

Macrophage infiltration of human adipose tissue and its association with systemic
inflammation, obesity, and metabolic disease

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A thesis

submitted in partial fulfillment of the
requirements for the degree of

Master of Public Health

University of Washington

2012

Committee:

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Program Authorized to Offer Degree:

Public Health - Epidemiology

University of Washington

Abstract

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Macrophages accumulate in expanding adipose tissue where they produce and secrete inflammatory cytokines known to impair insulin action. “Classically activated” (i.e., pro-inflammatory) adipose tissue macrophages (ATM) are therefore thought to play a crucial role in the development of insulin resistance. The Fc γ receptor III, CD16, is considered a marker for inflammatory activation in peripheral blood monocytes and macrophages in synovial fluid and associated membranes. Increased CD16 expression in these cells has been linked to cardiovascular disease, rheumatoid arthritis, and inflammatory diseases of the skin and bowel. We therefore sought to determine whether CD16 is a marker for “classically activated”, pro-inflammatory macrophages in adipose tissue associated with insulin resistance. In an initial cross-sectional study of 18 men and women varying widely in age, adiposity, and metabolic health, we observed that the number of CD16^{pos}-ATM within the subcutaneous adipose tissue was associated with body mass index (BMI; $r=0.60$, $p=0.009$) and insulin resistance measured by homeostasis model assessment (HOMA; $r=0.65$, $p=0.004$). Moreover, the number of CD16^{pos}-ATM was strongly associated with adipose tissue gene expression of key mediators of

inflammation, including intracellular adhesion molecule-1 (ICAM-1; $r=0.72$, $p=0.001$) and tumor necrosis factor- α (TNF- α ; $r=0.67$, $p=0.002$), and circulating levels of the inflammatory mediators interleukin-6 (IL-6; $r=0.51$, $p=0.029$) and C-reactive protein (CRP; $r=0.66$, $p=0.003$), while also being negatively correlated with both plasma ($r=-0.70$, $p=0.001$) and adipose tissue adiponectin expression ($r=-0.57$, $p=0.014$). However, these associations were not present in two additional study populations ($n=36$) in which we sought to confirm this initial finding. Of these, one consisted of 26 overweight to obese post-menopausal women while the other was comprised of 10 subjects, 8 of which were obese, undergoing abdominal surgery. Further, gene profiling of isolated CD16^{pos}-ATM revealed lower expression of the inflammatory genes TNF- α , IL-6 and interferon gamma (IFN γ) when compared to CD16^{neg}-ATM. We conclude therefore, that while CD16 may be a marker of inflammation in monocytes and synovial macrophages, it is not a marker for “classically activated” inflammatory macrophages in human subcutaneous adipose tissue.

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Chapter 1: Literature Review

Introduction

The global prevalence of obesity has reached epidemic proportions, serving as a harbinger of greater morbidity and mortality. The classification of individuals as either overweight or obese is based on a simple index of weight-for-height, otherwise known as the body mass index (BMI). Based on this index, the World Health Organization defines individuals with a BMI of greater than 25 kg/m² as being overweight, and those over 30 kg/m² as being obese (1). As of 2008, global estimates of obesity have doubled since 1980, with more than 500 million adults classified as obese, and 1.4 billion as overweight (1). Equally disturbing, as many as 40 million children under the age of five are overweight (1). In the United States alone, recent estimates indicate that more than 66% of adults (roughly 133 million) are either overweight, or obese (approximately 64 million) (2-4). By 2030 it is projected that more than 50% of American adults will be obese, representing an increase of 65 million (5, 6). Globally, projections based on secular trends translate into estimates of more than 2 billion overweight and 1 billion obese individuals by the year 2030 (7).

Excess weight is the fifth leading risk factor of global mortality (1). In the United States it is the third most common underlying preventable cause of death, behind only tobacco use and hypertension (8). The medical ramifications of this epidemic are staggering. Obesity is not only highly associated with the incidence of type 2 diabetes mellitus (T2DM) and prediabetes (8-10), as well as cardiovascular disease (CVD) (11), it is also an important risk factor for several cancers, including breast, colon, endometrium, esophagus, gall bladder, liver, kidney, pancreas

and prostate (12-15). Specifically, estimates of up to 44% of the diabetes burden, 23% of the CVD burden and between 7-41% of various cancers are attributable to being overweight or obese (1). Among those diagnosed with T2DM, nearly 50% will die prematurely due to complications associated with CVD and another 10% or so due to renal failure (16). By 2030, the cost of the ongoing obesity epidemic is estimated to result in an excess of 6 million new cases of diabetes, nearly 6 million cases of CVD, and 500,000 newly diagnosed cancers (5) with combined medical costs in excess of \$860 billion, or roughly 1 in every 6 dollars spent on healthcare (8).

There are several well-established risk factors for T2DM and prediabetes, CVD and cancer that accompany obesity. These include chronic low-grade inflammation, impaired glucose tolerance, insulin resistance, hyperinsulinemia, and diminished β -cell function (8, 17-21). Although the pathophysiology of T2DM ultimately leads to β -cell failure and frank diabetes (22), the disease itself is initiated by a combination of polygenic and environmental factors (20, 21, 23). Excess caloric intake and limited physical activity, the hallmarks of the western lifestyle, are key factors in excess weight gain, particularly in the abdominal region, which contributes to impaired glucose tolerance and insulin resistance (23-25). This in turn leads to an exacerbating spiral of hyperinsulinemia, insulin resistance and weight gain (24). Systemic insulin resistance is likely caused by low-grade chronic inflammation in adipose tissue and the liver, the ultimate cause of which is not known (25-28). As long as the pancreatic β -cells can produce more insulin to compensate for the insulin resistance, glucose homeostasis can be maintained. However, in some individuals, the β -cell fails to produce sufficient amounts of insulin at some point (' β -cell failure'), leading to glucose intolerance and T2DM (22).

The obesity-associated low-grade chronic inflammatory state is reflected by elevated concentrations of circulating inflammatory cytokines such as tumor necrosis factor (TNF)- α and interleukin (IL)-6, or the chemokine monocyte chemoattractant protein (MCP)-1 as well as acute phase proteins like C-reactive protein (CRP) (11, 18, 28-33). To a large extent, the inflammatory cytokines and chemokines are likely derived from adipose tissue-resident leukocytes, particularly macrophages and T-cells. Circulating inflammatory cytokines such as IL-6 also induce the expression of acute phase proteins such as CRP in the liver (34). As discussed in the next section, the specific activation status and phenotype of leukocytes, particularly macrophages, plays a major role in the inflammatory process in adipose tissue.

Macrophages and Adipose Tissue: the Ying and Yang

Macrophages are localized in tissues, having differentiated from circulating monocytes. Their primary role is to engulf and digest cellular debris and invading pathogens, where they play key roles in both the adaptive and innate immune responses (35). In adipose tissue, macrophages play an important physiological role by facilitating angiogenesis in expanding tissue and the removal of dead adipocytes (36, 37). And yet, intriguingly, the infiltration of macrophages into the adipose tissues of obese mice and humans results in the release of pro-inflammatory factors such as TNF- α , IL-6, visfatin, and resistin (19, 27, 31, 38-42). This paradox was resolved in part by the revelation that lean and obese mice have strikingly dissimilar macrophage populations in their adipose tissues that differ both phenotypically and functionally. Specifically, it was shown that the adipose tissue of lean animals contains “alternatively activated” or anti-inflammatory, insulin-sensitizing macrophages more suited for tissue remodeling (36, 37, 41). With the onset of diet-induced obesity, the adipose tissue

macrophage population shifts to a more “classically activated” or inflammatory phenotype (26, 27) that expresses high levels of the inflammatory mediators TNF- α , IL-6 and inducible nitric oxide synthase (iNOS) (41) (Figure 1.1).

As outlined in Figure 1.1, this shift from low numbers of anti-inflammatory, insulin-sensitizing (IL-10 secreting) macrophages to greater numbers of TNF- α and IL-6 inflammatory cytokine-secreting phenotype results initially in localized insulin resistance in the affected adipose tissue (9). Both TNF- α and IL-6 down-regulate adiponectin expression and secretion by adipocytes (33, 43), likely through the activation of the JNK and NF- κ B pathways (41, 44). Subsequently, this inflammation of the adipose tissue affects the release of fatty acids and other adipokines such as, leptin, and resistin from adipocytes (9, 27, 45). Specifically, adipocyte insulin resistance triggers increased lipolysis and impaired lipogenesis, resulting in elevated serum free fatty acid levels, which when combined with reduced concentrations of adiponectin (46-48), produces insulin resistance in liver and muscle (28, 35, 45, 48).

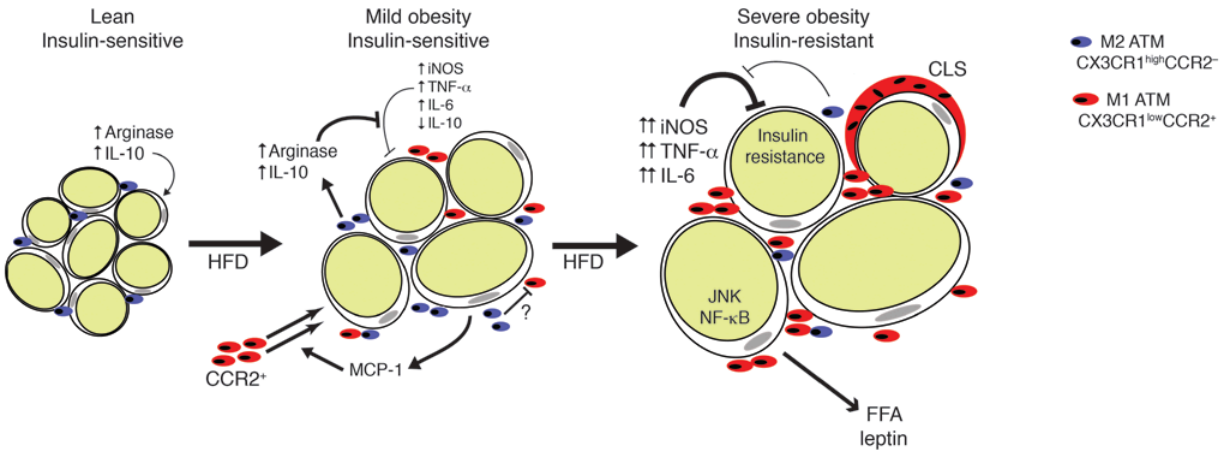


Figure 1.1: Diet induced obesity changes the composition of the macrophage population in adipose tissue. Lean insulin-sensitive adipose tissue contains relatively small numbers of non-inflammatory or M2 tissue macrophages, depicted in blue. As adipocytes enlarge and the surrounding tissue expands in response to diet-induced obesity (i.e., nutrient overload), M1 tissue macrophages expressing inflammatory markers (shown in red) are recruited. The increasing numbers of these M1 macrophages both displaces the resident M2 macrophages, and results in the production and secretion of the inflammatory cytokines IL-6 and TNF- α that directly contribute to adipocyte insulin resistance.

Figure taken from Lumeng et al. (2007) *JCI* 117(1):175-184 (41)

Linking Macrophages and Insulin Resistance

In mice, the phenotype of “classically activated” or inflammatory adipose tissue macrophages (ATM) is based largely on the expression of the integrin CD11c (41). Several studies have shown that CD11c^{pos}-ATM either secrete pro-inflammatory cytokines, or induce cytokine secretion by adipocytes (11, 29, 40, 49). Importantly, ablation of CD11c^{pos}-macrophages largely normalizes insulin sensitivity and glucose tolerance in insulin resistant, obese mice (50). Several other mouse knockout models lend additional evidence supporting pro-inflammatory macrophage infiltration of adipose tissue as a key link between obesity and

insulin resistance. For example, mice lacking I κ B kinase (51), a protein involved in the regulation of the inflammatory response in myeloid cells (e.g., monocytes, macrophages and dendritic cells), remain insulin sensitive and glucose tolerant despite diet-induced obesity (51). In addition, the absence of MCP-1 (52) or its receptor, the C-C motif chemokine receptor 2 (53), both of which are essential for macrophage recruitment, makes mice resistant to the development of obesity-induced insulin resistance. Finally, the loss of the toll-like receptor 4 (TLR4) (54), which is a key component in the activation of macrophages, or TNF- α (55), results in mice that are resistant to the development of obesity-induced insulin resistance. Collectively, these data strongly suggest that pro-inflammatory macrophages play an integral role in the development of obesity-induced insulin resistance (41, 51, 56).

Does the Anatomical Distribution of Fat Matter?

As in mice, human ATM have been shown to accumulate in response to increasing body fat mass (BFM) (31, 38-40). Importantly, studies in both rodent models and humans demonstrate that body fat distribution matters. Visceral fat, subcutaneous fat and ectopic fat are all linked to obesity-associated disease risk (57, 58). However, discrepancies exist with respect to the significance by which each of these different fat depots impacts insulin sensitivity. In mice, the intraperitoneal transplantation of subcutaneous fat, but not epididymal fat, improves insulin sensitivity (59, 60). In addition, targeted removal or expansion of visceral fat in rodents has significant impacts on metabolic function and disease risk (57).

However, in humans, such distinctions appear to be less clear, particularly given that the targeted removal of subcutaneous fat by liposuction failed to improve obesity-associated metabolic abnormalities (61). Still, despite discrepancies in adiponectin expression (46, 47), differences in metabolic function and secretory capacity have been reported for subcutaneous and visceral fat depots (62-64). Further, prospective studies have strongly linked central adiposity with metabolic disease, more so than total adiposity (58, 65). Along these lines, Klötting et al. (66) compared insulin sensitive and insulin resistant morbidly obese individuals and observed that macrophage infiltration of visceral fat was among the strongest predictors of insulin resistance. Intriguingly however, while increased inflammatory activity in visceral fat is associated with insulin resistance in obese women (67), this does not appear to be the case with pre-pubescent overweight or obese children and young men (68, 69). Furthermore, ectopic fat deposition in the skeletal muscle and liver appears to contribute to lipotoxicity and impairment of insulin action (57). In contrast, there is evidence to suggest that the expansion of the subcutaneous adipose tissue depot may actually confer a degree of protection from metabolic disruption by acting as a sink wherein excess lipid can be diverted from systemic circulation and ectopic deposition (57). Nevertheless, Klimcakova et al. (70) recently observed in both subcutaneous and visceral fat depots that genes involved in metabolic pathways, such as those pertaining to energy/electron transport, lipid, fatty acid, amino acid and pyruvate metabolism, were negatively associated with BFM and insulin resistance, while the opposite was true for immune response genes (proliferation, activation, cell death, NF- κ B). They further observed that ATM gene expression was essentially identical among the two depots, noting that overall ATM gene expression increased with increasing adiposity (71). Overall, this suggests

that in spite of potential limitations, assessments of inflammation in subcutaneous fat is associated with the degree of adipose tissue inflammation in intraabdominal adipose tissue depots.

