A Pilot Study of Sampling Subcutaneous Adipose Tissue to Examine Biomarkers of Cancer Risk

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Abstract

Examination of adipose tissue biology may provide important insight into mechanistic links for the observed association between higher body fat and risk of several types of cancer, in particular colorectal and breast cancer. We tested two distinct methods of obtaining adipose tissue from healthy individuals. Ten overweight or obese (body mass index, 25-40 kg/m²), postmenopausal women were recruited. Two subcutaneous abdominal adipose tissue samples were obtained per individual (i.e., right and left lower abdominal regions) using two distinct methods (method A: 14-gauge needle with incision, versus method B: 16-gauge needle without incision). Gene expression was examined at the mRNA level for leptin, adiponectin, aromatase, interleukin 6 (IL-6), and tumor necrosis factor-α (TNF-α) in flash-frozen tissue, and at the protein level for leptin, adiponectin, IL-6, and TNF-α following short-term culture. Participants preferred biopsy method A and few participants reported any of the usual minor side effects. Gene expression was detectable for leptin, adiponectin, and aromatase, but was below detectable limits for IL-6 and TNF-α. For detectable genes, relative gene expression in adipose tissue obtained by methods A and B was similar for adiponectin (r = 0.64, P = 0.06) and leptin (r = 0.80, P = 0.01), but not for aromatase (r = 0.37, P = 0.34). Protein levels in tissue culture supernatant exhibited good intra-assay agreement [coefficient of variation (CV), 1-10%], with less agreement for intraindividual agreement (CV, 17-29%) and reproducibility, following one freeze-thaw cycle (CV, >14%). Subcutaneous adipose tissue biopsies from healthy, overweight individuals provide adequate amounts for RNA extraction, gene expression, and other assays of relevance to cancer prevention research.

Higher levels of physical activity and lower body weight or body fat are associated with a decreased risk of several types of cancer, in particular colorectal and breast cancer (1, 2). Possible mechanisms for reducing cancer risk via changes in body composition involve inflammatory factors, steroid hormones, insulin-like growth factors, and insulin resistance (3–5). These mechanisms have been investigated to a limited degree in blood, yet even less so directly in adipose tissue.

Adipose tissue plays a critical role in energy homeostasis, contributing to the regulation of metabolism, energy intake, and fat storage (6). In addition to adipocytes, which account for most of adipose tissue mass, adipose tissue also contains preadipocytes, endothelial cells, fibroblasts, leukocytes, and macrophages (7). Adipose tissue secretes adipokines (e.g., leptin, adiponectin, and resistin) as well as cytokines [e.g., interleukin (IL)-1, IL-6, IL-10, and tumor necrosis factor-α (TNF-α)], which can act in an autocrine, paracrine, or endocrine manner to affect metabolic functions (8) and initiation and progression of cancer (9, 10). Adipose tissue is also important for the conversion of androgens to estrogens by aromatase, which provides a mechanistic basis for the associations between adiposity and risk of estrogen-related cancers (11, 12).

We are interested in examining the effect of lifestyle changes on adipose tissue biology to clarify the link between adipose tissue, cancer risk, exercise, and diet. The effect of change in energy balance on adipose tissue biology has been primarily examined, in the short term, in response to consuming very low calorie diets (13–16) and, to a lesser degree, in exercise interventions (17–19). These studies provide intriguing initial evidence that such interventions may affect mRNA expression of cytokines and adipokines, particularly IL-6 and leptin (13–16). Subcutaneous biopsies have been used to examine adipose tissue biology in a number of fields, such as obesity (20–22), lipoatrophy with antiretroviral therapy in HIV infection.
samples are gathered either during an unrelated surgical procedure, which allows for collection of both visceral and subcutaneous adipose tissue and greater yield (15, 21, 27, 28), or by needle biopsy, which is used with intervention or longitudinal analyses (13, 14, 16, 22, 23). Here we investigate the use of a minimally invasive biopsy technique in healthy volunteers to determine the appropriate analysis methods for adipose tissue that have relevance to cancer biomarker studies.

