**Role of phosphorylated fetuin-A in insulin action, insulin resistance, obesity, and moderate weight loss**

by

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Abstract

Fetuin-A (Fet-A), secreted by the liver into circulation, inhibits insulin-stimulated insulin receptor tyrosine kinase and is positively associated with obesity, insulin resistance, and incident diabetes. Though Fet-A exists in both phosphorylated and dephosphorylated forms in circulation, there are limited studies on its phosphorylation status or molecular characterization. The goal of this study was to investigate the role of phosphorylation of Fet-A in insulin action, insulin resistance, obesity and weight loss. We hypothesized that Ser312 phosphorylation status of Fet-A (pFet-A) is (a) critical for inhibition of insulin signaling and (b) associated with obesity and insulin resistance. While wild-type phosphorylated Fet-A blunted insulin signaling (IR, AKT, and MAPK, GLUT4 translocation), glucose uptake, and glycogen synthesis, single (Ser312Ala) and double (Ser312Ala+Ser120Ala) phospho-defective Fet-A mutants were without effect. To investigate the role of phosphorylation of Fet-A in insulin resistance, obesity and weight loss, we recruited 31 age-matched obese and 11 normal weight men. Circulating Fet-A and pFet-A were significantly elevated in obese individuals. Only pFet-A was significantly associated with serum glucose, insulin, HOMA, QUICKI and glucose-to-insulin ratio (G:I ratio). Following (24 hours after) a single bout of exercise, the area under the curve (AUC) for glucose, insulin, Fet-A and pFet-A were significantly lower. Next, 16 obese subjects took part in a modest weight loss study (8 - 10% of initial body weight) over a 6 to 10 month time period. Weight loss significantly decreased waist circumference, percent body fat, serum insulin, HOMA, QUICKI, and G:I ratio. While Fet-A and pFet-A levels were significantly lower, only pFet-A levels were correlated with

insulin, HOMA, QUICKI, and G:I ratio. Taken together, we demonstrate for the first time that phosphorylation status of Fet-A (Ser312) is critical for its inhibitory effects on insulin action. Elevated serum pFet-A concentrations observed in obese individuals correlate positively with surrogate markers of insulin resistance. Alterations in pFet-A following a single bout of exercise or with modest weight loss are associated with the improvement of insulin sensitivity. Our studies suggest a key role for pFet-A in the modulation of insulin action.

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List of Abbreviations

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| AHSG | Alpha-2-Heremans Schmid glycoprotein |
| AMPK | Adenosine monophosphate-activated protein kinase |
| AUC | Area under the curve |
| CVD | Cardiovascular disease |
| Fet-A | Fetuin-A |
| GLUT4 | Glucose transporter 4 |
| HDL-C | High-density lipoprotein cholesterol |
| HOMA | Homeostasis model assessment |
| IR | Insulin receptor |
| IRS-1 | Insulin receptor substrate-1 |
| LDL-C | Low-density lipoprotein cholesterol |
| MAPK | Mitogen-activated protein kinase |
| NEFA | Non-esterified fatty acid |
| PAI-1 | Plasminogen activator inhibitor |
| pFet-A | Phosphorylated Fetuin-A (Ser312) |
| PI3K | Phosphatidylinositol 3-kinase |
| QUICKI | Quantitative insulin sensitivity check index |
| T2DM | Type 2 diabetes |
| TG | Triglycerides |
| TLR-4 | Toll-like receptor-4 |
| TNFα | Tumor necrosis factor α |

# Chapter 1: Introduction

Obesity has become a global epidemic. Combined with a greater risk of chronic diseases, and a reduction in life expectancy, obesity currently threatens over 500 million adults around the world (Finucane et al., 2011; World Health Organization, 2013). The Centers for Disease Control and Prevention (CDC) report that more than one-third of adults (over 78 million) and nearly 17% of children and adolescents in the U.S (12.5 million) were obese in 2009–2010 (Ogden et al., 2012). An increase in each BMI unit is reported to be associated with an increased risk for death (Peeters et al., 2003). Obesity is considered an influential predictor of death for humans. It shortens life span by 7 years in 40 year-old adults, which is the same mortality risk for a smoker (Peeters et al., 2003; Tobias et al., 2014). Various diseases, such as hypertension, cardiovascular disease (CVD), and type 2 diabetes (T2DM) have been shown to be closely correlate with obesity (Della Corte et al., 2013). Recently, the American Medical Association adopted a new policy recognizing obesity as a disease (American Medical Association, 2013). This official policy requires medical interventions to advance obesity prevention and treatment, and encourages physicians and scientists to focus on decreasing obesity-related public health issues (American Medical Association, 2013).

With a direct link to obesity, diabetes, especially T2DM, is considered as an important contributor to the predicted decline in human life expectancy (Campbell, 2009). Currently, diabetes is the most common chronic disease around the world. International Diabetes Federation (IDF) predicts the global prevalence of people with diabetes will rise from 382 million in 2013 to 592 million by 2035 (International Diabetes

Federation, 2013). Of these, one-third of the adults are unaware that they have diabetes (International Diabetes Federation, 2011).

Several theories have been proposed to explain the relationship between obesity and T2DM (Johnson and Olefsky, 2013). The overflowing free fatty acids (FFAs) theory posit that increased dietary intake without adequate energy expenditure decreases the lipid storage potential of the adipose tissue and causes more FFAs to be secreted (Goossens, 2008). The acute, elevated plasma FFAs stimulate insulin secretion through direct effects on the pancreas, and decrease insulin clearance (Crespin et al., 1973; Kotronen et al., 2008; Valera Mora et al., 2003). Further, chronically elevated FFAs trigger gluconeogenesis, impairing insulin-stimulated glucose uptake and glycogen synthesis, resulting in insulin resistance in liver and skeletal muscle (Bays et al., 2004).

The recent discoveries of organokines provide another important theory linking obesity to T2DM. These organokines include adipokines secreted from adipose tissue (Ouchi et al., 2011), myokines secreted from skeletal muscle cells (Pedersen and Febbraio, 2012) and hepatokines secreted from hepatocyte (Stefan and Haring, 2013). After release, these proteins play important roles through paracrine and endocrine signaling pathways (Hotamisligil, 2006). Some of these biologically active proteins, including adiponectin, resistin, TNFα, leptin, angiotensinogen, plasminogen activator inhibitor-1, and interleukin-6, have been shown to affect insulin sensitivity, glucose metabolism, inflammation, and atherosclerosis, and functionally link obesity to T2DM and CVD (Bays et al., 2004; Inadera, 2008; Ouchi et al., 2011).

The benefits of regular physical activity and maintenance of a healthy weight in preventing obesity and T2DM have been well documented (Schulze and Hu, 2005). On

one hand, lifestyle modification decreases adiposity and improves dyslipidemia, hypertension, hyperglycemia and insulin resistance (Cornier et al., 2008; Piemonti et al., 2003). On the other hand, physical activity increases glucose uptake, through increased expression of GLUT4 in skeletal muscle (Chibalin et al., 2000; Hjeltnes et al., 1998; Hughes et al., 1993; Ren et al., 1994). Elegant studies by Kristiansen *et al.*. and Lund *et al.*. have shown that, immediately after a single bout of exercises, GLUT4 translocates to the cell surface, resulting in an increased glucose uptake in skeletal muscle (Kristiansen et al., 1996; Lund et al., 1995). Further, moderate intensity physical activity was shown to be independently associated with visfatin, apelin, adiponectin and leptin, indicating a relationship between physical activity, adipokines and inflammation factors (Gleeson et al., 2011; Kadoglou et al., 2012; Pedersen and Febbraio, 2012).

Our research is focused on one such organokine, fetuin-A (Fet-A), also called α2-Heremans-Schmid Glycoprotein (AHSG). It is a 60 kDa protein, primarily secreted by the liver into circulation. Average serum concentrations of Fet-A range from 300 - 800 μg/ml (Dziegielewska et al., 1990; Lebreton et al., 1979; Putman, 1984). Currently, several research groups are working on unraveling Fet-A's role in metabolism. Besides its functions as an inhibitor of ectopic calcification (Jahnen-Dechent et al., 2011; Mori et al., 2012; Mori et al., 2010; Schafer et al., 2003; Westenfeld et al., 2009) and a natural modulator of hepatocyte-growth factor (HGF) (Ohnishi et al., 1997), this 60 kDa glycoprotein was found to specifically inhibit insulin-stimulated insulin receptor (IR) tyrosine kinase and IR autophosphorylation (Auberger et al., 1989; Mathews et al., 2000; Srinivas et al., 1993). In animal studies, fetuin null mice demonstrated improved insulin sensitivity and resistance to diet-induced obesity (Mathews et al., 2002). Compared to

wild-type littermates, fetuin null mice were shown to increase both basal and insulin-stimulated phosphorylation of IR, MAPK and Akt in liver and skeletal muscle (Mathews et al., 2002). Further, fetuin-A null mice were protected against obesity and insulin resistance associated with aging (Mathews et al., 2006a). Recently, Fet-A was identified as an endogenous ligand of Toll-like receptor 4 (TLR4) linking fatty acids to induce inflammation and insulin resistance (Pal et al., 2012). TLR4 activates downstream inflammatory and stress signaling pathways, such as NF-κB and JNK, which further interferes with insulin sensitivity (Holzer et al., 2011; Wong et al., 2009). In clinical studies, Ix et al. found that Fet-A concentrations were associated with incident diabetes in older individuals (Ix et al., 2008). Another study by Stefan *et al.*. demonstrated that elevated plasma Fet-A positively correlated with the risk for diabetes independent of other established risk factors (Stefan et al., 2008). Recent studies demonstrate that Fet-A levels are elevated in NAFLD of children and adults, and correlate with critical enzymes involved in glucose and lipid homeostasis (Haukeland et al., 2012; Ix and Sharma, 2010; Kahraman et al., 2013; Ou et al., 2012b; Reinehr and Roth, 2008; Stefan and Haring, 2013). These studies support the hypothesis that Fet-A may play a role in the development of T2DM. Thus, decreasing Fet-A concentrations may provide a therapeutic approach in the managing insulin resistance and T2DM.

Human Fet-A has 2 polypeptide chain with an A chain of 282 amino acids (1-182) and a B chain of 28 amino acids (323-349), with six-disulfide (–S–S–) bonds and a 40 residue connecting peptide (283-321) (Araki et al., 1989; Nawratil et al., 1996). Fet-A is both a glycosylated and phosphorylated protein. There are two phosphorylation sites on human Fet-A: Ser120 in A chain and Ser312 in connecting peptide (Jahnen-Dechent et

al., 1994). Haglund *et al.*. previously demonstrated that Ser 312 was the dominant phosphorylation site, which provided around 77% phosphorylation, based on relative peak height using mass spectroscopy studies (Haglund et al., 2001). Further, Haglund et al. reported that approximately 20% of the circulating Fet-A was phosphorylated, which corresponds to circulating phosphorylated fetuin-A concentrations ranging from approximately 1.4 μM to 2.8 μM (Haglund et al., 2001; Mathews et al., 2000). There are two N-linked glycosylation sites (Asp138 and Asp158) and two O-linked glycosylation sites (Thr238 and Thr252) on A-chain. Another O-glycan is attached to B-chain at Ser328 for the trisaccharide moiety (Haglund et al., 2001; Sparrow et al., 2008). While the Asp residues in A-chain demonstrate 100% glycosylation, Ser328 on B-chain show either glycosylated or non-glycosylated forms (Haglund et al., 2001).

A few studies suggest that phosphorylation status of Fet-A is required for its IR-TK inhibitory activity (Auberger et al., 1989; Haglund et al., 2001; Kalabay et al., 1996; Kaushik et al., 2009; Ohnishi et al., 1997). However, there are no reports on Fet-A phosphorylation status in insulin resistant conditions or on its molecular characterization.

The goal of this study was to characterize the role of phosphorylation of Fet-A in insulin action, insulin resistance, obesity, and weight loss.

We hypothesized that Ser312 phosphorylation status of Fet-A (pFet-A) is (a) critical for inhibition of insulin signaling and (b) is associated with obesity and insulin resistance.

The following objectives were proposed for this study. (i) To characterize the effects of pFet-A in molecular mechanisms mediating insulin-stimulated glucose uptake; (ii) To examine alterations of circulating Fet-A and pFet-A (Ser312) in obese and normal

weight individuals, and their association with metabolic characteristics and insulin sensitivity indices; (iii) To understand Fet-A and pFet-A responses to a single bout of exercise (expending 500 kcal) in obese and normal weight individuals, and their relationship to changes in insulin sensitivity, and (iv) To investigate effect of incremental bodyweight loss (2-4%, 4-6%, 6-8%, and 8-10%) on alterations in Fet-A and pFet-A, markers of insulin sensitivity, and other physiological & metabolic characteristics in obese individuals.

To achieve the goals outlined in objective (i), we used site-directed mutagenesis and generated phospho-defective Ser312Ala and Ser120Ala mutant plasmids of Fet-A. We examined the role of wild type, Ser312Ala Fet-A single mutant and Ser312Ala & Ser120Ala double mutants on insulin action and glucose homeostasis.

To characterize objectives (ii), (iii), and (iv), we obtained approval from Auburn University Institutional Review Board (07-210 MR 0710). A total of 31 age-matched obese and 11 normal weight men were recruited from Auburn-Opelika area for this study. All participants underwent a single bout of treadmill walking at 60-70% VO2max, expending 500 kcals. For objective iv, 16 obese subjects took part in a modest weight loss study (8 - 10% of initial body weight) over a 6 to 10 month time period.

The significance of this study is in its potential to contribute to our understanding of (a) the mechanistic basis of Fet-A's role in insulin action, i.e. role of phosphorylation; (b) alterations in Fet-A and pFet-A in obese versus normal body weight individuals; (c) the effects of a single bout of exercise on Fet-A and pFet-A and other surrogate markers of insulin sensitivity and (d) alterations in Fet-A and pFet-A after a modest weight loss (8-10% of body weight) in the context of improvement of insulin sensitivity.

# Chapter 2: Review of Literature

## 2.1 Obesity

Obesity was first described in Paleolithic Stone Age (24,000–22,000 BC) in a series of artifacts across Europe and was considered as a serious disease extending to other diseases during the times of Greco-Roma medicine (Beller, 1977; Bray, 1998). Currently, World Health Organization (WHO) uses Body Mass Index (BMI) to define obesity. For adults, BMI equal to or over 25 $kg/m^2$ is considered as overweight; and BMI equal to or more than 30 $kg/m^2$ defines obesity (World Health Organization, 2000, 2003). For children, obesity, as defined by the Centers of Disease Control (CDC), and based on growth charts, is BMI greater than the $95^{th}$ percentile adjusted for age and sex (Kuczmarski et al., 2000).

Since 1980, obesity has become an epidemic, first in high-income countries, and shortly thereafter, enveloping the entire world. Within the last 30 years, obesity has become a global problem (Finucane et al., 2011; World Health Organization, 2008). According to the World Health Organization, 1.4 billion adults over 20 years old were *overweight*, including more than 200 million *obese* men and nearly 300 million *obese* women (World Health Organization, 2013). In 2011, more than 40 million children (under the age of 5 years) were overweight (World Health Organization, 2013). The prospective numbers of obesity and overweight for 2015 will be greater, with 2.3 billion adults expected to be overweight and 700 million expected to be obese (Finucane et al., 2011; World Health Organization, 2012).

Obesity is caused by chronic energy imbalance, including increased dietary intake and low physical activity. The rapid increase in the availability of inexpensive energy-dense foods (added sugar, fats, salt, and flavor enhancers) drives weight gain in populations, including a higher rate of childhood obesity (Cutler et al., 2003; Finucane et al., 2011; Mendez et al., 2005; World Health Organization, 2012)

Obesity is a major health hazard. High BMI is closely associated with type 2 diabetes, cardiovascular diseases (mainly heart disease and stroke), and many cancers (American Institute for Cancer Research, 2007; Australian Institute of Health and Welfare and Australia, 2004; Calle and Kaaks, 2004; Ezzati et al., 2004; Kahn et al., 2006; Van Gaal et al., 2006). In 2004, the disability caused by obesity and its consequences affected more than 36 million people (Ezzati et al., 2004). The risk of death has been shown to increase with each unit increment of BMI, with a resulting decline in life expectancy by 7 years (Peeters et al., 2003). Although it is not known that intentional weight loss in obese patients will prolong life or decrease the risks of death, preliminary evidence suggests that less than 10% of body weight loss would help to reduce obesity-related mortality risk by 30 - 40% (Williamson et al., 1995). For individuals who were newly diagnosed with diabetes, a 10 kg weight loss during their first year, can potentially extend 4 years of life (Lean et al., 1990).

**2.2 Diabetes**

The American Diabetes Association defines diabetes as a group of metabolic diseases resulting from defects in insulin secretion, insulin action or both (American Diabetes Association, 2014). Diabetes is the most common chronic disease around the world. The data from International Diabetes Federation (IDF) indicate that the global

prevalence of people with diabetes will rise from 382 million in 2013 to 592 million by 2035 (International Diabetes Federation, 2013). IDF also declared that globally, there are another 183 million people who are unaware that they have diabetes (International Diabetes Federation, 2011).

There are two major categories of diabetes, which affect most of diabetic patients. Type 1 diabetes is caused by absolute deficiency of insulin secretion and is thought to be a heterogeneous autoimmune disease resulting from both genetic and environmental factors (ADA, 2014). Studies on monozygotic twins (MZ) in Finland demonstrated that the incidence of type 1 diabetes is much higher for monozygotic twins (MZ) compared to dizygotic (DZ) twins when one twin is diagnosed early (Hyttinen et al., 2003). Other twin studies also predicted a higher heritability rate for early onset type 1 diabetes. (Arner, 2001; Kumar et al., 1993; Kyvik et al., 1995; Olmos et al., 1988) Evidence from US and UK population studies have revealed over 20 genetic loci which are closely associated with type 1 diabetes (Concannon et al., 1998; Cox et al., 2001; Mein et al., 1998). The human leukocyte antigen (HLA) genes in the major histocompatibility complex (MHC) on chromosome 6p21 (Concannon et al., 1998; Mein et al., 1998), were the first set of genes found to be associated with type 1 diabetes, (Davies et al., 1994). HLA genes have two major classes, class I and class II; the HLA-DR3/4-DQ8 genes have been reported for a high genetic risk for islet autoimmunity and type 1 diabetes. (Redondo et al., 2001). Environmental factors such as viruses and dietary factors also contribute to type 1 diabetes susceptibility. Kaufman *et al.*. found a similar peptide in both glutamate decarboxylase (GAD) and Coxsackie virus, suggesting that a molecular mimicry may cause the pathogenesis of type 1 diabetes (Hyoty and Taylor, 2002; Kaufman et al., 1992;

Tuomi et al., 1999). As dietary factors, some population studies suggest that exposure to cow's milk may trigger type 1 diabetes (Virtanen et al., 1993). Later studies identified albumin and β-casein as dietary factors that induce an immune response in the pathogenesis of type 1 diabetes (Cavallo et al., 1996; Levy-Marchal et al., 1995).

Type 2 diabetes contributes 90% cases of diabetes around the world. WHO predicted the number of people with type 2 diabetes will be at least 350 million worldwide by 2030 unless appropriate action is taken, which is double the current estimates for diabetes prevalence (World Health Organization, 2003). From the American Diabetes Association definition, type 2 diabetes results from a combination of resistance to insulin action and an inadequate compensatory insulin secretory response (ADA, 2014). The interaction between genetic and environmental factors contributes to the pathogenesis of type 2 diabetes. These factors cause insulin resistance, disorder of insulin secretion, defects in of β-cell mass, interfering with fat metabolism and resulting in obesity. Research studies based on population-based Danish Twin Register provides strong heritability estimates for type 2 diabetes. MZ twins in this study have much higher concordance for abnormal glucose tolerance (63%), which was significantly different from DZ twin pairs (43%) (Poulsen et al., 1999) The results from Pima Indians of Arizona demonstrated that 38-49% of the disorder of insulin action was independent of the effect of obesity. The young-onset diabetes is strongly familial; therefore, genetic factors play an important role in the age of onset of type 2 diabetes. (Baier and Hanson, 2004). Currently, it is understood that type 2 diabetes is a polygenic disease. In 1980s and 1990s, many genome-wide linkage studies were conducted to identify loci influencing type 2 diabetes. Unlike type 1 diabetes HLA region, no single region was

identified as influencing type 2 diabetes (Florez et al., 2003). Loci with significant linkages with type 2 diabetes include chromosomes 1q21-124, 1q25.3, 2q37.3, 3p24.1, 3q28, 10q26.13, 12q24.31, and 18p11 (Florez et al., 2003). The strongest association for type 2 diabetes was the Pro12Ala polymorphism in the peroxisome proliferator-activated receptor-γ (PPARγ) (Altshuler et al., 2000). PPARγ is related to high BMI, insulin resistance (Beamer et al., 1997; Deeb et al., 1998) and plays an important role in adipocyte development (Spiegelman, 1998). PPARγ is the target for the thiazolidiedione class of drugs, used in the treatment of diabetes. Besides genetic factors, environmental factors, especially lifestyle-related risk factors, have been repeatedly confirmed to determine the development of type 2 diabetes. From the Nurses' Health Study, Hu *et al.* found that BMI over 25 was the single most important factor of determining type 2 diabetes, especially for women (Hu et al., 2001). The second factor that influences type 2 diabetes is a sedentary lifestyle, with many cohort studies showing less physical activity was significantly associated with a higher risk for type 2 diabetes (Hu et al., 2003; Hu et al., 2001; Hu et al., 1999; Hu et al., 2004; Wannamethee et al., 2000).

**2.3 From obesity to diabetes: insulin resistance**

Several large population group studies provided strong observational evidence to support the link between obesity and diabetes. A ten-year follow-up (1986-1996) of middle-aged women in the Nurses' Health Study, and men in the Health Professionals Follow-up Study, showed that the risk of diabetes was increased with the severity of overweight and obesity in both women and men compared to their leaner peers (Field et al., 2001). In addition, the subsequent risk of diabetes (1996-2000) among 22,171 men in the Health Professionals Follow-up Study demonstrated that diabetes risk increased by

7.3% for every kilogram of weight gained, especially the gain in abdominal fat (Koh-Banerjee et al., 2004). Also in the Pima Indian study, investigators found a negative, linear association between changes in body weight and changes in insulin-stimulated glucose disposal in subjects with either normal glucose tolerance or impaired glucose tolerance (Weyer et al., 2000b).

Johnson and Olefsky (2013) systemically summarized three major mechanisms involved in insulin resistance: (i) inflammation; (ii) lipid metabolism and (iii) microbiota. All of these components were shown to interact with each other (Johnson and Olefsky, 2013).

It is now believed that, chronic, low-grade inflammation associated with obesity and occurring in different tissues plays a key role in the etiology of insulin resistance (Gregor and Hotamisligil, 2011; Johnson and Olefsky, 2013; Lumeng and Saltiel, 2011). Several studies have indicated a critical role for adipose tissue in the pathogenesis of insulin resistance (Bluher et al., 2002; Rosen and Spiegelman, 2014). The abnormalities in adipose tissue were observed earlier than the impairments of insulin action, insulin secretion and glucose tolerance (Weyer et al., 1999).

Besides the traditional view that adipose tissue is used to store energy, it is now well recognized that the adipose tissue is a complex endocrine organ that secretes several adipokines (Fruhbeck et al., 2001; Kershaw and Flier, 2004; Ouchi et al., 2011). Adipokines including resistin, TNFα, leptin, angiotensinogen, plasminogen activator inhibitor-1, and IL-6 were significantly elevated in obese and type 2 diabetes patients compared to healthy individuals (Bays et al., 2004; Ouchi et al., 2011).

In both animal models and human subjects, adiponectin levels were found to be

positively associated with insulin sensitivity in muscle (Arita et al., 1999; Hotta et al., 2000; Hotta et al., 2001; Weyer et al., 2001) and liver (Bajaj et al., 2004; Bajaj et al., 2003). Evidence from obese patients after bariatric surgery also suggested that the expression of adiponectin was under feedback inhibitions in obesity (Yang et al., 2001). The Pima Indian studies also indicated that adiponectin was involved in the development of type 2 diabetes and negatively correlated with markers of inflammation (Krakoff et al., 2003; Lindsay et al., 2002). The identification of two adiponectin receptors, AdipoR1 and AdipoR2 have helped to understand the role of adiponectin in modulating insulin sensitivity (Yamauchi et al., 2003). Activation of AdipoR1 was found in stimulate the AMPK signaling pathway and further regulate glucose production. AdipoR2 was shown to play an important role in glucose uptake, which was controlled by PPARα target genes (Yamauchi et al., 2007). Adiponectin was also shown to lower triglyceride levels in liver and circulation and further prevent both alcoholic and non-alcoholic hepatic steatosis (Xu et al., 2003; Yamauchi et al., 2001; Yamauchi et al., 2007). Furthermore, several studies have documented the association of decreased adiponectin concentrations with atherosclerosis (Ouchi et al., 1999) and inflammation (Fantuzzi, 2008). In human serum, adiponectin forms aggregate of 90 kDa homotrimers, 180 kDa hexamers and ~300 kDa 12-18mers; the last one called high-molecular weight (HMW) adiponectin (Trujillo and Scherer, 2005). Current findings indicate that HMW adiponectin is the form that associates with inflammation, insulin resistance, type 2 diabetes and CVD (Hirose et al., 2010; Murdolo et al., 2011; Rizza et al., 2010; Zhu et al., 2010).

Besides adiponectin, leptin is another important adipokine. Leptin and its receptors were first found in obese (ob) and diabetes (db) mutant mice, and this hormone

was predicted to affect energy homeostasis, neuroendocrine function and traditional endocrine systems (Chen et al., 1996; Kershaw and Flier, 2004; Lee et al., 1996; Zhang et al., 1994). Masuzaki *et al.*. demonstrated for the first time from their hyperleptinemic animal models, that high levels of leptin in circulation could delay the onset of impaired glucose metabolism and accelerate the recovery from diabetes (Masuzaki et al., 1999). Human studies also showed that the mutations in leptin gene were strongly associated with obesity, impaired glucose tolerance and insulin resistance (Clement et al., 1998). Therapeutic treatment with recombinant human leptin, reversed from the syndrome of obesity (Farooqi et al., 1999). The secretion of leptin is influenced by various factors, especially TNFα. TNFα was previously described as a potent proinflammatory cytokine (Locksley et al., 2001; Uzui et al., 2002). TNFα, also considered an adipokine, blunts insulin sensitivity and causes severe insulin resistance (Hotamisligil et al., 1995; Hotamisligil et al., 1993; Ryden et al., 2002). It increases lipolysis (Ryden et al., 2002), stimulates several inflammatory pathways such as MAPK, c-Jun N-terminal kinase and p38 (Hotamisligil et al., 1993; Ruan and Lodish, 2003) and inhibits insulin action by influencing the serine phosphorylation of insulin receptor substrate 1 (IRS-1) (Hofmann et al., 1994). Although the correlation between plasma levels of TNFα and insulin resistance is not significant (Zinman et al., 1999), it has been shown that localized insulin resistance in target tissues is associated with its own TNFα concentrations (Spiegelman, 1998). Currently, TNFα is implicated in the pathogenesis of obesity and insulin resistance (Hotamisligil, 2003; Hotamisligil et al., 1993; Johnson and Olefsky, 2013; Ruan and Lodish, 2003)

Like TNFα, IL-6 is another proinflammatory cytokine, that plays an important

role in inflammation and the regulation of T cell and β-cell apoptosis (Shimabukuro et al., 1998; Van Snick, 1990). One third of IL-6 is secreted from adipocyte, and it circulates mostly in bloodstream (Fernandez-Real and Ricart, 2003). In humans with type 2 diabetes, IL-6 was positively correlated with the severity of inflammation and glucose intolerance (Pickup et al., 2000; Pradhan et al., 2001).

Plasminogen activator inhibitor (PAI-1) is a serine protease inhibitor which inhibits the fibrinolytic cascade (Binder et al., 2002). PAI-1 is expressed by various cell types: adipocytes being one of the major sources of PAI-1 (Fain et al., 2004). Both type 2 diabetes and obese subjects had elevated PAI-1 levels (Yudkin, 1999). PAI-1 was also shown to be positively correlated with hyperinsulinemia and provided predictions for type 2 diabetes and cardiovascular disease (Juhan-Vague et al., 2003; Mertens and Van Gaal, 2002). In addition, treatment with metformin and thiazoladinediones (TZDs), significantly decreased circulating PAI-1 levels with concomitant insulin sensitivity improvements (Mertens and Van Gaal, 2002).

Resistin is a novel protein found in a screen for targets of insulin-sensitizing drugs (Steppan et al., 2001). Overexpression of resistin in animal models showed that resistin significantly reduced insulin sensitivity. On the other hand, low resistin levels improved insulin sensitivity and glucose homeostasis (Lazar, 2007). Studies of obese patients after bariatric surgery indicated that serum resistin levels decreased in the first 3-6 months (Edwards et al., 2011; Moschen et al., 2009) but some studies showed contrary findings (De Luis et al., 2010). Other comprehensive studies showed that the development of type 2 diabetes was associated with elevated serum resistin (Chen et al., 2009; Heidemann et al., 2008). Mechanistically, resistin caused vascular smooth muscle proliferation and

endothelial dysfunction (Calabro et al., 2004; Kougias et al., 2005; Verma et al., 2003). It was also found to inhibit AMPK signaling and negatively impact insulin signaling pathways (Banerjee et al., 2004; Muse et al., 2004; Satoh et al., 2004).

Besides the inflammation caused by adipokines, free fatty acids (FFA)-induced lipotoxicity also plays an important role in the pathogenesis of type 2 diabetes by directly interfering with several signaling pathways and producing metabolites to induce insulin resistance (Glass and Olefsky, 2012; Jornayvaz and Shulman, 2012). The acute elevation of plasma FFA levels stimulates insulin secretion through a direct effect on the pancreas. Elevation of plasma FFAs also decreases insulin clearance (Crespin et al., 1973; Peiris et al., 1986). Chronically elevated FFA triggers gluconeogenesis and induces insulin resistance in liver and skeletal muscle by interfering with the insulin-stimulated glucose uptake and glycogen synthesis (Bays et al., 2004). In addition, plasma FFAs induce TLR4-induced activation of NF-kB signaling pathway, stimulate inflammation and inhibit insulin signaling by phosphorylating serine sites of IRS-1 (Glass and Olefsky, 2012). Elevated plasma FFAs also increased the accumulation of triglycerides and other fat-derived metabolites, such as diacylglycerol, acyl coenzymes A and ceramides (Hirose et al., 1996). All of these metabolites can potentially decrease tyrosine phosphorylation of insulin-receptor substrate (Hirose et al., 1996). In Zucker diabetic fatty (ZDF) rats studies, FFAs induced pancreatic β cell apoptosis, which played an important role in the pathogenesis of type 2 diabetes (Shimabukuro et al., 1998).

Besides adipokines and FFAs, the pattern of body fat distribution plays a role in predicting insulin sensitivity. People who have upper body fat accumulation or high visceral fat mass have more insulin resistance, hyperlipidemia, impaired glucose

tolerance and hyperinsulinemia than people who have predominantly lower body fat accumulation (Clausen et al., 1996; Kissebah and Peiris, 1989). It has been shown that, compared to subcutaneous fat, visceral fat cells produced excessive amounts of proinflammatory adipokines, such as TNFα, IL-6, PAI-1, and resistin (Arner, 2001; Bjorntorp, 1990; Motoshima et al., 2002; Sewter et al., 2002). Recent data on adipose tissue cellularity indicated that abdominal adipocyte size in obese individuals is much larger than normal individuals, which suggests that hyperinsulimia, insulin resistance and type 2 diabetes maybe related to abdominal adipocyte size (Kim and Reaven, 2010; Kursawe et al., 2010; Weyer et al., 2000a).

