

2015

Depot differences in adipokine secretion from human omental and abdominal subcutaneous adipose tissues: potential role of adiporedoxin

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BOSTON UNIVERSITY
SCHOOL OF MEDICINE

Thesis

**DEPOT DIFFERENCES IN ADIPOKINE SECRETION FROM HUMAN
OMENTAL AND ABDOMINAL SUBCUTANEOUS ADIPOSE
TISSUES: POTENTIAL ROLE OF ADIPOREDOXIN**

by

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B.S. LIC., Universidad Francisco Marroquin, Guatemala, 2013

Submitted in partial fulfillment of the
requirements for the degree of
Master of Science

2015

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ACKNOWLEDGMENTS

First, I would like to thank Dr. Susan K Fried and the lab for all of the encouragement, support, and guidance provided to me throughout my time here. To Dr. Lee, thank you for teaching me every detail of the experiments and for the guidance in each experiment we run together. To Kalypso for being my guide in the beginning of the thesis and showed me how to run a clinical research and being supportive in every step I made. Also, for all of you for helping me to finish mi thesis. Finally, I would like to thank both Dr. Fried and Dr. Moore of the Nutrition and Metabolism program here at BU. First for letting me participate in this master degree that is awesome, for your guidance and patience with my English. I actually, feel more ready and with more experience to go back to my country, Guatemala and start doing research and help my country. I am so lucky to have you both as teachers and directors, I have learned so much.

Outside of the lab, there are a number of individuals I am eternally grateful for. To my family, thank you for supporting me and giving me the opportunity to make my masters degree in the US. Also to my husband for being so supportive in the master and always being so helpful in every step I did. Dad and mom, you inspired me to finish my masters degree. Finally, thanks to all my family and closest friends from back home, who prove that distance doesn't mean anything and my support, network is unbreakable.

**DEPOT DIFFERENCES IN ADIPOKINE SECRETION FROM HUMAN
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ABSTRACT

Adiporedoxin (Adrx) is an adipose tissue specific protein discovered by the Pilch lab. It is a member of the peroxiredoxin family and localized in the endoplasmic reticulum (ER). Previous studies showed that Adrx is involved in ER redox regulation and disulfide bond formation and secretion of adipokines. Further, Adrx mRNA expression and protein levels in human abdominal adipose tissue of young, healthy subjects, ranging in levels of obesity, correlated positively with adiponectin mRNA and protein, and negatively with adipose tissue inflammation (as indicated by phospho-Jun kinase).

Since previous studies have shown depot differences in adipokine release, we wanted to determine the differences in Adrx expression in human adipose tissues depots. There are no data on depot differences in Adrx expression and its association with adipokine release from human adipose tissue.

It is well known that omental (Om) adipose tissue is more inflamed and secretes higher amount of interleukin-6 (IL-6) while secreting lower amount of leptin than abdominal subcutaneous (Abdsc) fat. depot. Reports on depot differences in adiponectin secretion are inconsistent. Adipokine release measured from fresh obtained adipose tissues during a short-time incubation reflects a more physiologic state and the

characteristics of the subjects compared to cultured cells and thus, we measured the rates of adipokine secretion during acute incubation.

Adiponectin is an insulin-sensitizing protein, exclusively produced by mature adipocytes and highly expressed in by adipose tissue. The native adiponectin exists as low molecular weight, middle-molecular weight and high molecular weight (HMW). The potency of adiponectin is linked to the HMW isoform. There are no previous reports on the secretion of adiponectin isoforms from human Om and Abdsc adipose tissues.

In this study the goal is to determine the depot differences in Adrx expression and adipokine release in human Abdsc and Om adipose tissue in obese and morbidly obese subjects and to determine the relationship between Adrx protein expression and adipokine release in Abdsc versus Om and circulating levels of adipokines, primarily total and HMW adiponectin. To determine whether Adrx expression is correlated in the release in adipose tissue and secretion of circulating adipokines.

Adrx protein levels (assessed by Western blot) were ~72% higher in Abdsc than Om ($p < 0.05$; paired t-test values). As expected, secretion per gram of tissue in 3hr incubation of total adiponectin (~55%) and leptin (~134%) was higher, and IL-6 secretion was lower (~56%) in Abdsc compared to Om. Total adiponectin secretion measured with quantitative Western blotting, is higher in Abdsc by 19% compared to Om. However, HMW adiponectin by 29% and % HMW are higher by 30% in Om.

Adrx protein levels were positively correlated with total adiponectin and HMW release in Abdsc and only with HMW in Om. Adrx levels were negatively correlated with % HMW release in Abdsc but not in Om. Since in Adrx KO mice presented lower total

adiponectin in circulation, it was hypothesized that Adrx were positively correlated with circulating adiponectin, and wanted to study the correlation with serum adipokines. Adrx protein levels in Abdsc showed no correlation with total circulating adiponectin levels (ng/ml measured by ELISA) and a positive trend in Om. Total serum adiponectin, HMW and %HMW by Western blot showed no correlation with Adrx levels in both Abdsc and Om. None of the correlations with Adrx and adipokines in serum were statistically significant possibly due to a small sample size.

These data suggest that depot differences in Adrx expression may influence depot differences in adipokine secretion. The mechanism of higher HMW adiponectin secretion in Om with low Adrx levels needs further study.

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LIST OF ABBREVIATIONS

AA.....	African American
Abdsc	Abdominal subcutaneous
Adrx	Adiporedoxin
AT	Adipose tissue
AU.....	Arbitrary Unit
BMI.....	Body Mass Index
BSA.....	Bovine serum albumin
BU.....	Boston University
CAU	Caucasian
CI.....	Confidence interval
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
Fig.	Figure
HbA1C	Hemoglobin A1C
HIS	Hispanic
HMW	High molecular weight
HSP90	Heat shock protein 90
IL-6	Interleukin 6
KO.....	Knockout
LMW.....	Low molecular weight
MCP-1	Monocyte Chemoattractant Protein 1

MMW	Medium molecular weight
ng/g/3hr	nanogram per gram per 3 hours
ng/ml	nanogram per ml
Om.....	Omental
PAMM	Peroxiredoxin-like 2 protein
Prx	Peroxiredoxin
r^2	Pearson Regression coefficient squared
RBP4	Retinol Binding Protein 4
ROS.....	Reactive oxygen species
SAA	Serum Amyloid A1
SAT	Subcutaneous adipose tissue
SEM	Standard error of the mean
TNF- α	Tumor Necrosis Factor Alpha
VAT	Visceral adipose tissue

INTRODUCTION

Obesity is a major public health problem in the United States. The prevalence of childhood and adult obesity in the United States and worldwide has increased dramatically in recent years and remains high, reaching 16.9% in youth and 34.9% in adults in the United States in 2011-2012.¹ Overweight and obesity are positively associated with risk for chronic diseases such as type 2 diabetes and heart disease. Obesity increases risk of high blood pressure, stroke, nonalcoholic fatty liver disease, osteoarthritis, some types of cancer, including breast, colon, endometrial and kidney, leading to high mortality rates.¹⁻⁶

Fat distribution is independently associated with metabolic diseases. A central fat distribution, a large waist due to accumulation of visceral and abdominal subcutaneous (Abdsc) fat, is classified as android (apple-shape) and is associated with higher metabolic disease risk. In contrast, accumulation of gluteal-femoral fat gynoid (pear-shape) is associated with lower risk. Therefore to decrease the rates of type 2 diabetes and cardiovascular disease (CVD) it is important to determine the mechanisms associated with fat distribution and the development of metabolic diseases in obesity.^{6,7,8,9}

Endocrine functions of adipose tissue

Adipose tissue is a highly active metabolic and endocrine organ. Adipocytes express and secrete various types of bioactive molecules, well known as adipokines. The adipokines establish an important role of adipose tissue as an endocrine organ that is involved in the integration and coordination of a variety of biological processes including

energy metabolism, glucose and lipid metabolism, neuroendocrine function and immune function.^{9,10-12}

Adipokines can act in local (autocrine/paracrine) and systemic (endocrine) levels. The hormones leptin and adiponectin and acute phase response proteins including Serum Amyloid A1 (SAA) and Retinol Binding Protein 4 (RBP4), are produced in adipocytes. Tumor necrosis factor alpha (TNF- α), interleukin 6 (IL-6), monocyte chemoattractant protein 1 (MCP1), among others, are produced in non-adipocytes within the adipose tissue.^{9,10, 11-13}

Leptin and adiponectin play a role in appetite and satiety, fuel metabolism, innate immune function and reproduction. Leptin is a cytokine that provides signals to the organism regarding the energy status, especially fat stores, in the body. Leptin regulates appetite and satiety, food intake, energy expenditure and reproduction.^{12,14} Adiponectin plays a role as an insulin sensitizer predominantly in the liver and has anti-inflammatory and anti-apoptotic actions, so is anti-atherogenic.^{12,15,16,19} The proinflammatory cytokines, TNF α and IL-6, have paracrine effects on adipocyte metabolism and endocrine function. Both may link obesity and insulin resistance.^{13,17-19} IL-6 can impair insulin action on target tissues, by decreasing the insulin effects on glucose uptake into skeletal muscle and hepatic glucose output.¹³

Regulation of adipokine expression.

