Assessment of potential novel immune biomarkers to identify obese persons at increased risk for cardiometabolic disease (second year). Elisa Fabbrini, M.D. Ph.D.

Progress report

1. ABSTRACT

Although obesity is associated with multiple metabolic risk factors for cardiovascular disease, including insulin resistance, diabetes, dyslipidemia, and non-infectious inflammation, about 30% of obese adults do not have obvious metabolic abnormalities. We have found that excessive intrahepatic triglyceride (IHTG) content, independent of body mass index (BMI), percent body fat, and visceral fat mass, is a robust marker of obese persons who have metabolic dysfunction (insulin resistance in liver, muscle and adipose tissue and increased VLDL-triglyceride [VLDL-TG] secretion rate). Conversely, obese persons who have normal IHTG content appear to be resistant to developing obesity-related "cardiometabolic" complications. This observation has important implications for health and life insurance providers because excess IHTG identifies people at increased risk for future medical complications. However, the mechanisms responsible for the accumulation of ectopic fat (IHTG) and the development of cardiometabolic abnormalities in some, but not all, obese persons are not known.

In the first eight months of funding, we performed sophisticated metabolic phenotyping and collected blood samples and tissues biopsies on 20 obese subjects, who were divided into metabolically abnormal (MAO) or metabolically normal (MNO) obese according to their IHTG content at screening (MAO=IHTG ≥10%; MNO=IHTG ≤5%). By using a two-stage hyperinsulinemic clamp procedure, in conjunction with stable isotopically labeled tracer infusion, we found that MAO subjects are more insulin resistant at the liver, skeletal muscle and adipose tissue levels compared with MNO subjects.

We also made some novel observations that could have considerable physiological and clinical implications. We found that MAO is associated with TH17/Th22 CD4 T cell polarization in adipose tissue, and that the T cell polarization observed in adipose tissue is also seen in peripheral blood mononuclear cells (PBMCs). Furthermore, we found that adipose tissue 5-lipoxygenase (ALOX5) and its activating protein FLAP gene expression were increased in MAO compared with MNO subjects. Expression of ALOX5 was also found in PBMC from MAO. These finding are new and suggest that alterations in lymphocyte function and the prostanoid system are associated with metabolic dysfunction. In addition, our data demonstrate that PBMCs might be a useful marker of adipose tissue immune system function and pro-inflammatory leukotriene content, which can be used to identify MAO without the need for adipose tissue biopsies. Therefore, during the first eight months of this project we have made considerable progress towards completing our specific aims and were able to identify potential biomarkers produced by adipose tissue and traceable into the bloodstream that are specific of the metabolically-abnormal obese phenotype.

The overall goals for the second year of funding are to focus the future direction of this research project in conducting studies to: i) determine whether insulin action, adipose tissue T cell polarization and adipose tissue pro-inflammatory leukotriene in MNO are different than these outcome measure in lean, healthy individuals; and ii) understand the physiological and potentially clinical significance of the key observations made in year 1. This will be accomplished by: a) evaluating multiple-organ insulin sensitivity, fatty acid metabolism, and bloodstream and adipose tissue markers of inflammation and immune profile of T cells, in a group of lean, healthy subjects; b) determining the effect of IL-17, IL-22 and 5-lipoxygenase products on insulin sensitivity in primary human myocytes and hepatocytes, which are the main organs involved in metabolic dysfunction associated with obesity.

Our overall hypothesis is that targeted characterization of the factors and metabolic pathways that distinguish lean-healthy, MNO and MAO subjects will make it possible to 1) get new insight into the pathophysiology of metabolic dysfunction, and 2) better characterize putative biomarkers to assess and monitor metabolic health.

2. SUMMARY AND DESCRIPTION IN LAY LANGUAGE

Obesity is associated with a constellation of health complications, including diabetes, alterations in blood lipids, inflammation, and heart disease. However, not all obese people are at risk to develop these abnormalities, and approximately 30% of obese adults do not suffer from the complications.

Therefore, the purpose of the present proposal is to better understand the reasons why some but not all obese people develop metabolic complications and if possible to see if specific tests in the blood can help identify these people. Also, we want to understand if obese people who do not develop metabolic complications (metabolically-normal obese) are as metabolically "healthy" as lean people. This will be accomplished by studying a group of lean subjects and comparing this group to the two groups of obese subjects (metabolically normal and metabolically abnormal obese) that we studied during the first year of funding. With the funds obtained during the first year, we were able to show that fat tissue of metabolically abnormal obese subjects is "inflamed", and that produces inflammatory markers that are released from fat tissue into the bloodstream. We now want to understand if these inflammatory markers released by fat tissue into the bloodstream are responsible for causing the alterations in sugar and fat metabolism that we observed in the metabolically abnormal obese subjects. If this is the case, these inflammatory molecules present in the blood could be used as markers to identify those people that are at high risk to develop metabolic and cardiovascular disease.

