"ESTIMATION OF ISCHEMIA MODIFIED ALBUMIN IN ACUTE STROKE WITH AND WITHOUT DIABETES MELLITUS: A ONE YEAR CROSS SECTIONAL STUDY"

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LIST OF ABBREVIATIONS USED

ABBREVIATION	EXPANSION
DM	Diabetes Mellitus
IMA	Ischemia Modified Albumin
ABSU	Absorbance units
ROC	Receiver operating Characteristic curve
AUC	Area under the curve
FDA of US	Food and drug administration of United States of America
WHO	World Health Organization
CNS	Central Nervous System
IS	Ischemic Stroke
HS	Hemorrhagic Stroke
DALY	Disability adjusted life years
MRI	Magnetic Resonance Imaging
CT	Computed Tomography
T2DM	Type 2 Diabetes Mellitus
NO	Nitric Oxide
PKC	Protein Kinase C
HAS	Human Serum Albumin
ACB test	Albumin Cobalt Binding test

MI	Myocardial Infarction
DTT	Dithiothreitol
SPSS	Statistical Package for Social Sciences
S.D	Standard Deviation
ACS	Acute Coronary Syndrome
CV	Coefficient of Variation
CAD	Coronary Artery Disease
ANOVA	Analysis of Variance
RBS	Random Blood Sugar
FBS	Fasting Blood Sugar
CI	Confidence Interval
HTN	Hypertension
HPLC/MS	High Performance Liquid Chromatography/ Mass Spectrometry
PPV	Positive Predictive Value
NPV	Negative Predictive Value

ABSTRACT

Background and Objectives:

Stroke is a leading cause of mortality and morbidity all over the world. Diabetes Mellitus is frequently associated with it. Currently the diagnosis of stroke is mainly based on neuro imaging techniques which are not devoid of limitations. Thus the alternate approach would be using biomarkers to support the clinical diagnosis of acute stroke. Ischemia Modified Albumin is one such marker of ischemia approved by FDA of US.

Primary objective of the present study was to estimate and compare the IMA levels in 'Stroke with DM', 'Stroke without DM' and 'Healthy Control' groups. Secondary objective was to assess the diagnostic efficacy of the IMA in both the patient groups.

Materials and methods:

This one year cross sectional study included 90 subjects of either sex, of which 30 cases of acute stroke with diabetes, 30 cases of acute stroke without diabetes and 30 normal healthy controls after obtaining informed and written consent.

Blood sample was collected from stroke patients soon after the clinical diagnosis and included in the study after radiological confirmation of acute stroke. Blood sample from healthy volunteers attending the blood bank was taken as controls. IMA was estimated using Albumin Cobalt Binding test using a spectrophotometer. Results of IMA were expressed in absorbance units (ABSU). Serum albumin was also

estimated in all the subjects involved in the study. Results were tabulated and subjected to appropriate statistical analyses.

Results:

Mean IMA values were high in 'Stroke with DM' (0.23± 0.03) and 'Stroke without DM' (0.21 ± 0.03) groups when compared with the 'Controls' (0.16 ± 0.03). There was a statistically significant difference between the three groups when compared by ANOVA ($p < 0.001$). Comparison of IMA values of three groups with each other by Post-hoc Bonferroni test showed statistically significant difference between 'Stroke with DM' and 'Controls'($p < 0.001$), 'Stroke without DM' and 'Controls'($p < 0.001$). However the difference in mean IMA levels between 'Stroke with DM' and 'Stroke without DM' was not significant statistically ($p = 0.116$). There was no significant correlation between serum albumin and IMA in all the three groups ($p > 0.05$). In 'Stroke with DM' group, area under the ROC curve was **0.968** and at a cut off of **0.196 ABSU**, sensitivity was **90%** and specificity was **87%**. In 'Stroke without DM' group, area under the ROC curve was **0.917** and at a cut off of **0.192 ABSU**, sensitivity was **87%** and specificity was **83%**.

Interpretation and conclusion:

Ischemia Modified Albumin levels were found to be significantly higher in 'Stroke with DM' and 'Stroke without DM' patients than healthy controls. Though there was no statistically significant difference in IMA levels between the two groups, 'Stroke with DM' patients had higher IMA levels than 'Stroke without DM' patients. ROC analysis showed higher AUC for IMA in 'Stroke with DM' than 'Stroke without DM' group. In both the groups IMA was found to be having excellent diagnostic efficacy for the diagnosis of acute stroke. According to the present study there was no

significant correlation of serum albumin with IMA levels in both stroke patients and controls.

Hence we suggest that estimation of IMA could serve as an aid in the diagnosis of acute stroke in patients both with and without DM. Albumin cobalt binding test can be used for the estimation of IMA, which is a simple and cost-effective method.

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INTRODUCTION

Acute stroke is defined as an abrupt onset of a neurologic deficit that is attributable to a focal vascular cause which may be either ischemic or hemorrhagic in nature.[1] Acute ischemic stroke, most common of the two types, results from prolonged cerebral ischemia leading to cerebral neuronal death.

Stroke is a major contributor to the burden of morbidity and mortality all around the world. According to World Health Organization (WHO), stroke is the second leading cause of death after ischemic heart disease in high and middle income countries while sixth leading cause in low income countries.[2]

At present the diagnosis of acute stroke is based on history, clinical examination and neuro-imaging techniques. The clinical manifestations of stroke are highly variable because of the complex anatomy of the brain and its vasculature. Thus radiological evidence is almost always needed for the diagnosis and proper classification of the stroke. But these facilities are time consuming, costly and not widely available. More importantly radiological evidences are often absent in the early stages of stroke.[3] So we need markers which can be easily estimated at bedside or in laboratory which could help clinician in supporting the diagnosis of acute stroke. Thus identifying a biochemical marker which appears early after the ischemic event, cost effective and having rapid and easy measurement method is of immense help in rapid diagnosis and initiating proper treatment at the earliest.

Several studies have been published correlating several biochemical markers to stroke but none are approved for clinical use. Ischemia modified albumin (IMA) is the only ischemic marker which has been approved by Food and Drug Administration

of US for early diagnosis of Myocardial ischemia.[4] Serum albumin loses its divalent metal binding capacity due to the damage to its N- terminus as a result of ischemia producing Ischemia Modified Albumin. Ischemia modified albumin is a novel marker which can be estimated indirectly by quantifying the decrease in cobalt metal binding capacity of albumin. Several attempts have been made to understand the usefulness of Ischemia modified albumin in context with acute ischemic conditions other than Myocardial infarction like acute stroke,[5,6] pulmonary embolism[7] etc. but the data shows variable results[8] and the available data is insufficient and inconclusive.

There are many factors which could affect the serum IMA levels in stroke patients and Diabetes mellitus (DM) is one among them.[9] Diabetes mellitus is also known to accelerate the atherosclerosis process and hence increases the risk of stroke. There exist no sufficient literatures on the role of IMA levels in acute stroke especially in association with diabetes mellitus and effect of DM on IMA levels particularly in the setting of acute stroke. Therefore the present study is undertaken to estimate the serum IMA level in stroke patients with and without diabetes.

AIM AND OBJECTIVES

The aim of the present study is to know whether the Ischemia Modified Albumin (IMA) level is elevated in acute stroke and to compare the Ischemia Modified Albumin levels in acute stroke patients with and without Diabetes Mellitus (DM) to know the effect of DM on IMA levels.

OBJECTIVES

1. To estimate and compare Ischemia Modified Albumin in acute stroke patients with and without diabetes mellitus and healthy controls.
2. To evaluate the diagnostic efficacy of IMA in acute stroke patients with and without diabetes mellitus.

REVIEW OF LITERATURE

ACUTE STROKE

Definition

World Health Organization defines stroke as 'rapidly developing clinical signs of focal (or global) disturbance of cerebral function, lasting more than 24 hours or leading to death, with no apparent cause other than that of vascular origin'. This definition was introduced in 1976 and is still in use.[10, 11]

American Heart Association (AHA)/American Stroke Association (ASA) published an Expert Consensus Document in 2013 which includes a new definition of stroke.[12] According to that, stroke is "An episode of rapidly developing neurological dysfunction caused by either CNS infarction or attributable to a focal collection of blood (hemorrhage) that is not caused by trauma".

Types of Stroke:

- Ischemic stroke (IS): Most common type of stroke (85%)
- Hemorrhagic stroke (HS): (15%)

Disease burden:

Stroke is a major contributor to the burden of morbidity and mortality all over the globe. According to WHO, stroke stands at the second position in the list of leading causes of death after ischemic heart disease in high and middle income countries while sixth in low income countries.[2]

According to an estimate, annually about 15 million people worldwide suffer a stroke. Of these, 6 million die and another 5 million are left permanently disabled, placing a burden on family, community and the country.[13, 14] Stroke is the second leading cause of disability, after dementia. Disability may include loss of vision and / or speech, paralysis and confusion

The incidence of stroke is declining in most of the developed countries even though the actual number of stroke cases is increasing because of the ageing population. But in the developing world, however, the incidence of stroke is increasing.

According to a study, over the 1990–2013 period, there was a significant increase in the absolute number of Disability-adjusted life years (DALYs) due to Ischemic Stroke, and of deaths from IS and Hemorrhagic Stroke, survivors and incident events for both IS and HS. The preponderance of the burden of stroke continued to reside in developing countries, comprising 75.2% of deaths from stroke and 81.0% of stroke-related DALYs.[15] The burden has been projected to rise from 38 million DALY in 1990 to 61 million DALY in 2020.

Risk factors for acute stroke:

Non-Modifiable Risk Factors

- Age
- Gender (male > female, except in the very young and very old)
- Race (Afro-Caribbean > Asian > European)
- Heredity
- Previous vascular event, e.g. myocardial infarction, stroke or peripheral embolism

Modifiable Risk Factors

- High blood pressure
- Heart disease (atrial fibrillation, heart failure, endocarditis)
- Diabetes Mellitus
- Hyperlipidemias
- Smoking
- Excess alcohol consumption
- Polycythemia
- Oral contraceptives
- Social deprivation

ISCHEMIC STROKE:

Etiology:

Common causes

- Thrombosis
    - Lacunar stroke (small vessel)
    - Large-vessel thrombosis
    - Dehydration
- Cardioembolic occlusion
    - Atrial fibrillation
    - Mural thrombus
    - Myocardial infarction
    - Dilated cardiomyopathy
    - Valvular lesions

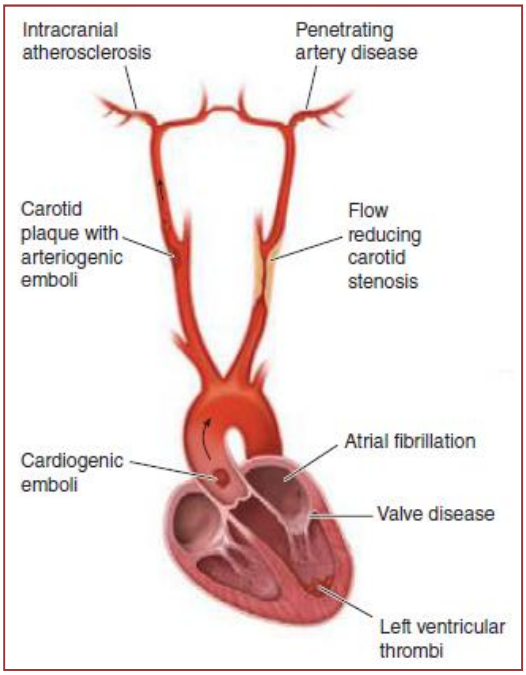


Figure 1: Common causes of ischemic stroke

Uncommon causes

- Hypercoagulable disorders
    - Protein C deficiency
    - Protein S deficiency
    - Antithrombin III deficiency
    - Hyperhomocysteinemia etc.
- Venous sinus thrombosis
- Fibromuscular dysplasia
- Vasculitis
- Cardiogenic
    - Mitral valve calcification
    - Atrial myxoma
- Eclampsia

Pathophysiology of ischemic stroke:

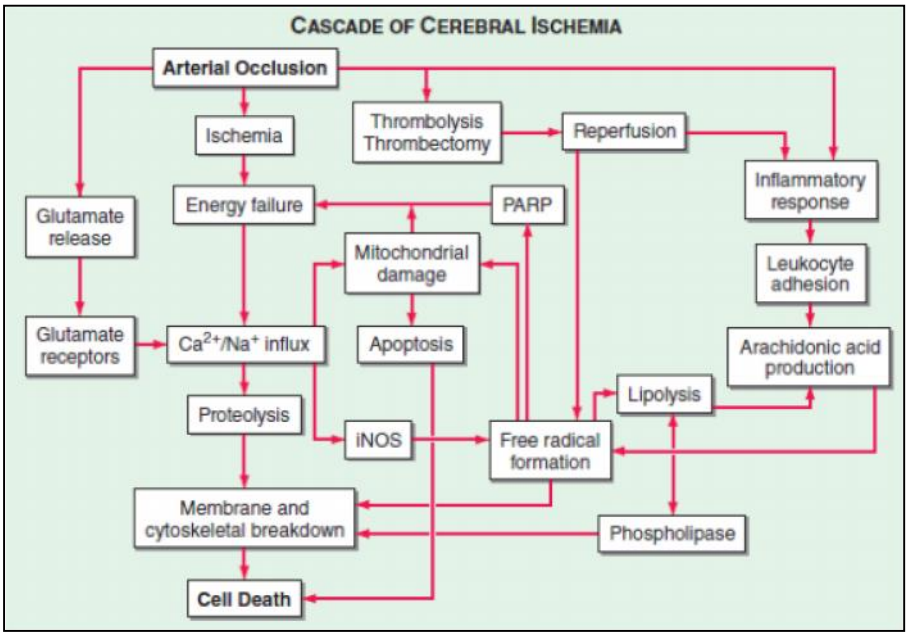


Figure 2: Cascade of cerebral ischemia[(1)]

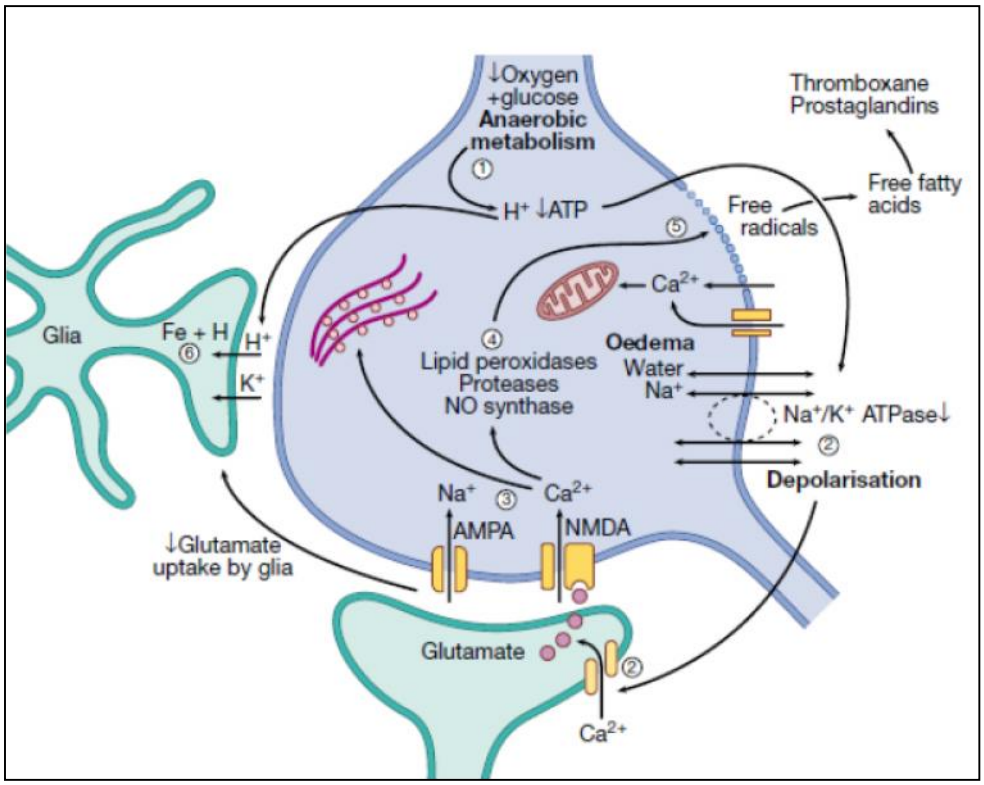


Figure 3: The process of neuronal ischemia and infarction[16]

Steps in the process of neuronal ischemia and infarction: (Refer Figure 3)

1. Decreased blood flow to a part of brain due to occlusion of an artery (may be due to any of the above causes) leads to decreased supply of glucose and oxygen to that part. ATP production in the neuronal cells is diminished and anaerobic glycolysis starts as a result of lack of oxygen. Hence lactic acid production increases leading to increased [H^+] i.e. intracellular acidosis.
2. Membrane ionic pumps which are energy dependent stop functioning leading to influx of Na^+ and water causing cytotoxic edema and membrane depolarisation with the release of glutamate.
3. Calcium enters the cell through glutamate gated channels.
4. Calcium thus entered activates intracellular destructive enzymes.

5. Intracellular destructive enzymes then destroy intracellular organelle and cell membrane with the release of free radicals. Free fatty acids thus released from membrane lipids further activate pro-coagulant pathways exacerbating ischemia.
6. Because of decreased glutamate uptake and increased H^+ entry, glial cells also succumb to cell death leading to necrosis.

Fever and hyperglycemia worsens the condition and leads to more widespread brain damage even at higher threshold for blood flow. Thrombolysis or thrombectomy done as a therapeutic measure may lead to hemorrhage into the ischemic area leading to reperfusion injury & hemorrhagic transformation producing more free radicals and more damage.

HEMORRHAGIC STROKE

Etiology of Intracerebral hemorrhage:

- Head trauma
- Hypertensive hemorrhage
- Transformation of prior ischemic infarction
- Metastatic brain tumor
- Coagulopathy
- Arteriovenous malformation
- Aneurysm
- Drugs (Cocaine, amphetamine)

Pathophysiology of hemorrhagic stroke:

Hemorrhagic stroke usually results from rupture of a blood vessel within the brain parenchyma but may also occur in a patient with a subarachnoid hemorrhage. Hemorrhage frequently occurs into an area of brain infarction (hemorrhagic transformation). The explosive entry of blood into the brain parenchyma causes immediate stoppage of function in that area because of structural disruption and white matter fibre tracts are split apart. The hemorrhage itself may expand over the first minutes or hours, or it may be associated with a rim of cerebral edema, which, along with the haematoma, acts like a mass lesion to cause progression of the neurological deficit.

Diagnosis of Acute Stroke:

Early recognition of acute stroke is very critical for the successful treatment and patient recovery. It is important to limit the brain damage and improve outcomes.

According to the standard guidelines 'Neuro-imaging' is the mainstay in the diagnosis of acute stroke. Neuro-imaging includes Computed Tomography (C.T.) and Magnetic Resonance Imaging (M.R.I.). C.T. is the most preferred method over M.R.I.[(17)] All suspected stroke patients have to be subjected to either of the neuro-imaging modalities to confirm the diagnosis of acute stroke irrespective of etiology. CT radiographic images identify or exclude hemorrhage as the cause of stroke, and they identify extra parenchymal hemorrhages, neoplasms, abscesses, and other conditions which have similar presentations as stroke.

Brain CT scans obtained in the first several hours after an infarction generally show no abnormality, and the infarct may not be seen reliably for 24–48 hours. CT

may fail to show small ischemic strokes in the posterior fossa because of bone artifact; small infarcts on the cortical surface may also be missed. Intracranial hemorrhage is often discovered on non-contrast CT imaging of the brain.

MRI reliably identifies the extent and location of infarction in all areas of the brain. It also detects hemorrhages. But MRI is more expensive and time consuming than CT and not widely available. Claustrophobia and the logistics of imaging acutely critically ill patients also limit its application. Most acute stroke protocols use CT because of these limitations.

Cerebral Angiography, Ultrasound Techniques, Perfusion Techniques are some of the sophisticated techniques which could aid in the diagnosis of acute stroke.

Neuro-imaging is considered to date the only tool available for differentiating between ischemic stroke and hemorrhagic stroke, as the symptoms of the two conditions overlap substantially. Since the treatment modalities differ in different stroke types, it is very important to identify the stroke type. But ischemia is visible in Brain CT scan in the first 3 hours after the onset in only a third of the cases.[18]

Thus there exists a clear need for other diagnostic tools and strategies to identify the stroke at the earliest and also to make the differential diagnosis between ischemia and hemorrhage. Diagnosis using biomarkers provides an alternative strategy.

Treatment of Acute Stroke:

- INTRAVENOUS THROMBOLYSIS: If onset of symptoms to time of drug administration is equal to or less than 4.5 hours in acute ischemic stroke. Recombinant Tissue Plasminogen Activator (rTPA) is the most commonly used thrombolytic agent.
- ENDOVASCULAR REVASCULARIZATION: Endovascular mechanical thrombectomy can be tried in patients with contraindications for thrombolysis in acute ischemic stroke.
- ANTITHROMBOTIC TREATMENT: Using anti-platelet drugs and anticoagulation drugs.
- Treatment for the comorbidities like hypertension, Diabetes Mellitus etc.