Leptin levels are variable depending on sex, depot and positively associated with fat mass and percentage of body fat, body mass index (BMI) and circulating inflammatory markers.^{12, 14, 34} Leptin levels are higher in women than men and increase with weight gain. Serum leptin is usually elevated in obesity within a wide range. Maffei et al, 1995, found that in humans at each BMI category, there was variability in plasma leptin levels suggesting that there are differences in its secretion rate from fat. He mentions, “leptin levels secretion has different mechanisms other than absolute fat mass per se”.³⁵

Serum adiponectin levels are decreased in obesity and inversely correlated to systemic oxidative stress. Reactive oxygen species (ROS) exposure in adipocytes suppresses adiponectin mRNA expression and secretion and increases the mRNA expression of pro-inflammatory cytokines, IL-6 and MCP-1. Oxidative stress may play a role in reducing adiponectin levels production that can contribute to obesity-associated disease pathogenesis.²¹

In obesity, oxidative stress contributes to a vicious cycle that promotes increased inflammation in adipose tissue. Macrophage infiltration into adipose tissue (AT) increases the production of inflammatory cytokines. Cell culture studies show that inflammatory cytokines, possibly through ROS production, may decrease expression of adiponectin and therefore are hypothesized to contribute to the lower adiponectin expression in obesity.

Depot differences in the endocrine function

Subcutaneous and visceral adipose tissue depots are well characterized due to their differential contribution to metabolic diseases.^{6,9} Adipose tissue depots present unique adipokine expression and secretion profiles. Visceral adipose tissue is associated with increased risk of metabolic diseases due to the differences in the endocrine function among adipose tissue depots.

Adipocyte size varies between abdominal subcutaneous and visceral adipose tissue. During weight gain adipocytes can be generated more rapidly in some depots: Abdsc fat provides more long-term nutrient storage because it increases in fat cell number first and then in fat cell size. Omental depot increases in fat cell size rather than number. Also, the number of macrophages in omental is higher compared to subcutaneous adipose tissue, being one of the reasons why there is an increased production of cytokines from this depot but other stromal cells may also contribute.

(Table 1)

The levels of proinflammatory cytokines and anti-inflammatory cytokines are variable depending on fat depot and obesity. It is well known that proinflammatory cytokines, such as tumor necrosis factor (TNF- α) and IL-6 are secreted at higher levels while anti-inflammatory cytokines, like adiponectin and IL-10, are lower in obese vs lean individuals, and in visceral compared to subcutaneous depot.¹³ A study by *Fontana et al (2007)* determined arteriovenous concentration differences across visceral fat, by obtaining portal vein and radial artery blood samples.⁴⁶ They concluded that IL-6

concentration in omental adipose tissue is an important site for IL-6 secretion. These data measured *in vivo* by ELISA in Abdsc and omental are more physiologically relevant compared to other studies that measure secretion of IL-6 in longer time ex vivo incubations.⁴⁶

Subcutaneous adipocytes secrete higher levels of leptin and present higher levels of leptin mRNA compared to omental adipocytes.³⁴ *Russel et al (1998)* investigated leptin secretion in incubation medium (incubated in M199 + 1% BSA for 3 hr) of omental and Abdsc fat in severely obese women and men. Leptin secretion was measured with RIA and was 2 fold higher in subcutaneous than omental tissue expressed as ng/ 10⁶ cells/3hr.^{34,44} As well in other study, *Harmelen et al (1998)* analyzed leptin secretion (ng/g/2hr incubation) in Abdsc and omental fat in obese and nonobese subjects. They found that leptin secretion were 2-3 times higher in subcutaneous than omental and per fat cell and the values were positively correlated with BMI.⁴⁵

The secretion of adiponectin by fat depot, visceral and subcutaneous, presents inconsistent results and is unclear.¹⁶ The study by *Kovacova et al*, showed no significant difference in the secretion of total adiponectin in omental and abdominal subcutaneous adipose tissue explants (4 hr incubation in Krebs/Ringer phosphate buffer with BSA) measured by Western Blot.³¹ *Drolet et al*, measured total adiponectin release in Abdsc and omental in 2 hour incubation (Krebs/Ringer/Henseleit buffer) expressed in ng/ mg lipids and showed not significantly difference between depots.³⁵ *Motoshima et al*, showed that in conditioned media samples for 12 and 24 hr basal adiponectin secretion was not significantly different between omental and Abdsc fat cells.³⁶ The study by *Perrini et al*,

determined total adiponectin releases in human omental and subcutaneous fat (incubated for 2h) and concluded that omental release higher adiponectin levels than subcutaneous, while adiponectin mRNA expression was higher in subcutaneous than omental by quantitative real-time PCR.³⁷ In the study by *Swensson et al*, total adiponectin release measured by ELISA was higher in Abdsc than omental at different time points of incubation in M199-1% BSA (2h, 4h, 6h)⁴⁷.

Since the data on total adiponectin secretion are inconsistent, this study focuses in the secretion of adipokines in subcutaneous vs omental fat. In addition, there is no report on the depot differences in the release of adiponectin multimers. Thus, we also measured HMW, MMW, and LMW adiponectin levels with quantitative Western blotting. It is known that the expression of adiponectin is regulated by proinflammatory cytokines, TNF- α and IL-6, which suppress adiponectin production. In obesity adiponectin production are downregulated and proinflammatory cytokines are upregulated.^{17,30} IL-6 secretion rates as well as pJNK levels in adipose tissue were determined to assess the inflammatory states.

Table 1. Depot differences in adipokine production

Adipokine	Depot	Sex differences	Obesity	Reference
Leptin	Subcutaneous > omental	Women > men	Increase	Russell et al (1998) Harmelen et al (1998)
Adiponectin	Subcutaneous > omental	Women > men	Decrease	Swensson et al
	No differences between depots			Motoshima et al (2002) Kovacova et al (2011) Drolet et al (2009)
	Omental > Subcutaneous			Perrini et al (2007)
IL-6	Visceral > subcutaneous		Increase	Fontana et al (2007)

Adiponectin production is downregulated in obesity

Adiponectin is an endocrine factor released exclusively by mature adipocytes and highly secreted by adipose tissue.^{12,15,31,32} In obesity, the circulating levels of adiponectin are decreased. It is well known that adiponectin plays a role as insulin sensitizing predominantly in the liver, has anti-inflammatory, anti-apoptotic protein on various different cell types and is anti-atherogenic.^{12,15,16}

The native adiponectin exist as low-molecular weight trimmers (LMW), middle-molecular weight hexamers (MMM) and high molecular weight multimers (HMW), which are composed by 12-18 monomers.^{15,31-33} The formation of MMM and HMW is favored by the lumen of endoplasmic reticulum (ER) by the disulfide bonds formation between the trimmers. These three are the major forms that circulate in plasma.^{16,32} The adipocytes are the major determining factor that influences the production and secretion of the isoforms.³³

The potency of adiponectin is linked to high molecular weight (HMW) isoform. The major biological effects of HMW are on liver, muscle and endothelium.^{15, 32} HMW isoform levels in plasma and the ratio of HMW to total adiponectin are associated with increased glucose tolerance and insulin sensitivity rather than total adiponectin, suggesting that HMW is the active form of the protein.^{15, 16, 32}

Total and HMW adiponectin levels are higher in women compared to men. Meyer et al, found that serum total and HMW adiponectin do not differ significantly between sexes but there is a tendency for being higher in females. Levels of both are decreased in obesity, which is related to conditions associated with insulin resistance, hyperlipidemia type 2 diabetes, atherosclerosis, nonalcoholic steatohepatitis (NASH) and metabolic disorders.^{12,16,31, 32}

It has been previously shown that insulin-resistant obese subjects present low HMW and LMW levels compared to insulin-sensitive lean subjects. Adipocyte size affects the secretion of adiponectin being meaningful to the metabolic and hormonal function of adipose tissue.¹⁶

