Ser312 FETUIN-A PHOSPHORYLATION AND ITS ASSOCIATION

WITH SERUM LIPIDS IN METABOLIC SYNDROME

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Ser312 FETUIN-A PHOSPHORYLATION AND ITS ASSOCIATION WITH SERUM LIPIDS IN METABOLIC SYNDROME

Shalini Kaushik

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WITH SERUM LIPIDS IN METABOLIC SYNDROME

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Shalini Kaushik daughter of Vijai Bhushan & Indu Kaushik was born in Meerut, UP, India on July 15, 1977. She completed Masters in Food Science & Nutrition from Nagpur University, Maharashtra, India in the year 2000. She completed a total of seven and half months training as Dietitian at renowned hospitals in India, including All India Institute of Medical Sciences (AIIMS) and APOLLO, New Delhi, India. She served as a Dietitian at one of the prominent cardiac clinic at Nagpur, India. She entered Auburn University's Graduate School in Fall 2006, and pursued a Master of Science Degree in Nutrition in the Department of Nutrition and Food Science under the direction of Dr. Suresh T. Mathews.

THESIS ABSTRACT

Ser312 FETUIN-A PHOSPHORYLATION AND ITS ASSOCIATION

WITH LIPID LEVEL IN METABOLIC SYNDROME

Shalini Kaushik

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Fetuin-A (also known as alpha 2-HS-glycoprotein), secreted by the liver into circulation, interacts with the insulin receptor, and is a physiological inhibitor of insulin receptor tyrosine kinase activity *in vitro*, in intact cells and *in vivo* in animals. Serum fetuin-A levels have been shown to be associated with insulin resistance, obesity, and metabolic syndrome in animals and humans. Fetuin-A null mice demonstrate improved insulin sensitivity and resistance to diet-induced obesity. Phosphorylation status of fetuin-A has been shown to be critical for its inhibitory activity on insulin action. However, there are no reports on fetuin-A phosphorylation status in insulin resistant conditions or on the molecular characterization of two phosphorylation sites (Ser120 and Ser312). This study examines serum total fetuin-A and Ser312-fetuin-A phosphorylation status in individuals with metabolic syndrome, both before and after treatment with the lipid-lowering drug, Niaspan, and correlates these with changes in serum lipids and markers of

insulin sensitivity and inflammation. Additionally, using mutation analysis, we have examined the role of phosphorylation on Ser312-fetuin-A on insulin action. Fifteen sedentary, male participants, who met the NCEP ATP III criteria for metabolic syndrome, were treated with extended-release niacin (Niaspan) for 6 weeks. Serum concentrations of phosphorylated (Ser312) fetuin-A were positively correlated with triglycerides in MetS. Serum total fetuin-A and phosphofetuin-A concentrations decreased significantly following Niaspan treatment. Changes in fetuin-A concentrations were correlated with changes in triglyceride concentrations after Niaspan treatment. Additionally, the change in high density lipoprotein cholesterol following Niaspan intervention was negatively correlated with the change in serum fetuin-A and phosphorylated fetuin-A concentrations. Interestingly, serum cortisol levels were significantly elevated following Niaspan intervention. The change in cortisol was significantly correlated with the change in serum non-esterified fatty acids (NEFA), suggesting that increased cortisol and NEFA concentrations may contribute to the mild hyperglycemia and insulin resistance observed in niacin-treated individuals. Molecular characterization using Ser312Ala-fetuin-A mutant, which was devoid of phosphorylation, suggested that phosphorylation on Ser312-fetuin-A is critical for fetuin-A's inhibition of insulin signaling through MAPK and Akt. This is the first report demonstrating that serum total- and phosphorylated Ser312-fetuin-A levels are amenable to intervention by Niaspan treatment and are correlated with changes in serum lipids. We demonstrate that while phosphorylation on Ser312 site of fetuin-A is critical, phosphorylation on Ser120 may be required for fetuin-A's full inhibitory activity.

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CHAPTER 1: INTRODUCTION

Western lifestyle has led to a substantial increase in the incidence of obesity and its associated metabolic complications. “Obesity” specifically refers to an excess amount of body fat with a BMI of 30 or higher. Health risks linked to obesity include type 2 diabetes, heart disease, high blood pressure, and stroke.

Obesity and type 2 diabetes represent a serious threat to the health of the population of almost every country in the world. The World Health Organization projects that globally approximately 400 million adults were obese in 2005 (1). Nearly 385,000 deaths each year may be attributed to obesity related disorders (2). According to the World Health Organization and United Nations, there were approximately 171 million people with type 2 diabetes mellitus in 2005, and this number is predicted to increase to 366 million by 2030 (3). This escalation is due in part to increasing rates of childhood obesity. Until recently, type 2 diabetes was a disease that afflicted only adults. Now, a growing number of children are being diagnosed with obesity-related type 2 diabetes.

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (4)

Type 2 diabetes is sometimes defined as a form of diabetes that develops when the body does not respond properly to insulin, a condition referred to as insulin resistance. At first, the pancreas keeps up with the added demand by producing more insulin. In time, however, it loses the ability to secrete enough insulin in response to meals. In type 1 diabetes, the pancreatic β cells produce little or no insulin.

Parallel to the rise in obesity is the increasing incidence of 'metabolic syndrome' (MetS), also known as syndrome X, the insulin resistance syndrome, and the deadly quartet (5-7). MetS is a complex condition that is characterized by a cluster of closely related clinical features linked to obesity, including insulin resistance, dyslipidaemia and hypertension. Using data from NHANES IV, the age-adjusted prevalence of metabolic syndrome in Americans is 27% (8). Metabolic syndrome is associated with an increased risk of cardiovascular disease, which is ultimately responsible for a considerable proportion of diabetic mortality. The most accepted and unifying hypothesis to describe the pathophysiology of the metabolic syndrome is insulin resistance. Various physiological regulators of insulin action, including tumar necrosis factor-alpha (TNF-α) (9) leptin (10), resistin (11), adiponectin (12), free fatty acids (13, 14, 15), and protein tyrosine phosphatase 1B (16, 17) have been identified. Alterations in serum concentrations of these humoral factors have been shown to influence whole body insulin sensitivity and insulin action, suggesting a potential role for these molecules in the development of insulin resistance and diabetes.

Fetuin-A, a liver secreted glycoprotein, is a novel humoral factor that has been implicated in the modulation of insulin sensitivity (18-20). Fetuin-A has been shown to influence a wide variety of biological processes, including insulin and growth factor signaling (21-24), lipid transport (25, 26), opsonization (27, 28), and tissue mineralization (23, 31-35). The distribution and expression of fetuin-A has been shown to have a wide-spread intracellular presence in many developing tissues including the central nervous system. It has been studied in the developing immune and hemopoietic organs of fetal and adult sheep (29). In adults, fetuin-A is expressed and secreted predominantly by the liver.

Among the earliest described biological effect of fetuin-A was the inhibition of insulin receptor tyrosine kinase activity (IR-TKA) (21, 22, 36-41). Fetuin-A's inhibition of the proximal steps of insulin signal transduction (IR autophosphorylation and TK activity) leads to inhibition of Ras-Raf-MAPK arm of the insulin signaling pathway (39). Additionally, data from several recent publications, including data from fetuin-A null mice (20, 40), and recent data generated from our laboratory indicate that fetuin-A may regulate glucose uptake and glycogen synthesis in skeletal muscle and adipose tissue. Additionally, several recent findings indicate that serum fetuin-A concentrations are strongly associated with insulin resistance. Using euglycemic-hyperinsulinemic clamp studies, Stefan *et al* demonstrated that serum fetuin-A levels are correlated with whole-body insulin resistance and liver fat (18). Studies by Mori *et al* and the landmark Heart and Soul study indicate that fetuin-A is associated with insulin resistance, metabolic syndrome, and an atherogenic lipid profile (23). Recently, Haglund *et al* (41) have shown

that circulating human plasma fetuin is ~20% phosphorylated, implying that the effects of phosphorylated fetuin-A glycoprotein on insulin signal transduction seen in different cell systems could be relevant to its physiological function *in vivo*. These authors have demonstrated that human fetuin-A is phosphorylated on serine120 located in the A-chain and on serine312 located in the connecting peptide. These phosphorylation sites are conserved in rats, sheep, pig and mouse bovine forms of fetuin-A (36, 45-49). Studies from several investigators show that phosphorylation of fetuin-A is required for it to be physiologically active, because the dephosphorylated fetuin-A was devoid of any inhibitory effects on insulin receptor tyrosine kinase activity (41-44). However, there are no studies examining fetuin-A phosphorylation status in insulin resistant conditions or characterizing the role of specific phosphorylation sites on fetuin-A.

The overall goal of this research was to examine the role of fetuin-A phosphorylation on insulin action using a combination of clinical, cellular and molecular analysis. Our first objective was to examine the association of serum fetuin-A and phosphorylated fetuin-A levels with serum lipids, markers of inflammation, and insulin sensitivity, in individuals with metabolic syndrome. Our second goal was to analyze whether fetuin-A and phosphorylated fetuin-A concentrations were amenable to therapeutic intervention by extended-duration niacin, one of the most effective agents currently available for clinical use for decreasing serum triglyceride and increasing levels of HDL-cholesterol. Finally, using mutational analyses, we sought to evaluate the role of Ser312 phosphorylation on fetuin-A's regulation of insulin action.

CHAPTER 2: REVIEW OF LITERATURE

"*As the food so the mind, as the mind so the man*"

- Bhagvad Geeta

Everyone loves to eat and doing so we may be eating ourselves into the twin-epidemics of obesity and diabetes. Western lifestyle has considerably increased the occurrence of obesity and its related metabolic problems, including insulin resistance and diabetes. Obesity and type 2 diabetes signify a severe threat to the health of the population of almost every country in the world. According to the International Diabetes Federation (IDF), diabetes is expected to cause 3.8 million deaths worldwide in 2007, roughly 6% of total world mortality. Today, there are almost 246 million people with diabetes worldwide. Within 20 years, this number is expected to rise to 380 million (50). Data from surveys conducted by National Health and Nutrition Examination Survey (NHANES) in the year 2003-2004 show the increase in the prevalence of obesity from 15.0% to 32.9% among adults aged 20–74 years (51).

2.1 Obesity

Obesity is the most common and important risk factor for the development of type 2 diabetes mellitus (T2DM). According to the Centers for Disease Control (CDC),

obesity is the increase in body weight due to increased accumulation of adipose tissue (body fat) relative to body mass (52). A body mass index (BMI) of 31 results in a 40-fold increase in the risk of T2DM, whereas a BMI greater than 35 leads to a 90-fold increase in the risk for T2DM in contrast with a BMI of 22 (53). When chronic energy intake exceeds energy expenditure, the mass of the white adipose tissue, that represents the vast majority of adipose tissue, expands, which is the primary cause for obesity (54). Obesity leads to the reduction in the sensitivity to the biological actions of insulin, a pathophysiological state known as insulin resistance (55). According to the NHANES data, obesity is the most significant risk factor for the development of insulin resistance among children (56, 57). Moreover, obesity is most commonly associated with low-grade systemic inflammation, characterized by elevated levels of inflammatory markers in blood, including C-reactive protein (CRP), interlukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α) (58-61). The degree of adiposity and BMI also strongly affects the serum level of many adipokines, which indicates that the synthesis and secretion of these signaling molecules is dynamic and modifiable. This suggests that the risk for diseases such as type 2 diabetes and cardiovascular disease in the obese is increased by the altered secretion of adipokines, and in particular those that affect systemic insulin sensitivity and inflammation (61-65). Primarily, as a consequence of the dysfunction of adipose tissue that develops with obesity, imbalances occur in the secretion of proinflammatory/pro-diabetic and anti-inflammatory/anti-diabetic adipokines that change systemic energy homeostasis and vascular function, which may lead to the development of insulin resistance, type 2 diabetes and cardiovascular diseases.

2.2 Diabetes:

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (4). The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels. Patients with type 2 diabetes have normal or even elevated levels of insulin in their blood, and normal insulin receptors, but due to faulty binding of insulin to their cell receptors, impaired transduction of the intracellular signaling, and/or an altered adipokine/inflammatory marker profile, they are unable to regulate normal glucose homeostasis (66). Type 1 diabetes, usually diagnosed in childhood, is a disorder of the endocrine system that is characterized by absolute lack of insulin production due to autoimmune destruction of the pancreatic ß cell. Features of both type 1 and type 2 are also seen in some patients and thus their disease is diagnosed as type 1.5 diabetes mellitus (67).

Gestational diabetes mellitus (GDM) is detected during pregnancy and is characterized by glucose intolerance of various degrees. There is strong evidence to support the fact that persons exposed to maternal diabetes *in utero* have an increased risk of obesity as well as abnormal glucose tolerance, and metabolic syndrome as children and young adults (68).

2.3 Insulin Resistance:

Insulin resistance plays a critical role in the development of type 2 diabetes (69). Insulin resistance is a decrease in the ability of insulin to metabolize glucose, and it is characterized by glucose intolerance and hyperglycemia followed by an increase in plasma concentrations of insulin, dyslipidemia (elevated levels of triglycerides and diminished HDL cholesterol), elevation of blood pressure, abdominal obesity, and elevated tendency for thrombosis (70, 71)

Obesity, which is usually polygenetic and environmental in origin, is the most important factor associated with insulin resistance (72, 73). In insulin resistance there is excessive release of free fatty acids (FFA), due to increased resistance of adipose tissue to the antilipolytic effect of insulin. This is a trigger in the progress towards insulin resistance.

Additionally, various hormones and cytokines from adipose tissues (adipokines), genetic factors and environmental factors such as food intake, reduced physical activity, aging, smoking or administration of drugs, including thiazide diuretics, beta-adrenergic antagonists, and glucocorticoids, can also contribute to insulin resistance (72). On the contrary, restricted calorie intake, weight reduction, and increased physical activity has been shown to improve insulin sensitivity (72, 74, 75).

2.4 Insulin action and signal transduction

Insulin elicits a diverse role in biological responses such as maintaining glucose homeostasis and regulating carbohydrate, protein and lipid metabolism. Circulating glucose levels are tightly regulated by mechanisms involving a sophisticated and coordinated role of insulin in reducing hepatic glucose production and stimulating glucse transport into muscle and fat (76, 77). Recent evidence points to the role of the hypothalamus and brain in the regulation of blood glucose (78-80). Several studies have demonstrated alterations of receptor synthesis, degradation, and function through certain mutations in the insulin receptor gene in patients with severe insulin resistance (81). The insulin receptor is a heterotetrameric protein, containing two trans-membrane β-subunits and two α-subunits, which are extracellular. Insulin action begins with the binding of insulin to the α-subunit of its receptor which (Fig.1). This binding stimulates the tyrosine kinase activity which is intrinsic to the β-subunit of the receptor.

The first step of insulin receptor activation is the autophosphorylation of tyrosine residues which is permitted by the joint participation of two α-subunits in the insulin binding domain and by kinase domains in the two β-subunits situated at the juxtamembrane position (82-83). Insulin binding results in autophosphorylation of the tyrosine residues, and the kinase domain undergoes a conformational change, which acts as an origin for activation of the kinase and interaction of downstream molecule that participates in the insulin signaling cascade. This autophosphorylation is followed by phosphorylation of the insulin-receptor substrates (IRS) (84-85). IRS contain an NH2

terminal phosphotyrosine-binding domain, a COOH-terminal domain with tyrosine residues involved in Src homology 2 (SH2) protein-binding sites, a Src homology 3 (SH3) domain that bind proline-rich ligands and serine-threonine-rich regions that then bind to other proteins. Insulin receptor trans-phosphorylates several substrates on Tyr residues including IRS1-4, Shc, and Gab1, Cbl, APS, and p60dok. Each of these provide specific docking sites for other signaling proteins containing SH2 domains (86). These events lead to the activation of downstream signaling molecules, orchestrated through three main pathways: the IRS/phosphatidylinositol 3- (PI3) kinase pathway; (RAS)/mitogen-activated protein kinase (MAPK) pathway; and the Cbl-associated protein (CAP)/Cbl pathway. The PI3 kinase is a heterodimeric enzyme containing the p85 regulatory subunit and p110 catalytic subunit. Inositol phosphate [I(3)P], phosphatidylinositol bisphosphate [PI(3,4,5)P2] and phosphatidylinositol-trisphosphate (PIP3) are produced by phosphorylated PI substrates by activated PI3Kinase (phosphoinositide 3-kinases). These phospholipids act as second messengers and employ the PI3K- dependent serine/threonine kinases (PDK1) and Akt (protein-serine/threonine kinase) which translocates from the cytoplasm to the plasma membrane. Akt is phosphorylated on Thr 308 and Ser 473 by PDK-1, and a conformational change takes place in Akt through the lipid binding and membrane translocation (39, 87-88). The activated Akt then regulates and phosphorylates components of the glucose transporter 4 (GLUT4) complexes, protein kinase C (PKC) isoforms, glycogen synthase kinase (GSK3) and p70S6kinase (87-91). Thus activation of the PI3K-Akt pathway plays a critical role in glycogen synthesis by mediating the insulin induced phosphorylation and inhibition of glycogen synthase kinase 3(GSK-3) (92, 93). In muscle and adipose tissue,

glucose uptake is facilitated by insulin through the stimulation of GLUT4 from intracellular sites to the plasma membrane (Fig 1). Mild hyperglycemia, cardiac and adipose abnormalities and short lifespan have been demonstrated in whole body GLUT4 homozygous knockout mice (94). Moreover, disruption of GLUT4, in muscle, leads to glucose intolerance and insulin resistance which indicate that glucose transport mediated by GLUT4 is essential in maintaining muscle glucose homeostasis (95). Also disruption of GLUT4 in adipose tissue of mice results in secondary insulin resistance and impaired glucose tolerance, indicating that alteration in expression of GLUT 4 could lead to the insulin resistance and diabetes (96). Recently a new 160-kDa Akt substrate called AS160 has been identified in 3T3-L1 adipocytes that contain Rab GTP-activating protein (GAP) domain, which is linked to glucose transport (96).

The *second pathway*, signaling through Grb2/Sos/Ras/Raf/MAPK, leads to insulin's mitogenic effects including DNA synthesis, cell growth, and gene expression (97). In the liver, insulin represses the transcription of gluconeogenic genes phosphoenol pyruvate carboxykinase (PEPCK) (98) and glucose 6-phosphatase (G6Pase) (99-101).

In metabolically responsive cells, c-Cbl undergoes tyrosine phosphorylation by insulin, which constitutes the *third signaling pathway*. C-Cbl makes a complex with insulin receptor through an adaptor protein called C-Cbl-associated protein (CAP) (102). When Cbl is phosphorylated, the Cbl/CAP complex translocates to the plasma membrane domain enriched in lipid rafts (103,104,105).

Expression of dominant *negative* CAP completely blocks insulin stimulated glucose uptake and GLUT4 translocation (106). This suggests that Cbl/CAP complex and PI3 kinase/Akt pathway are two compartmentalized parallel pathways that lead to GLUT4 translocation.

INSULIN SIGNAL TRANSDUCTION

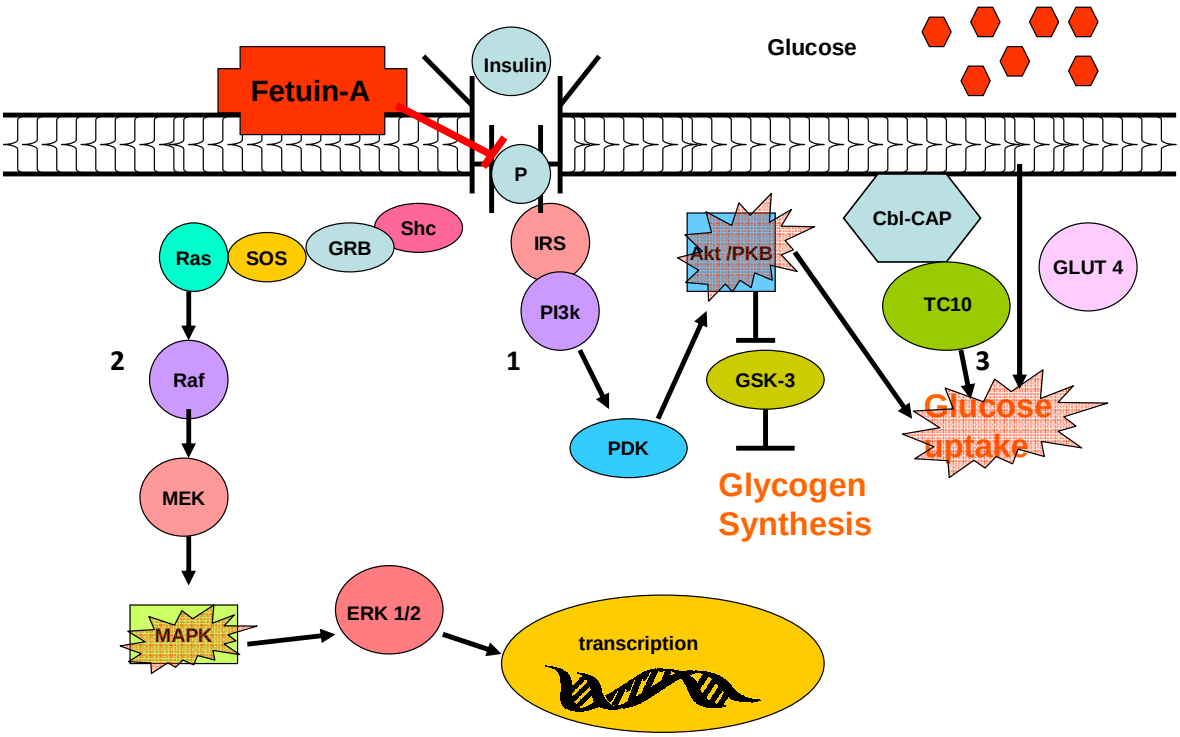

Fig. 1: Metabolic and mitogenic responses to insulin. *The binding of insulin to the α-subunit of insulin receptor (IR) concentrates insulin at its site of action and induces conformational changes in the receptor, which in turn stimulates the tyrosine activity intrinsic to the β-subunit of the IR and triggers the signaling cascades. Fetuin-A is shown here inhibiting the IR autophosphorylation and tyrosine kinase activity, thus repressing insulin-mediated events. 1, 2 and 3 refers to first, second and third signal pathways as described.*

2.5 Negative regulators of insulin signaling cascade:

Negative regulatory roles are played by certain humoral factors, metabolites, protein phosphatases, and other signaling proteins resulting in the mitigation of insulin's signal. These regulators, under pathological conditions, can impair insulin signaling leading to insulin resistance. Interleukin 1 (IL-1), IL-6 and TNF-α are pro-inflammatory cytokines implicated in the progression of obesity and insulin resistance (107). TNF-α impairs insulin-stimulated tyrosine phosphorylation of IRS molecules and decreases insulin signaling through phosphorylation of IRS-1 Ser307 and the stimulation of SOCS (suppressor of cytokine signaling) proteins (108).

