from nonatherogenic to atherogenic pathway. Proportion of elevated Lp(a) levels associated with glycated apoB-100 was significantly higher in diabetic patients compared to nondiabetic patients. AGEs and endothelial cell mediated oxidative modification enhances production of plasminogen activator inhibitor-1 (PAI-1) and attenuates the synthesis of tissue plasminogen activator (t-PA) induced by Lp(a) in the blood vessels [85]. Hyperglycemia and hyperlipoprotein(a) reduce fibrinolytic activity and promote thrombosis and atherosclerosis in diabetic patients[86].

3.5.6 – Measurement [87]:

Different methods to measure Lp(a) are as follows:

1. Enzyme linked Immunosorbent assay (ELISA)
2. Immunoturbidimetry
3. Immunonephelometry
4. Fluorescence assays
5. Latex immunoassays

Lp(a) is not measured routinely in any patients. These many years neither any prevention societies like American Heart Association (AHA), American College of Cardiology (ACC) and lipid association recommended for Lp(a) screening. In 2011, National lipid association, European atherosclerosis society consensus panel and Canadian cardiovascular society recommended Lp(a) measurement in patients with familial hypercholesterolemia, strong family history of CVD, personal history of premature CVD and inadequate response to statins [88,89].

3.5.7 - Measures to reduce Lp(a) levels:

At present there are no drugs available to decrease the Lp(a) levels. Apheresis decreases the Lp(a) levels by removing Lp(a) from the circulation. Aspirin used in CAD patients reduce the expression of LPA itself. Recent report by Hoover-Plow J et al in 2013, commented niacin reduces the Lp(a) levels to some extent. It lowers the diacylglycerol acyltransferase-2 that decreases TG synthesis and VLDL assembly resulting in increased post-translational intrahepatic apoB degradation [90]. It also decreases LDL and increases HDL which is beneficiary to the patients. A meta-analysis by Bruckert E et al in 2010, documented administration of niacin 1-3 g/day reduces the coronary events by 25% and

cardiovascular events by 27%[91]. Another drug in phase-II trial preprotein convertase subtilisin/kexin type 9 serine protease (PCSK9) inhibitor is also effective in reducing the Lp(a) levels. PCSK9 promotes hepatic LDLR degradation by reducing the LDLR density and clearance. PCSK9 inhibitor reduces the plasma Lp(a) levels.

3.5.8 - Lp(a) in DM without CAD patients

In current strategies of global risk assessment, lipid profile is the most commonly done blood test. Estimation of serum Lp(a) levels helps in early prediction of CAD risk in DM patients. Increase in Lp(a) levels may be due to formation of advanced glycation end products (AGE) in diabetes mellitus patients. These AGEs induce glycated Lp(a) and contribute to the alterations in the generation of plasminogen activator inhibitor-1 (PAI-1) and tissue plasminogen (t-PA).

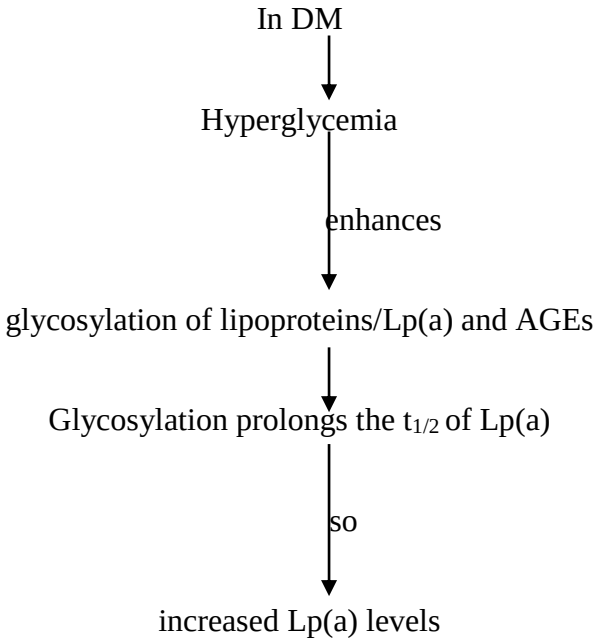


Figure-3.11: Flow chart showing the increased Lp(a) levels in DM patients [92]

A study done on Asian Indians reported high Lp(a) levels and low HDL-C levels may contribute to atherosclerosis at young age. Lp(a) appears to be useful marker of CAD in NIDDM patients independent of age, sex, BMI, smoking, hypertension and other lipid parameters. Mohan V et al, also commented that DM patients have very high prevalence rate of CAD[93].

A south Indian study on 300 type-2 DM subjects, showed Lp(a) to have an independent association with CAD. An increase Lp(a) was found to be associated with increase in carotid intimal thickness, which is a preclinical atherosclerotic marker. Hence Lp(a) is associated with CAD even at an early stage of atherosclerosis[94]. One more south Indian study by Mohan V et al in 2010, states an independent association of Lp(a) with CAD in type-2 diabetic patients[95].

A cross sectional study carried out in 2010 at Nigeria on type 2 DM patients by Anthonia O O et al[96] reported that elevated serum Lp(a) levels were higher in DM patients. They also showed positive association with most of the atherogenic profile parameters in type 2 DM individuals.

A study done by Gazzaruso C et al[97] reported that estimation of Lp(a) and apo (a) phenotypes may be used as a predictor of CAD as well as reliable predictor of CAD severity in type 2 diabetic patients.

Lp(a) which is an important risk factor, showed a significant rise in the serum levels in DM patients compared to controls but its levels were not significantly associated with HbA1C levels which is a standard risk factor for CHD[98].

Another study by Habib SS et al[99] in 2013, at Saudi on 203 participants, showed diabetic patients had higher Lp(a) levels compared to healthy controls. Lp(a) evaluation may have

the potential to improve cardiovascular risk prediction models when used in addition to traditional lipid profiles.

Whereas, according to Haffner et al [100] slightly lower Lp(a) levels were seen in diabetic patients than non diabetic subjects but there was no statistical significance.

3.5.9 - Lp(a) in DM with CAD patients

Human and animal studies have shown that Lp(a) can enter intima of arteries[101]. Hence it may have role in inflammation of intima, thrombosis, and also promotes foam cell formation and cholesterol deposition in plaque by binding to macrophages, which are involved in pathogenesis of atherosclerosis[102,103].

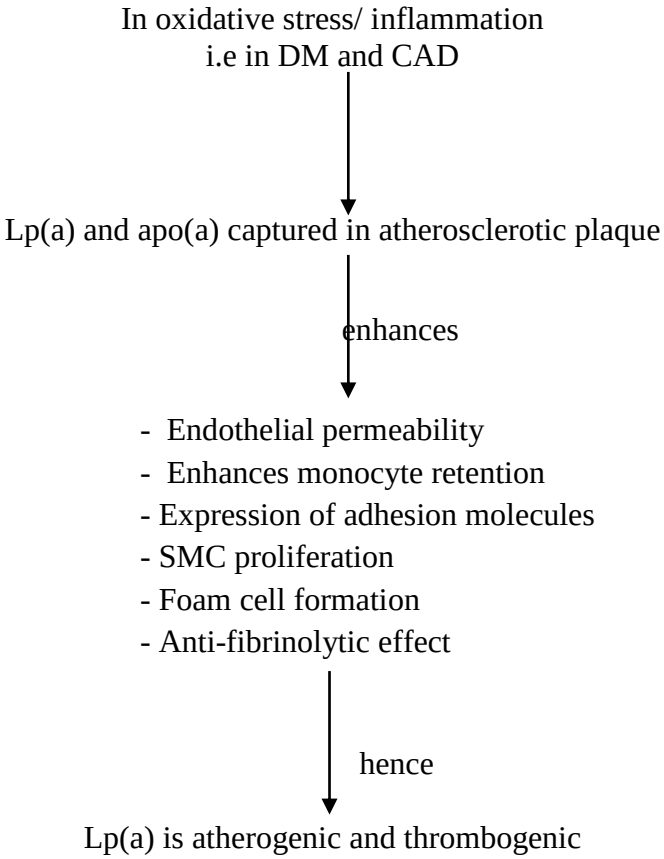


Figure-3.12: Flow chart showing the atherogenic and thrombogenic effect of Lp(a) in DM and CAD [104]

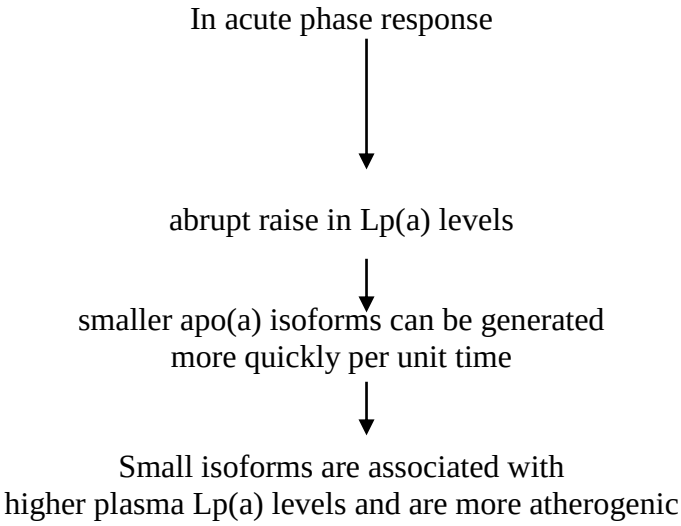


Figure-3.13: Flow chart showing the sequence resulting in elevated Lp(a) levels in DM with CAD patients [104]

Hoogeveen RC, et al[105] documented that conventional risk factors for coronary heart disease (CHD) on 103 subjects, do not completely account for increase in premature CHD in people from the Indian subcontinent. They documented that elevated Lp(a) levels confer a genetic predisposition to CHD in Asian Indians. Lp(a) level more than 30mg/dL is accepted cutoff point for CHD risk among Asian Indians. The synergistic effect of nutritional and environmental factors combined with genetic predisposition may put the Asian Indians living in the USA at increased risk for premature CHD. Estimating Apo(a) phenotyping and determination of plasma Lp(a) concentration, as part of a routine screening procedure of independent risk factors for CHD may aid in the identification of individuals who are genetically predisposed to CHD.

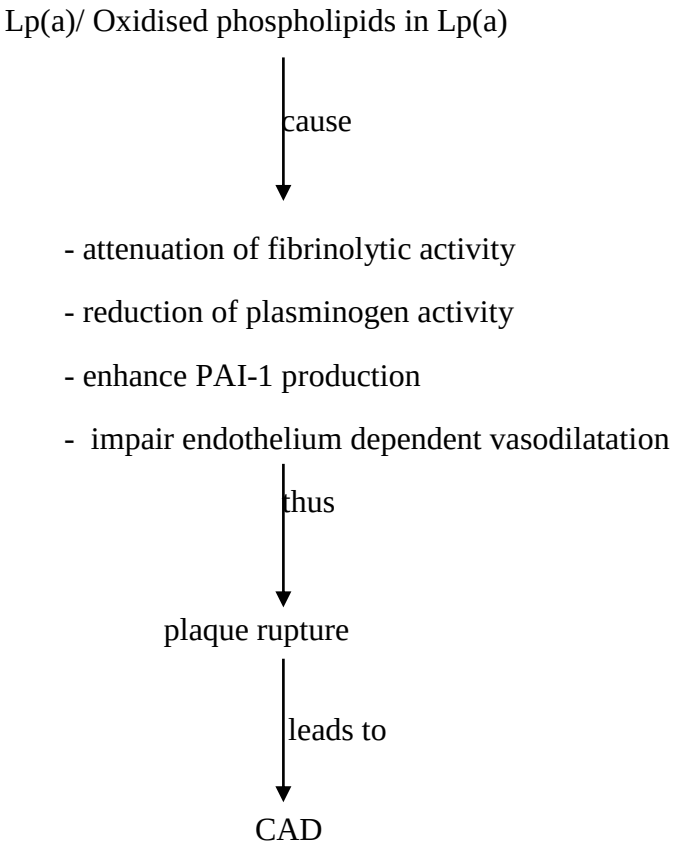


Figure-3.14: Flow chart showing Lp(a) as a cause for rupture of plaque and cause for CAD[104]

The largest study from North India by Yusuf J, et al[106] on 450 subjects reported that Lp(a) should be routinely assessed in all subjects with multiple risk factors for CAD and has been recommended by The European Atherosclerosis Society and The National Lipid Association to better predict the risk of developing CAD. Raised Lp(a) level is also associated with increased risk of ACS and multivessel CAD. Lp(a) above 40 mg/dL assessed by an isoform insensitive assay is an independent risk factor for CAD.

A study by Vandana Saini et al[107] evaluated the significance of the novel biomarkers Lp(a), homocystine and endothelin-1 in CAD patients. There was significant increase in these biomarkers in CAD patients as compared to controls. They reported that Lp(a) levels above 45 mg/dL can predict the risk of premature CAD in Indian population.

Evaluation of Lp(a) and hsCRP levels may have potential to improve cardiovascular risk prediction when it is used in addition to traditional lipid profile[108]. Along with routine lipid profile, Lp(a) should be assessed in all CAD patients. Statins used in dyslipidaemia, along with fibrates and Ezetimibe has unfortunately no effect on Lp(a) levels[109,110].

Administration of niacin 2g/day has shown to decrease Lp(a) by 25% and increase HDL-C by 40%[111]. Novel evidence suggests information on extreme Lp(a) levels to conventional risk factors may substantially improve MI and CHD risk prediction. Individuals at increased risk may hopefully benefit from aggressive prophylactic measures, including statins for LDL-C lowering and possibly niacin treatment for Lp(a) lowering as recently recommended[112].

Endothelial dysfunction is key factor in vascular dysfunction in type-2 DM patients. This can assessed indirectly by nitric oxide estimation.

3.6 - Nitric oxide (NO)

Nitric oxide (NO) is diatomic gaseous signalling molecule which plays an important role in biological processes [113]. It was first proposed by Robert F Furchgott and Louis J Ignarro in 1986[114,115].

3.6.1 - Synthesis of nitric oxide [116]:

NO is synthesised from L-arginine by the family of enzyme nitric oxide synthase (NOS). L-arginine is converted to N-hydroxy-L-arginine (NOHLA) as an intermediate molecule in the reaction which requires oxygen, NADPH, tetrahydrobiopterin (BH4), flavin mononucleotide (FMN) and heme. In the second step, oxidation of N-hydroxy-L-arginine (NOHLA) leads to formation of NO and citrulline. It is synthesized in neurons, macrophages and endothelial cells.

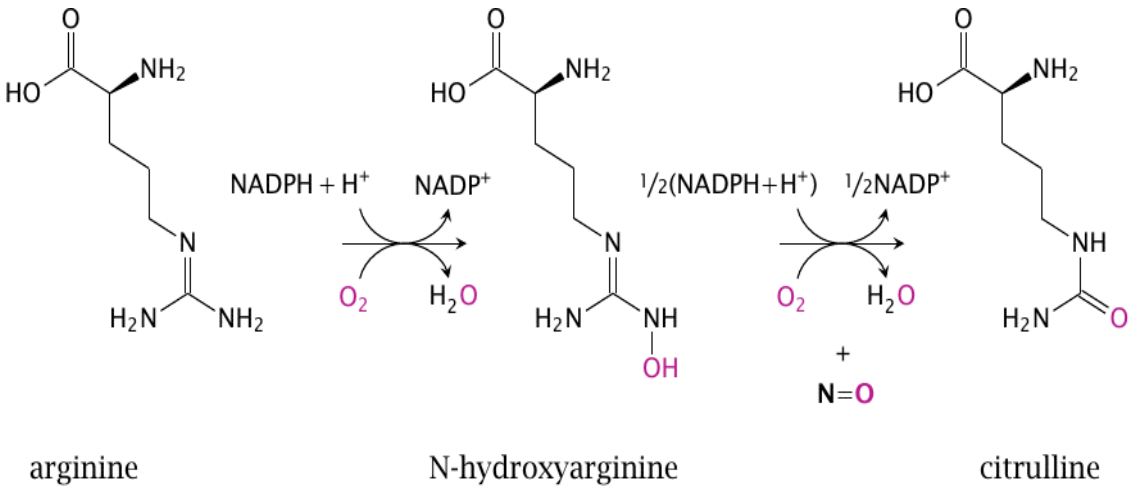


Figure-3.15: Synthesis of nitric oxide from arginine

3.6.2 - Nitric oxide synthase [117]:

There are three distinct isoforms of NOS which have been identified. They are neuronal (nNOS/NOS-1), inducible (iNOS/NOS-2) and endothelial (eNOS/NOS-3). Comparisons of three iso-forms are shown in the table.

Table-3.3: Characteristics of different NOS isoforms [118]

Character	**nNOS**	**iNOS**	**eNOS**
Other names	NOS-1	NOS-2	NOS-3
Gene	Chromosome-12	Chromosome-17	Chromosome-7
Size	161 kDa	131kDa	133kDa
Location	Neurons Smooth muscle, Skeletal muscle	Macrophages Immune system Smooth muscle	Endothelial cells Platelets smooth muscle
Ca^{2} dependency	Ca^{+2} dependent	Ca^{+2} independent	Ca^{+2} dependent
Gene expression	Constitutive	Inducible	Constitutive
Protein-protein interaction	hsp90, Caveolin	-	hsp90, Caveolin
Covalent modification	Phosphorylation	-	Palmitoylation Phosphorylation
Function	Signal transduction Neurotransmission	Defense against pathogens, Inflammation	Vasodilatation, modulation of platelet aggregation, modulation of leukocyte-endothelial interactions

3.6.3 - Mechanism of action of NO [119]:

NO acts through the activation of soluble guanylate cyclase (cGMP). Thus cGMP activates protein kinase G, which leads to reuptake of calcium and opening of calcium- activated potassium channels.

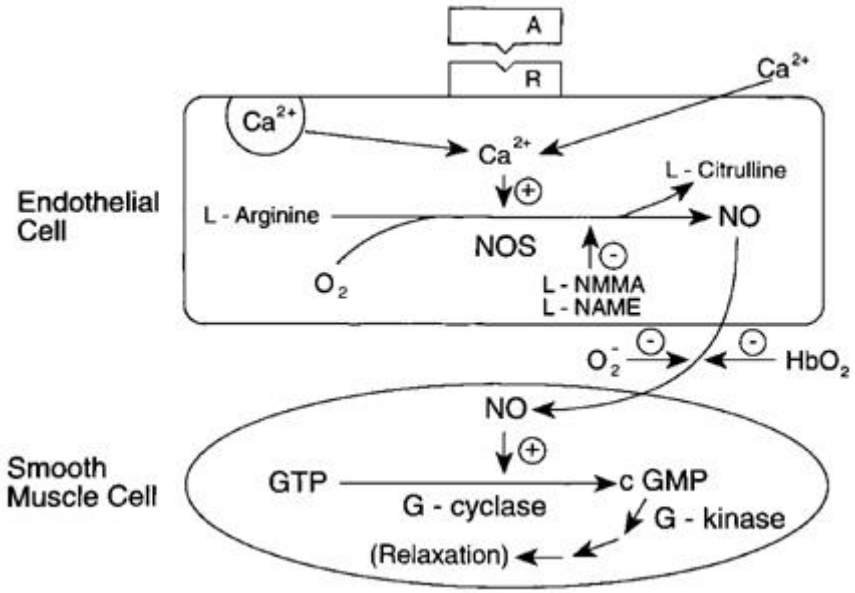


Figure-3.16: Synthesis and mechanism of action of nitric oxide
Source: http://www.downstate.edu

Half-life of nitric oxide is very short. NO is rapidly oxidised to nitrite (NO_2) in plasma and nitrate (NO_3) in whole blood[120]. Determination of NO is very difficult because of its very short half-life. So, its metabolites like nitrites and nitrates are measured to assess NO production since these are main stable breakdown end products of NO [121]. Studies showed that there is a strong correlation between endogenous NO production and serum NO levels [122,123]. Determination of these NO metabolites in the biological samples has been considered as an index of generalised NO production[124]. Measurement of these metabolites are indirect measure of endothelial dysfunction[125].

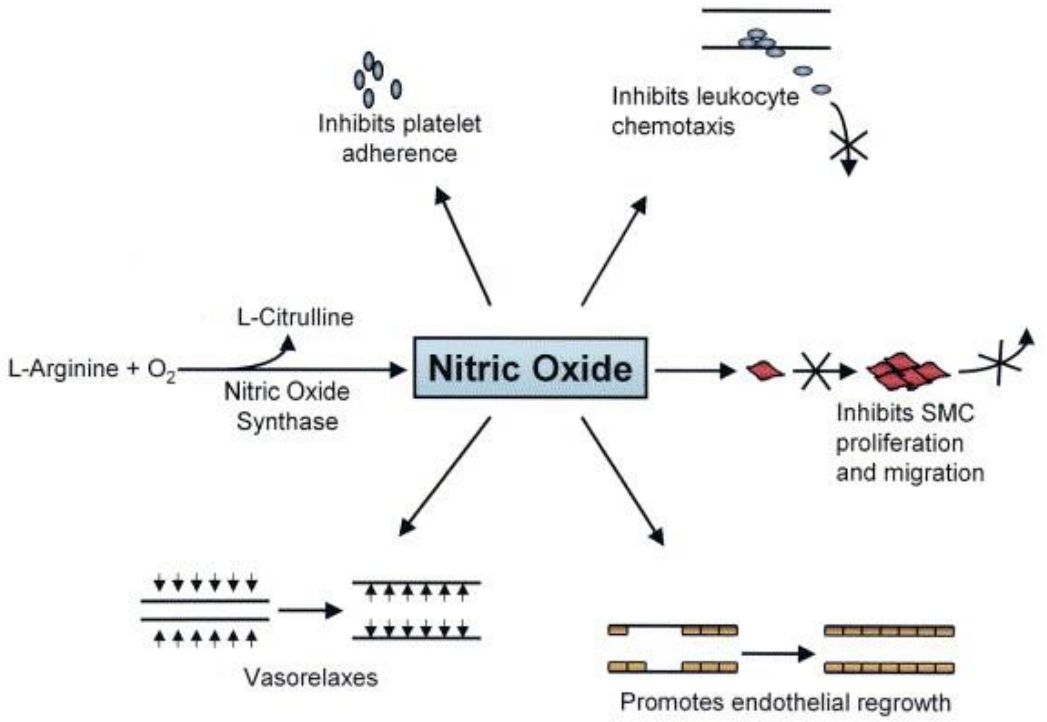


Figure-3.17: Functions of nitric oxide
Source: http://www.researchgate.net

NO is a vasodilator synthesised by eNOS in endothelial cells. This NO is immediately converted into nitrite (NO2) and nitrate (NO3) which are stable forms. It has important role in cardiovascular system like blood pressure regulation, inhibits thrombocyte aggregation, leukocyte adhesion, SMC proliferation and LDL oxidation[126].

3.6.4 - Methods of determination of NO[127,128]

There are several methods to determine the NO levels like ultraviolet absorbance, gas chromatography –Mass Spectroscopy (GC-MS), high performance liquid chromatography (HPLC), Chemiluminescence assay and florescent assay. Among these Griess assay was most popular, simple, rapid and cost effective method. In 1879 John Peter Griess described this for the first time. It has been shown that NO measured by Griess method has a good correlation with gas chromatography mass spectrometry. Hence, this method is considered

as most accurate quantitative method in serum[128]. Currently, modified Griess method is most frequently used.

3.6.5 - NO in DM without CAD patients

NO is an endothelium derived relaxing factor produced in the vascular endothelial cells. Type-2 DM increases the risk of CVD. Endothelial dysfunction is a potential contributor to vascular changes in DM[129].

Vascular changes in diabetes mellitus:[130]

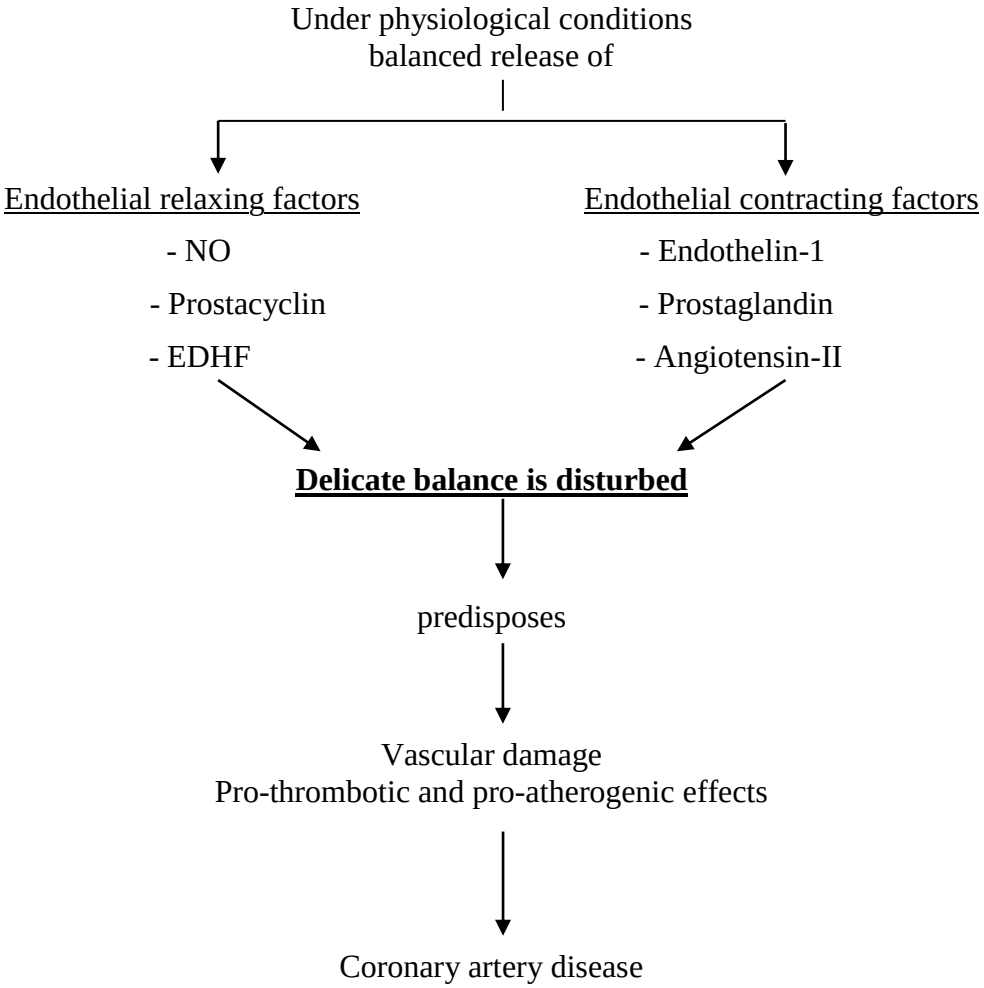


Figure-3.18: Flow chart showing the vascular changes leading to CAD in DM

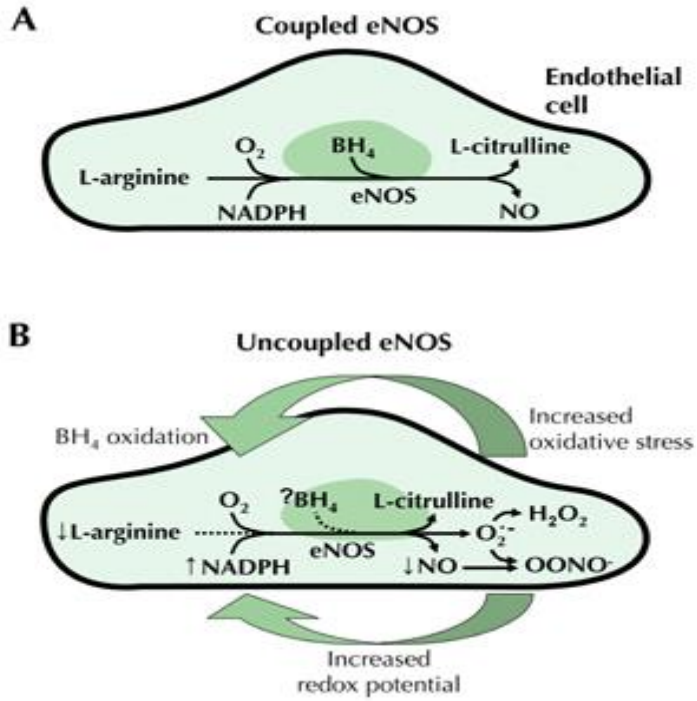


Figure-3.19: Uncoupling of eNOS in diabetes mellitus patients
Source: Hamilton SJ, Watts GF. Endothelial dysfunction in Diabetes: Pathogenesis, Significance and Treatment. Rev Diabet Stud 2013;10(2-3):133-56.

In normal endothelial cells, eNOS produces NO from L-arginine in highly coupled manner as shown in figure 3.19 part A. In DM patients, increased NADPH and decreased tetrahydrobiopterin leads to uncoupling of NO production. Thus there will be transfer of electrons to O_2 to form superoxide, which inturn reacts with NO to form peroxynitrite ($OONO^-$) [131, 132].

Mechanisms that cause dysfunction of NO in DM patients[133,134]

- Hyperglycemia activates protein kinase C (PKC) thus increases advanced glycation end products (AGEs), in turn oxidative destruction of NO molecule.
- Normally, insulin is known to stimulate the NO production via phosphotidyl inositol 3-kinase and protein kinase. Thus insulin resistance in DM could impede positive effect of insulin on NO release.

- Dyslipidemia in DM, where modification of lipoproteins i.e oxidation and glycation can diminish endothelial relaxation.
- In diabetic renal disease, microalbuminuria is most commonly associated with abnormal endothelial dysfunction in vascular beds of the kidney and in cardiac vascular beds.
- It has been proposed that genetic component may influence the status of the vascular NO in diabetes.

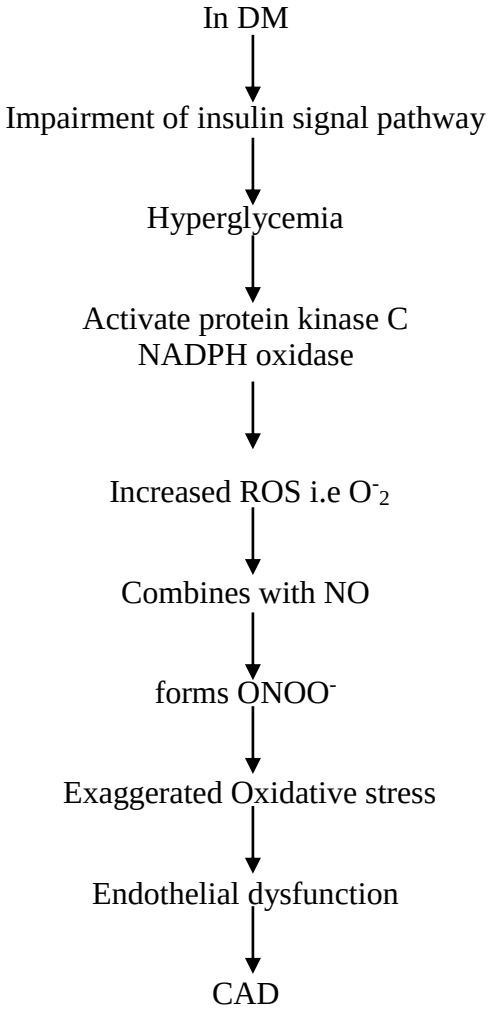


Figure-3.20: Flow chart showing the reduced NO levels due to oxidative stress responsible for CAD [135]

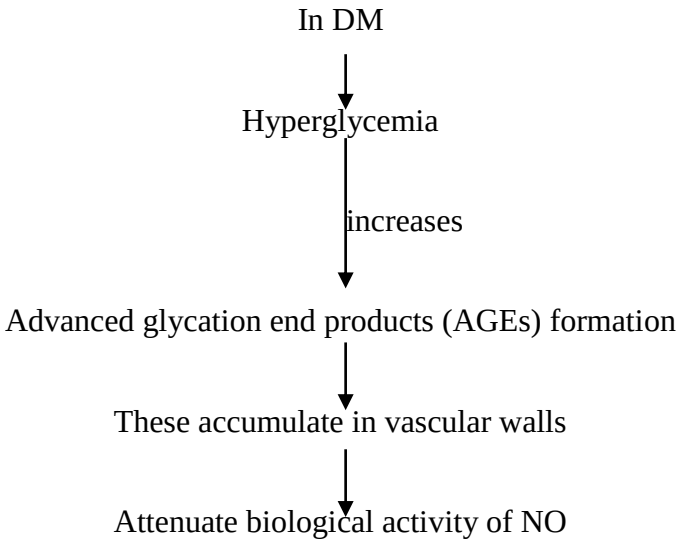


Figure-3.21: Flow chart showing the reduced NO activity due to advanced glycation end products [136]

There will be altered NO system in diabetes mellitus. Hyperglycemia is one of the important cause of vascular NO system dysfunction in DM. Studies revealed that glycemic control may improve vascular NO function[137,138].