DIABETES MELLITUS

Definition

Diabetes mellitus (DM) is a group of metabolic disorders that are characterized by a common feature “hyperglycemia”. It may be because of defects in insulin secretion, insulin action, or both.

Classification based on the etiology:

Depending on the etiology of the Diabetes Mellitus, it can be classified into different types.

i. Type I DM
ii. Type II DM
iii. Other specific types of DM (Due to various genetic defects, drugs, endocrinopathies, infections etc.)
iv. Gestational DM (GDM)

Major types of Diabetes Mellitus include Type1, Type 2 and Gestational Diabetes Mellitus (GDM). Type 2 Diabetes Mellitus (T2DM) accounts for at least 90% of all cases of diabetes mellitus.[19] It has become a major public health problem as the proportion of population having type 2 diabetes mellitus is constantly rising all over the globe.

Disease Burden:

The number of adults having diabetes has almost quadrupled since 1980 to 422 million adults according to the WHO Global report on diabetes, published in 2016. This drastic rise is mainly because of the rise in type 2 diabetes and factors driving it include overweight and obesity.

Diabetes is a major cause of mortality, but many studies show that diabetes is likely underreported as a cause of death. According to an estimate, about 1.5 million deaths were directly caused by diabetes mellitus in 2012 and another 2.2 million deaths were attributable to high blood glucose level.[20] Almost half of all deaths attributable to high blood glucose occur before the age of 70 years.(20) WHO projects that diabetes will be the 7^{th} leading cause of death in 2030 .[21]

Risk factors for Type 2 DM[22]:

- Family history of diabetes (i.e., parent or sibling with type 2 diabetes)
- Obesity (BMI $\geq$25 kg/m^2 or ethnically relevant definition for overweight)
- Physical inactivity
- Race/ethnicity
- Previously identified with Impaired Fasting Glucose, Impaired Glucose Tolerance

- History of Gestational Diabetes Mellitus or delivery of baby >4 kg
- Hypertension (blood pressure ≥140/90 mmHg)
- HDL cholesterol level <35 mg/dL and/or a triglyceride level >250 mg/dL
- Polycystic ovary syndrome or acanthosis nigricans
- History of cardiovascular disease

Pathophysiology of Type 2 Diabetes Mellitus:

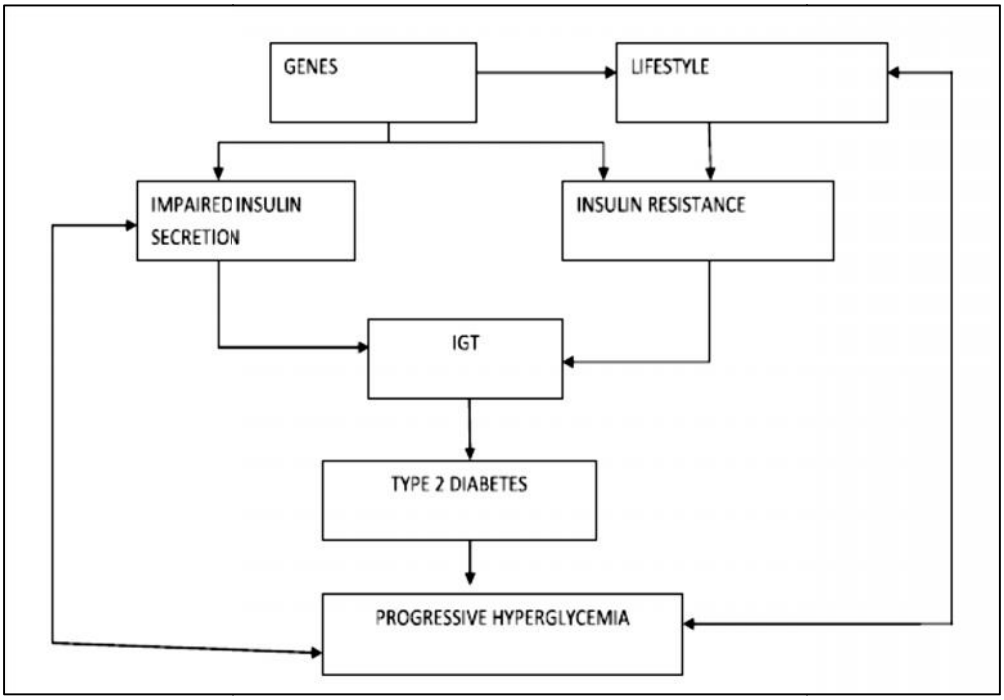


Figure 4: Pathophysiology of Type 2 DM[23]

Diagnosis:

American Diabetic Association has given recommendations for the diagnosis of DM and is widely used all over the world.

Table 1: Criteria for the diagnosis of diabetes mellitus[24]

1. Symptoms of diabetes plus casual plasma glucose concentration ≥200 mg/dl (11.1 mmol/l). Casual is defined as any time of day without regard to time since last meal. The classic symptoms of diabetes include polyuria, polydypsia, and unexplained weight loss.
OR
2. FPG ≥126 mg/dl (7.0 mmol/l). Fasting is defined as no caloric intake for at least 8 h.
OR
3. 2-h post load glucose ≥200 mg/dl (11.1 mmol/l) during an OGTT. The test should be performed as described by WHO, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.
OR
4. Hemoglobin A1c ≥ 6.5%

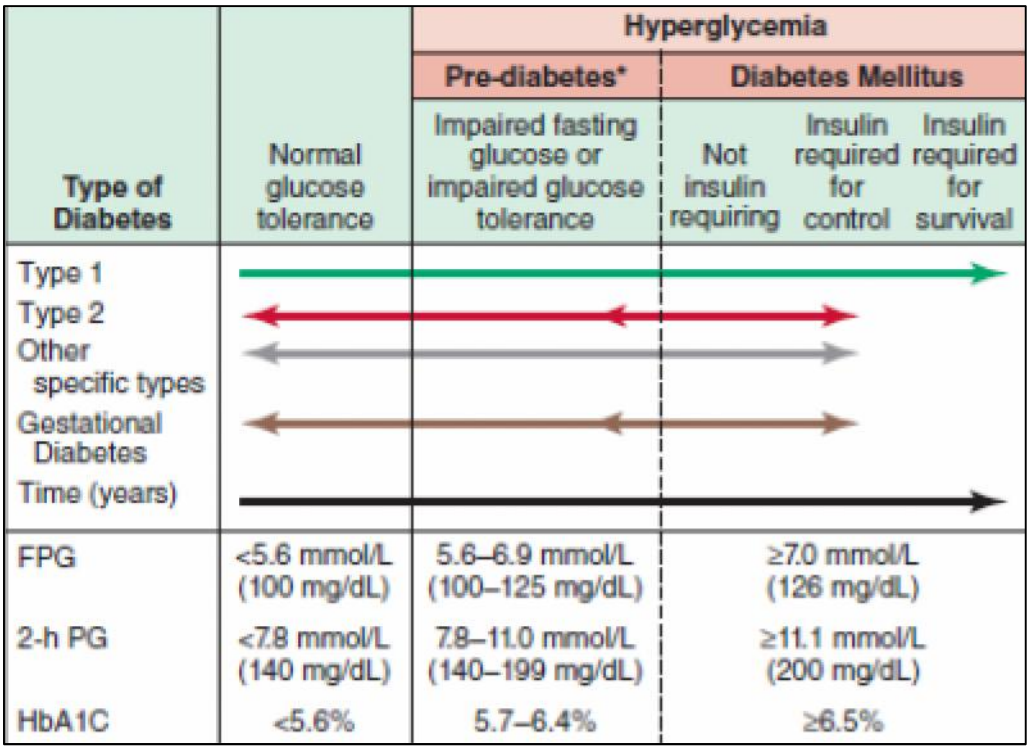


Figure 5: Spectrum of glucose homeostasis and diabetes mellitus[(1)]

Complications of Diabetes Mellitus:

Diabetes mellitus is known to affect nearly all systems of the body due to the underlying dysregulation of metabolic processes. Complications due to DM can be either acute or chronic. Diabetic ketoacidosis is a very important acute complication of DM which is potentially fatal if untreated. Chronic complications include macrovascular and microvascular complications. Retinopathy, neuropathy and nephropathy are microvascular complications. Macrovascular complications include coronary artery disease, peripheral vascular disease and cerebrovascular diseases. The risk of chronic complications depends on the duration and severity of hyperglycemia. They usually do not become apparent until the second decade of hyperglycemia. Since type 2 DM often has a long asymptomatic period of hyperglycemia, many individuals with type 2 DM have complications at the time of diagnosis.

ASSOCIATION OF DIABETES MELLITUS WITH ACUTE STROKE

The most common cause of mortality in diabetic patients is macro-vascular complications such as myocardial infarction, stroke.

There are indications that the macrovascular changes in diabetes are initiated before the manifestation of overt diabetes in the form of oxidative damage and sub-endothelial low grade systemic inflammation. These changes slowly progress to peripheral vascular insufficiency, nephropathy, retinopathy and neuropathy. The sub-endothelial inflammation is associated with oxidative stress and the resultant chronic ischemia as an underlying etiology.[25]

The micro and macro-vascular complication risk in type 2 diabetes has been found to be related to extent and duration of hyperglycemic state. The control of hyperglycemia in DM patients has been shown to be reflected in improvement in the lipid profile as well as other components of pathogenesis of atherosclerosis and hence reduction in the associated cardiovascular risk. Various studies done on glycemic control and risk projections in diabetic patients have shown that improvement of glycemic control improves most of the components of risk associated with diabetes.[26] The etiopathology of diabetic complications involves the development of reactive oxygen species (ROS). Plasma levels of ischemia modified albumin (IMA) in T2DM are connected with parameters of oxidative processes such as advanced oxidation protein products (AOPP) and thiol groups.[27, 28]

Pathophysiology of increased risk of Stroke in DM

1. Decreased endothelium-dependent vasodilation:

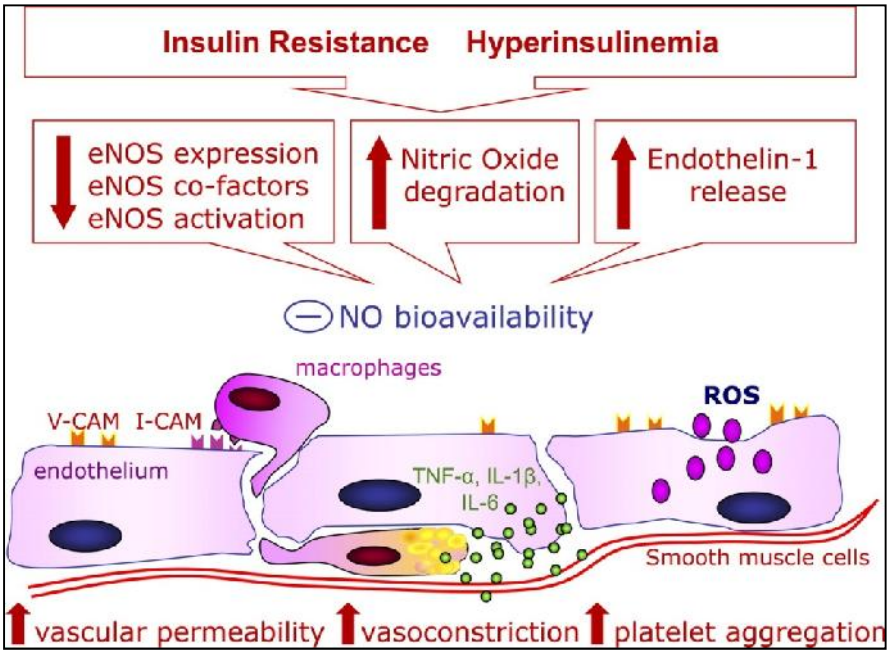


Figure 6: Endothelial dysfunction [29]

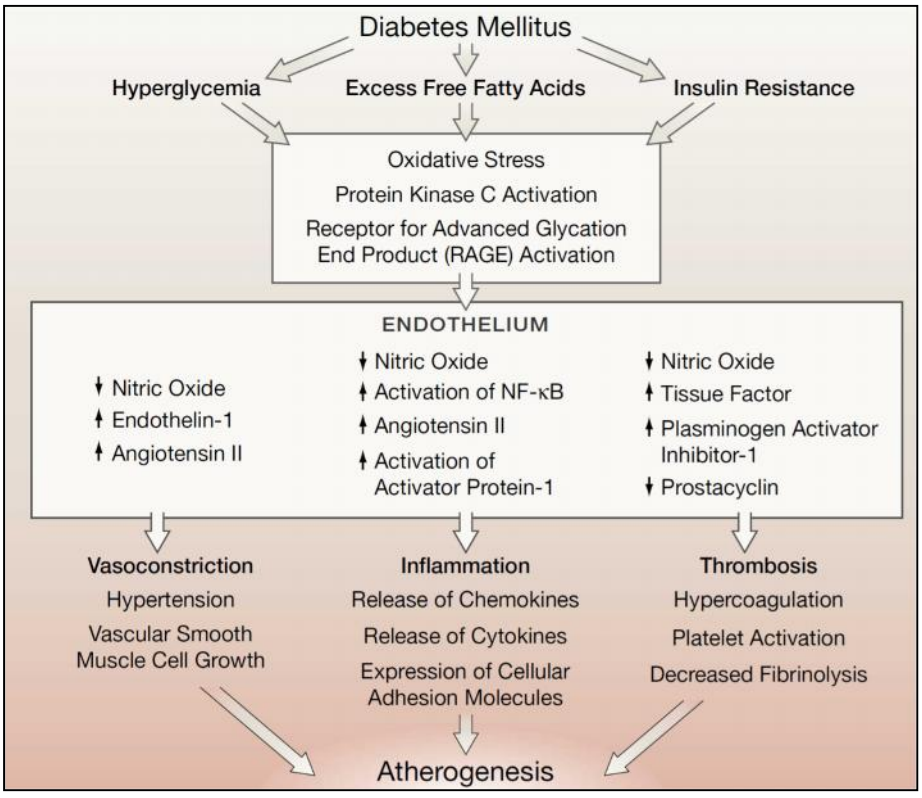


Figure 7: Mechanism of Endothelial dysfunction leading to atherogenesis [30]

Endothelium modulates blood flow, delivery of nutrients, coagulation and leukocyte diapedesis. It also produces nitric oxide (NO), prostaglandins, endothelin etc. NO is responsible for vascular relaxation. NO also known to inhibit platelet activation, inflammation and diminishes vascular smooth muscle cell proliferation. All these mechanisms finally protect the blood vessel. Hyperglycemia seen in DM decreases the NO synthesis by inhibiting NO synthase of endothelium. Hyperglycemia also increases the production of reactive oxygen species in endothelial and smooth muscle cells which also diminishes the bioavailability of NO. Insulin resistance leads to liberation of free fatty acids from adipose tissue thereby activating PKC which increases the production of reactive oxygen species. DM also increases the production of endothelin- 1 which is a potent vasoconstrictor. In addition it also leads to hypertrophy of vascular smooth muscle cell. Diabetes also aggravates the ongoing atherosclerotic mechanisms including monocyte migration into the intima and secretion of cytokines. Hyperglycemia via decreased NO synthesis, increased oxidative stress, activation of receptors for advanced glycated end products increases the gene expression of mediators of atherosclerosis through activation of nuclear factor – kB. DM is also known to decrease collagen formation and increase collagen breakdown which leads to plaque instability which ultimately triggers thrombosis.[28, 30]

2. Vascular smooth muscle dysfunction:

Hyperglycemia activates protein kinase C (PKC), Receptor for advanced glycated end products (RAGE) and nuclear factor-kB in vascular smooth vessel cells as well leading to increased oxidative stress. Smooth muscle cells migrate to intimal

lesions and lay down a complex extracellular matrix. Thus DM stimulates atherogenic activity and plaque instability.

3. Impaired platelet functions:

Impaired platelet function exacerbates the progression of atherosclerosis. In hyperglycemic conditions, intra-platelet glucose concentration is also increased which leads to activation of PKC, decreased NO production (platelet derived) and increased formation of reactive oxygen species similar to endothelial cells. Platelets also tend to have abnormal calcium metabolism which affects its secretion and aggregating properties. Increased expression of glycoprotein Ib (GpIb) and Gp IIb/IIIa on platelet surface leads to thrombosis. Decreased endothelial production of anti-aggregants, increased fibrinogen production may further increase the propensity towards platelet aggregation.

4. Hypercoaguable state of diabetic patients

DM impairs the fibrinolytic activity by elevating plasminogen activator inhibitor- 1 in atheromas. DM also increases the expression of tissue factor, coagulation factor VII and decreases the levels of antithrombin III, protein C, potent endogenous anticoagulants. Thus DM leads to hypercoaguability which ultimately leads to macrovascular complications including stroke.

ISCHEMIA MODIFIED ALBUMIN

Human Serum Albumin (HSA)

Human Serum Albumin is the most abundant plasma protein. It is synthesized by the liver. Various functions of albumin are known today including maintenance of colloidal osmotic pressure. The Human Serum Albumin also has a nutritive role. It

also binds to various ligands including copper, cobalt, free fatty acids, unconjugated bilirubin, thyroid hormones, various drugs and helps in their transportation in the blood.

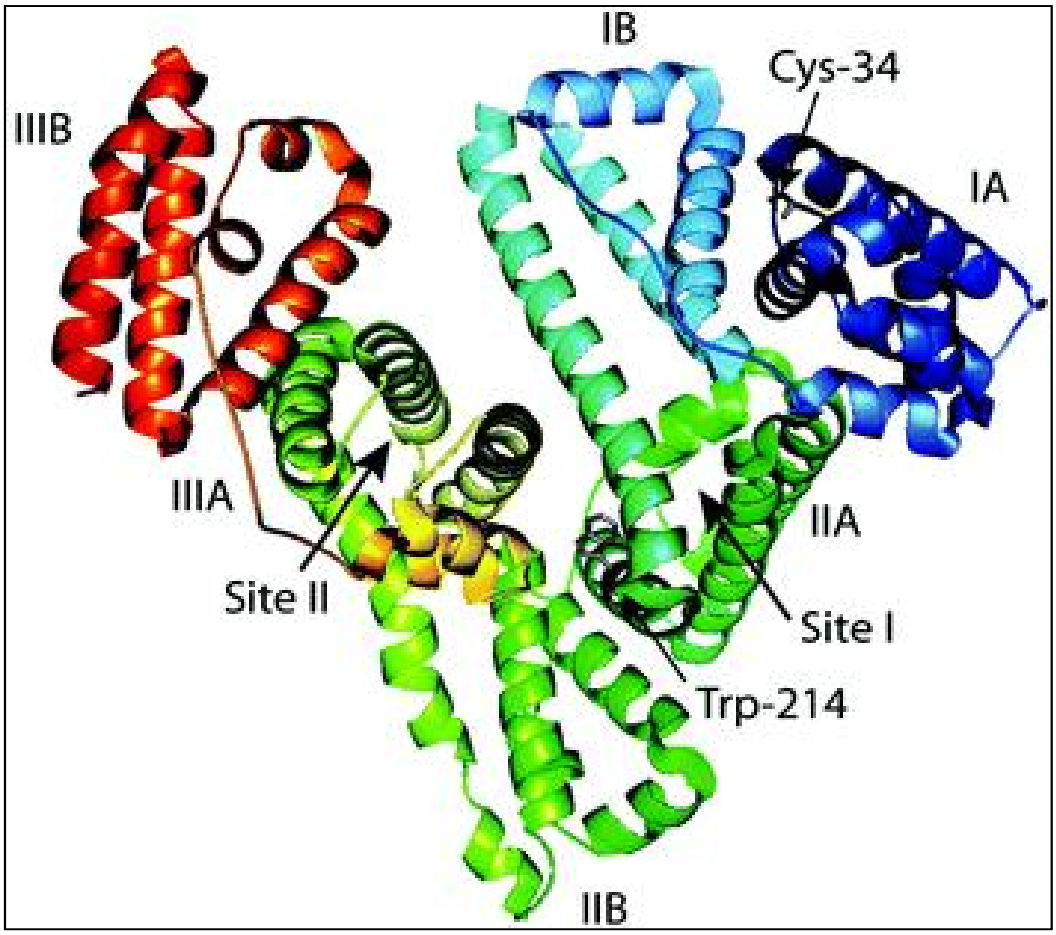


Figure 8: 3D structure of Human Serum Albumin (HSA)

Albumin is encoded by the *ALB* gene which is located on the chromosome 4 which codes for the precursor of albumin having 609 amino acid residues. It is then cleaved in the endoplasmic reticulum and Golgi apparatus to form a mature albumin molecule which is then released to the blood stream. The half life of albumin in the plasma is about 20 days.