Plasma free fatty acids (FFA) levels are elevated in both obesity and type 2 diabetes. Free fatty acids affect insulin action at the peripheral target tissues. FFA impairs glucose uptake into muscle, phosphorylation of glucose by glucose-6-phosphate and glycogen synthesis (109-110). It has also been proposed that the reduction in plasma free fatty acids levels observed with thiazolidinedione treatment (TZDs), a class of anti-diabetic drugs that are ligands for the nuclear receptor, peroxisome proliferator-activated receptor-gamma (PPARγ), may contribute to the improvement in insulin sensitivity (111, 112).

Protein tyrosine phosphatase-1B (PTP1B), which is ubiquitously expressed, has been shown to function as a negative regulator by dephosphorylating the insulin receptor both *in vitro* and also in intact cells and regulates both mitogenic and metabolic actions of insulin (113, 114). Additionally, elevated levels and activity of PTP1B in tissue culture

models has been shown to be associated with insulin resistance, stimulated by exposure to high glucose levels (17). Furthermore, a number of studies have revealed that SHP-2, a cytosolic PTPase, participates as a positive mediator of mitogenic action of insulin and other growth factors in Ras and mitogen activated protein kinase-dependent pathways (115, 116)

Fetuin-A, a liver-secreted humoral factor, has been identified as a physiological inhibitor of the insulin receptor autophosphorylation and tyrosine kinase activity (21, 22, 36-38). This proximal inhibition results in decreased insulin-stimulated activation of the Ras-Raf-MAPK pathway (38) and the PI3K-Akt-GSK3 pathway resulting in impaired DNA synthesis, glucose uptake, and glycogen synthesis (22). Altered serum fetuin-A concentrations have been reported in obesity, insulin resistance, and metabolic syndrome (18-19).

2.6 Fetuin-A

Fetuin-A, also called α2-Heremans-Schmid glycoprotein (AHSG) from the fetuin family of proteins, was first described simultaneously by Joseph Heremans and Karl Schmid. Fetuin A is a 49-kDa plasma protein that originates mainly in liver (36) and which is abundant in plasma and mineralized bone. Several functions have been described, including bone mineralization (23, 31-35) and osteogenesis (27-28), regulation of the insulin and hepatocyte growth factor receptors (21-24) and the response to inflammation (18, 19).

Fetuin-A in human plasma is one of the few negative acute-phase proteins. During an inflammatory response its level decreases drastically (117). Its concentration is also decreased in certain malignancies and Paget's disease (118). Phosphorylation has been shown to be critical for fetuin-A to inhibit IR-TK activity. Only 20% of the circulating fetuin-A pool is phosphorylated on Ser120 and Ser312 and considered biologically active. The rest (80%) is present in dephosphorylated form (41-44). In humans, the fetuin-A gene is localized on 3q27. This locus has been described as a a susceptibility locus for metabolic syndrome and type 2 diabetes mellitus by Kissebah *et al* (119) and Vionnet *et al* (120). Enhanced plasma level of fetuin-A has also been demonstrated clinically in pregnant women with insulin resistance and in gestational diabetes (121). Fetuin-A null mice demonstrate improved insulin sensitivity and resistance to weight gain. Additionally, these mice demonstrate improved basal and insulin stimulated phosphorylation of the insulin receptor and downstream signaling molecules in skeletal muscle and liver (20, 40).

2.6.1 Structure of fetuin-A

Human fetuin-A, secreted by the liver into circulation, is a glycoprotein consisting of two subunits, an A chain or heavy chain which is comprised of 282 amino acids and a B chain or light chain made up of 27 amino acids (Fig. 2). The A and B chain are connected via a 40 amino acid single interchain disulphide bridge called the 'connecting peptide'. The polypeptide moiety of B chain consists of 27 amino acid residues. Neutral and charged amino acid residues are distributed unequally (122-123).

The first 20 residues are uncharged; on the other hand, the carboxyl-terminal heptapeptide includes all charged residues. The carbohydrate units consist of sialic acid, galactose, and N-acetylgalactosamine O-glycosidically linked to the 6th serine residue. The B chain polypeptide has two β-turns. The second β-turn containing cystine residue links the B-chain to the A-chain. The B-chain lacks β-conformation but possesses a short α-helical region (124). The fetuin-A inserts in cDNA clones extend approximately 1.5 kilo base pairs and include the entire fetuin-A coding sequence for the A and B chains, indicating that both the chains are encoded by a single mRNA transcript (122). In a post translation step before mature fetuin-A is released into the circulation, the connecting sequence is cleaved by limited proteolysis (124-125).

Mammalian fetuins are characterized by a tripartite structure: two similar sequences of cystatin-like structures (‘cystatin domains’) comprising 116-118 amino acids that are positioned at the amino terminus of the proteins (Domains A and B, Fig.2). These are followed by a unique sequence of 110-112 residues, containing a proline-rich (Pro) region (126). Native fetuin-A undergoes a series of post-translational modifications including proteolytic processing, phosphorylation, multiple N-glycosylations and O-glycosylations, and sulfation of the carbohydrate side chains (127). At positions 81, 138 and 158 Asn-Xaa-Ser/Thr types of three potential N-glycosylation sites have been shown to be present in the mature sheep and pig fetuin molecules. Asparagine residues in bovine fetuin at these positions have been assigned as sites for N-glycosylation (49). The first of these (AS81) is absent in human a2-HS glycoprotein. The other two sites; AS138 and AS158 are conserved in all the fetuin and are known to be N-glycosylated in human

fetuin-A (123). Twelve half-cystine residues are present on fetuin-A, and 11 of them are positioned in the heavy chain and a single one in the light chain of the molecule; they form six disulfide bridges. The first and the last half-cystine residues of the amino acid sequence of fetuin-A are engaged in the formation of a loop spanning the extreme NH2 and COOH-terminal portions of the molecule, thereby connecting the heavy and light chains . The other half-cystine residues are linked consecutively in the heavy chain and form five loops which span 4-19 amino acid residues (128). Comparison of the deduced amino acid sequences of sheep and pig fetuin showed an extensive sequence identity between them (75%) and with other proteins of the mammalian fetuin family, i. e. human fetuin-A, and bovine and rat fetuin. Twelve cystine residues were found at invariant positions in all fetuin sequences, suggesting strongly that the arrangement of disulphide bridges identified in human fetuin-A is common to the members of the family (Fig. 3).

STRUCTURE OF HUMAN FETUIN-A

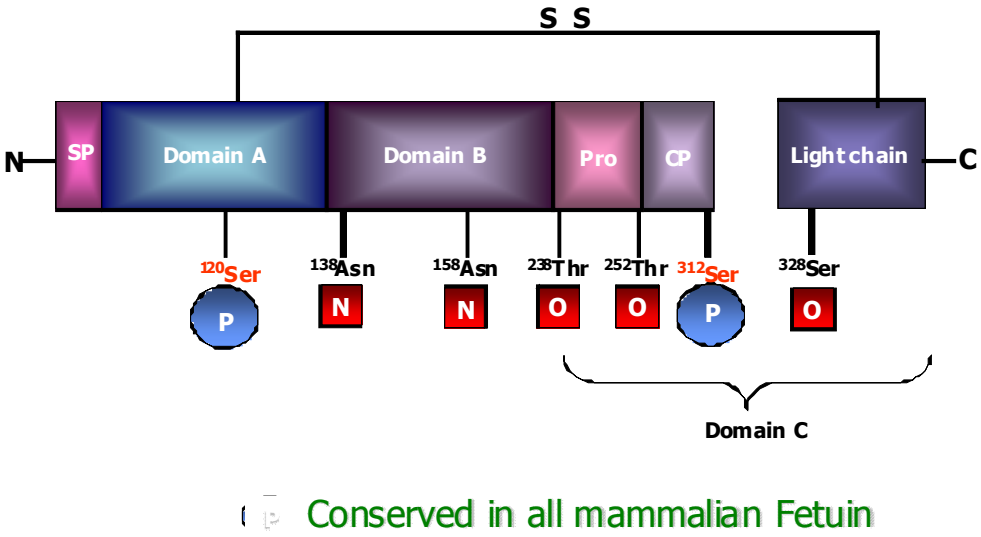

Fig.2: Fetuin-A structure*. Fetuin-A is comprised of two tandem cystatin-like domains A and B, and a small B-chain connected through a disulfide bond The A-chain, comprising the signal peptide (SP), domains A and B, a proline rich segment (Pro) and 39 of the 40 amino acids of the connecting peptide (CP), is linked to the B-chain (light chain) by a single disulfide bond (S-S). The conserved N- and O-linked glycosylation and phosphorylation sites are represented by N, O, and P, respectively.*

CONSERVED PHOSPHORYLATION SITES ON FETUIN-A

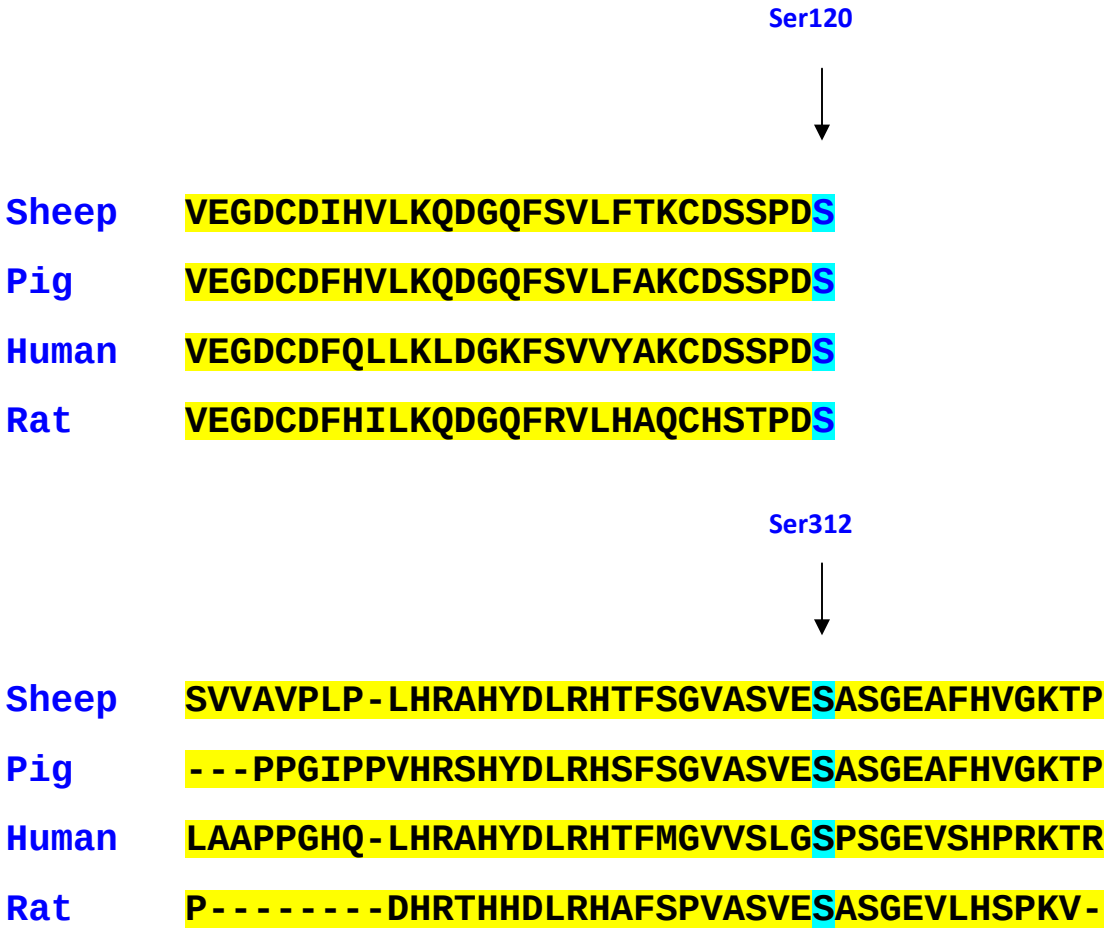

Fig. 3: Conserved serine residues. *Haglund et al (41) have identified that human fetuin-A is phosphorylated on Ser120 and Ser312. The sequence alignment of sheep, pig, human and rat are shown. Conserved Ser120 and Ser312 residues are indicated in blue and by arrows.*

2.6.2 Fetuin-A: An inhibitor of insulin receptor tyrosine kinase activity

Human fetuin-A is reported to be a natural inhibitor of the insulin-stimulated insulin receptor tyrosine kinase (21, 22, 36-38). Fetuin-A does not require the proximal 576 amino acids of the alpha subunit of IR and primarily interacts with the activated IR, and inhibits IR autophophorylation and IR tyrosine kinase activity (21). Fetuin-A also inhibits the association of IRS-1 with the p85 subunit of phosphatidylinositol-3 kinase in H-35 hepatoma cells. Fetuin-A does not affect insulin-stimulated induction of the metabolic enzyme tyrosine amino transferase but it inhibits insulin-dependent mitogenesis (21). Srinivas *et al* have shown complete inhibition of insulin receptor autophosphorylation, its tyrosine kinase activity towards an exogenous substrate Glu80Tyr20, tyrosine phosphorylation of IRS1, Shc (p52 kDa), and insulin-induced association of tyrosine phosphorylated IRS-1 with GRB2, with the 10μM of human fetuin. They also showed the complete inhibition of insulin stimulated MEK activity when cells were preincubated with 10μM of human fetuin-A. In CHO cells, fetuin-A has also been shown to inhibit the activation of insulin induced Ras-GTPase (22). Several earlier studies indicate that fetuin-A (even at the high concentration such as 10 uM) does not inhibit insulin-induced metabolic effects such as glycogen synthesis, glucose uptake, glycogen synthase activity. Recombinant fetuin also did not affect insulin incorporation of [14C] glucose into glycogen into two different rat cells muscle preparation (22). Similar findings have been shown by Auberger *et al* using rat fetuin-A (36). However, several human studies, data from fetuin-A null mice and recent studies in our laboratory using L6-GLUT4myc skeletal muscle cells and primary human skeletal muscle cells

indicate that fetuin-A inhibits insulin-stimulated glucose uptake and glycogen synthesis (20,40). The mechanism of action of fetuin-A is not found to be consistent among various cells types. Fetuin-A inhibits the insulin rector tyrosine kinase in hepatic cells but does not affect the same in adipose cells. On the other hand it inhibits mitogenesis in adipose cells (21). Le cam *et al* have shown that only phosphorylated form of pp63/fetuin is active and inhibits tyrosine kinase inhibition and partially phosphorylated form is 10-fold less active (128). That this protein should be phosphorylated to be physiologically active and that the nonphosphorylated form is ineffective has been confirmed by several studies (36, 42-44). Haglund *et al* identified two phosphorylation sites; serine 120 located in the A chain and on serine 312 located in the connecting peptide (41). The average degree of phosphorylation in plasma was at least 0.2 mol of phosphate/mol of fetuin-A (41).

2.6.3 Fetuin-A in animal models of obesity and diabetes

Acute injection of recombinant fetuin-A has been shown to inhibit the insulin stimulated tyrosine phosphorylation of insulin receptor and insulin receptor substrate-1 in rat liver and skeletal muscle (36). In a model of diet-induced obesity, which generally exhibits fatty liver, fetuin-A mRNA expression was increased (129). Fetuin-A null mice displays dramatically improved insulin sensitivity and are resistant to weight gain on a high fat diet (129). These mice also demonstrate improved glucose clearance in liver and muscle with lower levels of fasting plasma triglycerides. Increased downstream signaling molecules Akt and mitogen activated protein kinase (MAPK) have also been demonstrated in liver and skeletal muscle of fetuin knockout mice (40). Additionally

Papizan and Mathews (130) have shown increased plasma fetuin-A phosphorylation (^{312}Ser) in insulin resistant, leptin-signaling impaired Zucker diabetic fatty (ZDF) rats and in leptin-deficient *ob/ob* mice, compared to lean controls, suggesting roles for leptin and insulin in the regulation of ^{312}Ser-fetuin-A phosphorylation.

2.6.4 Fetuin-A and insulin resistance in humans

Sequencing of the fetuin-A gene promoter region and exons indicated the presence of nine common single nucleotide polymorphisms (SNPs). Siddiq *et al* carried out a detailed genetic association study of the contribution of these common fetuin-A SNPs to genetic susceptibility of type 2 diabetes in French Caucasians. The major allele of a synonymous coding SNP in exon 7 (rs1071592) presented significant evidence for association with type 2 diabetes. Two other SNPs in strong linkage disequilibrium with rs1071592 showed evidence approaching significance (131). In another study done by Osawa *et al* to identify the origin of two common alleles, fetuin-A*1 and *2, six single nucleotide differences were identified in comparison with the original sequence. Among the samples exhibiting phenotype 2–1 or 2, the nucleotide substitutions of C to T at amino acid position 230 and C to G at position 238 were very common (132). Genomic DNA of 68 individuals was studied, since these substitutions might give rise to a NlaIII site and a SacI site, respectively, for the potential fetuin-A*2. The results indicated that fetuin-A*1 was characterized by ACG (Thr) at position 230 in exon 6 and ACC (Thr) at position 238, on exon 7 and that fetuin-A*2 was characterized by ATG (Met) at position 230 and AGC (Ser) at position 238 (132). Lavebratt *et al* evaluated the association of rs4917 (Thr230Met) variation in the fetuin-A gene with fat cell function by investigating

subcutaneous fat cell lipolysis in 93 healthy obese and nonobese male subjects (133). They conclude that a common variation (Thr230Met) in the fetuin-A gene was associated with a marked increase in β2-adrenoceptor sensitivity in subcutaneous fat cells, which may be of importance in body weight regulation. Moreover, an association has been reported between a common SNP located in the 5'-region of the fetuin-A gene (rs2077119) and insulin mediated regulation of lipolysis in the adipose tissue from obese and nonobese women (134). A consequence of increased body fat is the development of resistance to insulin-stimulated glucose uptake. Indeed, previous studies have implicated a pathophysiologic role of fetuin-A in regulating insulin sensitivity. Serum levels of fetuin-A have been found to be increased in women with gestational diabetes mellitus and to correlate with maternal insulin resistance parameters (122). High fetuin-A plasma measures at baseline were found to be coupled with less increase in insulin sensitivity in the subjects who undergo a lifestyle intervention program with diet and increase in physical activity (18). Stefan *et al* have shown by cross sectional analysis, that fetuin-A plasma concentrations were elevated in subjects with high liver fat. In longitudinal analysis, with weight loss, relatively large mean decreases were observed in liver fat and in fetuin-A plasma levels, which suggest that the change in liver fat was associated with the change in fetuin-A plasma levels (18). This also suggests that fat accumulation in the liver may result in increased secretion of fetuin-A. Hennige *et al* have demonstrated that fetuin-A induces low-grade inflammation and represses adiponectin production in animals and in humans. These data suggest an important role of fetuin-A in the pathophysiology of insulin resistant conditions (135).

2.6.5 Fetuin A and serum lipids

Kumbla *et al* have shown a strong association of fetuin-A with variety of lipids such as cholesterol, cholesteryl ester and with decreased amounts of phospholipids, triglycerides, and free fatty acids (26). Additionally, Kumbla *et al* have shown that in comparison with albumin, fetuin-A is 50-fold more effective in incorporating exogenous fatty acid into cultured cells (25). They have demonstrated the capability of fetuin-A in inducing cholesterol efflux from both human fetal skin fibroblasts and HepG2 cells as effectively as HDL (26). These results suggest that fetuin-A might play a multifunctional role in lipid transport during development. Fetuin-A also showed concentration dependent significant increase in the incorporation of [^{14}C] oleic acid into triglycerides (26).

2.6.6 Regulation of fetuin-A expression

Synthesis of fetuin-A was found to be downregulated by recombinant human interleukin-1, interleukin-6, and tumor necrosis factor-α and partial hepatectomy. On the other hand, treatment of hepatocellular carcinoma cells with thyroid hormones (136), over-expression of signal transducer and activator of transcription-3 (137), or treatment with glucocorticoids or dexamethasone has been shown to upregulate the mRNA and protein expression of fetuin-A (138).

2.7 Metabolic Syndrome (MetS):

2.7.1 Definition and Prevalence

The definitions of metabolic syndrome (MetS) include those proposed by the World Health Organization (WHO) (139), the European Group for the Study of Insulin Reistance (EGIR) (140), the US National Cholesterol Education Program (ATPIII) (141) and International Diabetes Federation (IDF) (142) (Summarized in Table 1).

The National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) released a report identifying a state of central obesity in association with risk factors for CHD as the metabolic syndrome (143). In men, a waist circumference >40 inches and in women, a waist circumference >35 inches is defined as central obesity by this panel. Other risk factors of the MetS include dyslipidemia [high triglycerides, low high-density lipoprotein-cholesterol (HDL-C) and small low-density lipoprotein (LDL) particles], elevated fasting blood glucose (with or without glucose intolerance) and blood pressure (144). In addition MetS may be associated with pro-thrombotic and pro-inflammatory states.

A recent analysis of data from 8,814 men and women in the Third NHANES study (1988 to 1994) that used the ATP III definition of MetS indicates that the unadjusted and age-adjusted prevalence of the syndrome are 21.8% and 23.7%, respectively, in the US population aged ≥20 years (145). This finding indicates that on the basis of 2000 census data, approximately 47 million people in the United States have MetS. The presence of MetS is estimated to increase the risk of coronary heart disease by

1.6- to 3.0-fold (146). The prevalence of high fasting glucose in men and women with the metabolic syndrome is 15.6% and 10%, respectively (147). Interestingly, a vast number of studies have shown that lifestyle modification, together with weight reduction, reduces risk of development of type 2 diabetes. For example, the Diabetes Prevention Program has shown that the metformin-treated group had a 31% reduction in the incidence of diabetes compared with placebo whereas lifestyle intervention condensed the incidence of diabetes by 58% (148).