A study by Parineeta Samant et al[139], in 2012, showed low levels of serum NO in diabetic patients with hyperlipidemia. Decrease in NO may be due to inactivation of NO and decreasing its bioavailability or uncoupling of nitric oxide synthase complex. Hence, more ROS and superoxide are produced instead of NO.

In 2018, according to Siva Prasad Palem et al[140], there was a significant decrease in NO levels in type-2 diabetic patients compared to healthy controls.

Studies reported reduced NO bioavailability in diabetic patients. Decreased L-arginine causes decreased NO synthesis and results in increased cardiovascular risk[141,142].

A north Indian study in 2016 by Sanjeev kumar et al [143], on 223 type-2 diabetic patients conclude that NO level was significantly lower in diabetic patients compared to controls. They suggest there is definite role of NO in the pathogenesis of type-2 DM and there is poor correlation between NO and oxidative stress parameters.

According to Shahid SM et al[144] in 2009, there was significant increase in FBG, HbA1c and significant low levels of NO in diabetic patients compared to controls. A significant negative correlation of NO with serum glucose and HbA1c levels in diabetic normotensive patients. This suggests that disturbed glycemic control and HbA1c contribute to impaired NO metabolism or vice versa.

According to Leuiene de Carvalho Cardoso Weide et al[145] in 2014, it was concluded that low NO levels were seen in diabetic patients with HbA1c more than 9% and associated with worse prognosis. Increased oxidative stress due to excessive ROS production and low antioxidants levels is associated with microvascular and macrovascular complications in type-2 DM.

3.6.6 - NO in DM with CAD patients

NO is a potent endogenous vasodilator that decreases formation of vascular lesion in pathogenesis of atherosclerosis. Its availability in different tissues depends on the production and consumption of endothelial NO. Oxidative stress, lipid infiltration and inflammation contribute to markedly reduction of NO availability[146]. This is responsible for endothelial dysfunction and CAD. For the first time, Ludmer et al[147] in 2018 described the endothelial dysfunction and its association with NO bioavailability in atherosclerotic coronary artery. Decreased production and bioavailability of NO accelerates the atherosclerosis process, like vasoconstriction, thrombocyte aggregation, monocyte aggregation, oxidation of lipoproteins (Lp(a) and LDL-C) and foam cell formation.

Mechanisms that decrease NO bioavailability contributing to CAD

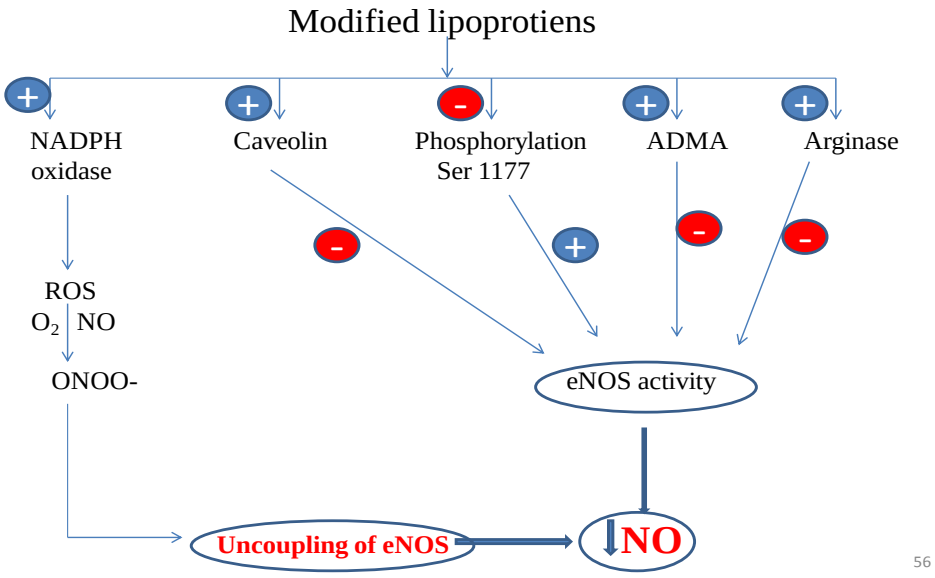


Figure-3.22: Diagram showing mechanisms that reduce the NO levels in DM and CAD

Source: Jing-Yi Chen, Zi-xin Ye, Xiu-fen Wang, Jian Chang, Mei-wen Yang, Hua-hua Zhong et al. Nitric oxide bioavailability dysfunction involves in atherosclerosis. Biomedicine and Pharmacotherapy 2018;97:423-8.

Oxidative modification of lipoproteins triggers the NADPH oxidase, thus leads to release of superoxide. This superoxide rapidly quenches the NO and forms $ONOO^-$ (peroxinitie), which upregulates the expression of caveolin, a negative regulator of eNOS. Caveolins are cholesterol rich invaginations present on endothelial as well as vascular smooth muscle cells. Phosphorylation of serine residue of 1177 upregulates the eNOS thus enhances the NO release and maintain normal vascular tone. Modified lipoproteins inhibit this eNOS ser 1177 phosphorylation thus uncoupling eNOS and reducing the bioavailability of NO in the endothelium [148].

Normally arginine is catabolised into arginine N-mono methyl arginine (NMMA), symmetric dimethyl arginine (SDMA) and asymmetric dimethyl arginine (ADMA) by protein arginine methyl transferase (PAMT). L-NMMA and ADMA competes with arginine for eNOS to reduce NO synthesis. It is postulated that ADMA concentration increased in pathophysiological condition which inhibits eNOS [149]. This abnormal signalling pathway responsible for decreased NO level contribute to CAD. A study proposed that the ratio of ADMA/NO was better predictor for severity of coronary atherosclerosis rather than serum ADMA or NO alone [150]. A study demonstrated increased ADMA production in hypercholesterolemia, which competitively inhibits NOS [151].

Increase in arginase activity causes uncoupling of eNOS, leading to reduced availability of NO responsible for various cardiovascular diseases [152].

Monitoring of long-term glycemic control can be determined by estimation of glycated hemoglobin (HbA1C) which is an alternate to blood glucose estimation. Recently it is recommended diagnosis of DM and its complications are well predicted by measuring the HbA1c. Hence HbA1c is estimated in the present study.

3.7 - Glycated haemoglobin (HbA_1c)

The non-enzymatic addition of sugar to amino group of proteins is called glycation. "Condensation of glucose with amino terminal of valine residue of β-chain of haemoglobin to form aldimine schiff base. This is very unstable intermediate product and immediately undergo an Amadori rearrangement to form stable product called glycated hemoglobin" [153].

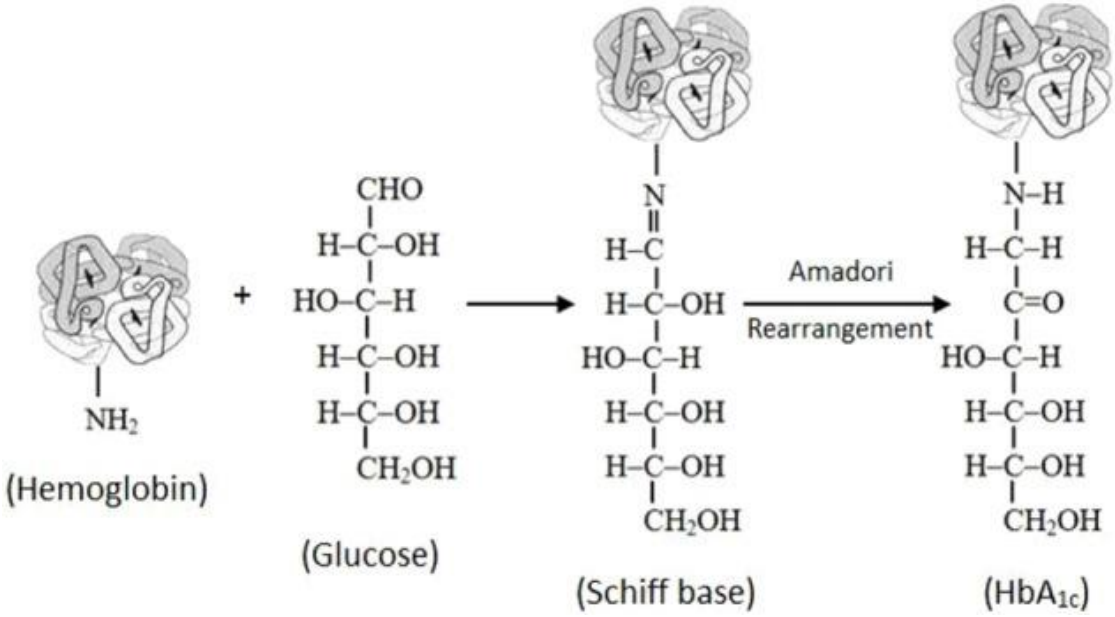


Figure-3.23: Formation of HbA1c

HbA1c was first isolated by Huisman [154] in 1958. In 1976, Koenig proposed HbA_1c as a biomarker for monitoring the levels of glucose in diabetic patients for the first time [155]. Formation of HbA_1c is irreversible. Concentration of HbA_1c in the circulation depends on amount of blood glucose and life span of erythrocytes. Rate of concentration is directly proportional to concentration of blood glucose. HbA_1c represents the average blood glucose over a period of 8 to 12 weeks [156]. Its values are not affected by diet and exercise.

HbA_1c provides a reliable measure of chronic hyperglycemia and also correlates well with the risk of long-term diabetes mellitus complications.

Reference range [153]

Normal range – 4 – 5.6%

High risk of developing DM – 5.7 – 6.4%

Diabetes mellitus - > 6.5%

Decreased HbA_1c levels found in the following conditions:

- Sickle cell anemia
- Hemolytic anemia
- Spherocytosis
- Eliptocytosis

Increased HbA_1c levels seen in :

- Recent significant blood loss
- Iron deficiency anemia

Elevated HbA_1c has also been regarded as an independent risk factor for coronary heart disease in subjects with or without diabetes [157]. An International expert committee advised HbA_1c more than 6.5% can be used for diagnosis of DM [158]. As per American Diabetes Association (ADA) recommendation 7% should be the goal of treatment and HbA_1c should be monitored every six months in stable DM patients and every three months in patients who are not on target[5]. Every 1% change in HbA_1c levels represents 30 mg/dL change in average blood glucose level.

3.8 - C-reactive protein (CRP)

Inflammation plays very important role in the initiation and progression of atherosclerosis. Many inflammatory molecules like interleukin, tumor necrosis factors, Lp(a) and hsCRP are studied to understand their role in DM and CAD. Among them hsCRP has identified has one of the vital marker in predicting the future CAD in DM patients. Estimation of CRP as a marker of inflammation in chronic inflammatory diseases, whereas hsCRP measured in cardiac diseases which is more specific.

C-reactive protein is an acute phase plasma protein. It is a highly conserved protein that participates in systemic response to inflammation mainly synthesized by the liver[159]. Tillett and Francis discovered C-reactive protein in 1930. In 1941 it was shown to be a protein and given the name C-reactive protein. This was so named because CRP as a trace protein binds to the C-polysaccharide of the streptococcal pneumonia cell wall [160]. It is a single polypeptide chain with 187 amino acids. It belongs to the family of pentraxins (penta=five; ragos=berries). It is made up of five identical non-glycosylated subunits linked non-covalently to form a disc like pentagonal ring. It has a plasma half life of 19 hours [160].

Functions of CRP:

It is an activator of classical complement pathway. It can initiate opsonisation, phagocytosis and lysis of invading cells as a response to inflammatory reaction. It binds to foreign and damaged cells and enhances phagocytosis by macrophages (opsonin mediated phagocytosis), hence contributes to host defense and plays a crucial role in the first line of innate host defense. Perhaps the main role of CRP is to recognize potentially toxic autogeneous substances released from damaged tissue, to bind them and then to detoxify them [160]. It is a marker of inflammation that has been associated with increased risk for cardiovascular diseases (CVD) [162,163]. CRP has been proposed to promote atherosclerosis

and atherothrombosis by stimulating chemotactic recruitment of monocytes and up regulating endothelial-leukocyte adhesion molecules and tissue factor expression [164].

High sensitivity C-reactive protein (hsCRP):

Lower detection limit using highly sensitive assay is called hsCRP. Standard assays for CRP lack sensitivity needed to determine the cardiovascular risk and thus clinical utility of standard CRP evaluation is limited. Recent improvements have resulted in new generation of highly sensitive assays that detect CRP at levels hundred folds lower than earlier assays.

Measurement of hsCRP may be considered as marker for better prediction of cardiovascular risk[165]. Framingham heart study was the first to provide the conceptual basis for cardiovascular risk factors in the early 1960 [166]. In current practice, those with 10-year Framingham coronary heart disease risk estimates that are less than 5% are considered to be at low risk, those with 10-year estimates between 6% and 20% are considered at intermediate risk and those with 10-year risks of 20% and higher or who have diabetes (diabetes is itself a CHD equivalent) are considered to be coronary risk equivalents [167].

Prospective epidemiological studies consistently demonstrate that hsCRP adds independent prognostic information at all levels of LDL-C and at all levels of Framingham risk score[168]. Data showed levels of hsCRP <1, 1 to 3, and >3mg/L have been defined as low, moderate and high cardiovascular risk. Taking a conservative approach, the CDC / AHA report suggested that the best use of CRP was in patients at intermediate Framingham risk[168]. A review report by Salazar et al in 2014, commented hsCRP levels more than 3 mg/L is associated with double risk of development of CVD when compared to individuals with hsCRP levels less than 1mg/L[169].

3.8.1 - hsCRP in DM without CAD patients

hsCRP act as inflammatory marker. It activates complement pathway, it induces adhesion molecule, enhances LDL uptake by macrophages and transform into foam cell[170]. It also induces plasminogen activator inhibitor-1[171], inturn enhances endothelial damage[172]. In type-2 DM patients, hsCRP stimulates the TNF-α and IL-1by tissue macrophages and amplifies the inflammatory response[173]. IL-6 is the main stimulus for the production of hepaic CRP.

SS Habib[174] in his study reported that diabetic patients have higher levels of Lp(a) and hsCRP. Diabetic patients with poor glycemic control patients have significantly higher hsCRP levels compared to good glycemic control DM patients.

3.8.2 - hsCRP in DM with CAD patients

A prospective cohort study on women by Ridker, reported hsCRP was the most strongest cardiovascular risk predictor among other parameters like lipid profile, Lp(a), homocystiene and hsCRP. He also proposed estimation of hsCRP improve cardiovascular risk prediction when combined with routine lipid profile[175].

A cross sectional study conducted in the year 2011 by Shahid HS et al, concluded significant increase in serum hsCRP levels in angiographically defined CAD. Measurement of hsCRP may be considered optional markers for better prediction of cardiovascular risk [176]. A study in 2018 by Kavitha MM et al[177]and Dambal A et al[178] in their studies also reported significant increase in hsCRP levels in CAD patients compared to controls and act as marker of inflammation.

With this background, we made an attempt to find the role of lipid profile, Lp(a), hsCRP and NO in diabetes mellitus with and without coronary artery disease patients.

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Material and methods

The study was approved by institutional ethics committee (IEC) Shri B M Patil Medical college and research centre, BLDE (DU), Vijayapura (Ref No- 1192015-16) and S Nijalingappa medical college and Hanagal Sri Kumareshwara hospital and research centre, Bagalkot, (SNMCIECHSR2014-15A-18-1.1). Informed written consent was taken from all the participants at the beginning of the study.

4.1 - Study design:

This was a hospital based observational, case-control study.

4.2 - Study Period:

The study was conducted from 2015 to 2017 for a period of three years.

4.3 - Source of data:

The study was conducted in the department of Biochemistry and General Medicine, at S. Nijalingappa medical college and Hanagal Sri Kumareshwara hospital and research centre in Bagalkot, Karnataka. The patients who attended the General Medicine OPD (out patient department) and also who were admitted in intensive cardiac care unit were selected for the study.

4.4 - Study group:

Age group between 30 to 75 years were included in the study.

4.5 - Sample size:

Sample size was calculated by using the software open epi version 2.3.1, at 95% confidence interval and 80% power, by taking mean values of triglyceride levels from Mohan V et al study[1].

4.6 - Sample size calculation:

$$n = \frac{2(Z_\alpha + Z_\beta)^2 \sigma^2}{d^2}$$

n= number of sample

Z_α – Z equivalent of α at 95% confidence interval = 1.96

Z_β – Z equivalent of β at 80% power of the study = 0.84

σ – Standard deviation

d – Mean difference of triglycerides [Control= 124±63, DM with CAD cases =175±121]

After calculation sample size came around 57 in each group. Extra dropouts 20% was added i.e 5. Hence total 62 participants thus rounded off to 65 in each group were selected.

Study comprises of total 195 participants who were divided into three groups:-

Group-1: 65 – Healthy controls

Group-2: 65 – DM without CAD patients

Group-3: 65 – DM with CAD patients

4.7 - Inclusion criteria:

The diagnosis of type-2 diabetes mellitus was based on WHO criteria. Fasting blood glucose more than 126 mg/dL or post-prandial more than 200 mg/dL or HbA1c more than 6.5% were diagnosed as diabetes mellitus.

The newly diagnosed CAD patients were enrolled. CAD was diagnosed based on angiography, ECG findings, cardiac markers, echocardiography and medical records.

Age and sex matched healthy controls with no history of DM, HTN and cardiac diseases were selected. DM was ruled out by estimating fasting blood glucose. Presence of any cardiac disease was ruled out by history, ECG findings and medical records.

4.8 - Exclusion criteria:

The patients with following diseases were excluded from the present study

- Hypertension
- Stroke
- Malignancy
- Nephrotic syndrome
- Thyroid disorders
- Pregnant women
- Chronic liver and kidney disease
- Patients on lipid lowering drugs
- Women on hormone replacement therapy
- Other cardiac diseases

4.9 - Study protocol:

History and General physical examination:

Detailed history was noted, height was measured in meter using standard measuring tape, weight in kilogram (Kg) using weighing machine in all the participants. Body mass index (BMI) was calculated using the formula Wt in Kg/Ht in m^2, blood pressure (BP) was recorded in supine position using sphygmomanometer. Both systolic (SBP) and diastolic blood pressure (DBP) were recorded. Mean arterial blood pressure (MAP) was calculated using the formula MAP=DBP+1/3(SBP-DBP).

Biochemical estimation:

Under aseptic precaution, 6 mL fasting venous blood was drawn from cubital vein. Two mL of blood was transferred to EDTA tube and was used for estimation of glycosylated hemoglobin (HbA_1c). Remaining 4 mL was allowed to clot and serum was separated. The serum was used for estimation of fasting glucose, lipid profile, Lp(a), NO and hsCRP.

Table 3.1: List of biochemical tests, principle and instrument used in the study:

S N	Test	Method	Instrument
1	FBG mg/dL	Glucose oxidase and peroxidase	Biosystem A25
2	HbA_1c %	HPLC	Bio-Rad, D-10
3	Total Cholesterol mg/dL	Cholesterol oxidase and peroxidase	Biosystem A25
4	Triglyceride mg/dL	Trinder method	Biosystem A25
5	HDL-C mg/dL	Direct method	Biosystem A25
6	Lipoprotein (a) mg/dL	Immunoturbidemeric method	Erba Chem 5 plus Transasia
7	NO μmol/L	Modified Griess method	UV, visible, double beam spectrophotometer (UV-1800, Schimadzu)
8	hsCRP mg/L	Turbidemetry method	Erba Chem-5 plus Transasia

4.10 - ESTIMATION OF BLOOD GLUCOSE[2]

Blood glucose was estimated in fully automated analyser using commercially available kit from Bio-Rad company by glucose oxidase peroxidase method an end point colorimetric method.

Principle:

Glucose is determined by enzymatic oxidation method in the presence of glucose oxidase (GOD) and peroxidase method. The hydrogen peroxide thus formed reacts with phenol and 4-aminoantipyrine (4AAP) catalysied by peroxidase to form a red coloured quinoneimine compound, color produced is directly proportional to the amount of glucose present in the given sample. Optical density was measured at 500 nm.

$$\text{Glucose} + 1/2O_2 + H_2O \xrightarrow{\text{Glucose oxidase}} \text{Gluconate} + H_2O_2$$

$$2H_2O_2 + 4\text{AAP} + \text{phenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 4H_2O$$

Reagents:

Reagent 1: Glucose mono reagent

Phosphate buffer (pH 7.5)	=	200 mmol/L
Glucose oxidase	≥	20000 U/L
4 AAP	=	0.3 mmol/L
Phenol	=	10 mmol/L
Peroxidase	≥	2000 U/L

Reagent 2: Glucose standard 100 mg/dL

Assay

Mode : End point method

Wavelength : 500 nm (480-520nm)

Optical path : 1 cm

Temperature: 37^0C

Measurement against blank sample

Procedure:

Reagents	Blank	Standard	Test
Serum	-	-	10µL
Standard	-	10µL	-
Glucose reagent	1000µL	1000µL	1000µL

Mix well and incubate at 37^0C for 10 minutes. Measure the absorbance of standard and test against blank at 500nm within one hour.

Calculation

$$\text{Concentration of Glucose (mg/dL)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 100$$

Reference values

Plasma glucose (fasting) = 70-110 mg/dL

4.11 - ESTIMATION OF GLYCOSYLATED HEMOGLOBIN (HbA_1c) BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) METHOD[3]

Glycemic status of diabetic patients was assessed by HbA_1c levels. It was measured by commercially available kit from Bio-Rad by HPLC method.

Principle: Glycosylated hemoglobin was estimated based on the principle of chromatographic separation of the analyte by ion-exchange high performance liquid chromatography method.

Kit components

Elution Buffer: Two bottles containing 2000mL of a Bis-Tris phosphate buffer, pH 6. Contains <0,05% sodium azide as a preservative.

Elution Buffer: One bottle containing 1000mL of a Bis-Tris phosphate buffer, pH 6.7. Contains <0,05% sodium azide as a preservative.

Wash/Diluent set: One bottle containing 1600 mL of deionized water with <0,05% sodium azide as a preservative.

Analytical cartridge: One cation exchange cartridge 4.0 ID X 30mm

HbA1c Calibrator: Consist of 3 vials of calibrator level 1, 3 vials of calibrator level 2 and bottle of calibrator diluents.

Specimen type: Whole blood

Preservative: The whole blood specimen should be collected in a vacuum collection tube containing EDTA

Storage: Stored for one day at room temperature i.e 15 to 30^0 C or upto 4 days at 2-8^0 C.

Specimen preparation: The sample tubes are loaded into the D-10 sample rack and placed in it. Ensure that sample barcodes are facing the back of the instrument. If the sample is less than 2 mL then it should be prediluted.

Procedure:

The samples are diluted automatically in the D-10 instrument and injected into the analytical cartridge. The D-10 delivers buffer to the cartridge on gradient basis with increasing the ionic strength. Then hemoglobin variants are separated depending on their ionic interaction with the cartridge. Later these separated hemoglobin pass through the flow cell photometer. The absorbance is measured at wavelength of 415 nm.

Interpretation of the results:

Reportable range – 3.8 -18.5 %

Reference values:

4-5.6%	Normal range
5.7 – 6.5%	High risk of developing DM
>6.5%	Diabetes mellitus

4.12 - ESTIMATION OF TOTAL CHOLESTEROL[4]

Lipid profile includes total cholesterol, triglyceride and high density lipoprotein. These were measured in fully automated analyser Biosystem A-25 using commercially available kits from the company Biosysatem. VLDL-C and LDL-C was calculated.

Method: Cholesterol oxidase and peroxidase

Principle:

The free and esterified cholesterol in the given sample is determined by enzymatic method

Cholesterol ester + H_2O $\xrightarrow{\text{cholesterol esterase}}$ cholesterol + Fatty Acid

Cholesterol + $1/2O_2$ + H_2O $\xrightarrow{\text{cholesterol oxidase}}$ cholesteone +H_2O_2

$2H_2O_2$ + 4AAP + phenol $\xrightarrow{\text{Peroxidase}}$ $4H_2O$ + quinoneimine

Absorbance of quinoneimine formed is directly proportional to the concentration of cholesterol in the given sample and absorbance measured at 480 to 520 nm of wavelength.

Reagents:

1. Cholesterol reagent contains:

Sodium cholate	-	0.5mmol/L
Cholesterol esterase	≥	0.2U/mL
Cholesterol oxidase	≥	0.1U/mL
Peroxidase	≥	0.8U/mL
4AAP	-	0.5mmol/L
pH	-	7

2. Cholesterol standard:

   Cholesterol - 200 mg/dL

Assay:

Mode : End point method

Wavelength : 500nm (480-520nm)

Optical path length : 1cm

Procedure:

Reagents	Blank	Standard	Test
Serum	-	-	10µL
Standard	-	10µL	-
Cholesterol reagent	1000µL	1000µL	1000µL

Mix well. Incubate all the tubes for 10 minutes at room temperature or at 37^0C for 5 minutes.

Calculation:

$$\text{Cholesterol concentration} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 200$$

Reference range: (As per NCEP ATP III guidelines)

<u>For total cholesterol</u>

Desirable : < 200mg/dL

Borderline high : 200-239mg/dL

High : ≥ 240mg/dL

4.13 - ESTIMATION OF TRIGLYCERIDES [5]

Method: By Glycerol phosphate oxidase/ peroxidase method

Principle:

Triglycerides in the blood sample can be measured by spectrophotometrically. The intensity of color formed is directly proportional to the triglyceride concentration in the sample when measured at 500nm.

Triglycerides + H_2O $\xrightarrow{\text{lipase}}$ Glycerol + FFA

Glycerol + ATP $\xrightarrow{\text{Glycerol kinase}}$ glycerol 3-phosphate + ADP

Glycerol 3-phosphate + O_2 $\xrightarrow{\text{Glycerol phosphate oxidase}}$ DAP + H_2O_2

H_2O_2 +4AAP +4-Cholorophenol $\xrightarrow{\text{peroxidase}}$ quinoneimine + $2H_2O$

DAP – Dihydroxyacetone phosphate

4AAP - 4 Aminoantipyrine

Reagents:

1. Triglyceride mono reagent:

Pipes buffer	-	45 mmol/L
4-chlorophenol	-	6 mmol/L
Magnesium chloride	-	5 mmol/L
ATP	-	1 mmol/L
Lipase	>	100 U/mL
Peroxiadse	≥	0.8 U/mL
Glycerol kinase	≥	1.5 U/mL
4 AAP	-	0.75mmol/L

Glycerol 3 phosphate oxidase ≥ 4 U/mL

2. Triglyceride standard:

Triglyceride - 200 mg/dL

Assay:

Mode : End point

Wavelength : 500nm

Optical path length : 1 cm

Procedure:

	Blank	**Standard**	**Test**
Serum	-	-	10µL
Standard	-	10µL	-
Reagent	1000µL	1000µL	1000µL

Mix well. Incubate at tubes for 15 minutes at room temperature for 5 minutes.

Calculation:

$$\text{Triglyceride concentration (mg/dL)} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 200$$

Reference range: (as per NCEP ATP III guidelines)

Normal : < 150 mg/dL

Borderline high : 150-199 mg/dL

High : 200-499 mg/dL

Very high : ≥ 500 mg/dL

4.14 - ESTIMATION OF HDL CHOLESTEROL[6]

Principle:

The cholesterol from LDL-C, VLDL-C and chylomicrons, is broken down by the cholesterol oxidase in an enzymatic reaction. The detergent in reagent B, will solubalizes cholesterol from HDL-C in the given sample. The HDL-C is measured at 600 to 700 nm. Absorbance of quinoneimine formed is directly proportional to amount of HDL-C present in the given sample.

Cholesterol ester + H_2O $\xrightarrow{\text{cholesterol esterase}}$ cholesterol + Fatty Acid

Cholesterol + $1/2O_2$ + H_2O $\xrightarrow{\text{cholesterol oxidase}}$ cholestenone + H_2O_2

$2H_2O_2$ + 4AAP + DSBmT $\xrightarrow{\text{peroxidase}}$ $4H_2O$ + quinoneimine

Reagents:

1. Reagent:

   Cholesterol oxidase < 1U/mL

   Peroxidase < 1U/mL

   N,N-bis(4-sulfobutyl)-m-toluidine (DSBmT) - 1mmol/L

   Accelerator - 1mmol/L

2. Reagent:

   Cholesterol esterase < 1.5 U/mL

   4-aminoantipyrine - 1 mmol/L

   Ascorbate oxidase < 3KU/L

Assay:

Mode : End point

Wavelength : 600-700nm

Optical path length : 1cm

Procedure:

Pipette into tube marked	Test
Serum	7 µL
Reagent A	750 µL
Mix well and insert the cuvette in the photometer. After 5 minutes read the absorbance (A1) at 600/700 nm against distilled water	
Reagent B	250µL
Mix well. Incubate at 37^0C for 5 minutes and read the absorbance (A2) at 600/700 nm.	

Calculation:

$$\text{Concentration of HDL} = \frac{\text{Absorbance of (A2-A1) sample}}{\text{Absorbance of calibrator (A2-A1)}} \times \text{Calibrator}$$

Reference range: (As per NCEP ATP III guidelines)

For HDL cholesterol

Low risk : ≥ 60 mg/dL

High risk :< 35 mg/dL

4.15 - ESTIMATION OF LDL CHOLESTEROL [7]

Low density lipoprotein was calculated using "Friedwald's equation" and was applied for those TG levels which were less than 400 mg/dL.

$$\text{LDL-C} = \text{Total cholesterol} - \frac{\text{triglycerides}}{5} - \text{HDL-C}$$

Reference range: (As per NCEP ATP III guidelines)

Optimal < 100mg/dL

Near optimal : 100-129mg/dL

Borderline high : 130-159mg/dL

High : 160-189mg/dL

Very high ≥190mg/dL

4.16 - ESTIMATION OF HIGH SENSITIVE C-REACTIVE PROTEIN [8]

Inflammatory marker hsCRP was measured by Latex high sensitivity method.

Principle:

CRP present in the serum causes agglutination of the latex particles coated with antihuman CRP antibodies. The agglutination of the latex particles is directly proportional to the amount of CRP present in the given sample. It can be measured by turbidimetry method.

Reagents:

A. Reagent contains Glycine buffer 0.1 mol/L, sodium azide 0.95 g/L

B. Reagent contains suspension of latex particles coated with anti-human CRP antibodies, sodium azide 0.95 g/L

C. hs-CRP standard

Procedure:

Bring all the working reagent and instrument to 37^0C.

a. Zero the instrument with distilled water.

b. Pipette the reagents into a cuvette:

Working reagent	1.5 mL
Blank/standard/sample	20µL

Mix and immediately insert cuvette into instrument. Start stop watch. Record the absorbance at 540 nm after incubating for 5 minutes at 37^0C.

Reference Range for cardiovascular risk stratification:

Low risk :< 1mg/L

Moderate risk: 1-3 mg/L

High risk :> 3mg/L

4.17 - ESTIMATION OF LIPOPROTEIN (a) BY TURBIDEMETRIC METHOD [9]

Special investigation Lp(a) levels were measured using commercially available kit from Erba company. Standardization was done before estimation.

Principle:

Amount of lipoprotein (a) in human serum was by turbidimetric immunoassay method. Latex particle coated with antibodies anti-Lp(a) are agglutinated when mixed with samples containing Lp(a) levels. The agglutination formed changes the absorbance and depends on Lp(a) levels in the given sample. This is quantified by comparison with calibrator of known concentration.