It is known that the N- terminus of HSA containing amino acid sequence N-Asp-Ala-His-Lys is the strong binding site for the divalent metal ions like Co^{2+}, Ni^{2+}, Cu^{2+}[31, 32]

Ischemia modified albumin (IMA)

Ischemia modified albumin is a term used to describe the human serum albumin having decreased binding capacity to the divalent metal ions like Co^{2+}, Ni^{2+} etc. This change in the binding ability after exposure to the ischemic conditions was first observed in 1990 by an emergency physician. N-terminus of albumin appears to be the bonding site for these metals. Initially the presence of ischemia modified albumin was utilized to detect acute myocardial ischemia. Estimation of ischemia modified albumin can be done using Albumin Cobalt Binding test.[33]

Bar-Or and others studied the binding of divalent metal ions like Co^{2+}, Ni^{2+}, Cu^{2+} to the HSA. They used HPLC, LC- MS and ^{1}H- NMR techniques to study the binding under the conditions artificially created to simulate the process of ischemia. Their study showed the decrease in the binding capacity of N- terminus of HSA to the divalent metal ions. They also demonstrated that the first three amino acids in the N-terminus Asp-Ala-His are essential for this binding. They modified the N- terminal of peptide with N- acetylation and deletion of one or more amino acids. In both the cases the binding of Cobalt is decreased.[34]

Bhagavan et. al. conducted a study on the efficacy of ACB test for assessing MI patients. They also studied Co^{2+} binding sites on the purified serum albumin by N-terminal amino acid sequencing. The results showed no change in the amino acid residues for six of seven high-ABSU (≥0.70) individuals. However, only one

individual with a high ABSU (0.80) had two missing amino acid residues from the N-terminal region.[35]

Mechanism of formation of IMA

The mechanism of formation of IMA is not known yet. The proposed mechanisms for conversion of serum albumin to ischemia modified albumin include hypoxia, acidosis, superoxide radical injury, exposure to free iron and copper etc. Reactive oxygen species are well known as factors responsible for chemical and molecular damage of many biological molecules. Most of the recent studies reported that IMA levels increased in many diseases where oxidative stress is proposed as the main mechanism supporting the hypothesis that IMA generation depends strongly on the high oxidative stress state.

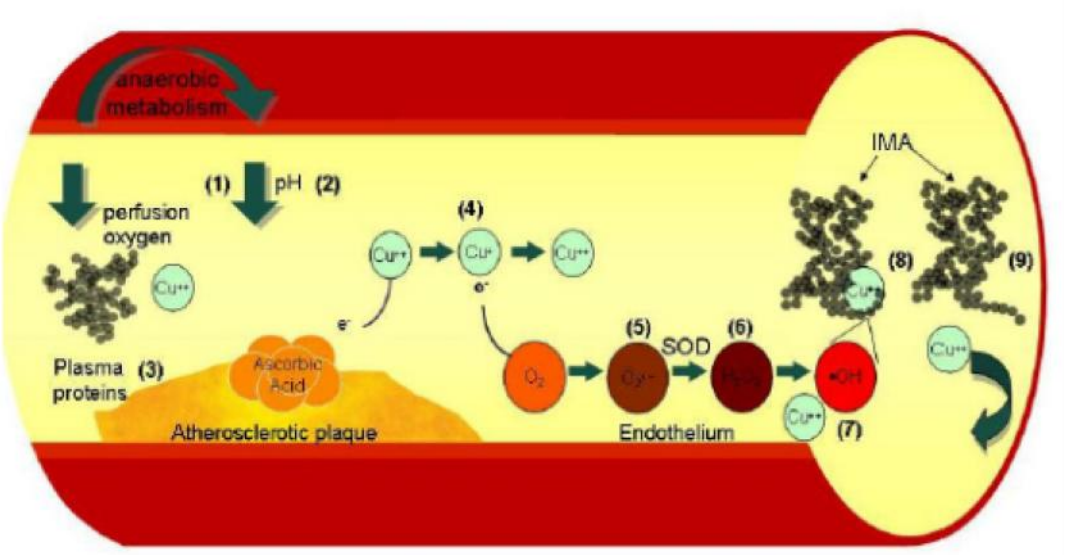


Figure 9: Postulated mechanism of IMA generation[36]

Kinetics of IMA:

It is reported that the IMA levels may be reversible which increase within 6-10 minutes of ischemia and seems to be produced continually during the acute phase of vascular injury and remain elevated during an ischemic event and return to normal level within few hours (12-24 hrs) with the removal of free radicals.[34, 37]

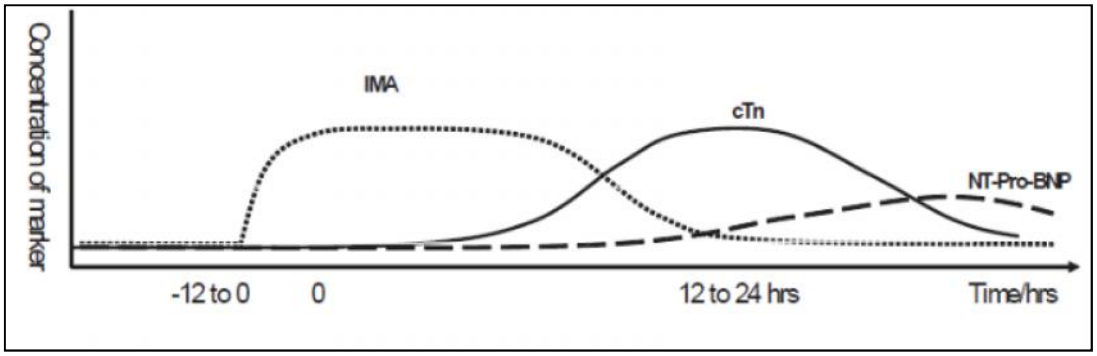


Figure 10: Kinetics of IMA along with other cardiac ischemia markers[36]

Factors affecting IMA measurement:

The stability of IMA appears to be two hours at 4°C or 20°C. Values increase significantly after four hours irrespective of storage temperature.(38) The change may be due to in vitro pH change that would alter the metal binding capacity of albumin. Samples frozen at −20 °C are stable although values have been reported slightly higher compared to freshly analyzed samples.[39]

It is known that there are many factors which can affect the IMA levels in healthy individuals. IMA levels may vary in different population. The IMA concentration may depend on the ethnic diversity depending upon geographic, dietary or environmental factors but gender differences and age differences are not known to occur in IMA levels according to a study.[40]

Serum albumin values could be expected to affect IMA estimation. A relationship exists between IMA and serum albumin levels although this is much less marked across the normal range for albumin.[41] Thus to remove the effect serum albumin concentrations use of an albumin adjusted correction has been proposed.[42] However a reference interval study found albumin correction to have little impact compared to other analytical factors.[43]

Methods for estimation of Ischemia Modified Albumin:

Albumin Cobalt Binding (ACB) test is the commonly used test to estimate indirectly the IMA. Most of the studies done in the past on the role of IMA in different clinical conditions have used this method. In this method a known amount of exogenous cobalt will be added to the patient's serum. As the ability of the HSA to bind to the divalent metal ions is decreased in ischemia, the binding of the cobalt to albumin is decreased. Then concentration of unbound cobalt is estimated using a coloring agent, Dithiothreitol (DTT). Thus the concentration of unbound cobalt is inversely proportional to the concentration of IMA.

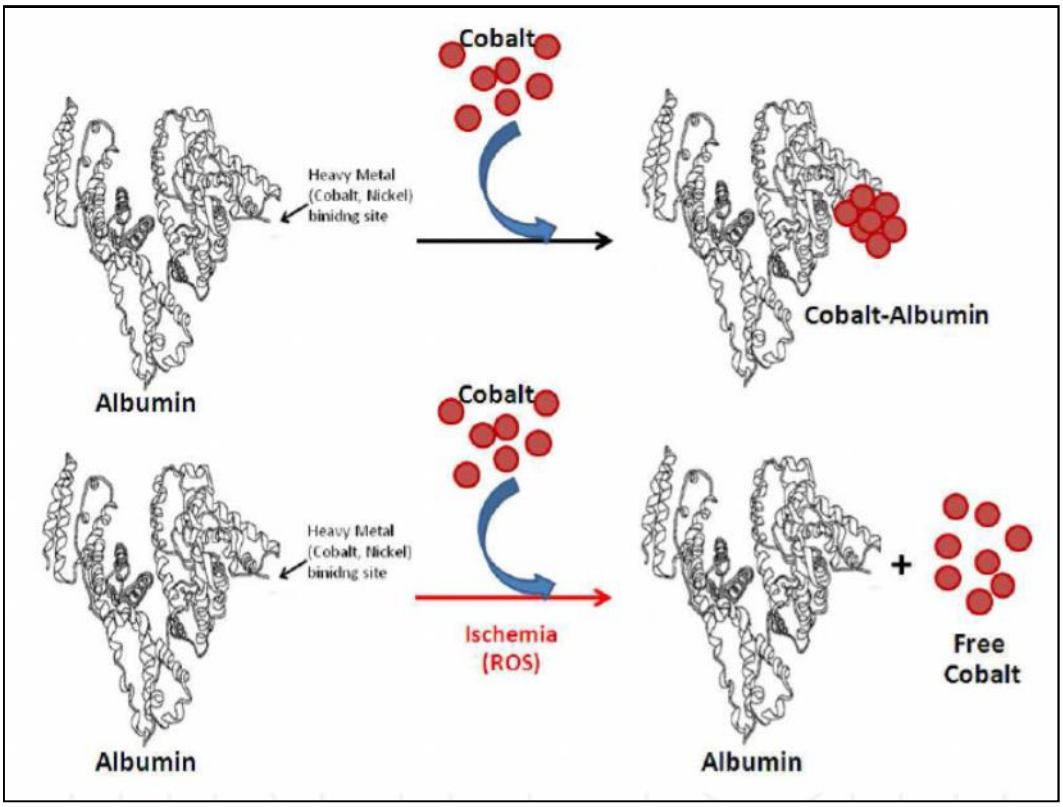


Figure 11: Principle of Albumin cobalt binding (ACB) test

This method for estimation of IMA is approved for clinical use by the Food and Drug Administration of US. Many companies have adopted this method for their automated clinical chemistry platforms. Though POCT device for estimation of IMA is not available yet for the commercial purposes, Microwells Biotechnology Co. Ltd, Shanghai, China has developed a portable spectrophotometer with IMA assay in which the DTT has been replaced with azo dye chromogen. Moreover it should be kept in mind that ACB test is an indirect measure of IMA and such albumin has not been isolated yet.

Another method using the similar principle has been reported by da Silva and others in which the binding capacity of HSA to the Nickel (Ni^{2+}) is assessed instead of cobalt. They estimated IMA concentrations in the acute coronary syndrome (ACS) patients. There was a significant correlation of the nickel binding assay with the

cobalt binding assay. Area under the curve (AUC) for nickel binding assay (0.75) was higher than the cobalt binding assay (0.72) which indicates nickel binding assay is superior in discriminating ACS patients from non- ACS patients.[44]

Eom et. al. tried to establish Albumin Copper Binding (ACuB) assay in their study. The observed stronger binding of copper ion to the HSA N-terminal peptide than cobalt ion by HPLC and ESI-TOF mass spectrometric analyses. The copper ion was employed with lucifer yellow (LY), a copper-specific reagent to develop a new albumin copper binding (ACuB) assay. Estimation of serum IMA in rats (healthy and acute stroke) showed that ACuB assay is a fairly reliable and sensitive method for the detection of ischemic states.[45]

Many other methods for the estimation of IMA have been reported in the literature. A study done by Oh et. al. utilized an immunoassay method. The method was based on the principle of sandwich ELISA. They compared the conventional colorimetric assay with the immunoassay method and found no significant correlation between the IMA values obtained from the two methods.[46]

Another study conducted by Bar-Or et. al. used High pressure liquid chromatography coupled to positive electrospray ionization mass spectrometry (HPLC/MS) to study cobalt binding to HSA. They compared the IMA concentration as estimated by the ACB test with that of HPLC/MS in ischemic and non ischemic group. They observed no difference in the Cobalt- HSA binding between the two groups when HPLC/MS is used though there was a significant difference when estimated using ACB test. They concluded that the proportion of intact N-terminus of HSA, HSA concentration, plasma cysteine/cystine ratio, plasma pH determine the

cobalt- HSA binding and hence these factors should be considered to develop improved assay methods for IMA.[47]

Luo et al. developed a rapid method using quantum dots-coupled X-ray Fluorescence Spectroscopy (Q-XRF). In this method quantum dot-coupled sandwich immunoassay is used to quantify serum total HSA, and intact HSA (iHSA) is determined using a XRF spectroscopy, by measuring XRF intensity of Co (II) bonded to iHSA. IMA concentration is by calculating the difference between total HSA and iHSA by an automated procedure. They concluded that this method can effectively eliminate the interference by the HSA concentration, high levels of cholesterol, triglycerides and hemolysis. This method showed improved sensitivity than ACB assay (95.9% vs. 82.9%) in differentiating ischemic patients from health individuals.[48]

ISCHEMIA MODIFIED ALBUMIN AS A MARKER OF ACUTE ISCHEMIA

IMA originally was studied as a marker of cardiac ischemia. There are a number of studies available in literature which have studied role of IMA in identifying acute coronary syndrome and myocardial injury at the earliest. Many studies have also tried to look into the properties of IMA, mechanisms of its generation, and compared it with already available markers of myocardial injury like CK-MB, troponins. Much of the details about IMA known today is the result of studies on IMA in ACS patients.

IMA has performed well in clinical trials studying its ability to characterize ACS patients. Blood levels of IMA rise promptly during myocardial ischemia triggered by a primary reduction of blood flow, as seen in patients undergoing

percutaneous coronary intervention (PCI), stay elevated for about 6 hours and return to baseline within 12 hours.[49, 50]

Roy and colleagues in 2004 studied the role of IMA in patients presenting with features of ACS such as acute chest pain but normal or non-diagnostic 12-lead electrocardiograms and negative cardiac troponin T. They recruited 131 patients of mean age 58.5 years. They noticed IMA levels were higher in patients with ACS (98.3 ± 11 U/ml) compared to patients without ACS (85.5 ± 15 U/ml). The difference was statistically significant ($p< 0.0001$). They further studied the diagnostic efficacy of IMA in ACS patients by plotting ROC curve in which area under the curve was 0.78 (95% CI: 0.70-0.85). At a cut off of 85 U/ml, the sensitivity was 90.6% with a specificity of 49.3% (negative predictive value = 84.6%). They concluded that IMA can be used for diagnosis of ACS in patients presenting with chest pain who have normal or non- diagnostic ECG pattern.[51]

A study done by Sinha et al. in 2004 in London evaluated the role of IMA in 208 patients presenting to the Emergency department within three hours of onset of chest pain. Results of IMA, ECG, and cTnT in those patients were analysed after final diagnosis. IMA alone showed a sensitivity of 82%, compared with 45% for ECG and 20% for cTnT alone. When used IMA with ECG or cTnT sensitivity raised to 92%. In patients with unstable angina the sensitivity of IMA was highest. Hence they concluded that IMA can be of help in identifying ACS patients particularly with Unstable Angina.[52]

Bhagavan and others evaluated the Albumin Cobalt Binding test for assessment of Myocardial ischemia in 2003. The recruited 167 patients treated at an emergency department. The study was double blinded. They estimated the IMA

concentration by Albumin Cobalt Binding (ACB) test. Measures of diagnostic efficacy were evaluated using ROC curve analysis. They expressed the concentration of IMA in mean ABSU. 0.43 ± 0.10 and 0.63 ± 0.25 were the IMA values in non-ischemic and ischemic patients respectively. There was a statistically significant increase in the IMA values in ischemic patients ($p< 0.0001$). AUC after ROC analysis was 0.95 [95% confidence interval (CI), 0.92–0.99] and sensitivity and specificity were 88% (78–94%) and 94% (86–98%), respectively, at a cutoff value of 0.50 ABSU. They concluded that ACB test could be a useful diagnostic test especially in emergency settings.[35]

Numerous studies have been reported just after the approval of IMA as an marker of cardiac ischemia by US Food and Drug Administration in 2004. A study conducted by Bakula et al. was reported in 2015 in which they tried to analyze the kinetics of IMA by using a model of exercise-stress induced transitory ischemia. They included 43 patients with positive exercise stress test and confirmed coronary artery disease and 22 healthy volunteers with negative exercise stress test. IMA levels in plasma were measured before and at nine time points after exercise over a 6-hour period. IMA levels peaked between the 3rd and 4th hour after exercise. Though they could not define an optimum time for the detection of recent myocardial ischemia by a single IMA sampling, they concluded that serial measurements of IMA can be a useful biochemical tool for the detection of myocardial ischemia.[53]

Few studies on IMA estimation in ischemic conditions other than the cardiac origin have also been reported.

Gunduz et. al. studied the role of IMA in mesenteric ischemia patients. Preliminary report of their study showed that IMA levels were statistically higher in

the thromboembolic occlusion of the superior mesenteric artery (SMA) group than 0.163 ± 0.025 ABSU in the control group. This study shows that IMA may have a place in the diagnosis of acute mesenteric embolism.[54]

Falkensammer et al. published a study in 2007 which shows the effect of skeletal muscle ischemia on serum IMA levels in healthy volunteers. In the study 12 healthy volunteers underwent standardized exercise. Ischemic conditions were achieved by inflating a femoral blood pressure cuff at incremental pressures. Magnetic Resonance Spectroscopy, intracellular concentrations of phosphocreatine (PCr) and inorganic phosphate (Pi) were used to identify calf muscle ischemia. In addition, IMA was also measured at various intervals. In their study IMA concentrations increased significantly after cuff release and returned to baseline within 30 min.[55]

Roy et. al. have also shown that change in the serum IMA levels is also associated with Peripheral vascular disease.[56] Turedi et. al. have demonstrated IMA to be having The value in the diagnosis of pulmonary embolism.[57]

IMA is not specific for ischemia. Many studies have also correlated the elevated IMA levels with the oxidative stress markers. Recently IMA has been used to differentiate different seizure types also.

Reddy and colleagues published a study in 2015 in which they recruited 23 non-diabetic subjects, 16 DM patients without any complications and 18 DR patients with an aim of assessing the association between oxidative stress and IMA levels. They measured Glutathione levels along with IMA and found out that IMA was increased in patients with high oxidative stress.[58] Similarly the serum IMA levels

have been correlated with oxidative stress markers in many diseases like chronic obstructive lung disease, ankylosing spondylitis, chronic kidney disease, acute pancreatitis, sepsis, ovarian torsion, hypothyroidism, thalassaemia and malignancy etc.(59-61). Pregnancy and pre-eclampsia is also a condition in which IMA appears to be elevated. Many studies have revealed a positive association between markers of oxidative stress and IMA.[62, 63]

Sinha et. al. published a study in 2006 in which they tried to evaluate the effects of balloon occlusion during percutaneous coronary intervention on circulating Ischemia Modified Albumin level along with transmyocardial lactate extraction. In their study IMA level was related to the number, pressure and duration of inflations which suggested IMA level is not merely a marker of free radical damage but it reflects the magnitude and duration of ischemia.[64] IMA is also known to be elevated in most patients with liver cirrhosis, acute infections and advanced cancers as all these conditions are potent producers of free radicals. IMA is also known to be increased in Diabetes Mellitus which will be discussed later in this part.

ISCHEMIA MODIFIED ALBUMIN IN ACUTE STROKE

Acute stroke is one of the many ischemic conditions wherein IMA appears to be elevated. There are a few studies available which studied the significance of IMA in different aspects in acute stroke patients. Most of the studies reported have studied the role of IMA in the diagnosis of acute stroke while some others studied its prognostic significance.

The first study reported in literature is of Abboud and colleagues. They estimated serum IMA levels in 118 consecutive patients including 84 brain infarctions

(BI), 18 brain hemorrhages (ICH) and 16 transient ischemic attacks lasting less than 1 h. They included only those patients who presented within three hours of onset of symptoms. The used Albumin Cobalt Binding test for IMA estimation. They observed increased serum IMA levels in brain infarction and intracerebral hemorrhage patients than others. Baseline serum IMA levels significantly correlated with the National Institutes of Health Stroke Scale in both infarction and hemorrhage patients. The also observed increase in IMA levels in first 24 hours in ischemic stroke patients.(65)

Gunduz et. al. published their study conducted on 106 consecutive patients involving 43 with brain infarction (BI), 11 with brain hemorrhage (ICH), 52 with sub-arachnoid hemorrhage (SAH), and a 43-member control group. Mean serum IMA levels of all the three patients groups were higher than control group. Further IMA level of brain infarction group was significantly higher than hemorrhage groups. They concluded that IMA can serve as a diagnostic marker in cerebrovascular accidents in emergency department.(66)

Nayak et. al. conducted a study to assess the prognostic significance of IMA in acute stroke patients. They recruited five patients of acute ischemic stroke and collected blood samples at 5 time intervals i.e at admission, 24^{th} hour, 48^{th} hour,72^{nd} hour and 144^{th} hour. They found elevated IMA levels at all time intervals in stroke patients than controls. They also found gradual decrease in IMA levels at 24^{th}, 48^{th} and 72^{nd} hours than 0^{th} hour which may be due to decreased oxidative stress because of treatment. But IMA level again increased at 144^{th} hour after stroke. This suggests further worsening of condition after initial improvement.(67)

Though the mechanism of generation of IMA in acute stroke is not completely understood as with the other ischemias, it appears to be the result of oxidative stress

generated during the cerebral ischemia. Several studies have shown that free radical production increases in acute stroke, particularly with ischemia and reperfusion and proposed oxidative stress as a fundamental mechanism of brain damage in ischemic as well as hemorrhagic stroke.[68, 69] Reactive oxygen species, produced during ischemia generate highly reactive hydroxyl free radicals with an impaired brain-blood barrier, resulting in site-specific modification to the N terminus of the albumin moiety, especially at the N-Asp-Ala-His-Lys sequence.