Adiporedoxin, a novel protein that may regulate adipokine secretion

Adiporedoxin is a protein expressed at high levels in fat cells. The protein is present in the adipocyte endoplasmic reticulum and was found by Paul F. Pilch and his colleagues.^{38,39} Adiporedoxin is a member of the peroxiredoxin (Prx) family, redox-regulated proteins, that its nominative activity is reduction of peroxides in various cell types.³⁸ In previous studies, adiporedoxin has been reported as peroxiredoxin-like 2 protein (PAMM) with the function of a redox regulatory protein in bone marrow monocyte that modulated osteoclast differentiation *in vitro*.⁴⁰

Adiporedoxin and other proteins similar to Adrx are expressed in the ER. Alterations in adiporedoxin levels can affect proteins that contain disulfide bond such as adiponectin.³⁸ *In vitro*, higher expression of adiporedoxin increases the secretion of adiponectin and vice versa.^{39,40}

One study in mice found that adiporedoxin knockout (KO) mice present with reduced levels of circulating HMW adiponectin, increased intracellular adiponectin retention and normal glucose tolerance, mild hyperinsulinemia.³⁹ When KO mice were fed on high fat diet for 6 weeks, they develop glucose intolerance/insulin resistance and present a further decrease in HMW adiponectin levels. KO mice were found to be free of fibrosis, apparently due to impaired collagen secretion. Wild type mice fed in a longer period of high fat diet showed decreased cellular adiporedoxin, and lower HMW adiponectin levels.^{39,40}

Data from human preadipocytes show that during differentiation, adiporedoxin mRNA and protein are upregulated.³⁹ It is shown in preliminary data in humans that

mRNA for adiporedoxin and adiponectin, and protein expression for adiporedoxin and adiponectin are positively correlated in both sexes, independent of BMI, which has an independent negative influence on adiponectin expression.⁴¹

Obese subjects present variation in the magnitude of inflammation of adipose tissues and insulin resistance that may affect adiporedoxin levels. In response to inflammation and to metabolic status of the adipose tissue in obesity, Adrx levels are down regulated, consequently decreasing adiponectin levels. A lower level of adiponectin in obesity may be one of various factors, which remain unknown.⁴¹

Summary and Goals of the thesis:

Studies by the Pilch and Fried labs indicate that Adrx is an important regulator of adipokine secretion. Data from cell culture models shows that knockdown of Adrx in cultured human adipocytes decreases and overexpression increases the secretion of adiponectin. However, no information was available on possible depot differences in Adrx expression, and whether it is associated with changes in adipokine release in intact human adipose tissue. Although studies document differences in the metabolism and endocrine function of Abdsc and Om depots of humans, data on adiponectin release are inconsistent and no previous studies measured the secretion of different adiponectin isoforms.

Therefore the goals of the thesis were to:

1. Determine the fat depot differences in adipokine release (adiponectin (total and HMW), leptin and IL-6) during short-term incubations of human Abdsc versus omental adipose tissues from obese subjects.
2. Determine if Adrx expression differs between human Abdsc versus omental sample of obese and morbidly obese subjects, mostly females.
3. Determine the correlation between Adrx protein expression and adipokine release from human Abdsc and omental adipose tissue.
4. Determine the relationship between Adrx protein expression and circulating serum levels of adipokines, primarily of total and HMW adiponectin.

METHODS AND PROCEDURES

Subjects

Participants were recruited from subjects undergoing elective surgery, such as laparoscopic sleeve gastrectomy, gastric bypass laparoscopic, nephrectomy donor laparoscopic and laparoscopic cholecystectomy. Adipose tissue samples were obtained from intra-abdominal (omental and mesenteric) and subcutaneous abdominal fat of a total of 44 samples from 35 females and 9 males that were selected for this study due to the availability of acute conditioned media samples, blood samples and fat tissue for protein expression.

Each participant provided written informed consent. The study was approved by the Institutional Review Board of the Boston University School of Medicine (Nutritional Regulation of Leptin Production, IRB Number H-28139). Information on the height and weight of volunteers, medications, medical conditions and serum levels of glucose, HbA1c, blood pressure were obtained from the medical records and recorded in lab records with a code number.

Body Measurements

On the day of the surgery, participants were given a consent form. Body measurements were obtained from the chart, weight and height, which were used to determine the body mass index (BMI) calculated as weight in kilograms divided by height in meters squared.

Blood Sampling

Blood samples were taken on the day of the surgery in the overnight fasting state. Serum was separated with centrifugation for 15 min at 2500 rpm and 4 °C and stored at -80 °C for further analyses. Serum samples were used to measure glucose and insulin levels, and adipokines: leptin, total adiponectin and HMW adiponectin.

Adipose Tissue Samples

Fat tissue, Abdsc and omental, was excised in the operating room, and portions were immediately frozen in the operating room in liquid nitrogen or transferred to the lab in room temperature in media 199. Frozen tissue was stored at -80 °C for later use.

Measurement of Adipokine Secretion

Adipokine Secretion

Upon arrival in the laboratory, within 30 minutes of excision, adipose tissue samples, transferred in M199 were minced into 5 to 10 mg fragments with surgical grade scissors, rinsed with PBS, and incubated in M199 with 1% BSA for 3 hours with shaking 100 rpm at 37°C in duplicates (~100 mg in 1.5 ml incubation media). After incubation, the media samples were collected and stored in aliquots at -80 °C for further analyses for adipokine secretion such as leptin, total adiponectin and IL-6.

Acute Media Analyses

Leptin, IL-6 and adiponectin concentration was measured in acute media from adipose tissue using an enzyme-linked immunosorbent assay (ELISA). Standard and acute media measurements were performed in duplicate. A non-linear fit curve of the adipokine standards was performed to recalculate the adipokine concentration values using the Graphpad Prism 5.0b software.

To measure samples within the range of the standard (Leptin: 0.5–1.5 ng/mL; adiponectin: 0.5–3 ng/mL, IL-6: 0.18-3 ng/ml) Abdsc and omental samples were used, 1 and 1/2 dilution for leptin, 1/50 and 1/100 for adiponectin, 1/1.25 dilution and 1/2.5 in Abdsc and 1/2 and 1/4 dilution in omental for IL-6 quantification.

In acute samples the intra-assay coefficient of variation values were $9.0 \pm 0.7\%$ for IL6 and $9 \pm 1.5\%$ for leptin. Adiponectin intra-assay coefficient of variation values were $2 \pm 0.5\%$; $7 \pm 2.3\%$; and inter-assay coefficient of variation values were $5 \pm 1.4\%$.

Acute media leptin levels:

For detection of natural and Recombinant Human Leptin in serum, the samples were analyzed with DuoSet ELISA Development kit (Catalog # DY398 from R&D Systems) and the manufacturer's instructions were followed (DuoSet ELISA, R&D Systems Inc, Minneapolis, MN).

Acute media total adiponectin and HMW levels:

For detection of Human Total Adiponectin/Acrp30, acute samples were analyzed with Human Total Adiponectin/Acrp30 Quantikine ELISA Kit (Catalog DRP300 R&D Systems, Minneapolis, USA).

For acute media Western Blot analysis was also performed to measure adiponectin multimers (description below).

Acute media IL-6 levels:

For detection of Human IL-6, the samples were analyzed with DuoSet ELISA Human IL-6 (Catalog DY206 R+D Systems, Minneapolis, USA).

Serum Hormone Measurements

Standard and serum analysis measurements were performed in duplicates.

Serum insulin levels:

For detection of serum insulin concentration, the serum samples were analyzed by Ultrasensitive Insulin Enzyme-linked immunosorbent assay (ELISA) kit for the quantitative determination of insulin in serum and plasma (Catalog 80-INSHU-E01.1) and following the manufacturer's instruction (ALPCO Diagnostics, Salem, NH).

Serum leptin levels:

For detection of human leptin in serum, the samples were analyzed with DuoSet ELISA Development kit (Catalog # DY398 from R&D Systems) and the manufacturer's instructions were followed (DuoSet ELISA, R&D Systems Inc, Minneapolis, MN).

Serum total adiponectin and HMW levels:

For detection of Human Total Adiponectin/Acrp30, serum samples were analyzed with Human Total Adiponectin/Acrp30 Quantikine ELISA Kit (Catalog DRP300 R&D Systems, Minneapolis, USA). For serum Western Blot analysis was also performed (description below).

A non-linear fit curve of the adipokine standards was performed to recalculate the adipokine concentration values using the Graphpad Prism 5.0b software.

To measure samples within the range of the standard (Leptin: 0.5–1.5 ng/mL; Adiponectin: 0.5–3 ng/mL, insulin ranges: 5 – 25 μ IU/mL) serum samples were diluted 1/100 for leptin, 1/5151 for adiponectin quantification and no dilution for insulin.

