"Activities of Fetuin-A protein in type-2 diabetic nephropathy patients"

Thesis submitted for the award of the degree of
Doctor of Philosophy
in
Medical Biochemistry

By

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ACKNOWLEDGEMENT

My deepest gratitude to **God Almighty** for illuminating my path with wisdom, resilience, and the determination to complete this work.

I express my heartfelt gratitude to all those who have contributed immensely in my work without whose support it would have been impossible to complete the project.

I would like to express my deepest gratitude to my respected supervisor, **Dr. Nilima Dongre,** Professor Department of Biochemistry, for her unwavering support, invaluable mentorship, and constant encouragement throughout the course of my research. Her insightful guidance, patience, and dedication have been a source of inspiration and motivation at every stage of this work. She has not only enriched my academic journey with her profound knowledge and experience but has also helped me grow as a researcher and an individual. Her constructive feedback and thoughtful suggestions have been instrumental in shaping the direction and quality of this thesis. It has been a true privilege to work under her guidance, and I am sincerely thankful for the opportunity. I look forward to continuing my research journey under her esteemed guidance in future endeavours as well.

I also thank **Dr. J G Ambekar** former guide for his guidance in my work. I am indebted to **Dr. S M Goornavar**, Professor, Department of Medicine, S Nijalingappa Medical College, Bagalkot, Karnataka, my coguide, who has always supported me unconditionally and guided me enthusiastically.

I express my sincere thanks to **Dr. Nandish Kadakol**, Assistant Professor, Medical genetics. I am thankful to **Dr. Deepa Sajannar**, Professor & Head, Department of Biochemistry, as well as to the esteemed faculty members, along with the staff of **BLDE (Deemed to be University)** for their constant support and cooperation. Their encouragement and collaborative spirit greatly contributed to the successful completion of my research.

I take this opportunity to thank the Chairman of Doctoral Committee, **Prof. Kusal K. Das,** Distinguished Chair Professor, Laboratory of Vascular Physiology and Medicine, Department of Physiology, Member Secretary of Doctoral committee and other Doctoral committee members, **Dr. Tejaswini Vallabha** Dean, Faculty of Medicine, BLDE (Deemed to be University)'s Shri B. M. Patil Medical college. Hospital and Research Centre, Vijayapura, **Dr. Shailaja Patil** Professor, Department of community medicine, BLDE (Deemed to be University's Shri B. M. Patil Medical college, Hospital and Research Centre, Vijayapura, for the valuable suggestions and timely advice which were vital for the completion of my research work.

I am grateful to **Dr. R. S. Mudhol**, **Vice-Chancellor**, BLDE (Deemed to be University), Pro-Chancellor, **Dr. Y. M. Jayaraj**, **Dr. Arun C. Inamdar, Pro- Vice-Chancellor,** BLDE (Deemed

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Hospital and Research Centre, Vijayapura, **Mr. Satish Patil, Deputy Registrar**, BLDE (Deemed to be University), Vijayapura, for their valuable support and encouragement.

I thank **Dr. Prasanna Kumara B.M,** Chief Librarian and **Shri. Shivakumar Acharya,** Assistant librarian of BLDE (Deemed to be University)'s Shri B. M. Patil Medical college, Hospital and Research Centre, Vijayapura for their timely help.

I am extremely grateful to the Chairman of BVV Sangha, Bagalkot. **Dr. Veeranna C Charantimath, Shri. Ashok M. Sajjan (Bevoor)** Governing Council Chairman, Dean, **Dr. Bhuvaneshwari C Yelamali** for their continuous support .

I am grateful to my department Professor & Head **Dr. S V Kashinakunti** for his constant support and also **Dr Basavaraj D** , **Dr Shankarprasad DS**, **Dr Sunitha H, Dr Neela M, Dr Kavitha M M, Ms. Meetu** and **Mrs.Supriya** for giving me constant support and encouragement to complete this project.

I also thank technical and clerical staff of Biochemistry and Central clinical laboratory, S. Nijalingappa Medical College, Bagalkot, Karnataka, for providing the facilities, timely help and support in every aspect. It gives me immense pleasure to express my utmost sincere gratitude to **Dr. Sanjeev Ratna,** Professor and **Dr. Mahantesh B Bhutal,** Associate Professor of Biochemistry. S. Nijalingappa Medical College, Bagalkot, Karnataka, for guiding and supporting me at every step of my work.

I am grateful to my husband, **Arunkumar B R**., my daughter **Ritanya** and my parents for supporting me unconditionally at every step. I thank all my family members for their support and encouragement throughout my course. I thank all my friends who have helped and supported me in completing this work

I thank all the participants of the study who have been very kind and co-operative.

Finally, I thank every person who has helped me directly or indirectly throughout the course.

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LIST OF ABBREVIATIONS

Abbreviations	Full form
ADA	American diabetes association
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
DKD	Diabetic kidney disease
FBS	Fasting blood sugar
PPBS	Post prandial blood sugar
HbA1c	Glycated hemoglobin
DN	Diabetic nephropathy
IR	Insulin resistance
GBM	Glomerular Basement Membrane
ESRD	End stage renal disease
KDIGO	Kidney disease improving global outcomes
eGFR	Estimated glomerular filteration rate
AHSG	A-2 hereman schimid glycoprotein
NEFA	Non esterified fatty acids
CRP	C-reactive protein
HOMA-IR	Homeostatic model assessment- Insulin resistance
TC	Total cholesterol
TG	Triglycerides
HDL	High density lipoproteins
LDL	Low density lipoproteins
VLDL	Very low density lipoproteins
ROC	Receiver operating curve
AUC	Area under curve
AGEs	Advanced glycation end products
RAAS	Rennin angiotensin aldosterone system
SGLT-2	Sodium glucose co-transporter-2
GBM	Glomerular basement membrane

TLR	Toll like receptor
RTK	Receptor tyrosine kinase
PTM-Fetuin-A	Post translationally modified fetuin-A
FFA	Free fatty acids
ELISA	Enzyme linked immunosorbent assay
CLIA	Chemilumino immuno assay
TMB	Tetramethylbenzidine
HRP	Horseradish peroxidase
OD	Optical density
NCEP	National cholesterol education programme
NF-kB	Nuclear factor kappa B
SD	Standard deviation
SPSS	Statistical package social system

ABSTRACT

ABSTRACT

Background: Diabetic nephropathy (DN) is a serious complication of type 1 and type 2 diabetes mellitus (T2DM). It is also known as diabetic kidney disease (DKD). It is characterized by albuminuria and decreased eGFR. Excess blood sugar leads to damage of endothelial cells, renal glomerular cells along with central and peripheral nervous system involvement.. C-reactive protein (CRP) is a systemic inflammation marker that has been revealed to be correlated with DN development. Based on albuminuria and eGFR, diabetic nephropathy is divided into 5 grades/stages by KDIGO (Kidney Disease Improving Global Outcomes) guidelines. It is a terminal disease requiring early diagnostic markers to protect renal function and individual life. Many studies have shown CRP levels increased in DN cases, showing its relation to proportional rise as the disease progress. Traditional biomarkers like serum creatinine, eGFR, albuminuria are used routinely. They have limitations in detecting early renal changes as they are influenced by age, sex, and muscle mass. Fetuin-A is an acute phase protein, inhibits receptor tyrosine kinase leading to insulin insensitivity. The Fetuin-A gene (Thr256Ser) plays a important role in Diabetes and its complications. Free fatty acids have a role in glucose intolerance resulting in diabetes which is reported by many authors. Studies have demonstrated high serum Fetuin-A levels and free fatty acids along with the elevated urinary Fetuin-A levels as early predictor of diabetic nephropathy in comparison to the usage of the traditional biomarkers like creatinine, albuminuria. Therefore, study was done to determine how Fetuin-A and its gene relate to the severity of various diabetic nephropathy stages.

Aim: To estimate serum Fetuin-A levels and assess the severity of diabetic nephropathy.

Objectives: This study was under taken to estimate and compare the glycemic, renal parameters, CRP and lipid profile in controls and different stages DN cases. To estimate and compare the serum Fetuin-A, free fatty acid and urinary Fetuin-A levels in controls and various stages of diabetic nephropathy. To study the polymorphism of Fetuin-A gene in diabetic nephropathy cases. To find the best cut-off value by ROC curve analysis of these parameters for early diagnosis of diabetic nephropathy and prevent the further complications.

Methods: This is a hospital based comparative study, done at medicine department, HSK hospital, in Bagalkot, Karnataka. Approval for the study was taken from institution ethical committee. Consent was taken by study participants. Confidentiality of the participants was maintained as per Helsinki declaration. 40 healthy controls and 40 type 2 diabetic nephropathy cases in each first 3 stages and 19 cases in 4^{th} stage of diabetic nephropathy were selected between the age group of 35-65 yrs based on KDIGO guidelines for nephropathy. Serum FBS, PPBS levels were estimated by spectrophotometric method, HbA1c by HPLC, fasting insulin by CLIA, HOMA-IR by formula. Serum urea, creatinine, uric acid, urine microalbumin were estimated by spectrophotometric method, eGFR by MDRD equation. Serum TC,TGL, HDL-C, and VLDL-C were estimated by spectrophotometric method LDL-C by Friedwald formula. Serum CRP is estimated by latex turbidometric method. Serum and urinary Fetuin-A, Serum free fatty acids were estimated by ELISA method. Fetuin-A gene polymorphism study was done. Statistical analysis was done using SPSS software version 19.

- **Results:** Serum glycemic parameters showed significant elevation in all the stages of diabetic nephropathy cases compared to controls ($p<0.001$). Serum and urinary renal parameters increased significantly in all the stages of diabetic nephropathy cases in comparison to controls ($p<0.001$) except eGFR which showed gradual decline as the

stages of DN progressed. Serum CRP also showed significant increase in all the stages of diabetic nephropathy compared to controls ($p<0.001$). Serum lipid profile showed increased levels in all the stages of DN compared to controls except the HDL-C which decreased as the stages of DN progressed ($p<0.001$). Serum Fetuin-A level was significantly increased in first 2 stages ($p=0.003$) and decreased in 3rd and 4th stages of diabetic nephropathy indicating the urinary loss of this protein. Urinary Fetuin-A was significantly increased ($p=0.001$) in all the stages of diabetic nephropathy. There was significant gene polymorphism noted with change in the frequency of G allele (G>C) which denotes the alteration of serum Fetuin-A levels in diabetic nephropathy cases. Serum free fatty acids also significantly increased in all the stages of diabetic nephropathy ($p=0.000$). Best cut-off value of serum Fetuin-A, Urinary Fetuin-A and serum free fatty acids were 48.21ng/ml, 41.28ng/ml and 395.4mmol/L respectively and area under the curve 0.802, 0.947 and 0.979.

Conclusion: Serum Fetuin-A levels were significantly increased in first two stages of diabetic nephropathy but decreased in 3rd and 4th stages, whereas urinary Fetuin-A levels increased in all the stages compared to controls. There was significant positive correlation of serum and urinary Fetuin-A levels with parameters like urea, creatinine, microalbuminuria, and CRP and lipid profile observed in cases. eGFR was decreased in all the stages of diabetic nephropathy cases compared to controls, showed negative correlation with serum Fetuin-A, FFA and urinary Fetuin-A levels.Gene polymorphism showed missense mutation for the Fetuin-A gene with the 100% frequency of G allele which can be one of the factor affecting the concentration of Fetuin-A in diabetic nephropathy. Cut-off values of serum and urinary Fetuin-A, serum free fatty acids can be

used in predicting the severity of diabetic nephropathy compared to other routine biomarkers of DN.

Keywords: Diabetic nephropathy, Fetuin-A, Fetuin-A gene (Thr256Ser), HOMA-IR, eGFR , microalbuminuria, Free Fatty Acids.

Chapter I

INTRODUCTION

INTRODUCTION

Diabetes mellitus is a major health problem which is expanding quickly on a global basis. It is caused by reduction in insulin synthesis and also due to insulin resistance. This is classified into type 1 (T1DM) and type 2 diabetes mellitus (T2DM) as per ADA guidelines.[1]

90% of diabetes cases belong to type 2 diabetes. T2DM presents with insulin resistance, which is characterized by hyperinsulinemia and hyperglycemia, increased HbA1C, hyperlipidemia, and increased inflammatory markers. Environmental factors and genetic factors such as family history, obesity, age > 40 yrs play vital role as risk factors for the development of diabetes mellitus.[2]

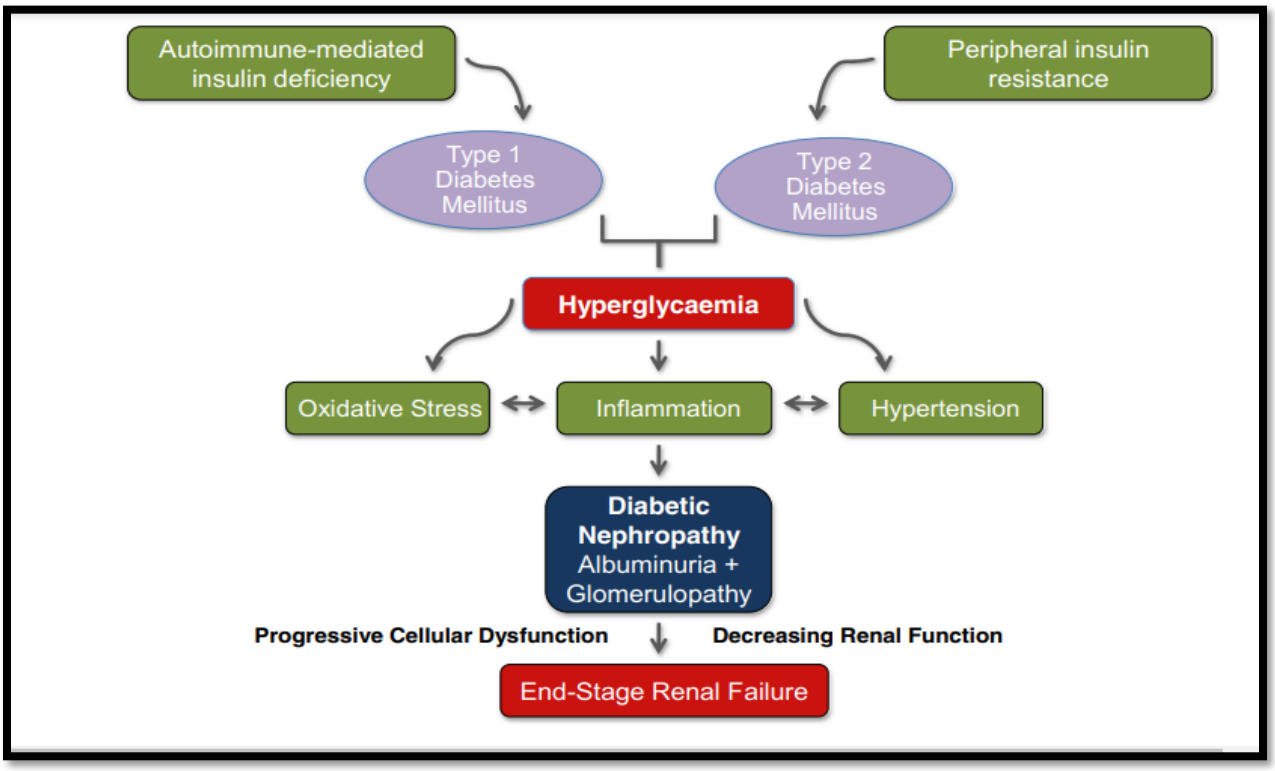
The persistently high blood glucose levels in T2DM cause generalized vascular damage affecting the kidneys, leading to nephropathy and neurological problems like peripheral neuropathy. It also affects vision by affecting the fundus, leading to retinopathy.[3]

Diabetes nephropathy (DN), also referred to as diabetic kidney disease, is characterised by a chronic decline in renal function that is observed in individuals with diabetes. [4] The metabolism of carbohydrates, lipids, and proteins is impaired by diabetic complications, which may affect the vascular system of the kidney [5].

Diabetic nephropathy is a major global public health concern. About 10% of people worldwide suffer from DN, which significantly increases morbidity and mortality, particularly in those who also have co-morbid condition like hypertension. As it worsens, it often results in End-Stage Renal Disease, which needs dialysis of kidney, which is expensive procedure that places a significant strain on healthcare systems and individuals [6].

Twenty to forty percent (20-40%) of diabetic people have diabetic kidney damage. Nephropathy can result from type 1 and type 2 diabetes; however, a lower percentage of patients with type 2 diabetes go on to develop ESRD. 20% of DN patients have microalbuminuria within ten years of onset [7].

Figure 1.1: Role of hyperglycemia in diabetic nephropathy [8]



In DN assessment of renal function is primarily based on use of traditional biomarkers such as serum creatinine, eGFR, and albuminuria. Their ability to identify early renal abnormalities is limited. Age, sex, and muscle mass all affect serum creatinine and eGFR sensitivity and specificity, restricting its application in the early identification of kidney disease. Similar to this, water levels, physical activity, and infections can all have an impact on albuminuria, which can cause measurement variability and lower its validity as a standard indicator. [9,10]

As these parameters have certain limitations in assessing the kidney functions so there is a need for the novel biomarker which can detect the renal damage earlier compared to other parameters and help in predicting the complications associated with disease and aids in prevention.

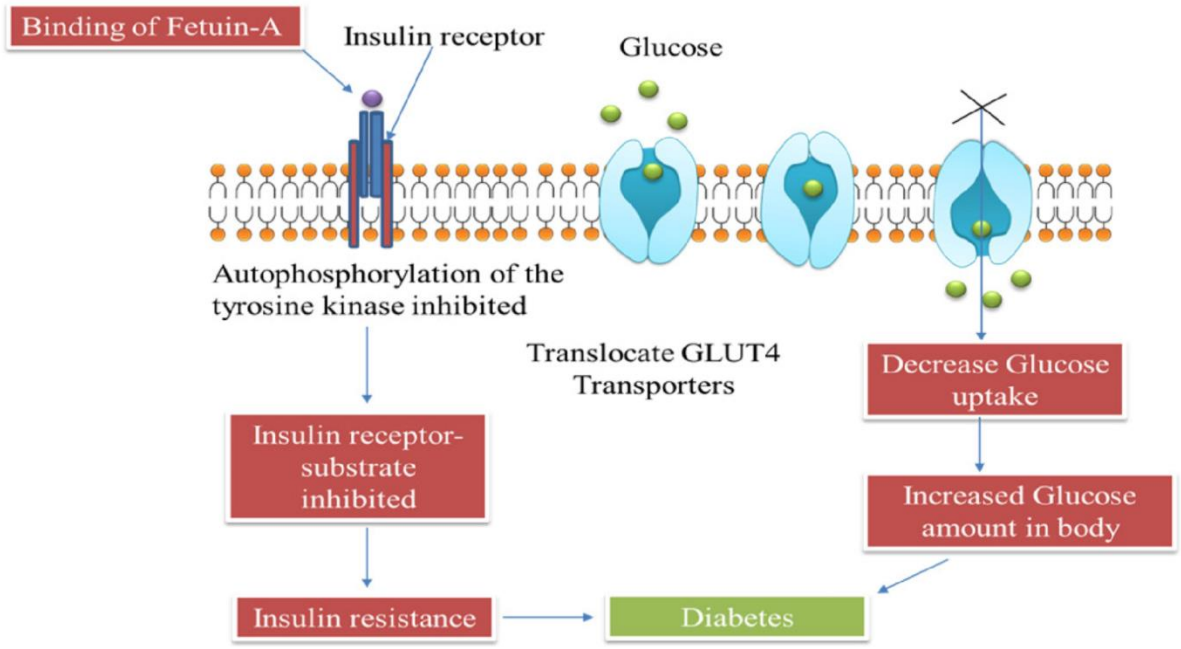
Current research showed the association of serum Fetuin-A and free fatty acid levels in diabetes and its complications.

First discovered in fetal bovine serum, α2-Heremans-Scmid glycoprotein (AHSG) is the human homologue of Fetuin-A. [11] Adipocytes, monocytes/macrophages, and other cells generate a lower amount of fetuin-A, a hepatokine mostly (above 95%) synthesized in the adult liver [12–15]. AHSG gene, which is found on the 3q27 human chromosomal locus, encodes this heterodimeric globular protein, which has six introns and seven exons and is about 8.2 kb long [16].

Fetuin-A is a multifunctional protein that has both physiological and pathological roles. This is done by binding of Fetuin-A with multiple receptors such as insulin, toll-like receptors, and transforming growth factors.[17]

Several experimental studies suggest the role of Fetuin-A in developing T2DM, like in regulation of inflammation, insulin sensitivity, and dyslipidemia. Insulin resistance is increased and glucose clearance is decreased when Fetuin-A inhibits the insulin receptor tyrosine kinase [18,19].

Figure 1.2: Role of Fetuin-A in insulin resistance [20,21]



FFA additionally referred to as non-esterified fatty acids. A main source of lipids is plasma FFA, which are produced by hydrolysis of TGL stored in adipocytes and liver. Free fatty acids (NEFA) have been implicated by numerous studies in developing diabetes due to glucose intolerance [22]. Insulin resistance (IR) in the liver and muscle is caused by elevated serum free fatty acids or persistent hyper free fatty acidemia, and vice versa, insulin resistance in T2DM may also result in elevation of free fatty acids. [23, 24].The association between serum FFA levels, IR, and diabetes incidence has been demonstrated by other epidemiologic research [25].

More precise and sensitive biomarkers that can reliably represent early kidney damage and forecast the course of the disease are desperately needed in light of these constraints. Ideally, these biomarkers would identify minor alterations in the kidneys before serious structural harm arises, enabling prompt treatment and better patient outcomes. [26,27]

Therefore, present research has been performed for assessing levels of serum Fetuin-A and free fatty acids, as well as urine Fetuin-A, in type 2 DN patients. Also an attempt was made to see the genetic polymorphism of Fetuin-A if any in these cases. This will help with early management of nephropathy and can detect and predict renal damage earlier than other biomarkers mentioned above.

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Chapter 2

AIM AND OBJECTIVE

AIM AND OBJECTIVES

2.1. AIM: To assess the role of Fetuin-A in the severity of diabetic nephropathy.

2.2. Objective:

Primary Objectives:

1 To estimate routine glycemic parameters (FBS,PPBS,HbA1c, Insulin, HOMA-IR), renal function tests (Creatinine, Urea, Uric acid, eGFR), microalbuminuria, CRP, lipid profile (TC, TGL, HDL, LDL,VLDL) in the study group and healthy controls.

2 To quantitate and compare the levels of serum free fatty acids, serum and urinary Fetuin-A protein in various stages of diabetic nephropathy.

3 To study polymorphism of Fetuin-A gene in diabetic nephropathy subjects.

Secondary objectives:

1 To find correlation of serum Fetuin-A with glycemic status, renal parameters like urea, creatinine, eGFR, microalbuminuria, CRP and lipid profile, serum free fatty acids at various stages of diabetic nephropathy.

2.3. Hypothesis:

Null hypothesis:

There is no difference in serum and urinary Fetuin-A levels in diabetic nephropathy cases and controls.

Alternate hypothesis:

Serum and urinary Fetuin-A levels in diabetic nephropathy cases is increased when compared to controls. In diabetes, Fetuin-A is linked to insulin resistance as it inhibits insulin receptors.

Chapter 3

REVIEW OF LITERATURE

REVIEW OF LITERATURE

3.1.Diabetes:

Patients with T1DM and T2DM may develop diabetic nephropathy, sometimes referred to as diabetic kidney disease, a microvascular complication. Prolonged albuminuria and a steadily declining glomerular filtration rate (GFR) are the condition's hallmarks. There is strong evidence that intensive, early treatment can stop or slow the disease course. Although both T1DM and T2DM can cause diabetic nephropathy, more than 90% of all instances of T2DM are characterised by IR. [1, 2]

The main pathological characteristics of DN are mesangial matrix growth, podocyte foot process effacement, glomerular hypertrophy, and glomerular basement thickening. [3,4]

3.1.1. Etiology of Diabetes:

In type 2 diabetes, hyperglycemia causes reactive oxygen species to be produced and activates a number of molecular pathways, like generation of advanced glycemic end products (AGEs), oxidation, cytokine activation, etc. DN is a clinical disorder that exhibits a typical pattern of glomerular disease and is characterised by progressive deterioration in renal function and chronic microalbuminuria [5]. Both renal structure and function are altered in DN [6].

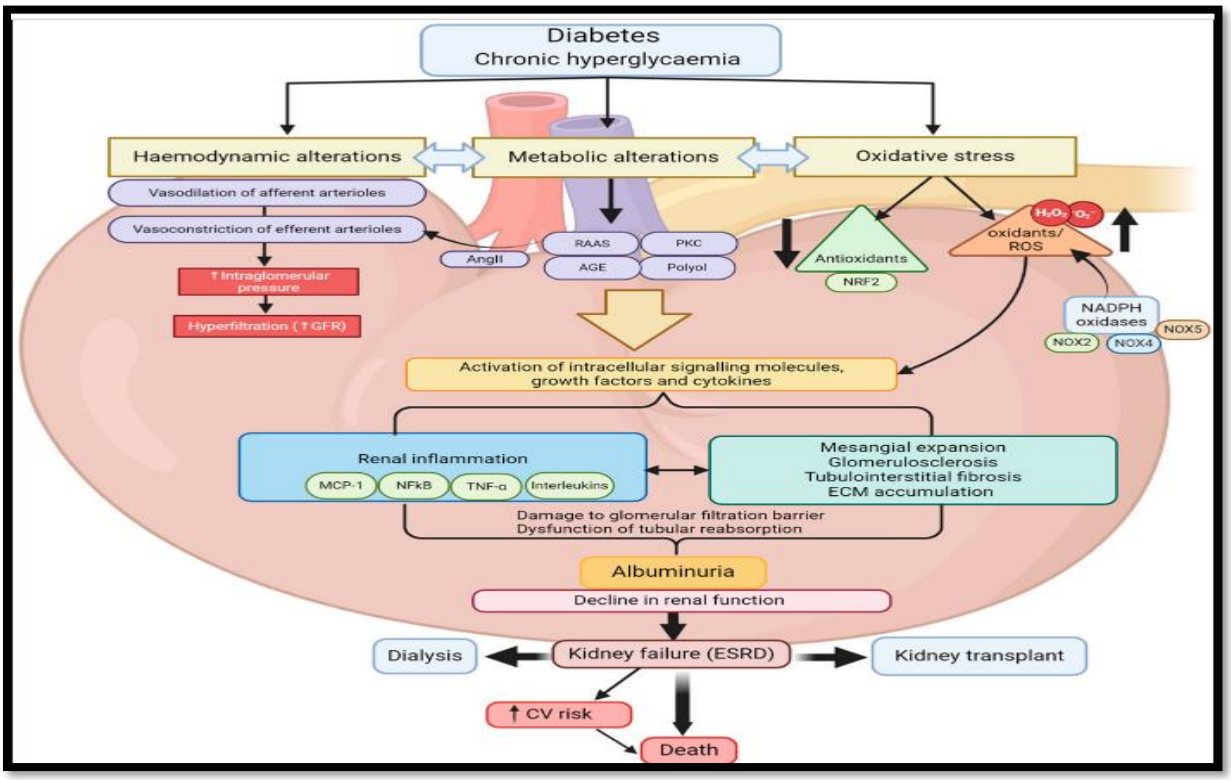
3.1.2. Pathogenesis of Diabetic nephropathy:

Haemodynamic and metabolic variables are hypothesised to work together to develop diabetic nephropathy. Afferent and efferent renal arterioles, interstitium, glomerulus, renal tubules, and other kidney components are all impacted [7].The final common mechanism for DN is renal fibrosis. [8, 9] This fibrosis is caused by a variety of causes, including an overactive renin-angiotensin-aldosterone system (RAAS), inflammatory

processes, disturbed renal haemodynamics, and abnormal glucose metabolism related to oxidative stress. [10]

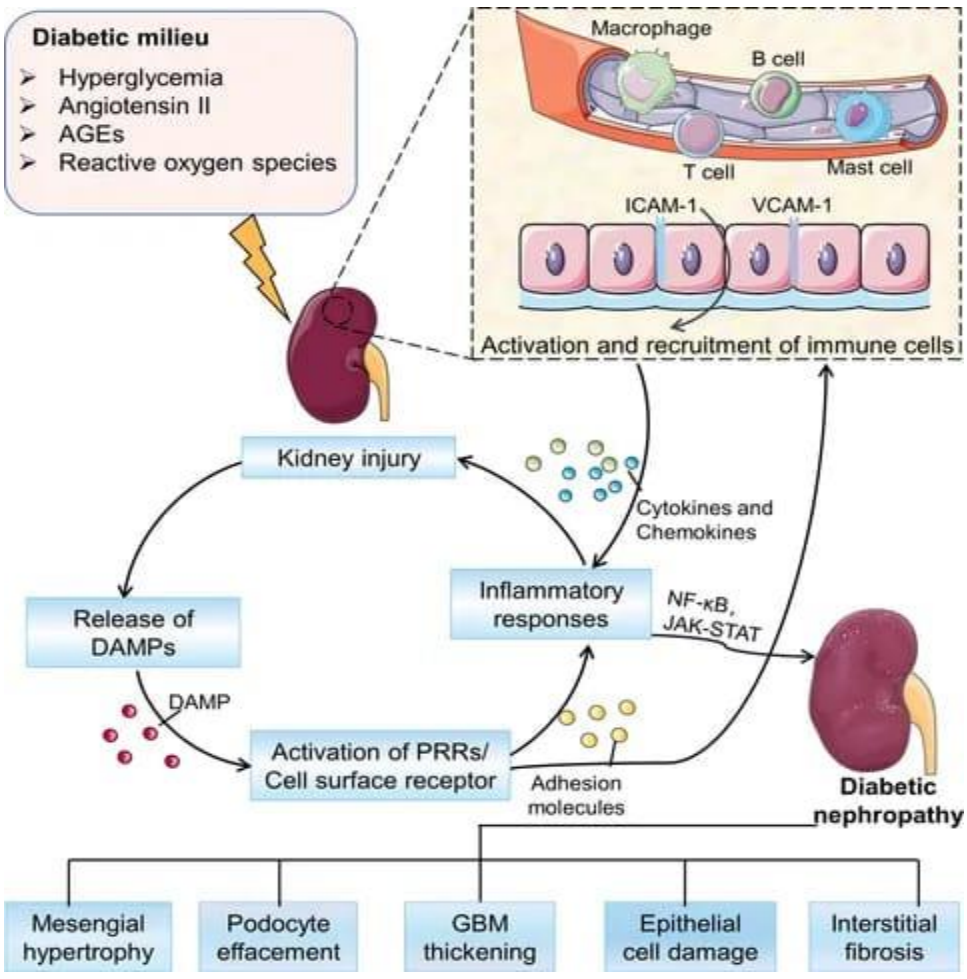
Among the haemodynamic factors are the over-activation of RAAS and an increase in intraglomerular and systemic pressure. Research has demonstrated that RAAS is one of primary pathways involved in pathophysiology of diabetic nephropathy, and a variety of factors can activate it. Higher filtered glucose load causes the proximal tubules to up-regulate SGLT2, which cotransports sodium and glucose back into circulation. Consequently, macula densa of distal tubules receives a reduced amount of sodium chloride, leading to overactivation of RAAS and promotion of renin release. [11, 12]

Figure 3.1: Pathogenic pathways of diabetic nephropathy.[13]



Hyperfiltration is one of the initial features of DN. Numerous mechanisms have been suggested as the likely cause of hyperfiltration. Among these is the increase in filtration surface area that occurs with hypertrophied glomeruli. Afferent glomerular arteriolar resistance is thought to decrease, and efferent glomerular arteriolar resistance is thought to increase as a result of diabetic nephropathy, which in turn increases renal blood flow (RBF) and glomerular filtration rate (GFR) [14]. Abnormal RAAS regulation and glomerular hyperfiltration induce elevated intraglomerular pressure, which places stress on mesangial, endothelial, and podocyte cells.This worsens the impairment brought on by hyperglycemia's metabolic consequences. [15]

Figure 3.2: The metabolic etiology of diabetic nephropathy [16]



In T2DM, oxidative stress, angiotensin II, hyperglycemia, and AGE's trigger a number of signalling cascades that recruit and activate immune cells to support the development of inflammation and ultimately result in several pathological alterations in DN. (AGEs- advanced glycation end products; GBM- glomerular basement membrane; DAMPs- damage-associated molecular patterns; PRRs- pattern recognition receptors.)

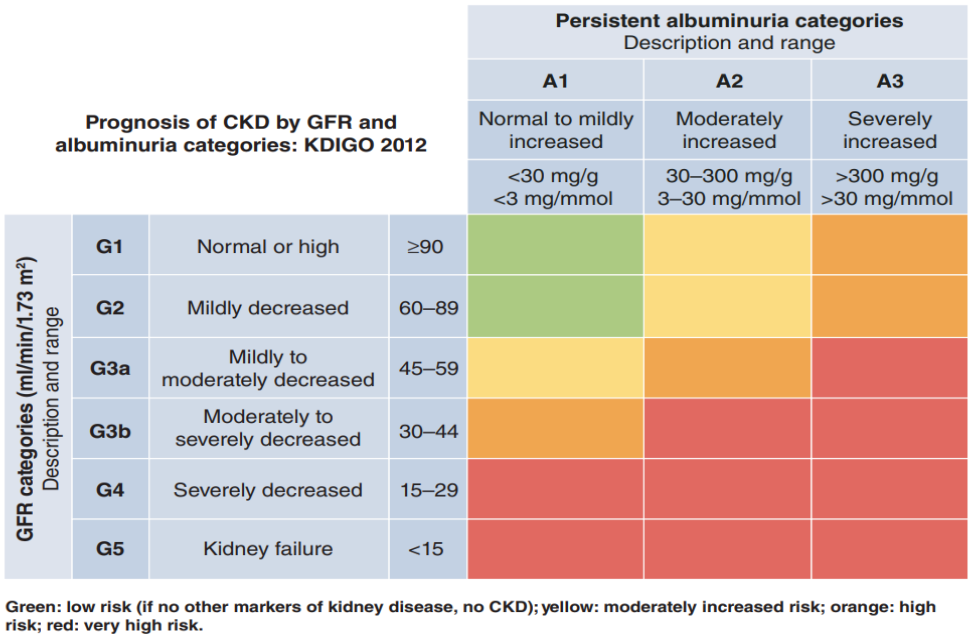
A metabolic component is the generation of AGE's, which are critical to pathophysiology of several complications associated with diabetes mellitus, namely cardiovascular difficulties. [17]. The primary mechanism by which AGE's interact occurs via receptor for advanced glycation end products (RAGE). As damage occurs and kidney function declines, the glomerular basement membrane (GBM) becomes more porous and less effective at filtering. Renal function gradually deteriorates en route. [18]

3.1.3. Diagnostic criteria :

The diagnostic parameters include microalbuminuria, decreased eGFR, elevated serum creatinine and urea levels, elevated fasting blood sugar > 126 mg%, PPBS > 200 mg%, and HbA1c > 6.5%. The degree of damage to any glomeruli that are still working is reflected in the level of proteinuria. eGFR, or estimated GFR, is a measure of proportion of glomeruli that are no longer filtrating blood, can be computed using the serum creatinine measurement [19].

3.1.4. Diabetic kidney disease is classified into a variety of grades/stages as per Kidney Disease Improving Global Outcomes (KDIGO) guidelines.

Figure 3.3: KDIGO grades /stages of Diabetic kidney disease[20]



Diabetic nephropathy is diagnosed by using traditional biomarkers like serum creatinine, urea, albuminuria and eGFR. As a result of the non specificity, late detection of DN by the above markers, other molecules have been identified as key markers which will detect early kidney damage compared to the traditional biomarkers.

3.2.Fetuin-A :

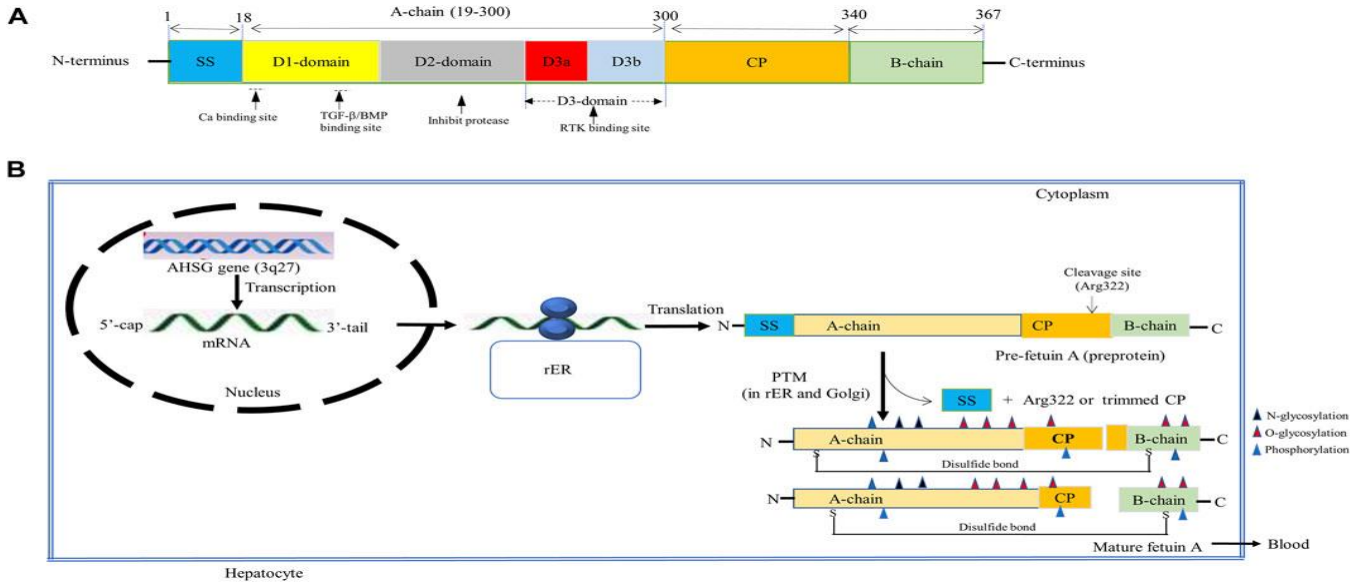
Fetuins are cystatin superfamily of cysteine protease inhibitors of cathepsin, papain, metalloprotease, calpain, and caspase. A domain of conserved cysteine residues is accountable for their capacity to block proteases [21].In addition to unclassified cystatins, cystatin superfamily includes type 1 (mostly intracellular proteins), type 2 (primarily extracellular proteins), and type 3 (plasma proteins) proteins [22].

K. Pedersen first identified fetuin-A in foetal calf blood in 1944, which is why the term "fetuin" was coined [23]. In 1960 and 1961, J.F. Heremans and K. Schmid conducted

independent investigations with W. Burgi that purified Fetuin-A in humans. Consequently, it is now commonly referred to as α2-Heremans Schmid glycoprotein (AHSG) in honour of the discoverers. The prefix "α2" is derived from comigration of Fetuin-A with α2-globulin fraction of plasma proteins during electrophoresis, which is official name of the protein, AHSG [24].

In humans, gene for Fetuin-A is found on chromosome 3q27. This chromosome is also known as the MetS susceptibility and T2DM locus. T2DM, dyslipidaemia, adipocyte insulin action, and many nucleotide polymorphisms are all connected. Despite the fact that precursor proteins of Fetuin-A consist of three chains, the active protein is composed of only two chains, A & B, encoded by single mRNA transcript. Chain A comprises two cystatin-like domains, D1 and D2, and is thus longer. It contains 26% reverse turns, 24% beta pleated sheets, and 29% alpha helices. It is made up of 282 residues of amino acids. The charged and neutral portions of these residues are not uniformly distributed [25, 26].

Figure 3.4: Structure of Fetuin-A [27]



3.2.1. Fetuin-A structure :

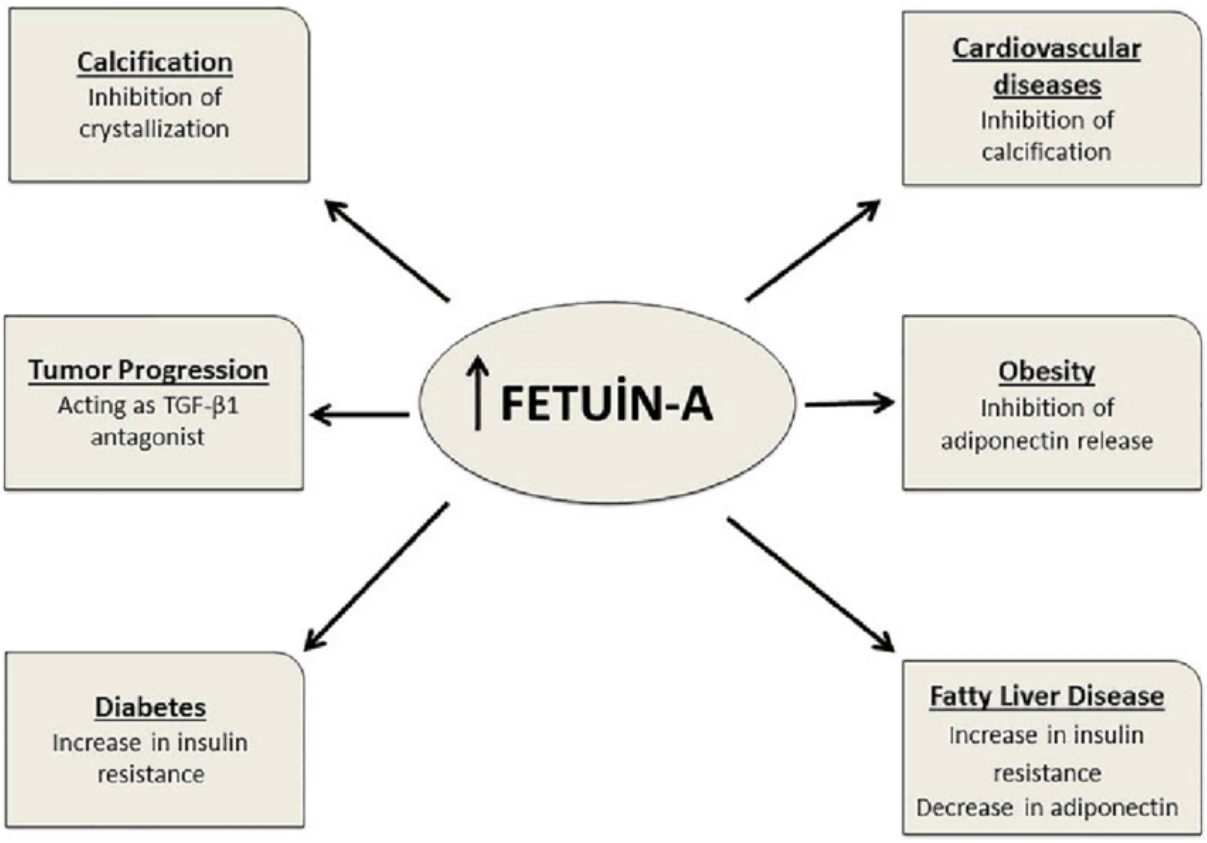
The 367 amino acids that make up pre-Fetuin-A are divided into 18 small SS amino acids, 282 A-chain amino acids (19–300), 40 connecting peptide (CP) amino acids (301–340), and 27 long B-chain amino acids (341–367). The A-chain comprises two cystatin-like domains (D1 and D2) plus a variable non-cystatin domain (D3a and D3b). D1 offers binding sites for calcium, TGF-β, and Bone morphogenic proteins, D3 interacts with the insulin receptor, and D2 suppresses cysteine protease.

The AHSG (α2-Heremans-Schmid glycoprotein) gene, which yields a single copy of mRNA encoding a pre-Fetuin-A, encodes the hepatokine Fetuin-A, which is primarily (more than 95%) synthesised in the adult liver and to a lesser extent in adipocytes, monocytes, and macrophages. Post translationally modifications (glycosylation, proteolysis, folding, and phosphorylation) are performed on the pre-Fetuin-A, which is composed of the signal sequence, A-chain, and connecting peptide. Asp (N-glycosylation), Thr, and Ser (O-glycosylation) residues are the sites of glycosylation. A mature Fetuin-A with a full length of CP except for Arg322 or C-terminally trimmed CP, connected by a disulphide bond created between Cyst-14 and Cys-340, will then be generated by proteolyzing SS and Arg322 using an unidentified proteinase. Then phosphorylation at multiple Ser and Thr residues, mostly in plasma, occurs by FAM20C. [28-31]

3.2.2. Role of Fetuin-A :

Fetuin A functions by binding with several receptors, including insulin, growth hormone, growth factors (including platelet-derived growth factor (PDGF), TGF-β2, and basic fibroblast growth factor (bFGF), nerve growth factor (NGF), transforming growth factor (TGF)-II, and several toll-like (TLR) receptors can fulfill its biological functions[32,33].

Figure 3.5: Biological roles of Fetuin-A [34].

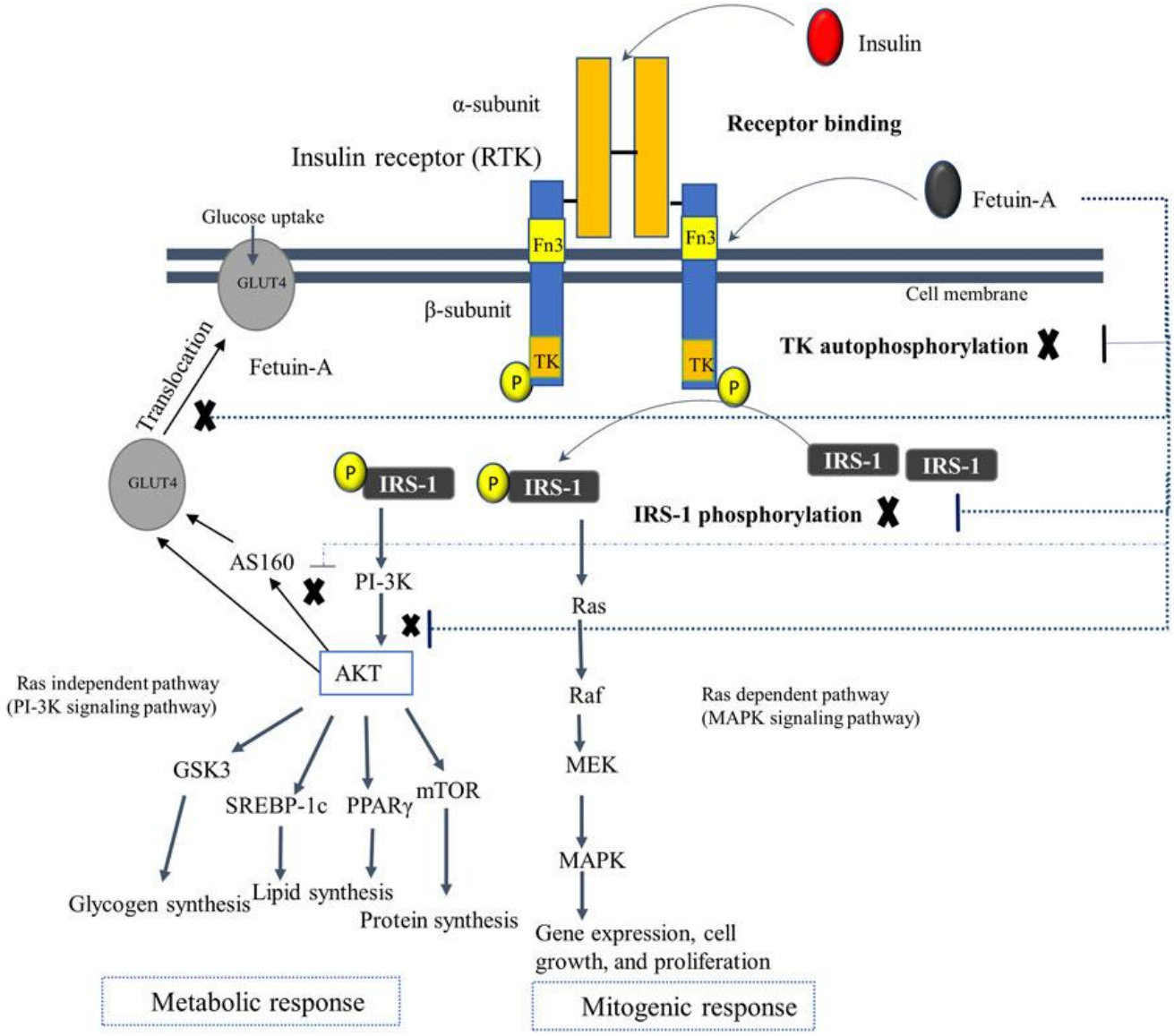


3.2.3. Diabetes and Fetuin-A :

Fetuin-A regulates insulin receptor sensitivity and the insulin signalling pathway as demonstrated by a numerous studies in vivo and in vitro[35,36]. The receptor tyrosine kinase (RTK), is naturally inhibited by Fetuin-A. Two alpha subunits that bind to ligands (insulin) and two beta subunits that mediate intracellular insulin signals are present in RTK, widespread in peripheral tissues such as the liver, muscle, and adipose tissue. Although the specific domain of Fetuin-A that interacts with RTK is not yet well understood, it has been determined that it binds with RTK in a location distinct from the insulin binding site. This interaction between Fetuin-A and the RTK inhibits the autophosphorylation of tyrosine kinase and insulin receptor substrate-1

(IRS-1), deactivating the intracellular insulin signalling pathway. Furthermore, Fetuin-A has the potential to influence the insulin signalling pathway by inhibition of the AKT, AS160, and GLUT4 phosphorylation, thereby reducing the uptake of glucose by cells [37].

Figure 3.6: Fetuin A role in insulin signaling pathway [38].



In 2008, Norbert Stefan and his associates demonstrated that increased level of circulating Fetuin-A is associated with insulin resistance in people. The similar result has also been seen by other studies, indicating that elevated levels of Fetuin-A might be a novel mechanism for the onset of diabetes. Disease pathogenesis is mostly influenced by decreased insulin sensitivity along with impairment of insulin release from the liver's beta cells. [39,40]

In 2009, Banine and colleagues discovered Fetuin-A is involved in inflammatory reactions. According to their findings, Fetuin-A activates transcriptional processes that raise the expression of a number of cytokines, such as IL-1, IL-6,IL-12 and tumour necrosis factor-α [41].

According to the results of a research on Chinese men and women, patients with type II DM have much greater serum Fetuin-A concentrations than people with normal glucose levels. Higher Fetuin-A concentrations were independently linked to an increased IR risk in T2DM patients and non-diabetic subjects. The glycoprotein showed a favourable correlation with both HOMA-IR and fasting serum insulin. In T2DM, both show signs of insulin resistance [42].

A pilot investigation on Fetuin-A in patients with renal disease due to diabetic and non-diabetic aetiology, Musolino et al. (2024) revealed that patients having DKD had considerably higher levels of Fetuin-A than patients with other causes. In situations when renal biopsy is impractical, the authors proposed Fetuin-A may be utilised as a potential biomarker to distinguish between diabetic and non-diabetic kidney disease [43].

According to a recent study by Kumar Bandi et al.(2022), post translationally modified (PTM)-Fetuin-A and albuminuria are significantly correlated, and PTM-Fetuin-A performs better than more conventional indicators like microalbumin in predicting the stages of diabetic kidney disease [44].

Further research on the possibility of Fetuin-A as a biomarker that can forecast renal function decrease in patients with T2DM from various ethnic backgrounds was conducted by Chuanga et al. in (2024). Regardless of other conventional risk variables including albuminuria, eGFR, and HbA1c levels, their results showed that elevated Fetuin-A levels were linked to an improved risk of renal function decrease [45,46]. Fetuin-A can be a useful marker for the early identification of progression of DN, as evidenced by its additive predictive power over conventional markers.[47,48]

Fetuin-A levels in individuals with T1DM and T2DM, both with and without low-grade albuminuria, were examined in a cohort study of Bulgerian patients. Fetuin-A as a marker for early DKD detection and its predictive value in forecasting the decrease of kidney function were determined by comparing it with conventional markers such as albuminuria and eGFR [49]

According to a study conducted by Manal et al.,(2018) the association between Fetuin-A and microalbuminuria may be attributed to its role in mediating insulin resistance. Endothelial dysfunction may result from compensatory hyperinsulinemia, which is a defining feature of IR. This is achieved by altering the metabolism of intracellular calcium and magnesium, as well as by enhancing the accessibility of endothelin-1.This phenomenon is also associated with decreased nitric oxide availability. Hyperinsulinemia additionally boosts sodium counter-transport activity and sodium reabsorption in kidneys, which may lead to hypertension, hyper adrenergic state and volume expansion. Additionally, progressive kidney injury can be directly influenced by impaired insulin sensitivity, associated with proliferation of endothelial cell , renal hypertrophy, and lipid deposition in renal matrix and inner medulla.[50 , 51].