3. NIH BIOGRAPHICAL SKETCH

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EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing,*

INSTITUTION AND LOCATION	DEGREE	YEAR(s)	FIELD OF STUDY
La Sapienza Univ. School of Medicine, Rome, Italy	M.D.	1998	Medicine
La Sapienza Univ. School of Medicine, Rome, Italy	Ph.D.	2006	Endocrinology

A. Positions and Honors

Postdoctoral training and professional experience:

1998 Rotating Medical Internship, University Hospital, La Sapienza University School of Medicine, Rome, Italy
1998-2003 Medical Internship and Residency, Endocrinology and Metabolism, Tor Vergata University School of Medicine, Rome, Italy
2000-03 Resident Fellow, Endocrinology and Bone Diseases Unit, Tor Vergata University School of Medicine, Rome, Italy
2003 Certificate of Specialization in Endocrinology and Metabolism, Tor Vergata University School of Medicine, Rome, Italy
2003-05 Endocrinologist, Endocrine Clinic, Local Health Authority, 2nd District, Rome, Italy
2003-05 Endocrinologist, Endocrine Clinic, Local Health Authority, 3rd District, Rome Italy
2003-05 Endocrinologist, Bone Disease Clinic, Villa Mafalda Institute, Rome, Italy
2003-05 Endocrinologist, Obesity Clinic, Bios Institute, Rome, Italy
2005-06 PhD Fellowship, Nutrition and Metabolism, Washington University School of Medicine, St. Louis, MO
2006-08 Postdoctoral Research Associate, Veronica Atkins International Fellow in Obesity Research, Nutrition and Metabolism, Washington University School of Medicine, St. Louis, MO

Board Certification:

1999 Italian Medical License
2003 Italian Board of Endocrinology and Metabolism

Honors:

1994 Fellowship Award, Institute of Human Anatomy, La Sapienza University School of Medicine, Rome, Italy
1995 Fellowship Award, Institute of Biochemistry, La Sapienza University School of Medicine, Rome, Italy
1996 Fellowship Award, Institute of Neurology, La Sapienza University School of Medicine, Rome, Italy
1997 Fellowship Award, Department of Medical Pathophysiology, La Sapienza University School of Medicine, Rome, Italy
1998 Magna Cum Laude, MD, La Sapienza University School of Medicine, Rome, Italy
2003 Magna Cum Laude, Residency, Tor Vergata University School of Medicine, Rome, Italy
2006 Magna Cum Laude, PhD, La Sapienza University School of Medicine, Rome, Italy
2007 Young Investigator Travel Grant Award, Annual Meeting of The Obesity Society, New Orleans, LA
2008 Visiting Professor, Department of Internal Medicine, Indiana University School of Medicine, Indianapolis, IN
2009 Visiting Professor, Division of Digestive and Liver Disease, Obesity Alliance Seminar Series, UT Southwestern Medical Center in Dallas, TX

2009 Invited Speaker at the Pan-American Endocrinology Conference, Margarita, Venezuela. Title of presentation: “Nonalcoholic fatty liver disease: a growing health concern”

2009 Invited Speaker at the American Diabetes Association Annual Meeting, New Orleans, LA. Title of presentation: “Integrated pathophysiology of nonalcoholic fatty liver disease”

2009 Invited speaker at the 1st Venezuelan Conference on Obesity, Metabolic syndrome, and Prediabetes, Caracas, Venezuela. Title of presentation: “Effect of weight loss on nonalcoholic fatty liver disease”

2009 Invited Speaker at The Obesity Society’s Annual Meeting, Washington, DC. Title of presentation: “Metabolic effects of gastrointestinal bypass surgery in humans”

2010 Awarded with the “Ethan Sims Young Investigator Award”. The Obesity Society’s Annual Meeting, Orlando, FL. Title of presentation: “Surgical removal of visceral fat does not improve insulin sensitivity and cardiovascular risk factors in obese patients”

2010 Invited speaker at the 2nd Venezuelan Conference on Obesity, Metabolic syndrome, and Prediabetes, Caracas, Venezuela. Title of presentation: “Metabolically normal and metabolically abnormal obesity.”

B. Publications:

1. Fabbri A, Giannini D, Aversa A, De Martino MU, Fabbrini E, Franceschi F, Moretti C, Frajese G, Isidori A: Body-fat distribution and responsiveness of the pituitary-adrenal axis to corticotropin-releasing-hormone stimulation in sedentary and exercising women. J Endocrinol Invest. 1999 May;22(5):377-85. PMID: 10401712
2. Polito A, Fabbri A, Fertro-Luzzi A, Cuzzolaro M, Censi L, Ciarapica D, Fabbrini E, Giannini D: Basal metabolic rate in anorexia nervosa: relation to body composition and leptin concentrations. Am J Clin Nutr. 2000 Jun;71(6):1495-502. PMID: 10837290
3. Aversa A, Isidori AM, De Martino MU, Caprio M, Rocchietti.March M, Fabbrini E, Fabbri A: Androgens and penile erection: evidence for a direct relationship between free testosterone and cavernous vasodilation in men with erectile dysfunction. Clin Endocrinol (Oxf). 2000 Oct;53(4):517-22. PMID: 11012578
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C. Research Support

2003-05 Merck industry-initiated trial; "A Study to Determine the Effects of MK0557 in Obese Subjects"; PI: G. Spera, Co-I: E. Fabbrini.