Table 1: Current definition of Metabolic Syndrome (MetS)

WHO (1999)	***EGIR (1999)***	***(NCEP ATPIII) 2001***	***(IDF) 2005***
Diabetes/impaired glucose tolerance /insulin resistance + two or more of the following:	***Insulin resistance /hyperinsulinemia + two or more of the following:***	***Three or more of following:***	
Obesity: BMI >30kg/m^2 or waist: Hip>0.9 (M), > 0.85 (F)	***Central obesity: Waist Circumference ≥ 94 cm (M), ≥80 cm (F)***	***Central obesity: Waist Circumference ≥102 cm (M), ≥88 cm (F)***	***Central obesity + any two of the following:***
Dyslipidemia: TG≥1.7 mmol/L, HDL-C <0.9 mmol/L	***Dyslipidemia: TG ≥2.0 mmol/L, HDL-C <1.0 mmol/L***	***Hypertriglyceridemia: TG ≥2.0 mmol/L***	***Raised triglyceride ≥1.7 mmol/L or Specific treatment***
Hypertension: Blood Pressure ≥140/90 mmHg or medication	***Hypertension: Blood Pressure ≥140/90 mmHg or medication***	***Low HDL: HDL-C <1.03 mmol/L (M), 1.29 mmol/L (F)***	***Reduced HDL <1.03 mmol/L (M), <1.29 mmol/L (F), or Specific treatment***
Microalbuminuria: Albumin excretion ≥ 2.5 mg/mmol (M), ≥3.5 mg/mmol (F)	***Fasting plasma glucose: ≥6.1 mmol/L***	***Hypertension: Blood Pressure ≥130/85 mmHg or medication***	***Hypertension: Blood Pressure ≥135/80 mmHg or medication***
		Fasting plasma glucose: ≥6.1 mmol/L	***Fasting plasma glucose: ≥5.6 mmol/L or previously diagnosed type 2 diabetes***

WHO, World Health Organization (139); EGIR, the European Group for the Study of Insulin Resistance (140); ATPIII, National Cholesterol Education Program Expert Panel on Detection Evaluation , and Treatment of High blood Cholesterol in Adults(Adult Treatment Panel III) (141): IDF, Internation Diabetes federation (142); F, female; and M, male

2.7.2 MetS and inflammation

Both experimental data in human and animals and epidemiological studies have shown convincing evidence linking inflammation to insulin resistance. It is well known that the prevalence of diabetes, obesity, and MetS all increase with age. In a cross-sectional study of 70 healthy individuals aged 21– 94 years, increase in age was found to be negatively correlated with whole-body glucose disposal and positively associated with plasma concentrations of tumor necrosis factor-alpha (TNF-α). Additionally, a significant negative correlation was noted between whole-body glucose disposal and plasma TNF-α. In 439 non-diabetic women followed in the Women's Health Study, fasting insulin was strongly associated with plasma concentrations of the acute-phase reactant C-reactive protein (CRP) and the pro-inflammatory cytokine like interleukin-6 (IL-6) (149). Furthermore, a linear rise in CRP levels was noted with increasing numbers of MetS components, dyslipidemia, abdominal obesity, insulin resistance, and hypertension). Using the ATP-III definition of MetS, similar results were reported among 14,719 non-diabetic women enrolled in the Women's Health Study; median CRP concentrations increased from 0.68 mg/L in women with no characteristics of MetS, to 5.75 mg/L in those with five characteristics (150).

2.7.3 Obesity, atherogenic dyslipidemia and MetS

High serum cholesterol has been acknowledged as being a major risk factor for coronary heart disease (CHD). As much of the serum cholesterol is transported by LDL, most people with high serum cholesterol also have elevated LDL. The National Cholesterol Education Program (NCEP) specifically targeted LDL cholesterol as the

chief goal of cholesterol-lowering therapy (151). Statin (HMG CoA reductase inhibitors) therapy has produced marked reductions in new coronary events; these reductions almost definitely were connected to decreases in LDL levels (152-154). Strong evidence also indicates that high LDL concentrations initiate atherogenesis and promote atherosclerosis (152). A strong link between increased abdominal (visceral) fat and hyperinsulinemia, insulin resistance, elevated plasma free fatty acid (FFA) levels, hypertension, predisposition to thrombosis, hypertriglyceridemia, small, dense LDL particles, and reduced HDL has been established (146). There is also evidence that increased abdominal adipose tissue is associated with physical inactivity, increased plasma cortisol, and intrauterine environment (155). The changes in lipid metabolism seen with abdominal fat accumulation have been well characterized and include hypertriglyceridemia, reduced HDL cholesterol, and increased numbers of small, dense LDL particles. Elevated LDL cholesterol is not a feature of the dyslipidemia seen with abdominal obesity. Other features of the dyslipidemia of abdominal adiposity include elevated very low density lipoproteins (VLDL), and reduced HDL2, which are the large buoyant antiatherogenic subspecies of total HDL. In some individuals, apo B levels may be elevated, reflecting an increase in the number of small, dense lipoprotein particles (VLDL and LDL) (146).

2.8 Niacin, a lipid-lowering drug

Niacin (Nicotinic acid, Vitamin B3) is a water-soluble vitamin. The major metabolic role of niacin is that it serves as a precursor for two essential coenzymes, nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). Both NAD and NADP can be reduced to NADH and NADPH,

respectively, and these coenzymes participate in oxidation-reduction reactions catalyzed by dehydrogenase and oxidoreductase enzymes. These NAD/NADP linked enzyme systems are involved in virtually every aspect of metabolic processes. Clinically, niacin deficiency causes pellagra characterized by dermatitis, diarrhea and dementia. (156, 157).

NIACIN: CHEMICAL STRUCTURE

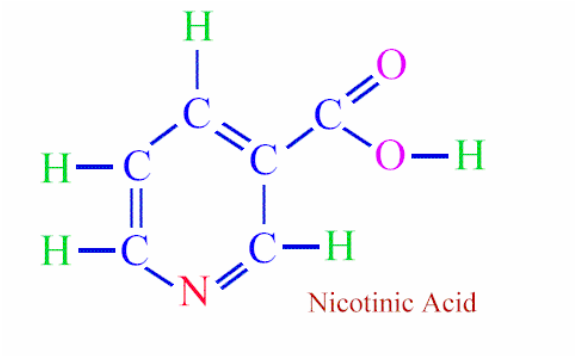

Fig.4: Chemical structure of niacin*. Nicotinic acid, a 3-pyridine carboxylic acid, widely known as niacin, has a molecular formula $C_6H_5NO_2$ and a molecular weight of 123.*

In 1955 Altshulm Hoffer and Stephen reported niacin as a pharmacologic agent (158), and currently it is a widely used agent in the treatment of dyslipidemia (159-160). Niacin reduces concentrations of total plasma cholesterol (TC), apolipoprotein (apo) B, triglyceride, VLDL, LDL, and lipoprotein(a) [Lp (a)], and increases HDL levels in pharmacologic doses of 1-3 g/day (reviewed in 161). Several clinical trials (secondary prevention and angiographic studies) indicate that the treatment with niacin considerably reduces coronary events, retards the progression and induces regression of coronary atherosclerosis and reduces total mortality. Although the use of niacin in the past has

been associated with adverse effects like flushing and hepatic toxicity, recent studies utilizing novel formulations of niacin have shown decreased flushing with almost no hepatic toxicity with similar effects on plasma lipid profile (161).

Although the exact mechanism is not understood completely, the probable primary action of niacin is to restrain recruitment of free fatty acids from peripheral adipose tissue to the liver. This results in a reduced synthesis of VLDL and triglycerides from liver. Further, as less VLDL is accessible as a substrate, LDL-cholesterol (LDL-C) levels decrease (162). Niacin is believed to increase serum HDL-C levels by blocking hepatic uptake of apolipoprotein A-I, a major component of HDL-C (162). Niacin also increases a cardioprotective subfraction of HDL, which improves reverse cholesterol transport (163). Niacin decreases levels of LDL-C by 5% to 25%, triglycerides by 20% to 50%, Lp (a) by 34%, (164) and the TC/HDL-C ratio by 27% (165); niacin increases levels of HDL-C by 15% to 35% (166). Niaspan is a prolonged release (PR) preparation of niacin with absorption rates between immediate release (IR) and sustained release (SR) preparation. It has been used in clinical trials in USA since the 1990s. In a dose–response, placebo-controlled study with a daily dose of Niaspan of 1000 and 2000 mg, a clear dose-dependent effect was observed on lipid/lipoprotein levels (23). HDL-cholesterol increased by 17 and 23%, while plasma triglycerides decreased by 21 and 29%, respectively, with the two doses of Niaspan. Both LDL-cholesterol and Lp(a) were also reduced in a dose-dependent manner (164).

2.9 Study Objectives

Fetuin-A has been shown to influence a wide variety of biological processes, including insulin and growth factor signaling, lipid transport, opsonization fetal development and tissue mineralization. Among the earliest described biological effect of fetuin-A was the inhibition of the insulin receptor tyrosine kinase. Activation of the insulin receptor tyrosine kinase, one of the most proximal events in insulin signaling, is thought to be required for most, if not all of the biological actions of insulin. It has also been shown that the ser120 and ser312 are the critical sites for the phosphorylation of fetuin A and that this protein is biological active only when it is phosphorylated. Fetuin-A has been shown to be strongly associated with insulin resistance, metabolic syndrome and an atherogenic lipid profile. However, there are no reports of fetuin-A phosphorylation status and its association with insulin resistance in humans. Also, there are no reports on the molecular characterization of the Ser312 phosphorylation site of fetuin-A. Therefore, the goal of the present study was to examine alterations in serum total fetuin-A and phosphorylated (Ser312) fetuin-A concentrations in individuals with metabolic syndrome treated with Niaspan, a lipid-lowering drug, and correlate these with changes in serum lipids, and markers of insulin sensitivity and inflammation. Additionally, to characterize the significance of the fetuin-A-Ser312 phosphorylation site, we have examined the effects of both wild-type and mutant Ser312Ala-fetuin-A (defective in phosphorylation on Ser312) in the inhibition of insulin signaling and glucose uptake into skeletal muscle cells.

2.10 Hypothesis

Since phosphorylation of fetuin-A has been shown to be critical for its IR-TK inhibitory activity, and given that serum fetuin-A is associated with obesity, insulin resistance, and MetS, it is our hypothesis that elevated levels of the phosphorylated form fetuin-A (Ser312) in MetS are associated with serum lipid metabolism, insulin resistance, and inflammatory markers. It is anticipated that therapeutic intervention with Niaspan may lower serum fetuin-A levels and be correlated with a decrease in serum triglycerides. Since the majority of phosphorylation of fetuin-A is on Ser312, we hypothesize that phosphorylation on Ser312 is critical for fetuin-A's physiological effects.

CHAPTER 3: MATERIALS AND METHODS

3.1 Recruitment of Subjects

The study was approved by the Auburn University Institutional Review Board (IRB) for the Protection of Human Subjects in Research. Fifteen males, who were sedentary, obese (body mass index ≥30 kg/m2, waist girth >88 cm), hypertriglyceridemic (triglycerides≥150 mg/dL), and nonsmokers, between the ages of 30 and 65 years were recruited from the local community and surrounding areas by Dr. Peter W. Grandjean, Associate Professor, Exercise Technology Laboratory, Department of Health, Human Performance and Kinesiology, Auburn University, AL. Individuals with known history of active gout, peptic ulcer disease, diabetes, liver disease, or, who were on medications for any of the diseases mentioned above were not included in the study. Selected volunteers were called to the laboratory and preliminary screenings were done and were asked to complete an institutionally approved informed consent. Venous blood samples were collected from each individual and were sent to the Centers for Disease Control and Prevention-certified laboratory to assay blood glucose, serum insulin, and circulating levels of liver enzymes. Before the clearance for entrance in the study, participants were fully examined by an attending physician of the Exercise Technology Laboratory.

3.2 Niaspan intervention and blood sampling

Three days before starting the niacin intervention, all physical activity and daily food consumption was recorded by the participants. To estimate baseline fetuin-A, glucose and insulin concentrations, fasting blood samples were collected and evaluated from each participant. All the participants were then provided with extended-release niacin (*Niaspan,* Abbott Laboratories, Abbott Park, IL) as prescribed by the attending physician. Niaspan was taken by each participant once a day in the evening along with a low-fat snack before bedtime at a dose of 500 mg/day during the first week, 1000 mg/day during the second week and 1500 mg/day during weeks 3 through 6. Additionally, to minimize the flushing induced by the intake of Niapan, each participant was asked to take an enteric-coated aspirin (300 mg) 30 to 60 minutes before the evening dose of Niaspan. Blood samples from each participant was obtained following the 6-week Niaspan intervention, after an 8-12 hours overnight fast between 8:00 and 9:00 a.m. Serum was separated from the collected blood samples by centrifuging whole blood for 20 minutes at 1500g, aliquoted and stored at -80^{o}C.

3.3 Analytical Procedures

Anthropometric measurements, body fat analysis by Dual-energy X-ray absorptiometry (DEXA), blood glucose, serum levels of insulin, total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, free fatty acids, and adiponectin were previously assayed by Dr. Grandjean and colleagues. In this study, we have analyzed serum concentrations of fetuin-A by a sandwich ELISA (BioVendor LLC, Candler, NC),

according to the manufacturer's recommended protocol. To determine serum C-reactive protein concentrations, serum samples were diluted in 1:100 dilutions and estimated using a sandwich ELISA (United Biotech Inc., Mountain View, CA). The antibodies were highly specific for the human C reactive protein. Serum TNF-α (assay sensitivity: 0.5-5.5 pg/ml) and IL-6 (less than 0.70 pg/ml) concentrations were analyzed by sandwich ELISA (R&D systems, Inc., Minneapolis, MN) according to the manufacturer's recommendations.

3.4 Human fetuin-A full-length cDNA construction

Full-length human fetuin-A cDNA, cloned into pCMV6-XL4 vector and driven by a CMV promoter, was purchased from Origene Inc., Rockville, MD (NCBI Reference Sequence ID # NM _001622.2). Next, a 3x FLAG® peptide (22 amino acids) affinity tag (Sigma Chemical Company, St. Louis, MO) was cloned into the C-terminus end of fetuin-A cDNA with the goal of producing recombinant FLAG-tagged human fetuin-A. The C-terminus site was specifically chosen for the FLAG-tag, since the N-terminus of fetuin-A encodes a signal peptide, which is critical for its processing as a secreted protein.

3.4.1 Plasmid DNA isolation

The FLAG-tagged fetuin-A cDNA clone was transformed into OneShot Top10 competent cells (Invitrogen Corporation, Carlsbad, CA). Plasmid DNA was prepared by inoculating a single colony of transformed bacteria into 5 mL LB/Amp (50 µg/mL)

culture. After 8 hours, 4 mL of this culture was used to inoculate a 500 mL LB/Amp culture. The remaining 1 ml culture was saved as glycerol stock for future use. Plasmid DNA was isolated using QIAfilter Plasmid Maxi kit (Qiagen Sciences, Maryland, USA) according to the manufacturer's recommendations.

3.4.2 Restriction analysis and DNA sequencing

Purified plasmid DNA (1µg) was incubated with Not1 restriction enzyme and REact 3 buffer (Invitrogen Corporation, Carlsbad, CA), at 37 ^{o}C for 6 hours. Next, 20µl of the undigested and digested DNA was mixed with 2µl of DNA loading dye (0.25% Bromophenol blue, 0.25% Xylene cyanole FF, 40% sucrose in water; Sigma Chemical Company, St. Louis, MO) and electrophoresed at 100 volts. The gel was stained in 1% ethidium bromide solution, and visualized using an UV trans-illuminator (UVP LLC, Upland, CA). The full-length fetuin-A cDNA was sequenced using a custom-designed "primer-walking" approach (GenScript Corp., Piscataway, NJ). Briefly, a primer that matched the beginning of the fetuin-A cDNA insert sequence was used to synthesize an oligonucleotide strand adjacent to the unknown sequence, starting with the primer. This new, short DNA strand was sequenced by standard DNA sequencing methodology (167). Next, the end of the sequenced strand was used as a primer for the next part of the DNA sequence. This was continued until the entire cDNA insert was sequenced. These fragments of DNA sequences were then compiled to confirm the full-length fetuin-A cDNA sequence.

3.5 Generation of Ser312Ala Fetuin-A: Site-directed mutagenesis

To understand the significance of phosphorylation on Ser312 residue of fetuin-A, this site (Ser312) was mutated to alanine using a custom-designed PCR-based site-directed mutagenesis approach (GenScript Corp., Piscataway, NJ). The location of the Ser312 residue (S in bold letter) and amino-acids adjacent to this site are shown below:

Location	Position	Amino acid sequence
Connecting Peptide	300-319	HTFMGVVSLGSP**S**(P04)GEVSHPR

Briefly, oligonucleotide primers with the Ser312Ala mutation and complementary to the opposite strands of the vector was extended using Pfu Turbo DNA polymerase. The product was then treated with Dpn I endonuclease, which is specific for methylated and hemi-methylated DNA and digests the parental DNA template. Since DNA isolated from all *E.coli* strains is methylated and therefore susceptible to Dpn I endonuclease, this allows the selection of mutation-containing synthesized DNA. The nicked vector DNA containing the Ser312Ala mutation was transformed into super component cells. DNA sequencing was performed to identify and confirm the Ser312Ala mutation.

3.6 Functional Studies: Transfection of COS-7 and HIRc B cells

African green monkey kidney cells (COS-7), and rat fibroblast cells overexpressing human insulin receptors (HIRcB) were cultured in complete Dulbecco's Modified Eagle Medium, (DMEM) (Invitrogen, Grand Island, NY, USA) media containing 10% FBS and penicillin-streptomycin as antibiotics. After reaching confluence, cells were washed with phosphate buffered saline (PBS) (Invitrogen

Corporation, Carlsbad, CA) and transfected with 4µg of either wild type fetuin-A or Ser312Ala fetuin-A plasmid DNA, diluted in opti-MEM media and 10µl of lipofectamine and were incubated at 37 ^{o}C. Media was changed to complete DMEM after 6 hours of incubation followed by the overnight incubation. The next day, media was changed to serum-free DMEM media (Invitrogen Corporation, Carlsbad, CA) containing 0.1% BSA. Twenty-four hours later, the media was assayed for the presence of secreted fetuin-A and phosphorylated fetuin-A (wild-type and Ser312Ala) by Western blotting analysis.

3.6.1 Transfection efficiency: β-Galactosidase staining

Transfection efficiency was determined by transfecting the cDNA for β-galactosidase using lipofectamine, as described above. β-galactosidase was visualized with the In Situ β-Galactosidase Staining kit (Stratagene, Ceder Creek, TX). Briefly, cells were washed three times in ice-cold PBS followed by fixation using 1x fixing solution for 10 minutes. Next, the cells were stained with X-gal diluted in staining solution. Cells expressing β-galactosidase appeared blue, which was visualized and captured using a Canon PowerShot S31S-attached Nikon TS100-F inverted microscope.

3.6.2 Transfection assays: Activation of MAPK and Akt

HIRcB cells were transfected with 4µg wild type fetuin-A or Ser312Ala fetuin-A DNA as described above. Following transfection, the media was changed to serum-free DMEM containing 0.1% BSA and incubated at 37^{o}C overnight in a CO_2 incubator. The next day, cells were stimulated with 100nM of insulin for 10 minutes. The cells were then

washed with ice-cold PBS three times and lysed using cell lysis buffer (50mM HEPES, 100 mM sodium pyrophosphate, 100 mM NaF, 10mM EDTA, 2 mM PMSF, 2 mM sodium orthovanadate, 1% Triton X-100), sonicated for one minute (Sonics and Material Inc., Newtown, CT, USA) and centrifuged for 30 minutes at 14,000 rpm at 4^{o}C. An aliquot of the supernatant, normalized for protein, was separated by SDS-PAGE on 4-20% Tris-glycine gel (NuSep Inc., Lawrenceville, GA). Gels were then transferred to nitrocellulose membrane (BioRad Laboratories, Hercules, CA, USA) by using semi-dry transfer method using 10% transfer buffer made up of 25 mM Tris, 192 mM glycine and 20% methanol. Proteins were blocked in either 5% non fat dry milk (Bio-Rad, Hercules, CA, USA) or 5% BSA (Fisher Scientific, Fairlawn, NJ, USA).

Fetuin-A, secreted into the media, was detected using goat anti-human fetuin-A/alpha2-HS glycoprotein antibody (IncStar, Stillwater, MN). Since the cDNA construct was engineered to produce recombinant FLAG-tagged human fetuin-A, FLAG antibodies (Sigma Chemical Company, St. Louis, MO) were also used to detect fetuin-A, in conjunction with the anti-fetuin-A antibody. Phosphorylated Ser312-fetuin-A was analyzed using a custom-generated affinity-purified antibody (Affinity BioReagents, Golden, CO) against the phosphorylated Ser312-fetuin-A epitope "HTFMGVVSLGSP**S**(PO_4)GEVSHPR". This antibody specifically recognized phosphorylation on ^{312}Ser-fetuin-A.

Western blotting for intracellular signaling molecules was performed using antibodies specific for ERK2 (BD Transduction Laboratories, Lexington, KY, USA),

phospho-p44/42 MAPK, phospho-Akt (Ser473), and phospho-GSK3 (Cell Signaling Technology, Danvers, MA) and Akt (Upstate biotechnology, Lake Placid, NY). Western blotting for GAPDH (AbCam PLC, Cambridge, MA) was performed to assess equal loading of proteins. The membranes were developed using either SuperSignal West Femto Maximum Sensitivity substrate (Thermo Scientific, Rockford, IL) or SuperSignal West Pico chemiluminescence substrate (Thermo Scientific, Rockford, IL), and chemiluminescence was captured and analyzed using UVP Bioimaging System and Labworks Software package (UVP, Upland, CA). Area densities of the bands were analyzed using the Un-Scan-It software package (Silk Scientific, Orem, UT, USA).

3.7 Recombinant human fetuin-A: Production and purification

3.7.1 CHO-S cell culture

Chinese Hamster Ovary cells adapted to suspension culture (CHO-S) were thawed from liquid nitrogen and transferred into 125 ml polycarbonate, disposable, sterile Erlenmeyer spinner flask (Invitrogen Corporation, Carlsbad, CA) containing 60ml of pre-warmed FreeStyle^{TM} CHO Expression medium (Invitrogen Corporation, Carlsbad, CA) supplemented with 8mM L-glutamine. Cells were incubated in a 37 ^{o}C incubator containing a humidified atmosphere of 8% CO2, on an orbital shaker at 125 rpm.

3.7.2 Transfection of CHO-S cells

One day before transfection, CHO-S cells were resuspended in 60 ml of fresh FreeStyle^{TM} CHO Expression medium without serum and antibiotics. CHO-S cells were transfected when cells reach a density of 1x10^{6} cells/ml. 75µg of plasmid DNA (wild

type or Ser312Ala-fetuin-A) was diluted into OptiPro SF Media (Invitrogen Corporation, Carlsbad, CA) in a total volume of 0.6 ml and mixed into a total volume of 0.6 ml of 75µl of FreeStyle MAX transfection reagent diluted in OptiPro serum-free media to obtain a total volume of 1.2 ml. This mixture was incubated for 10 minutes at room temperature and added slowly to the flask containing cells while slowly swirling the flask. Transfected cells were incubated at 37 ^{o}C, 8% CO2 on an orbital shaker platform rotating at 135 rpm.

3.7.3 Recombinant human fetuin-A purification

Five days after transfection of CHO-S cells, the media was collected and stored at -80^{o}C until further purification. The media was purified on an agarose-bound jacalin column (Vector Laboratories, Burlingame, CA, USA) that has been shown to specifically bind human fetuin-A. Wild-type fetuin-A and Ser312Ala fetuin-A bound to the jacalin column were eluted using 0.1M melibiose in six different fractions. Purified fetuin-A fractions were diluted 1:50 ratio with Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA) and protein concentration was analyzed using a UV spectrophotometer (Beckman Coulter, Fullerton, CA). Purity was determined using SDS-PAGE and Western blotting techniques.