With this pilot study, we aimed to (a) compare two subcutaneous adipose tissue biopsy methods for participant preference and sample yield; (b) examine mRNA expression of IL-6 (IL6), TNF-α (TNF), leptin (LEP), adiponectin (ADIPOQ), and aromatase (CYP19A1); and (c) develop tissue culture methods to examine the levels of proteins of interest (i.e., leptin, adiponectin, IL-6, and TNF-α) in supernatant. The results will inform methods that can be used to examine the effects of lifestyle or genetics on biomarkers of cancer risk related to adipose tissue biology.

### Materials and Methods

#### Subjects

Ten overweight and obese (body mass index, 25-40 kg/m²), postmenopausal women, ages 50 to 75 y, were recruited to participate in this pilot study. They had been deemed ineligible (i.e., too physically active, time commitment) for a large randomized, controlled trial of physical activity and/or diet and breast cancer biomarkers.

#### Biopsy method

Participants completed an 8-h fast before the biopsy procedure. Subcutaneous abdominal adipose tissue samples were obtained by the same trained physician (K.F.S.) from superficial abdominal adipose tissue. The biopsy methods and sample processing steps are outlined in Fig. 1. A sample was collected from an area in the lower quadrant (10-12 cm from the umbilicus) by one method and repeated on the contralateral side in succession, using the second method. The method done first (i.e., A or B) and the location (i.e., right or left) were alternated and recorded. Method A used a 14-gauge needle and required a <0.5-cm scalpel incision, whereas method B used a 16-gauge needle without incision. In both cases, the Yale needle was attached to a 20-mL syringe filled with sterile saline and was passed through the subcutaneous fat several times while applying negative pressure. The collected adipose tissue was processed immediately following sampling. From method A, approximately half of the sample was flash frozen on dry ice, whereas the remainder was processed for tissue culture. From method B, the entire sample was flash frozen on dry ice.

Subjects provided informed consent and the study was approved by the Fred Hutchinson Cancer Research Center Institutional Review Board. Potential side effects were reviewed by participants and included some discomfort, small risk of bleeding, mild bruising, and a small risk of infection. Participants reported method preference, extent of side effects, and any unanticipated symptoms in a follow-up phone call (7-10 d after procedure) by one of the investigators (K.C.). This scripted interview asked about the presence and extent of potential side effects and also biopsy method preference (i.e., the sample done on the right or left).

#### Tissue yield

Tissue mass for RNA isolation was determined by weighing the tube containing the flash-frozen tissue on an analytic balance and
subtracting out the mass of the preweighed tube. Similarly, tissue mass for in vitro culture experiments was determined with an analytic balance to weigh the tissue culture plate containing the harvested and washed tissue immediately before culture and subtracting out the weight of the plate. The total yield per biopsy method was calculated by adding together the total net tissue mass for all aliquots of tissue obtained from that biopsy.

RNA analysis of gene expression

Total RNA was extracted from the flash-frozen tissues using the Absolutely RNA Miniprep Kit (Stratagene) and stored at −80°C. RNA was quantitated by the Ribogreen RNA quantitation assay (Invitrogen). Expression of mRNA was determined by quantitative real-time PCR using the TaqMan one-step reverse transcription-PCR master mix and inventoried gene expression assays for IL-6 (IL6), TNF-α (TNF), leptin (LEP), adiponectin (ADIPOQ), aromatase (CYP19A1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Applied Biosystems). GAPDH expression was measured as a housekeeping gene for normalization across samples. Fifty nanograms of RNA were loaded per well. Samples were batched together and run in triplicate on an ABI 7900HT sequence detection system. Relative expression levels were calculated using the ddCt method (Applied Biosystems).

Tissue culture

Adipose tissue for culture was immediately placed in 10-mL sterile PBS with 1% bovine serum albumin after biopsy. Samples were incubated for 30 to 60 min on ice with occasional mixing to wash away blood and then centrifuged at 250 × g for 5 min to pellet debris. In a sterile tissue culture hood, the upper floating layer of tissue was harvested with sterile forceps, split into two separate preweighed 24-well tissue culture plates, and weighed on an analytic balance. An average of 140 mg of tissue were cultured per well in sterile DMEM with 1% bovine serum albumin. Culture conditions were normalized across samples by adjusting the amount of media added to obtain 100 mg adipose tissue/mL of media based on the net weight of the tissue. Cultures are incubated at 37°C, 5% CO2 for 3h. Following incubation, supernatant was harvested in aliquots and stored at −80°C until analysis.