Pack Size:

2 x 25 mL - Lp(a) Buffer

1 x 5 mL - Lp(a) latex

1 x 1 mL - Lp(a) calibrator

Reagents:

1. Buffer

   Sodium chloride – 9g/L

   Sodium azide – 0.95 g/L

   Detergent – 0.01%

2. Latex

   Glycine buffer pH 7.3

   Rabbit anti-human Lp(a) antibody sensitized latex – 0.5%

   Sodium azide – 0.95%

3. Calibrator:

   Defibrinated and delipidated human plasma, liquid stabilized and with 0.09% sodium azide as preservative.

Reagents are ready to use

Storage: 2-8 C

Reagents required : Saline (NaCl) 9g/L

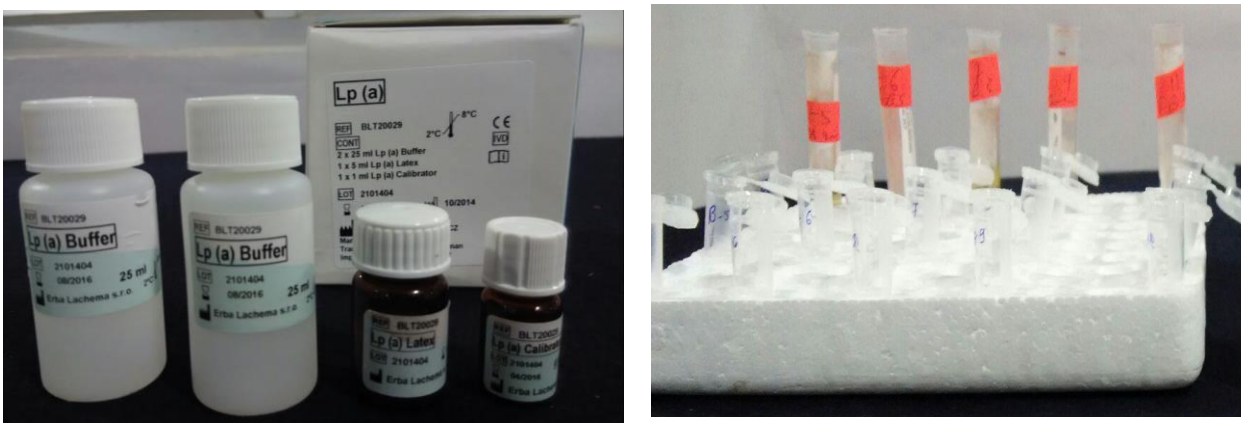

Figure-4.1: Lipoprotein (a) kit and its estimation

Sample collection:

Fresh serum

May be stored for 48 hrs

Frozen sample can be stored for long period

Normal range: 0-30 mg/dL

Measuring range: 0-80 mg/dL

Detection limit: 1.25 mg/dL

Hook effect: No risk

Sensitivity: 0.0147 ABS units/concentration unit

Specificity: Monospecific

Interferences:

Bilirubin > 30mgdL

Apolipoprotein B -200mgdL

Haemoglobin – 500 mg/dL

Rheumatoid factor – 500 IU/mL

Procedure:

Steps:

1) Sample preparation
2) Reagent preparation

I. Sample preparation:

- Preparation of diluted sample.
- Dilute he sample with normal saline (0.9% NaCl) i.e 1:10 ratio

  100μL sample+900μL NS

II. Reagent preparation:

Take 2 test tubes, add the reagents as follows

	Sample bank	Sample
Normal saline	490 μL	-
Diluted sample	15 μL	15μL
Latex	-	40 μL
Buffer	-	450 μL

Keep at room temperature for 5 minutes. Take the reading at 600 nm wavelength to get the direct value.

Calibration table

Standard	Concentration	Absorbance
S1	0	0.7861
S2	6.75	0.5914
S3	13.5	0.4922
S4	27	0.3128
S5	54	0.2019
S6	108	0.0928

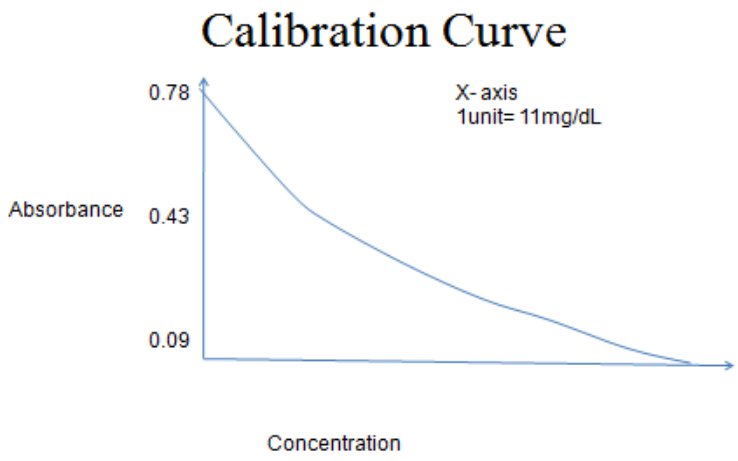

Figure-4.2: Lp(a) calibration curve

Reference range:

Normal – 0-30 mg/dL

4.18 - ESTIMATION OF NITRIC OXIDE (NO) BY GRIESS METHOD [10]

Endothelial dysfunction was indirectly assessed by measuring nitric oxide levels by modified Griess method.

Principle:

Nitric oxide (NO) is oxidized to nitrite (NO_2) and nitrate (NO_3). Nitrate was reduced to nitrite by vanadium chloride. This nitrite was determined by diazotization of sulfanilamide and coupling to naphthyethyline diamine. The color complex is measured at 540nm wavelength.

Reagent preparation

1. Griess reagent

A. Sulphanil amide:

2.5g Sulphanil amide + 250 mL 3M HCl

B. 50 mg Naphthyl ethylene diamine dihydrochloride (NED) + 250 mL DW.

2. Vanadium chloride:

8 mg of vanadium chloride in 1 mL distilled water.

3. Ethanol:

For precipitation of protein in the serum. (Protein precipitation)

4. Standard solution

Sodium nitrate (NaNO3) - 21.2475 mg of sodium nitrate in 250 mL of distilled water.

Sodium nitrite (NaNO2) – 17.25 mg of sodium nitrite in 250 mL of distilled water.

SL No	Working standard		Vanadium chloride (mL)	Griess reagent			OD at 540 nm
	Standard (μL)	DW(μL)		Sulphanilamide (mL)	NED(mL)		
S1	10	490	0.5	0.25	0.25		
S2	15	485	0.5	0.25	0.25	Incubate	
S3	20	480	0.5	0.25	0.25	30 min	
S4	25	475	0.5	0.25	0.25	at	
S5	30	470	0.5	0.25	0.25	37 C	
S6	35	465	0.5	0.25	0.25		
S6	40	460	0.5	0.25	0.25		
S7	45	455	0.5	0.25	0.25		
S8	50	450	0.5	0.25	0.25		
S9	55	445	0.5	0.25	0.25		
S10	60	440	0.5	0.25	0.25		
Test	0.5 mL supernatant		0.5	0.25	0.25		

Calculation:

$$\text{Cons of NO} = \frac{\text{OD of T} - \text{OD of S}}{\text{OD of S} - \text{OD of B}} \times 100$$

$$= \text{---------- } \mu\text{mol/L}$$

Figure-4.3: Reagents prepared for nitric oxide estimation

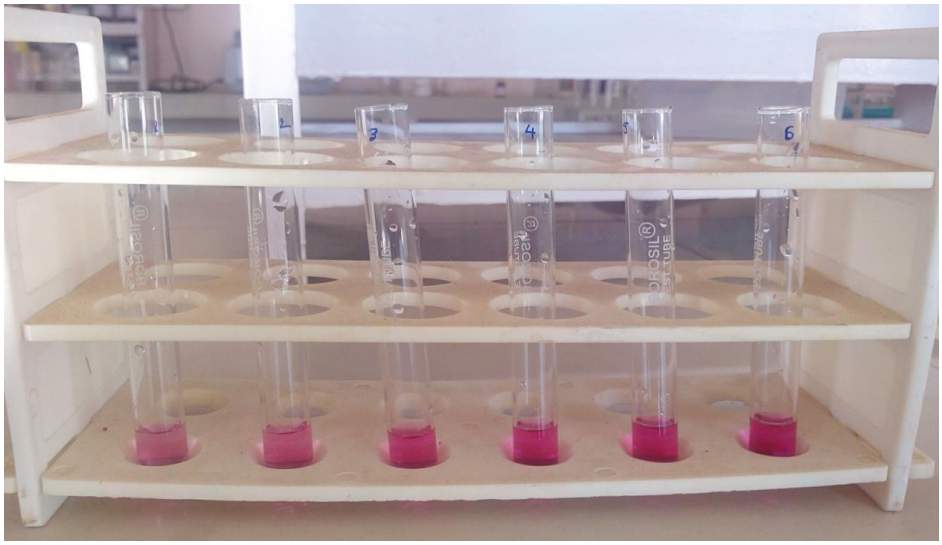

Figure-4.4: Estimation of nitric oxide by modified Griess method

Figure-4.5: Spectrophotometer (Model UV-1800, Shimadzu)

Figure-4.6: Fully automated analyser Biosystem A-25 for biochemical investigations

4.19 - Statistical analysis:

Data obtained from the study was summarized in microsoft excel sheet and analysis was done. The excel and SPSS (SPSS Inc, Chicago version v.23.0) software packages were used for data entry and analysis. Results were expressed as mean ± standard deviation (SD). The difference of the means between three independent groups was tested by analysis of variance (ANOVA) and F-test of testing of equality of variance. Correlation analysis was done using Pearson's correlation coefficient (r) to test the strength and direction of relationships between the interval levels of variables. Receiver operating curve (ROC) analysis for sensitivity and specificity was done to check relative efficiency and to find the best cut-off value. Post-hoc test was applied for comparison in different groups. Logistic linear regression analysis was done and odds ratio (OR) was calculated to predict CAD in diabetes mellitus patients. The p-value < 0.05 was considered to be statistically significant.

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Results

Total 195 subjects participated in the present study. The subjects were divided into three groups as shown in figure-5.1.

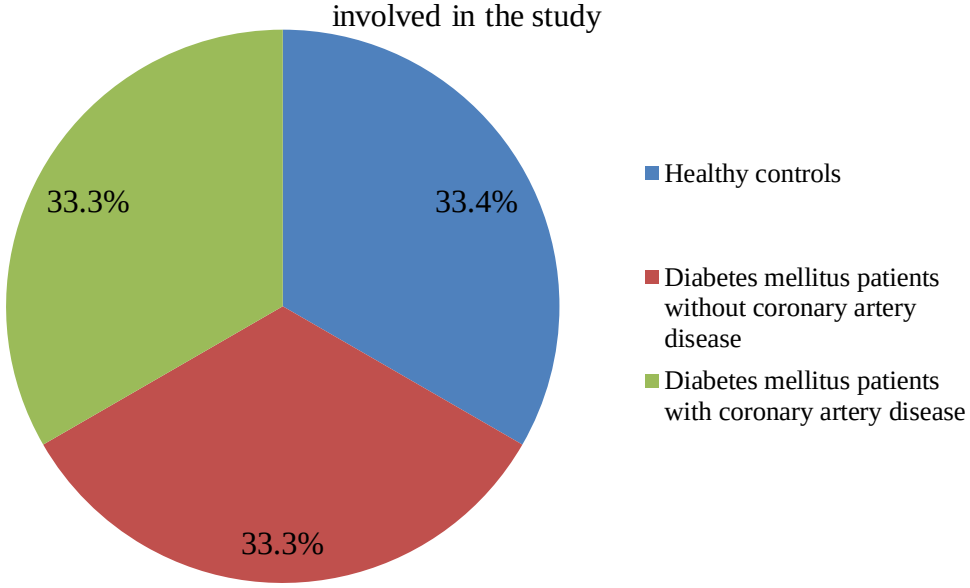
5.1 – Classification of Groups

Healthy controls: 65

Diabetes mellitus patients without coronary artery disease - 65

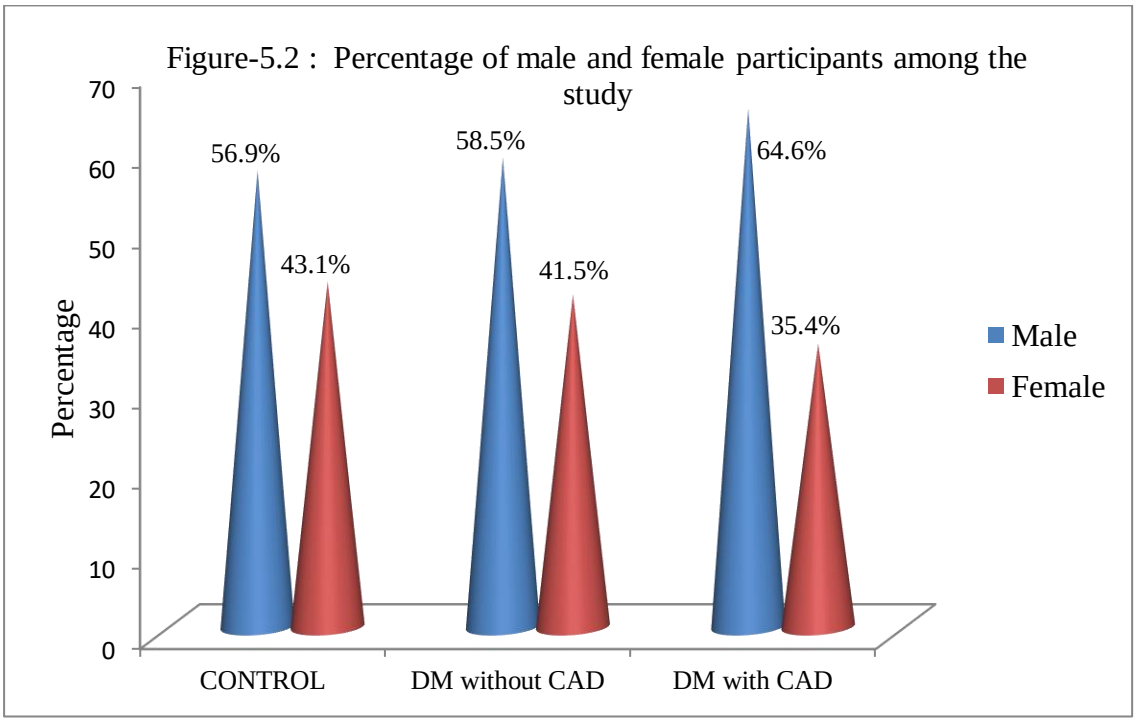
Diabetes mellitus patients with coronary artery disease – 65

Figure-5.1: Pie chart showing the percentage of participants involved in the study



5.2 – Gender distribution of participants:

Among the participants males were more compared to females with male to female ratio of 1.5:1. Gender distribution of the participants is shown below in the figure 5.2.



In DM without CAD patients, there were 38 males and 27 females. Among them 23.1% of the patients were in the age group of 51 to 60 years and 23.1% were in 61 to 70 years shown in table 5.1. There were 42 males and 23 females in DM with CAD group. Among them 29.2% of the patients were in the age group of 51 to 60 years and also the same in 61 to 70 years, followed by age group of above 70 years accounted for 23.1% shown in table 5.2. CAD risk was more in the higher age group.

Table-5.1: Distribution of age and gender among DM without CAD patients

Age(year)	Male		Female		Total	
	N	%	N	%	N	%
31-40	9	23.7	4	14.8	13	20.0
41-50	7	18.4	5	18.5	12	18.0
51-60	8	21.1	7	25.9	15	**23.1**
61-70	7	18.4	8	29.6	15	**23.1**
>70	7	18.4	3	11.1	10	15.4
Total	38	100.0	27	100.0	65	100.0

Table-5.2: Distribution of age and gender among DM with CAD patients

Age(yrs)	Male		Female		Total	
	N	%	N	%	N	%
31-40	3	7.0	1	4.5	4	6.1
41-50	5	11.6	3	13.6	8	12.3
51-60	12	27.9	7	31.8	19	**29.2**
61-70	11	25.6	8	36.4	19	**29.2**
>70	11	25.6	4	18.1	15	**23.1**
Total	42	100.0	23	100.0	65	100.0

In our study male to female ratio, percentage of smokers and percentage of family history of CAD in the different groups is depicted in table 5.3. Duration of diabetes mellitus was 6.8 ± 2.3 years in DM without CAD patients and 7.3 ± 3.2 years in DM with CAD patients. All sixty five (100%) DM without CAD patients were on oral hypoglycemic drugs. Among 65 DM with CAD patients, 60 (92.3%) were on oral hypoglycemic drugs and only five (7.6%) patients were on insulin.

Table-5.3: Characteristics of the groups involved in the study

PARAMETERS	**CONTROL**	**DM without CAD**	**DM with CAD**
Male: Female	37 : 28 (56.9: 43.1%)	38:27 (58.5:41.5%)	42:23 (64.6:35.3%)
Smokers (%)	10 (15%)	12 (18%)	12 (18%)
Family history of CAD (%)	8 (12%)	9 (13.84%)	10 (15 %)
Duration of DM years	-	6.8 ± 2.3	7.3 ± 3.2
Patients on oral hypoglycemic drugs (%)	-	65 (100%)	60 (92.3%)
Patients on insulin (%)	-	-	5 (7.6%)

5.3 – Baseline characteristics:

In the present study, mean age of the participants were 56.12±14.63, 55.97±14.82 and 61.15±12.51 years in controls, DM without CAD group and DM with CAD group respectively. Body mass index was 24.73±1.25, 26.47±2.18 and 26.37±3.43 Kg/m^2 respectively. Our findings showed statistically significant difference (ANOVA, $p<0.001$) in mean arterial pressure (MAP), fasting blood glucose (FBG) and glycated haemoglobin (HbA1c) among the three groups which is shown in table 5.4.

Table- 5.4: Baseline characteristics in the three groups

PARAMETERS	**CONTROL**	**DM without CAD**	**DM with CAD**	**ANOVA F**	**p value**
Age years	56.12±14.63	55.97±14.82	61.15±12.51	2.9	0.06
BMI Kg/m^2	24.73±1.25	26.47±2.18	26.37±3.43	10.3	<0.001*
MAP mm of Hg	87.6 ± 6.73	92.15 ± 7.81	108.03 ± 6.89	36.4	<0.001*
FBG mg/dL	81.49±16.09	158.2±44.04	162.42±48.93	88.2	<0.001*
HbA_1c %	5.19±0.59	7.81±1.58	8.97±1.76	131.7	<0.001*

Note: * statistical significance ($p<0.001$)

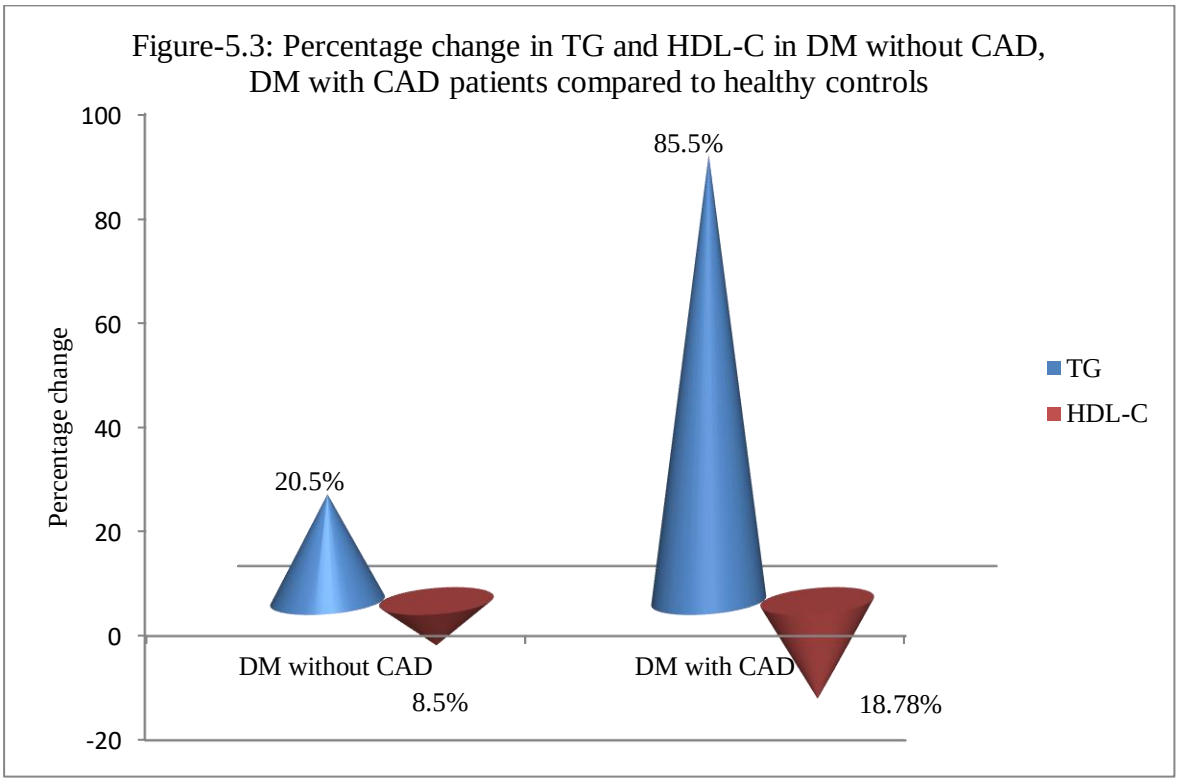
5.4 - Lipid parameters:

In the present study we measured lipid parameters in all the participants. Table-5.5 shows lipid profile among all the three groups. Our results showed statistically significant difference ($F=9.6$, $p<0.001$) in triglycerides (TG) and very low density lipoprotein (VLDL) among all the three groups. There was 20.5% and 85.5% increase in TG levels in DM without CAD patients and DM with CAD patients respectively when compared to healthy controls. At the same time there was significant difference in high density lipoprotein (ANOVA, $F=15.4$, $p<0.001$) among the groups. HDL-C was decreased more in DM with CAD patients i.e 18.5% compared to healthy controls whereas in DM without CAD patients it was 8.5% showed in figure 5.3. Even though the total cholesterol and LDL-C were increased in the DM patients it was not statistically significant ($p>0.05$).

Table-5.5: Comparison of mean values of lipid profile in the three groups

PARAMETERS	**CONTROL**	**DM without CAD**	**DM with CAD**	**ANOVA F**	**p value**
TC mg/dL	153.6±26.81	158.41±41.22	162.91±41.46	1.6	0.262
TG mg/dL	97.52±23.7	117.54±46.77	141.12±83.26	**9.6**	**<0.001***
HDL-C mg/dL	39.69±7.42	36.2±8.35	32.22±7.23	**15.4**	**<0.001***
LDL-C mg/dL	94.41±24.09	98.7±37.92	102.13±35.25	1.63	0.206
VLDL-C mg/dL	19.5±4.74	23.51±9.35	28.62±19.06	**8.7**	**<0.001***

Figure-5.3: Percentage change in TG and HDL-C in DM without CAD, DM with CAD patients compared to healthy controls



5.5 - Lp(a) levels:

In the study we measured novel marker of coronary artery disease Lp(a) in all the study participants. Mean Lp(a) levels in healthy controls was 15.4±3.89 mg/dL, whereas in DM without CAD patients it was 28.74±14.12 mg/dL and DM with CAD patients had 47.99±20.55 mg/dL. We noted there was statistical significant difference (ANOVA, F=82.3 and p<0.001) in serum Lp(a) levels among study groups shown in figure-5.4. We noticed huge increase in Lp(a) levels in DM with CAD patients i.e 211.6 % and 86.6% DM without CAD patients respectively when compared to healthy controls which is depicted in the figure 5.5.

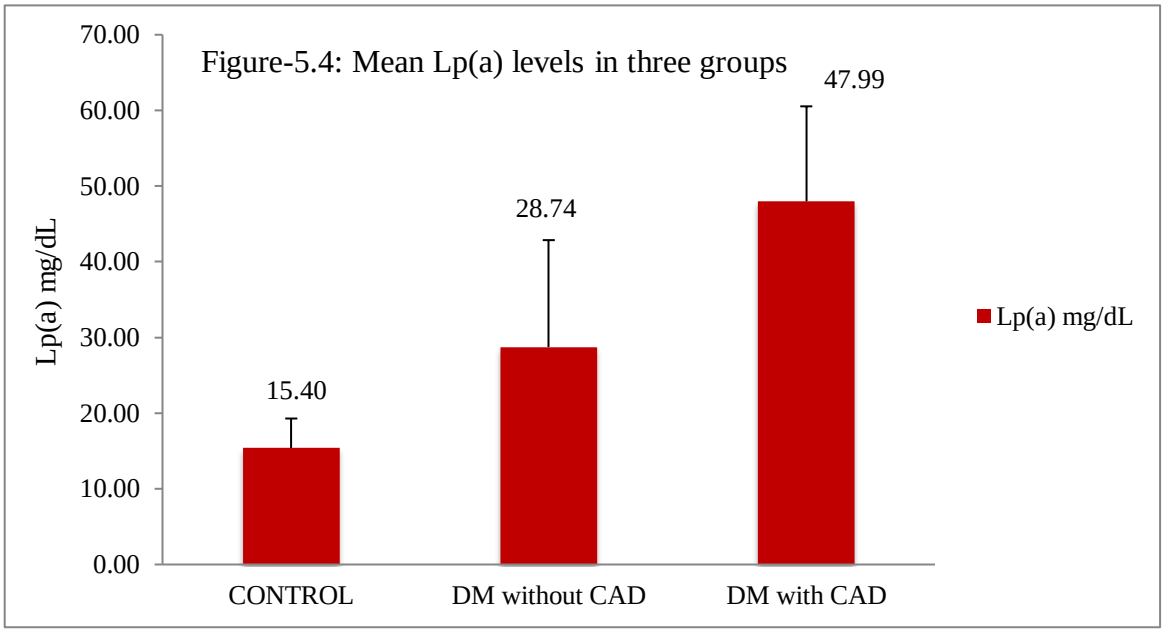
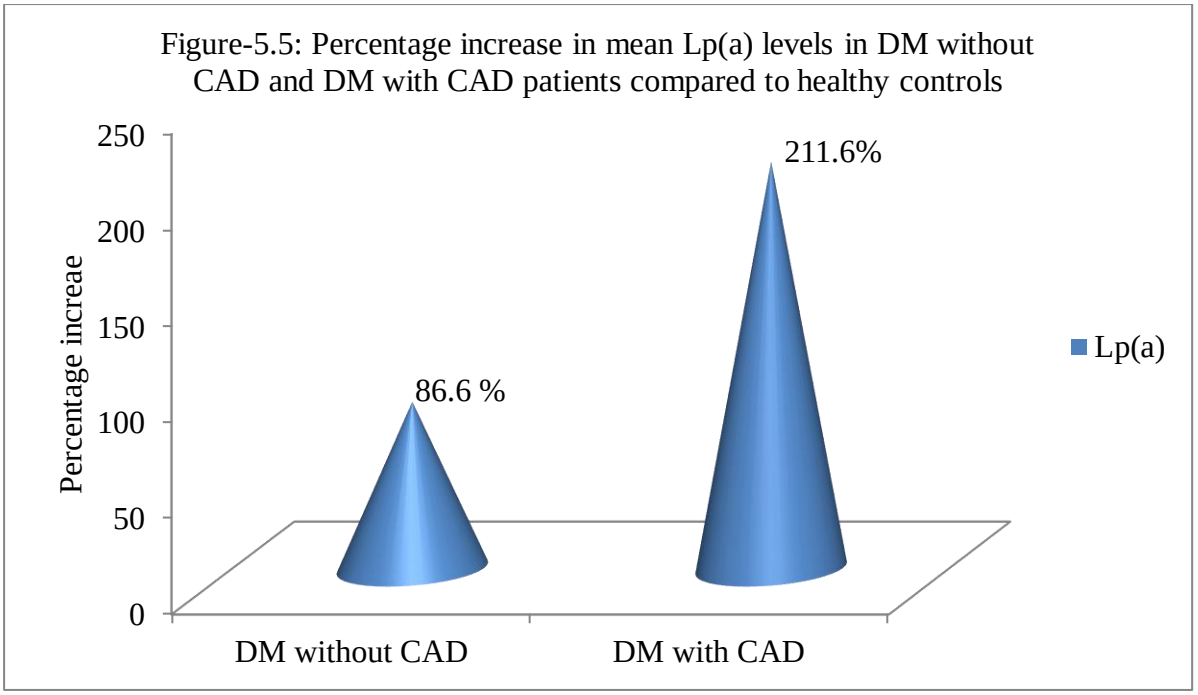
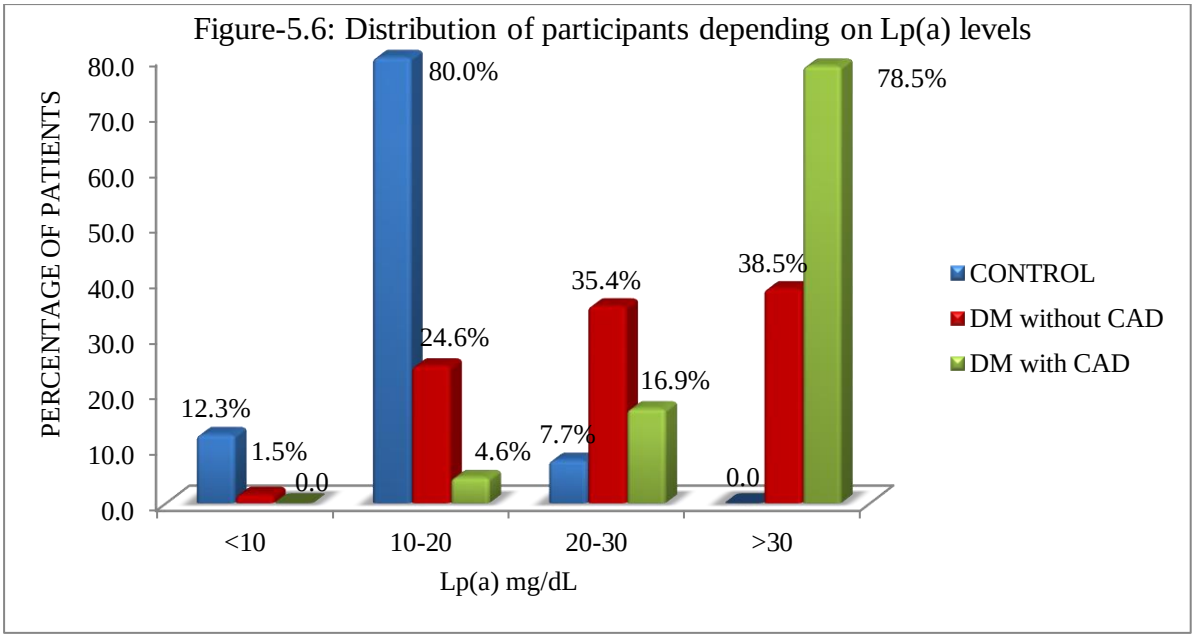


Figure-5.5: Percentage increase in mean Lp(a) levels in DM without CAD and DM with CAD patients compared to healthy controls



We also distribute the participating individuals into four categories depending on their Lp(a) levels as individuals whose Lp(a) levels were less than 10 mg/dL, 10-20 mg/dL, 20-30 mg/dL and participants with Lp(a) levels more than 30 mg/dL. We observed eighty percent of healthy controls had Lp(a) levels in the range of 10 to 20 mg/dL, whereas 78.5% of DM with CAD patients had Lp(a) levels above 30 mg/dL (Figure-5.6).

Figure-5.6: Distribution of participants depending on Lp(a) levels



We also compared the Lp(a) levels in patients with well controlled and poorly controlled diabetic patients, who were grouped depending on their HbA_1c levels. HbA_1c levels 7.5% was taken as cut-off levels. Individuals having HbA_1c levels > 7.5% were labelled as poorly controlled and those having HbA_1c levels < 7.5% labelled as well controlled diabetic patients. We noticed that Lp(a) levels increased with an increase in HbA_1c level that is in poorly controlled patients compared to well controlled patients but it was not statistically significant ($p>0.05$) in our study.