3.8 Glucose uptake assay

Rat L6-GLUT4 skeletal muscle cells were cultured to confluence in complete DMEM (high glucose media) (Invitrogen, Grand Island, NY, USA) containing 10% FBS. Cells were then washed with PBS and starved for five hours in 1 ml serum free DMEM

(low glucose) media (Invitrogen, Grand Island, NY, USA). Cells were again washed with PBS and incubated for 30 minutes in 500 µl of glucose free KRH buffer (50mM HEPES, pH 7.4, 136mM NaCL, 4.7mM KCL, 1.25mM $MgSO_4$, 1.25mM $CaCl_2$, 0.1% BSA). Cells were then treated with various concentrations of wild type fetuin-A or Ser312Ala fetuin-A for 20 min and incubated at 37^{o}C. Finally, the cells were treated with 100 nM insulin for 30 min. 10X START buffer (100µM 2-deoxyglucose, 0.5µCi/ml [^{3}H]-2-deoxyglucose) was added to each well and incubated for 10 min at room temperature. Next, the cells were gently washed three times with ice-cold PBS and solubilized in 0.2N NaOH. [^{3}H]-2-deoxyglucose taken up by the cells were counted in a liquid scintillation counter for 2 minutes (Packard Instrument Company, Downers Grove, IL).

3.9 Statistical Analysis

Data are presented as means ± S.D. All variables were normally distributed; therefore, a paired Student *t* test was performed. Relationships between the variables of interest were determined using Pearson product-moment correlation coefficients. All data were analyzed using the Statistical Analysis System (SAS for Windows, version 9.1; SAS Institute, Cary, NC). The level of significance was taken as $p<0.05$. Quantification of data from Western blots was done using UN-SCAN-IT Gel Digitizing System. (Silk Scientific, Orem, UT, USA).

CHAPTER 4: RESULTS

4.1 Anthropometric measurements in MetS: Association with fetuin-A and phosphorylated fetuin-A

Individuals who met the NCEP ATP III criteria for MetS had an average BMI of 34.0 ± 3.15 with a waist circumference of 107.9 ± 7.96 cm (Table 1). DEXA analyses indicated that individuals with MetS had a percent body fat of 35.13 ± 4.79, with an android percent fat distribution of 46.83 ± 4.93. Serum total fetuin-A and phosphorylated fetuin-A did not show an association with the above measurements. Fetuin-A levels demonstrated a negative correlation (p = 0.05) with systolic blood pressure (Table 1). However, phosphorylated fetuin-A did not demonstrate an association with systolic blood pressure.

4.2 Serum fetuin-A and phosphorylated fetuin-A concentrations in Niaspan-treated individuals with MetS

Serum fetuin-A levels, assayed by a sandwich ELISA method, demonstrated a detection limit of 3.5 ng/ml. Taking the dilution of the samples into consideration, this translated into an assay sensitivity of 3.5 µg/ml (3.5 ng/ml x 1000 = 3.5 µg/ml) (Fig. 5). Serum fetuin-A concentrations ranged from 118.9 µg/ml to 348.6 µg/ml in individuals with MetS and the data showed a normal distribution (Fig.6A). Serum levels of

phosphorylated fetuin-A in MetS individuals were alsonormally distributed (Fig.6B). Additionally, serum phosphorylated fetuin-A levels were tightly correlated ($r = 0.84$, $p < 0.0001$) with serum fetuin-A levels (Fig.6C). MetS individuals treated with Niaspan for a period of six weeks demonstrated a 20% decrease ($p < 0.001$) in serum fetuin-A concentrations (208.25 ± 57.3 µg/ml) compared to levels before treatment (259.82 ± 66.62 µg/ml) (Fig.7). Interestingly, Niaspan treatment decreased serum fetuin-A concentrations in 13 of the 15 participants, suggesting that Niaspan-treatment effectively decreases serum fetuin-A levels in individuals with MetS.

Since phosphorylation has been shown to be critical for several of fetuin-A's functions, and considering that Niaspan treatment decreases fetuin-A levels, we postulated that Niaspan might decrease phosphorylated fetuin-A levels as well. Therefore, serum levels of phosphorylated fetuin-A were assayed by Western blotting in MetS individuals before and after treatment with Niaspan. While the results were not significant ($p = 0.08$), 12 of the 15 participants demonstrated a lowering of phosphorylated fetuin-A following the six weeks Niaspan treatment (Fig.8).

4.3 Association of serum fetuin-A and phosphorylated fetuin-A concentrations with serum triglycerides levels

An elevated serum triglyceride concentration is a criterion for the diagnosis of MetS. Currently, Niaspan is the most effective therapeutic agent in lowering serum triglycerides. In this study, as was expected, Niaspan treatment significantly decreased ($p < 0.01$) triglyceride levels by 36.8% in individuals with MetS from 292.9 ± 142.9 mg/dl

to 185.1 ± 66.2 mg/dl (Fig.9 and Table 3). Serum triglyceride levels were not associated with serum fetuin-A levels in individuals with MetS before Niaspan treatment ($r = 0.43$, $p = 0.12$). However, fetuin-A concentrations were significantly correlated with serum triglyceride concentrations after Niaspan treatment ($r = 0.59$, $p < 0.05$) (Fig.10). Additionally, the changes in fetuin-A concentrations with Niaspan treatment were significantly correlated with the changes in serum triglycerides with Niaspan treatment ($r = 0.62$, $p = 0.01$).

In individuals with MetS, phosphorylated fetuin-A concentrations were significantly correlated with serum triglycerides ($r = 0.69$, $p = 0.005$) before Niaspan treatment (Fig.11). However, phosphorylated fetuin-A levels were not correlated with serum triglycerides after Niaspan treatment. Additionally, the changes in phosphorylated fetuin-A concentrations with Niaspan treatment were also not correlated with the changes in serum triglycerides with Niaspan treatment ($r = 38$, $p = 0.15$).

4.4 Association of serum fetuin-A and phosphorylated fetuin-A concentrations with serum high density lipoprotein (HDL) and non-esterified fatty acid (NEFA) levels

In this study, we observed that 6-weeks Niaspan treatment induced a significant increase in HDL-cholesterol ($p = 0.02$) in individuals with MetS (Table 3). Though fetuin-A or phosphorylated fetuin-A levels were not associated with serum HDL-cholesterol concentrations, either before or after Niaspan treatment, the percent change with Niaspan treatment of serum fetuin-A was significantly correlated with the percent change in HDL-cholesterol ($r = -0.57$, $p = 0.03$) (Table 3). Similarly, the percent change

with Niaspan treatment of phosphorylated fetuin-A was significantly correlated with the percent change in HDL-cholesterol ($r = -0.58$, $p = 0.02$) (Table 3).

Niaspan treatment significantly lowered serum levels of total cholesterol ($p = 0.01$), without a significant change in serum LDL-cholesterol or NEFA concentrations Table 2). However, percent changes with Niaspan treatment in fetuin-A or phosphorylated fetuin-A concentrations were not associated with percent changes of serum total cholesterol and LDL-cholesterol. Interestingly, the percent change in serum NEFA levels were significantly correlated with the percent change in serum fetuin-A concentrations with Niaspan treatment ($r = 0.52$, $p = 0.05$) (Table 3).

4.5 Association of serum fetuin-A and phosphorylated fetuin-A concentrations with markers of insulin sensitivity

Niaspan treatment stimulated a significant increase in serum insulin levels ($p = 0.05$), without a concomitant increase in blood glucose levels (Table 4). HOMA values tended to increase but was not statistically significant ($p = 0.09$, Table 4). Serum concentrations of total adiponectin, high molecular weight adiponectin, and low molecular weight adiponectin were significantly elevated with Niaspan treatment (Table 4). However, serum total fetuin-A or phosphorylated fetuin-A did not show an association with these measurements.

4.6 Association of serum fetuin-A and phosphorylated fetuin-A concentrations with serum cortisol levels

Niaspan treatment induced a significant increase in serum cortisol levels ($p = 0.049$) in individuals with MetS (Table 4, Fig 12). Serum cortisol levels were found to be significantly correlated with phosphorylated fetuin-A after Niaspan treatment ($r = 0.55$, $p < 0.05$) (Fig 13). Additionally, the percent change in serum cortisol levels with Niaspan treatment were tightly correlated with the percent change in serum fetuin-A concentrations ($r = 0.78$, $p = 0.001$) and phosphorylated fetuin-A ($r = 0.72$, $p = 0.004$) (Table 4). Another important finding was that the percent change with Niaspan treatment in cortisol levels were significantly correlated with the percent change in serum NEFA concentrations ($r = 0.83$, $p = 0.0003$). Additionally, the percent change in serum cortisol levels with Niaspan treatment was negatively correlated with the percent change in serum HDL levels ($r = 0.78$, $p = 0.001$).

4.7 Association of serum fetuin-A and phosphorylated fetuin-A concentrations with inflammatory markers

Niaspan treatment demonstrated a significant decrease in serum concentrations of C-reactive protein ($p = 0.007$) and IL-6 ($p = 0.0004$) (Table 5) in individuals with MetS. Moreover, all the 15 participants demonstrated decreased levels of C-reactive protein following Niaspan treatment (Fig.14). Serum fetuin-A concentrations were negatively correlated with C-reactive protein in MetS individuals before Niaspan treatment ($r = -0.57$, $p = 0.025$) (Fig 15). Conversely, the percent change in serum fetuin-A with Niaspan treatment was positively associated with the percent change in C-reactive

protein (*r = 0.58, p = 0.03*). Niaspan treatment decreased IL-6 levels in 13 of the 15 subjects with MetS (Fig.16). Serum concentrations of TNF-α demonstrated a increasing trend with Niaspan treatment (p = 0.095, Fig.17). Serum total fetuin-A and phosphorylated fetuin-A were not associated with serum IL-6 or TNF-α concentrations (Table 4).

4.8 Full-length human fetuin-A cDNA construction, restriction analysis, and DNA sequencing

To characterize the role of fetuin-A and its phosphorylation status at the molecular level, several studies were conducted as detailed below. A 3X-FLAG-tag was cloned into the C-terminal end of a full-length human fetuin-A cDNA clone, purchased from Origene Inc., Rockville, MD. The C-terminus site was specifically chosen for the 3X-FLAG-tag, since the N-terminus of fetuin-A encodes a signal peptide sequence, which is critical for the processing of fetuin-A as a secreted protein (Fig.18). To confirm the size of the fetuin-A-3X-FLAG insert, a restriction analysis was performed with *Not* I, which produced an insert size of 1.2 kb (Fig.19), confirming the presence of the 2 *Not* I sites on the fetuin-A-3X-FLAG cDNA construct. DNA sequencing of the cDNA construct was performed first with the T7 and M13 primer sets. However, this approach was not successful because of the long poly(A) tail. Therefore, a custom-designed "primer-walking" approach was used for sequence analysis**.** The fragments of DNA sequences were compiled to confirm the full-length fetuin-A-3X-FLAG cDNA sequence

(Fig.20). The full-length human fetuin-A cDNA sequencing data was identical to the sequence in the NCBI database (NCBI Reference Sequence ID # NM_001622.2).

4.9 Generation of Ser312Ala-Fetuin-A mutant: Site-directed mutagenesis

To understand the significance of phosphorylation on the Ser312 residue of fetuin-A, a site-directed mutagenesis approach was taken to mutate Ser312 to Ala. Oligonucleotide primers with the Ser312Ala mutation and complementary to the opposite strands of the vector, were extended, treated with Dpn I, and transformed into ultracompetent cells. DNA sequencing was performed, which confirmed that the Ser312 codon was changed to Ala.

4.10 Ser312Ala-fetuin-A: Functional studies in COS7 cells

Transformed African Green Monkey kidney fibroblast cells (COS-7 cells), designed to achieve high transfection efficiency, were transiently transfected with 4µg of either wild type fetuin-A, Ser312Ala fetuin-A DNA or β-galactosidase cDNA. Transfection efficiency was estimated and confirmed by β-galactosidase staining procedure. Cells that took up the blue stain indicated that β-galactosidase was expressed. The resulting transfection efficiency, based on β-galactosidase staining was 50-70% (Fig 21). COS-7 cells, transfected with wild type or Ser312Ala-fetuin-A were changed to serum-free media. The next day, the media was collected and analyzed for fetuin-A secretion into the media.

Our data demonstrated that both the wild type and Ser312Ala-fetuin-A-FLAG tag cDNA construct effectively synthesized and secreted fetuin-A into the media. This was confirmed by immunoblotting with FLAG and fetuin-A antibodies. Phosphorylation status of fetuin-A was assayed using antibodies specific for Ser312-phosphorylated fetuin-A. As expected, wild type fetuin-A was phosphorylated, while no phosphorylation was detected on Ser312Ala-fetuin-A (Fig 22). Since COS-7 cells lack insulin receptors and do not respond well to insulin stimulation, it was of interest to examine the role of Ser312 phosphorylation in other cell lines.

4.11 Ser312Ala-fetuin-A: Functional studies in HIRcB cells

Rat1 fibroblasts overexpressing human insulin receptors (HIRcB cells) were transiently transfected with 4µg of wild type fetuin-A or Ser312Ala fetuin-A DNA. Transfection efficiency was estimated and confirmed by transfecting HIRcB cells with the cDNA for β-galactosidase. Transfection efficiency observed in this cell line ranged from 20-30% (Fig 23).

Following transfection, the media was collected and stored in -80^{o}C. An aliquot of the media was separated on SDS-PAGE. FLAG-tagged wild type and Ser312Ala-fetuin-A was synthesized and secreted by the transfected HIRc B cells, as detected by the FLAG antibody. Transfection with the pCMV6-XL4 vector served as control. While wild type fetuin-A was phosphorylated on Ser312, the mutant Ser312Ala-fetuin-A was not phosphorylated (Fig 24).

To analyze the significance of Ser312 phosphorylation, transiently transfected HIRc B cells were serum-starved for 24 hours following which the cells were stimulated with insulin, but without a change of media. This was done such that wild type or Ser312Ala-fetuin-A secreted into the media would be available to act in a 'paracrine' manner. We demonstrate that insulin-stimulated phosphorylation of MAPK (pMAPK) in the presence of wild type fetuin-A was noticeably inhibited compared to the vector-transfected or Ser312Ala-fetuin-A-transfected cell lysates (Fig 24). A similar pattern was observed on the effect of fetuin-A on activation of Akt. Unlike vector-transfected or Ser312Ala-fetuin-A-transfected cell lysates, wild type fetuin-A inhibited insulin-stimulated phosphorylation of Akt (pAkt) (Fig 24).

4.12 Recombinant human fetuin-A: Production and purification

To address our goal of characterizing the significance of Ser312 phosphorylation, we produced recombinant wild type human fetuin-A and Ser312Ala-fetuin-A using transiently-transfected Chinese Hamster Ovary-Suspension (CHO-S) cell culture. CHO-S cells, grown in suspension culture in spinner flasks, were transfected with either wild type fetuin-A or Ser312Ala fetuin-A. Five days after transfection, the media was collected and purified using agarose-bound jacalin column. Purified recombinant wild type and Ser312Ala-fetuin-A fractions were analyzed for protein concentrations. We observed the highest protein concentrations in fraction #3 for both wild type (Fig 25) and Ser312Ala-fetuin-A (Fig 26). No phosphorylation was indicated on Ser312Ala, whereas wild type fetuin-A demonstrated the presence of Ser312 phosphorylation. Also, highest phosphorylation was confirmed on the fraction #3 of the wild type fetuin-A (Fig 27).

4.13 Functional studies using recombinant wild type and Ser312Ala-fetuin-A

HIRc B cells were preincubated with purified wild type or Ser312Ala-fetuin-A for 20 min, followed by insulin-treatment (100 nM) for 10 min. Cells were lysed in buffer containing protease and phosphatase inhibitors, and lysates were immunoblotted for pMAPK, ERK-2, pAkt, Akt, and GAPDH. Wild-type fetuin-A, at the highest dose (3.2 μM) inhibited insulin-stimulated phosphorylation of MAPK compared to no inhibition by Ser312Ala-fetuin-A at the same dose (Fig.28). Similarly, unlike Ser312Ala-fetuin-A, wild type fetuin-A marked inhibited insulin-stimulated phosphorylation of Akt (Fig 29).

Next, we examined the effects of wild type and Ser312Ala-fetuin-A on insulin-stimulated glucose uptake. Surprisingly, both wild type and Ser312Ala-fetuin-A induced a dose-dependent inhibition of insulin-stimulated glucose uptake in the L6-GLUT4myc skeletal muscle cells (Fig 30). However, the magnitude of inhibition was greater with wild type fetuin-A compared to Ser312Ala-fetuin-A.

CHAPTER 5: DISCUSSION

Fetuin-A has been shown to have an inhibitory effect on insulin action by interacting with the activated insulin receptor and inhibiting insulin receptor autophosphorylation and IR-TK activity *in vitro*, in intact cells, and in peripheral tissues in animals (21, 22, 36-38). Only the phosphorylated form of fetuin-A has been shown to be active as an IR-TK inhibitor. Haglund *et al* have shown that serum fetuin-A was partly phosphorylated with the majority (80%) of the circulating fetuin-A being non-phosphorylated (41). Further, Haglunc *et al* (41) identified that human fetuin-A was phosphorylated on two sites - Ser120 and Ser312. Of these two sites, Ser312 was identified as the major phosphorylation site (nearly 77% as determined from relative peak heights in the mass spectrum) (41). In humans, the fetuin-A gene has been localized on 3q27, which was recently been shown to be linked to metabolic syndrome (9). Serum fetuin-A levels have been found to be strongly correlated to metabolic syndrome. In particular, a strong association of human fetuin-A with an atherogenic lipid profile has been reported (10). Additionally, fetuin-A levels have been shown to be positively correlated to insulin resistance and the accumulation of liver fat (18). However, there are no reports on the phosphorylation status of fetuin-A and its association with insulin resistance in humans. Accordingly, in the present study we have examined alterations in serum fetuin-A and phosphorylated (Ser312) fetuin-A concentrations in Niaspan-treated

individuals with metabolic syndrome and have correlated these with changes in serum lipids, insulin sensitivity and inflammatory markers. Niacin, which has been used to treat dyslipidemia for >40 years, improves all serum lipid and lipoprotein subfractions (162). Niaspan, the extended-release niacin formulation has been shown to retain the traditional efficacy of immediate-release niacin on serum lipids and lipoproteins, while minimizing coetaneous flushing and avoiding the hepatotoxicity associated with other long-acting (sustained-release) niacins. Several clinical trials have identified Niaspan as a treatment option for dyslipidemia either as a stand-alone or as combined niacin-statin regimens in patients with dyslipidemia and also with type 2 diabetes (167, 168). Niacin has been shown to decrease triglyceride synthesis and hepatic atherogenic lipoprotein secretion by inhibiting diacylglycerol acyltransferase 1 (DGAT-1), the last committed enzyme for triglyceride synthesis (169). These earlier findings led us to hypothesize that fetuin-A and phosphorylated fetuin-A (Ser312) concentrations may be amenable to therapeutic intervention with extended-release niacin formulation (Niaspan), the most effective agent currently available for clinical use for decreasing serum triglyceride and increasing levels of HDL-cholesterol.

We provide evidence for the first time that six weeks of Niaspan treatment significantly decreases total fetuin-A levels in MetS. Additionally, we report a novel association of phosphorylated fetuin-A with serum lipid levels and inflammatory markers in individuals with MetS treated with Niaspan. Among the components of MetS, we found a particularly strong positive association of phosphorylated fetuin-A (Ser312) with

serum triglyceride levels in individuals with MetS, before treatment with Niaspan. Niaspan treatment significantly decreased serum triglycerides and serum fetuin-A in individuals with MetS. Additionally, the changes in serum triglyceride concentrations were significantly correlated with the changes in serum fetuin-A levels. Recent findings by Stefan *et al* (18) demonstrate that elevated plasma levels of fetuin-A in humans were associated with increased fat accumulation in the liver and whole-body insulin resistance. Additionally, these authors show that when C57Bl6 mice were fed a high-fat diet, the mice became obese, insulin-resistant, and showed increased accumulation of fat in the liver. This was accompanied by an increase in fetuin-A gene expression. Liver fat content has been shown to be significantly increased in metabolic syndrome (170-172) Our data demonstrating that the changes in serum triglycerides with Niaspan treatment are associated with the changes in serum fetuin-A, are consistent with Niaspan's mechanism of action, and further lend strength to the hypothesis that decreased fetuin-A levels with Niaspan treatment are associated with decreased liver fat.

We observed that the changes in fetuin-A and phosphorylated fetuin-A with Niaspan treatment were strongly associated with the beneficial changes in HDL. Low HDL cholesterol has been shown to be an independent risk factor for CAD and premature atherosclerosis, independent of serum LDL or triglyceride levels (173). The primary mechanism by which HDL exerts its atheroprotective efficacy is reverse cholesterol transport, a process by which cholesterol is extracted from macrophages, foam cells, and atherosclerotic plaque, and delivered back to the liver for elimination as bile salts or

biliary cholesterol. However, several other anti-inflammatory, antithrombotic, and antiproliferative functions for HDL have also been identified (174). Hypertriglyceridemia may lead to decreased high density lipoprotein, enhancing HDL clearance from the circulation and in turn leading to atherogenic lipid profile (175). It is possible that the decrease in serum triglyceride concentrations after Niaspan treatment may be associated with a decrease in hepatic fetuin-A gene synthesis and an increase in serum HDL levels. Kuvin *et al* have shown that extended-release niacin treatment results in an improvement in the size distribution of HDL and LDL particles and inflammatory markers in patients with coronary artery disease (176). One major limitation of our study is the small sample size of our study group. This limits our ability to effectively address the association of fetuin-A with total cholesterol, LDL and other components of MetS.

Increased levels of free fatty acids are known to occur in patients with type 2 diabetes or insulin resistance, because of the reduced ability of insulin to suppress lipolysis (177). Our leading hypothesis is that fetuin-A may directly or indirectly mediate these changes through its inhibitory effects on the insulin receptor tyrosine kinase. In the adipose tissue, increased fetuin-A concentrations may result in the impaired ability of insulin to suppress lipolysis, resulting in increased free fatty acids (178). Preliminary findings from our lab indicate that in primary human adipocytes, fetuin-A impairs insulin's ability to inhibit lipolysis (personal communication). Resistance to the suppressive effect of insulin on the production of VLDL in the liver is another postulated defect in insulin-resistant states (179). This may additionally lead to increased production

of apolipoprotein B-containing very low density lipoprotein (VLDL), IDL and LDL in MetS and insulin resistance. Ganji *et al* suggest that by inhibiting DGAT, niacin decreases triglyceride synthesis resulting in increased apoB degradation and subsequent decreased secretion of VLDL/LDL particles (169). Aktories *et al* on the other hand have shown niacin inhibited adenylate cyclase activity in adipocytes resulting in reduced concentrations of c-AMP, which in turn regulates the activation of hormone sensitive lipase; an enzyme that controls the adipose tissue lipolysis (180).

Adipose tissue, in addition to being a fat storage organ, secretes a number of hormones and proteins collectively termed adipokines, including adiponectin, leptin, interleukin-6 (IL-6), C-reactive protein (CRP), and TNF-α. Thoel *et al* have demonstrated an anti-atherogenic effect of extended-release niacin in MetS by improving endothelial function and decreasing vascular inflammation. They have suggested that extended-release niacin therapy affects a regression of carotid intimal medial thickness and improvement in metabolic parameters (increased HDL and reduced triglycerides) (181). Though the mechanisms by which niacin is able to affect this change are not entirely understood, Benjo *et al* (182) suggests that, extended release niacin treatment results in improvements in flow-mediated dilatation. Mori *et al* have found that fetuin-A levels are positively correlated with arterial stiffness in healthy subjects (183).