**2.4 Insulin action and signal transduction**

Insulin is the most active physiological agent ever known. Insulin promotes glucose uptake and storage in skeletal muscle, decreases endogenous glucose production in liver, stimulates storage and synthesis of lipids and inhibits lipolysis in adipose tissue (Brownlee, 2005; Saltiel and Kahn, 2001). At the molecular level, insulin signaling cascade begins by insulin binding to the extracellular α subunits insulin receptor (IR). This binding promotes the activation of an intrinsic tyrosine kinase activity, leading to a transphosphorylation of the tyrosine residues of one intracellular β subunit by the other. This insulin-stimulated IR autophosphorylation increase catalytic activity of the tyrosine kinase, and finally, both of the β subunits become phosphorylated on their 3 tyrosine residues – Tyr 1131, Tyr 1135, and Tyr 1136 (Saltiel and Pessin, 2003; Watson et al., 2004; White, 1998; Withers and White, 2000). The phosphorylated β subunits facilitate recruitment of adapter proteins such as the insulin receptor substrate (IRS) proteins which creates docking sites for other signaling proteins that contain src homology 2 (SH2)

domains. The most important SH2 proteins are lipid kinase phosphoinositide-3 (PI-3), and adaptor molecule growth factor receptor binding protein 2 (Grb2) (Fritsche et al., 2008). PI-3 kinase binds to IRS proteins and catalyzes the second messengers from phosphoinositol 4,5 bisphosphate (PIP2) to phosphoinositol 3,4,5 trisphosphate (PIP3) (Vanhaesebroeck et al., 2001). PIP3 is an essential protein, which is important in recruiting downstream kinases, for example, the 3-phosphoinositide-dependent protein kinase 1 (PDK-1). PDK1 is an important controller protein which regulates various downstream kinases including isoforms of protein kinase B (PKB)/Akt, p70 ribosomal S6 kinase (S6K), serum- and glucocorticoid-induced protein kinase (SGK) and protein kinase C (PKC) (Mora et al., 2004). In all of these proteins, Akt/PKB is suggested to be one of the most essential agents in regulating glycogen synthesis, glucose uptake and protein synthesis. Akt phosphorylates and inactivates GSK-3, decreases the rate of phosphorylation of glycogen synthase and stimulates it into an active state (Cross et al., 1995; Sutherland et al., 1993). It has also been shown that Akt plays an important role in hepatic gene transcription, Forkhead transcription factor, Foxo1, is stimulated by Akt (Nakae et al., 2001; Rena et al., 1999). FOXO1 plays an important role in hepatic gene transcription, especially in the genes of PEPCK and G6Pase, because they determine the expression of the enzymes which take part in the critical steps in gluconeogenesis (Lochhead et al., 2001; Onuma et al., 2006). Activation of Akt also induces the translocation of glucose transport GLUT-4 vesicles from the intracellular pool to the plasma membrane, where they increase the glucose uptake into cells (Cong et al., 1997; Kohn et al., 1998; Kohn et al., 1996; Wang et al., 1999). In addition, Akt leads to mTOR-mediated activation of protein synthesis by PHAS/elf4 and p70s6k, and control of cell

growth pathways (Potter et al., 2002; Vander Haar et al., 2007). Besides the IRS/PI(3)K/Akt pathway, GLUT-4 is also regulated by cbl/CAP signaling pathway, which is phosphorylated and binds with insulin receptors, then becomes a protein complex Cbl/CAP (Ribon et al., 1998). CAP helps to target Cbl to the lipid raft micro domains; it contains three Src3 homology (SH3) domains at its C terminus that bind to Cbl (Lin et al., 2001). Cbl/CAP complex then provides docking sites and recruits CkrII, C3G, and further activates the GTP binding protein TC10 (Chiang et al., 2001). Finally, the Cbl/CAP/TC10 complex is translocated to the plasma membrane to induce GLUT4 to the adipocyte membrane (Chang et al., 2002). Insulin regulates another signaling pathway named Ras/MAPK pathway through the IRS proteins' autophosphorylation. Phospho-IRS then binds to Grb2/mSOS; Grb2 is an adaptor protein with SH3 domain, which recruits the guanine nucleotide exchange factor mSOS, that exchanges GDP for GTP on the small G-protein Ras activation (McKay and Morrison, 2007). Activation of Ras leads to activation of RAF, phosphorylation of MAP/ERK kinase (MEK) and phosphorylation of ERK1/2 (Kyriakis et al., 1992; Payne et al., 1991). Activated ERK1/2 can be translocated into the nucleus and then the phosphorylated transcription factors, such as P62TCF, will cause cell proliferation and differentiation (Boulton et al., 1991). Insulin regulates lipogenic gene expression by activating the sterol regulatory element binding protein (SREBP)-1c in a PI3K dependent pathway (Matsumoto et al., 2003; Taniguchi et al., 2006b), leading to transcription of various key enzymes in fatty acid synthesis, such as fatty acid synthase and acetyl-CoA carboxylase (Horton et al., 1998).

**2.5 Tyrosine kinase inhibitors**

Insulin stimulated tyrosine phosphorylation of IRS proteins is the key in

mediating insulin action (Hotamisligil, 2006; Taniguchi et al., 2006a). For people who have insulin resistance, tyrosine kinase activity and its autophosphorylation effects are significantly reduced (Comi et al., 1987; Maegawa et al., 1991; Nyomba et al., 1990). Various kinases including lipid cytokines (TNFα), stress activated protein kinase, c-Jun N-terminal kinase (JNK), IKK and PKC can phosphorylate IRS-1,2 on specific serine/threonine residues, then inhibit IRS activity and finally block downstream insulin signaling (Aguirre et al., 2000; Hotamisligil et al., 1996; Paz et al., 1997). Another mechanism for these kinases to inhibit IRS activity is related to the induction of suppressors of cytokine signaling (SOCS-1,3). SOCS-1 and 3 were shown to compete with IRS-1 in binding with insulin receptors, and caused a proteasome-mediated degradation of IRS-1 in blocking insulin signaling (Emanuelli et al., 2001; Kile et al., 2002; Rui et al., 2002). In addition, several members of protein tyrosine phosphatases (PTPs) play a negative role in insulin signaling. PTP LAR is a subfamily of PTPs which is highly expressed in adipose tissue (Chagnon et al., 2004). Animal experiments have shown that insulin treatment increased the amount of IR/LAR complexes. Overexpression of LAR negatively regulates phosphorylation of insulin receptor, IRS and SHC, and affects both PI3K and MAPK pathways (Kulas et al., 1996; Kulas et al., 1995; Li et al., 1996; Zhang et al., 1996). SHP-2 is an intracellular PTP which has two SH2 domains. Some studies showed that SHP-2 acts on IRS-1, blocking several pathways, such as Grb2/SOS and MAPK in muscle cells (Hayashi et al., 2004). Most studies of PTP family have focused on PTP1B, a 50 kDa enzyme localized mainly in the endoplasmic reticulum (ER) and which functions as the major negative regulator of the insulin signaling pathway (Frangioni et al., 1992). Both the IR and IRS-1 are substrates of

PTP1B (Goldstein et al., 2000). Over expression of PTP1B blocked the tyrosine phosphorylation in IR and IRS-1 in skeletal muscle and liver cells, inhibited PI3K, Akt and MAPK phosphorylation, decreased glycogen synthesis (Egawa et al., 2001; Venable et al., 2000). GLUT4 translocation to the cell membrane was also reduced (Chen et al., 1997). In animal studies, PTP1B-deficient mice were shown to be leaner, had less adipose stores and were more insulin sensitive (Elchebly et al., 1999; Klaman et al., 2000). Tyrosine kinase inhibitors, can serve as potential therapeutic targets in the treatment of insulin resistance, obesity and type 2 diabetes (Srinivas and Grunberger, 1994).

**2.6 Fetuin-A**

Fetuin was first discovered in 1944 from studies on the fractionation of bovine serum with ammonium sulphate (Pedersen, 1944). In the 1960s, Schmid and Bürgi in Boston, and Heremans in Belgium, independently discovered the human isoform of fetuin and named it α2-Heremans-Schmid Glycoprotein (AHSG) (Heremans, 1960; Schmid and Burgi, 1961). Human AHSG is primarily released from the liver and presents in the blood of adults at an average concentration ranging from 300 - 800 μg/ml (Dziegielewska et al., 1990; Lebreton et al., 1979; Putman, 1984). Initially AHSG was studied for its function of mineralizing human bones (Dickson et al., 1975), because it was observed to be taken up by bone, where it was concentrated 30-300 fold compared with other plasma proteins (Triffitt et al., 1976).

Besides bone mineralization, AHSG was shown to promote various physiological processes in humans. For example, van Oss *et al.* found 0.1% of AHSG increased the phagocytosis of *E.coli* in human neutrophils and caused hydrophobicity of

microorganisms in human body (van Oss et al., 1974). Kumbla *et al.* showed AHSG's role in regulating the lipid transport, such as the delivery of fatty acids to cells and cholesterol efflux from cells (Kumbla et al., 1991). Schafer *et al.* demonstrated another important role of AHSG as an inhibitor of ectopic calcification at systemic levels, which may help to prevent various diseases (Schafer et al., 2003). Recently, fetuin-A was shown to be beneficial in coronary artery and the vasculature because it prevented calcification of soft tissue (Jahnen-Dechent et al., 2011; Mori et al., 2010; Westenfeld et al., 2009). Ohnishi *et al.* suggested a novel role of phosphorylated rat fetuin as a natural modulator of hepatocyte-growth factor (HGF) (Ohnishi et al., 1997). In the next section, I will focus on the function of fetuin-A as a regulator of insulin action.

In 1985 Le Cam *et al.* discovered the 63 kDa rat hepatic glycoprotein; they called it phosphorylated protein 63 (pp63) (Le Cam and Le Cam, 1985). Then in 1989, Auberger *et al.* found that pp63 could potently inhibit the insulin receptor autophosphorylation (Auberger et al., 1989). They also sequenced pp63 and found that it belongs to the rat feuin family. In the same era, Dziegielewska *et al.* sequenced a bovine fetal protein, that was similar to rat pp63 and human α2-HS glycoprotein, and named it bovine fetuin (Dziegielewska et al., 1990).

Later in 1993, Srinvas *et al.* successfully cloned the full length human AHSG cDNA from human liver cells and confirmed that AHSG and rat pp63 have high amino acids homology (Srinivas et al., 1993). In 2000, the second member of the mammalian fetuin family was identified in the rat, mouse and human on the basis of domain homology; the investigators named this protein fetuin-B (Olivier et al., 2000). Therefore, the previously known AHSG was renamed "Fetuin-A" (Fet-A).

The human Fet-A gene is localized on human chromosome 3q27 (Cintron et al., 2001; Magnuson et al., 1988; Rizzu and Baldini, 1995; Siddiq et al., 2005). This loci was also shown to be associated with type 2 diabetes and metabolic syndrome (Kissebah et al., 2000; Vionnet et al., 2000). Fet-A gene consisted of a 48 bp 5' untranslated region, 54 bp encoding a putative 18 amino acid leader sequence, a 1047 bp open reading frame for amino acid sequence and 389 bp of 3' untranslated region (Lee et al., 1987). Human and bovine mature, Fet-A contains 2 chains, an A chain of 282 amino (1-182) and a B chain of 28 amino acids (323-349). Mature Fet-A in circulation features six-disulfide (–S–S–) bonds which contains 40 residues as a connecting peptide (283-321) (Araki et al., 1989; Nawratil et al., 1996).

There are two phosphorylated sites in human Fet-A, Ser120 in the A chain and Ser312 in the connecting peptide (Haglund et al., 2001; Jahnen-Dechent et al., 1994). Approximately 20% of the circulating Fet-A is phosphorylated, with the majority of phosphorylation (~77%) on Ser312 and approximately 20% on Ser120 (Haglund et al., 2001). Using recombinant human Fet-A, Mathews *et al.* showed that α2-HSG (1.8 μM) inhibited insulin-induced IR autophosphorylation by over 80% both *in vitro* and *in vivo* in skeletal muscle and liver tissue (Mathews et al., 2000). Compared to a normal human circulating Fet-A concentration of 0.3 - 0.6 mg/ml, the phosphorylated Fet-A (pFet-A) concentrations in human plasma range from 1.4 to 2.8 μM (Haglund et al., 2001; Mathews et al., 2000). Human Fet-A also undergoes N- and O- glycosylated modification. Two N-linked glycosylation sites (Asp138, Asp158) and two O-linked glycosylation sites (Thr238, Thr252) were found in A chain. Another O-glycan attached site was also found in B-chain at Ser328 for the trisaccharide moiety (Haglund et al.,

2001; Sparrow et al., 2008). While the Asp residues in A-chain demonstrate 100% glycosylation, Ser328 on B-chain show either glycosylated or non-glycosylated forms (Haglund et al., 2001).

From euglycemic hyperinsulinemic clamp studies, Fet-A null mice on a high fat diet were shown to be more insulin sensitive than littermate control mice and displayed increased basal and insulin-stimulated phosphorylation of IR, Akt and MAPK in liver and skeletal muscle. Further, Fet-A null mice showed lower free fatty acid and triglyceride levels compared to wild type mice (Mathews et al., 2006b; Mathews et al., 2002). These studies indicate that Fet-A may play a key role in the modulation of insulin sensitivity and insulin action.

Hennige *et al.* provided the first evidence of the relationship between Fet-A and adipokines. Their research showed that Fet-A induced TNFα and IL-1B mRNA expression and decreased circulating adiponectin levels (Hennige et al., 2008). The study conducted by Bluher *et al.* in the 2-year Dietary Intervention Randomized Controlled Trial (DIRECT) demonstrated that various biomarkers, including adiponectin and Fet-A levels display a continued long-term improvement despite partial weight regains (Bluher et al., 2012). All of the adipokines, as previous described, play a critical role in regulating the insulin resistance and type 2 diabetes. The newest and important discovery was that of to Pal *et al.*, who showed that Fet-A served as an adapter protein liking free fatty acid to TLR-4, leading to NF-κB activation (Pal et al., 2012). These studies suggested that Fet-A may be "the missing link in lipid-induced inflammation" (Heinrichsdorff and Olefsky, 2012).

In 2008, two landmark clinical studies on Fet-A and diabetes were published. The

first study conducted by Ix *et al*. found that serum Fet-A levels were associated with incident diabetes in older persons (Ix et al., 2008). They measured Fet-A levels in 406 randomly selected participants from 3075 well-functioning persons aged 70 to 79 years and followed these individuals for 6 years. Individuals with the highest Fet-A levels were observed to have an increased risk of incident diabetes. This association was independent of age, sex, race, waist circumference, body weight, physical activity, blood pressure level, fasting glucose level, high-density lipoprotein cholesterol concentration, triglyceride concentration, and C-reactive protein level(Ix et al., 2008). Another case-cohort study of 2,500 individuals in the EPIC-Postsdam study was published by Stefan *et al.* in 2008. They followed 2,164 individuals who were diabetes free at baseline for 7 years and found 849 incident diabetes cases. After adjustment for glucose, triglycerides, HDL cholesterol, A1C, γ-glutamyltransferase, or high-sensitivity C-reactive protein, they also found that plasma Fet-A levels were positively associated with the risk of diabetes (Stefan et al., 2008). In 2011, a community-based cross-sectional study was completed by Song et al. among 5,227 Chinese adults (Song et al., 2011). In this study, serum Fet-A was positively associated with elevated HOMA-IR and fasting serum insulin both among the individuals with or without type 2 diabetes; however, the relationship between serum Fet-A and impaired glucose regulation was not significant. Reinehr and Roth in 2008 showed that Fet-A levels were high in children with nonalcoholic fatty liver disease, a feature of metabolic syndrome. After a 1 year weight loss study using exercises, behavior and nutrition therapy, substantial weight loss led to a significant reduction of Fet-A and the prevalence of nonalcoholic fatty liver disease (Reinehr and Roth, 2008). In another clinical study studying the feuin-A concentrations in 75 morbidly obese patients (BMI

over 45) before and after weight loss induced by gastric bypass (Brix et al., 2010), the investigators found that Fet-A levels were significantly higher in morbidly obese patients compare to normal weight individuals. Gastric bypass surgery caused significant weight loss and reductions of serum Fet-A concentrations, fasting insulin and HOMA IR. In total, Fet-A is an independently factor related to type 2 diabetes, and therefore the reduction of Fet-A concentrations may provide a therapeutic method of type 2 diabetes, obesity and metabolic syndrome.

Previously, AHSG Gene Polymorphisms and circulating Fet-A concentrations have been found to be associated with cardiovascular diseases (CVD) (Fisher et al., 2009). Recently, while Jesen *et al.* showed that higher Fet-A levels were related with lower CVD risks in healthy individuals without type 2 diabetes, obesity or insulin resistance, they did observe an opposite direction in which high circulating Fet-A was positively associated with CVD among individuals with type 2 diabetes (Jensen et al., 2013). The newest clinical study was conducted by Samocha-Bonet *et al.*. They overfed 40 healthy men and women with 1100 ± 100 kcal/day above baseline energy requirement for 28 days. The circulating Fet-A, FFAs, and proinflammatory markers were significantly increased after overfeeding, while Fet-A showed significant association with MCP-1, this relationship was independent with FFAs (Samocha-Bonet et al., 2014).

The precise mechanisms regulating Fet-A expression and secretion are largely unknown. In 2006, Woltje *et al.* found that dexamethasone could up-regulate hepatic Fet-A mRNA, which then binds to the hepatocyte nuclear factor 3β and CCAAT enhancer binding the protein β (C/EBP-β). This up-regulation was also related to glucocorticoid hormones (Woltje et al., 2006). Free fatty acids had also been found to activate the

ERK1/2 signaling pathway and further increase the Fet-A secretion (Dasgupta et al., 2010; Takata et al., 2009). Recently, Kahraman *et al.* observed that NEFA up-regulated Fet-A's mRNA expression in human primary hepatocytes (Kahraman et al., 2013). Ou *et al.* reported that endoplasmic reticulum (ER) stress caused by palmitate and high glucose further increased expression of Fet-A and decreased the insulin sensitivity (Ou et al., 2012a). In our group, Kaushik *et al.* in 2009 reported that niacin treatment lowed serum levels total and phosphorylated Fet-A in individuals who have metabolic syndrome. This modification was also associated with changes in triglyceride, C-reactive protein, HDL, and serum cortisol (Kaushik et al., 2009).

**2.7 Weight loss, exercise and risk of type 2 diabetes**

The benefits of regular physical activity and maintenance of a healthy weight have been well established through various prospective studies, demonstrating a decrease in the incidence and the development of type 2 diabetes and improving the other factors of metabolic syndrome (Schulze and Hu, 2005). Previously, four, large population cohort studies have been done to test if physical activity and weight change could delay or prevent the transition from prediabetes to type 2 diabetes (Sherwin et al., 2004). The results from two early studies, the Da Qing study and Malmo feasibility study indicated that the lifestyle modification can prevent diabetes, but these studies were limited in their general relevance (Eriksson and Lindgarde, 1991; Pan et al., 1997). Later, two randomized controlled trials reported and confirmed the same conclusion of the effect of lifestyle modification in preventing type 2 diabetes. In the Finnish study, after 3.2 years follow up of 522 obese subjects with IGT, the investigators found 58% relative reduction of incidence of diabetes in groups of people who achieved 5% weight loss, ate low fat

diet and exerciseed more (Tuomilehto et al., 2001). In the Diabetes Prevention Program (DPP), 3,234 obese patients were recruited and divided into 3 different groups, including lifestyle group, with goal of over 7% weight loss based on at least of 150/min/week of moderately intense activity, the metformin treatment group and the control group. After an average 2.8 years follow up, the lifestyle group showed a significant 58% reduction in progressing from prediabetes to type 2 diabetes compare to control group; the metformin group achieved 31% relative reduction in the incidence of diabetes (Knowler et al., 2002; The Diabetes Prevention Program Research Group, 2000). Recently, the results of "Action for Health in Diabetes (Look AHEAD) Trial" have been launched. It is the first study to determine whether an intensive lifestyle intervention (ILI group) (calorie control and increased physical activity for weight loss) could reduce cardiovascular morbidity and mortality in overweight individuals with type 2 diabetes compared to the impact of diabetes support and education (DSE group) (Ryan et al., 2003; Vitolins et al., 2009; Wadden et al., 2006). Unfortunately, after 4 years follow up, while the participants in ILI group maintained greater fitness, lost more weight, had lower hemoglobin A1c levels, and improvements in blood pressure and high-density lipoprotein cholesterol levels (Wing, 2010), the investigators could not find significant difference in cardiovascular events between the ILI group and DSE group finally, and this program was stopped (Look Ahead Research Group et al., 2013).

It is well known that physical activity provides a physiological tool to enhance insulin action (Seals et al., 1984). One of the molecular mechanisms related to physical activity is the regulation of various signaling proteins involved in the glucose uptake, such as GLUT4 in skeletal muscle (Chibalin et al., 2000; Hjeltnes et al., 1998; Hughes et

al., 1993; Ren et al., 1994). Immediately after a single bout of exercise, GLUT4 expression is increased and it is translocated to the cell surface in skeletal muscle (Kristiansen et al., 1996; Lund et al., 1995). Physical activity also change AMPK expression in skeletal muscle. Even low intensity aerobic exercises induce active form of AMPK (Fujii et al., 2000). Mu *et al.* used transgenic mice, which express a dominant inhibitory mutant of AMPK in skeletal muscle, and found that it completely blocked the ability of hypoxia or AICAR to activate hexose uptake (Mu et al., 2001). Recently, accumulating evidence indicates that physical activity plays a key role in the modification of energy expenditure and impact on various hormones (Jakicic and Otto, 2005). Kadoglou *et al.* recently published a report showing that even moderate intensity physical activity is independently associated with visfatin, apelin, adiponectin and leptin, indicating a relationship between physical activities and various adipokines (Kadoglou et al., 2012). In addition, peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) may provide another interesting mechanism (Gurd, 2011; Summermatter and Handschin, 2012). PGC-1α is an important regulator in mitochondrial biogenesis which activates various genes both in oxidative energy production and in fiber type transformation (Eckardt et al., 2011). During the exercises, PGC-1α controls the exercise adaptation, regulates the metabolic circuits and increases the thermogenic capacity in skeletal muscle (Summermatter and Handschin, 2012).

**2.9 Study goals and hypothesis**

Recent studies have indicated that Fet-A concentrations are lowered with exercise and weight loss (Brix et al., 2010; Jenkins et al., 2011; Reinehr and Roth, 2008). However there are no studies that explore the effect of a single bout of exercise on

alterations in Fet-A and pFet-A and its association with improvements in insulin sensitivity. Further, our understanding of Fet-A and pFet-A alterations with incremental weight loss (2-4%, 4-6%, 6-8% and 8-10%) is limited. We hypothesized that pFet-A would be the metabolically active form of Fet-A, which would be associated with insulin resistance and impaired glucose metabolism, and be more "sensitive" to a single bout of exercise and moderate weight loss. Therefore, the significance of this study is in its potential to contribute to our understanding of (a) the mechanistic basis of Fet-A's role in insulin action, i.e. role of phosphorylation; (b) alterations in Fet-A and pFet-A in response to an oral glucose load in obese versus normal body weight individuals; (c) the effects of a single bout of exercise on Fet-A and pFet-A and other surrogate markers of insulin sensitivity and (d) a modest weight loss (8-10% of body weighty loss) and alterations in Fet-A and pFet-A in the context of improvement of insulin sensitivity.

Our results might provide a potential target for measurement of insulin resistance and a therapeutic approach in treating obesity and type 2 diabetes.

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**Chapter 3: Phosphorylation status of fetuin-A is critical for its inhibitory effect on insulin-stimulated GLUT4 translocation, glucose uptake, and glycogen synthesis**

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**contributed to Fig.1, 2, and Fig.3B,D,E*

## Abstract

Fetuin-A, primarily secreted by the liver into circulation, is a negative regulator of insulin action. Elevated levels of serum fetuin-A are associated with insulin resistance, obesity and incident diabetes. While the majority of fetuin-A in circulation is dephosphorylated, approximately ~20% is in the phosphorylated form. However, studies on molecular characterization of fetuin-A phosphorylation status are limited. We hypothesized that phosphorylation status of fetuin-A is critical for its inhibitory action on insulin signaling and glucose uptake. We generated single (Ser312Ala) and double (Ser120Ala+Ser312Ala) mutants of human fetuin-A, which was partly or totally devoid of phosphorylation. Transfection of these plasmids into fibroblast cells that overexpress human insulin receptors (HIRcB cells) showed that only wild-type (WT) phosphorylated fetuin-A inhibited insulin signaling through AKT and MAPK. The single mutant (Ser312Ala) partially reversed the inhibition, and the double phospho-defective mutant was without effect. Further, we generated recombinant, wild-type human fetuin-A and phospho-defective fetuin-A (Ser312Ala and Ser120Ala+Ser312Ala) proteins using Chinese

Hamster Ovary-suspension (CHO-S) cells. Treatment of HIRcB and L6GLUT4myc cells showed that while WT phosphorylated fetuin-A impaired insulin stimulated insulin receptor phosphorylation, pAKT, pMAPK, GLUT4 translocation, glucose uptake and glycogen synthesis, the single Ser312Ala fetuin-A mutant partially reversed the inhibition. Full reversal of inhibition was observed with the Ser120Ala+Ser312Ala double mutant of fetuin-A. In conclusion, we report for the first time that phosphorylation on both Ser120 and Ser312, is critical for fetuin-A's full inhibitory activity on insulin signaling, glucose uptake, and glycogen synthesis.

## Introduction

According to the World Health Organization (WHO), in 2008, an estimated 1.46 billion adults were overweight or greater; of these 200 million men and nearly 300 million women were obese (Finucane et al., 2011; World Health Organization, 2013). Obesity is associated with an increased risk of developing insulin resistance, type 2 diabetes, cardiovascular diseases (CVD), and many cancers (Calle and Kaaks, 2004; Kahn et al., 2006; Van Gaal et al., 2006) Several mechanisms have been proposed that link obesity to insulin resistance and diabetes, including elevated plasma free fatty acids (FFAs) (Despres and Lemieux, 2006), body fat distribution (Arner, 2001; Bjorntorp, 1990; Motoshima et al., 2002; Sewter et al., 2002) and altered secretion of adipokines (Ouchi et al., 2011), myokines (Pedersen and Febbraio, 2012) and hepatokines (Stefan and Haring, 2013b).

Fetuin-A, earlier known as phosphorylated protein 63 (pp63) in rats and α2 Heremans-Schmid glycoprotein (AHSG) in humans, is a novel hepatokine secreted primarily by the liver that modulates insulin action by inhibiting insulin-stimulated IR autophosphorylation and TK activity (Auberger et al., 1989; Goustin et al., 2013; Mathews et al., 2000; Srinivas et al., 1993). Circulating concentrations of human fetuin-A range from 0.3 - 0.6 mg/ml, and exist in both phosphorylated (approximately 20%) and dephosphorylated (approximately 80%) forms (Dziegielewska et al., 1990; Haglund et al., 2001; Putman, 1984). Human fetuin-A has 2 chains; a 282 amino acid A chain (1-182), a 28 amino acid B chain (323-349) and a 40 amino acid connecting peptide (283-321) (Araki et al., 1989; Nawratil et al., 1996). Fetuin-A undergoes posttranslational

modifications including phosphorylation (~20% phosphorylation on Ser120 and ~77% on Ser312) and N- and O- glycosylation, on two N-linked glycosylation sites (Asp138 and Asp158) and two O-linked glycosylation sites (Thr238 and Thr252) on A-chain. Another O-glycan is attached to B-chain at Ser328 for the trisaccharide moiety (Haglund et al., 2001; Sparrow et al., 2008).

Several elegant studies, in animal models and humans, have shown that serum concentrations of fetuin-A are elevated with insulin resistance, obesity, incident type 2 diabetes, endoplasmic reticulum stress, fatty liver, high glucose, and elevated free fatty acids (Hennige et al., 2008; Ix et al., 2008; Lin et al., 1998; Ou et al., 2012; Pal et al., 2012; Srinivas et al., 1996; Stefan et al., 2008). Recent studies suggest that fetuin-A induces cytokine expression and serves as a novel adaptor protein linking free fatty acids to Toll-like receptor 4 (TLR4)-mediated inflammation and insulin resistance (Heinrichsdorff and Olefsky, 2012; Pal et al., 2012). Consistent with these findings, we and others have shown that mice lacking fetuin-A demonstrate improved insulin sensitivity and resistance to diet-induced obesity (Mathews et al., 2006; Mathews et al., 2002; Pal et al., 2012).

While the above studies seem to suggest a role for fetuin-A in modulating insulin sensitivity, studies on the molecular characterization of fetuin-A, in particular, studies on phosphorylation status of fetuin-A are limited (Akhoundi et al., 1994; Auberger et al., 1989). In this study we sought to characterize the role of phosphorylation stats on Ser120 and Ser312-fetuin-A on insulin-stimulated IR autophosphorylation and signaling through MAPK and Akt pathways, GLUT4 translocation, glucose uptake, and glycogen synthesis.

## Materials and Methods

### Human fetuin-A full-length cDNA construction

Human fetuin-A cDNA (NCBI Reference Sequence ID # NM _001622.2) cloned into pCMV6-XL4 vector, was purchased from Origene Inc, (Rockville, MD, USA). We cloned a 3x FLAG® peptide (22 amino acids) affinity tag (Sigma Chemical Company, St. Louis, MO) into the C-terminus end of fetuin-A cDNA. The C-terminus site was chosen for FLAG-tag because N-terminus encodes a signal peptide, which is required for fetuin-A to be secreted from the liver (Araki et al., 1989; Haglund et al., 2001; Nawratil et al., 1996).

### Generation of single and double fetuin-A mutant plasmids: Site-directed mutagenesis

To characterize the role of phosphorylation on Ser120 and Ser312 of fetuin-A, these sites (Ser120, Ser312) were mutated to "Alanine" using a PCR-based, custom-designed, site-directed mutagenesis approach (GenScript Corp., Piscataway, NJ). Briefly, oligonucleotide primers with the Ser120Ala, Ser312Ala, and Ser120Ala+Ser312Ala mutations and complementary to the opposite strands of the vector, were extended using Pfu turbo DNA polymerase, treated with Dpn I endonuclease to digest parental DNA template, and the nicked vector DNA incorporating the Ala mutations were transformed into ultracompetent cells. DNA sequencing was performed, which confirmed that the Ser312 codon was changed to Ala. Both single ($^{312}$Ala) and double ($^{120, 312}$Ala) mutants

were generated using the above approach. The location of the Ser120 residue and Ser312 residue (S in bold letter) and amino-acids adjacent to this site are shown below:

| Location | Position | Amino acid sequence |
|---|---|---|
| A chain | 110-130 | VYAKCDSSPD**S**(P04)AEDVRKVCQ |
| Connecting Peptide | 300-319 | HTFMGVVSLGSP**S**(P04)GEVSHPR |

**Cells culture and transfection**

Rat-1 fibroblast cells overexpressing human insulin receptors (HIRcB) were grown in complete Dulbecco's Modified Eagle Medium, (DMEM) (Invitrogen, Grand Island, NY, USA) containing 10% FBS (Invitrogen Corporation, Carlsbad, CA, USA). After confluence, HIRcB cells were transfected with 4 μg wild type (WT), single mutant ($^{312}$Ala) and double mutant ($^{120, 312}$Ala) fetuin-A plasmid DNA. using lipofectamine Plus$^{TM}$ reagent (Invitrogen Corporation, Carlsbad, CA) following the manufactory's recommended protocol. in 37 $^{o}$C. The media was tested for used for the presence of secreted fetuin-A using FLAG antibody (Sigma Chemical Company, St. Louis, MO, USA), phosphorylation status ($^{312}$Ser) was detected using anti phosphofetuin-A antibody.

**HIRcB cells transfection assays: MAPK and Akt stimulation**

Transfected cells were serum-starved for 6 hours and then treated with 100 nM insulin for 10 minutes. After washing with DPBS (Invitrogen Corporation, Carlsbad, CA) Cell were lysed sonicated (Sonics and Material Inc., Newtown, CT, USA) and the supernatant was obtained by centrifugation for 30 minutes at 14,000 rpm at 4 $^{o}$C. Proteins were separated on a 4-20% gradient SDS-PAGE gel (NuSep iGel, Austell, GA, USA), transferred to nitrocellulose, and detected using antibodies specific for phospho-Akt (Ser473) (Cell Signaling Technology, Danvers, MA, USA), Akt (Cell Signaling

Technology, Danvers, MA, USA), phospho-p44/42 MAPK (Thr202/Tyr204) (Cell Signaling Technology, Danvers, MA, USA) and GAPDH (AbCam PLC, Cambridge, MA). The membranes were developed by either SuperSignal West Femto Maximum Sensitivity substrate (Thermo Scientific, Rockford, IL) or SuperSignal West Pico chemiluminescence substrate (Thermo Scientific, Rockford, IL). Chemiluminescence was detected using UVP Bioimaging System (UVP, Upland, CA). Area densities of the bands were analyzed using the Un-Scan-It software package (Silk Scientific, Orem, UT, USA).

**Recombinant human fetuin-A protein production and purification**

**Transfection of CHO-S cells**

Chinese Hamster Ovary cells adapted to suspension culture (CHO-S) were purchased from Invitrogen (Invitrogen Corporation, Carlsbad, CA). CHO-S cells were thawed from liquid nitrogen and transferred into 70 ml of pre-warmed serum and antibiotic-free FreeStyle CHO Expression medium (Invitrogen Corporation, Carlsbad, CA), containing 8 mM L-glutamine. Cells were cultured in 125 ml Erlenmeyer spinner flasks (Invitrogen Corporation, Carlsbad, CA, USA) at 100 rpm in a 37 $^{o}$C incubator and a humidified atmosphere of 8% $CO_2$.