The intra-assay coefficient of variation values were $9.9\pm4\%$ for insulin, $6\pm3.9\%$ for adiponectin and for leptin serum intra-assay coefficient of variation values were $1.9\pm0.5\%$; $7.7\pm1.2\%$; and inter-assay coefficient of variation values were $4\pm0.9\%$.

Fasting glucose levels were determined by YSI 2300 STAT Plus Glucose & Lactate Analyzer. Precision as specified in YSI 2300 Stat Plus Specification Sheet is $\pm 2\%$ or 2.5 mg/L for glucose.⁴²

Western blot analysis of total adiponectin and HMW in serum and acute samples

Adiponectin, high, medium and low molecular weight isoforms were measured in serum and 3 hr secretion media using native PAGE electrophoresis. The samples were thawed on ice. 6% PAGE gels were prepared (Tris-Acetate (23.6 ml of ddH₂O, 10 ml of Tris-Acetate 1.5M, 6 ml of Acrylamide 40% 37:1, 400 µl of 10% APS and 48 µl of TEMED) and stacking solution 3% (7.94 ml ddH₂O, 0.78 ml of Acrylamide 40% 37:1, 0.78 ml Tris-acetate, 100 µl of 10% APS and 16 µl of TEMED).

Serum samples were diluted with PBS 100 times and mixed with native sample buffer (Life Technologies) and 10 µl were loaded per well. 30 µl acute media samples were mixed with 10 µl of native sample buffer and 20 µl of the mixture was loaded into each well. In each gel, control serum samples were loaded in 3 different amounts. Protein samples were resolved with electrophoresis in Tris-Glycine running buffer (25 mM Tris Base, 190 mM glycine) and transferred to PVDF membranes (3 hours at 40 V).

After transfer, membranes were blocked in 3% milk/ Tris Buffer Saline and 0.1% Tween 20 (TBS/T) for 1 hr at room temperature and incubated with anti-adiponectin antibody (1/5000 in 3% BSA in TBS/T, BD Bioscience, Catalog # 611644) overnight at 4 °C. The next day membranes were washed 3 times for 5 minutes in TBS/T and incubated with anti-mouse IgG, HRP-linked Antibody (1/6000, Cell Signaling Technologies, #7076) for 1 hour at room temperature. The membranes were washed three times with TBS/T, 5 minutes each wash and used for signal detection with a highly sensitive enhanced substrate for detecting horseradish peroxidase (HRP) on immunoblots, SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL).

Chemiluminescence images were captured using Luminescent Image Analyzer (LAS4000, Fuji) and band densities were quantitated using Multi Gauge Image software. Western Blot analysis in acute samples and serum samples were made in duplicate. The control was a pool of the samples that were run in each gel. The samples were normalized for each gel.

Western blot analysis of adiporedoxin protein expression in adipose tissue

Adipose tissue samples were homogenized with glass on glass tissue grinder (Kontes, Kimble Chase North America), in 3 times volume of lysis buffer (Cell Signaling Technology, Triton X-100 (polyethylene glycol octylphenol ether 1%, Danvers, MA) or (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na₃VO₄, 1 µg/ml leupeptin, catalog number 9803) with 15 strokes. Homogenates were centrifuged for 10 minutes, 14,000g at 4 °C and the supernatant below the fat cake was used for Western blotting. Supernatant were mixed with LDS sample buffer (Life Technologies) and denatured by boiling for 5 minutes.

For adiporedoxin measurement 10 µl of the sample was loaded in SDS-PAGE along with molecular weight markers. Gels were prepared as follows: 10% Tris-HCl (19.2 ml of ddH₂O, 10 ml of Tris-HCl 1.5M, 10 ml of Acrylamide 40% 29:1, 400 µl of 10% SDS, 400 µl of 10% APS and 48 µl of TEMED) and stacking solution 3% (7.3 ml ddH₂O, 1.25 ml of Acrylamide 40% 29:1, 1.26 ml Tris-HCl, 100 µl of 10% SDS, 100 µl of 10% APS

and 16 µl of TEMED). 3 different amounts of pooled adipose tissue lysates were run on each gel.

After resolving in Tris/Glycine/SDS running buffer, protein samples were transferred to PVDF membranes (60 V for 100 minutes at 4 °C). After transfer, membranes were blocked with 3% milk in TBS/T for 1 hour at room temperature. Membranes were then probed with the primary rabbit anti FAM213A antibody (1/1000, Sigma #HPA009025) and a loading control, HSP90 (1/2000, Santa Cruz Biotechnology, Cat sc-69703/ sc-101494) overnight at 4°C. After overnight incubation, membranes were washed with TBS/T 3 times and incubated with anti-rabbit IgG-HRP antibodies (1/2000, Cell Signaling Technologies, Cat #7074) for 1h at room temperature, followed by 3 times of washing in TBS/T and signal detection with SuperSignal West Femto Maximum Sensitivity Substrate for adiporedoxin or West Pico Maximum Sensitivity Substrate for HSP90 (Pierce Biotechnology, Rockford, IL). Chemiluminescence images were captured using Luminescent Image Analyzer (LAS4000, Fuji) and band densities were quantitated using Multi Gauge Image software. Adrx levels were presented relatively to HSP90. Western Blot analysis in Adrx protein levels was made once in each depot and if samples were not well expressed or if the membrane presented contamination samples were excluded. The control was a pool of the samples that were run in each gel. The samples were normalized for each gel.

Fat cell size and number calculation:

Adipocytes were isolated with collagenase digestion of adipose tissue [Type 1 collagenase (Worthington Biochemicals), 1 mg/ml in modified Krebs Henseleit Buffer (KHB), pH 7.4, containing 4% bovine serum albumin and 5 mM glucose] at 37 °C for 1 h. The digest was filtered through 250 micron mesh and adipocytes are washed by floatation or low speed centrifugation and then resuspended in buffer. Cells were sized after digital photography using imaging software (Image J, NIH). The mean fat cell volume was calculated according to Goldrick formula.⁴³ The mean fat cell weight was calculated as fat cell volume multiplied by the density of triglycerides (0.915 g/ml). The percentage of lipid in each fat depot was assessed by dividing lipid weight by wet tissue weight. Total fat cell number was calculated as depot weight multiplied by percentage of lipid and divided by the mean fat cell weight.

Statistical Analyses

Paired t-tests were conducted using JMP Pro 11.2.1 to assess differences in the expression of adiporedoxin and adipokine secretion in human omental (Om) and abdominal subcutaneous (Abdsc) adipose tissues of this study. For the correlation between adiporedoxin (Adrx) and adipose tissue and serum adipokines, a bivariate correlation was used. A p-value less than 0.05 was considered to be statistically significant. The differences between depots were calculated in each paired samples and the average of all the samples was the differences between each depot in %.

RESULTS

Subject characteristics

Forty-four samples were analyzed for the study, 80% were females (n=35) and 20% (n=9) males. The study included 7 (F6 M1) African Americans, 24 (F19, M 5) Caucasian, and 13 (F10, M3) Hispanics (Table 2).

20% from all subjects were classified as diabetic (9 subjects, 5 F: 4 M). Of the diabetic subjects, 4 were classified as ‘treated’ based on medications listed in the medical record and 4 of them were not taking any medication for diabetes at the time of surgery. HbA1C data were taken from the medical record prior to the surgery. The characteristics of these subjects are shown in Table 3. In 2 subjects, there was no blood sample available and insulin and glucose could not be determined. All the subjects were included in the analysis since excluding the diabetic subjects had no effect on the analysis. Subject 1 and 9 are the same subject that had the same surgery 2 times in less than one year. In between the subject did loose weight, improved HbA1C and had new medications. The subject were included as two different persons.(Table 3).