2005-07 Merck industry-initiated trial; "A Two Year Study to Assess the Efficacy, Safety, and Tolerability of MK-0364 in Obese Patients"; PI: G. Spera, Co-I: E. Fabbrini.

2006-08 Sanofi-Aventis Investigator-Initiated Research Award; "Effect of plasma uric acid on insulin sensitivity, endothelial function, and inflammation"; PI: S. Klein, Co-I: E. Fabbrini.

2010 Nutricia Research Foundation; "Effect of Endoplasmic Reticulum Stress on Metabolic Function" PI: E. Fabbrini

2010 Institute for Clinical and Translational Research Core Funding: Effect of bariatric surgery-induced weight loss on glucose homeostasis and taste perception. PI: E. Fabbrini

2011 Longer Life Foundation Developmental Research Award (RGA/Washington University Partnership): "Assessment of potential novel immune biomarkers to identify obese persons at increased risk for cardiometabolic disease" PI: E. Fabbrini

4. RESEARCH PLAN.

i. Specific Aims

The overall goals of this proposal are to elucidate the cellular and organ system mechanisms responsible for the development of metabolic/immune dysfunction in metabolically abnormal obese (MAO) persons, and therefore to identify potential bloodstream biomarkers specific of the MAO population. Our central hypothesis is that MAO subjects have increased adipose tissue lipolytic activity and inflammation ("sick" adipose tissue), which results in increased release of free fatty acids (FFA) into plasma, and systemic inflammation (increased circulating pro-inflammatory proteins, metabolites and cells), which in turn cause insulin resistance (diabetes) and increases cardiometabolic disease risk.

In our first year of funding we made the novel observation that adipose tissue and PBMC of MAO subjects show: i) a specific T-cell polarization, and ii) the activation of the ALOX5 pathway, therefore suggesting that these could be used as biomarkers for metabolic abnormalities.

We now propose for the second year of funding to focus the future direction of this research project on the following specific areas: 1) perform metabolic and immune system phenotyping studies of a group of lean, healthy subjects to determine whether MNO subjects are actually "abnormal" compared with lean subjects; 2) conduct studies to understand the physiological and potentially clinical significance of the key observations made in year 1, namely T-cell polarization and the ALOX5 system.

Accordingly, we propose the following specific aims:

Aim 1. Determine multi-organ insulin sensitivity, adipose tissue and PBMC T-cell polarization, and adipose tissue and PBMC prostanoid system in lean, healthy subjects. Results obtained from this lean population will be compared with data previously collected in MNO and MAO obese subjects. This will allow us to better characterize putative biomarkers involved in the pathogenesis and progression of cardiometabolic diseases.

The following factors will be evaluated:

a. Insulin action: insulin sensitivity in the liver, skeletal muscle and adipose tissue will be assessed *in vivo* by using a two-stage euglycemic hyperinsulinemic clamp procedure with stable isotope tracer infusion and cellular factors that influence insulin action, including components of the insulin signaling cascade and factors that inhibit insulin signaling will be evaluated in skeletal muscle biopsies.

b. Peripheral blood mononuclear cell (PBMC) immune profile and secreted immunoregulatory cytokines. We will determine the immune profile of circulating T cells.

c. Adipose tissue inflammation: adipose tissue assessment of inflammatory and resident monocytes, gene expression of monocyte/macrophage and T cell inflammatory pathways and pro-inflammatory cytokines and chemokines.

Hypothesis: We hypothesize that: 1) lean subjects will be more insulin sensitive than both MAO and MNO subjects, 2) lean subjects will show a more favorable inflammatory profile than MNO subjects, 3) fatty acid metabolism and insulin sensitivity will correlate with the inflammatory markers we identified across the groups. Therefore, this aim will allow us to complete a comprehensive metabolic and immune system analysis from three distinct groups of lean, MNO and MAO subjects to help us characterize circulating inflammatory markers that could predict metabolic health or disease.

Aim 2. Evaluate the effects of IL-17, IL-22 and leukotriene products of 5-lipoxygenase on metabolic function (insulin action in primary human hepatocytes and myocytes).

Insulin action will be assessed *in vitro* by evaluating insulin-sensitive metabolic responses in primary cultures of human hepatocytes and myocytes incubated with insulin, with or without IL-17, IL-22, or products of ALOX-5.

Hypothesis: We expect that incubation of hepatocytes and myocytes with IL-17, IL-22 or ALOX-5 products will reduce glucose uptake, glycolysis and glucose production (hepatocytes only) and will activate the JNK MAPK pathway, therefore providing a molecular link between these biomarkers and insulin resistance.

ii. Relevance of the project to the Mission of the Foundation and Scope of Work

This project is relevant to the Mission of the Foundation for several reasons:

1) This study has the potential to generate important new findings that could improve our ability to prognosticate disease risk in obese people. Accordingly, the data from this study may lead to new biomarkers that could be used to recognize obese people who are at higher risk of developing cardiometabolic complications; these biomarkers might allow a better assessment of disease and mortality.
2) The data from this study will provide preliminary data for a larger NIH R01 grant application, which is an important mission of the P and F program.
3) This project focuses on obesity and health which is listed as one of the "Areas of Interest" of the Foundation.
4) This is the first study to use sophisticated *in vivo* methods in human subjects to very carefully evaluate the metabolic profile of lean subjects and compare with MNO and MAO, in conjunction with an extensive characterization of bloodstream and adipose/muscle tissues inflammatory and immunological markers. We hope these results will be published in a high-impact journal, which will highlight support from LLF.