ISCHEMIA MODIFIED ALBUMIN IN DIABETES MELLITUS

IMA is known to be elevated in certain non ischemic conditions also and Diabetes Mellitus is one among them. Most of the studies reported in the literature have evaluated the association of IMA with Type 2 DM.

Chawla et. al. studied IMA as a marker of glycemic control and vascular complications in Type 2 DM patients in 100 patients. IMA was found to be elevated significantly in patients with poor glycemic control than good glycemic control as indicated by the HbA1c levels. There was a significant positive correlation between IMA and glycated hemoglobin (r^2= 0.14). IMA appeared to be related to even the lipid profile in that study. IMA level was marginally higher in diabetic patients with micro vascular complications (retinopathy and neuropathy) than without though the association was not significant.[70]

IMA was reported as an indicator of widespread endothelial damage and ischemia in diabetic patients in few studies. Ukinc et. al. recruited 50 diabetic patients without history of macrovascular complications and age matched 30 healthy individuals. IMA level was increased in diabetic than controls. IMA was also high in

nephropathy patients than without. Elevated IMA levels correlated with inflammation marker hsCRP and microalbuminuria in diabetic patients. It may thus indicate subclinical vascular pathology in diabetics.

Reddy and others performed a meta-analysis of five observational studies on the significance of ischemia modified albumin in diabetic retinopathy (DRP) patients. They observed increased mean IMA values in diabetic retinopathy patients than controls. They also observed a strong correlation with oxidative stress markers and concluded that IMA can act as a simple marker in monitoring of oxidative stress status in DRP.[71]

Few studies suggest IMA to be a glycemic marker. Ischemia modified albumin has been shown to correlate well with the fasting glucose concentration, HbA1c levels. Kaefer and others showed significant correlation of IMA with fasting glucose and hs CRP.[72] Significant correlations between IMA and total cholesterol, LDL cholesterol, oxidized low-density lipoprotein (ox-LDL) antibodies and hs-CRP levels have also been noted.[73]

It is clear from the above discussion that IMA appears to be elevated in DM and the severity, duration, glycemic control and associated complications of DM are also correlated with the IMA levels. Hyperglycemia, hypercholesterolemia and inflammation via oxidative stress and chronic hypoxia may modify albumin and hinder its capacity to bind cobalt, resulting in higher IMA levels. Hence in this study we tried to evaluate the association of IMA in DM patients who were also ischemic patients.

IMA IN STROKE IN ASSOCIATION WITH DIABETES MELLITUS

Ma et. al. conducted a study on 97 diabetic patients in which 47 were with symptomatic lacunar infarction (SLI), and 45 healthy controls. Serum IMA and homocysteine were estimated and analysed. They found increased IMA levels in patients with SLI than without. This study concluded that IMA can distinguish between SLI and nonslip patients. This was the only study which analyzed IMA level in stroke patients with DM. But they have not compared the results with non-diabetic stroke patients.

We know from the previous discussion that Ischemia Modified albumin (IMA) is elevated during many ischemic conditions such as acute coronary syndrome, acute stroke etc. So it is clear that IMA is a non specific ischemic marker. Further, IMA has been found to be increased in few non ischemic conditions as well and Diabetes Mellitus is one among them. Serum IMA levels also suggest the level of glycemic control in diabetics. Hence it can be postulated that IMA level will be more in the acute stroke patients with DM than without DM.

Stroke is one of the major macro vascular complications of DM, and poor glycemic control is a major risk factor for stroke. This further would increase the possibility of elevated IMA levels. There are no published studies in the literature which have studied this particular relation of DM with the stroke. Hence to test this hypothesis the present study was undertaken.

MATERIALS AND METHODS

Study design: Cross sectional study

Study period: One year. January 2015 to December 2015

Source of data:

This study comprises of the confirmed cases of acute stroke (diagnosed clinically and radiologically) with and without diabetes mellitus, attending Neurology Out Patient Department or Emergency and Intensive Care Unit of KLES' Dr. Prabhakar Kore Hospital and Medical Research Centre, Belagavi.

Blood samples of normal healthy volunteers who attended the blood bank of Dr. Prabhakar Kore Hospital and Medical Research Centre were taken as controls.

Sample size Calculation (n):

Considering the mean and standard deviation values from previous studies done, at 95% confidence limit and 5% tolerance level, the sample size will be 21. Considering contamination and loosing results, a sample size of 30 was taken in each of the groups.

A total of 90 subjects were recruited for the study.

- 30 Acute stroke patients of either sex with Type 2 Diabetes mellitus
- 30 Acute stroke patients of either sex without Diabetes mellitus
- 30 Normal healthy volunteers of either sex in Control group

CRITERIA FOR SELECTION OF THE STUDY GROUP

Inclusion Criteria:

- Patients presenting with the symptoms of acute stroke within 12 hours of onset of symptoms
- A confirmed diagnosis of stroke after radiological investigations like Computed Tomography (C.T.) and/ or Magnetic Resonance Imaging (M.R.I.)
- Age > 18 years
- Acute stroke patients with confirmed Type 2 Diabetes Mellitus who are on treatment for hyperglycaemia (Either oral hypoglycaemic drugs or Insulin) were included in Diabetic group.
- Acute stroke patients without the history of Type 2 Diabetes Mellitus were included in Non Diabetic group.
- Healthy subjects without known ischemic diseases like Myocardial infarction, peripheral vascular diseases, and with conditions wherein the analyte of the study may be increased like sepsis, diabetes were included in Control group

Exclusion Criteria:

- Acute stroke patients presenting after 12 hours of onset of symptoms.
- Any patient with history suggestive of recent ischemic events like acute coronary syndrome, myocardial infarction, pulmonary embolism, peripheral vascular disease etc.
- Patients with liver diseases and renal failure and acute infections, cancer and pregnancy.
- Type 1 Diabetes Mellitus
- Type 2 Diabetic patients who are not on any medication but only on diet modification therapy.

APPROVAL FROM THE AUTHORITIES:

Permission to conduct the study was obtained from all the concerned authorities viz.

1. Institutional Ethics Committee on human subjects' research of Jawaharlal Nehru Medical College, Belgaum. **(Annexure I).**
2. Director– clinical services for medical director and chief executive of KLE'S Dr. Prabhakar Kore Hospital and Medical Research Centre, Belagavi**. (Annexure II).**

OBTAINING INFORMED CONSENT

Informed consent was taken from all the participants in the study**. (Annexure III).**

METHOD OF COLLECTION OF DATA:

A. Acute stroke cases group:

- Patients with symptoms suggestive of acute stroke presenting within 12 hours of onset of symptoms were recruited initially for the study immediately after clinical diagnosis by the Neurologist.
- They were requested to participate in the study after briefing about the nature of the study and interventions needed.
- Informed consent was taken from either the patient or the relative of the patient accompanying him/ her.
- Clinical details with other relevant information were obtained.
- Blood sample was drawn from the patient.

- Patients with confirmed diagnosis of acute stroke by brain computerised tomography (C.T) and/or Magnetic Resonance Imaging (M.R.I.) were included in the case group.
- Eligible patients fulfilling all criteria for cases group were included in the study.
- Detailed history and examination details were collected as per the structured proforma**. (Annexure IV)**
- Diabetes Mellitus history was taken and included them in different groups accordingly.

B. Control group:

- Subjects who have come to the blood bank of Dr. Prabhakar Kore Hospital and Medical Research Centre for voluntary blood donation were approached.
- Informed consent was taken from the subjects.
- Detailed history was taken and examination was done.
- Eligible subjects fulfilling all criteria for control group were included in the study.
- A blood sample was drawn.

Collection and Storage of Blood Samples:

Under strict aseptic precautions, 3.5 ml of venous blood was collected into two vacutainer tubes each containing serum separator gel along with clot activator. The vacutainer tubes were allowed to stand at room temperature for 30 minutes. Then centrifugation at a speed of 5000 rpm for 10 minutes was done to separate serum from cellular fractions. Samples were analysed immediately whenever possible. All

samples were analysed in Clinical Biochemistry laboratory of Dr. Prabhakar Kore Hospital and Medical Research Centre, Belagavi and Department of Biochemistry, J.N.Medical College, Belagavi.

If the biochemical analysis was not possible immediately, the samples were stored at -20^{0}C until the biochemical evaluation. The biochemical analyses of all samples were completed within 72 hours of collection.

MATERIALS USED IN THE STUDY

1. Gold top vacutainer tubes containing serum separator gel along with clot activator
2. 10 ml Disposable syringe
3. Tourniquet, Gloves.
4. Automated pipettes and tips
5. Distilled water

INSTRUMENTS USED IN THE STUDY

1. Centrifuge machine
2. UV- Spectrophotometer
3. -20^{0}C Refrigerator
4. SIEMENS Dimension® RxL Max Clinical chemistry system

ESTIMATION OF ISCHEMIA MODIFIED ALBUMIN

Estimation of IMA was carried out using Albumin Cobalt Binding (ACB) test as described by Bar-Or et. al.[(74)]

Principle:

Human serum albumin (HSA) when comes into contact with ischemic conditions, loses its capacity to bind to the Cobalt (Co^{2+}). The decrease in the binding ability of HSA is determined by adding a known amount of exogenous cobalt to the serum. A colouring agent, Dithiothreitol (DTT) when added to the reaction tube, binds with the unbound cobalt to produce a brown colour. The intensity of the colour developed is directly proportional to the unbound cobalt. Ischemia modified albumin (IMA) can thus be estimated indirectly as the unbound cobalt increases with increase in IMA.

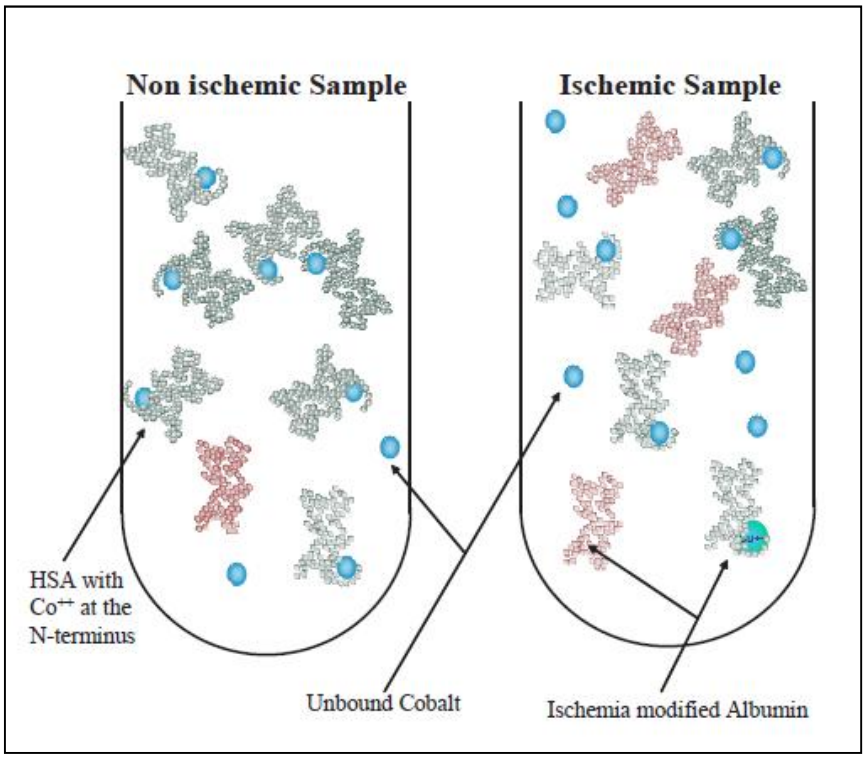


Figure 12: Principle of Albumin cobalt binding (ACB) test

Reagents:

1. Cobalt chloride solution (1 Gm/L):

100 mg of Cobalt chloride in 80 mL of distilled water and volume is made upto 100 mL.

2. Dithiothreitol solution (1.5 Gm/L):

150 mg of Dithiothreitol in 80 mL of distilled water and volume is made upto 100 mL.

3. Sodium Chloride solution (9 Gm/L):

0.9 Gm of pure Sodium Chloride in 80 mL of distilled water and volume is made upto 100 mL.

Procedure:

Table 2: Procedure of Albumin Cobalt Binding (ACB) test

Particulars	**Test**	**Blank**
Serum	200 microlitre	200 microlitre
Cobalt chloride (1 Gm/L)	50 microlitre	50 microlitre
Followed by vigorous mixing and kept 10 min for incubation at room temperature		
Dithiothreitol (1.5 Gm/L)	50 microlitre	-
Distilled water	-	50 microlitre
Incubation for 2 min at room temperature		
Sodium Chloride (9 Gm/L):	1 ml	1 ml

The absorbance of the assay mixture read at 470 nm using a spectrophotometer. Concentration of IMA is expressed in Absorbance units (ABSU)

All samples were run in duplicate and average of the two readings was taken as the IMA value.

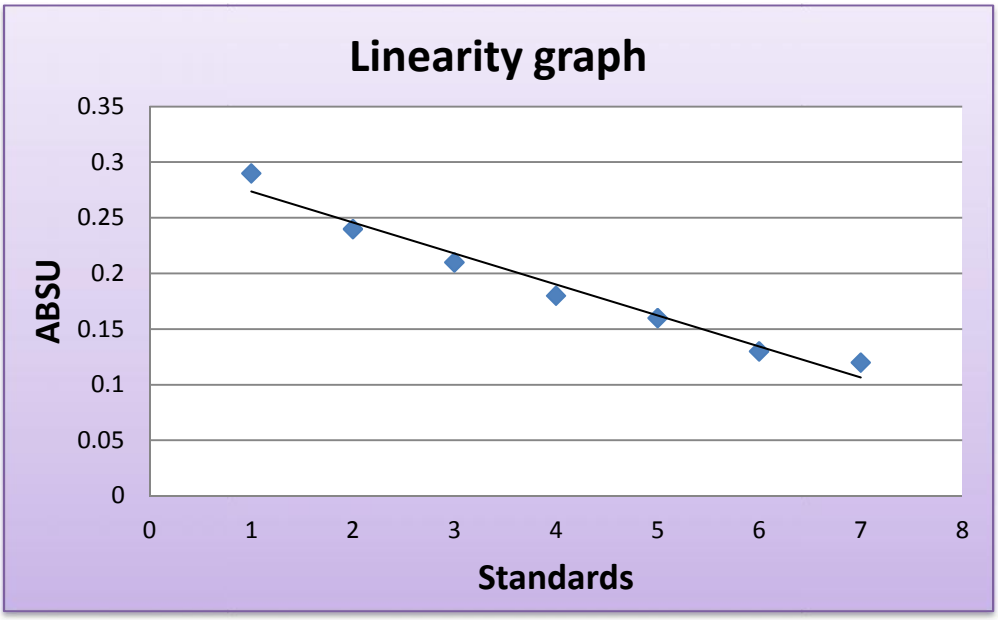
PERFORMANCE DATA OF ALBUMIN COBALT BINDING (ACB) TEST:

Study of Linearity:

Table 3: Linearity study over varied concentrations of HSA (Graph 1)

Particulars	S1	S2	S3	S4	S5	S6	S7
Vol. of HSA standard (µL)	200	200	200	200	200	200	200
Concentration of HSA (g/dL)	1.6	2.4	3.2	4.0	4.8	5.6	6.4
Cobalt chloride (1 Gm/L): µL	50	50	50	50	50	50	50
	Followed by vigorous mixing and kept **10 min** for incubation						
Dithiothreitol (1.5 Gm/L): µL	50	50	50	50	50	50	50
	Incubation for **2 min**						
Sodium Chloride (9 Gm/L): mL	1	1	1	1	1	1	1
	Absorbance of the assay mixture read at **470 nm**						
O.D (ABSU)	0.29	0.24	0.21	0.18	0.16	0.13	0.12

Graph 1: Linearity graph



Study of Precision:

Intra-day precision:

Within run (n=05)	Mean (ABSU)	SD (ABSU)	CV (%)
Pooled serum sample	0.194	0.014	7.32

Inter-day precision:

Run to run (n=08)	Mean (ABSU)	SD (ABSU)	CV (%)
Pooled serum sample	0.192	0.011	6.24

ESTIMATION OF BLOOD GLUCOSE:

Estimation of blood glucose was done using GOD- POD (Glucose oxidase-peroxidase) method on automated SIEMENS Dimension® RxL Max Clinical chemistry system. The principle of the method is as follows.

Principle:

The aldehyde group of β-D- glucose is oxidized by glucose oxidase to give gluconic acid and hydrogen peroxide.

$$\beta\text{-D- glucose} + H_2O + O_2 \xrightarrow{\boxed{\text{GOD}}} \text{Gluconic acid} + H_2O_2$$

Addition of enzyme peroxidase and a chromogenic oxygen acceptor such as 4-aminoantipyrine results in the formation of a colored compound that can be measured.

$$H_2O_2 + \text{phenol l+ 4-aminoantipyrine} \xrightarrow{\boxed{\text{PO}}} \text{Quinoninamine dye} + H_2O$$

(Colorless compound) (Coloured compound)

ESTIMATION OF SERUM ALBUMIN:

Serum Albumin was estimated using automated SIEMENS Dimension® RxL Max Clinical chemistry system with Albumin Flex@ reagent cartridge (ALB) which is based on the bromocresol purple (BCP) dye-binding method reported by Carter and Louderback, et al. In this assay serum or plasma albumin quantitatively binds to BCP to form an albumin-BCP complex that is measured as an endpoint reaction at 596/694 nm.

STATISTICAL ANALYSIS:

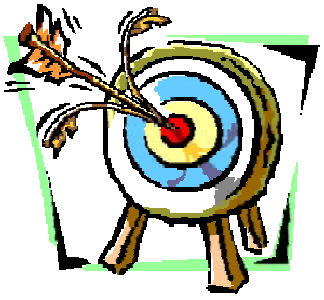
Data obtained in the study was tabulated using Microsoft Excel. All the numerical values of a group were summarized as mean± SD. Statistical analyses of the data was done using Statistical Package for Social Sciences, version 20.0 software (IBM- SPSS, Chicago, USA).

Two way ANOVA with post hoc Bonferroni test and students unpaired 't' tests were used to compare the mean and standard deviations of the three groups. p-value < 0.05 was considered as the significance level.

Pearson's correlation analyses were carried out to assess the correlation of serum albumin and Ischemia Modified Albumin in all the three groups at 5% level of significance. Pearson's correlation coefficient (r) was expressed along with the p value.

Receiver Operating Characteristic curve was drawn using SPSS software for IMA in both 'Stroke with DM' and 'Stroke without DM' groups. AUC was calculated and measures of diagnostic accuracy were also calculated at a specific cut-off.

Introduction



Objectives

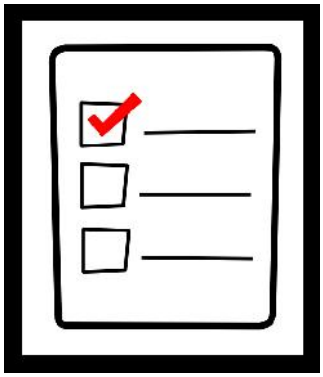
Review of Literature

Methodology

Results

Discussion

Conclusion

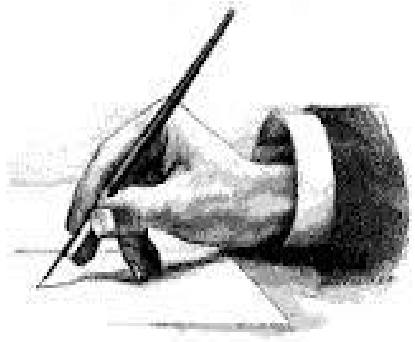


Limitations

Scope of the Study

Summary

Bibliography



Annexures

RESULTS

This cross sectional study consisted of three groups each having 30 participants. 30 patients with confirmed acute stroke based on radiological findings having a history of Diabetes Mellitus were recruited in '**Stroke with DM'** group. 30 confirmed acute stroke patients without the history of Diabetes Mellitus were recruited in '**Stroke without DM'** group. 30 apparently healthy subjects were recruited as **'Control'** group.