Of Mice and Men: All Macrophages are not Phenotypically or Functionally Identical

In humans, specific macrophage phenotypes akin to those identified in mice have yet to be definitively characterized (42). There are several reasons for this. First, cell surface markers traditionally used to characterize immune cells from isolated or cultured peripheral blood leukocytes often exhibit different patterns than those present in tissues (31, 42, 72). Second, results in a given model, such as the mouse, do not necessarily translate directly to humans (73). Third, although many antigens are considered telltale markers of cell function or lineage, they are in fact often non-specific cell markers (74). Taken together, human ATM have paradoxically been described as phenotypically anti-inflammatory, based upon constitutive expression of CD206, yet capable of producing large amounts of pro-inflammatory cytokines (31, 75). Although not definitive, one potential limitation to these studies was the inclusion of only metabolically healthy and/or non-obese subjects. This could limit the ability to delineate a pro-inflammatory macrophage phenotype that may be more abundant in metabolically unhealthy or more obese individuals. Adding to the confusion, two relatively recent studies discrepantly characterized inflammatory macrophages as either CD11c^{pos}CD206^{pos} (76) or CD40^{pos}CD206^{neg} (77). Nevertheless, there remains a significant emphasis on efforts to identify and characterize a pro-inflammatory, cytokine-secreting ATM that is associated with obesity and metabolic disease.

In order to determine whether distinct ATM populations relevant to insulin sensitivity and inflammation exist in humans, we set out to isolate leukocytes from the adipose tissue of human subjects across a wide range of adiposity. The intent was to identify a pro-inflammatory, “classically activated” macrophage whose numbers in adipose tissue increase with increasing body mass, exhibit an inflammatory phenotype and are associated with insulin resistance in humans. For this study we elected to focus on the expression of the cell surface antigen Fcγ receptor III (CD16) (75), which is thought to be a marker of pro-inflammatory activation in blood monocytes and macrophages (78-82). The overall objective was to ascertain whether the number of CD16^{pos} macrophages in adipose tissue was related to clinically relevant endpoints, such as BMI or insulin resistance, and explain the variability in adipose tissue gene expression and plasma concentrations of inflammatory mediators.

Specific Aims

We are utilizing fasting plasma samples and adipose tissue specimens collected from 54 subjects recruited into three separate cross-sectional studies, exhibiting a wide range of adiposity and metabolic health.

Specific Aim 1: To assess the relationship between the number of CD16^{pos}-ATM and BMI. We hypothesize that a greater number of ATM expressing the Fc γ receptor III (CD16) is positively correlated with BMI.

Specific Aim 2: To examine the relationship between CD16^{pos}-ATM and plasma concentrations and adipose tissue gene expression of pro-inflammatory cytokines, acute phase proteins, and the anti-inflammatory and insulin-sensitizing adipokine, adiponectin. We hypothesize that the number of CD16^{pos}-ATM will correlate positively with the plasma concentrations and the adipose tissue gene expression of CRP, TNF- α , IL-6, MCP-1, and ICAM-1, and negatively with adiponectin.

Specific Aim 3: To assess the relationship between the number of CD16^{pos}-ATM and insulin sensitivity. We hypothesize that an increase in the number of inflammatory CD16^{pos}-ATM will negatively correlate with insulin sensitivity.

Public Health Impact

Today, preventable illness comprises more than 70 percent of the burden of illness and associated costs to the U.S. health care system, with preventable deaths in excess of 1 million per year (83, 84). Among the most prominent of these are obesity and diabetes, which account for more than \$200 billion, or roughly 12 percent of all health expenditures (85-90). Compared to normal weight individuals, obese people command more medical care, more frequent and longer hospitalizations and greater pharmacological intervention leading to 25-45 percent greater annual total costs (85, 90). Obese individuals are also more likely to develop diabetes and are at greater risk for developing CVD. Evidence, supported by claims data, overwhelmingly indicates that well-formulated health-promotion (i.e. prevention) programs have the potential to significantly reduce health care costs. By decreasing sick leave, outpatient services, and hospitalizations, prevention efforts have the potential of reducing costs up to three or more times the investment in program costs (83).

Clearly, improving our understanding of those factors involved in the pathogenesis of obesity and the associated chronic state of inflammation could result in significantly better public health options to combat the growing obesity epidemic. Specifically, the identification of immune cell lineages that are directly linked to the development of obesity-associated insulin resistance and systemic inflammation would provide an endpoint for intervention studies. In this context, our study has the potential to expand our understanding of the relationship between inflammation and obesity and ultimately the etiology of metabolic disease.

Chapter 2: Methods

Study Design

This study was comprised of 54 subjects that had previously enrolled into one of three cross-sectional studies: the Fat and Inflammation (F&I) study (n=18), the Nutrition and Exercise in Women (NEW) study (n=26) and the Inflammation in Diabetes Etiology Assessment (IDEA) study (n=10). The primary aim of these studies was to ascertain potential associations between macrophage infiltration and state of activation (pro-inflammatory vs. anti-inflammatory) as it pertains to overall metabolic health, based on assessments of body composition, insulin sensitivity and systemic markers of inflammation.

For F&I and NEW studies, participants were asked to attend a single clinic visit at the Fred Hutchinson Cancer Research Center (FHCRC). During the visit, participants provided a 33 mL fasting blood sample, drawn by a trained staff phlebotomist, a subcutaneous adipose tissue biopsy, performed by the study physician of record, and an anthropometric assessment that included a whole-body dual-energy X-ray absorptiometry (DEXA) scan to measure body composition. The IDEA study included individuals undergoing either bariatric surgery or some other elective abdominal procedure at the University of Washington Medical Center (UWMC). During their respective surgical procedures participants provided a 33 mL fasting blood sample along with two ~5 g adipose tissue specimens, one each from the subcutaneous and omentum depots.

Subjects

Recruitment for the F&I study ran from June 2008 through May of 2009, with 124 potential volunteers responding to flyers (see Appendix 1) posted at the FHCRC or the University of Washington. Following phone screening, 72 of these potential recruits were disqualified based on the inclusion and exclusion criteria outlined in Table 2.1. Of 52 qualified candidates, 5 declined participation, while 30 were recruited, into three groups of normal weight, overweight and obese subjects. Ten subjects were enrolled into each BMI category split evenly among men and women. This left 17 eligible candidates who were not enrolled after the recruitment goals for each BMI category had been met.

Table 2.1 Inclusion and exclusion criteria for the Fat and Inflammation (F&I) study.

Inclusion Criteria	Exclusion Criteria
<ul style="list-style-type: none">• Age 18-65 years at time of enrollment• BMI: 20-35 kg/m²• Weight stable within 5 pounds for 6 months prior to entering the study, and within 10 pounds of lifetime maximum weight• Ability to be admitted for 5 hours to the CRC at the UW Med Center on three occasions• Ability to provide informed written consent	<ul style="list-style-type: none">• Chronic medical condition, such as hypertension, cardiovascular disease, or autoimmune disease• Current or recent (within one month) use of medications likely to interfere with study endpoints (includes: lipid-lowering drugs, thiazolidinediones, metformin, sulfonylureas, corticosteroids, β-blockers, thyroid hormone, non-steroidal anti-inflammatory drugs, selective serotonin reuptake inhibitors, antibiotics and probiotics)• Pregnancy• Regular use of tobacco or current abuse of alcohol, prescription or recreational drugs

Recruitment for the NEW study ran from 2005 through 2009. A total of 126,802 women were sent a mass mailing about the study (Appendix 2), with 5,621 (4.4%) responding and another 2,048 calling in response to other media and community outreach efforts. Of these,

929 were deemed eligible after phone screening (12%) and 703 of these (~76%) subsequently attending an information session. Of these 684 were screened, with 245 either not meeting the eligibility criteria [191] or declined to participate [54]. The remaining 439 eligible and willing participants were then randomized into one of four arms. A diagram depicting the subject recruitment and study breakdown can be viewed in Appendix 3. Of the 439 subjects enrolled into the parent study, 48 were recruited into a small ancillary pilot study between February 2008 and April 2009. The inclusion and exclusion criteria are outlined in Table 2.2.

Table 2.2 Inclusion and exclusion criteria for the Nutrition and Energy in Women (NEW) study.

Inclusion Criteria	Exclusion Criteria
<ul style="list-style-type: none"> • Post-menopausal women, aged 50-70 years • BMI: >25 kg/m² (>23 kg/m² for Asian-Americans) • Current level of activity includes <100 minutes per week of moderate-vigorous exercise • Willingness to participate in a moderate exercise or calorie restriction program for 1 year • Ability to provide informed written consent 	<ul style="list-style-type: none"> • Current use of hormone replacement therapy • History of breast cancer or other serious medical conditions • Diagnosed diabetes, fasting blood glucose ≥126 mg/dl or use of diabetes medications • Current smoker or alcohol intake > 2 beverages per day • Current or recent (within one month) use of medications likely to interfere with study endpoints • Any other contraindication to participation, including abnormal exercise tolerance tests, participation in another weight loss program, use of weight loss drugs, inability to participate in the intervention program

Recruitment for the IDEA study ran from January 2010 through March 2011. Initially, UWMC staff screened a total of 1163 individuals undergoing abdominal surgery at the UWMC. Of these, 157 potential candidates were forwarded to the study coordinator for further

screening using the inclusion/exclusion criteria listed in Table 2.3. Out of this pool, 47 candidates were deemed eligible. Forty-two were contacted, with the permission of the lead surgeon, to ascertain interest in study participation. Twenty-five subjects either declined participation or could not participate for other reasons, such that 17 of these subjects were enrolled into the IDEA pilot study.

Table 2.3 Inclusion and exclusion criteria for the Inflammation and Diabetes Etiology Assessment (IDEA) study.

Inclusion Criteria	Exclusion Criteria
<ul style="list-style-type: none"> • Age 21-65 years at time of enrollment • BMI of <30 kg/m² for non-obese (controls), metabolically healthy controls and BMI ≥40 kg/m² for morbidly obese (bariatric) subjects • Undergoing an elective (control) or bariatric (Roux-en-Y gastric bypass or adjustable gastric banding) surgery at the UWMC • Ability to provide informed written consent 	<ul style="list-style-type: none"> • Presence of history of diabetes [controls], hypertension, cardiovascular disease, autoimmune disease, or any other chronic inflammatory disease (Crohn’s Disease or Ulcerative Colitis) • Current or recent (within one month) intake or administration of insulin, glucose-lowering or lipid-lowering drugs, thiazolidinediones, metformin, sulfonylureas, corticosteroids, sex steroids, β-blockers, thyroid hormone, non-steroidal anti-inflammatory drugs, selective serotonin reuptake inhibitors, antibiotics, or probiotics • Smoking (current or within past year) • Pregnancy

Following phone screening, all volunteers were sent study documents and consent forms. Subjects were then enrolled after returning signed consent forms and scheduled for clinic visits at the FHCRC (F&I and NEW studies) or study staff attended the surgical procedures (IDEA study) as scheduled by the UWMC.

Of the 95 subjects enrolled into all three studies, 54 of the subjects were included in the final analysis. Reasons for exclusion included the following: technical problems with the flow

cytometry protocol/antibody staining in 11 (5 in the F&I study, 6 in the NEW study); insufficient tissue harvested from biopsy to perform flow cytometry in 13 (4 from the F&I study, 5 in the NEW study, and 4 in the IDEA study); insufficient cell yield for flow cytometry following tissue digestion in 15 (3 in the F&I study, 11 in the NEW study, 1 in the IDEA study); no blood collected for plasma analysis (2 in the IDEA study). Thus, for the final analysis, a total of 54 subjects from all three studies were included, broken down into 18 subjects from the F&I study, 26 in the NEW study and 10 in the IDEA study.

Clinic Visits

For the F&I and NEW studies, participants were admitted to the FHCRC at 8:00 a.m. following an overnight (>10-hour) fast. Height, weight and vital signs were recorded. A fasting blood sample was drawn, collected in EDTA tubes and placed on ice. Blood samples were then immediately centrifuged for 10 minutes at 3000 rpm at 4°C. Plasma was separated, aliquoted and frozen at -80°C for later analyses. Following the fasting blood draw, a small aspiration biopsy was performed near the umbilicus to collect approximately 1 g of subcutaneous adipose tissue. Briefly, with the subject in the supine position, 1 ml of a 2% lidocaine solution in saline was injected into the skin, followed by a small incision with a scalpel. A 16-gauge spinal needle was then used to inject 10 ml of a 0.12% lidocaine in saline solution under the skin. A 14-gauge needle was inserted, and negative pressure was used to remove adipose tissue. Adipose tissue samples were then placed on Telfa pads to remove contaminating blood and excess fluid, with separate portions either snap frozen on dry ice for gene expression analysis or incubated for 30-60 minutes in a PBS + 1% BSA solution prior to collagenase digestion and analysis by flow cytometry. After cleaning the incision site, an antibiotic ointment was applied to the wound and

a sterile butterfly bandage applied as a de facto suture. The subject was then instructed to keep the area dry for the next 24 hours and not to remove the butterfly bandage for 72 hours. Upon conclusion of the biopsy procedure, body composition (i.e., body fat mass, lean mass and bone density) was assessed by a dual-energy X-ray absorptiometry (DEXA)-scan.

For the IDEA study, participants were admitted to the UWMC surgical center. Immediately prior to the surgical procedure, a fasting blood sample was drawn into EDTA and serum tubes. These samples were then processed as described above. During the procedures, the attending surgeons removed small (<5 g) portions of subcutaneous and omental adipose tissue. As before, adipose tissue samples were both snap frozen on dry ice for gene expression analysis and incubated for 30-60 minutes in a PBS + 1% BSA solution prior to collagenase digestion and analysis by flow cytometry.