iii. Background and Significance

Inflammation in obesity. Obesity is associated with chronic low-level inflammation which can lead to the development of cardiovascular disease, metabolic dysfunction, and certain types of cancers. Adipose tissue monocyte/macrophage content is increased in obesity, and these cells are active participants in obesity-induced inflammation which can induce metabolic disease (1, 2). However, little is known on the role of other immune cell types in the development and maintanence of inflammation in obesity. Recently, data from studies conducted in animal models have indicated that the distribution of T cell subsets is altered during obesity (3, 4). Emerging evidence in rodents also supports the potential contribution of the proinflammatory lipoxygenase-5 (ALOX-5) pathway to adipose tissue inflammation and lipid dysfunction in experimental obesity (5, 6).

Metabolically normal and metabolically abnormal obesity. The recognition that a subset of obese persons are resistant to the typical metabolic complications of obesity has led to several studies that have tried to characterize the distinguishing features between metabolically abnormal obesity (MAO) and metabolically normal obesity (MNO), also known as metabolically healthy but obese (7), uncomplicated obesity (8), and metabolically benign obesity(9). In general, the data from these studies found MNO persons had similar percent body fat but less visceral and liver fat compared with MAO persons, and had normal insulin sensitivity, blood pressure, lipid profile, and inflammatory profile (plasma CRP concentration) (712).

Summary of year 1 of funding and implications for year 2 proposal. We had previously found that

increased IHTG content is a robust marker of metabolic dysfunction (insulin resistance in liver, muscle and adipose tissue and increased VLDL-triglyceride [VLDL-TG] secretion rate), independent of body mass index (BMI), percent body fat, and visceral fat mass in obese persons (1316). During the first year of funding, we used IHTG content to identify persons who have metabolically normal or metabolically abnormal obesity, and we found that metabolically abnormal obese subjects have adipose tissue immunological dysfunction that can be identified by biomarkers found in blood, and that the profile of circulating T-lymphocytes in the blood reflects the changes in adipose tissue T-cell profile and function. Also, we have found an increase in the expression of the key leukotriene producing enzyme, lipoxygenase-5 (ALOX5), and its activating protein, in adipose tissue from MAO subjects. However, it is not known if T-cell profile and lipoxygenase-5 pathway are similar in MNO and in lean healthy individuals, or if MNO, despite being the "healthy" obese phenotype, are still at greater risk than lean subjects to develop cardiometabolic complications. Also, it is not known if T-lymphocyte polarization and ALOX-5 pathway are innocent bystanders or active contributors of metabolic abnormalities in MAO subjects.

iv. Progress Report

1. Insulin action in the liver, adipose tissue and muscle

During the first eight months of funding we studied a total of 20 obese subjects (13 women and 7 men, age 42 ± 12 yrs; BMI: 39.3 ± 8.6 kg/m^2; mean ± SD), divided according to IHTG content into metabolically normal (MNO, n=10, IHTG ≤5%) or metabolically abnormal (MAO, n=10, IHTG≥10) obese (Table 1). No subject took medications that can affect metabolism or cause hepatic abnormalities, consumed more than 20 g/d of alcohol, or had diabetes. Each subject underwent comprehensive body composition analyses (including dual-energy X-ray absorptiometry [DXA] to evaluate total body fat and fat-free masses, magnetic resonance imaging [MRI] to evaluate total, subcutaneous and intra-abdominal fat volumes, and magnetic resonance spectroscopy [MRS] to evaluate intrahepatic triglyceride content) and a two-stage hyperinsulinemic-euglycemic clamp procedure in conjunction with stable isotope labeled tracer infusions to evaluate liver and adipose tissue insulin sensitivity (low-dose insulin infusion) and skeletal muscle insulin sensitivity (high-dose insulin infusion) In addition, skeletal muscle (quadriceps femoris) and adipose tissue (subcutaneous abdominal) biopsies were obtained to evaluate cell and tissue factors involved in regulating metabolic function.

Table 1. Characteristics of the study subjects

	MNO	MAO
N (F:M)	10 (7:3)	10 (6:4)
Age	44 ± 11	38 ± 11
Weight (kg)	100 ± 19	120 ± 24
Fat mass (%)	44 ±7	46 ± 7
Intrahepatic triglyceride content (%)	3.9 ± 3.7	13.6 ± 9.6
Visceral fat (cm^3)	1,412 ± 631	1,882 ± 748
Subcutaneous abdominal fat (cm^3)	3,835 ± 1,427	4,364 ± 877
Glucose (mg/dL)	94 ± 8	93 ± 8
Insulin (mU/L)	5.6 ± 2.1	19.6 ± 7.9
HOMA-IR	1.29 ± 0.53	4.57 ± 2.04
Total cholesterol (mg/dL)	181 ± 25	161 ± 22
HDL-cholesterol (mg/dL)	48 ± 13	35 ± 7
LDL-cholesterol (mg/dL)	110 ± 19	97 ± 17
Triglyceride (mg/dL)	116 ± 51	141 ± 46
C-reactive protein (µg/ml)	3.8 ± 2.8	5.9 ± 4.2

Values are mean±SD.