The plasma concentrations of C-reactive protein start rising within a few hours of onset of pain continuing to rise over 1-3 days with a magnitude related to the degree of tissue injury and begin to fall 4-6 days after acute myocardial infraction (AMI) (184). The plasma concentrations of α_1-acid glycoprotein rise after AMI, with peak concentrations occurring 8-10 days after MI (185). Mathews *et al* have shown that in patients with AMI, fetuin-a/α_2-HSglycoprotein concentrations fall with the onset of pain and are significantly increased in matched-pair patient sample in the recovery phase (186). In this study we have demonstrated a significant decrease in CRP levels followed by the Niaspan treatment. We also saw a negative correlation of CRP serum fetuin-A in individuals with MetS before Niaspan treatment. Fetuin-A has been identified as a negative acute phase protein (120). Previously, we have shown that with acute myocardial infarction, serum concentrations of fetuin-A are significantly decreased. Additionally, the kinetics of fetuin-A are in contrast to those observed for positive acute phase reactants, such as C-reactive protein and alpha1-acid glycoprotein.

Our study also shows an increase in adiponectin levels (total, LMW and HMW), an atheroprotective substance after extended-release Niaspan. Similar results were observed by Westphal *et al*, who report that short-term treatment with extended-release niacin causes a pronounced increase in adiponectin but fails to improve atheroprotective functions attributed to adiponectin, such as insulin sensitivity, anti-inflammation and endothelial function (187). However, our studies indicate an increase in high molecular weight adiponectin (HMW). This suggests that at least part of the cardioprotective

benefits of niacin may be attributed to a shift in the HMW/LMW adiponectin ratio in obese men with the metabolic syndrome (188)

In our study, we observed a significant decrease in serum IL-6 with Niaspan treatment in individuals with MetS. A recent comprehensive review that took account of a range of *in vitro, in vivo*, and genetic studies, together with studies on diverse tissues such as liver, muscle, adipose tissue, and pancreas, concluded that chronically elevated IL-6 may indeed contribute to development of type 2 diabetes via mechanisms including altered insulin signaling in hepatocytes/adipocytes and effects on the central nervous system to impair energy regulation (182). Additionally, high IL-6 may also drive hepatic fatty acid synthesis and cause endothelial dysfunction (182). In the present study, TNF-α concentrations were not significantly altered ($p = 0.095$) with Niaspan treatment. This is consistent with studies by Muller *et al* who demonstrate that impaired glucose tolerance was associated with increased serum concentrations of interleukin 6 and co-regulated acute-phase proteins but not with TNF-α or its receptors (189). Our findings that Niaspan treatment improves inflammatory markers are in contrast to the findings of Westphal *et al* (187), who show that 6 weeks Niaspan treatment did not alter CRP, IL-6, or TNF-α. Conversely, the decrease in inflammatory markers observed in this study may partly be due to administration of aspirin, an anti-inflamatory drug, 30 minutes to 1 hour before Niaspan treatment, to reduce flushing (190). However, fetuin-A or phosphorylated fetuin-A levels were not correlated these inflammatory markers either before or after Niaspan treatment.

In our study, we observed that Niaspan treatment significantly increased serum insulin concentrations. HOMA values, though not significantly different, tended to increase with Niaspan treatment ($p = 0.09$). These findings confirm several earlier findings that show a decrease in insulin sensitivity and an increase in glucose levels (191,-193) The increase in insulin concentrations may be explained by the pharmacokinetics of extended-release niacin. For example, extended-release niacin has been shown to reduce adipose tissue lipolysis for at least 4 hours after administration. This is followed by a 3-fold rebound in lipolysis for up to 5 hours as niacin levels diminish in the blood. It is possible that the increase in serum insulin concentrations may be attributed to the increase in serum NEFAs as niacin concentrations diminish in the blood because serum NEFAs would be expected to increase hepatic glucose production and reduce hepatic and skeletal muscle glucose oxidation. Though NEFA levels are not altered with Niaspan treatment in our studies, possibly because of the kinetics of the NEFA rebound, we observed a novel and strong positive correlation of the percent change in serum NEFA concentrations with the percent change in serum fetuin-A following Niaspan treatment. Additionally, in this study, we show that Niaspan treatment significantly increases serum cortisol levels. Since elevated cortisol, and low sex-steroid and growth hormone secretions, probably direct storage fat to visceral depots, these hormonal abnormalities most likely at least contribute to the creation of insulin resistance with additional effects of elevated fatty acids from central fat depots, which are sensitive to lipid mobilization agents. Interestingly, we have observed that the percent change in serum cortisol levels with Niaspan treatment were tightly correlated with the percent change in serum fetuin-A and phosphorylated fetuin-A concentrations. This is consistent

with the data from our laboratory and previous studies (152) showing that both dexamethasone and endogenous glucocorticoids upregulate fetuin-A gene expression and serum concentrations of fetuin-A and phosphorylated fetuin-A. Further, the percent change with Niaspan treatment in cortisol levels were significantly correlated with the percent change in serum NEFA concentrations. *In vitro* studies have shown the regulation of desnutrin gene expression by dexamethasone, suggesting that glucocorticoids could mediate the increase in desnutrin mRNA during fasting. Villena *et al* (194) have shown that ectopic overexpression of desnutrin acts as a lipase in adipose tissue and increases triglyceride hydrolysis in the cell, which may in turn lead to increased efflux of free fatty acids from adipose tissue and again give an evidence of increased insulin resistance after Niaspan intervention. These data suggest that the worsening of insulin sensitivity may be associated with multiple mechanisms, including elevated cortisol levels, fetuin-A and the NEFA rebound.

Our study has several limitations. The first is that the sample size is of this study group is rather small. BMI, waist circumference, waist to hip ratio, percent body-fat, android, and gynoid fat distribution, etc., were not associated with fetuin-A. It is possible that this is because of the small sample size. Previously, the Heart and Soul study, examining 711 individuals with MetS, demonstrated significant association with the components of MetS in the higher quartiles (195). Another limitation of this study is the absence of a placebo-control group. Also, this study would have benefited if subjects with normal BMI were included as lean controls. Further studies are therefore required

to elucidate the significance and role of phorphorylated fetuin-A with these markers of obesity and atherogenic lipid profile in MetS. Among the earliest described biological effect of fetuin-A is the inhibition of the insulin receptor tyrosine kinase activity (IR-TKA) (21, 22, 36, 44). The importance of phosphorylation was suggested by Kalabay *et al* (43). They demonstrated that recombinant fetuin-A produced in insect cells was phosphorylated and to possess the connecting peptide between the A and the B chains. Additionally, the authors show that the phosphorylated fetuin-A inhibits insulin-stimulated IR autophosphorylation and IR-TK, while fetuin-A purified from normal human plasma was not phosphorylated and unable to inhibit IR autophosphorylation and TK activity. Further, the connecting peptide was clipped, suggesting that biological activity of this protein may be associated with its single chain form together with its phosphorylation.

Recently, Haglund *et al* (41) demonstrated that circulating human plasma fetuin-A was phosphorylated (approximately 20%), implying that the effects of phosphorylated fetuin-A glycoprotein on insulin signal transduction seen in different cell systems could be relevant to its physiological function *in vivo*. They have also shown that human fetuin-A is phosphorylated on serine120 located in the A-chain and on serine312 located in the connecting peptide. We have therefore characterized the effect of wild type and phospho-defective Ser312Ala fetuin-A in the inhibition of insulin action. By using site-directed mutagenesis, the 312 serine residue was mutated to alanine (Ser312Ala-fetuin-A) and the functionality of the construct was tested by transfecting wild type and Ser312Ala-fetuin

A into COS7 kidney cells. While the wild type fetuin-A cDNA construct synthesized and secreted phosphorylated (Ser312) fetuin-A, the Ser312Ala mutant which was synthesized and secreted fetuin-A which was devoid of phosphorylation. Srinivas *et al* have suggested that recombinant fetuin-A produced from insect cells using the baculovirus system was 100 times more potent than serum fetuin-A, suggesting that phosphorylation is critical for insulin's mitogenic effects (22). Similarly we treated HIRcB cells with recombinant wild type fetuin-A and Ser312Ala-fetuin-A produced from CHO purification. The wild type fetuin-A was found to be phosphorylated while no phosphorylation was detected on Ser312Ala-fetuin-A. Also in parallel to the results shown by Srinivas et al, only wild type fetuin-A have been found to inhibit the phosphorylation of MAPK considerably at 3.2 (μM). Further in contrast with the results presented by Srinivas *et al* 3.2 (μM) concentrations we have also demonstrated the considerable inhibition of Akt and glucose uptake by L6-GLUT4myc cells. While at the same concentration Ser312Ala-fetuin-A could only partially inhibit the MAPK and Akt phosphorylation.

In summary, this study provides evidence for the first time that serum total- and phosphorylated fetuin-A (Ser312) levels are amenable to intervention by extended-release niacin treatment and are correlated with changes in serum triglycerides. Results from mutational analyses indicate that phosphorylation on Ser312 site of fetuin-A is only partially required for its inhibitory activity, suggesting that phosphorylation on Ser120 may be important. These findings are consistent with earlier findings that fetuin-A may

be involved in lipid metabolism and modulation of inflammatory pathways. The increase in serum cortisol following niacin treatment may also provide further insight into potential mechanisms by which niacin reduces insulin sensitivity. Additionally, these observations offer insights into potential mechanisms and targets for the prevention and treatment of MetS particularly dyslipidemia.

CHAPTER 6: CONCLUSION

A substantial increase in the incidence of obesity and its related metabolic disorders has been observed, primarily due to an increased consumption of a diet rich in saturated fats and refined sugar, coupled with a sedentary lifestyle. Obesity and type 2 diabetes represent a serious threat to the health of the population of almost every country in the world. According to World Health Organization and United Nations, approximately 171 million people were afflicted with type 2 diabetes mellitus in 2005 and this is predicted to increase to 366 million by 2030. Alabama leads the nation with over 440,000 cases of diabetes, resulting in an estimated $3 billion dollars annually in health care costs in Alabama alone. Metabolic syndrome is associated with an increased risk of cardiovascular disease, which is ultimately responsible for a considerable proportion of diabetic mortality. Various novel physiological regulators of insulin action, including TNF-alpha, leptin, resistin adiponectin, free fatty acids, protein tyrosine phosphatase1B have been shown to influence whole body insulin sensitivity and insulin action, suggesting a potential role of these molecules in the development of insulin resistance and diabetes.

Fetuin-A, a liver secreted glycoprotein, is a humoral factor that has been implicated in the modulation of insulin sensitivity. Phosphorylation status of fetuin-A has

been shown to be critical for its inhibitory activity. Human data demonstrate that circulating levels of total fetuin-A positively correlate with BMI, insulin resistance, fatty liver, and an atherogenic blood profile. Furthermore, the gene for fetuin-A is localized on chromosome 3q27, a locus that has been shown to be associated with metabolic syndrome and the development of type 2 diabetes. Human fetuin-A is phosphorylated on serine120 located in the A-chain and on serine312 located in the connecting peptide. While previous human studies have investigated the associations of fetuin-A in disease conditions, there are no reports on the phosphorylation status of fetuin-A and its association with insulin resistance, alterations lipid profile and markers of inflammation in MetS in humans. Also, there are no reports on the molecular characterization of the Ser312 phosphorylation site of fetuin-A.

Therefore, the goal of the present study was to examine alterations in serum total fetuin-A and phosphorylated (Ser312) fetuin-A concentrations in individuals with metabolic syndrome treated with Niaspan, a lipid-lowering drug, and correlate these with changes in lipid metabolism, insulin sensitivity and inflammation. Additionally, to characterize the significance of the fetuin-A-Ser312 phosphorylation site, we have examined the effects of both wild-type and mutant Ser312Ala-fetuin-A (defective in phosphorylation on Ser312) in the inhibition of insulin signaling, and in the uptake of glucose into skeletal muscle cells.

Our findings suggest the involvement of fetuin-A in the regulation of lipid metabolism and inflammatory pathways. We demonstrate, for the first time, that serum

total and phosphorylated fetuin-A concentrations are amenable to therapeutic intervention by Niaspan. While, phosphorylation on fetuin-A has been shown to be critical, our studies on the molecular characterization, indicate that phosphorylation on Ser312 is only partially required for fetuin-A's physiological effects, suggesting that Ser120 may also be critical. These observations offer critical insights into potential mechanisms of fetuin-A action and suggest strategies for lowering fetuin-A and phosphorylated fetuin-A, which are associated with dyslipidemia in MetS. Additionally, this opens new avenues for future research as several other intervention studies have shown that liver fat can be decreased by weight loss, PPARgamma agonists, and insulin therapy.

Table 2: Association of anthropometric measurements with fetuin-A and phosphofetuin-A in MetS subjects before (baseline) and after treatment with Niaspan

Variables	*MetS (n=15)*	*Fetuin-A*		*Phosphofetuin-A*	
		MetS (r value)	*Niaspan (r value)*	*MetS (r value)*	*Niaspan (r value)*
Age (years)	*45.5 ± 7.5*	*-0.37*	*-0.06*	*-0.14*	*0.06*
BMI (kg/m²)	*34.0 ± 3.15*	*-0.06*	*0.21*	*-0.28*	*-0.13*
Waist circumference (cm)	*107.9 ± 7.96*	*0.06*	*0.05*	*-0.09*	*-0.05*
WHR	*0.95 ± 0.04*	*0.05*	*-0.03*	*-0.03*	*-0.01*
Systolic BP (mm Hg)	*128.9 ± 13.7*	*-0.52**	*-0.30*	*-0.32*	*-0.25*
Diastolic BP (mm Hg)	*82.7 ± 8.3*	*-0.18*	*-0.31*	*-0.20*	*-0.30*

Data expressed as mean ± SD; BMI – Body mass index, WHR-Waist hip ratio, BP- Blood pressure; 'r value' represents Pearson product-moment correlation coefficient; * $P < 0.05$.

Table 3: Association of serum lipid concentrations with fetuin-A and phosphofetuin-A in MetS subjects before (baseline) and after-treatment with Niaspan

Variables	*MetS*	*Niaspan*	*Fetuin-A*		*Phosphofetuin-A*		*Fetuin-A % Change from baseline (r value)*	*Phosphofetuin-A % Change from baseline (r value)*
			MetS (r value)	*Niaspan (r value)*	*MetS (r value)*	*Niaspan (r value)*		
Total cholesterol (mg/dl)	*226.1 ± 31.6 (n=15)*	*209.4 ± 35.4* (n=15)*	*-0.35*	*0.34*	*-0.03*	*0.34*	*0.15*	*0.25*
LDL cholesterol (mg/dl)	*135.2 ± 33.2 (n=13)*	*126.4 ± 28.7 (n=13)*	*-0.59**	*0.23*	*-0.48*	*0.28*	*-0.15*	*0.06*
HDL cholesterol (mg/dl)	*39.9 ± 8.01 (n=15)*	*46.3 ± 8.9* (n=15)*	*-0.41*	*-0.37*	*-0.26*	*-0.11*	*-0.57**	*-0.58**
Triglyceride (mg/dl)	*292.9 ± 142.9 (n=15)*	*185.1 ± 66** (n=15)*	*0.42*	*0.59**	*0.69***	*0.35*	*0.62**	*0.39*
NEFA (mM)	*0.53 ± 0.125 (n=15)*	*0.46 ± 0.1 (n=15)*	*0.06*	*0.13*	*0.12*	*0.107*	*0.52**	*0.42*

Data are expressed as mean ± SD; LDL – Low density lipoprotein, HDL- High density lipoprotein, NEFA- Non esterified fatty acids;'r value' represents Pearson product-moment correlation coefficient; * $P < 0.05$, ** $P < 0.01$.

Table 4: Association of markers of insulin sensitivity with fetuin-A and phosphofetuin-A in MetS subjects treated with Niaspan

Variables	MetS	Niaspan	Fetuin-A		Phosphofetuin-A		Fetuin-A % Change from baseline (r value)	Phosphofetuin-A % Change from baseline (r value)
			MetS (r value)	Niaspan (r value)	MetS (r value)	Niaspan (r value)		
Insulin (µU/ml)	15.6 ± 11.9 (n=15)	18.9 ± 10.8* (n=15)	-0.43	0.32	-0.29	0.05	-0.23	-0.39
Glucose (mg/dl)	102.9 ± 25.7 (n=15)	103.6 ± 17 (n=15)	0.24	-0.14	0.11	-0.01	0.01	-0.25
HOMA	3.9 ± 2.85 (n=14)	4.57 ± 2.7 (n=14)	-0.39	0.19	-0.25	-0.04	-0.25	-0.43
Cortisol (µg/dl)	14.06 ± 7.7 (n=15)	21.5 ± 9.2* (n=15)	0.28	0.27	0.42	0.61*	0.78***	0.72**
Adiponectin (HMW) (µg/ml)	2.40 ± 0.88 (n=15)	3.87 ± 1.3*** (n=15)	0.44	0.10	0.12	0.11	-0.15	-0.17
Adiponectin (LMW) (µg/ml)	3.37 ± 0.92 (n=13)	4.48 ± 1.5** (n=13)	0.42	0.08	0.16	0.20	-0.21	-0.39
Adiponectin (Total) (µg/ml)	5.73 ± 5.29 (n=14)	8.4 ± 2.7*** (n=14)	0.44	0.09	0.14	0.16	-0.16	-0.29

Data are expressed as mean ± SD; 'r value' represents Pearson product-moment correlation coefficient; HMW – High molecular weight Adiponectin, LMW- Low molecular weight Adiponectin; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$.

Table 5: Association of inflammatory markers with fetuin-A and phosphofetuin-A in MetS subjects before (baseline) and after treatment with Niaspan

Variables	*MetS*	*Niaspan*	*Fetuin-A*		*Phosphofetuin-A*		*Fetuin-A % Change from baseline (r value)*	*Phosphofetuin-A % Change from baseline (r value)*
			Mets (r value)	*Niaspan (r value)*	*MetS (r value)*	*Niaspan (r value)*		
C-Reactive Protein (mg/l)	*45.46 ± 32.4 (n=14)*	*18.71 ± 20.9** (n=14)*	*-0.57**	*-0.30*	*0.06*	*0.08*	*0.58**	*0.45*
TNF-α (pg/ml)	*44.8 ± 30.0 (n=12)*	*69.9 ± 52,4 (n=12)*	*0.16*	*-0.29*	*0.27*	*-0.36*	*-0.17*	*-0.42*
IL-6 (pg/ml)	*151.61 ± 67.8 (n=15)*	*58.65 ± 29.3** (n=15)*	*0.10*	*0.14*	*0.16*	*-0.19*	*0.04*	*0.11*

Data are expressed as mean ± SD; TNF-α – Tumor necrosis factor, IL-6- Interleukin-6; 'r value' represents Pearson product-moment correlation coefficient; * $P < 0.05$, ** $P < 0.01$.

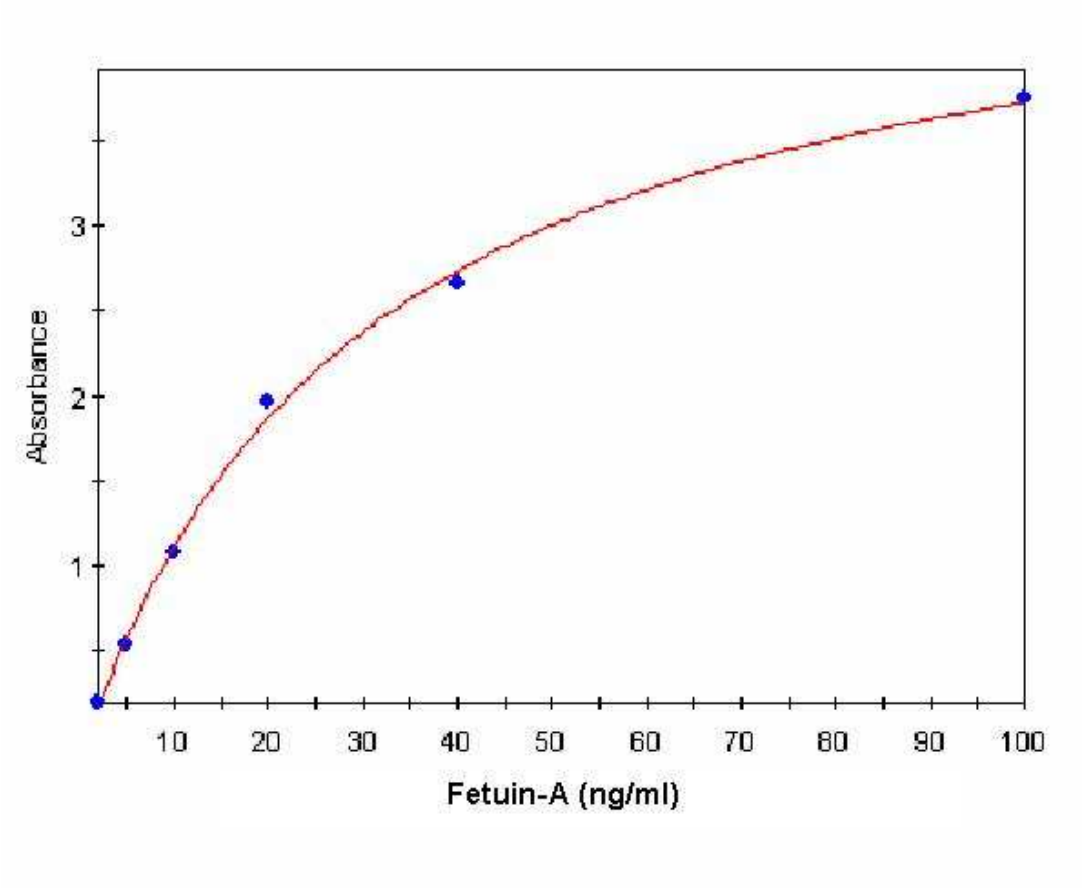

Fig.5. Fetuin-A standard curve. Serum fetuin-A levels were assayed by a sandwich ELISA method (BioVendor LLC, Candler, NC). The antibodies were highly specific for the human fetuin-A protein. The assay had a detection limit of 3.5 ng/ml and an assay sensitivity of 3.5 µg/ml.