Laboratory Analyses

Fasting glucose, insulin and high-sensitivity C-reactive protein (hsCRP) concentrations were measured at the Northwest Lipid Research Laboratories, Seattle, WA. All samples from each study were measured in the same assay. Glucose concentrations were determined enzymatically on a Hitachi 917 Autoanalyzer (Roche Diagnostics, Mannheim, Germany). Insulin was measured by immunoenzymatic assay on an AIA 600 II auto-analyzer (Tosoh Bioscience Inc., San Francisco, CA). Immunonephelometry with a Behring Nephelometer II (Behring Diagnostics, Somerville, NJ) was used to measure hsCRP. The cytokine IL-6 (R&D Systems, Minneapolis, MN) and plasma adiponectin in the F&I and IDEA studies (Alpco, Salem, NH) were measured by ELISA. For the NEW study, adiponectin was measured in serum by

radioimmunoassay (Millipore Inc., Billerica, MA) using 125 I-labeled murine adiponectin as described previously (91). Intra- and inter-assay coefficients of variation (CV) were less than 15% for all analytes. All samples within a study were batched together on the same ELISA plates or instrument runs, but separately for each individual study.

Flow cytometric analysis was performed on whole blood and stromavascular cells (SVC) freshly isolated from adipose tissue. Briefly, 100 ml of whole blood was labeled with fluorescently conjugated antibodies (see below) and incubated at room temperature for 20 min. This was followed by BD Pharm Lyse™ Lysing Buffer (BD Biosciences, San Jose, CA), according to the manufacturers protocol, and resuspension in 150 ml of 0.2% BSA / 0.09% NaN₃ / PBS staining buffer with 5 mM EDTA. Isolation of SVC from adipose tissue was carried out by a 1 hour digestion at 37°C in a 0.035 mg/ml solution of Liberase Blendzyme 3 (Roche Diagnostics, Indianapolis, IN) in PBS on a rocking platform. The digest was passed through a 180 mm mesh filter and rinsed twice with PBS + 1% BSA and then resuspended in 300 ml of staining buffer containing 1 mg/ml of human IgG1 to block Fc receptors.

Whole blood and SVC were stained with a combination of five directly conjugated primary antibodies for CD3, CD14, CD15, CD16, CD24, CD206 and human leukocyte antigen (HLA-DR) or their respective isotype controls purchased from either BD Pharmagen (San Jose, CA) or BioLegend (San Diego, CA). Samples were analyzed immediately following staining using a LSR II flow cytometer (Beckton Dickinson, San Jose, CA) to collect up to 30,000 events in a broad gate, defined by forward- and side-scatter attributes. Individual cell populations were selectively isolated using a FACS Aria™ III cell sorter (Beckton Dickinson). Analyses were

conducted with FlowJo version 8.8.2 (TreeStar, Ashland, OR) using histograms and dot plots. Positive staining was determined by comparison to staining with the appropriate isotype controls. Specifically, positive expression was defined such that the false-positive rate in the isotype control tube equaled 2%. The expression of various combinations of the above markers was used to identify specific sub-populations of myeloid cells.

Total RNA was extracted from adipose tissue and sorted cell populations using RNeasy[®] mini kits (Qiagen, Hilden, Germany) and quantified using the RiboGreen[®] RNA Quantitation Kit (Invitrogen, Carlsbad, CA). cDNA synthesis was carried out on 0.5-1.5 µg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and PCR performed using pre-designed ABI TaqMan[®] Gene Expression Assays on an Mx3005P[®] Multiplex QPCR System (Stratagene, Cedar Creek, TX). For whole adipose tissue, target genes were adiponectin, MCP-1, IL-6, IL-10, intercellular adhesion molecule 1 (ICAM-1), interferon (IFN) γ and TNF- α , while for isolated cell analyses these were restricted to IL-6, IL-10, IFN γ and TNF- α . All results are expressed as target gene copy number per ng RNA, based on the conversion of Ct values into gene copy number using the standard curve. Gene expression in sorted cell populations was normalized to a housekeeping gene (18s rRNA) and the number of cells the RNA was isolated from. For whole adipose tissue, a normalization factor was applied to each gene using three housekeeping genes, glucuronidase- β , phosphoglycerate kinase 1, and 18s rRNA using the previously freely available GeNorm application (<http://medgen.ugent.be/~jvdesomp/genorm/>, accessed 01/2010) (92). Stable expression of all three of these housekeeping genes in adipose tissue was previously demonstrated (93).

As a surrogate for formal quantification of insulin sensitivity, we calculated the homeostasis model assessment insulin resistance index (HOMA-IR) using the following formula: [fasting insulin (mU/l) x fasting glucose (mmol/l)] / 22.5 (94).

Statistical Analyses

For all variables, we assessed whether they were consistent with a normal distribution by checking normal plots and histograms, and by using Kolmogorov-Smirnov and Shapiro-Wilk tests. Those variables not normally distributed were logarithmically transformed prior to analyses. Pearson's correlation coefficients were calculated for normally distributed variables, and Spearman's correlation coefficients for non-normally distributed variables. Descriptive data are displayed as mean \pm standard deviation for normally distributed data and the median and interquartile range for non-normally distributed data. Statistical analyses were performed using SPSS software (version 16.0, SPSS Inc., Chicago, IL), and the α -error level was set to 5% for all analyses.

Chapter 3: Results

Subject Characteristics

Overall, 95 subjects were enrolled into the three cross-sectional studies presented herein. Of these, 41 subjects were excluded either because insufficient tissue or cells were available for subsequent analysis, or due to technical complications with the analytical methods. The results are presented in a chronological order that reflects the completion of the initial cross-sectional F&I study, and its preliminary findings. This was then followed by the inclusion of the additional subjects drawn from the other two studies as a confirmatory analysis and the gene expression analysis of ATM either expressing or not expressing CD16.

Anthropometric and clinical parameters of the three study populations are presented in Table 3.1. The non-obese and obese groups showed predictable differences with regard to fasting glucose, insulin, hsCRP, and adiponectin plasma concentrations. Of note, the higher adiponectin values reported for the NEW study likely stem from the use of different assays to measure this parameter. Also, the higher hsCRP concentrations in the NEW study may be attributable in part to the fact that subjects in this study were on average both 10 years older (95), and among the non-obese, more overweight (34) than participants in the other two studies.

Phenotyping Adipose Tissue Macrophages

As described previously (31, 42, 75), the combination of the monocyte/macrophage marker CD14 and the mannose receptor CD206 were used to identify ATM ($CD14^{pos}CD206^{pos}$; Figure 3.1, panels A and C). The $CD14^{neg}CD206^{pos}$ cells were identified as neutrophils based on positive expression of both CD15 and CD24 (Figure 3.2). ATM were further subdivided into two

subpopulations (Figure 3.1, panels B and D) (75), based on the expression of CD16, which exhibited considerable inter-individual variability. The absolute number of CD16^{pos}-ATM was generally lower among lean subjects (Figure 3.1, panel B) relative to obese subjects (Figure 3.1, panel D).

Table 3.1 Baseline characteristics of study participants.*

	F&I Study (n=18)		NEW Study (n=26)		IDEA Study (n=10)	
	Non-obese (BMI 22-29 kg/m ²)	Obese (BMI 30-35 kg/m ²)	Non-obese (BMI 25-29 kg/m ²)	Obese (BMI 30-43 kg/m ²)	Non-obese (BMI 24-29 kg/m ²)	Obese (BMI 30-78 kg/m ²)
n (male/female)	9 (4/5)	9 (6/3)	14 (0/14)	12 (0/12)	2 (2/0)	8 (3/5)
Age (yr)	41.6 ± 12.1	45.4 ± 10.7	57.6 ± 5.3	58.2 ± 5.1	41.0 ± 15.6	43.9 ± 9.1
BMI (kg/m ²)	25.0 ± 2.2	32.5 ± 1.9	28.0 ± 1.5	34.6 ± 4.2	24.9 ± 0.5	49.6 ± 15.1
Fasting Glucose (mmol/l)	5.3 ± 0.5	6.0 ± 0.8	5.1 ± 0.3	5.3 ± 0.4	5.3 ± 0.1	6.8 ± 2.5
Fasting Insulin (μU/ml)	5.9 ± 1.5	14.7 ± 6.1	11.1 ± 5.0	16.9 ± 8.4	8.8 ± 5.2	11.7 ± 7.8
HOMA-IR	1.4 ± 0.4	4.0 ± 1.9	2.5 ± 1.2	4.0 ± 1.8	2.0 ± 1.2	3.9 ± 3.5
Adiponectin (μg/ml)	8.0 ± 3.5	5.5 ± 2.2	13.0 ± 5.7	10.8 ± 4.1	4.9 ± 0.8	4.2 ± 1.5
hsCRP (mg/l)	0.7 ± 0.4	3.1 ± 1.8	2.8 ± 3.2	4.1 ± 3.0	0.4 ± 0.04	4.7 ± 3.9
IL-6 (pg/ml) [†]	0.7 (0.6 – 1.6)	1.7 (1.1 – 3.1)	1.4 (0.8 – 1.9)	1.6 (1.2 – 2.4)	10.4 (0.5 – 20.4)	3.2 (1.9 – 6.3)

* Data are means ± SD or medians (interquartile range)[†], BMI: body mass index, HOMA-IR: homeostasis model assessment insulin resistance index, hsCRP: high sensitivity C-reactive protein, IL: interleukin.

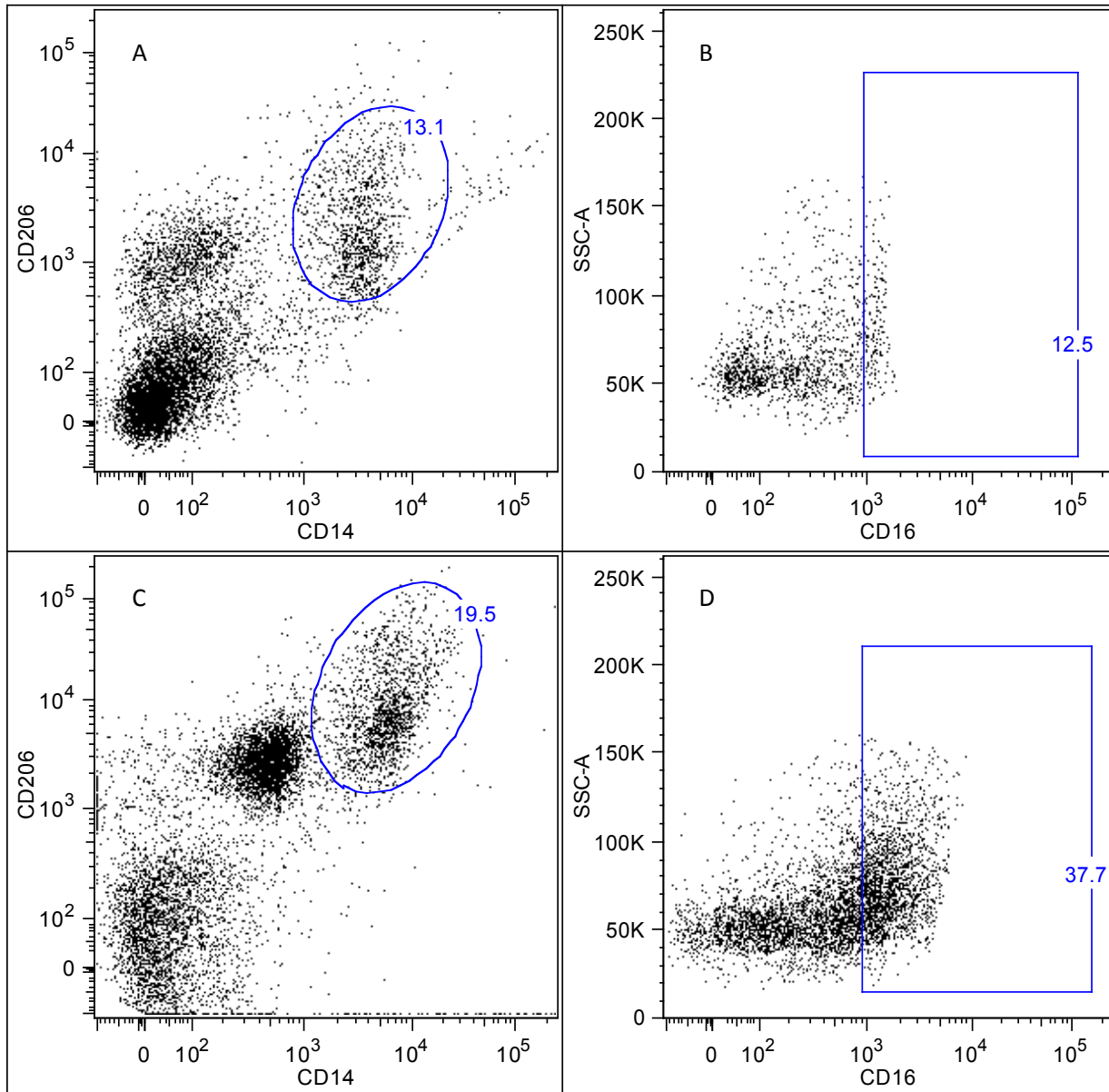


Figure 3.1. Identification of adipose tissue macrophages (ATM). Expression of the monocyte/macrophage marker CD14 and co-expression of the mannose receptor (CD206) identifies the upper right cell population as tissue macrophages (panels A and C). Within this population the expression of the inflammatory marker CD16 is highly variable, but generally lower among leaner, insulin-sensitive subjects (panel B) and increases with increasing subject adiposity (panel D).

Accumulation of CD16^{pos}-ATM Correlates with Body Mass Index, Insulin Resistance and Measures of Adipose Tissue and Systemic Inflammation

Overall, we observed that the number of CD16^{pos}-ATM (normalized per g of adipose tissue) was associated with BMI (Figure 3.3; $r=0.60$, $p=0.009$) and also correlated strongly with insulin resistance (HOMA: $r=0.65$, $p=0.004$; Figure 3.4). CD16^{neg}-ATM exhibited slightly weaker, yet statistically significant, associations with BMI ($r=0.54$, $p=0.02$) and HOMA ($r=0.49$, $p=0.04$). We similarly assessed these associations between CD16^{pos}-ATM normalized to total live cell number, shown in Table 3.2, which produced similar results. All subsequent associations are therefore reported as normalized per g adipose tissue.