Table-5.6: Mean Lp(a) levels in relation to HbA_1c levels

GROUPS	**HbA1C**				**p value**
	< 7.5 %		**> 7.5 %**		
	Mean Lp(a) mg/dL	**SD**	**Mean Lp(a) mg/dL**	**SD**	
CONTROL	15.40	3.89	-	-	-
DM without CAD	25.41	15.64	31.26	12.49	0.098
DM with CAD	28.14	7.3	32.4	7.54	0.075

In addition to this we compared Lp(a) levels in stratified variables among DM without CAD and DM with CAD patients it showed Lp(a) levels were affected by hsCRP in DM without CAD patients. Lp(a) levels were affected by HDL-C and hsCRP levels in DM with CAD patients; Whereas age, gender, BMI, smoking, family history, HbA_1c, TC, TG and LDL-C did not have any effect on the Lp(a) levels in both the groups.

Table-5.7: Comparison of Lp(a) levels in stratified variables among DM without CAD and DM with CAD patients

Variables		**DM without CAD**		**DM with CAD**	
		Mean± SD **Lp(a) mg/dL**	p-value	Mean± SD **Lp(a) mg/dL**	p-value
Age in years	<50	30.9 ± 17.14	0.34	33.7 ± 13.7	0.17
	>50	27.51 ± 12.87		33.7 ± 13.7	
Gender	Male	28.46± 13.67	0.85	23.3 ± 6.07	0.53
	Female	29.13 ± 14.98		25.06±8.4	
Smoking	Present	25.9 ± 16.9	0.52	58.5 ± 24.6	0.07
	Absent	29.2 ± 13.7		46.1 ± 19.4	
Family history of CAD	Positive	29.9 ±16.8	0.75	52.4 ± 21.7	0.41
	Negative	27.35 ± 13.6		47.0 ± 20.4	
BMI (Kg/m^2)	<25	32.97 ± 16.81	0.17	22.49 ± 6.7	0.5
	>25	27.36 ± 13.04		23.8 ± 8.4	
HbA_1c (%)	<7.5	25.41 ± 15.64	0.09	28.14 ± 7.3	0.07
	>7.5	31.26 ±12.49		32.4 ± 7.5	
TC (mg/dL)	<200	29.09 ±15.08	0.63	48.91 ± 10.53	0.26
	>200	26.78 ±7.92		39.02 ± 10.19	
TG (mg/dL)	<150	28.72 ±15.0	0.97	46.57 ±10.41	0.25
	>150	28.89 ±6.9		53.71 ± 10.8	
HDL-C (mg/dL)	<40	28.09 ± 12.81	0.61	50.93 ± 10.78	**0.009***
	>40	30.01 ± 16.65		33.53 ±11. 6	
LDL-C (mg/dL)	<100	29.75 ± 12.06	0.98	47.05 ± 18.86	0.62
	>100	36.8 ± 13.18		49.72 ± 13.69	
hsCRP (mg/L)	<3	18.9± 6.91	**0.003***	19.2 ± 9.0	**0.001***
	>3	31.45 ± 14.43		48.44 ± 10.39	

5.6 – Association of Lp(a) with other parameters:

Further we also correlated Lp(a) with other parameters to find the association between serum Lp(a) and lipid parameters, HbA_1c, hsCRP and NO. We applied Pearson's correlation coefficient analysis. We found that Lp(a) shows positive correlation with HbA1c and hsCRP in DM without CAD patients which was statistically significant ($p<0.05$), whereas negative correlation was seen with NO which was also statistically significant ($p<0.05$ and $r= -0.31$). In DM with CAD patients Lp(a) shows positive correlation with hsCRP ($r=0.64$) and negative correlation with HDL-C ($r=-0.355$) and both were statistically significant with p value less than 0.05. (Shown in table 5.8 and figure number 5.7 to 5.11)

Table-5.8: Correlation between Lp(a) and other parameters in DM without CAD and DM with CAD patients

PARAMETERS	DM without CAD		DM with CAD	
	r value	p value	r value	p value
Age in years	-0.006	0.963	-0.071	0.572
BMI Kg/m^2	-0.185	0.141	-0.169	0.178
MAP mm of Hg	-0.16	0.203	-0.051	0.688
FBG mg/dL	0.005	0.998	-0.102	0.42
TC mg/dL	0.013	0.917	0.037	0.768
TG mg/dL	0.053	0.675	0.082	0.517
HDL-L mg/dL	0.092	0.467	-0.355	**0.004***
LDL-C mg/dL	-0.013	0.917	0.058	0.649
VLDL-C mg/dL	0.053	0.675	0.103	0.414
NO μmol/L	-0.311	**0.01***	0.027	0.834
HbA_1c %	0.372	**0.002***	-0.043	0.732
hsCRP mg/L	0.623	**0.001***	0.641	**0.001***

Note: * ($p<0.05$) level of significance

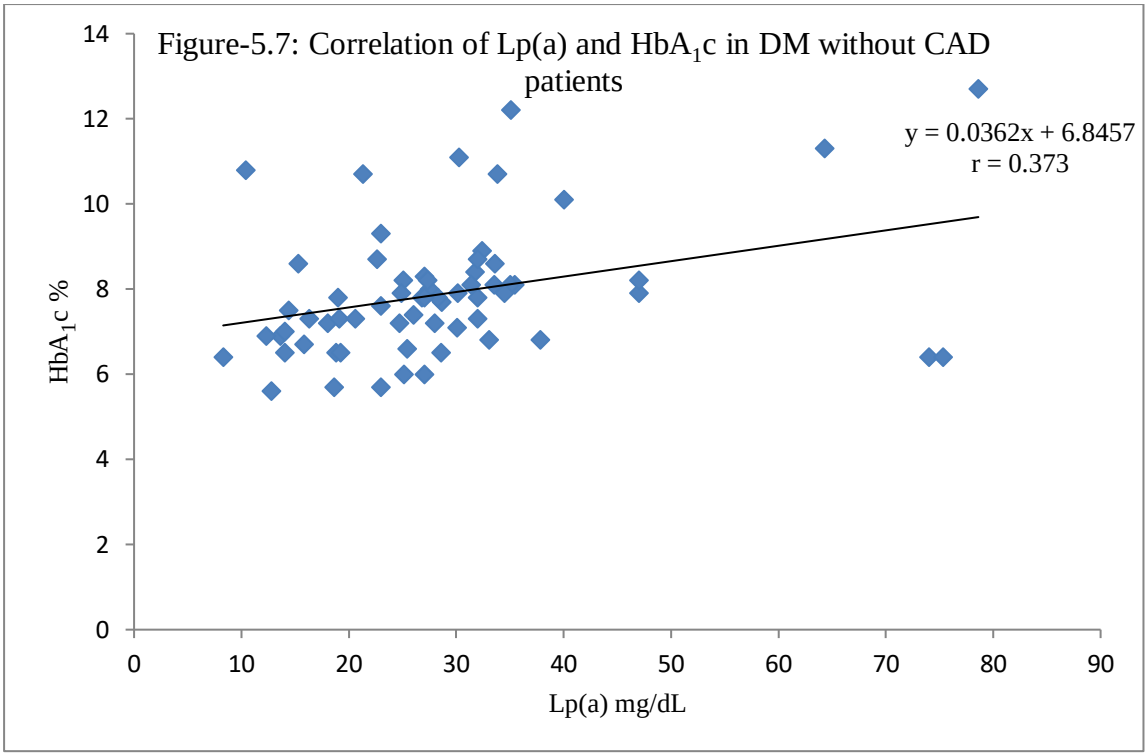


Figure-5.7: Correlation of Lp(a) and HbA1c in DM without CAD patients
y = 0.0362x + 6.8457
r = 0.373
HbA1c %
Lp(a) mg/dL
0
2
4
6
8
10
12
14
0
10
20
30
40
50
60
70
80
90

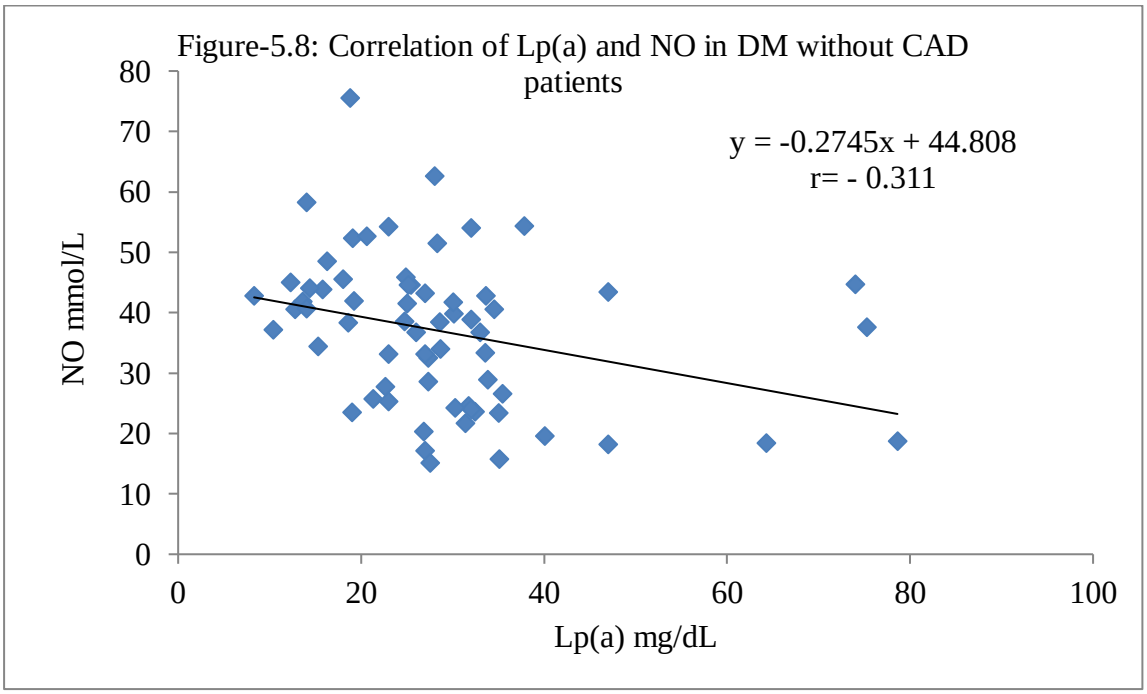


Figure-5.8: Correlation of Lp(a) and NO in DM without CAD patients
y = -0.2745x + 44.808
r= - 0.311
NO mmol/L
Lp(a) mg/dL
0
10
20
30
40
50
60
70
80
0
20
40
60
80
100

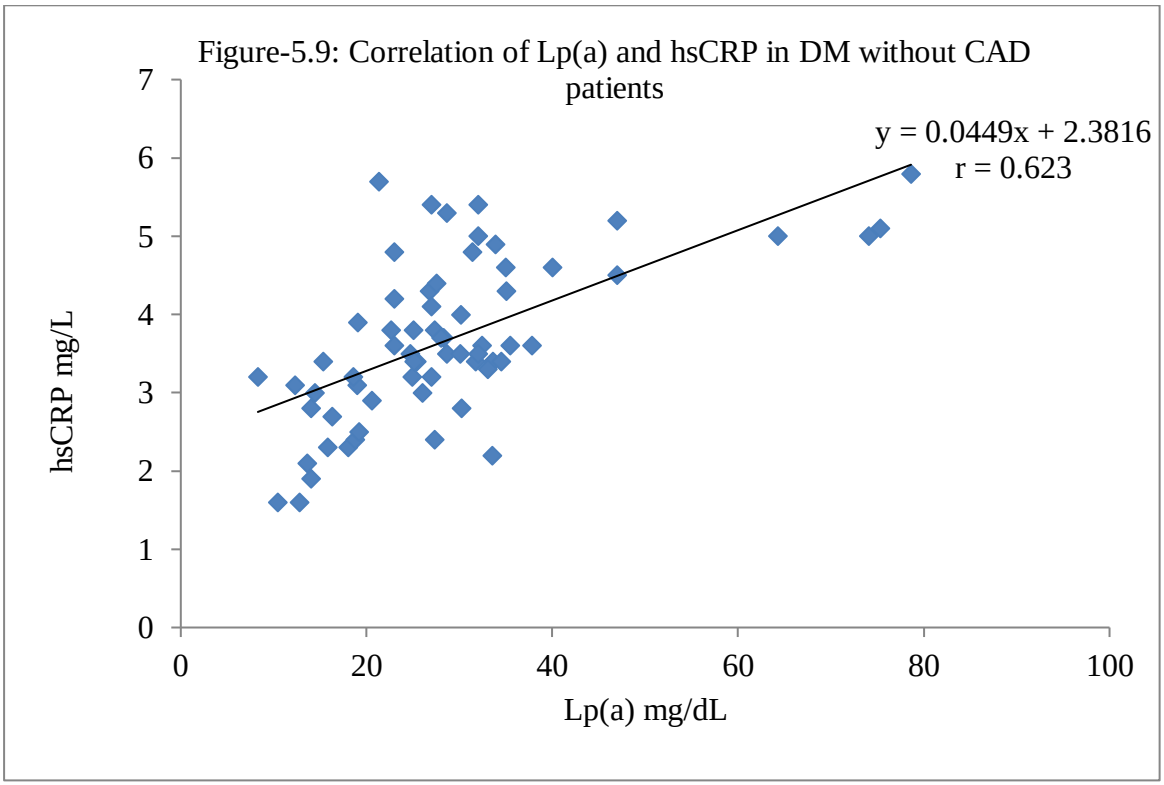


Figure-5.9: Correlation of Lp(a) and hsCRP in DM without CAD patients

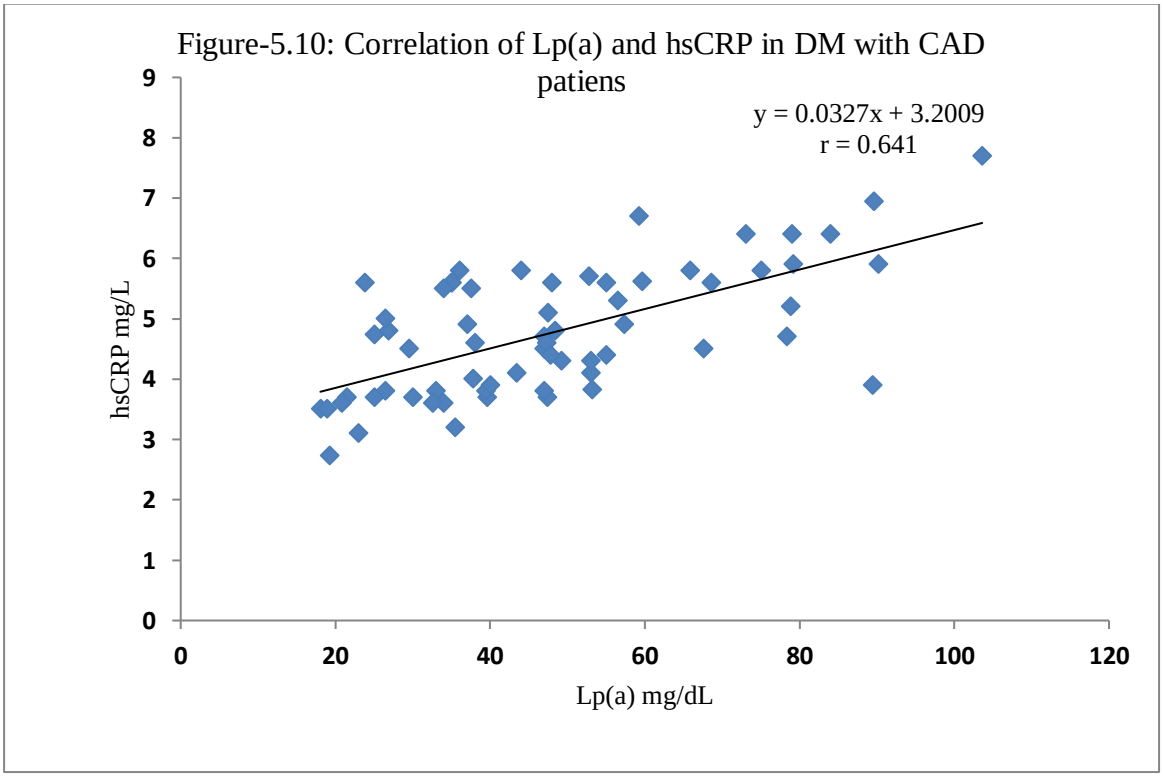
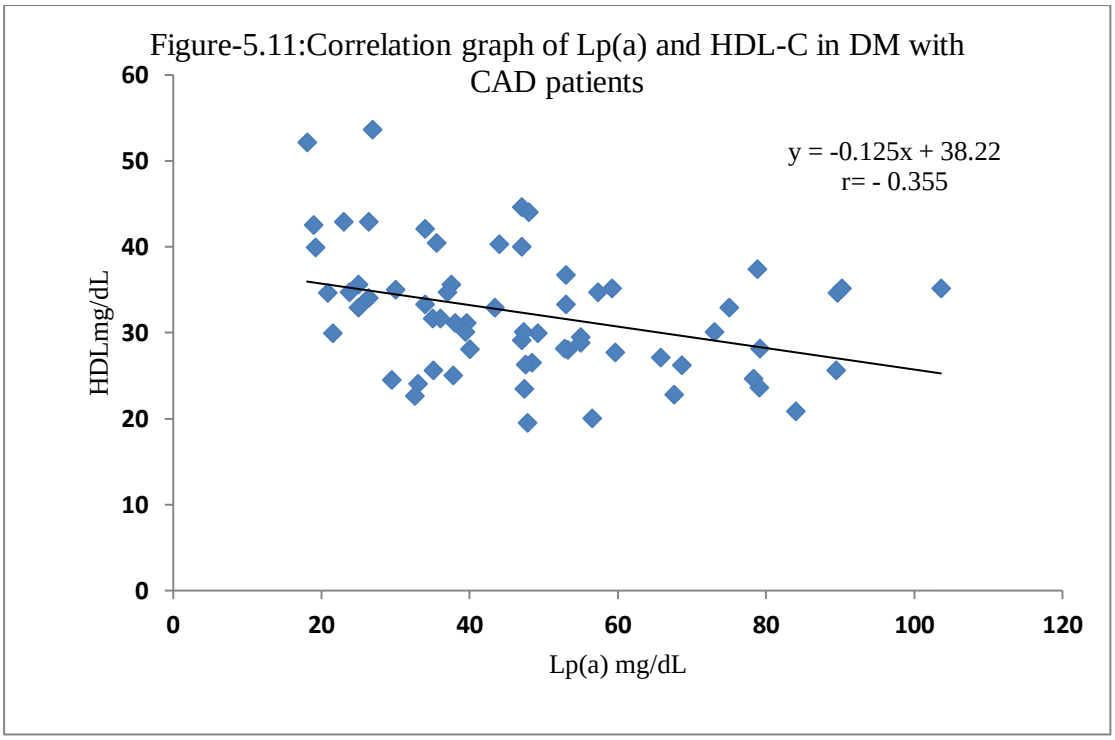


Figure-5.10: Correlation of Lp(a) and hsCRP in DM with CAD patiens



5.7 - Lp(a) best cut-off value

The review of literature shows that Lp(a) levels have world-wide ethnic variation, we made an attempt to find out Lp(a) levels in and around Bagalkot population in north Karnataka. For this, receiver operating curve (ROC) was drawn to find the best cut-off value of Lp(a). In our population cut-off value of Lp(a) was 18.3 mg/dL in DM without CAD patients with 81.5% of sensitivity and 81.5% of specificity and area under the curve (AUC) was 0.8 as shown in table 5.9 and figure 5.12. In DM with CAD patients cut-off value was 21.6 mg/dL at 93.8% sensitivity and 92.3% specificity and AUC was 0.9 as depicted in table 5.10 and figure 5.13. Hence Lp(a) can be used as early indicator to evaluate risk of CAD in DM patients.

Table-5.9: Area under ROC to detect best cut-off values of Lp(a) levels in DM without CAD patients

Area Under the Curve	Std. Error	p value	95% Confidence Interval	
			Lower Bound	Upper Bound
0.867	0.034	<0.001*	0.8	0.934

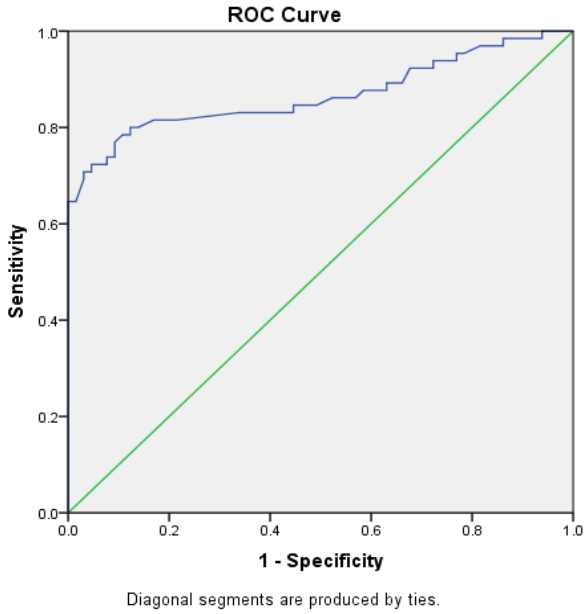


Figure-5.12: ROC to detect best cut-off values of Lp(a) levels in DM without CAD patients

Positive if Greater Than or Equal To	Sensitivity	Specificity
9.45	98.5%	10.8%
15.90	84.6%	50.8%
18.32	**81.5%**	**81.5%**
19.04	76.9%	90.8%
24.06	64.6%	100.0%

Table-5.10: Area under ROC to detect best cut-off value of Lp(a) levels in DM with CAD patients

Area Under the Curve	Std. Error	p value	95% Confidence Interval	
			Lower Bound	Upper Bound
0.991	0.005	<0.001*	0.981	1

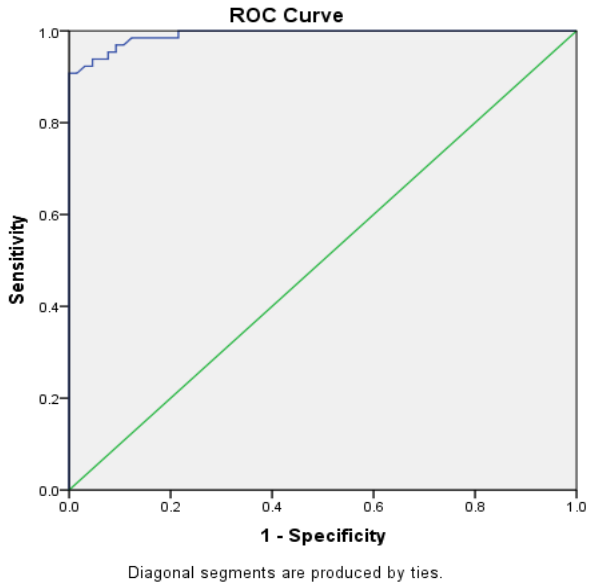


Figure-5.13: ROC to detect best cut-off values of Lp(a) levels in DM with CAD patient

Positive if Greater Than or Equal To	Sensitivity	Specificity
9.45	100.0%	10.8%
18.03	100.0%	78.5%
21.6	**93.8%**	**92.3%**
23.21	90.8%	98.5%
29.75	80.0%	100.0%

In our study, logistic linear regression analysis was done and odds ratio (OR) was calculated to predict CAD in diabetic patients. The logistic regression table 5.11 results show the adjusted effect of Lp(a) on the risk of DM without CAD and DM with CAD by controlling the effect of age, BMI and lipid profile variables.

Lp(a) was found to have positive effect on the chance of developing CAD in diabetes mellitus patients. With unit increase in Lp(a), the chance of CAD was 1.33 times higher in DM without CAD patients, while in the DM with CAD patients it was 2.82 times higher.

Table-5.11: Logistic regression analysis and Odds ratio (OR) of Lp(a) in the DM without CAD and DM with CAD group

Predictors	DM without CAD				DM with CAD			
	Adjusted Odds ratio	p-value	95% CI		Adjusted Odds ratio	p-value	95% CI	
			Lower	Upper			Lower	Upper
Lp(a) mg/dL	1.33	<0.001*	1.179	1.493	2.82	0.008*	1.316	6.035

5.9 - Nitric oxide (NO):

We also studied the NO levels, which is an indirect measure of endothelial dysfunction. Mean ± SD of NO levels in healthy controls was 43.01±10.1 μmol/L. NO levels in DM without CAD patients had 36.92±12.47 μmol/L and DM with CAD patients had 23.49±7.66 μmol/L. There was statistical significant difference (ANOVA, F=62.54, p<0.001) in NO levels in all the three groups, shown in figure 5.14. More reduced NO levels were observed in DM with CAD group compared to DM without CAD and healthy controls. It was observed 14.2% decrease values in DM without CAD patients compared to healthy controls whereas it was 45.37% decrease in DM with CAD patients (Figure 5.15).

Figure-5.14: Mean NO levels in different groups

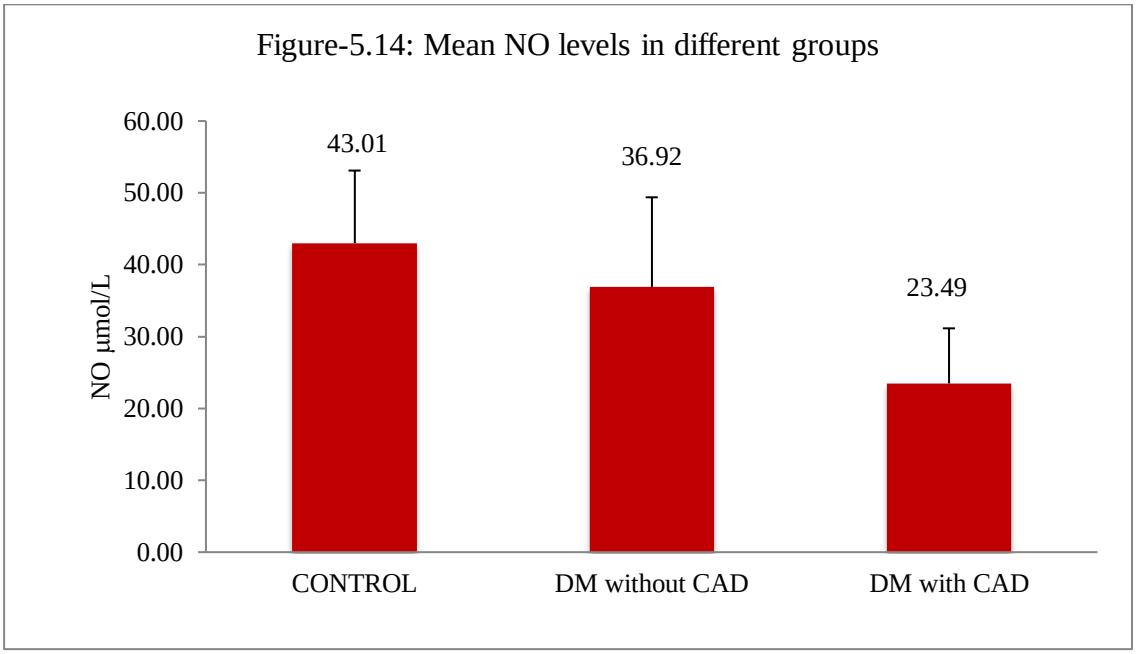
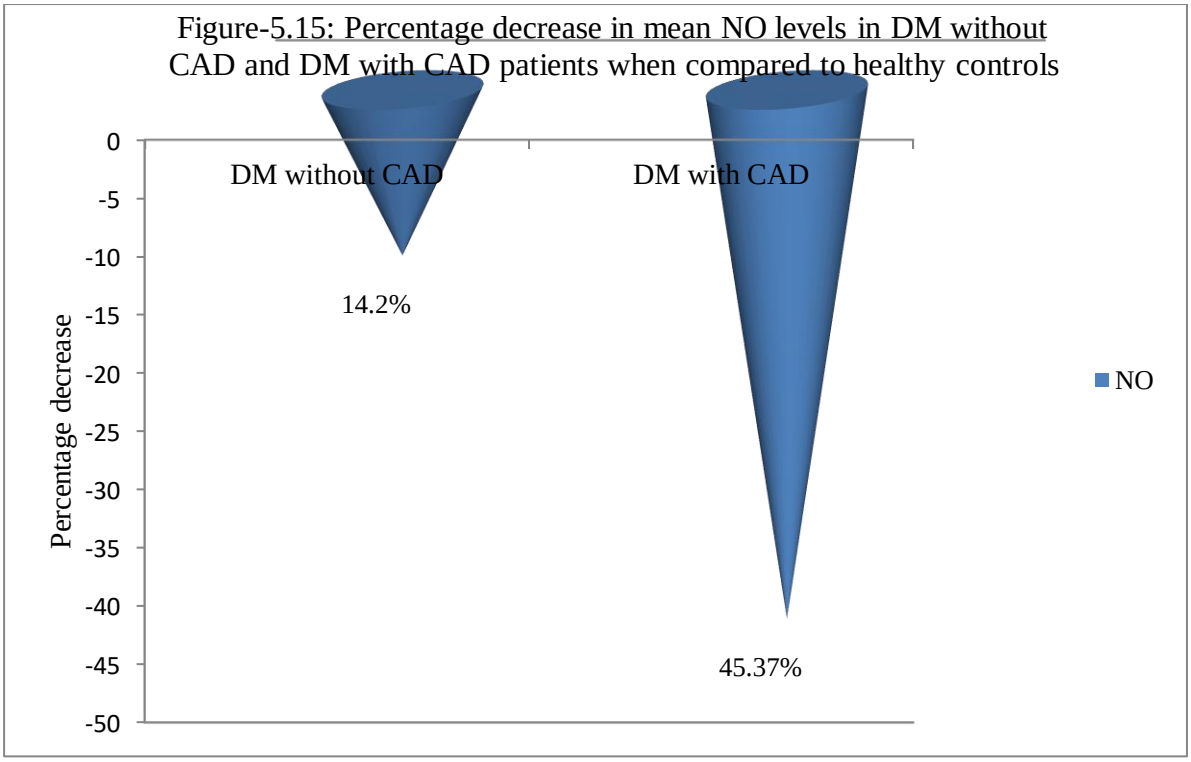
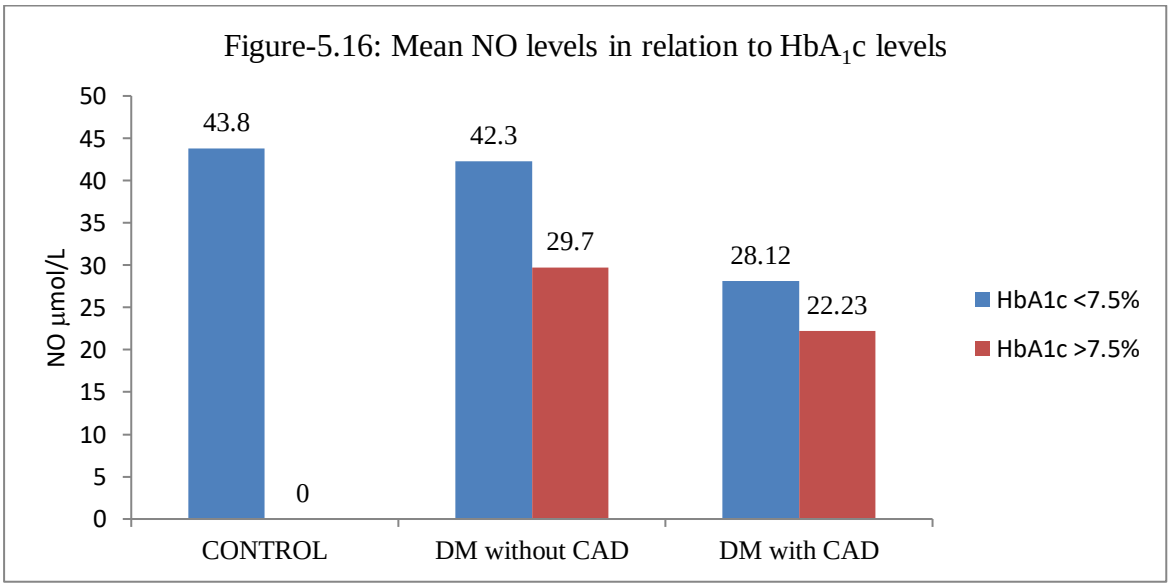


Figure-5.15: Percentage decrease in mean NO levels in DM without CAD and DM with CAD patients when compared to healthy controls



We examined the impact of glycemic control on vascular endothelial system by estimating the NO in the study group. We noticed poor glycemic control patients (HbA_1c >7.5%) had reduced NO levels compared to good glycemic control patients (HbA_1c < 7.5%) both in DM without CAD patients and DM with CAD patients (Figure 5.16), which was statistically significant ($p<0.001$).