C reactive protein is an acute phase protein, released in infection and inflammatory conditions. In kidney diseases, CRP is highly expressed by many inflammatory cells like macrophages and kidney cells along with tubular and endothelial cells. It is secreted at the initial 4-10 hrs of inflammation, peaks at 48 hr and has a short half life of 19hrs. Continuous high level of CRP results in chronic inflammation which is reported in patients with Diabetic kidney disease.[52]

Several studies have been done to know the association of serum Fetuin-A and CRP in diabetic kidney disease. As the inflammatory state of nephropathy increases serum Fetuin-A and CRP are both elevated. They also showed that the Fetuin-A levels decrease in later stages of diabetic nephropathy but not CRP which shows consistent elevation in all the stages indicating the positive correlation with the severity of nephropathy. Where as serum Fetuin-A showed positive correlation with CRP in early stages of DN but in later stages shows negative correlation with CRP. [53]

3.2.4. Fetuin-A gene:

The gene for Fetuin-A is found on chromosome 3q27. This region of DNA contains genetically predisposed loci for metabolic syndrome and type 2 diabetes. According to many investigations, a greater proportion of individuals with GG genotypes (91.2%) had lower serum Fetuin-A levels than the other subject group ($p<0.001$). Mean and median serum Fetuin-A levels were greatest in the CC genotype group of patients.[54]

Maharem et al.(2013) analysed Thr256Ser gene polymorphism in CKD patients on conservative therapy and compared them with healthy control subjects in an effort to

comprehend the pathophysiology of vascular calcification elevated risk. In every group, they found no difference between 3 Fetuin-A genotypes (C→G).[55]

However, distribution of Fetuin-A (C →G) revealed a significant correlation between low serum Fetuin-A levels and Thr256Ser gene polymorphism, with CG and GG participants having lower serum fetuin levels than CC patients. Axelson and colleagues (2008) discovered that the polymorphism of the Fetuin-A Thr256Ser gene (C-G) influenced the level of circulating serum Fetuin-A. On the other hand, Zeidan et al.(2012) and Cozzolino et al. (2001) in Italy came to the conclusion that there was no association between the distribution of Fetuin-A gene and the serum Fetuin-A level in either the control group or kidney disease patients. [56,57,58]

3.3.Free Fatty acids

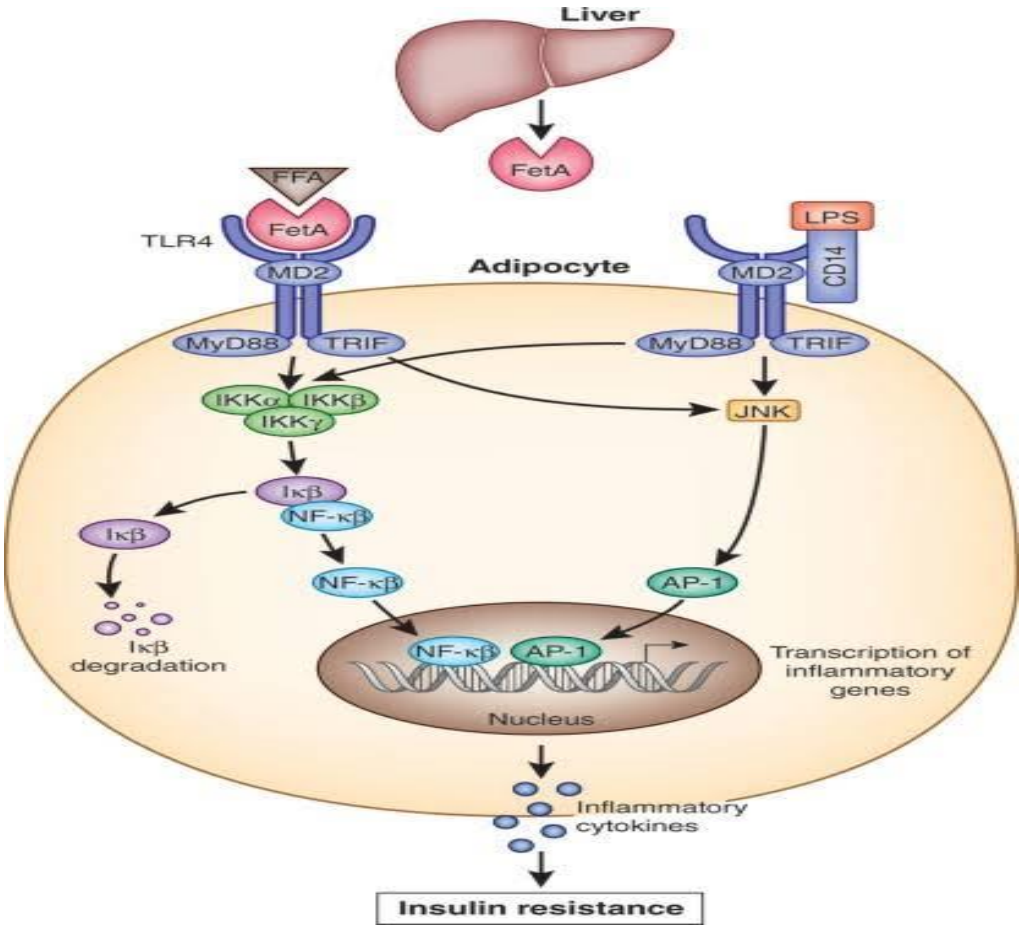
Free fatty acids are the compounds which are produced by the breakdown of triacylglycerol in adipose tissue and other sites. Fatty acids that are free biomolecules known as lipids are necessary for cell viability. The relevance of lipids in the control of cellular homeostasis is highlighted by their involvement in a variety of cellular processes, including intracellular signalling, transport, immunity, cell shape maintenance, and metabolism [59,60]. Lipids and triglycerides are stored in adipocytes, which function as fuel tanks.

Free fatty acids also known as NEFA are stored in adipose tissue, primarily as subcutaneous belly fat, and are carried to their site of utilization by plasma linked to albumin [61]. The liver and myocardium, two highly metabolic organs, preferentially use FFA as an energy source. Among other factors, gender, psychological stress, and fasting status all influence plasma FFA concentrations. [62,63].

3.3.1. Diabetes and free fatty acids:

T2DM is also linked to plasma FFA levels. Therefore, regardless of the presence of prior insulin resistance or abnormalities in insulin secretion, elevated fasting plasma FFA concentrations are linked to glucose intolerance [64,65]. As a result, elevated plasma FFA concentration is linked to T2DM and is considered a separate risk factor for insulin resistance in obese individuals [66]. Increased plasma FFA concentrations and an imbalance between pro- and anti-inflammatory adipokines are symptoms of adipose tissue malfunction brought on by dyslipidaemia and insulin resistance [67].

Figure 3.7: Inflammatory role of Fetuin A and FFA [68]

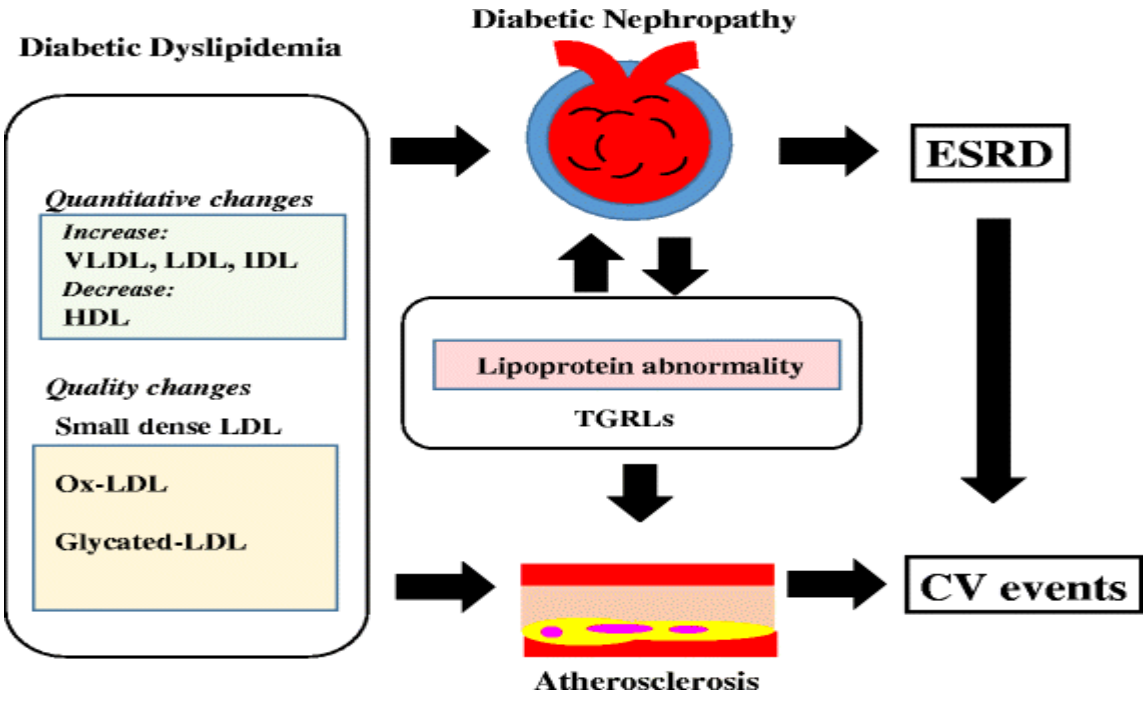


3.3.2. Dyslipidemia and diabetic nephropathy:

For those with type 2 diabetes, the main cause of mortality and morbidity is involvement of cardiovascular system, which is frequently associated with dyslipidaemia in diabetic nephropathy [66]. The development of renal injury is significantly influenced by abnormalities in lipid and lipoprotein metabolism, are correlated with the severity of proteinuria, resulting in both quantitative and qualitative changes [69].

These abnormalities in lipid metabolism encourage atherogenicity and the advancement of kidney injury in the hyperglycemic, inflammatory, and oxidising environment of DN [70]. The aberrant production, transport, and clearance of lipids and lipoproteins in DN are attributed to a number of processes. As a result, DN patients have lower lipoprotein lipase expression, impaired reverse cholesterol transport, and fewer receptors mediating lipid uptake [71].

Figure 3.8: Relation of dyslipidemia and diabetic nephropathy [72].



Lipid accumulation in the renal parenchyma is the hallmark of this pathological condition, which may eventually impair renal function by harming proximal tubular epithelial cells, podocytes, and tubulointerstitial tissue through a variety of mechanisms [73].

However, albuminuria, a known risk factor for the advancement of renal disease, is typically present in DN patients. Apart from its direct harmful impact, albumin may also transport fatty acids in the urine. Therefore, in DN patients, albuminuria may promote tubular injury by favouring a significant accumulation of fatty acids in the kidney [74] In this regard, it has been shown that fatty acids attached to albumin, but not albumin itself, stimulate oxidative stress and tubular cell death [75,76].

According to a study by Ninomiya et al.,(2009) serum free fatty acid levels were significantly elevated in patients with advanced diabetic nephropathy.[77]

With this background , we made an attempt to find the association of serum, urinary Fetuin-A and FFA in the different stages of type 2 Diabetic nephropathy and also found out the relation of Fetuin-A gene polymorphism in development of type 2 diabetic nephropathy.

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Chapter 4

MATERIALS AND METHODS

MATERIALS AND METHODS:

4.1. Study design: This is a hospital based comparative study.

4.2. Study Duration: This was conducted from 2020 to 2022.

4.3. Source of data: Type 2 Diabetic nephropathy cases from department of medicine OPD and IPD, Hanagal Shri Kumareshwar Hospital. Bagalkot. Karnataka.

4.4. Sample size: Calculation was done by using the software open epi version 2.3.1, at 99% confidence interval and 90% power, by taking correlation coefficient of serum Fetuin-A with serum creatinine: r=-0.61 from the article by Sadeghi et.al.[1].

The following formula was used:

$N= ([Z\alpha+Z_\beta]/C) + 3$. Where $C = 0.5* \ln ([1+ r] /[1-r])$.

n= number of sample

Z_α– Z equivalent of α at 99% confidence interval=$\alpha= Z\alpha = 2.54$

Z_β– Z equivalent of β at 90% power of the study = $\beta = Z_\beta = 1.64$

The sample size calculated was = 41, which is rounded off to 40 in each group

Controls (40), Cases (40 in each stages)

Ethical Clearance: Approval for the study was given by institutional ethics committee (IEC) of BLDE (Deemed To Be University), Vijayapura (BLDE(DU)/IEC/399/2020) and SNMC and HSK hospital, Bagalkot (SNMC/IECHSR/2020/A-71/1.1).

Informed written consent was taken from all the study participants. All the demographic and other information of the participants was obtained through questionnaire and the confidentiality of the participants was maintained as per the Helsinki Declaration.

Study place: The study was conducted in the department of Biochemistry and General Medicine, at S.Nijalingappa Medical College and HSK hospital in Bagalkot and CAMER BLDE(DU) Vijaypura, Karnataka. The patients who attended the General Medicine OPD and IPD were selected for the study.

4.6. Inclusion Criteria:

- Diabetic patients with onset of up to 5 years, type 2 diabetic nephropathy patients having microalbuminuria with decreased eGFR
- Age group of 35-65 years, attending medicine OPD & IPD were included in the study.

4.7. Exclusion criteria:

Patients having -

- Systemic diseases such as hypertension on treatment, and also diabetic patients on treatment with GLP-1 receptor agonists –Liraglutide and Thiazolidinedones drugs-Pioglitazone, hypothyroidism, hyperthyroidism, cardiovascular diseases, pregnancy, malignancy, autoimmune diseases and alcoholics were excluded from the study

Table 4.1: As per KDIGO guideline diabetic nephropathy subjects are divided in to five different stages based on eGFR and microalbuminuria. [2]

Parameters	Stage 1 (Grade 1)	Stage 2 (Grade 2)	Stage 3 (Grade 3)	Stage 4 (Grade 4)	Stage 5 (Grade 5)
eGFR(ml/min/1.73m2)	$\geq$ 90	60-89	30-59	15-29	<15
Albuminuria (mg/day)	< 30	<30	30-300	>300	>300

Study participants were divided as follows:

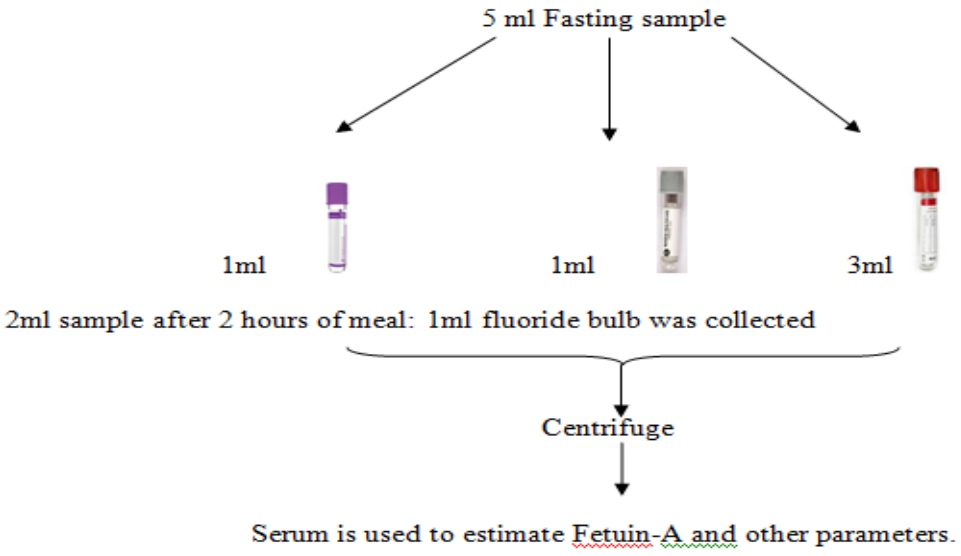
- 40 – Controls (diabetic patients with less than 5 years of diabetes mellitus)
- 40 – Stage 1 Diabetic nephropathy (Grade 1)
- 40 – Stage 2 Diabetic nephropathy (Grade 2)
- 40 – Stage 3 Diabetic nephropathy (Grade 3)
- 19 – Stage 4 Diabetic nephropathy (Grade 4)

4.8. Study protocol:

Detailed history was taken and clinical examination was done of the patients attending the medicine OPD, S.Nijalingappa Medical College and Hanagal Shri Kumareshwara Hospital and Research Centre Bagalkot at the time of diagnosis.

5ml Fasting blood sample was collected under aseptic precautions after an overnight fast, out of which 1ml was transferred in EDTA-containing vacutainers for HbA1C and 1ml was transferred in fluoride-containing vacutainers for fasting blood sugar estimation, 3ml in plain vacutainers for estimation of other biochemical parameters. Blood sample (2ml) collected 2 hours post-meals in fluoride containing vacutainers for PPBS estimation. After centrifugation of all the vacutainers at 3000 rpm for 20 minutes, plasma/serum was obtained. The separated serum was used to estimate fasting insulin , Fetuin-A levels, free fatty acids and other biochemical parameters like urea, creatinine, uric acid, CRP, total cholesterol(TC), triglyceride (TGL) and high density lipoprotein (HDL-C), LDL-C by Friedwald formula and VLDL-C.

Figure 4.1: Sample collection in different tubes.



10 ml urine was collected after overnight fasting from the study participants in a sterile container for the estimation of urinary Fetuin-A and microalbumin within 2 hours.

Blood glucose, serum creatinine, urea, uric acid, CRP and lipid profile levels were analyzed by spectrophotometric method using auto analyzer BA 400 Biosystem. Serum insulin was estimated by Autolomo1000 using CLIA methodology. HOMA-IR was estimated by formula (Fasting blood glucose X fasting insulin) / 22.5 , eGFR was calculated by using MDRD formula (175 X (S Cr) – 1.154 X (age) – 0.203 X (0.742 in females).

Serum Fetuin-A, urinary Fetuin-A and serum FFA levels were determined using the ELISA method (Robotic) using Bioassay Technologies kits. K3 EDTA sample stored at -20^{0}C was used for the polymorphism analysis and the DNA isolation was done by using Bioanalysis kits.

4.9 ESTIMATION OF BLOOD GLUCOSE [3]

Method: End point method

Principle:

Glucose present in sample is oxidized to gluconic acid in the presence of glucose oxidase(GOD). The hydrogen peroxide so formed reacts under catalysis of peroxidase (POD) with phenol and 4-Aminoantipyrine (4AAP) to form a red coloured quinoneimine compound, which is measured at 500 nm and is directly proportional to the glucose concentration

$$\text{Glucose} + 1/2O_2 + H_2O \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2$$

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

Procedure:

Pipette into tubes marked	Blank	Standard	Test
Serum	-	-	10µL
Standard	-	10µL	-
Glucose reagent	1000µL	1000µL	1000µL

Mix well and incubate at 37^0C for 10 minutes. Measure the absorbance of standard and test against blank at 500nm within 60 minutes.

Calculation

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

Reference values

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

PPBS = 70-140 mg/dL

RBS = < 140 mg/dL

4.10 ESTIMATION OF GLYCOSYLATED HEMOGLOBIN (HbA1c) [4]

Method: High Performance Liquid Chromatography (HPLC) method

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

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

Procedure:

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

Interpretation of the results:

Reportable range – 3.8 -18.5 %

Reference values:

HbA1c in %	**Degree of glucose control**
<5.7%	Non-diabetic
< 5.7-6.4%	Prediabetic
>6.5 %	Diabetic

4.11 ESTIMATION OF FASTING SERUM INSULIN [5]

Method: Sandwich CLIA

Principle: The assay begins with microtitre plate precoated with capture antibody that is specific to insulin antigen. Sample containing the insulin is added to the wells and the sample insulin binds to the capture antibody on the microplate. This is followed by addition of a biotin conjugated detection antibody which binds to a different site on the captured antigen forming a sandwich complex. Following wash an HRP enzyme conjugate (avidin) is added which binds to the complex on the detection antibody. The chemiluminescent substrate is added in presence of enzyme a reaction occurs that produces light. The emitted light is measure by luminometer and intensity of light is directly proportional to the insulin concentration.

Procedure:

1. Secure the desired number of coated wells in the holder. Dispense 25µl of Standards and Serums into the appropriate wells.
2. Dispense 50µl of Enzyme Conjugate into each well. Incubate at room temperature (18-25°C) for 30 minutes.
3. Remove the incubation mixture by emptying the plate content into a waste container. Rinse and empty the microtiter plate 5 times with Wash Buffer (1X). Strike the microtiter plate sharply onto the absorbent paper or paper towels to remove all residual water droplets.
4. Add 50µl of working Substrate (A+B) in all the micro-wells. Keep away from direct light while adding the substrate. TM
5. Cover the microplate and incubate for 10 minutes at room temperature (18-25°C) in dark.

6. The micro-plate is read at 10 minutes in Analyzer.

Normal levels:

Fasting insulin: 4.03-23.45 µIU/ml

4.11 HOMEOSTATIC MODEL ASSESSMENT - INSULIN RESISTANCE (HOMA-IR) [6]:

HOMA-IR is calculated using the following formula,

$$\text{HOMA-IR} = \frac{\text{(Fasting blood glucose X Fasting insulin)}}{22.5}$$

Normal range: 0.5-1.4

>1.9 = Early IR

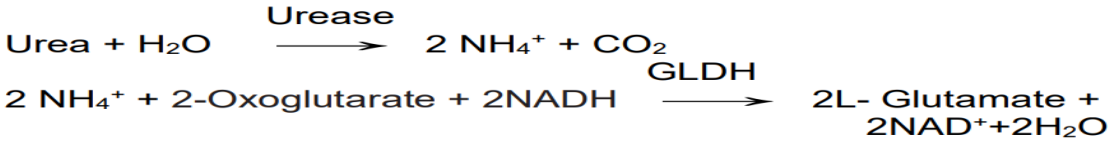
$\geq$ 2.9 = IR

4.12 ESTIMATION OF BLOOD UREA GLDH METHOD [7]

Method : Urease / GLDH (Glutamate dehydrogenase)

Principle:

In presence of urease and water, urea hydrolyses to produce ammonia and carbon dioxide. The Ammonia produced in the reaction combines with 2-Oxoglutarate and NADH in the presence of Glutamate dehydrogenase to yield glutamate and NAD+. The NADH/NAD+ change in absorbance at 340nm, which correlates with the concentration of urea nitogen in the sample. Reaction produces a unique change in absorbance at 340nm, which correlates with the concentration of urea nitrogen in the sample.



PROCEDURE

The samples and reagents should be brought to room temperature prior to use.

	Standard	Sample
Reagent	100µl	100µl
Standard	10 µl	-
Sample	-	10 µl

Mix & read immediately at 340 nm wavelength.

CALCULATION

$$\text{Urea Conc (mg/dL)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Conc. of Standard (mg/dL)}$$

Normal range: 15-45 mg/dl

4.13 ESTIMATION OF SERUM CREATININE [8]

Method: Modified Jaffe's method.

Principle :

Creatinine reacts with alkaline picrate to produce an orange yellow color product (Jaffe's reaction). Specificity of the assay has been improved by the introduction of an initial rate method. The absorbance of the orange yellow color formed is directly proportional to the creatinine concentration and is measured photometrically at 500-520nm.

Procedure

1. Take the working reagent and the photometer to 37^0C
2. Pipette into a cuvette

Working reagent	1.0ml
Standard or Sample	0.1ml

3. Mix and insert cuvette into the photometer. Start stopwatch.
4. Record the absorbance at 500 nm after 30 seconds ($\mathbf{A_1}$) and after 90 seconds ($\mathbf{A_2}$).

Calculations:

$\Delta A = A_2 - A_1$

ΔA of test / ΔA of standard × concentration of standard (mg/dl)

Serum creatinine concentration = $\frac{\text{Absorbance of test}}{\text{Absorbance of Standard}}$ X Concentration of standard. (mg/dl).

Normal reference range-

Males – 0.7-1.4 mg/dl

Females – 0.6- 1.2 mg/dl

4.14 ESTIMATION OF eGFR

Estimated glomerular filtration rate (eGFR) is used to assess the tubular function of kidney.

eGFR is calculated by using (Modification of Diet in Renal Diseases) MDRD formula

(175 X (S Cr) – 1.154 X (age) – 0.203 X (0.742 in females).

Normal ranges:

>90mL/min/1.73m^2 = Normal or High GFR

60-89 mL/min/1.73m^2 = Mildly decreased GFR

30-59 mL/min/1.73m^2 = Moderately decreased GFR

15-29 mL/min/1.73m^2 = Severely decreased GFR

$\leq$ 15mL/min/1.73m^2 = Kidney failure

4.15 ESTIMATION OF SERUM URIC ACID [9]

Method: Uricase - perioxidase enzymatic method.

Principle: Uric acid is oxidized to allantoin and hydrogen peroxide by the enzyme uricase. In presence of peroxidase, released hydrogen peroxide is coupled with aniline derivative and 4-amino antipyrine to form coloured chromogen complex. Absorbance of coloured dye is measured at 550nm and is proportional to the uric acid concentration in the sample.

- Uric acid + $2H_2O$ $\xrightarrow{\text{Uricase}}$ Allantoin + CO_2 + H_2O_2

- H_2O_2 + Aniline derivative + 4-AAP $\xrightarrow{\text{Peroxidase}}$ Chromogen complex + H_2O_2

Procedure:

Equipment used: Stat Fax 3300 Semi-automated analyzer

1) The dilutions of Reagent 2 from 1 to 5 were selected to prepare the calibration curve.

2) Plane reagent was considered as blank.

3) 1000 µL Reagent 1 was pipetted in the measuring cuvette and incubated for five minutes at 37^0C.

4) 20µl of calibrator 5 was mixed with 1000µl of the above preparation gently and the absorbance (A) was read.

Normal reference range-

Males : 2.7-7.0 mg/dl, Females : 2.5-6.0 mg/dl.

4.16 ESTIMATION OF C -REACTIVE PROTEIN (CRP) [10]

Method: Latex Turbidometry.

Principle:

Serum CRP causes agglutination of the latex particles coated with antihuman CRP . the agglutination of the latex particles is directly proportional to CRP concentration and can be measured by turbidometry.

Procedure:

Working reagent	1.0ml
Standard or sample	7µl.

Mix and immediately read the absorbance at 540nm after 2minutes.

CALCULATION

$$\text{CRP Conc (mg/L)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Conc. of Standard (mg/L)}$$

Normal Range: 0 -6 mg/L

4.17 ESTIMATION OF URINE MICROALBUMIN [11]

Method: Pyrogallol red method.

Principle: Protein in the sample reacts with pyrogallol red and molybdate in acid medium forming a colored complex measured by spectrophotometry.

Procedure:

	Blank	Standard	Sample
DH_2O	20µl	-	-
Protein Standard	-	20µl	-
Sample	-	-	20µl
Reagent	1ml	1ml	1ml

Mix thoroughly and incubate for 10min at 37^0C and read absorbance at 600nm against blank.

CALCULATION

$$\text{Urine microalbumin Conc (mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Conc. of Standard (mg/dl)} \times \text{24hour urine volume}$$

Normal range : 0-25 mg/dl

Interpretation :

< 30 mg/day: Normal

30-300 mg/day: Microalbuminuria

>300 mg/day : Macroalbuminuria or overt proteinuria

4.18 ESTIMATION OF TOTAL CHOLESTEROL[12]

Method: CHOD-POD method

Principle: Cholesterol ester is hydrolyzed to free cholesterol then this free cholesterol is oxidized by cholesterol oxidase producing hydrogen peroxide. Finally the hydrogen peroxide reacts with 4-aminoantipyrine and phenol in presence of peroxidase forming a colored compound (quinoneimine) whose absorbance is measured to determine the cholesterol concentration.

The estimation of cholesterol involves the following reactions.

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

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

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

Absorbance of quinoneimine so formed is directly proportional to the cholesterol concentration in the specimen.

Procedure:

Pipette into tubes marked	Blank	Standard	Test
Serum	-	-	10µL
Reagent 2	-	10µL	-
Reagent 1	1000µL	1000µL	1000µL

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

Calculation:

$$\text{Cholesterol concentration} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{Concentration of std.}$$

Reference range: Less than 200 mg/dl

(As per NCEP ATP III guidelines)

Desirable : < 200 mg/dL

Borderline high : 200-239 mg/dL

High risk : ≥ 240mg/dL

4.19 ESTIMATION OF TRIGLYCERIDES[13]

Method: Glycerol phosphate oxidase/ peroxidase .

Principle: Determination of triglycerides after enzymatic splitting with lipoprotein lipase. Indicator is quinoneimine which is generated from 4-amino antipyrine and 4-chlorophenol by hydrogen peroxide under the catalytic action of peroxidase.

The estimation of triglycerides involves the following reactions.

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

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

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

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

DAP – Dihydroxyacetone phosphate

4AAP - 4 Aminoantipyrine

The intensity of chromogen (quinoneimine) formed is proportional to the triglyceride concentration in the sample when measured at 500nm.

Procedure:

Pipette into tube marked	Blank	Standard	Test
Serum	-	-	10µL
Standard	-	10µL	-
Reagent	1000µL	1000µL	1000µL

Mix well. Incubate at tubes for 15 minutes at room temperature 16^0-25^0C or 37^0C for 5 minutes.

Calculation:

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

Reference range: 0-150 mg/dl

(as per NCEP ATP III guidelines)

Normal : < 150 mg/dL

Borderline high : 150-199 mg/dL

High : 200-499 mg/dL

Very high : ≥ 500 mg/dL

4.20 ESTIMATION OF HDL - CHOLESTEROL [14]

Method: Direct method

Principle:

The cholesterol from low density lipoprotein (LDL-C), very-low density lipoprotein (VLDL) and chylomicrons, is broken down by the cholesterol oxidase in an enzymatic accelerated non-color forming reaction. The detergent present in the reagent B, solubalizes cholesterol from high density lipoprotein (HDL) in the sample. The HDL cholesterol is the spectrophotometrically measured by means of the coupled reactions described below.

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

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

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

Absorbance of quinoneimine so formed is directly proportional to the HDL cholesterol concentration in the specimen.

Procedure:

Bring the reagents and photometer to 37^0 C

Pipette into tube marked	Test
Serum	7 µL
Reagent A	750 µL

Mix well and insert the cuvette into the photometer. After 5 minutes, read the absorbance (A1) at 600/700 nm against distilled water.

Pipette into a cuvette

Pipette into tube marked	Test
Reagent B	250µL

Mix well. Incubate at 37^0C for 5 minutes and read the absorbance (A2) at 600/700 nm.

Calculation:

$$\frac{\text{Absorbance of (A2-A1) sample}}{\text{Absorbance of calibrator (A2-A1)}} \times \text{C. calibrator}$$

Reference range: 30 -60 mg/dl

(As per NCEP ATP III guidelines)

Low risk : ≥ 60mg/dL

High risk :< 35mg/dL

4.21 ESTIMATION OF LDL CHOLESTEROL [15]

LDL-C is calculated using Friedwald's equation:

$$\text{LDL-C} = \text{Total cholesterol} - \left(\frac{\text{Triglycerides}}{5} + \text{HDL-C}\right)$$

Reference range: 0 - 130 mg/dl

(As per NCEP ATP III guidelines)

Optimal < 100mg/dL

Near optimal : 100-129mg/dL

Borderline high : 130-159mg/dL

High : 160-189mg/dL

Very high ≥190mg/dL

4.22 ESTIMATION OF VLDL CHOLESTEROL [16]

VLDL is calculated by following formula,

$$\textbf{VLDL-C} = \frac{\textbf{Triglycerides}}{\textbf{5}}$$

Reference range: 0-30 mg/dl

4.23 SERUM AND URINARY FETUIN-A ESTIMATION [17]

Method: Sandwich ELISA

Test Principle:

A specific polyclonal anti human Fetuin-A antibody is attached to the wells of a microplate. The sample containing the target antigen (Fetuin-A) is added to the wells and then the antigen binds to antibody coated to microplate forming a complex. A second polyclonal anti-human Fetuin-A antibody, conjugated with horseradish peroxidase is added to the wells which binds to the different epitope of the antigen forming a sandwich with the antigen and the capture antibody coated to microplate.TMB substarte specific for the enzyme is added and the enzyme catalysis the reaction and produces a detectable signal(color change). The intensity of signal is measured and is proportional to the amount of Fetuin-A present in the sample.

Preparation of standards, standard curve, dilution buffers.

Volume of Standard	Dilution Buffer	Concentration
Stock	-	100ng/ml
200µl of stock	300µl	40ng/ml
100µl of stock	400µl	20ng/ml
50µl of stock	450µl	10ng/ml
25µl of stock	475µl	5ng/ml
10µl of stock	490µl	2ng/ml

ASSAY PROCEDURE

1. Pipette 100 µl of diluted Standards, Quality Controls, Dilution Buffer (=Blank) and samples, preferably in duplicates, into the appropriate wells.

2. Incubate the plate at room temperature (25°C) for 1 hour, shaking at 300 rpm on an orbital microplate shaker.

3. Wash the wells 3-times with Wash Solution (0.35 ml per well). After final wash, invert and tap the plate strongly against paper towel.

4. Add 100 µl of Conjugate Solution into each well.

5. Incubate the plate at room temperature (25°C) for 1 hour, shaking at 300 rpm on an orbital microplate shaker.

6. Wash the wells 3-times with Wash Solution (0.35 ml per well). After final wash, invert and tap the plate strongly against paper towel.

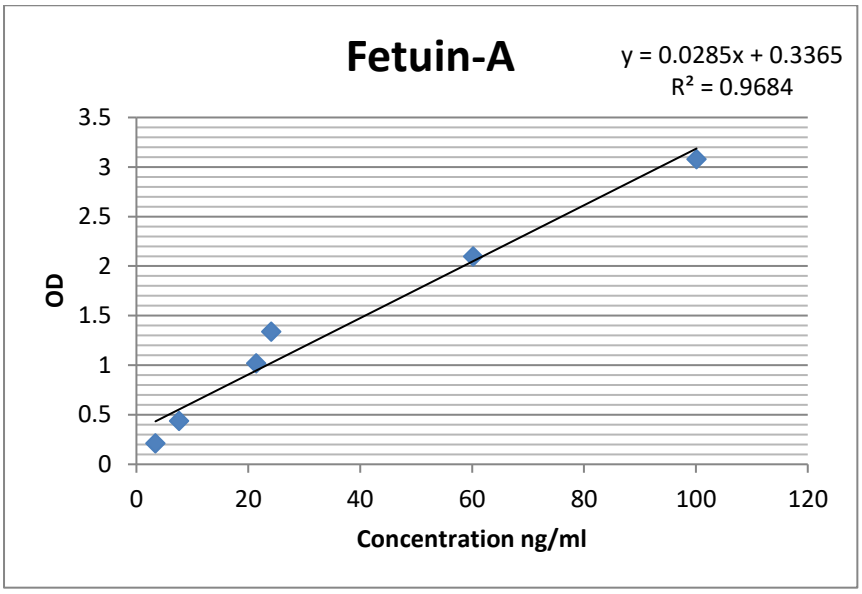
7. Add 100 µl of Substrate Solution into each well. Avoid exposing the microtiter plate to direct sunlight. Covering the plate with e.g. aluminium foil is recommended.

8. Incubate the plate for 10 minutes at room temperature. The incubation time may be extended [up to 20 minutes] if the reaction temperature is below than 20°C. Do not shake the plate during the incubation.

9. Stop the colour development by adding 100 µl of Stop Solution.

10. Determine the absorbance by reading the plate at 450 nm. The absorbance should be read within 5 minutes following step 9.

Figure 4.2: Fetuin-A standard curve



4.24 ESTIMATION OF SERUM FREE FATTY ACIDS [18]

Method : Sandwich ELISA

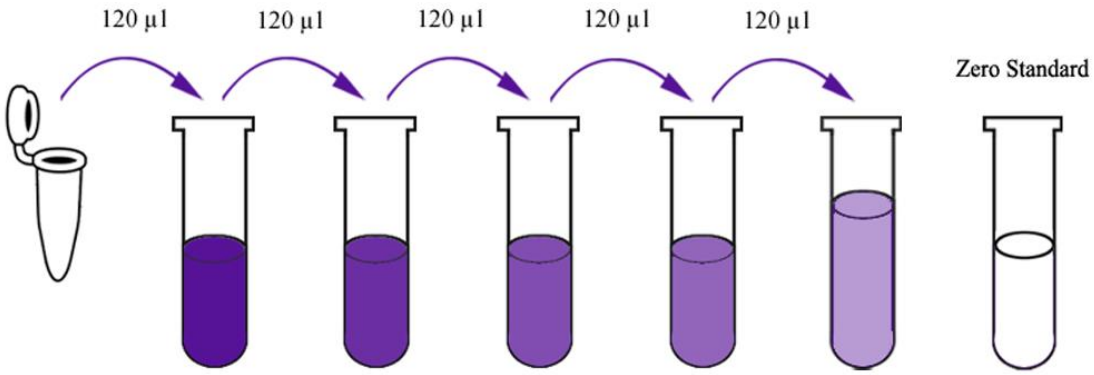
Principle:

A specific polyclonal FFA antibody is attached to the wells of a microplate. The sample containing the target antigen (FFA) is added to the wells and then the antigen binds to antibody coated to microplate forming a complex. A second biotinylated human FFA antibody,conjugated with horseradish peroxidase (HRP) is added to the wells which binds to the different epitope of the antigen forming a sandwich with the antigen and the capture antibody coated to microplate.TMB substarte specific for the enzyme is added and the enzyme catalysis the reaction and produces a detectable signal(color change). The intensity of signal is measured at 450nm and is proportional to the amount of FFA present in the sample.

Reagent Preparation :

- All reagents should be brought to room temperature before use.
- Standard Reconstitute the 120µl of the standard (6400mmol/ml) with 120µl of standardλ diluent to generate a 3200mmol/ml standard stock solution. Allow the standard to sit for 15 mins with gentle agitation prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (3200mmol/ml) 1:2 with standard diluent to produce 1600mmol/ml, 800mmol/ml, 400mmol/ml and 200mmol/ml solutions. Standard diluent serves as the zero standard(0 mmol/ml). Any remaining solution should be frozen at -20℃ and used within one month. Dilution of standard solutions suggested are as follows:

3200mmol/ml	Standard No.5	120µl Original Standard +120µl Standard Diluent
1600mmol/ml	Standard No.4	120µl Standard No.5 + 120µl Standard Diluent
800mmol/ml	Standard No.3	120µl Standard No.4 + 120µl Standard Diluent
400mmol/ml	Standard No.2	120µl Standard No.3 + 120µl Standard Diluent
200mmol/ml	Standard No.1	120µl Standard No.2 + 120µl Standard Diluent



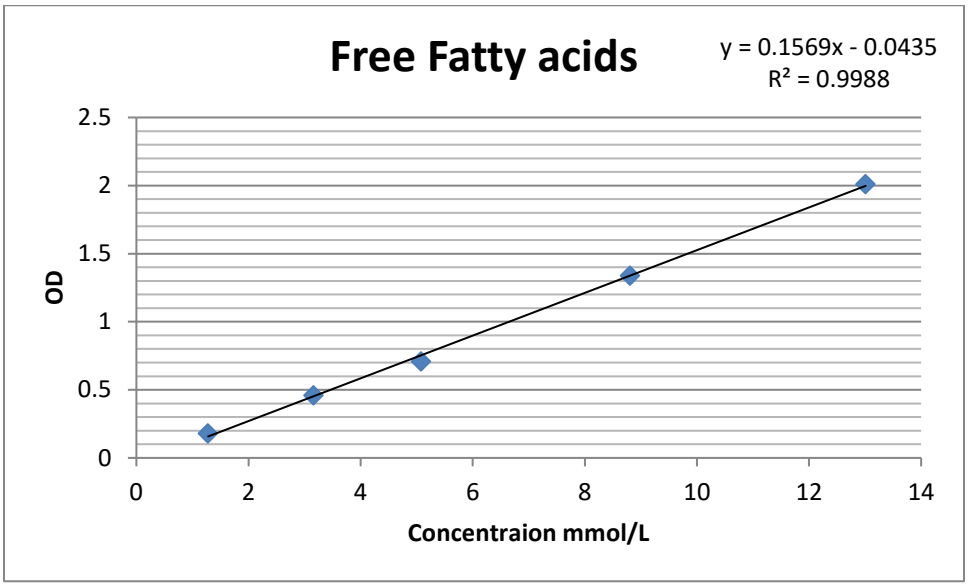
Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
6400mmol/ml	3200mmol/ml	1600mmol/ml	800mmol/ml	400mmol/ml	200mmol/ml

Wash Buffer Dilute 20ml of Wash Buffer Concentrate 30x into deionized or distilled water to yield 500 ml of 1x Wash Buffer. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

Assay Procedure

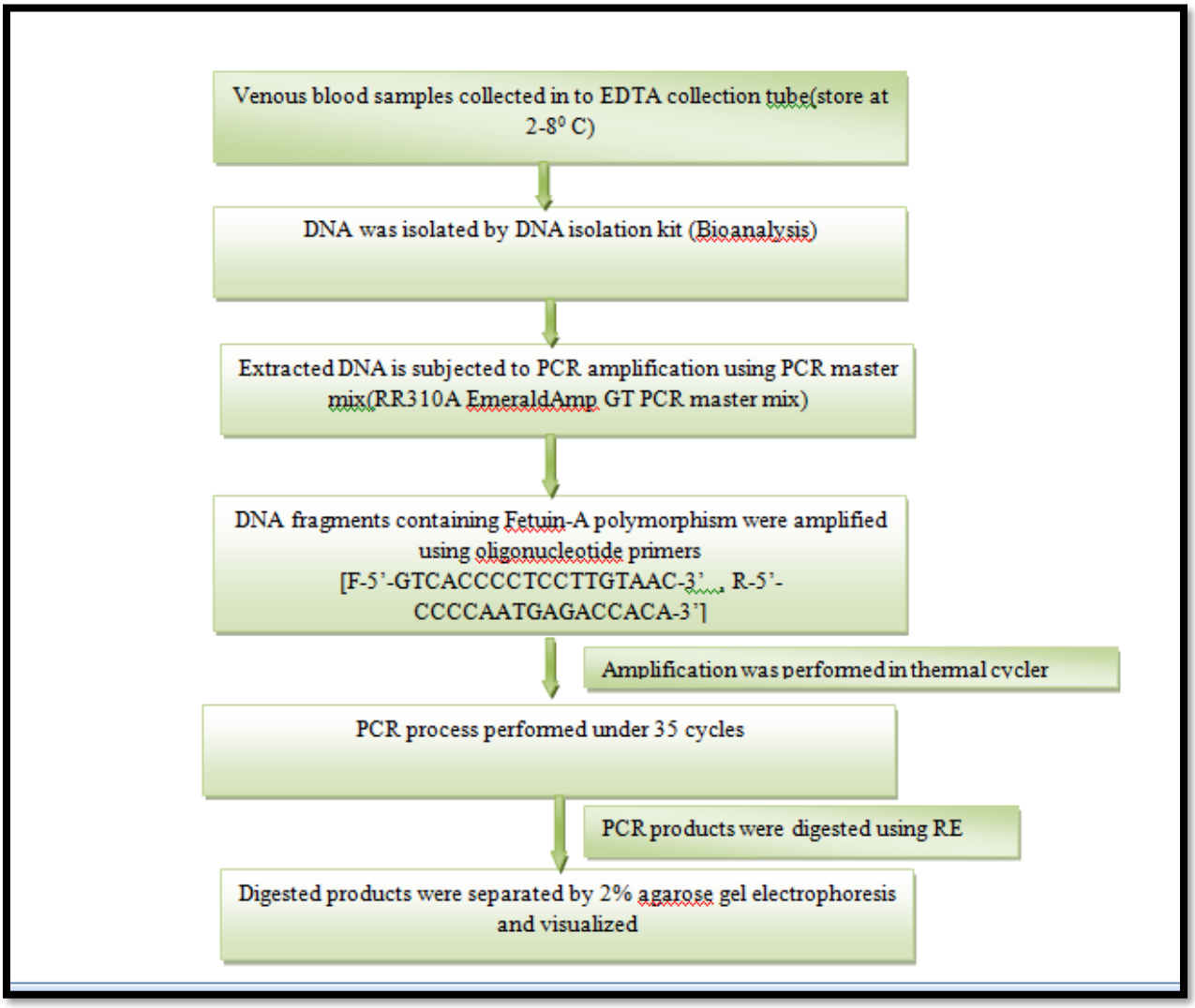
1. Prepare all reagents, standard solutions and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature.
2. Determine the number of strips required for the assay. Insert the strips in the frames for use. The unused strips should be stored at 2-8°C.
3. Add 50µl standard to standard well. Note: Don't add antibody to standard well because the standard solution contains biotinylated antibody.
4. Add 40µl sample to sample wells and then add 10µl anti-FFA antibody to sample wells, then add 50µl streptavidin-HRP to sample wells and standard wells (Not blank control well). Mix well. Cover the plate with a sealer. Incubate 60 minutes at 37°C.
5. Remove the sealer and wash the plate 5 times with wash buffer. Soak wells with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirate all wells and wash 5 times with wash buffer, overfilling wells with wash buffer. Blot the plate onto paper towels or other absorbent material.
6. Add 50µl substrate solution A to each well and then add 50µl substrate solution B to each well. Incubate plate covered with a new sealer for 10 minutes at 37°C in the dark.
7. Add 50µl Stop Solution to each well, the blue color will change into yellow immediately.
8. Determine the optical density (OD value) of each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

Figure4.3: Standard curve for free fatty acids:



4.25 FETUIN-A GENOTYPING [19]

4.4: Procedure of genotyping:



Fetuin-A genotyping was done using the forward and reverse primers F-5'-GTCACCCCTCCTGTAAC-3', R-5'-CCCCAATGAGACCACA-3'. Using NCBI and kit literature the procedure was used to determine the polymorphism. Forward and reverse primers were prepared as per the reference of study by Manal et al.[19]

PCR products were produced as per the chart and the products were analyzed for gene polymorphism .

4.26. Statistical Analysis:

SPSS software version 19 was used for statistical analysis. ANOVA, unpaired "t" test and Pearson's correlation tests were used for quantitative data. The same version 19 was used for Receiver operating curve (ROC curve) analysis. The data was analyzed using the mean ± standard deviation for age (years), FBS (mg/dl), PPBS (mg/dl), Insulin (µIU/l), HOMA-IR, Urea(mg/dl), Creatinine (mg/dl), Uric acid (mg/dl), microalbumin (mg/day), eGFR (ml/min/1.73m^2), serum CRP(mg/L), TC, TGL,HDL-C,LDL-C,VLDL-C, serum Fetuin-A (ng/ml), urinary Fetuin-A (ng/ml), and serum FFA (mmol/L) levels.

Frequency analysis was used for the Fetuin-A gene polymorphism data. The validity tests such as sensitivity, specificity and diagnostic accuracy of serum FFA, Fetuin-A and urinary Fetuin-A levels were used to determine the best cut-off value for assessing the severity of diabetic nephropathy.

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Chapter 5

RESULTS

RESULTS

5.1. Classification of groups:

This study included 40 controls (Diabetic with duration of <5years) and 40 cases in 1st stage , 40 in 2nd stage, 40 in 3rd stage, 19 in 4th stage of diabetic nephropathy (stage 1 to stage 4) . Stage 5 diabetic nephropathy cases were not included.

5.2. Age distribution of participants:

The range of age of the participants (35-65 years). The mean±SD age of participants in each stage is given in table 5.1.

Table 5.1: Mean age of controls and cases in different stages of diabetic nephropathy.

	Controls N=40	Stage 1 N=40	Stage 2 N=40	Stage 3 N=40	Stage 4 N=19	p Value
Age (years) Mean ± SD	51.54 ± 8.79	53.32 ± 8.61	53.93 ± 8.02	58.85 ± 5.06	57.4 ± 7.02	0.079

p value<0.05* significant , <0.001 **High significant is considered.

5.3. Glycemic status and insulin levels of participants:

Table 5.2 : Mean±SD of glycemic status & insulin levels in study groups.

Table 5.2 shows the mean FBS, PPBS, Insulin, HbA1c, HOMA-IR level are increased in cases compared to controls and is highly statistically significant (p<0.001).

In diabetic nephropathy cases glycemic parameters levels showed significant elevation as the stage advances with p< 0.001.

Table 5.2 : Mean±SD of glycemic status & insulin levels in study groups.

	Controls (Mean± SD) (n=40)	**Cases (Mean ± SD)**				**ANOVA**	
		Stage 1 (n=40)	**Stage 2 (n=40)**	**Stage 3 (n=40)**	**Stage 4 (n=19)**	**F value**	**p value**
FBS (mg/dl)	**101.08±5.70**	**109.18±10.20**	**151.36±12.05**	**169.96±16.59**	**189.96±18.62**	**314.75**	**<0.001**
PPBS(mg/dl)	**124.84±8.83**	**146.93±28.24**	**174.47±26.13**	**245.15±70.00**	**285.11 ±80.02**	**67.78**	**<0.001**
Insulin (µIU/l)	**8.91 ± 0.97**	**18.05 ± 1.59**	**23.26 ± 1.99**	**27.38 ± 1.38**	**32.26 ± 2.22**	**1089.18**	**<0.001**
HbA1C (%)	**5.34 ± 0.43**	**6.49 ± 0.41**	**8.21 ± 0.64**	**10.89 ± 1.55**	**12.98 ± 2.35**	**293.71**	**<0.001**
HOMA-IR	**2.20 ± 0.24**	**4.89 ± 0.64**	**8.70 ± 0.94**	**11.50 ± 1.29**	**13.42 ± 3.12**	**878.18**	**<0.001**

p value<0.05* significant , <0.001 **High significant is considered.

5.4. Renal parameters:

Table 5.3: Mean±SD of renal parameters between controls and cases.

Table 5.3 shows significant elevation of renal parameters in cases compared to controls, except eGFR which shows significant decreased levels in cases comparison to controls.

This table shows the highly significant elevation of urinary microalbumin, serum creatinine, urea in cases compared to controls (p <0.001). So as the disease progress these parameters serum level also increased. Uric acid levels in stage 1,2,3 were less compared to controls and in the

final 4th stage its level increased (**5.77 ± 1.20)** compared to controls (**5.06 ± 1.21)**. This was not statistically significant with (p = 0.101). eGFR showed highly significant reduction in cases as the stage advances in comparison to controls (p<0.001). As the disease progressed eGFR decreased and is inversely related to the progression of the DN stages. Urine microalbumin shows highly significant elevation in diabetic nephropathy cases compared to controls and also increased as the stage advances (p<0.001).

Table 5.3: Mean±SD of renal parameters between controls and cases.

	Controls (Mean ± SD) (n=40)	**Cases (Mean ± SD)**				**ANOVA**	
		Stage 1 (n=40)	**Stage 2 (n=40)**	**Stage 3 (n=40)**	**Stage 4 (n=19)**	**F value**	**p value**
Urinary Microalbumin (mg/day)	**24.68 ± 4.38**	**27.47 ± 2.41**	**45.32±49.67**	**234.57±36.05**	**356.34±38.76**	**895.22**	**<0.001**
eGFR (ml/min)	**103.35±11.61**	**103.08±10.81**	**55.55 ±10.99**	**28.68 ± 5.61**	**22.42±4.53**	**540.6**	**<0.001**
Urea (mg/dl)	**32.88 ± 8.11**	**38.42 ± 8.96**	**54.23 ± 6.19**	**73.78 ± 6.52**	**81.25± 5.72**	**237.5**	**<0.001**
Creatinine (mg/dl)	**0.71 ± 0.13**	**0.71 ± 0.11**	**1.32 ± 0.29**	**2.39 ± 0.34**	**3.67 ± 0.48**	**438.44**	**<0.001**
Uric acid (mg/dl)	**5.06 ± 1.21**	**4.77 ± 1.26**	**4.33 ± 1.35**	**4.76 ± 1.40**	**5.77 ± 1.20**	**2.109**	**0.101**

p value<0.05* significant , <0.001 **High significant is considered.

5.5. CRP levels:

Table 5.4: Mean ± SD of CRP levels in study groups.

Table 5.4 shows CRP levels are increased significantly in cases compared to controls (p=0.001). It shows elevated levels with the stage advancement of type 2 diabetic nephropathy (p=0.001) .

Table 5.4: Mean ± SD of CRP levels in study groups.

	Controls (n=40)	Stage1 (n=40)	Stage 2 (n=40)	Stage 3 (n=40)	Stage 4 (n=19)	p Value
C-reactive protein (mg / L)	6.20±1.90	15.83±4.65	28.42±5.30	40.05±5.98	52.01±6.67	0.001

p value<0.05* significant , <0.001 **High significant is considered.

5.6 Lipid profile in study participants:

Table 5.5: Lipid profile between the study groups.

Table 5.5 shows highly significant elevation of serum TG, TC , LDL , VLDL levels in cases compared to controls and shows positive correlation with advancement of stage with p<0.001. And HDL-C is significantly decreased in cases as compared to controls with p < 0.001. As the stages progressed the HDL-C levels in serum declined. Hence this HDL-C is negatively correlated with the stages of diabetic nephropathy.

Table 5.5: Lipid profile between the study groups.