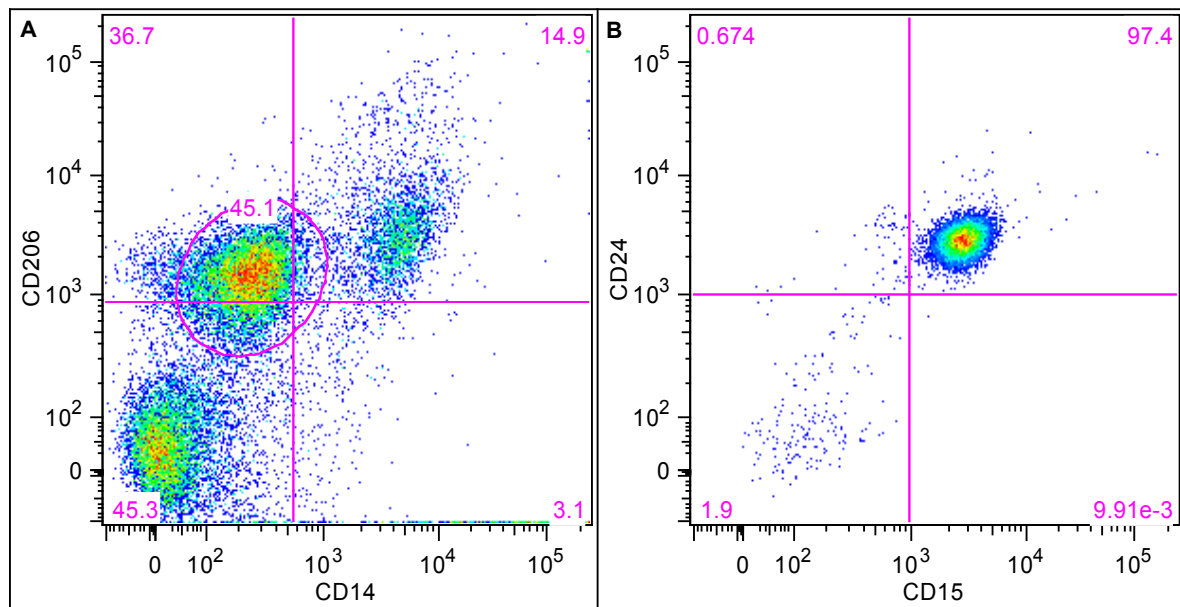


Figure 3.2. Adipose tissue neutrophils are CD14^{neg/lo}CD206^{pos}. Co-expression of both CD15 and CD24 (panel B) among the identified CD14^{neg/lo}CD206^{pos} population (panel A) confirms that these are in fact neutrophils.

Inflammatory gene expression within adipose tissue revealed that the number of CD16^{pos}-ATM correlated with the expression of TNF- α ($r=0.67$, $p=0.002$; Figure 3.5) and ICAM-1 ($r=0.72$, $p=0.001$; Figure 3.6), but less so with the expression of MCP-1 ($r=0.46$, $p=0.057$; Figure 3.7) and IL-6 ($r=0.29$, $p=0.242$; Figure 3.8). Curiously, CD16^{neg}-ATM number correlated only with ICAM-1 expression ($r=0.59$, $p=0.01$), but none of the rest: TNF- α ($r=0.37$, $p=0.127$), MCP-1 ($r=0.27$, $p=0.274$) and IL-6 ($r=0.26$, $p=0.301$). Furthermore, adiponectin expression was highly and inversely correlated with adipose tissue TNF- α expression ($r=-0.81$, $p<0.001$) and the number of CD16^{pos}-ATM ($r=-0.70$, $p=0.001$; Figure 3.9). Here again, the number of CD16^{neg}-ATM did not correlate with tissue adiponectin expression ($r=-0.37$, $p=0.129$). CD16^{pos}-ATM number also correlated inversely with plasma adiponectin concentrations ($r=-0.57$, $p=0.014$; Figure 3.11), and positively with CRP ($r=0.66$, $p=0.003$; Figure 3.12) and IL-6 ($r=0.60$, $p=0.009$; Figure 3.13). For CD16^{neg}-ATM there was a similarly strong association with CRP ($r=0.66$, $p=0.003$), but none with plasma adiponectin ($r=-0.24$, $p=0.341$) or IL-6 ($r=0.46$, $p=0.057$) levels.

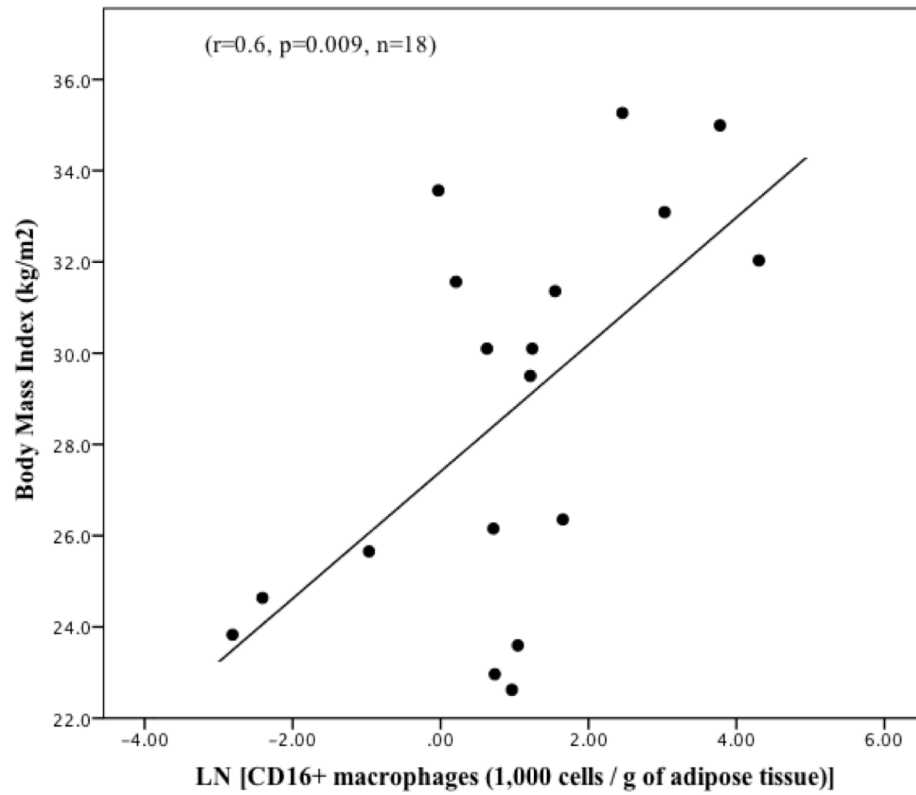


Figure 3.3 Association between CD16^{pos}-ATM and BMI.

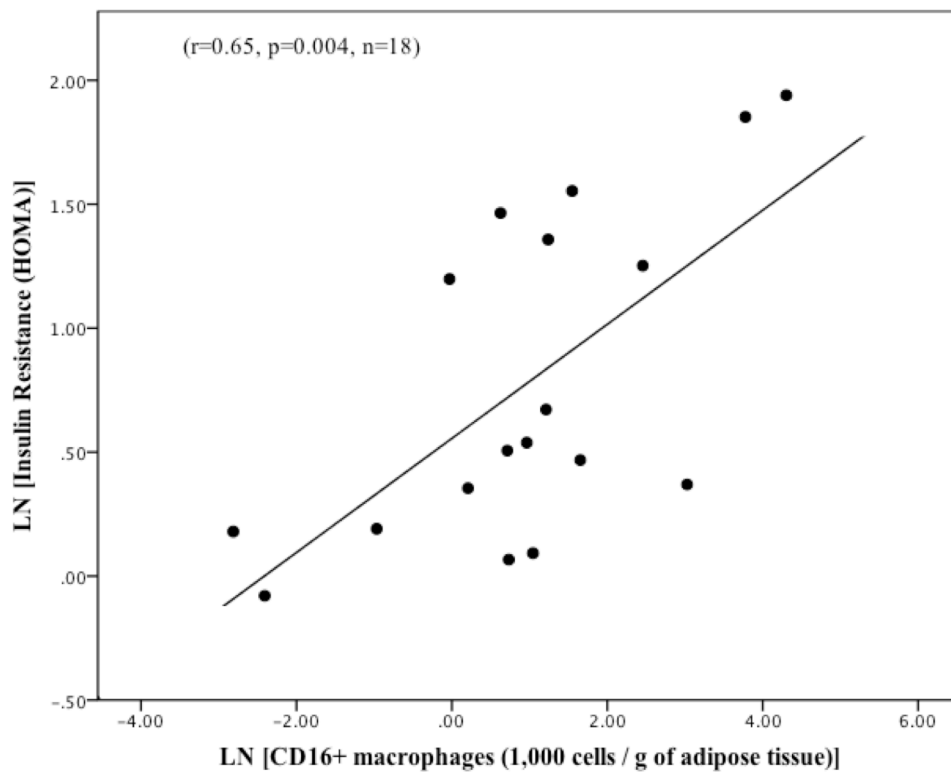


Figure 3.4 Association between CD16^{pos}-ATM and insulin resistance (HOMA).

Table 3.2 Pearson Correlations between CD16^{pos}-ATM and CD16^{neg}-ATM normalized to total live cells and measured outcome variables (n=18).

	CD16 ^{pos} -ATM	CD16 ^{neg} -ATM
BMI (kg/m ²)	r=0.55, p=0.019	r=0.53, p=0.023
HOMA-IR	r=0.58, p=0.011	r=0.38, p=0.124
AT TNF- α mRNA copy number per ng RNA	r=0.83, p<0.001	r=0.75, p<0.001
AT IL-6 mRNA copy number per ng RNA	r=0.24, p=0.338	r=0.28, p=0.261
AT ICAM-1 mRNA copy number per ng RNA	r=0.74, p<0.001	r=0.75, p<0.001
AT Adiponectin mRNA copy number per ng RNA	r=-0.79, p<0.001	r=-0.59, p=0.011
Plasma adiponectin (μ g/ml)	r=-0.64, p=0.004	r=-0.34, p=0.163
Plasma hsCRP (mg/l)	r=0.60, p=0.009	r=0.68, p=0.002
Plasma IL-6 (pg/ml)	r=0.60, p=0.009	r=0.51, p=0.032

BMI: body mass index, HOMA-IR: homeostasis model assessment insulin resistance index, AT: adipose tissue, TNF- α : tumor necrosis factor, IL: interleukin, ICAM: intercellular adhesion molecule, hsCRP: high sensitivity C-reactive protein.

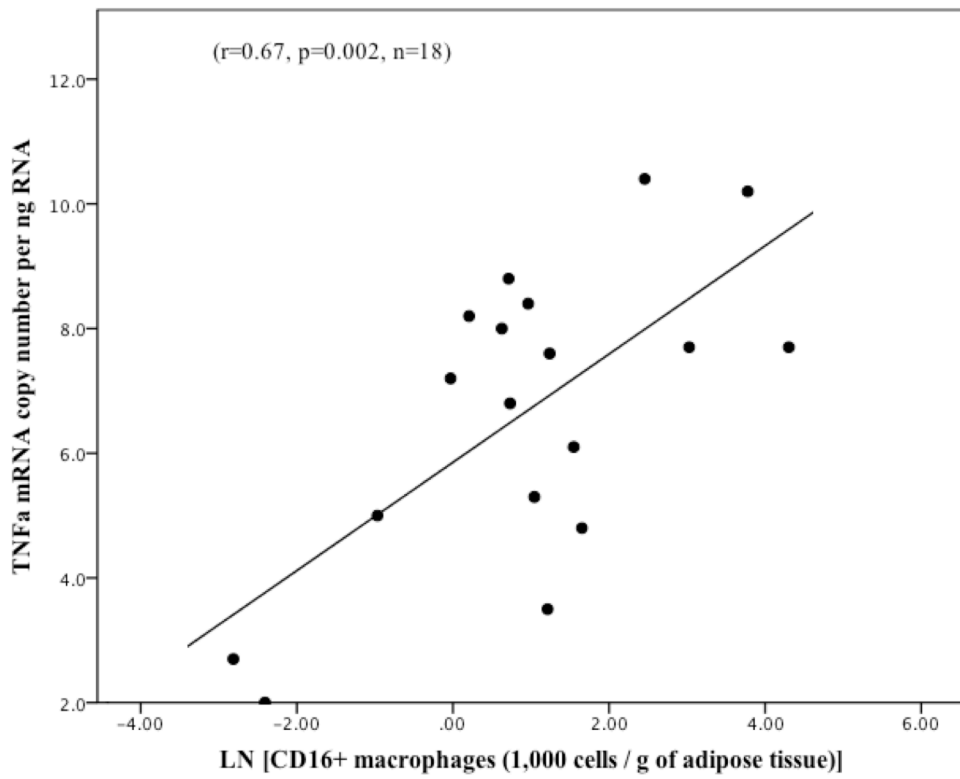


Figure 3.5 Association between CD16^{pos}-ATM and TNF- α mRNA expression.

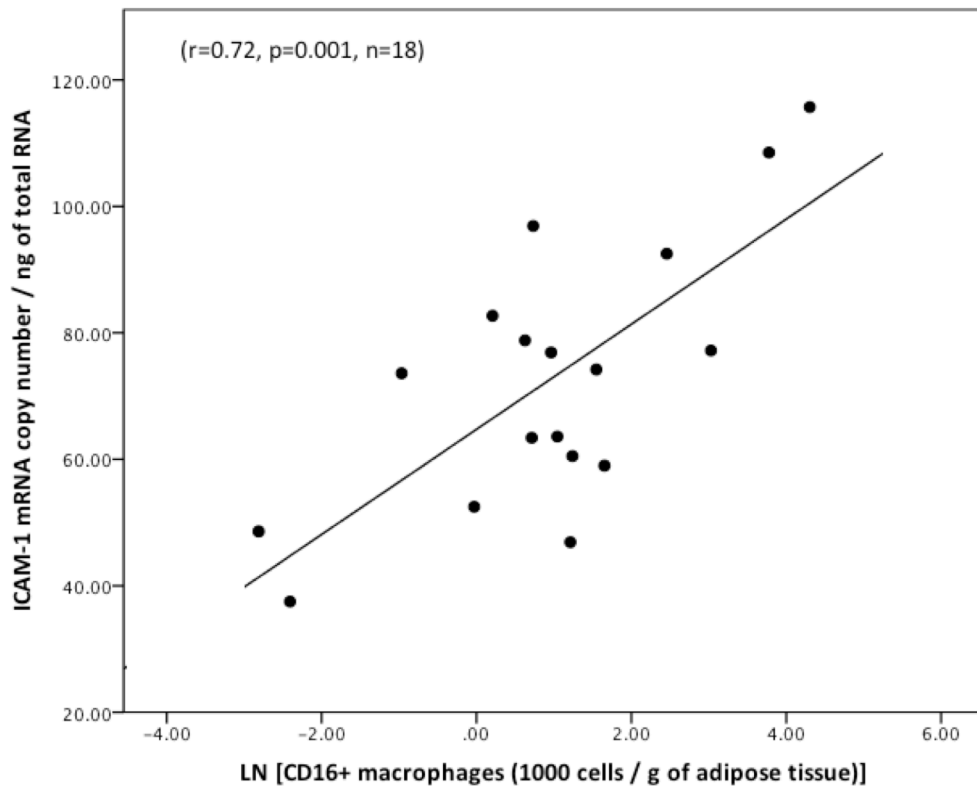


Figure 3.6 Association between CD16^{pos}-ATM and ICAM-1 mRNA expression.