Figure-5.16: Mean NO levels in relation to HbA_1c levels



Comparison of NO levels in stratified variables among DM without CAD and DM with CAD patients shown in table 5.12. NO levels were affected by HbA_1c and hsCRP levels in DM without CAD patients and DM with CAD patients. NO levels were also affected by TC levels in DM with CAD patients. Age, gender, BMI, smoking, family history, TG, HDL and LDL levels did not have any effect on NO levels in both the groups.

Table-5.12: Comparison of NO levels in stratified variables among DM without CAD and DM with CAD patients

Variables		**DM without CAD**		**DM with CAD**	
		Mean± SD NO μmol/L	p-value	Mean± SD NO μmol/L	p-value
Age in years	<50	35.14 ± 12.7	0.4	33.7 ± 13.7	0.17
	>50	37.89 ± 12.37		33.7 ± 13.7	
Gender	Male	37.87 ± 10.4	0.98	24.3 ± 6.07	0.53
	Female	36.95 ± 13.8		23.06 ± 8.4	
Smoking	Present	38.2 ± 9.2	0.73	23.3 ± 5.2	0.93
	Absent	36.7 ± 13.0		23.5 ± 8.1	
Family history of CAD	Positive	33.0 ±12.0	0.23	20.8 ± 6.2	0.18
	Negative	37.8 ± 12.5		24.1 ± 7.9	
BMI (Kg/m^2)	<25	33.7 ± 13.7	0.24	22.49 ± 6.7	0.5
	>25	37.9 ± 12.4		23.8 ± 8.4	
HbA_1c (%)	<7.5	42.3 ± 8.9	**0.00***	28.14 ± 7.3	**0.05***
	>7.5	29.6 ±9.6		22.3 ± 7.5	
TC (mg/dL)	<200	36.4 ±11.8	0.41	23.86 ± 7.23	**0.04***
	>200	39.8 ±15.72		12.7 ± 11.23	
TG (mg/dL)	<150	36.9 ±12.0	0.93	23.9 ± 7.1	0.35
	>150	36.59 ±15.9		21.7 ± 9.8	
HDL-C (mg/dL)	<40	35.6 ± 13.94	0.26	23.3 ± 7.8	0.67
	>40	39.3 ± 8.56		24.03 ± 6.9	
LDL-C (mg/dL)	<100	36.9 ± 12.06	0.98	23.8 ± 7.57	0.56
	>100	36.8 ± 13.18		22.7 ± 8.13	
hsCRP (mg/L)	<3	44.1± 12.43	**0.01***	28.3 ± 8.35	**0.03***
	>3	34.95 ± 11.8		17.5 ± 7.69	

5.9: Association of NO with other parameters:

Correlation of NO with different parameters is depicted in table 5.13 and figure numbers 5.17 to 5.22. NO showed significant negative correlation ($p<0.05$) with FBG, HbA_1c and hsCRP in DM without CAD patients. Whereas in DM with CAD patients, NO showed significant negative correlation ($p<0.05$) with HbA_1c, hsCRP and MAP. There was significant ($p<0.05$) negative correlation between NO and HbA_1c in DM without ($r=-0.728$) and DM with CAD patients ($r=-0.43$). Poor glycemic patients had decreased NO levels. NO had no correlation with lipid parameters in both the groups.

Table-5.13: Correlation of NO with other parameters in DM without CAD and DM with CAD patients

PARAMETERS	DM without CAD		DM with CAD	
	r value	p value	r value	p value
Age in years	0.116	0.358	0.024	0.85
BMI Kg/m^2	0.03	0.81	0.131	0.299
MAP mm of Hg	0.03	0.815	-0.243	**0.05***
FBG mg/dL	-0.247	**0.047***	-0.226	0.07
TC mg/dL	0.018	0.886	-0.188	0.133
TG mg/dL	0.15	0.232	-0.128	0.308
HDL-C mg/dL	0.149	0.237	0.081	0.523
LDL-C mg/dL	-0.037	0.767	-0.134	0.286
VLDL-C mg/dL	0.15	0.232	-0.152	0.227
Lp(a) mg/dL	-0.311	**0.01***	0.027	0.834
HbA_1c %	-0.728	**0.001***	-0.439	**0.001***
hsCRP mg/L	-0.467	**0.001***	-0.261	**0.05***

Figure-5.17: Correlation between NO and MAP among DM with CAD patients

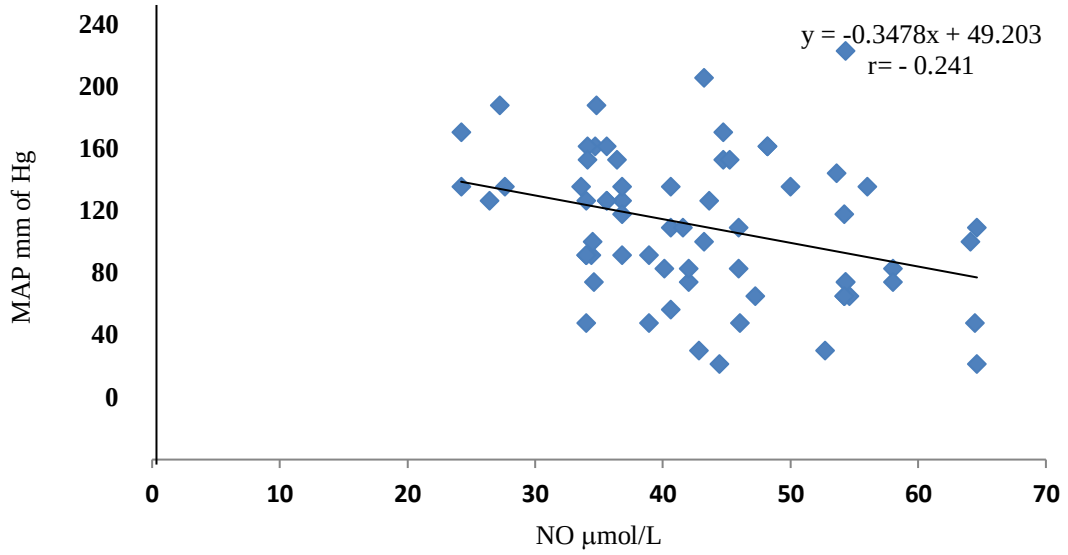


Figure-5.18: Correlation of NO and FBG in DM without CAD patients

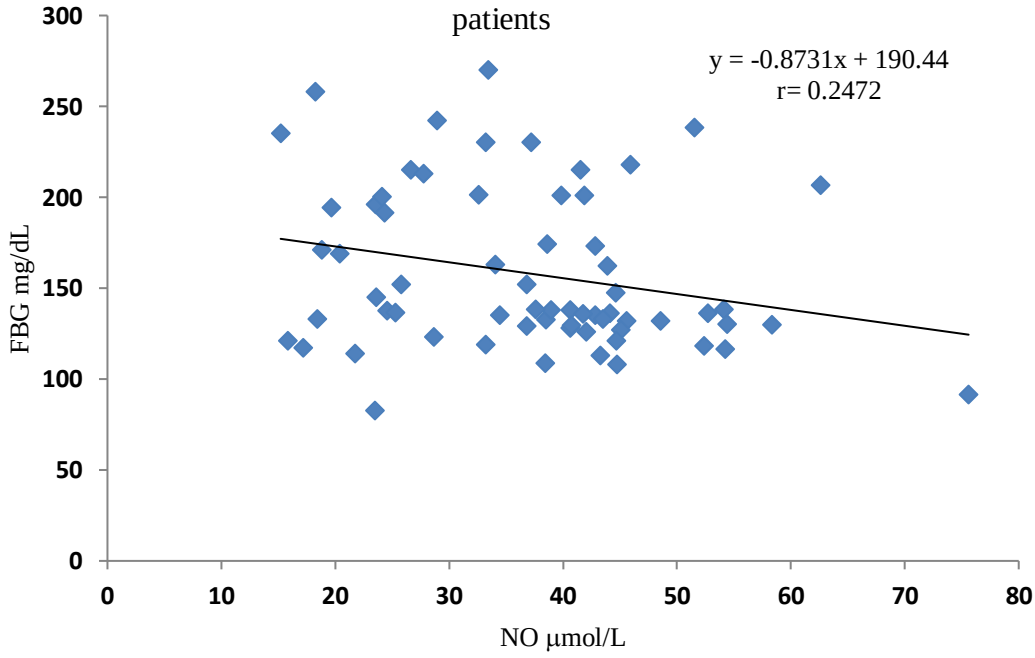


Figure-5.19: Correlation of NO and HbA_1c in DM without CAD patients

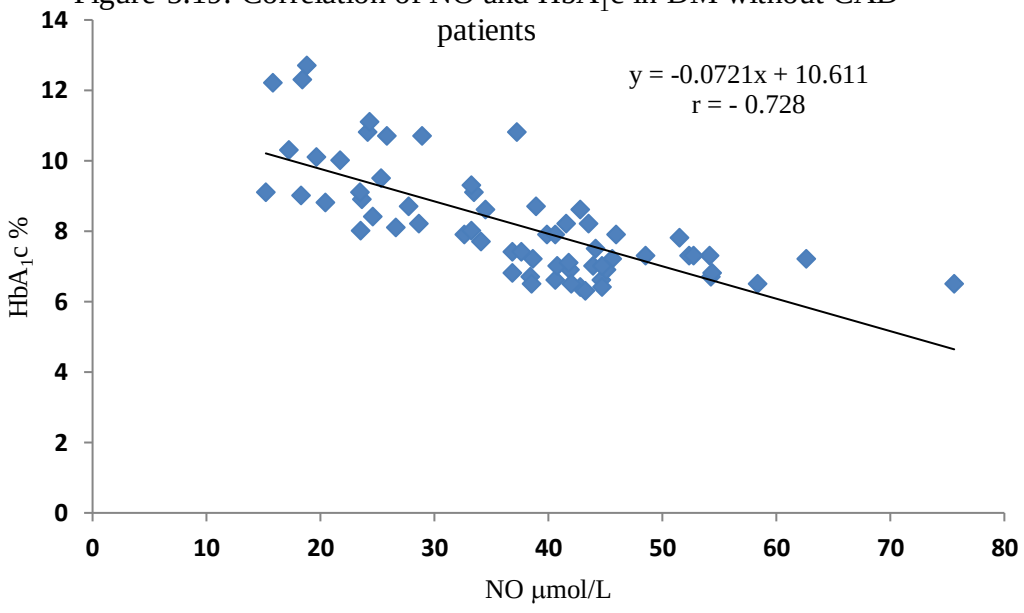
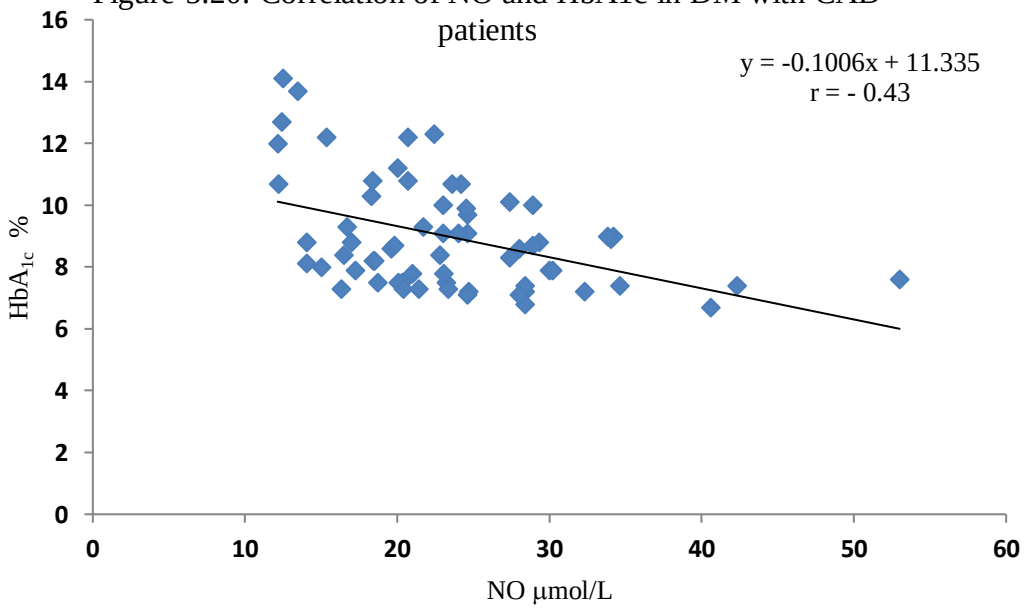


Figure-5.20: Correlation of NO and HbA1c in DM with CAD patients



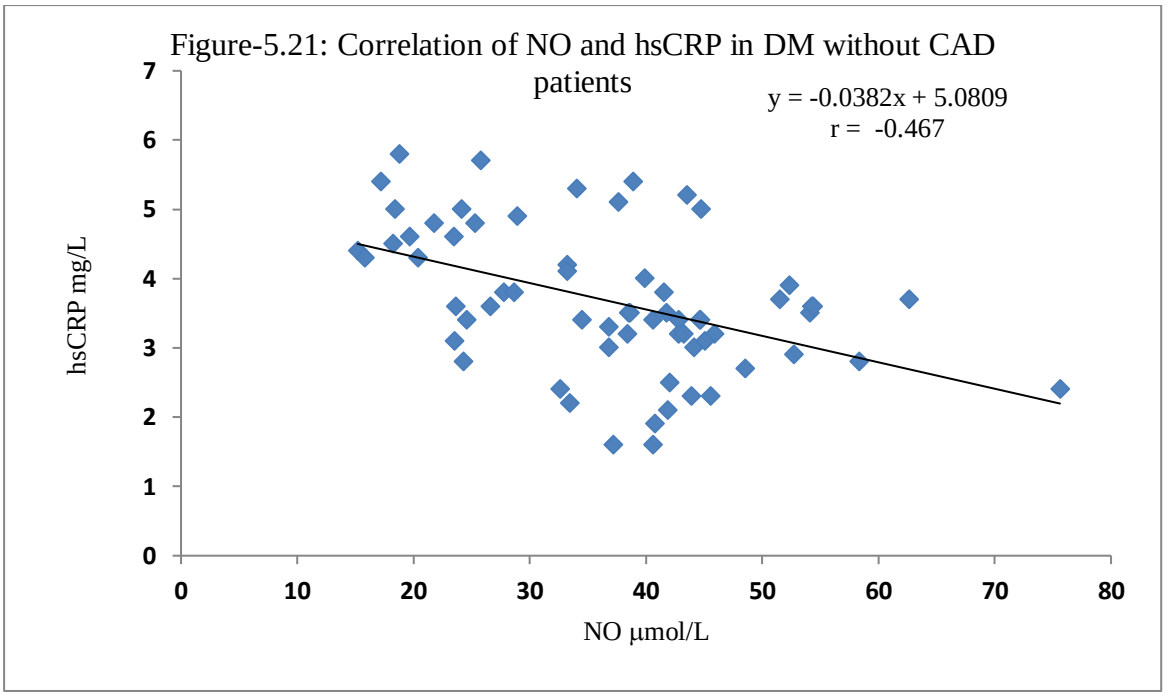


Figure-5.21: Correlation of NO and hsCRP in DM without CAD patients

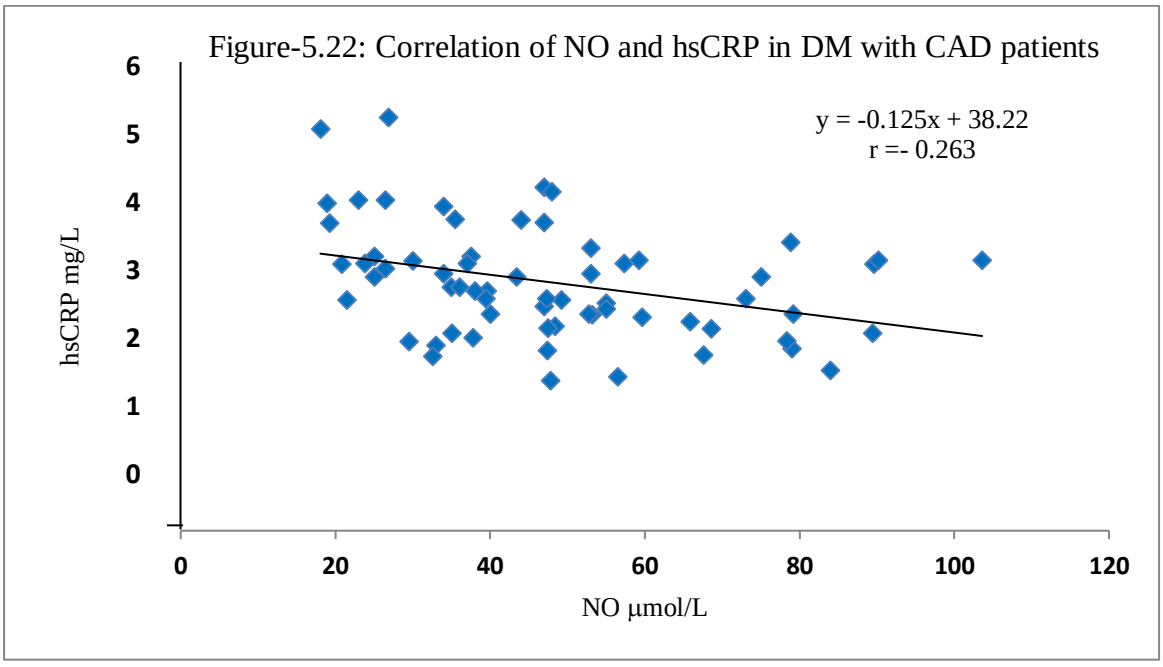


Figure-5.22: Correlation of NO and hsCRP in DM with CAD patients

The logistic regression table results show the adjusted effect of NO on the risk of DM without CAD and DM with CAD by controlling the effect of age, BMI and lipid profile variables. NO had negative effect on the chance of developing CAD in diabetes mellitus patients. While one unit increases in NO, the chance of developing CAD in DM without CAD was 6% less whereas in DM with CAD patients it was 26% less chance which is shown in table 5.14.

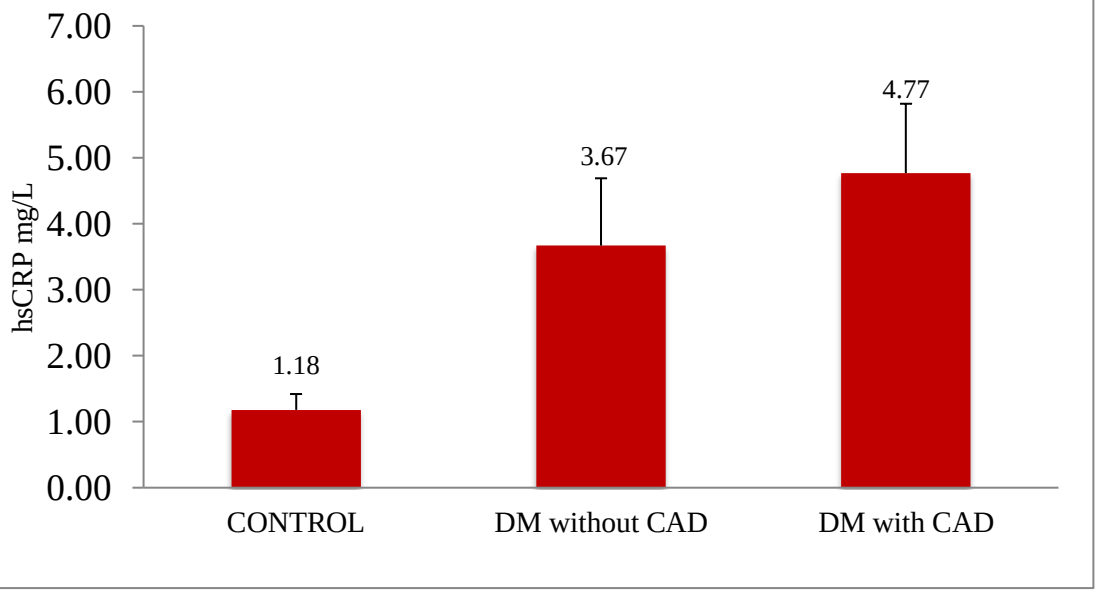
Table-5.14: Table showing the logistic regression analysis and Odds ratio (OR) of NO in DM without CAD and DM with CAD patients

Predictors	DM without CAD				DM with CAD			
	Adjusted Odds ratio	p-value	95% CI		Adjusted Odds ratio	p-value	95% CI	
			Lower	Upper			Lower	Upper
NO μmol/L	0.94	0.002*	0.896	0.975	0.74	<0.001*	0.645	0.837

5.10-hsCRP levels

Inflammation is key component in pathogenesis and complications of DM as well as in CAD. We estimated serum hsCRP levels, an inflammatory marker in diabetic subjects with and without CAD patients. In the present study, we found that mean hsCRP levels were 1.18±0.24, 3.74 ± 1.02, 4.7 ± 1.04 mg/L in healthy controls, DM without CAD and DM with CAD patients respectively as shown in figure 5.23. It showed significant difference in hsCRP levels among the three groups which was statistically significant with p value < 0.001.

Figur-5.23: Mean hsCRP levels in the three groups



In order to study the effect of inflammation on Lp(a) and NO levels, we grouped the participants into two groups one with hsCRP levels < 3mg/L and another with hsCRP levels >3mg/L. We observed there was statistically significant increase (p<0.001) in Lp(a) levels with increase in hsCRP levels in DM without CAD patients and DM with CAD patients depicted in table 5.15. At the same time NO levels decreased with increase in hsCRP levels, which was also statistically significant with p < 0.05 as shown in table 5.16.

Table-5.15: Mean Lp(a) levels in relation to hsCRP levels

GROUPS	**hsCRP mg/L**				**p value**
	<3.0		**≥3.0**		
	Mean Lp(a) mg/dL	**SD**	**Mean Lp(a) mg/dL**	**SD**	
CONTROL	15.40	3.89	0.00	0.00	-
DM without CAD	18.90	6.91	31.45	14.43	0.003*
DM with CAD	19.20	9.00	48.44	10.39	0.001*

Table-5.16: Mean NO (μmol/L) levels in relation to hsCRP levels

GROUPS	**hsCRP mg/L**				**p value**
	<3.0		**≥3.0**		
	Mean NO μmol/L	**SD**	**Mean NO μmol/L**	**SD**	
CONTROL	44.01	10.10	0.00	0.00	-
DM without CAD	42.10	12.43	34.95	11.86	0.014*
DM with CAD	28.30	8.10	17.57	7.69	0.03*

To summarize, comparison of all the parameters among the three groups with their F value by ANOVA and comparison between the groups with their p values by post-hoc test is shown in the table 5.17.

Table-5.17: Comparison of parameters in the three groups using ANOVA, Post-hoc for comparison of two groups and their p-values

Parameters	Controls	DM without CAD	DM with CAD	ANOVA "F"	p-value	DM without CAD & control p-value	DM with CAD & control p-value	DM with & without CAD p-value
Age in Year	56.12±14.63	55.90 ± 14.82	61.09 ± 12.52	2.9	0.06	0.94	0.06	0.06
BMI Kg/m^2	24.73±1.25	26.47 ± 2.18	26.37 ± 3.43	**10.3**	**0.001**	**0.001**	**0.001**	0.849
MAP mm of Hg	87.6 ± 6.73	92.15 ± 7.81	108.03 ± 6.89	**36.4**	**0.001**	0.31	**0.001**	**0.001**
Duration of DM in years	-	6.3 ± 2.3	7.2 ± 2.7		-	-	-	0.08
FBG mg/dL	81.49±16.09	158.2 ± 44.03	162.47 ± 48.93	**88.2**	**0.01**	**0.001**	**0.001**	0.54
HbA1c %	5.19±0.59	7.8 ± 1.58	8.9 ± 1.76	**131.7**	**0.01**	**0.01**	**0.001**	**0.002**
TC mg/dL	153.6±26.81	158.41 ± 41.23	162.91 ± 41.4	1.6	0.26	0. 15	0.94	0.17
TGs mg/dL	97.52±23.7	117.54 ± 46.75	141.12 ± 83.25	**9.6**	**0.001**	**0.04**	**0.001**	**0.019**
HDL-C mg/dL	39.69±7.42	36.2 ± 8.35	32.16 ± 7.23	**15.4**	**0.001**	**0.01**	**0.001**	**0.004**
LDL-C mg/dL	94.41±24.09	98.79 ± 37.92	102.13 ± 35.25	1.63	0.21	0.14	0.86	0.106
Lp(a) mg/dL	15.4±3.89	28.74 ± 14.12	47.95 ± 20.54	**82.3**	**0.001**	**0.001**	**0.001**	**0.000**
hsCRP mg/L	1.18±0.24	3.74 ± 1.02	4.7 ± 1.04	**299.5**	**0.001**	**0.001**	**0.001**	**0.000**
NO µmol/L	43.01±10.2	36.91 ± 12.47	23.48 ± 7.65	**62.45**	**0.001**	**0.001**	**0.001**	**0.000**

Discussion

In this case control study there are three groups, healthy controls, DM without CAD and DM with CAD group. These three groups were homogenous in terms of age, gender, smoking and family history of CAD. Male participants were more than the female participants. Duration of diabetes was 6.8 ± 2.3 years in DM without CAD group and 7.3 ± 3.2 years in DM with CAD group. This shows DM patients with CAD had DM for longer duration compared to DM patients without CAD. All sixty five patients of the DM without CAD group were on oral hypoglycemic drugs. Among 65 patients in DM with CAD group, sixty were on oral hypoglycemic drugs and only five patients were on insulin. (Table-5.3)

6.1 - Comparison of demographic characteristics in all the three groups

Mean age was 56.12±14.63 in healthy controls, 55.97±14.82 in DM patients without CAD and 61.15±12.51 years in DM with CAD patients. In the present study, highest occurrence of CAD (29.2%) was reported in the age group of 51 to 60 years and followed by 61 to 70 years age group around 29%. An INTERHEART study by Yusuf S et al[1] reported first presentation of acute myocardial infarction (MI) in the south Asians was at the age of 53 years, while in western population it was at the age of 63 years. A study conducted by Deepak Kumar et al[2], in Haryana, mean age was 50 years which is lesser than mean age of present study. CAD strikes the Indian population early at younger age and kills in their productive years. Epidemic transition in lifestyle, urbanization, mechanization and environmental factors may be some of the reasons for early presentation of CAD[3].

Body mass index (BMI) in Kg/m^2 was 24.73±1.25, 26.47±2.18 and 26.37±3.43 in healthy controls, DM without CAD and DM with CAD patients respectively (Table-5.4). There was statistically significant difference ($p<0.001$) in mean arterial pressure (MAP), fasting

blood glucose (FBG) and HbA_1c among the three groups and all three parameters were significantly increased in study groups compared to healthy controls. DM with CAD group had significant increase in BMI, FBG and HbA_1c compared to remaining other two groups. Mean arterial pressure (MAP) was more in DM with CAD patients i.e 108.03± 6.89 mm of Hg compared to healthy controls which was 87.6 ± 6.73 mm of Hg (Table-5.4).

Elevated FBG and HbA_1c were observed in DM without CAD and DM with CAD patients compared to healthy controls. Since both groups were known diabetic patients obviously their fasting glucose as well as HbA_1c was raised above the WHO cut-off values. Similar findings were documented by the previous studies[4-5].

6.2 – Lipid profile among the three groups

On assessing the lipid profile in the participants, we observed significant difference in TGs and HDL-C levels between healthy controls, DM without CAD patients and DM with CAD patients. There were increased TGs and decreased HDL-C levels. This shows that dyslipidemia is prevalent in diabetic patients. Our findings are in accordance with previous study by Mohan V et al[4]. Even though the TC and LDL-C levels increased in type-2 DM with and without CAD groups it was not statistically significant when compared with healthy controls as shown in table 5.5.

In the present study we observed significant increase in TGs i.e 20.5% in DM without CAD patients and 85.5% in DM with CAD patients when compared to healthy controls as shown in figure 5.3. A study in 2013 by Ashfaq F et al[5], states that TG levels more than 150 mg/dL had positive predictive value of 70% to identify the individuals at risk for insulin resistance and CAD. Similar results were observed by previous studies [6,7]. In type-2 DM, high TGs tend to coexist with low HDL-C levels[8]. Framingham Heart Study report

states increased prevalence of hypertriglyceridemia, low HDL-C levels but TC and LDL-C remain same as that of non-diabetic counterparts[9]. Similar type of altered lipid profile pattern was reported by UK Prospective Diabetes Study (UKPDS)[10]. In DM with CAD patients there were increased TGs and decreased HDL levels when compared to healthy controls. This could be due to reduced lipoprotein lipase function in damaged endothelial vessels in DM patients. A large cross-sectional study conducted in north India by Ashfaq F et al[5] found increased TG and very low HDL-C levels in DM with CAD patients as compared to those with normal coronary status. Low levels of HDL-C are reported to increase the risk of cardiovascular disease even when total cholesterol levels are within normal range[11,12]. HDL-C levels reduced more in DM with CAD patients compared to other groups. HDL-C levels reduced 18.7% in DM with CAD patients and 8.5% in DM without CAD patients compared to healthy controls as shown in figure 5.3. TGs try to change the composition of LDL-C and convert them into small, dense and highly atherogenic particles[13]. An eight year follow-up study on male patients revealed that estimation of TGs gives an indirect picture of measurement of LDL-C size. An increase in TGs from 90 to 180 mg/dL doubles the risk of incidence of CAD[14]. Low HDL-C levels are an independent risk factor for CAD[15]. An epidemiologic study by Austin M et al[16] in 1999 documented that hypertriglyceridemia is usually associated with CAD patients.

In current strategy of global risk assessment, simple blood investigations like lipid parameters are routinely recommended. Alone with lipid profile estimation, we cannot completely explain the increasing burden of the CAD in DM patients. Thus, estimation of other newer parameter like Lp(a) may throw light and have potential to improve cardiovascular risk prediction when used along with traditional lipid profile.

6.3 – Lipoprotein (a) among the three groups

In the current study we evaluated the significance of the novel biomarker Lp(a) in DM and CAD patients. Changes in Lp(a) levels in relation to DM and CAD disease have shown discordant results. Studies by Enas EA et al[17] in 2006, Geethanjali FS et al[18] and Fayyaz I et al[19] in 2009 reported increased Lp(a) levels , Haffner[20] and Ding L[21] showed decreased Lp(a) levels, and still few reports also commented no change [22,23] in Lp(a) levels at all. Hence, the present study was undertaken to estimate the Lp(a) levels and also to find the best cut-off value to predict CAD in type-2 DM patients in and around Bagalkot population.

In our study we did not find change in the Lp(a) levels in the different age groups among study groups. Lp(a) attains normal level at the age of two and remains constant in the circulation throughout life. We noticed female DM patients with and without CAD group to have higher Lp(a) levels compared to males but it was not statistically significant as shown in table 5.7. Increase in Lp(a) levels in females could be due to hormonal regulation. Estrogen acts on responsive element in LPA promoter gene and reduce the Lp(a) levels. In postmenopausal women, decreased estrogen leads to increased Lp(a) levels[24]. As mean age of our study group was between 55 to 60 years, this may be true in our case. Jenner JL mentioned, post menopausal women on hormone replacement therapy i.e on estrogen observed decreased serum Lp(a) levels[25]. In our study none of the women were on hormone replacement therapy. Henrikson et al[26] reported higher Lp(a) levels in females compared to males which may be due to lowering effect of testosterone in males. In the year 2007, Pedreno J et al[27] observed no change in Lp(a) levels among males and females.

In the present study, even though increased Lp(a) levels were observed in one with positive family history of CAD compared to one with negative family history, there was no significant difference among them in both the groups since the number of positive family history cases were very few compared to negative family history. Our findings are in accordance with Von Eckardstein A et al[11]. They found that elevated Lp(a) increases the coronary risk even in patients without family history. Due to small sample size they were unable to make a conclusion on role of Lp(a) on cardiovascular risk in patients with positive family history for myocardial infarction. An Iranian study by Nila AF et al[28] in 2007, observed elevated Lp(a) levels in children of patients with premature MI. Few other previous studies also had similar findings with significant higher Lp(a) levels in off-springs of parents with past history of premature MI compared to healthy controls[29-31]. There was no significant change in Lp(a) levels in smokers and non-smokers. There was no much significant difference in the mean Lp(a) levels with change in BMI, HbA_1c, TC, TG and LDL-C in both DM without CAD patients and DM with CAD patients showed in table 5.7.