Table 2. Subject characteristics

	n	Mean ± SD	Range (av. range)	SEM
Age (years)	44	39.8± 11.7	20-69 (49)	1.76
BMI (kg/m²)	44	42.1± 6.8	25.6-57.5 (31.8)	1.02
Glucose (mg/dl)	31	98.8± 50.1	70.1-341 (270.9)	9.0
Insulin (μU/ml)	31	8.5± 4.12	1.0-19.0 (18.0)	0.73
HbA1C (%)	43	5.9± 1.5	4.6-12.6 (8.0)	0.22

Table 3. Characteristics of diabetic subjects

Age years	Sex	Race	BMI kg/m ²	DM	Glu mg/dl	INS μ IU/ml	HbA1C %	MEDICATION
55	M	CAU	52.3	DM	178	4.5	11.3	N/A
40	F	CAU	34.3	DM	151	13.5	7.6	Metformin, Drisdol
45	F	HIS	41.5	DM	96.4	12.9	5.9	Aspirin, Lisinopril, Toprol, Pravastatin, Amoxicillin, Biaxin
61	F	AA	38.8	DM	99	9.4	6.5	Drisdol, Lancets
35	F	HIS	39.9	DM	81.2	1.7	5.2	Ranitidine, Ursodiol
40	M	HIS	43.7	DM	94.7	7.6	6.5	Percocet
43	F	CAU	43.6	DM	N/A	N/A	6.8	Glucophage, Lantus, BD pen needle, Victoza, Phentemine
40	M	HIS	43.5	DM	341	10.1	12.6	Lisinopril, Simvastatin, Glucotrol, Januvia, Gabapentin, Metoprolol, Diclofenac, Metformin, Adipex-P, Amoxicillin, Levaquin, Omeprazole
56	M	CAU	38.4	DM	N/A	N/A	6.8	Allopurinol, omeprazole, carvedil, prozac, oxycodone, carafati

Abbreviations: DM (Diabetes Mellitus), Glu (Glucose), INS (Insulin)

Note: Subject 1 and 9 are same subject that had the same surgery 2 times in one year.

Adipokine secretion from human omental and Abdsc adipose tissues

Higher leptin secretion in Abdsc than omental adipose tissues

The rates of adipokine secretion were measured by incubating adipose tissue fragments in M199+1% BSA for 3 hours using fresh obtained tissue (within 30min of excision).

Leptin levels in the incubation media was measured by ELISA (n=43). As expected, leptin secretion from Abdsc, expressed as nanogram per gram of tissue for 3 hr incubation, was ~134% higher compared to omental adipose tissue (Figure 1A). Because fat cell size is known to affect leptin secretion and is lower in omental than Abdsc, we also calculated leptin release per cell. Leptin secretion, expressed as ng/million fat cells/3h, was 3 fold higher in Abdsc than omental adipose tissue (Figure 1B).

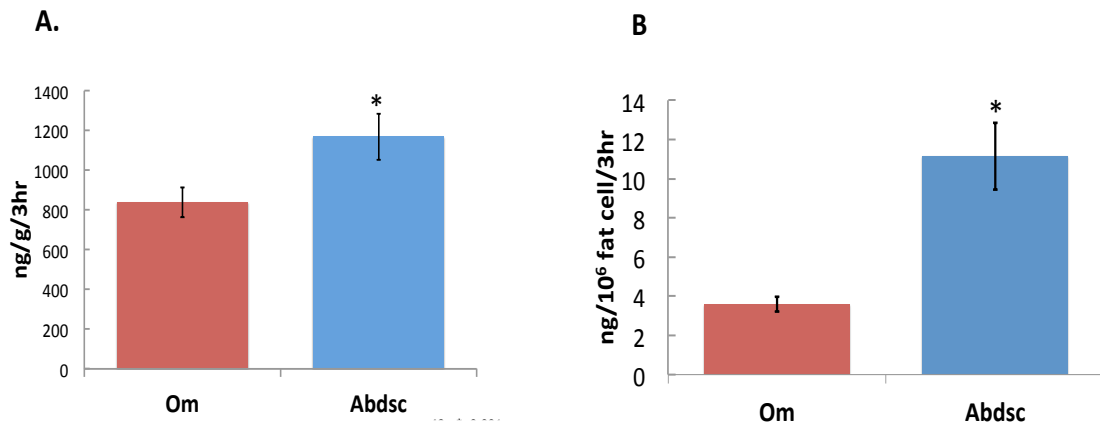


Figure 1. A. Leptin release (ng/g/3hr) from forty-three paired samples in omental (Om) and abdominal subcutaneous (Abdsc). **B.** Leptin release (ng/10⁶ fat cell/3hr) from 22 paired samples in omental and Abdsc. Fragments of adipose tissue were incubated for 3hr in M199+1% BSA pH 7.4 under 95% of Oxygen and 5% CO₂. Leptin levels were determined with ELISA. *, p<0.05 Om vs. Abdsc with paired t test.

Higher Total adiponectin secretion in Abdsc than omental adipose tissues

Total adiponectin release during the 3h incubation, expressed as nanogram per gram of tissue for 3 hr incubation was ~55% higher in Abdsc compared to omental adipose tissue. Because adiponectin expression is affected by fat cell size and fat cell size is different between Om and Abdsc, we calculated adiponectin release per cell in each depot. When adiponectin release was calculated per million fat cells, it was 1.8 fold higher in Abdsc than omental with $p < 0.001$ (Figure 2).

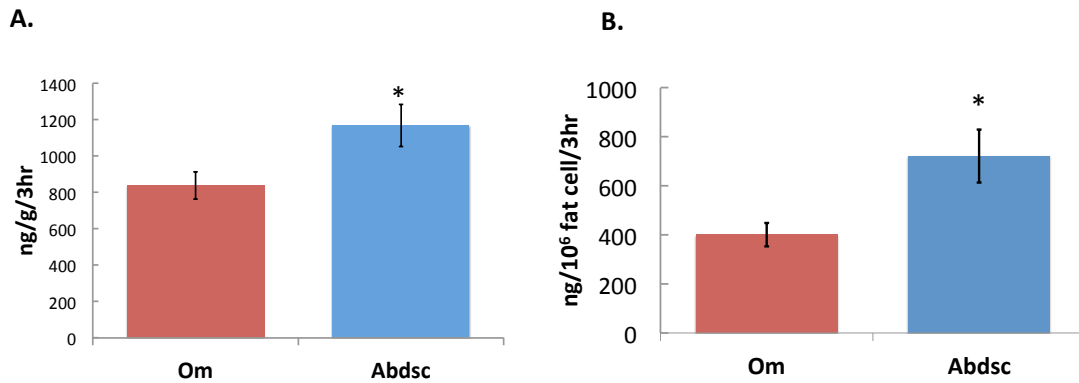


Figure 2. A. Total adiponectin release (ng/g/3hr) from forty-three paired samples in omental and abdominal subcutaneous adipose tissue. **B.** Total adiponectin release, expressed as ng/10⁶ fat cell/3hr, from 22 paired samples in omental and Abdsc. Fragments of adipose tissue were incubated for 3hr in M199+1% BSA pH 7.4 under 95% of Oxygen and 5% CO₂ and adiponectin levels were measured with ELISA. *, $p < 0.05$ Om vs. Abdsc with paired t test.

Western analysis of the secretion of adiponectin multimers from Om and Abdsc adipose tissues

Adiponectin circulates in multiple isoforms, high, medium and low molecular weight. The HMW form is of most interest because it is the most active, and its assembly requires intermolecular disulfide bonds and would therefore be expected to be impaired in adipose tissue with higher levels of inflammation. We therefore measured the release of adiponectin isoforms with Western Blotting in the same acute incubation samples (n=43). Total adiponectin, the sum of HMW, MMW and LMW, is significantly higher, by ~19%, in Abdsc (Figure 3A), similar to the results measured with ELISA. The secretion of HMW adiponectin isoform, however, is ~29% higher in omental than Abdsc with a p value of 0.008 (Figure 3B). Percent of HMW/total adiponectin secreted was also higher in omental by ~30% than Abdsc ($p < 0.001$) (Figure 3C). Representative images of native Western blotting are shown in Figure 4.