One day before transfection, CHO-S cells were re-suspended in 70 ml of pre-warmed fresh FreeStyle$^{TM}$ medium without antibiotics. The next morning, CHO-S cells were counted to ascertain cells reached a density of $1x10^6$ cells/ml. Then 75 μg of plasmid DNA (wild type, $^{312}$Ala and $^{120, 312}$Ala fetuin-A) was diluted into 0.7 ml of OptiPro Media (Invitrogen Corporation, Carlsbad, CA, USA), mixed with 0.7 ml of 75 μl of FreeStyle MAX transfection reagent (Invitrogen Corporation, Carlsbad, CA, USA). Spinner incubated for 10 minutes at room temperature, and then added slowly to the flask

containing cells with swirling. Transfected cells were returned to the incubator and placed on an orbital shaker at 100 rpm and cultured at 37 $^{o}$C and in a humidified atmosphere of 8% $CO_2$.

**Recombinant human fetuin-A purification**

Five days after transfection, the media was collected; cells and cell debris were removed by centrifugation at 2,500 rpm for 10 min at 4 $^{o}$C. Supernatant was then purified by jacalin column chromatography (Vector Laboratories, Burlingame, CA, USA) (Hortin and Trimpe, 1990; To et al., 1995). WT, $^{312}$Ala and $^{120, 312}$Ala fetuin-A proteins bound to the jacalin column were eluted by 0.1 M melibiose, in 1 ml fractions. The fraction containing the highest concentrations of protein was further concentrated using Micosep$^{TM}$ Advance Centrifugal Devices 10K MWCO (Pall Corporation, Ann Arbor, MI, USA), at 3000 g, 90 minutes.

Purity was determined using SDS-PAGE and Western blotting techniques to detect FLAG and phosphofetuin-A. Wild type, $^{312}$Ala and $^{120, 312}$Ala fetuin-A purified proteins were separated by SDS-PAGE on 4 - 20% tris-glycine gel (NuSep Inc., Lawrenceville, GA, USA). Gels were then transferred to nitrocellulose membrane (BioRad Laboratories, Hercules, CA, USA) by using semi-dry transfer method with 20% transfer buffer made up of 25 mM Tris, 192 mM glycine and 20% methanol. Proteins were blocked in 5% non fat dry milk (Bio-Rad, Hercules, CA, USA) FLAG antibodies (Sigma Chemical Company, St. Louis, MO, USA) were used to detect fetuin-A and mutants. Phosphorylated Ser312 fetuin-A was detected using a custom generated affinity-purified antibody (Affinity BioReagents, Golden, CO, USA). This antibody specifically detected the phosphorylated Ser312 fetuin-A epitope:

"HTFMGVVSLGSP**S**(PO4)GEVSHPR". WT, phosphorylated fetuin-A, and phospho-defective mutant proteins ($^{312}$Ala and $^{120, 312}$Ala) were aliquoted and stored in -80 $^{o}$C for future use.

**Functional Studies for fetuin-A mutant protein:**

**Cell culture and treatment with fetuin-A mutant protein:**

Confluent HIRcB cells were serum-starved in DMEM media containing 0.1% BSA. The next morning, cells were treated with recombinant WT, $^{312}$Ala and $^{120, 312}$Ala fetuin-A for 20 minutes, followed by stimulation with 100 nM of insulin for 10 minutes. The cells were then lysed, proteins separated by gel electrophoresis in a 4-20% Tris-glycine gel, and immnuoblotting with antibodies against phospho-Akt (Ser473) (Cell Signaling Technology, Danvers, MA, USA), Akt (Cell Signaling Technology, Danvers, MA, USA), phospho-p44/42 MAPK (Thr202/Tyr204) (Cell Signaling Technology, Danvers, MA, USA) and GAPDH (AbCam PLC, Cambridge, MA). Insulin receptors were first immunoprecipitated using insulin receptor β-subunit (C-19) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and immunoblotted with anti-phospho-IR/IGF 1R (Tyr1158/Tyr1162/Tyr1163) (Upstate Millipore, Temecula, CA, USA).

**Glucose uptake assay**

2-Deoxy glucose uptake in rat L6- GLUT4myc cells was assayed as described previously (Ueyama et al., 1999). Briefly, confluent L6- GLUT4myc cells were treated with WT, $^{312}$Ala and $^{120, 312}$Ala fetuin-A proteins for 20 minutes at 37 $^{o}$C. Next, cells were stimulated with 100 nM insulin for 30 minutes, following by the addition of START buffer containing 100 μM 2-deoxyglucose and 0.5 μCi/ml [$^{3}$H]-2-deoxyglucose

(American Radiolabeled Chemicals. Inc, St Louis, MO, USA) for 10 minutes at room temperature. Cells were immediately washed three times with ice-cold PBS and solubilized in 0.2 N NaOH. The solubilized cells were mixed with ScintiVerse (TM) BD cocktail (Fisher Scientific, Fair Lawn, NJ, USA) and 2-deoxyglucose taken up by the cells was counted for 2 minutes in a liquid scintillation counter (Packard Instrument Company, Downers Grove, IL, USA).

**Glycogen synthesis assay:**

Gycogen synthesis was assayed according to Brady (Brady, 2003). Briefly, WT, $^{312}$Ala and $^{120, 312}$Ala fetuin-A purified proteins were pre-incubated for 15 minutes prior to the addition of 100 nM insulin for 30 min. Next, 1 μCi of $^{14}$C-D-glucose solution was added into each well, and incubated at 37 $^{o}$C for 1.5 hours. Cells were washed, lysed, and cellular glycogen was precipitated with ethanol. Glycogen pellets were washed and dried overnight and the $^{14}$C-glucose incorporated into glycogen was measured by liquid scintillation counting (Packard Instrument Company, Downers Grove, IL, USA).

**GLUT4 translocation assay:**

Cell surface myc-tagged GLUT4 was quantitated as described earlier (Wang et al., 1998). Confluent rat L6-GLUT4 skeletal muscle cells were treated with WT, $^{312}$Ala and $^{120, 312}$Ala fetuin-A purified proteins for 30 minutes, followed by the addition of 100 nM insulin for 20 minutes. Next, cells were washed three times with ice cold DPBS, incubated with 3% paraformaldehyde (PFA) (Electron microscopy sciences, Hatfield, PA, USA), at 4 $^{o}$C. and blocked with 5% goat serum (GIBCO Invitrogen, Grand Island, NY, USA). Next, cells were incubated with anti-myc monoclonal antibody (1:100 dilution) (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) in DPBS containing 3% goat

serum for 60 minutes at 4 $^{o}$C. After incubation, cells were washed with DPBS, incubated in the dark with freshly prepared OPD reagent for 30 minutes at room temperature. Finally, the reaction was stopped by adding 3M HCl, and the absorbance was measured at 492 nm in a microplate reader (Bio-Tek, Winooski, VT, USA).

## Results

### Generation, purification, and detection of wild-type and mutated forms of recombinant human fetuin-A

Fetuin-A plasmid with a 3X-FLAG-tag, attached to the 3'-end, was constructed as shown in Fig.1A. Restriction digestion using *NotI* confirmed the 1.2 kb product size of the fetuin-A-3X-FLAG cDNA (Fig.1B). Next, using site-directed mutagenesis, we generated Ser312Ala fetuin-A mutant ($^{312}$Ala ) and Ser120Ala+Ser312Ala double mutant of fetuin-A ($^{120, 312}$Ala). A custom-designed "primer-walking" approach confirmed that the full-length fetuin-A cDNA sequence was identical to the one in NCBI database (NCBI Reference Sequence ID # NM_001622.2) (Fig.1C). Next, CHO-S cells were transiently transfected with either WT, $^{312}$Ala, or $^{120, 312}$Ala-fetuin-A cDNA (Fig.2A,B). Culture media, obtained after 5 days, was purified by jacalin column chromatography, and column eluates were analyzed by Western blotting for fetuin-A expression using FLAG antibodies. Typically, column eluates #2 and #3 were enriched for fetuin-A ((Fig.2C,D). Following concentration using a 10 kD-MW cutoff centrifugal device (Fig.2E), fetuin-A was separated on 8% SDS-PAGE. Western blot analysis using a Ser312-phosphorylated fetuin-A antibody demonstrated that secreted recombinant human WT fetuin-A was phosphorylated on Ser312, while both $^{312}$Ala and $^{120, 312}$Ala fetuin-A mutants were not phosphorylated on Ser312 (Fig. 2F).

**Effect of phosphorylated fetuin-A on IR, Akt and MAPK-mediated insulin signal transduction**

To investigate the role of fetuin-A phosphorylation status on insulin signal transduction, we first transiently transfected WT, $^{312}$Ala, or $^{120, 312}$Ala-fetuin-A cDNA into rat fibroblast cells that overexpress human insulin receptors (HIRcB cells). The next day, media was collected and analyzed by SDS-PAGE to verify expression and secretion of WT and mutant fetuin-A. As expected, while WT and mutant fetuin-A were detected by FLAG antibody, only WT fetuin-A was phosphorylated on Ser312 (Fig.3A). Cells lysates were collected and separated by 4-20% SDS-PAGE gel. We observed, as expected, that insulin significantly stimulated phosphorylation of Akt and MAPK. HIRc B cells transfected with WT fetuin-A showed a marked blunting of insulin-stimulated phosphorylation of Akt and MAPK (Fig.3A). However, cells transfected with phosphodefective fetuin-A single ($^{312}$Ala) and double ($^{120, 312}$Ala) mutant plasmids showed no inhibition of insulin-stimulated phosphorylation of Akt and MAPK (Fig.3A).

Next, to further characterize the effects of fetuin-A phosphorylation status on insulin signaling pathways, confluent, serum-starved HIRcB cells were preincubated with purified recombinant wild type, $^{312}$Ala and $^{120, 312}$Ala fetuin-A proteins for 20 min followed by insulin (100 nM) stimulation for 10 min incubation. Cell lysates were immunoblotted with antibodies against pIR, IR- β subunit, pAkt, Akt, pMAPK and GAPDH. WT fetuin-A significantly blocked the insulin stimulated phosphorylation of IR, Akt and MAPK. While $^{312}$Ala showed comparatively less magnitude of inhibition compared to WT fetuin-A, $^{120,312}$Ala fetuin-A had no inhibitory effect on insulin-stimulated pIR, pAkt, and pMAPK (Fig.3B).

**Effect of phosphorylated fetuin-A on insulin-stimulated GLUT4 translocation, glucose uptake, and glycogen synthesis**

To test whether the inhibitory effect of fetuin-A on insulin signaling pathways was associated with skeletal muscle insulin action, we used rat L6 myoblasts stably transfected with a GLUT4 cDNA harboring exofacial myc epitope tag (L6-GLUT4myc myoblasts) to examine the effects of WT fetuin-A and its dephosphorylated mutants in insulin stimulated glucose uptake, glycogen synthesis and GLUT4 translocation. We observed that 0.2 μM WT fetuin-A significantly inhibited insulin-stimulated GLUT4 translocation, glucose uptake, and glycogen synthesis (Fig.3C,D,E). Compared to WT fetuin-A, $^{312}$Ala-fetuin-A showed a partial reversal of its inhibitory effects on insulin-stimulated GLUT4 translocation, glucose uptake and glycogen synthesis. The dephosphorylated double mutant $^{120,312}$Ala fetuin-A did not show any inhibitory effects on insulin-stimulated GLUT4 translocation, glucose uptake, and glycogen synthesis (Fig.3C,D,E).

## Discussion

While fetuin-A is considered a novel hepatokine in regulating insulin action and glucose homeostasis (Auberger et al., 1989; Kaushik et al., 2009; Mathews et al., 2000; Mathews et al., 2006; Stefan and Haring, 2013b) and accumulating evidence supports the association between fetuin-A and insulin resistance (Brix et al., 2010; Ix et al., 2006; Ix et al., 2009; Ix et al., 2008; Liu et al., 2012; Reinehr and Roth, 2008; Stefan and Haring, 2013a, b; Stefan et al., 2008; Stefan et al., 2006), there are two critical issues in this area that are still poorly understood. First, fetuin-A is the second abundant protein in circulation, after albumin, with concentrations ranging from 0.3 - 0.6 mg/ml (Dziegielewska et al., 1990; Putman, 1984). Thus, how fetuin-A potentially functions as a negative regulator of insulin action is a key question. Second, the role of fetuin-A in modulating the metabolic arm of insulin signaling is not clear. Previously fetuin-A was identifed to be a specific inhibitor of insulin's mitogenic action, with no effects on its metabolic action (Akhoundi et al., 1994; Auberger et al., 1989; Srinivas et al., 1993), a recent study seems to indicate that fetuin-A antagonizes metabolic functions initiated by insulin receptor activation (Goustin et al., 2013).

In this study, we addressed these two issues by characterizing the role of phosphorylated fetuin-A in insulin action and insulin signal transduction. Although the initial findings by Auberger *et al.* and a few other studies claimed that phosphorylation of fetuin-A may be important to exert its inhibitory effects, (Auberger et al., 1989; Kalabay et al., 1996; Ohnishi et al., 1997), this was not extensively characterized. Our findings have shown that phosphorylated protein is the active isoform of fetuin-A, exerting an

inhibitory effect on IR autophosphorylation and negatively impacting downstream insulin signaling and blunting insulin-stimulated GLUT4 translocation, glucose uptake and glycogen synthesis.

These findings have important implications. First, this offers a better understanding of how a protein that is second largest in concentration next to albumin could have regulatory effects on insulin action. Using site-directed mutagenesis, we produced single mutant of Ser312Ala, which predictably lost over 75% of its phosphorylation status and double mutant Ser120Ala+Ser312Ala fetuin-A, which, predictably, was 100% dephosphorylated (Haglund et al., 2001). Our findings that the double-mutant was unable to inhibit insulin signaling through Akt and MAPK, suggested that phosphorylation may be important for its inhibitory action. Next, we treated HIRc B cells with recombinant WT or mutant fetuin-A protein, which confirmed that while the single Ser312Ala mutant partially reversed inhibition, full inhibition of IR, MAPK, and Akt phosphorylation was observed with the phospho-defective fetuin-A Ser120Ala+Ser312Ala double mutant. This suggests that, if only 20% of fetuin-A is phosphorylated in circulation, then, that phosphorylated fetuin-A may exist at a concentration of 60 – 120 μg/ml (1.4 - 2.8 μM ) in circulation (Haglund et al., 2001; Mathews et al., 2000). Our studies with HIRc B cells show that 0.2 μM WT fetuin-A, which demonstrated phosphorylation, was able to inhibit insulin signaling by 50-80%, which was similar to our earlier *in vitro* and findings with recombinant human fetuin-A (Srinivas et al., 1995). However, *in vivo*, the concentrations required to achieve blunting of insulin effects may be closer to ~2 μM (Mathews et al., 2000).

Secondly, this study demonstrates that fetuin-A inhibits both the mitogenic and the metabolic arms of insulin signaling. We show that fetuin-A, in particular, phosphorylated fetuin-A was able to inhibit insulin-stimulated glucose uptake and glycogen synthesis. These findings are consistent with our earlier findings on fetuin-null mice that showed improved insulin sensitivity, and increased insulin-stimulated glucose incorporation into glycogen in skeletal muscle (Mathews et al., 2002), and another recent report which shows that fetuin inhibits insulin-stimulated GLUT4 translocation in C2C12 myotubes (Goustin et al., 2013). The novel aspect of this study is that $^{312}$Ala fetuin-A showing partially inhibitory effects, while $^{312, 120}$Ala fetuin-A significantly blunted the inhibitory effects of fetuin-A, suggesting that phosphorylation on Ser120 and Ser312 is critical for fetuin-A inhibitory effects on insulin-stimulated IR, Akt, and MAPK phosphorylation, GLUT4 translocation, glucose uptake and glycogen synthesis.

GLUT4 has been described as a key signaling molecule regulated by both insulin-dependent and insulin-independent signaling pathways (Haruta et al., 1995; Kennedy et al., 1999; Wallberg-Henriksson and Holloszy, 1984). AMP-activated kinase (AMPK) signaling pathway was shown as a critical regulator of GLUT4 and glucose uptake (Dreyer et al., 2006; Pedersen and Febbraio, 2012). We have not determined the relationship between fetuin-A and AMPK activation in the present study.

Our study has several limitations: 1. We have not examined, directly, the effects of Ser120 phosphorylation, but indirectly, using the $^{120,312}$Ala double mutant. Our studies suggest that phosphorylation on both Ser120 and Ser312 are important. It is preliminary to specifically rate the significance, order, or differential effects of phosphorylation of either of the 2 sites. 2. We did not examined the mechanism of inhibition of

phosphorylated fetuin-A. Previously studies have shown that fetuin-A reduces Vmax of the insulin-stimulated IR-TK reaction and increases $S_{0.5}$ for ATP and the exogenous substrate, without affecting insulin binding (Goustin et al., 2013; Grunberger et al., 2002). Further, we have shown that fetuin-A interacts with the activated insulin receptor (Mathews et al., 2000). It may be speculated that phosphorylated fetuin-A preferentially interacts with the insulin receptor and affects kinetics of the IR-TK reaction.

In conclusion, we have presented key evidence to indicate that fetuin-A inhibits both the mitogenic and metabolic arm of insulin signaling. Secondly, our studies have revealed a critical role for phosphorylation status of fetuin-A for its inhibition of insulin-stimulated glucose uptake and glycogen synthesis. These studies raise the possibility that Ser120,312-phosphorylated fetuin-A may be the active isoform of fetuin-A. Further, modulation of phosphorylation status of fetuin-A may provide potential strategies for treatment of insulin resistance and type 2 diabetes.

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## Figure Legends

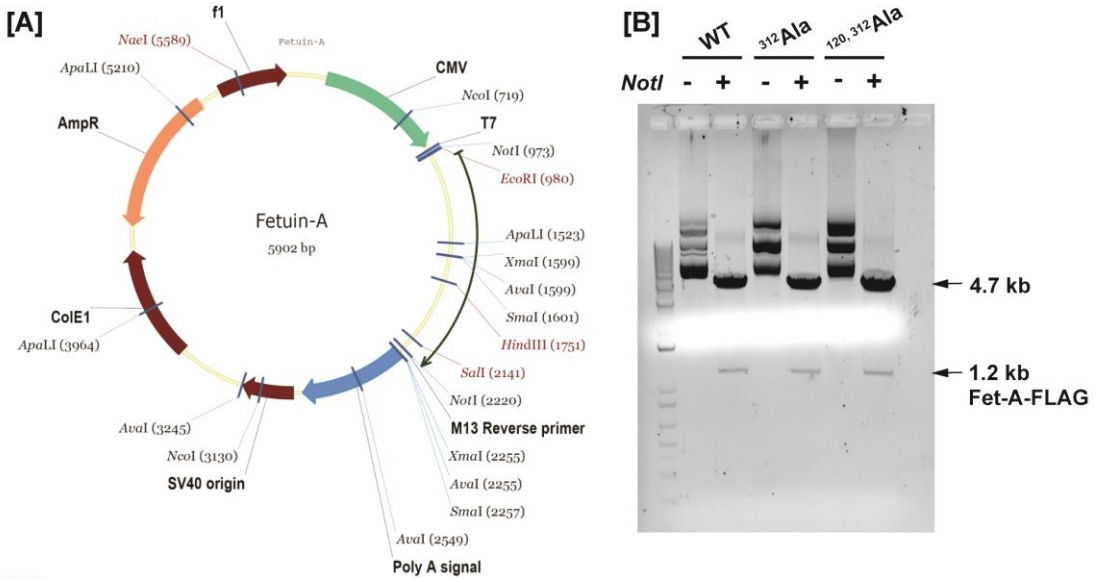
Fig.1. Full-length fetuin-A plasmid map and nucleotide sequence. Plasmid map of full length human fetuin-A cDNA cloned into pCMV6-XL4 vector (Origene Inc., Rockville, MD, USA) (A) and human fetuin-A cDNA sequence showing the start and stop codons in red, the signal peptide in gray, and Ser120 and Ser312 highlighted in yellow (C). Plasmid fetuin-A (WT, 312Ala and 120, 312Ala) was digested with Not I restriction enzyme (Invitrogen Corporation, Carlsbad, CA), for 6 hours at 37$^{o}$C, separated by electrophoresis at 160 Volts for 30 min, stained with 1% ethidium bromide, and imaged using UVP BioImaging system (UVP LLC, Upland, CA) (B).

Fig.2. Recombinant human fetuin-A protein production and purification. FLAG-tagged WT, $^{312}$Ala and $^{120, 312}$Ala fetuin-A plasmids were transfected into CHO-S cells and grown for 5 days in a spinner flask at 100 rpm, in a 37$^{o}$C incubator with 8% humidified $CO_2$ atmosphere (A, B). Media was spun down to remove cells and cell debris. WT and mutant fetuin-A proteins secreted into the culture media, were purified by jacalin column (Vector Laboratories, Burlingame, CA, USA) chromatography (C). Fractions containing fetuin-A were identified by Western blotting using FLAG antibody (D). Proteins containing highest concentrations of fetuin-A were concentrated using MicosepTM Advance Centrifugal Devices 10K MWCO (Pall Corporation, Ann Arbor, MI) (E). Custom-generated affinity-purified antibody (Affinity BioReagents, Golden, CO) against the phosphorylated Ser312-fetuin-A epitope was used to detect wild type, $^{312}$Ala and $^{120,}$ $^{312}$Ala recombinant human fetuin-A proteins purified from CHO-S cells. FLAG antibody

(Sigma Chemical Company, St. Louis, MO, USA) was used to detect the 3XFLAG tag in recombinant fetuin-A proteins (F).

Fig.3. Effects of phosphorylation status of fetuin-A on insulin signal transduction, insulin-stimulated GLUT4 translocation, glucose uptake, and glycogen synthesis. HIRcB cells were transfected with 4 μg of recombinant human fetuin-A WT, $^{312}$Ala, $^{120, 312}$Ala or empty vector using Lipofectamine Plus$^{TM}$ reagent. Media were collected to analyze FLAG and fetuin-A phosphorylation status. Cells were stimulated with 100 nM insulin and analyzed for expression of pAKT, AKT, pMAPK and GAPDH (A). HIRcB cells were treated with 0.2 μM purified human recombinant WT, $^{312}$Ala and $^{120, 312}$Ala fetuin-A proteins for 20 minutes followed by insulin (100 nM) stimulation for 10 minutes. Cells were collected for Western blot analyses of pIR, IR-β subunit, pAKT, AKT, pMAPK and GAPDH (B). Rat L6-GLUT4myc myoblasts were treated with 0.2 μM WT or mutant fetuin-A proteins for 30 minutes and then stimulated with 100 nM insulin. Cells were collected for analyses of insulin-stimulated GLUT4 translocation (C), glucose uptake (D), and glycogen synthesis (E). Representative blots are depicted for (A) and (B). All experiments were run in triplicate. *Compared to insulin treatment group. * $P < 0.05$; ** $P < 0.01$

**Fig.1**




[C]