We also observed highly significant difference in serum Lp(a) levels between healthy controls verses DM without CAD patients and DM with CAD patients. Our findings are in accordance with other studies [32-34]. Studies by Mohan V et al[4], Gazzaruso C et al[33] in 2002, found an independent association of Lp(a) with CAD in type-2 DM patients. We observed there is increased Lp(a) levels in DM without CAD patients compared to healthy controls. There was 86.6% increase in Lp(a) levels in DM without CAD patients compared to healthy controls (figure 5.5). Similar findings were documented by previous researchers too[35,36]. The increased Lp(a) levels in DM subjects may be due to increased glycosylation of Lp(a) in the circulation[37,11]. Study by Gazzaruso C et al[38] in 2006 reported that estimation of Lp(a) and apo (a) phenotypes may be used as better predictor of CAD and

also reliable predictor of CAD severity in DM patients. But, Haffner et al[39] in his study observed slightly lower Lp(a) levels in DM patients compared to non-diabetic subjects but it was not significant. Recently Lp(a) is emerging as a strong biomarker of CAD. We found higher Lp(a) levels in DM with CAD patients, our results are in agreement with other studies[1-3]. A south Indian study by Rajashekhar D[29] et al in 2004, reported significant difference in Lp(a) levels in coronary artery diseased vessels compared to normal coronaries. In 2014, north Indian studies by Yusuf J et al[40] and Gupta et al[41] showed high Lp(a) levels in DM with CAD patients compared to controls. CAD patients showed almost five fold higher Lp(a) levels when compared to controls. Smooth muscle cells of the vessel wall undergo proliferation due to the influence of Lp(a) which is present in atherosclerotic plaque[42]. There is impaired fibrinolysis because of Lp(a), which competitively inhibits plasminogen. Smaller the apo(a), higher will be Lp(a) levels, which are more atherogenic. It also enhances the oxidation of lipoproteins and foam cell formation. Oxidized phospholipids of the Lp(a) may be proatherogenic and are potentially taken up by extracellular matrix of the vessel wall[40]. Zampoulkis et al[43] in 2000, studied the relationship of excess Lp(a) with extent and severity of atherosclerosis in CAD patients.

We also correlated Lp(a) with other parameters to find the association between serum Lp(a) and lipid parameters, HbA_1c, hsCRP and NO. We applied Pearson's correlation coefficient analysis. In the present study we witnessed statistically significant ($p<0.05$) positive correlation of Lp(a) with HbA_1c and hsCRP in DM without CAD patients whereas negative correlation seen with NO which was also statistically significant (Table-5.8 and Figure 5.7 to 5.11) ($p<0.01$ and $r= -0.31$). In DM without CAD patients, we observed positive correlation between Lp(a) and HbA_1c ($r=0.372$, $p= 0.002$). Association between Lp(a) and glycemic status still remains controversial. An Arab study in 2001 on pediatric

patients by Alsaedi M et al[44], reported elevated Lp(a) levels in children with poor glycemic control compared to good glycemic controlled children. Many studies did not show any association between Lp(a) and HbA_1c,[7,8,45]. Even we also did not find association in DM with CAD patients. In our study we did not find any correlation between Lp(a) and lipid parameters in DM without CAD patients. In 2010 as per Ogbera AO et al[46] Lp(a) showed positive correlation with TG and LDL-C. Habib SS et al[36] and Heller et al[47] observed positive correlation between Lp(a) and TC and LDL. In DM with CAD patients we observed negative correlation between Lp(a) and HDL-C (r= -0.355, p=0.004) (Figure-5.11). A French study by Ben Hamda K et al[48] also reported the same (r= -0.13, p=0.03). A study by Cobbaert C et al[49], suggested that atherogenicity of Lp(a) was more marked in the presence of decreased HDL cholesterol levels. Our data showed negative correlation between Lp(a) and NO in DM without CAD patients. The increase of Lp(a) levels may be responsible for the impaired coronary endothelial function[50]. Oxidatively modified lipoproteins inhibit eNOS or inactivate the existing NO levels[51]. Hence Lp(a) is negatively correlated with NO in our study. A cross sectional study conducted in Nuremberg and Regensburg, in Germany by Schlaich MP et al[52] states that Lp(a) levels does not affect the NO levels.

Lp(a) levels have shown worldwide ethnic variation, with different levels associated with CAD in different population [53,54]. In the present study, we made an attempt to predict the CAD in type-2 DM patients in our study population by drawing ROC (receiver operating curve). Our findings showed cut-off value for Lp(a) was 18.3 mg/dL in DM without CAD patients with 81.5% sensitivity and 81.5% specificity, area under the receiver operating curve (AUROC) was 0.8 as shown in table 5.9 and figure 5.12. In DM with CAD patients, Lp(a) cut-off value was found to be 21.6 mg/dL with 93.8% sensitivity and 92.3%

specificity and AUROC was 0.9 (Table-5.10 and Figure-5.13) which suggests that, estimation of Lp(a) can be used to find out the risk of CAD in DM patients. Table 6.1 shows few Indian and western studies with their Lp(a) cut-off values. A north Indian study in 2014 by Yusuf J et al[40] reports Lp(a) levels above 40 mg/dL assessed by an isoform insensitive assay was an independent risk factor for CAD. A cross sectional study conducted by Gladder et al[55], said Lp(a) levels above 30 mg/dL independently predicts the CAD mortality. Nearing to our Lp(a) cut-off value, study by Hoogeveen RC et al[56] had proposed a cut off value above 19 mg/dL to be associated with risk of CAD in 103 DM with CAD subjects. Saini V et al[57] showed Lp(a) to be an independent predictor of CAD irrespective of traditional risk factors with relatively good sensitivity and specificity. The best cut-off value of Lp(a) came out to be 45 mg/dL in her study conducted in 2013 in north Indian population. Even other studies supported that, Lp(a) level exceeding 30 mg/dL indicates increased risk of CAD by about three folds[58-60].

Table-6.1: Lp(a) cut-off value in different studies compared with the present study

Author	**Year**	**Lp(a) mg/dL cut-off levels**	**Study area**
Present study	2019	21.6	Karnataka
Rajashekhar D, et al[29]	2004	25	Andhrapradesh
Yusuf J et al[40]	2014	40	New Delhi
Fauzia Ashfaq et al[5]	2013	21.0	Lucknow
Vandana Saini et al[57]	2013	45	New Delhi
Sharma A et al[54]	2012	20	Deharadun
Maranhao R et al[59]	2011	30	North India
Hoogevean RC et al[56]	2001	19	North India
Erbagci AB et al[60]	2002	22.6 –Women 9.8 –Men	Turkey
Gladder et al[55]	2002	30	Sweden

In addition to above findings, logistic regression analysis was also carried out to calculate the odds ratio (OR) for Lp(a) to predict the CAD in type-2 DM patients in our population. Lp(a) was found with positive effect on the chance of developing CAD in type-2 DM patients. Odds ratio for Lp(a) in DM without CAD was found to be 1.3 and was 2.82 in DM with CAD patients. From this, we could state that with each unit increase in Lp(a) levels, the chance of developing CAD was 33% higher in DM without CAD patients with p value <0.001, while in the DM with CAD it was almost three times higher with p value less <0.002. To support our findings, study by Saini V et al[57], Yusuf J et al[40] and Mohan V

et al[4] documented the odds ratio for Lp(a) was 4.8, 1.7 and 1.5 respectively and concluded unit change in Lp(a) can have significant impact on occurrence of CAD. According to Gazzaruso C et al[33], odds ratio for Lp(a) was found to be 2.62 with 95% CI and showed independent predictor of asymptomatic CAD.

We noted, raised Lp(a) levels to be associated with increased risk of CAD in type-2 DM patients. It is an independent risk factor for CAD in diabetic patients. Lp(a) cut-off level 18.3 mg/dL in DM without CAD patients and 21.6 mg/dL in DM with CAD patients predicts the risk of CAD in our population. From regression analysis findings Lp(a) was having positive effect on chance of developing CAD in DM patients with odds ratio (OR) 1.33 in DM without CAD patients and 2.82 in DM with CAD patients.

As earlier discussed, endothelium plays an very important role in maintaining the normal structure and tone of the blood vessels. In DM, endothelial dysfunction is a key factor and early indicator of vascular damage. To assess the vascular changes we measured the NO levels in our participants.

6.4 – Endothelial marker nitric oxide (NO) to assess vascular changes in the three groups

Cardiovascular risk is markedly increased in type-2 DM patients. DM increases the risk of endothelial dysfunction which is an early indicator of vascular diseases. This endothelial damage can be indirectly assessed by estimating the many clinical markers like nitric oxide (NO), endothelin-1 (ET-1), Von Willebrand factor (vWF), tissue plasminogen activator (t-PA), vascular cell adhesion molecules (VCAM) and high sensitive C - reactive protein (hsCRP) etc[61]. Among them estimation of NO which is released from endothelial cells will approximately measure the extent of endothelial dysfunction.

Endothelium is the main source for NO in blood vessels. Alteration in NO levels in diabetic patients changes endothelial function and leads to vascular complications[62]. In this study, NO level in controls was 43.01±10.1 μmol/L, DM without CAD patients had 36.92±12.47 μmol/L and DM with CAD patients had 23.49±7.66 μmol/L as shown in figure 5.14. There was statistical significant difference (ANOVA, $p<0.001$) in NO levels among the three groups. Acute exposure of endothelial cells to high glucose concentration results in significant blunting of NO[63]. In diabetic patients, hyperglycemia induces oxidative stress and accumulation of NADH and NADPH in the vascular intima[64]. This oxidative stress neutralizes antioxidants like glutathione and catalase[65]. We observed decreased NO levels in both the groups. Our findings are consistent with other studies like Amrita Ghosh et al[66] in 2011, Sanjeev Kumar[67] in 2016, Manju M et al[68] in 2014 and Tessari P et al[69] in 2010. A study by Sanjeev Kumar et al[67] in 2016,on 223 type-2 DM patients reported reduced NO levels in DM patients compared to controls and also commented that NO plays an important role in the pathogenesis of type-2 DM. A cross sectional study conducted in 2018 by Jayashri SJ et al[70], observed reduced NO levels in type-2 DM patients with microalbuminuria having duration of diabetes for more than

seven years. She concluded measurement of NO may be used as prognostic factor for predicting the onset of vascular complications. She also proposed, very soon estimation of NO levels may be diagnostic tool for assessment of endothelium function. Reduced NO levels in type-2 DM may be due to, hyperglycemic stimulation leads to advanced glycation end products (AGE) formation[71]. AGEs enhance polyol pathway and activates protein kinase C resulting in formation of reactive oxygen species (ROS) thus increasing oxidative stress. These ROS immediately reacts with NO, to form peroxynitrite which is highly cytotoxic. Peroxynitrite degrades the tetrahydrobiopterin (BH_4) which is one of the cofactor of eNOS leading to uncoupling of eNOS [72]. Thus there is reduced NO levels in DM. As per Rodriguez–Mannas[73], AGEs are important modulators of NO activity and impair endothelial function in poorly controlled DM patients. In 2010, an Italian study by Tessari P[69] reported that decreased NO synthesis due to reduced arginine levels in type-2 DM with nephropathy patients. Reduced NO availability leads to the development of atherosclerosis[74].

Few studies also reported increased NO levels in DM[75,76]. According to Ramu A et al[77], an experimental study in 2015, to find the effect of NO production after exposing the endothelial cells to hyperglycemia, human umbilical vein endothelial cells (HUVECs) are treated with high concentration of glucose. As per this experiment, he reported increased NO levels in HUVEC cells when exposed to high glucose at initial period but subsequently reduced NO production after exposure for longer duration. He also reported that increased NO levels in DM patients in the first five years, but reduced NO levels in DM patients who were having diabetes history of more than five years. This could be the reason for decreased NO levels in our DM patients as most of the diabetic patients participated in the present study were above six years.

We found decreased NO levels in DM with CAD patients. We observed 14.2% reduction in DM without CAD patients and 45.37% in DM with CAD patients compared to healthy controls (Figure-5.15). Endothelial dysfunction (ED) is an early change that contributes to the pathogenesis of CVD. ED is seen in coronary as well as peripheral circulation in diabetic patients [78]. In the year 2004, Davignon J et al[79] concluded ED is associated with microangiopathy and atherosclerosis in diabetic patients. Impaired endothelium dependent vasodilatation due to reduced availability of NO is the key role in the ED and play an important role in atherosclerosis. Studies reported intercellular adhesion molecule-1(ICAM-1), VCAM-1, vWF, E-selectin can also be used to assess the endothelial dysfunction[80]. Dyslipidemia, oxidative stress and inflammation are main mechanisms that decrease NO production by endothelial cells via uncoupling of eNOS activity or quenching NO by reactive oxygen species in DM patients was observed by Pittaco D et al[81] in the year 2013. Markedly reduced NO availability enhances initiation, progression and complications of atherosclerosis. A recent study in 2018 by Jing-Yi Chen et al, concluded that lack of NO bioavailability in the coronary and peripheral artery has been regarded to be result in prospective cardiovascular events[82].

We also found levels of serum NO to have significant negative correlation ($p<0.05$) with FBG, HbA1c, hsCRP and Lp(a) in DM without CAD patients. In DM with CAD patients NO shows significant negative correlation ($p<0.05$) with MAP. It shows that alteration in NO levels may have an impact on endothelium of blood vessel. Our study is in accordance with Manju M [68]. He noticed that, as the severity of DM increases there will be increased blood pressure due to reduced NO levels thus vasoconstriction. Hence NO and MAP are negatively correlated. Another study by Song-Tao AN et al[83] observed severity of CAD can be predicted by measuring central fractional systolic and diastolic pressure. Studies by Ghosh A et al[66], Manju M et al[68] and Johnstone MT et al[84] have shown negative

correlation between serum NO levels with glucose and HbA_1c levels which is concurrent with the present study . Poorly controlled DM patients (whose HbA_1c >7.5%) had decreased NO levels compared to good glycemic (whose HbA_1c <7.5%) controlled DM patients in our study. As per Leuiene de Carvalho Cardoso Weide et al[85] in 2014, lower NO levels were reported in type-2 DM patients with HbA_1c >9%. These patients have increased risk of vascular complications. In the year 2009, a study conducted in Karachi by Shahid SM[86], on DM patients also reported negative correlation between NO and serum glucose and HbA_1c. He suggested that HbA_1c can modulate NO metabolism and vice-versa. Even in DM with CAD patients we observed negative correlation between NO and HbA_1c.

There was no much difference in nitric oxide levels with change in age, gender, BMI, smokers, family history of CAD, TG, HDL and LDL levels in both DM without CAD patients and DM with CAD patients. NO levels decreased with increase in TC levels in DM with CAD patients (Table-5.12).

To predict the risk of CAD in DM patients regression analysis for NO was carried out. Results showed for every one unit increase in NO, the chance of developing CAD in DM without CAD patients decreases by 6%, whereas in DM with CAD it was 26% less chance of developing CAD shown in table 5.14. Overall, it shows there will be decreased NO levels in DM patients as well as DM with CAD patients. Poorly controlled diabetic patients have decreased NO levels and inflammation decreases the NO levels. NO had negative effect on the chance of developing CAD in DM patients.

6.5-Inflammatory marker hsCRP in the three groups

Inflammation plays very important role in the initiation and progression of atherosclerosis. Many inflammatory molecules like interleukin, tumor necrosis factors and hsCRP are studied to understand their role in DM and CAD. Among them hsCRP as identified has one of the vital marker in predicting the future CAD risk in DM patients. Our results showed significant rise in hsCRP levels in DM without CAD and DM with CAD patients compared to healthy controls (Figure-5.23). Our study is in accordance with other studies. As per Mirza Sharif AB et al[87] in 2013, hsCRP was much higher in DM patients with complications compared to other two groups, DM patients without complications and healthy controls. Hence, they concluded estimation of serum hsCRP appears to be useful marker in diabetic patients with complications and provide more valuable information for medical intervention. Other studies by Simin Liu et al[88], reported hsCRP levels significantly associated with age and positively related to insulin resistance, BMI, and blood pressure. It is also observed that hsCRP levels are the sensitive marker for inflammation. A study by Sanchez PL et al[89] in 2004, on type-2 DM patients with acute coronary syndrome showed raised hsCRP levels and seemed to be an independent predictor for cardiovascular death. From this we conclude that evaluation of hsCRP may have potential to improve cardiovascular risk prediction when used along with other traditional lipid profile. It was considered as single strongest predictor of CAD risk[90].

In DM without CAD patients as well as DM with CAD patients Lp(a) showed positive correlation with hsCRP (Table-5.8 and Figure 5.9). As the Lp(a) increases hsCRP levels also increased, which was significant ($p<0.001$). SS Habib et al[91] in 2011 in his study reported that diabetic patients have higher levels of Lp(a) and hsCRP. Diabetic patients

with poor glycemic status have significantly higher hsCRP levels compared to those with good glycemic control.

The present study showed negative correlation of NO with hsCRP (Table-5.13). As the inflammation increases hsCRP levels were increased and NO levels reduced, which was statistically significant in both DM without CAD and DM with CAD patients (Figur-5.21 and 5.22). Prasad K et al[92] in their study in 2015, showed increased AGEs in DM have pro-inflammatory effect and progress the atherosclerotic process. Oxidative stress exaggerates the inflammatory process by activating transcription factor NF-kB[93]. Reduced NO may stimulate the synthesis and release of endothelin, proinflammatory cytokines, proliferation and migration of smooth muscle cells. Thus progresses the atherosclerotic process. So reduced NO levels may stimulate the release of inflammatory marker i.e hsCRP[94]. Thus NO was negatively correlated with hsCRP, observed in tour study. In 2013, Libby P et al[95] also proposed, it may be due to opsonizing property of the hsCRP and recruitment of monocytes into atheromatous plaque and also inducing endothelial dysfunction by suppressing basal and induced NO release.

Thus, there is increased serum FBG, HbA_1c, TG, Lp(a), hsCRP levels and decreased HDL-C, NO levels in DM without CAD patients and DM with CAD patients compared to healthy controls.

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Summary

This is a hospital based, observational case-control study. Study comprised of 195 participants, 65 in each group i.e healthy controls, type-2 DM without CAD patients and type-2 DM with CAD patients. Serum FBG, lipid profile, Lp(a), NO, hsCRP and HbA_1c were estimated in all the participants.

In the present study, maximum occurrence of DM without CAD (23%) and DM with CAD (29%) was found in the age group of 51-70 years. Serum FBG, HbA_1c, TGs, VLDL, Lp(a) and hsCRP were significantly increased in DM without CAD patients and DM with CAD patients as compared to normal healthy controls. HDL-C and NO significantly decreased in DM without CAD patients and DM with CAD patients as compared to healthy controls. Raised serum Lp(a) level is associated with increased risk of CAD in DM patients. Lp(a) cut-off value above 18 mg/dL in DM without CAD and above 21.6 mg/dL in DM with CAD patients and AUROC more than 0.8, suggests that Lp(a) can be used to evaluate risk of CAD in DM patients. Lp(a) was found with positive effect on the chance of developing CAD in DM patients. With unit increase in Lp(a) level, the chance of CAD was 1.3 times higher in DM without CAD patients, while in the DM with CAD patients it was 2.82 times higher compared to healthy controls.

Decreased NO levels may be a potential contributor to the pathogenesis of early vascular changes in DM without CAD patients and DM with CAD patients. Hence it can be used as marker of endothelial dysfunction. Poorly controlled diabetic patients have decreased NO levels. As the inflammation increases NO level decreases. NO had negative effect on the chance of developing CAD in diabetic patients.

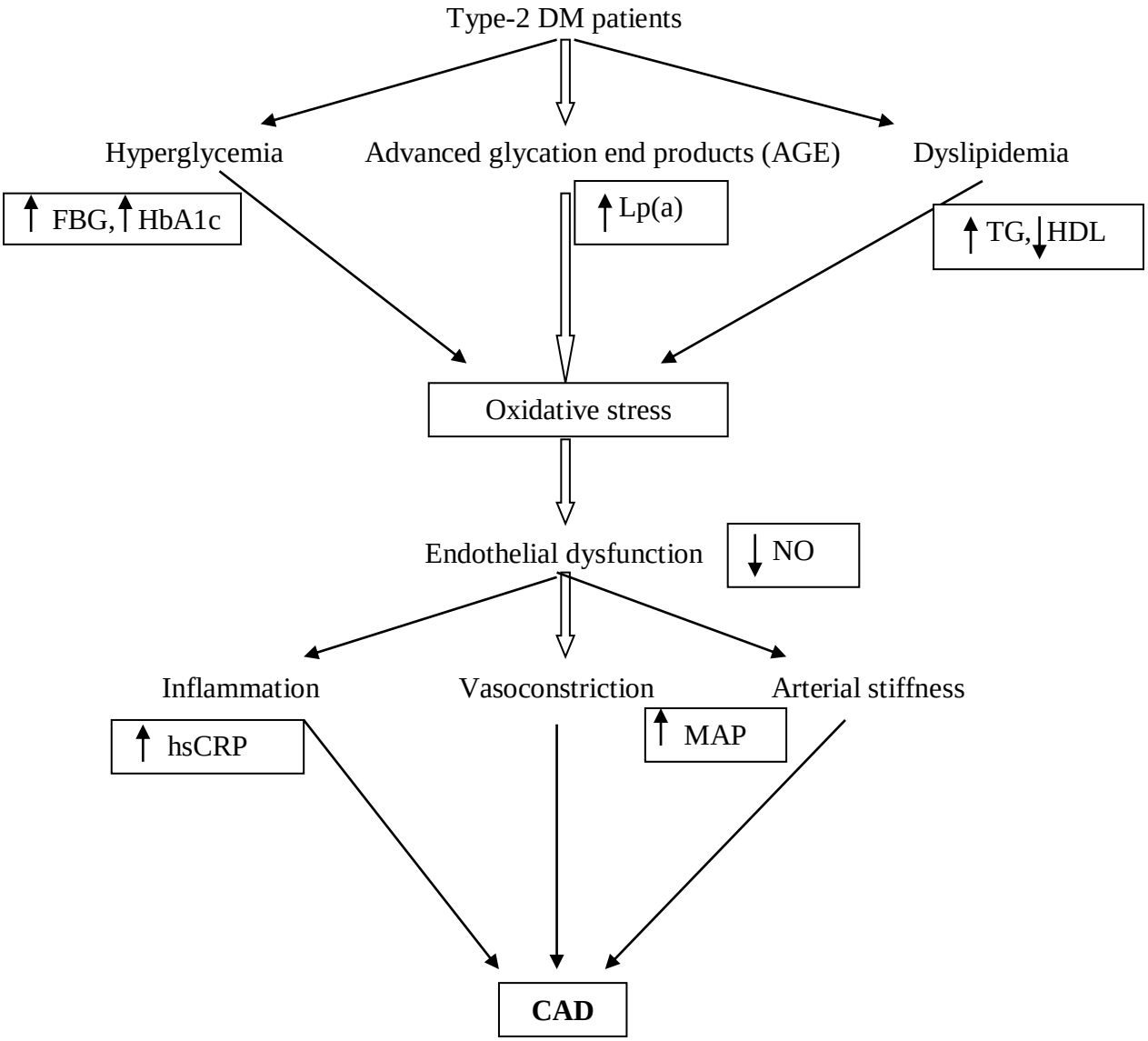

Figure-7.1: Graphical presentation of the study

Conclusion

There was significant increase in serum FBG, HbA_1c, TG, Lp(a) and hsCRP and significant decrease in NO and HDL-C levels in DM without CAD patients, DM with CAD patients compared to healthy controls. Lp(a) was positively correlated with hsCRP and NO was negatively correlated with HbA_1c and hsCRP in both DM without CAD patients and DM with CAD patients. Lp(a) level above 18.3 mg/dL in DM without CAD patients and above 21.6 mg/dL in DM with CAD patients predicts the risk of CAD. Odds ratio showed Lp(a) was found with positive effect on the chance of developing CAD in DM patients. With unit increase in Lp(a) levels, the chance of CAD was 1.33 times higher in DM without CAD patients, while in the DM with CAD patients it was 2.82 times higher compared to healthy controls. NO was found with negative effect on the chance of developing CAD in DM patients. With unit increase in NO levels, the chance of developing CAD in DM without CAD patients was 6% less whereas in DM with CAD patients it was 26% less.

Elevated Lp(a) levels is associated with increased risk of CAD, hence it can be used as early marker of CAD in type-2 DM patients. Thus estimation of Lp(a), NO and hsCRP serve as markers of CAD in type-2 DM patients where lipid profile was within normal range. Thus, these tests should be included in panel of investigations for early diagnosis of CAD in DM patients.

Limitation

1. Small sample size.
2. Large cross sectional study can be carried out.
3. Molecular study for nitric oxide synthase (NOS-3) can be done.
4. Studies of apo(a) isoform can be undertaken.

INFORMED CONSENT FORM

Study Title - "Study of dyslipidemia, glucose homeostasis and nitrosative stress in Diabetic patients with and without Coronary artery disease"

Study Number: _____

Subject's Full Name: _____

Date of Birth/Age: _____

Address:

1) I confirm that I have read the information in this form (or if has been read to me). I was free to ask any questions and they have been answered.

2) I have read and under stood this consent form and information provided to me.

3) I have been explained above the nature of the study.

4) I have been explained about duration of participation with number of participants.

5) I have been explained about procedures to be followed and about investigations, if any to be performed. I have been explained that I don't have to pay or bear the cost of procedure/investigations.

6) My rights and responsibilities have been explained to me by the investigators.

7) I have been adequately explained risks and discomforts associated with my participation in the study.

8) I have been explained about benefits of my participation in the study to myself, community and to medical profession.

9) If despite following the instructions I am physically harmed because of any substances or any procedures as stipulated in the study plan my treatment will be carried out free of cost at investigational site and the sponsor will bear all the expenses, If they are not covered by insurance agency or by Government program or any third party. I have had my questions, answered to my satisfaction

10) I have been explained about available alternative treatments.

11) I understand that my participation in the study is voluntary and I am free to withdraw at any time, without giving any reason and without my medical care or legal rights being affected.

12) I hereby give permission to the investigators to release information obtained from me as result

of participation in the study to the sponsors, representatives of sponsors, regulatory authorities, Government agencies & ethics committee. I understand that they may inspect my original records. However, I understand that my identity will not be revealed in any information released to third parties or published.

13) I agree not to restrict the use of any data or results that arise from the study provided such a use is only for scientific purpose (S).

I am exercising my free power of choice, hereby give my consent to be included as participant in the present study.

I agree to co-operate with investigator and I will inform him/her immediately.

By signing this consent form I attest that this document has been clearly explained to me and understood by me.

14. Contact details of Chairman of the IEC for appeal against violation of rights.
Dr. S.L. Hoti, Sc. F, Deputy Director, RMC (ICMR), Belgaum- 590010
Phone No. 0831-2477477
Fax. 0831-2475479

Signature of the Investigator – Dr. Kavitha M M

Department of Biochemistry, SNMC, Bagalkot.

Contact no - 9481137713

Signature (or Thumb impression) of the Subject/Legally Acceptable

Representative: _____

Signatory's Name_____ Date_____

Title: **Study of dyslipidemia, glucose homeostasis and nitosative stress in diabetic patients with and without coronary artery disease**

Name: DOA:

Age: DOE:

Sex: M / F OPD No:

Occupation: IPD No:

Income: Address:

Educational Status: Literate / Illiterate:

Clinical diagnosis:

Duration of DM - Years

Treatment: Regular / Irregular

Drugs / Insulin -

Chief complaints:

- Polyuria
- Polyphagia -
- Polydipsia
- Chest pain
- Weight loss
- Paresthesia

History of presenting illness:

CVS: - Breathlessness

- Angina
- Syncope
- Paroxysmal nocturnal dyspnoea
- Swelling of the feet

Renal symptoms:

- UTI symptoms
- Flank pain
- Fever with chills & rigors
- Puffiness of face
- Distention of abdomen

CNS: - Giddiness

- Loss of consciousness
- Altered sensorium
- TIA
- Stroke

Visual symptoms:

- Blurring of vision
- Sudden blindness
- Progressive loss of vision

Peripheral vascular disease: - Gangrene
- Claudication pain

PAST HISTORY:

1. Routine investigation
2. Other complications
3. Treatment – regular / irregular
4. Drugs used
5. Follow up – regular / irregular

FAMILY HISTORY:

- H/o Diabetes in family – Yes / No
- Family h/o HTN / IHD / CVA / Obesity / Sudden death etc

PERSONAL HISTORY:

Diet - Veg / Mixed

Appetite - Normal/Increased/Decreased

Bowel/Bladder – Regular/ Altered

Sleep – Sound / Disturbed

Menstrual history -

Smoking – Yes/ No

Alcoholic – Yes/ No

Other Habits -

Marital status – Married / Unmarried

GENERAL EXAMINATION:

Height: ……mts

Weight: ……...Kg

BMI:

Waist/Hip ratio

BP: ……mm of Hg

Pulse: …….beats/min

Pallor -

Icterus -

Clubbing -

Cyanosis -

Lymphadenopathy -

Oedema -

Febrile / Afebrile

SYSTEMIC EXAMINATION:

Cardiovascular system:

Inspection –

Palpation –

Auscultation -

Respiratory system:

Abdomen:

Central Nervous system:

INVESTIGATIONS:

1. Fasting blood glucose -
2. HbA1C -
3. Serum total cholesterol -
4. Serum triglycerides –
5. Serum LDL-C -
6. Serum HDL-C -
7. VLDL -
8. Lipoprotein (a), Lp(a) -
9. hsCRP -
10. Serum nitric oxide -
11. Other investigations

BLDE (DEEMED TO BE UNIVERSITY) Annexure -I

PLAGIARISM VERIFICATION CERTIFICATE

1. Name of the Student: Dr. Kavitha M M, Reg No: 12PHD003

2. Title of the Thesis: Study of dyslipidemia, glucose homeostasis and nitrosative stress in diabetes mellitus patients with and without coronary artery disease

3. Department: Biochemistry

4. Name of the Guide & Designation: Dr. Jeevan G Ambekar, Professor

5. Name of the Co Guide & Designation: Dr. S V Kashinakunti, Professor

The above thesis was verified for similarity detection. The report is as follows:

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The plagiarism report of the above thesis has been reviewed by the undersigned.

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The similarity index is above accepted norms, because of following reasons:

.....The thesis may be considered for submission to the University. The software report is attached.

Signature of the Guide **Signature of Co-Guide** **Signature of Student**

Name & Designation **Name & Designation**

Verified by (Signature)

Name & Designation

B.L.D.E. UNIVERSITY

(Declared vide notification No. F.9-37/2007-U.3 (A) Dated. 29-2-2008 of the MHRD, Government of India under Section 3 of the UGC Act,1956)

The Constituent College

SHRI. B. M. PATIL MEDICAL COLLEGE, HOSPITAL AND RESEARCH CENTRE

IEC Ref No-119/2015-16 April 10, 2015.

INSTITUTIONAL ETHICAL CLEARANCE CERTIFICATE

The Ethical Committee of this University met on 16th March 2015 at 11 AM to scrutinize the Synopsis / Research projects of Postgraduate student / Undergraduate student / Faculty members of this University / college from ethical clearance point of view. After scrutiny the following original / corrected & revised version synopsis of the Thesis / Research project has been accorded Ethical Clearance.

Title "Study of dyslipidemia, glucose homeostasis and nitrosative stress in diabetic patients with and without Coronary artery disease"

Name of Ph.D./ P. G. / U. G. Student / Faculty member. Miss. Kavitha M M. Department of Biochemistry.

Name of Guide : Dr. J.G.Ambekar. Professor Department of Biochemistry.

Dr. Sharada Metgud
Chairperson, I.E.C
BLDE University,
VIJAYAPUR – 586 103

Institutional Ethical Committee, BLDE University, BIJAPUR

Dr.G.V.Kulkarni
Secretary, I.E.C
BLDE University,
VIJAYAPUR – 586 103.