MNO and MAO subjects were well matched on percent body fat. However, IHTG content, visceral fat volume, plasma insulin and C-reactive protein concentrations and HOMA-IR were greater in the MAO than the MNO subjects. Basal plasma glucose concentration was not different between MNO and MAO subjects.

Basal glucose rate of appearance (Ra) into plasma (i.e., endogenous glucose production) was not different between MNO and MAO subjects. However, glucose Ra during insulin infusion was higher in MAO compared with MNO (Figure 1). Basal free fatty acid (FFA) Ra into plasma either expressed per kg of fat mass (an index of adipose tissue lipolysis) or per kg of fat free mass (an index of FFA availability to tissues) was not different between MNO and MAO subjects. However, FFA Ra during insulin infusion was higher in MAO compared with MNO (Figure 2).

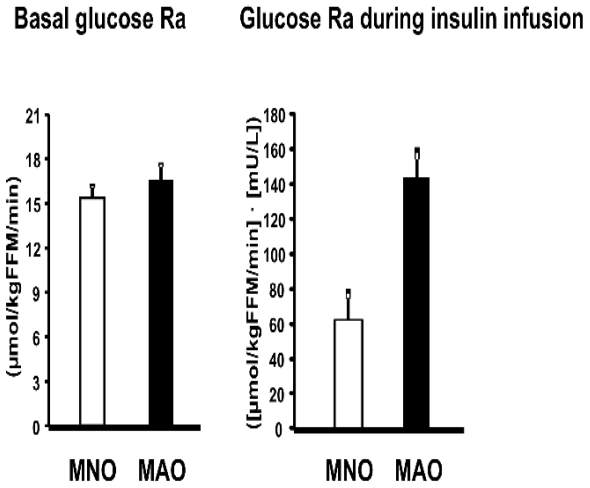

Figure 1. Hepatic glucose production during basal conditions and during insulin infusion in MNO and MAO subjects

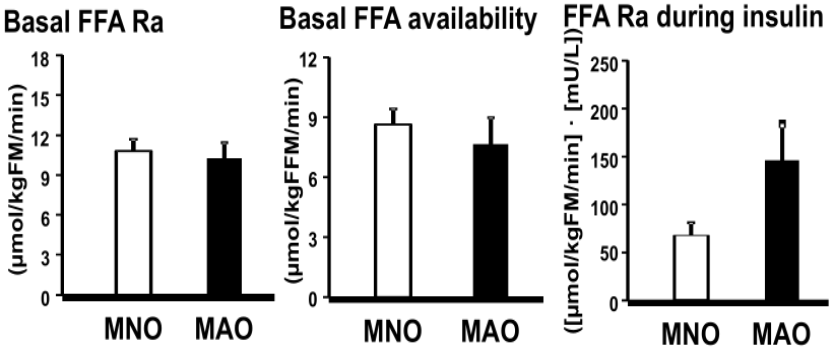

Figure 2. Free fatty acid release into plasma during basal conditions and during insulin infusion in MNO and MAO subjects.

Insulin-mediated glucose rate of disappearance (Rd) from plasma, an index of skeletal muscle glucose insulin sensitivity, was blunted in MAO compared with MNO (Figure 3).

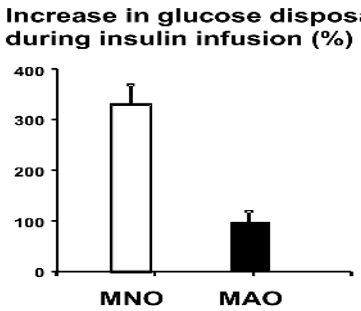

Figure 3. Percent increase in glucose disposal from basal to insulin infusion in MNO and MAO subjects.

2. Markers of adipose tissue and bloodstream inflammation.

a) Adipose tissue gene expression: We performed gene expression of subcutaneous abdominal adipose tissue samples from MNO and MAO subjects. Real time qPCR was used to measure expression of genes that encode for key proteins involved in pathways for inflammation; target gene expression was normalized to at least one housekeeping gene (acidic ribosomal phosphoprotein 36B4, beta-actin or 18s) and followed the 2-ΔCt method.

These analyses resulted in the following observation:

▪*Macrophages markers:* Gene expression of markers of macrophages infiltration into adipose tissue, EMR1 and CD68, were higher in biopsies obtained from MAO subjects compared to adipose tissue biopsies from MNO (Figure 4).

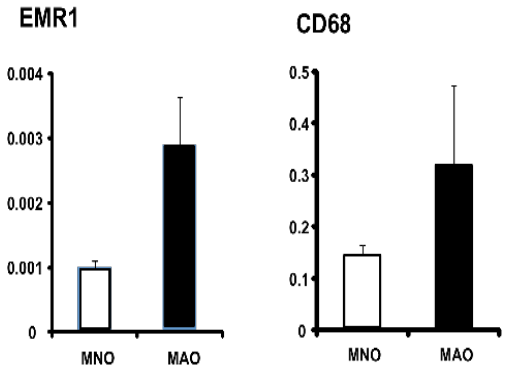

Figure 4. Gene expression of macrophages markers in adipose tissue biopsies of MNO and MAO subjects

▪*Lipoxygenase 5*. Lipoxygenase 5 (ALOX5) is a key enzyme in the conversion of arachidonic acid to the bioactive leukotrienes, which act to sustain inflammatory reactions. The mRNA expressions of ALOX5 and the ALOX5 activating protein (ALOX5AP) were significantly greater in adipose tissue from MAO than MNO subjects (Figure 5). These data are consistent with a potential pro-inflammatory role of adipose tissue in MAO subjects.