	Controls (Mean ± SD) (n=40)	Cases (Mean ± SD)				ANOVA	
		Stage 1 (n=40)	Stage 2 (n=40)	Stage 3 (n=40)	Stage 4 (n=19)	F value	p value
TC (mg/dl)	178.74±17.97	172.84±59.23	244.23±64.54	255.46±55.09	293.42±76.54	26.9	<0.001
TGL (mg/dl)	128.83±10.93	162.44±45.83	165.68±33.65	174.86±35.71	184.96±25.21	13.99	<0.001
LDL (mg/dl)	84.83 ±9.96	138.30±55.48	150.86±41.84	160.45±46.11	182.65±36.28	25.85	<0.001
HDL(mg/dl)	50.17 ±9.09	37.60 ± 4.42	40.96 ± 3.85	37.30 ± 5.06	26.32 ± 8.32	40.47	<0.001
VLDL(mg/dl)	23.96 ±4.18	32.15 ± 7.22	34.57 ± 8.10	37.45 ± 8.45	41.21 ± 7.32	26.07	<0.001

p value<0.05* significant , <0.001 **High significant is considered.

5.7. Serum Free Fatty Acids:

Table 5.6: Serum Free Fatty Acid levels in study groups

Table 5.6 shows serum free fatty acids are highly significantly increased in cases compared to controls and also showed positive correlation with stage progression (p< 0.000).

Table 5.6: Serum Free Fatty Acid levels in study group.

	Controls (Mean±SD) (n=40)	**Cases (Mean + SD)**				**ANOVA**	
		Stage 1 (n=40)	**Stage 2 (n=40)**	**Stage 3 (n=40)**	**Stage 4 (n=19)**	**F value**	**p value**
Serum FFA (mmol/l)	**99.44±16.43**	**117.88±19.7**	**306.36±56.77**	**574.05±135.33**	**588.34±146.36**	**3.65**	**0.000**

p value<0.05* significant , <0.001 **High significant is considered.

5.8. Serum and urinary Fetuin-A levels:

Table 5.7: Serum and urinary Fetuin –A levels in study groups.

Table 5.7 denotes there was highly significant elevation of serum Fetuin-A levels in cases of stage 1 and stage 2 of diabetic nephropathy compared to controls. It also shows positive correlation with stages. Serum Fetuin-A levels in stage 2 was more than stage 1 and controls (p=0.003).

But serum Fetuin-A showed highly significant lower values in stage 3 and stage 4 of diabetic nephropathy compared to the previous stage 1 and stage 2 (p=0.003) .

Simultaneously urinary Fetuin-A also showed highly statistically significant increased levels in all the stages of diabetic nephropathy compared to controls (p=0.001).

Table 5.7: Serum and urinary Fetuin –A levels in study groups.

	Controls (Mean±SD) (n=40)	Cases (Mean + SD)				ANOVA	
		Stage 1 (n=40)	Stage 2 (n=40)	Stage 3 (n=40)	Stage 4 (n=19)	F value	p value
Serum Fetuin- A (ng/ml)	**38.66±4.77**	**82.35±6.63**	**85.72±5.93**	**38.16 ±4.36**	**32.26± 3.21**	**670.97**	**0.003**
Urinary Fetuin-A (ng/ml)	**36.46 ±3.79**	**42.48±4.44**	**51.77±5.20**	**70.05 ±9.29**	**82.63±10.32**	**233.21**	**0.001**

p value<0.05* significant , <0.001 **High significant is considered.

5.9. a. ROC curve analysis:

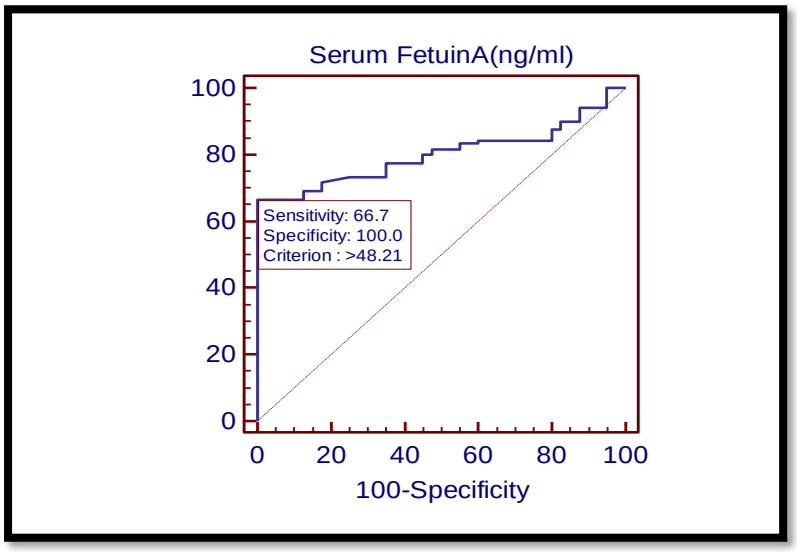
Table 5.8: ROC curve of serum Fetuin -A

Table 5.8 shows Receiver operating characteristics (ROC) curve used to define the best cut off value of serum Fetuin-A which was 48.21 ng/ml with sensitivity of 67.7%, specificity of 100% and area under the curve showed 0.802.

Table 5.8: ROC curve of serum Fetuin -A

Serum Fetuin-A (ng/ml)	
Area under curve (AUC)	**0.802**
Sensitivity	**67.7**
Specificity	**100.0**
Best cut off	**48.21**

Figure 5.1: ROC curve for serum Fetuin-A.



5.9. b ROC for urinary Fetuin-A:

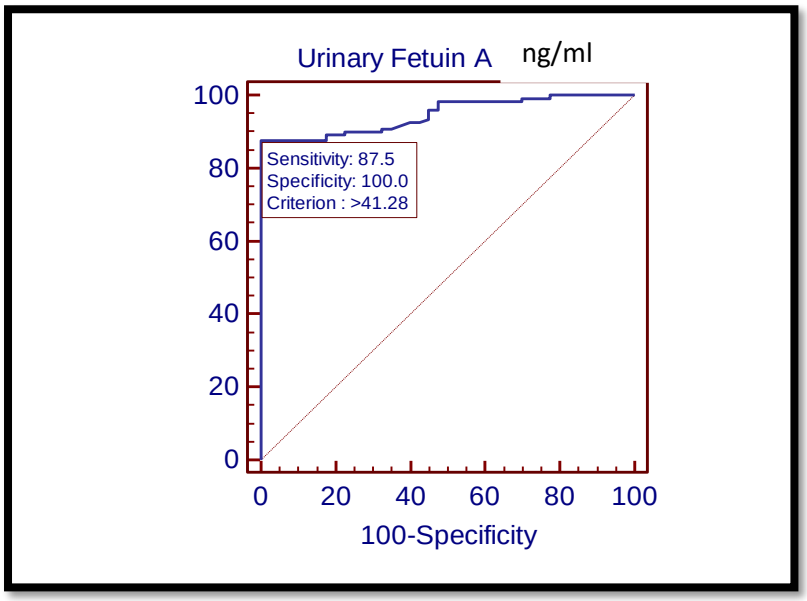
Table 5.9: ROC curve of urinary Fetuin-A:

Table 5.9 shows ROC curve, used to define the best cut off value of urinary Fetuin-A which was 41.28 ng/ml with sensitivity of 87.5%, specificity of 100% and area under the curve showed 0.947.

Table 5.9: ROC curve of urinary Fetuin-A:

Urinary Fetuin-A (ng/ml)	
Area under curve (AUC)	**0.947**
Sensitivity	87.5
Specificity	100.0
Best cut off	**41.28**

Figure 5.2: ROC curve for urinary Fetuin-A.



5.9. c. ROC for Serum FFA:

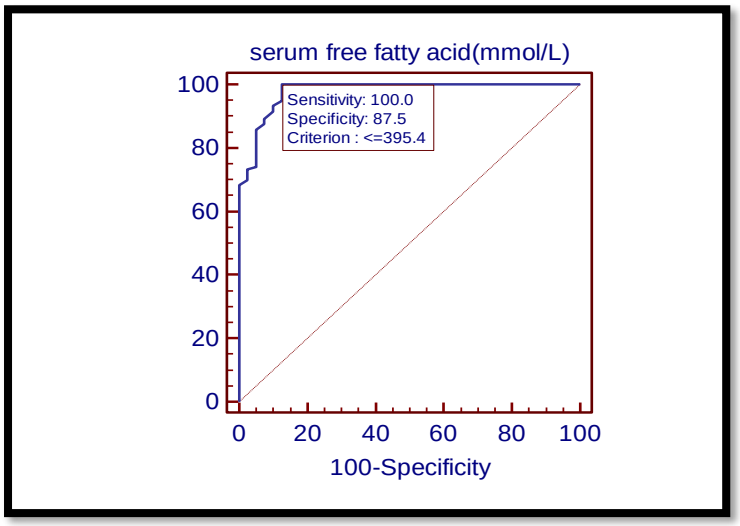
Table 5.10: ROC curve of Serum Free Fatty Acid (FFA)

Table 5.10 shows ROC curve, used to define the best cut off value of serum free fatty acid levels, which was 395.4 mmol/L with sensitivity of 100 %, specificity of 87.5 % and area under the curve showed 0.979.

Table 5.10: ROC curve of Serum Free Fatty Acid (FFA)

Serum Free Fatty Acids (mmol/l)	
Area under curve (AUC)	**0.979**
Sensitivity	**100.0**
Specificity	**87.5**
Best cut off	**395.4**

Figure 5.3: ROC curve for serum FFA.



5.10. Correlation analysis of Serum Fetuin-A with all the parameters :

Our study showed significant elevation of the serum Fetuin-A levels in diabetic nephropathy cases compared to controls. We noticed that serum Fetuin-A levels increased in 1st and 2nd stage but started to decline in 3rd and 4th stage of diabetic nephropathy. But the Fetuin-A levels in 3rd and 4th stage of diabetic nephropathy were significantly elevated compared to controls $p<0.003$.

Correlation study was done between serum Fetuin-A and FBS, PPBS, Insulin, HOMA-IR, HbA1c, blood urea, creatinine, uric acid ,CRP eGFR, urine microalbumin, urine Fetuin-A, free fatty acids, TC,TGL,HDL,LDL, and VLDL which are shown in the following tables

Table 5.11: Correlation of serum Fetuin-A with renal parameters,CRP, glycemic status, urinary Fetuin-A and lipid profile in controls and stages of diabetic nephropathy cases.

Parameters	Controls	Stage 1	Stage 2	Stage 3	Stage 4
	r – value				
Blood urea	0.192	0.261	0.069	0.32	0.352
S. Creatinine	0.141	0.342	0.018	0.022	0.247
Uric acid	0.206	0.16	0.12	0.092	0.101
eGFR	-0.202	-0.312	-0.054	-0.088	-0.051
Urine MA	0.205	0.014	0.059	0.201	0.37
Urine Fetuin-A	0.115	0.218	0.053	0.208	0.241
CRP	0.216	0.241	0.183	0.196	0.172
FBS	0.329	0.29	0.148	0.103	0.148
PPBS	0.267	0.092	0.133	0.125	0.114
HbA1c	0.022	0.044	0.109	0.133	0.125
Insulin	0.195	0.085	0.45	0.302	0.161
HOMA-IR	0.403	0.241	0.474	0.207	0.251
Sr.FFA	0.03	0.178	0.189	0.143	0.135
T.Chol	0.38	0.101	0.14	0.008	0.091
TGL	0.003	0.116	0.026	0.14	0.132
HDL	-0.536	-0.121	-0.018	-0.026	-0.035
LDL	0.178	0.339	0.014	0.092	0.063
VLDL	0.163	0.145	0.141	0.112	0.127

Table 5.11 shows significant positive correlation of serum Fetuin-A with all the glycemic , renal parameters, CRP, lipid profile and FFA in 1st and 2nd stage of DN. It showed negative correlation with eGFR and HDL in all the stages.

Correlation of Serum Fetuin-A with Glycemic status, renal parameters, CRP, lipid profile parameters and serum FFA in Stage 1:

Figure 5.4: Correlation of serum Fetuin-A and Creatinine

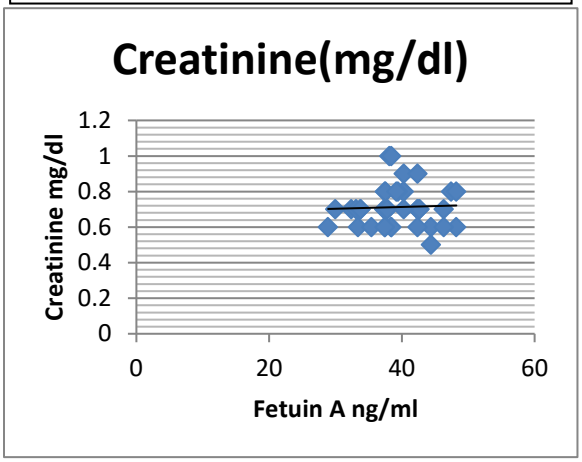


Figure 5.5: Correlation of serum Fetuin-A and urea

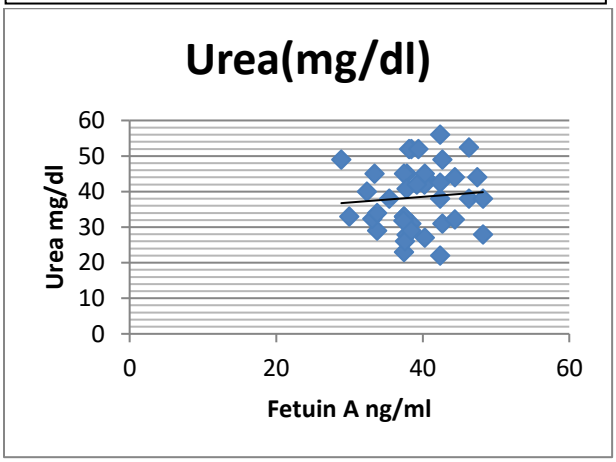


Figure 5.6: Correlation of serum Fetuin-A and uric acid

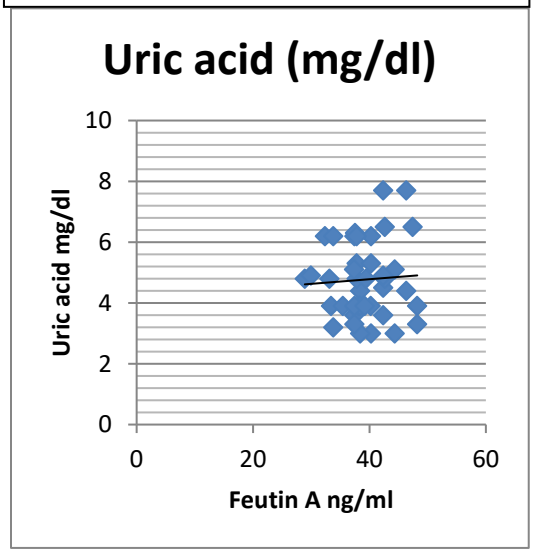


Figure 5.7: Correlation of serum Fetuin-A and eGFR

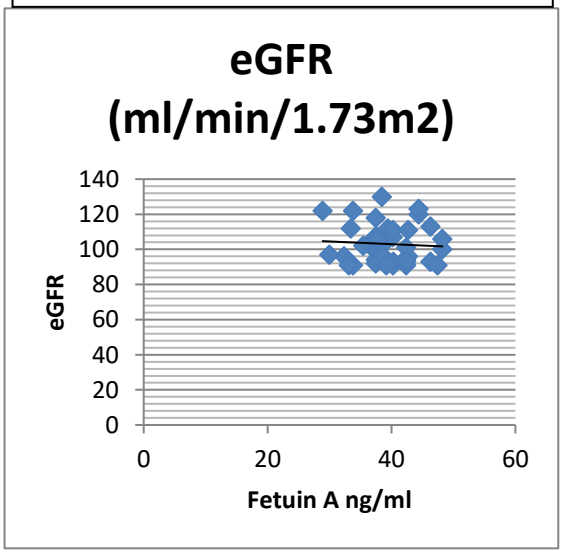


Figure 5.8: Correlation of serum Fetuin-A and Microalbumin

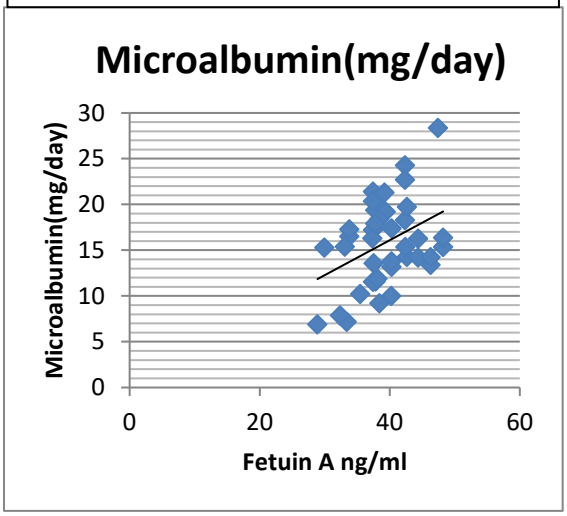


Figure 5.9: Correlation of serum Fetuin-A and CRP

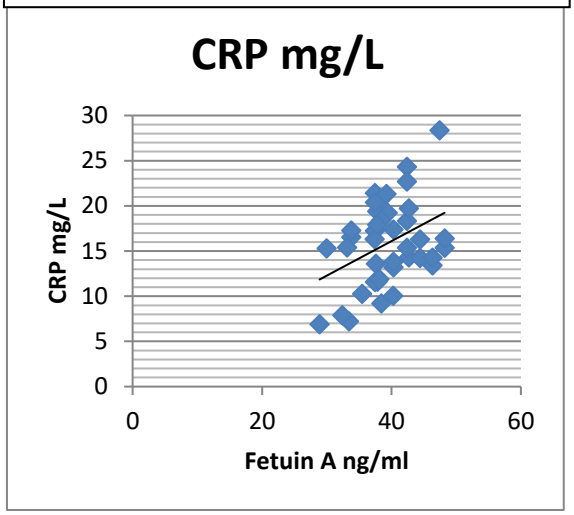


Figure 5.10: Correlation of serum Fetuin-A and FBS

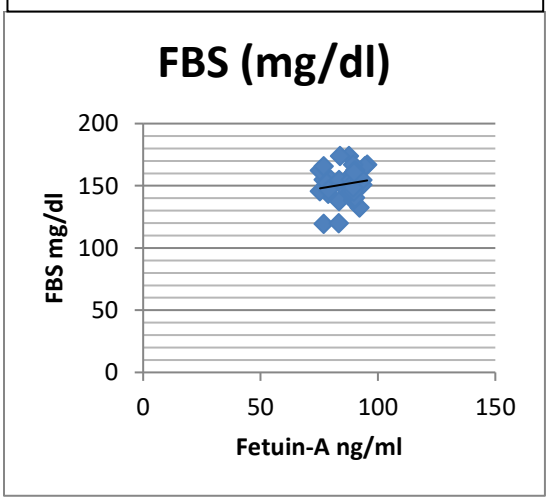


Figure 5.11: Correlation of serum Fetuin-A and PPBS

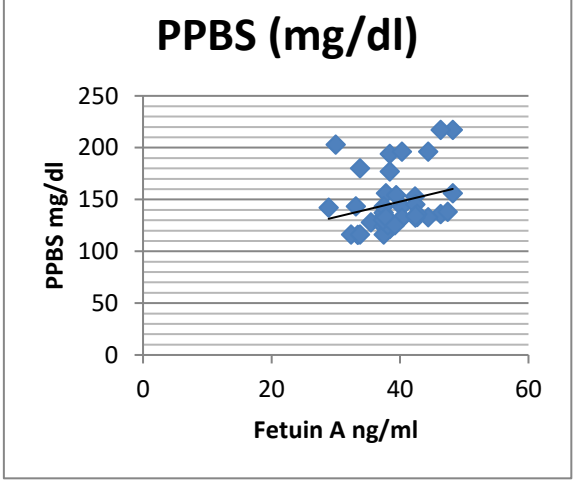


Figure 5.12: Correlation of serum Fetuin-A and Insulin

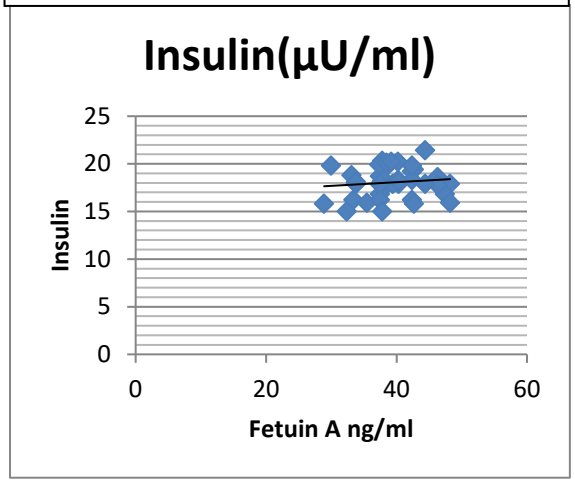


Figure 5.13: Correlation of serum Fetuin-A and HOMA-IR

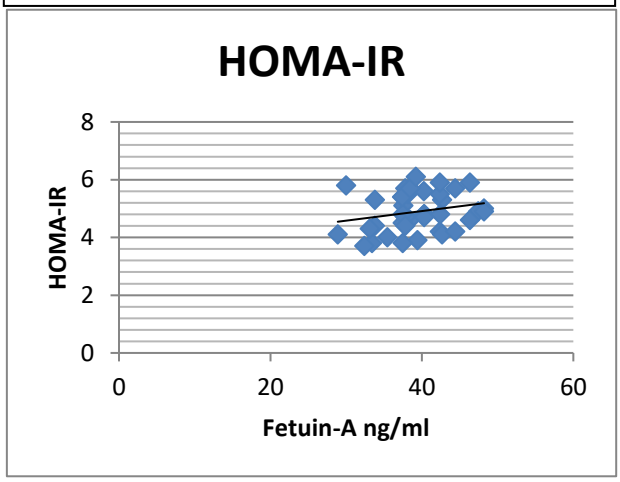


Figure 5.14: Correlation of serum Fetuin-A and HbA1c

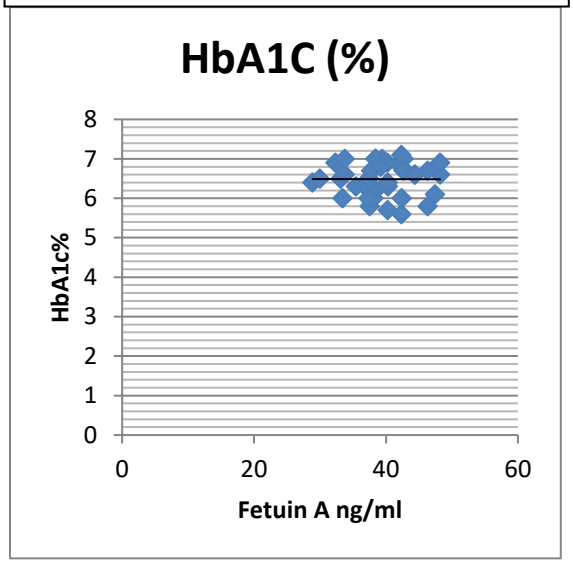


Figure 5.15: Correlation of serum Fetuin-A and FFA

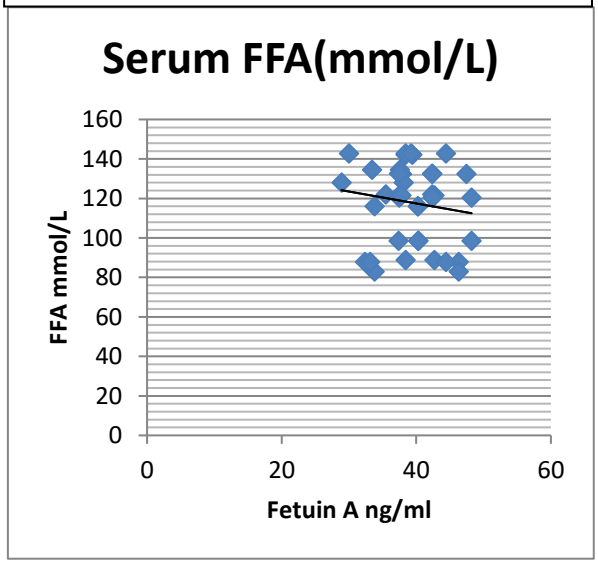


Figure 5.16: Correlation of serum Fetuin-A and total cholesterol

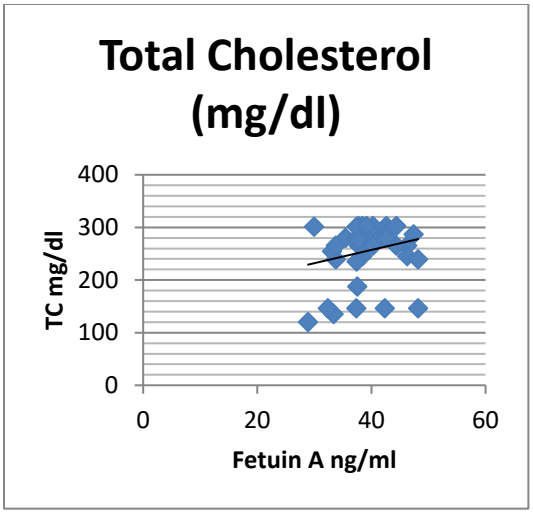


Figure 5.17: Correlation of serum Fetuin-A and triglyceride

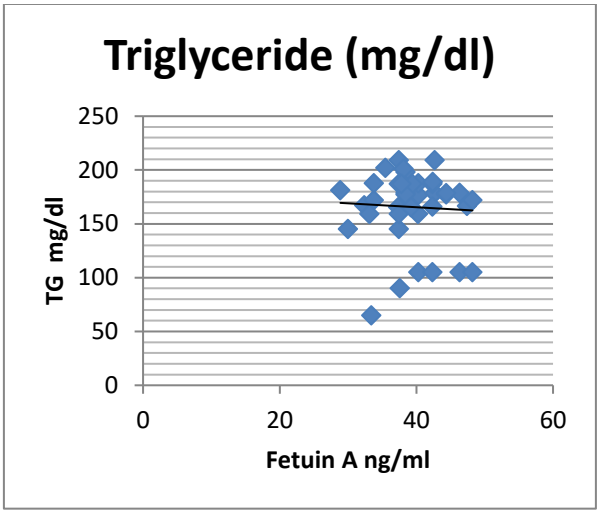


Figure 5.18: Correlation of serum Fetuin-A and LDL

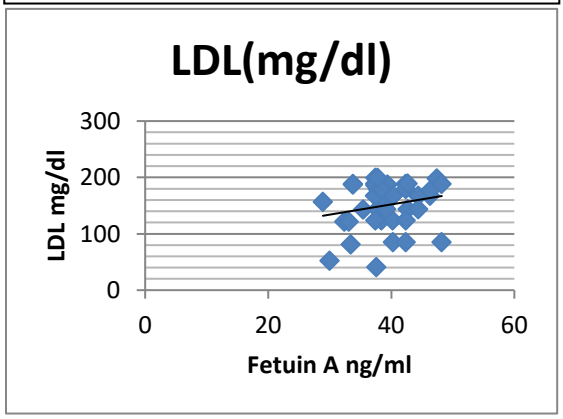


Figure 5.19: Correlation of serum Fetuin-A and V LDL

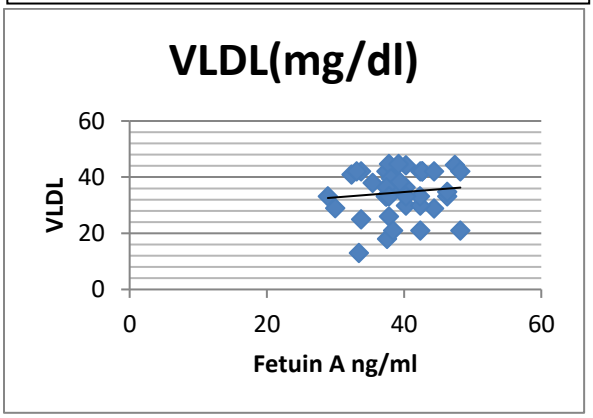
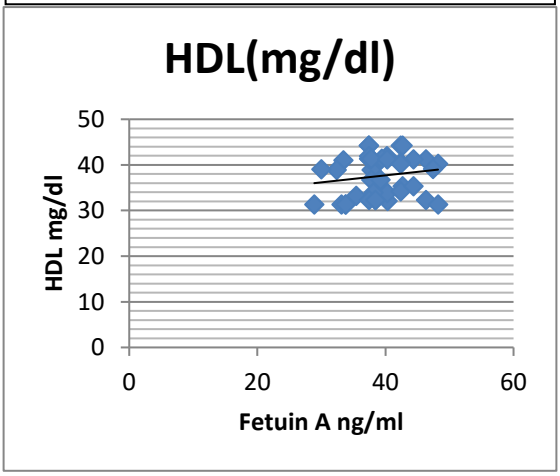


Figure 5.20: Correlation of serum Fetuin-A and HDL



Correlation of serum Fetuin-A with Glycemic status, renal parameters, CRP, lipid profile parameters and serum FFA in Stage 2:

Figure 5.21: Correlation of serum Fetuin-A and Creatinine

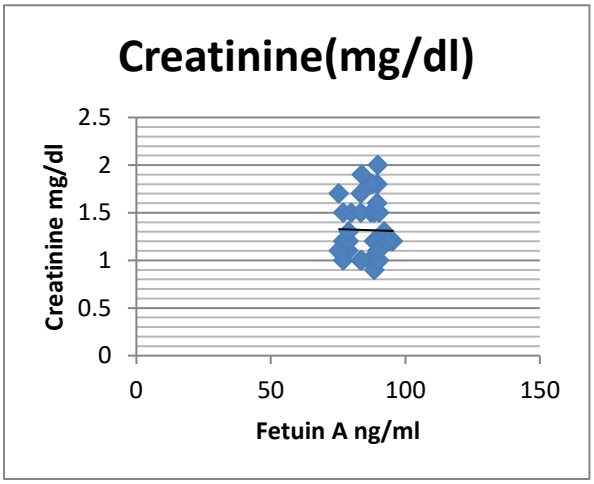


Figure 5.22: Correlation of serum Fetuin-A and Urea

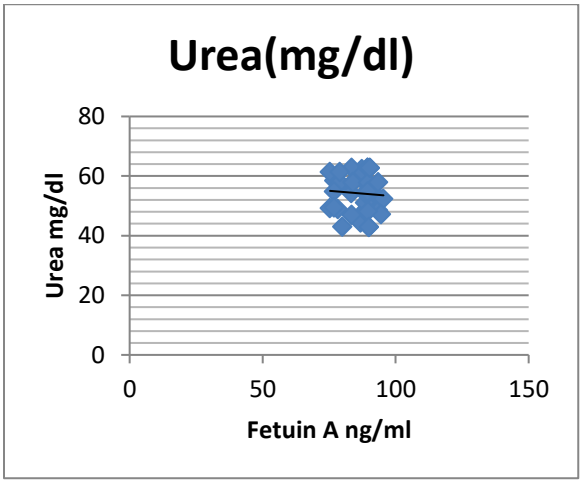


Figure 5.23: Correlation of serum Fetuin-A and eGFR

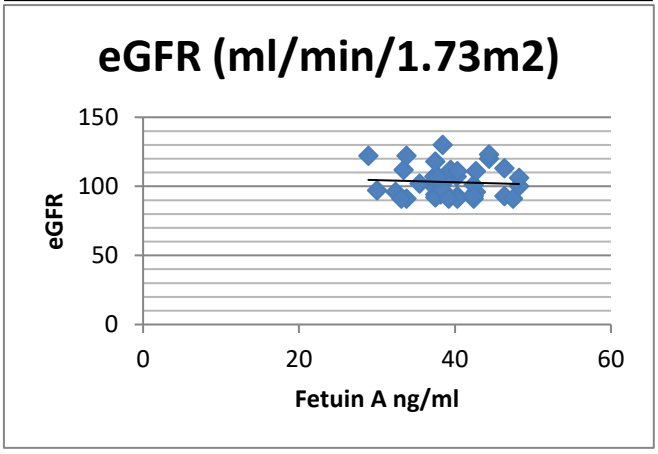


Figure 5.24: Correlation of serum Fetuin-A and Uric acid

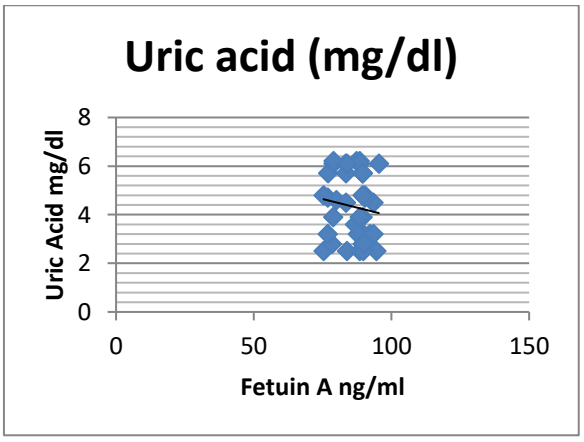


Figure 5.25: Correlation of serum Fetuin-A and microalbumin

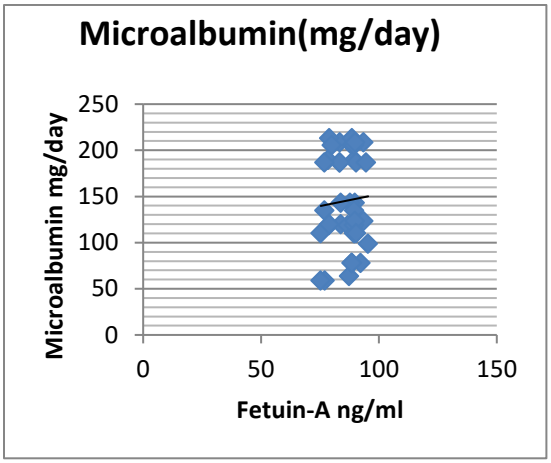


Figure 5.26: Correlation of serum Fetuin-A and CRP

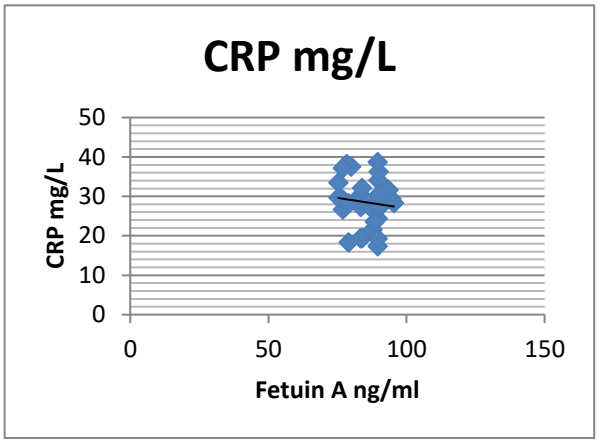


Figure 5.27: Correlation of serum Fetuin-A and FBS

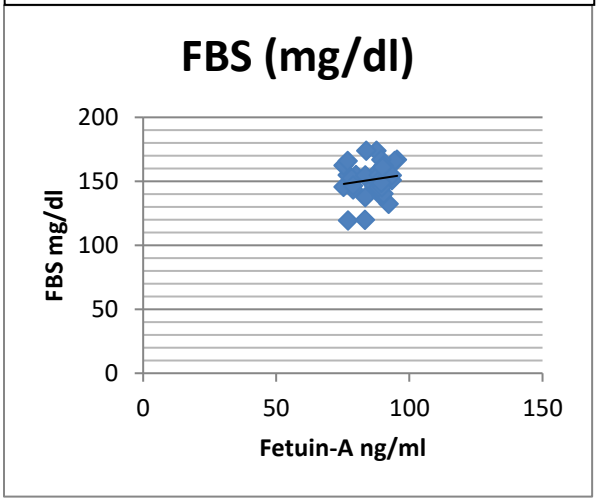


Figure 5.28: Correlation of serum Fetuin-A and PPBS

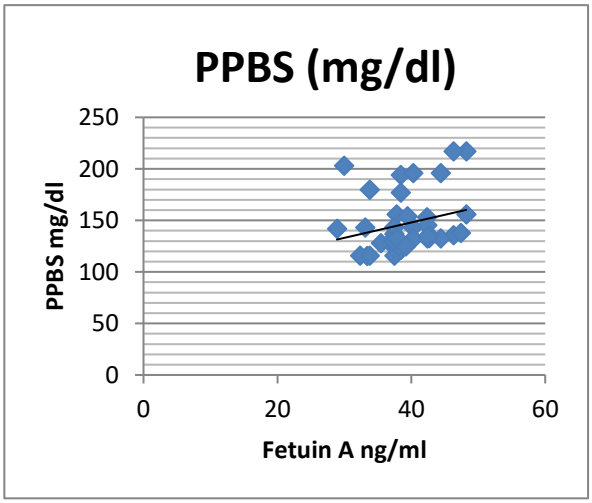


Figure 5.29: Correlation of serum Fetuin-A and HbA1c

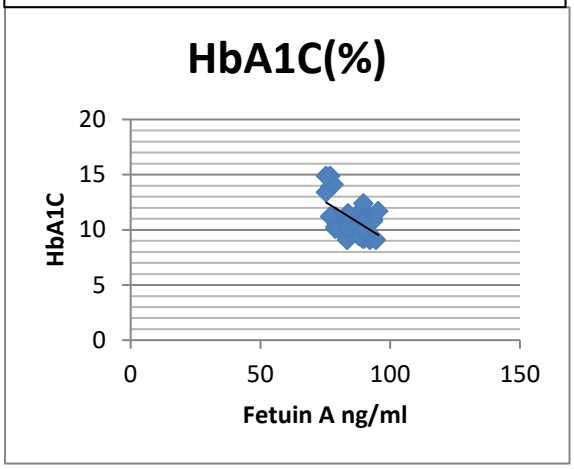


Figure 5.30: Correlation of serum Fetuin-A and HOMA-IR

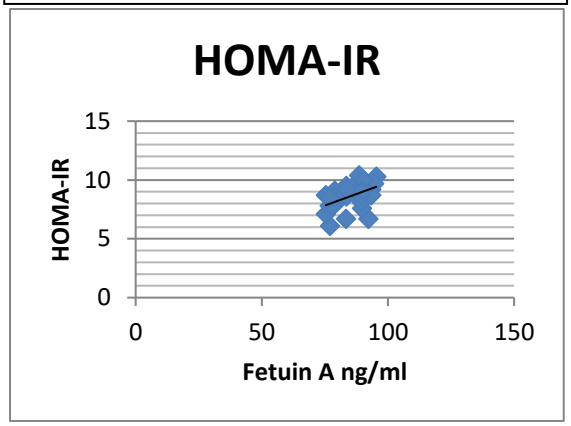


Figure 5.31: Correlation of serum Fetuin-A and Insulin

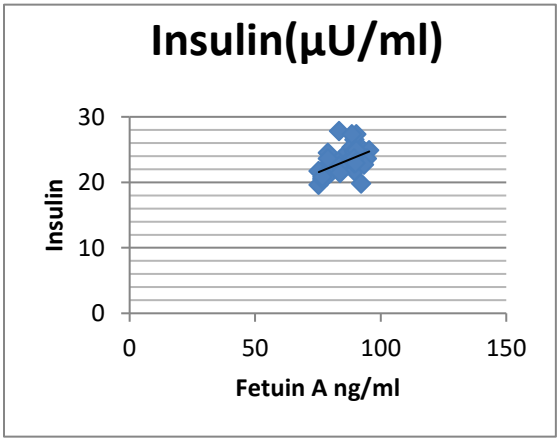


Figure 5.32: Correlation of serum Fetuin-A and FFA

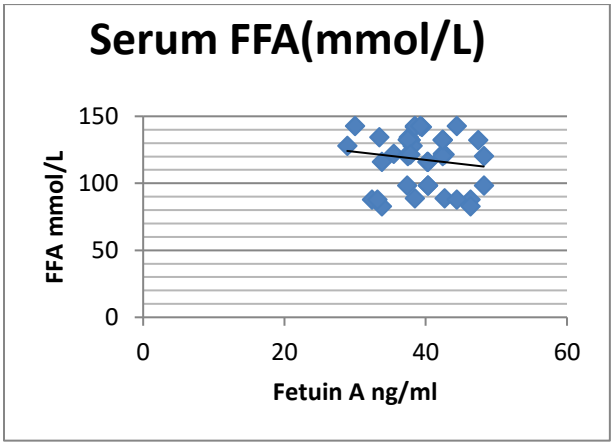


Figure 5.33: Correlation of serum Fetuin-A and TC

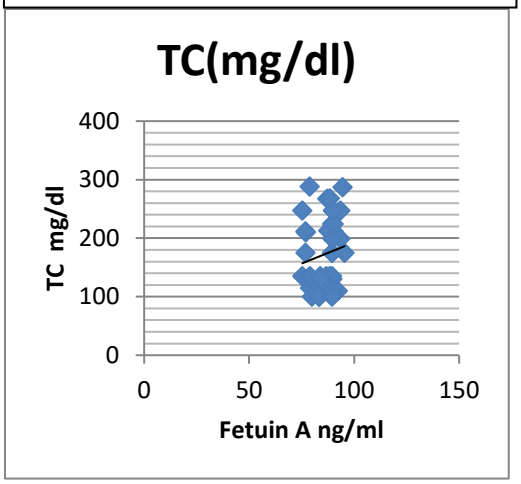


Figure 5.34: Correlation of serum Fetuin-A and TG

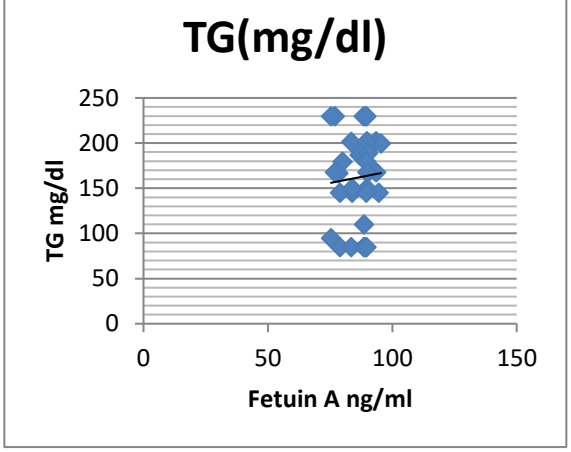


Figure 5. 35: Correlation of serum Fetuin-A and LDL

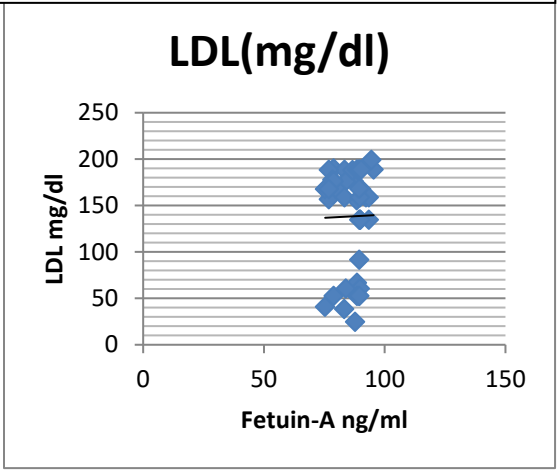


Figure 5.36: Correlation of serum Fetuin-A and HDL

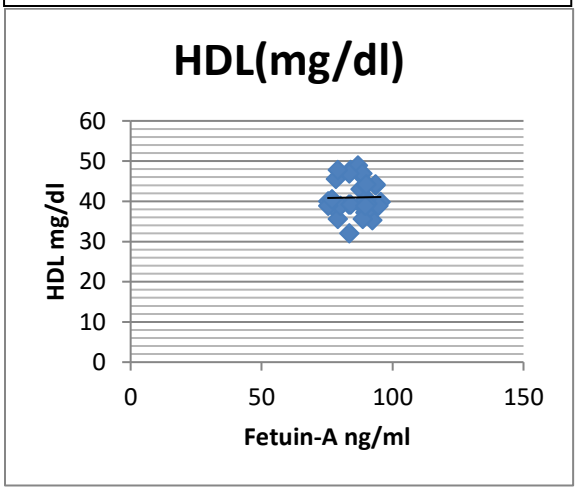
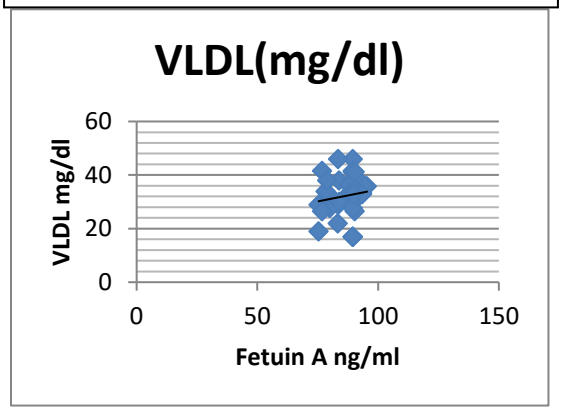


Figure5. 37: Correlation of serum Fetuin-A and VLDL



Correlation of serum Fetuin-A with Glycemic status, renal parameters, CRP, lipid profile parameters and serum FFA in Stage 3:

Figure 5.38: Correlation of serum Fetuin-A and Creatinine

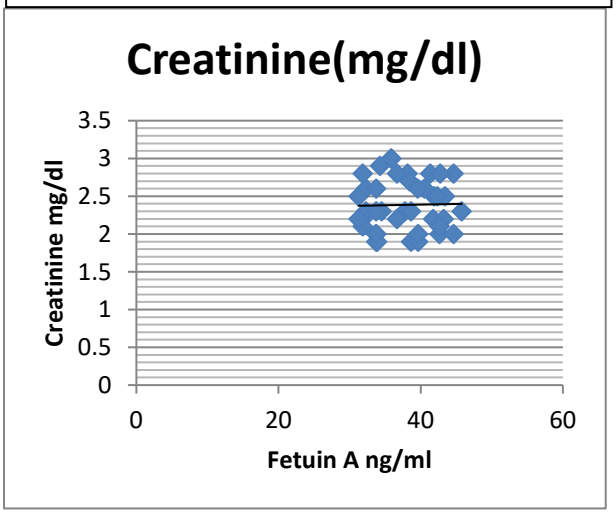


Figure 5.39: Correlation of serum Fetuin-A and Urea

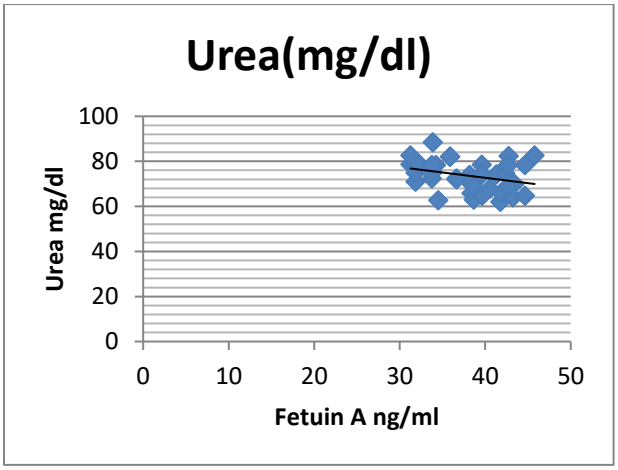


Figure 5.40: Correlation of Fetuin-A and Uric acid

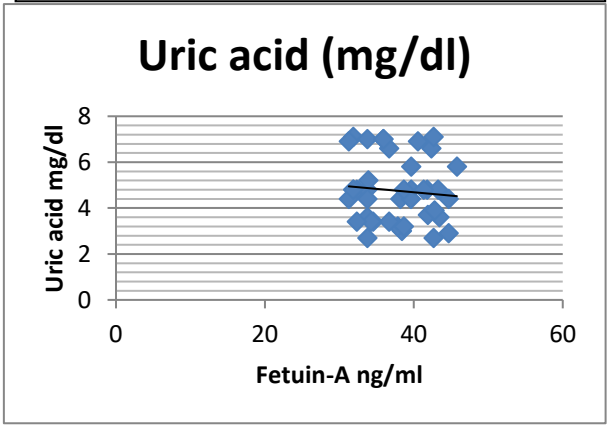


Figure5. 41: Correlation of Fetuin-A and CRP

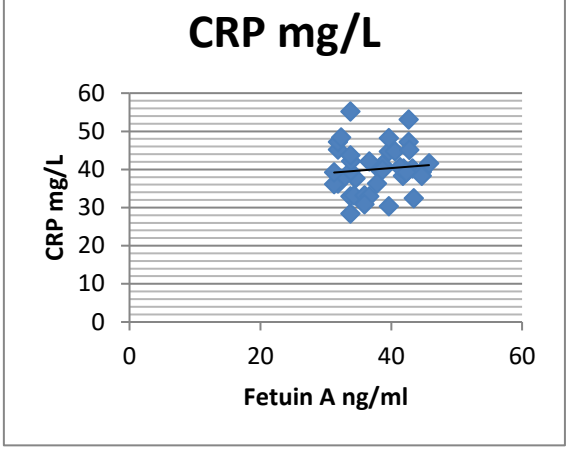


Figure 5.42: Correlation of serum Fetuin-A and eGFR

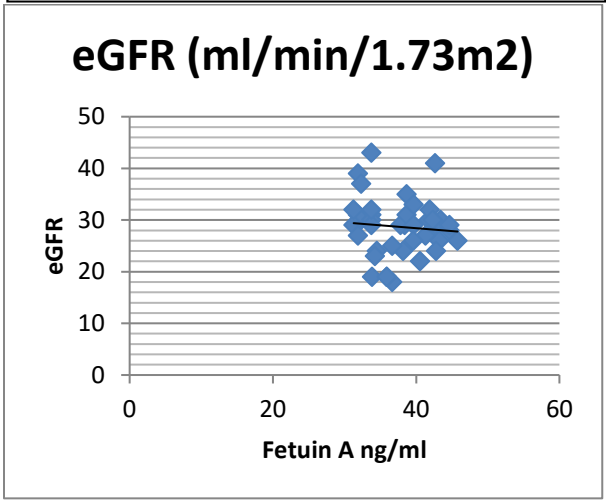


Figure 5.43: Correlation of serum Fetuin-A and Microalbumin

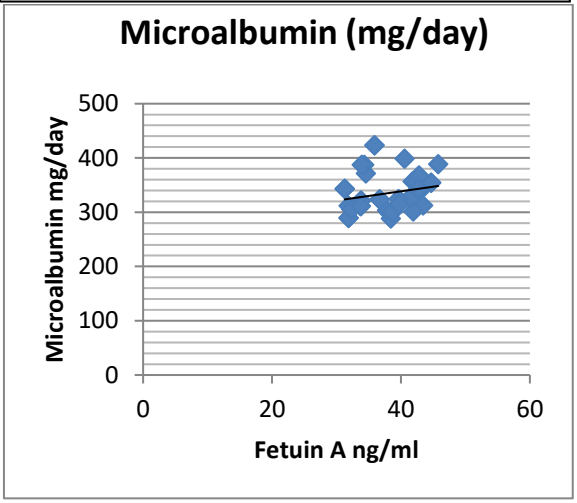


Figure 5.44: Correlation of serum Fetuin-A and HbA1c

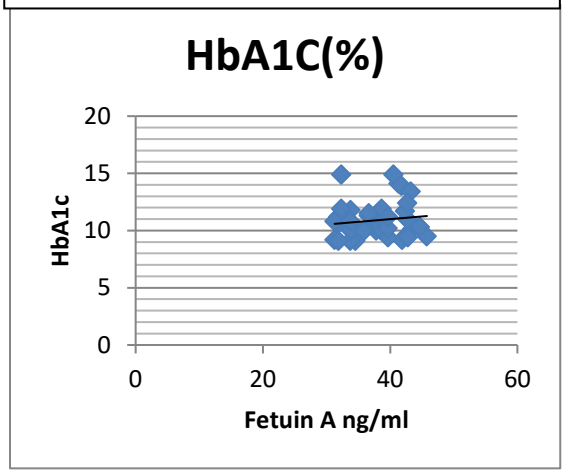


Figure 5.45: Correlation of serum Fetuin-A and FBS

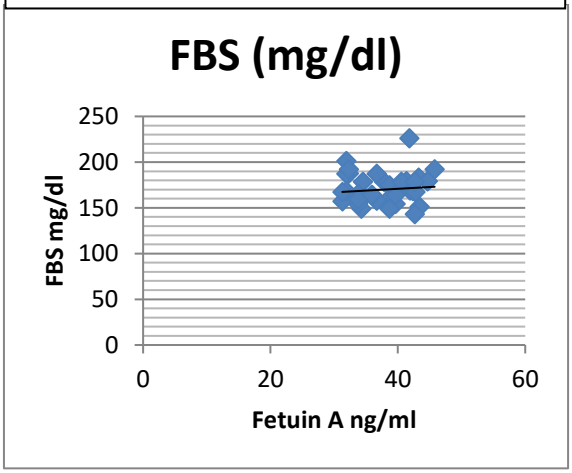


Figure 5.46: Correlation of serum Fetuin-A and Insulin

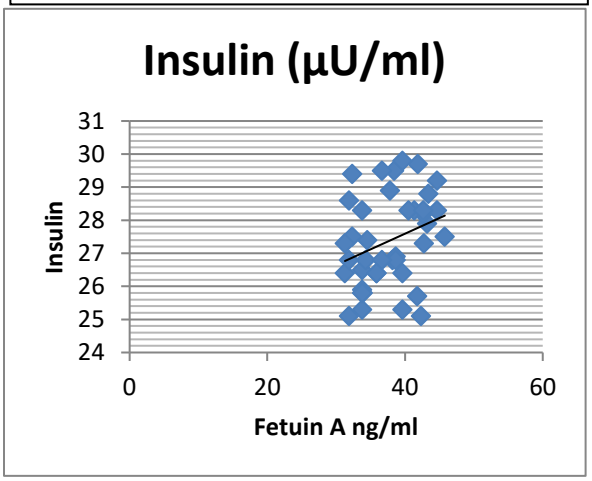


Figure 5.47: Correlation of serum Fetuin-A and PPBS

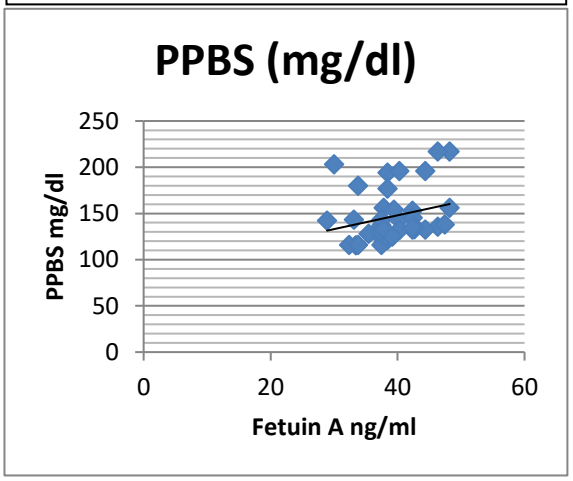


Figure 5.48: Correlation of serum Fetuin-A and HOMA-IR

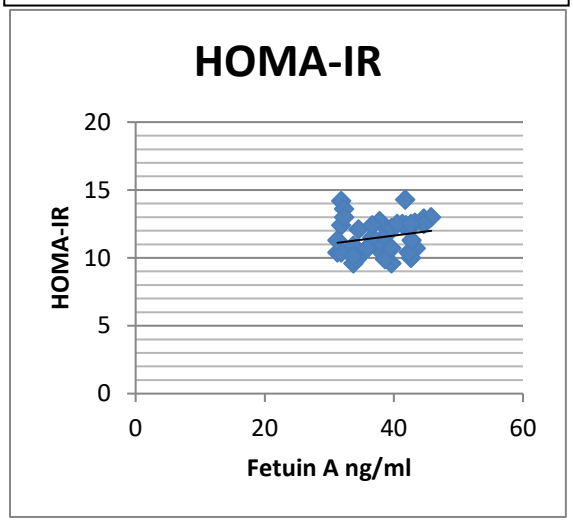


Figure 5.49: Correlation of serum Fetuin-A and serum FFA

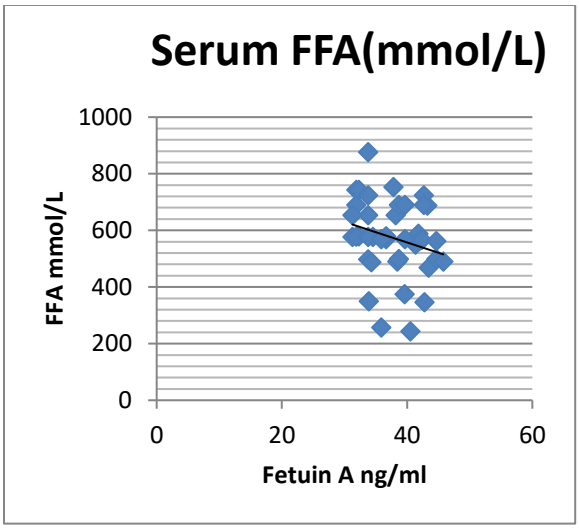


Figure 5.50: Correlation of serum Fetuin-A and TC

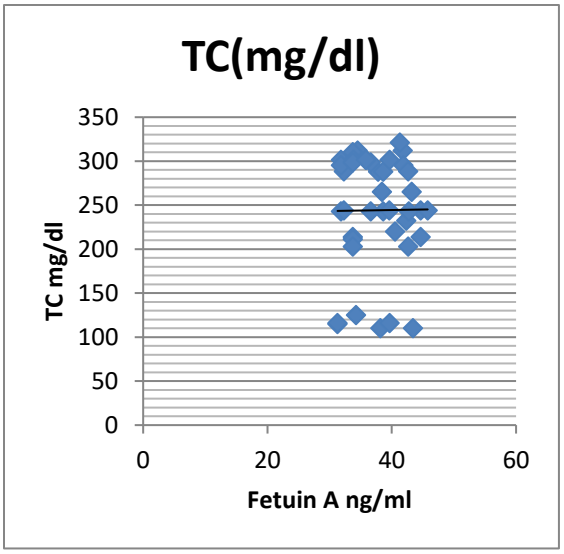


Figure 5.51: Correlation of serum Fetuin-A and TG

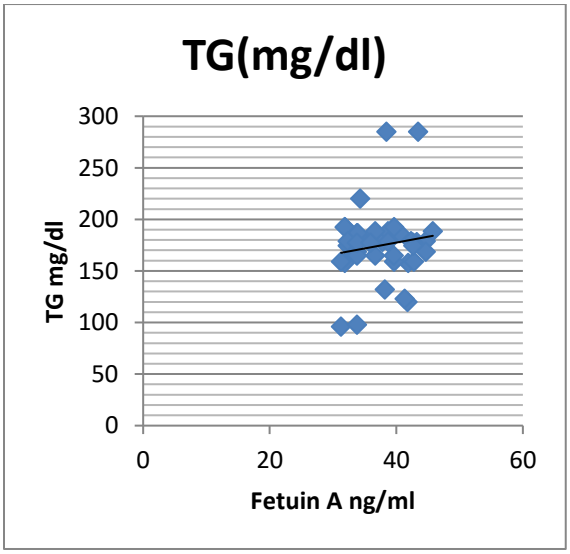


Figure 5.52: Correlation of serum Fetuin-A and HDL

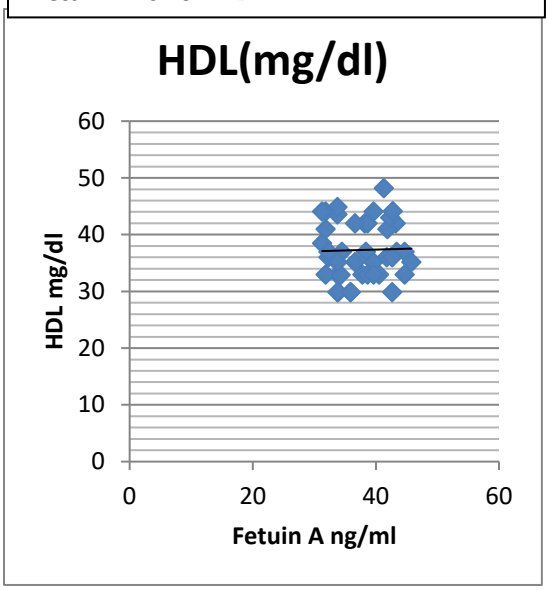


Figure 5.53: Correlation of serum Fetuin-A and LDL

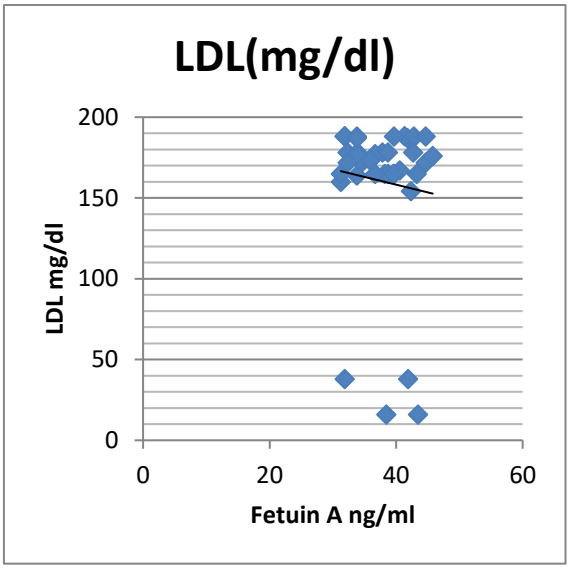
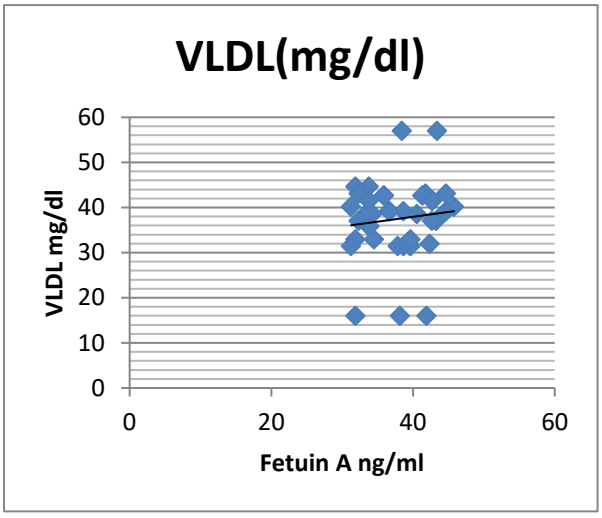


Figure 5.54: Correlation of serum Fetuin-A and VLDL



Correlation of serum Fetuin-A with Glycemic status, renal parameters, CRP, lipid profile parameters and serum FFA in Stage 4:

Figure 5.55: Correlation of serum Fetuin-A and Creatinine

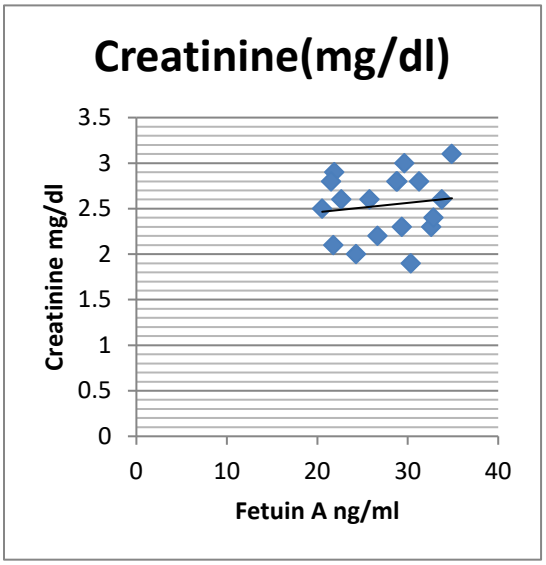


Figure 5.56: Correlation of serum Fetuin-A and Urea

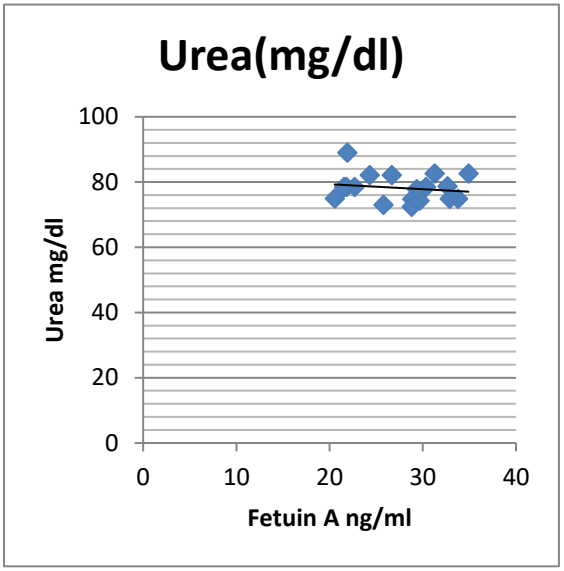


Figure 5.57: Correlation of serum Fetuin-A and Uric acid

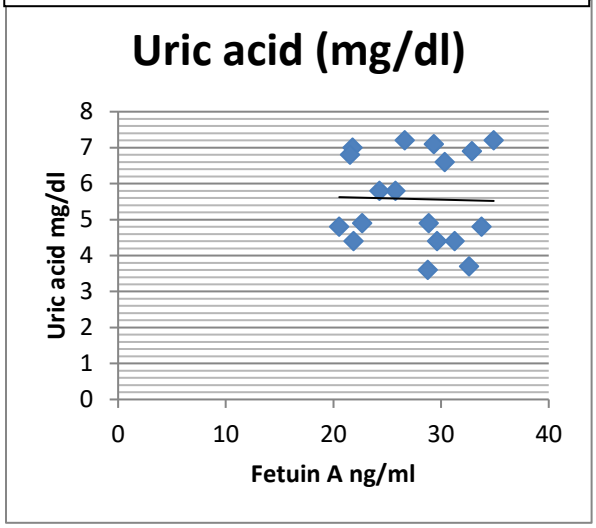


Figure 5.58: Correlation of serum Fetuin-A and CRP

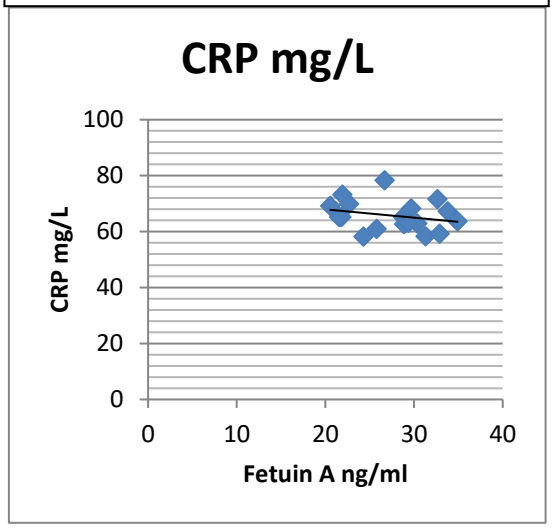


Figure 5.59: Correlation of serum Fetuin-A and eGFR

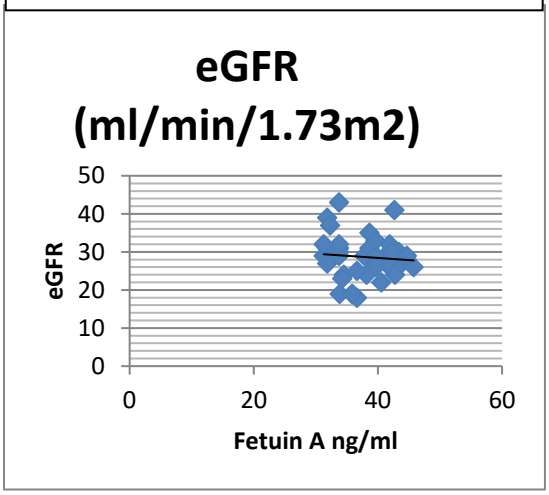


Figure 5.60: Correlation of serum Fetuin-A and Microalbumin

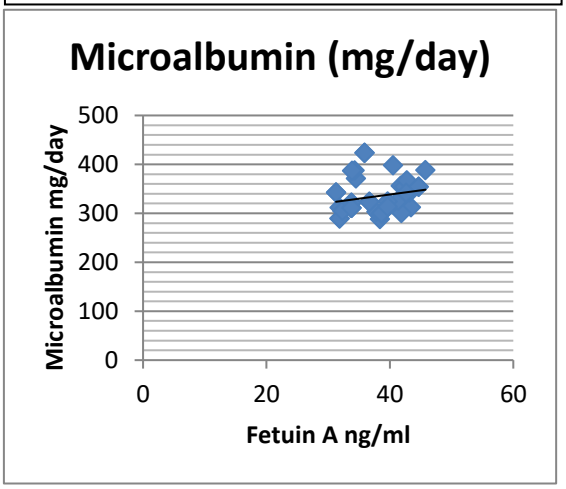


Figure 5.61: Correlation of serum Fetuin-A and FBS

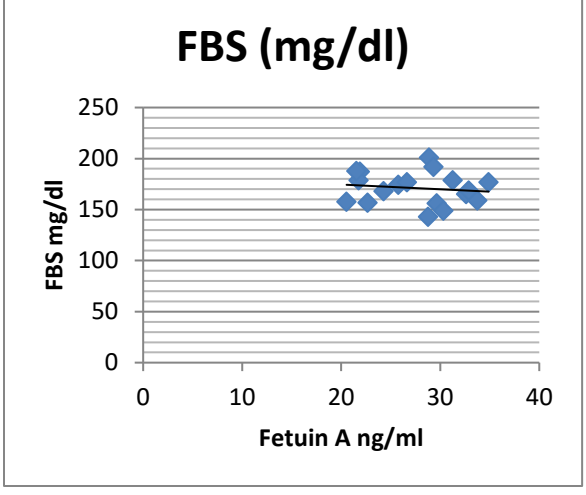


Figure 5.62: Correlation of serum Fetuin-A and PPBS

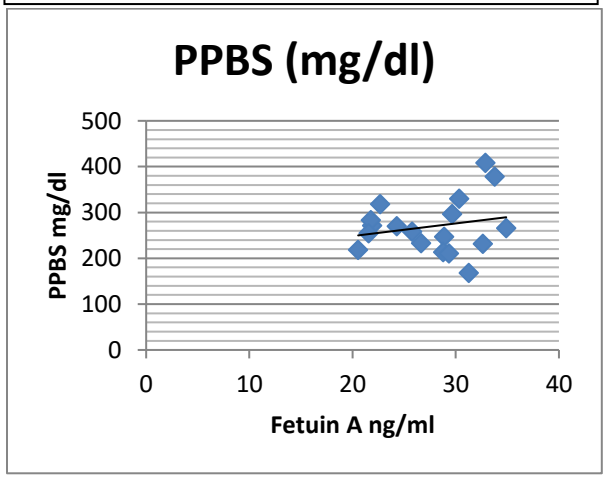


Figure5. 63: Correlation of serum Fetuin-A and Insulin

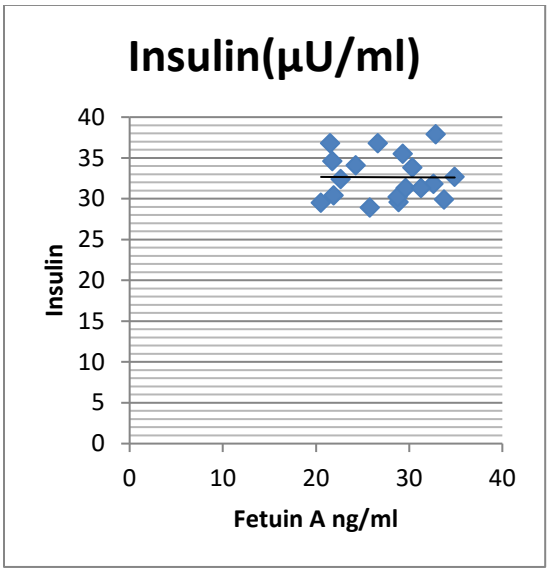


Figure5. 64: Correlation of serum Fetuin-A and HOMA-IR

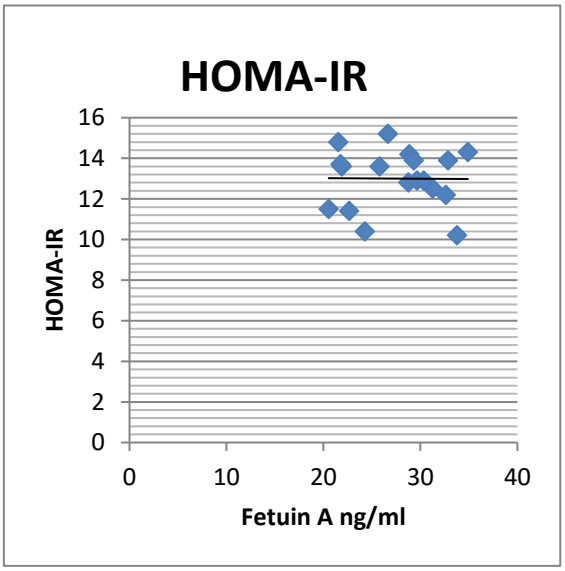


Figure 5.65: Correlation of serum Fetuin-A and serum FFA

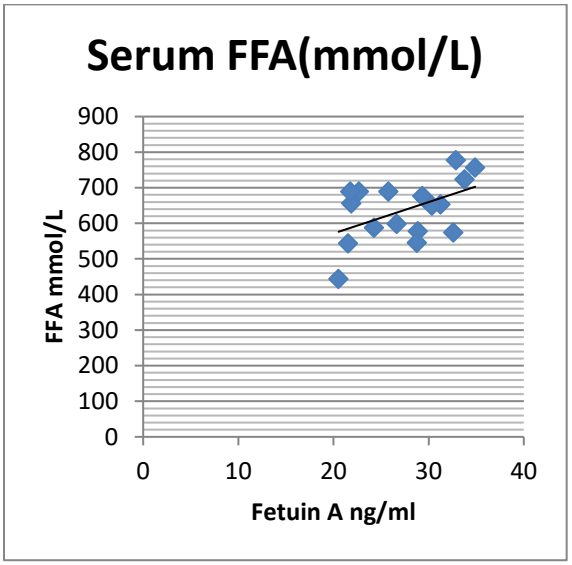


Figure 5.66: Correlation of serum Fetuin-A and TC

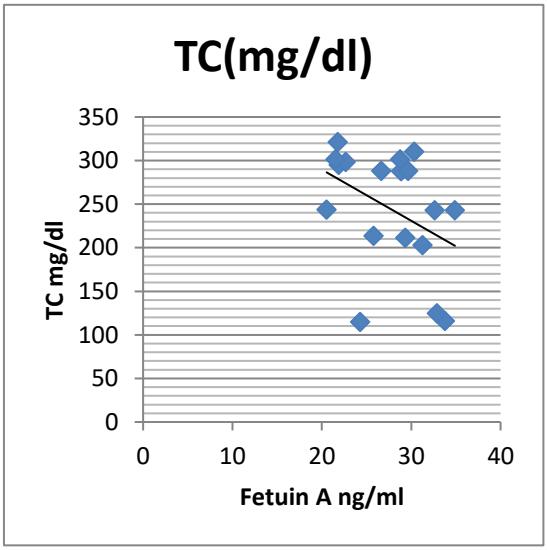


Figure 5. 67: Correlation of serum Fetuin-A and TG

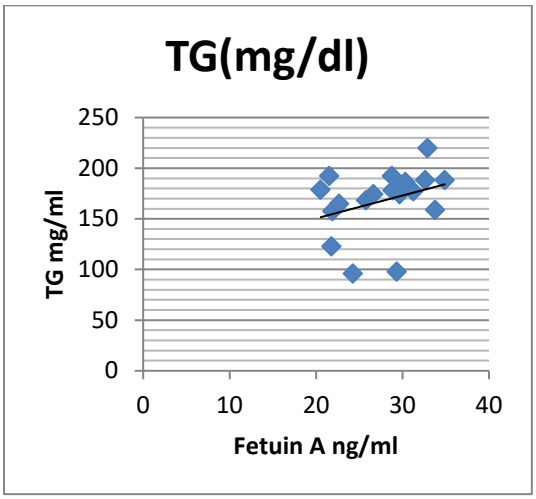


Figure 5.68: Correlation of serum Fetuin-A and HDL

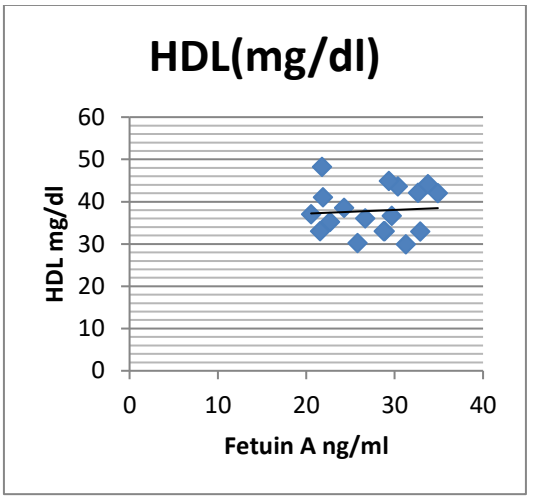


Figure 5.69: Correlation of serum Fetuin-A and LDL

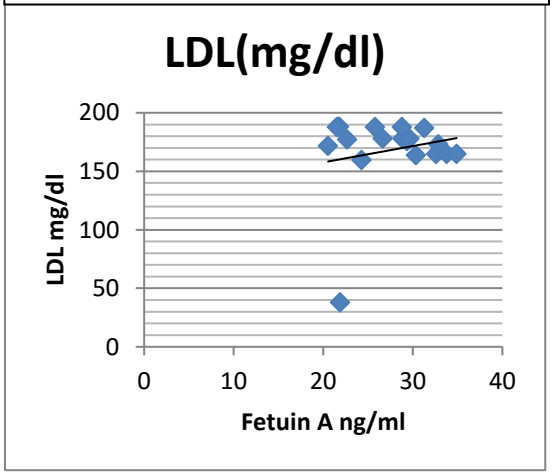
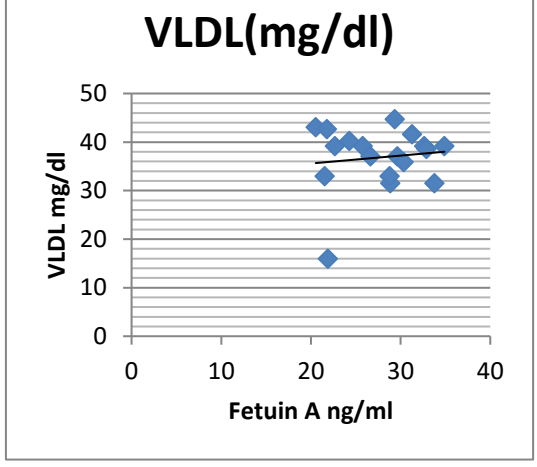


Figure 5.70: Correlation of serum Fetuin-A and VLDL



5.11. Urinary Fetuin-A correlation with all the parameters:

Urinary Fetuin-A levels were significantly increased in diabetic nephropthy cases of all stages compared to controls $p<0.001$. Urinary levels correlated positively with severity of stages in diabetic nephropathy.

Table 5.12 : Correlation of urinary Fetuin-A with renal parameters, CRP, glycemic status and lipid profile in controls and stages of diabetic nephropathy cases.

Parameters	Controls	Stage 1	Stage 2	Stage 3	Stage 4
	r – value				
Blood urea	0.259	0.223	0.056	0.091	0.312
Serum Creatinine	0.213	0.382	0.080	0.034	0.163
Uric acid	0.036	0.026	0.265	0.213	0.11
eGFR	-0.024	-0.006	-0.099	-0.108	-0.062
Urine MA	0.005	0.152	0.059	0.111	0.172
SerumFetuin-A	0.115	0.218	0.053	0.208	0.215
CRP	0.201	0.193	0.181	0.173	0.131
FBS	0.166	0.181	0.222	0.277	0.172
PPBS	0.194	0.291	0.296	0.042	0.052
HbA1c	0.058	0.160	0.171	0.079	0.121
Insulin	0.239	0.126	0.154	0.20	0.17
HOMA-IR	0.159	0.21	0.279	0.141	0.21
Sr.FFA	0.175	0.157	0.103	0.186	0.112
T.Chol	0.012	0.082	0.055	0.209	0.072
TGL	0.075	0.315	0.133	0.247	0.109
HDL	-0.116	-0.094	-0.10	-0.057	-0.017
LDL	0.09	0.288	0.142	0.009	0.041
VLDL	0.191	0.062	0.156	0.306	0.10

Table 5.12 shows the significant positive correlation of urinary Fetuin-A levels with the above parameters in diabetic nephropathy cases compared to controls except HDL and eGFR in all the stages of diabetic nephropathy.

Correlation of Urinary Fetuin-A with renal parameters, CRP, lipid profile parameters and serum FFA in stage 1:

Figure 5.71: Correlation of Urinary Fetuin-A and Creatinine

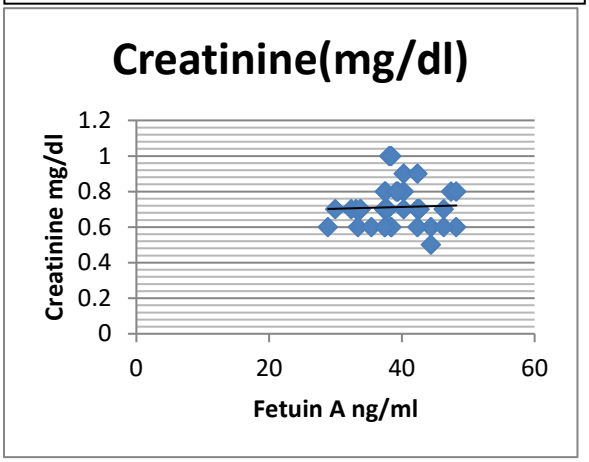


Figure 5.72: Correlation of Urinary Fetuin-A and urea

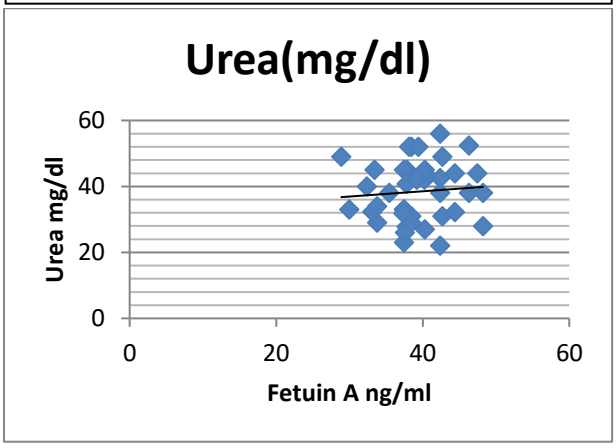


Figure 5.73: Correlation of Urinary Fetuin-A and uric acid

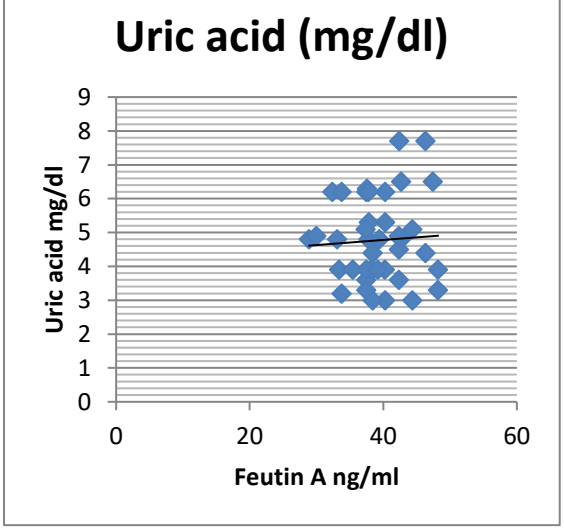


Figure 5.74: Correlation of Urinary Fetuin-A and eGFR

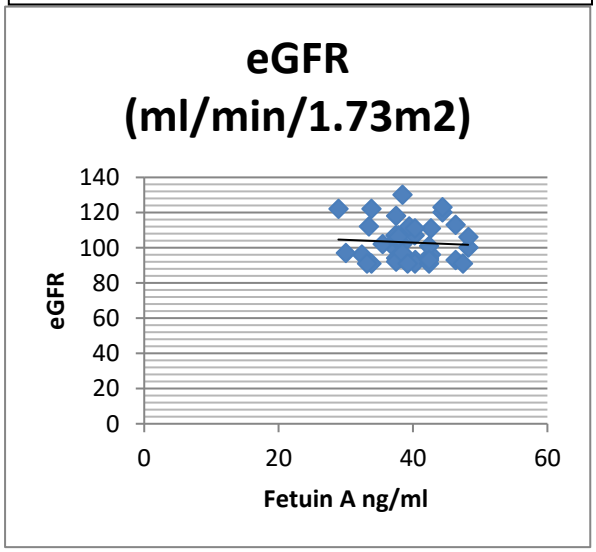


Figure 5.75: Correlation of Urinary Fetuin-A and Microalbumin

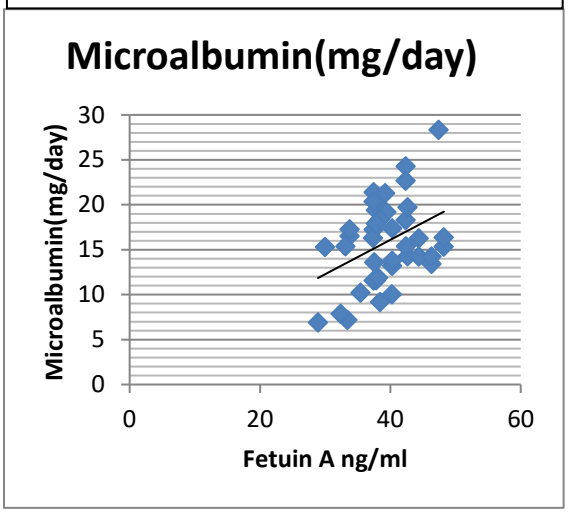


Figure 5.76: Correlation of Urinary Fetuin-A and CRP

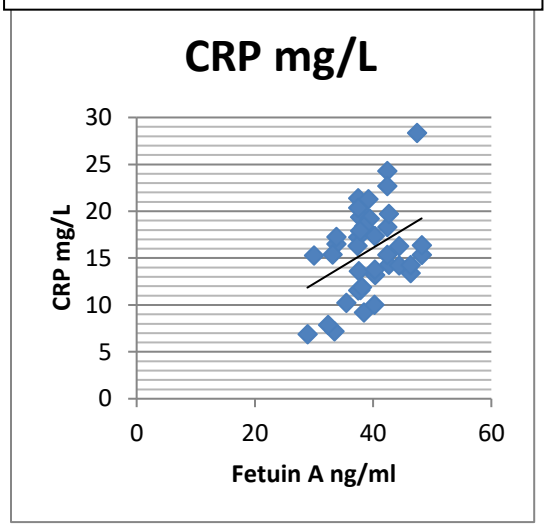


Figure 5.77: Correlation of Urinary Fetuin-A and FBS

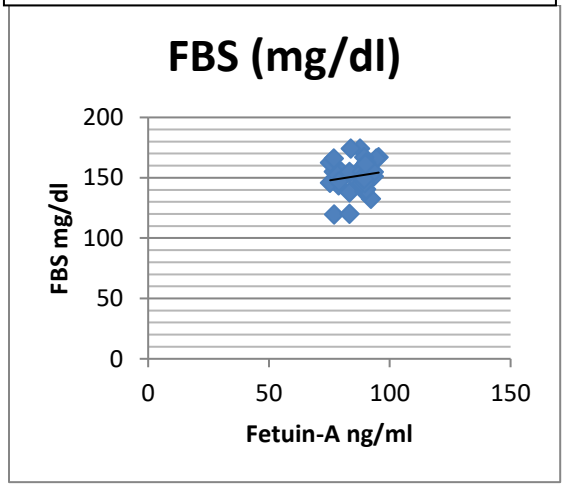


Figure 5.78: Correlation of Urinary Fetuin-A and PPBS

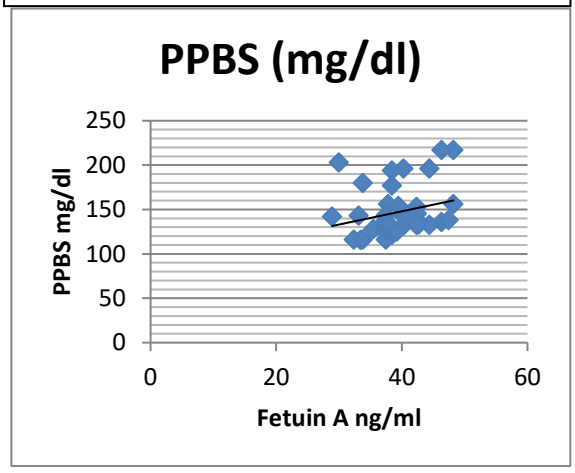


Figure 5.79: Correlation of Urinary Fetuin-A and Insulin

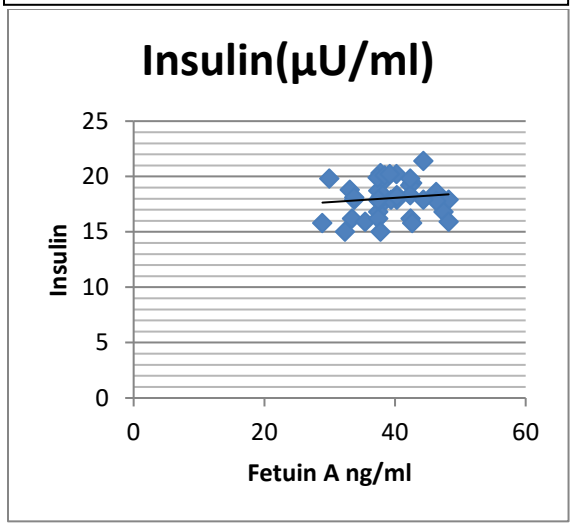


Figure 5.80: Correlation of Urinary Fetuin-A and HOMA-IR

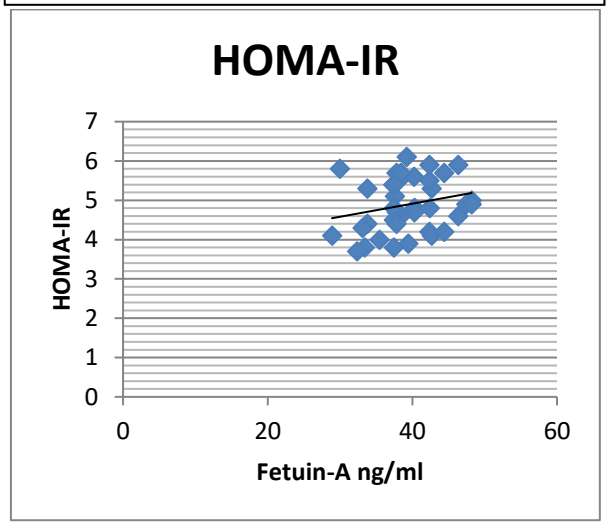


Figure 5.81: Correlation of Urinary Fetuin-A and HbA1c

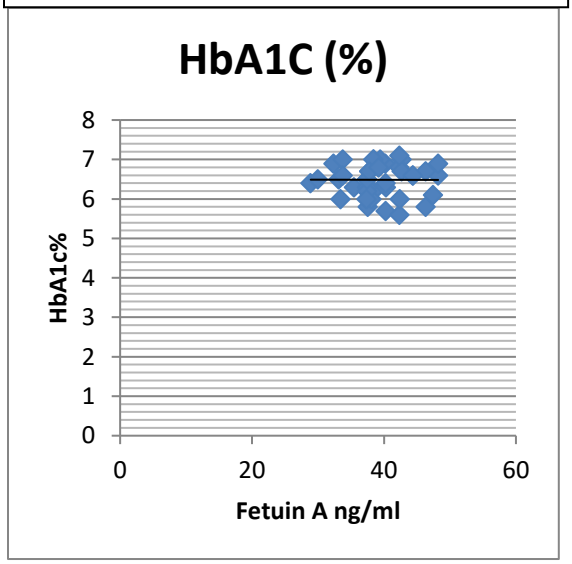


Figure 5.82: Correlation of Urinary Fetuin-A and FFA

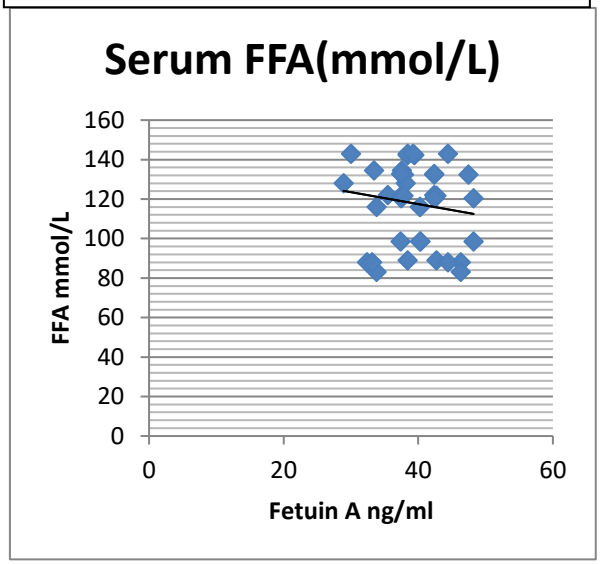


Figure 5.83: Correlation of Urinary Fetuin-A and total cholesterol

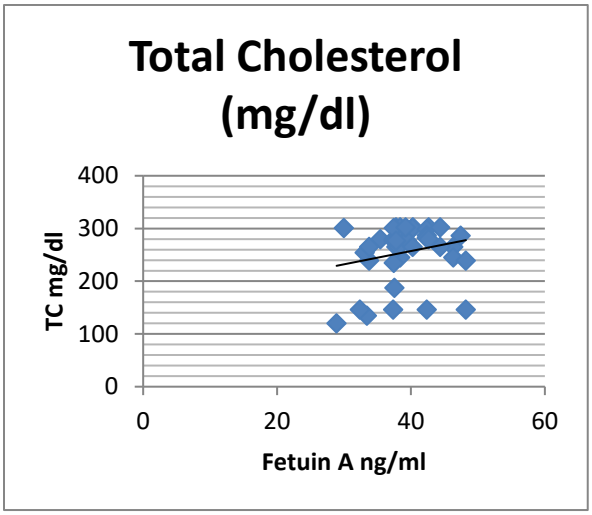


Figure 5.84: Correlation of Urinary Fetuin-A and triglyceride

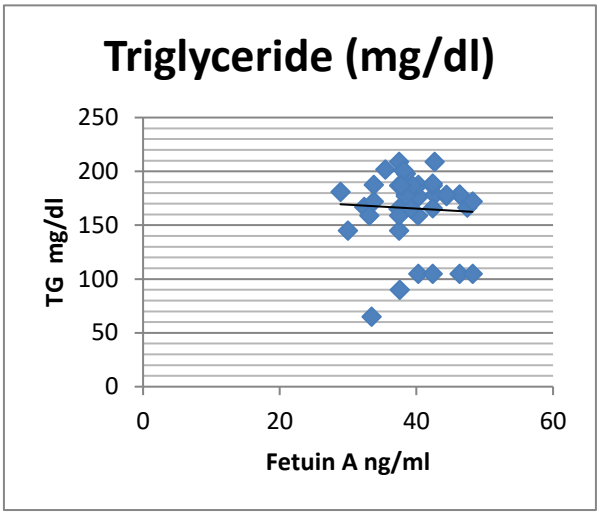


Figure 5.85: Correlation of Urinary Fetuin-A and LDL

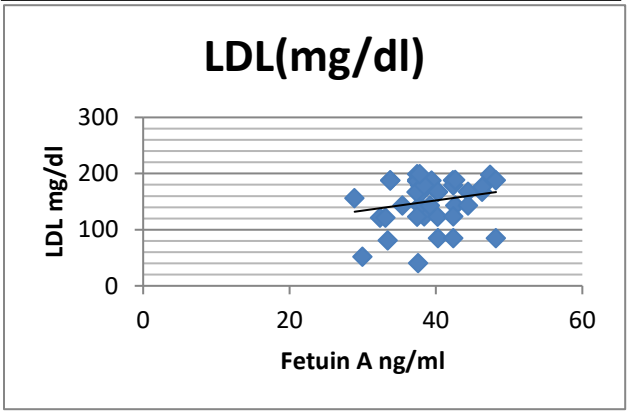


Figure 5.86: Correlation of Urinary Fetuin-A and V LDL

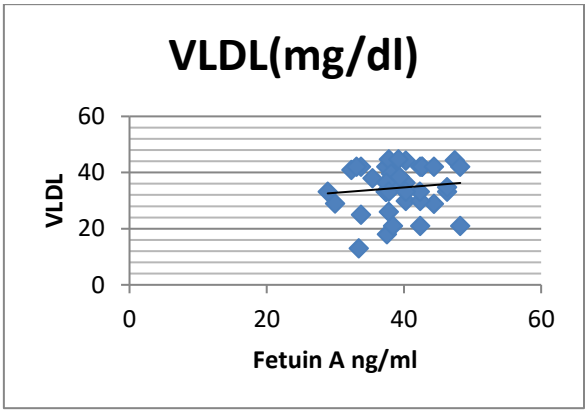
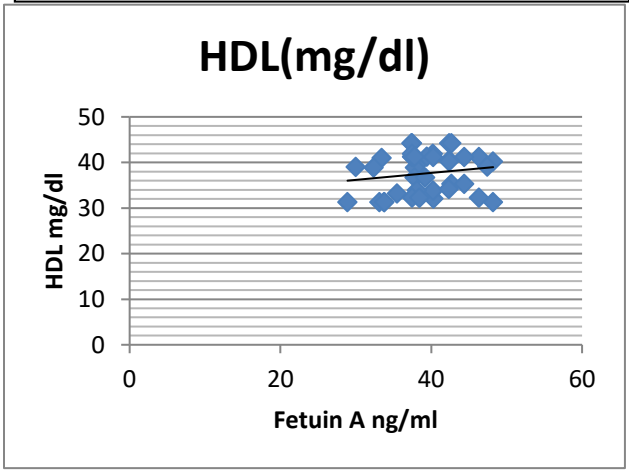


Figure 5.87: Correlation of Urinary Fetuin-A and HDL



Correlation of Urinary Fetuin-A with renal parameters, CRP, lipid profile parameters and serum FFA in stage 2:

Figure 5.88: Correlation of Urinary Fetuin-A and Creatinine

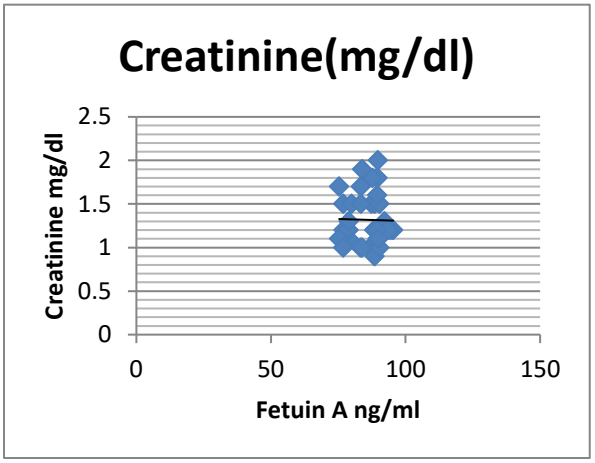


Figure 5.89: Correlation of Urinary Fetuin-A and Urea

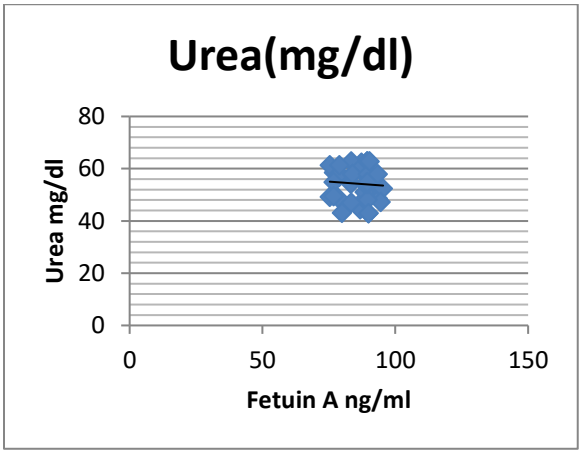


Figure 5.90: Correlation of Urinary Fetuin-A and eGFR

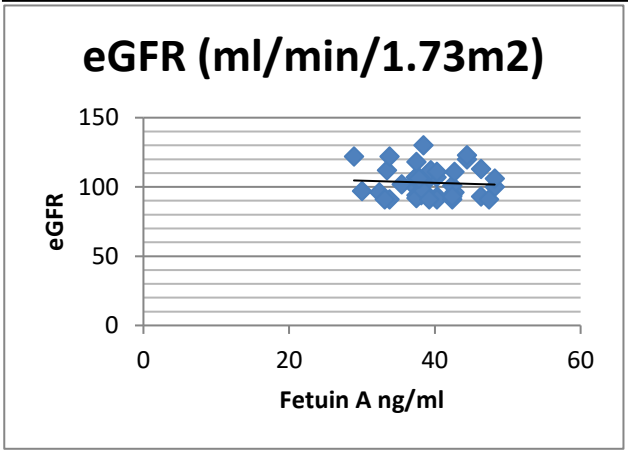


Figure 5.91: Correlation of Urinary Fetuin-A and Uric acid

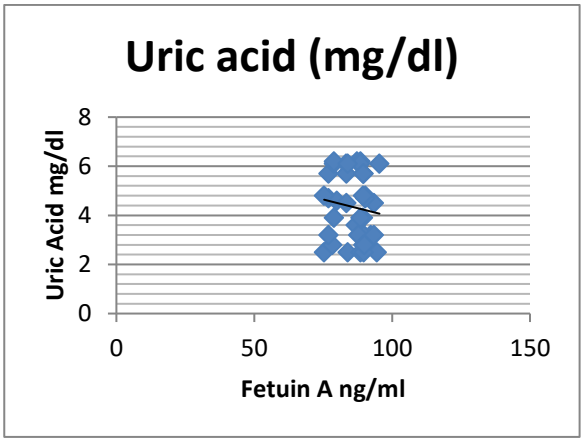


Figure 5.92: Correlation of Urinary Fetuin-A and microalbumin

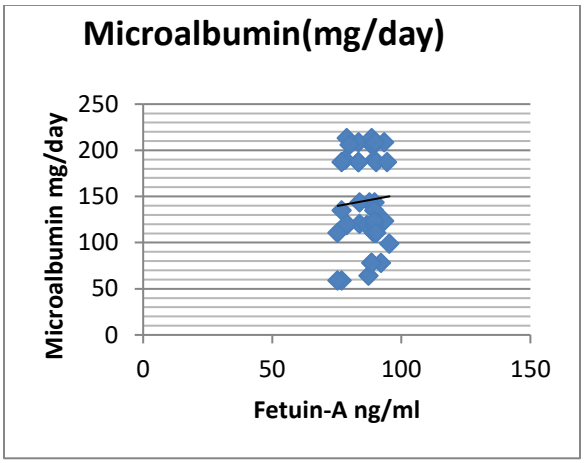


Figure 5.93: Correlation of Urinary Fetuin-A and CRP

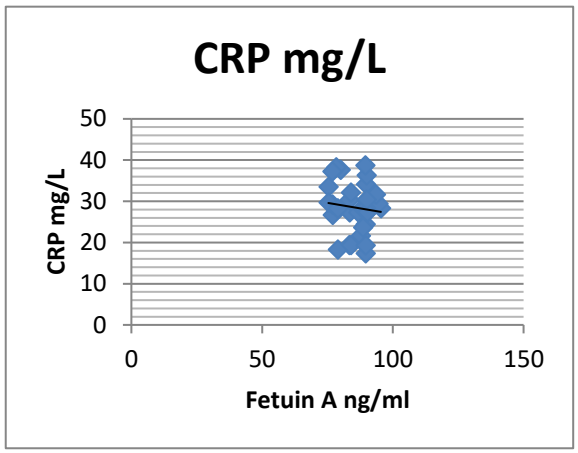


Figure 5.94: Correlation of Urinary Fetuin-A and FBS

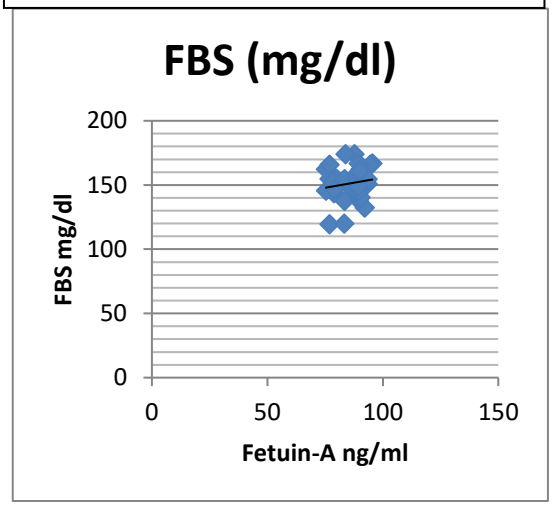


Figure 5.95: Correlation of Urinary Fetuin-A and PPBS

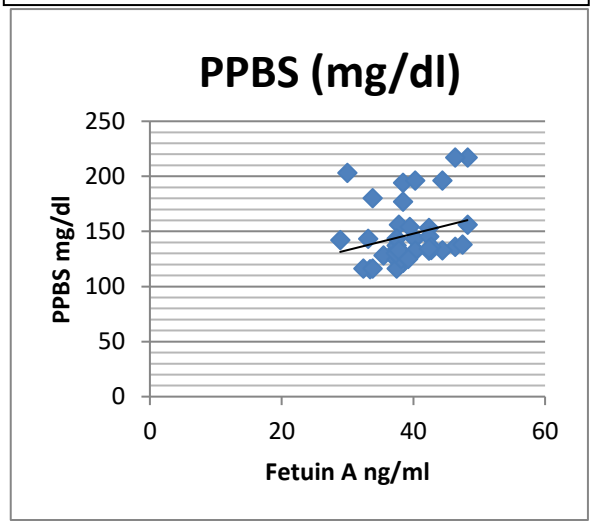


Figure 5.96: Correlation of Urinary Fetuin-A and HbA1c

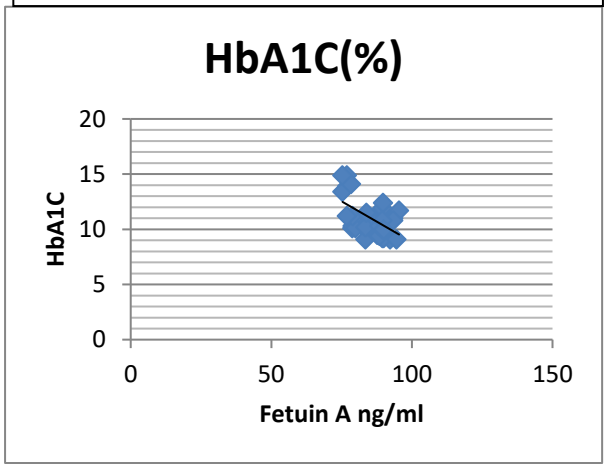


Figure 5.97: Correlation of F Urinary Fetuin-A and HOMA-IR

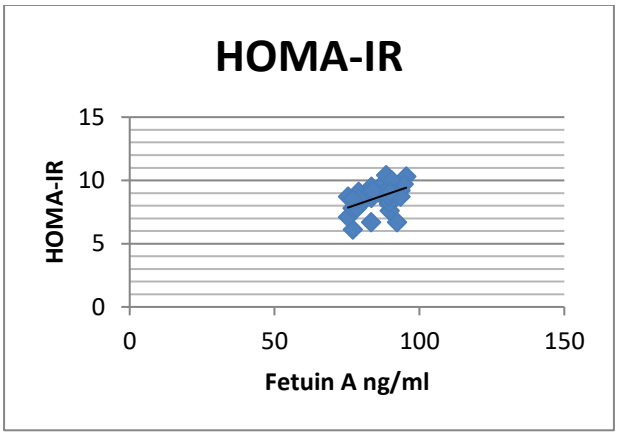


Figure 5.98: Correlation of Urinary Fetuin-A and Insulin

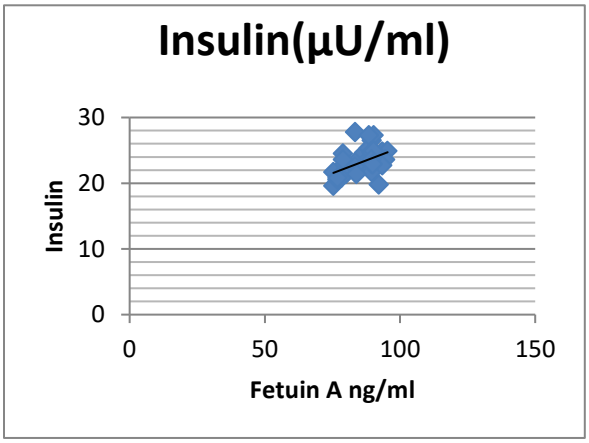


Figure 5.99: Correlation of Urinary Fetuin-A and FFA

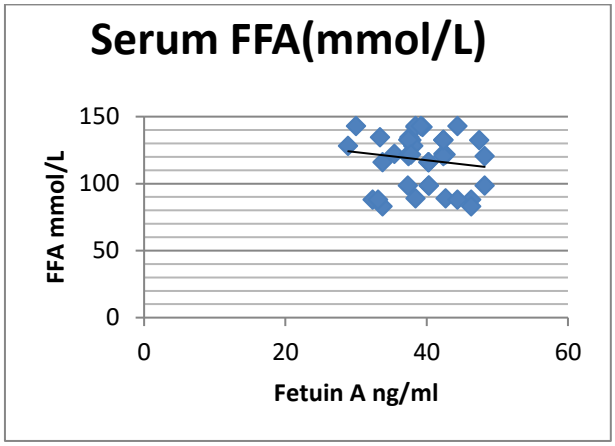


Figure 5.100: Correlation of Urinary Fetuin-A and TC

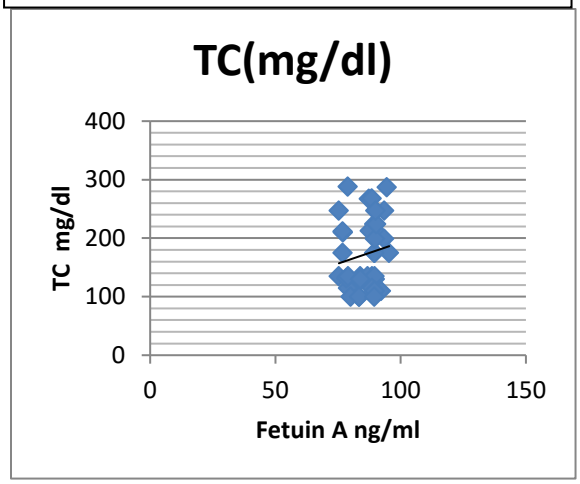


Figure 5.101: Correlation of Urinary Fetuin-A and TG

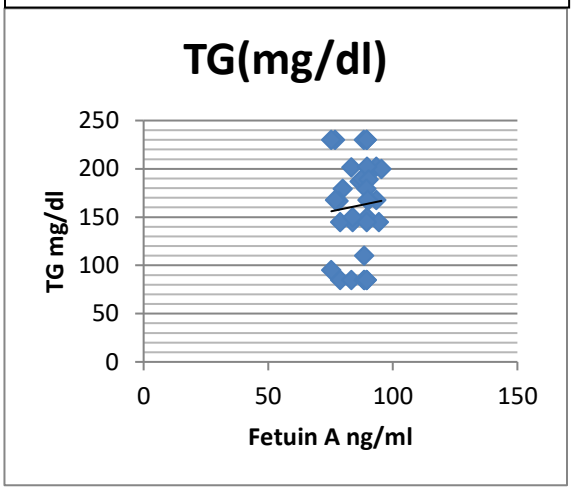


Figure 5.102: Correlation of Urinary Fetuin-A and LDL

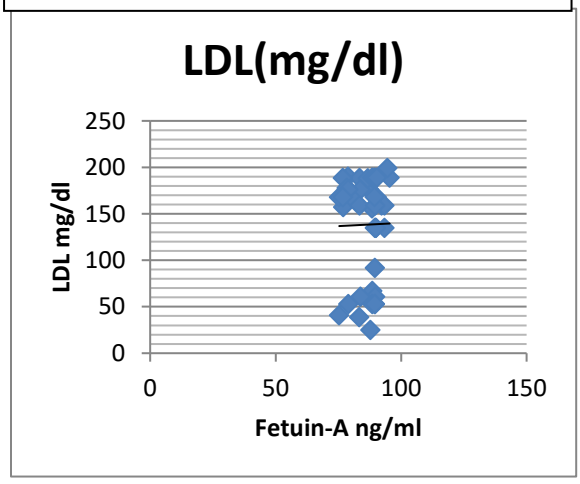


Figure 5.103: Correlation of Urinary Fetuin-A and HDL

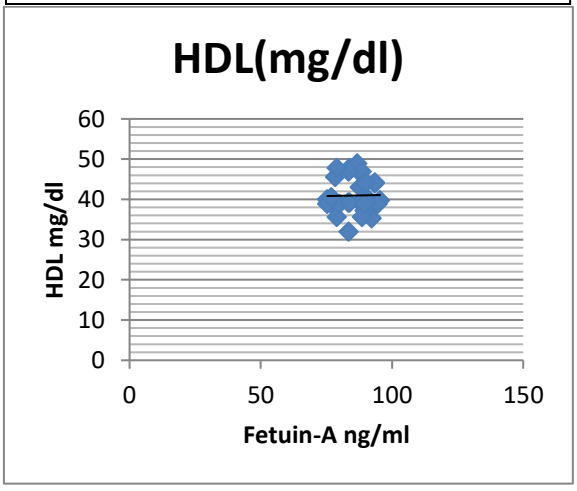
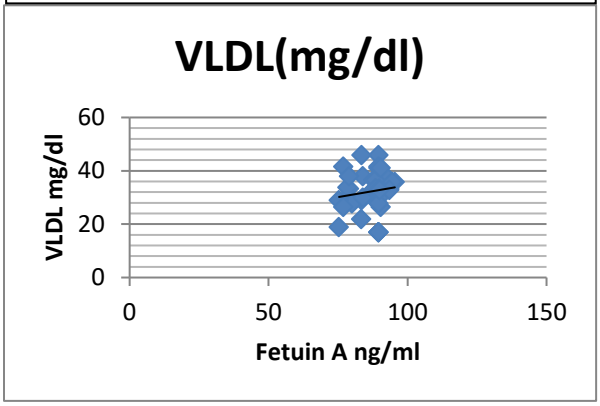


Figure 5.104: Correlation of Urinary Fetuin-A and VLDL



Correlation of Urinary Fetuin-A with renal parameters, CRP, lipid profile parameters and serum FFA in stage 3:

Figure 5.105: Correlation of Urinary Fetuin-A and Creatinine

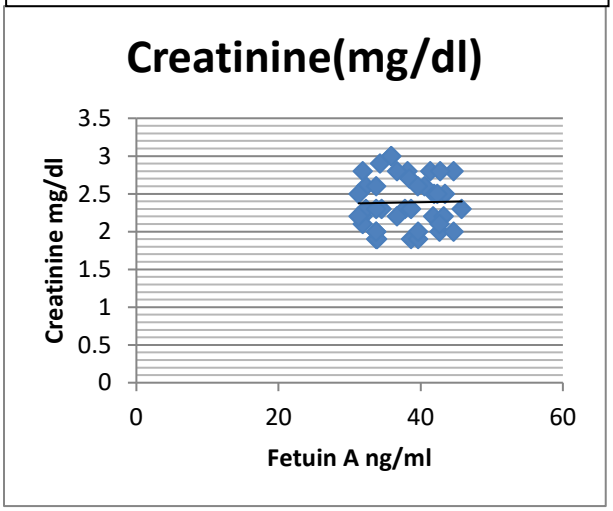


Figure 5.106: Correlation of Urinary Fetuin-A and Urea

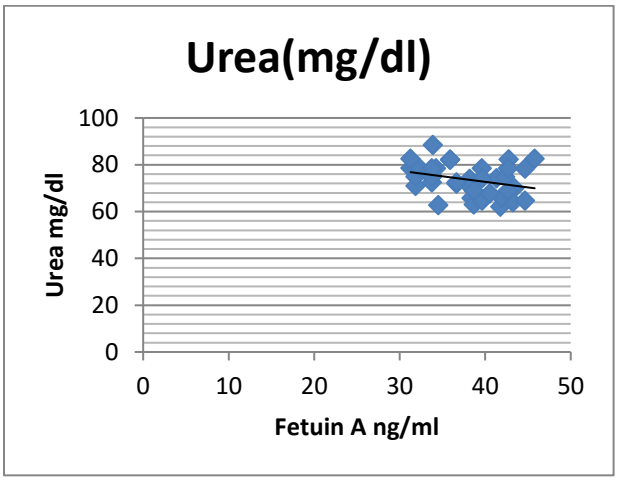


Figure 5.107: Correlation of Urinary Fetuin-A and Uric acid

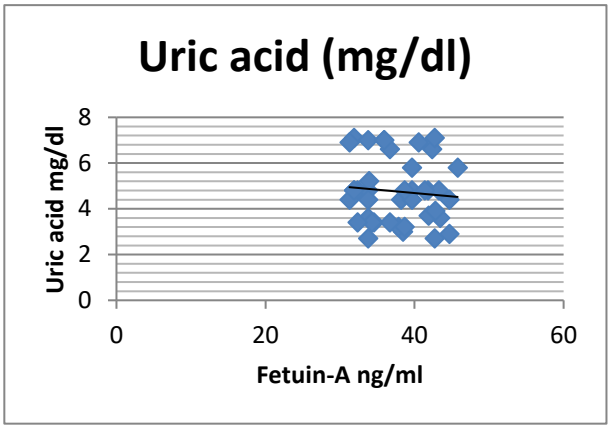


Figure 5.108: Correlation of Urinary Fetuin-A and CRP

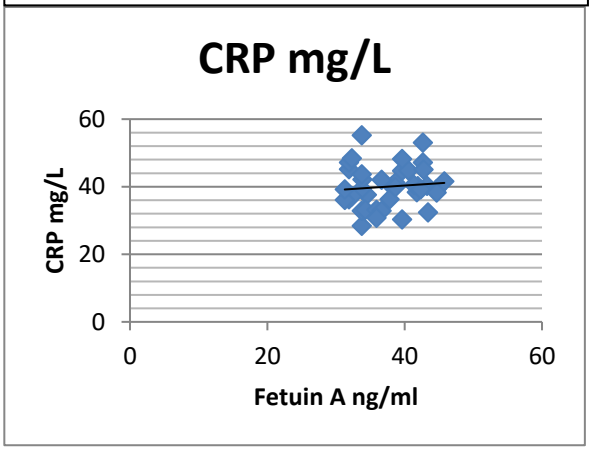


Figure 5.109: Correlation of Urinary Fetuin-A and eGFR

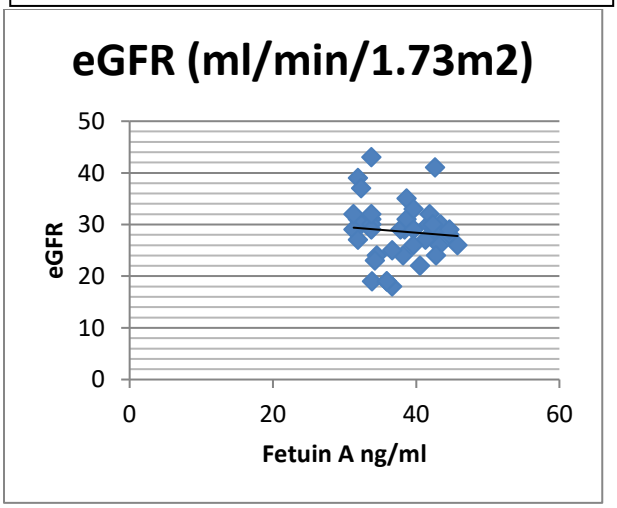


Figure 5.110: Correlation of Urinary Fetuin-A and Microalbumin

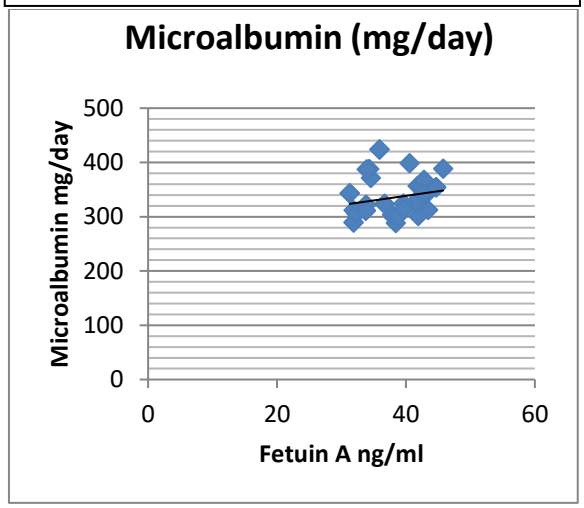


Figure 5.111: Correlation of Urinary Fetuin-A and HbA1c

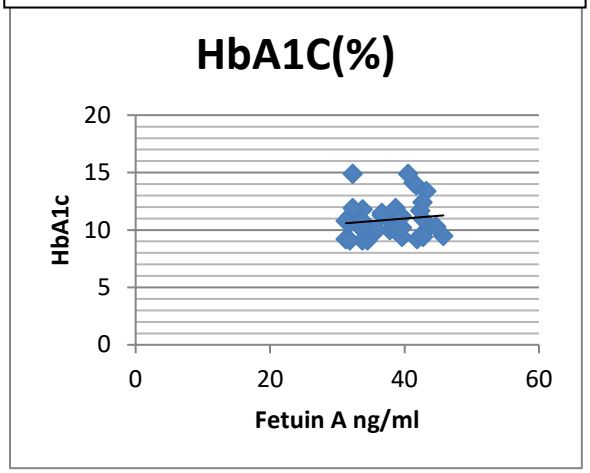


Figure 5.112: Correlation of Urinary Fetuin-A and FBS

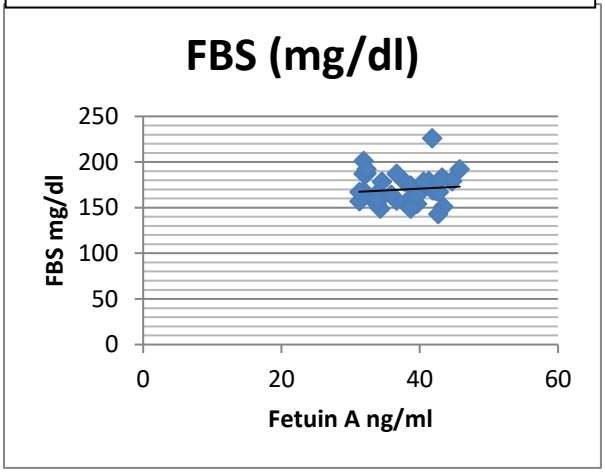


Figure 5.113: Correlation of Urinary Fetuin-A and Insulin

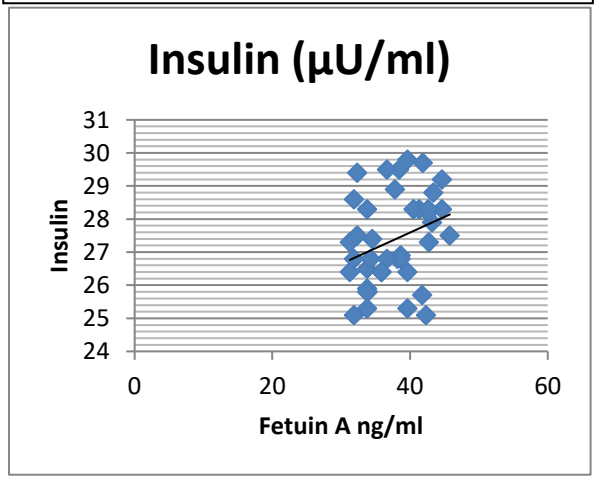


Figure 5.114: Correlation of Urinary Fetuin-A and PPBS

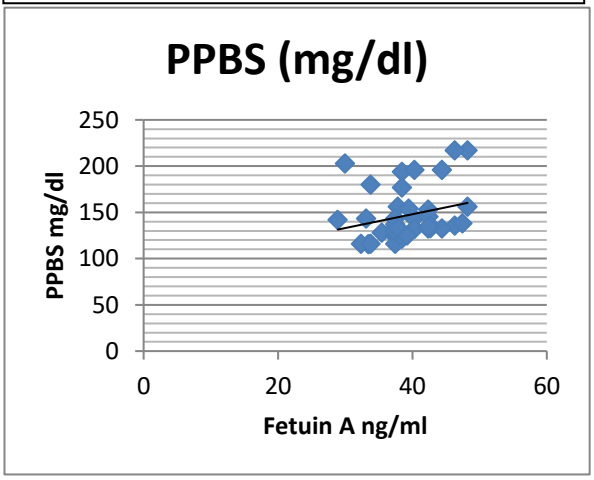


Figure 5.115: Correlation of Urinary Fetuin-A and HOMA-IR

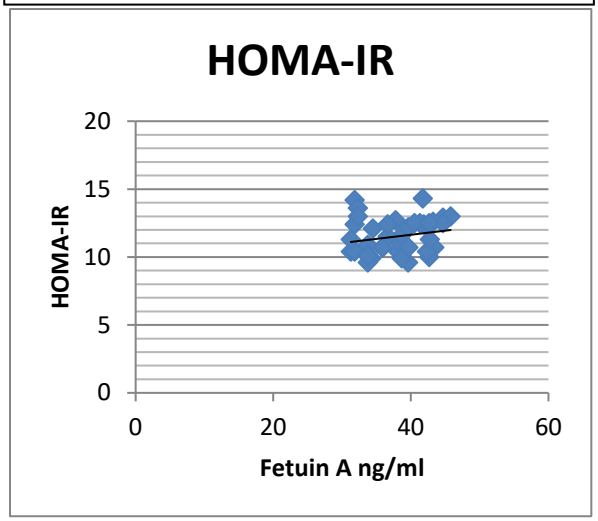


Figure 5.116: Correlation of Urinary Fetuin-A and serum FFA

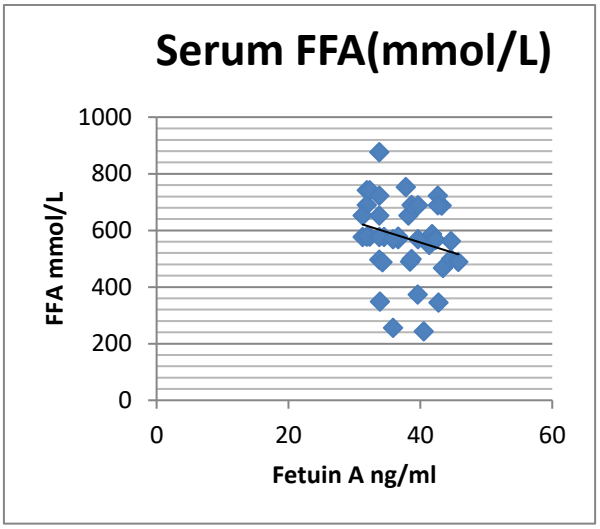


Figure 5.117: Correlation of Urinary Fetuin-A and TC

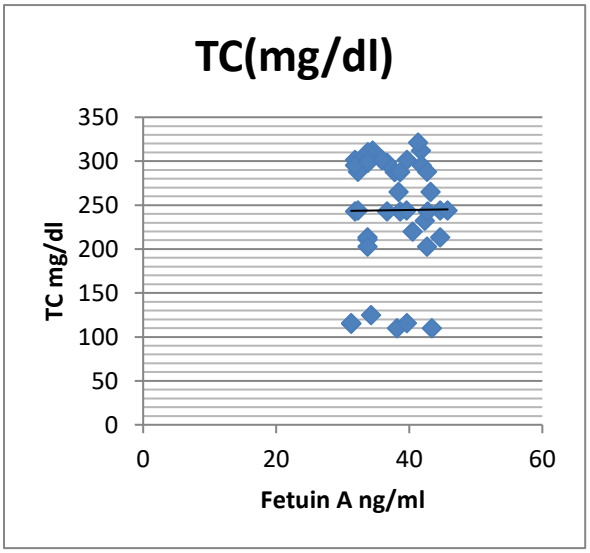


Figure 5.118: Correlation of Urinary Fetuin-A and TG

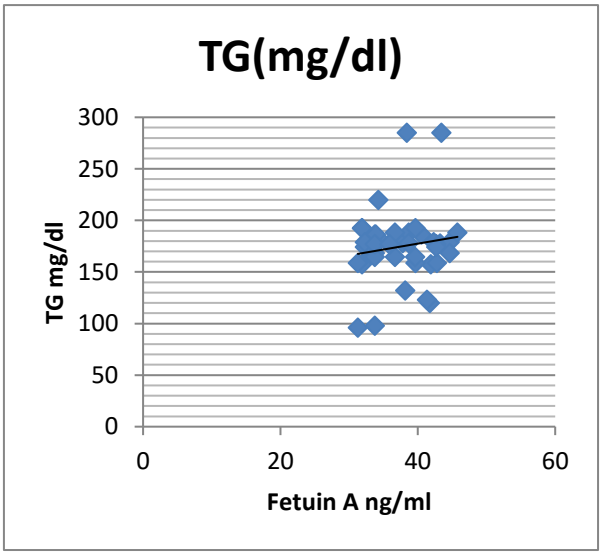


Figure 5.119: Correlation of Urinary Fetuin-A and HDL

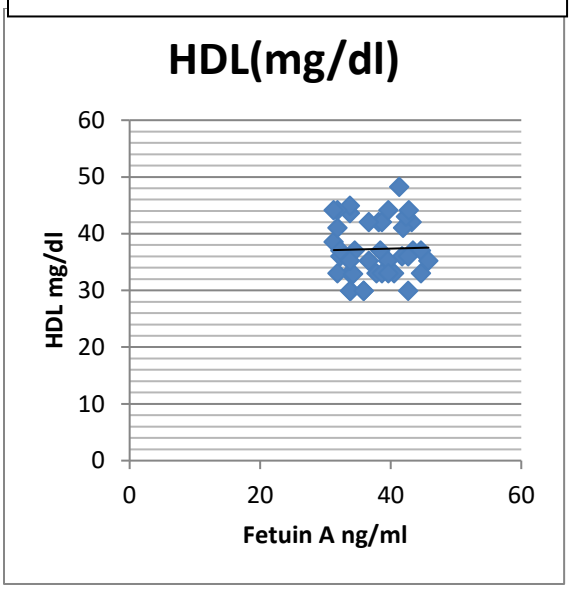


Figure 5.120: Correlation of Urinary Fetuin-A and LDL

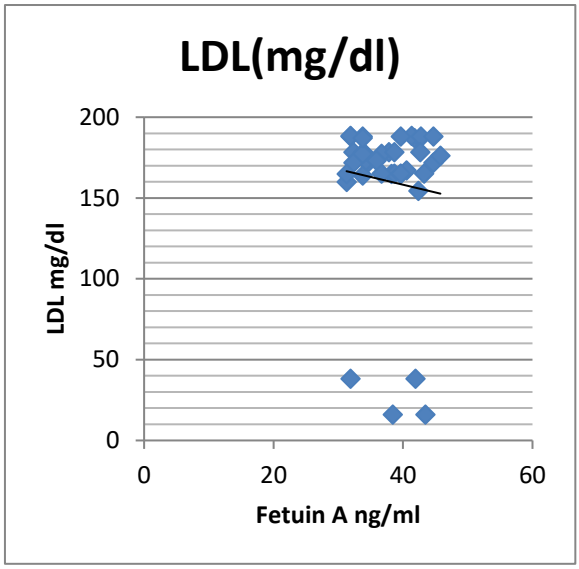
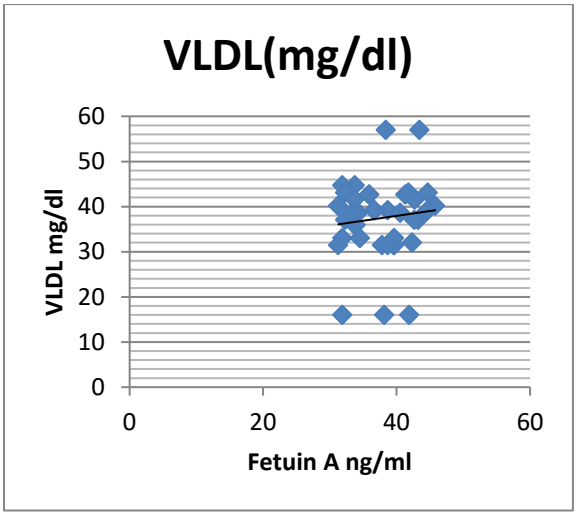


Figure 5.121: Correlation of Urinary Fetuin-A and VLDL



Correlation of Urinary Fetuin-A with renal parameters, CRP, lipid profile parameters and serum FFA in stage 4:

Figure 5.122: Correlation of Urinary Fetuin-A and Creatinine

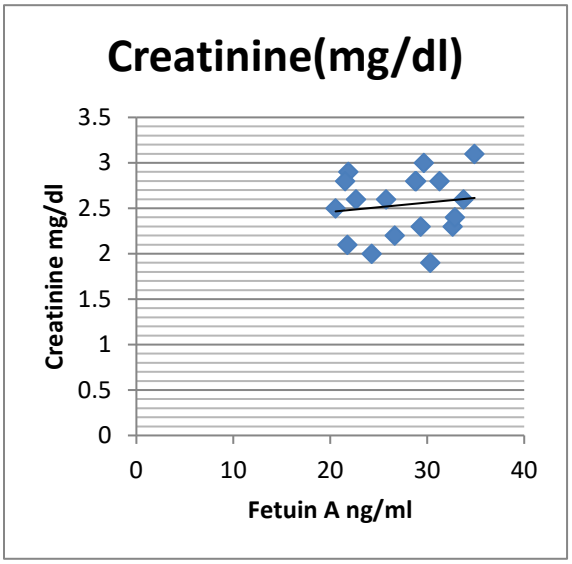


Figure 5.123: Correlation of Urinary Fetuin-A and Urea

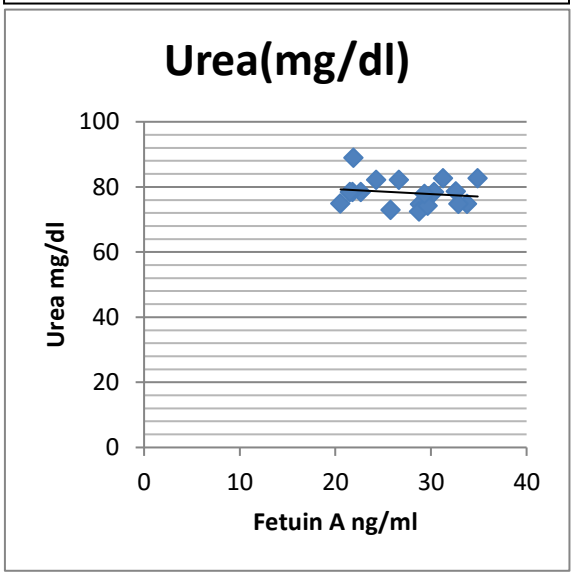


Figure 5.124: Correlation of Urinary Fetuin-A and Uric acid

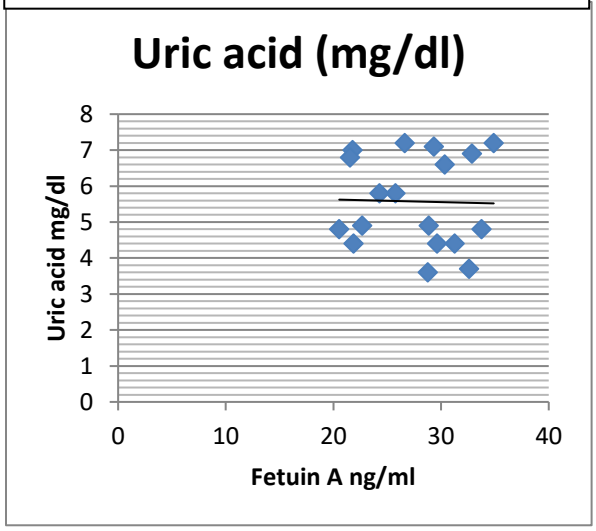


Figure 5.125: Correlation of Urinary Fetuin-A and CRP

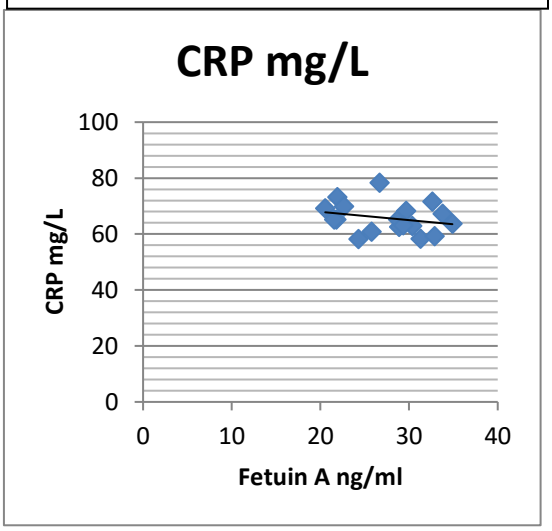


Figure 5.126: Correlation of Urinary Fetuin-A and eGFR

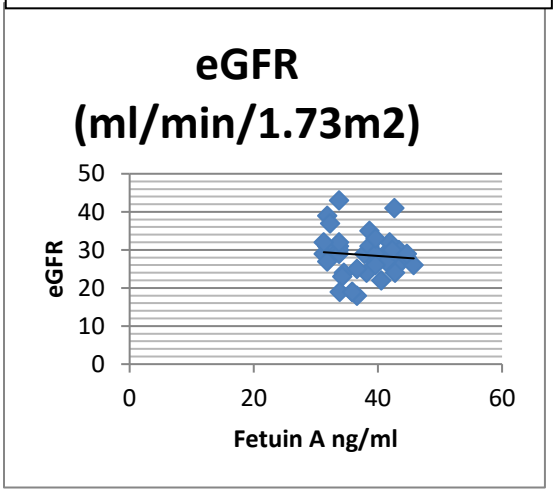


Figure 5.127: Correlation of Urinary Fetuin-A and Microalbumin

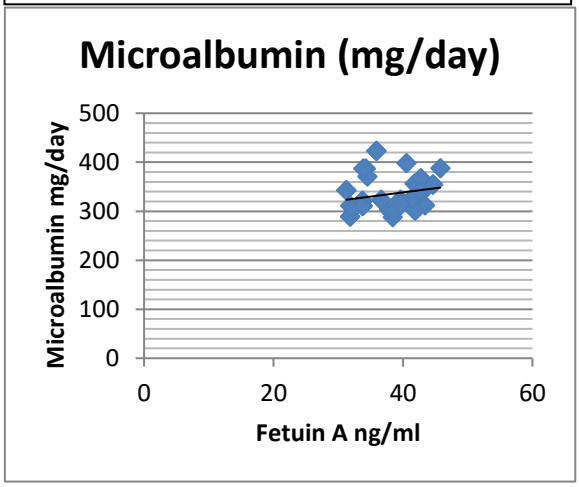


Figure 5.128: Correlation of Urinary Fetuin-A and FBS

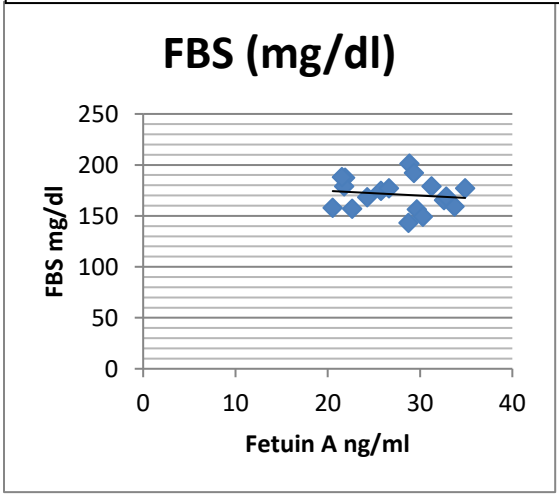


Figure 5.129: Correlation of Urinary Fetuin-A and PPBS

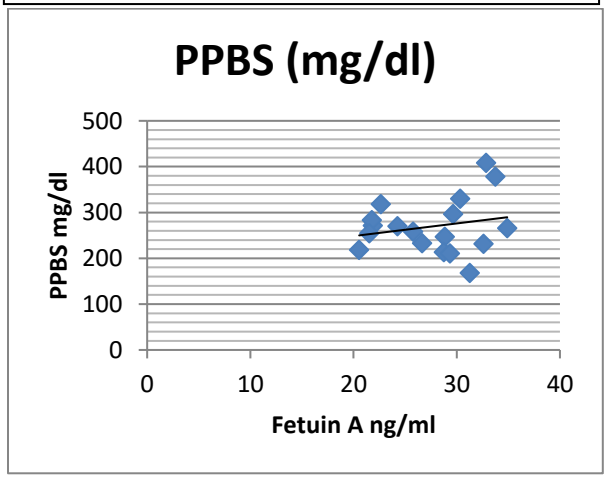


Figure 5.130: Correlation of Urinary Fetuin-A and Insulin

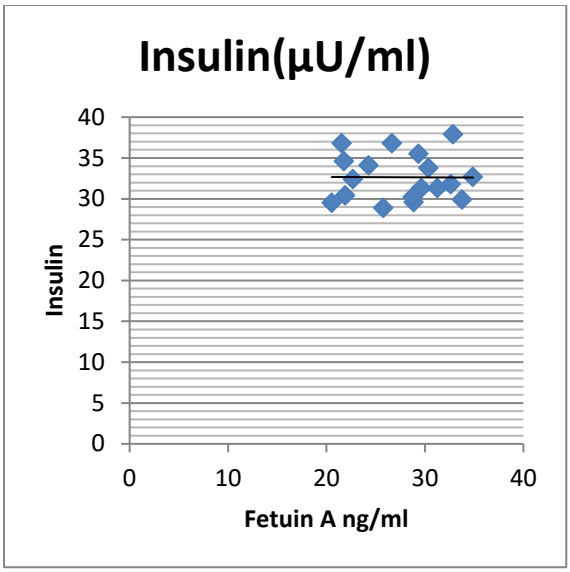


Figure 5.131: Correlation of Urinary Fetuin-A and HOMA-IR

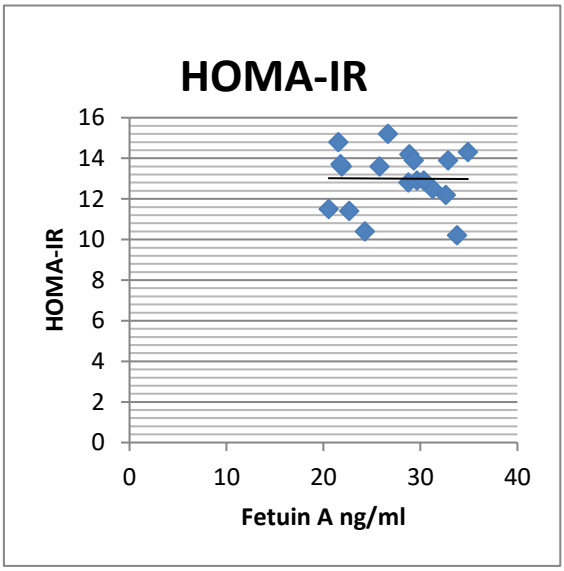


Figure 5.132: Correlation of Urinary Fetuin-A and serum FFA

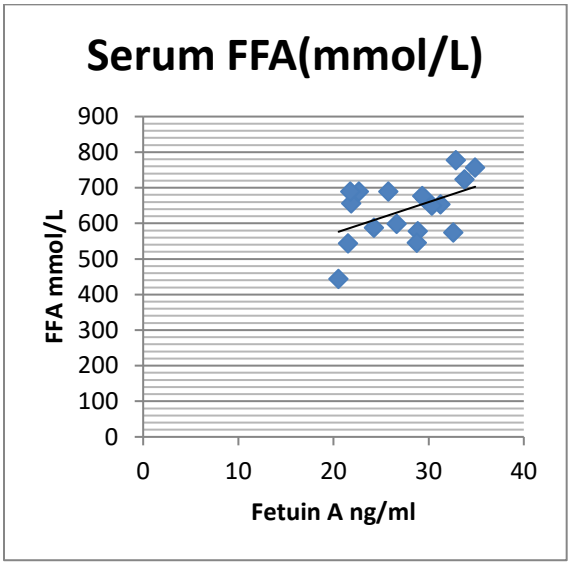


Figure 5.133: Correlation of Urinary Fetuin-A and TC

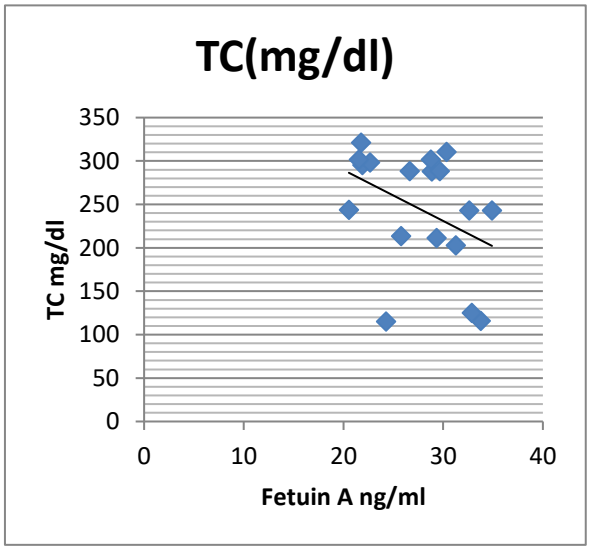


Figure 5.134: Correlation of Urinary Fetuin-A and TG

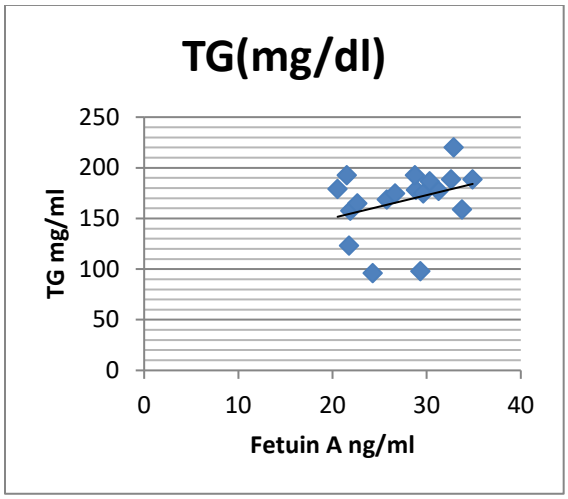


Figure 5.135: Correlation of Urinary Fetuin-A and HDL

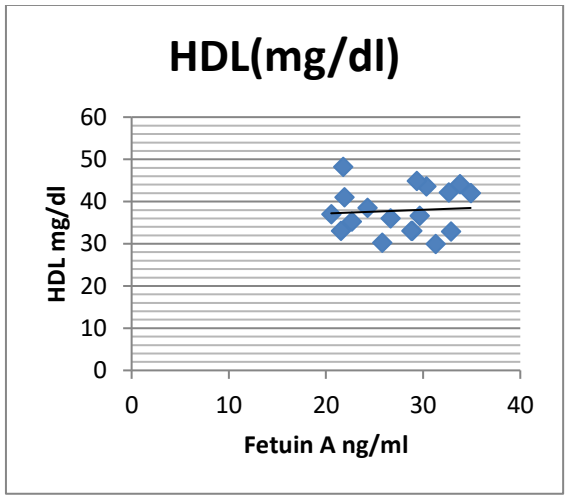


Figure 5.136: Correlation of Urinary Fetuin-A and LDL

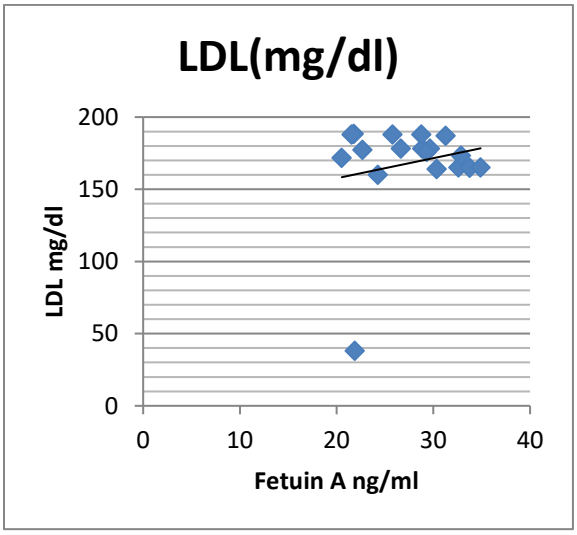
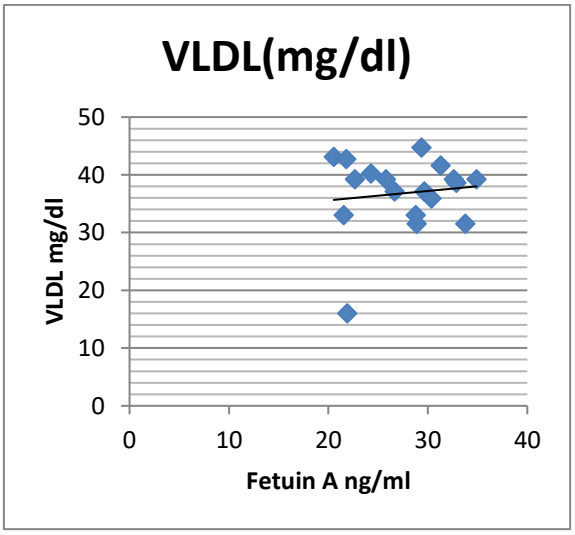


Figure 5.137: Correlation of Urinary Fetuin-A and VLDL



5. 12. Correlation of serum free fatty acids with controls and DN cases

Serum free fatty acids are also significantly increased in all the stages of diabetic nephropathy compared to controls p<0,001.

Table 5.13: Correlation of free fatty acids with renal parameters, CRP, glycemic status, serum and urinary Fetuin-A levels and lipid profile in controls and stages of diabetic nephropathy cases.

Parameters	Controls	Stage 1	Stage 2	Stage 3	Stage 4
	r – value				
Blood urea	0.374	0.089	0.281	0.227	0.184
Serum Creatinine	0.096	0.067	0.104	0.094	0.117
Uric acid	0.138	0.156	0.104	0.086	0.101
eGFR	-0.073	-0.175	-0.016	-0.297	-0.042
Urine MA	0.226	0.164	0.265	0.129	0.151
SerumFetuin-A	0.030	0.178	0.187	0.147	0.182
Urine fetuinA	0.175	0.158	0.103	0.180	0.211
CRP	0.217	0.175	0.149	0.162	0.171
FBS	0.130	0.136	0.078	0.054	0.191
PPBS	0.060	0.164	0.037	0.064	0.072
HbA1c	0.246	0.097	0.059	0.111	0.15
Insulin	0.058	0.131	0.142	0.096	0.121
HOMA-IR	0.013	0.185	0.048	0.002	0.085
T.Chol	0.015	0.197	0.191	0.004	0.016
TGL	0.013	0.388	0.109	0.049	0.110
HDL	-0.025	-0.054	-0.076	-0.074	-0.012
LDL	0.347	0.278	0.048	0.12	0.211
VLDL	0.017	0.465	0.087	0.080	0.541

Table 5.13 shows positive correlation of serum free fatty acids was seen with all the above parameters in DN cases compared to controls except HDL and eGFR which show negative correlation as the stage advances.