Note:-Kindly send Quarterly progress report to the Member Secreatary.

Member Secretary, Institutional Ethical Committee, BLDE University, VIJAYAPUR.

Following documents were placed before Ethical Committee for Scrutinization:

- Copy of Synopsis / Research project
- Copy of informed consent form
- Any other relevant documents.

B.V.V. Sangha's
S. Nijalingappa Medical College & Hanagal Shri Kumareshwar Hospital & Research Centre
Navanagar, Bagalkot-587102, Karnataka State, India.
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SNMC-INSTITUTIONAL ETHICS COMMITTEE ON HUMAN SUBJECTS RESEARCH
☎08354-235340 Fax: 08354-235360 Website: www.snmcbgk.in Email: iechsrsnmcbgk@gmail.com

Office of the Institutional Ethice Committee

Ref. No. :
File No: SNMC/IECHSR/2014-15/A-18-1.1

Date:
Date: 22/01/2015

Certificate of Ethical clearance

This is to certify that **Dr. Kavitha M M**, working as Assistant Professor in the Department of Biochemistry, S. Nijalingappa Medical College, Bagalkot-587102. The Institutional Ethics Committee on human subject research has decided to issue Ethical Clearance in its meeting on **07.01.2015** for the Research Protocol entitled "**Study of dyslipidemia, glucose homeostasis and nitrosative stress in Diabetic patients with and without Coronary artery disease(CAD)**" for her Ph.D. thesis. She is permitted to carryout the research work.

You are requested to report to the Ethics Committee the Following:

1. Progress of the study at the end of year.
2. Provide a report to the Ethics Committee on completion of the study.

The Ethical Committee of SNMC follows procedures that are in compliance with the requirements of ICH (international Conference on Harmonization) guidance related to GCP (Good Clinical Practice), schedule Y and all other applicable Indian regulations.

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SRatna. 22/1/15
(Dr. Sanjeev Ratna)
Member Secretary,
IEC
S.N. Medical College
BAGALKOT

CERTIFICATE OF PARTICIPATION

AMBICON 2014

XXII Annual National Conference of
Association of Medical Biochemists of India
Sri Venkateswara Institute of Medical Sciences, Tirupati
(A university established by an act of Andhra Pradesh Legislature)
14th - 16th November 2014

This is to certify that Dr KAVITHA HIREMATH has participated in the conference as delegate / faculty. He / She has participated in PG Quiz / PG debate / Faculty debate / Chaired a Session / Delivered an Oration / Lecture / Presented a paper (Platform / Poster) tilted,

"Kavitha MM, Kashinakunti SV, Ambekar JG, Nilima, Hiremath CS. Study of lipid profile and lipid indices in diabetes mellitus - A case control study."

Dr E. Ravindra Reddy Chairman AP Medical Council	**K. Satyanarayana Murthy** Registrar AP Medical Council	**Dr Jasbinder Kaur** President AMBI	**Dr P.V.L.N. Srinivasa Rao** Organizing Secretary AMBICON- 2014	**Dr B. Vengamma** Director cum Vice Chancellor SVIMS University

This conference has been allotted **5 credit hours** by Andhra Pradesh Medical Council (Vide letter no. APMC/CME/203/2014 Dated : 05-09-2014)

AMBKCCON-2015

RAJARAJESWARI MEDICAL COLLEGE & HOSPITAL

Bengaluru, Karnataka

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Department of Biochemistry

This is to Certify that Dr. **Kavitha M M** bearing Registration No **67381** registered with **Karnataka** Medical Council, has attended the Conference held on 11th & 12th Sept. 2015, as a Delegate/ Speaker / Chairperson/ Organizing Committee Member/ Chaired a session/ Presented a Paper /Postertitled **Serum lipoprotein(a) and lipid profile in Diabetes mellitus with and without coronary artery disease**

Dr. H.V. Shetty	Dr. S.M.R Usha	Dr. Prabhakara G.N	Dr. Shivakumar Veeraiah
Organizing Chairman	Organizing Secretary	Zonal Chairman. KMC	Dean

Karnataka Medical Council is granted Four credit hours for Delegates Vide letter No: K.M.C/C.M.E/ 583/2015 dated 25/07/2015).

Silver Jubilee Year

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CERTIFICATE

This is to Certify that

Dr. Kavitha Hiremath bearing MCI/State Reg.No. 67381.

Address S Nijalingappa Medical College, Bagalkot has participated as

Delegate/~~Speaker~~/~~Chairperson~~ and has presented an **Oral & Poster** presentation in the **"25th Annual Conference of Association of Medical Biochemists of India"** held from 17th to 19th November 2017. The Karnataka Medical Council has granted 5 Credit hours for delegates with Vide letter No.K.M.C./C.M.E./547/2017 dated 28-10-2017.

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Role of serum lipoprotein (a) in type 2 diabetes mellitus and its association with glycemic control

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Abstract

Diabetes mellitus (DM) is an established risk factor for cardiovascular disease. At present global risk assessment, lipid profile is only the blood test routinely recommended most commonly. Increased lipoprotein (a) [Lp(a)] contributing factor to accelerated development of macrovascular complications in DM. However Lp(a) and hsCRP evaluation may have the potential to improve cardiovascular risk prediction when used in addition to traditional lipid profile. Hence the study was undertaken to estimate the serum lipoprotein (a), lipid profile, lipid ratios and hsCRP in diabetic patients and their association with glycemic control. Within the diabetic patients we examined Lp(a) levels among controlled and uncontrolled diabetic individuals.

Materials and Methods: An observational study comprises of 150 participants. 75 were type 2 diabetic patients and 75 were healthy controls. Diabetic patients were further sub classified into good and poor glycemic control group depending on their HbA1c levels 7.5% as cut-off value. Fasting venous blood sample was collected and used for analysis of fasting glucose, lipoprotein (a), lipid profile, high sensitive C reactive protein (hsCRP) and glycosylated hemoglobin (HbA1c).

Results: A significant increase ($p<0.05$) in Lp(a), TG, LDL, hsCRP and Lipid ratios and significant decrease in HDL in diabetic patients compared to controls. Subjects with poor glycemic control subjects showed significant increase in FBS, HbA1c, TG and hsCRP. Even though, the Lp(a) increased in poor controlled diabetics, but it was not statistical significant. HbA1c shows positive correlation with FBS, TG, HDL, hsCRP and TG/HDL in diabetic patients.

Conclusion: The study helps in identification of at risk individuals for CAD beyond the routinely done lipid profile especially in insufficient resource situations. In our study we have higher levels of Lp(a), hsCRP and other atherogenic risk factors cluster more in diabetic patients compared to healthy controls. FBS, TG, hsCRP and TG/HDL are positively correlated with HbA1c. Thus Lp(a) and hsCRP evaluation may have the potential to improve cardiovascular risk prediction when used in addition to traditional lipid profile in diabetic patients. Poor glycemic control subjects have significantly higher hsCRP and TGs compared to good glycemic controls. There is no effect of glycemic control on Lp(a) levels, hence Lp(a) is independent risk factor for CAD in diabetic patients.

Keywords: Lipoprotein (a), Glycemic contol, Diabetes mellitus.

Introduction

Diabetes mellitus (DM) is a chronic non-communicable metabolic disorder and is also known as "Iceberg" disease. India has the largest number of diabetic people with 50.8 million and is said to be diabetes capital of the world.[1] Among non-communicable diseases diabetes is considered as high in morbidity and mortality rates. According to WHO reports diabetes will be the seventh cause of death by the end of 2030. As per study 50% of diabetics die due to cardiovascular disease. Diabetic subjects are known to have a two to four times increased risk of developing coronary artery disease (CAD) and CAD occurs two to three decades earlier in diabetic subjects as compared to nondiabetic subjects.[2] It was considered DM is an established risk factor for cardiovascular disease (CVD) and is considered to be CVD equivalent. Thus WHO in 2016 put an effort for sensitizing and bringing awareness about diabetes among people all over the world with theme "Beat Diabetes".

Abnormalities in lipid metabolism i.e dyslipidemia and lipoproteins are the established traditional risk factors for CAD. Elevated cholesterol (TC), triglyceride (TG), low density lipoprotein cholesterol (LDL-C) and low levels of high density lipoprotein cholesterol (HDL-C) are characteristic of typical dyslipidemia. Several intervention studies reveled reduction of serum TC and TG levels inturn reduction of mortality due to CAD.[3,4]

Lipoprotein (a) [Lp(a)] type of low density lipoprotein cholesterol (LDL-C) called little rascal. It was first identified by K Berg in 1963.[5] Lp(a) composed of an LDL-C like particle and apolipoprotein(a) which is covalently bound to apo B of LDL-C particle. Lp(a) is structural analogue and competitive inhibitor of plasminogen leading to impaired fibrinolysis. Lp(a) accumulates in the vessel wall; inhibits binding of plasminogen to cell surface. This inhibition also promotes proliferation of smooth muscle cells. Hence Lp(a) has atherogenic and thrombotic properties.[6] Studies reveal that diabetic patients are reported to have higher Lp(a) values than non diabetic controls and still higher values found in diabetics with complications.[7,8] Increased Lp(a) levels

confer genetic predisposition to CVD and may be related to accelerated atherogenesis in DM.

Recent data support that lipid ratios are more meaningful than individual lipid parameters. They help in early prevention and diagnosis of CHD than the individual serum lipids.[9]

High sensitivity C-reactive protein (hsCRP) is an acute phase protein, biomarker of inflammation and as an independent predictor for CAD. hsCRP levels increase with inflammation and insulin resistance. Measurement of hsCRP may be considered marker for better prediction of cardiovascular risk.[10]

Glycosylated hemoglobin (HbA1c) reflects the cumulative blood glucose over period of 8-10 weeks. It is an important indicator of long term glucose control and thus part of continuing care in diabetics.[11,12] Elevated HbA1c concentration has been considered an independent risk factor for coronary artery disease in diabetic patients.[13] It is reported that good glycemic control is associated with reduction in CVD.[14] Studies reveal that decrease Lp(a) levels with improved glycemic control in diabetic patients.[15]

There are very few and controversial data on Lp(a) in diabetics and its relationship with glycemic control in Indian population. Hence the present study was conducted to estimate the serum Lp(a) levels, lipid profile, lipid ratios and hsCRP in diabetic patients and to compare with healthy controls. Within the diabetic patients we examined Lp(a) levels among controlled and uncontrolled diabetic individuals by taking HbA1c 7.5% as cut-off value and also to see the relationship between Lp(a), lipid profile, lipid ratios and hsCRP with glycemic control.

Materials and Methods

This is a hospital based observational case-control study, conducted in the department of Biochemistry, S Nijalingappa Medical College and Hanagal Sri Kumareshwara Hospital Research Centre, Bagalkot, a teaching Centre in Karnataka, India. The study protocol was approved by institutional ethics committee (IEC). Study was conducted over a period of one year from January to December 2016. The study comprises of 150 subjects, 75 were clinically diagnosed diabetic patients attending the medical OPD and admitted patients. Age and sex matched 75 healthy controls of the age group between 30 to 60 years. Diabetes was diagnosed on the basis of WHO criteria (FBS > 126 mg/dL or PPBS > 200 mg/dL or HbA1c > 6.5%). Diabetic patients were further subdivided into two groups as good and poor glycemic control depending on HbA1c levels 7.5% as cut-off levels. Patients with thyroid disorders, nephrotic syndrome, chronic liver, renal diseases, diabetics with any complications and pregnant ladies were excluded from the study.

Informed written consent was taken from all participants. All care was taken to maintain the confidentiality of the patients as per Helsinki declaration. For each patient detail history was taken, general physical examination and anthropometric measurements like height and weight are recorded. Body mass index (BMI) was calculated using the formula Wt (Kg)/Ht (m).

Under aseptic precaution 5 mL of fasting venous blood was drawn. Out of this 2 mL was EDTA sample used for estimation of HbA1c by high performance liquid chromatography (HPLC) method in D-10 Bio-Rad instrument. Remaining 3 mL was allowed to clot and serum was separated. The serum was used for analysis of biochemical parameters like fasting blood glucose (FBS), lipid profile, lipoprotein (a) and hsCRP. Glucose was estimated by glucose oxidase and peroxidase method. TG's by glycerol phosphate method, HDL-C estimated by direct method. LDL-C was calculated by Friedewald's formula i.e LDL=TC-TG/5-HDL. Lp(a) and hsCRP was estimated by immunoturbidemetric method. All parameters were measured in fully automated analyser, Biosystem A25 using Biosystem kits. Lipid ratios were calculated i.e TC/HDL, LDL/HDL, and TG/HDL.

Statistical Analysis

Statistical analysis was carried out using software SPSS version 19. Quantitative data were expressed as mean±SD. Student 't' test was applied for comparison of two groups and Pearson's correlation test was applied for correlation. $p < 0.05$ is considered as statistically significant.

Results

Demographic characters of cases and control was shown in table 1. There was significant increase in BMI, FBS and HbA1c in cases compared to controls. There was no difference (p=0.16) in age among two groups.

Table 2 shows the comparison of lipid profile, Lp(a), hsCRP and lipid ratios between cases and controls. In the present study diabetic subjects have significantly higher ($p<0.05$) serum TG's, LDL-C, Lp(a) and hsCRP compared to controls. There was significant decrease in HDL-C levels in cases compared to controls. There was significant increase ($p<0.000$) in lipid ratios in diabetics compared to controls.

Comparison of lipid profile, Lp(a), hsCRP and lipid ratios among type 2 diabetic patients with good and poor glycemic control are depicted in table 3. Diabetic patients with poor glycemic control showed significant (p=0.0001) increase in FBS, HbA1c, TGs, and hsCRP compared to good glycemic control group. Although, the Lp(a) levels in poor glycemic patients are increased compared to good glycemic control subjects (29.8 ± 16.47, vs 23.69 ± 14.23) it was not statistical significant.

Pearson correlation of HbA1c with FBS, lipid profile, Lp(a), hsCRP and lipid ratios was shown in table 4. There was significant positive correlation between HbA1c with FBS (r = 0.38, p=0.001) Fig. 1, TGs (r= 0.234, p=0.04), HDL (r=0.277, p=0.01) hsCRP (r=0.780, p=0.0001) Fig. 2 and TG/HDL (r=0.14, p=0.001).

Table 1: Table showing clinical characteristics, FBS and HbA1c in controls and cases

	Control N=75	Cases N=75	t	p-value
Age in Year	52.74 ± 16.69	56.45 ± 16.3	1.39	0.16
BMI Kg/m^2	24.98 ± 1.41	26.34 ± 2.08	4.61	0.000**
FBS mg/dL	87.79 ± 17.37	165.73 ± 58.91	10.98	0.000**
HbA1c %	5.28 ± 0.61	8.51 ± 1.96	13.58	0.000**

** - Highly significant

Table 2: Table showing the lipid profile, Lp(a), hsCRP and lipid ratios in control and cases

Parameters	Control N=75	Cases N=75	t	P value
TC mg/dL	153.97 ± 35.1	163.49 ± 47.10	1.45	0.16
TGs mg/dL	112.32 ± 54.95	128.98 ± 41.26	2.09	0.03*
HDL-C mg/dL	39.2 ± 9.69	33.17 ± 9.88	3.71	0.002**
LDL-C mg/dL	92.3 ± 28.74	104.52 ± 42.1	2.07	0. 03*
Lp(a) mg/dL	17.20 ± 3.98	26.87 ± 15.63	5.19	0.001**
hsCRP mg/L	1.28 ± 0.37	3.93 ± 0.98	21.71	0.001**
TC/HDL	4.04 ± 0.95	5.22 ± 1.73	5.19	0.001**
LDL/HDL	2.43 ± 0.81	3.37 ± 1.51	4.75	0.001**
TG/HDL	3.01 ± 1.5	4.27 ± 1.84	4.11	0.001**

Table 3: Table showing the lipid parameters, Lp(a), hsCRP and lipid ratios in good and poor glycemic control diabetic patients

Parameters	HbA1c < 7.5 % N=36	HbA1c > 7.5% N=39	t value	P value
FBS mg/dL	136.81 ± 31.92	182.41 ± 65.46	4.2	0.0001 *
HbA1c %	6.98 ± 0.45	9.84 ± 1.65	13.4	0.0001 *
TC mg/dL	162.06 ± 48.31	160.78 ± 46.83	1.06	0.84
TG mg/dL	125.74 ± 32.83	137.36 ± 47.38	2.08	0.03 *
HDL-C mg/dL	35.83 ± 8.64	30.18 ± 9.68	1.25	0.49
LDL-C mg/dL	101.13 ± 61.03	103.2 ± 52.67	1.34	0.37
Lp(a) mg/dL	23.69 ± 14.23	29.8 ± 16.47	1.257	0.49
hsCRP mg/L	3.29 ± 0.56	4.57 ± 0.93	2.758	0.003 *
TC/HDL	4.52 ± 1.83	5.32 ± 1.4	1.653	0.1
LDL/HDL	2.82 ± 0.94	3.42 ± 1.0	1.403	0.3
TG/HDL	3.51 ± 1.29	4.51 ± 2.1	3.062	0.001**
* - Statistical significant, **- Highly significant				

Table 4: Correlation of HbA1c with other lipid parameters

	Parameters	r	p-value
HbA1c	FBS	0.380	0.001 *
	TC	0.182	0.114
	TG	0.234	0.043 *
	HDL-C	0.277	0.01*
	LDL-C	0.142	0.218
	hsCRP	0.780	0.000 *
	Lp(a)	0.125	0.287
	TC/HDL	0.019	0.873
	LDL/HDL	0.018	0.87
	TG/HDL	0.243	0.03*
* - Statistical significance			

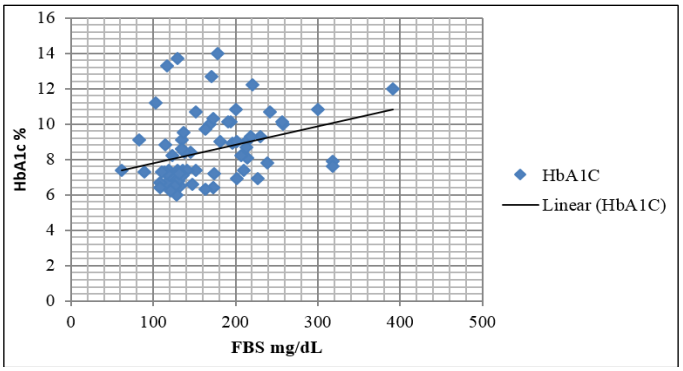

Fig. 1: Correlation graph of HbA1c with FBS in diabetic patients

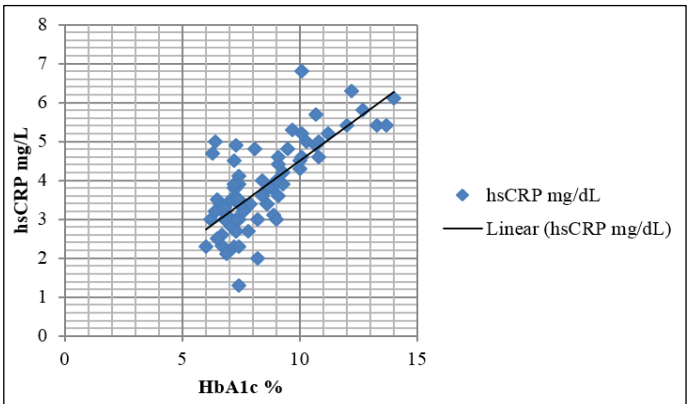

Fig. 2: Correlation graph of hsCRP with HbA1c in diabetic patients

Discussion

In our study FBS and HbA1c are significantly increased in diabetic patients compared to controls. Increased TG's, LDL-C and decreased HDL-C are well known risk factors for CAD in diabetic patients. In our study we did not find any difference in TC. High prevalence of dyslipidemia in subjects with type 2 diabetes mellitus, our study is in accordance with other studies.[16,17] In diabetic subjects, high TG levels tend to coexist with low HDL levels.[18,19]

Lp(a) is variant of LDL-C emerging as a strong biomarker of CAD. Our results show that diabetic patients have high Lp(a) levels compared to controls. The increased Lp(a) levels in diabetic subjects may be due to increased concentration of glycated Lp(a) in the circulation.[20,21] Glycosylation increases the half-life of lipoproteins i.e Lp(a). Reports say that Lp(a) levels above 20 mg/dL are associated with high risk of CAD.[22] A south Indian study on 300 participants, reports Lp(a) had an independent association with CAD in type 2 diabetes patients. An increase Lp(a) levels was found to be associated with increase in carotid intimal thickness. Hence, Lp(a) is associated with CAD even at an early stage of atherosclerosis.[23]

A cross sectional study by Anthonia OO[24] et al carried out on 200 Nigerian patients with type 2 DM, reported that elevated serum Lp(a) levels are higher in subjects with DM than in people without DM. It also showed positive association with most of the atherogenic profile parameters in type 2 DM individuals. A study by Gazzaruso C et al[25] reported that estimation of Lp(a) and apo (a) phenotypes may be used not only as predictor of CAD and also predictor of CAD severity in type 2 diabetic patients. A south Indian study by Mohan V et al,[26] reports that Lp(a) is an independent association with CAD in type-2 diabetic patients. Haffner et al[14] says contradictory statement that, slightly lower Lp(a) levels in diabetic patients than non diabetic subjects and there was no statistical significance.

Data support that lipid ratios have been reported more sensitive in reflecting the morbidity and severity of CHD than individual lipid parameters. Our study shows that there was significant increase in lipid ratios in diabetic patients compared to controls. Among the lipid ratios TG/HDL showed positive correlation with HbA1c. According to Shai et al [27] TC/HDL, LDL/HDL and apoB/apoA ratios are more susceptible to increased CVD mortality than individual lipid. According to Imran AS et al[28] atherogenic index (AI) can be used to indicate the presence of increased cardiovascular risk in patients with type 2 DM and as a guide for the aggressive therapeutic approach. The TG/HDL ratio is cheap and easy to calculate and is a good predictor of LDL size. In current strategies of global risk assessment, lipid profile is only the blood test routinely recommended most commonly in all setups. Calculation of lipid ratios is very easy and can be done

routinely from lipid profile parameters especially in Indian set up where new tests are not routinely possible because of cost factor.

We know that hsCRP is the inflammatory biomarker. In our study there was significant increase in hsCRP in diabetic patients compared to controls. Evaluation of hsCRP may have potential to improve cardiovascular risk prediction when used along with other traditional lipid profile. It was considered as single strongest predictor of risk.[29] In our study it was also increased in poorly controlled diabetic individuals compared to good glycemic diabetic individuals. It was positively correlated with HbA1c levels. Many trials and studies reveal that hsCRP is an independent predictor of future cardiovascular events in diabetes and significantly correlates with HbA1c. [30,31]

Hyperglycemia enhances glycosylation of proteins particularly lipoproteins. Glycosylation of LDL has been shown to enhance its susceptibility to oxidation, which triggers the atherosclerotic processes. In present study subjects with poor glycemic control showed significantly higher levels of FBS, HbA1c and TGs compared to good control diabetics. Dyslipidemia increases with increasing blood glucose levels and thus cardiovascular risk would be high with poor glycemic control.[32] Data revels 25% reduction in microvascular complications with improved blood glucose control in type 2 diabetics was demonstrated in United Kingdom Prospective Diabetic Study (UKPDS).[33]

In our study there is relatively higher Lp(a) levels in poorly controlled glycemic diabetics compared to good controls but it is not statistically significant. SS Habib [34] in his study reported that diabetic patients have higher levels of Lp(a) and hsCRP. Diabetic patients with poor glycemic subjects have significantly higher hsCRP levels compared to those with good glycemic control and there is no effect of glycemic control on Lp(a) levels. Our results are in accordance with few other studies.[35,36] Poorly controlled diabetes mellitus is associated with high Lp(a) levels and this metabolic abnormality contributes to elevated coronary risk in diabetic individuals. According to few hypotheses there is defect in the clearance of the apoprotein B-100 in diabetic individuals. Few say decrease in LDL-C in cellular metabolism in diabetes mellitus is proposed to be due to glycation of LDL particle and LDL receptors.[35] In our study there is no significant association of Lp(a) with glycemic control which is in accordance with Smaoui et al.[37]

In our study there was significant positive correlation of HbA1c with FBS, TGs hsCRP and TG/HDL which are significantly associated with CVD risk factors. The findings by Habib[30] and Heller [7] showed that positive correlation of Lp(a) with TC and LDL-C but not with TGs and HDL-C. Study by Elizabath et al[38] LDL and HDL cholesterol significantly associated with HbA1c. HDL-C was inversely associated with HbA1c and LDL-C positively associated with HbA1c in diabetic patients.

Conclusion

Increased levels of Lp(a), hsCRP and dyslipidemia in diabetic patients compared to healthy controls. FBS, TG, HDL-C and hsCRP are positively correlated with HbA1c. Thus Lp(a) and hsCRP evaluation may help in early cardiovascular risk prediction when used in addition to other traditional lipid parameters in diabetic patients. Poor glycemic control subjects have significantly higher hsCRP and TGs compared to good glycemic controls. There is no effect of glycemic control on Lp(a) levels, hence Lp(a) is an independent risk factor for CAD in diabetic patients.

Limitation of the Study: Small sample size.

Further Scope of the Study: Genetic factor should be taken into consideration while estimating the Lp(a) levels.

Conflict of Interest: There is no conflict of interest among the authors.

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ORIGINAL ARTICLE

Correlation of Serum Nitric Oxide, High Sensitivity C-reactive Protein and Lipid Parameters in Diabetics with and without Coronary Artery Disease

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Abstract:

Background: Coronary Artery Disease (CAD) and Diabetes Mellitus (DM) top the list among non-communicable diseases. Nitric Oxide (NO) preserves normal vascular physiology. Uncoupling of endothelial nitric oxide synthase enzyme occurs in the blood vessels of diabetics leading to endothelial dysfunction and excessive production of superoxide anion causing decreased bioavailability of NO. *Aim and Objectives:* To assess the serum NO levels, high-sensitive C-reactive Protein (hsCRP), lipid parameters and their association with CAD in diabetics. *Material and Methods:* The study comprises total 195 participants. There are three groups, each group consist of 65 participants. Three groups were diabetes with CAD, diabetes without CAD and control. NO assessed by modified Griess method. hsCRP by immunoturburdimetric method FBS and lipid parameters were analysed in fully automated analyzer. *Results:* There was a significant decrease in NO levels and significant increase in hsCRP levels in diabetes without CAD and diabetes with CAD patients compared to controls. NO showed negative correlation with Fasting Blood Sugar (FBS) and hsCRP in DM without CAD patients. NO showed negative correlation with DBP in DM with CAD patients. NO showed negative correlation with HbA1c in both the groups. *Conclusion:* The study concludes that estimation of NO and hsCRP along with lipid profile, help in early detection of endothelial dysfunction in diabetese patients. Reduced NO and increased hsCRP levels in diabetese patients may be strong indicator of coronary artery disease.

Keywords: Nitric oxide, high-sensitive C-reactive protein, Diabetes mellitus, Coronary artery disease

Introduction:

Cardiovascular Disease (CVD) is the leading cause of death and disability in developing countries [1]. In India three million deaths per year are due to cardiovascular diseases [2]. Coronary Artery Disease (CAD) and Diabetes Mellitus (DM), top the list among non-communicable diseases. Increasing incidence of morbidity and mortality due to cardiovascular complications including CAD has been observed in DM. The risk of CAD is two to four times higher in diabetic subjects. CAD strikes Indians at younger age that is in their productive age group. DM and CAD share several common risk factors like age, dyslipidemia, obesity, life style, environmental & genetic factors, hypertension and stress [3]. According to National Cholesterol Education Program (NCEP) guidelines, diabetes has been considered as cardiovascular risk equivalent [4]. Complications of DM include macrovascular i.e CAD, peripheral vascular disease, stroke and microvascular complications include retinopathy, nephropathy and neuropathy.

Among all these, endothelial dysfunction is more common [5]. Endothelium secretes many substances like Nitric Oxide (NO), endothelin-1. Impairment of endothelial function is the early feature of cardiovascular disease in DM [6].

NO is an endogenous gaseous molecule secreted by endothelium by a family of Nitric Oxide Synthase (NOS) enzymes. It is a key signaling messenger in the cardiovascular system [7]. It is having very short half life of few seconds, with a potent vasodilator and endothelial relaxing factor. NO produced in endothelium controls vascular tone and permeability, maintains vascular integrity by inhibiting the platelet aggregation, leukocyte endothelium adhesion and vascular smooth muscle proliferation [8]. Diabetes mellitus, atherosclerosis, hypertension, stroke and congestive heart failure have been linked to abnormalities in NO signaling [9, 10]. Adequate levels of NO preserve normal vascular physiology. Uncoupling of endothelial nitric oxide synthase enzyme occurs in the blood vessels of diabeteses leading to endothelial dysfunction and excessive production of superoxide anion causing decreased bioavailability of NO [11].

Pro-inflammatory markers like Tumor Necrosis Factor alpha (TNF-a), CRP and interleukin-6 are strongly associated with development of CAD. C-reactive protein (CRP) is a biomarker of inflammation due to acute-phase response. High-sensitive C-reactive protein (hsCRP) is considered as an important, powerful predictor of future cardiovascular disease [12]. Measurement of hsCRP helps to quantify low grade inflammation, in the absence of overt systemic inflammation and immunologic disorders. Asian Indians were shown to have elevated CRP levels, suggesting that pro-inflammatory factors may contribute to increased risk for diabetes and CAD [13].

There is paucity as well as controversial data were reported on NO levels in DM and CAD patients. Hence the present study was undertaken to assess the serum NO levels, hsCRP, lipid parameters and their association with CAD in diabetes.

Material and Methods:

Study design:

The research work was carried out in the department of Biochemistry, S Nijalingappa Medical College and Hanagal Sri Kumareshwara Hospital Research centre, Bagalkot, Karnataka. It was hospital based observational study. Study was conducted for two year 2015-16. The study protocol was approved by the Institutional Human Ethics Committee (SNMC/IECHSR/2014-15/A-18-1.1). Informed consent was taken from all patients and healthy controls at the beginning of the study.

Sample size:

Sample size was calculated by using the software, at 95% confidence interval and 80% power, it was calculated by taking into consideration of mean values from study by [14]. Study comprises total 195 participants, among them 65 were diabetes with coronary artery disease patients. CAD was documented on the basis of angiographically confirmed cases diagnosed by cardiologists i.e stenosis in major vessels, ECG findings, cardiac markers, previous medical records. DM was confirmed by WHO criteria (FBG >126 mg/dL or PPBG >200 mg/dL or HbA1c >6.5%). Sixty five were diabetese patients without CAD. Age and sex matched 65 healthy controls were taken who were free of any clinical manifestations i.e., diabetes, hypertension and cardiac disease. Smokers without any cardiac event were taken as controls.

Smokers with cardiac event were taken as cases. Patients with chronic liver and kidney diseases, nephrotic syndrome, thyroid disorders, pregnant women, cancer, patients on OCP's steroids and statins were excluded from the present study.

Methodology:

In all the patients, detailed history was taken, anthropometric characteristics like height, weight, blood pressure were noted and Body Mass Index (BMI) was calculated. Six mL of fasting venous blood sample was collected from all subjects by venipuncture. Two mL of EDTA sample used for estimation of HbA1c, determined by High Performance Liquid Chromatography (HPLC) principle, using Bio-Rad D-10 instrument. Remaining 4mL plain blood was allowed to clot and serum was separated. The blood was centrifuged at 3000 revolution per minute for 10 minutes. Serum was used for analysis of nitric oxide, hsCRP, lipid profile and Fasting Blood Glucose (FBG). Serum nitric oxide was estimated by Griess method. FBG by Glucose oxidase and peroxidase (GOD-POD) method, Total Cholesterol (TC) by cholesterol oxidase-peroxidase method, Triglycerides (TG) by glycerol phosphate oxidase-peroxidase method and High Density Lipoprotein-cholesterol (HDL-c) by direct method were assessed. Low Density Lipoprotein-cholesterol (LDL-c) was calculated by using Friedwald's formula. hsCRP by immunoturbidemetric method. All these were analysed in fully automated analyzer in Biosystem A-25 using commercially available kits.