The data obtained was tabulated and subjected to proper statistical analyses. The results obtained were systematically described here along with the tables and graphs wherever required.

- **BASELINE CHARACTERISTICS OF THE THREE GROUPS**

Gender: Out of 30 patients in each group, in 'stroke with DM' group, 23 were males and 07 were females. Whereas in 'Stroke without DM' group, 27 were males and only 03 were females. Control group consisted of 25 males and 05 females. **(Refer Table 4 & Graph 2)**

Age: There was a statistically significant difference between the mean age of 'Stroke with DM' group and 'Stroke without DM' group when compared with the mean age of 'controls' ($p < 0.001$). However there was no statistically significant difference between two when compared with each other. **(Refer Table 4 & Graph 3)**

Hypertension (HTN) & Coronary Artery Disease (CAD): In 'Stroke with DM' group, 66% patients had history of HTN and 36% had history of CAD whereas in

'Stroke without DM' group, 53% patients had history of HTN and only 23% had history of CAD.

Duration of DM: Mean duration of DM in 'Stroke with DM' group was 7.35 ± 4.8 years

Type of Stroke: In 'Stroke with DM' group, 83% patients had ischemic stroke and 16% had hemorrhagic stroke. While in 'Stroke without DM' group, 73% patients had ischemic stroke and 26% had hemorrhagic stroke. The frequency of hemorrhagic stroke was high in non diabetic patients. . **(Refer Table 4 & Graph 4 and 5)**

Systolic and Diastolic Blood Pressure: Statistically significant difference was seen in both mean systolic & diastolic BP between controls and other two groups ($p < 0.01$). But the difference between 'Stroke with DM' group and 'Stroke without DM' group was not significant ($p = 0.521$). **(Refer Table 4)**

Mean sampling time: The time gap between the onset of symptoms of stroke and collection of blood was astonishingly same (7.98 ± 3 hours) in both 'Stroke with DM' and 'Stroke without DM' groups ($p = 1.0$) indicating there was no significant difference between the two groups. **(Refer Table 4 & Graph 6)**

- **COMPARISON OF THREE GROUPS WITH RESPECT TO ISCHEMIA MODIFIED ALBUMIN (IMA)**

Mean IMA values were high in 'Stroke with DM' (0.23± 0.03) and 'Stroke without DM' (0.21 ± 0.03) groups when compared with the 'Controls' (0.16 ± 0.03). There was a statistically significant difference between the three groups when compared by ANOVA ($p < 0.001$).

Comparison of IMA values of three groups with each other by Post-hoc Bonferroni test showed statistically significant difference between 'Stroke with DM' and 'Controls'($p < 0.001$), 'Stroke without DM' and 'Controls'($p < 0.001$). However the difference in mean IMA levels between 'Stroke with DM' and 'Stroke without DM' was not significant statistically ($p = 0.116$). **(Refer Table 5, 6 and Graph 7)**

Distribution of IMA values of all the 30 patients in 'Stroke with DM', 'Stroke without DM' and 'Control' groups can be seen in graphs 7-9 respectively. **(Refer Graphs 8, 9 and 10)**

Graph 10 shows the comparison of the distribution of IMA values of all the groups with each other. **(Refer Graph 11)**

- **COMPARISON OF THREE GROUPS WITH RESPECT TO RANDOM BLOOD SUGAR (RBS)**

Mean RBS value of 'Control' group was lower (110 ± 14.54 mg/dL) than 'Stroke with DM' (208 ± 87.68 mg/dL) and 'Stroke without DM' (131 ± 50.09 mg/dL) groups. Also the range of RBS values was very wide in 'Stroke with DM' group as evidenced by large SD. When all the three groups were compared with ANOVA, significant difference was seen.

Further the RBS values of three groups were compared with each other by Post-hoc Bonferroni test. There was a statistically significant difference between 'Controls' and 'Stroke with DM' ($p<0.001$). Also the 'Stroke with DM' group had higher mean RBS than 'Stroke without DM' and there was a statistically significant difference ($p<0.001$). No significant difference was observed in RBS values between

'Controls' and 'Stroke without DM' groups (p= 0.459). **(Refer Table 7, 8 and Graph 12)**

- **COMPARISON OF TWO PATIENT GROUPS WITH RESPECT TO FASTING BLOOD SUGAR (FBS)**

Mean FBS value of 'Stroke with DM' group (192 ± 83.53mg/dL) was higher than the 'Stroke without DM' (130 ± 40.61 mg/dL) group. Also the range of FBS values was very wide in 'Stroke with DM' group as evidenced by large SD. There was a statistically significant difference between 'Stroke with DM' and 'Stroke without DM' groups (p= 0.001). **(Refer Table 9 and Graph 13)**

- **COMPARISON OF THREE GROUPS WITH RESPECT TO SERUM ALBUMIN:**

There was a statistically significant difference between the three groups with respect to mean serum albumin values when compared with ANOVA (p< 0.01). Mean serum albumin value of 'Control' group (4.0 ± 0.4 g/dL) was higher than the 'Stroke with DM' (3.6 ± 0.6 g/dL) and 'Stroke without DM' (3.8 ± 0.4 g/dL) groups. Comparison of serum albumin values of three groups with each other by Post-hoc Bonferroni test showed significant difference between 'Control' and 'Stroke with DM' groups (p< 0.01). **(Refer Table 10, 11 and Graph 14)**

- **CORRELATION OF ISCHEMIA MODIFIED ALBUMIN WITH SERUM ALBUMIN:**

There was no statistically significant correlation between IMA and serum albumin levels in all the three groups.

Stroke with DM group: There was a statistically insignificant negative correlation between IMA values and serum albumin values (r = -0.103, p = 0.58). **(Refer Graph 15)**

Stroke without DM group: There was a statistically insignificant positive correlation between IMA values and serum albumin values (r = 0.111, p = 0.56) **(Refer Graph 16)**

Control group: There was a statistically insignificant negative correlation between IMA values and serum albumin values (r= -0.007, p = 0.96) **(Refer Graph 17)**

STUDY OF DIAGNOSTIC EFFICACY OF ISCHEMIA MODIFIED ALBUMIN

- **DIAGNOSTIC EFFICACY OF IMA IN 'STROKE WITH DM' GROUP**

Considering radiological investigations (CT/MRI) as the 'reference standard test' and IMA as the 'index test' in 30 patients of 'Stroke with DM' group and healthy 'controls' the diagnostic accuracy parameters were analyzed. Receiver Operating Characteristic (ROC) curve was plotted using SPSS software version 20.

Area under the ROC curve (AUC) was **0.968** (95% CI: 0.933- 1.000) which shows IMA is an excellent diagnostic test in stroke with DM group (p< 0.001). **(Refer Table 12 & Graph 18)**

A 2X2 contingency table was drawn and the patients were classified and arranged in the table according to the reference as well as index test results into True Positives (TP), False Positives (FP), False Negatives (FN) and True Negatives (TN). **(Refer Table 13)**

An arbitrary cut-off of **0.196 ABSU** of IMA was chosen based on the ROC curve analysis and diagnostic accuracy measures were calculated. At this cut- off sensitivity was **90%**, specificity was **87%**, Positive predictive value was **87%** and Negative predictive value was **90%. (Refer table 14).**

- **DIAGNOSTIC EFFICACY OF IMA IN 'STROKE WITHOUT DM' GROUP**

Considering radiological investigations (CT/MRI) as the 'reference standard test' and IMA as the 'index test' in 30 patients of 'Stroke without DM' group and healthy 'controls' the diagnostic accuracy parameters were analyzed. Receiver Operating Characteristic (ROC) curve was plotted using SPSS software version 20.

Area under the ROC curve (AUC) was **0.917** (95% CI: 0.847- .987) which shows IMA is an excellent diagnostic test in stroke without DM group as well ($p < 0.001$). **(Refer Table 15 & Graph 19)**

A 2X2 contingency table was drawn and the patients were classified and arranged in the table according to the reference as well as index test results into True Positives (TP), False Positives (FP), False Negatives (FN) and True Negatives (TN). **(Refer Table 16)**

An arbitrary cut-off of **0.192 ABSU** of IMA was chosen based on the ROC curve analysis and diagnostic accuracy measures were calculated. At this cut- off sensitivity was **87%**, specificity was **83%**, Positive predictive value was **84%** and Negative predictive value was **86%**. **(Refer table 17).**

TABLES

Table 4: Baseline characteristics of the three groups

Parameters	Stroke with DM	Stroke without DM	Control	P value
Total Participants	30	30	30	-
Gender				
Male	23	27	25	-
Female	07	03	05	-
Age (Mean years ± SD)	61.7 ± 10.03	60.2 ± 15.4	34.6 ± 8.44	a < 0.001 b < 0.001 c = 0.657
H/o HTN No.(Frequency)	20 (66%)	16 (53%)	-	-
H/o CAD No.(Frequency)	11 (36%)	7 (23%)	-	-
Duration of DM (Mean years ± SD)	7.35 ± 4.8	-	-	-
Type of Stroke				
Ischemic No.(Frequency)	25 (83%)	22 (73%)	-	-
Hemorrhagic No.(Frequency)	05 (16%)	08 (26%)	-	-
Systolic BP (mmHg) (Mean ± SD)	150 ± 24	146 ± 22	125 ± 10	a < 0.01 b < 0.01 c = 0.521
Diastolic BP (mmHg) (Mean ± SD)	94 ± 12	96 ± 10	82 ± 8	a < 0.01 b < 0.01 c = 0.71
Mean sampling time (Mean hours± SD)	7.98 ± 3.79	7.98 ± 3.36	-	1.00

***a** = Stroke with DM vs Control, **b** = Stroke without DM vs Control, **c** = Stroke with DM vs Stroke without DM*

Table 5: Mean and SD values of Ischemia Modified Albumin (IMA) in the three groups and results of ANOVA

Groups	N	Mean	Standard Deviation	95% Confidence Interval for Mean		ANOVA
				Lower Bound	Upper Bound	
Controls	30	0.16	0.03	0.15	0.17	F-value – 52.50 p-value - <0.001
Stroke with DM	30	0.23	0.03	0.22	0.24	
Stroke without DM	30	0.21	0.03	0.20	0.22	

Table 6: Comparison of IMA values of three groups with each other by Post-hoc Bonferroni test

Groups		Mean Difference	Significance (p- value)	95% Confidence Interval	
				Lower Bound	Upper Bound
Controls	Stroke with DM	-0.07	< 0.001	-0.08	-0.05
	Stroke without DM	-0.05	< 0.001	-0.07	-0.04
Stroke without DM	Stroke with DM	-0.01	0.116	-0.03	0.00

Table 7: Mean and SD values of Random Blood Sugar (RBS) in the three groups and results of ANOVA

Groups	N	Mean	Standard Deviation	ANOVA
Controls	30	110.0	14.54	F-value – 23.07 p-value - <0.001
Stroke with DM	30	208.4	87.68	
Stroke without DM	30	131.9	50.09	

Table 8: Comparison of Random Blood Sugar (RBS) of three groups with each other by Post-hoc Bonferroni test

Groups		Mean Difference	Significance (p- value)
Controls	Stroke with DM	98.4	<0.001
	Stroke without DM	21.93	0.459
Stroke without DM	Stroke with DM	76.47	<0.001

Table 9: Comparison of Fasting Blood Sugar (FBS) of Stroke with DM and without DM by student t-test

Groups	N	Mean	Std. Deviation	T test
Stroke with DM	30	192.03	83.53	value= 0.001*
Stroke without DM	30	130.07	40.61	

* $p < 0.01$

Table 10: Mean and SD values of Serum Albumin (HSA) in the three groups and results of ANOVA

Groups	N	Mean (g/dL)	Standard Deviation	ANOVA
Controls	30	4.0	0.40	
Stroke with DM	30	3.59	0.60	F-value – 5.432
Stroke without DM	30	3.80	0.41	p-value - 0.006*

* $p < 0.01$

Table 11: Comparison of Serum Albumin values of three groups with each other by Post-hoc Bonferroni test

Groups		Mean Difference	Significance (p- value)
Controls	Stroke with DM	0.41	0.004*
	Stroke without DM	0.20	0.326
Stroke without DM	Stroke with DM	0.21	0.293

* $p < 0.01$

Table 12: Area under the curve (AUC) for IMA in stroke with DM group

Area	Std. Error [a]	Asymptotic Significance [b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.968	.018	.000	.933	1.000

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

Table 13: 2 X 2 Contingency table for IMA in Stroke with DM group

IMA in Stroke with DM (At cut-off 0.196 ABSU)		Results as per CT/MRI	
		Stroke	No stroke
Results as per IMA test	Stroke Positive	27 (TP)	04 (FP)
	Stroke Negative	03 (FN)	26 (TN)

Table 14: Diagnostic efficacy of IMA in stroke with DM group

Diagnostic Accuracy Measures	**At a Cut off: 0.196 ABSU**
Sensitivity (%)	90%
Specificity (%)	87%
Positive Predictive value (PPV) (%)	87%
Negative Predictive value (NPV) (%)	90%

Table 15: Area under the curve (AUC) for IMA in stroke without DM group

Area	**Std. Error [a]**	**Asymptotic Significance [b]**	**Asymptotic 95% Confidence Interval**	
			Lower Bound	**Upper Bound**
.917	.036	.000	.847	.987

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

Table 16: 2 X 2 Contingency table for IMA in Stroke without DM group

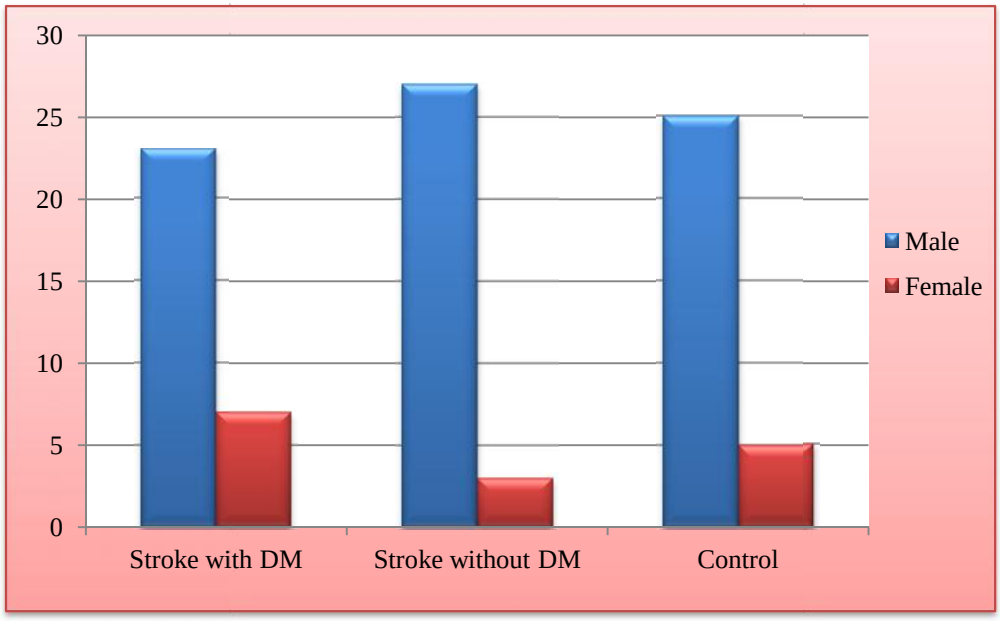
IMA in Stroke without DM (At cut-off 0.192 ABSU)		Results as per CT/MRI	
		Stroke	No stroke
Results as per IMA test	Stroke Positive	26 (TP)	05 (FP)
	Stroke Negative	04 (FN)	25 (TN)

Table 17: Diagnostic efficacy of IMA in stroke without DM group

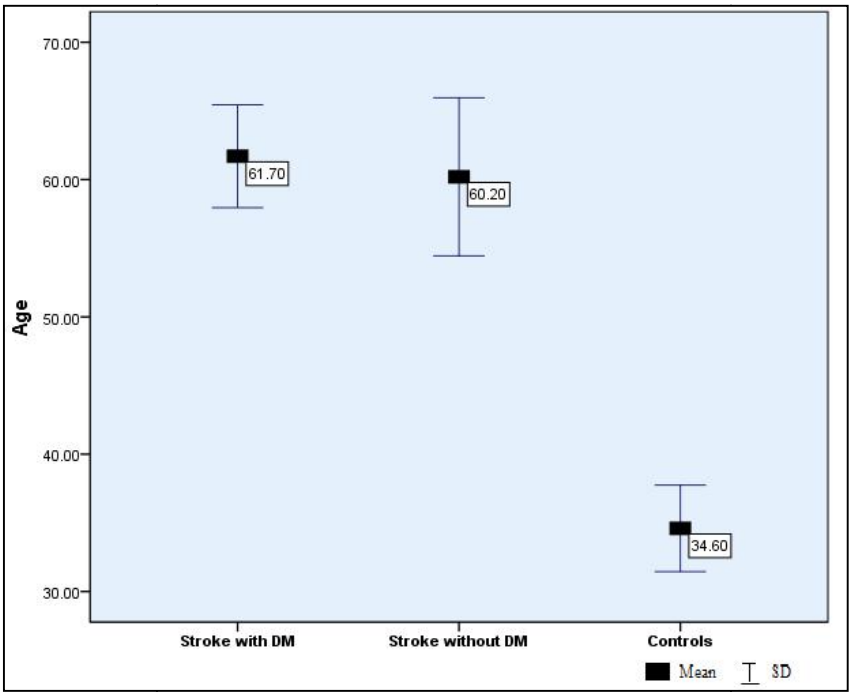
Diagnostic Accuracy Measures	**At a cut off: 0.192 ABSU**
Sensitivity (%)	87%
Specificity (%)	83%
Positive Predictive value (PPV) (%)	84%
Negative Predictive value (NPV) (%)	86%

GRAPHS

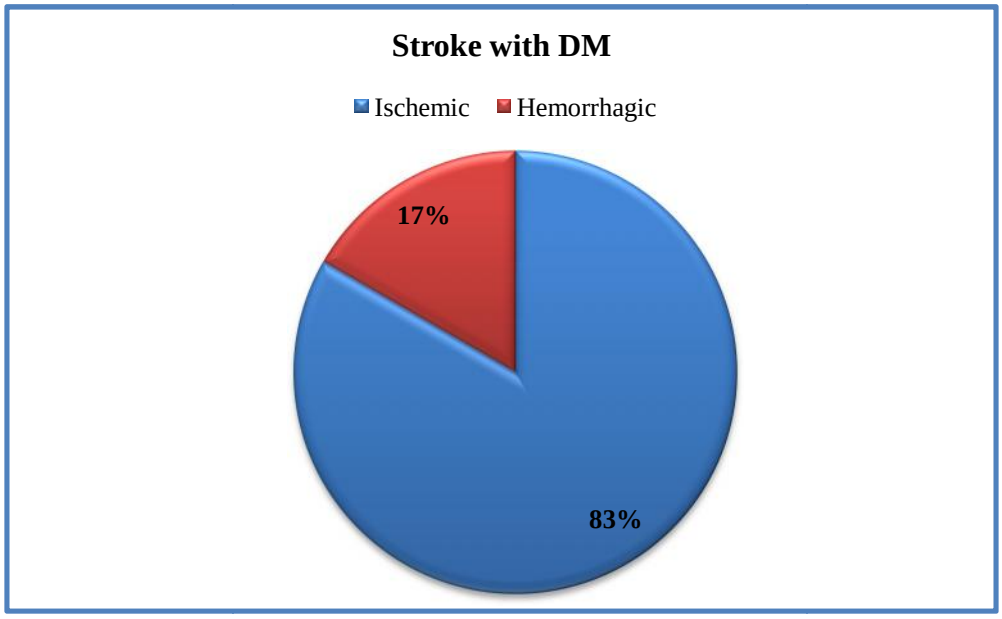
Graph 2: Gender distribution in Stroke with DM, Stroke without DM and control groups