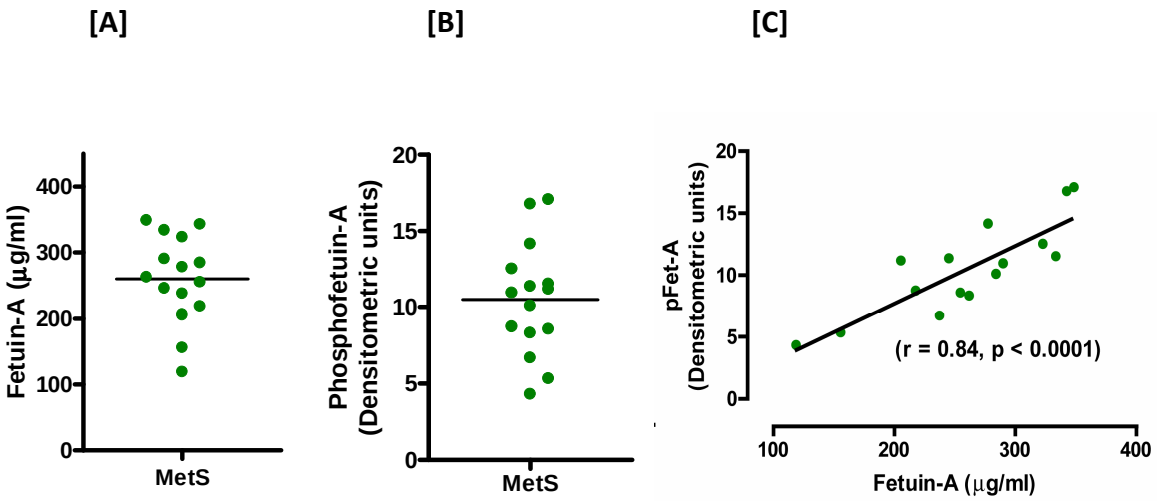

Fig.6. Serum fetuin-A and phosphorylated fetuin-A levels in individuals with metabolic syndrome (MetS). Blood samples were obtained from fifteen individuals who met the NCEP ATP III criteria for MetS. Serum fetuin-A levels were assayed by a sandwich ELISA (enzyme-linked immunosorbent assay) method (BioVendor LLC, Candler, NC). Fetuin-A phosphorylation status (pFet-A) was assayed by Western blot analysis. Serum fetuin-A (A) and phosphorylated fetuin-A concentrations (B) in MetS are depicted as scatter diagrams. Mean values are indicated by a horizontal line. Correlation of phosphorylated fetuin-A with fetuin-A was determined using Pearson product-moment correlation coefficient (C).

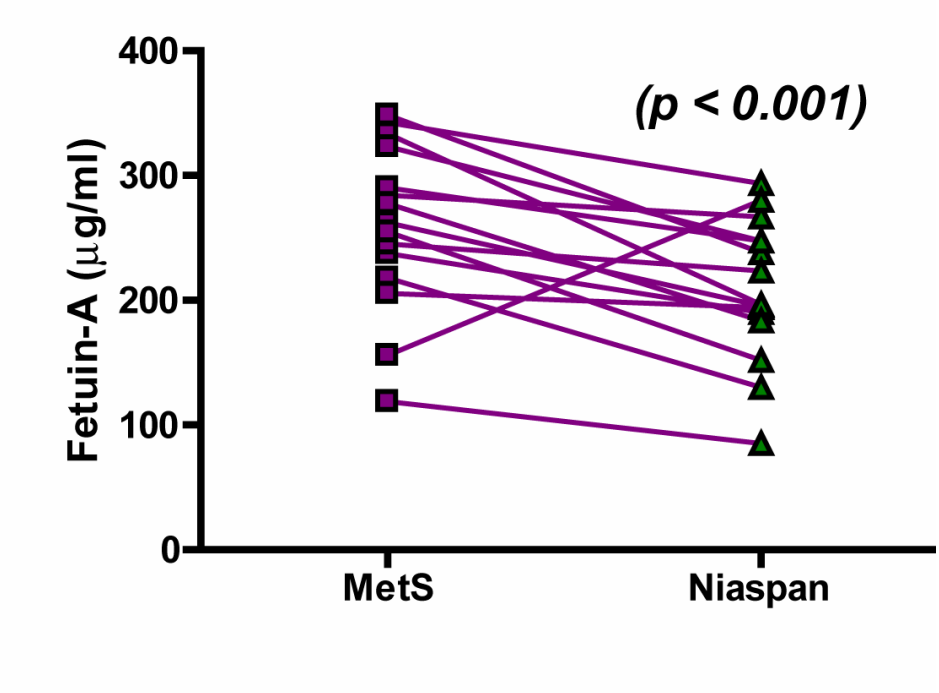

Fig.7: Serum fetuin-A concentrations in individuals with metabolic syndrome (MetS) before and after Niaspan treatment. Fifteen individuals who met the NCEP ATP III criteria for MetS were treated with Niaspan for a period of six weeks. Plasma samples were analyzed for fetuin A concentration by using sandwich ELISA (BioVendor LLC, Candler, NC). Values shown are fetuin-A (μg/ml) for each group (n=15).

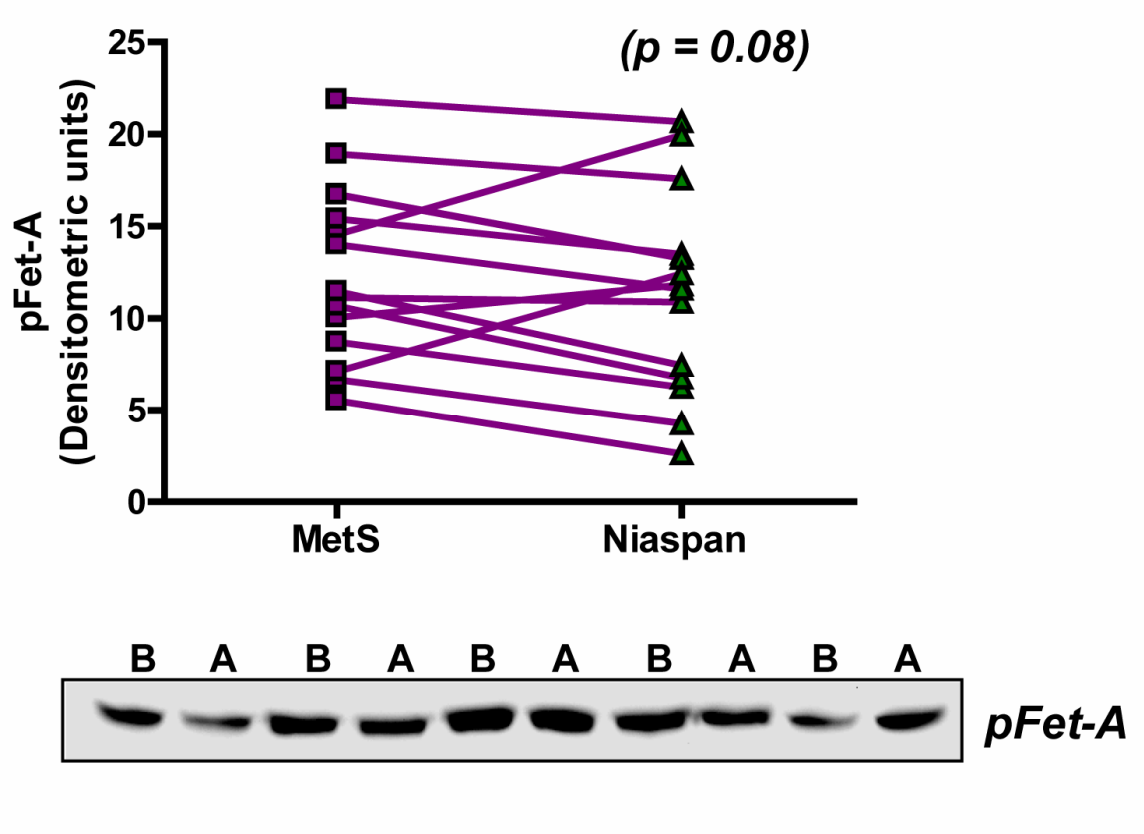

Fig.8: Serum phosphorylated fetuin-A (pFet-A) levels in individuals with metabolic syndrome (MetS) before and after Niaspan treatment. Fifteen individuals who met the NCEP ATP III criteria for MetS were treated with Niaspan for a period of six weeks. Plasma samples diluted 1:100 in saline were separated on 4-20% SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with custom-generated and affinity-purified antibodies specific for human 312Ser-Fetuin-A (Affinity BioReagents, Golden, CO). Data shown are densitometric scan units of changes in phosphorylated fetuin-A with Niaspan treatment for each participant (*top panel*), based on Western blot analysis data (*bottom panel-Representative blot*) (n=15, for each group). B=Before Niaspan treatment, A=After Niaspan treatment

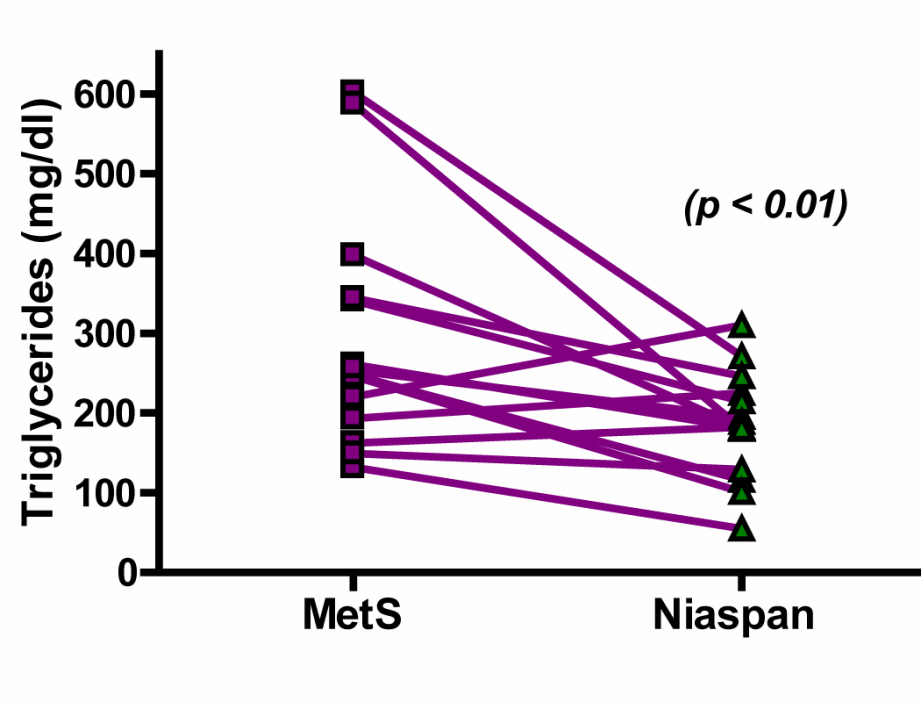

Fig.9: Serum triglyceride levels in individuals with metabolic syndrome (MetS) before and after treatment. Fifteen individuals who met the NCEP ATP III criteria for MetS were treated with Niaspan for a period of six weeks**.** Data shows changes in serum triglyceride concentrations with Niaspan treatment for each participant (n = 15, for each group).

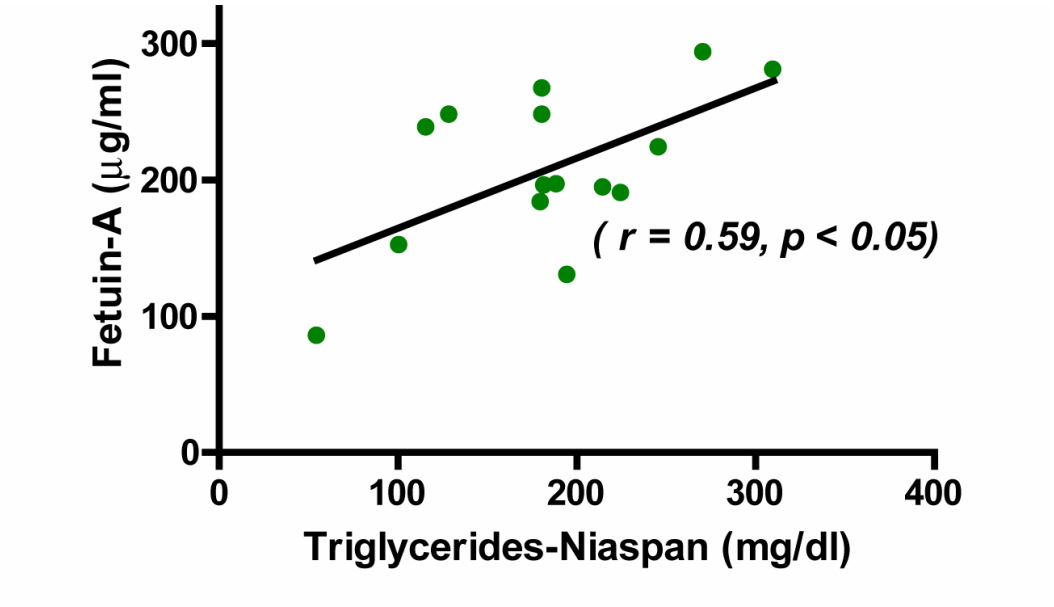

Fig.10: Correlation of fetuin-A with triglyceride levels in metabolic syndrome (MetS) individuals after six weeks of Niaspan treatment. Fifteen individuals who met the NCEP ATP III criteria for MetS were treated with Niaspan for a period of six weeks. Data show the correlation of serum fetuin-A concentrations with serum triglycerides in individuals with MetS following Niaspan treatment. Correlation was determined using Pearson product-moment correlation coefficient (n=15, for each group)

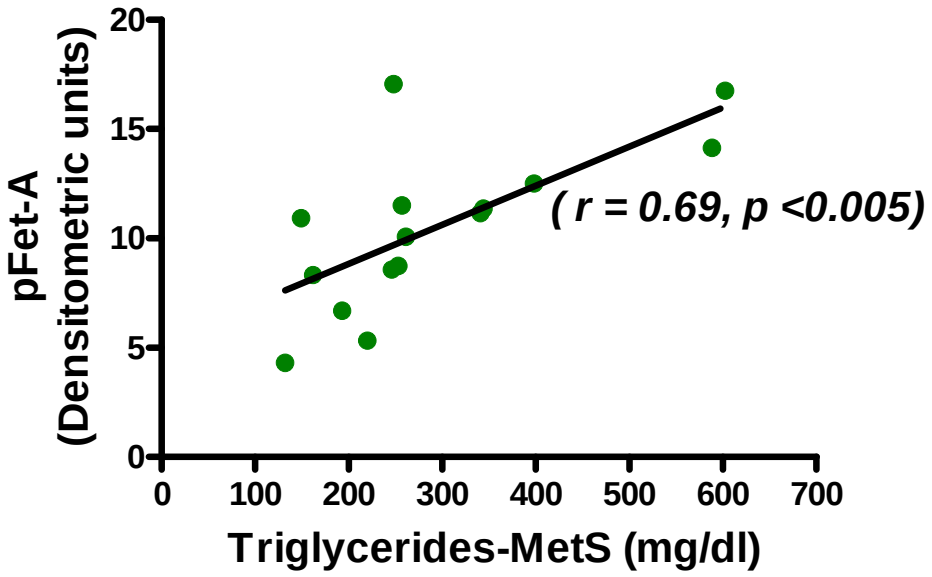

Fig.11: Correlation of phosphorylated fetuin-A with serum triglyceride levels in metabolic syndrome (MetS) individuals before Niaspan treatment. Blood samples were obtained from fifteen individuals who met the NCEP ATP III criteria for MetS. Data show the correlation of phosphorylated fetuin-A concentrations with serum triglycerides in individuals with MetS before Niaspan treatment. Correlation was determined using Pearson product-moment correlation coefficient (n=15, for each group)

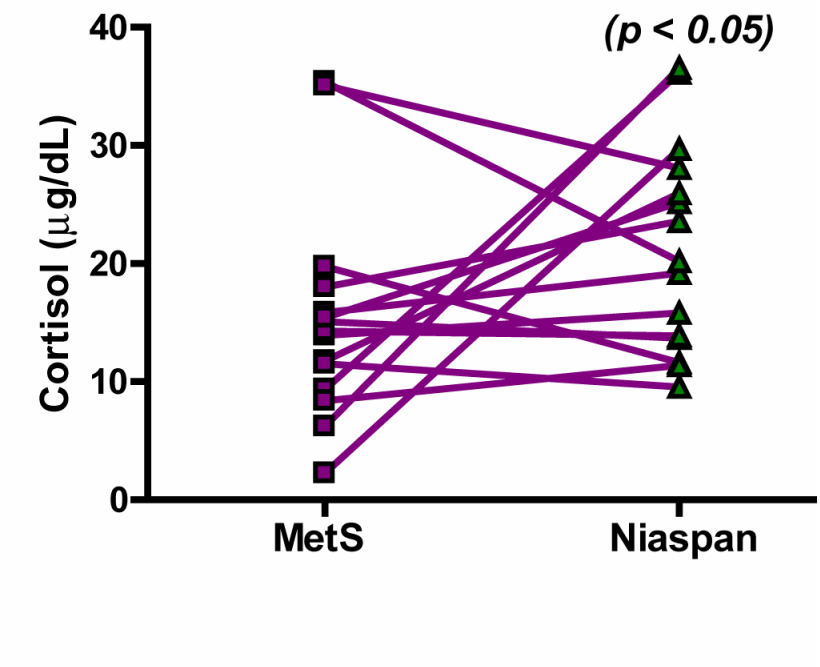

Fig.12: Serum cortisol levels in individuals with metabolic syndrome (MetS) before and after Niaspan treatment. Fifteen individuals who met the NCEP ATP III criteria for MetS were treated with Niaspan for a period of six weeks. Serum samples were analyzed for cortisol concentration by a sandwich ELISA (Diagnostic Systems Laboratories, Inc., TX). Data shows changes in serum cortisol concentrations with Niaspan treatment for each participant (n=15, for each group).

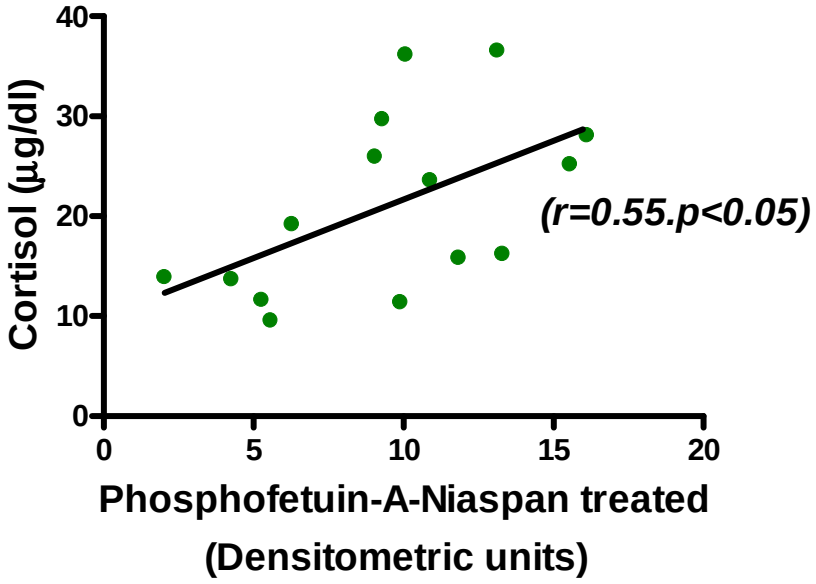

Fig.13: Correlation of phosphorylated fetuin-A with serum cortisol levels in metabolic syndrome (MetS) individuals after Niaspan treatment. Fifteen individuals who met the NCEP ATP III criteria for MetS were treated with Niaspan for a period of six weeks. Data shows the correlation of phosphorylated fetuin-A concentrations with serum cortisol in individuals with MetS after Niaspan treatment. Correlation was determined using Pearson product-moment correlation coefficient (n=14, for each group)

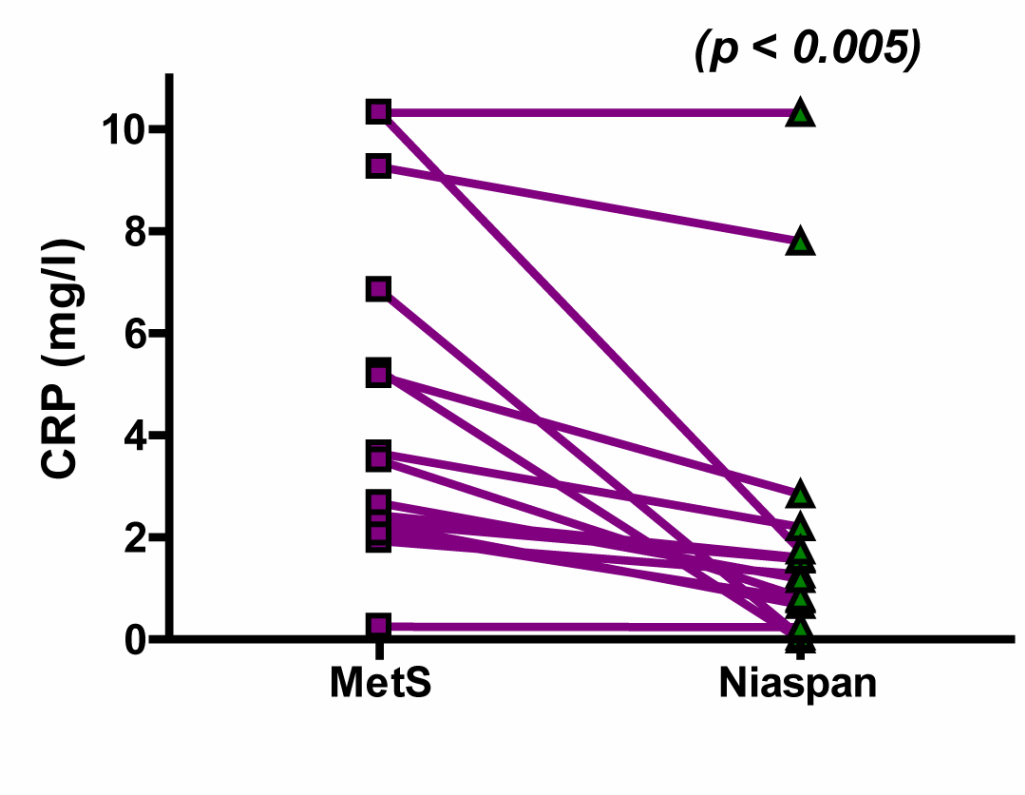

Fig.14: Serum C-reactive protein levels in individuals with metabolic syndrome (MetS) before and after Niaspan treatment. Fifteen individuals who met the NCEP ATP III criteria for MetS were treated with Niaspan for a period of six weeks. Samples were diluted in 1:100 dilution ratio in saline and serum C-reactive protein (CRP) concentrations were determined using sandwich ELISA (United Biotech Inc, Mountain View, CA). Data shows changes in serum CRP concentrations with Niaspan treatment for each participant (n=15, for each group).