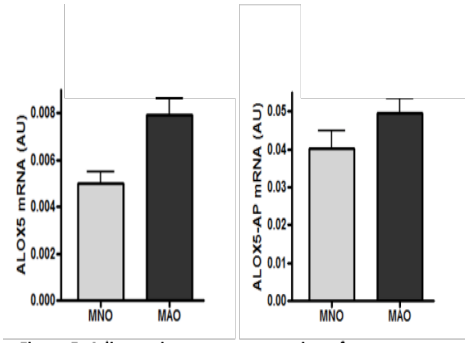

Figure 5: Adipose tissue gene expression of enzymes involved in leukotrienes synthesis pathway.

b) Plasma prostaglandins. Prostaglandins are products of arachidonic acid metabolism and participate in inflammatory responses and have been found to be up-regulated in some cancers. We measured Prostaglandin E2 (PGE2) in plasma and we found that was higher in MAO subjects compared to MNO (Figure 6).

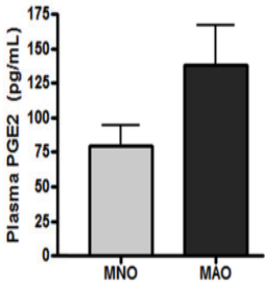

Figure 6: Plasma concentration of Prostaglandin E2.

c. *Systemic and adipose tissue CD4 T-cell polarization*. We expanded lymphocytes and the adipose tissue stromal-vascular fraction (SVF) which were extracted from blood and adipose tissue samples from MNO and MAO subjects to evaluate the systemic and local polarization of CD4 T-cells by intracellular staining and flow cytometry for the production of cytokines associated with the Th1 (IFNγ), Th2 (IL-13), Th17 (IL-17), and Th22 (IL-22) subsets of CD4 T cells. We observed increased IL-22 and IL-17 peripheral blood CD4 T-cell polarization in MAO compared to MNO subjects. Examination of CD4 T cell polarization in adipose tissue revealed that Th17 polarization was enhanced primarily in MAO compared to MNO subjects (p=0.051) (Figure 8) and adipose tissue Th17 was negatively correlated with glucose infusion rate during the clamp procedure (p=0.038, data not shown). These results suggest that both local (adipose tissue) and systemic (PBMC) CD4 T cells have increased polarization towards the Th17 and Th22 subsets in MAO compared to MNO subjects.

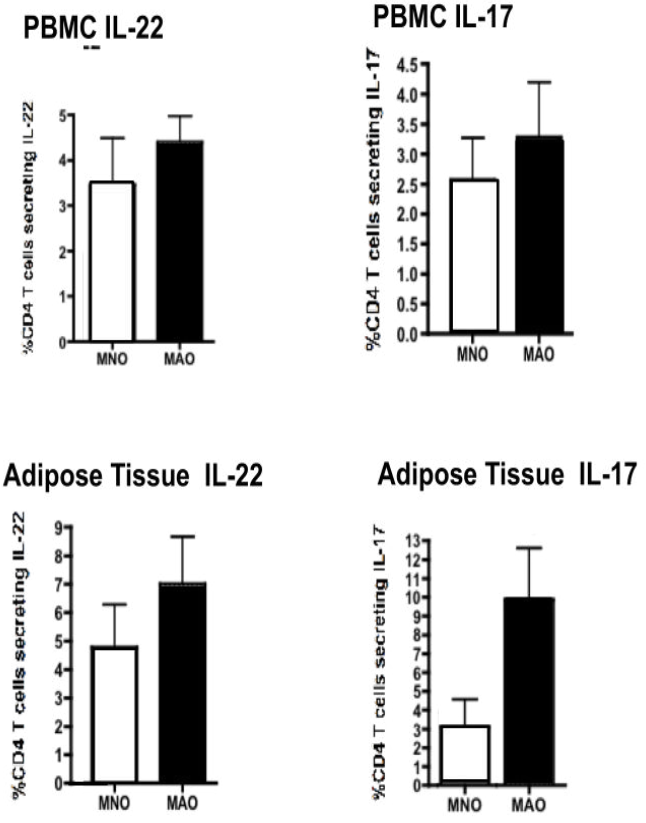

Figure 8. Adipose tissue and PBMC CD4 T-cell polarization

v. Research Design and Methods

1. Study Protocol

Subjects: The study population will consist of 12 lean, healthy subjects who are 18-55 years old. Volunteers will be screened with a history and physical examination, routine blood tests, lipid panel, liver biochemistries, 12-lead electrocardiogram, and an oral glucose tolerance test. Women who are able to bear children will have a pregnancy test. Subjects who have active or previous history of other liver diseases, history of alcohol abuse, currently consuming ≥20 g alcohol/day, diabetes, severe hypertriglyceridemia (>300 mg/dL), smoke tobacco, or take medication that might confound the study results will be excluded. Women who are pregnant or lactating will also be excluded.