Correlation of serum free fatty acids with all the study parameters:

Stage 1:

Figure 5.138: Correlation of FFA and Creatinine

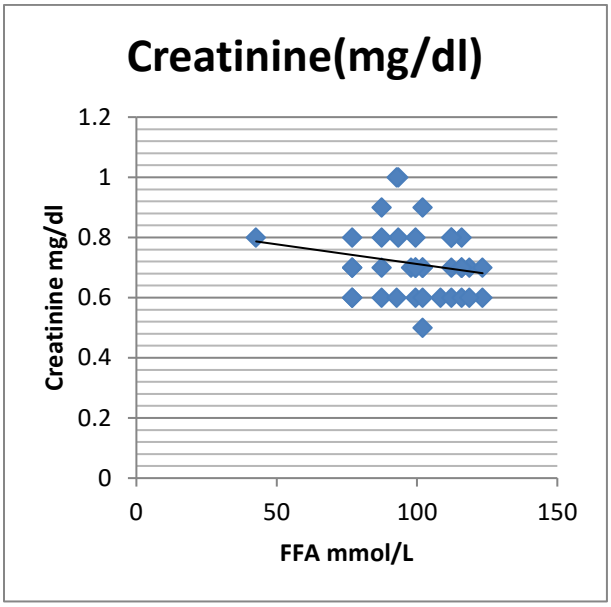


Figure 5.139: Correlation of FFA and Urea

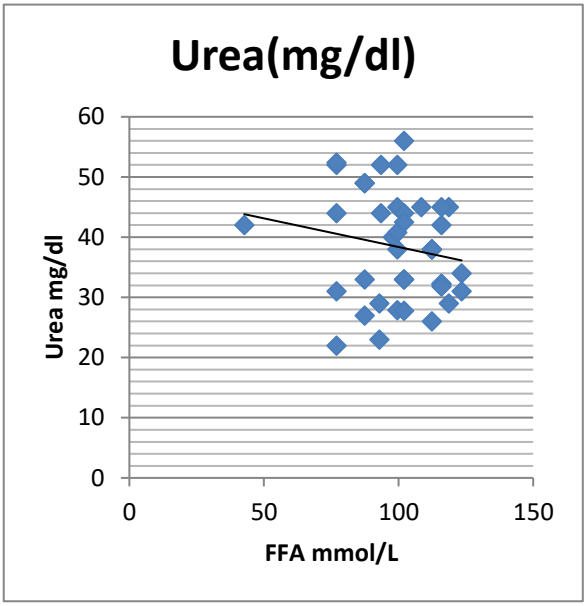


Figure 5.140: Correlation of FFA and uric acid

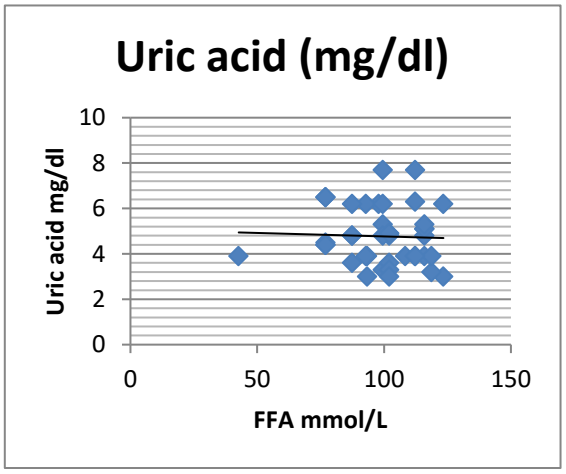


Figure 5.141: Correlation of FFA and uric acid

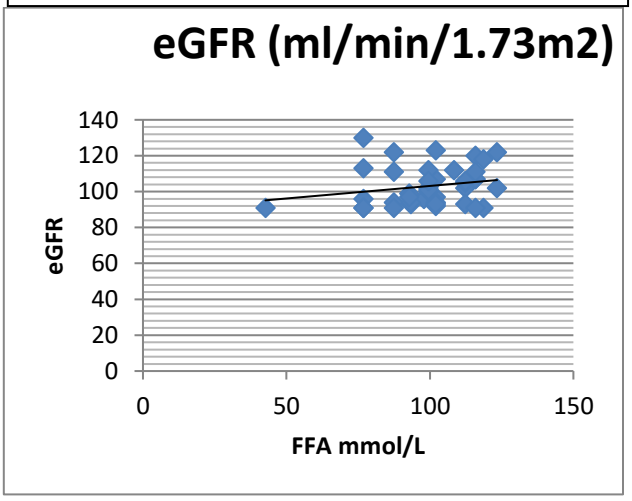


Figure 5.142: Correlation of FFA and microalbumin

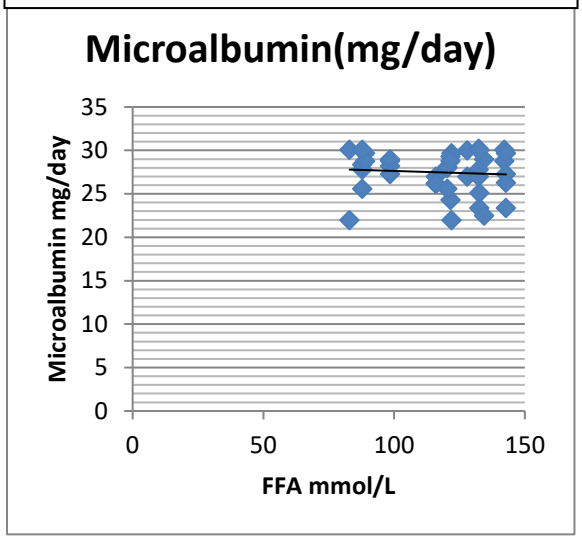


Figure 5.143: Correlation of FFA and CRP

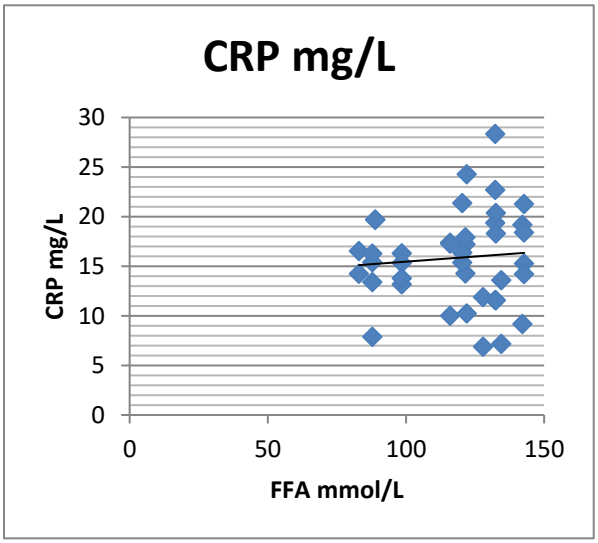


Figure 5.144: Correlation of FFA and FBS

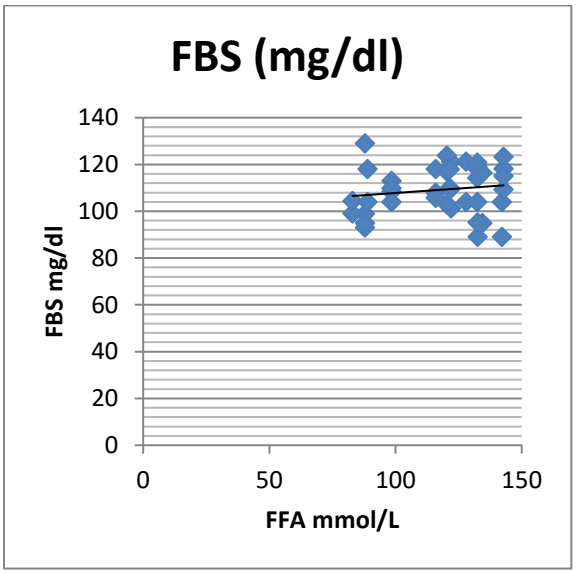


Figure 5.145: Correlation of FFA and PPBS

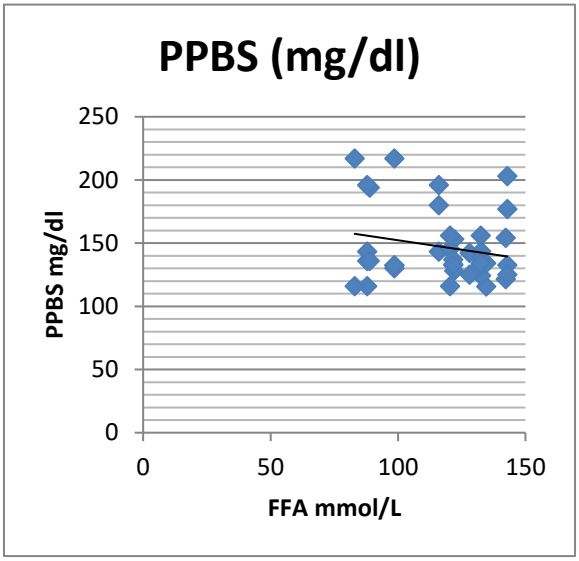


Figure 5.146: Correlation of FFA and HbA1c

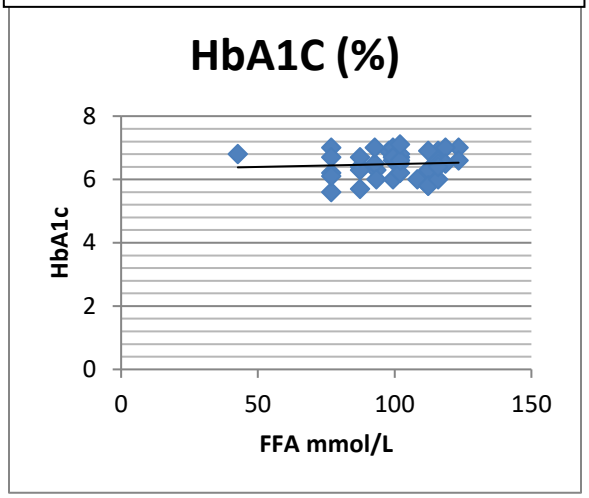


Figure 5.147: Correlation of FFA and Insulin

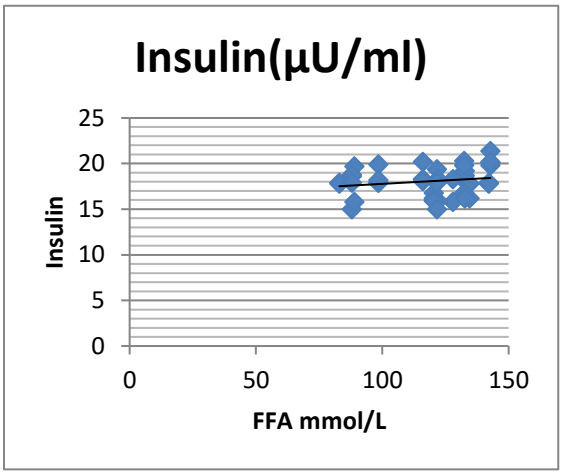


Figure 5.148: Correlation of FFA and HOMA-IR

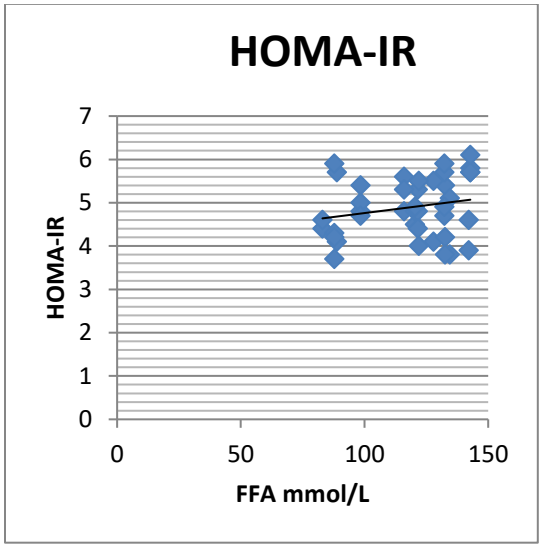


Figure 5.149: Correlation of FFA and Serum Fetuin-A

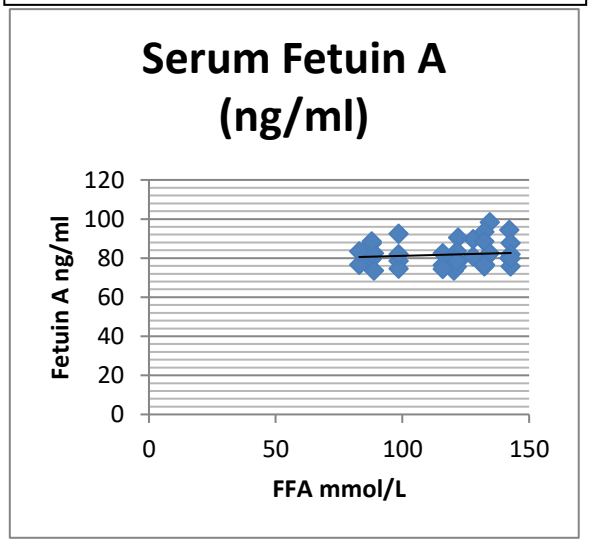


Figure 5.150: Correlation of FFA and Urinary Fetuin-A

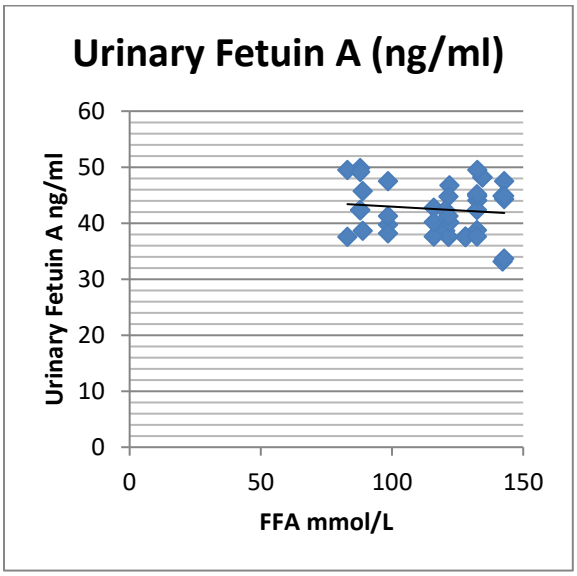


Figure 5.151: Correlation of FFA and TC

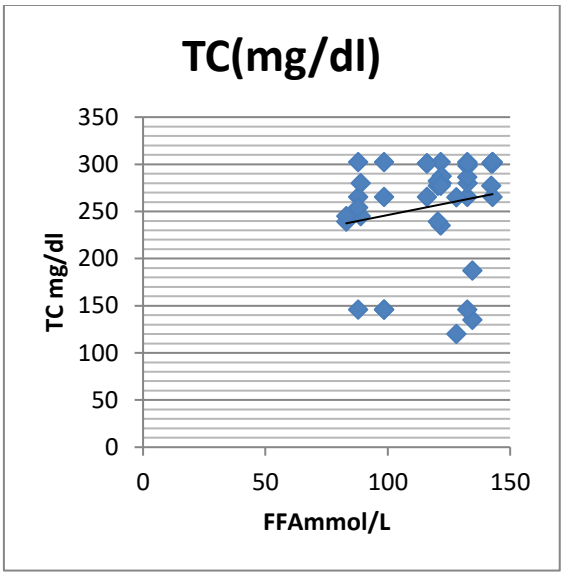


Figure 5.152: Correlation of FFA and TG

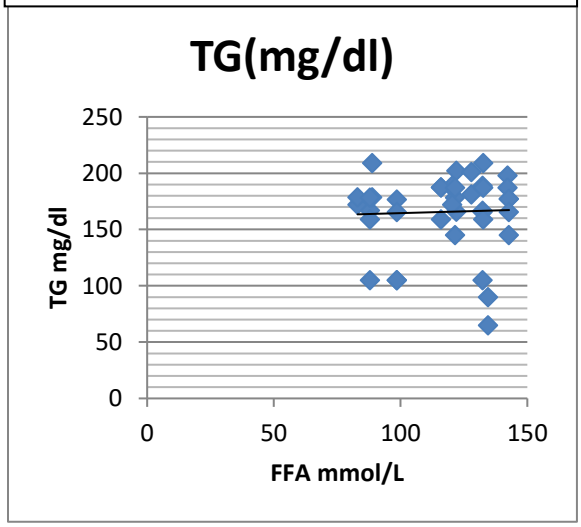


Figure 5.153: Correlation of FFA and

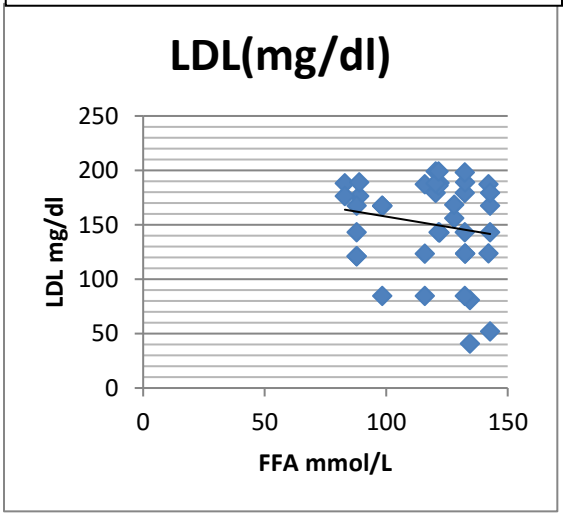


Figure 5.154: Correlation of FFA and HDL

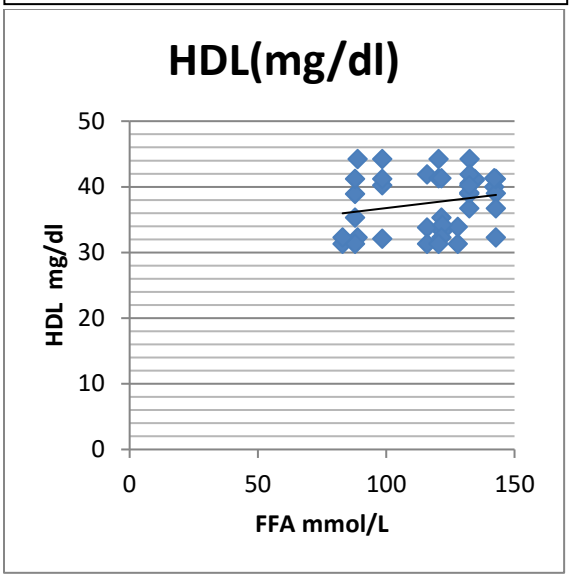
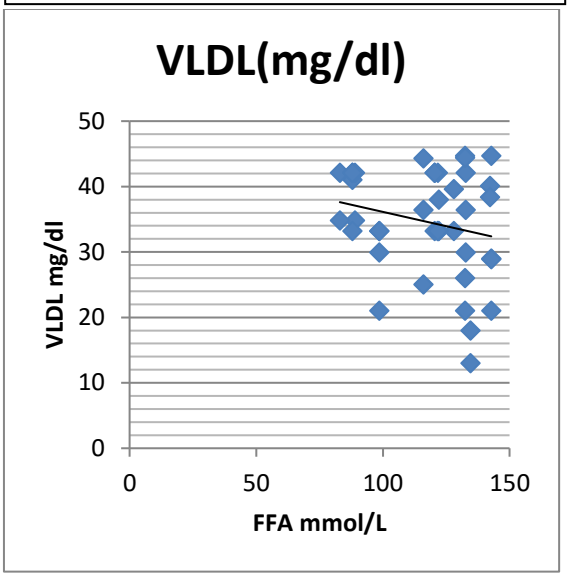


Figure 5.155: Correlation of FFA and VLDL



Correlation of serum free fatty acids with all the study parameters:

Stage 2:

Figure 5.156: Correlation of FFA and Creatinine

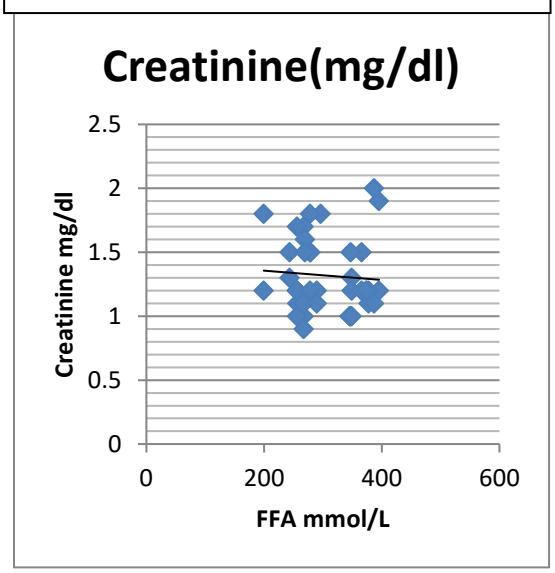


Figure 5.157: Correlation of FFA and Urea

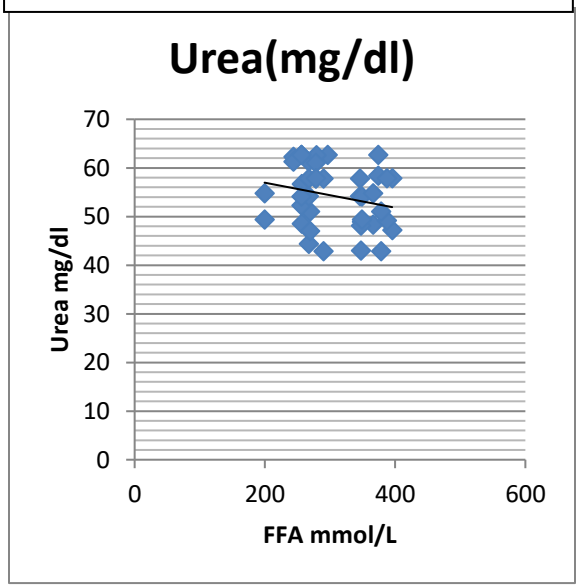


Figure 5.158: Correlation of FFA and Uric acid

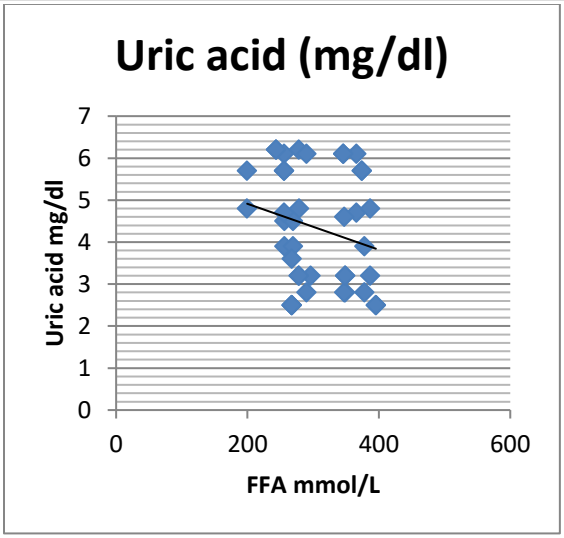


Figure 5.159: Correlation of FFA and eGFR

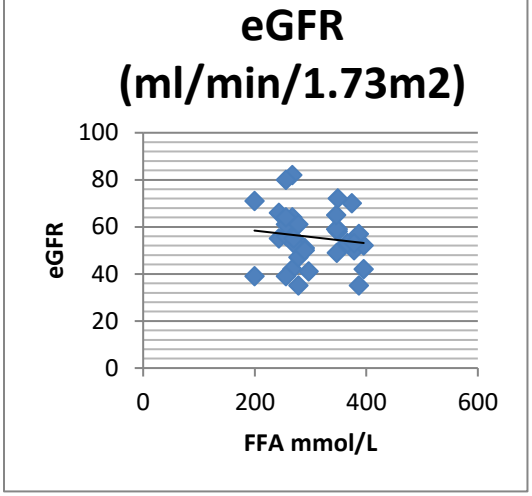


Figure 5.160: Correlation of FFA and Microalbumin

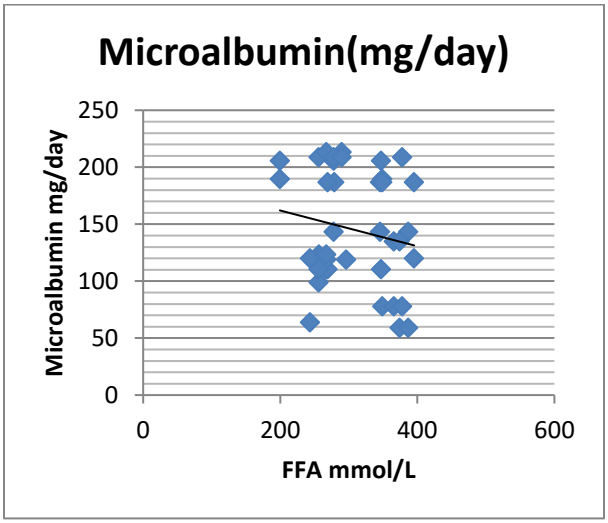


Figure 5.161: Correlation of FFA and CRP

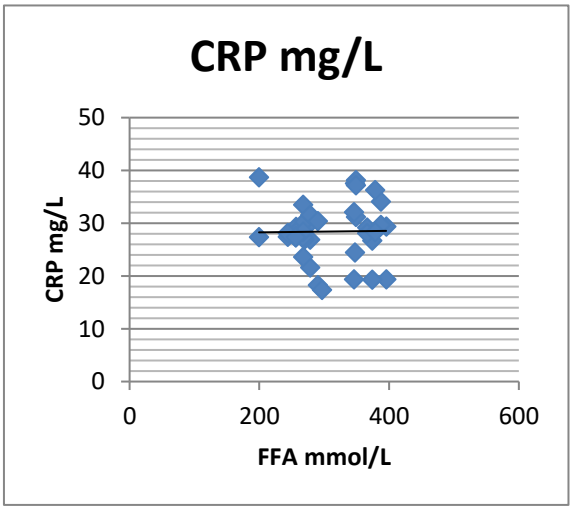


Figure 5.162: Correlation of FFA and FBS

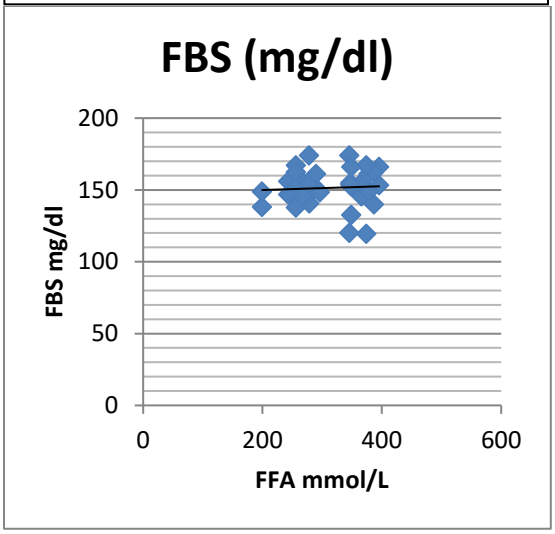


Figure 5.163: Correlation of FFA and PPBS

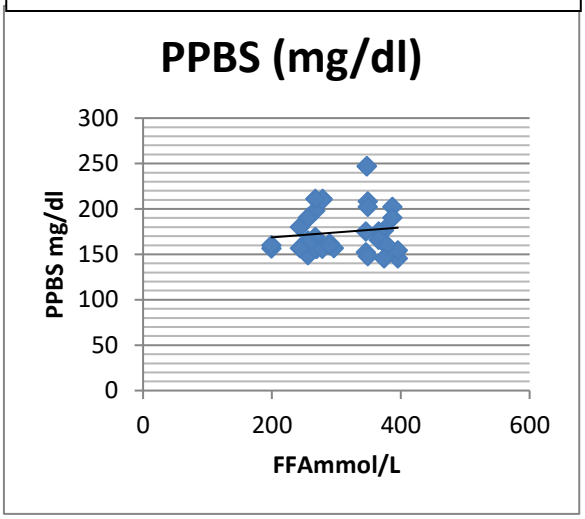


Figure 5.164: Correlation of FFA and HbA1c

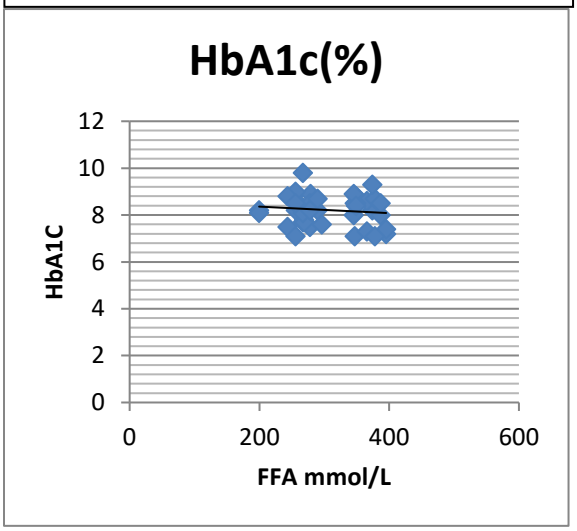


Figure 5.165: Correlation of FFA and Insulin

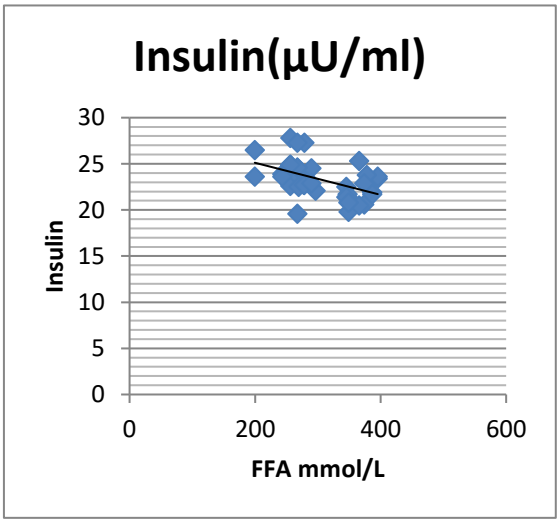


Figure 5.166: Correlation of FFA and HOMA-IR

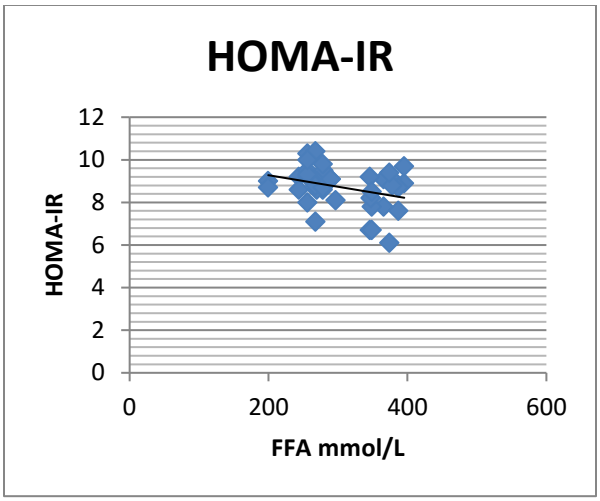


Figure 5.167: Correlation of FFA and Serum Fetuin-A

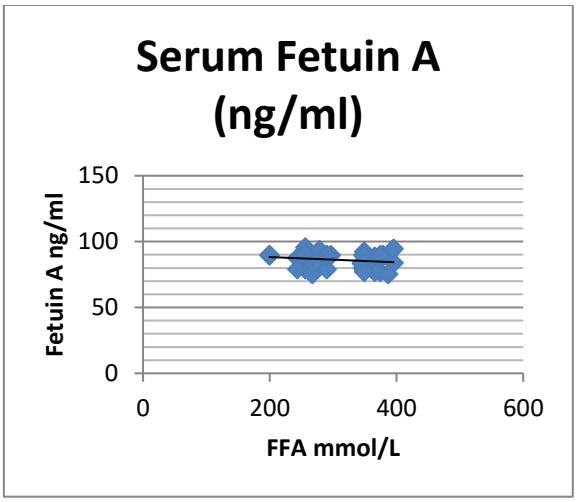


Figure 5.168: Correlation of FFA and Urinary Fetuin-A

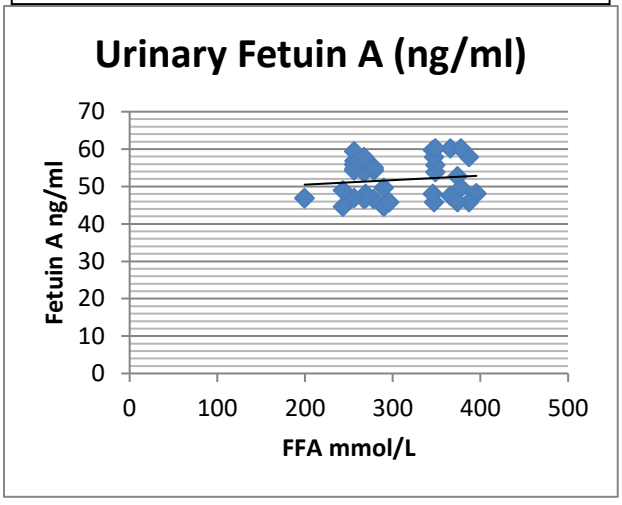


Figure 5.169: Correlation of FFA and TC

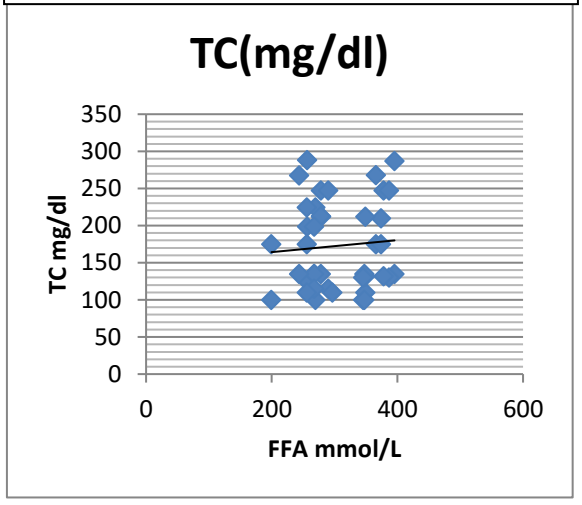


Figure 5.170: Correlation of FFA and TG

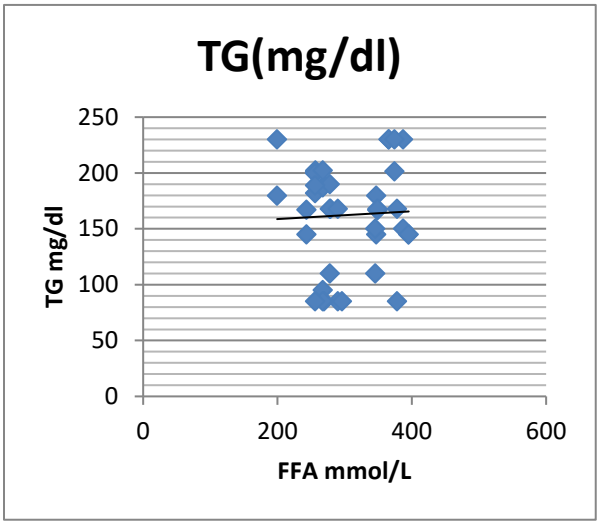


Figure 5.171: Correlation of FFA and LDL

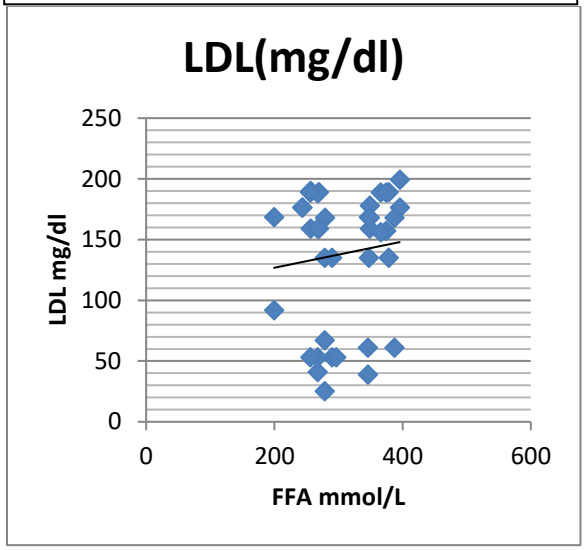


Figure 5.172: Correlation of FFA and HDL

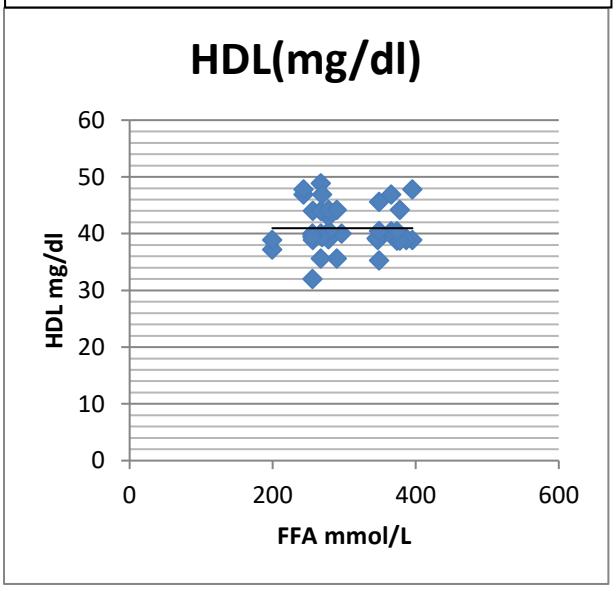
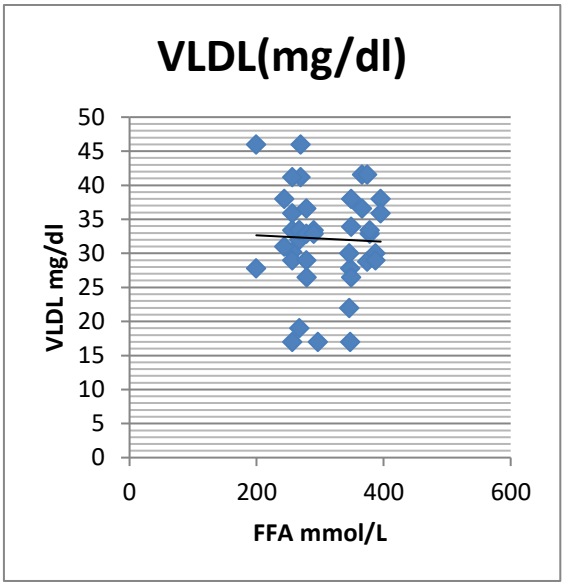


Figure 5.173: Correlation of FFA and VLDL



Correlation of serum free fatty acids with all the study parameters:

Stage 3:

Figure 5.174: Correlation of FFA and Creatinine

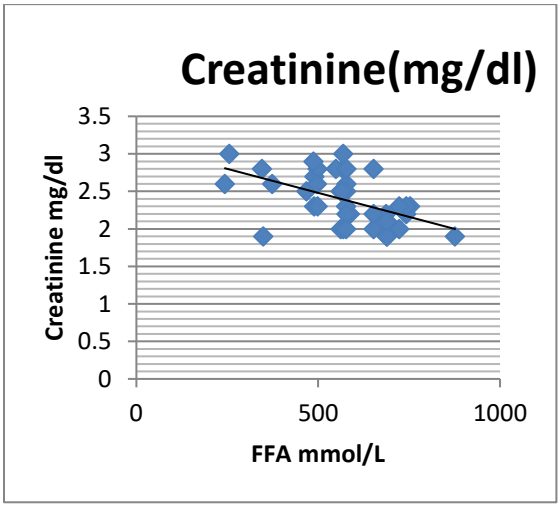


Figure 5.175: Correlation of FFA and Urea

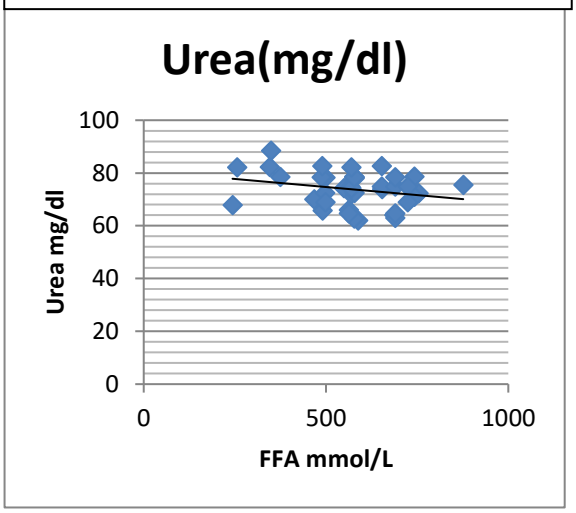


Figure 5.176: Correlation of FFA and Uric acid

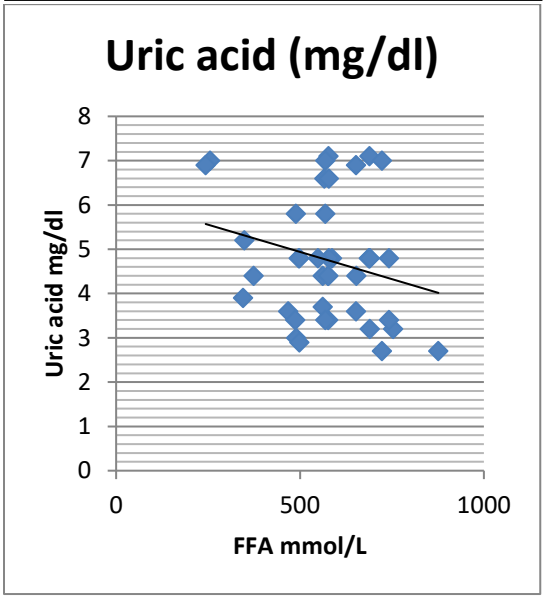


Figure 5.177: Correlation of FFA and eGFR

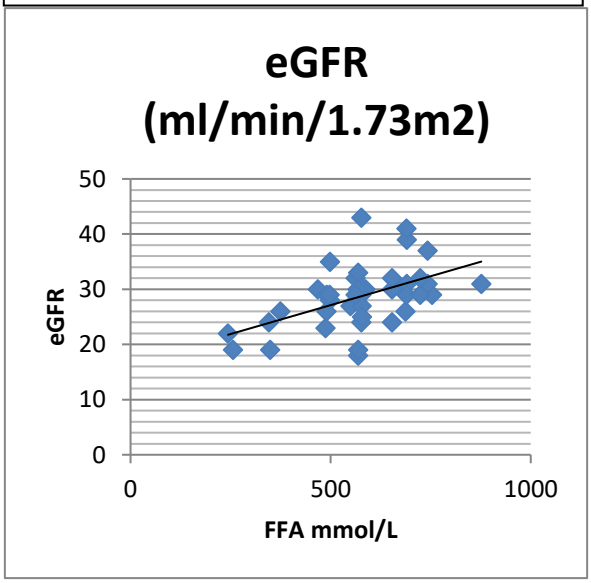


Figure 5.178: Correlation of FFA and Microalbumin

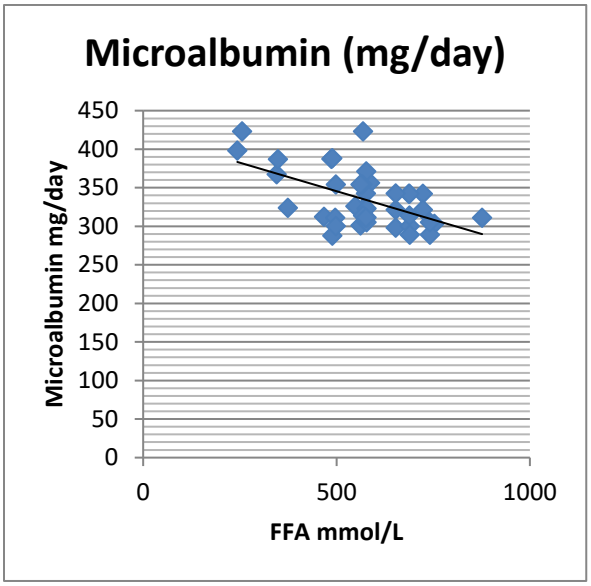


Figure 5.179: Correlation of FFA and FBS

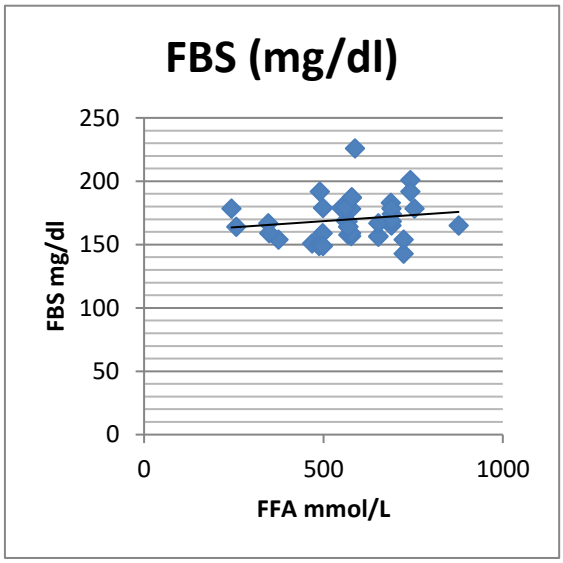


Figure 5.180: Correlation of FFA and PPBS

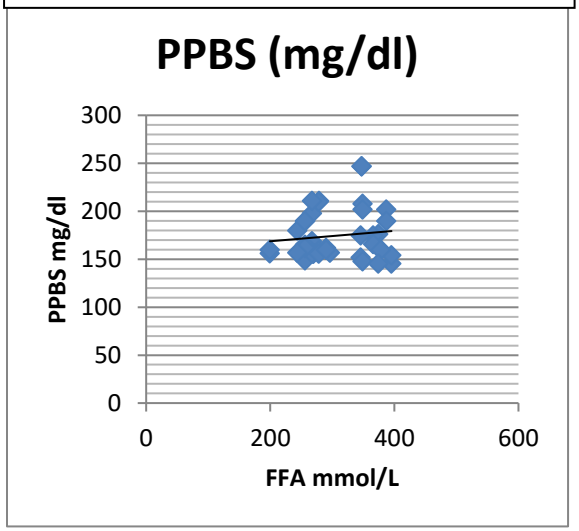


Figure 5.181: Correlation of FFA and HbA1c

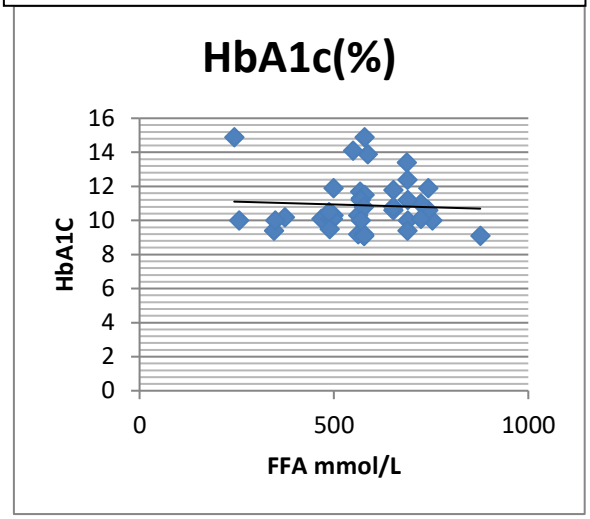


Figure 5.182: Correlation of FFA and Insulin

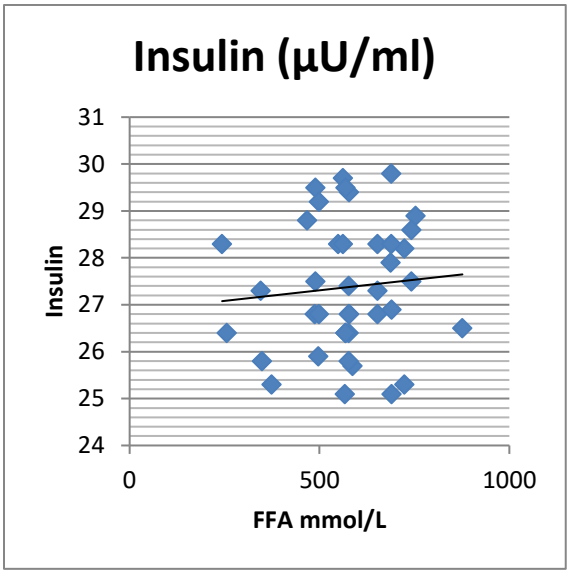


Figure 5.183: Correlation of FFA and HOMA-IR

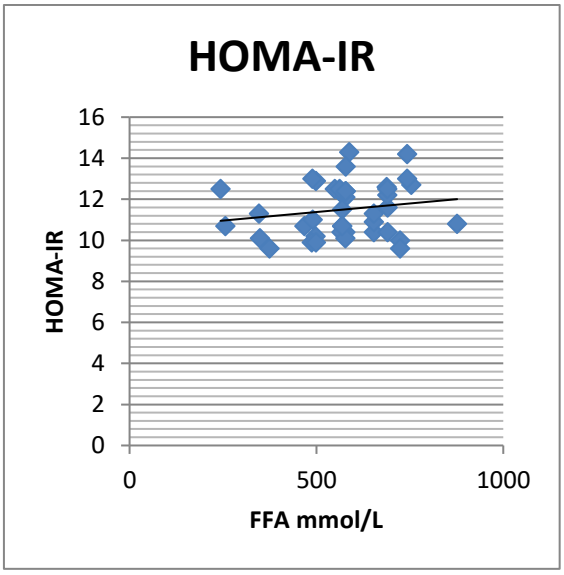


Figure 5.184: Correlation of FFA and Serum Fetuin-A

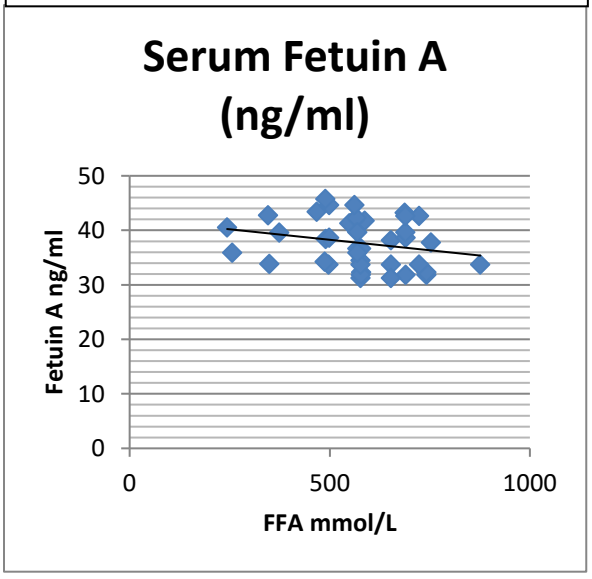


Figure 5.185: Correlation of FFA and Urinary Fetuin A

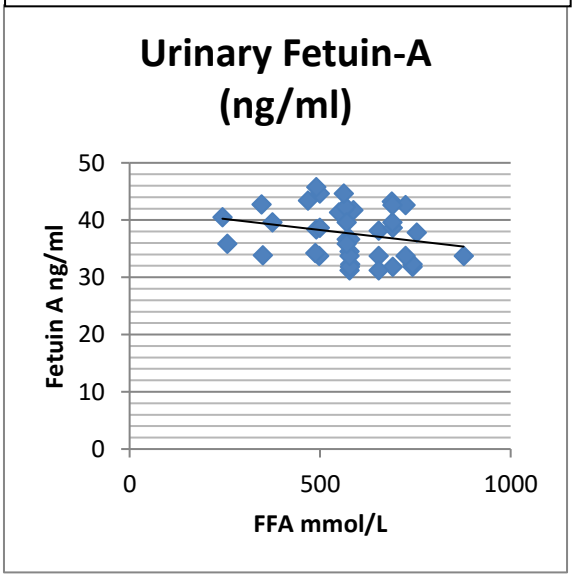


Figure 5.186: Correlation of FFA and TC

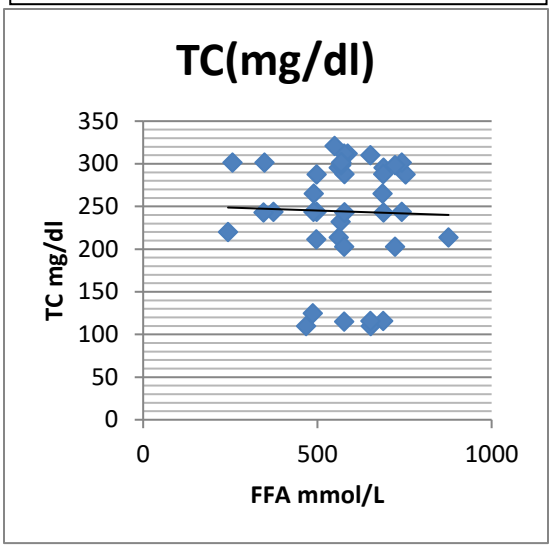


Figure 5.187: Correlation of FFA and TG

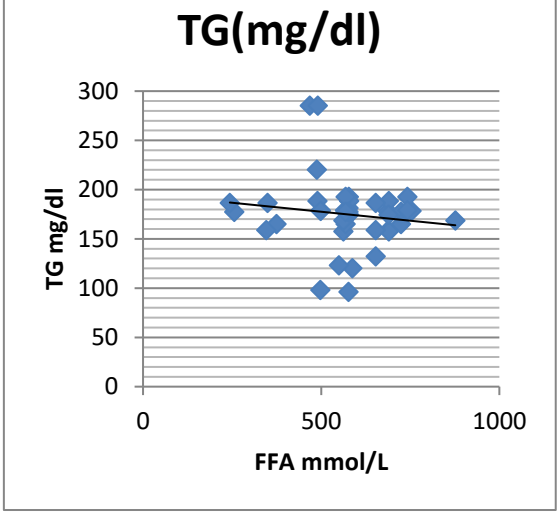


Figure 5.188: Correlation of FFA and LDL

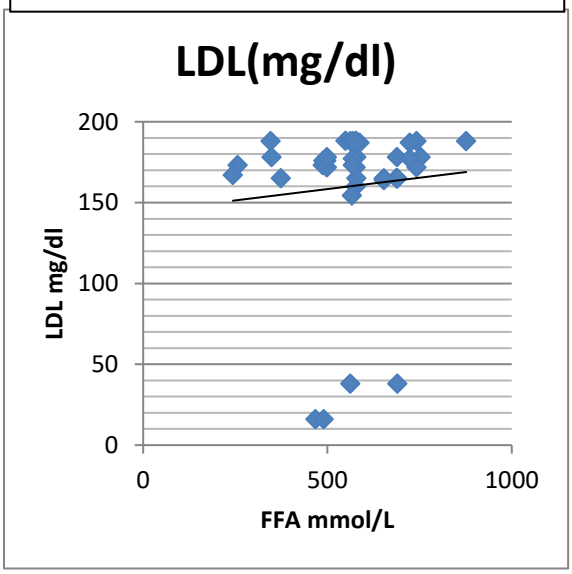


Figure 5.189: Correlation of FFA and HDL

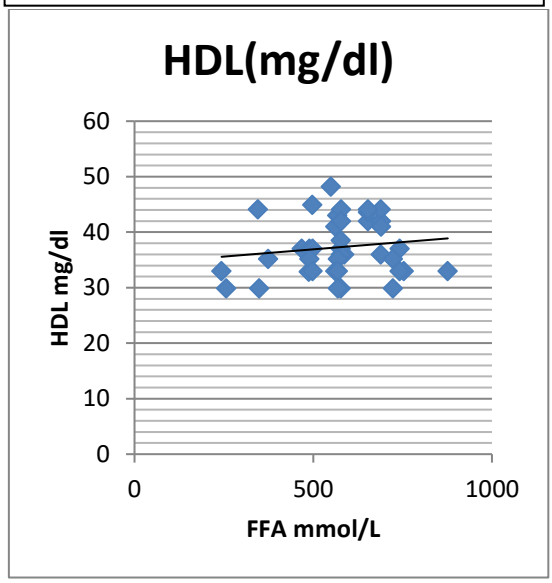
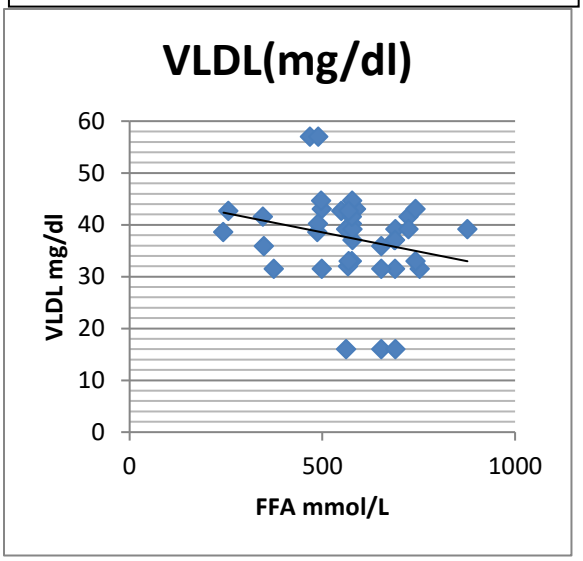


Figure 5.190: Correlation of FFA and VLDL



Correlation of serum free fatty acids with all the study parameters:

Stage 4:

Figure 5.191: Correlation of FFA and Creatinine

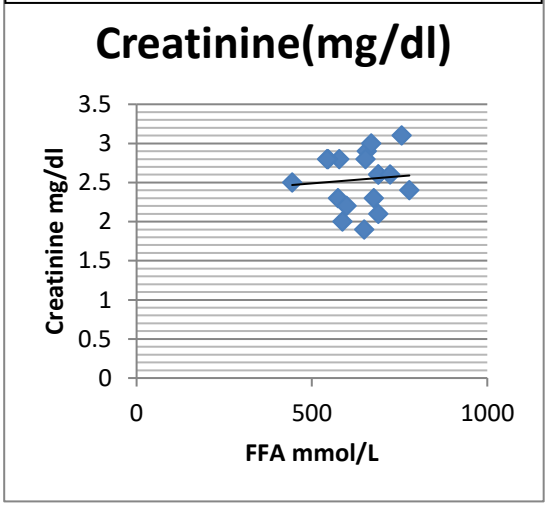


Figure 5.192: Correlation of FFA and Urea

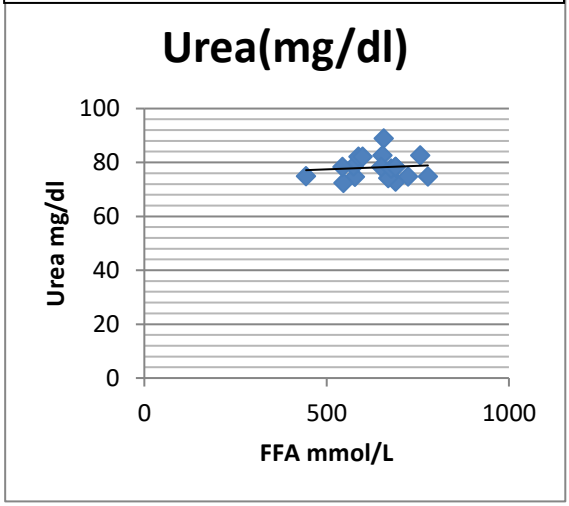


Figure 5.193: Correlation of FFA and Uric acid

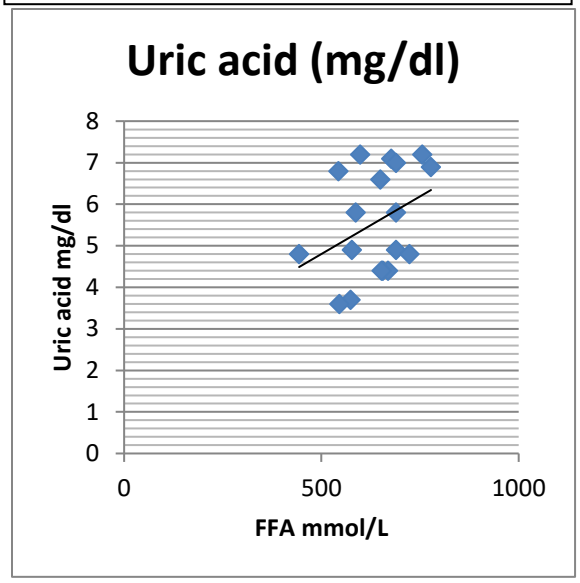


Figure 5.194: Correlation of FFA and eGFR

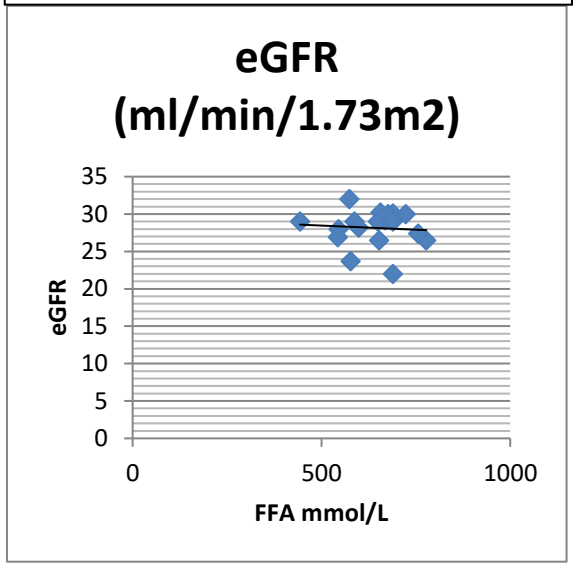


Figure 5.195: Correlation of FFA and Microalbumin

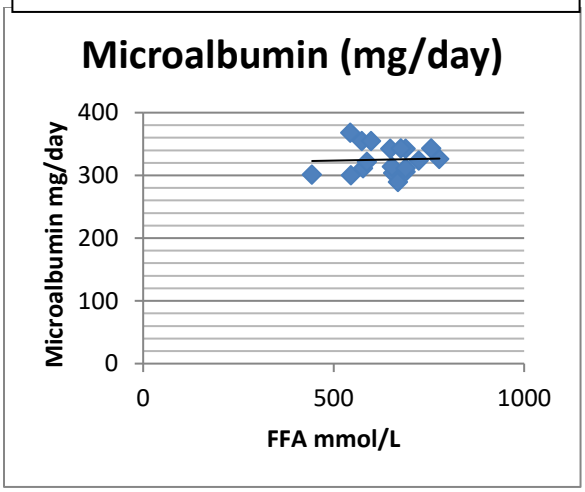


Figure 5.196: Correlation of FFA and CRP

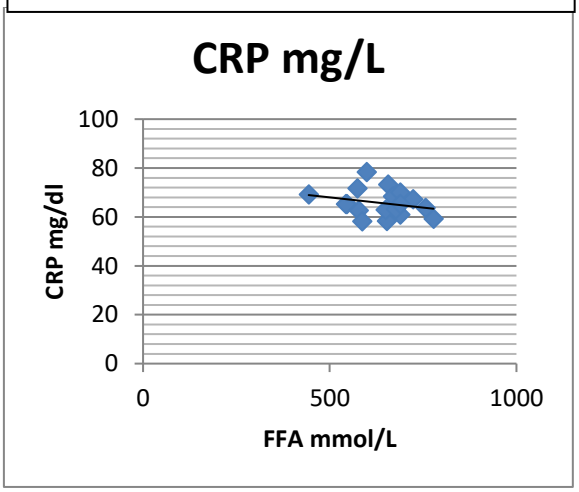


Figure 5.197: Correlation of FFA and HbA1c

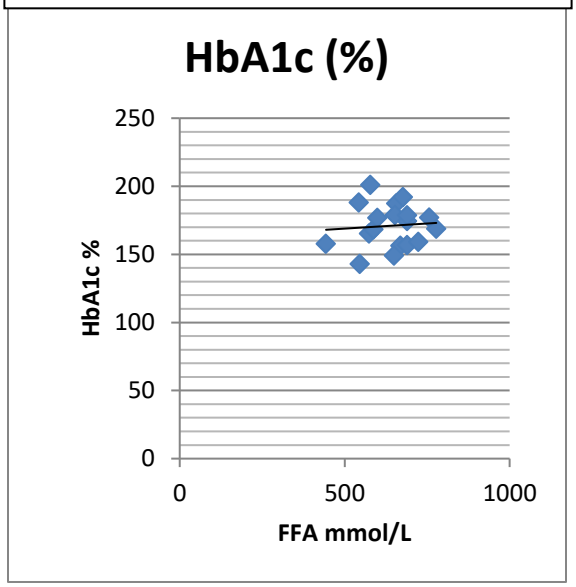


Figure 5.198: Correlation of FFA and FBS

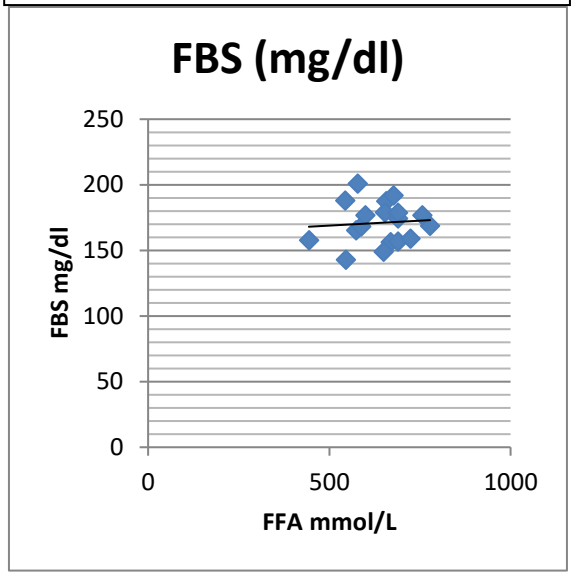


Figure 5.199: Correlation of FFA and PPBS

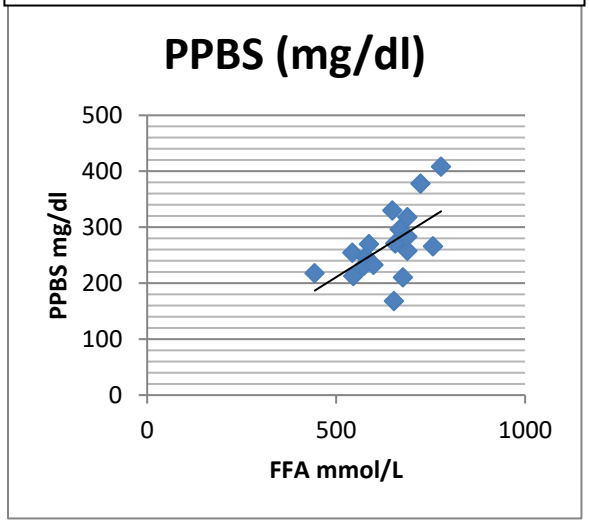


Figure 5.200: Correlation of FFA and Insulin

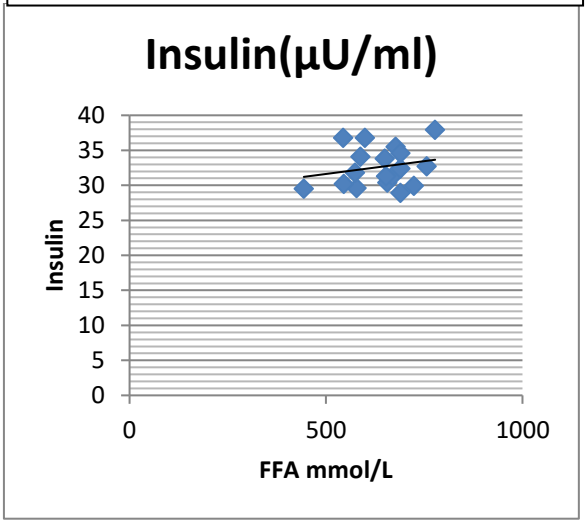


Figure 5.201: Correlation of FFA and HOMA-IR

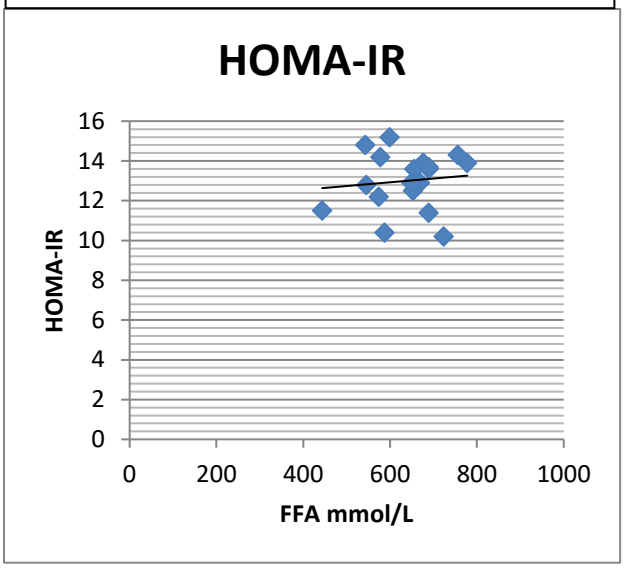


Figure 5.202: Correlation of FFA and Serum Fetuin-A

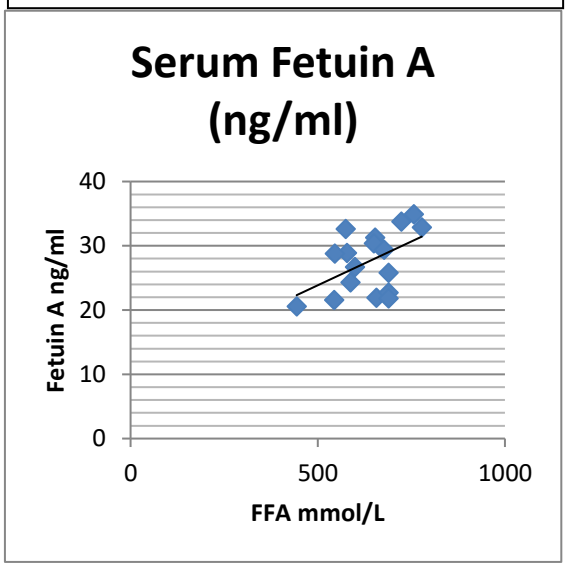


Figure 5.203: Correlation of FFA and Urinary Fetuin-A

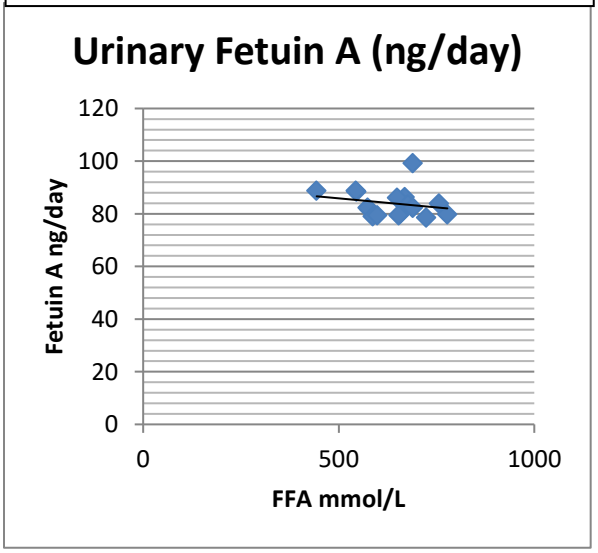


Figure 5.204: Correlation of FFA and Urinary Fetuin-A

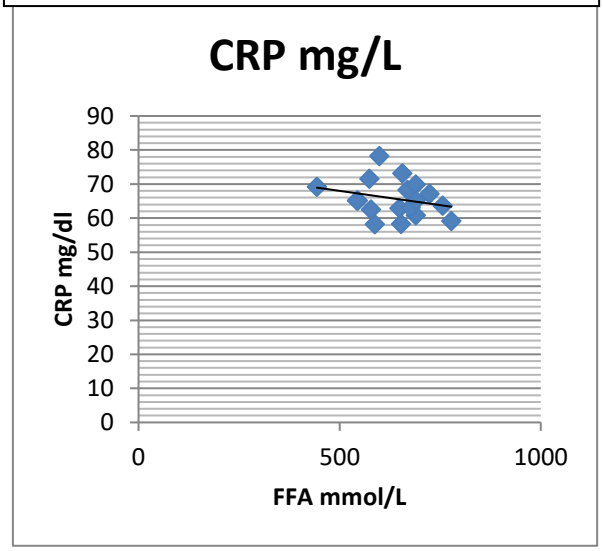


Figure 5.205: Correlation of FFA and TC

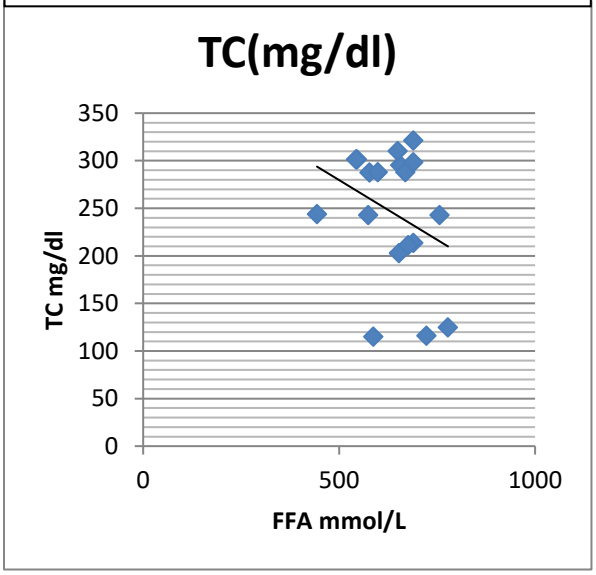


Figure 5.206: Correlation of FFA and TG

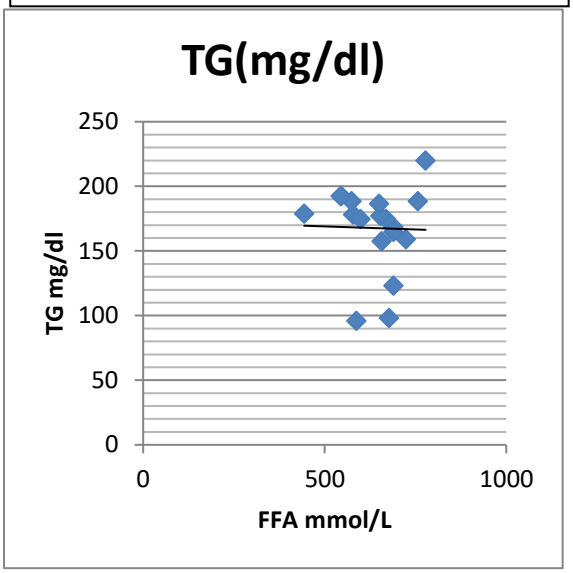


Figure 5.207: Correlation of FFA and LDL

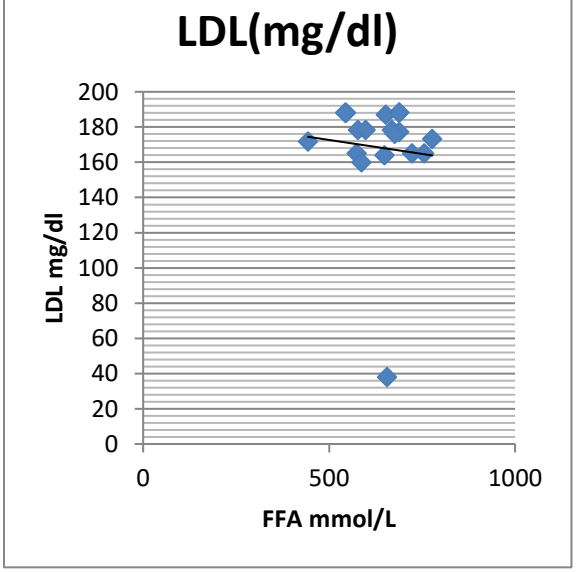


Figure 5.208: Correlation of FFA and HDL

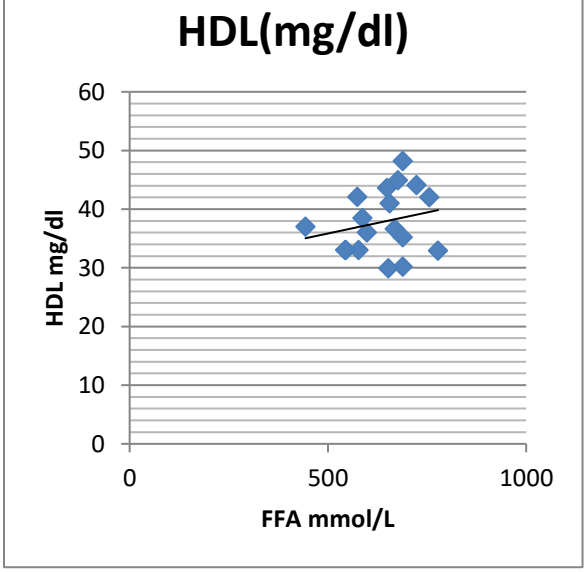
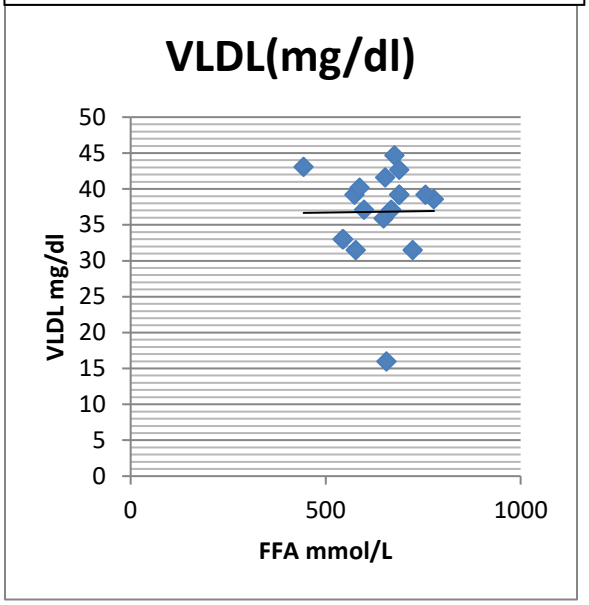


Figure 5.209: Correlation of FFA and VLDL

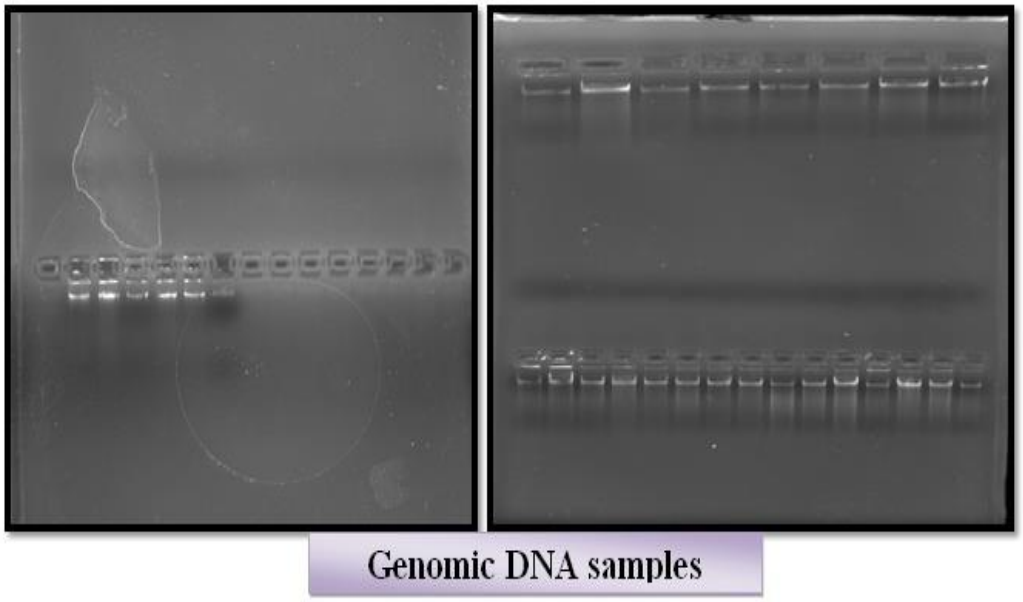


Our study also showed the significant elevation of serum free fatty acids levels in all the stages of diabetic nephropathy compared to controls $p<0.001$.

5.13 GENE POLYMORPHISM ANALYSIS.

Genotyping for the Fetuin-A gene (thr256Ser) polymorphism (C→G) was performed by PCR-RFLP technique .

Figure 5.210: Gel electrophoresis of Genomic DNA.



Gel electrophoresis shows the bands of standard DNA on left side whereas right side gel electrophoresis shows the bands of genomic DNA.

Figure 5.211: PCR setting.

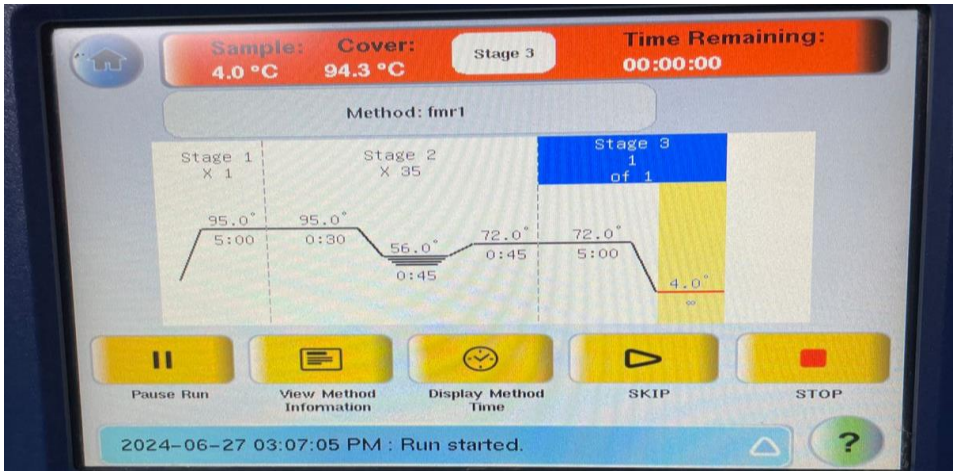
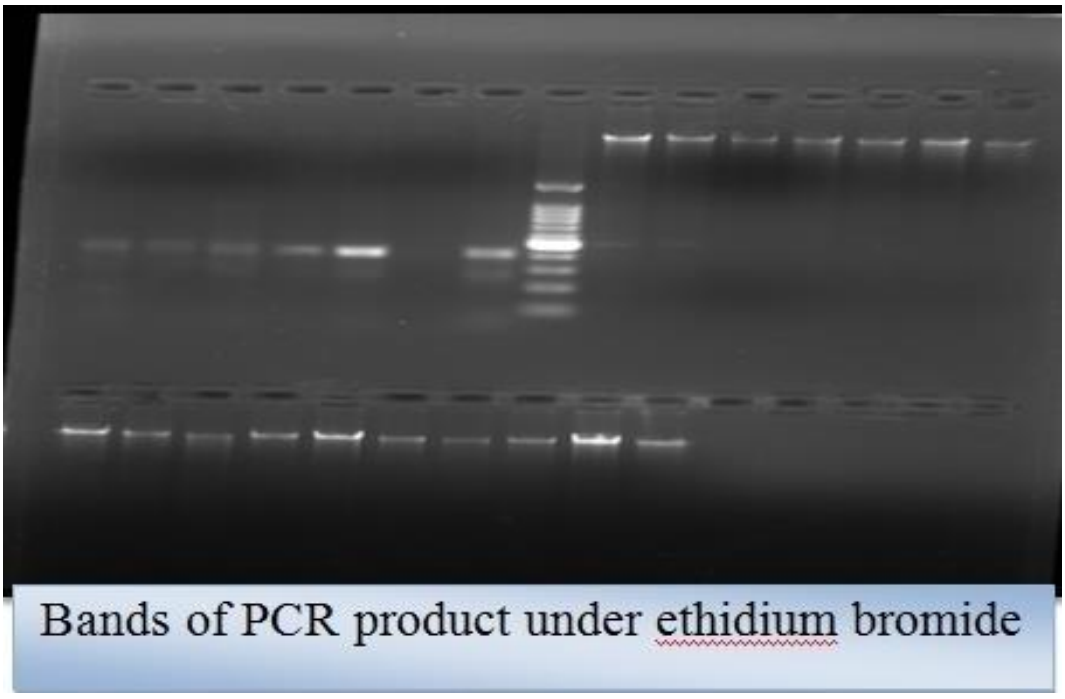


Figure 5.211 shows the PCR amplification setting of single cycle completely from DNA denaturation to elongation.

Figure 5.212: PCR products.



5.14. Polymorphism analysis.

Table 5.14 : Data analysis of polymorphism

Sl.No	gDNA position	cDNA position	Amino acid Position	Variant type
1	g. 7452 G>C	c. 767 G>C	p. Thr256Ser	Missense variant
2	g.7495 A>C	c. 810 A>C	p. Thr270Thr	Synonymous variant

Figure 5.213: Mutation graph.

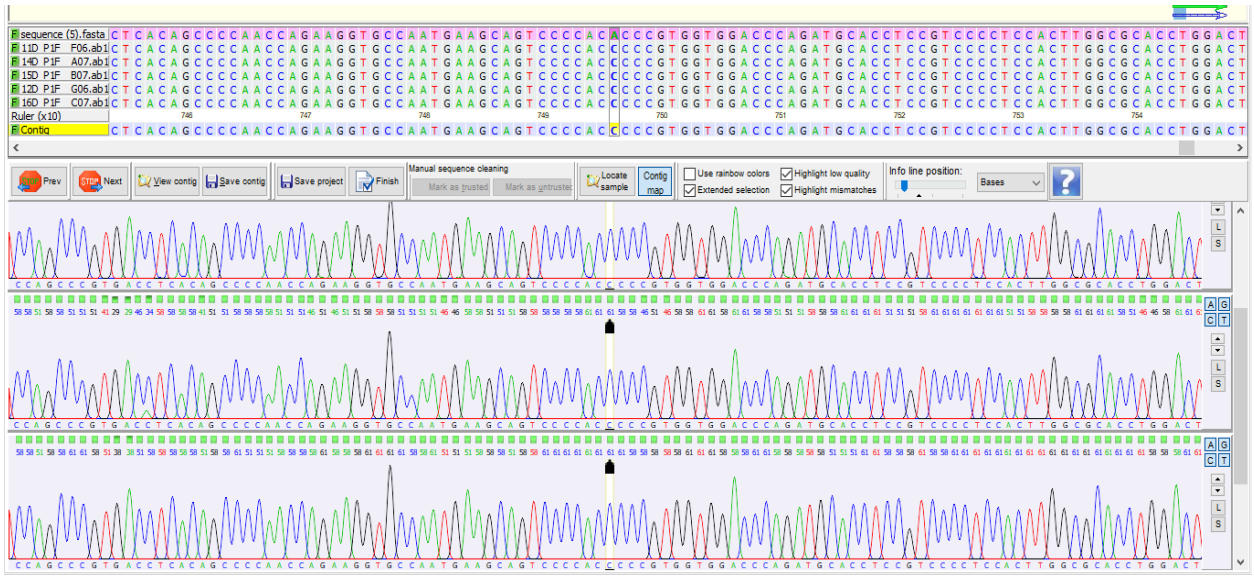


Figure 5.214: Mutation graph.

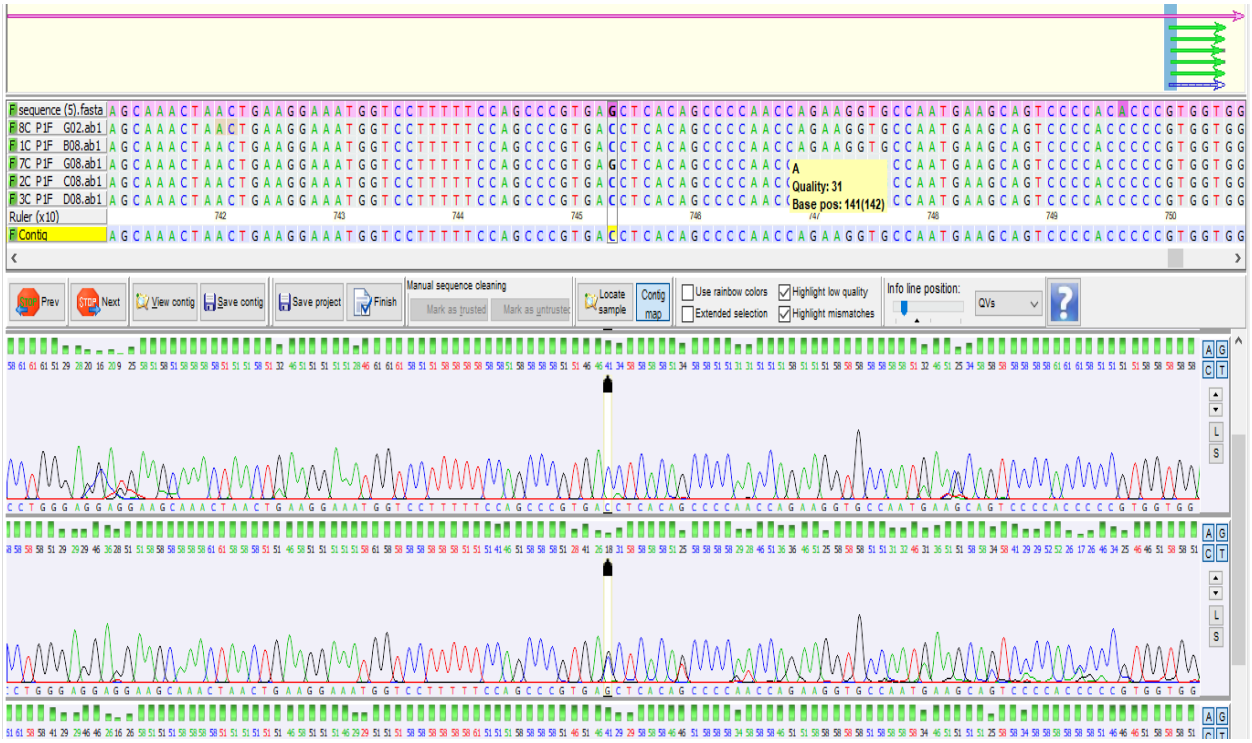


Table 5.15 : Frequency of alleles

Genotype distribution and alleles frequencies of Fetuin-A		Frequency
g.7452 A>C	Wild A	0%
	Mutant C	**100%**
g.7495 A>C	Wild A	0%
	Mutant C	**100%**

- Thr256Ser is the most common gene responsible for T2DN.
- As a result of missense mutation the frequency of G allele is 100% which can be one of the factor affecting the concentration of Fetuin-A in diabetic nephropathy.

Chapter 6

DISCUSSION

DISCUSSION

Our study included total number of 40 healthy controls and 40 cases in each group of diabetic nephropathy (DN) for the study. Study participants involved controls and DN cases of 1st 2nd 3rd and 4th stage. Exception to this is- our study couldn't involve 5th stage of DN as we were unable to get the cases belonging to this stage. Comparison between these groups with respect to gender, age was done, which showed no statistical significance.(Table no 5.1)

6.1 Study of glycemic parameters:

This study showed the glycemic parameters like FBS, PPBS, HbA1c, HOMA-IR are significantly increased in all the stages of diabetic nephropathy cases compared to controls $p<0.001$. Hyperglycemic state results in generation of free radicals, AGEs and activates inflammatory pathways resulting in onset of DN. As stage progressed, the glycemic parameters also increased proportionally compared to their previous stage significantly. (Table no.5.2)

6.2 Renal parameters and CRP:

Our study showed the significant elevation of renal parameters like serum creatinine ,urea, uric acid, urine micro-albumin along with CRP indicating the ongoing inflammation and damage within the kidney involving the glomerular membrane and mesangial cells expansion leading to elevation of the above parameters in serum and urine within all the stages of DN compared to controls ($p <0.001$) , whereas eGFR significantly decreased as the stages progressed to 4th stage compared to previous stages and also controls ($p<0.001$) (Table no. 5.3)

Serum CRP levels in our study is significantly ($p=0.001$) (Table no. 5.4) elevated in all the stages of diabetic nephropathy as the severity of the disease progress indicating the high inflammatory

state. Our study also showed positive correlation of CRP with serum and urinary Fetuin-A in all the stages of DN .

Similar study by Lebreton JP et al.(1979) [1] showed, elevated CRP levels in nephropathy cases down regulate or inhibit Fetuin-A synthesis leading to low levels of circulating Fetuin-A in CKD.

Along with this we found the significant elevation of TGL, TC, LDL-C, VLDL-C in cases than controls (p=0.000). HDL-C significantly reduced as the stages of DN progressed to end stage compared to controls and previous stages. (Table no. 5.5)

This explains in Diabetic nephropathy, insulin resistance promotes the release of FFA from adipose tissue which are then processed by the liver resulting in increased production of TGL and VLDL.

6.3 Serum Fetuin A levels:

Many authors compared the serum Fetuin-A levels in controls and diabetic nephropathy cases in general without considering the stages of DN. Here in our study we evaluated Fetuin-A levels in various stages of DN cases and controls. Comparison was done in between the stages..

In the present study we found a significant ($p<0.001$) elevation in serum Fetuin-A levels in DN cases compared to controls. Serum Fetuin-A levels increased in first two stages and started to decline in 3^{rd} and 4^{th} stages of DN. (Table no. 5.7)

Serum Fetuin-A levels showed positive correlation with the renal parameters like serum creatinine, urea, albuminuria but showed negative correlation with eGFR in $1^{st}$and 2^{nd} stages.

(Table no. 5.3) (Figure 5.7, 5.23). We observed decreased serum Fetuin- A levels in 3rd and 4th stage of DN compared to previous stages 1st and 2nd stages. (Table no. 5.3) (Figure 5.184, 5.185).

Similarly we saw the significant positive correlation of serum Fetuin-A levels with the FBS, PPBS, HbA1c, HOMA-IR in first two stages of DN cases. (Table no. 5.11) (Figure 5.27, 5.28, 5.29 and 5.30).

Serum Fetuin-A levels also showed positive correlation with lipid markers like TGL, TC, LDL-C, VLDL-C except HDL-C in 1st and 2nd stage of DN (Figure 5.36) but showed negative correlation with these lipid parameters in 3rd and 4th stage of DN compared to previous two stages. (Table no. 5.11) (Figure 5.33, 5.34, 5.35)

This study also showed the statistically significant elevation of urinary Fetuin-A levels in all the stages of diabetic nephropathy cases (Stage1-4) compared to controls (p=<0.001). (Table no. 5.12). This elevated urinary Fetuin-A levels also showed positive correlation with the glycemic parameters like FBS, PPBS, HbA1c, HOMA-IR (Table no. 5.12) (Figure 5.111-5.115) and renal parameters serum creatinine, urea, albuminuria and lipid profile except HDL in all the stages of DN. But this elevated urinary Fetuin-A showed negative correlation with eGFR as the DN stages progressed. (Table no. 5.12) (Figure 5.109, 5.126)

In the present research, we noticed a significant (p=0.001) elevation in serum Fetuin-A levels in first 2 stages of diabetic nephropathy and this result is in accordance with study by Akbari et. al (p=0.001),(2018)[2]. Wang Y et al(2019) showed increase in serum Fetuin-A level in diabetic nephropathy[3]. Similar findings were also seen in study by Musolino et. al (2024) reported elevated serum Fetuin-A levels in DKD compared to non diabetic CKD[4].

In accordance to our findings, a study by Al-Said (2018) and his colleagues who found that mean levels of serum Fetuin-A are significantly increased in Diabetic patients with nephropathy compared to healthy control group [5].

Similarly study by El-Batch (2015) also showed significant elevated serum Fetuin-A levels in microalbuminuria patients than controls. Their findings showed the association of Fetuin-A in insulin insensitivity, lipid abnormalities along with endothelial damage [6].

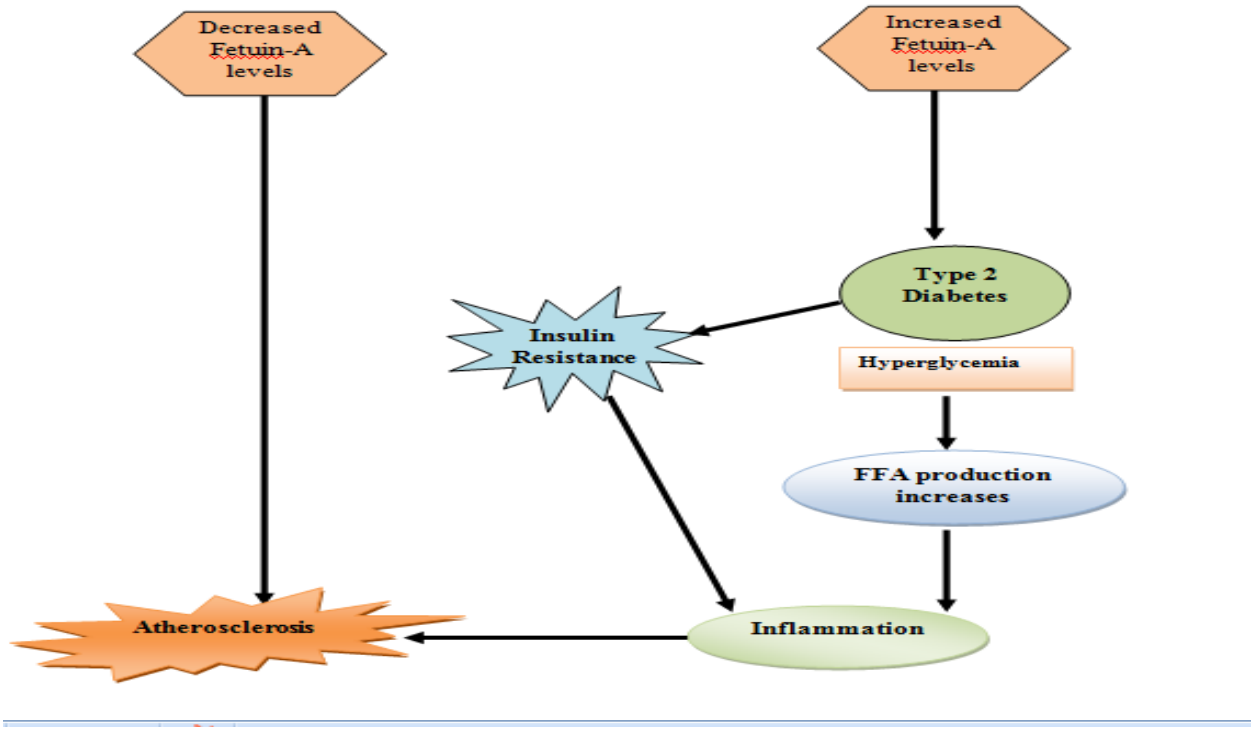
Another study by Budoff and his colleagues (2011) found elevated serum Fetuin-A levels among Latinos with DN when compared to normal control groups [7] .

In contrast to our findings study by Koulman et al (2013) reported that serum Fetuin-A is lower in microalbuminuria patients compared to normo-albuminuria patients [8].

A study by A mitkees et al.(2020) showed significant (p=0.001) decreased serum Fetuin-A levels and also study by Philip Bassey et.al. (2022), showed slight elevation of serum Fetuin-A in early stages of CKD but with the disease progression serum Fetuin-A significantly decreased in end stage of disease, which is in accordance to our finding where first two stages showed elevated serum Fetuin-A levels and subsequently decreased serum levels in 3rd and 4th stage of CKD [9, 10].

6.4 Fetuin-A and its relation with diabetes:

Figure 6.1: Fetuin A status and its relation to type 2 diabetes.[2]



In relation to the low levels of serum Fetuin-A study by R Mehrotra (2007) in his research concluded saying that low Fetuin-A levels are observed in nephropathy patients. Reason being that Fetuin-A acts as negative phase protein and others like IL-1 beta mediates in the decreased production of Fetuin-A. Uremia with inflammation is an important reason for low Fetuin-A levels in nephropathy patients [11].

Another study by Westenfeld et al. (2005) showed that in patients of early diabetic nephropathy Fetuin-A levels are not low, Fetuin-A levels will be higher in serum of early stages or early phase of diabetic nephropathy and as the disease progress to severity the serum levels of Fetuin-A comes down and the reason is unknown [12**]**.

Similarly study by Mitkees et al (2020) showed that there is significant reduction in serum Fetuin-A levels in controlled and uncontrolled diabetic patients [13].

In accordance to our study, research by El batch et al (2015) showed a positive correlation of increased sr. Fetuin-A with fasting insulin and HOMA–IR indicating the involvement of this protein in insulin resistance [6] .

In addition study by Ix and Sharma et. al (2010) found that raised Fetuin-A levels leads to suppression of adiponectin transcription and this lowers 5'AMP activated protein kinase in podocytes leading to foot process effacement and albuminuria [14].

6.5 ROC analysis for serum Fetuin-A:

In the present study we also calculated the cut off value of the above mentioned parameters. Our study showed **AUC for serum Fetuin-A was 0.802** and cut off value found to be **48.21ng/ml.** (Table no. 5.8 Figure 5.1). Study by Kanjanabuch T et al (2023) showed AUC 0.79 and cut off value was found to be 41.0 ng/ml [15].

Another study by Noura et al. (2020) Receiver operating characteristics (ROC) curve was used to define the best cut off value of serum Fetuin A which was >294.2 µg/dl with sensitivity of 93.3 %, specificity of 93 %. [13]

6.6 Urinary Fetuin-A:

Our study showed statistically significant raise in urinary Fetuin-A levels in diabetic nephropathy cases compared to controls. Urinary Fetuin-A levels gradually increased from the stage 1 to stage 4 of diabetic nephropathy indicating the severity of damage to the glomerulus and nephron part

leading to loss of Fetuin-A in urine. It showed positive correlation as the stage severity progressed.

In accordance of our study, Pedro Magalhese et al (2021) identified 14 different urinary protein fragments belonging to Fetuin-A and showed their significant increase in urine levels of T2DM with nephropathy. They showed the association of urinary Fetuin-A with eGFR decline need not be clinically meaningful but demonstrated that Fetuin-A peptide levels increase earlier than albuminuria during progression of Diabetic kidney disease[16]

Study by Inoue et al (2013) showed the Fetuin-A is secreted in the urine during advanced diabetic nephropathy. These stated that urinary Fetuin-A levels in these patients correlate with microalbuminuria, indicating leakage of Fetuin-A through defective glomerulus and also inability to get reabsorbed in proximal tubules [17,18].

Study by Dautova et al (2014) analyzed that raised urinary Fetuin-A levels in abnormal kidney function can be because of many reasons. Fetuin-A protein of 51 to 67 kDa weight faces difficulty while going through normal glomerulus. In case of DN disruption of filtration barriers has lead to increase protein leakage into the urine. Another mechanism includes the upregulation of protease specific to cleaving of Fetuin-A in kidneys of proteinuria patients enable its passage through the glomerulus [19,20,21] .

Study by Ming et al (2024) highlighted the significant association between urinary Fetuin-A levels and albuminuria, explaining the role of disrupted filtration barriers in most cases of glomerular diseases. They showed the reduced staining of Fetuin-A and Megalin in affected kidney tubules may indicate or emphasis the significance of tubular reabsorption/uptake impairment leading to elevated urinary Fetuin-A levels in CKD[22]

Another explanation for increased urine Fetuin-A levels given by Matsui et al (2013)[23,24,25] is that in normal healthy patients proteins like retinol binding protein, alpha1 microglobulin and Fetuin-A are being reabsorbed in proximal tubules and gets degraded by Megalin. But in case of diabetic nephropathy /CKD there is decreased expression of megalin in proximal kidney tubules which leads to elevation of urinary Fetuin A.

Study by Maghalies et al (2021) reported urinary Fetuin-A beyond its prognostic value in CKD addresses the limitations of eGFR and proteinuria enabling early CKD detection [16].

In accordance to our study Kentaro Inoeu (2013) in his research showed the urinary excretion of Fetuin-A is positively correlated with serum creatinine, albuminuria and also showed negative correlation with HDL-C and eGFR with statistical differences $p<0$. [17].

6.6 ROC analysis for urinary Fetuin-A:

Our study found AUC for urinary Fetuin-A was 0.947 and cut off value found to be 41.28 ng/ml**.** (Table no. 5.9, figure 5.2) in accordance to our study Li F et al (2021) in their study found the urine Fetuin A cut off value 43.3ng/ml and sensitivity 81.8%, specificity 73.4% [26].

Several studies have shown the association of anti diabetic drugs influencing the serum Fetuin-A levels. Drugs like pioglitazone significantly reduce this protein levels in T2DM. Akinobu et al. (2014) found pioglitazone treatment resulted in suppression of mRNA coding for Fetuin-A possibly through PPARλ activation. This finding imply that pioglitazone lowers blood levels of Fetuin-A and directly inhibits its expression in the liver, hence partially improving insulin resistance [27].

Suhalya K et al. (2021) in their study investigated the effects of metformin on proteins like Fetuin A, Fbxw7, pentraxin 3 in T2DM and they found non significant effect of metformin therapy duration on levels of Fbxw7, Fetuin A, pentraxin3[28].

In contrast to the above study Koel Dutta et al. (2023)] showed a significant reduction in serum Fetuin-A was seen with both Metformin and Dapagliflozin treatment, previously not reported for latter drug. [29]

Study by Chan Jung et al. (2013) made comparison between two study groups , those taking ACE inhibitors or angiotensin receptor blockers for diabetic nephropathy and those who do not take. Comparison of mean Fetuin-A was done between two groups. There was no significant difference seen in serum Fetuin A levels between the diabetic nephropathy patients not taking ACEI/ARB versus those on ACEI/ARB ($p = 0.736$) [30].

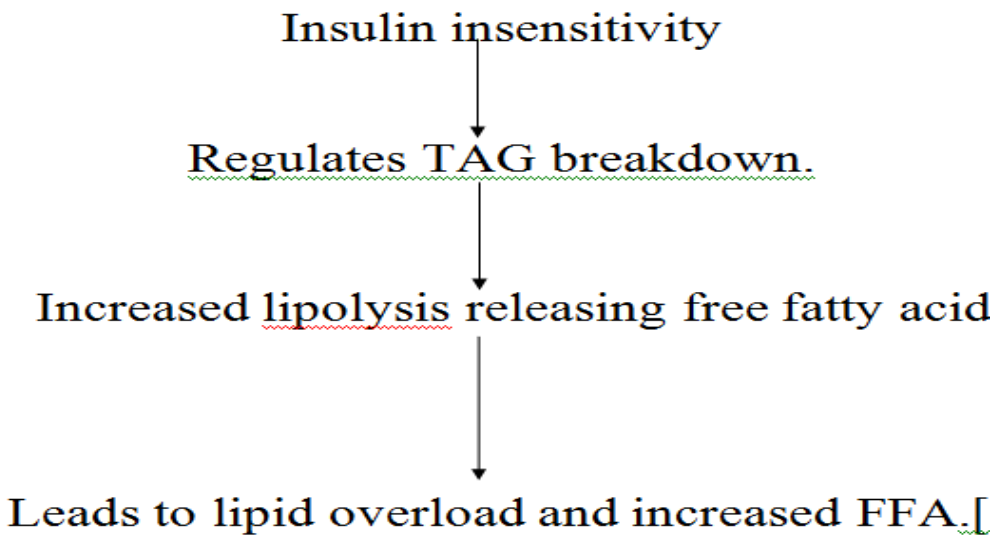
6.8 Serum free fatty acids:

In our study serum free fatty acids levels were increased in all the stages of diabetic nephropathy cases compared to controls. As the progression of stage increased there is significant elevation of the serum free fatty acids in cases with p value <0.001.(Table no 5.13)

Our study also showed the significant positive correlation of serum fatty acids with the glycemic status, creatinine, urea, albumiuria and also with TGL,TC, LDL-C,VLDL-C. But it showed negative correlation with eGFR, HDL-C as the severity of the disease progressed. (Table no 5.13)(Figure 5.205-5.207).

6.9 Diabetes and Free Fatty Acids:

Figure 6.2: Free fatty acids in diabetes mellitus. [26]



In accordance of our study, Xin Y et al [31] and Liu ZX et al .[32] showed elevated serum free fatty acids in diabetic nephropathy cases indicating the insulin resistance.

But in contrast to our findings, study by Li F et al. showed decreased serum free fatty acid levels [26].

ROC curve was used to give the best cut off value of serum free fatty acid levels, which was 395.4 mmol/L with sensitivity of 100 %, specificity of 87.5 % and area under the curve showed 0.979. (Table no.5.10)(Figure 5.6)

Study by Li F et al showed cut off value for serum free fatty acids is 573 mmol/day , sensitivity 72%, specificity 62%, and area under the curve as 0.71 [26].

6.10 Gene polymorphism:

In our study **polymorphism of Fetuin-A gene (Thr256ser)** showed higher frequency of G allele indicating the increased levels of Fetuin-A in early stages of nephropathy. (Table no.5.15), (Figure 5.214) Whereas study by Ma et. al found higher frequency of G allele [33].

The "G" allele on AHSG exon-7 (C/G) SNP is highly concurrent and confers risk for people with normo-albuminuria, according to a study by Damodharan Umapathy et al. The 'G' allele indicated the risk of chronic macroalbuminuria in the DN individuals. Among South Indian T2DM subjects, the AHSG Thr256Ser (rs4918) SNP was linked to renal problems [34].

According to a 2013 study by Dalia A. M., patients with the CG and GG genotypes had lower serum Fetuin-A levels than those with the CC genotype, indicating a statistical significant relation between serum Fetuin-A levels and its gene polymorphisms [35].

In contrast to our study, El. Batch et. al. showed synonymous mutation in all the DN cases[6].

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Chapter 7

SUMMARY AND CONCLUSION

SUMMARY

This was a hospital based comparative study. Study comprised of 179 participants divided into controls (40) and DN different stages stage1 (40), stage 2(40), stage 3(40), stage 4(19). DN stages were done as per KDIGO classification.

Serum glycemic parameters like FBS, PPBS, HbA1c, insulin, HOMA-IR were estimated and compared between controls and cases in all the study participants .

Renal parameters like serum creatinine, urea, uric acid, eGFR, urine microalbumin were estimated and compared between controls and cases in all the study participants along with these serum CRP levels were also estimated and compared between the study groups.

Serum lipid profile parameters like Total cholesterol, TGL, HDL, LDL, VLDL also were estimated and compared between controls and cases in all the study participants .

Serum Fetuin-A levels were estimated in all the study groups. Study showed statistically significant elevation of serum Fetuin-A in all the stages of DN compared to controls $p<0.001$. In DN cases serum Fetuin-A levels increased in stage 1 and 2 proportionally but declined in stage 3 and 4 compared to stage 1 and 2.

Urinary Fetuin-A level was also estimated simultaneously in study groups. Study showed significant elevation of urinary Fetuin-A $p<0.001$ levels in all the stages of DN cases compared to controls showing proportional increase as the stage advanced .

Serum free fatty acids levels were estimated in controls and DN cases. Serum FFA showed statistically significant elevation in DN cases compared to controls $p<0.001$. Serum FFA levels increase in linear to the stage advancement of DN.

Our study also found the polymorphism of Fetuin-A gene in DN cases. This **polymorphism of Fetuin-A gene (Thr256Ser)(C→G)** showed higher frequency of G allele indicating the increased levels of Fetuin-A in early stages of nephropathy.

Best Cut off value for the serum Fetuin-A level in our study was 48.21ng/ml, urinary Fetuin-A level was 41.28 ng/ml and serum free fatty acids was 395.4 mmol/L .

Clinical implications:

Serum Fetuin-A level assessment in DN will help us to know the severity of DN and we noticed Fetuin-A levels declined in 3rd and 4th stages of DN compared 1st and 2nd stage. This reduced Fetuin-A level in final stages of DN indicates the severity of illness and also Fetuin-A reduction will lead to increased vascular calcification of cardiac vessels, resulting in moratility of the DN cases. So estimating this parameter will help us to consider this as a reliable predictor of severity of DN and prevent the morbidity and mortality related to severity of DN.

Inclusion of serum & urinary Fetuin-A levels in to the current clinical assessment could achieve an earlier diagnosis of severity of the diabetic nephropathy and prevent the further progression to end stage renal disease and mortality.

Our study also showed the elevation of serum free fatty acids in DN cases, indicating its role in predicting the severity of DN.

CONCLUSION

- There was significant increase in serum Fetuin-A levels in first two stages and decreased in stage 3 and 4 of diabetic nephropathy.
- Urinary Fetuin -A level was also increased significantly in all the stages of diabetic nephropathy which is directly proportional to severity of the disease.
- Serum Fetuin-A is directly proportional to CRP in early 2 stages and is negatively correlated in stage 3 and 4, indicating loss of Fetuin-A in urine.
- Serum Fetuin-A level is positively correlated with FBS, PPBS, HbA1C, micro-albuminuria, triglyceride and free fatty acids levels in 1^{st} and 2^{nd} stage but showed negative correlation with all these in stage 3 and 4 of diabetic nephropathy.
- Cut off value for serum Fetuin-A level found to be 48.21 ng/ml and urinary Fetuin- A level was 41.28 ng/ml.
- Cut of value for serum free fatty acid was 395.4 mmol/ l.
- There was significant gene polymorphism noted with change in the frequency of G allele (G>C) which denotes the alteration of serum Fetuin-A levels in diabetic nephropathy cases.
- From the observation of the study we conclude that Fetuin-A can be an effective biomarker for the early detection of severity of DN. Thus our proposed null hypothesis is rejected and alternate hypothesis is proved.

Research implications:

- Better understanding of pathogenesis of diabetic nephropathy in type 2 DM and its severity at early stages.

- Identification of the status of renal damage at the earliest by using this novel marker Fetuin-A in blood and urine, as well at molecular level.