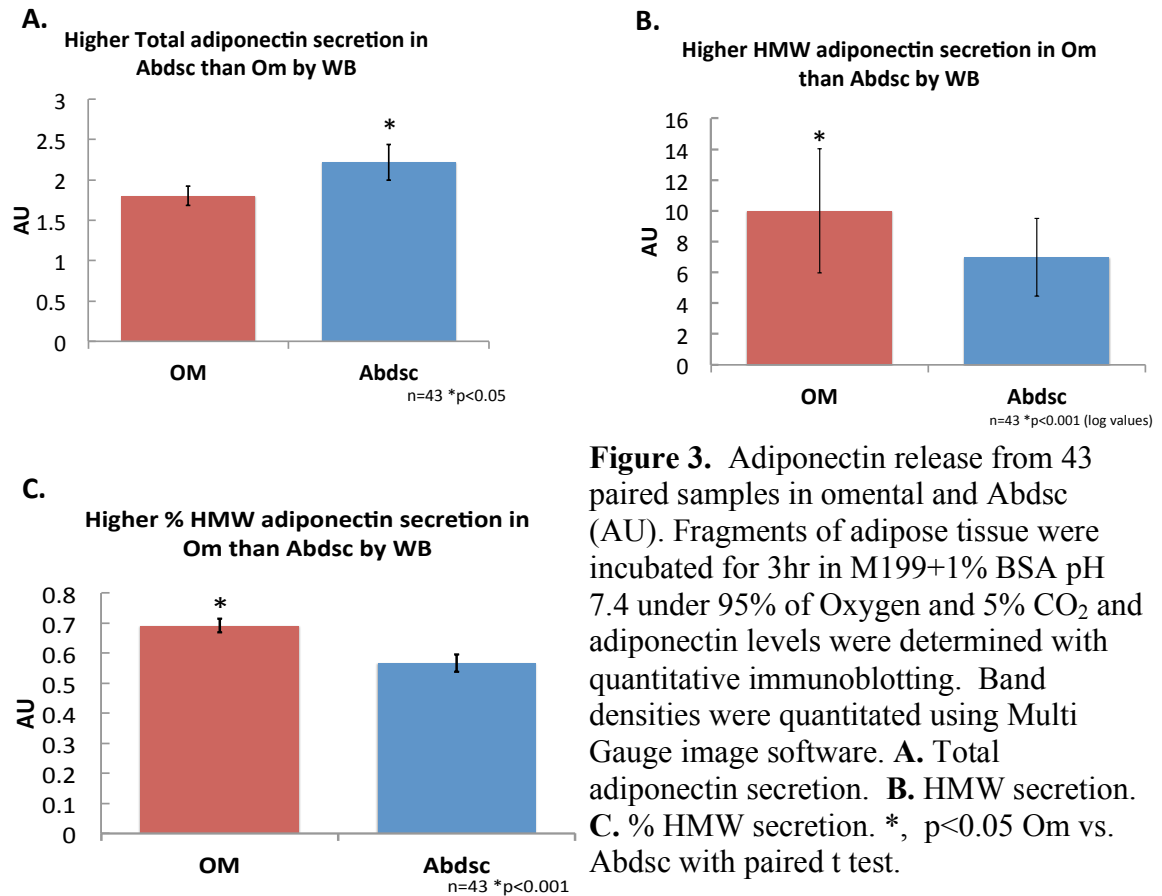


Figure 3. Adiponectin release from 43 paired samples in omental and Abdsc (AU). Fragments of adipose tissue were incubated for 3hr in M199+1% BSA pH 7.4 under 95% of Oxygen and 5% CO₂ and adiponectin levels were determined with quantitative immunoblotting. Band densities were quantitated using Multi Gauge image software. **A.** Total adiponectin secretion. **B.** HMW secretion. **C.** % HMW secretion. *, p<0.05 Om vs. Abdsc with paired t test.

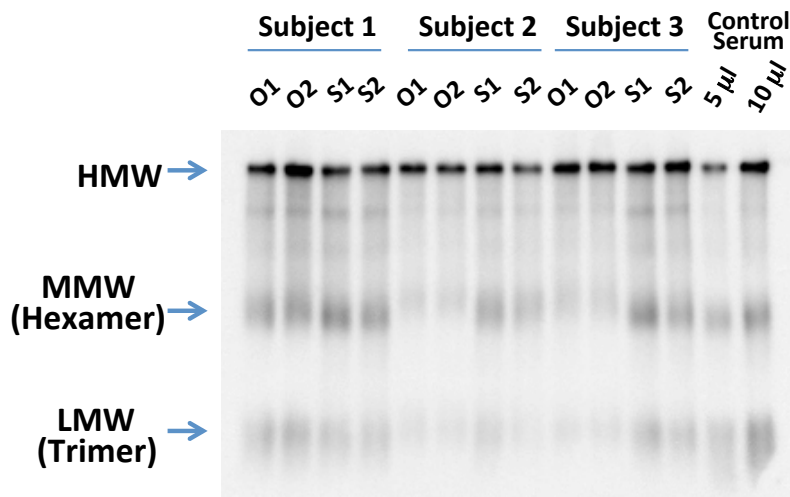


Figure 4: Representative images of Western blotting Native PAGE gels 3 different subjects for HMW, MMW and LMW adiponectin levels. Abbreviations: O (Om) S (Abdsc)

Higher IL-6 secretion in Om than Abdsc adipose tissues

Because IL-6 production by macrophages and other cell types within adipose tissue is a marker for inflammation, and inflammation is known to affect adipokine expression and release, we measured IL-6 secretion in the acute media. IL-6 secretion was ~56% higher in omental compared to Abdsc ($p=0.03$, paired t test on log transformed values, Figure 5), as expected.

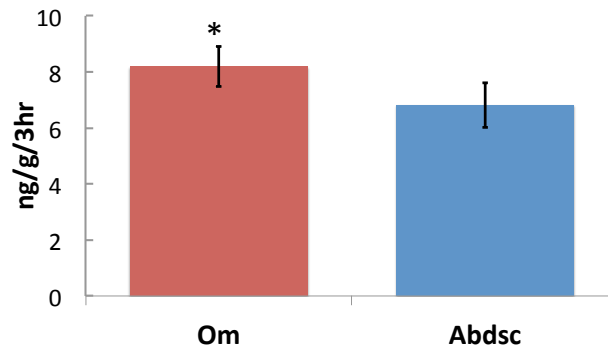


Figure 5. IL-6 release (ng/g/3hr) from forty-three paired samples in omental and abdominal subcutaneous. Fragments of adipose tissue were incubated for 3hr in M199+1% BSA pH 7.4 under 95% of Oxygen and 5% CO₂. IL-6 levels were determined with ELISA. *, $p<0.05$ Om vs. Abdsc with paired t test.

Higher expression of Adiporedoxin in Abdsc than omental adipose tissues

To test whether Adrx expression levels are related with adipokine secretion, we determined its protein levels in nineteen paired samples from omental and Abdsc adipose tissues with immunoblotting [mean BMI 43, $n=19$ (2 M, 16F), 6 with diabetes; 5F, 1M]. Adrx protein bands were quantified and relative expression levels were calculated using heat shock protein 90 (HSP 90) as a loading control. Adrx protein levels are 1.4 fold higher in Abdsc compared to omental with a p value <0.05 analyzed with paired t-test

(Figure 6A). Phospho-JNK levels were also measured as a marker of tissue inflammation and were higher in omental than Abdsc, similar to higher IL-6 expression in the omental depot (Figure 6B).

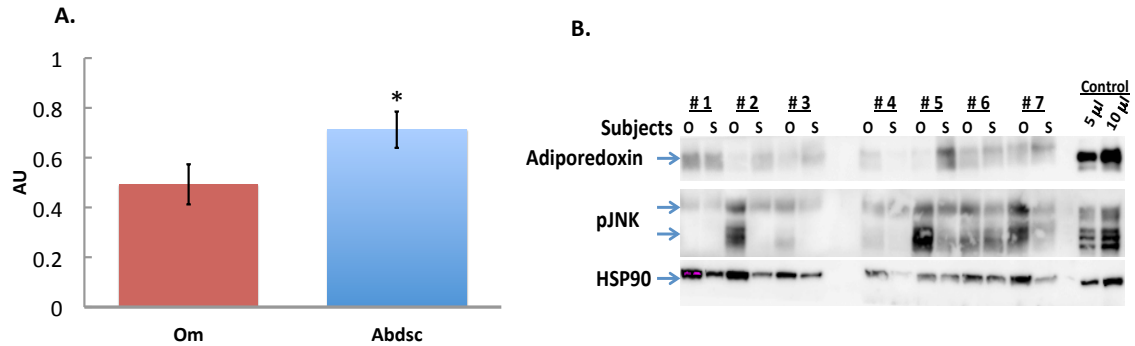


Figure 6. Adrx levels (AU) from nineteen paired samples in omental and Abdsc human adipose tissue. **A.** Adrx levels were measured by Western Blot and the band densities were quantitated using Multi Gauge image software and Adrx expression is presented as relative to HSP90, a loading control. The data are presented in bars indicating the mean values for each nineteen set of samples. *, $P < 0.05$. **B.** Representative images of Western blotting from 7 subjects. HSP90, a loading control. Expression levels of phospho-JNK are also measured as a marker of tissue inflammatory status. Abbreviations: O (Om) S (Abdsc)

Correlation of Adiporedoxin (Adrx) with adipokine release in adipose tissues

The protein expression levels of Abdsc and omental Adrx were not correlated to the releases of leptin and IL-6 release in either depot. Total adiponectin release, measured by ELISA, were not correlated with Adrx protein levels in both depots with a $p = 0.3781$ in Abdsc and $p = 0.3829$ in Om.