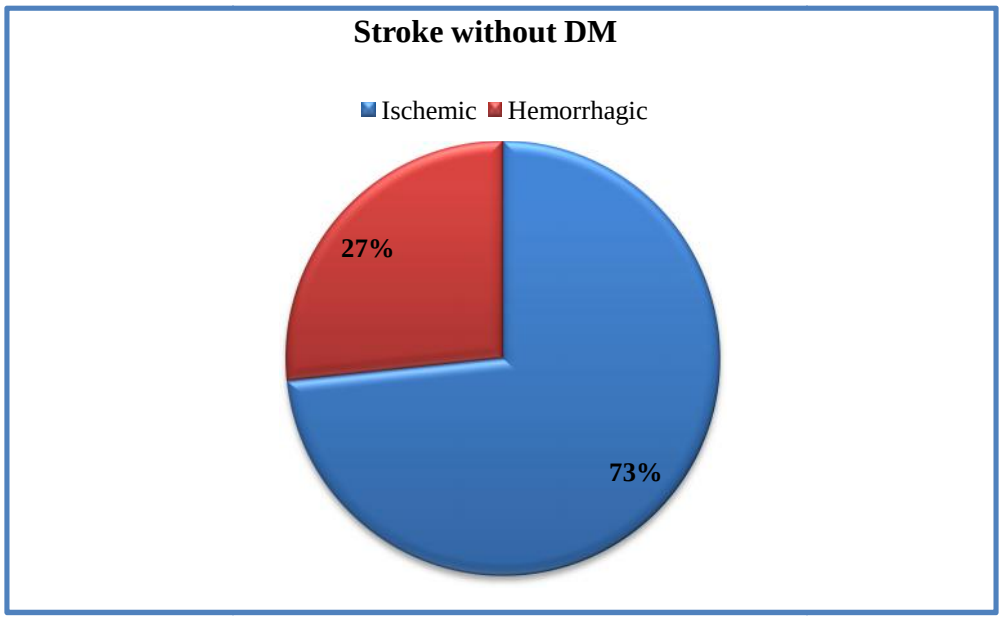
Graph 3: Age distribution in Stroke with DM, Stroke without DM and control groups



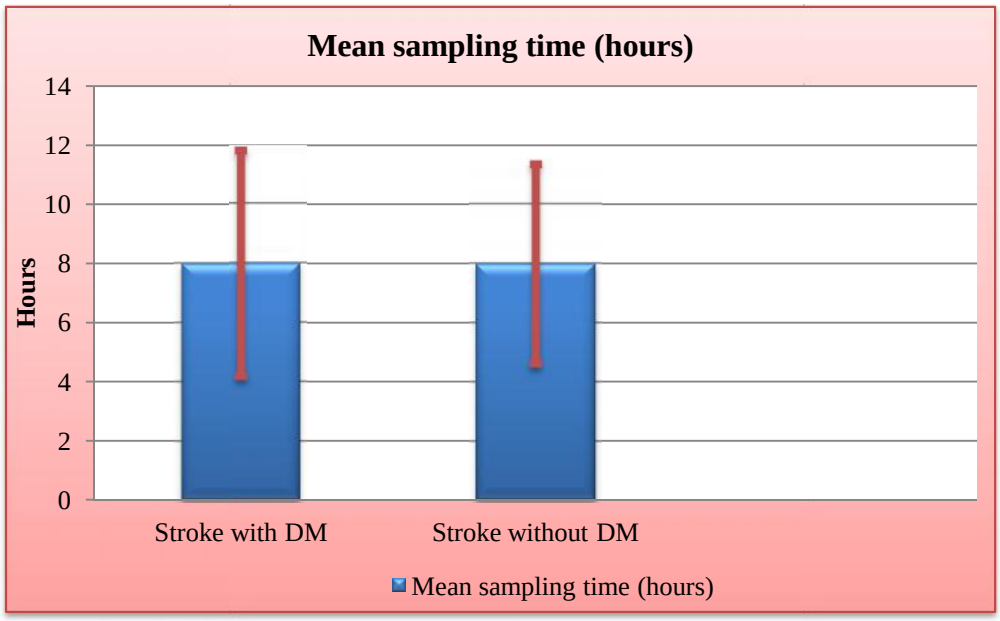
Graph 4: Frequency of different types of stroke in 'Stroke with DM' group



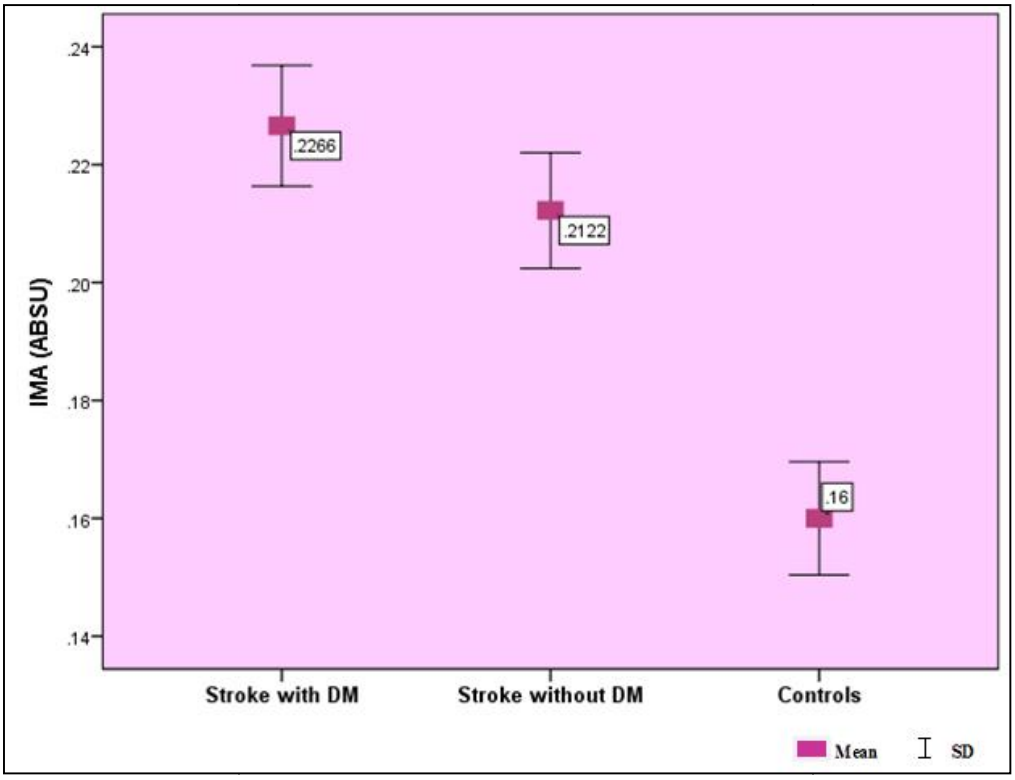
Graph 5: Frequency of different types of stroke in 'Stroke without DM' group



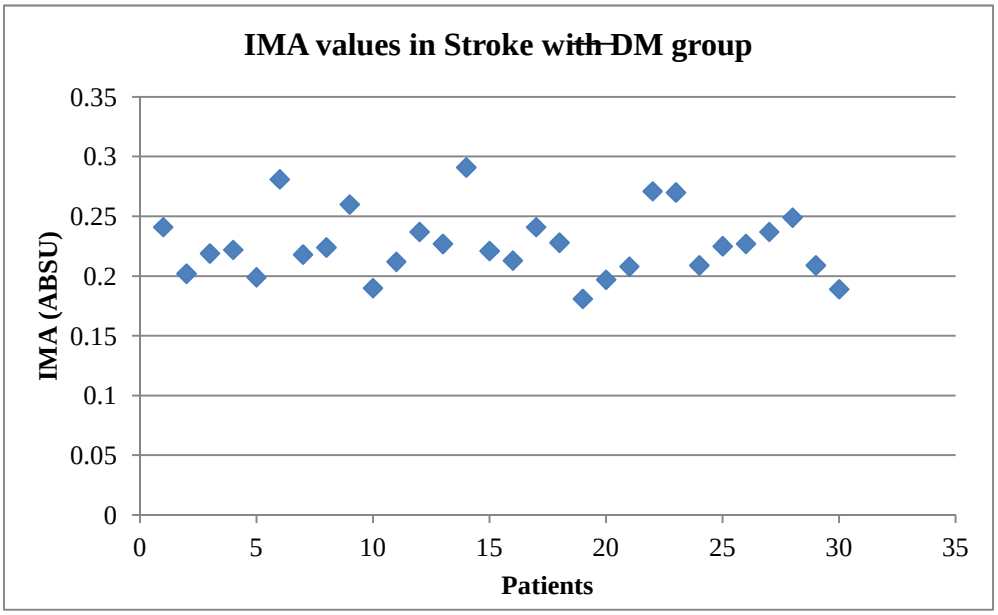
Graph 6: Mean sampling time in stroke with DM and stroke without DM groups



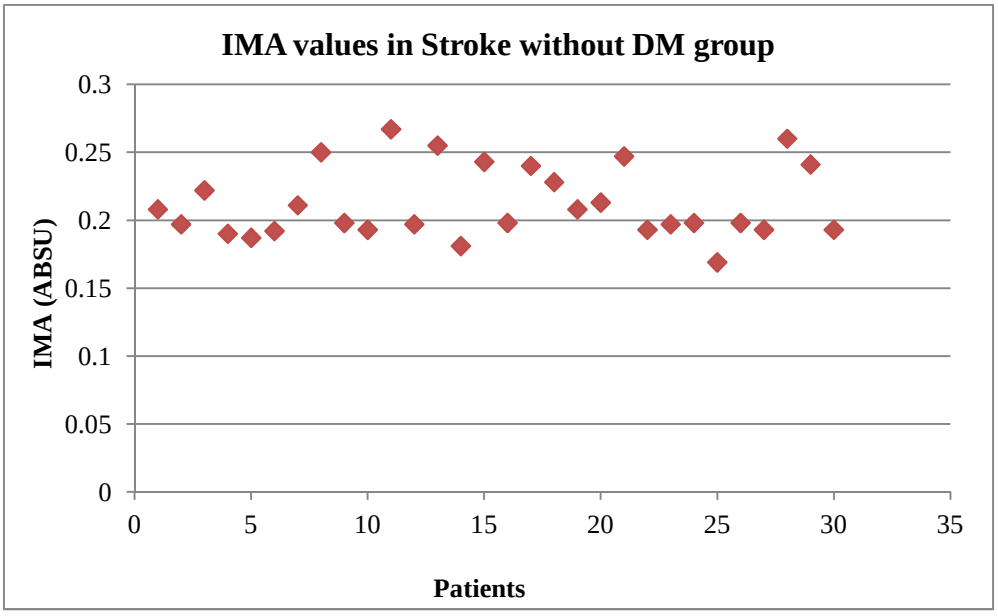
Graph 7: Comparison of Ischemia Modified Albumin (IMA) values in 'Stroke with DM', 'Stroke without DM' and 'Control' groups



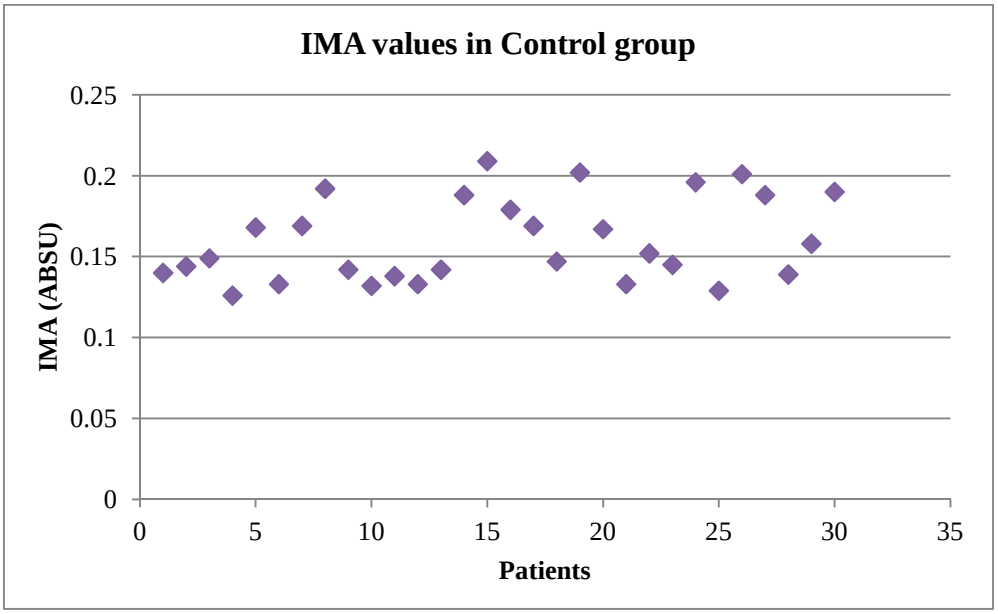
Graph 8: Distribution of Ischemia Modified Albumin (IMA) values in 'Stroke with DM', group



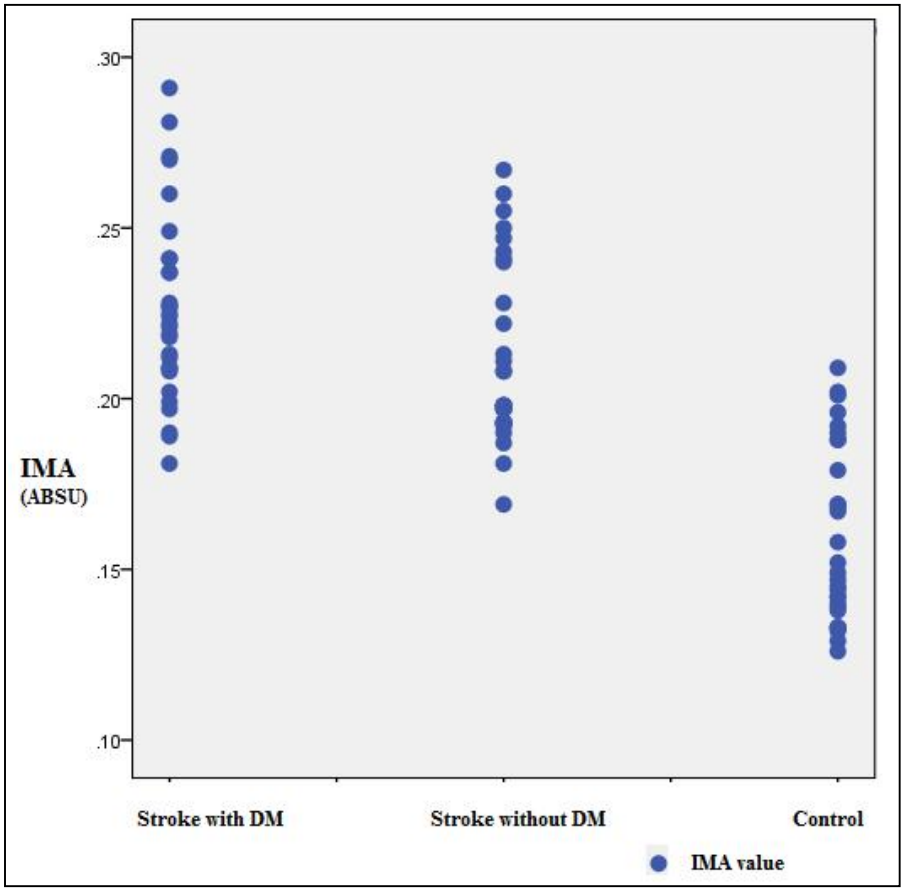
Graph 9: Distribution of Ischemia Modified Albumin (IMA) values in 'Stroke without DM' group



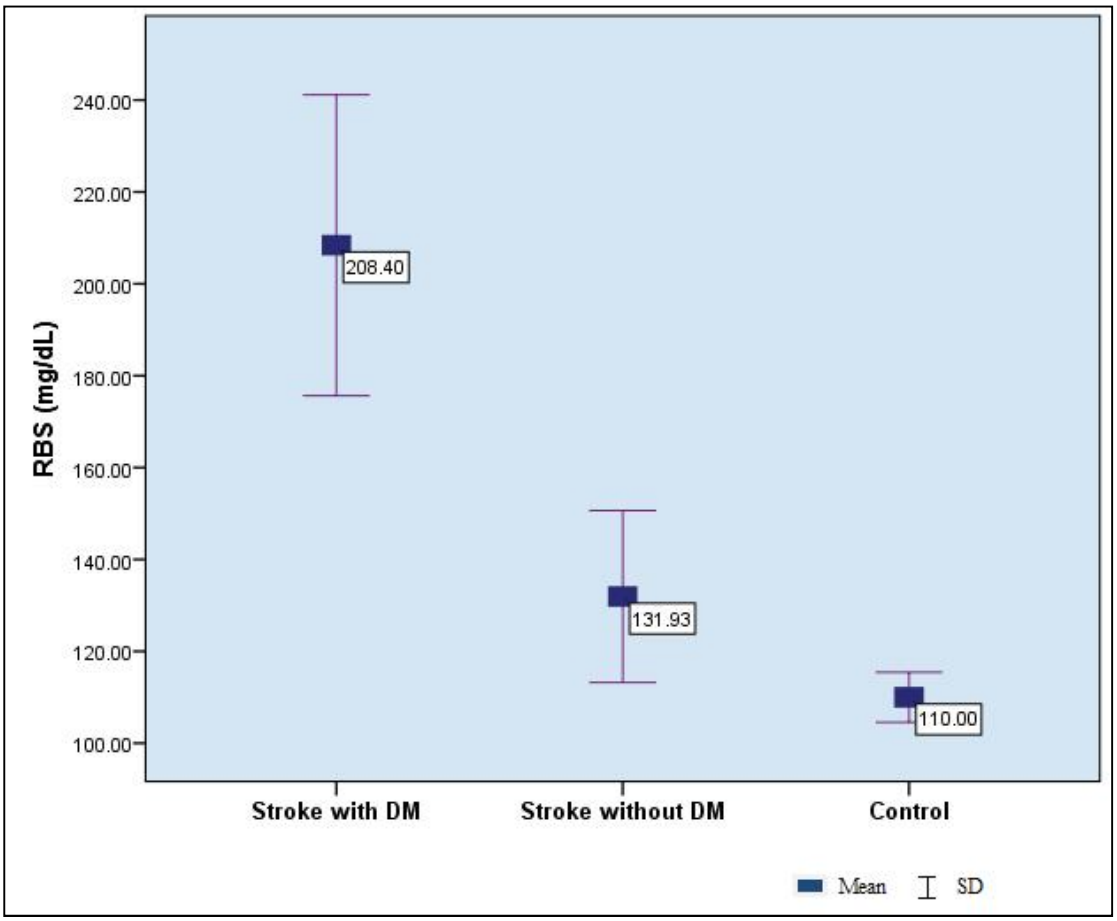
Graph 10: Distribution of Ischemia Modified Albumin (IMA) values in 'Control' group



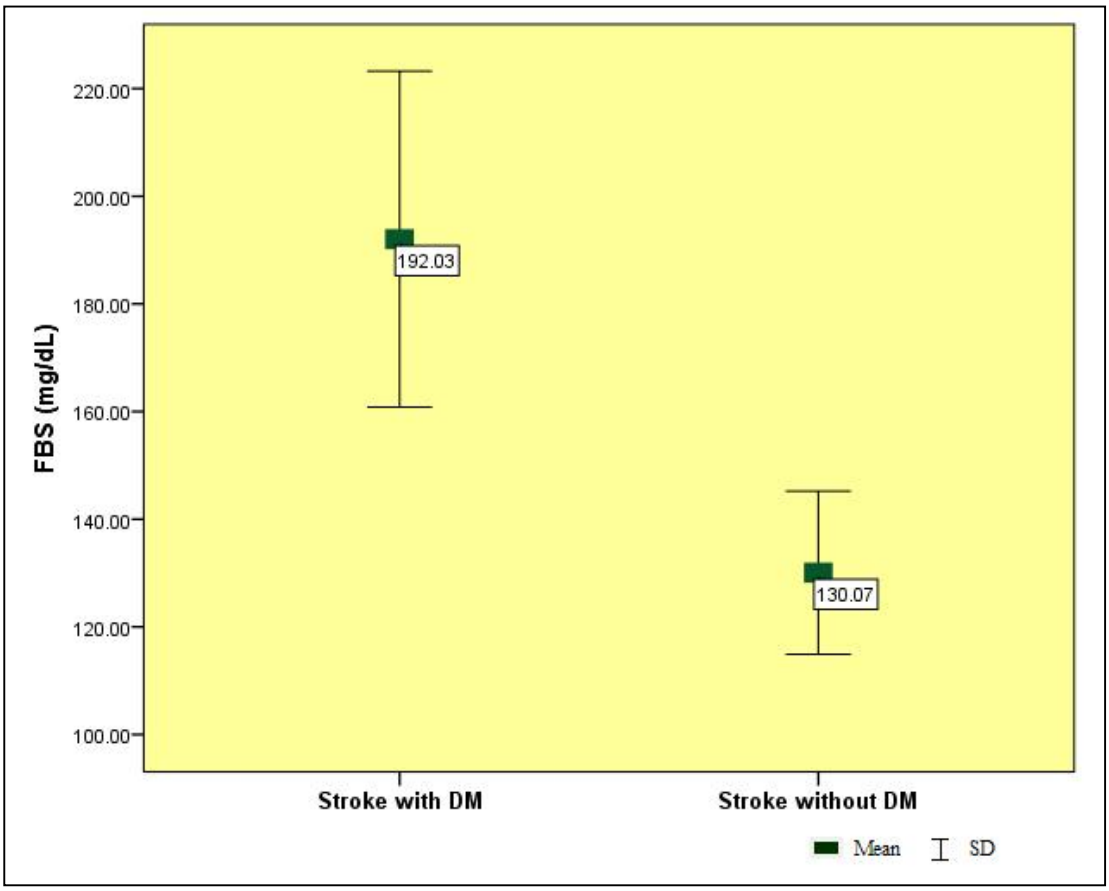
Graph 11: Distribution of Ischemia Modified Albumin (IMA) values in 'Stroke with DM', 'Stroke without DM' and 'Control' groups