Statistical analysis:

Quantitative data was expressed Mean ± SD. Comparison of three groups by Analysis of Variance (ANOVA) test and followed by *Post-hoc* multiple comparison tests. Correlation of NO with other parameters was done by Pearson correlation. The data was analyzed by using software SPSS version 17. $p < 0.05$ was considered as statistical significant.

Results:

Baseline characters of the study group are shown in table-1. There was no difference in the percentage of smokers and family history of CAD in different groups. The study groups were age and sex matched. There was significant increase ($p<0.001$) in blood pressure, BMI, fasting glucose and HbA1c in cases compared to controls shown in table-2. Diabeteses with CAD had significant increase ($p<0.001$) in systolic as well diastolic blood pressure compared to controls.

Table 1: Baseline Characters in the Different Groups

Parameters	Control (n=65)	DM without CAD (n=65)	DM with CAD (n=65)
Male: Female	37:28	38:27	42:23
Smoking (%)	13 (20%)	12 (18.76%)	12 (18.76%)
Family history of CAD (%)	8 (12%)	9(13.84%)	13 (20%)
Duration of DM in year	-	6.8 ± 2.3	8.3 ± 3.2

CAD = Coronary Artery Disease, DM = Diabetes Mellitus

Table 2: Anthropometric and Biochemical Parameters in Different Groups

Parameters	Control (n=65)	DM without CAD (n=65)	DM with CAD (n=65)	F	p- value
Age in Year	056.12 ± 14.62	056.00 ± 15.60	061.09 ± 14.72	2.87	0.06
SBP (mm of Hg)	112.00 ± 10.00	124.00 ± 10.00*	136.00 ± 10.00*#	70.939	0.00
DBP (mm of Hg)	078.00 ± 06.00	084.00 ± 08.00	094.00 ± 06.00*#	68.128	0.00
BMI (Kg/m^2)	023.73 ± 01.25	026.47 ± 02.18*	026.37 ± 03.43*	10.27	0.00
FBG (mg/dL)	081.49 ± 16.08	158.20 ± 44.03*	162.47 ± 48.93*	88.10	0.00
HbA1c (%)	005.10 ± 0.50	008.21 ± 01.57*	008.90 ± 01.75*	131.66	0.00

DM = Diabetes mellitus, CAD = Coronary artery disease, SBP = Systolic blood pressure, DBP = Diastolic blood pressure, BMI = Body mass index, FBG = Fasting blood glucose, HbA1c = Glycosylated hemoglobin
** Comparison with control, # comparison between DM without CAD and DM with CAD, P < 0.05 statistical significance*

Table 3: Comparison of Lipid Parameters, hsCRP and NO Levels in Different Groups

Parameters	Control (n=65)	DM without CAD (n=65)	DM with CAD (n=65)	F	p-value
TC (mg/dL)	153.60 ± 26.81	163.41 ± 48.23	154.08 ± 37.10	1.35	0.262
TG (mg/dL)	097.52 ± 23.70	117.54 ± 46.75*	141.23 ± 83.25*#	9.59	**0.001**
HDL-C (mg/dL)	039.68 ± 07.40	036.20 ± 8.35*	032.16 ± 05.78*#	15.36	**0.001**
LDL-C (mg/dL)	094.41 ± 24.08	103.69 ± 47.38	093.29 ± 34.16	1.59	0.21
VLDL-C (mg/dL)	019.50 ± 04.74	023.51 ± 09.35	028.62 ± 19.06*#	8.61	**0.001**
hsCRP (mg/L)	001.10 ± 0.24	003.67 ± 01.02*	004.80 ± 1.04*#	301.04	**0.001**
NO (mmol/L)	045.01 ± 10.2	036.2 ± 12.47*	023.48 ± 7.65*#	61.48	**0.001**

*DM = Diabetes mellitus, CAD = Coronary artery disease, TC = Total cholesterol, TG = Triglyceride, HDL-C =High density cholesterol, LDL-C = Low density cholesterol, VLDL-C = Very low density cholesterol, hsCRP = High sensitive C-reactive protein, NO = Nitric oxide, * comparison with control # comparison between DM without CAD and DM with CAD P < 0.05 statistical significance*

Summary of lipid parameters, hsCRP and NO levels in three groups is shown in table-3. There was significant increase ($p<0.001$) in TG, VLDL and hsCRP and significant decrease ($p<0.001$) in HDL and NO levels in diabetese without CAD patients and diabetes with CAD group than compared to controls. Even though TC and LDL increased in cases, the difference was not statistically significant. Inflammatory marker hsCRP was 4.8 ± 1.04 mg/L in DM with CAD patients, 3.6 ± 1.02 mg/L in DM without CAD patients and 1.1 ± 0.2 mg/L in controls. NO in DM with CAD group was drastically reduced 23.48 ± 7.66 mmol/L, compared to 36.92 ± 12.47 mmol/L in DM without CAD and in controls 45.01 ± 10.1 mmol/L.

Comparison between DM without CAD and DM with CAD group

As expected, both diabetes groups had higher ($p<0.001$) BMI, fasting glucose and HbA1c as compared to controls. There was no significant difference in BMI, FBG and HbA1c between two diabetes groups. DM with CAD patients had a longer duration of diabetes than compared to diabetes without CAD. TG, VLDL, hsCRP were significantly increased ($p<0.001$), HDL and NO was significantly decreased ($p<0.001$) in DM with CAD group compared to DM without CAD group. Correlation of NO with different parameters is depicted in table-4. NO shows significant negative correlation ($p<0.05$) with FBG, HbA1c and hsCRP

Table 4: Correlation of NO with Other Parameters in DM without CAD and DM with CAD Patients

Parameters	DM without CAD		DM with CAD	
	r-value	p-value	r-value	p-value
SBP (mm of Hg)	0.12	0.83	0.009	0.94
DBP (mm of Hg)	- 0.13	0.27	-0.295	0.01*
FBG (mg/dL)	- 0.247	0.05*	-0.226	0.07
HbA1c (%)	- 0.728	0.00*	-0.439	0.00*
TC (mg/dL)	0.018	0.87	-0.188	0.13
TG (mg/dL)	0.15	0.23	-0.128	0.31
HDL-C (mg/dL)	0.149	0.23	0.081	0.52
LDL-C (mg/dL)	-0.037	0.76	-0.134	0.28
hsCRP (mg/L)	-0.467	0.00*	- 0.235	0.08

*DM = Diabetes mellitus, CAD = Coronary artery disease, SBP = Systolic blood pressure, DBP = Diastolic blood pressure, FBG = Fasting blood glucose, HbA1c = Glycosylated hemoglobin, TC = Total cholesterol, TGs = Triglyceride, HDL-C =High density cholesterol, LDL-C = Low density cholesterol, hsCRP = High sensitive C-reactive protein, NO = Nitric oxide, * Comparison with control, P < 0.05 statistical significance*

in DM without CAD patients. In DM with CAD group NO shows significant negative correlation (P<0.05) with DBP and HbA1c. Even though there was negative correlation between NO with FBG and hsCRP in DM with CAD group, it was not statistically significant. NO had no correlation with lipid parameters in both the groups.

Table-5 shows the mean values of serum NO levels in DM without CAD patients in different biochemical parameters. Poorly controlled diabetes has decreased NO levels compared to good glycemic controlled diabetics. As the inflammation increases NO levels are reduced which was statistically significant (p<0.01) in DM without CAD patients

Serum NO levels was significantly decreased (p<0.05) with increase in HbA1c levels in DM with CAD patients shown in Table-6. NO levels also decreased with increase in TC levels in DM with CAD patients. There was not much difference in NO levels with change in BMI, TG, HDL and LDL levels.

Table 5: Serum NO Levels in DM without CAD Patients in Different Biochemical Parameters

Parameters		N	Mean ± SD	p-value
BMI (Kg/m^2)	<25	19	35.3 ± 12.7	0.5
	>25	46	37.5 ± 12.4	
HbA1c (%)	<7.5	29	46.3 ± 8.7	**0.00***
	>7.5	36	29.3 ±9.4	
TC (mg/dL)	<200	55	36.4 ±11.8	0.4
	>200	10	39.8 ±15.72	
TG (mg/dL)	<150	56	36.9 ±11.9	0.9
	>150	9	36.5 ±15.9	
HDL-C (mg/dL)	<40	43	35.6 ± 9.34	0.2
	>40	22	39.3 ± 8.56	
LDL-C (mg/dL)	<100	36	36.9 ± 12.06	0.9
	>100	29	36.8 ± 13.18	
hsCRP (mg/L)	<3	16	43.6 ± 11.72	**0.01***
	>3	49	34.7 ± 12.03	

*BMI = Body mass index, HbA1c = Glycosylated hemoglobin, TC = Total cholesterol, TG = Triglyceride, HDL-C =High density cholesterol, LDL-C = Low density cholesterol, hsCRP = High sensitive C-reactive protein, * Comparison with control, P < 0.05 statistical significance*

Table 6: Serum NO Levels in DM with CAD Patients in Different Biochemical Parameters

Parameters		N	Mean ± SD	p-value
BMI (Kg/m^2)	<25	21	22.6 ± 5.7	0.6
	>25	44	23.8 ± 8.4	
HbA$_1$c (%)	<7.5	18	26.4 ± 7.2	**0.05***
	>7.5	47	22.3 ± 7.5	
TC (mg/dL)	<200	60	24.2 ± 7.5	**0.01***
	>200	5	15.2 ± 2.3	
TG (mg/dL)	<150	52	23.9 ± 7.1	0.3
	>150	13	21.7 ± 9.8	
HDL-C (mg/dL)	<40	55	23.3 ± 7.8	0.8
	>40	10	24.03 ± 6.9	
LDL-C (mg/dL)	<100	43	23.8 ± 7.4	0.5
	>100	22	22.7 ± 8.13	
hsCRP (mg/L)	<3	1	18.3	0.5
	>3	64	23.5 ± 7.69	

*BMI = Body mass index, HbA1c = Glycosylated hemoglobin, TC = Total cholesterol, TG = Triglyceride, HDL-C =High density cholesterol, LDL-C = Low density cholesterol, hsCRP = High sensitive C-reactive protein, * Comparison with control, P < 0.05 statistical significance*

Discussion:

Endothelial dysfunction and reduced production or bioavailability of NO is an important factor in the pathogenesis of diabetic vascular complications. In our study, we observed decrease NO levels in both DM without CAD and DM with CAD patients. Reduced NO levels in diabetes can be explained as due to, hyperglycemic stimulation leading to formation of Advanced Glycation End (AGE) products which enhances polyol pathway and activates protein kinase C. This leads to oxidative stress and formation of reactive oxygen species. These ROS rapidly quench NO, leading to formation of peroxynitrite which is a cytotoxic oxidant. Reduced NO availability leads to the development of atherosclerosis [15]. Peroxynitrite degrades the Tetrahydrobiopterin (BH4) which is one of the cofactor of eNOS leading to uncoupling of eNOS [16]. Peroxynitrite is one of the mediators of oxidation of LDL and in turn accelerates the pathogenesis of atherosclerosis [17]. A study by Rodriguez–Mannas *et al*., observed that AGEs are important modulators of NO activity and relevant

to the impairment of endothelial function in poorly controlled diabetes patients [18].

According to Manju *et al.*, significant decrease in the serum NO values was observed in diabetese patients compared to controls [19]. They also observed that diabetes mellitus affects the vascular endothelium, vascular tone by affecting NO levels, as the severity of diabetes increases there will be increase in BP, due to decrease in serum NO levels. A study in Italy by Tessari *et al*. concluded that NO production from arginine decreased in type 2 diabetes and nephropathy [20]. A study at north-eastern part of India by Ghosh *et al*., sum up that NO was lower in diabetes patients which was statistically significant. Due to wide variations in NO levels, they proposed the need for the standardization of NO estimation, by conducting multi centric study [21]. Reduced NO levels in diabetes may be due to inactivation of NOS. Diabetes accelerates kidney dysfunction; it may prevent the elimination of NOS competitive inhibitors like Asymmetric Dimethyl Arginine (ADMA) which is and thus limits the NO production [22].

Few studies also showed increase in serum NO level; this could be due to diabetic complications and the stage of the disease [23, 24]. Study by Adela *et al*, even though showed increased NO levels in DM, significant low levels of NO in diabetes who were more than five years compared to the subjects having diabetes history of less than five years. Their cell culture data confirmed that high glucose exposure enhanced NO production at early time point but reduced subsequently after exposure for longer duration [23]. This holds good for us, as all diabetic patients in our study group were above six years of duration. Weide *et al*., found lower levels of NO in diabetes patients with HbA1c > 9% and associated with development of vascular complications in type-2 diabetes mellitus [25].

In our study, we found decreased NO levels in DM with CAD patients. Reduced NO availability engages in initiation, progression and complications of atherosclerosis. Lack of NO bioavailability in the coronary and peripheral artery has been regarded to be prospective cardiovascular events [26].

The alteration in endothelial function is due to imbalance between endothelial vasoprotective factors like NO, endothelium-dependent hyperpolarization, oxidative stress state and generated vasoconstrictors [27]. Hyperlipidemia i.e oxidized-LDL brings endothelial dysfunction by uncopling the eNOS, which results in increase in superoxide anions (O_2^-) production. This superoxide spontaneously reacts with NO to form peroxynitrite anion ($ONOO^-$) which is highly reactive and cytotoxic induces lipid peroxidation and endothelial dysfunction [28].

In our study there was negative correlation between NO and Diastolic Blood Pressure (DBP) in DM with CAD patients. A study by Manju *et al*, on diabetes patients observed, there was negative correlation between NO with HbA1c and Mean Arterial Pressure (MAP). They also showed as the severity of diabetes increases there was increase in BP, due to marked reduction in NO levels [19].

In our study there was negative correlation between NO with HbA_1c in DM without CAD and DM with CAD patients, which is in accordance with other studies [19, 25, 29]. Study found that serum NO was significantly low in diabetes normotensive as well as diabetese hypertensive patients as compared to controls. A negative

correlation was found between serum NO with glucose and HbA1c [29]. Poorly controlled diabetics have decreased NO levels.

In our study there was significant increase in hsCRP in diabetics without CAD as well as diabetics with CAD patients compared to controls, which is in accordance with other studies [30-32]. A south Indian study on 150 participants showed CRP levels were higher among diabetes with and without CAD compared to non diabetic subjects [31]. Elevated hsCRP levels associated with diabetes and CVD, but had no correlation with disease duration or glucose control [32]. hsCRP act as inflammatory marker by activating complement pathway, induces adhesion molecule, enhances LDL uptake by macrophages and induces plasminogen activator inhibitor-1 [33], inturn enhances endothelial damage [34]. We observed negative correlation of hsCRP with NO in diabetes without CAD patients. This may be due to opsonizing property of the hsCRP and recruitment of monocytes into atheromatous plaque and also inducing endothelial dysfunction by suppressing basal and induced NO release [33].

The study concludes that estimation of NO and hsCRP along with lipid profile, help in early detection of endothelial dysfunction in diabetes patients. Reduced NO and increased hsCRP levels in diabetes patients may be a strong indicator of coronary artery disease. Estimation of NO and hsCRP levels may contribute to early detection of coronary artery disease and in turn may reduce the morbidity and mortality associated with it in diabetes.

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Original Article

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Serum Lipoprotein (a) as a Diagnostic Marker of Coronary Artery Disease: A Case-Control Study

Biochemistry Section

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ABSTRACT

Introduction: Coronary Artery Disease (CAD) is a major cause of mortality in India. Conventional risk factors fail to explain the increasing burden of CAD, thus necessitating to search for newer risk factors like Lipoprotein (a) {Lp(a)}. Lp(a) is considered as one of the risk marker of CAD. Lp(a) levels have shown wide ethnic variation among human population.

Aim: The study was aimed to estimate Lp(a) levels in CAD patients and to compare it with healthy controls. To see the correlation between Lp (a) with other lipid parameters and also to find the best cut-off value for Lp(a) in CAD patients in North Karnataka population.

Materials and Methods: This was hospital based, observational, case-control study done on 120 participants, with 60 CAD patients and 60 healthy controls. Lipid profile, Lp(a) and FBS (Fasting Blood Sugar) was analysed along with anthropometric measurements. Quantitative data expressed as mean±SD. Student's t-test for comparison, Pearson correlation for correlation and Receiver Operating Curve (ROC) to get the best cut-off value for Lp(a) was used.

Results: Lp(a) levels were significantly increased ($p<0.001$) in CAD patients compared to controls with mean values 52.34±24.87 and 16.81±3.91 respectively. Triglyceride (TG) was significantly increased ($p<0.001$) and High Density Lipoprotein (HDL) was significantly decreased in CAD patients. Area under the curve is >0.8 with 78% sensitivity and 86% specificity. The best cut-off value is 23.7 mg/dL. Lp(a) positively correlated with Total Cholesterol (TC), Low Density Lipoprotein cholesterol (LDL) and negatively correlated with HDL.

Conclusion: Raised Lp(a) levels is associated with an increased risk and may serve as marker of CAD. When Lp(a) is above 23.7 mg/dL. it acts as a predictor for CAD even when lipid levels are in normal range. It is better to assess the Lp(a) along with routine lipid profile in all patients with CAD in north Karnataka.

Keywords: Dyslipidemia, Low density lipoprotein variant, Risk factor

INTRODUCTION

Cardiovascular Disease (CVD) is the leading cause of death and accounts for 30% of all deaths and 10.3% of disability adjusted life year loss. By the year 2020, it is estimated that CVD will surpass infectious diseases as the world's leading cause of death and disability. India is predicted to bear the greatest CAD burden according to the estimates from Global Burden of Disease Study [1]. CAD strikes Indian population at younger age and many will be killed in their potential productive years [2].

Risk factors play an important role in initiating and accelerating the complex process of CAD. The modifiable risk factors are dyslipidemia, diabetes mellitus, hypertension, and smoking. Unmodifiable risk factors include age, gender and family history. These conventional risk factors have failed to explain the increasing burden of CAD. Inspite the usage of newer drugs to lower the lipid concentration, CVD continue to be the main cause of death [3]. Thus, necessitating the need to search for newer risk factors like lipoprotein (a) {Lp(a)}, homocysteine, inflammatory markers, endothelin and thrombogenic factors.

Lipoprotein (a) {Lp(a)} is a LDL like particle formed by the association of the highly polymorphic glycosylated apolipoprotein (a) with apolipoprotein B100 attached through a single disulphide bond. It is located on chromosome 6 (6q26-27) on the telomeric region [4]. Lp(a) has emerged as a powerful genetic risk factor for CAD [5]. Many studies documented that Lp(a) in excess increases the risk of premature CAD even in the presence or absence of concomitant risk factors [6,7]. Lp(a) is structural analogue and competitive inhibitor of plasminogen leading to impaired fibrinolysis. Lp(a) accumulates in the vessel wall and inhibits binding of plasminogen to cell surface and promotes proliferation of smooth muscle cells. Hence Lp(a) has atherogenic and thrombotic properties [8]. Different population across the globe have shown Lp(a) worldwide ethnic variation with different levels associated with CAD [9,10].

Hence, this study was aimed to estimate Lp(a) levels in CAD patients and to compare with healthy controls. To see the correlation between Lp(a) with other lipid parameters and also, to find the best cut-off value for Lp(a) in CAD patients in North Karnataka population.

MATERIALS AND METHODS

This was hospital based observational case control study conducted in the Department of Biochemistry, tertiary care teaching hospital in Karnataka. The study protocol was approved by Institutional Ethics Committee (IEC) with reference number SNMC/IECHSR/2014-15/A-18-1.1. Study was conducted over a period of one year from January to December 2016.

The study comprises of 120 subjects. Sample size was calculated using open-epi version 2.3.1 software. Taking Mohan V et al., reference, at 95% confidence interval and 80% power, sample size was 57 in each group [11]. Sixty patients who were clinically diagnosed of CAD coming to Medicine, Cardiology OPD and admitted patients were chosen for the study. The CAD patients were selected on the basis of coronary angiography i.e., presence of atleast 50% stenosis in major coronary arteries, ECG, Echocardiograph and previous medical records. Age and sex matched 60 healthy controls in the age group of 40-80 years, who were non-diabetic, non-hypertensive and nonsmokers were enrolled

in the study. Presence of CAD in controls was ruled out by detailed clinical history, ECG pattern, echocardiography and family history. Patients with thyroid disorders, nephrotic syndrome, stroke, chronic liver and renal diseases, cancer, pregnant women and women on oral contraceptives, hormone replacement therapy were excluded from the study group.

Informed consent was taken from all participants before the study. Care was taken to maintain the confidentiality of the patients as per Helsinki declaration. For each patient detailed history was taken on smoking, family history, medication and medical records. General physical examination and anthropometric measurements like height and weight were recorded. Body Mass Index (BMI) was calculated using the formula Wt (Kg)/Ht (m^2).

Under aseptic precaution 6 mL of fasting venous blood was drawn and serum was separated by centrifugation. The serum was used for estimation of biochemical parameters like blood glucose (FBS), lipoprotein (a) and lipid profile fasting. Two mL blood was collected in sodium floride bulbs for glucose estimation. Glucose was determined by glucose oxidase and peroxidase (GOD –POD) method. Total cholesterol by cholesterol oxidase and peroxidase method, TG's by glycerol phosphate method. HDL-C estimated by direct method and LDL-C was calculated by Friedewald's formula i.e., LDL=TC-TG/5-HDL. Lp(a) was estimated by immuno-turbidemetric method [12]. All parameters were measured in fully automated analyser, Biosystem A25 using Biosystem kits on the same day. Lipid ratios were calculated by using formulae TC/HDL, LDL/HDL, and TG/HDL.

STATISTICAL ANALYSIS

All statistical analysis was done using software Statistical Package for the Social Sciences (SPSS) version 13. Quantitative data has been expressed in terms of mean±SD. Student's t-test was used for comparison of groups and Pearson's correlation test was applied for correlation of Lp(a) with other parameters. Receiver Operating Curve (ROC) was put to find the best cut off value for Lp(a) in CAD patients. A p<0.05 was considered as statistically significant.

RESULTS

The 60 CAD patients were in the age group of 40 to 80 years. Among them 38 were male and 22 were female patients and there was no significant difference in age and sex ratio between cases and controls. Males are affected more in our study, this may be due to (29) 48% of CAD patients were smokers. The BMI, systolic and diastolic blood pressure and fasting blood glucose were significantly (p<0.05) higher in CAD patients as compared to controls as depicted in [Table/Fig-1].

There was statistically significant increase (p<0.001) in TGs and VLDL and significant decrease (p<0.001) in HDL in CAD patients compared to control group. Even though, there was difference in TC and LDL but it was not statistical significant. There was significant increase (p<0.001) in Lp(a) in CAD patients compared to controls. Mean Lp(a) levels in CAD patients were 52.34±24.87 which was highly significant (p<0.001) compared to controls 16.81±3.91. There was significant increase in lipid ratios TC/HDL, TG/HDL and LDL/HDL in cases compared to controls as shown in [Table/Fig-2]. Distribution of Lp(a) among the CAD patients was shown in [Table/Fig-3]. Majority of the CAD patients i.e., 43.3% had Lp(a) levels in the range of 41-60 mg/dL followed by 31.6 % in the range of 21-40 mg/dL. The cut-off value for Lp(a) was 23.7 mg/dL in CAD patients in our study. Most of CAD patients had Lp(a) levels were above this value.

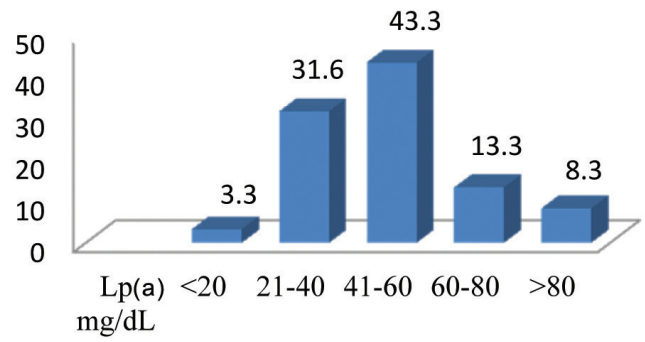
[Table/Fig-4] shows the correlation of Lp(a) with other lipid parameters. Lp(a) shows significant positive correlation with TC and LDL where as negative correlation with HDL. There was also positive correlation with TC/HDL and LDL/HDL. ROC for Lp(a) in CAD patients is shown [Table/Fig-5]. Area under the curve is >0.8 which is highly significant (p<0.001). In our study we found cut-off value was 23.7 mg/dL with 78% sensitivity and 86% specificity shown in [Table/Fig-6]. This suggests that Lp(a) above this cut off value can be used to evaluate risk of CAD in our region.

Parameters	Cases n = 60	Controls n =60	t	p-value
Male: Female	38:22	35:25	-	-
Age in year	58.85± 12.82	55.72 ± 16.24	1.17	0.243
BMI Kg/m^2	26.37± 3.54	24.87 ± 1.32	3.06	0.003
SBP mm of Hg	134±10	112± 9	12.52	<0.001
DBP mm of Hg	94 ± 6	78 ± 6	13.57	<0.001
FBS mg/dL	135.84 ± 42.92	85.45 ± 16.04	10.38	<0.001

[Table/Fig-1]: Baseline characters in cases and controls.
BMI= Body mass index, SBP=Systolic blood pressure DBP= Diastolic blood pressure, FBS= Fasting blood sugar.
p<0.05 statistical significant

Parameters	Cases	Controls	t	p-value
T cholesterol mg/dL	153.69±39.63	145.78±23.41	1.333	0.185
Triglyceride mg/dL	139.06±80.41	92.01±22.46	4.366	<0.001
HDL mg/dL	30.86±5.62	38.76±7.36	6.604	<0.001
LDL mg/dL	94.59±32.07	88.62±23.51	1.18	0.24
VLDL mg/dL	28.24±18.7	18.4±4.49	3.95	<0.001
Lp(a) mg/dL	52.34±24.87	16.81±3.91	10.93	<0.001
TC/HDL	5.21±1.96	3.8±0.93	4.73	<0.001
TG/HDL	4.78±3.46	2.46±0.77	5.053	<0.001
LDL/HDL	3.23±1.45	2.38±0.83	3.91	<0.001

[Table/Fig-2]: Comparison of lipid profile and Lp(a) in cases and controls.
HDL=High density lipoprotein, LDL=Low density lipoprotein, VLDL= Very low density lipoprotein, TG=Triglyceride
p<0.05 considered as statistical significant



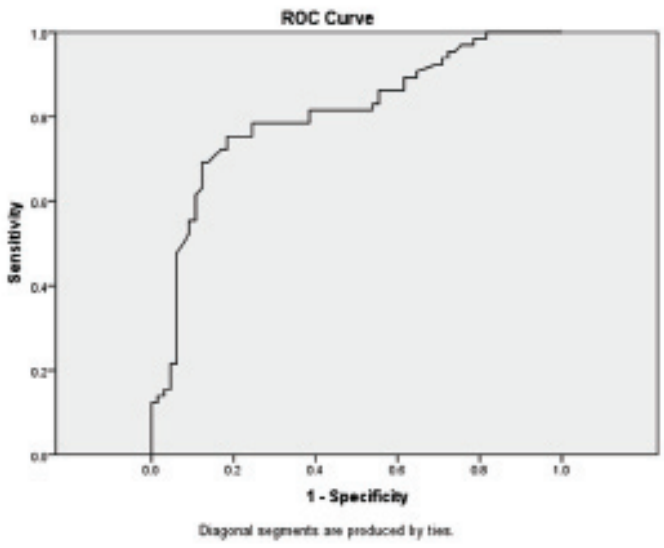
[Table/Fig-3]: Percentage of CAD patients depending on Lp(a) levels.

	Parameters	r-value	p-value
Lp(a)	TC	0.385	0.002
	TG	0.101	0.442
	HDL	- 0.455	<0.001
	LDL	0.494	<0.001
	VLDL	0.104	0.428
	TC/HDL	0.549	<0.001
	TG/HDL	0.227	0.08
	LDL/HDL	0.623	<0.001

[Table/Fig-4]: Correlation of Lp(a) with other lipid parameters in CAD patients.
p<0.05 statistical significance

DISCUSSION

In the present study we evaluated the significance of novel biomarker i.e., Lp(a) in CAD patients. Recently Lp(a) is emerging as a strong biomarker of CAD. Our result shows high levels of TG and low levels



[Table/Fig-5]: ROC curve of Lp(a) to detect the best cut-off value in CAD patients.

	Sensitivity	Specificity	AUROC	Cut-off value	p-value
Lp(a) mg/dL	78.3 %	86.4%	0.8	23.7 mg/dL	< 0.001

[Table/Fig-6]: Sensitivity, specificity and AUC of Lp(a) in CAD patients.

of HDL and is strongly associated with CAD. A large cross-sectional study conducted in North India by Ashfaq F et al., found increased TG and very low HDL levels in diseased vessel patients as compared to those with normal coronary status [13]. They also indicated that usefulness of estimation of TG to identify the presence of CAD even when the patients are receiving treatment i.e., hypolipidemic drugs like statins. Austin MA et al., reported TG levels more than 130 mg/dL are strongly associated with the extent of patient's coronary atherosclerosis and states that hypertriglyceridemia is commonly associated with CAD patients [14]. Low levels of HDL are reported to increase the risk of cardiovascular disease even when cholesterol is not elevated [15,16]. Low HDL level is an independent risk factor for CAD [17]. In our study, we did not find any statistical difference in TC and LDL levels in CAD patients compared to controls. Atherogenicity of Lp(a) is more marked with concomitant decrease in HDL levels which was also seen in our study [18].

In this study we estimated Lp(a) levels in CAD patients and controls. We also made an attempt to find out cut-off value for Lp(a) levels using ROC. We found higher levels of Lp(a) in CAD patients, our results are in agreement with other studies [19-21]. Ashfaq F et al., reported that Lp(a) levels correlated positively with severity of atherosclerosis [19]. Lp(a) levels of 21mg/dL are associated with presence of CAD even when cardiovascular risk factors and specific treatments were taken. In north Indian studies by Yusuf J et al., and Gupta R et al., showed highest Lp(a) levels were observed in triple vessel disease, followed by double and single vessel disease [20,21]. CAD patients showed almost five fold higher Lp(a) levels as compared to controls [22]. Zampoulkis JD et al., studied the relationship of excess Lp(a) with extent and severity of atherosclerosis in CAD patients. They revealed that Lp(a) is related to diffuse lesions covering large part of coronary vasculature [23]. Lp(a) competitively inhibit plasminogen activity, leading to impaired fibrinolysis. The smaller the apo (a), the higher are the Lp(a) levels and the risk for CAD. It enhances the oxidation and foam cell formation.

In our study, cut-off value was 23.7 mg/dL with 78% sensitivity and 86% specificity. A study on 151 south Indian patients, by Rajashekhar D et al., shown Lp(a) >25 mg/dL is associated independently with around two fold risk of CAD [10]. A north Indian study by Hoogeveen RC et al., had proposed a cut off value was >19 mg/dL in 103 subjects [24]. Another largest north Indian study by Yusuf J et al., showed Lp(a) above 40 mg/dL assessed by an isoform insensitive assay is an independent risk factor for CAD [20]. According to Saini V et al., Lp(a) showed independent predictor of CAD irrespective of traditional risk factor with good sensitivity and specificity. The best cut-off value of Lp(a) came out to be 45 mg/dL [9]. Lp(a) levels above 20mg/dL are reported to be associated with a high risk of CAD [25]. Studies found that patients with Lp(a) levels more than 30 mg/dL had risk of CAD increased by about three fold [26,27].

We noted there was significant positive correlation of Lp(a) with TC, LDL, TC/HDL and LDL/HDL and negative correlation with HDL which are in agreement with other studies [28,29]. A large cohort study from five European countries comprising of 56,804 individuals reported that elevated Lp(a) was robustly associated with an increased risk of major coronary events. Total cholesterol correlated positively with Lp(a) levels [28]. A study by Pedreno J et al., reported Lp(a) levels associated with LDL cholesterol [29].

LIMITATION

Small sample size, genetic data is not provided. It is recommended to conduct study in larger sample size.

CONCLUSION

In this study we were able to show Lp(a) as one of the reliable marker, as Lp(a) was found to be raised significantly in CAD patients compared to controls. Since Lp(a) levels show wide ethnic variation, an attempt has been made to derive the best cut-off value for Lp(a) levels in CAD patients in North Karnataka population.

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