Total adiponectin measured by quantitative Western blotting tend to be positively correlated with Adrx protein expression levels in Abdsc and but not in Om ($n = 19$: $p = 0.0787$ in Abdsc and $p = 0.1982$ in Om). There was one outlier for total adiponectin, identified with a box plot. When the outlier was excluded, the correlation between Adrx

protein expression and adiponectin secretion in Abdsc was statistically significant ($r^2=0.23$, $p=0.044$). HMW adiponectin secretion was positively correlated with Adrx protein levels in Abdsc and there was a positive trend in Om adipose tissues ($n=19$: $p=0.0136$ in Abdsc and $p=0.069$ in Om). % HMW adiponectin secreted and Adrx levels were negatively correlated in Abdsc ($p=0.0173$) but not in Om ($p=0.846$) (Table 4 and Figure 7).

Table 4. Correlation of Adrx protein levels with adipokine release in Abdsc and Om in human adipose tissues.

Adrx (AU) n=19	Abdsc		Om	
	r^2	p	r^2	p
Leptin (ng/g/3hr)	0.0056	0.75	0.04	0.376
Total Adiponectin (ng/g/3hr)	0.0459	0.3781	0.045	0.3829
IL-6 (ng/g/3hr)	0.08	0.2273	0.043	0.3901
Total adiponectin (AU)	0.1706	0.0787	0.095	0.1982
HMW adiponectin (AU)	0.308	0.0136*	0.17	0.069
% HMW adiponectin	0.29	0.0173*	0.002	0.846

Simple bivariate correlation of Adrx levels in Abdsc and Om ($n=19$) with adipokines in both depots. P values are consider statistical significant with a $p < 0.05$

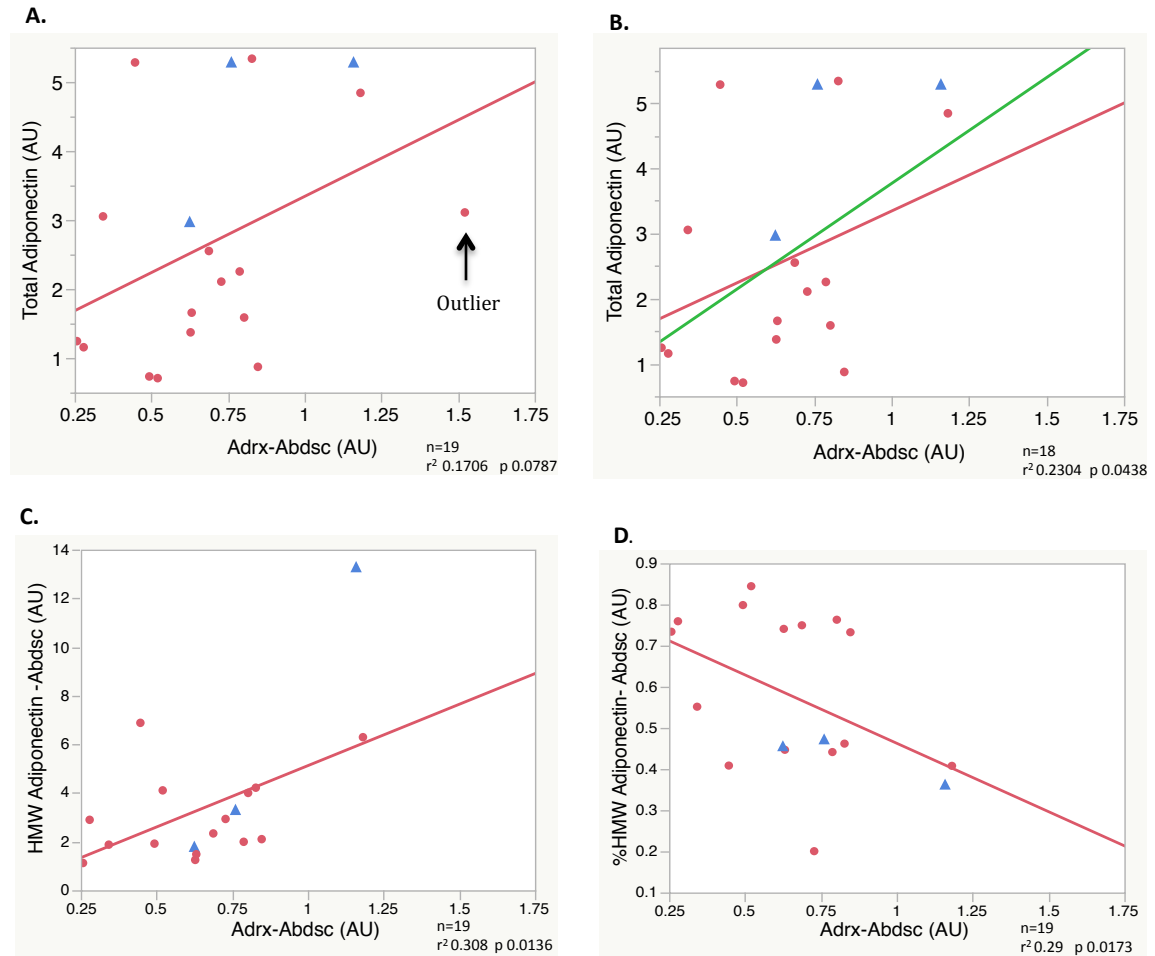


Figure. 7: Correlation of Adrx protein levels in Abdsce and the secretion in Abdsce of total (the sum of HMW+MMW+LMW), HMW and % of HMW (HMW/total adiponectin) levels determined with Native PAGE **A. Correlation between** total adiponectin secretion and Adrx protein expression levels in Abdsce **B. Correlation between** total adiponectin release and Adrx expression levels in Abdsce with adjusted line (AU) excluding one outlier (green line) **C. Correlation between** HMW adiponectin secretion and Adrx protein expression in Abdsce **D. Correlation between** % of HMW (HMW/total adiponectin) and Adrx expression levels in Abdsce tissue. Red dots = females, blue triangle = males.

Correlation of Adrx expression levels in adipose tissues and serum adipokines levels

Adrx protein levels in Abdsce, not omental, adipose tissue tended to be negatively correlated with serum leptin levels. Total serum adiponectin levels, measured by ELISA

were not correlated with Adrx protein levels in Abdsc, while tend to be positively correlated in Om (p=0.1116). Total serum adiponectin, HMW and % HMW levels, determined by native Western blotting, were not correlated with Adrx protein levels in both Abdsc and Om human adipose tissues (Table 5)

Table 5. Correlation of Adrx protein levels with serum adipokine levels.

Adrx (AU) n=11	Abdsc		Om	
	r ²	p	r ²	p
Leptin (ng/ml)	0.32	0.0672	0.006	0.8191
Total Adiponectin (ng/ml)	0.16	0.2216	0.2568	0.1116
Total adiponectin (AU)	0.222	0.6615	0.095	0.2561
HMW adiponectin (AU)	0.037	0.5673	0.1486	0.2415
% HMW adiponectin	0.028	0.6214	0.026	0.6336

Simple bivariate correlation of Adrx protein levels in Abdsc and Om (n=11) with serum adipokine levels. P values are consider statistical significant with a p <0.05.

DISCUSSION

Depot differences in Adrx protein expression levels

It was hypothesized that Adrx, a protein highly enriched in adipocytes and ER resident that is likely to facilitate protein assembly and secretion would be decreased in omental depot compared to Abdsc. Work from our lab (KK, MJL, SKF) showed that Adrx mRNA and protein levels in human Abdsc adipose tissue of young, healthy subjects, ranging in level of obesity, correlated positively with adiponectin mRNA and protein, and negatively with adipose tissue inflammation (as indicated by phospho-Jun kinase) in a statistical model in which BMI was accounted for.³⁹ In the current study of nineteen paired samples of Om and Abdsc from obese men and women, we found that Adrx protein expression was 1.4 fold higher in Abdsc than omental adipose tissues. Adrx protein expression in Om may be lower than Abdsc, due to higher inflammation and higher oxidative stress, as it was previously show.^{39,46} Accordingly, we found higher pJNK, a marker of tissue inflammation; in omental than Abdsc, but the results was not quantificated. 6 subjects were included and 1 was underloaded.