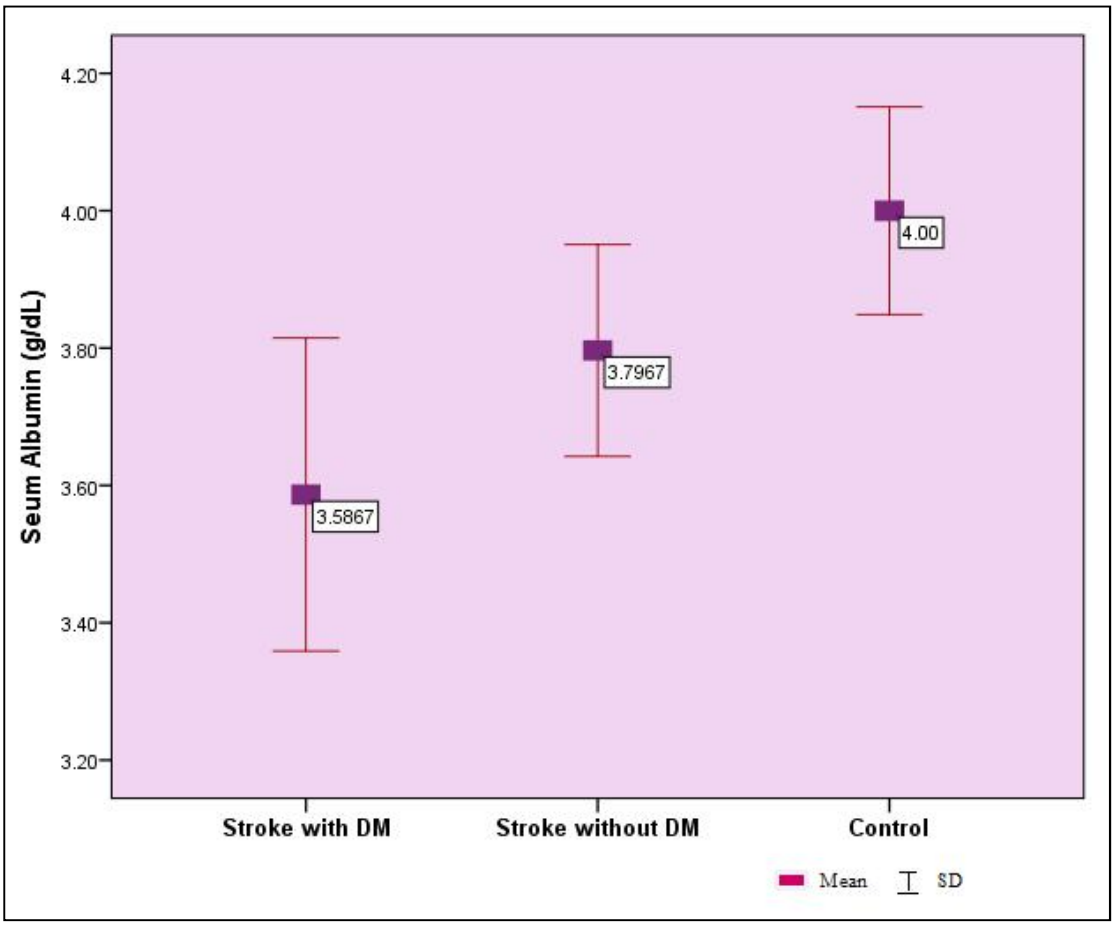
Graph 12: Distribution of Random Blood Sugar (RBS) values in 'Stroke with DM', 'Stroke without DM' and 'Control' groups



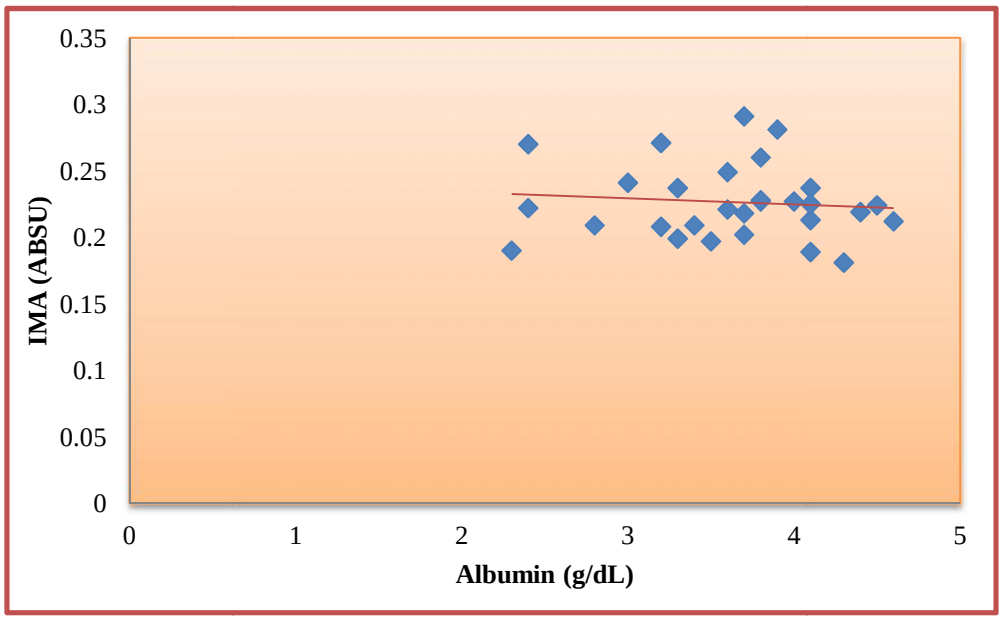
Graph 13: Distribution of Fasting Blood Sugar (FBS) values in 'Stroke with DM' and 'Stroke without DM' groups



Graph 14: Distribution of Serum Albumin (HSA) values in 'Stroke with DM', 'Stroke without DM' and 'Control' groups

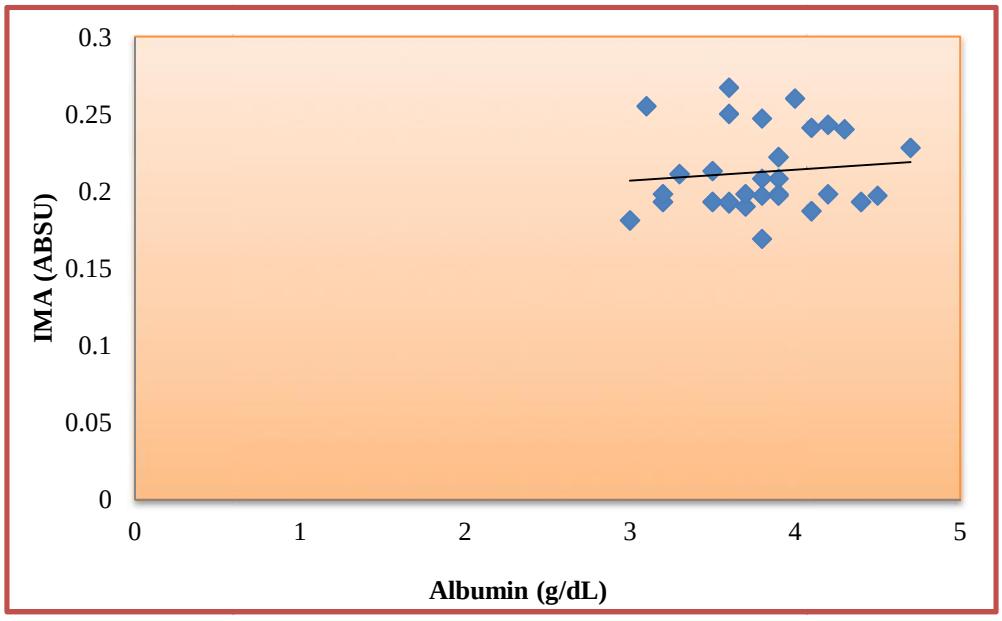


Graph 15: Correlation of Ischemia Modified Albumin with Serum Albumin in 'Stroke with DM' group



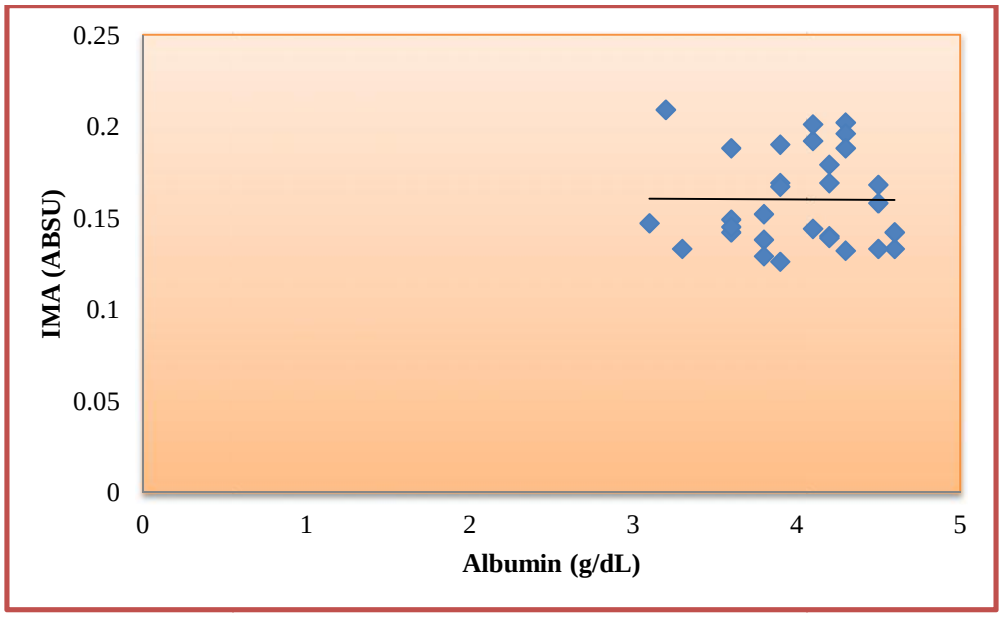
r (Correlation coefficient)= -0.103, *P* = 0.58

Graph 16: Correlation of Ischemia Modified Albumin with Serum Albumin in 'Stroke without DM' group



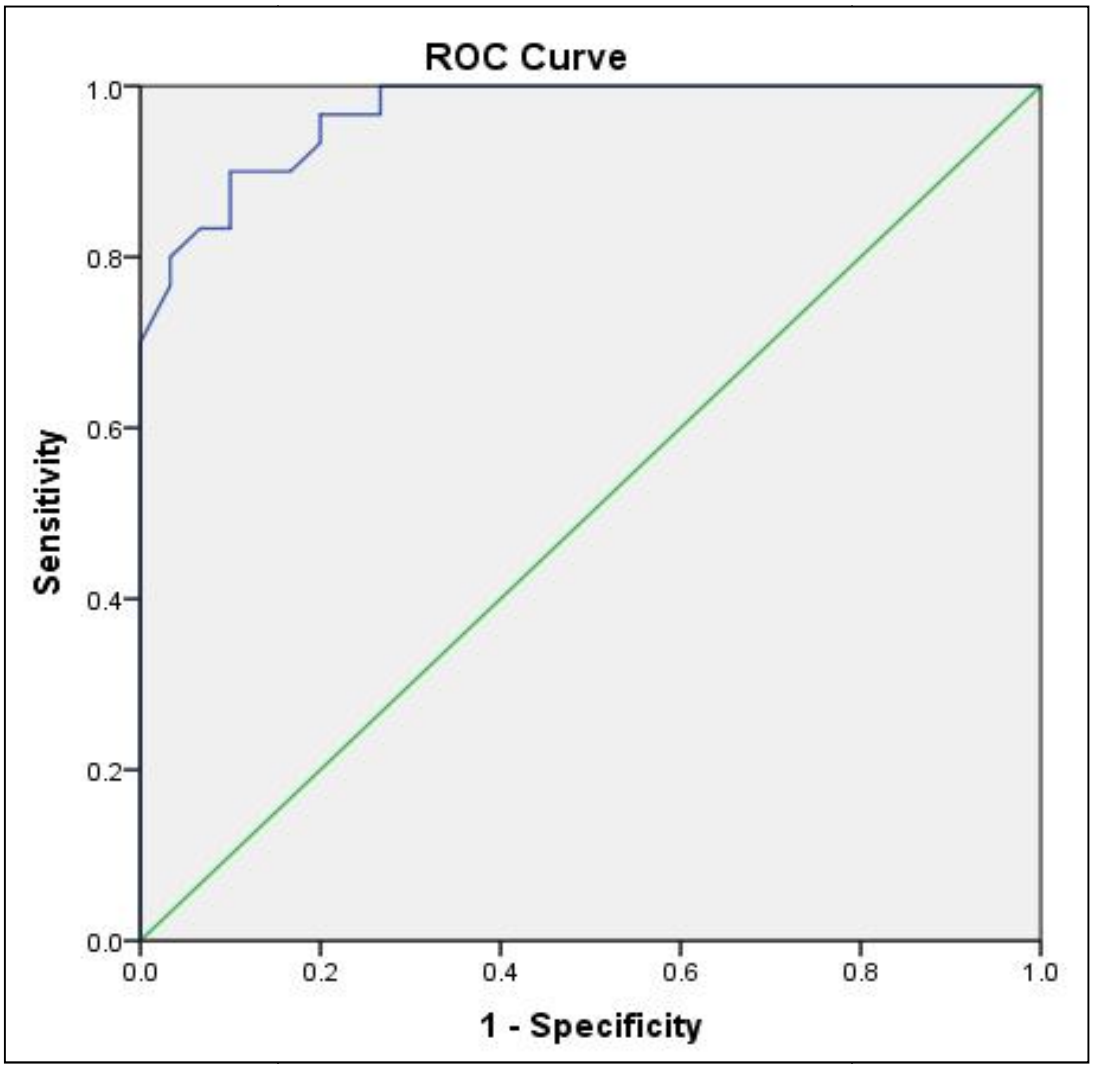
r (Correlation coefficient)= 0.111, *P* = 0.56

Graph 17: Correlation of Ischemia Modified Albumin with Serum Albumin in 'Control' group

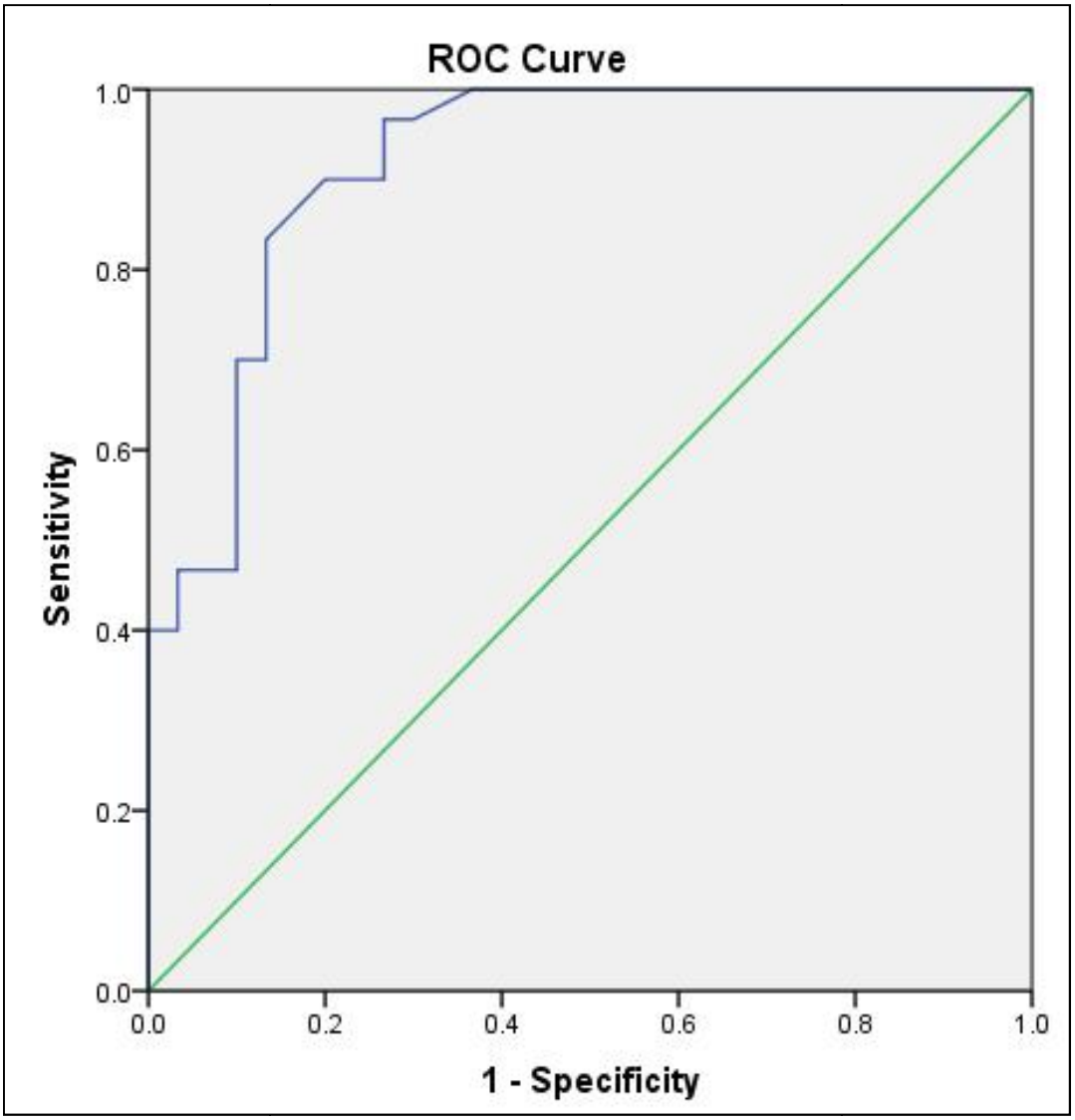


r (Correlation coefficient)= -0.007, $P = 0.96$

Graph 18: Receiver Operating Characteristic Curve for IMA in 'Stroke with DM' group



Graph 19: Receiver Operating Characteristic Curve for IMA in 'Stroke without DM' group



DISCUSSION

Ischemia Modified Albumin, the only FDA approved biomarker for ischemia has been shown to increase in various ischemic conditions including coronary artery disease, acute stroke, and mesenteric ischemia. Originally IMA was studied in acute coronary syndrome (ACS) patients for early diagnosis along with other specific markers like CK-MB, Troponins. Following the promising results in various studies of ACS patients and FDA approval for the diagnosis of myocardial injury there has been an upsurge in the studies involving IMA. Further IMA has also been shown to increase in response to non-ischemic conditions like diabetes mellitus. There are few studies on IMA levels in stroke with respect to different stroke types. But still the role of IMA in diagnosis of stroke is unclear especially in association with DM. Hence this study was undertaken to estimate and compare the IMA values in acute stroke patients with and without DM along with apparently healthy controls.

In the present study the controls were not age matched with that of stroke patients as seen by the significant difference between the mean age of patients in both 'stroke with DM' and 'stroke without DM' groups and controls. But a study conducted by Govender et al has shown that the effect of age and sex on IMA levels appears to be minimal or nil. [40]

In this study mean IMA values of both 'stroke with DM' and 'stroke without DM' groups were significantly higher than the 'control' group. This is in accordance with the many previous studies done in acute stroke patients though they did not take the effect of DM into consideration.[65, 66] In almost all of the studies which estimated and compared IMA levels in acute stroke with the controls have shown statistically

significant increase in stroke patients indicating IMA as a marker of cerebral ischemia. Most of the studies have compared the IMA levels between different stroke types. Gunduz and colleagues estimated IMA in ischemic and hemorrhagic strokes and showed that the IMA values in ischemic strokes were significantly higher than the hemorrhagic. A study conducted by Dalsania and colleagues also showed similar results wherein IMA levels were significantly higher in ischemic strokes than hemorrhagic. Only one study published by Herisson and colleagues showed the results contradicting the many other study results. In their study, they found no difference between hemorrhagic and ischemic strokes. They also found no correlation between IMA levels and Stroke severity and also the prognosis.[75] In the present study we did not separate the stroke cases based on the stroke type as the aim of our study was to evaluate the effect of DM in stroke patients on IMA values.