Body composition analyses: Total body fat and fat-free mass will be determined by using dual energy x-ray absorptiometry (DXA; General Electric iDEXA, Madison, WI). Abdominal (subcutaneous and intra-abdominal) adipose tissue mass will be quantified by magnetic resonance imaging, as we have previously described (14). Intrahepatic TG content will be determined by proton MRS (3T whole-body system; Magneton Vision Scanner; Siemens, Erlanger, Germany) as we have previously described (17).

Two-stage euglycemic-hyperinsulinemic clamp procedure: Studies will be performed in the Clinical Research Unit (CRU). At 1800 h on the day of admission to the CRU, subjects will consume a standard dinner. After this meal, subjects will fast, except for water, until completing the metabolic study the next day. At 0600 h, a catheter will be inserted into a radial artery to obtain arterial blood samples and into an antecubital vein of the contralateral arm to infuse isotope tracers, dextrose and insulin. At 0700 h, a two-stage hyperinsulinemic euglycemic clamp procedure with stable-isotopically labeled tracer will be initiated to determine hepatic (suppression of glucose production), skeletal muscle (stimulation of glucose disposal), and adipose tissue (suppression of lipolysis) insulin sensitivity. After baseline blood samples are obtained, a primed-constant infusion of [6,6-$^{2}H_2$]glucose (22 μmol/kg prime and 0.22 μmol·kg^{-1}·min^{-1} constant infusion) will be started (t=0 min) and continued throughout the clamp procedure. After 90 min (0830 h), a constant infusion of [U-^{13}C]palmitate (6 nmol·kg FFM^{-1}·min^{-1}) will be started to determine the rate of appearance (Ra) of plasma palmitate. At 1030 h, after the basal period is completed, insulin will be infused at a rate of 7 mU·m^{2}·min^{-1} for 3 h (stage 1), and will be infused at a rate of 50 mU·m^{2}·min^{-1} for the following 3 h (stage 2). Euglycemia will be achieved by a variable rate infusion of 20% dextrose enriched to approximately 2.5% with [6,6-$^{2}H_2$]glucose to minimize changes in glucose isotopic enrichment (18)). The plasma insulin concentrations achieved with these insulin infusion rates provide an optimal range for evaluating insulin's effect on adipose tissue lipolysis and hepatic glucose production (low-dose insulin infusion) and glucose uptake by skeletal muscle (moderate dose insulin infusion).

Blood and tissue sampling: Plasma samples will be taken before beginning the isotope infusion to obtain baseline measurements of substrate enrichment. Plasma samples will be taken every 10 min during the last 30 min of the basal period and each stage of the insulin clamp to determine glucose and FFA concentrations and kinetics, and plasma insulin concentrations. Plasma samples will be obtained every 10 min at the end of the basal period and throughout the entire clamp period to monitor plasma glucose concentration. Samples will be obtained from subcutaneous abdominal adipose tissue and the *vastus lateralis* muscle 60 min after starting the glucose tracer infusion (basal stage of the clamp procedure). Muscle and fat biopsies will also be obtained 60 minutes after starting stage 2 of the clamp procedure to determine the effect of insulin infusion. All tissue samples will be immediately and gently rinsed with ice-cold saline. Adipose tissue samples will be divided into 2 aliquots: 1) immediately frozen in liquid nitrogen for subsequent determination of cell factors and quantitative PCR; and 2) placed in PBS for immunological analyses. Muscle samples will be immediately frozen in liquid nitrogen and stored at -80°C.

2. Sample Analyses

The following factors will be evaluated:

Insulin action: i) insulin sensitivity in the liver (suppression of glucose production), skeletal muscle (glucose uptake) and adipose tissue (suppression of lipolysis) assessed *in vivo* by using a two-stage euglycemic hyperinsulinemic clamp procedure with stable isotope tracer infusion; ii) cellular factors that influence insulin action, including components of the insulin signaling cascade and factors that inhibit insulin signaling (i.e., IRS [total and phospho Tyr and phospho Ser]; Akt [total and phospho Thr^{308} and phosphor Ser^{473}], JNK [total and phosphor Thr^{183}], mTOR [total and phosho Ser^{2448}] in skeletal muscle biopsies.

Selected markers of inflammation in blood: i) inflammatory and immunoregulatory cytokines:TNF-α, IL-6, IL-17, IL-22, IL-26, ii) adipokines: adiponectin; iii) peripheral blood mononuclear cell (PBMC) immune profile: CD4 T cells ($CD3^+CD4^+$), CD8 T cells ($CD3^+CD8^+$), Tregs ($CD4^+CD25^+Foxp3^+$), NK cells ($CD3^-CD56^+$), NK-T cells ($CD3^+CD56^+$), B cells ($CD19^+$), resident monocytes ($CD14^+CD16^+$), inflammatory monocytes ($CD14^+CD16^-$) and granulocytes ($CD16^+$, SSC^{hi}).