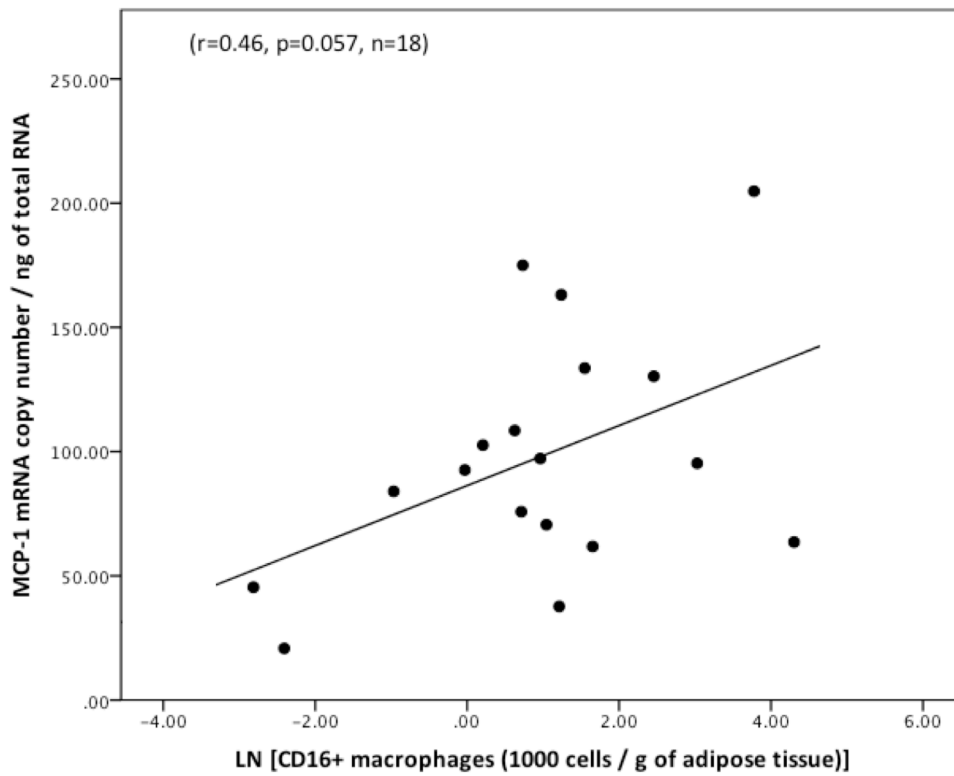


Figure 3.7 Association between CD16^{pos}-ATM and MCP-1 mRNA expression.

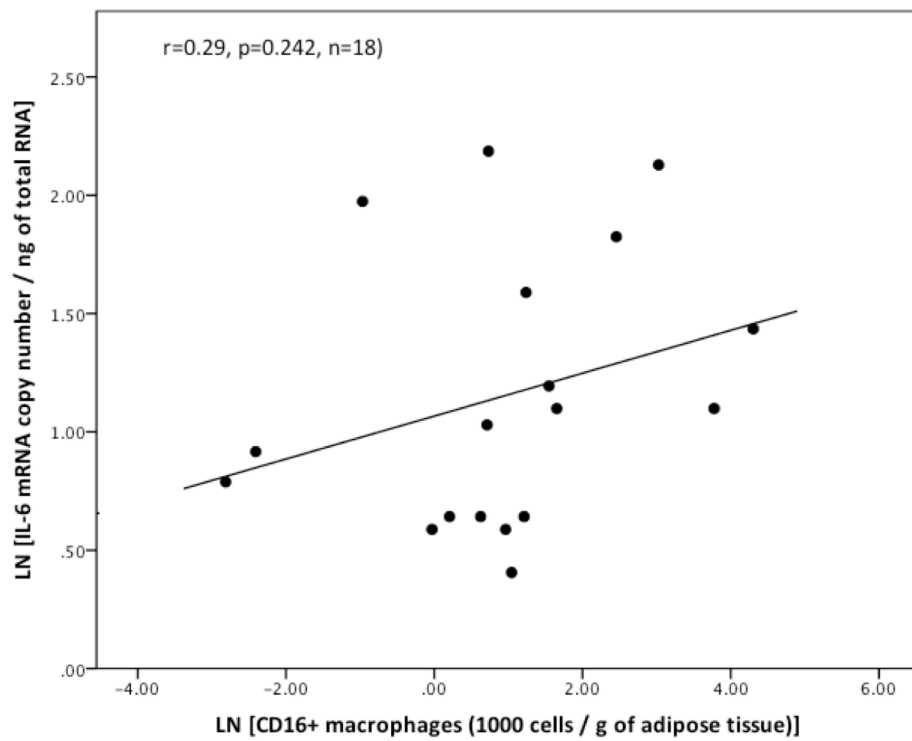


Figure 3.8 Association between CD16^{pos}-ATM and IL-6 mRNA expression.

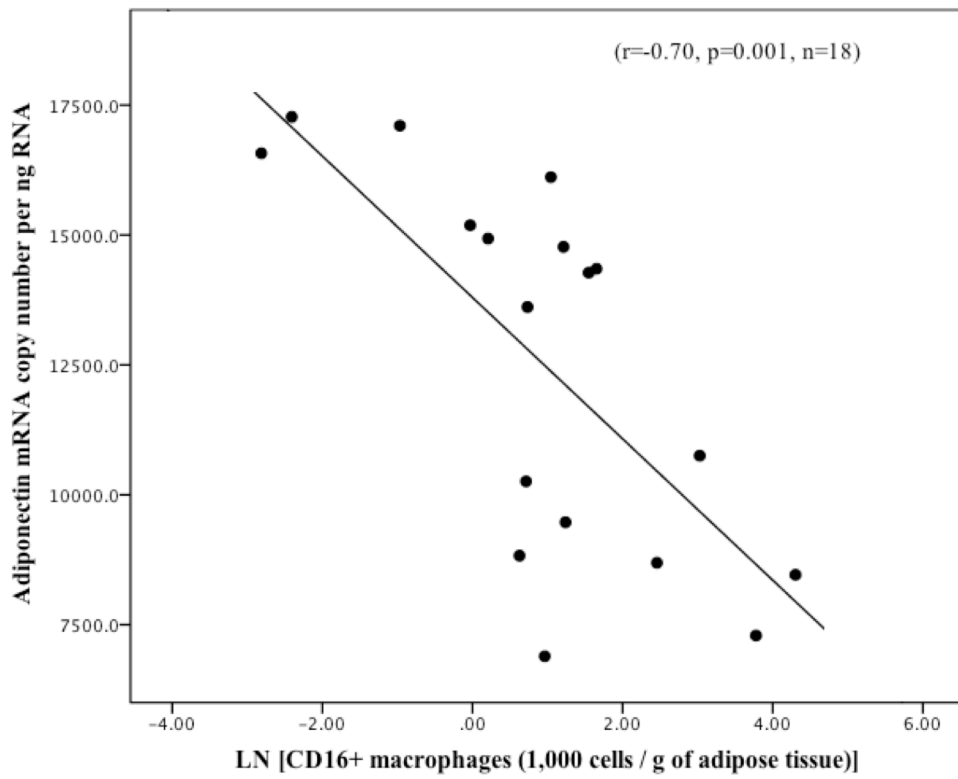


Figure 3.9 Association between CD16^{pos}-ATM and adiponectin mRNA expression.

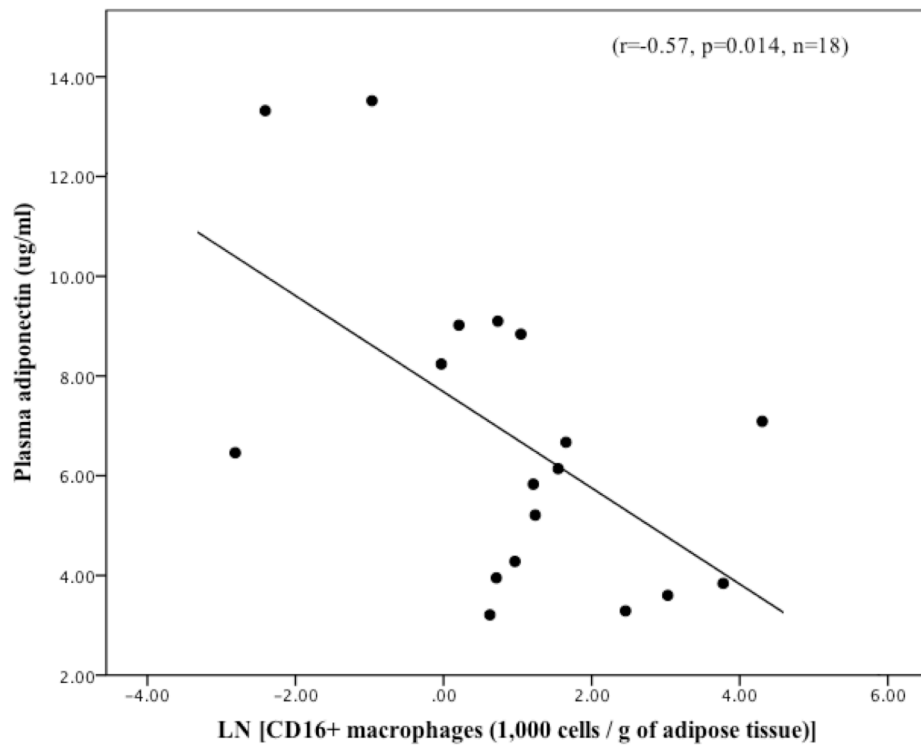


Figure 3.10 Association between CD16^{pos}-ATM and plasma adiponectin concentration.

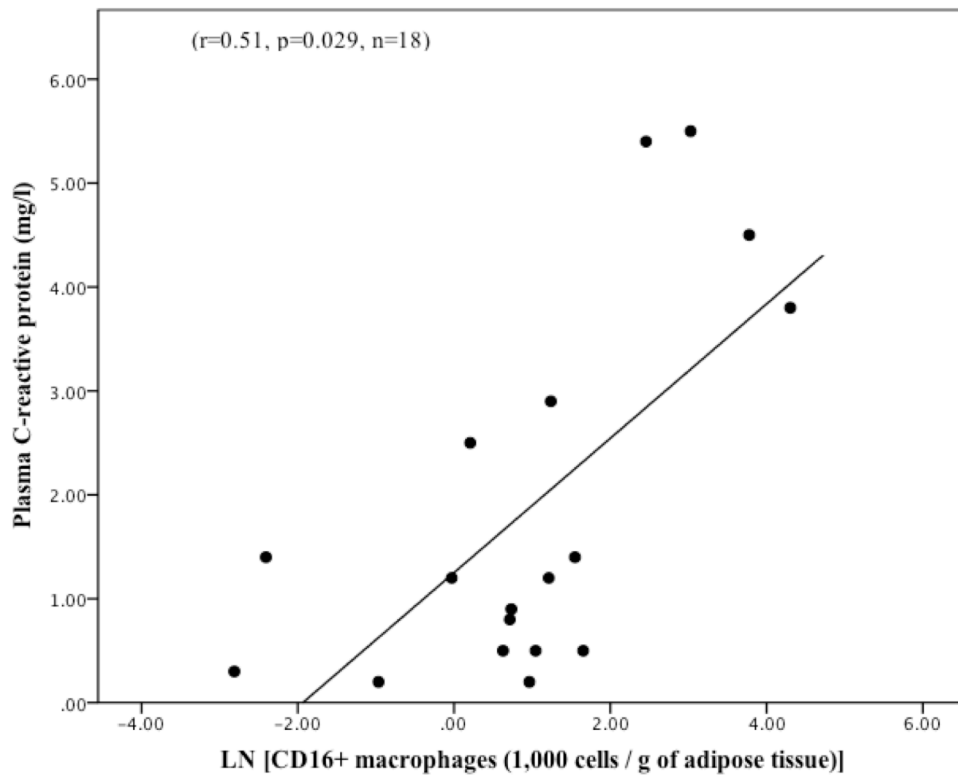


Figure 3.11 Association between CD16^{pos}-ATM and plasma CRP concentration.

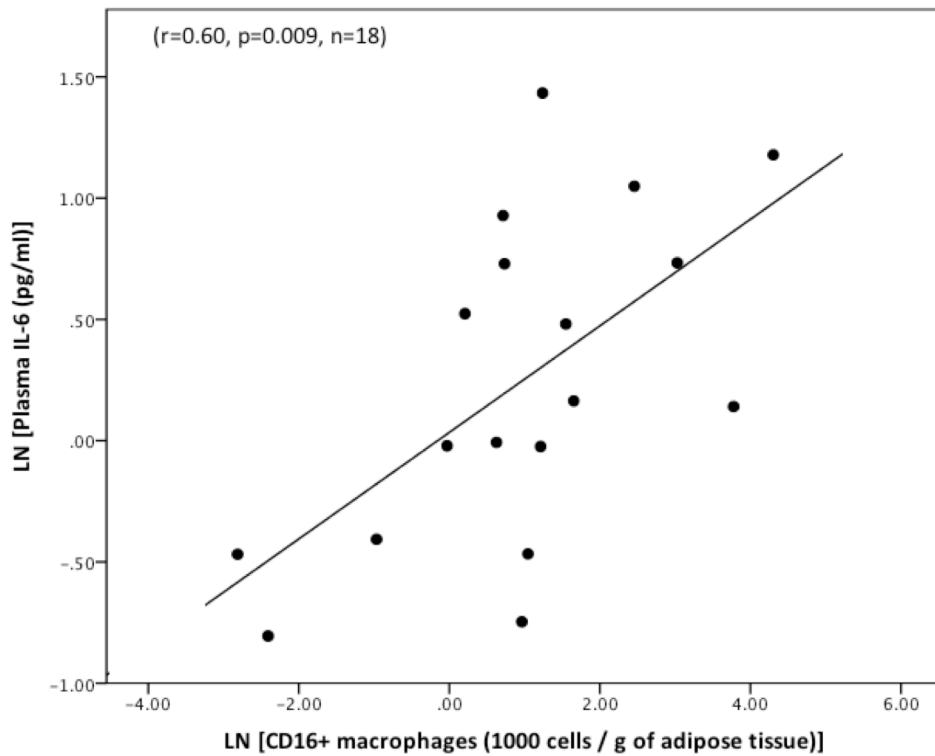


Figure 3.12 Association between CD16^{pos}-ATM and plasma IL-6 concentration.