Limitations of the study:

1. Could not get the 5^{th} stage of diabetic nephropathy cases.
2. Less number of participants in the study group to validate the effectiveness of the parameters externally.

Future perspective:

1. Need extensive research on this protein and find the exact correlation between Fetuin-A and the severity of complications of T2DM like nephropathy.
2. This can be used as best predictor of progressive diabetic nephropathy and can be regularly utilised for diagnostic and prognostic purpose.

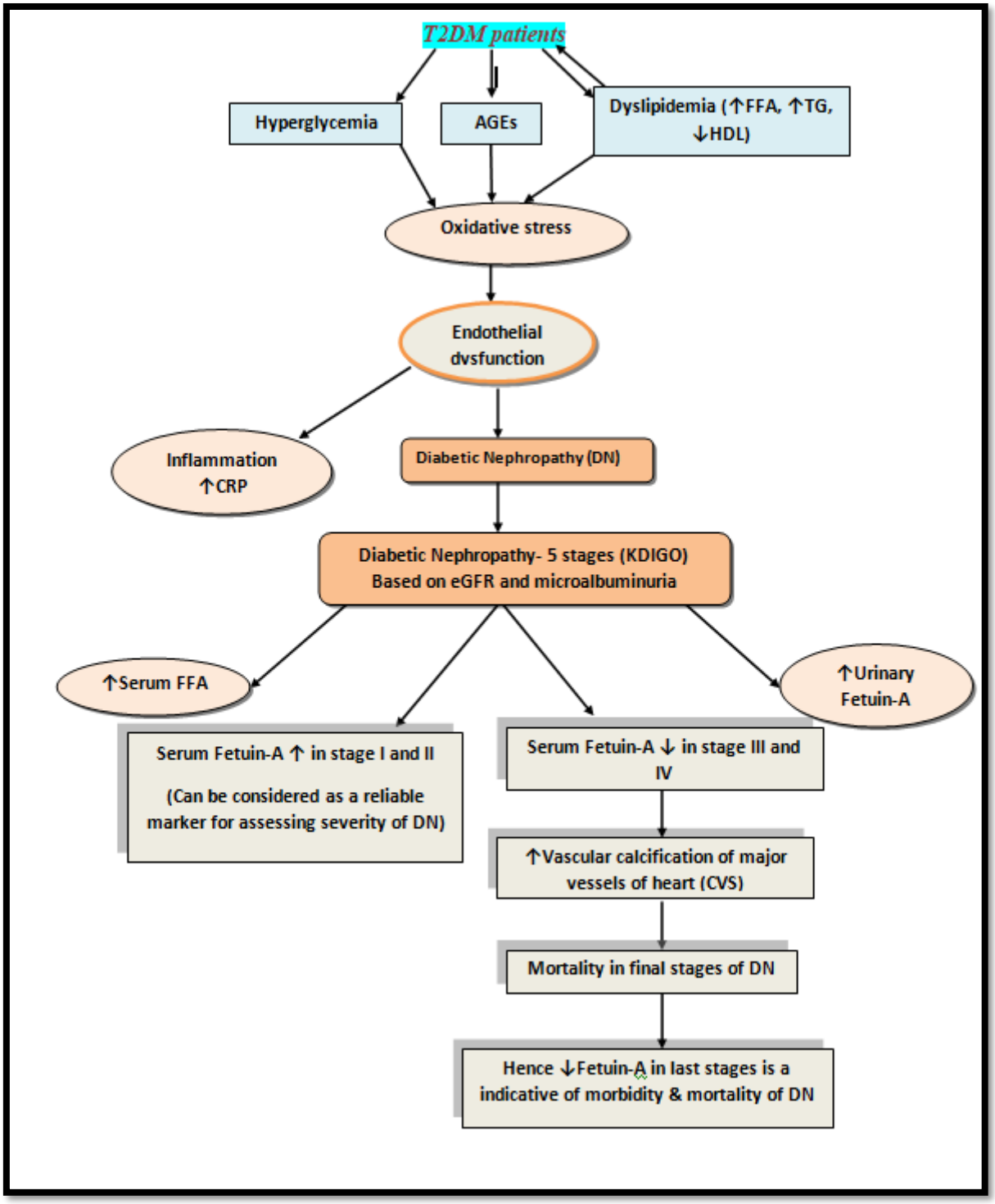

Figure 7.1: Summary of the study.

Chapter 8

ANNEXURES

CONSENT FORM I

INFORMATION FOR PARTICIPANTS OF THE STUDY

1. Title of the project	**"Activities of Fetuin-A protein in type-2 diabetic nephropathy patients".**
2. Name of the PI/Ph.D student and Department	**Deepali S M** Lecturer, Department of Biochemistry. S Nijalingappa Medical College, Navanagar Bagalkot.-587102, Karnataka, India. E-mail:malgavkardeepali@yahoo.in Cell No: 9743760123.
3. Name, Designation, Address, Phone No Email ID of the Guide	**Dr. Nilima Dongre** Professor Department of Biochemistry Shri B M Patil Medical College BLDE(Deemed to be University) Vijaypura, Karnataka Cell. No. 9480031381 Email:nilimadongre@gmail.com
4. Name of the Co-guide/ coinvestigator with designation, Department,Phone No. Email ID	**Dr. S M Goornavar** Professor Department of Medicine S Nijalingappa Medical College Bagalkot. 587102 Phone: 9845977783 Email: drshiv_77@rediffmail.com
5. Purpose / objective of this project/ study	• To estimate routine glycemic status and insulin levels, renal function tests, CRP, microalbuminuria, lipid profile in the study group. • To quantitate the levels of serum free fatty acids, serum and urinary Fetuin-A protein and at various stages of diabetic nephropathy. • To study polymorphism of Fetuin-A gene in diabetic nephropathy subjects. • To find correlation of Fetuin-A with glycemic status, renal parameters like urea creatinine, eGFR, microalbuminuria, CRP and also

lipid profile, serum free fatty acids at various stages of diabetic nephropathy.

6. Procedure/ method of the study

This was a hospital based comparative study conducted at tertiary care hospital, in Karnataka India. The study was approved by Institutional ethics committee. Informed consent was obtained from all the participants. 40 healthy controls and 40 type2 diabetic nephropathy cases in each stages of diabetic nephropathy were selected between the age group of 35-70 yrs based on KDIGO guidelines for nephropathy.

Lab investigation:

5ml Fasting blood sample was collected under aseptic precautions after an overnight fast, out of which 1ml was transferred in EDTA-containing vacutainers for HbA1C and 1ml was transferred in fluoride-containing vacutainers for fasting blood sugar estimation, 3ml in plain vacutainers for estimation of other biochemical parameters. Blood sample (2ml) collected 2 hours post-meals in fluoride containing vacutainers under aseptic precautions for PPBS estimation. After centrifugation of all the vacutainers at 3000 rpm for 20 minutes, plasma/serum was obtained. The separated serum was used to estimate Fetuin-A levels and other biochemical parameters like urea, creatinine, uric acid, TG and HDL.

At the same time 10 ml of urine sample was collected after overnight fasting from the same

	patients in a sterile container and estimated for microalbumin within 2 hours.
7. Expected duration of the subject participation	1 Hour
8. Expected benefits from the research to the participants	Results of the study will help in the further management of severity of diabetic nephropathy.
9. Any risks expected from the study to the participants.	Minimal risk.
10. Maintenance of confidentiality of records	Confidentiality will be maintained.
11. Provision of free treatment for research related injury	Free treatment will be given by the institution.
12. Compensation of the participants for Disability of death resulting from such inury	Compensation for any unforceable research related injury or death resulting from such injury will be duly given to you through hospital insurance policy number 68040236180200000009.
13. Freedom to withdraw from the study at any time during the study period without the loss of benefits that the participant would otherwise be entitled.	Yes, participants can withdraw from the study whenever they wish.
14. Possible current and future uses of the biological material and of the data to be generated from the research and if the material is likely to be used for secondary purposes or would be shared with others, this should be mentioned.	Not applicable.
15. Contact details of Chairman of IEC for Appeal against violation of rights	**Dr. S L. Hoti.** Director Grade Scientist (Scientist G)

ICMR- National Institute of Traditional Medicine.
(Formerly RMRC)
Belagavi- 590010
Phone No. 0831-2477477
Fax. 0831-2475479.

CONSENT FORM II

PARTICIPANT CONSENT FORM

Participant's Name:

Address:

Phone No.:

Email ID:

Title of the Project: Activities of Fetuin-A protein in type-2 diabetic nephropathy patients.

The details of the study have been provided to me in writing and explained to me in my own language. I confirm that I have understood the above study and had the opportunity to ask questions. I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without medical care that will normally be provded by the hospital being affected. I agree not to restrict the use of any data or results that arise from this study provided. Such use is only for scientific purpose(s).

I have been given an information sheet giving details of the study. I fully consent to participate in the above study.

Signature of the participant: Date:

Signature of the investigator: Date:

ಒಪ್ಪಿಗೆ ಪತ್ರ

ಹೆಸರು :

ವಿಳಾಸ :

ದೂರವಾಣಿ :

ಶಿರ್ಷಿಕೆ : **ಟೈಪ್ – 2 ಡಯಾಬಿಟಿಕ್ ನೆಫ್ರೋಪತಿ ರೋಗಿಗಳಲ್ಲಿ ಫೆಟುಯಿನ್ – ಎ ಪ್ರೋಟಿನ್‌ನ ಚಟುವಟಿಕೆಗಳು.**

ಅಧ್ಯಯನದ ವಿವರಗಳನ್ನು ಬರವಣಿಗೆಯ ರೂಪದಲ್ಲಿ ಮತ್ತು ನನ್ನ ಭಾಷೆಯಲ್ಲಿ ತಿಳಿಸಿದ್ದಾರೆ. ನಾನು ಅಧ್ಯಯನವನ್ನು ಅರ್ಥಮಾಡಿಕೊಂಡಿರುತ್ತೇನೆ ಹಾಗೂ ಪ್ರಶ್ನೇ ಕೇಳುವ ಅವಕಾಶವನ್ನು ಹೊಂದಿದ್ದೇನೆ. ಅಧ್ಯಯನದಲ್ಲಿ ನನ್ನ ಪಾಲ್ಗೊಳ್ಳುವಿಕೆ ಸ್ವಯಂಪ್ರೇರಿತವಾಗಿದೆ ಮತ್ತು ಯಾವುದೇ ಸಮಯದಲ್ಲಿ ಅಧ್ಯಯನದಿಂದ ಹಿಂದೆ ಸರಿಯಲು ಮುಕ್ತವಾಗಿದ್ದೇನೆ. ಅಧ್ಯಯನದ ಡೇಟಾ ಹಾಗೂ ಅಂಶಗಳನ್ನು ನಿರ್ಬಂಧಿಸಲಾಗುವುದಿಲ್ಲ ಎಂದು ಒಪ್ಪಿರುತ್ತೇನೆ. ಈ ಮಾಹಿತಿಯನ್ನು ವೈಜ್ಞಾನಿಕ ಕಾರಣಗಳಿಗೆ ಬಳಸತಕ್ಕದ್ದು. ಈ ಅಧ್ಯಯನಕ್ಕೆ ನನ್ನ ಸಂಪೂರ್ಣ ಒಪ್ಪಿಗೆ ಇರುತ್ತದೆ.

ಅಭ್ಯರ್ಥಿಯ ಸಹಿ : ದಿನಾಂಕ :

ಸಾಕ್ಷಿದಾರರ ಸಹಿ : ದಿನಾಂಕ :

MODEL OF QUESTIONAIRE TO THE PARTICIPANTS

Serial number:

Personal Details:

Name (In Full): _____

Age: _____ Sex: _____

Contact Number: _____ Religion: _____

Address: _____

Personal History:

a. Do you take meals regularly? Yes ☐ No ☐

b. Do you get proper sleep ? Yes ☐ No ☐

c. Do you have feeling of weakness ? Yes ☐ No ☐

d. Do you have swelling in hand, feet or on face ? Yes ☐ No ☐

e. Do you have any disease? Yes ☐ No ☐

f. What disease do you have? _____

g. Do you have any of the following disease:

i. Hypertension ☐ iv. Arthritis ☐

ii. Liver disease ☐ v. Diabetes mellitus ☐

iii.Cardiovascular disease ☐ vi. Tuberculosis ☐

h. Duration of Diabetes _____

i. Family history of kidney disease _____

Investigations:

FBS

Fasting Insulin

Insulin Resistance

Urinary Microalbuminuria

Urea

Creatinine

eGFR

Serum free fatty acids

Serum and Urinary levels of *Fetuin A* protein

Investigator's Signature **Participant's Signature**

BLDE (DEEMEDTO BE UNIVERSITY)

PLAGIARISM VERIFICATION CERTIFICATE

1. Name of the Student: **Deepali S M** Reg No:**18PHD001**
2. Title of the Thesis: **"Activities of Fetuin-A protein in type 2 diabetic nephropathy patients".**
3. Department: **Biochemistry**
4. Name of the Guide & Designation: **Dr.Nilima Dongre Professor,** Dept of Biochemistry, Shri B M Patil Medical College, BLDE (DU), Vijayapura.
5. Name of the Co-Guide & Designation: **Dr. S M Goornavar,** Professor of Medicine, S Nijalingappa Medical College Navanagar, Bagalkot.

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

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Signature of Guide Name & Designation	Signature of Coguide Name & Designation	Signature of Student Name & Designation	Verified By (Signature) Name & Designation
Professor Dept. of Biochemistry BLDE (Deemed to be University) Shri B.M.Patil Medical College Vijayapur-586103.	Professor, Dept. of General Medicine S. Nijalingappa Medical College & H.S.K. Hospital & Research Centre BAGALKOT - 587 102.		24/6/25

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BLDE (DU)/IEC/ 399/2019-20 27th December, 2019

INSTITUTIONAL ETHICAL CLEARANCE CERTIFICATE

The ethical Committee of this University met on 27th December, 2019 at 11.00 a.m. scrutinizes the Synopsis/ Research Projects of Post Graduate Student / Under Graduate Student /Faculty members of this University /Ph.D. student College from ethical clearance point of view. After scrutiny, the following original/ corrected and revised version synopsis of the thesis/ research projects has been accorded ethical clearance.

Title: *Activities Of Fetuin A Protein In Type -2 Diabetic Nephropathy Patients.*

Name of the Principal Investigator: Deepali S. M., PhD Scholar (18PHD001),Biochemistry.

Dr. Santoshkumar Jeevanagi
Chair person
IEC, BLDE (DU),
VIJAYAPURA

Chairman,
Institutional Ethical Committee,
BLDE (Deemed to be University)
Vijayapura

Dr.Akram A. Naikwadi
Member Secretary
IEC, BLDE (DU),
VIJAYAPURA

MEMBER SECRETARY
Institutional Ethics Committee
BLDE (Deemed to be University)
Vijayapura-586103, Karnataka

Following documents were placed before Ethical Committee for scrutinization.

- Copy of Synopsis/Research Projects
- Copy of inform consent form
- Any other relevant document

Smt. Bangaramma Sajjan Campus, B. M. Patil Road (Sholapur Road), Vijayapura - 586103, Karnataka, India.
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SNMC-INSTITUTIONAL ETHICS COMMITTEE ON HUMAN SUBJECTS RESEARCH
☎08354-235340 Fax: 08354-235360
Website: www.snmcbgk.in **Email:** iechsrsnmcbgk@gmail.com

Office of the Institutional Ethics Committee

File No: SNMC/IECHSR/2019-20/A-71/1.1 Date: 05/02/2020

To:

Deepali

Lect dept of biochemistry

SNMC, Bagalkot

1. **Topic of Protocol**: **Activities of Fetuin-A protein in type-2 diabetic nephropathy patients.submitted** by SNMC, Bagalkot
2. **Subject**: Approval for conducting the above mentioned study & related documents by IEC.

Dear Deepali

The Ethics Committee (EC) meeting of SNMC was held on 25-01-2020 from 10.30 AM onwards in the Hall of Medical Education Department of S. Nijalingappa Medical College & Hanagal Shri Kumareshwar Hospital & Research Centre, Bagalkot.

Following members of the committee were present:

1. Dr. S. L Hoti, Scientist-G,Director grade scientist ICMR-NITN, Belgaum.	**Chairman**
2. Dr.Yasmeen Maniyar , Professor & HOD of Pharmacology , SNMC, Bagalkot.	**Member**
3. Dr Anita Herur Professor of Physiology,SNMC,Bagalkot	**Member**
4. Dr Ashalata Mallapur Prof &HoD OBG, SNMC,Bagalkot	**Member**
5. Dr Chandrashekar V M Professor of Pharmacology HSK Pharmacy college	**Member**
6. Dr. Chandrashekharayya S. Hiremath, Professor of ENT, SNMC, Bagalkot	**Member**
7. Dr Manjula R Associate professor of Community Medicine	**Member**
8. Mr. Vittal Kamble , Near Vallabhbai chowk, Bagalkot.	**Member**
9 Mr.Jagdeesh, Budihal, advocate Navanagar , Bagalkot.	**Member**
10. Mr. D. G. Bannur, Holebasaveshwar Nilaya, 10th Cross, Vidyagiri, Bagalkot.	**Member**
11. Dr.Vijayamahantesh SN Professor of Forensic Medicine, SNMC, Bagalkot.	**Member Secretary**

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Email: iechsrsnmcbgk@gmail.com

Office of the Institutional Ethics Committee

The Ethical Committee of SNMC reviewed the following documents:

1. **Research Protocol entitled** Activities of Fetuin-A protein in type-2 diabetic nephropathy patients submitted by Deepali lect dept of biochemistry SNMC, Bagalkot
2. **Information sheet for participants of the study (Consent Form –I) and (Consent Form –I) of the protocol** Activities of Fetuin-A protein in type-2 diabetic nephropathy patients

NOTE: It is to be noted that neither PI nor any of the proposed study team members were present during the decision-making procedures of the Ethics Committee, and members who are independent of the Investigator, have voted/ provided opinion on the trial.

Discussion points:
After reviewing the documents submitted by the Principal Investigator, the Committee has decided to grant approval for conducting the above mentioned study.

You are requested to report to the Ethics Committee the Following:
1. Progress of the study at the end of 4 months.
2. Provide a report to the Ethics Committee on completion of the study.

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

If you have any Questions concerning the above, please feel free to contact the undersigned.

Thanks & Regards,

(Dr. Vijayamahantesh SN)
Member Secretary p 2/2

Member Secretary,
IEC
S. N. Medical College
BAGALKOT

PAPER PRESENTATIONS

VINAYAKA MISSION'S RESEARCH FOUNDATION
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Certificate of Appreciation

This certificate is presented to

Dr. Deepali .S.M.

*for making an oral paper Presentation - (Faculty Category) for his/her paper titled "**Study of Fetuin-a levels in different stages of diabetic nephropathy(DN)**" in **BMS e-CON**,organized by the Departments of Anatomy, Biochemistry, Physiology and Center for Biomedical Research, AVMCH, Puducherry, India held from 25th to 27th November, 2021.*

Dr. T. Rajan	Dr. Lakshmi Jatiya	Dr. M. Manju	Dr. P.F. Kotur
Organizing Secretary	Organizing Chairperson	Scientific Committee Head	Dean, AVMC

REVA UNIVERSITY
Bengaluru, India

Certificate of Appreciation

School of Allied Health Sciences

This is to certify that

Dr./Ms./Mr. Deepali. S. M

of Shri BM Patil Medical College

has presented Oral/Poster presentation titled Fetuin A as a risk factor ..... A case control study

in the International Conference on **"Recent Advances in Allied Health and Biological Sciences Research"** held on 18th and 19th November 2022 .

Convener
School of Allied Health Sciences

Registrar
REVA University

Vice Chancellor
REVA University

PUBLICATIONS

Research article

Role of serum free fatty acids in determining severity of diabetic nephropathy: A case control study

Deepali S. M.[1], J. G. Ambekar[2], S. M. Goornavar[3], Nilima Dongre[4], Sanjeev Ratna[5]

[1,2,4] Department of Biochemistry, Shri B M Patil Medical College, BLDE University, Vijayapur-586101, Karnataka, India
[1,5] Department of Biochemistry, [3]Department of Medicine, S. Nijalingappa Medical College, Navanagar, Bagalkot-587102 Karnataka, India

(Received: December 2022 Revised: February 2023 Accepted: February 2023)

Corresponding author**: Deepali S. M.** Email: malgavkardeepali@yahoo.in

ABSTRACT

Introduction and Aim: The Diabetic Nephropathy (DN) is a result of impaired renal function in Type 2 diabetes. At the onset of diabetes, microvascular complication increases due to accumulation of free fatty acids (FFA) causing renal damage. A study was conducted to estimate the concentration of serum FFA causing severity of diabetic nephropathy.

Materials and Methods: 90 Type 2 diabetic subjects and 30 study controls (age group 35 to 65 years) were selected from the medicine OPD of S N Medical College and HSK Hospital, Bagalkot. Based on the presence of microalbuminuria, the 90 Type 2 diabetic patients were equally divided in to 3 groups, named as stage I to stage III. The serum FFA was estimated by ELISA method in these three groups and control subjects. The statistical analysis was done using SPSS software version 19 utilizing unpaired "t" test for quantitative data and Pearson's correlation tests.

Results: The estimated serum FFA levels in stage I to III was found to be higher and highly significant as compared to control (p=0.001). We find the best cut off value of serum FFA was 4.75 mmol/L, causing severity of diabetic nephropathy. The area under the curve (AUC) is 0.92 with the specificity of 86%, sensitivity 89% and the diagnostic accuracy was found to be of 87%.

Conclusion: Serum free fatty acid levels were higher in diabetic nephropathy subjects, which could be used as diagnostic marker for the severity of renal damage with cut off value of 4.75 mmol/L.

Keywords: Type 2 diabetes; diabetic nephropathy; serum free fatty acids; microalbuminuria.

INTRODUCTION

Diabetes mellitus is the most common disease worldwide. Insulin resistance and insulin deficiency are the most common causes for Type 2 diabetes (1). Long term diabetes causes impairment and dysfunction of organs like eye, kidney, nerves, heart etc. In diabetes the excess glucose binds to circulating free amino acids and tissue proteins by non-enzymatic reaction, produces early glycation products giving rise to advanced glycation end products (AGE's), which causes micro vascular complications (2).

Diabetic nephropathy is characterized by microalbuminuria (excretion of albumin in urine) and loss of glomerular filtration rate (GFR) due to glomerular lesions. In many cases the terminal stage of life in diabetic subjects with diabetic nephropathy is caused due to complete loss of renal function (3). Therefore, early diagnostic markers for monitoring and predicting the development of diabetic nephropathy are needed to protect the renal function and life of individual. Hyperglycemia mainly causes endothelial dysfunction ultimately leading to albumin loss (4). As the insulin inhibits the hormone sensitive lipase, mobilization of free fatty acids from fat depot takes place in diabetes (5).

Many authors have reported the role of free fatty acids in glucose intolerance causing diabetes (6). The increased serum free fatty acids or sustained hyper-free fatty academia causes insulin resistance (IR) in the liver and muscle (7,8). However, relatively other longitudinal epidemiologic studies have shown the relationship between serum FFA levels and incidents of diabetes (9). Non esterified fatty acids (NEFA) also called as Free Fatty Acids (FFA) corresponds to IR and Type 2 diabetes. Insulin level regulates release of free fatty by breakdown of TAG. Insensitivity to insulin by adipose tissue leads to lipid overload in liver and pancreas due to excess FFA, causing development of Type 2 diabetes by impaired functioning of islets of β-cells of pancreas (10).

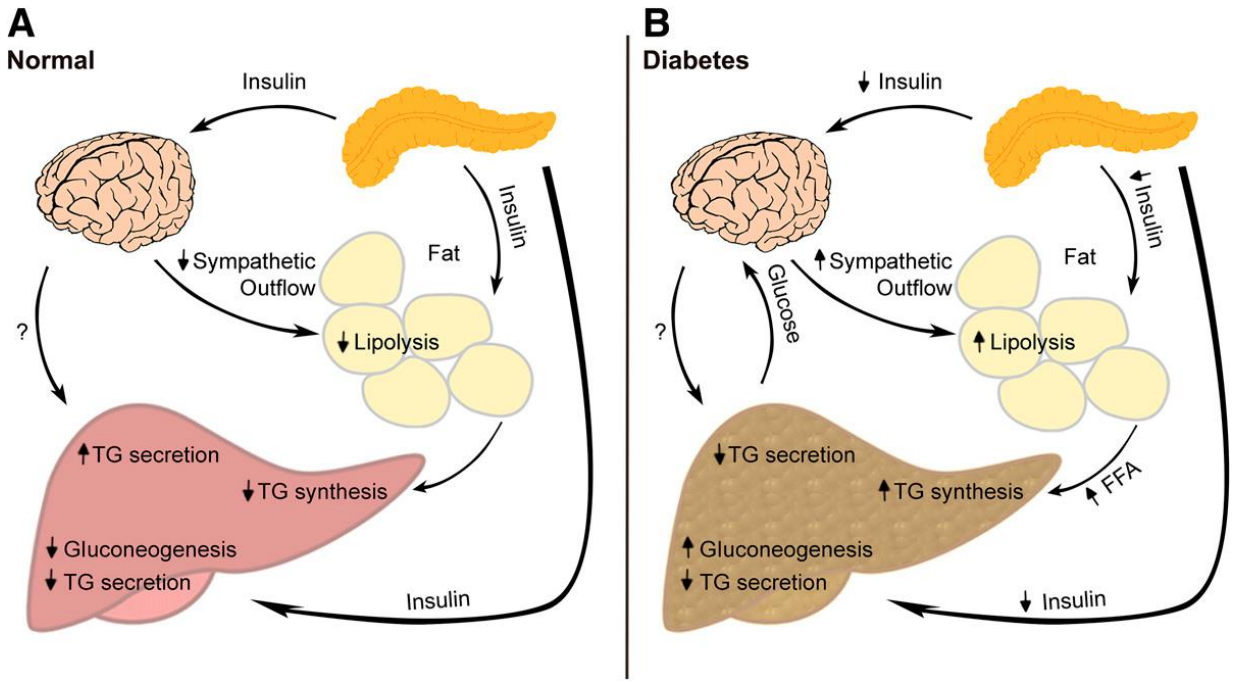

Fig. 1: Showing increased formation of free fatty acids and insulin resistance in diabetes (11)

To correlate the serum free fatty acids (FFA) levels and severity of DN, we estimated serum FFA levels in 3 stages of DN and in the study control subjects, in the present study. With the data analysis, we also tried to find the cut off value of serum FFA concentration responsible to cause severity of diabetic nephropathy.

MATERIALS AND METHODS

The present study was conducted in Medicine and Biochemistry department of S. N. Medical College and HSK Hospital and Research Centre, a tertiary care hospital in Bagalkot, Karnataka, India. Institutional ethics committee approval was taken for the study. Informed consent was obtained from all the study participants.

Ninety type 2 diabetic subjects with the onset of disease for more than 5 years, within the age group of 35-65 years, were divided 30 each and classified in to mild, moderate, and severe (stage I, II and III) diabetic nephropathy based on the presence of microalbuminuria, were selected for the study. 30 Subjects with the same age group not having diabetes were considered as healthy control group. Subjects with the age < 35 years or > 65 years, systemic diseases (hypothyroidism or hyperthyroidism), cardiovascular diseases, pregnancy, malignancy and systemic drug or alcohol abuse were excluded from the study.

To separate the serum and plasma, 5 ml of fasting blood sample was drawn under aseptic conditions and transferred into plane (3 ml) and EDTA coated vacutainer tubes (2 ml), mixed gently and then centrifuged at 3000 rpm for 20 minutes. The separated serum and plasma samples were stored at -20^0 C until assayed for serum glucose, plasma $HbA1_C$ and serum FFA. At the same time, 10 ml of urine sample were collected from the same subjects in a sterile container and assayed within 2 hours for microalbuminuria (12). Serum glucose, (Ba 400 Biosystem), plasma HbA1C (D10 Biorad machine) was estimated using Biosystem kits (13, 14). The serum FFA was estimated by ELISA method (Robonic) using kits of Bioassay Technologies (15).

Sample size calculation was done by open Epi software version 2.3:1, retrospectively with 90% power of the study; the sample size calculated was 28-33. Hence, 30 cases in each group (stage I, II and III) of DN and 30 healthy controls were taken for the study.

The data was analyzed by taking mean$\pm$ SD for age (years), microalbuminuria (mg%), FBS (mg%) $HbA1_C$ (%) and FFA (mmol/L). Statistical analysis was done by using ANOVA, unpaired "t" test for quantitative data and Pearson's correlation tests. The SPSS software version 19 was used for ROC curve analysis, Tests of validity, viz.- sensitivity, specificity, positive predictive value, negative predictive value, and diagnostic accuracy of serum FFA to find optimum cut off value for severity of Diabetic nephropathy.

RESULTS

The data given in Table 1 shows mean $\pm$ SD of age, microalbuminuria, FBS, $HbA1_C$ and ANOVA f value.

Table 1: Demographic characteristics of cases and controls

	Controls (Mean ± SD)	Cases (Mean ± SD)			ANOVA	
		Stage-I	Stage-II	Stage- III	F value	p value
Age (in years)	51.54 ± 8.79	53.32 ± 8.61	53.93± 8.02	58.85± 5.06	2.378	0.079
Microalbuminuria (mg/dl)	23.31 ± 5.17	34.41 ± 5.92	137.93 ±54.55	118.28 ± 11.67	90.298	**0.001**
FBS (mg%)	98 ±5.89	120.53± 15.64	152.81 ± 18.54	163.96 ±18.65	76.394	**0.001**
HbA1$_C$ (%)	5.09 ± 0.48	6.92 ± 0.68	6.79 ± 0.52	8.32 ± 0.60	97.933	**0.001**

The data given in Table 1 doesn't show any statistical significance for age (p=0.079). Microalbuminuria, FBS, HbA1$_C$ were found greater in all the stages of DN as compared to healthy controls and it was found to be highly significant (p=0.001)

Table 2 shows serum FFA (mmol/L) in control and all the stages of DN, suggests highly significant (p=0.001) when compared to healthy controls.

Table 2: Serum free fatty acid in cases and controls

	Controls (Mean ± SD)	Cases (Mean ± SD)			ANOVA	p value
		Stage-I	Stage- II	Stage-III	F value	
SerumFFA (mmol/L)	0.59 ± 0.27	6.16 ± 1.85	6.68 ± 1.75	7.10 ± 1.40	99.631	**0.001**

The best cut off value for serum FFA (4.75mmol/L) was obtained from ROC curve given in figure 2 and the sensitivity, specificity, positive predictive value, negative predictive value, diagnostic accuracy and AUC of serum FFA is given in Table 3.

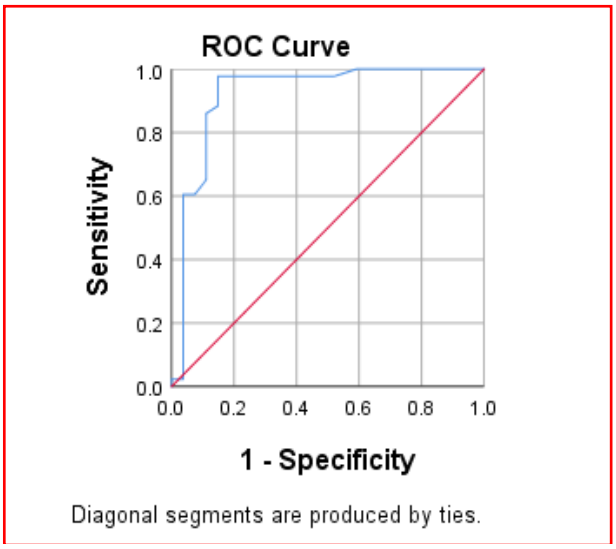

Fig. 2: The best cutoff value of serum free fatty acids for diabetic nephropathy by ROC curve.

Table 3: Sensitivity, Specificity, Positive Predictive Value, Negative Predictive Value and Diagnostic Accuracy of serum free fatty acids in diabetic nephropathy stages

Serum free fatty acid (mmol/L)	
Sensitivity	89%
Specificity	86%
PPV	80%
NPV	92%
Diagnostic Accuracy	87%
AUC	92%

DISCUSSION

Triglycerides on hydrolysis produce FFA. Many biological processes require FFA as important intermediary metabolite. Free Fatty acids act as important key component of glycolipid and phospholipid in cell structure and function. Energy for cell is provided by fatty acids in between the meals and during starvation. Fatty acid metabolism, when abnormal, leads to conditions like hyperthyroidism, obesity, severe liver dysfunction, insulin resistance and Type 2 DM. The serum lipid and lipoprotein abnormality occur in nephrotic syndrome due to impaired clearance and biosynthetic alterations (16).

In our study, the statistical difference in age group was not found to be significant between cases and healthy controls (p = 0.079), whereas the cases with diabetic nephropathy showed higher levels of microalbuminuria, FBS, HbA1c and serum FFA, when compared to healthy controls (p=0.001) was found to be highly significant.

In previous study carried out by Xin *et al.*, the process of glucose induced insulin secretion by free fatty acid is explained. Free fatty acid levels when elevated, offset of insulin resistance compensates for acutely elevated insulin secretion. So, function of insulin is not only reducing blood sugar but also inhibit breakdown of fat and promotes fat synthesis (16).

In our study there were low levels of serum free fatty acids in healthy controls as compared to DN subjects, the study with similar findings carried out by Zhang *et al.*, showed association of increased levels of FFA and proteinuria that increases the risk for kidney

damage, inflammation, oxidative stress, activated RAS and impairs insulin signal transduction. The effects of nitric oxide synthesis and endothelial programmed cell death are some mechanisms, affecting endothelial dysfunction carried due to increased accumulation of free fatty acids causing renal injury (17).

Xin *et al.,* reported that diabetic person with microalbuminuria had significant increase in fasting blood glucose levels and AGE's. Microalbuminuria is associated with progression end stage renal disease and CVD indicating early clinical marker for diabetic nephropathy (16). In a study by Ninomiya *et al.,* patients with advanced diabetic nephropathy (macroalbuminuric diabetic patients) had higher significant value of serum FFA than fasting blood glucose (18).

In our study we find that the best cut off value of serum FFA (4.75 mmol/L) for severity of diabetic nephropathy. The sensitivity, specificity and diagnostic accuracy was found to be at par with the study reported by Zhang *et al.,* (17). Further studies need to be done with larger sample size for better understanding the role of FFA in early diagnosis of diabetic nephropathy.

CONCLUSION

Serum FFA was found to be increased in all the stages of diabetic nephropathy. Hence, it can be used as an early diagnostic marker to prevent the severity of diabetic nephropathy with the cut off value of 4.75 mmol/L.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Frontiers in Health Informatics
ISSN-Online: 2676-7104
www.healthinformaticsjournal.com
2024; Vol 13: Issue 3
Open Access

Assessment Of Serum Fetuin-A Levels In Diabetic Nephropathy. A Case Control Study.

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Cite this paper as: Deepali S. Malgavkar, Nilima Dongre, S M. Goornavar, J G. Ambekar, Sanjeev Ratna, Manjula R. (2024) Assessment Of Serum Fetuin-A Levels In Diabetic Nephropathy. A Case Control Study.*Frontiers in Health Informatics, 13* (3), 10358-10367

Abstract

Introduction: *Diabetic Nephropathy (DN) is a most common vascular complication of Type 2 diabetes. Fetuin-A, after binding with Tyrosine kinase, acts as endogenous inhibitor of insulin receptor in diabetic subjects, causing insulin resistance (IR).*

Aim and objective: *This study was aimed to estimate the serum concentration of fetuin-A levels in the subjects having diabetic nephropathy and healthy controls. To determine cut off value of fetuin-A levels to prevent severity of renal damage in Diabetic Nephropathy subjects.*

Methods: *30 subjects having diabetic nephropathy and 30 healthy controls (age group 35 to 65 years) were selected from the medicine OPD of S. N. Medical College and HSK Hospital, Bagalkot. Based on the presence of microalbuminuria and eGFR levels, 30 subjects of nephropathy secondary to diabetes were selected. Cases having other systemic illnesses like hypertension, thyroid disorders and cardiac disorders were excluded from the study. The serum fetuin-A levels were estimated by the*

ELISA method in the cases and controls. The statistical analysis was done using SPSS software version 19, utilizing unpaired "t" test and Pearson's correlation tests for quantitative data.

Results: *The estimated serum fetuin-A levels in the cases were found to be higher as compared with control subjects and were found to be highly significant (p=0.001). We found the best cut-off value of serum fetuin-A was ≤ 36.66 ng/ml in the subjects having diabetic nephropathy. The area under the curve (AUC) was 0.515 with a specificity of 75 % and sensitivity of 41%.*

Conclusion: *Serum fetuin-A levels were higher in cases compared to controls, which could be used as a diagnostic marker for the severity of renal damage with a cut-off value of ≤ 36.66 ng/ml and could be used to control the nephropathy secondary to the patients suffering with diabetes.*

Categories: *Diabetes*

Keywords: *Type 2 diabetes Mellitus (T2DM), diabetic nephropathy (DN), Fetuin-A-cut-off value, microalbuminuria (MA), eGFR.*

Introduction

The most common cause of End-Stage Renal Disease (ESRD) is diabetic nephropathy (DN) which has become a major global predictor of mortality and morbidity in patients with diabetes [1,2]. The severity of renal damage along with cardio vascular disease linked to type 2 Diabetes mellitus (T2DM) can be reduced by early detection of patients at raised risk for microalbuminuria (MA) and by its treatment. As a renal indicator of widespread vascular endothelial damage, increased MA might be a useful early marker of atherosclerosis and cardiovascular death. Therefore, more precise and targeted biomarkers are required to detect MA early for enhance clinical care and enable timely intervention to prevent further complications. Diabetic kidney disease (DKD) is a syndrome characterized by a gradual rise in the excretion of albumin in urine linked to glomerular lesions, which ultimately results in the loss of glomerular filtration and ESRD. About 20-40% of diabetic patients have DKD which is a major global cause of ESRD [3,4]. Despite being just one component of renal excretory function, GFR is considered the best overall indicator of kidney function in chronic kidney disease (CKD) due to its tendency to decrease with extensive renal structural damage [5].

Fetuin-A, AHSG (α-2 Heremans schmid glycoprotein), has been a multifunctional glycoprotein which causes insulin resistance (IR). Fetuin-A after binding with tyrosine kinase, functions as main hepatokine and endogenous inhibitor of the insulin receptor in the skeletal muscle and liver, causing insulin resistance [6].

The human fetuin-A gene is situated on the chromosome 3q27, which also co-exists genetic susceptibility loci for T2DM as well as metabolic syndrome. Fetuin-A, prevents phosphorylation of insulin receptors in the muscle and liver, lowers insulin signaling and IR. Consequently, elevated levels of fetuin-A have been linked to the onset of T2DM and IR. Insulin resistance is a pathological mechanism of T2DM that may contribute to its onset and consequences [7,8].

Many research workers have depicted that fetuin-A levels influence the severity of diseases, such as diabetes and its consequences. Therefore, the current study was planned to investigate the relationship between microvascular issues like diabetic nephropathy and fetuin-A levels in individuals having T2DM.

Materials and Methods

Patient selection

The current study was carried out at the Medicine and Biochemistry Department of S N Medical College and HSK Hospital and Research Centre, a tertiary care hospital in Bagalkot, Karnataka, India. An institutional ethical committee approvals and certificates were obtained (SNMC/IECHSR/2020/A-71/1.1) & (BLDE(DU)/IEC/399/2020) from S. N. Medical College and BLDE(Deemed to be University), respectively. Before collecting blood samples, the study's purpose and procedures were thoroughly explained to the patients and their families, ensuring informed and written consent, taken from all the participants of the study, conducted in between January - October 2022.

Inclusion Criteria

Type 2 diabetic nephropathy patients characterized by the presence of microalbuminuria and eGFR, within the age group of 35-65 years, attending medicine OPD, were included in the study. Subjects in the same age group without diabetes were referred to as healthy controls.

Exclusion Criteria

Patients having other systemic diseases such as hypothyroidism or hyperthyroidism, cardiovascular diseases, pregnancy, malignancy and systemic drug or alcohol abuse were excluded from the study.

Sample Size calculation

In the present study, sample size was calculated to 60 (30 cases and 30 controls) using the study by Karajibani et. al [9]. The correlation coefficient of serum fetuin-A with serum creatinine was r= -0.61, the sample size calculated using the formula, $N= ([Z\alpha+Z_\beta]/C) + 3$. Where $C = 0.5^* \ln ([1+ r] /[1-r])$. At 99% confidence level $\alpha= Z\alpha = 2.54$, 90% power of study $\beta = Z_\beta = 1.64$.The sample size calculated was = 31, which is round off to 30.

Clinical sample collection

5ml Fasting blood sample was collected under aseptic precautions after overnight fast, out of which 1ml was transferred in EDTA containing vacutainers for HbA1c and 1ml was transferred in fluoride containing vacutainers for fasting blood sugar estimation, 3ml in plain vacutainers for estimation of other biochemical parameters. 2ml blood sample was collected, 2 hours after the meals, in fluoride containing vacutainers under aseptic precautions for PPBS estimation. After centrifugation of all the

vacutainers, at 3000 rpm for 20 minutes, plasma/serum was obtained. The separated serum was used to estimate fetuin-A levels and other biochemical parameters.

At the same time, 10 ml of urine sample was collected after overnight fast from the same patients in a sterile container and estimated for microalbumin within 2 hours. Biosystem kits [10,11] were used to estimate serum glucose, urea, creatinine, uric acid and insulin by auto analyzer BA 400 Biosystem. The serum fetuin-A level was determined using the ELISA method (Robotic) using Bioassay Technologies kits.

Statistical Analysis

The data was analyzed using the mean ± standard deviation for age (years), Microalbumin (mg/dl), FBS (mg/dl), PPBS (mg/dl), Insulin (µIU/l), HOMA-IR, Urea(mg/dl), Creatinine (mg/dl), eGFR (ml/min/1.73m^2), Uric acid (mg/dl) and Fetuin-A (ng/ml). The ANOVA, the unpaired "t" test and Pearson's correlation tests were used for quantitative data and statistical analysis. The SPSS software version 19 was used for Receiver operating curve (ROC curve) analysis. The validity tests such as sensitivity, specificity and diagnostic accuracy of serum fetuin-A were used to determine the best cut-off value for assessing the severity of diabetic nephropathy.

Results

This study included 60 participants classified into two groups. Group 1: Age and sex matched healthy controls. Group 2: Diabetic nephropathy Subjects. Demographic data of studied group is shown in Table 1, which is not statistically significant.

Table 1: Demographic data of study groups.

	Group 1 (n=30) **Mean ± SD**	**Group 2 (n=30)** **Mean ± SD**	**p Value**
Age (years)	51.34 ± 8.73	53.41 ± 10.17	0.176

Table 2: Comparison of glycemic status & insulin levels between the study groups

Parameters	**Healthy controls (n=30)** **Mean ±SD**	**DN Cases (n=30)** **Mean ± SD**	**t value**	**p value**

FBS (mg/dl)	101.68± 6.03	114.60 ± 31.03	3.086	0.003*
PPBS (mg/dl)	124.76 ± 9.20	165.55 ± 39.02	-5.478	0.001**
HbA1c (%)	5.31± 0.45	8.02 ± 0.58	-19.479	0.000**
Insulin (µIU/ml)	9.64 ± 1.63	27.62 ± 1.41	2.187	0.001*
HOMA-IR	2.39 ± 0.36	11.1 ± 1.27	3.164	0.001*

FBS, PPBS, HbA1c, Insulin and HOMA-IR were significantly increased in DN cases when compared to healthy controls which is highly significant ($p<0.001$).

Table 3: Serum fetuin-A levels in DN cases and healthy controls

Parameters	Healthy controls Mean ± SD	DN Cases Mean ± SD	t value	p Value
Fetuin–A (ng/ml)	38.66 ± 4.77	85.72 ± 5.93	-33.267	0.001**

Fetuin A level was significantly elevated in diabetic nephropathy cases compared to healthy controls.

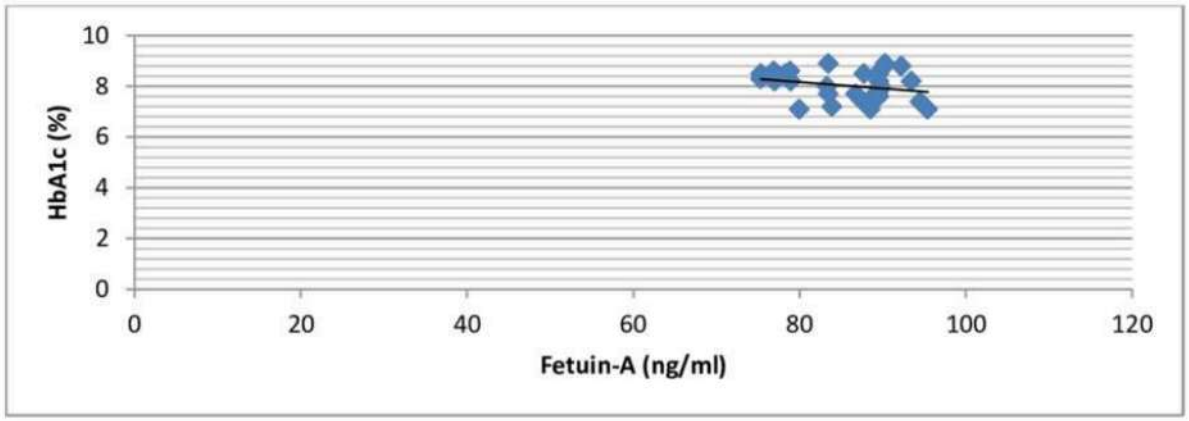
Table 4: Shows comparison between renal parameters and fetuin-A.

Parameters	Healthy controls (n=30) Mean ±SD	DN Cases (n=30) Mean ± SD	t value	p value
Urea (mg/dl)	47.13 ± 10.61	38.11 ± 8.26	3.612	0.001*
Creatinine (mg/dl)	0.74 ± 0.11	1.39 ± 0.29	-11.036	0.000**
eGFR (ml/min/1.73m²)	100.48 ± 10.59	50.93 ± 7.74	20.336	0.000**
Uric acid (mg/dl)	4.90 ± 1.21	4.33 ± 1.32	1.703	0.094

Microalbumin (mg/dl)	24.41 ± 4.73	136.94 ± 52.98	-11.393	0.000**

*significant, ** highly sign

Figure 1: Correlation between serum fetuin-A (ng/ml) and HbA1c (%).



Positive correlation was seen between the HbA1c and serum fetuin-A levels in cases.

Figure 2: Correlation between serum fetuin-A (ng/ml) and microalbumin (mg/dl)

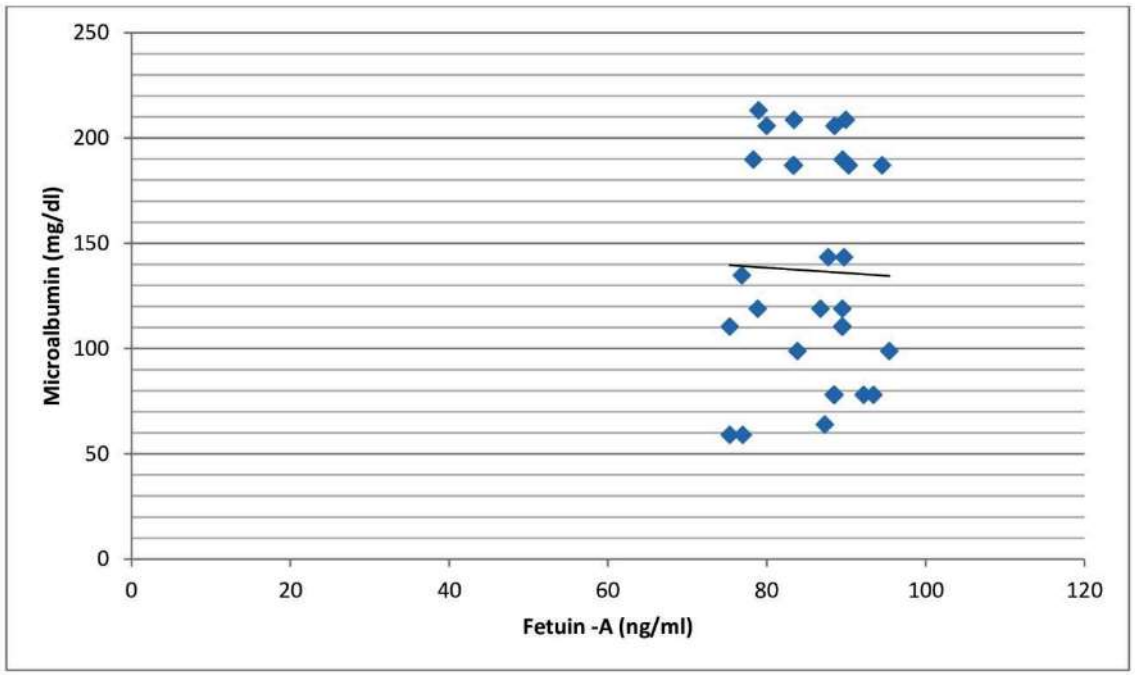
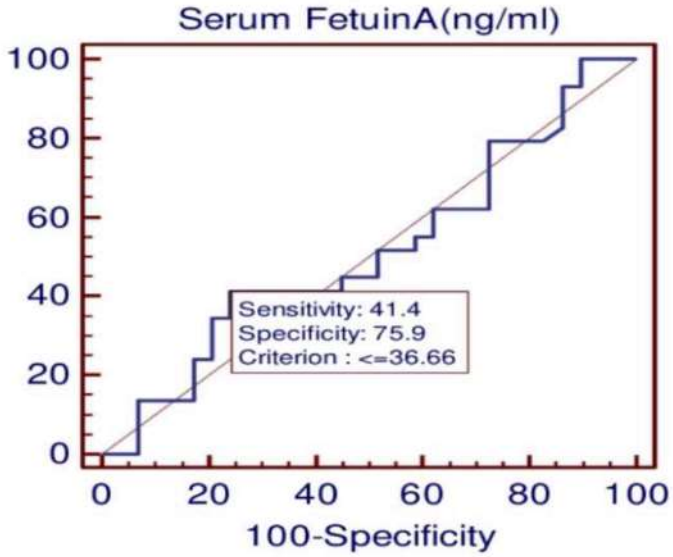


Figure 3: ROC curve analysis for fetuin-



ROC curve analysis for fetuin-A levels showed AUC= 0.515 ($p<0.001$), sensitivity 41.4 % and specificity 75.9% in diabetic nephropathy cases. With this curve fetuin-A cut off value was found to be ≤ 36.66 ng/ml in diabetic nephropathy cases.

Discussion

The most frequent consequences of diabetes that causes renal failure and other issues, in adults, is diabetic nephropathy. Early detection and prevention can decrease the severity of the complications caused by T2DM. The objective of the study was to ascertain fetuin-A levels as a preliminary diagnostic indicator for severity of diabetic nephropathy.

The primary discovery of our research was a noteworthy distinction in fetuin-A concentrations between the DN cases and healthy controls. Fetuin-A has been observed to have a highly significant positive association with FBS, PPBS, HbA1c, Insulin and HOMA-IR.

These results are in accordance with the research conducted by Atef Farouk et al. who also reported increased fetuin-A levels before hemodialysis and after hemodialysis [12].

Nonetheless, our analysis revealed a statistically significant distinction between the levels of fetuin-A and microalbumin. El-Batch et al. found that patients having microalbuminuria had significantly higher serum fetuin-A levels than both the normoalbuminuria patients and the control group [13]. The connection between fetuin-A and aberrant albuminuria may be explained by the function it plays in mediating IR, abnormalities of lipid profile and dysfunction of endothelium. According to Mitkees et al., diabetic patients with microalbuminuria had significantly higher serum fetuin-A levels than those in the control group. This may be because the healthy control group was less likely to experience vascular complications from fetuin-A than that of diabetic patients [14].

Al-Said et al.'s research supports our findings, demonstrating that the group of diabetics with nephropathy had significantly higher mean levels of fetuin-A, HOMA-IR, and insulin than the other groups. There has been a strong positive correlation observed among fetuin-A and renal parameters [15], which also supports our findings. The mechanism that fetuin-A plays a role in the pathophysiology of T2DM was supported by the outcomes of the study conducted by Ahn M B et al. In this study, which led to IR, they demonstrated elevated levels of fetuin-A in obese adolescents with the T2DM [16]. A conflict of results was observed by Sherif et al., who discovered that low levels of fetuin-A appeared to be linked to common micro and macrovascular problems in T2DM [17].

The study limitation is measurement of fetuin-A levels at a single point in time, which may not be accurately reflect its long-term levels. Furthermore, the absence of crucial data regarding the family history of T2DM introduces the possibility of residual confounding factors. Further studies correlating fetuin-A, with the severity of DN and other complications of the disease, are required with the larger sample size.

Conclusion

In diabetic patients with nephropathy, elevated fetuin-A levels may cause endothelial dysfunction and microvascular complications. Fetuin-A may therefore can be used as predictor for the diagnosis and management of diabetic nephropathy. Additional research is necessary to clarify the role of fetuin-A in T2DM using a larger sample size and at various stages of diabetic nephropathy

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