Adipose tissue inflammation: i) cell content: inflammatory and resident monocytes and CD4 T cells [T_H1, T_H2, Treg]; ii) gene expression of monocyte/macrophage and T cell inflammatory pathways; iii) expression of pro-inflammatory cytokines and chemokines.

Effects of IL-17, IL-22 and leukotrienes on insulin action: Primary human hepatocytes, adipocytes, and skeletal myocytes will be treated with IL-17, IL-22, LTB4, LTD4, 5-HETE, or PGE2 and insulin action (glucose production, glucose uptake and insulin intra-cellular signaling) will be evaluated.

2.1. Methods

Plasma glucose and palmitate kinetics. Our laboratory has streamlined methods to process plasma to recover these substrates and derivatize them for GC/MS analyses (14, 15, 1921). Steele's steady state equation(22) will be used to calculate substrate kinetics.

Tissue metabolic factors; gene expression; content of specific proteins. Gene expression will be measured by quantitative real-time PCR. Protein content of insulin signaling pathways will be measured by western blot as we have described (23).

Plasma adipokines. TNF-α, IL-6 and adiponectin will be determined by commercially available ELISA kits.

Insulin action in primary human cells. Primary cultures of human hepatocytes, myocytes, and adipocytes will be incubated with insulin, with or without IL-17, IL-22, LTB4, LTD4, 5-HETE, or PGE2. Insulin action will be measured by measuring i) glucose uptake by using 2-[1-^{14}C]deoxy-D-glucose (2-DG), ii) glycolysis with ^{3}H-glucose, iii) glucose production (hepatocytes only) by using a colorimetric glucose assay, iii) insulin signaling by immunoblot analysis using antibodies specific for phosphotyrosine or phosphoserine IRS, Akt serine/threonine phosphorylation, glycogen synthase (GS) and GLUT4.

3. Statistical Analyses

Analysis of variance will be used to evaluate differences between groups. In all analyses, we will ensure that the conditions necessary for the valid use of a proposed statistical procedure (e.g., normal distribution and equal variance) are satisfied. When conditions are violated, we will use data transformations intended to produce data that satisfy normality and equal variance assumptions. If an appropriate transformation cannot be identified, we may use non-parametric methods as an alternative to the more standard analyses. In addition, we may perform correlation analyses to determine the relationship between variables.

5. PLANS FOR OBTAINING ADDITIONAL EXTRAMURAL FUNDING ARISING FROM THE PROPOSED STUDIES

The results from this study will provide pilot data for an R01 application to further explore the mechanisms responsible for the differences in cardiometabolic risk factors between different obese populations. This study will help launch my independent research career at Washington University in St. Louis.

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7. BUDGET AND BUDGET JUSTIFICATION

Budget category	**Totals**
Personnel Costs	$15,068
Supplies	$26,536
Other expenses	$8,400
Total Direct Costs	**$50,000**

Budget Justification

Personnel

(Salary plus Fringe Benefits)

Elisa Fabbrini, MD, PhD – PI	(5% effort)	$4,546
Zhouji Chen, PhD	(10% effort)	$10,522

Elisa Fabbrini, MD, Research Assistant Professor of Medicine. As Principal Investigator, Dr. Fabbrini will devote 20% (2.5 calendar months per year) of her time to this project. However, only 5% is requested from the LLF foundation grant. The remain of her salary is supported bu other sources. She will be responsible for all aspects of this project, including subject recruitment, metabolic studies and oversight of sample processing and analyses. Dr. Fabbrini will analyze and interpret the data, and write manuscripts for publication. Support for 5% salary and fringe is requested.

Zhouji Chen, PhD, Research Instructor of Medicine. Dr Chen will devote 20% (2.5 calendar months per year) of his time to this project. However, only 10% is requested from the LLF foundation grant. The remain of his salary is supported by other sources. He will be working on the in vitro studies. Support for 10% salary and fringe is requested.

Equipment

All necessary equipment for the completion of the proposed study is available.

Supplies

Clamp study supplies:
Each subject will undergo a Clamp metabolic study with a budgeted cost of $750 per subject to cover the cost of tracers, insulin, IV supplies, blood drawing supplies, etc. $750 x 12 subjects = $9,000
Tissue culture reagents, cytokines, and radiolabeled glucose $4,000
Western blotting reagents and general lab supplies $3,532
Human hepatocytes, adipocytes, and skeletal myocytes $10,000

Total Supplies **$26,532**

Other expenses

Body composition analyses:

Each subject will have tests to determine body composition (total and abdominal fat mass, intrahepatic triglyceride content, etc.). These tests are DXA scan, MRI, and MRS. $400 per subject is budgeted for the three tests for 12 subjects for a total of $4,800.

Subject reimbursement:

Each subject will be reimbursed $50 for each body composition test (DXA, MRI, MRS) = $150 and $150 for completion of the clamp study for a total of $300 per subject x 12 subjects = $3,600.

Total Other Expenses **$8,400**

Total Direct Costs **$50,000**

Recruitment of subjects, tracer mixing and analysis of substrates isotope enrichment by mass spectrometry will be covered by the Clinical Science Research Core, provided by the Washington University Nutrition and Obesity Research Center.