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+1    Met Lys Ser Leu Val Leu Leu Leu Cys Leu Ala Gln Leu Trp Gly Cys His Ser Ala Pro His Gly Pro Gly Leu Ile Tyr Arg Gln Pro Asn Cys Asp Asp
1     ATGAAGTCCC TCGTCCTGCT CCTTTGTCTT GCTCAGCTCT GGGGCTGCCA CTCAGCCCCA CATGGCCCAG GGCTGATTTA TAGACAACCG AACTGCGATG
      TACTTCAGGG AGCAGGACGA GGAAACAGAA CGAGTCGAGA CCCCGACGGT GAGTCGGGGT GTACCGGGTC CCGACTAAAT ATCTGTTGGC TTGACGCTAC
+1    Asp Pro Glu Thr Glu Glu Ala Ala Leu Val Ala Ile Asp Tyr Ile Asn Gln Asn Leu Pro Trp Gly Tyr Lys His Thr Leu Asn Gln Ile Asp Glu Val Lys
101   ATCCAGAAAC TGAGGAAGCA GCTCTGGTGG CTATAGACTA CATCAATCAA AACCTTCCTT GGGGATACAA ACACACCTTG AACCAGATTG ATGAAGTAAA
      TAGGTCTTTG ACTCCTTCGT CGAGACCACC GATATCTGAT GTAGTTAGTT TTGGAAGGAA CCCCTATGTT TGTGTGGAAC TTGGTCTAAC TACTTCATTT
+1    Lys Val Trp Pro Gln Gln Pro Ser Gly Glu Leu Phe Glu Ile Glu Ile Asp Thr Leu Glu Thr Thr Cys His Val Leu Asp Pro Thr Pro Val Ala Arg Cys
201   GGTGTGGCCT CAGCAGCCCT CCGGAGAGCT GTTTGAGATT GAAATAGACA CCCTGGAAAC CACCTGCCAT GTGCTGGACC CCACCCCTGT GGCAAGATGC
      CCACACCGGA GTCGTCGGGA GGCCTCTCGA CAAACTCTAA CTTTATCTGT GGGACCTTTG GTGGACGGTA CACGACCTGG GGTGGGGACA CCGTTCTACG
+1    Ser Val Arg Gln Leu Lys Glu His Ala Val Glu Gly Asp Cys Asp Phe Gln Leu Leu Lys Leu Asp Gly Lys Phe Ser Val Val Tyr Ala Lys Cys Asp Ser
301   AGCGTGAGGC AGCTGAAGGA GCATGCTGTC GAAGGAGACT GTGATTTCCA GCTGTTGAAA CTAGATGGCA AGTTTTCCGT GGTATACGCA AAATGTGATT
      TCGCACTCCG TCGACTTCCT CGTACGACAG CTTCCTCTGA CACTAAAGGT CGACAACTTT GATCTACCGT TCAAAAGGCA CCATATGCGT TTTACACTAA
+1    Ser Ser Pro Asp Ser Ala Glu Asp Val Arg Lys Val Cys Gln Asp Cys Pro Leu Leu Ala Pro Leu Asn Asp Thr Arg Val Val His Ala Ala Lys Ala Ala
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+1    Ala Leu Ala Ala Phe Asn Ala Gln Asn Asn Gly Ser Asn Phe Gln Leu Glu Glu Ile Ser Arg Ala Gln Leu Val Pro Leu Pro Pro Ser Thr Tyr Val Glu
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+1    Phe Thr Val Ser Gly Thr Asp Cys Val Ala Lys Glu Ala Thr Glu Ala Ala Lys Cys Asn Leu Leu Ala Glu Lys Gln Tyr Gly Phe Cys Lys Ala Thr Leu
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+1    Leu Ser Glu Lys Leu Gly Gly Ala Glu Val Ala Val Thr Cys Thr Val Phe Gln Thr Gln Pro Val Thr Ser Gln Pro Gln Pro Glu Gly Ala Asn Glu Ala
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+1    Ser His Pro Arg Lys Thr Arg Thr Val Val Gln Pro Ser Val Gly Ala Ala Ala Gly Pro Val Val Pro Pro Cys Pro Gly Arg Ile Arg His Phe Lys Val
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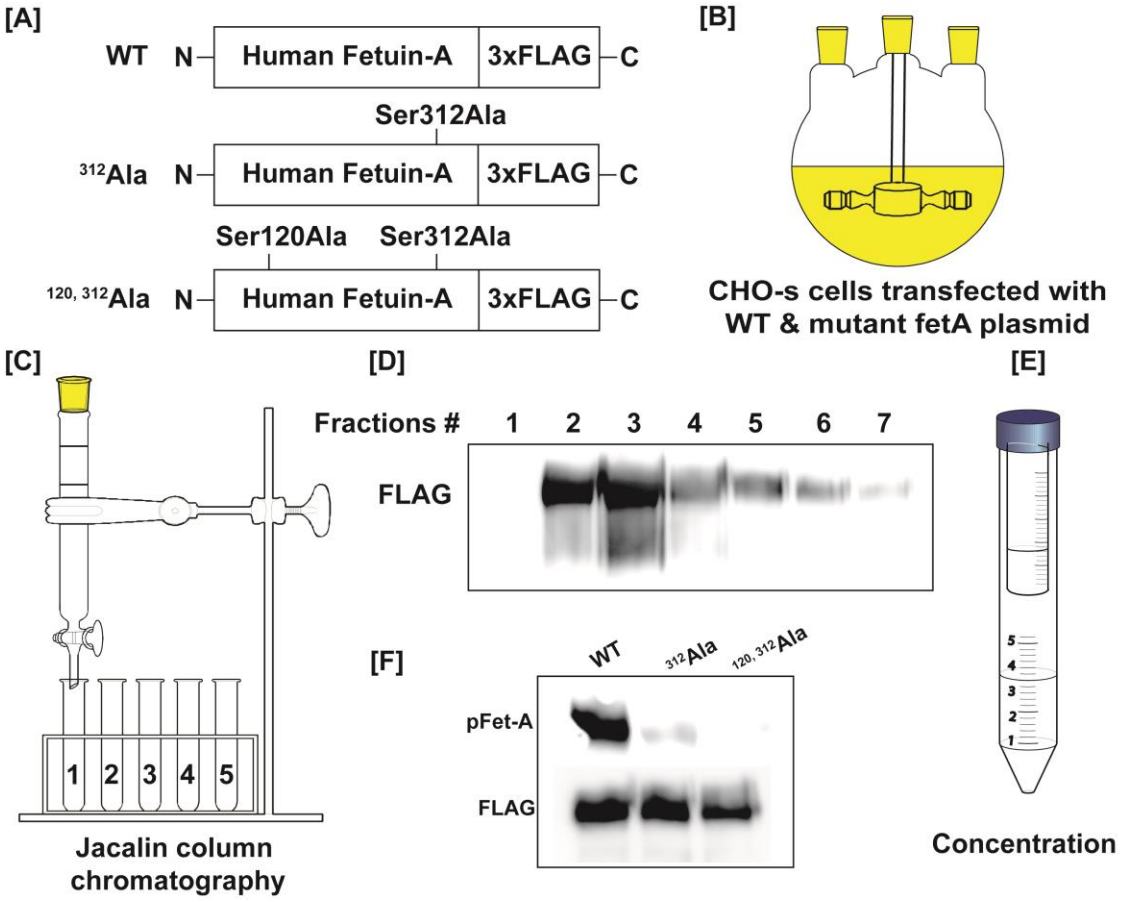
+1    Val ***
1101  CTAG
      GATC
```

**Fig.2**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Human Fetuin-A
3xFLAG
C
Ser312Ala
312Ala
Ser120Ala
Ser312Ala
120, 312Ala
[B]
CHO-s cells transfected with
WT & mutant fetA plasmid
[C]
1 2 3 4 5
Jacalin column
chromatography
[D]
Fractions # 1 2 3 4 5 6 7
FLAG
[E]
5
4
3
2
1
Concentration
[F]
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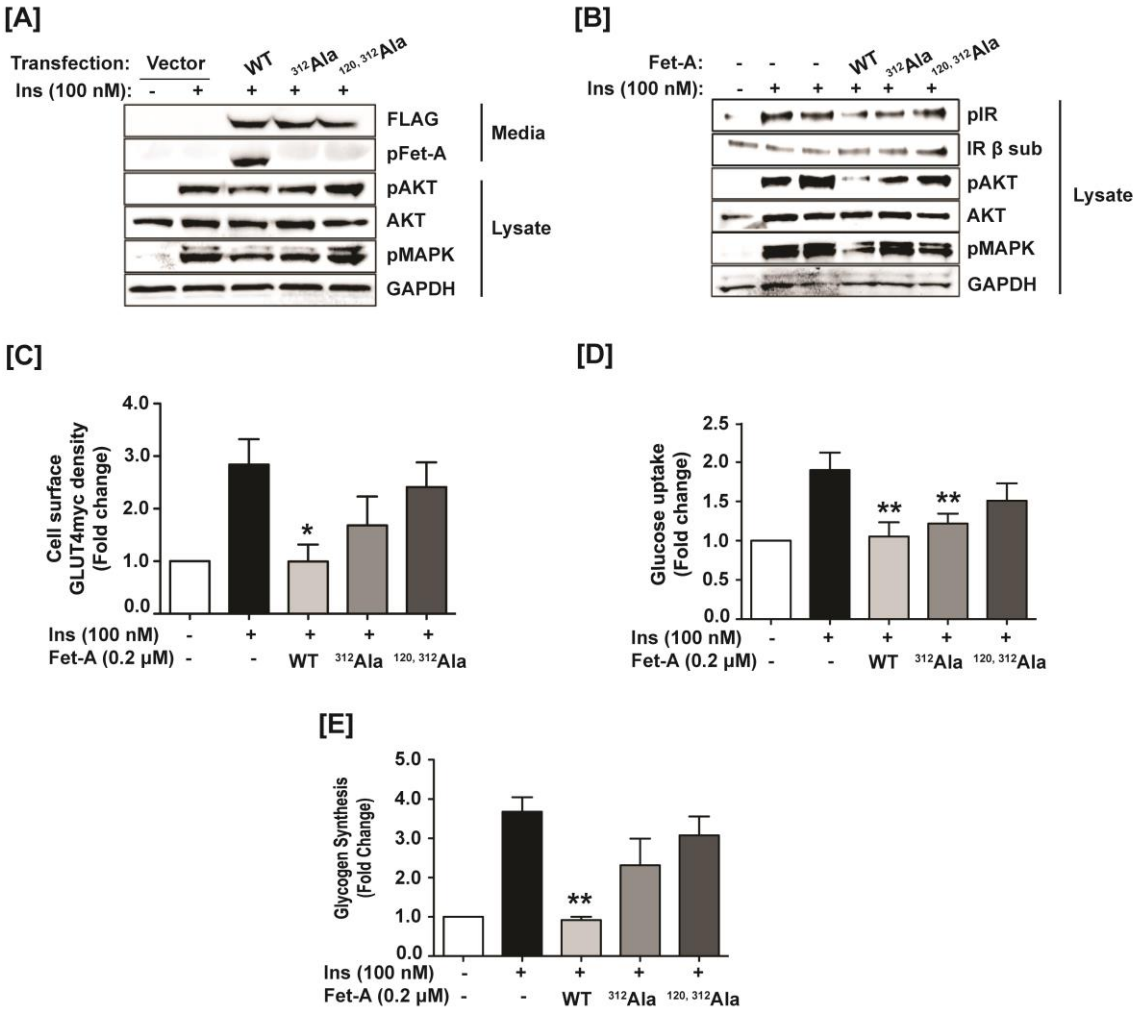
120, 312Ala
pFet-A
FLAG

**Fig.3**


[A]
Transfection: Vector WT 312Ala 120, 312Ala
Ins (100 nM): - + + + +
FLAG
pFet-A
Media
pAKT
AKT
pMAPK
GAPDH
Lysate
[B]
Fet-A: - - - WT 312Ala 120, 312Ala
Ins (100 nM): - + + + + +
pIR
IR β sub
pAKT
AKT
pMAPK
GAPDH
Lysate
[C]
Cell surface GLUT4myc density (Fold change)
4.0
3.0
2.0
1.0
0.0
*
Ins (100 nM) - + + + +
Fet-A (0.2 μM) - - WT 312Ala 120, 312Ala
[D]
Glucose uptake (Fold change)
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2.0
1.5
1.0
0.5
0.0
**
**
Ins (100 nM) - + + + +
Fet-A (0.2 μM) - - WT 312Ala 120, 312Ala
[E]
Glycogen Synthesis (Fold Change)
5.0
4.0
3.0
2.0
1.0
0.0
**
Ins (100 nM) - + + + +
Fet-A (0.2 μM) - - WT 312Ala 120, 312Ala

## Chapter 4: Phosphorylation status of fetuin-A (Ser312) is positively associated with insulin resistance and obesity

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*lead graduate student - contributed to Table 1, Fig.1-4

#contributed to recruitment of subjects, sample collection

[‡]contributed partially to assay of serum fetuin-A

[##]Co-Principal Investigator

[‡‡]Principal Investigator

### Abstract

Fetuin-A (Fet-A) is a negative regulator of insulin-stimulated insulin receptor tyrosine kinase and inhibitor of insulin signaling. Previously, the phosphorylated form of Fet-A was shown to be 20 to 100-fold more potent than dephosphorylated form. While several landmark studies have shown that elevated levels of Fet-A are associated with insulin resistance, obesity, metabolic syndrome, fatty liver, and incident diabetes, there are no clinical studies that have explored Fet-A phosphorylation status. In this study, we recruited 31 obese and 11 normal weight men. Of these 23 were diagnosed with metabolic syndrome. All the participants underwent an OGTT and blood samples were

obtained every 30 minutes for 2 hours, to analyze serum Fet-A, Ser312-phosphorylated fetuin-A (pFet-A) and other metabolic parameters. As expected, serum Fet-A and pFet-A concentrations were significantly higher in obese individuals compared to normal weight men. However, unlike Fet-A, pFet-A showed a significant correlation with BMI, waist circumference, percent total fat, blood pressure and markers of insulin sensitivity including glucose, insulin, HOMA-IR, adipose-IR, QUICKI, and glucose-to-insulin ratio. During an oral glucose tolerance test (OGTT), $insulin_{AUC}$, $glucose_{AUC}$, IR index, Fet-$A_{AUC}$ and pFet-$A_{AUC}$ were significantly elevated in obese individuals. Further, we observed a temporal reduction of Fet-A and pFet-A concentrations in 30 and 60-minute respectively during OGTT in obese individuals. Our findings indicate that pFet-A is strongly associated with insulin resistance and may be dynamically involved in insulin response.

## Introduction

Although the reason for the parallel accelerated global epidemic of type 2 diabetes mellitus (T2DM) and obesity is complex and not yet fully understood, it is well known that insulin resistance associated with obesity, triggers the pathogenesis of type 2 diabetes mellitus (T2DM) (Johnson and Olefsky, 2013). The etiology of insulin resistance is complex. It is now believed that, chronic, low-grade inflammation associated with obesity and occurring in different tissues plays a key role in the etiology of insulin resistance (Gregor and Hotamisligil, 2011; Johnson and Olefsky, 2013; Lumeng and Saltiel, 2011). Organokines, including adipokines secreted from adipose tissue (Ouchi et al., 2011), myokines secreted from skeletal muscle cells (Pedersen and Febbraio, 2012) and hepatokines secreted from hepatocyte (Stefan and Haring, 2013b) are important mediators of insulin resistance. After secretion, these proteins play paracrine or endocrine roles or both to affect insulin sensitivity, glucose metabolism, inflammation, and atherosclerosis, and functionally correlate with insulin resistance, and link obesity to T2DM (Bays et al., 2004; Hotamisligil, 2006; Inadera, 2008; Ouchi et al., 2011).

Fetuin-A (Fet-A), a 60 kDa phosphorylated glycoprotein, and primarily produced by the liver, is an acute negative phase protein (Auberger et al., 1989; Le Cam et al., 1985). In circulation, Fet-A concentrations range from 0.3 – 0.6 mg/ml (Dziegielewska et al., 1990; Haglund et al., 2001; Putman, 1984). Fet-A was recently described as one of the most important hepatokines inducing insulin resistance and inflammation (Stefan and Haring, 2013b). Fet-A was initially identified as a physiological inhibitor of insulin receptor tyrosine kinase (IR-TK) and IR-autophosphorylation in skeletal muscle and liver tissue (Auberger et al., 1989; Goustin and Abou-Samra, 2011; Goustin et al., 2013; Mathews et al., 2000; Mathews et al., 1997; Srinivas et al., 1993). Currently, a large body of evidence has accumulated that demonstrate that Fet-A may play a major role in regulating insulin sensitivity. Single nucleotide polymorphisms (SNP) of human

Fet-A were found to be significantly associated with T2DM (Andersen et al., 2008; Siddiq et al., 2005). High circulating Fet-A concentrations in humans were observed to be strongly associated with insulin resistance, metabolic syndrome, atherogenic lipid profile, incident diabetes, and an increased risk of myocardial infarction and ischemic stroke (Ishibashi et al., 2010; Ix et al., 2006; Ix et al., 2008; Mori et al., 2006; Stefan et al., 2008a; Weikert et al., 2008). The association of Fet-A and incident diabetes was independent of age, sex, race, waist circumference, body weight, physical activity, blood pressure level, fasting glucose level, high-density lipoprotein cholesterol concentration, triglyceride concentration, and C-reactive protein level (Ix et al., 2008). Human circulating Fet-A levels were also found to be independently related with liver fat content and nonalcoholic fatty liver disease (NAFLD) in adults (Haukeland et al., 2012; Stefan et al., 2006) and children (Reinehr and Roth, 2008), which are important driving forces for development of T2DM.

In circulation, human Fet-A exits in both phosphorylated (approximately 20%) and dephosphorylated (approximately 80%) forms (Dziegielewska et al., 1990; Haglund et al., 2001). A few preliminary reports suggest that phosphorylation status of Fet-A may be important to exert its inhibitory effects (Auberger et al., 1989; Kalabay et al., 1996; Ohnishi et al., 1997). Our studies confirm that phosphorylation on Ser120 and Ser312 is critical for Fet-A to inhibit insulin signaling, and insulin-stimulated glucose uptake and glycogen synthesis (Ren, 2014). However, clinical studies on the phosphorylation status of Fet-A are limited. In this study we assayed Ser312-fetuin-A phosphorylation status in obese and normal-weight individuals and examined its association with anthropometric, physiological, and metabolic indices.

## Methods

### Recruitment of subjects:

The study included 31 obese and 11 normal-weight individuals. Obese volunteers met the following criteria for recruitment: 1) male between 30 to 65 years old; 2) body mass index (BMI) $\geq$ 30 kg/m$^2$ and/or body fat $\geq$ 30% of total body weight and waist circumference $\geq$ 40 inches; 3) without previously diagnosed cardiovascular or metabolic disease; 4) weight stable over previous 6 months; 5) no signs or symptoms of latent cardiovascular disease; 6) no regular exercise over the past 6 months and did not have a job that required strenuous physical activity job or program 7) non-smoker; 8) did not take medication known to alter lipid or glucose metabolism; 9) had no physical disability that would impede treadmill walking. Control subjects exhibited the same characteristics shown above, except number 2. Control subjects had a BMI $\leq$ 27 kg/m$^2$. This study was approved by the Auburn University Institutional Review Board (07-210 MR 0710).

### Preliminary screening procedure:

Volunteers were recruited through recruitment fliers placed around the Auburn-Opelika local area, and by Auburn University's daily faculty/staff announcement e-mail. The preliminary screening procedures included completion of a health history and physical activity questionnaire. Next, their height, weight, waist circumference and resting blood pressure were measured. If all criteria were met, individuals were scheduled to return to the lab for a further exam. On this scheduled visit, a blood sample was obtained via venipuncture from an antecubital vein. Next, volunteers took part in a body

composition assessment using whole body dual energy e-ray absorptiometry (DEXA) to determine body fat levels and fat distribution patterns.

**Baseline blood sampling and oral glucose tolerance test**

After completion of preliminary screening, obese and normal-weight volunteers who met the criteria, as shown above, were asked to return to the lab after two days of stable diet and physical activity and after a 10 to 12 hour overnight fast (restricted to water only). Following measurement of body weight and blood pressure, a blood sample was obtained via venipuncture in an antecubital vein. This fasting blood sample, collected into one 10 ml serum vacutainer tube (Becton Dickinson Vacutainer, Franklin Lakes, NJ) was used as baseline sample to assess all biochemical variables. Next, participants were administered a standard 75-g oral glucose tolerance test (OGTT). Blood samples were drawn at 30, 60, 90, and 120 min and allowed to clot for 30 minutes. Following this, samples were centrifuged for 10 minutes to separate the serum. Aliquots of serum were transferred into 2 ml micro-centrifuge tubes and subsequently stored at -80℃ for future analyses.

**Serum Fet-A and Ser312-phosphorylated Fet-A**

Serum concentrations of Fet-A were measured using an ELISA kit (BioVendor, LLC, Candler, NC). Serum samples were assayed in duplicate, using human Fet-A standards ranging from 2 - 100 ng/mL. Standards, quality controls, and 1:10,000 diluted serum samples were added to anti-human Fet-A antibody-coated microtiter strips. After incubation, washing, addition of conjugate solution and substrate, the absorbance was read in a microplate reader at 450 nm.

Serum pFet-A was assayed by Western blotting. Serum samples and quality control serum were diluted 1:100 in sterile saline and proteins were separated on a 4 ~ 20% gradient SDS-PAGE gel (Bio-Rad, Hercules, CA). Proteins were transferred to nitrocellulose membrane, blocked in 5% non-fat dry milk (Bio-Rad, Hercules, CA, USA). Serum pFet-A was detected using a custom-generated affinity-purified antibody (Affinity BioReagents, Golden, CO) against the phosphorylated Ser312-fetuin-A epitope "HTFMGVVSLGSPS(PO4)GEVSHPR". Chemiluminescence was imaged with UVP BioImaging system (UVP, Upland, CA). Area densities of the bands were analyzed using the UnScan-It software package (Silk Scientific, Orem, UT, USA). All samples were calculated by comparing pixels to the quality control serum sample, loaded in duplicate. Fold changes were used for statistical analysis.

**Biochemical analysis, insulin resistance, and glucose tolerance**

Serum glucose was measured using a glucose hexokinase assay kit (Cliniqa Corporation, San Marcos, CA). Serum insulin concentrations were measured by a human insulin-specific radioimmunoassay (Millipore Corporation, Billerica, MA). Serum non-esterified fatty acid (NEFA) concentrations were assayed using a 96-well NEFA assay kit (Wako, Richmond, VA). Blood samples were also sent to a Centers for Disease Control and Prevention-certified laboratory to verify blood chemistries, lipids, and liver enzymes.

The homeostasis model assessment of insulin resistance (HOMA-IR), which reflects hepatic insulin resistance was calculated using the following formula: [fasting insulin (μU/mL) x fasting glucose (mmol/L)/22.5] (Matthews et al., 1985). Adipose insulin resistance (Adipose-IR) was calculated as follows: [fasting NEFA mEq/L x fasting insulin μU/mL] (Abdul-Ghani et al., 2008). The quantitative insulin sensitivity

check index (QUICKI), which is useful for measuring insulin sensitivity, was calculated by the following: [1 / (log (fasting insulin μU/mL) + log (fasting glucose mg/dL))] (Katz et al., 2000); Glucose to insulin ratio (GIR) was calculated as glucose (mg/dL)/insulin (μU/mL).

Glucose, insulin, Fet-A, and pFet-A area under the curve (AUC) during the oral glucose tolerance test were calculated using the trapezoidal method. Insulin resistance index, which primarily reflects skeletal muscle glucose uptake, was estimated by multiplying $insulin_{AUC}$ and $glucose_{AUC}$ and dividing by $10^6$ (Evans et al., 2001).

**Statistical Analysis:**

Multiple statistical procedures were used to analyze our data. An unpaired student t-test was employed to determine differences in baseline physiological and biochemical parameters between obese and normal-weight participants. For OGTT analysis, a repeated measure ANOVA was used to analyze differences among different time points. Further, an unpaired t test was used to analyze differences in $glucose_{AUC}$, $insulin_{AUC}$, Fet-$A_{AUC}$ and pFet-$A_{AUC}$ between normal and obese individuals. Pearson's product moment correlation was used to determine the relationship associations, and significance was accepted as $\alpha \leq 0.05$.

# Results

## Baseline anthropometric, physiological and metabolic indices

Of the 31 obese participants, 27 were Caucasian and 4 were of African-American origin. Among normal-weight participants, 6 were Caucasian, 1 was African-American, and 2 were of Asian origin. Ethnicity was not reported by 2 normal-weight individuals. The baseline physiological characteristics from 31 obese participants and 11 age-matched normal weight controls are shown in Table 1. Twenty-three of the 31 obese participants satisfied the criteria for clinical identification of metabolic syndrome (MetS), as described by NCEP ATP III (Grundy et al., 2005). Among obese individuals, 29 subjects had a waist circumference above 40 inches; 12 had systolic blood pressure over 130 mm Hg; 13 had diastolic blood pressure over 85 mm Hg; 21 participants had blood glucose levels above 100 mg/dl; 16 exhibited triglyceride levels above 150 mg/dl; and 17 participants had HDL cholesterol concentrations below 40 mg/dl. Normal weight participants in this study were sedentary, but healthy individuals, with an average BMI of 23.9 $kg/m^2$.

Obese individuals, as expected, demonstrated significantly increased body weight, BMI, waist circumference, percent total fat, fat mass, lean mass, percent android fat, percent gynoid fat, and blood pressure compared to age-matched normal-weight participants. However, serum total cholesterol, triglycerides, HDL cholesterol and LDL cholesterol were not significantly different between obese and normal-weight subjects.

Further, the lipid profile of obese individuals did not indicate the presence of hyperlipidemia.

Fasting blood samples were analyzed for NEFA, glucose and insulin. HOMA-IR, insulin resistance index, and adipose-IR were calculated as surrogate measures of insulin resistance. QUICKI and glucose/insulin ratio were calculated to assess insulin sensitivity. Compared to normal weight individuals, obese individuals had significantly higher fasting glucose (Mean ± SD, 107.3 ± 16.1 vs 95.9 ± 7.2 mg/dL, $p = 0.03$), insulin (36.3 ± 20.1 vs 10.5 ± 8.6 μU/ml, $p = 0.0002$), NEFA (0.73 ± 0.32 vs 0.36 ± 0.17 mEq/L, $p = 0.0008$), and HOMA (9.92 vs 2.53 ± 2.12, $p = 0.0008$), indicating insulin resistance (Fig.1). Normal-weight individuals showed significantly elevated QUICKI (0.36 ± 0.05 vs 0.29 ± 0.02, $p < 0.0001$) and GIR (18.8 ± 14.6 vs. 3.6 ± 1.7, $p < 0.0001$), suggestive of increased insulin sensitivity compared to obese individuals (Fig.1).

Both serum Fet-A and pFet-A concentrations followed normal distributions (Fig.1.G and H). Serum total Fet-A concentrations were significantly higher in obese individuals (358.1 ± 69.1, ranging from 235.6 - 535.6 μg/ml) compared to normal weight individuals (296.0 ±48.9, ranging from 219.8 - 387.1 μg/ml) ($p < 0.01$) (Fig.1). Serum pFet-A concentrations were 3.3-fold higher in obese individuals (3.82 ± 1.56 vs 1.16 ± 0.45 scan units) compared to normal weight individuals ($p < 0.0001$) (Fig.1).

**Correlation of fasting serum Fet-A and pFet-A with anthropometric, physiological, and metabolic indices**

Bivariate correlation analyses between fasting serum Fet-A and pFet-A with anthropometric, physiological and metabolic indices are shown in Fig2. Serum Fet-A

showed significant positive correlations with NEFA (r = 0.39; p = 0.01) and tended to associate with adipose IR (r = 0.30, p = 0.053), percent total fat (r = 0.30, p = 0.057) and percent android fat (r = 0.30, p = 0.063). Interestingly, fasting pFet-A demonstrated a strong positive association with body weight (r = 0.56; p = 0.0001), BMI (r = 0.56; p =0.0001), waist circumference (r = 0.56; p = 0.0001) (Fig.2H), percent total fat (r = 0.54; p = 0.0003), fat mass (r = 0.54; p = 0.0002), lean mass (r = 0.48; p = 0.0013), percent android fat (r = 0.37; p = 0.019), percent gynoid fat (r = 0.39; p = 0.015), systolic blood pressure (r = 0.32; p = 0.037), diastolic blood pressure (r = 0.36; p = 0.019), fasting glucose (r = 0.31; p = 0.049), fasting insulin (r = 0.48; p = 0.001), adipose IR (r = 0.35, p = 0.02) and HOMA-IR (r = 0.47; p = 0.002). Further, pFet-A showed significant negative correlation with $VO_{2max}$ (r = -0.44; p = 0.003), QUICKI (r = -0.49; p = 0.001) and glucose/insulin ratio (r = -0.41; p = 0.006).

**Fet-A and pFet-A response to an oral glucose challenge**

Since Fet-A and pFet-A were altered in obese individuals, it was of significant interest to characterize Fet-A and pFet-A changes in response to an oral glucose load. As expected, obese individuals demonstrated significantly higher 2-h serum insulin and NEFA (Table 1), and significantly elevated $glucose_{AUC}$ ($p < 0.01$), $insulin_{AUC}$ ($p < 0.001$), and insulin resistance index ($p < 0.001$) compared to normal-weight individuals (Fig. 3E,F,I), indicating a lower glucose tolerance and insulin resistance. Consistent with these observations, elevations in 2-h Fet-A (p = 0.066), 2-h pFetA ($p < 0.05$), Fet-$A_{AUC}$ ($p < 0.05$) and pFet-$A_{AUC}$ ($p < 0.05$) were observed in obese individuals (Table 1, Fig. 2G,H). Further, while Fet-$A_{AUC}$ tended to positively associate with $insulin_{AUC}$ (r = 0.30, p = 0.055), but not with $glucose_{AUC}$ or insulin resistance index (Fig.4A,B,C), pFet-$A_{AUC}$ was

significantly correlated with $insulin_{AUC}$ ($r = 0.34$, $p = 0.028$) and insulin resistance index ($r = 0.36$, $p = 0.02$), and tended to correlate with $glucose_{AUC}$ ($r = 0.30$, $p = 0.055$) (Fig.4D,E,F). Interestingly, we also observed that obese individuals demonstrated temporal changes in both Fet-A and pFet-A at 30 and 60 min respectively ($p < 0.05$) (Fig.3B,D) compared to the zero time point, during an OGTT. Though a similar trend was observed for Fet-A, but not pFet-A in normal-weight individuals, these were not statistically significant.

## Discussion

Earlier, Haglund et al. showed that Fet-A secreted from liver into circulation existed in both phosphorylated and dephosphorylated forms (Haglund et al., 2001). Using mass spectrometry, these investigators identified Ser312 as the predominant phosphorylation site in addition to phosphorylation on Ser120. Accordingly we sought to assess the clinical relevance of Ser312-fetuin-A phosphorylation in obesity and insulin resistance in humans. In this study, we report for the first time that pFet-A was elevated in obese individuals compared to normal-weight participants. While both Fet-A and pFet-A were significantly elevated, and showed a normal distribution, only pFet-A was positively correlated with BMI, waist circumference, percent total fat, fat mass, lean mass, and blood pressure. Further, pFet-A also showed significant associations with fasting glucose, insulin, and indices of insulin resistance (HOMA-IR and adipose-IR). These findings are consistent with our previous report showing that phosphorylated Fet-A, but not dephosphorylated Fet-A, inhibited insulin-stimulated glucose uptake and glycogen synthesis in L6-GLUT4myc cells, suggesting that the phosphorylated form of Fet-A interferes with insulin action (Ishibashi et al., 2010). Previously, several investigators have shown that the phosphorylated form of Fet-A exhibited 20 to100-fold higher potency compared to dephosphorylated form of Fet-A, in inhibiting insulin-stimulated IR autophosphorylation and DNA synthesis (Auberger et al., 1989) (Kalabay et al., 1997; Srinivas et al., 1995). Inflammatory cytokines such as IL-6, IL-1α, and TNFα was shown to decrease the rate of synthesis of phosphofetuin, while insulin and EGF stimulated phosphofetuin synthesis (Ohnishi et al., 1994). The phosphorylated form of Fet-A was

also shown to dose-dependently decrease human hepatocyte-growth factor-stimulated DNA synthesis (Ohnishi et al., 1997). The Ser312 site is conserved in rat, mice, and bovine species, with bovine fetuin having 4 phosphorylation sites: Ser138, Ser320, Ser323, and Ser324 (Wind et al., 2003). Further, phosphorylated Fet-A containing calciprotein particles were associated with aortic stiffness in patients with pre-dialysis CKD. Recently, we have shown that niacin-treatment lowers Ser312-phosphofetuin-A in individuals with metabolic syndrome correlating with beneficial changes in serum lipids (Kaushik et al., 2009).