A few studies have been reported in the literature which evaluated the effect of DM on IMA levels though not in association with stroke. A study done by Piwowar et al revealed 75% higher IMA levels in diabetics than non diabetics.[76] They also showed that poor glycemic control is associated with higher IMA values. Hence they concluded that hyperglycemia and associated oxidative stress could be the reason for the elevated IMA levels in patients with DM. Many other studies have supported this observation. [72, 77] Contrary to this observation, Dahiya et al study showed no effect of DM on IMA levels. Their study included newly diagnosed Diabetes Mellitus patients and hence the concluded that increase in IMA is not associated with DM before the vascular complications. [9]

There were no studies in the literature reported on the effect of DM on the IMA levels in association with acute stroke. Mishra et al studied IMA levels in CAD patients with and without DM. They observed a significant increase in IMA values in CAD patients with DM when compared with non diabetic CAD patients.[78] We also observed increased IMA levels in stroke with DM patients than stroke without DM patients though the difference was not statistically significant. A number of reasons could be considered for this observation. Here we have not taken into account of the history of medication for Diabetes Mellitus while interpreting the results. Some patients of stroke with DM were on oral hypoglycemic agents like Metformin while most others were on insulin. This could have decreased the actual effect of Diabetes Mellitus.

In our study, we also estimated and compared serum albumin levels in all the three study groups. We also tried to assess the correlation of serum albumin with IMA levels in the three groups separately. Serum albumin values were higher in 'Control' group than 'Stroke with DM' and 'Stroke without DM' groups. This is in line with results of many studies which analyzed the role of albumin in acute stroke. The First National Health and Nutrition Examination Survey (NHANES I) Epidemiologic Follow-up Study showed lower serum albumin level is associated with higher stroke incidence and death risk due to stroke.[79] Xu and colleagues also observed in their Northern Manhattan Study, lower albumin levels in stroke patients with various etiologies.[80] Folsom et al reported lower serum albumin levels in diabetics than non diabetics which was also observed in our study.[81]

Gaze and others studied the correlation between serum albumin and IMA in healthy non ischemic patients and observed that there was a significant ($r = –0.888$, $p < 0.0001$) negative correlation between IMA and albumin both over the entire range of albumin concentrations and in the low albumin concentration subgroup ($r = –0.85$, $p < 0.0001$) but there was a less significant negative correlation between the two in the group with albumin levels within the reference interval.[41] A study done by Arun Kumar et al. revealed a significant negative correlation ($r = -0.473$, p value <0.001)between serum albumin levels and IMA levels in acute myocardial infarction patients.[82] No study evaluated the correlation of IMA with serum albumin in acute stroke patients. In our study, there was a negative correlation in 'Stroke with DM' and 'Control' groups though it was statistically not significant. In 'Stroke without DM' group there was a statistically not significant positive correlation.

Though there are a number of studies which have evaluated the diagnostic efficacy of IMA in acute coronary syndrome patients, studies analyzing the diagnostic efficacy measures in acute stroke patients are limited. Moreover no study has been reported in the literature evaluating the diagnostic efficacy of IMA in stroke patients grouped based on the presence of DM.

In the present study, Area Under the Curve (AUC) obtained by plotting ROC curve in both 'Stroke with DM' and 'Stroke without DM' groups showed estimation of IMA as an excellent diagnostic test with AUC being 0.968 and 0.917 respectively. At a cut off of 0.196 ABSU, sensitivity was 90%, specificity was 87% in 'Stroke with DM' group. Whereas in 'Stroke without DM' group, at a cut off of 0.192 ABSU, we reported sensitivity 87%, specificity 83%. Ahn and colleagues also reported AUC of 0.928 (95% CI: 0.857–0.999) in acute stroke group which increased to 0.990 (95%

CI: 0.970–1.000) after adjusting IMA values with the albumin values.[83]This study reported sensitivity of 87.5% & specificity of 89.3%. A study conducted by Dalsania et. al. also showed excellent diagnostic value of IMA in stroke patients with AUC of 0.951 (95% CI: 0.889- 0.984). This study predicted that IMA has a sensitivity of 94% and specificity 90%. [84]

CONCLUSION

In the present study Ischemia Modified Albumin levels were found to be significantly higher in 'Stroke with DM' and 'Stroke without DM' patients than healthy controls. Though there was no statistically significant difference in IMA levels between the two groups, 'Stroke with DM' patients had higher IMA levels than 'Stroke without DM' patients. ROC analysis showed higher AUC for IMA in 'Stroke with DM' than 'Stroke without DM' group. In both the groups IMA was found to be having excellent diagnostic efficacy for the diagnosis of acute stroke. According to the present study there was no significant correlation of serum albumin with IMA levels in both stroke patients and controls.

Hence we suggest that estimation of IMA could serve as an aid in the diagnosis of acute stroke in patients both with and without DM. Albumin cobalt binding test can be used for the estimation of IMA, which is a simple and cost-effective method. Hence IMA could be used as an early biomarker for acute stroke in various clinical settings including resource-poor settings. Further large scale studies are needed in order to confirm the above findings and to choose clinically appropriate cut-off for serum IMA levels. It is also important to standardize and validate appropriate method for IMA estimation for better comparison of results all around the world.

LIMITATIONS OF THE PRESENT STUDY:

- Sample size was less for the results to be generalized for acute stroke diagnosis.

- We did not consider the effect of treatment (either oral hypoglycemic drugs or insulin) in stroke with diabetic patients on IMA levels which could be the reason for insignificant difference between the stroke patients with and without DM.

- In our study, the time gap between onset of symptoms and collection of blood sample (mean sampling time) was slightly above (~8 hours) the reported peak time of IMA (~6 hours).

SCOPE FOR FURTHER STUDY:

- Reduction in the time gap between onset of symptoms and collection of blood sample would give better results.
- The comparison of IMA levels between hemorrhagic and ischemic stroke patients would be useful clinically for appropriate treatment.
- Further studies are required to elucidate the exact mechanism of IMA generation during acute stroke.
- Standardization of method of IMA estimation and units of expression of IMA levels will help in comparing different studies.

SUMMARY

Stroke is a leading cause of mortality and morbidity all over the world. Diabetes Mellitus is frequently associated with it. Currently the diagnosis of stroke is mainly based on neuro imaging techniques which are not devoid of limitations. Thus the alternate approach would be using biomarkers to support the clinical diagnosis of acute stroke. Ischemia Modified Albumin is one such marker of ischemia.

Primary objective of the present study was to estimate and compare the IMA levels in 'Stroke with DM', 'Stroke without DM' and 'Healthy Control' groups. Secondary objective was to assess the diagnostic efficacy of the IMA in both the patient groups. This one year cross sectional study was conducted in KLES Dr Prabhakar Kore Hospital and Medical Research Centre, Belagavi after obtaining required clearances. This study included 90 subjects of either sex, of which 30 cases of acute stroke with diabetes, 30 cases of acute stroke without diabetes and 30 normal healthy controls after obtaining informed and written consent.

Blood sample was collected from stroke patients soon after the clinical diagnosis and included in the study after radiological confirmation of acute stroke. Blood sample from healthy volunteers attending the blood bank was taken as controls. IMA was estimated using Albumin Cobalt Binding test using a spectrophotometer. Results of IMA were expressed in absorbance units (ABSU). Serum albumin was also estimated in all the subjects involved in the study. Results were tabulated and subjected to appropriate statistical analyses.

Few important observations of the present study are as follows;

- IMA levels in stroke patients both with and without DM were significantly higher than the controls.
- There was no statistically significant difference in IMA levels between 'Stroke with DM' and 'Stroke without DM' groups though the IMA levels were higher in the former group.
- The time gap between the onset of symptoms of stroke and collection of blood was ~8 hours in both the patient groups.
- There was no significant correlation between serum albumin and IMA in all the three groups.
- In 'Stroke with DM' group, area under the ROC curve was **0.968** and at a cut off of **0.196 ABSU**, sensitivity was **90%** and specificity was **87%**.
- In 'Stroke without DM' group, area under the ROC curve was **0.917** and at a cut off of **0.192 ABSU**, sensitivity was **87%** and specificity was **83%**.

Thus we conclude that estimation of IMA levels could serve as an aid in the diagnosis of acute stroke in patients both with and without Diabetes Mellitus.

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ANNEXURE I

INSTITUTIONAL ETHICAL COMMITTEE CLEARANCE

K.L.E.UNIVERSITY'S
JAWAHARLAL NEHRU MEDICAL COLLEGE,
NEHRU NAGAR, BELAGAVI-590010 (KARNATAKA-INDIA)
(Accredited 'A' Grade by NAAC)

Website: http//www.jnmc.edu
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Phone: (+ 91-(0)831 Office : 2471350
Principal: 2471701
Fax No. +91 (0)831 – 2470759

Ref: MDC/DOME/ 175 **Date: 13/11/2014**

To,

Sub: Institutional Ethical Clearance for the study.

With reference to the above, we wish to inform you that your proposed research project titled **"ESTIMATION OF ISCHEMIA MODIFIED ALBUMIN IN ACUTE STROKE WITH AND WITHOUT DIABETES MELLITUS: A ONE YEAR CROSS SECTIONAL STUDY",** is ethical and justifiable. The proposed research project has been cleared by the JNMC Institutional Ethics Committee on Human Subjects Research.

(Dr.Hema Dhumale)
Member Secretary
JNMC Institutional Ethics Committee
on Human Subjects Research,
J.N.Medical College, Belagavi.

(Dr.Ganga Pilli)
Chairman,
JNMC Institutional Ethics Committee
on Human Subjects Research,
J.N.Medical College, Belagavi.

ANNEXURE II

PERMISSION FROM HOSPITAL AUTHORITY

KLES
DR.PRABHAKAR KORE HOSPITAL
&
MEDICAL RESEARCH CENTRE
NEHRUNAGAR, BELGAUM-590 010
KARNATAKA-INDIA

ಕೆ. ಎಲ್. ಇ. ಸಂಸ್ಥೆಯ
ಡಾ. ಪ್ರಭಾಕರ ಕೋರೆ ಆಸ್ಪತ್ರೆ ಮತ್ತು
ವೈದ್ಯಕೀಯ ಸಂಶೋಧನಾ ಕೇಂದ್ರ
ನೆಹರುನಗರ, ಬೆಳಗಾವಿ–590 010 ಕರ್ನಾಟಕ, ಇಂಡಿಯಾ

Phone : 0831-2473777 (16 lines)
Fax : 0831-2470732
E-Mail : klehosp@satyam.net.in
Website : www.klehospital.org

REF. NO: KLES/DRPKHOSP/ADM/12-13/ 11961 DATE: 16/03/2015

To,

SUB: Permission to collect blood samples from Stroke patients. in the Hospital

1. Refer to your letter dated 09/03/2015 on above subject.
2. I am pleased to inform you that the Medical Director & Chief Executive has permitted you to collect 5 ml blood sample & clinical details from Stroke patients and normal healthy controls.
3. You have been granted 20% of discount for the same.
4. As per hospital policy, you are not permitted to give any information / materials / data of the hospital to be sent for publication in any form to any individual / organization, or modify, copy reproduce, republish, upload, post, transmit or distribute materials of hospital documents in any form for commercial or business use, without the prior permission of the Head of the Institution as they are intellectual property of the KLES Dr Prabhakar Kore Hospital & MRC, Belagavi.

Asst. Administrator (CS)

Copy to:-
MD & CE : Sir, for your kind information.
Chief Consultant-Neuro Med : for information & necessary action
I/C Hi-Tech Lab : ---do---

Shilpa/Adm

ANNEXURE III

CONSENT FOR PARTICIPATION IN THE STUDY

TITLE: Estimation of Ischemia Modified Albumin In Acute Stroke With And Without Diabetes Mellitus: A One Year Cross Sectional Study

Study investigator: _____ Post Graduate student (M.D. Biochemistry), J. N. Medical College, Belagavi-10.

Guide: _____

OBJECTIVE AND PURPOSE: Your patient is being invited to participate in this study on estimation of ischemia modified albumin in stroke patients- a cross sectional study.

In this study we are going to estimate the levels of Ischemia modified albumin in diabetic and non-diabetic acute stroke patients. Stroke is a condition due to loss of blood supply to the brain leading usually to paralysis of the limbs and other complications. Ischemia modified albumin is a novel biomarker for the ischemia which was approved for clinical use in Acute coronary syndrome by FDA recently. We are investigating the role of IMA in stroke patients. Stroke is a common disease in India and lot of people suffer from it. We also want to investigate role of IMA in Diabetes Mellitus patients since it is most commonly associated with Stroke. This will help to identify a cost effective, simple biomarker for early identification of stroke and treat at the earliest.

EXPLANATION OF PROCEDURE: Under aseptic precautions, 5 ml of venous blood will be collected by using a thin 22 Gauge needle prick. The collected blood will be transferred to a plain vacutainer tube and sent to the biochemical laboratory.

POSSIBLE BENEFITS: The investigator does not promise or guarantee that your patient will receive any benefit by being in the study; however, it will be aimed at better understanding of the mechanism of a new biomarker, Ischemia Modified Albumin formation and its utility in acute stroke patients.

CONFIDENTIALITY: All information collected during the course of study will be kept confidential.

WITHDRAWAL: Participation in this study is voluntary. If you don't wish your patient to participate in this study; you will not lose benefits to which your patient is entitled. After starting the study, anytime during the study, if you feel to withdraw from the study, you are free to do so.

COST OF PARTICIPATION: The cost of the study will be borne by the researcher. There will be no additional cost to you for participation in the study.

PAYMENT OF PARTICIPATION: No incentive will be paid to the patient for participating in this study

QUESTIONS:

If you or your patient has any question about their rights as a study participant, you may also contact,

DR. (MRS) N.S. MAHANTASHETTI. Principal, J. N. Medical College, Belagavi

OR DR. GANGA S.PILLI, Chairman, Institutional Ethics Committee on human subjects' research, J. N. Medical College, Belagavi Phone no.:0831- 2471702(O).

LEGAL RIGHTS: By signing this consent form, you are not waiving any of your patient's legal rights.

PUBLICATION RIGHTS: The result of this study will be used for teaching and medical publication; however the patient's identity will be kept confidential.

Name of the patient: IP/OP No.:

CONSENT STATEMENT:

"I am giving consent on behalf of my patient that, my patient will volunteer and participate in this study. I have read the content or it has been read to me and my patient in the language I can understand. This study has been fully explained to me and my patient and I may ask any questions at any time."

Name of patient's Relative:

Signature/ Thumb impression of patient's Relative:

Relation to the patient:

Name of witness:

Signature of witness:

Date:

Place: Signature of the investigator

ANNEXURE IV

PROFORMA FOR PATIENT DATA

Name:

Age/sex:

Address:

Contact number:

Socioeconomic status:

IP / OP no.

Date of admission:

Admitted in:

Time elapsed since onset of symptoms:

(Time of sample collection)

Weight: Height: Body mass index:

History

Presenting symptoms & Brief History:

Past history:

Family history:

Personal history:

Treatment history:

General physical examination

Pulse: RR: BP:

Temperature:

C.N.S. Examination

Higher mental functions:

Cranial Nerve examination:

Sensory system:

Motor system:

Provisional Diagnosis:

CT/MRI:

Final diagnosis:

BIOCHEMICAL INVESTIGATION RESULTS:

Ischemia modified albumin (ABSU):

Serum albumin:

Random Blood Sugar:

Fasting Blood Sugar:

ANNEXURE V

MASTER CHART

'Stroke with Diabetes Mellitus' Group									
Sl. No.	**Age (Yrs)**	**Sex**	**Diabetes duration (Yrs)**	**Stroke Type**	**Time gap (Hours)**	**RBS (mg/dL)**	**FBS (mg/dL)**	**Albumin (g/dL)**	**IMA (ABSU)**
1	56	M	10	IS	9	240	240	3	0.241
2	63	M	2	HS	12	198	223	3.7	0.202
3	67	M	20	IS	12	192	109	4.4	0.219
4	64	M	15	IS	2	435	437	2.4	0.222
5	69	M	4	IS	6	112	110	3.3	0.199
6	68	M	10	IS	1	114	77	3.9	0.281
7	68	F	9	IS	12	156	135	3.7	0.218
8	44	M	2	IS	4	405	336	4.5	0.224
9	78	M	10	IS	12	113	121	3.8	0.26
10	60	M	8	IS	12	187	209	2.3	0.19
11	55	F	5	IS	12	158	116	4.6	0.212
12	70	M	6	HS	10	311	264	4.1	0.237
13	70	F	5	IS	10	215	231	3.8	0.227
14	56	M	0.9	IS	7	214	236	3.7	0.291
15	58	M	5	IS	3	244	302	3.6	0.221
16	65	F	10	IS	8	184	180	4.1	0.213
17	65	F	8	IS	12	252	196	3	0.241
18	71	F	20	IS	4.5	241	283	3.8	0.228
19	55	M	6	HS	12	178	114	4.3	0.181
20	65	M	7	HS	1	350	220	3.5	0.197
21	53	M	5	HS	12	203	191	3.2	0.208
22	72	M	10	IS	12	213	207	3.2	0.271
23	65	M	5	IS	8	308	293	2.4	0.27
24	69	F	8	IS	10	112	140	3.4	0.209
25	45	M	1.5	IS	5	93	90	4.1	0.225
26	42	M	1	IS	4	173	176	4	0.227
27	35	M	2	IS	7	283	173	3.3	0.237
28	65	M	8	IS	3	135	112	3.6	0.249
29	70	M	7	IS	8	132	110	2.8	0.209
30	68	M	10	IS	9	101	130	4.1	0.189

Sl. No.- Serial Number, M- Male, F- Female, IS- Ischemic Stroke, HS- Hemorrhagic Stroke, RBS- Random Blood Sugar, FBS- Fasting Blood Sugar, IMA- Ischemia Modified Albumin

MASTER CHART

'Stroke without Diabetes Mellitus' Group								
Sl. No.	**Age (Yrs)**	**Sex**	**Stroke Type**	**Time gap (Hours)**	**RBS (mg/dL)**	**FBS (mg/dL)**	**Albumin (g/dL)**	**IMA (ABSU)**
1	75	M	IS	12	160	138	3.9	0.208
2	63	M	IS	8	120	67	4.5	0.197
3	59	M	HS	9	111	109	3.9	0.222
4	86	M	IS	4	140	131	3.7	0.19
5	47	M	IS	6	126	118	4.1	0.187
6	62	M	IS	10	121	105	3.6	0.192
7	67	M	IS	5.5	163	146	3.3	0.211
8	28	M	IS	4.5	113	146	3.6	0.25
9	45	M	IS	3	114	77	3.9	0.198
10	28	F	IS	12	115	110	3.2	0.193
11	75	M	IS	1.5	193	184	3.6	0.267
12	81	M	IS	9	110	165	3.8	0.197
13	50	M	IS	12	112	122	3.1	0.255
14	80	M	HS	12	116	251	3	0.181
15	64	M	IS	4	90	120	4.2	0.243
16	43	M	IS	8	123	119	4.2	0.198
17	66	M	IS	7	146	131	4.3	0.24
18	58	F	HS	10	145	136	4.7	0.228
19	35	M	IS	7	118	108	3.8	0.208
20	50	M	HS	11.5	371	249	3.5	0.213
21	60	M	HS	10	126	99	3.8	0.247
22	60	M	HS	4	118	139	4.4	0.193
23	74	M	IS	12	102	125	3.9	0.197
24	56	M	IS	12	136	149	3.2	0.198
25	58	M	IS	10	132	101	3.8	0.169
26	76	M	IS	8	112	100	3.7	0.198
27	72	M	HS	8	95	95	3.6	0.193
28	48	M	IS	6	101	106	4	0.26
29	58	F	HS	1.5	128	136	4.1	0.241
30	82	M	IS	12	101	120	3.5	0.193

Sl. No.- Serial Number, M- Male, F- Female, IS- Ischemic Stroke, HS- Hemorrhagic Stroke, RBS- Random Blood Sugar, FBS- Fasting Blood Sugar, IMA- Ischemia Modified Albumin

MASTER CHART

'Control' group					
Sl No.	**Age (Yrs)**	**Sex**	**RBS (mg/dL)**	**Albumin (g/dL)**	**IMA (ABSU)**
1	40	F	98	4.2	0.14
2	31	M	103	4.1	0.144
3	33	M	111	3.6	0.149
4	48	M	89	3.9	0.126
5	46	M	109	4.5	0.168
6	43	M	98	4.6	0.133
7	30	M	107	3.9	0.169
8	38	M	121	4.1	0.192
9	49	M	112	3.6	0.142
10	24	F	109	4.3	0.132
11	23	M	130	3.8	0.138
12	35	M	126	3.3	0.133
13	34	M	97	4.6	0.142
14	30	M	123	3.6	0.188
15	24	M	115	3.2	0.209
16	21	M	108	4.2	0.179
17	39	F	100	4.2	0.169
18	32	F	104	3.1	0.147
19	50	M	81	4.3	0.202
20	37	M	110	3.9	0.167
21	29	M	109	4.5	0.133
22	32	M	94	3.8	0.152
23	32	M	107	3.6	0.145
24	38	M	124	4.3	0.196
25	41	M	135	3.8	0.129
26	23	M	119	4.1	0.201
27	24	F	109	4.3	0.188
28	27	M	150	4.2	0.139
29	38	M	113	4.5	0.158
30	47	M	89	3.9	0.19

Sl. No. - Serial Number, M- Male, F- Female, RBS- Random Blood Sugar, IMA- Ischemia Modified Albumin