CD16 is not an Inflammatory Marker in ATM

We sought to confirm our initial observed associations using subjects drawn from two other cross-sectional study populations, and to determine whether CD16^{pos}-ATM exhibit a more pro-inflammatory gene expression profile. Pooling 36 additional subjects drawn from two other study populations we observed that none of the original associations held between the number of CD16^{pos}-ATM in adipose tissue and BMI ($r=0.19$, $p=0.258$), HOMA ($r=-0.29$, $p=0.083$), plasma concentrations of CRP ($r=-0.10$, $p=0.547$) and IL-6 ($r=0.14$, $p=0.408$). Similarly, for CD16^{neg}-ATM correlations with BMI ($r=-0.01$, $p=0.953$), CRP ($r=0.06$, $p=0.729$) and IL-6 ($r=0.6$, $p=0.75$) were not significant. The Pearson correlations for both CD16^{pos}-ATM and CD16^{neg}-ATM are shown in Table 3.3. When expanded to include all 54 subjects the Pearson correlations for CD16^{pos}-ATM and HOMA ($r=0.15$, $p=0.277$) and plasma CRP ($r=0.15$, $p=0.271$) remained non-significant, while correlations between CD16^{pos}-ATM and BMI ($r=0.27$, $p=0.047$) and plasma IL-6 ($r=0.33$, $p=0.014$) were significantly correlated. For CD16^{neg}-ATM, only plasma CRP ($r=0.27$, $p=0.045$) was significant, while the others BMI ($r=0.17$, $p=0.208$), HOMA ($r=0.19$, $p=0.164$), and IL-6 ($r=0.26$, $p=0.063$) were not. This suggests that among these measured factors that there were no stronger associations for CD16^{pos}- than for CD16^{neg}-ATM.

Table 3.3 Pearson Correlations between CD16^{pos}-ATM and CD16^{neg}-ATM normalized by total live cells and measured outcome variables for the combined populations (n=36) of the NEW and IDEA Studies.

	CD16 ^{pos} -ATM	CD16 ^{neg} -ATM
BMI (kg/m ²)	r=0.19, p=0.258	r=-0.01, p=0.953
HOMA-IR	r=-0.29, p=0.083	r=-0.11, p=0.522
hsCRP (mg/l)	r=-0.10, p=0.547	r=0.06, p=0.729
IL-6 (pg/ml)	r=0.14, p=0.408	r=0.06, p=0.75

BMI: body mass index, HOMA-IR: homeostasis model assessment insulin resistance index, TNF- α : tumor necrosis factor, IL: interleukin, ICAM: intercellular adhesion molecule, hsCRP: high sensitivity C-reactive protein.

Additionally, based on the initial strong association between CD16^{pos}-ATM, insulin resistance and systemic inflammation, we sought to ascertain whether CD16^{pos}-ATM actually expressed more pro-inflammatory cytokines than CD16^{neg}-ATM. To this end we sorted T cells (CD3^{pos}), neutrophils (CD15^{pos}CD16^{hi}), and ATM (CD14^{pos}CD206^{pos}) that either expressed or did not express CD16 from the SVC liberated from adipose tissue. Along with whole adipose tissue and adipocytes, we then isolated the mRNA of each cell population and assessed the expression of the cytokines, TNF- α , IL-6, IL-10, and IFN γ . Consistently, CD16^{pos}-ATM exhibited lower mRNA copy numbers than CD16^{neg}-ATM, with individual cell populations normalized by 18s expression or by total cell number following isolation. For TNF- α , mRNA copy number among CD16^{pos}-ATM was 342 ± 262 vs. 1087 ± 1283 in CD16^{neg}-ATM ($p=0.133$; Figure 3.13) when normalized by 18s and 634 ± 65 vs. 130 ± 184 ($p=1.0$; Figure 3.14) when expressed in relation to total cell number. For IL-6 expression, CD16^{pos}-ATM expressed 25 ± 20 vs. 26 ± 28 ($p=1.0$; Figure 3.15) copies normalized by 18s or 5 ± 4 vs. 6 ± 8 copies per 1000 cells ($p=1.0$; Figure 3.16). Similarly, for IL-10 mRNA copy number was 69 ± 41 vs. 101 ± 95 ($p=1.0$; Figure 3.17) and 15 ± 15 vs. 24 ± 32 ($p=1.0$;

Figure 3.18), respectively. For IFN γ , CD3^{pos}-T cells, as expected, were the pivotal source of IFN γ ($p < 0.001$; Figures 3.19 & 3.20), regardless of the normalization approach. Nevertheless, CD16^{pos}-ATM expressed 1.4 ± 2.6 vs. 1.5 ± 1.9 copies of mRNA in CD16^{neg}-ATM, when normalized by 18s ($p = 1.0$; Figure 3.19), and 0.2 ± 0.4 vs. 0.3 ± 0.4 copies of mRNA normalized per 1000 cells ($p = 1.0$; Figure 3.20). This strongly suggests that CD16^{pos}-ATM are not a primary source of inflammatory mediators in human adipose tissue.

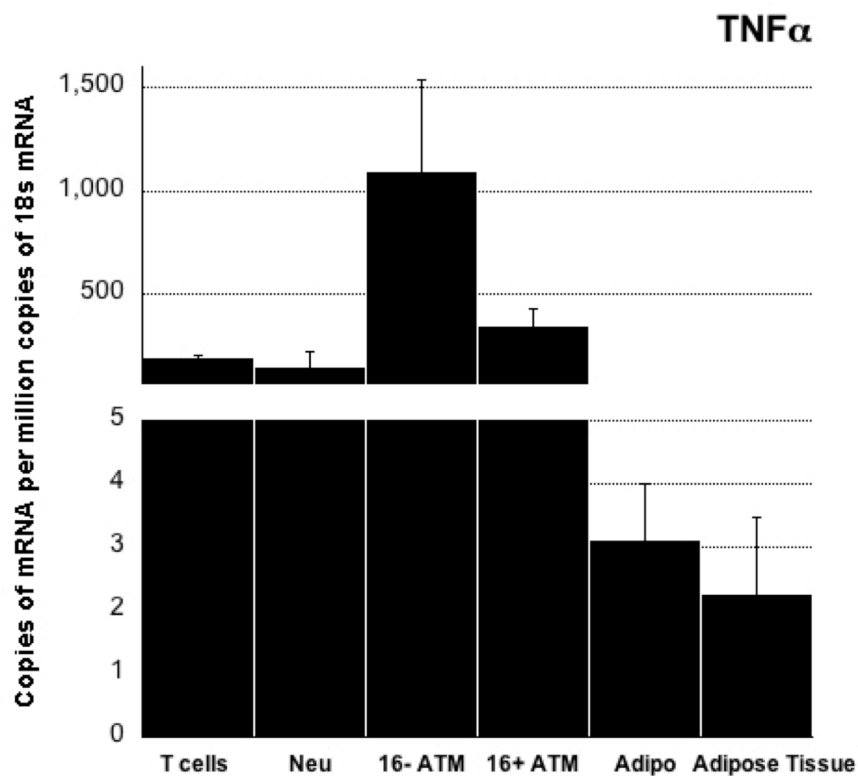


Figure 3.13 TNF- α mRNA expression in whole adipose tissue and isolated cell populations normalized by 18s expression levels.

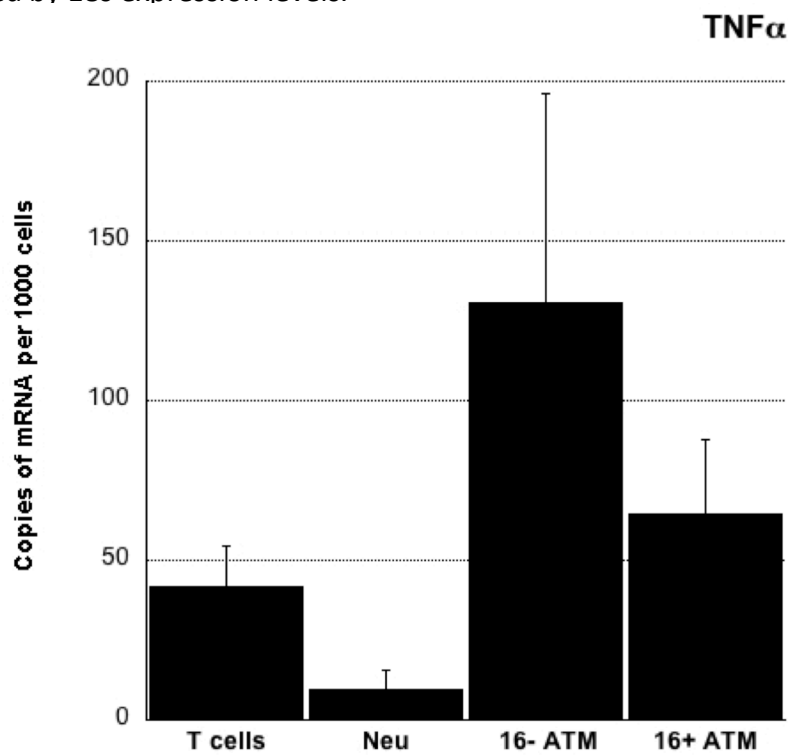


Figure 3.14 TNF- α mRNA expression normalized by cell number among isolated cell populations.

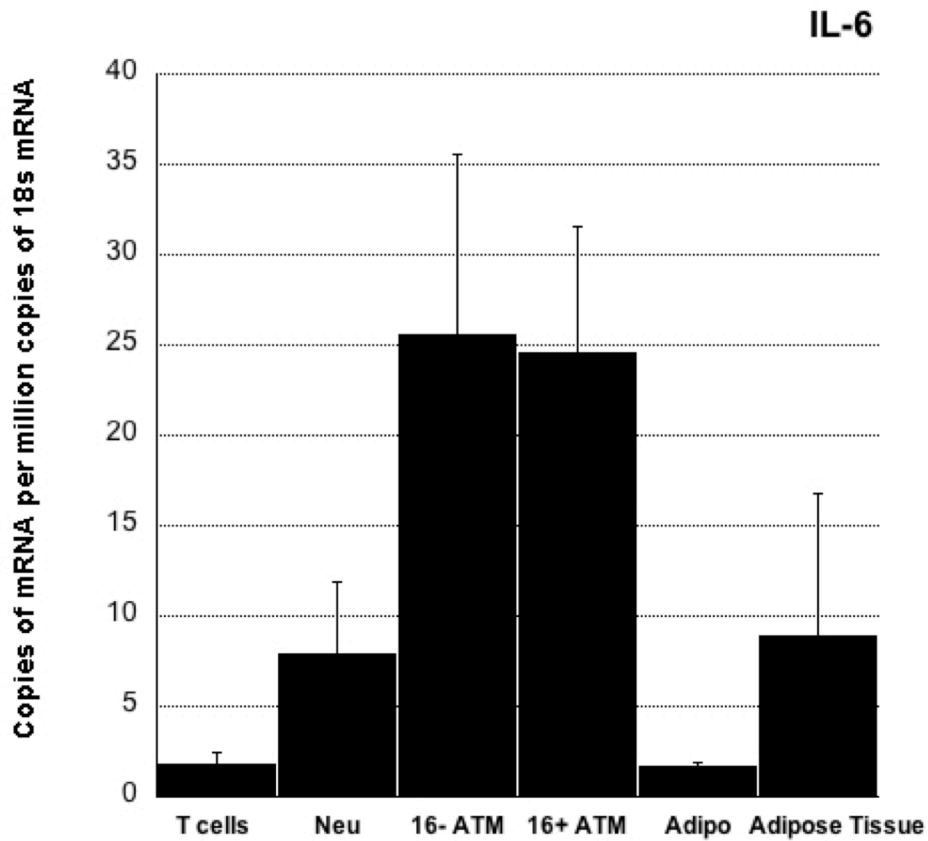


Figure 3.15 IL-6 mRNA expression in whole adipose tissue and isolated cell populations normalized by 18s expression levels.

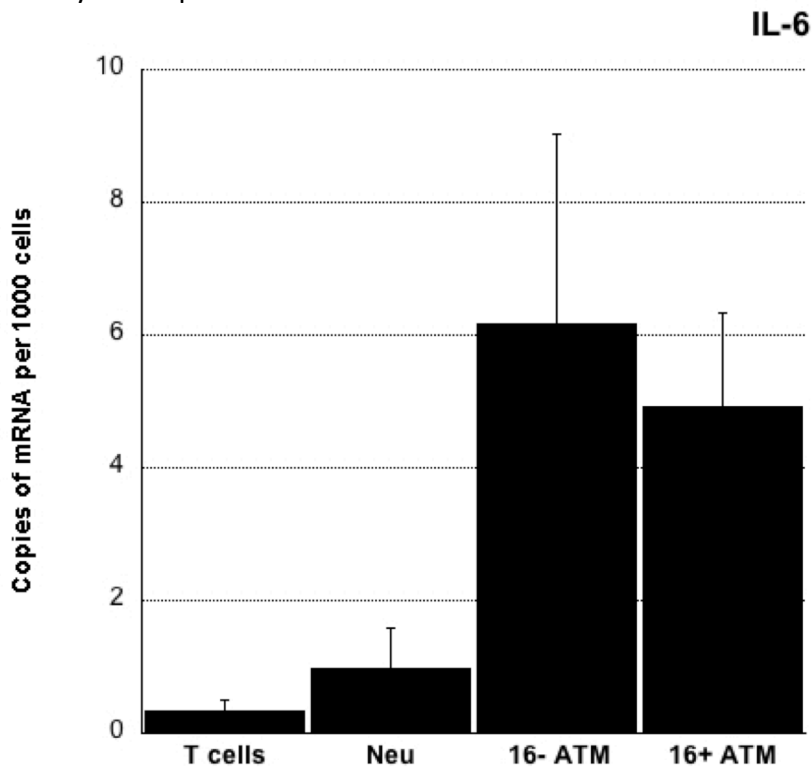


Figure 3.16 IL-6 mRNA expression normalized by cell number among isolated cell populations.