Unlike previous studies that showed that Fet-A was positively associated with insulin resistance (Ishibashi et al., 2010; Ix et al., 2006; Ix et al., 2008; Mori et al., 2006; Stefan et al., 2008b; Stefan et al., 2006) , our studies did not reveal an association of total Fet-A with insulin sensitivity indexes such as HOMA, QUICKI and glucose/insulin ratio. This may be due to the following differences. This may primarily be attributed to the relatively small sample size (n=42). Secondly, we did not categorize our subjects by tertiles or quintiles, based on Fet-A concentrations. Previous studies found an association between Fet-A and insulin resistance or diabetes incidences in people with high serum Fet-A concentrations, when grouped as tertiles or quintiles (Ix et al., 2006; Ix et al., 2008; Stefan et al., 2008b; Stefan et al., 2006).

To our knowledge, this study is the first to document Fet-A and pFet-A responses during an OGTT in obese and normal weight individuals. OGTT being a more sensitive method than fasting glucose for diagnosis of diabetes (DECODE Study Group on behalf of the European Diabetes Epidemiology Study Group, 1998; Resnick et al., 2001; Wahl et al., 1998), it is not surprising that obese individuals demonstrated higher $glucose_{AUC}$,

insulin$_{AUC}$ and IR index, compared to normal weight individuals after a glucose load. Obese individuals also showed significant higher Fet-A$_{AUC}$ and pFet-A-A$_{AUC}$. Interestingly, pFet-A, but not Fet-A, showed a significant association with insulin$_{AUC}$ and IR index. Our observations suggest that the increase in pFet-A$_{AUC}$ may contribute to the worsening of insulin action. Further, in our study, we observed a dynamic regulation of Fet-A and pFet-A during an OGTT. In obese individuals, but not in normal-weight individuals, both Fet-A and pFet-A demonstrated a temporal decrease at the 30' and 60' time-points, respectively. This response was in opposition to the temporal increase observed with glucose and insulin during the 30 and 60 min of OGTT, suggesting a worsening hepatic insulin resistance or impaired β cell function (Abdul-Ghani et al., 2007). Similar to glucose and insulin responses, but in the counter direction, Fet-A and pFet-A returned to levels similar to baseline at the 120' time-point. These findings of a temporal decrease in Fet-A and pFet-A at 30' and 60' time-points of OGTT were consistent with our previous results in diet-induced obese C57BL6 mice (Okerberg, 2012). While the observed dynamics of Fet-A and pFet-A may imply potential mechanisms in regulating insulin action, detailed mechanisms of Fet-A and pFet-A secretion and clearance are currently unclear. It can only be surmised that an glucose load either temperately increases secretion of the Fet-A and pFet-A, or stimulates its uptake into target cells. Previously, Wajih *et al.* found that Fet-A was rapidly internalized into cells and released back from the cells into which it was internalized; therefore, some receptor-mediated internalization may be involved in the utilization of Fet-A (Wajih et al., 2004). Further studies are needed to characterize Fet-A and pFet-A's secretion and

clearance. In addition, how Fet-A and pFet-A affect insulin receptor and the dynamics of regulation need to be understood.

Our data showed that serum Fet-A was significantly associated with NEFA concentrations and had a tendency to associate with adipose-IR ($p = 0.053$), percent total fat ($p = 0.057$) and percent android fat ($p = 0.063$), suggesting that Fet-A may play an important role in fatty acid metabolism. Recently, Pal *et al.* showed that Fet-A helped to build a "free fatty acid - fetuin-A - TLR-4" complex and induced NF-κB signaling pathway (Pal et al., 2012). Our clinical findings lend additional support to the view that Fet-A may be "the missing link in lipid-induced inflammation" (Heinrichsdorff and Olefsky, 2012). Recently, Stefan *et al.* reported similar findings that in subjects with elevated free fatty acids, Fet-A concentrations were associated with insulin resistance; also in subjects with high serum Fet-A concentrations, free fatty acids negatively associated with insulin sensitivity. However, subjects with low Fet-A concentrations or low free fatty acids did not show association with insulin resistance (Stefan and Haring, 2013a). Furthermore, these studies are consistent with our previous observation of decreased serum NEFA concentrations in the insulin-sensitive Fet-A null mice (Mathews et al., 2002) and studies showing that NEFA up-regulated Fet-A expression and secretion in human primary hepatocytes (Dasgupta et al., 2010; Kahraman et al., 2013).

Our study has several limitations. First, our sample size was small and was restricted only to men. Secondly, our exclusion criteria may impact generalization of these findings. Thirdly, for this study, we have combined individuals who were diagnosed with MetS with obese individuals without MetS. The limited sample size prevented us from running analyses for the two conditions separately. Fourthly, we have

used surrogate measures for assaying insulin resistance, viz., HOMA-IR, IR index, adipose-IR, QUICK-I, and GIR, and additional studies using euglycemic-hyperinsulinemic clamp would be needed to validate these findings. Further, additional studies would be required to characterize Ser120-fetuin-A phosphorylation status.

In conclusion, we have demonstrated, for the first time, that phosphorylation of Ser312-fetuin-A is associated with insulin resistance and glucose intolerance in obese individuals. Taken together with previous findings that phosphorylation status of Fet-A is critical for its inhibitory function on insulin action, our data raise the possibility that Ser312-phosphorylated fetuin-A might be the active form of Fet-A. Further, our studies have identified Ser312-phosphorylated fetuin-A as a potential therapeutic target for the treatment of insulin resistant conditions including diabetes.

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## Figure legends

**Fig.1**. Serum Fet-A, pFet-A, and metabolic indices of insulin sensitivity in normal-weight (n=11) and obese (n=31) individuals. Values are shown as scatter plots. Horizontal lines within scatter plots indicate mean values. P values are shown to indicate statistical significance. GIR = glucose to insulin ratio; HOMA-IR = homeostasis model assessment of insulin resistance [fasting insulin μU/mL x fasting glucose (mmol/L)]/22.5;QUICKI = quantitative insulin sensitivity check index [1 / (log (fasting insulin μU/mL) + log (fasting glucose mg/dL))].

**Fig.2**. Correlation of Fet-A and pFet-A with BMI, waist circumference, percent total fat, NEFA, glucose, insulin, HOMA-IR, and adipose-IR (fasting NEFA mEq/L x fasting insulin μU/mL). Correlation was determined using Pearson product-moment correlation coefficient (n = 42).

**Fig.3**. Temporal alterations in serum glucose, insulin , Fet-A, and pFet-A following an OGTT in normal-weight (n=11) and obese (n=31) individuals (A,B,C,D). $^{\#}p < 0.05$ for changes in Fet-A and pFet-A concentrations compared to 0 time-point during OGTT. The area under the curve (AUC) for glucose (E), insulin (F), Fet-A (G), pFet-A (H), and insulin resistance index (I) ($insulin_{AUC}$ x $glucose_{AUC}$ / $10^6$) were calculated. Data are shown as mean ± SEM. *p < 0.05, ** p < 0.01, *** p < 0.001 compared to normal-weight individuals.

**Fig.4**. Association of Fet-$A_{AUC}$ with $glucose_{AUC}$ (A), $insulin_{AUC}$ (B), insulin resistance index ($insulin_{AUC}$ x $glucose_{AUC}$ / $10^6$) (C), and pFet-$A_{AUC}$ with $glucose_{AUC}$ (D), $insulin_{AUC}$ (E), and insulin resistance index (F). Correlation was determined using Pearson product-moment correlation coefficient (n = 42).

**Table 1. Baseline anthropometric, physiological and metabolic characteristics**

| Variables | Normal (n = 11) Mean ± SD | Obese (n = 31) Mean ± SD | P value |
|---|---|---|---|
| Age | 43.3 ± 10.7 | 43.3 ± 9.2 | 0.9436 |
| Height (inches) | 68.8 ± 2.5 | 70 ± 2.9 | 0.1812 |
| Weight (lbs) | 160.1 ± 13.8 | 251.8 ±26.9 | <.0001 |
| BMI ($kg/m^2$) | 23.9 ± 2.0 | 36.0 ± 6.1 | <.0001 |
| Waist circumference (inches) | 34.5 ±2.7 | 46.5 ± 5.6 | <.0001 |
| Total fat % | 24.5 ± 4.8 | 38.2 ± 5.1 | <.0001 |
| Fat mass (kg) | 17.6 ± 4.1 | 43.9 ± 13.7 | <.0001 |
| Lean mass (kg) | 53.5 ± 3.7 | 69. 0 ± 9.8 | <.0001 |
| Android fat % | 33.4 ± 10.1 | 49.5 ± 4.7 | <.0001 |
| Gynoid fat % | 28.9 ± 5.4 | 40.2 ± 5.8 | <.0001 |
| Systolic blood pressure (mm Hg) | 115 ± 11 | 127.9 ± 12.6 | 0.0042 |
| Diastolic blood pressure (mm Hg) | 75 ± 6 | 82.5 ± 9.3 | 0.0187 |
| $VO_{2max}$ (ml/kg/min) | 36.0 ± 6.2 | 28.0 ± 6.1 | 0.0007 |
| $VO_{2max}$ absolute (L/min) | 2.59 ± 0.39 | 3.10 ± 0.53 | 0.0057 |
| Total cholesterol (mg/dL) | 184.7 ± 30.0 | 194.0 ± 42.4 | 0.5265 |
| Triglycerides (mg/dL) | 137.8 ± 64.1 | 191.6 ± 107.8 | 0.1448 |
| HDL cholesterol (mg/dL) | 45.2 ± 8.4 | 39.2 ± 10.8 | 0.1191 |
| LDL cholesterol (mg/dL) | 116.0 ± 5.5 | 111.8 ± 8.3 | 0.6995 |
| 2-h glucose (mg/dL) | 95.7 ± 22.5 | 111.3 ± 31.4 | 0.1390 |
| 2-h insulin (μU/ml) | 40.1 ± 28.9 | 109.8 ± 63.7 | 0.0012 |
| 2-h NEFA (mEq/L) | 0.11 ± 0.04 | 0.31 ± 0.21 | 0.0033 |
| 2-h Fet-A (μg/ml) | 293.9 ± 46.2 | 335.6 ± 12.1 | 0.0662 |
| 2-h pFet-A (Scan units) | 1.50 ± 0.44 | 2.51 ± 1.39 | 0.0245 |
| Insulin resistance index | 80.36 ± 15.30 | 306.1 ± 24.58 | <.0001 |
| Adipose-IR | 3.55 ± 1.02 | 26.15 ± 3.09 | 0.0001 |

**Fig.1**

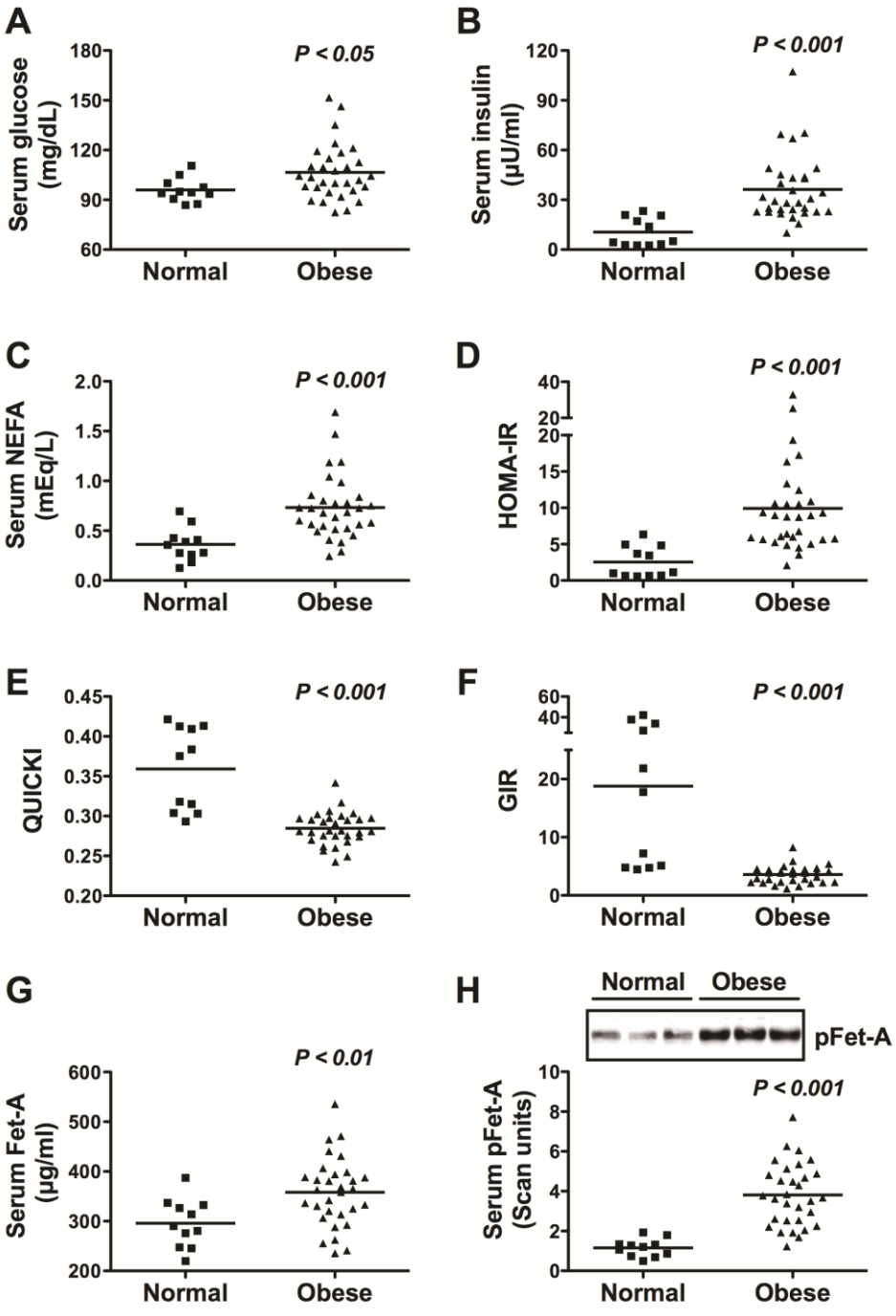

A
Serum glucose (mg/dL)
180
150
120
90
60
P < 0.05
Normal
Obese
B
Serum insulin (μU/ml)
120
90
60
30
0
P < 0.001
Normal
Obese
C
Serum NEFA (mEq/L)
2.0
1.5
1.0
0.5
0.0
P < 0.001
Normal
Obese
D
HOMA-IR
40
20
20
15
10
5
0
P < 0.001
Normal
Obese
E
QUICKI
0.45
0.40
0.35
0.30
0.25
0.20
P < 0.001
Normal
Obese
F
GIR
60
40
20
10
0
P < 0.001
Normal
Obese
G
Serum Fet-A (μg/ml)
600
500
400
300
200
P < 0.01
Normal
Obese
H
Normal
Obese
pFet-A
Serum pFet-A (Scan units)
10
8
6
4
2
0
P < 0.001
Normal
Obese

**Fig.2.**

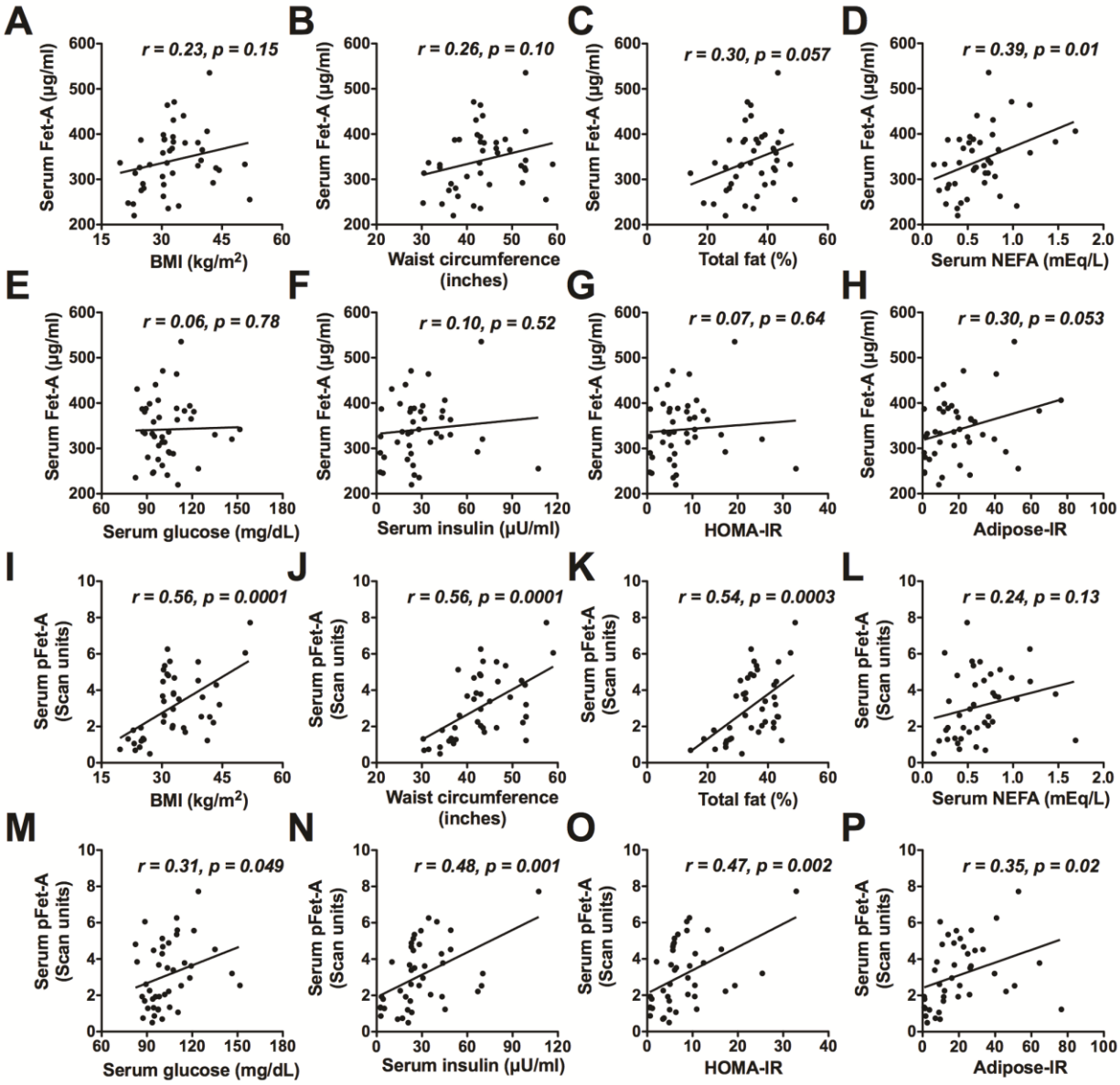

A
r = 0.23, p = 0.15
Serum Fet-A (µg/ml)
BMI (kg/m2)
B
r = 0.26, p = 0.10
Waist circumference (inches)
C
r = 0.30, p = 0.057
Total fat (%)
D
r = 0.39, p = 0.01
Serum NEFA (mEq/L)
E
r = 0.06, p = 0.78
Serum glucose (mg/dL)
F
r = 0.10, p = 0.52
Serum insulin (µU/ml)
G
r = 0.07, p = 0.64
HOMA-IR
H
r = 0.30, p = 0.053
Adipose-IR
I
r = 0.56, p = 0.0001
Serum pFet-A (Scan units)
BMI (kg/m2)
J
r = 0.56, p = 0.0001
Waist circumference (inches)
K
r = 0.54, p = 0.0003
Total fat (%)
L
r = 0.24, p = 0.13
Serum NEFA (mEq/L)
M
r = 0.31, p = 0.049
Serum glucose (mg/dL)
N
r = 0.48, p = 0.001
Serum insulin (µU/ml)
O
r = 0.47, p = 0.002
HOMA-IR
P
r = 0.35, p = 0.02
Adipose-IR

**Fig.3.**

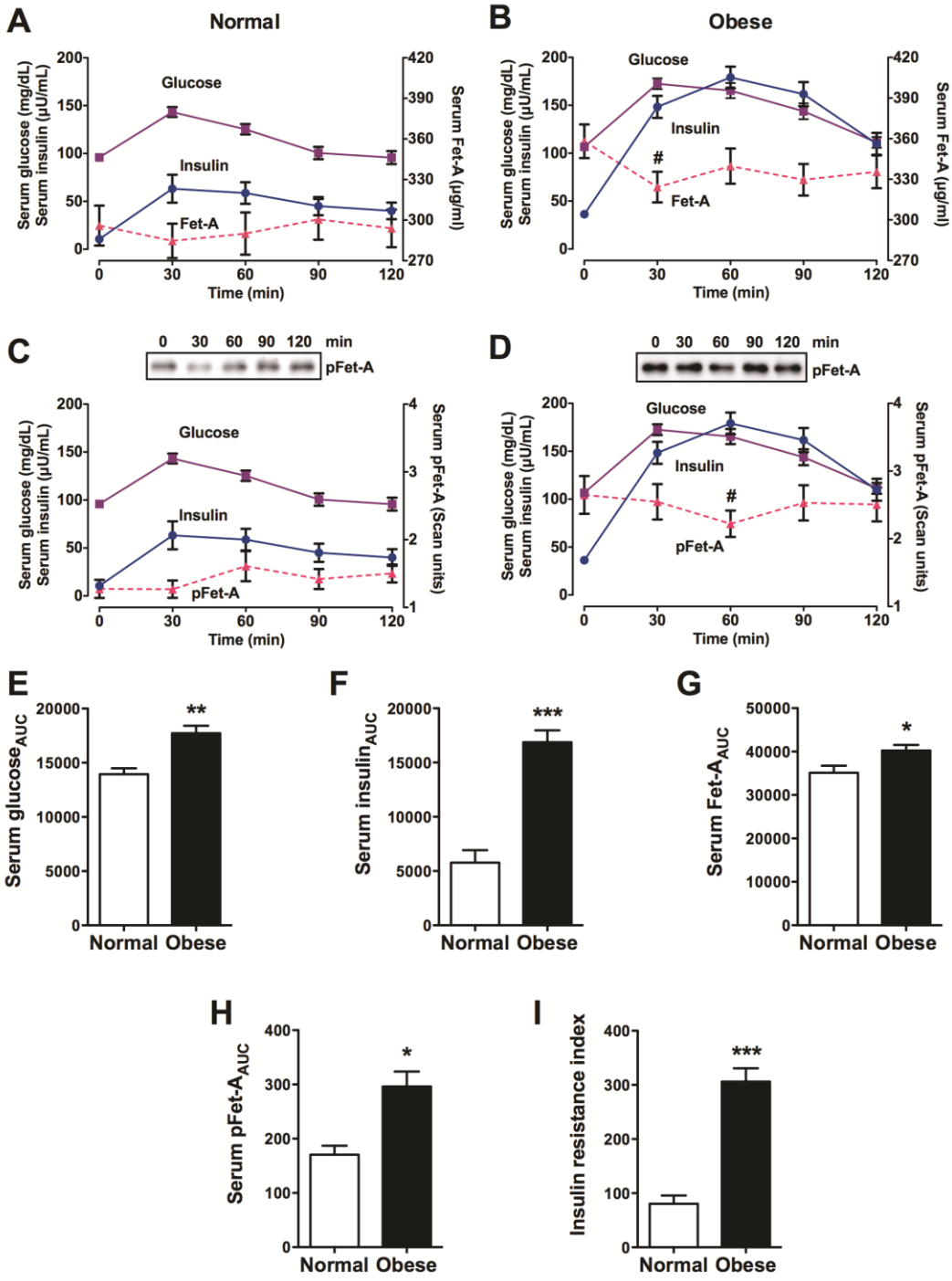
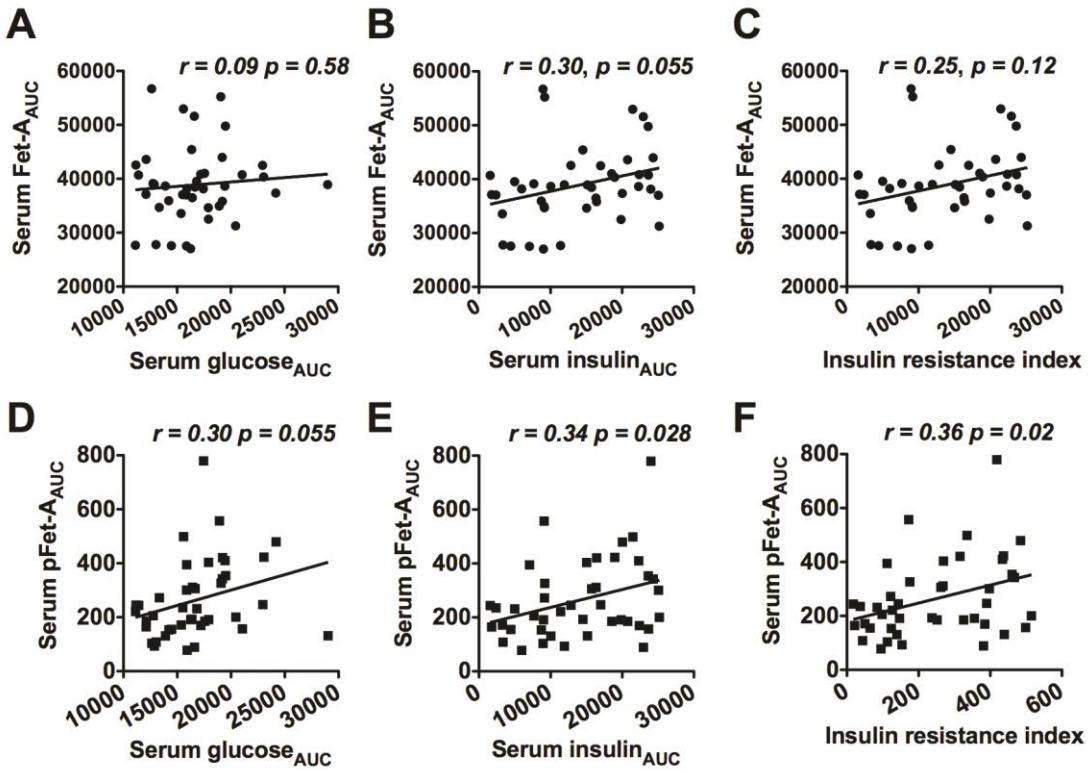

A
Normal
Glucose
Insulin
Fet-A
Serum glucose (mg/dL)
Serum insulin (μU/mL)
Serum Fet-A (μg/ml)
Time (min)
B
Obese
Glucose
Insulin
Fet-A
#
C
0 30 60 90 120 min
pFet-A
Glucose
Insulin
pFet-A
Serum pFet-A (Scan units)
D
E
Serum glucose AUC
F
Serum insulin AUC
G
Serum Fet-A AUC
H
Serum pFet-A AUC
I
Insulin resistance index
Normal Obese

**Fig.4**


A
r = 0.09 p = 0.58
Serum Fet-A$_{AUC}$
Serum glucose$_{AUC}$
B
r = 0.30, p = 0.055
Serum Fet-A$_{AUC}$
Serum insulin$_{AUC}$
C
r = 0.25, p = 0.12
Serum Fet-A$_{AUC}$
Insulin resistance index
D
r = 0.30 p = 0.055
Serum pFet-A$_{AUC}$
Serum glucose$_{AUC}$
E
r = 0.34 p = 0.028
Serum pFet-A$_{AUC}$
Serum insulin$_{AUC}$
F
r = 0.36 p = 0.02
Serum pFet-A$_{AUC}$
Insulin resistance index

**Chapter 5: Effects of single bout of endurance exercise on fetuin-A and Ser312 phosphorylated fetuin-A in obese and normal weight individuals.**

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## Abstract

Liver-secreted glycoprotein, fetuin-A (Fet-A), inhibits insulin receptor autophosphorylation, tyrosine kinase activity, and interferes with downstream insulin signaling. Several studies demonstrate that elevated serum Fet-A concentrations are associated with insulin resistance and incident diabetes. While lower serum Fet-A concentrations have been associated with the improvement of insulin sensitivity with short-term exercise and weight loss, there are no reports of alterations in Fet-A or its phosphorylated form, with a single bout of exercise. The objective of this study was to characterize the effects of a single bout of endurance exercise on serum Fet-A and Ser312-phosphorylated fetuin-A (pFet-A) in obese and normal weight individuals and correlate these changes with improvements in insulin sensitivity. Thirty one obese

individuals and 11 normal-weight men underwent a single bout of treadmill walking, expending 500 kcal at 60-70% $VO_{2max}$. Serum Fet-A and pFet-A concentrations were significantly elevated in obese individuals. Only pFet-A was correlated with fasting glucose, insulin, NEFA, HOMA-IR, adipose IR, and QUICKI. Following a single bout of endurance exercise, we did not observe alterations of fasting glucose, insulin, HOMA-IR, QUICKI, and G:I ratio, either immediately or 24 hours after exercise. Serum Fet-A concentrations were significantly elevated immediately after exercise, and then decreased to pre-exercise levels 24-h after the single bout of exercise. In obese individuals, area under the curve (AUC) for serum Fet-A, pFet-A, glucose, insulin, and insulin resistance index, during an oral glucose tolerance test, was significantly decreased 24-h after a single bout of exercise. Reductions in fasting pFet-A concentrations and Fet-$A_{AUC}$ 24-h after a single bout of exercise correlated with a reduction in insulin resistance. In conclusion, we report for the first time a transient elevation of serum Fet-A after a single bout of endurance exercise. Secondly, our data suggests that reductions in Fet-$A_{AUC}$ and pFet-A are associated with an improvement of insulin sensitivity following a single bout of exercise.

## Introduction

Physical activity is defined as any bodily movement produced by skeletal muscle that leads to energy expenditure above rest (Caspersen et al., 1985). The benefits of regular physical activity and maintenance of a healthy weight in glycemic control and prevention of obesity and type 2 diabetes have been well documented (Helmrich et al., 1994; Qiu et al., 2012; Schulze and Hu, 2005). Exercise is a subset of physical activity which is planned and structured (Caspersen et al., 1985). It has been well established that a single bout of endurance exercise effectively stimulates whole body insulin sensitivity and glucose tolerance, and that this effect can persist from 2-48 h post exercise (Devlin et al., 1987; Devlin and Horton, 1985; Mikines et al., 1988a; Perseghin et al., 1996). Several metabolic and hemodynamic factors can contribute to improvements in glucose homeostasis observed after an acute exercise in insulin resistant individuals, including enhanced insulin action on skeletal muscle glucose uptake, GLUT4 translocation, GLUT4 mRNA expression, reduced hepatic glucose production, and improved blood flow to skeletal muscle (Egan and Zierath, 2013; Henriksen, 2002; Kraniou et al., 2000; Thorell et al., 1999).

Organokines, secreted by specific tissues, with both paracrine and endocrine functions have been shown to regulate different signaling pathways and may play important roles in development of insulin resistance and type 2 diabetes (Hotamisligil, 2006). Several studies suggest that moderate intensity acute exercise does not significantly contribute to alterations in circulating adipokine concentrations of HMW adiponectin, leptin, or TNFα (Berggren et al., 2005; Magkos et al., 2010; Saghizadeh et

al., 1996). Interleukin (IL)-6 has been identified as the predominant myokine whose concentrations transiently increase upto 100-fold after prolonged exercise, and only modestly after shorter duration exercise (Fischer, 2006; Pedersen and Febbraio, 2008). However, the relationship between exercise and hepatokines is limited (Kim et al., 2013).

Human fetuin-A (Fet-A), a hepatokine, primarily released from the liver and present in the blood of adults at concentrations ranging from 300 - 600 μg/ml (Dziegielewska et al., 1990; Lebreton et al., 1979; Putman, 1984), was originally identified as a physiological inhibitor of insulin receptor tyrosine kinase (IR-TK) and IR-autophosphorylation in skeletal muscle and liver tissue (Auberger et al., 1989; Goustin and Abou-Samra, 2011; Goustin et al., 2013; Mathews et al., 2000; Mathews et al., 2006; Mathews et al., 1997; Srinivas et al., 1993). Recently, Fet-A was identified as an endogenous ligand of Toll-like receptor 4 (TLR4) that stimulates inflammation (Pal et al., 2012), and was described as a key hepatokine that regulates insulin action and inflammation (Stefan and Haring, 2013). Elevated circulating Fet-A levels were shown to be associated with obesity, insulin resistance and an increased risk for type 2 diabetes (Ishibashi et al., 2010; Ix et al., 2006; Ix et al., 2008; Mori et al., 2006; Stefan et al., 2008; Weikert et al., 2008).

Previously, it was shown that adults who were habitually sedentary had higher plasma Fet-A compared with physically active counterparts (Jenkins et al., 2011). Recent studies by Malin *et al.* suggested that short-term exercise training, without body weight changes, significantly decreased circulating Fet-A, which was correlated with the improvement of insulin regulated glucose uptake (Malin et al., 2013). Another recent study by the same authors indicate that 12 weeks of exercise training lowered Fet-A by

8%, and this was correlated with lower hepatic insulin resistance, increased metabolic flexibility, and HMW-adiponectin.

Human circulating Fet-A exists in both phosphorylated (approximately 20%) and dephosphorylated (approximately 80%) forms (Dziegielewska et al., 1990; Haglund et al., 2001). Fet-A is phosphorylated on Ser120 and Ser312, with the majority of phosphorylation on Ser312 (~77%) (Haglund et al., 2001). A few preliminary reports suggest that phosphorylation status of Fet-A (pFet-A) may be important to exert its inhibitory effects on insulin action (Auberger et al., 1989; Kalabay et al., 1996; Ohnishi et al., 1997). Our research has confirmed these findings that phosphorylation status of Fet-A is critical to exert its inhibitory effects on inhibit insulin signaling, and insulin-stimulated glucose uptake and glycogen synthesis (Ren, 2014).

To data, there are no studies that examine the effects of a single bout of exercise on both total Fet-A and phosphorylated Fet-A and other surrogate markers of insulin sensitivity in obese and normal weight individuals. Therefore, the purpose of this study was to determine the alterations of serum Fet-A and pFet-A before, immediately after, and 24-h after a single bout of exercise in normal weight and obese individuals. We hypothesized that a single bout of exercise would lead to alterations in serum Fet-A and pFet-A concentration and that these changes would be associated with the improvement of surrogate markers of insulin sensitivity.

## Methods

### Study population

A total 31 obese and 11 normal weight individuals were recruited for this study. All obese volunteers met the following criteria: male between 30 to 65 years old; body mass index (BMI) over 30 $kg/m^2$ and/or body fat over 30% of total body weight and waist circumference over 40 inches; without previously diagnosed cardiovascular or metabolic disease; weight stable over previous 6 months; no signs or symptoms of latent cardiovascular disease; did not participate in any regular physical activity in the previous 6 month period [regular physical activity defined as moderate or intense physical exertion (50 - 80% of $VO_{2max}$) for 20 minutes or more at least two times per week]; non-smoker; did not take medication known to affect lipid or glucose metabolism; without any physical impairment or conditions that would prevent regular treadmill walking. Subjects with normal body weight (control subjects) met the same criteria as above except that of BMI/percent body fat/waist circumference. Control subjects had BMI $\leq$ 27 $kg/m^2$. Informed consent was obtained from all subjects, and this study received approval from the Auburn University Institutional Review Board (07-210 MR 0710).

### Preliminary Screening and Baseline Procedures

The study was advertised to university faculty/staff through University email, and to the general public through fliers and ads in local newspapers. After registering, volunteers were asked to complete a health history questionnaire and physical activity questionnaire. Height, weight, waist circumference and hip circumferences, and relative

body fat were measured using a seven-site skinfold method. If all criteria were met, individuals were scheduled to return to the laboratory a second time for further physical exams. During this physical exam, a 5 ml blood sample was drawn via venipuncture from an antecubital vein to assay lipid and lipoprotein status. Following this, a body composition assessment was conducted using whole body dual energy x-ray absorptiometry (DEXA) to determine body fat levels and fat distribution patterns.

All subjects were asked to perform a maximal exercise test (GXT) using the standard Bruce protocol on a motor driven treadmill (Quinton Q-65) to assess maximal exercise capacity and determine cardiovascular fitness (Lear et al., 1999). Heart rate and rhythm were monitored continuously using a 12-lead electrocardiogram (Medical Graphics CPX/D Integrated Systems) at rest and throughout the exercise test. Blood pressure was obtained periodically throughout the GXT to determine cardiovascular responses to exercise. Respiratory gas exchange ($VO_2$ and $VCO_2$) was measured continuously and averaged over 15 sec intervals via open-circuit spirometry utilizing Medical Graphics Ultima metabolic unit (MedGraphics, St. Paul, MN). The peak oxygen uptake achieved during exercise was recorded as $VO_{2max}$ (L/min). The exercise tests were conducted and evaluated by the physician for cardiac contraindications to exercise training. Further, heart rate (HR) and ratings of perceived exertion (RPE) were monitored during the test to calculate 500 kcal energy expend for each individual.

After the preliminary screening procedures, subjects were asked to report to the laboratory on the 3$^{rd}$ day in the fasted state (12-h fast, restricted to water only). A standard oral glucose tolerance test (OGTT) was administered. After a fasting blood sample was obtained, participants received 75 g of standard carbohydrate syrup. Blood

samples were drawn at 30 min intervals for 2 hours following consumption of the glucose solution. All blood samples were obtained from venipuncture in an antecubital vein. Blood serum were drawn into one 10 ml "red top" serum vacutainer tube (Becton Dickinson Vacutainer, Franklin Lakes, NJ) and allowed to clot for 30 minutes, then centrifuged for 10 minutes to separate the serum. Aliquots of serum were transferred into 2 ml microcentrifuge tubes and subsequently stored at -80℃ freezer until further analyses.

**Single bout of exercise**

On day 7, both obese and normal-weight subjects were asked to return to the laboratory after a 12-hour fast (restricted to water only) for pre-exercise blood sampling (Pre-Ex). Next, subjects were asked to walk or jog on a motorized treadmill at 60-70% of their $VO_{2max}$ for the duration required to expend 500 kcals of energy. Blood samples were taken immediately after exercise (Post-Ex). To examine the sustained effects of the single bout of exercise, all subjects were asked to come to the laboratory the next day, i.e., day 8, to obtain a fasting blood sample (24-h Post-Ex). Immediately after obtaining the fasting blood sample, all subjects were administered a second OGTT following the same protocol reported previously. Serum samples were collected and stored in a -80℃ freezer until further analyses.

**Serum Fet-A and Ser312-phosphorylated Fet-A**

Serum concentrations of Fet-A were measured using an ELISA kit (BioVendor, LLC, Candler, NC). Serum samples were assayed in duplicate, using human Fet-A standards ranging from 2 - 100 ng/mL. Standards, quality controls, and 1:10,000 diluted serum samples were added to anti-human Fet-A antibody-coated microtiter strips. After

incubation, washing, addition of conjugate solution and substrate, the absorbance was read in a microplate reader at 450 nm.