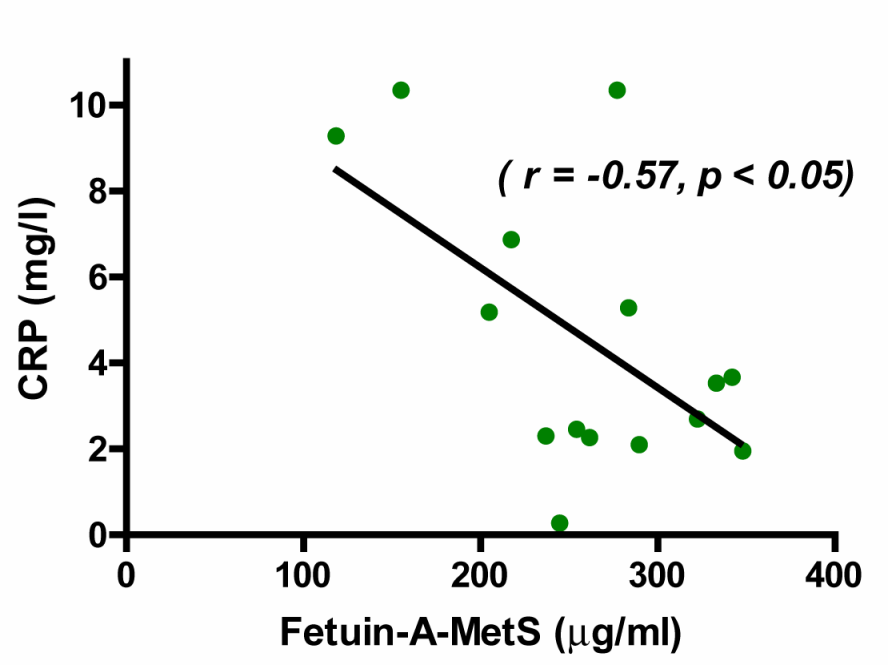

Fig.15: Correlation of serum C-reactive protein levels with serum fetuin-A concentrations in metabolic syndrome (MetS) individuals before Niaspan treatment. Blood samples were obtained from fifteen individuals who met the NCEP ATP III criteria for MetS. Data shows the correlation of serum C-reactive protein (CRP) levels with serum fetuin-A in individuals with MetS before Niaspan treatment. Correlation was determined using Pearson product-moment correlation coefficient (n=14, for each group)

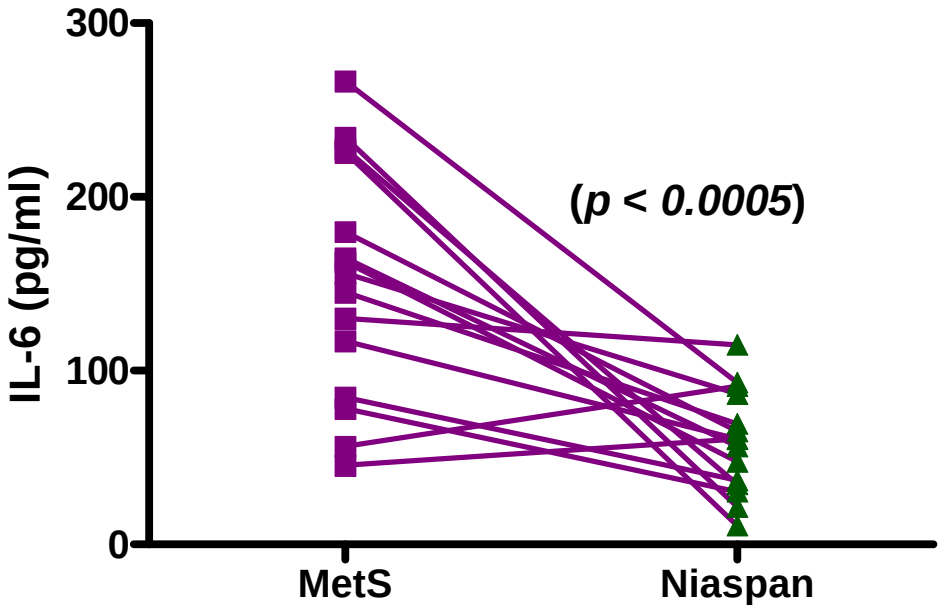

Fig.16: Serum IL-6 levels in individuals with metabolic syndrome (MetS) before and after Niaspan treatment. Fifteen individuals who met the NCEP ATP III criteria for MetS were treated with Niaspan for a period of six weeks. Samples were diluted 1:8 in saline and serum IL-6 concentrations were determined using sandwich ELISA (R&D Systems, Inc., MN). Data shows changes in serum IL-6 concentrations with Niaspan treatment for each participant (n=15, for each group)

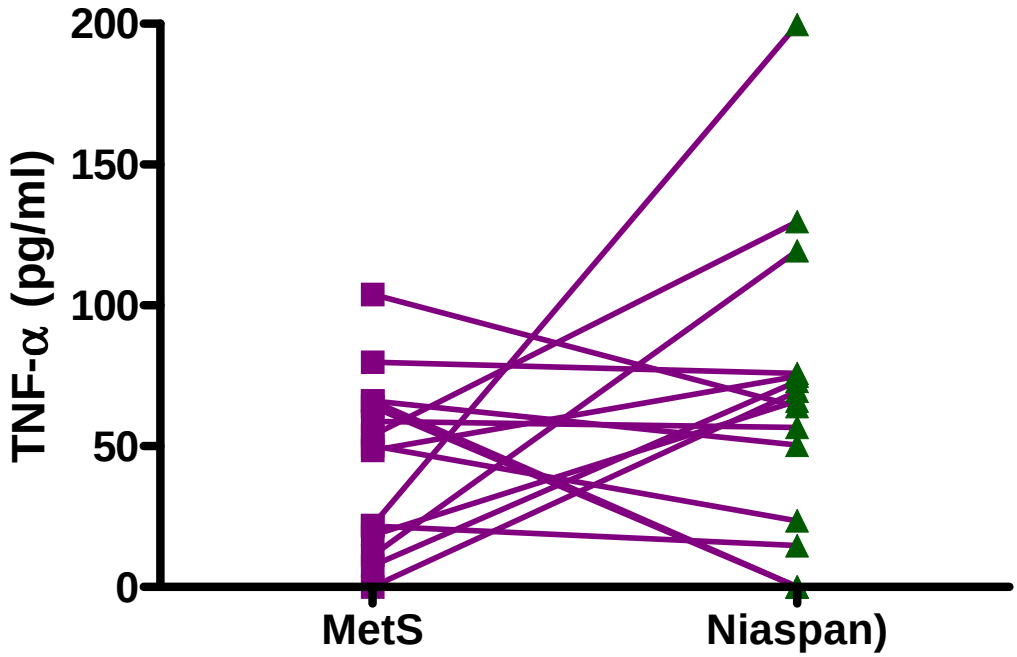

Fig.17: Serum TNF-α levels in individuals with metabolic syndrome (MetS) before and after Niaspan treatment. Fifteen individuals who met the NCEP ATP III criteria for MetS were treated with Niaspan for a period of six weeks. Samples were diluted in 1:8 dilution ratio in saline and serum TNF-α concentrations were determined using sandwich ELISA (R&D Systems, Inc., MN). Data shows changes in serum TNF-α concentrations with Niaspan treatment for each participant (n=15, for each group). No significant alterations were observed.

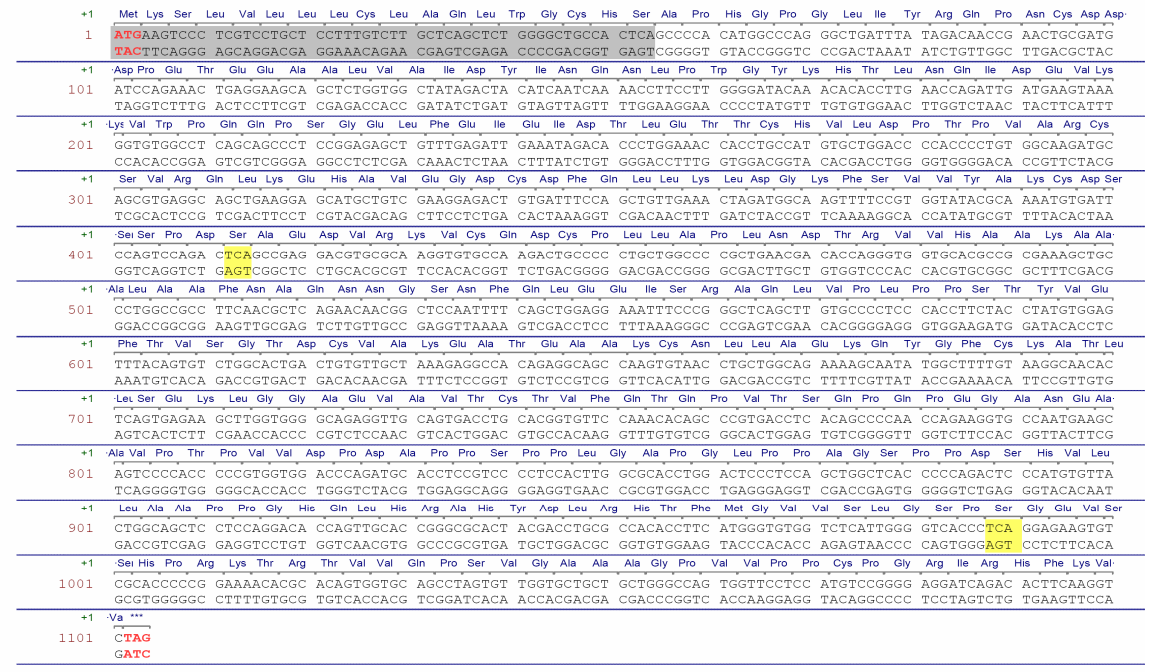

Fig.18: Sequence analysis. Human Fetuin-A cDNA sequence showing the start and stop codons in red, the signal peptide in gray, and Ser120 and Ser312 highlighted in yellow

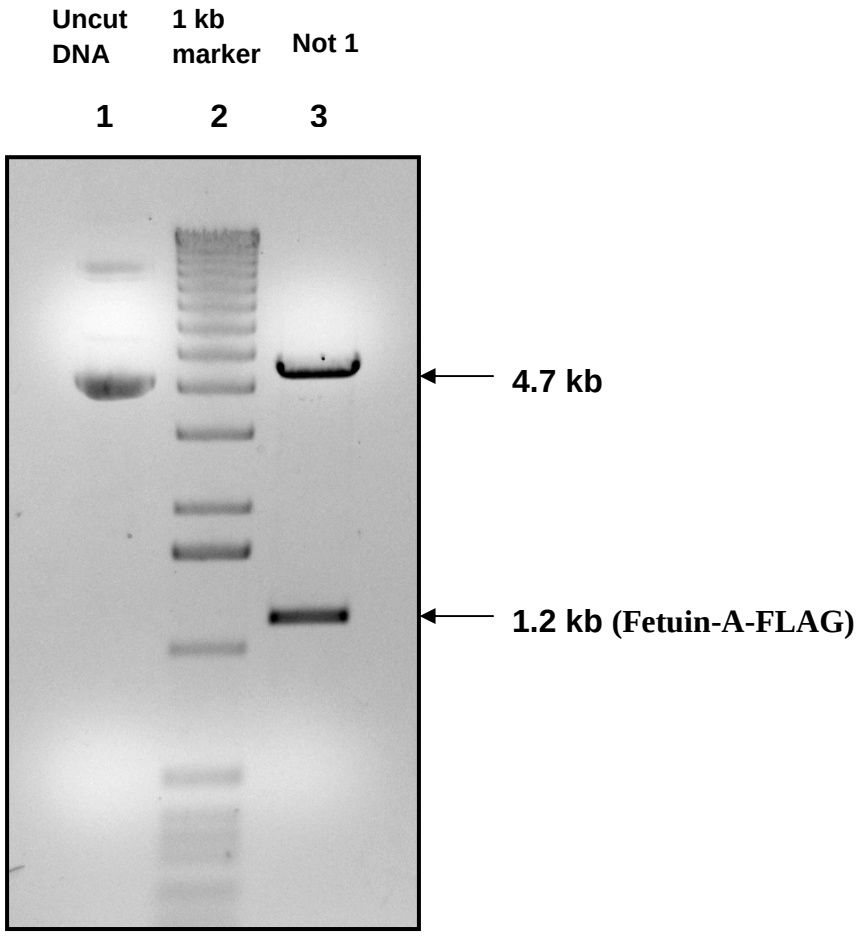

Fig.19: Restriction Analysis. Purified plasmid DNA was incubated with *Not1* restriction enzyme and REact 3 buffer (Invitrogen Corporation, Carlsbad, CA) at 37^{o}C for 6 hours. Undigested DNA (lane 1) and digested DNA (lane 3) were mixed with DNA loading dye and electrophoresed at 100 Volts. The gel was stained with 1% ethidium bromide and visualized under UV transilluminator (UVP LLC, Upland, CA). The 4.7kb DNA band represents the empty vector, pCMV6-XL4 and the 1.2kb Not I fragment represents fetuin-A-3X FLAG cDNA insert.

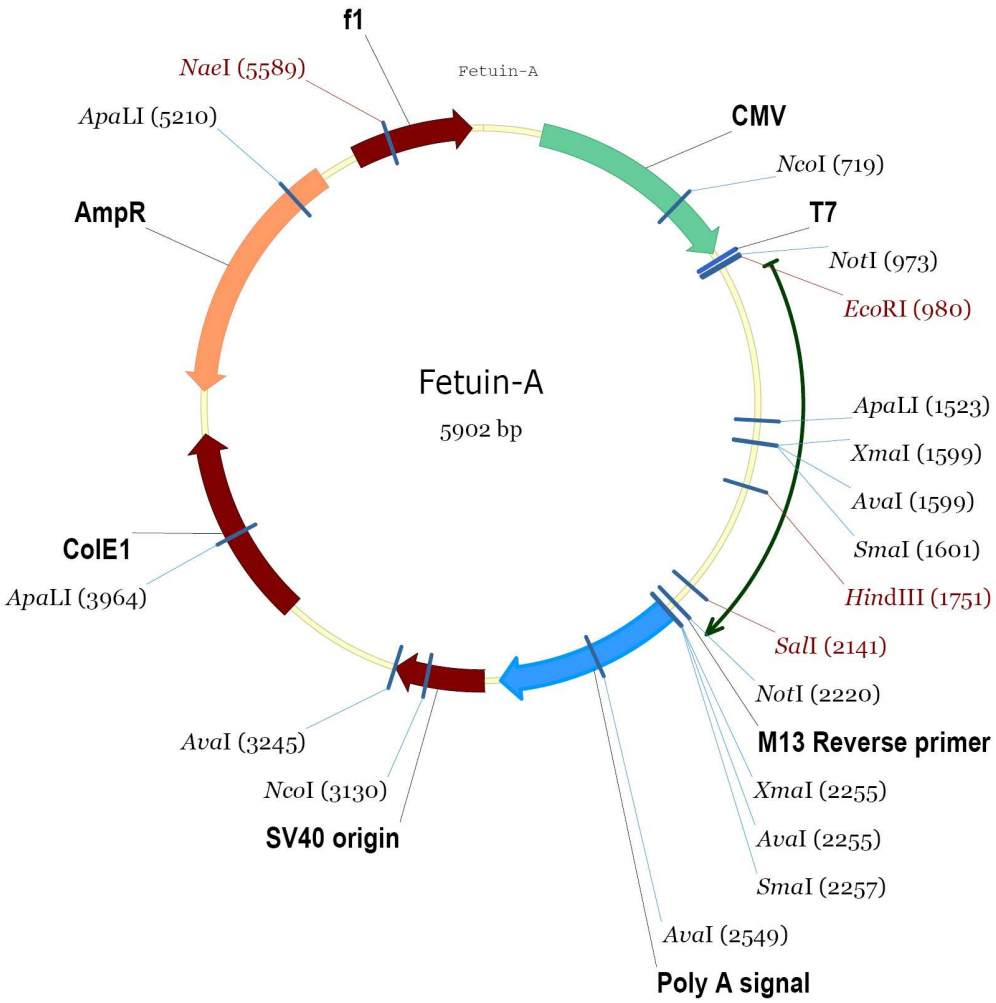

Fig.20: Human Fetuin-A cDNA Construction. Full length human fetuin-A cDNA, cloned in a pCMV6-XL4 vector, was purchased from Origene Inc., Rockville, MD. A 3X-FLAG-tag (FLAG-octapeptide/polypeptide protein tag) was cloned into the C-terminal end of a full-length human fetuin-A cDNA clone. The C-terminus site was specifically chosen for the 3X-FLAG-tag, since the N-terminus of fetuin-A encodes a signal peptide sequence, which is critical for the processing of fetuin-A as a secreted protein. Restriction digestion with Not I generates a 1.2kb insert of fetuin-A -3X FLAG cDNA

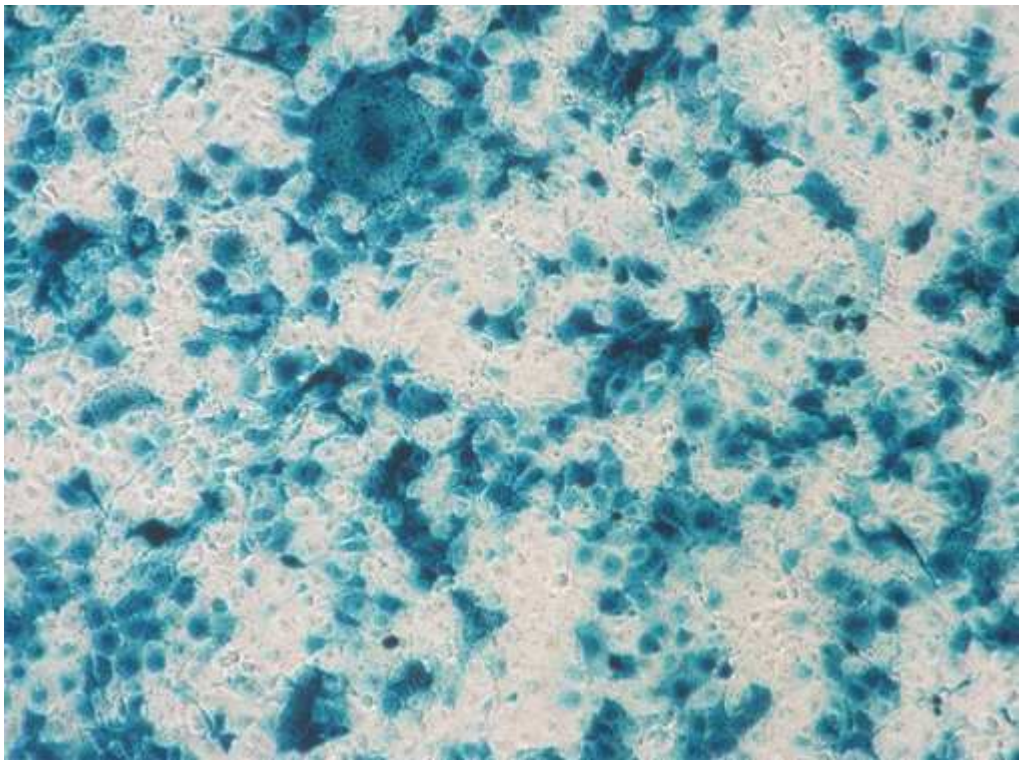

Fig.21: Transfection efficiency in African green monkey kidney cells (COS-7) cells. COS-7 cells were transiently transfected with 2µg of β-galactosidase cDNA. Cells were stained with In-situ β-galactosidase staining kit. Cells taking up the blue stain express β-galactosidase, which is used as an measure of transfection efficiency.

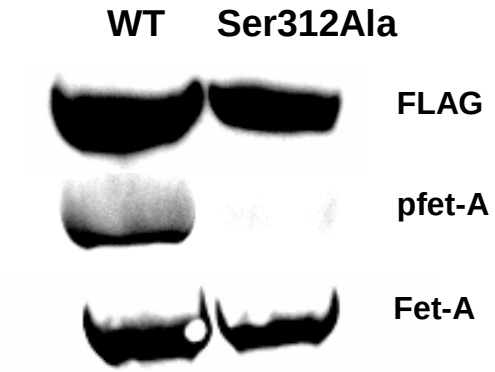

Fig.22: Functional studies: Transfection of COS-7 cells. 4µg of wild type (WT) or Ser312Ala-fetuin-A plasmid DNA was transfected using lipofectamine into COS-7 cells. Twenty four hours after transfection, the media was changed to complete DMEM. Next day, the media was again changed to serum-free DMEM. The following day, the media was collected and stored at -80^{o}C. An aliquot of the media was separated on 4-20% SDS-PAGE, transferred to nitrocellulose membrane, and developed with antibodies against human Ser312-fetuin-A, fetuin-A and FLAG-tag (FLAG-octapeptide/polypeptide protein tag). Western blot analysis indicated that while wild type fetuin-A was phosphorylated, the Ser312Ala fetuin-A was devoid of any phosphorylation.

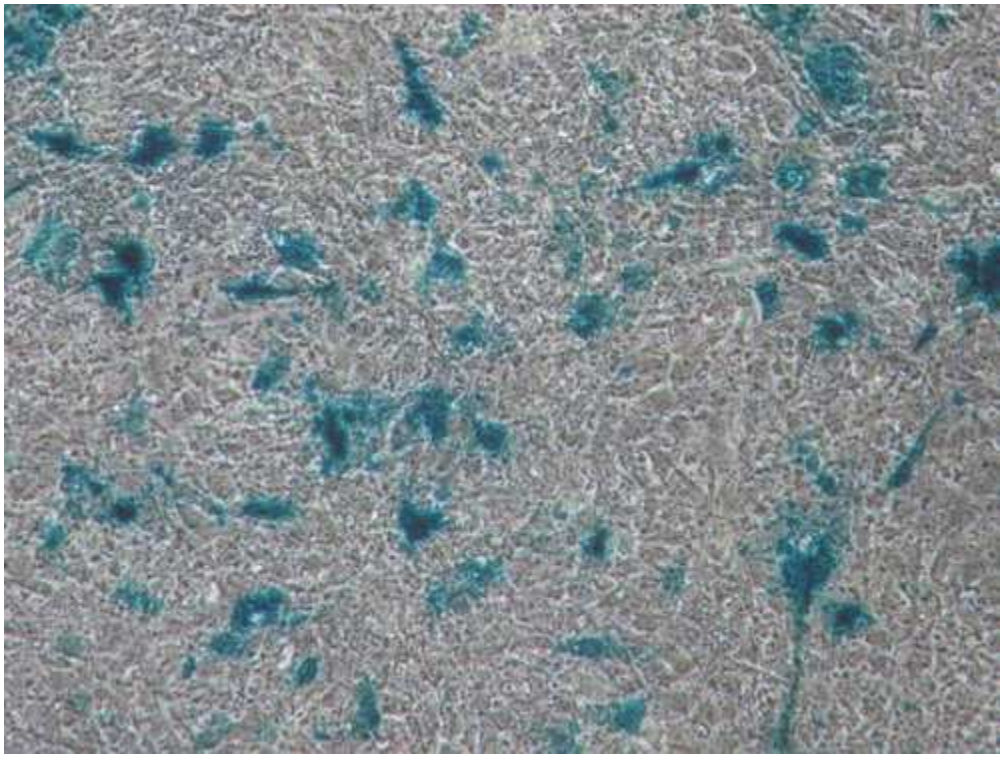

Fig. 23. Transfection efficiency in rat fibroblast (HIRcB) cells. HIRc cells were transiently transfected with 4µg of β-galactosidase cDNA. Cells were stained with In-situ β-galactosidase staining kit. Cells taking up the blue stain express β-galactosidase, which is used as an measure of transfection efficiency.