The release of adipokines, leptin, adiponectin and IL-6 is known to be different between the two depots although reports on adiponectin releases are inconsistent.^{31,35-37} Consistent with other results in the literature^{34,44,45}, leptin release was higher in Abdsc than omental while IL-6 was higher in omental than Abdsc. We measured the release in short term incubation (3hr) of freshly obtained adipose tissue within 30 min of excision because it is likely to reflect the in vivo characteristics of the subjects, in contrast to longer-term cultures. We also measured actual the release of adipokines rather than

mRNA expression or release in long-term incubations. In addition, we also expressed the release of leptin and adiponectin per cell number as well as gram tissue since leptin and adiponectin is produced by fat cells and the sizes of fat cells are different between adipose depots and known to affect adipokine secretion. We wanted to determine the adipokine secretion per fat cell to whether determine if the results from adipokine secretion per tissue weight were similar when it was measured with per fat cell. The results were attenuated. Leptin and total adiponectin were higher, in Abdsc than Om by 3 fold and 1.8, respectively.

The present study demonstrated that human adiponectin secretion is higher in Abdsc than Om adipose tissue. This finding is in contrast with previous studies by *Kovacova et al*, measured total adiponectin in adipose tissue explants incubated for 4hr, *Drolet et al*, measured total adiponectin in Abdsc and Om incubated for 2 hrs and the results were expressed as ng/mg lipids and *Motoshima et al*, measured total adiponectin in subcutaneous and Om incubated for 24 and 48 hrs. These three studies showed no depot difference in total adiponectin secretion in adipose tissue. However, *Swensson et al* showed significantly lower total adiponectin secretion in omental adipose tissue than Abdsc with short-time incubation (2, 4 and 6 hr). Thus, the present study confirms and strengthens the findings that omental secretes lower total adiponectin secretion compared to Abdsc. In addition, we measured adiponectin levels using both ELISA and quantitative native Western blotting, and both methods results in similar data, i.e. total adiponectin secretion is higher in Abdsc than omental adipose tissue.

Adiponectin circulates in multiple isoforms, HMW, MMW, and LMW and HMW adiponectin correlates better with insulin sensitivity in vivo.^{15, 16, 32} No previous studies however, examined the fat depot differences in the releases of adiponectin isoforms in human Abdsc versus omental adipose tissues. Using native polyacrylamide gel electrophoresis and quantitative immunoblotting, we compared the secretion of adiponectin multimers between the two depots. Similar to ELISA data, total adiponectin secretion was higher in Abdsc than omental adipose tissue. The secretion of HMW isoform as well as the % HMW however, was higher in Om than Abdsc. These results were not expected since it was hypothesized that HMW and % HMW would be higher in Abdsc than omental, since total adiponectin was higher in the former depots. In addition, because Adrx was higher in Abdsc and Adrx plays a role in the assembly of the disulfide bonds of the adipokine, adiponectin, it was thought that the secretion of HMW would be higher in Abdsc than omental adipose tissue.

Adrx correlation with adipokine release from human Om and Absc adipose tissue

To understand the relationship between Adrx protein expression and adipokine release in human Abdsc and omental adipose tissue, we tested whether Adrx protein levels were correlated with adipokines release. Leptin secretion and IL-6 secretion were not statistically significantly correlated with Adrx expression in either depot. It was expected that Adrx levels in adipose tissue would be positively correlated with total adiponectin, HMW and % HMW in both depots. Adrx protein levels were not correlated with total adiponectin measured by ELISA in both depots. Adrx protein levels positively correlated

with total and HMW adiponectin measured by Western blotting in Abdsc adipose tissue. Total adiponectin in Abdsc measured with Western blot was not statistically significant although there was a positive trend with a p value close to 0.05. When one outlier was excluded from the analysis, the correlation became statistically significant. Total adiponectin and HMW was positively correlated with Adrx levels in Abdsc. However, Om showed no correlation with total adiponectin, however a positive trend was shown with HMW but it was not statistically significant since the p value was close to 0.05. % HMW secretion was negatively correlated to Adrx levels in Abdsc. Om showed no correlation between Adrx protein expression and % HMW in both depots. This result was unexpected because Adrx play a role in the formation of disulfide bond of adiponectin, we hypothesized that total adiponectin, HMW and % HMW will be positively correlated. It may be that Adrx increases the production of LMW (trimmers) and MMW (hexamers) but not as much as HMW (increased in the production of LMW and MMW by 30% but the production of HMW is 20% lower in Abdsc than Om). There may be another enzyme or a regulatory step between MMW and HMW that play roles in the assembly of HMW adiponectin.

Adrx levels in Abdsc and omental adipose tissue did not correlate with serum adipokine levels.

Data from our lab found that knockdown of Adrx in human adipocytes decreased all isoforms of adiponectin secretion from Abdsc incubations, while increasing leptin secretion (unpublished data KK MJ SKF). C57BL/6J wild type mice exposed to high fat

diet showed decreased cellular Adrx and decreased circulating HMW adiponectin and Adrx knockout mice presented higher cellular adiponectin retention and lower circulating adiponectin levels (unpublished data). Based on these data we hypothesized that Adrx levels in Abdsc and omental would be positively correlated with total, HMW and % HMW adiponectin in serum. Thus, we determined whether adipose Adrx protein levels correlates with circulating levels of adipokines. The relationship between Adrx levels and the circulating adipokines in serum were not statistically significant. Circulating leptin levels, a maker of adiposity, have a negative trend to Adrx protein levels in Abdsc but there was not a trend with Adrx expression in Om. Total adiponectin in serum measured by ELISA were not correlated with Adrx levels in Abdsc while it showed a positive trend with Adrx levels in Om. The result with no correlation between circulating total adiponectin and Adrx protein levels in Abdsc are unexpected because the assembly of total adiponectin should be correlated with Adrx. However, the small sample size limits this analysis, and many factors that could affect adiponectin production were not assessed (e.g. inflammation, redox stress, body fat distribution). We also did not have sufficient numbers to assess the effect of sex and only studied obese subjects so the range of values was likely limited. Total adiponectin and HMW levels measured with quantitative Western blotting were not correlated with Adrx expression in Abdsc and Om. As well as the % HMW were not correlated with Adrx expression in either depots.

The differences between the results in total adiponectin measured by ELISA and Western Blot could be explained because Western Blot is a more sensitive assay than ELISA and we measured multiple isoforms with Western blotting, and the findings are

more exact with Western Blot than ELISA. To determine if the values were close enough we run a correlation between total adiponectin measured by ELISA and Western blotting and showed a positive correlation. We did tried to measure adiponectin multimers in serum with ELISA but got strange results because the % HMW was higher > 100% in most of the samples, did not make any sense. Also, it is not possible to use HMW multimer with ELISA kit in secretion media. So we decided to run the Western blotting for adiponectin isoforms.

Our results suggest that the assembly of HMW is not 100% controlled by Adrx and other proteins may play a role. In addition, the number of samples used in correlation studies was small, and most of our subjects were severely obese. It would be better to determine a relationship with a bigger sample size and wide range of subjects. We did not find positive correlation between Adrx protein levels and total adiponectin in Abdsc measured with ELISA although there was a positive trend, possibly due to a small sample size.

In conclusion, we have provided evidence that in obese subjects, depot differences in Adrx expression, lower in omental vs. Abdsc, may influence depot differences in adipokine secretion. Adrx may play a role in the assembly of LMW and MMW rather than HMW, since it was found that % HMW was negatively correlated with Adrx while there was a trend for total adiponectin secretion. The mechanism of higher HMW and % HMW in omental with lower Adrx levels needs further study, but appears to be related to depot differences in inflammation and oxidative stress (as indicated by JNK-P).

Limitations of the study

In the current study, one limitation was the sample size for Adrx levels (n=19) and when the correlation was calculated with adipokine release and serum the sample size was even smaller (n=11). Also, only obese subjects, mostly female, were included in this study. Another limitation is that the diabetic subjects some of them were classified as “diabetic” but some of them had a normal blood glucose levels, normal HbA1c and some of them did not have any medication for diabetes.

For future studies, a bigger sample size with same number of females and males including a variable range of BMI to determine sex differences and non-obese vs. obese in depot for Adrx expression and adipokine secretion will be required. In addition, a better analysis of the diabetic subjects, looking for more information about each diabetic subject, such as date of diagnosis, laboratories results, all medication listed for the day of the sample collection.

Future directions

As it is well known from the current study, Adrx levels are different between depots, i.e. higher in Abdsc than omental. It would be interesting to determine sex differences in Adrx expression levels as there are sex-dependent differences in leptin and adiponectin levels, and analyze the relationship between Adrx protein expression levels and phospho-JNK in the Western Blots. We are currently investigating this. It would be also interesting to do the correlation of Adrx expression with the other adiponectin

isoforms (MMW and LMW) to determine why the % HMW is negative correlated with Adrx and if there is another molecule playing a role in the conversion of MMW to HMW. The unexpected results between the negative correlation with Adrx in Abdsc and %HMW needs to be analyze more carefully.

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