Serum pFet-A was assayed by Western blotting. Serum samples and quality control serum were diluted 1:100 in sterile saline and proteins were separated on a 4 ~ 20% gradient SDS-PAGE gel (Bio-Rad, Hercules, CA). Proteins were transferred to nitrocellulose membrane, blocked in 5% non-fat dry milk (Bio-Rad, Hercules, CA, USA). Serum pFet-A was detected using a custom-generated affinity-purified antibody (Affinity BioReagents, Golden, CO) against the phosphorylated Ser312-fetuin-A epitope "HTFMGVVSLGSPS(PO4)GEVSHPR". Chemiluminescence was imaged with UVP BioImaging system (UVP, Upland, CA). Area densities of the bands were analyzed using the UnScan-It software package (Silk Scientific, Orem, UT, USA). All samples were calculated by comparing pixels to the quality control serum sample, loaded in duplicate. Fold changes were used for statistical analysis.

**Biochemical analysis, insulin resistance, and glucose tolerance**

Serum glucose was measured using a glucose hexokinase assay kit (Cliniqa Corporation, San Marcos, CA). Serum insulin concentrations were measured by a human insulin-specific radioimmunoassay (Millipore Corporation, Billerica, MA). Serum non-esterified fatty acid (NEFA) concentrations were assayed using a 96-well NEFA assay kit (Wako, Richmond, VA). Blood samples were also sent to a Centers for Disease Control and Prevention-certified laboratory to verify blood chemistries, lipids, and liver enzymes.

The homeostasis model assessment of insulin resistance (HOMA-IR), which reflects hepatic insulin resistance was calculated using the following formula: [fasting insulin (μU/mL) x fasting glucose (mmol/L)/22.5] (Matthews et al., 1985). Adipose

insulin resistance (Adipose-IR) was calculated as follows: [fasting NEFA mEq/L x fasting insulin μU/mL] (Abdul-Ghani et al., 2008). The quantitative insulin sensitivity check index (QUICKI), which is useful for measuring insulin sensitivity, was calculated by the following: [1 / (log (fasting insulin μU/mL) + log (fasting glucose mg/dL))] (Katz et al., 2000); Glucose to insulin ratio (GIR) was calculated as glucose (mg/dL)/insulin (μU/mL).

Glucose, insulin, Fet-A, and pFet-A area under the curve (AUC) during the oral glucose tolerance test were calculated using the trapezoidal method. Insulin resistance index, which primarily reflects skeletal muscle glucose uptake, was estimated by multiplying $insulin_{AUC}$ and $glucose_{AUC}$ and dividing by $10^6$ (Evans et al., 2001).

**Statistical Analysis**

An unpaired Student's *t*-test was used to determine statistical differences between obese and normal-weight individuals. A one-way ANOVA was used to examine the effects of single bout of exercise. The total area under the curve (AUC) during an OGTT was calculated. A paired Student's *t*-test was used to test statistical differences in AUCs between Pre-Ex OGTT and 24-h Post-Ex OGTT within groups. Pearson product moment correlation coefficients were used to examine associations and significance was accepted as $p \leq 0.05$. Data are expressed as Mean ± SD, unless noted otherwise.

## Results

### Anthropometric, physiological, and metabolic indices

As envisioned by study design, obese men demonstrated significantly higher body weight, BMI, waist circumference, percent total fat, fat mass, lean mass, percent android fat, percent gynoid fat, systolic and diastolic blood pressure compared to normal-weight individuals (Table 1). Obese individuals also showed poor aerobic capacity as evidenced by their $VO_{2max}$. Further, obese individuals demonstrated significantly higher fasting glucose, insulin, NEFA, HOMA-IR, adipose-IR, and significantly lower QUICKI, and G:I ratio. However, obese subjects did not exhibit hyperlipidemia. Fasting serum Fet-A and pFet-A concentrations were significantly elevated in obese subjects compared to normal-weight individuals (Table 1).

### Fet-A and pFet-A responses to an oral glucose tolerance test

As expected, prior to single bout of exercise, obese individuals demonstrated significantly elevated 2-h glucose, 2-h insulin (Table 2), and total area under the curve for glucose ($glucose_{AUC}$) and insulin ($insulin_{AUC}$) during an OGTT compared to normal-weight subjects (Fig.1A, B). Further, obese individuals exhibited significantly increased insulin resistance index (Fig.1C). Interestingly, we observed that fet-$A_{AUC}$, 2-h pFet-A, and pFet-$A_{AUC}$ were significantly increased in obese individuals compared to normal-weight individuals (Table1, Fig.1D,E).

**Effects of a single bout of exercise on Fet-A, pFet-A and surrogate markers of insulin sensitivity**

All participants underwent a 500 kcal single bout of exercise and fasting blood samples were collected prior to and immediately after exercise. Twenty-four hours after the single bout of exercise, another fasting blood sample was collected to determine the latent effects of single bout of exercise. After analyzing these blood samples, glucose, insulin, HOMA, QUICKI and G:I ratio were not altered immediately after or 24-h after the single bout of exercise in both obese and normal weight individuals (Table 2). As expected, serum NEFA significantly increased immediately after a single bout of exercise in normal-weight and obese individuals. Interestingly, we observed a transient significant increase in serum Fet-A immediately after exercise in both normal-weight and obese subjects ($p = 0002$, n=42). A similar trend of a temporary increase in pFet-A concentrations was observed immediately after exercise ($p = 0.077$, $n = 42$). This transient increase in Fet-A ($p < 0.0001$, n=42) and pFet-A ($p = 0.02$, $n = 42$)was reversed 24 hours after exercise.

Expending 500 kcals through a single bout of exercise reduced 24-h post-prandial glucose, insulin, insulin resistance index, Fet-A, and pFet-A responses in obese individuals as indicated by lower total AUC ($p < 0.05$) (Fig.1). Similarly, 2-h glucose, insulin, and pFet-A were significantly lower after a single bout of exercise in obese individuals (Table 2). In normal-weight individuals, a single bout of exercise significantly decreased 2-h insulin, $insulin_{AUC}$ and insulin resistance index, but was without effect on 2-h glucose, $glucose_{AUC}$, 2-h Fet-A, $Fet\text{-}A_{AUC}$, 2-h pFet-A, and $pFet\text{-}A_{AUC}$.

Consistent with these observations, prior to exercise, fasting pFet-A, but not Fet-A, was positively correlated to fasting glucose, insulin, and NEFA, HOMA-IR, adipose-IR, 2-h insulin, and negatively associated with QUICKI (Table 3). pFet-$A_{AUC}$ was correlated to $glucose_{AUC}$ ($r = 0.30$, $p = 0.05$), $insulin_{AUC}$ ($r = 0.34$, $p = 0.03$), and insulin resistance ($r = 0.36$, $p = 0.02$). On the other hand Fet-$A_{AUC}$ was only correlated with $insulin_{AUC}$ ($r = 0.30$, $p = 0.05$).

Immediately after exercise, pFet-A, but not Fet-A was positively associated with fasting glucose, insulin, HOMA-IR, and negatively with QUICKI. Changes in glucose were significantly correlated with changes pFet-A ($r = 0.45$, $p = 0.003$) and Fet-A ($r = 0.30$, $p = 0.05$).

Reductions in fasting pFet-A concentrations 24-h after a single bout of exercise correlated with a reduction in insulin resistance (Fig.2A) and tended to correlate with $insulin_{AUC}$ (Fig.2B). Further, reductions in $Fet_{AUC}$ 24-h after a single bout of exercise correlated with reductions of both insulin resistance (Fig.2C) and $insulin_{AUC}$ (Fig.2D).

## Discussion

Transient changes, caused by acute exercise, alters various aspects of muscle, adipose, and liver tissue function, independent of body weight loss, and exerts beneficial consequences in metabolism and improves insulin sensitivity (Egan and Zierath, 2013; Henriksen, 2002).

Several studies have documented that elevated Fet-A was associated with insulin resistance and risk for type 2 diabetes (Ishibashi et al., 2010; Ix et al., 2006; Ix et al., 2008; Mori et al., 2006; Stefan et al., 2008; Stefan et al., 2006; Sun et al., 2013). Further, weight-loss has shown to lower serum Fet-A, and which was shown to be correlated to the improvement of insulin action (Kahraman et al., 2013; Reinehr and Roth, 2008). Dramatic weight loss (~35% of their original body weight) following gastric bypass surgery was shown to lower serum Fet-A, which was associated with improvements in insulin sensitivity (Brix et al., 2010).

In this study, we report for the first time serum Fet-A and pFet-A responses to a single bout of endurance exercise in both normal-weight and obese individuals. This study is unique, because it examines (a) alterations of Fet-A, a negative acute phase reactant, immediately and 24-h after a single bout of exercise, (b) Fet-A and pFet-A responses independent of weight loss, and (c) the role of Fet-A and pFet-A in the improvement of insulin sensitivty after an acute exercise.

Fet-A is a negative acute phase reactant whose concentrations have been shown to decrease in inflammatory conditions (Lebreton et al., 1979; Ombrellino et al., 2001;

Wang et al., 2005). Several studies have examined the acute phase response to exercise. Acute exercise has been shown to temporarily increase circulating inflammatory markers including IL-1, IL-6, IL-8, tumor necrosis factor alpha (TNFα, and C-reactive protein (CRP) (Weight et al., 1991) (Kasapis and Thompson, 2005; Ostrowski et al., 1999). Contracting skeletal muscle plays a key role in transiently increasing plasma IL-6 concentrations, either modestly after short duration, or intermittent exercise protocols, and over 100-fold with prolonged exercise (Fischer, 2006; Meckel et al., 2009; Pedersen and Febbraio, 2008). The transiently increased IL-6 concentrations returned to baseline after 24 hours (McFarlin et al., 2004; Rhind et al., 2001). The transient rise in IL-6 was followed by the induction of anti-inflammatory cytokines, such as IL-10 and IL-1 receptor antagonist (IL-1RA) (Gleeson et al., 2011; Steensberg et al., 2003). Our studies indicate that a single bout of endurance exercise, expending 500 kcal, led to a significant transient increase in serum Fet-A. While liver has been identified as the key source for circulating Fet-A, a recent study has shown that adipocytes also secrete Fet-A (Chatterjee et al., 2013). Our studies have shown that Fet-A inhibits proximal insulin signaling and glucose uptake in L6GLUT4myc skeletal muscle cells and in muscle tissue (Mathews et al., 2000; Ren, 2014). Therefore, it may be surmised that Fet-A may be secreted by the liver, or adipocyte in response to muscle contraction or cytokine or hormone-mediated mechanisms. Further, Fet-A taken up by skeletal muscle cells to regulate insulin action, could potentially be secreted this back into circulation, by the contracting skeletal muscle tissue (Chen et al., 2007; Mathews et al., 2000; Ren, 2014).

Secondly, we speculate the Fet-A is secreted from hepatic tissue by acute exercise-induced increase in serum NEFA concentrations (Frayn, 2010; Noland et al.,

2003). This is consistent with the finding that treatment of human primary hepatocytes with NEFA up-regulated Fet-A mRNA expression (Kahraman et al., 2013). Further, a recent publication indicates that Fet-A serves as an endogenous ligand to TLR4 linking free fatty acids to TLR4 signaling (Pal et al., 2012). Thus, exercise-stimulated lipolysis resulting in raised plasma free fatty acids concentrations are in agreement with the transient increase in serum Fet-A. Previous studies also demonstrated that a single bout of exercise has long effects (up to 18 h) on postprandial response to food intake, such as reducing hepatic secretion of very-low-density lipoprotein, increasing triacylglycerol clearance, and increasing postprandial leg blood flow and glucose uptake (Gill et al., 2001a; Gill et al., 2001b; Malkova et al., 2000). The prolonged effects of single bout of exercise also have been found to alter trafficking of dietary fat and fat oxidation (Gill et al., 2001a; Votruba et al., 2002).

Recent studies by Malin *et al.* have shown that a 7-day short-term exercise training decreased Fet-A by 11% in obese adults, without a change in body weight or aerobic capacity (Malin et al., 2013). They newly reported that 12 weeks exercise training decreased Fet-A by ~8% in older adults (Malin et al., 2014). Similarly, our studies show significant reduction of Fet-$A_{AUC}$ and pFet-$A_{AUC}$ 24-h post-exercise, independent of body weight changes. Duncan *et al.* demonstrated that 6 months of exercise improved insulin sensitivity and several markers of lipid metabolism in sedentary men and women, without a corresponding change in BMI, waist circumference, or cardiorespiratory fitness (Duncan et al., 2003). This suggests that alterations in Fet-A, independent of body weight changes, may play a role in insulin sensitivity.

In the present study, we showed for the first time, that pFet-A, but not Fet-A was significantly correlated with surrogate measures of insulin resistance, in the pre-exercise and post-exercise groups. Therefore, this suggests that elevated serum pFet-A may contribute to the development of insulin resistance. Further, our studies shown that the reductions in 24-h Fet-$A_{AUC}$ and fasting pFetA concentrations 24-h after a single bout of exercise were associated with a reduction in insulin resistance in both normal-weight and obese individuals. The mechanisms of acute improvements in insulin sensitivity and glucose tolerance can be explained by insulin-dependent and insulin- independent glucose uptake pathways (Goodyear and Kahn, 1998; Jessen and Goodyear, 2005). Insulin-dependent GLUT4 translocation is the primary mechanism for increasing glucose tolerance during acute exercise (Kennedy et al., 1999; Wallberg-Henriksson and Holloszy, 1984). Fet-A was shown by various previous studies, as an inhibitor of insulin receptor tyrosine kinase (IR-TK) in muscle cells, adipocyte, hepatocyte and animal models (Auberger et al., 1989; Mathews et al., 2000; Mathews et al., 2006; Mathews et al., 2002; Mathews et al., 1997; Srinivas et al., 1993). Our previous studies have shown that phosphorylation on Ser120 and Ser312 is critical for Fet-A to inhibit insulin signaling, and insulin-stimulated GLUT4 translocation, glucose uptake, and glycogen synthesis. The reduction of postprandial Fet-$A_{AUC}$ and pFet-$A_{AUC}$ suggests that it may relieve the inhibitory effects of insulin receptor phosphorylation, and stimulate the downstream insulin signaling pathway resulting in GLUT4 translocation to the plasma membrane and increasing glucose uptake. Additionally, recent findings by Malin *et al.* and Goustin *et al.* indicate that Fet-A inhibits insulin-stimulated GLUT4 translocation into skeletal muscle cells (Goustin et al., 2013; Malin et al., 2013). Acute exercise has

also been shown to activate AMPK, which plays an important role in regulating glucose tolerance and energy metabolism (Dreyer et al., 2006; Pedersen and Febbraio, 2012). Recent studies by Jung *et al.* suggest that AMPK may modulate Fet-A expression (Jung et al., 2013). However, additional studies are needed to characterize the effects of AMPK activation on Fet-A synthesis and secretion.

One limitation of the study is the small sample size. Secondly, we only assayed Fet-A and pFet-A immediately after and 24-h after a single bout of exercise. Previous studies have shown an improvement of insulin sensitivity persisting for a period ranging from 2-h (Mikines et al., 1988b), 4-6 h (Wojtaszewski et al., 2000), 12-16 h (Devlin et al., 1987; Devlin and Horton, 1985; Heath et al., 1983), to 48-h post exercise (Mikines et al., 1988b; Perseghin et al., 1996). In future, it will be of significant interest to examine the relationship between Fet-A or pFet-A with other inflammatory cytokines, including IL-6, IL-10, and TNFα during a single bout of exercise.

In conclusion, we observe for the first time, that serum Fet-A concentrations are significantly elevated immediately after acute exercise. Reductions of both total and phosphorylated forms of fetuin-A are correlated with a reduction of insulin resistance, suggesting that alterations in Fet-A and pFet-A with acute exercise may play a role in the improvement of insulin sensitivity, independent of changes in body composition.

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## Figure legends

**Fig.1.** Timeline for blood sampling and single bout of exercise. On day 3, an oral glucose tolerance test was administered. On day 7, participants completed a single bout of endurance exercise expending 500 kcal, 60-70% $VO_{2max}$. Fasting blood samples were obtained pre-exercise, immediately post-exercise, and 24-h post-exercise to assess Fet-A, pFet-A, and markers of insulin sensitivity. An oral glucose tolerance test was administered 24-h post exercise to analyze area under the curve (AUC) for glucose, insulin, insulin resistance index, Fet-A and pFet-A.

**Fig.2.** Fet-A and pFet-A responses during an oral glucose tolerance test. Total area under the curve (AUC) for glucose (A), insulin (B), insulin resistance index ($insulin_{AUC}$ x $glucose_{AUC}$ / $10^6$), (C), Fet-A (D) and pFet-A (E), was calculated. Data are shown as Mean ± SEM. Different letters above histograms (for AUC) indicate statistical significance, $p \leq 0.05$.

**Fig.3**. Correlation between change (Δ) in pFetA with Δ in insulin resistance index ($insulin_{AUC}$ x $glucose_{AUC}$ / $10^6$) (A); Δ in pFetA with Δ in $insulin_{AUC}$ (B); Δ in Fet-$A_{AUC}$ and Δ in insulin resistance index (C); Δ in Fet-$A_{AUC}$ with Δ in $insulin_{AUC}$ (D). Correlation was determined using Pearson product-moment correlation coefficient (n = 42).

**Table 1. Baseline anthropometric, physiological and metabolic characteristics in normal-weight and obese individuals**

| Variables | Normal (n = 11) | Obese (n = 31) | P value |
|---|---|---|---|
| | Mean ± SD | Mean ± SD | |
| Age | 43.3 ± 10.7 | 43.3 ± 9.2 | 0.9436 |
| Height (inches) | 68.8 ± 2.5 | 70 ± 2.9 | 0.1812 |
| Weight (lbs) | 160.1 ± 13.8 | 251.8 ±26.9 | <.0001 |
| BMI ($kg/m^2$) | 23.9 ± 2.0 | 36.0 ± 6.1 | <.0001 |
| Waist circumference (inches) | 34.5 ±2.7 | 46.5 ± 5.6 | <.0001 |
| Total fat % | 24.5 ± 4.8 | 38.2 ± 5.1 | <.0001 |
| Fat mass (kg) | 17.6 ± 4.1 | 43.9 ± 13.7 | <.0001 |
| Lean mass (kg) | 53.5 ± 3.7 | 69. 0 ± 9.8 | <.0001 |
| Android fat % | 33.4 ± 10.1 | 49.5 ± 4.7 | <.0001 |
| Gynoid fat % | 28.9 ± 5.4 | 40.2 ± 5.8 | <.0001 |
| Systolic blood pressure (mm Hg) | 115 ± 11 | 127.9 ± 12.6 | 0.0042 |
| Diastolic blood pressure (mm Hg) | 75 ± 6 | 82.5 ± 9.3 | 0.0187 |
| $VO_{2max}$ (ml/kg/min) | 36.0 ± 6.2 | 28.0 ± 6.1 | 0.0007 |
| $VO_{2max}$ absolute (L/min) | 2.59 ± 0.39 | 3.10 ± 0.53 | 0.0057 |
| Total cholesterol (mg/dL) | 184.7 ± 30.0 | 194.0 ± 42.4 | 0.5265 |
| Triglycerides (mg/dL) | 137.8 ± 64.1 | 191.6 ± 107.8 | 0.1448 |
| HDL cholesterol (mg/dL) | 45.2 ± 8.4 | 39.2 ± 10.8 | 0.1191 |
| LDL cholesterol (mg/dL) | 116.0 ± 5.5 | 111.8 ± 8.3 | 0.6995 |
| NEFA (mEq/L) | 0.36 ± 0.17 | 0.73 ± 0.32 | 0.0008 |
| Fasting glucose (mg/dL) | 95.9 ± 7.2 | 107.3 ± 16.1 | 0.03 |
| Fasting insulin (μU/ml) | 10.5 ± 8.6 | 36.3 ± 20.1 | 0.0002 |
| G:I ratio | 18.8 ± 14.6 | 3.6 ± 1.7 | <.0001 |
| HOMA-IR | 2.53 ± 2.12 | 9.92 ± 6.58 | 0.0008 |
| QUICKI | 0.36 ± 0.05 | 0.29 ± 0.02 | <.0001 |
| Fet-A (μg/ml) | 296.0 ± 48.9 | 358.1 ± 69.1 | 0.0092 |
| pFet-A (Scan units) | 1.16 ± 0.45 | 3.82 ± 1.56 | <.0001 |

Data are expressed as Mean ± SD. BMI-body mass index; $VO_{2max}$ – maximum oxygen consumption measured over one minute of a standard Bruce protocol graded exercise test. NEFA: non-esterified fatty acids; Fet-A: fetuin-A; pFet-A-Ser312 phosphorylated fetuin-A; HOMA-IR: Homeostasis model assessment of insulin resistance; Adipose-IR: adipose insulin resistance, QUICKI: Quantitative insulin sensitivity check index, G:I ratio: glucose to insulin ratio.

**Table 2. Metabolic indices in normal weight (n = 11) and obese (n = 31) individuals before exercise (Pre-Ex), immediately after exercise (Post-Ex), and 24-h after a single-bout of exercise expending 500 kcal (24-h Post-Ex)**

| Variables | Normal weight (n = 11) | | | Obese (n = 31) | | |
|---|---|---|---|---|---|---|
| | Pre-Ex | Post-Ex | 24-h Post-Ex | Pre-Ex | Post-Ex | 24-h Post-Ex |
| Fasting glucose (mg/dL) | 97.2 ± 6.4 [a] | 94.9 ± 6.8 [a] | 96.4 ± 8.7 [a] | 102.0 ± 12.7 [a] | 103.9 ± 16.6 [a] | 104.2 ± 15.7 [a] |
| Fasting insulin (μU/ml) | 10.1 ± 7.5 [b] | 11.5 ± 7.8 [b] | 10.8 ± 8.4 [b] | 34.3 ± 14.4 [a] | 33.0 ± 14.9 [a] | 33.1 ± 16.4 [a] |
| Fasting NEFA (mEq/L) | 0.38 ± 0.21 [c] | 0.84 ± 0.50 [b] | 0.46 ± 0.29 [c] | 0.60 ± 0.26 [b] | 1.15 ± 0.34 [a] | 0.71 ± 0.34 [b] |
| Fasting Fet-A (μg/ml) | 298.4 ± 33.2 [c] | 321.8 ± 48.0 [c] | 305 8 ± 62.2 [c] | 347.5 ± 64.6 [b] | 386.2 ± 99.0 [a] | 337.6 ± 74.5 [b] |
| Fasting pFet-A (Scan units) | 1.50 ± 0.70 [c] | 1.61 ± 0.53 [c] | 1.65 ± 0.52 [c] | 2.71 ± 1.12 [a b] | 2.91 ± 1.04 [a] | 2.40 ± 0.84 [b] |
| 2-h glucose (mg/dL) | 95.7 ± 22.5 [b] | - | 92.7 ± 13.2 [b] | 111.3 ± 31.4 [a] | - | 101.2 ± 26.5 [b] |
| 2-h insulin (μU/ml) | 40.1 ± 28.9 [c] | - | 33.7 ± 29.0 [d] | 109.8 ± 63.7 [a] | - | 88.6 ± 62.0 [b] |
| 2-h Fet-A (μg/ml) | 293.9 ± 46.2 [a] | - | 295.4 ± 46.2 [a] | 335.6 ± 67.4 [a] | - | 328.7 ± 68.5 [a] |
| 2-h pFet-A (Scan units) | 1.50 ± 0.44 [b] | - | 1.53 ± 1.04 [b] | 2.51 ± 1.39 [a] | - | 1.65 ± 1.35 [b] |
| HOMA-IR | 2.4 ± 1.7 [b] | 2.5 ± 2.0 [b] | 2.6 ± 2.0 [b] | 8.8 ± 4.4 [a] | 8.7 ± 4.7 [a] | 8.7 ± 4.8 [a] |
| Adipose-IR | 3.4 ± 2.6 [d] | 8.6 ± 8.2 [c] | 4.8 ± 4.9 [d] | 20.7 ± 12.1 [b] | 36.1 ± 17.5 [a] | 22.8 ±12.6 [b] |
| QUICKI | 0.35 ± 0.04 [a] | 0.37 ± 0.08 [a] | 0.35 ± 0.05 [a] | 0.29 ± 0.02 [b] | 0.29 ± 0.02 [b] | 0.29 ± 0.02 [b] |
| G:I ratio | 16.5 ± 11.9 [a] | 13.5 ± 10.2 [a] | 16.1 ± 10.5 [a] | 3.4 ± 1.1 [b] | 3.6 ± 1.2 [b] | 4.0 ± 2.0 [b] |

Data are expressed as Mean ± SD. Different letters in superscript following values indicate statistical significance, $p < 0.05$. NEFA: non-esterified fatty acids; Fet-A: fetuin-A; pFet-A-Ser312 phosphorylated fetuin-A; HOMA-IR: Homeostasis model assessment of insulin resistance; Adipose-IR: adipose insulin resistance, QUICKI: Quantitative insulin sensitivity check index, G:I ratio: glucose to insulin ratio; 2-h: two hour time-point during an oral glucose tolerance test.

**Table 3. Correlation of Fet-A and pFet-A with metabolic indices before and after a single bout of exercise**

| Pre-Ex (n = 42) | Fet-A | | pFet-A | |
|---|---|---|---|---|
| | r value | p value | r value | p value |
| Fasting glucose (mg/dL) | -0.22 | 0.16 | 0.45 | 0.003 |
| Fasting insulin (μU/ml) | 0.02 | 0.88 | 0.31 | 0.04 |
| Fasting NEFA (mEq/L) | 0.25 | 0.11 | 0.36 | 0.02 |
| Fasting Fet-A (μg/ml) | 1.00 | - | 0.15 | 0.34 |
| Fasting pFet-A (Scan units) | 0.15 | 0.34 | 1.00 | - |
| 2-h glucose (mg/dL) | -0.02 | 0.91 | -0.06 | 0.71 |
| 2-h insulin (μU/ml) | 0.17 | 0.29 | 0.34 | 0.03 |
| 2-h Fet-A (μg/ml) | 0.63 | <.001 | 0.22 | 0.16 |
| 2-h pFet-A (Scan units) | -0.10 | 0.53 | 0.43 | 0.01 |
| HOMA-IR | -0.03 | 0.84 | 0.37 | 0.02 |
| Adipose-IR | 0.13 | 0.40 | 0.41 | 0.01 |
| QUICKI | -0.15 | 0.34 | -0.34 | 0.03 |
| G:I ratio | -0.21 | 0.19 | -0.19 | 0.23 |
| Post-Ex (n = 42) | | | | |
| Fasting glucose (mg/dL) | 0.10 | 0.53 | 0.50 | 0.001 |
| Fasting insulin (μU/ml) | 0.12 | 0.47 | 0.31 | 0.05 |
| Fasting NEFA (mEq/L) | 0.12 | 0.43 | 0.11 | 0.47 |
| Fasting Fet-A (μg/ml) | 1.00 | - | 0.20 | 0.21 |
| Fasting pFet-A (Scan units) | 0.20 | 0.21 | 1.00 | - |
| HOMA-IR | 0.13 | 0.42 | 0.42 | 0.01 |
| Adipose-IR | 0.15 | 0.34 | 0.22 | 0.16 |
| QUICKI | -0.20 | 0.20 | -0.37 | 0.02 |
| G:I ratio | -0.16 | 0.32 | -0.22 | 0.18 |
| 24-h Post-Ex (n = 42) | | | | |
| Fasting glucose (mg/dL) | 0.08 | 0.63 | 0.04 | 0.83 |
| Fasting insulin (μU/ml) | 0.08 | 0.63 | 0.14 | 0.36 |
| Fasting NEFA (mEq/L) | 0.08 | 0.63 | 0.15 | 0.35 |
| Fasting Fet-A (μg/ml) | 1.00 | - | 0.18 | 0.25 |
| Fasting pFet-A (Scan units) | 0.18 | 0.25 | 1.00 | - |
| 2-h glucose (mg/dL) | -0.15 | 0.36 | 0.22 | 0.17 |
| 2-h insulin (μU/ml) | 0.10 | 0.53 | 0.25 | 0.12 |
| 2-h Fet-A (μg/ml) | 0.74 | <.001 | 0.39 | 0.01 |
| 2-h pFet-A (Scan units) | -0.15 | 0.34 | 0.02 | 0.92 |
| HOMA-IR | 0.05 | 0.75 | 0.12 | 0.47 |
| Adipose-IR | 0.12 | 0.44 | 0.18 | 0.25 |
| QUICKI | -0.13 | 0.40 | -0.25 | 0.12 |
| G:I ratio | -0.13 | 0.43 | -0.26 | 0.10 |

Data are expressed as Mean ± SD. NEFA: non-esterified fatty acids; Fet-A: fetuin-A; pFet-A-Ser312 phosphorylated fetuin-A; HOMA-IR: Homeostasis model assessment of insulin resistance; Adipose-IR: adipose insulin resistance, QUICKI: Quantitative insulin sensitivity check index, G:I ratio: glucose to insulin ratio; 2-h: two hour time-point during an oral glucose tolerance test.

**Fig.1**

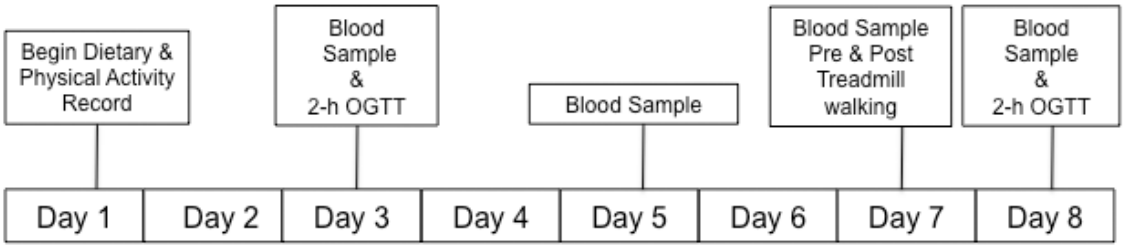

Begin Dietary & Physical Activity Record
Blood Sample & 2-h OGTT
Blood Sample
Blood Sample Pre & Post Treadmill walking
Blood Sample & 2-h OGTT
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8