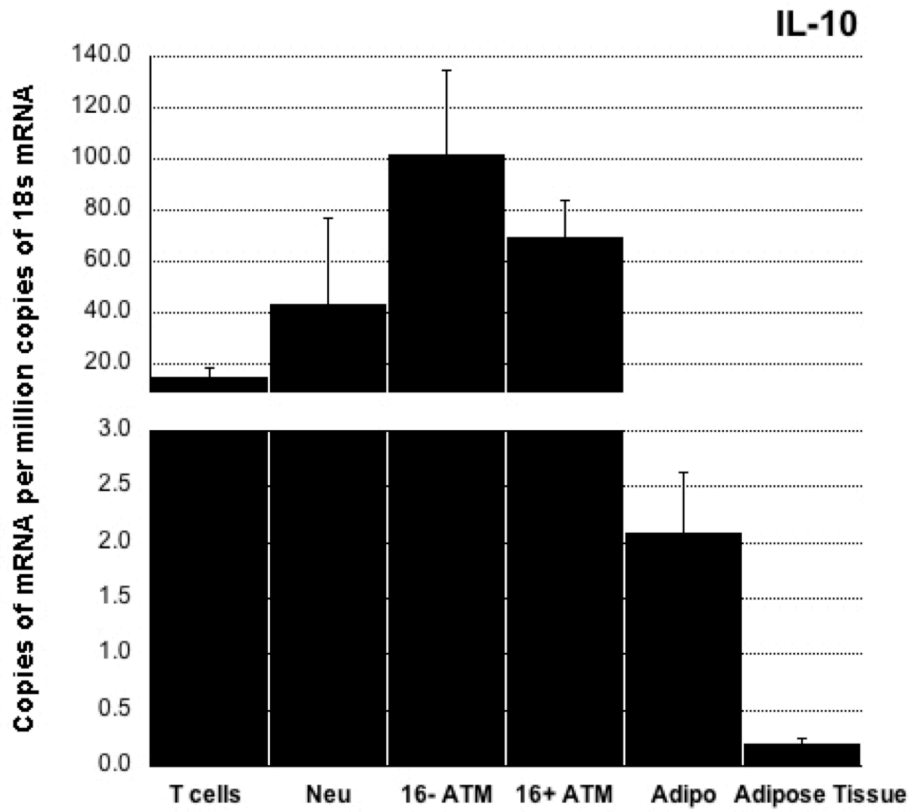


Figure 3.17 IL-10 mRNA expression in whole adipose tissue and isolated cell populations normalized by 18s expression levels.

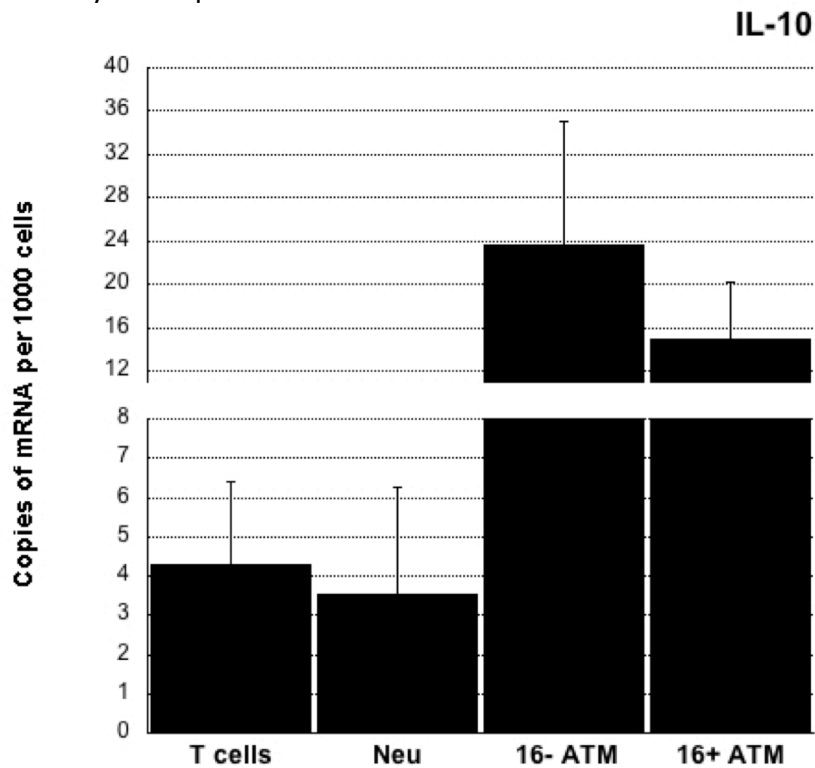


Figure 3.18 IL-10 mRNA expression normalized by cell number among isolated cell populations.

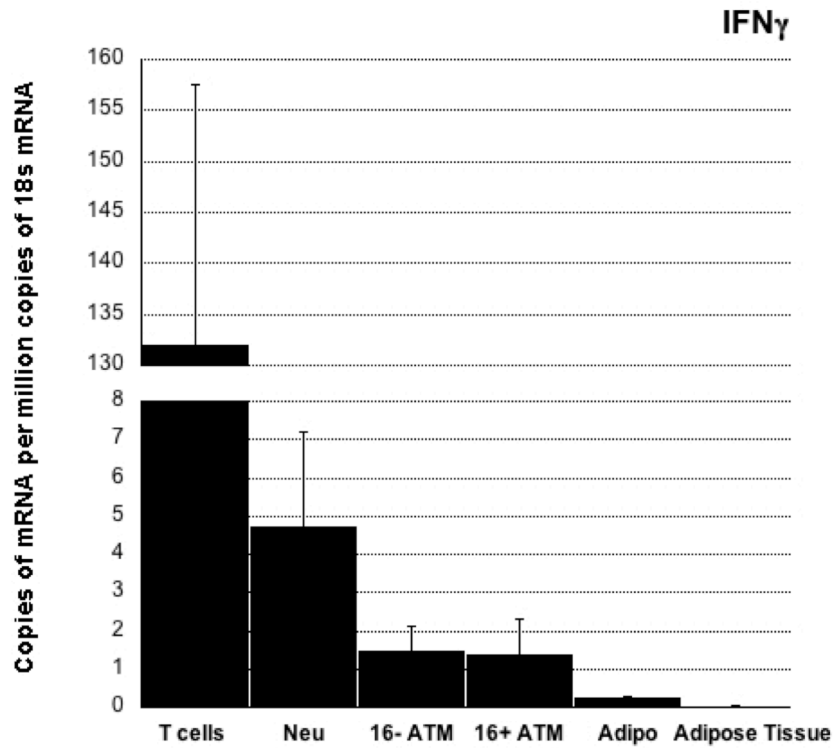


Figure 3.19 IFN γ mRNA expression in whole adipose tissue and isolated cell populations normalized by 18s expression levels.

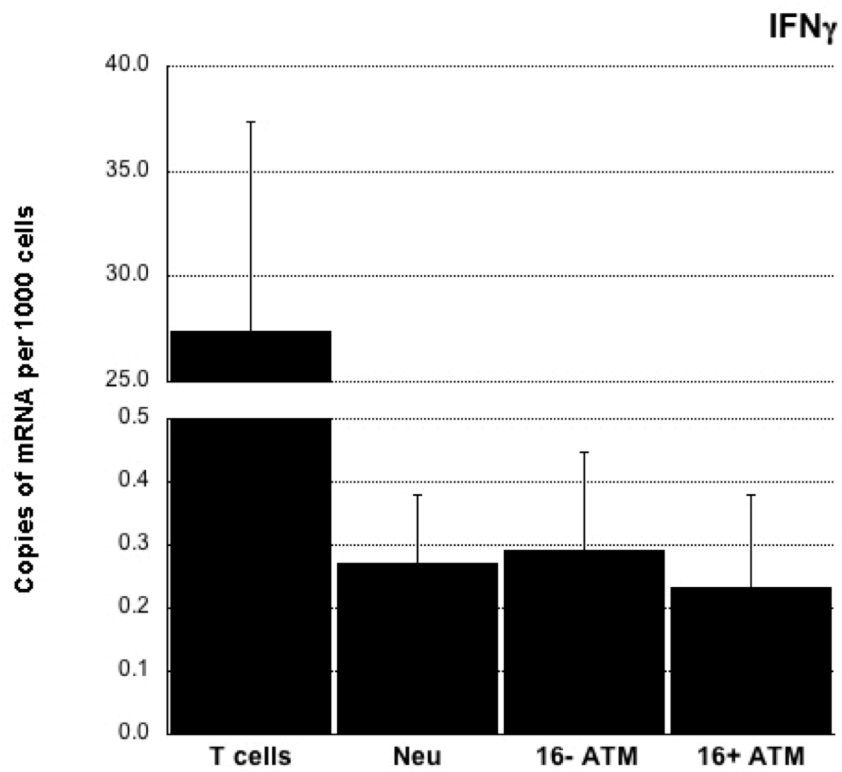


Figure 3.20 IFN γ mRNA expression normalized by cell number among isolated cell populations.

Chapter 4: Discussion

We set out to ascertain whether CD16 was a marker of “classically activated” pro-inflammatory “M1” ATM. In our initial study of men and women varying widely in age, adiposity, and metabolic health, we observed that CD16 expression on ATM was (a) greater within the subcutaneous adipose tissue of obese, insulin resistant subjects relative to leaner, more insulin sensitive subjects; (b) was positively associated with both the expression of mediators of inflammation in adipose tissue and with systemic inflammation; and (c) was negatively correlated with both adipose tissue expression and plasma concentrations of the key insulin sensitizing hormone adiponectin. This suggested that CD16^{pos}-ATM may be a strong candidate for an inflammatory macrophage subtype linking obesity and insulin resistance, consistent with data implicating CD16^{pos}-ATM in the etiology of other chronic inflammatory disease (96-98). However, further inquiry failed to substantiate this conclusion. First, evaluation of an additional 36 subjects failed to replicate any of the originally observed associations between CD16^{pos}-ATM and insulin resistance, BMI and several plasma markers of inflammation. Selective isolation of specific cell populations from adipose tissue following enzymatic digestion revealed that CD16^{neg}-ATM had a gene expression profile suggesting greater production of pro-inflammatory cytokines than CD16^{pos}-ATM.

Our rationale for focusing on CD16 expression in adipose tissue macrophages was based on a number of previous findings strongly suggesting that CD16 is a marker of inflammatory activation and enhanced TNF production in myeloid cells (78-82). For example, Cooper et al. (99) observed that CD16 expression on monocytes was significantly higher among patients with rheumatoid arthritis (RA) relative to healthy individuals, and that this shift in expression pattern

could be detected early during the onset of disease. Moreover, several other RA studies identified up-regulated CD16 expression on macrophages found in the synovial fluid and surrounding membrane-lining layers (96-98). In addition, CD16^{pos} monocytes also appear to play a role in the initiation and perpetuation of mucosal inflammation associated with inflammatory bowel disease (100), and the pathogenesis of coronary heart disease (101, 102). Finally, Powell et al. (103) recently demonstrated in obese glucose-tolerant men that expanding fat mass was associated with increased macrophage infiltration of adipose tissue, and that this correlated with increasing numbers of circulating CD16^{pos} monocytes. Unfortunately, one key limitation in all of the aforementioned studies is that they similarly relied on correlative associations between CD16^{pos} monocytes and measured outcomes. None of these studies sorted the CD16^{pos} or CD16^{neg} cell populations so as to ascertain whether CD16^{pos} monocytes/macrophages are indeed more pro-inflammatory.

In contrast, two studies examined macrophages in adipose tissue specifically stained for CD16 on ATM. In one of these, Zeyda et al. (31) reported that CD16 was not expressed on ATM. This clearly contradicts the results of both the present study and that of Bourlier et al. (75). And yet, in contrast to our initial findings, Bourlier et al. reported a stronger correlation between CD16^{neg}-ATM and BMI rather than with CD16^{pos}-ATM. Although their study population was large, one of the presumed limitations of this study was its failure to include obese individuals (75). Nevertheless, Bourlier et al. (75), reported that CD16^{neg}-ATM, exhibited higher mRNA expression of inflammatory markers IL-6, TNF- α , and MCP-1 compared to CD16^{pos}-ATM. Importantly, this study confirms these results and extends them with the inclusion of obese individuals, demonstrating that these associations hold across a wider range of BMI, further

supporting the assessment that CD16 is not a marker for distinguishing between a “classically activated” inflammatory (M1) or an “alternatively activated” anti-inflammatory (M2) ATM phenotype.

Study Strengths and Limitations

A key strength to the present study lies in our efforts to confirm the initial observed associations by expanding the study population, and the interrogation of inflammatory gene expression among sorted cell populations, including CD16^{pos}-ATM vs. CD16^{neg}-ATM. In addition, the population studied included a broad range of BMI and was well controlled by the exclusion of individuals with known inflammatory diseases or drug use likely to interfere with inflammatory processes.

Our study also had limitations. As an observational study there was no controlling for potential confounding due to inter-subject variability (i.e., no subject matching) that could account for discrepancies in ATM number and BMI or insulin sensitivity. This could potentially suggest correlations that otherwise do not exist. More to the point, the absence of associations between CD16^{pos}-ATM and the measured outcomes among the additional 36 subjects examined suggests that the observed associations in the original 18-member study population may represent a chance finding. On another front, BMI is not the best indicator of adiposity or metabolic health. In fact, several studies have observed that despite clear health risks associated with obesity, that up to 25% of obese subjects remain insulin sensitive or “metabolically healthy” (104-106). In this context, the study exclusion criteria, which selected against those with diagnosed metabolic disease, in combination with a small study population may have altered the distribution of metabolic status between overweight and morbidly obese

individuals. Such a bias may have limited the ability to identify associations between CD16^{pos}–ATM, BMI, insulin resistance and adipose tissue inflammation. Lastly, the enzymes used for tissue digestion and cell isolation differed between studies which has been shown to impact both cell recovery and cell surface marker expression (107), either of which could impact obtained results.

Conclusions

In summary, the conclusion drawn from the current and preceding studies is that while CD16 may represent an inflammatory marker in blood monocytes, it does not appear to serve as a marker of “classical activation” and pro-inflammatory activity in ATM.

References

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Are you willing to donate a small amount of Fat Tissue, Blood, and Stool?