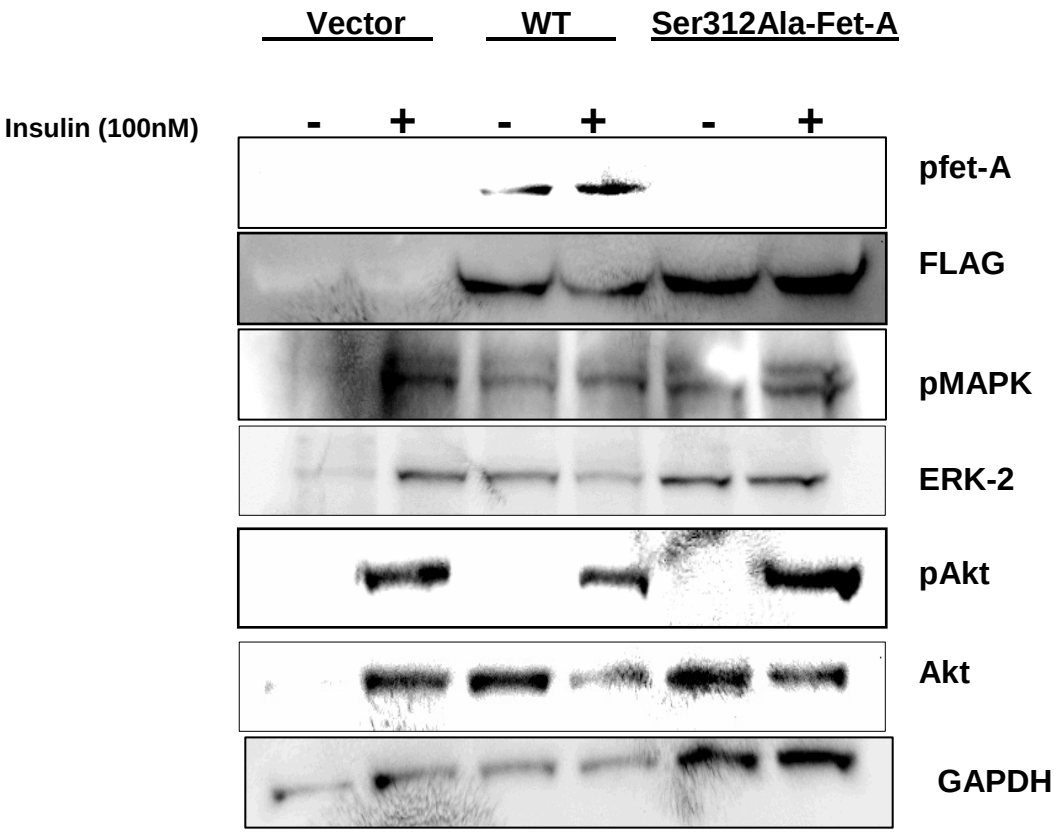

Fig.24: Significance of Ser312Ala-fetuin-A on insulin-stimulated phosphorylation of MAPK and Akt. 4µg of wild type (WT) or Ser312Ala-fetuin-A plasmid DNA was transfected using lipofectamine into rat 1 fibroblast cells overexpressing human insulin receptor (HIRcB). Twenty four hours after transfection, the media was changed to complete DMEM. Next day, the media was again changed to serum-free DMEM. The following day, the media was collected and stored at -80^{o}C. An aliquot of the media was separated on 4-20% SDS-PAGE, transferred to nitrocellulose membrane, and developed with antibodies against human Ser312-Fetuin-A, fetuin-A and FLAG-tag (FLAG-octapeptide/polypeptide protein tag), phosphorylated MAPK and phosphorylated-Akt. ERK-2 and Akt was loaded as a loading control using specific antibody against ERK-2 and Akt. Western blot analysis indicated that while wild type fetuin-A was phosphorylated, the Ser312Ala mutant secreted fetuin-A was devoid of any phosphorylation. Wild type fetuin-A inhibits insulin-stimulated phosphorylation of MAPK and Akt, while Ser312Ala-fetuin-A failed to inhibit the phosphorylation of MAPK and Akt.

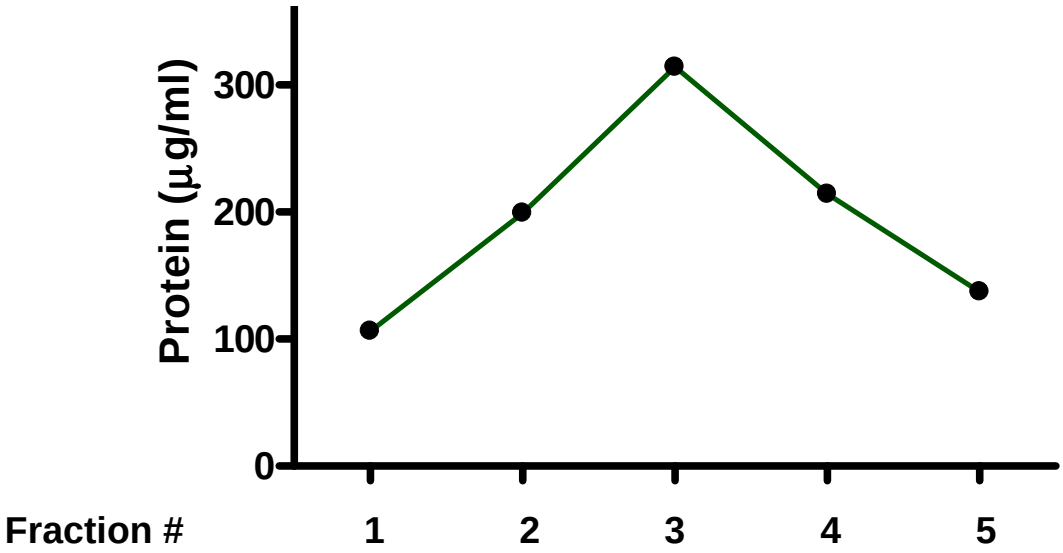

Fig.25: Recombinant human fetuin-A Wild type (WT) purification from CHO-cells. CHO cells were transfected with wild type fetuin-A cDNA using FreeStyle MAX transfection reagent diluted in OptiPro serum-free media (Invitrogen Corporation, Carsbad, CA) and cultured in a spinner-flask. Five days after transfection, the media was collected and purified using agarose bound jacalin column (Vector Laboratories, Burlingame, CA). Fetuin-A was eluted with 0.1 M melibiose in six fractions. Protein concentrations were analyzed using Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Fraction #3, which showed the highest protein concentration, was used for further analyses.

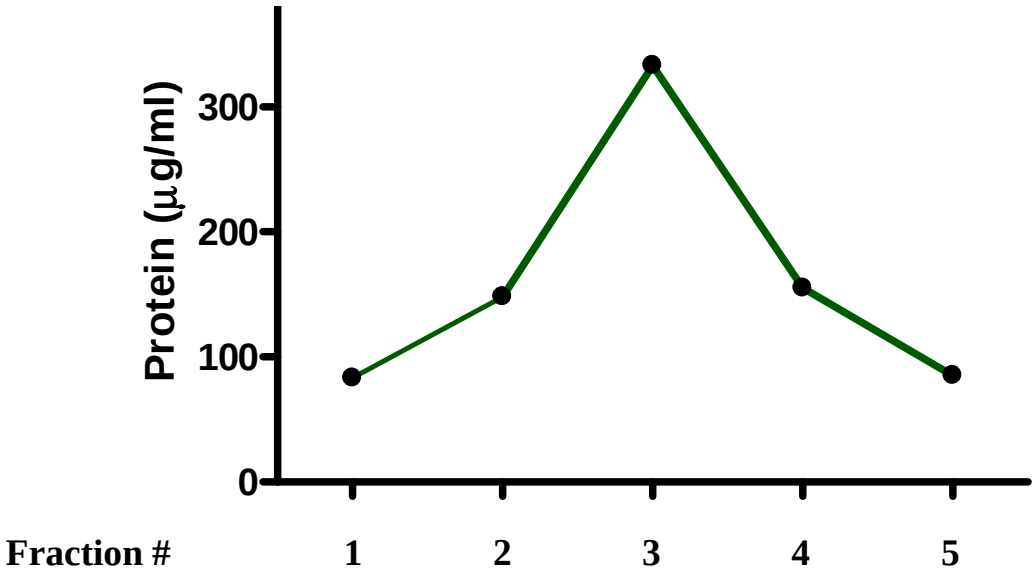

Fig. 26. Recombinant human Ser312Ala-fetuin-A purification from CHO-cells.
CHO cells were transfected with Ser312Ala-fetuin-A cDNA using FreeStyle MAX transfection reagent diluted in OptiPro serum-free media (Invitrogen Corporation, Carsbad, CA) and cultured in a spinner-flask. Five days after transfection, the media was collected and purified using agarose bound Jacalin Column (Vector Laboratories, Burlingame, CA). Fetuin-A was eluted with 0.1 M melibiose in six fractions. Protein concentrations were analyzed using Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Fraction #3, which showed the highest protein concentration, was used for further analyses.

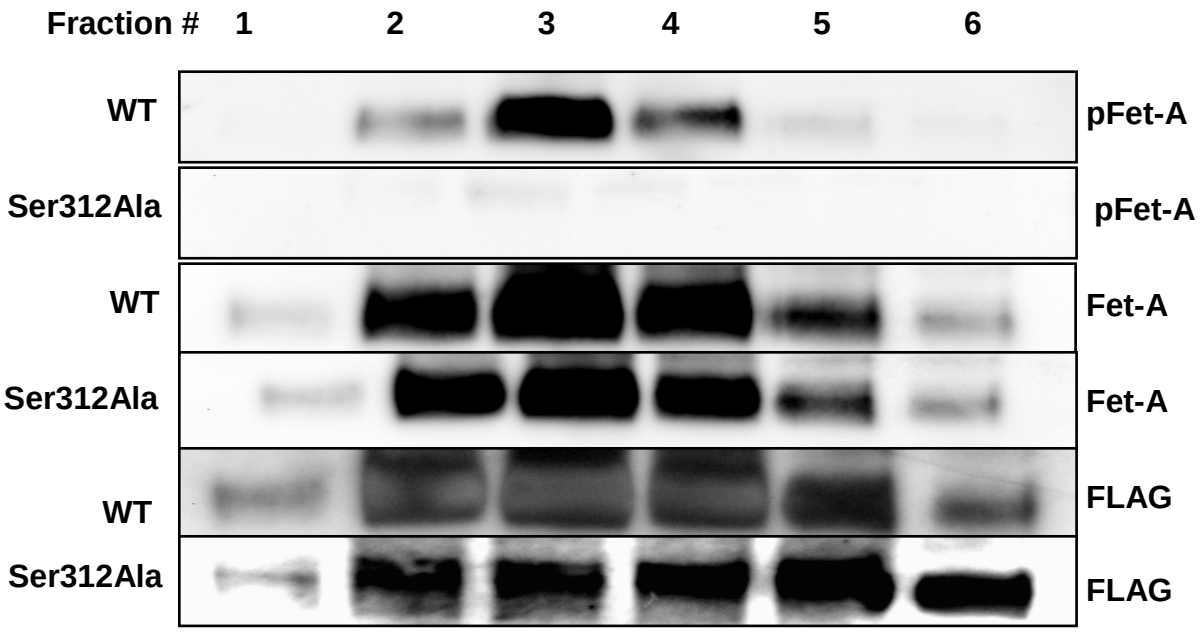

Fig.27: Phosphorylation status of purified recombinant human wild type (WT) and Ser312Ala-Fetuin-A. CHO cells grown in suspension culture in a spinner-flask, were transfected with either wild type or Ser312Ala-fetuin-A. Five days after transfection, media was collected and fetuin-A was purified on an agarose-bound Jacalin column. Eluted fractions from Jacalin column were assayed by Western blotting for Ser312-phosphorylated fetuin-A (pFet-A), fetuin-A (Fet-A) and FLAG. Western blot analysis indicated that fraction #3 of WT fetuin-A had the highest phosphorylation status. Ser312Ala was devoid of phosphorylation. A representative blot from 2 individual experiments is shown.

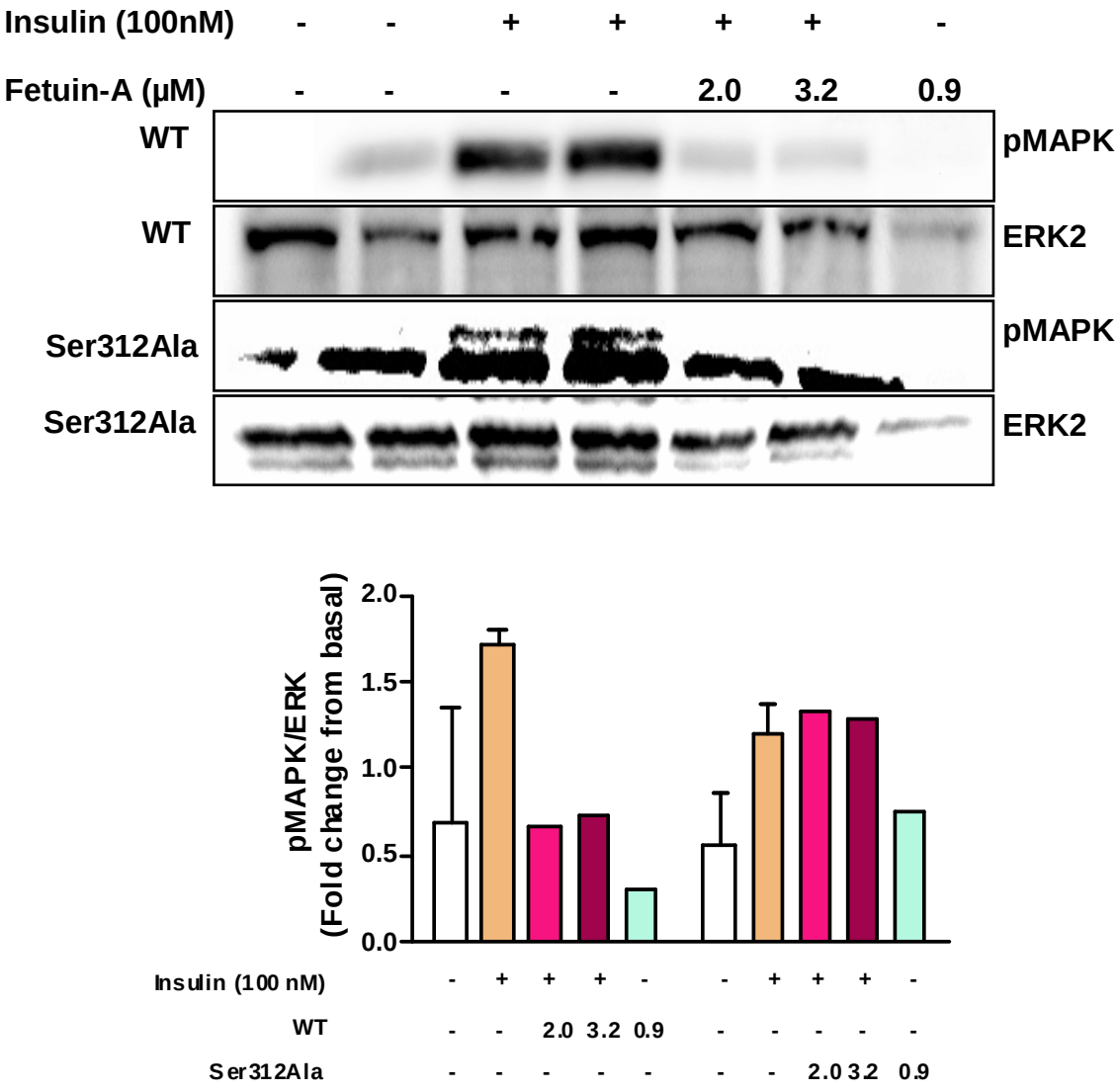

Fig.28: Significance of Ser312Ala-fetuin-A on insulin-stimulated phosphorylation of MAPK. Rat 1 fibroblast cells overexpressing human insulin receptor (HIRcB) were treated with recombinant human wild type fetuin-A and Ser312Ala-Fetuin-A for 20 minutes followed by insulin (100 nM) stimulation for 10 min. Western blot analysis indicated that unlike Ser312Ala-fetuin-A, wild type fetuin-A inhibited insulin-stimulated phosphorylation of MAPK. ERK2 blotting was included as loading control.

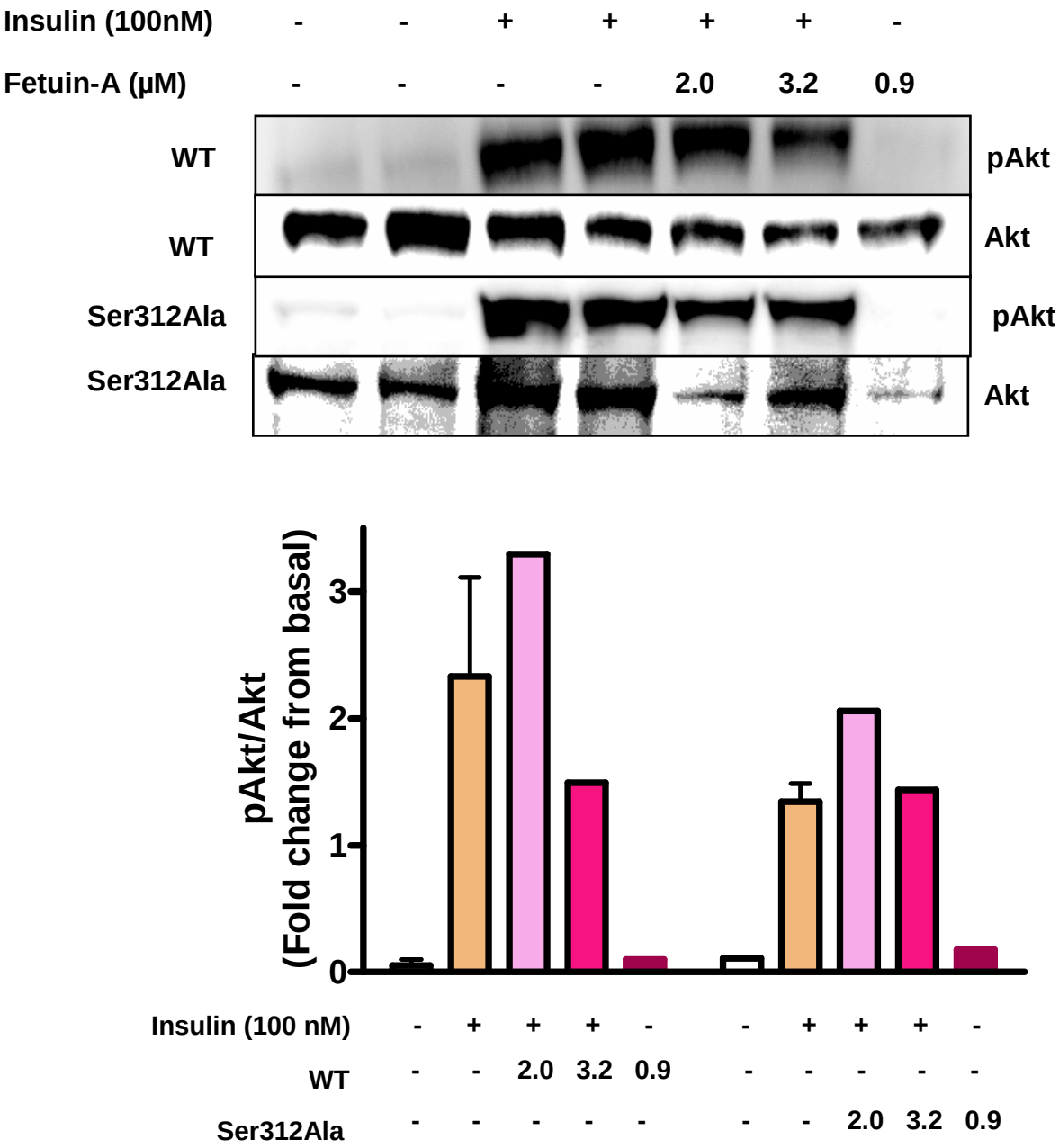

Fig.29: Significance of Ser312Ala-fetuin-A on insulin-stimulated phosphorylation of Akt. Rat 1 fibroblast cells overexpressing human insulin receptor (HIRcB) were treated with recombinant human wild type fetuin-A and Ser312Ala-Fetuin-A for 20 minutes followed by insulin (100 nM) stimulation for 10 min. Western blot analysis indicated that unlike Ser312Ala-fetuin-A, wild type fetuin-A inhibited insulin-stimulated phosphorylation of Akt. Akt blotting was included as loading control.

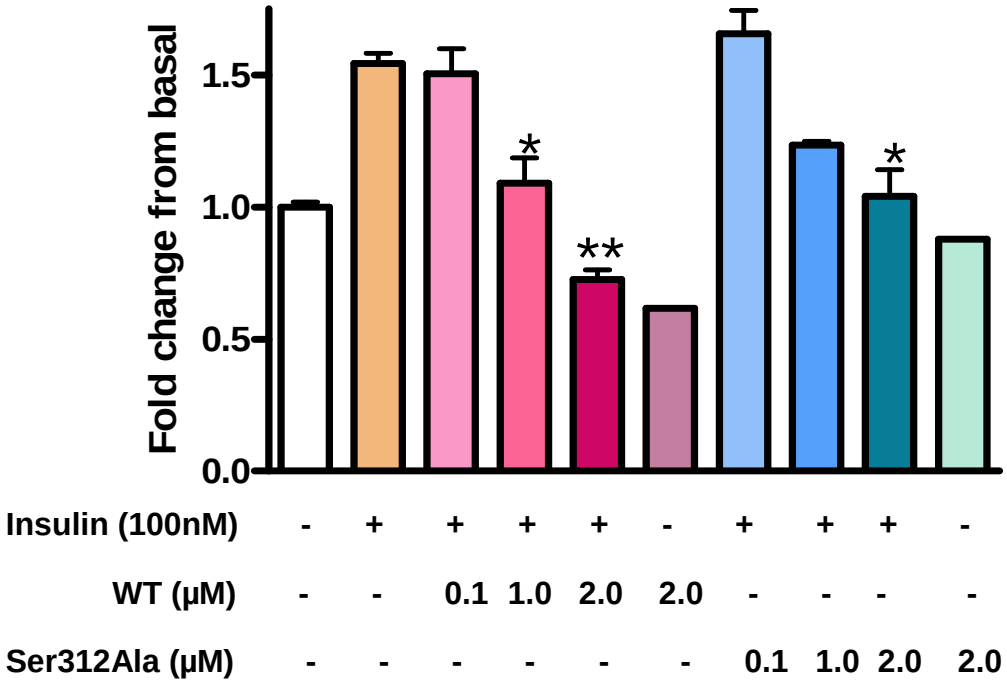

Fig.30: Significance of Ser312Ala-fetuin-A on insulin-stimulated glucose uptake by L6-GLUT4myc cells. Rat L6-GLUT-4Myc skeletal muscle cells were grown to confluence, serum-starved for five hours and treated in duplication with 0.1μM, 1.0μM, and 2.0μM of either recombinant wild type fetuin-A or Ser312Ala-fetuin-A followed by the incubation for 20 minutes and insulin stimulation (100 nM) for 30 minutes. Further, cells were treated and incubated for 10 minutes with 10X START buffer (100 μM 2-deoxyglucose, 0.5 μCi/ml [^{3}H]-2—deoxyglucose). [^{3}H]-2—deoxyglucose taken up by the cells were counted in liquid scintillation counter for 2 minutes. Values shown are fold change from basal. ** $p < 0.005$, *$p < 0.05$ compared to insulin.

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