**Fig.2**

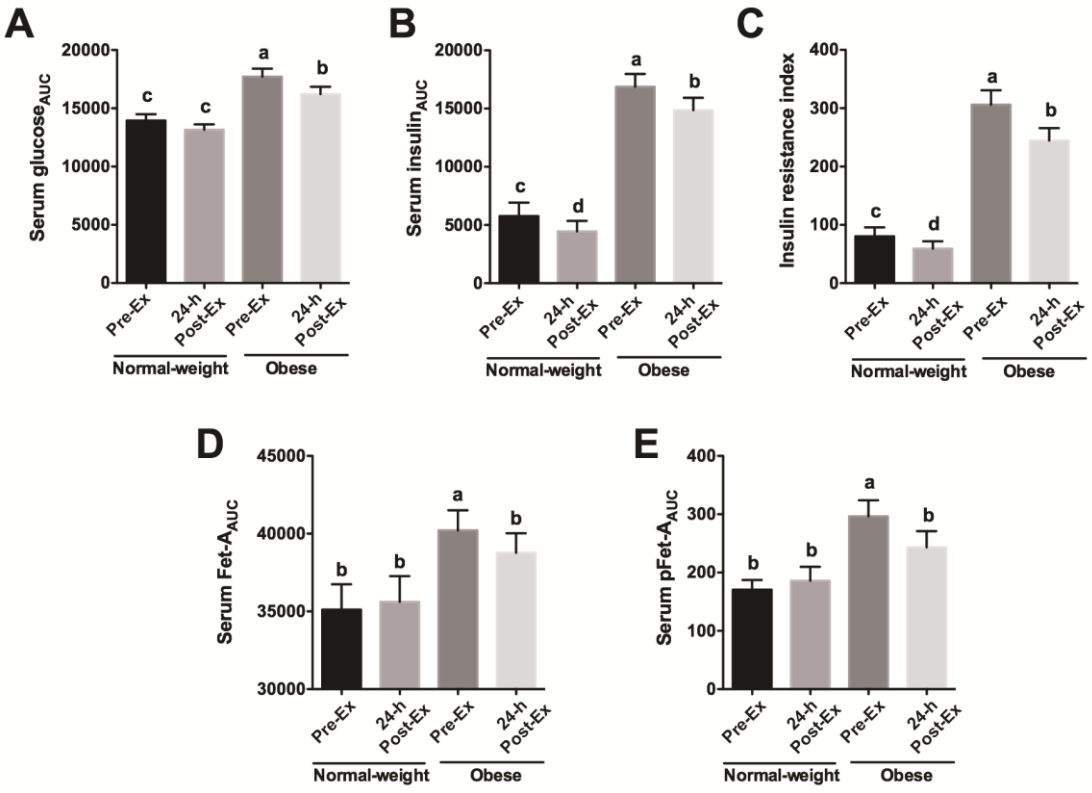
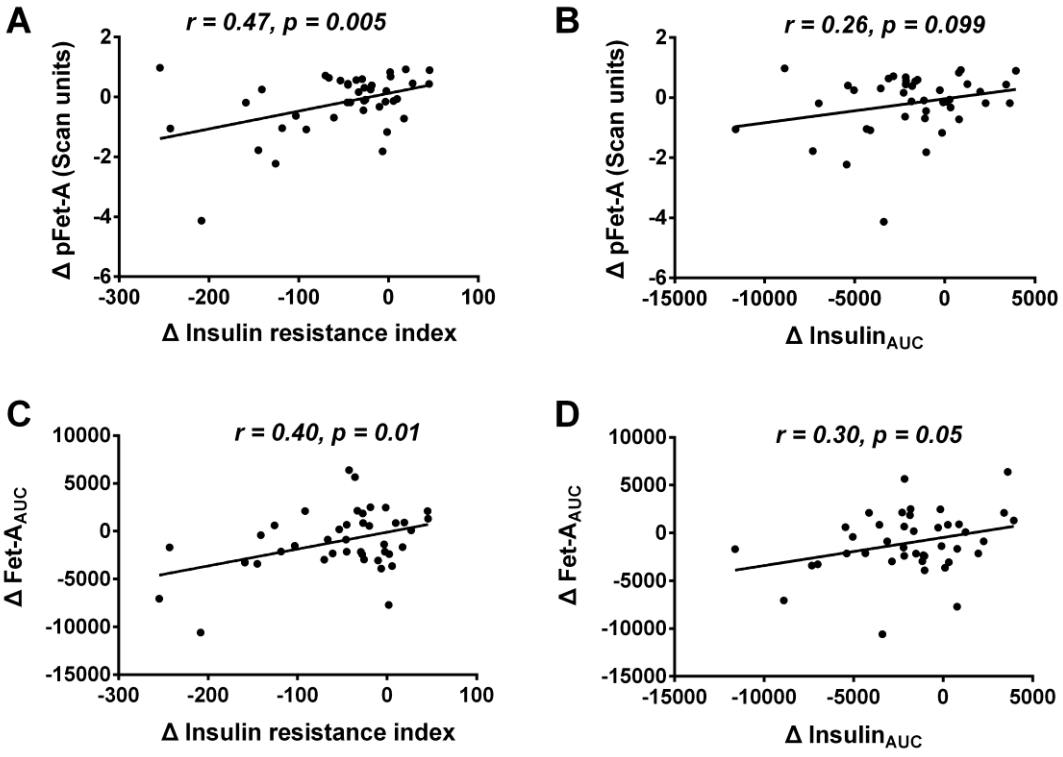

A
Serum glucose$_{AUC}$
20000
15000
10000
5000
0
c
c
a
b
Pre-Ex
24-h Post-Ex
Pre-Ex
24-h Post-Ex
Normal-weight
Obese
B
Serum insulin$_{AUC}$
20000
15000
10000
5000
c
d
a
b
Pre-Ex
24-h Post-Ex
Pre-Ex
24-h Post-Ex
Normal-weight
Obese
C
Insulin resistance index
400
300
200
100
0
c
d
a
b
Pre-Ex
24-h Post-Ex
Pre-Ex
24-h Post-Ex
Normal-weight
Obese
D
Serum Fet-A$_{AUC}$
45000
40000
35000
30000
b
b
a
b
Pre-Ex
24-h Post-Ex
Pre-Ex
24-h Post-Ex
Normal-weight
Obese
E
Serum pFet-A$_{AUC}$
400
300
200
100
0
b
b
a
b
Pre-Ex
24-h Post-Ex
Pre-Ex
24-h Post-Ex
Normal-weight
Obese

**Fig.3**


A
r = 0.47, p = 0.005
Δ pFet-A (Scan units)
Δ Insulin resistance index
B
r = 0.26, p = 0.099
Δ pFet-A (Scan units)
Δ Insulin$_{AUC}$
C
r = 0.40, p = 0.01
Δ Fet-A$_{AUC}$
Δ Insulin resistance index
D
r = 0.30, p = 0.05
Δ Fet-A$_{AUC}$
Δ Insulin$_{AUC}$

# Chapter 6: Alterations of serum fetuin-A and Ser312-phosphorylated fetuin-A to moderate body weight loss in obese individuals

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*lead graduate student - contributed to Table 1-3, Fig.1-4

#contributed to recruitment of subjects, sample collection, exercise protocol

[‡]contributed partially to assay of serum fetuin-A

[##]Co-Principal Investigator

[‡‡]Principal Investigator

## Abstract

Elevated serum fetuin-A has been shown to be positively associated with obesity, insulin resistance, and an increased risk for type 2 diabetes. The phosphorylated form of fetuin-A inhibits insulin receptor tyrosine kinase (IR-TK), and negatively impacts insulin-stimulated GLUT4 translocation and glucose uptake. Moderate to extreme weight loss has been shown to decrease serum fetuin-A (Fet-A) with a concomitant improvement of insulin sensitivity. However, the role of Ser312-phosphorylated fetuin-A (pFet-A) in the beneficial effects of a moderate weight loss is unknown. We hypothesized that the decrease in pFet-A with incremental weight loss would be associated with the improvement of insulin sensitivity. Sixteen obese men participated in this study to attain a targeted weight loss of 8-10% of their initial body weight in

6-10 months. Subjects underwent two monitored treadmill exercise per week, and additional exercise sessions, on their own, to achieve an energy expenditure/deficit of 2000-2500 kcal/week. Oral glucose tolerance tests (OGTT) were carried out before and after 8-10% weight loss. Blood samples were used to analyze Fet-A, pFet-A, glucose, insulin and non-esterified fatty acids (NEFA). Homeostasis model assessment of insulin resistance (HOMA-IR), adipose IR, quantitative insulin sensitivity check index (QUICKI) and glucose to insulin ratio (G:I ratio) were calculated as clinical indexes of insulin sensitivity. Weight loss (8-10%) decreased waist-circumference, percent total fat, fat distribution, blood pressure, fasting glucose, insulin, HOMA-IR, adipose IR, and insulin resistance index. Serum Fet-A and pFet-A concentrations were significantly decreased with incremental weight loss. Serum Fet-A concentrations was reduced with 2-4% weight loss (83 µg/ml or 15.1%), and decreased by 126 µg/ml or 24.0% with 8-10% body weight loss. Importantly, serum pFet-A was reduced by 19.6% with 2-4% weight loss, and 42.3% with target weight loss. Percent reductions of pFet-A, and not Fet-A, after 8-10% weight loss was significantly correlated with percent changes of insulin, HOMA-IR, and G:I ratio. Similarly, percent reduction of $insulin_{AUC}$ after 8-10% weight loss was associated with percent reduction of pFet-$A_{AUC}$ and not Fet-$A_{AUC}$. Taken together, our data suggest that improvements in insulin sensitivity with moderate weight loss may be due to reductions of Ser312 phosphorylated-fetuin-A.

## Introduction

Obesity is caused by a chronic energy imbalance as a result of increased dietary intake and low physical activity. Worldwide, in 2011, over 1.46 billion adults were overweight; of these 200 million men and nearly 300 million women were obese (Finucane et al., 2011; World Health Organization, 2013). Obesity is associated with an increased risk of developing insulin resistance, type 2 diabetes (T2DM), cardiovascular diseases (CVD), and cancer (Calle and Kaaks, 2004; Kahn et al., 2006; Van Gaal et al., 2006). The beneficial effects of moderate weight loss (5-10%) on hyperglycemia, hyperinsulinemia, hyperlipemia, and insulin resistance are well documented (Kelley et al., 2004; Olefsky et al., 1974; Petersen et al., 2005; Pi-Sunyer, 1993; Watkins et al., 2003). Several elegant studies including the Diabetes Prevention Program, Diabetes Prevention Study, Da Qing study, Indian Diabetes Prevention Program demonstrate that lifestyle intervention with modest weight loss decreased the incidence of T2DM and delayed the progression from obesity to T2DM (Diabetes Prevention Program Research et al., 2009; Eriksson and Lindgarde, 1991; Knowler et al., 2002a; Pan et al., 1997; Ramachandran et al., 2006; The Diabetes Prevention Program Research Group, 2000; Tuomilehto et al., 2001).

Several mechanisms have been suggested to illustrate the benefits of lifestyle intervention (both calorie restriction and physical activity) in obesity, T2DM and CVD, including improved insulin sensitivity and glucose homeostasis (Gower et al., 2002; Rector et al., 2007; Tuomilehto et al., 2001; Weiss et al., 2006), alterations in fatty acid mobilization and oxidation (Kelley et al., 2004; Solomon et al., 2008; Thompson et al., 2012) , intramyocellular lipid content (Petersen et al., 2012), reduced inflammation (Forsythe et al., 2008; Nicklas et al., 2004; Rokling-Andersen

et al., 2007), gut hormones (Asakawa et al., 2001; Peterli et al., 2012) and improved oxidative stress (Rector et al., 2007; Vincent and Taylor, 2006).

Fetuin was originally identified in bovine species (Pedersen, 1944), as phosphorylated protein 63 (pp63) in rats (Le Cam et al., 1985), countertrypin or mouse fetuin in mice (Yamamoto and Sinohara, 1993; Yang et al., 1992), and alpha2-HS glycoprotein in humans (Heremans, 1960; Schmid and Burgi, 1961). Identification of fetuin-B, led to renaming all members of the fetuin family, as fetuin-A (Fet-A) (Olivier et al., 2000). Fet-A is a 60 kDa glycoprotein, mainly secreted by the liver in both phosphorylated and dephosphorylated forms (Le Cam et al., 1985). Fetuin-A is a physiological inhibitor of insulin receptor tyrosine kinase (IR-TK) and IR-autophosphorylation in skeletal muscle and liver tissue (Auberger et al., 1989; Goustin and Abou-Samra, 2011; Goustin et al., 2013; Le Cam et al., 1985; Mathews et al., 2000; Mathews et al., 1997; Srinivas et al., 1993). In circulation, Fet-A concentrations range from 0.3 to 0.6 mg/ml (Dziegielewska et al., 1990; Haglund et al., 2001; Putman, 1984).

An accumulating body of clinical evidence suggests that high circulating Fet-A concentrations in humans are strongly associated with insulin resistance, metabolic syndrome, atherogenic lipid profile, incident diabetes, and an increased risk of myocardial infarction and ischemic stroke (Ishibashi et al., 2010; Ix et al., 2006; Ix et al., 2008; Mori et al., 2006; Stefan et al., 2008; Weikert et al., 2008). A few studies have explored the role of Fet-A with weight loss and insulin action. Serum Fet-A concentrations were significantly decreased following a gastric bypass surgery that resulted in a 35% body weight loss. This reduction was significantly correlated with fasting insulin, postprandial insulin, and HOMA-IR (Brix et al., 2010). Reinehr et al reported that substantial weight loss significantly decreased serum Fet-A in children with nonalcoholic fatty liver disease (NAFLD), which correlated with reductions of HOMA-IR, blood

pressure and waist circumferences (Reinehr and Roth, 2008). However, other studies report no change in serum fetuin-A concentrations with exercise training (Schultes et al., 2010; Yang et al., 2011).

However, there are no clinical studies that examine the effects of moderate incremental weight loss on Fet-A, its phosphorylation status on Ser312 (pFet-A) (Auberger et al., 1989; Haglund et al., 2001; Kalabay et al., 1996; Ohnishi et al., 1997; Ren, 2014), and their relationship with other surrogate markers of insulin sensitivity in obese individuals. Therefore, in this study we determined alterations of serum Fet-A and pFet-A with incremental (2-4%, 4-6%, 6-8% and 8-10%) weight loss in obese individuals. We hypothesized that moderate weight loss would decrease serum Fet-A and pFet-A concentrations and that these changes would be associated with improvements of surrogate markers of insulin sensitivity.

## Methods

### Participant recruitment & study population

The study was advertised through recruitment fliers placed around the Auburn-Opelika local area, and by Auburn University's daily faculty/staff email announcement. Approximately 200 individuals responded to these advertisements. Of these, a preliminary screening was conducted on 80 participants. Of the 51 individuals that met the criteria, 42 men (31 were obese and 11 were normal-weight) completed all baseline requirements for the study. All 31 obese men started the weight-loss protocol, but only 16 achieved the targeted 8-10% body weight loss. Fifteen participants withdrew from the study at various time points, either due to a lack of time/interest (n=13) or for medical reasons unrelated to the study (n=2).

All obese volunteers met the following criteria: male between 30 to 65 years old; body mass index (BMI) over 30 $kg/m^2$ and/or body fat over 30% of total body weight and waist circumference over 40 inches; without previously diagnosed cardiovascular or metabolic disease; weight stable over previous 6 months; no signs or symptoms of latent cardiovascular disease; did not participate in any regular physical activity in the previous 6 month period [regular physical activity defined as moderate or intense physical exertion (50 - 80% of $VO_{2max}$) for 20 minutes or more at least two times per week]; non-smoker; did not take medication known to affect lipid or glucose metabolism; without any physical impairment or conditions that would prevent regular treadmill walking. Subjects with normal body weight (control subjects) met the same criteria as above except that of BMI/percent body fat/waist circumference. Control subjects had BMI $\leq$ 27 $kg/m^2$. Of the 16 individuals recruited for this study, 13 were Caucasian, 1 was Caucasian/American Indian, and 2 were of African-American descent. Informed consent was

obtained from all subjects, and this study received approval from the Auburn University Institutional Review Board (07-210 MR 0710).

**Baseline procedures**

Subjects who met the recruitment criteria initially underwent a physical exam by a physician followed by a body composition assessment using dual energy x-ray absorptiometry (DEXA) scan to determine body fat levels and fat distribution patters. Next, volunteers were asked to perform a maximal exercise test (GXT) using standard Bruce protocol on a motor-driven treadmill to determine cardiovascular fitness (Lear et al., 1999). A 12-lead electrocardiogram was administered at rest and throughout the maximal exercise test. Respiratory gases were measured using a Medical Graphics Ultima metabolic unit (MedGraphics, St. Paul, MN). Further, blood pressure, heart rate, and perceived exertion ratings were monitored throughout the test.

After the preliminary screening procedures, subjects were asked to report to the laboratory on the 3$^{rd}$ day in the fasted state (12-h fast, restricted to water only) for an oral glucose tolerance test (OGTT). A fasting blood sample was obtained through venipuncture in an antecubital vein, which was used for the assessment of baseline biochemical variables. Immediately after the fasting blood sample was obtained, participants received 75 g of a standard carbohydrate syrup. Blood samples were drawn at 30 min intervals for 2 hours following consumption of the glucose solution. All blood samples were obtained from venipuncture in an antecubital vein. Blood serum were drawn into one 10 ml "red top" serum vacutainer tube (Becton Dickinson Vacutainer, Franklin Lakes, NJ) and allowed to clot for 30 minutes, then centrifuged for 10 minutes to separate the serum. Aliquots of serum were transferred into 2 ml microcentrifuge tubes and subsequently stored at -80℃ freezer until further analyses.

**Weight Loss Procedures**

Only obese subjects participated in the weight loss protocol. The overall targeted weight loss was 8-10% of the initial body weight over a 6- to 10-month period, following the guideline of the National Heart, Lung and Blood Institute (NHLBI) (National Institutes of Health, 1998). The weight loss intervention was designed to achieve a 2000 to 2500 kcal/week energy expenditure and/or energy deficit. Each obese subject was asked to visit the laboratory every 4 weeks for blood sampling and body composition analyses. During this visit, subjected were also asked to provide his 3-day diet and physical activity record for review and analysis. Based on the information, subjects were given recommendations and modifications for the next month. Once participants achieved the weight loss goal of 8-10%, another OGTT was administered following the same protocol as previously described.

**Exercise Intervention**

Participants were asked to perform physical exercise 4 to 5 times per week to achieve their weekly energy expenditure goal. Participants were required to complete two exercise sessions per week in the laboratory under supervision. During the lab exercise sessions, exercise intensity, blood pressure, and body weight were documented. To achieve the energy expenditure goal of 400-500 kcal/session in the laboratory, respiratory gases and heart rates were monitored. All subjects were trained for using heart rate monitor and rating of perceived exertion (RPE) scale for monitoring exercise intensity for the remaining exercise sessions, to achieve the weekly energy expenditure goal.

**Serum Fet-A and Ser312-phosphorylated Fet-A**

Serum concentrations of Fet-A were measured using an ELISA kit (BioVendor, LLC, Candler, NC). Serum samples were assayed in duplicate, using human Fet-A standards ranging from 2 - 100 ng/mL. Standards, quality controls, and 1:10,000 diluted serum samples were added to anti-human Fet-A antibody-coated microtiter strips. After incubation, washing, addition of conjugate solution and substrate, the absorbance was read in a microplate reader at 450 nm.

Serum pFet-A was assayed by Western blotting. Serum samples and quality control serum were diluted 1:100 in sterile saline and proteins were separated on a 4 ~ 20% gradient SDS-PAGE gel (Bio-Rad, Hercules, CA). Proteins were transferred to nitrocellulose membrane, blocked in 5% non-fat dry milk (Bio-Rad, Hercules, CA, USA). Serum pFet-A was detected using a custom-generated affinity-purified antibody (Affinity BioReagents, Golden, CO) against the phosphorylated Ser312-fetuin-A epitope "HTFMGVVSLGSPS(PO4)GEVSHPR". Chemiluminescence was imaged with UVP BioImaging system (UVP, Upland, CA). Area densities of the bands were analyzed using the UnScan-It software package (Silk Scientific, Orem, UT, USA). All samples were calculated by comparing pixels to the quality control serum sample, loaded in duplicate. Fold changes were used for statistical analysis.

**Biochemical analysis, insulin resistance, and glucose tolerance**

Serum glucose was measured using a glucose hexokinase assay kit (Cliniqa Corporation, San Marcos, CA). Serum insulin concentrations were measured by a human insulin-specific radioimmunoassay (Millipore Corporation, Billerica, MA). Serum non-esterified fatty acid (NEFA) concentrations were assayed using a 96-well NEFA assay kit (Wako, Richmond, VA). Blood samples were also sent to a Centers for Disease Control and Prevention-certified laboratory to verify blood chemistries, lipids, and liver enzymes.

The homeostasis model assessment of insulin resistance (HOMA-IR), which reflects hepatic insulin resistance was calculated using the following formula: [fasting insulin (μU/mL) x fasting glucose (mmol/L)/22.5] (Matthews et al., 1985). Adipose insulin resistance (Adipose-IR) was calculated as follows: [fasting NEFA mEq/L x fasting insulin μU/mL] (Abdul-Ghani et al., 2008). The quantitative insulin sensitivity check index (QUICKI), which is useful for measuring insulin sensitivity, was calculated by the following: [1 / (log (fasting insulin μU/mL) + log (fasting glucose mg/dL))] (Katz et al., 2000); Glucose to insulin ratio (GIR) was calculated as glucose (mg/dL)/insulin (μU/mL).

Glucose, insulin, Fet-A, and pFet-A area under the curve (AUC) during the oral glucose tolerance test were calculated using the trapezoidal method. Insulin resistance index, which primarily reflects skeletal muscle glucose uptake, was estimated by multiplying $insulin_{AUC}$ and $glucose_{AUC}$ and dividing by $10^6$ (Evans et al., 2001).

**Statistical Analysis**

A paired Student's *t*-test was used to determine statistical differences between pre- and post-weight loss in obese individuals. Repeated measures one-way ANOVA was used to examine the effects of OGTT, incremental weight loss. The total area under the curve (AUC) during an OGTT was calculated. A paired Student's *t*-test was used to test statistical differences in AUCs between Pre-weight loss OGTT and Post-weight loss OGTT. Pearson product moment correlation coefficients were used to examine associations and significance was accepted as $p \leq 0.05$. Data are expressed as Mean ± SD, unless noted otherwise.

## Results

### Anthropometric, physiological, and metabolic characteristics before and after 8-10% weight loss

Obese participants lost an average of 20.5 lbs or 8.8% of their initial body weight. Their weight loss ranged from 15.1 lbs to 27.9 lbs or from 7.6% to 10.9%. Most of the participants achieved their weight loss goal within 6 to 10 months. The anthropometric, physiological, and metabolic characteristics before and after weight loss are shown in Table 1.

As expected, 8-10% weight loss significantly decreased waist circumference, with a decrease of $3.7 \pm 2.8$ inches (Mean ± SD, $p = 0.0001$), percent total fat ($p < 0.0001$), fat mass ($p < 0.0001$), lean mass ($p = 0.0075$), percent android fat ($p < 0.0001$), percent gynoid fat ($p < 0.0001$), systolic blood pressure ($p < 0.0001$), diastolic blood pressure ($p = 0.0015$); and significantly increase maximal oxygen consumption ($p = 0.0002$) (Table 1).

### Alterations in serum insulin, glucose, and surrogate measures of insulin sensitivity with 8-10% body weight loss

Fasting glucose and NEFA concentrations were unaltered after 8-10% weight loss ($p > 0.05$) (Table 1, Fig.1A and 1C). Serum insulin, HOMA-IR, adipose-IR, quantitative insulin sensitivity check index (QUICKI) and G:I ratio significantly improved by 8-10% body weight loss. Post-weight loss, obese individuals decreased serum insulin concentrations by 21.4%, and markers of insulin resistance, viz., HOMA-IR by 17%, and adipose-IR by 14% (Table 1, Fig.1D). QUICKI, the indicator of insulin sensitivity, increased by 3.6% and G:I ratio by 41.7% (Table 1, Fig.1E, F).

**Alterations in Fet-A and pFet-A changes with 8-10% body weight loss**

Serum Fet-A and pFet-A concentrations were significantly decreased after 8-10% weight loss. Serum Fet-A concentrations ranged from 262.6 to 535.6 μg/ml in obese individuals before weight loss, and from 221.9 to 377.2 μg/ml after 8-10% weight loss (Table 1, Fig.1G, H). Serum Fet-A concentrations were decreased by 24.0% and pFet-A by 42.3% with target weight loss. In obese individuals before weight loss, serum fetuin-A concentrations were significantly correlated with NEFA (Table 2). Serum pFet-A demonstrated an association with adipose IR and tended to correlate with fasting insulin concentrations and G:I ratio (Table 2). After 8-10% body weight loss, unlike Fet-A, pFet-A was significantly correlated with insulin, HOMA-IR, QUICKI, and G:I ratio (Table 2). Consistent with these findings, percent changes of pFet-A after 8-10% weight loss, was significantly correlated with percent changes of insulin, HOMA-IR, and G:I ratio (Fig.3).

**Fet-A and pFet-A response to an oral glucose load before and after 8-10% body weight loss**

To analyze alterations of Fet-A and pFet-A responses to an oral glucose load, all subjects underwent oral glucose tolerance tests before and after 8-10% body weight loss. We observed significant reductions for pFet-$A_{AUC}$ ($p = 0.006$) after 8-10% weight loss, which accompanied significant reductions in $insulin_{AUC}$ ($p = 0.0002$) and insulin resistance index ($p = 0.0005$). Serum Fet-$A_{AUC}$ showed a strong trend ($p = 0.056$) of reduction after 8-10% body weight loss. Similarly, we observe a significant decrease in 2-h glucose, 2-h insulin, 2-h Fet-A and a trend to decrease 2-h pFet-A ($p = 0.067$). Percent reduction of Fet-A was correlated with reduction of 2-h insulin ($r = 0.61$, $p = 0.01$). Percent reduction of pFet-$A_{AUC}$ was significantly correlated with percent reduction of $insulin_{AUC}$ (Fig.3D), and tended to associate with percent change in insulin resistance index (Fig.3E) and G:I ratio (Fig.3F).

Surprisingly, we observed temporal changes in serum Fet-A and pFet-A during the OGTT. In obese individuals Fet-A concentrations decreased following an oral glucose load at the 30' ($p < 0.01$), 60' ($p < 0.05$), and 90' ($p < 0.05$) time points (Fig.2C). After 8-10% weight loss, Fet-A concentrations were significantly decreased only at the 30'-time point, compared to 0' time point. No change in pFet-A response was observed before weight loss. However, after weight loss, pFet-A concentrations decreased significantly at the 90' time point (Fig.2D).

**Alterations of Fet-A and pFet-A with incremental weight loss**

We observed a temporal decrease in waist circumference, percent total fat, fat mass, android, gynoid fat, and blood pressure starting with 2-4% weight loss and which continued through 4-6%, 6-8%, and 8-10% weight loss (Table 3). Similarly, HOMA-IR, Fet-A, and pFet-A demonstrated temporal reductions with 2-4%, 4-6%, 6-8%, and 8-10% weight loss. Serum glucose, NEFA, and adipose IR showed temporal decreases at 2-4%, 4-6% and 6-8% body weight loss. However, this was not sustained at 8-10% weight loss.

With 2-4% weight loss, we observe significant decreases in serum glucose (Fig.4A), NEFA (Fig.4C), HOMA-IR (Fig.4D), and adipose IR (Table 3). Furthermore, serum Fet-A and pFet-A were decreased significantly by 15.1 and 19.6% respectively, with 2-4% body weight loss. Serum NEFA concentrations following 2-4% weight loss were significantly correlated with Fet-A ($r = 0.51$, $p = 0.04$) and pFet-A ($r = 0.55$, $p = 0.03$).

On the other hand, we observed a significant decrease in serum insulin only with 6-8% and 8-10% weight loss (Fig.4B). Similarly, improvements in QUICKI were observed only with 6-8% and 8-10% weight loss (Fig.4E). G:I ratio showed significant improvement only with 8-10% body weight loss (Fig.4F).

## Discussion

In the present study, we present novel findings regarding fetuin-A and its phosphorylated counterpart, Ser312-phosphorylated fetuin-A, with incremental weight loss in obese subjects. We demonstrate, for the first time that significant reductions in pFet-A and pFet-$A_{AUC}$ were associated with an improvement of insulin sensitivity. In addition, we also report that serum Fet-A and pFet-A were decreased significantly with as little as 2-4% body weight loss, which was prior to the observed significant decrease in serum insulin and the improvements in QUICKI and G:I ratio.

Our results extend previous findings that Fet-A concentrations are strongly associated with insulin resistance (Brix et al., 2010; Ix et al., 2006; Ix et al., 2009; Reinehr and Roth, 2008; Stefan and Haring, 2013; Stefan et al., 2006) . We did not detect significant changes in Fet-A with the improvement of insulin sensitivity with moderate weight loss, but we observed that pFet-A concentrations decreased significantly in a parallel manner to the reduction in insulin, HOMA-IR, and percent increase of G:I ratio. These finding suggested an important role of phosphorylation status of Fet-A, in modulating insulin sensitivity through lifestyle intervention and moderate weight loss.