Then you might want to participate in a pilot study at the Fred Hutchinson Cancer Research Center to help us explore the role of fat tissue in causing low-grade inflammation which may affect the risk of several diseases.

We are looking for women and men who are:

- 21-65 years old
- Overall healthy
- Body Mass Index (BMI) between 22- 40 kg/m²
- Not currently dieting or involved in a weight loss program.
- Able to come to the Fred Hutchinson Cancer Research Center for one visit in the morning.
- Willing to donate one blood sample.
- Willing to donate two small fat samples.
- Willing to have your body composition measured by a low grade form of overall body X-ray called a DEXA-scan.
- Willing to collect and return one stool sample.

You will receive:

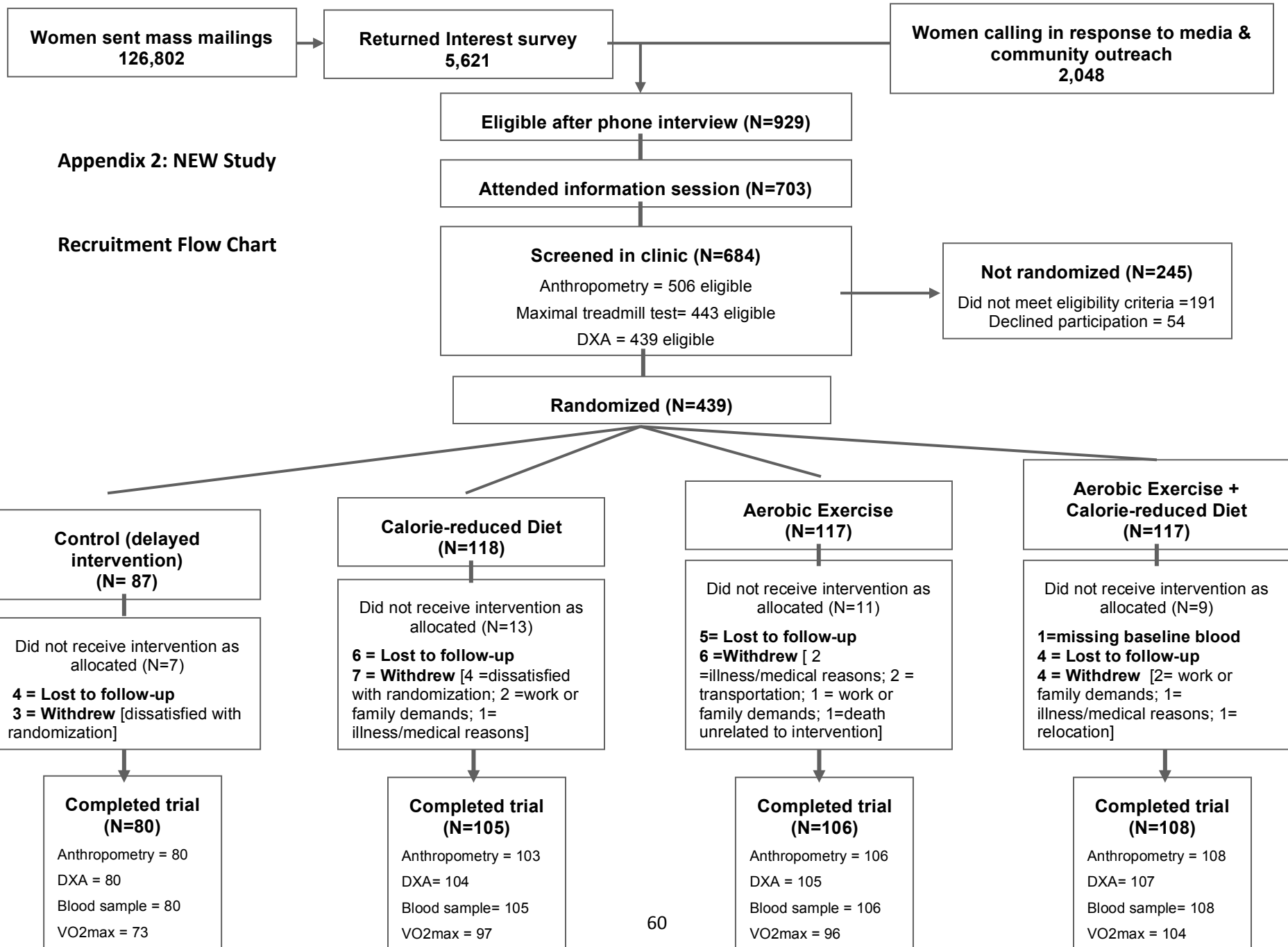
- A compensation of \$150 for your time, travel and donation after completion of the whole study.
- The results of the DEXA-scan, which will provide information about your body composition including your bone density.

Fred Hutchinson Cancer Research Center

Division of Public Health Sciences

Call: Ilona Larson, PhD, (206) 667-1116

IRB approval date: 2/20/08



Appendix 2: NEW Study

Recruitment Flow Chart

Appendix 3: Nutrition & Exercise for Women (NEW) Study Interest Survey



Nutrition & Exercise for Women (NEW) Study Interest Survey

OFFICE USE ONLY	
PARTICIPANT ID #:	_____
DATE RECEIVED/COMPLETED	____/____/____
STAFF ID #:	_____
STATUS: I or E	CODE: _____

If you are interested in joining the NEW Study please complete this form and return it in the postage-paid envelope enclosed.

The purpose of this breast cancer prevention study is to test the effectiveness of a diet and exercise program on weight loss in healthy postmenopausal women. All interested women will be screened for the study, and eligible participants will be enrolled by chance (like the toss of a coin) into one of these four study groups:

- A. Diet group - which will involve meeting regularly with a nutritionist for a year long weight loss program,**
- B. Exercise group - which will involve exercising at our FHCRC Exercise Research Facility, located on our Southeast Lake Union campus, for 45 minutes per session, 3 times per week under the supervision of an exercise specialist. Women assigned to this group will be asked to eventually exercise 2 additional days per week on their own, for a total of 5 days per week for 12 months,**
- C. Diet & Exercise group - which will involve meeting the requirements of both the diet and exercise group just mentioned,**
- D. Delayed group - which will involve not making any lifestyle, dietary, weight and exercise changes to your current lifestyle during the 12 month study enrollment. After completion of the 12 month study requirements, this group will receive 2 months of group exercise training at our exercise facility as well as 4 group meetings with the nutritionist. Members assigned to this group will also receive the study materials on healthy diet and exercise.**

All screening tests, nutrition meetings, exercise classes and personal training will be provided at no cost to study participants.

-
1. If you are eligible for the study, would you be willing to be assigned by chance to any of the four groups just described?

₁ No ₂ Yes

2. Would you be willing and able to meet regularly with a nutritionist and follow a diet program for 12 months that involves changing your diet and eating habits?

₁ No ₂ Yes

3. Would you be willing and able to follow an exercise program for 12 months that involves moderate to vigorous exercise, such as treadmill walking or stationary bicycling, for 45 minute sessions, 5 days per week?

₁ No ₂ Yes

4. Would you be available to attend exercise classes 3 times per week at the Southeast Lake Union Fred Hutchinson Cancer Exercise Research Center?
(Morning sessions and evening sessions will be available)

₁ No ₂ Yes

5. If you are assigned by chance to the delayed group, are you willing to maintain your current diet and exercise level and not participate in any other exercise/weight loss programs/studies during your 12 month enrollment in the NEW study?

₁ No ₂ Yes

The next set of questions ask about your health and medical history.

6. What is your current age? _____ years old

7. How tall are you? * _____ / _____ * These two amounts are required
feet inches

8. How much do you weigh? _____ pounds

9. How much did you weigh a year ago? _____ pounds

10. How much did you weigh three months ago? _____ pounds

11. When was your last menstrual period?
(If you can't remember the month, just indicate the year. If you can't remember the year, give your best guess)

____ / ____
month year

12. In the past 6 months have you taken any female hormones like estrogen (Premarin or Estrace), progesterone (Provera), Testosterone, Raloxifene (Evista), Tamoxifen, or herbal hormones (such as Dong Quai, Black Cohash, Wild Yam)? These might be pills, skin patches, implants, vaginal creams or suppositories, shots, or birth control pills.

₁ No ₂ Yes

13. Have you ever been told you had heart disease, angina, heart attack or heart failure?

₁ No ₂ Yes

13.1 If yes, what was the date of your last heart attack or hospitalization for heart disease?

____ / ____
month year

14. Have you ever been told you had a stroke, mini-stroke, or abnormal EKG?

₁ No ₂ Yes

14.1 If yes, what was the date of your last stroke? ____ / ____
month year

15. Do you currently have or have you ever been told that you have cancer (other than basal or squamous cell skin)?

₁ No ₂ Yes

15.1 If yes, please specify the type of cancer and date/year of diagnosis:

16. Do you currently have or have you ever been told that you have diabetes?

₁ No ₂ Yes

17. Do you currently have or have you ever been told that you have emphysema, asthma, or other lung disease?

₁ No ₂ Yes

18. Do you have fibromyalgia?

₁ No ₂ Yes

19. Do you have arthritis?

₁ No ₂ Yes

20. Do you have epilepsy?

₁ No ₂ Yes

21. Have you ever had a hip or joint replacement ?

₁ No ₂ Yes

22. Do you have bipolar disorder or depression?

₁ No ₂ Yes

(If yes, is it currently treated/controlled? ₁ No ₂ Yes)

23. Do you have any other chronic health problems?

₁ No ₂ Yes (If yes, Please specify: _____)

24. Do you smoke or use any tobacco products?

₁ No ₂ Yes

25. Do you drink alcohol?

₁ No ₂ Yes

(If yes, on the average, how many drinks of wine, beer or liquor do you have:

___ ___ per day or ___ ___ per week or ___ ___ per month ?

One drink is equal to one 12 oz beer or one 4-oz glass of wine or one ounce of liquor.)

26. Are you currently or have you in the past taken part in any other research studies that involve taking some type of medication or changing your diet or exercise pattern in any way?

₁ No ₂ Yes (If yes, please specify and give dates : _____)

27. Have you ever had any gastric altering surgery? (ie, stomach stapling, Rouen Y, gastric band)

₁ No ₂ Yes

28. During the past 6 months, have you had any serious medical problems or hospitalizations?

₁ No ₂ Yes (If yes, please specify: _____)

29. Are you currently on a diet or using any methods of weight loss such as diet pills or commercial programs (i.e., Weight Watchers, Jenny Craig, etc.)?

₁ No ₂ Yes

30. Are you currently on a medically prescribed diet?

₁ No ₂ Yes

31. Are you willing to eliminate other weight loss programs or weight loss products for the next 14 months other than what the study nutritionist prescribes for you?

₁ No ₂ Yes

32. Do you have any history of eating disorder or eating problems, such as binge eating, anorexia, bulimia?

₁ No ₂ Yes

The next set of questions ask about your ability to exercise.

33. In the past year, have you exercised at a gym (or "Curves") or outside doing activities such as jogging, aerobics, or fast walking that increase your heart rate and cause you to sweat?

₁ No ₂ Yes

(If yes, are you now regularly exercising 3 or more times per week? ₁ No ₂ Yes)

34. Do you have any health problems that significantly limit your ability to exercise (such as severe arthritis or bursitis, or asthma that worsens with exercise)?

₁ No ₂ Yes (If yes, please specify: _____)

35. When you exercise, walk, or walk up stairs, do you have any problems with your breathing (shortness of breath or wheezing)?

₁ No ₂ Yes

36. When you exercise, walk or walk up stairs, do you have any chest pain or discomfort in your chest, arms or neck?

₁ No ₂ Yes

37. Has a doctor ever told you that you should not exercise?

₁ No ₂ Yes (If yes, what was the reason? _____)

38. Have you ever had any of the following illnesses or conditions? (if yes, specify when diagnosed and if you have it now)

Plantar fasciitis (inflammation and pain on the bottom of your feet) No

Yes (dates: _____)

Hip problems (specify: _____) No Yes (dates: _____)

Knee pain/problems No Yes (dates: _____)

Neuroma of the feet No Yes (dates: _____)

Painful Bunions No Yes (dates: _____)

39. Do you plan to have surgery during the next 14 months?

₁ No ₂ Yes

(If yes, please describe the surgery that you plan to have, and when you plan to have it)

_____ / _____
month year

The next set of questions ask about your time commitments and travel.

40. Do you work for pay or do volunteer work?

₁ No ₂ Yes

(If yes, what is your job or volunteer title? _____)

41. How many total hours per week do you work and/or volunteer? _____ hours

42. Do you plan to live in the greater Seattle or Eastside area for the next 14 months?

₁ No ₂ Yes

43. Do you plan to be out of town for periods longer than 30 consecutive days during the next 14 months?

₁ No ₂ Yes

The next set of questions will help us focus our recruitment efforts

44. If you did not receive this survey as part of a mailing, how did you hear about the study?*

₁ Friend/Relative

₄ Radio

₇ Enrolled participant

₂ Flyer

₅ Newspaper

₈ Other: _____

₃ TV

₆ Newsletter

₉ Unknown/missing

45. How would you describe your racial group?*

₁ American Indian or Alaskan Native

₂ Asian or Asian-American

₃ Native Hawaiian or other Pacific Islander

₄ Black or African-American

₅ White

₆ Other: _____

₇ No Answer

* Answers will not affect eligibility

Please complete the following section so that study staff knows how and when you prefer to be contacted.

Name _____

(First)

(Last)

Home Telephone Number (_____) _____ - _____

Time of day you would prefer to be contacted _____ Morning _____ Afternoon
_____ Evening

If you would like us to contact you while at work:

Work Telephone Number (_____) _____ - _____

Time(s) of day you would prefer to be contacted _____ AM or _____ PM

Is it okay to contact you on the weekends? NO YES _____ AM or _____
PM

Email address (if available): _____

If you would like to be contacted by Dr. McTiernan or her staff about other studies, please check "YES" below and we will keep your name and number on file. If you check "NO," we will not contact you about our other studies.

YES—I would like to be contacted by Dr. McTiernan or her staff about other studies.

NO—I do not wish to be contacted by Dr. McTiernan or her staff about other studies.

Thank you for your time in completing this interest survey. Please return this completed questionnaire in the enclosed postage-paid envelope.

If you have questions about the study please call the Nutrition & Exercise for Women Study information line at **(206) 667-6444**. We look forward to hearing from you.

Please check this box If you do not want to be contacted about the NEW study in the future, and return this form in the postage-paid envelope enclosed.
(Address: _____)