While fetuin-A exists in circulation in both phosphorylated (~20%) and dephosphorylated (~80%) forms (Le Cam et al., 1985; Lebreton et al., 1979), only the phosphorylated form was shown to exert its inhibitory effects on insulin-stimulated IR autophosphorylation, IR-TK activity, DNA synthesis (Auberger et al., 1989; Kalabay et al., 1998; Srinivas et al., 1993). In 2001, Haglund et al detected two phosphorylation sites, Ser120 and Ser312, in human Fet-A. Of these, Ser312 was shown to be the dominant phosphorylation site displaying ~77%

phosphorylation compared to Ser120 (Haglund et al., 2001). Recently, we have demonstrated that mutation of Ser120 and Ser312 residues to alanine leads to a loss of the inhibitory effect of fetuin-A to inhibit insulin signal transduction, insulin-stimulated GLUT4 translocation, glucose uptake, and glycogen synthesis in skeletal muscle cells (Ren, 2014).

In this study we demonstrate that obese, insulin-resistant individuals have significantly higher serum Fet-A and its phosphorylated form, pFet-A. While this confirmed previous findings that obese, insulin resistant subjects have higher circulating Fet-A (Brix et al., 2010; Ix et al., 2006; Ix et al., 2009; Reinehr and Roth, 2008; Stefan et al., 2006), we provide novel evidence, showing that the Ser312-phosphorylated form of fetuin-A is significantly elevated in obese and insulin resistant individuals before weight loss, and indicating that pFet-A is associated with adipose IR with a trend to correlate with fasting insulin, 2-h insulin, and G:I ratio.

The Diabetes Prevention Program Research Group demonstrated that weight loss and physical activity lowered the incidence of type 2 diabetes in individuals with impaired glucose tolerance (Diabetes Prevention Program Research et al., 2009). In the current study, there was a remarkable decrease in Fet-A (24.0%) and pFet-A (42.3%), with 8-10% weight loss. In comparison, fasting insulin concentrations were decreased by 21.4%, HOMA-IR was lower by 17%, and adipose IR was lower by 14%. The improvement of insulin sensitivity after moderate weight loss was confirmed through a decrease in $insulin_{AUC}$ (30.3%) and insulin resistance index (33.8%). This suggested that moderate weight loss observed in our study resulted in the improvement of hepatic insulin sensitivity (Van Der Heijden et al., 2010a; Viljanen et al., 2009; Vitola et al., 2009), adipose insulin sensitivity (Goodpaster et al., 1999; Schenk et al., 2009), and skeletal muscle insulin sensitivity (Petersen et al., 2012; Wojtaszewski et al., 2002). These

findings are consistent with previous findings demonstrating improved insulin sensitivity with exercise and calorie restriction in adults (Ross et al., 2000a; Ryan et al., 2012; Straznicky et al., 2012; Trussardi Fayh et al., 2013; Yassine et al., 2009), adolescents (van der Heijden et al., 2010b), and children (Abrams et al., 2013; Uysal et al., 2013; Zeybek et al., 2010). Several studies have also shown that exercise alone (Bell et al., 2007; Ryan et al., 2013; Watkins et al., 2003), hypocaloric diets (Muzio et al., 2005; Xydakis et al., 2004), gastric banding or bypass surgery (Bojsen-Moller et al., 2013; Gazzaruso et al., 2002) and medication (Bougoulia et al., 2006; Kelley et al., 2004) exert beneficial effects and alters insulin sensitivity.

Substantial weight loss in children with NAFLD was shown to decrease serum fetuin-A (Reinehr and Roth, 2008). This decrease was shown to be associated with changes in HOMA-IR, blood pressure and waist circumference. Among older adults, a 12-week supervised exercise training was shown to lower serum fetuin-A by 8%, which was correlated with hepatic insulin resistance and HMW-adiponectin (Malin et al., 2014). Further, a 16-month longitudinal study of 75 morbidly obese patients after gastric bypass demonstrated that changes of serum fetuin-A were significantly correlated with changes in fasting insulin, 2-h insulin, and HOMA-IR (Brix et al., 2010). We demonstrate that with 8-10% weight loss, serum pFet-A was decreased (42.3%) considerably more than Fet-A (24.0%) in our study subjects. Importantly, our findings showed that changes in pFet-A and not Fet-A were correlated to the observed improvement of insulin sensitivity. This is consistent with findings that pFet-A but not the dephosphorylated form of fetuin-A inhibits GLUT4 translocation, glucose uptake & glycogen synthesis in skeletal muscle cells (Ren, 2014).

To understand the effects of incremental changes in body weight to alterations in Fet-A and pFet-A, and their role in improving insulin sensitivity, fasting blood samples were collected

once every month until the targeted 8-10% weight loss goal was achieved. We categorized their weight loss as percentages compared to pre weight loss, i.e., 2-4%, 4-6%, 6-8% and 8-10% weight loss. By analyzing fasting blood samples under this category, we sought to identify alterations in Fet-A and pFet-A with incremental weight loss and correlate these with improvement of surrogate markers of insulin sensitivity. We found, not surprisingly, that even a moderate weight loss could be an important factor to stimulate insulin action. Our studies show that with as little as 2-4% weight loss, serum NEFA, HOMA-IR, and adipose IR were decreased. Similarly Fet-A and pFet-A concentrations were significantly decreased with 2-4% weight loss, and continued to decrease with incremental weight loss. Further, we observed significant associations between serum Fet-A and pFet-A with NEFA after 2-4% weight loss. Previous studies have shown that in subjects with elevated free fatty acids, serum Fet-A was associated with insulin resistance (Stefan and Haring, 2013). Recently, Kahraman et al reported that NEFA up-regulated Fet-A's mRNA expression in human primary hepatocytes (Kahraman et al., 2013). Additionally, fetuin-A was identified as an adapter protein linking free fatty acids to TLR-4, resulting in inflammation and insulin resistance (Pal et al., 2012).

Our studies demonstrate that serum insulin significantly decreased during 6-8% weight loss, QUICKI significantly increased from 6-8% and glucose-to-insulin ratio increased in 8-10% weight loss, indicating insulin sensitivity enhancement from 6-8% weight loss. Our findings are fairly consistent with Diabetes Prevention Program (DPP) study, in which 7% weight loss can significantly improve insulin sensitivity and further decrease the risks of type 2 diabetes (Diabetes Prevention Program Research et al., 2009). Accordingly, numerous other clinical studies documented that slight weight loss, as little as 4-6%, begin to improve insulin sensitivity and decrease insulin resistant clinical markers (Chowdhury et al., 1993; Dumortier et al., 2003;

Goldstein, 1992; Goodpaster et al., 1999; Kerksick et al., 2010; Knowler et al., 2002b; Olefsky et al., 1974; Pasanisi et al., 2001; Rector et al., 2007; Ross et al., 2000b; Sciacqua et al., 2003; Stallone et al., 1991; Williams and Kelley, 2000; Wing et al., 1987). Since Fet-A and pFet-A concentrations are decreased with as little as 2-4% weight loss, we are tempted to speculate that alterations in this hepatokine may "predict" improvements in insulin sensitivity with weight loss.

A limitation of this study is that of the 31 obese men that were recruited only 16 completed the study. Thus, the small sample size may have influenced the outcomes of this study. Secondly, though alterations in liver fat, intramyocellular lipid content, inflammatory cytokines including TNFα, IL-6, and adiponectin, and decreased ER and oxidative stress have been implicated in the improvement of insulin sensitivity with weight loss, we have not examined the relationship of Fet-A and pFet-A with these markers (Bruun et al., 2003; Chae et al., 2013; Gregor et al., 2009; Ou et al., 2012; Petersen et al., 2012; Rector et al., 2007; Stefan et al., 2006).

In conclusion, our present study indicates that 8-10% body weight loss significantly decreases serum fetuin-A and its phosphorylated counterpart, Ser312-phosphofetuin A. Importantly, our findings showed that changes in Ser312-phosphofetuin-A and not fetuin-A were correlated to the observed improvement of insulin sensitivity. Furthermore, fetuin-A and Ser312-phosphofetuin A were significantly decreased with as little as 2-4% weight loss. Taken together, these results suggest that changes in Ser312-phosphofetuin-A with moderate weight loss may play a role in the improvement of insulin sensitivity.

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## Figure Legends

Fig.1. Fetuin-A (Fet-A), Ser312-phosphorylated fetuin-A (pFet-A), and metabolic indices before and after 8-10% body weight loss. Sixteen obese men took part in a weight loss program with a goal of losing 8-10% of initial body weight. Serum glucose (A), insulin (B), NEFA (C), HOMA-IR (D), QUICKI (E), glucose/insulin (G:I) ratio (F), fetuin-A (Fet-A) (G) and Ser312-phosphorylated fetuin-A (pFet-A) (H) were analyzed for each individual before (Pre-WL) and after weight loss (Post-WL) and are shown as before-after graph. Statistical significance was denoted using p values. For Fig.1H, B = Before weight loss, A = After weight loss.

Fig.2. Effects of 8-10% body weight loss during an OGTT in obese individuals. An oral glucose tolerance test (OGTT) was administered before (-○-) and after 8-10% body weight loss (-●-) in obese individuals. Blood samples were collected at 30 min intervals for 2 hours and were assayed for serum glucose (A), insulin (B), fetuin-A (Fet-A) (C) and Ser312-phosphorylated fetuin-A (pFet-A) (D). Area under the curve (AUC) during OGTT was calculated as $glucose_{AUC}$ (E), $insulin_{AUC}$ (F), insulin resistance indext (G), serum Fet-$A_{AUC}$ (H) and pFet-$A_{AUC}$ (I). Data are shown as Means ± SEM. * $p < 0.05$, ** $p < 0.01$, compared to 0' time-point in the before weight loss group; $^{\#}$ $p < 0.05$, compared to 0' time-point in the obese after weight loss group.

Fig.3. Association of Ser312-phosphorylated fetuin-A (pFet-A) with insulin resistance. Correlation of percent change (% Δ) in pFet-A with % Δ in serum insulin (A), HOMA-IR (B), glucose/insulin (G:I) ratio (C). Percent change in pFet-$A_{AUC}$ with % Δ $insulin_{AUC}$ (D), insulin

resistance index (E), and G:I ratio (F) after 8-10% weight loss results are depicted. Pearson correlation coefficient r and statistical significance, depicted as p values are shown..

Fig.4. Fetuin-A (Fet-A), Ser312-phosphorylated fetuin-A (pFet-A), and metabolic indices with incremental body weight loss. Sixteen obese individuals participated in the targeted 8-10% body weight loss program. Incremental weight loss was categorized as before weight loss (Pre-WL), 2-4 %, 4-6 %, 6-8 % and 8-10% weight loss. Alterations in serum glucose (A), insulin (B), NEFA (C), HOMA-IR (D), QUICKI (E), glucose/insulin (G:I) ratio (F), Fet-A (G) and pFet-A (H) were analyzed during the incremental weight loss period. Data are shown as Mean ± SEM. Bar diagrams with different letters indicate statistical significance between groups.

**Table 1. Baseline anthropometric, physiological and metabolic characteristics in obese individuals before and after moderate weight loss**

| Variable | Pre-WL (n = 16) | Post-WL (n = 16) | p value |
|---|---|---|---|
| | Mean ± SD | Mean ± SD | |
| Weight (lbs) | 234.3 ± 39.1 | 213.8 ± 36.3 | <.001 |
| Waist circumference (inches) | 44.5 ± 4.8 | 41.0 ± 4.8 | <.001 |
| Total fat % | 35.9 ± 4.3 | 32.4 ± 5.7 | <.001 |
| Fat mass (kg) | 37.5 ± 9.3 | 31.4 ± 10.0 | <.001 |
| Lean mass (kg) | 66.1 ± 10.0 | 64.2 ± 10.2 | 0.008 |
| Android fat % | 47.8 ± 4.6 | 43.5 ± 5.6 | <.001 |
| Gynoid fat % | 37.4 ± 5.5 | 34.2 ± 5.8 | <.001 |
| SBP (mmHg) | 131 ± 10 | 121 ± 11 | <.001 |
| DBP (mmHg) | 83 ± 8 | 75 ± 7 | 0.002 |
| $VO_{2max}$ (ml/kg/min) | 30.3 ± 4.4 | 35.7 ± 5.8 | <.001 |
| $VO_{2max}$ absolute (L/min) | 3.18 ± 0.43 | 3.49 ± 0.47 | 0.031 |
| Fasting glucose (mg/dL) | 107 ± 17 | 109 ± 15 | 0.640 |
| Fasting insulin (μU/ml) | 27.9 ± 11.0 | 20.3 ± 6.9 | 0.001 |
| Fasting Fet-A (μg/ml) | 374.3 ± 70.9 | 306.4 ± 41.3 | 0.002 |
| Fasting pFet-A (Scan units) | 4.26 ± 1.20 | 3.10 ± 1.51 | 0.006 |
| Fasting NEFA (mEq/L) | 0.72 ± 0.23 | 0.76 ± 0.23 | 0.696 |
| 2-h Glucose (mg/dL) | 118 ± 41 | 103 ± 39 | 0.048 |
| 2-h Insulin (μU/ml) | 90.0 ± 47.9 | 60.0 ± 37.4 | 0.001 |
| 2-h Fet-A (μg/ml) | 322.8 ± 58.4 | 292.1 ± 50.3 | 0.043 |
| 2-h pFet-A 2h(Scan units) | 2.85 ± 1.48 | 2.16 ± 1.95 | 0.067 |
| Adipose-IR | 19.9 ± 9.2 | 15.1 ± 5.6 | 0.025 |
| HOMA-IR | 7.6 ± 3.7 | 5.5 ± 2.2 | 0.005 |
| QUICKI | 0.29 ± 0.02 | 0.30 ± 0.01 | 0.031 |
| G:I ratio | 4.3 ± 1.5 | 5.9 ± 1.8 | 0.001 |

Data are expressed as Mean ± SD. BMI-body mass index; $VO_{2max}$: maximum oxygen consumption measured over one minute of a standard Bruce protocol graded exercise test; Fet-A: fetuin-A; pFet-A-Ser312 phosphorylated fetuin-A; Adipose-IR: adipose insulin resistance; 2-h: two hour time-point during an oral glucose tolerance test. NEFA: non-esterified fatty acids; HOMA-IR: Homeostasis model assessment of insulin resistance; Adipose-IR: adipose insulin resistance, QUICKI: Quantitative insulin sensitivity check index, G:I ratio: glucose to insulin ratio; 2-h: two hour time-point during an oral glucose tolerance test.

**Table 2. Correlation of Fet-A and pFet-A with metabolic indices in obese individuals before and after moderate weight loss**

| Pre-WL (n = 16) | Fet-A | | pFet-A | |
|---|---|---|---|---|
| | r value | p value | r value | p value |
| Fasting glucose (mg/dL) | -0.17 | 0.53 | 0.09 | 0.73 |
| Fasting insulin (μU/ml) | -0.13 | 0.63 | 0.45 | 0.08 |
| Fasting NEFA (mEq/L) | 0.60 | 0.01 | 0.27 | 0.32 |
| Fasting Fet-A (μg/ml) | 1.00 | - | 0.21 | 0.44 |
| Fasting pFet-A (Scan units) | 0.21 | 0.44 | 1.00 | - |
| 2-h glucose (mg/dL) | -0.15 | 0.58 | 0.02 | 0.95 |
| 2-h insulin (μU/ml) | -0.16 | 0.54 | 0.47 | 0.06 |
| 2-h Fet-A (μg/ml) | 0.18 | 0.50 | 0.36 | 0.18 |
| 2-h pFet-A (Scan units) | 0.06 | 0.81 | 0.46 | 0.07 |
| HOMA-IR | -0.15 | 0.58 | 0.37 | 0.16 |
| Adipose-IR | 0.35 | 0.19 | 0.56 | 0.02 |
| QUICKI | 0.18 | 0.51 | -0.40 | 0.12 |
| G:I ratio | 0.13 | 0.64 | -0.46 | 0.08 |
| Post-WL (n = 16) | | | | |
| Fasting glucose (mg/dL) | 0.25 | 0.35 | 0.07 | 0.80 |
| Fasting insulin (μU/ml) | 0.14 | 0.61 | 0.56 | 0.02 |
| Fasting NEFA (mEq/L) | -0.29 | 0.28 | -0.36 | 0.18 |
| Fasting Fet-A (μg/ml) | 1.00 | - | 0.46 | 0.07 |
| Fasting pFet-A (Scan units) | 0.46 | 0.07 | 1.00 | - |
| 2-h glucose (mg/dL) | 0.30 | 0.25 | 0.33 | 0.22 |
| 2-h insulin (μU/ml) | 0.22 | 0.41 | 0.47 | 0.07 |
| 2-h Fet-A (μg/ml) | 0.84 | <.001 | 0.52 | 0.04 |
| 2-h pFet-A (Scan units) | 0.10 | 0.72 | 0.31 | 0.24 |
| HOMA-IR | 0.23 | 0.39 | 0.53 | 0.04 |
| Adipose-IR | -0.05 | 0.86 | 0.24 | 0.38 |
| QUICKI | -0.24 | 0.38 | -0.58 | 0.02 |
| G:I ratio | -0.05 | 0.86 | -0.54 | 0.03 |

Data are expressed as Mean ± SD. NEFA: non-esterified fatty acids; Fet-A: fetuin-A; pFet-A-Ser312 phosphorylated fetuin-A; HOMA-IR: Homeostasis model assessment of insulin resistance; Adipose-IR: adipose insulin resistance, QUICKI: Quantitative insulin sensitivity check index, G:I ratio: glucose to insulin ratio; 2-h: two hour time-point during an oral glucose tolerance test.

**Table 3. Metabolic indices in obese individuals with incremental body weight loss**

| Variable (n = 16) | Pre-WL | 2-4% | 4-6% | 6-8% | 8-10% |
|---|---|---|---|---|---|
| Weight (lbs) | 234.3 ± 39.1 [a] | 227.7 ± 37.9 [b] | 222.2 ± 37.2 [c] | 217.7 ± 37.0 [d] | 213.8 ± 36.3 [e] |
| Waist circumference (inches) | 44.3 ± 4.8 [a] | 42.5 ± 4.6 [b] | 41.1 ± 4.8 [c] | 40.7 ± 4.6 [c] | 41.0 ± 4.8 [c] |
| Total fat % | 35.9 ± 4.3 [a] | 34.7 ± 5.0 [b] | 33.5 ± 5.2 [c] | 32.5 ± 5.5 [d] | 32.4 ± 5.7 [d] |
| Fat mass (kg) | 37.5 ± 9.3 [a] | 35.4 ± 10.2 [b] | 34.0 ± 10.0 [c] | 31.8 ± 10.1 [d] | 31.4 ± 10.0 [d] |
| Lean mass (kg) | 66.1 ± 9.9 [a b] | 65.4 ± 10.0 [b c] | 66.4 ± 9.1 [a] | 64.8 ± 10.4 [c d] | 64.2 ± 10.2 [d] |
| Android fat % | 47.8 ± 4.6 [a] | 46.5 ± 5.3 [b] | 44.7 ± 5.8 [c] | 43.6 ± 6.0 [d] | 43.5 ± 5.6 [d] |
| Gynoid fat % | 37.4 ± 5.5 [a] | 36.3 ± 5.7 [b] | 35.2 ± 5.5 [c] | 34.5 ± 5.0 [c d] | 34.2 ± 5.8 [d] |
| SBP (mm Hg) | 131 ± 10 [a] | 121 ± 8 [b] | 122 ± 11 [b] | 118 ± 8 [b] | 121 ± 11 [b] |
| DBP (mm Hg) | 83 ± 8 [a] | 77 ± 5 [b] | 77 ± 7 [a b] | 76 ± 6 [b] | 75 ± 7 [b] |
| Adipose-IR | 19.9 ± 9.2 [a] | 13.6 ± 6.7 [b] | 12.7 ± 5.7 [b] | 11.5 ± 4.5 [b] | 15.1 ± 5.6 [a] |

Data are expressed as Mean ± SD. Different letters in superscript following values indicate statistical significance, $p < 0.05$. SBP: Systolic blood pressure; DBP: Diastolic blood pressure; Adipose-IR: adipose insulin resistance

**Fig.1**

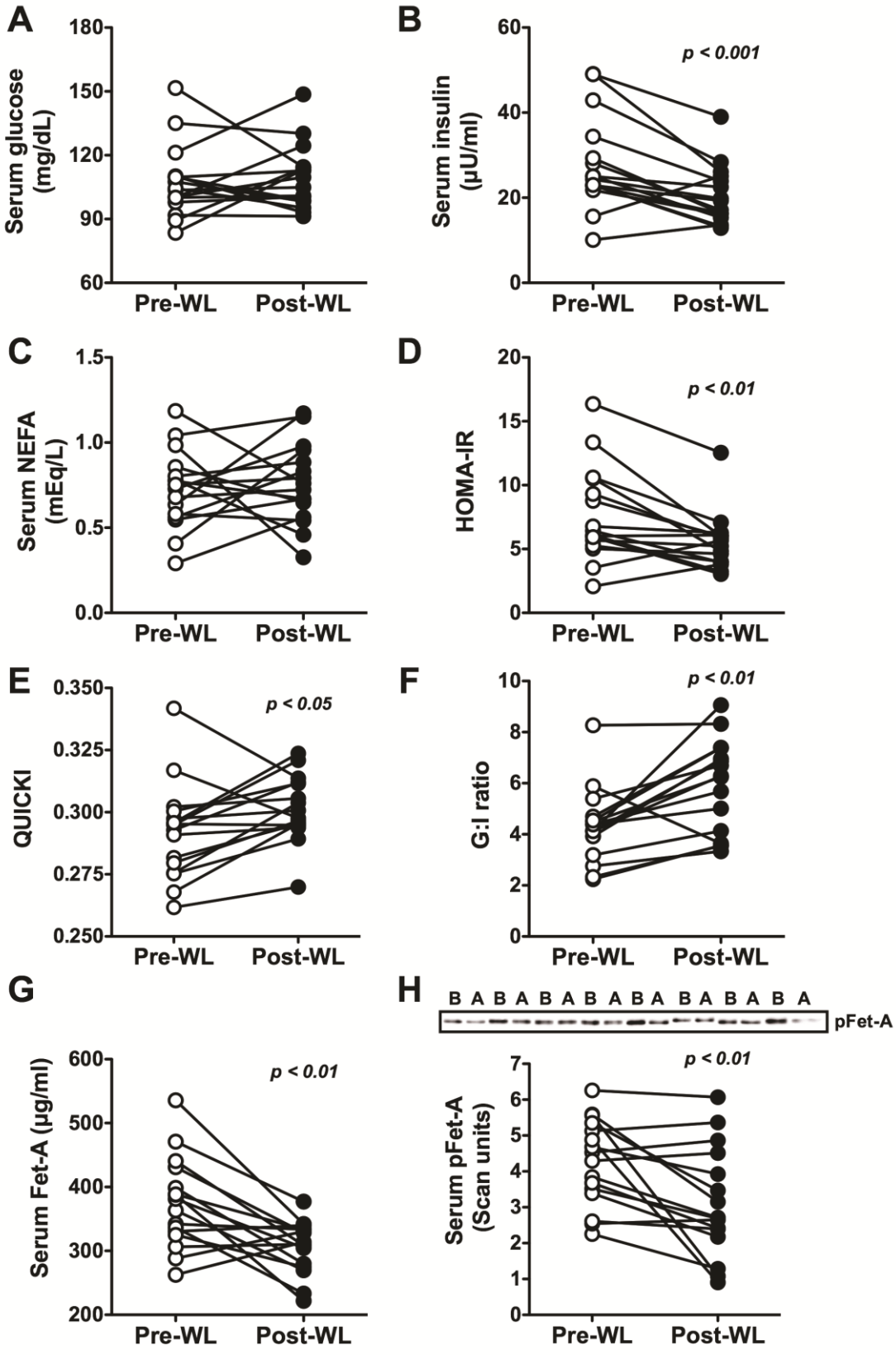

A
Serum glucose (mg/dL)
180
150
120
90
60
Pre-WL
Post-WL
B
Serum insulin (μU/ml)
60
40
20
0
p < 0.001
Pre-WL
Post-WL
C
Serum NEFA (mEq/L)
1.5
1.0
0.5
0.0
Pre-WL
Post-WL
D
HOMA-IR
20
15
10
5
0
p < 0.01
Pre-WL
Post-WL
E
QUICKI
0.350
0.325
0.300
0.275
0.250
p < 0.05
Pre-WL
Post-WL
F
G:I ratio
10
8
6
4
2
0
p < 0.01
Pre-WL
Post-WL
G
Serum Fet-A (μg/ml)
600
500
400
300
200
p < 0.01
Pre-WL
Post-WL
H
B A B A B A B A B A B A B A B A
pFet-A
Serum pFet-A (Scan units)
7
6
5
4
3
2
1
0
p < 0.01
Pre-WL
Post-WL

**Fig.2**

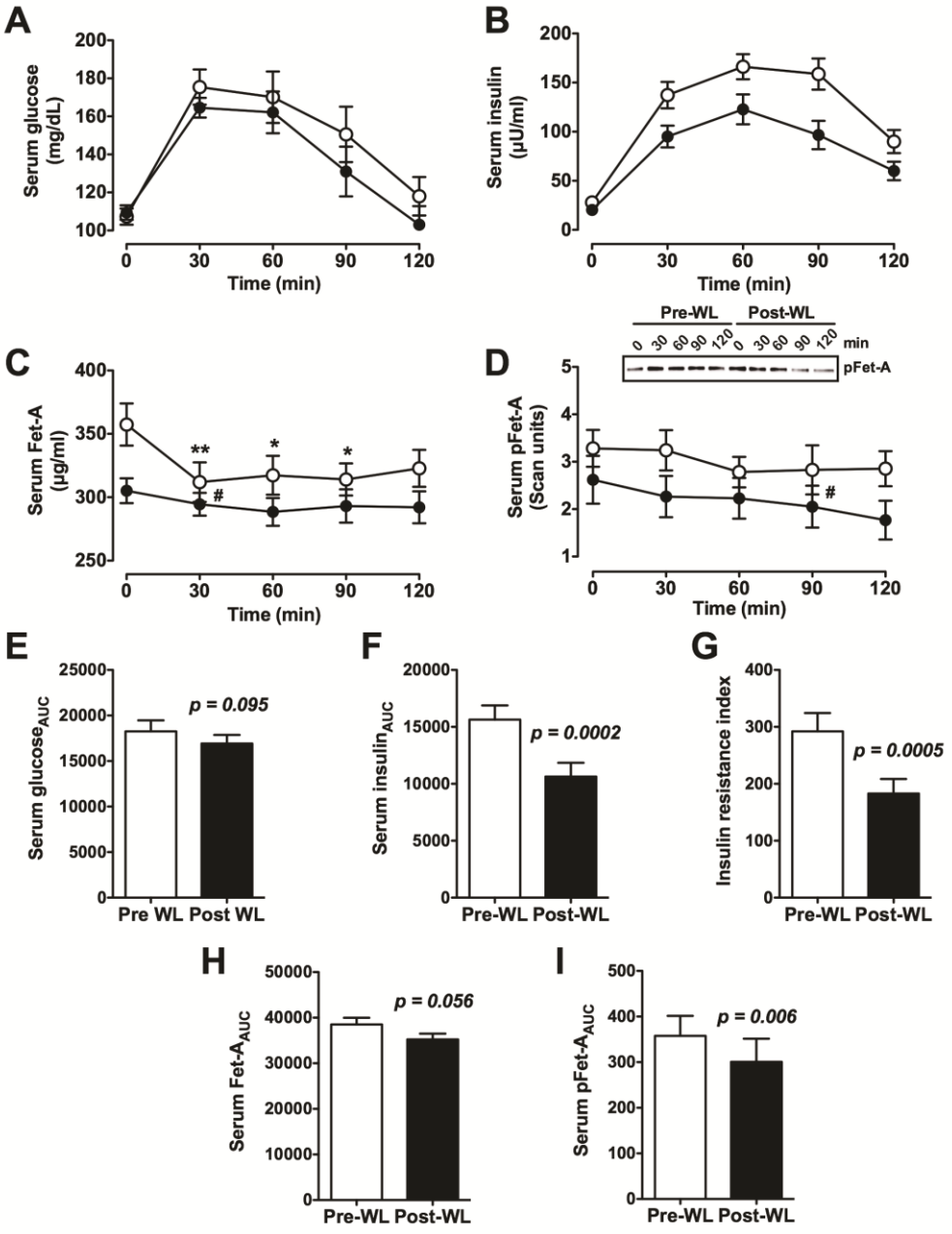

A
Serum glucose (mg/dL)
Time (min)
B
Serum insulin (µU/ml)
Time (min)
C
Serum Fet-A (µg/ml)
Time (min)
D
Pre-WL
Post-WL
min
pFet-A
Serum pFet-A (Scan units)
Time (min)
E
Serum glucose AUC
p = 0.095
Pre WL Post WL
F
Serum insulin AUC
p = 0.0002
Pre-WL Post-WL
G
Insulin resistance index
p = 0.0005
Pre-WL Post-WL
H
Serum Fet-A AUC
p = 0.056
Pre-WL Post-WL
I
Serum pFet-A AUC
p = 0.006
Pre-WL Post-WL

**Fig.3**

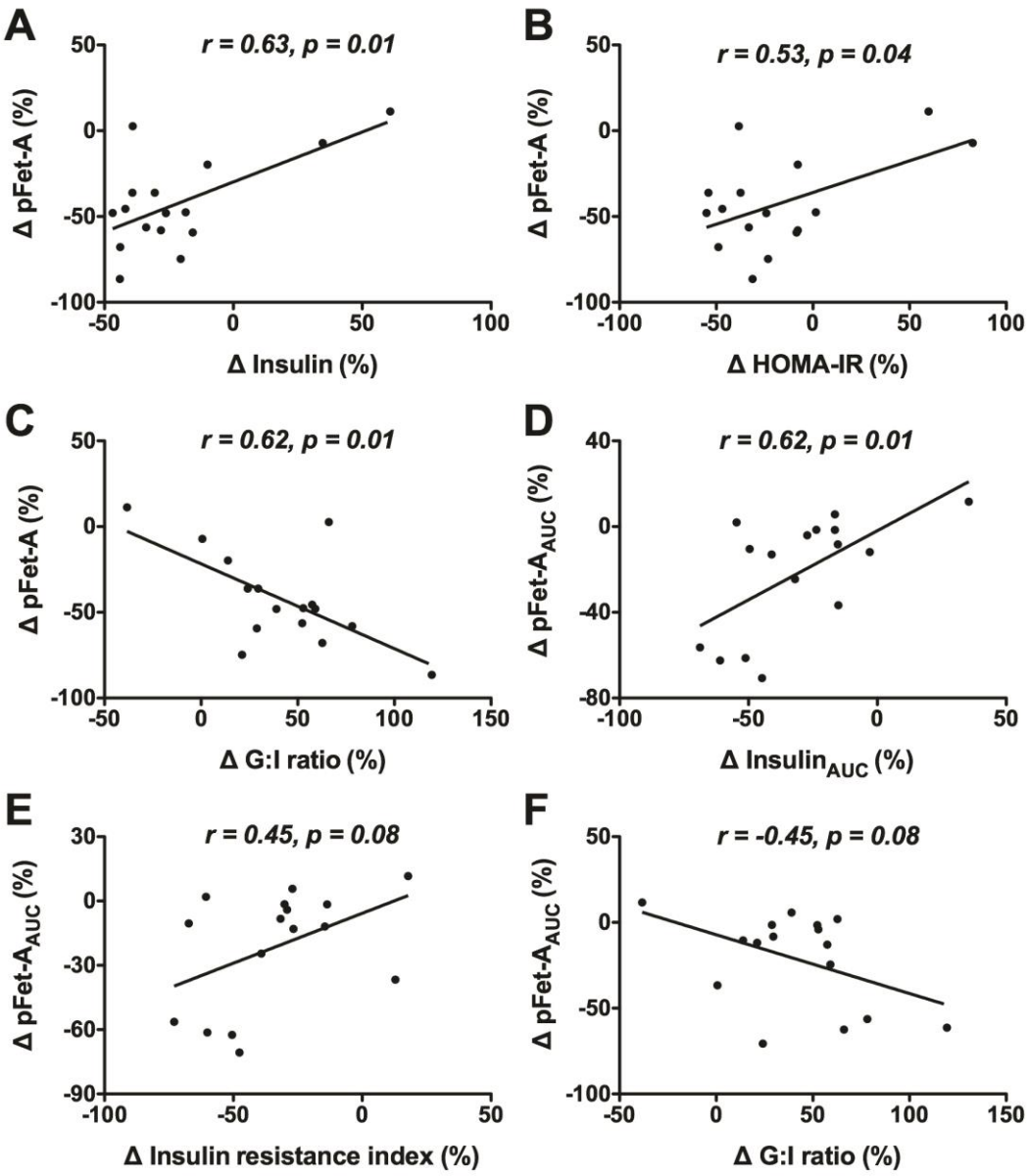
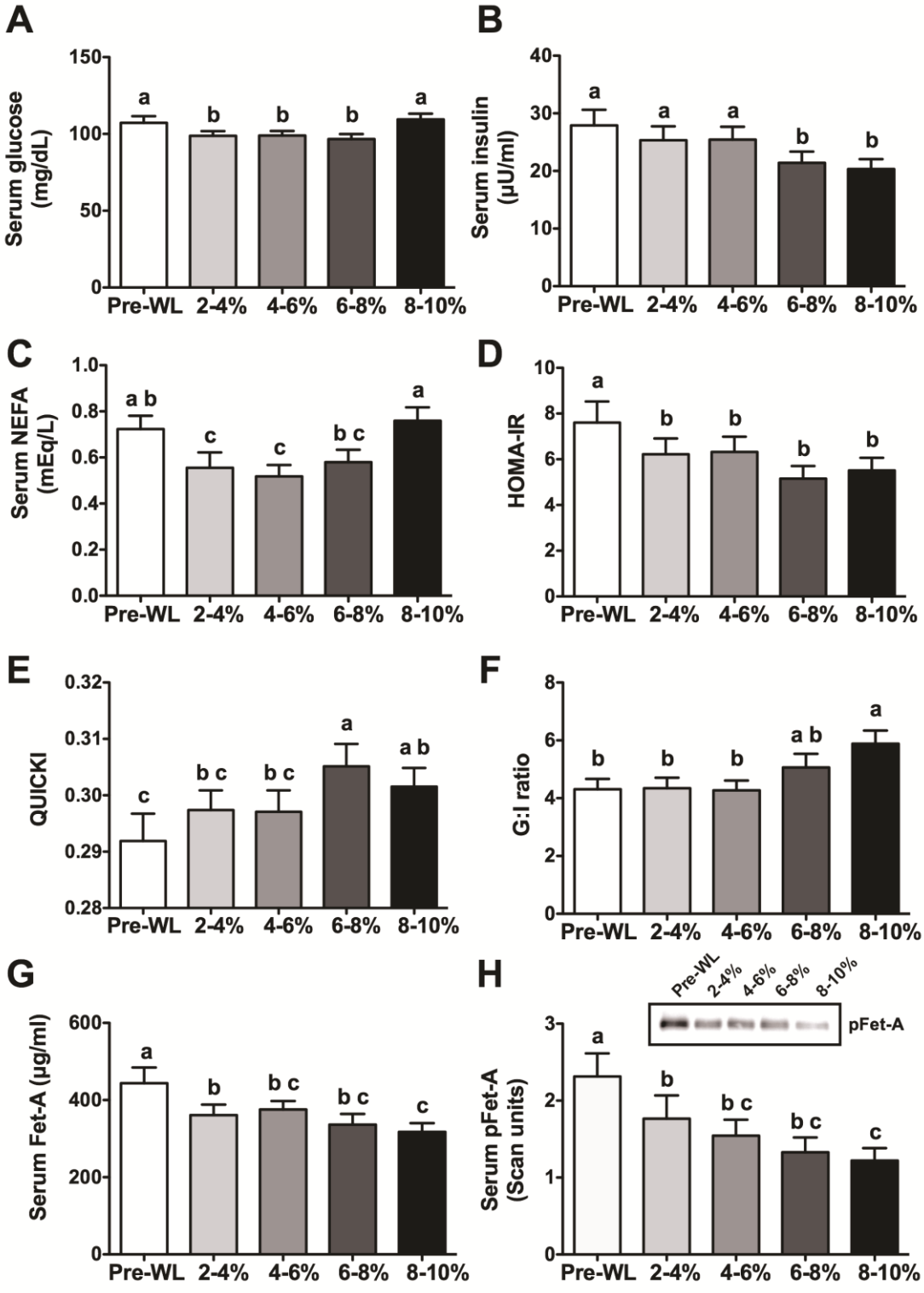

A
r = 0.63, p = 0.01
Δ pFet-A (%)
Δ Insulin (%)
B
r = 0.53, p = 0.04
Δ pFet-A (%)
Δ HOMA-IR (%)
C
r = 0.62, p = 0.01
Δ pFet-A (%)
Δ G:I ratio (%)
D
r = 0.62, p = 0.01
Δ pFet-A$_{AUC}$ (%)
Δ Insulin$_{AUC}$ (%)
E
r = 0.45, p = 0.08
Δ pFet-A$_{AUC}$ (%)
Δ Insulin resistance index (%)
F
r = -0.45, p = 0.08
Δ pFet-A$_{AUC}$ (%)
Δ G:I ratio (%)

**Fig.4**


A
Serum glucose (mg/dL)
150
100
50
0
a
b
b
b
a
Pre-WL
2-4%
4-6%
6-8%
8-10%
B
Serum insulin (μU/ml)
40
30
20
10
0
a
a
a
b
b
C
Serum NEFA (mEq/L)
1.0
0.8
0.6
0.4
0.2
0.0
a b
c
c
b c
a
D
HOMA-IR
10
8
6
4
2
0
a
b
b
b
b
E
QUICKI
0.32
0.31
0.30
0.29
0.28
c
b c
b c
a
a b
F
G:I ratio
8
6
4
2
0
b
b
b
a b
a
G
Serum Fet-A (μg/ml)
600
400
200
0
a
b
b c
b c
c
H
Serum pFet-A (Scan units)
3
2
1
0
a
b
b c
b c
c
pFet-A

## Conclusions

Fetuin-A, primarily secreted by the liver into circulation, is a negative regulator of insulin action. Elevated levels of serum fetuin-A are associated with insulin resistance, obesity and incident diabetes. The goal of this study was to investigate the role of phosphorylation of Fet-A in insulin action, insulin resistance, obesity and weight loss.

To characterize the role of fetuin-A phosphorylation, phospho-defective single (Ser312Ala) and double (Ser120Ala+Ser312Ala) mutants of human fetuin-A were generated. Transfection of HIRcB cells with wild-type and mutant fetuin-A showed that only wild-type (WT) phosphorylated fetuin-A inhibited insulin signaling through AKT and MAPK. The single mutant (Ser312Ala) partially reversed the inhibition, and the double phospho-defective mutant was without effect. Similarly, treatment of L6GLUT4myc cells with recombinant WT phosphorylated fetuin-A impaired insulin stimulated insulin receptor phosphorylation, GLUT4 translocation, glucose uptake and glycogen synthesis, while phospho-defective mutants were without effect, indicating that phosphorylation on both Ser120 and Ser312 was critical for fetuin-A's full inhibitory activity on insulin signaling, glucose uptake, and glycogen synthesis.

Next, we recruited 31 obese and 11 normal-weight men from the Auburn-Opelika local area, after receiving IRB approval. Serum Fet-A and Ser312-phosphorylated fetuin-A (pFet-A) concentrations were significantly higher in obese individuals compared to normal weight men. However, unlike Fet-A, pFet-A showed a significant correlation with BMI, waist circumference, percent total fat, blood pressure and markers of insulin sensitivity. During an oral glucose tolerance test (OGTT), $insulin_{AUC}$, $glucose_{AUC}$, IR

index, Fet-$A_{AUC}$ and pFet-$A_{AUC}$ were significantly elevated in obese individuals, indicating that pFet-A is strongly associated with insulin resistance and may be dynamically involved in insulin response.

All recruited subjects participated in a single bout of treadmill walking, expending 500 kcal at 60-70% $VO_{2max}$. In obese individuals, area under the curve (AUC) for serum Fet-A, pFet-A, glucose, insulin, and insulin resistance index, during an oral glucose tolerance test, was significantly decreased 24-h after a single bout of exercise. Reductions in Fet-$A_{AUC}$ and pFet-A twenty-four hours after a single bout of exercise correlated with an improvement of insulin sensitivity following a single bout of exercise.

Of the 31 recruited obese men who participated in the moderate weight loss study, only 16 men achieved a targeted weight loss of 8-10% of their initial body weight in 6-10 months. Serum Fet-A and pFet-A concentrations were significantly decreased with incremental weight loss. We observed that percent reductions of pFet-A and, but not Fet-A, were significantly correlated with percent changes of insulin, HOMA-IR, and G:I ratio. Similarly, percent reduction of $insulin_{AUC}$ after 8-10% weight loss was associated with percent reduction of pFet-$A_{AUC}$ and not Fet-$A_{AUC}$, suggesting that improvements in insulin sensitivity with moderate weight loss may be due to reductions of Ser312 phosphorylated-fetuin-A.

Taken together, our study indicates that phosphorylated status of fetuin-A is critical in regulating insulin signaling, glucose uptake, and glycogen synthesis. We demonstrate that Ser312-phosphorylated fetuin-A is increased in obese, insulin resistant individuals, and decreased with a single bout of exercise or moderate weight loss,

suggesting that phosphorylated fetuin-A plays a key role in the modulation of insulin action and insulin sensitivity.

## Future studies

Our studies have shown that phosphorylation status of fetuin-A is critical for the regulation of insulin action. Further, in human obesity Ser312-phosphorylated fetuin-A concentrations are elevated and correlated with insulin resistance. Improvements in insulin sensitivity associated with a single bout of exercise or weight loss were shown to be associated with lower serum Ser312-phosphorylated fetuin-A concentrations. These studies have set the stage for future investigations, both in exploring mechanistic basis and functional characterization.

While our studies using intact cells indicate that phosphorylation on both Ser120 and Ser312 are important, future studies would be needed to characterize significance, order, or differential effects of phosphorylation of either of the 2 sites. It will be of great interest to examine the role of Ser120 and Ser 312 phosphorylation on fetuin-A's interaction with the insulin receptor and how it affects the kinetics of the IR-TK reaction. Validation of these findings using animal models would be the next logical step.

Additional clinical studies exploring correlations of phosphorylated fetuin-A with insulin sensitivity using euglycemic-hyperinsulinemic clamp can contribute to a better understanding the role of the skeletal muscle versus the liver in modulating insulin sensitivity. Additionally, while a majority of our recruited subjects met the criteria for metabolic syndrome, some individuals did not. However, in this study all samples were grouped together based on BMI. For future studies, it may be important to separate obese individuals from individuals with MetS, to delineate potential differences.

For single bout exercise studies, it will be of significant interest to examine the relationship between Fet-A or pFet-A with other inflammatory cytokines, including IL-6, IL-10, and TNFα during a single bout of exercise. While alterations in liver fat, intramyocellular lipid content, inflammatory cytokines including TNFα, IL-6, and adiponectin, decreased ER stress, and oxidative stress have been implicated in the improvement of insulin sensitivity with weight loss, these were not explored in our studies. Such studies could pave the way for a better understanding of the relationship of Fet-A and pFet-A with these markers.

Further, we have only conducted these studies in men. Therefore, future studies will need to evaluate alterations in Fet-A and pFet-A and correlate these changes with insulin sensitivity, in obese women and children.

Some of these studies are currently being investigated in our laboratory.