Protein analysis

Analysis of human adipocyte IL-6, TNF-α, leptin, and adiponectin protein expression was measured in the supernatant of the adipose tissue explant culture. Proteins were detected using the custom multiplex SearchLight Assay System (Pierce/Endogen). Samples from each culture were shipped on dry ice to the SearchLight Sample Testing Service, who conducted the assays. This method of protein quantification is based on a traditional sandwich ELISA technique and integrates plate-based antibody arrays with chemiluminescent detection. All samples are run in duplicate and the assays are validated to ensure optimal sensitivity, specificity, linearity of dilution, and dynamic range.

Statistical analysis

Sample yield by biopsy method was compared by paired sample t test and reported as mean and SD. Tissue culture protein expression was examined for agreement by determining the coefficients of variation (CV). Intra-assay CV was calculated by comparing two aliquots harvested from one plate in the same assay run. Intra-individual CV was determined by comparing aliquots from each duplicate plate for each individual in the same assay run. A reproducibility CV was determined by repeating the analysis of supernatant samples following one freeze-thaw cycle and comparing results to the first run. Pearson correlations were used to examine the association between relative mRNA expression from two sites and also between protein levels and relative mRNA expression for a particular gene. Data were analyzed using SPSS version 14 (SPSS, Inc.).

Results

Biopsy method comparison

Participants were 60.4 ± 5.1 years of age and weighed 77.1 ± 7.9 kg (body mass index, 30.0 ± 4.9 kg/m²). The yield of adipose tissue is outlined in Fig. 2, and mean yield was greater for method A than for method B (497 ± 185 versus 111 ± 99 mg, P < 0.001). Five participants preferred method A, whereas three preferred method B and two had no preference. At the follow-up phone call, few participants reported experiencing any side effects; specifically, minor bleeding from the biopsy site resolving within 12 hours (n = 1; method A), moderate bruising (up to 10 cm diameter) resolving within 10 to 14 days (n = 1; method A), or discomfort with contact to the biopsy site resolving within 2 to 3 days (n = 1; method B). These three participants received an additional call 7 days later for follow-up on their symptoms.
and reported that these side effects had resolved. There was no pattern of participant preference or sample yield across the duration of the pilot (data not shown).

**Gene expression**

RNA yield per milligram of tissue was equivalent for methods A and B (14.2 ± 7.8 versus 16.2 ± 8.3 ng of RNA/mg of tissue, respectively; \( P = 0.95 \)) and RNA quality was good for both methods (data not shown). Gene expression data were not available for one participant due to GAPDH failure. Gene expression was detectable for ADIPOQ, LEP, and CYP 19A1, but below detectable limits for IL6 and TNF (Table 1). For detectable genes, relative gene expression in adipose tissue obtained by methods A and B (i.e., different abdominal sites) was similar for ADIPOQ (\( r = 0.64, P = 0.06 \)) and LEP (\( r = 0.80, P = 0.01 \)), but not for CYP 19A1 (\( r = 0.37, P = 0.34 \)).

**Protein production**

Table 1 also shows the protein expression results from the tissue culture and CVs. TNF-\( \alpha \) was below the level of quantitation in 7 of 10 samples, thus, results are not reported. Search-Light (SL) provided assay precision data for the assay. For adiponectin, leptin, and IL-6, the SL intra-assay CV was 9.4%, 8.6%, and 11.7%, respectively, and the SL inter-assay CV was 10.5%, 16.6%, and 10.4%, respectively. Agreement between relative gene expression and protein levels was examined for leptin (\( r = -0.30, P = 0.44 \)) and adiponectin (\( r = 0.40, P = 0.29 \)), where both gene expression and protein levels were available.

**Discussion**

Subcutaneous adipose tissue biopsies are feasible in a population of overweight, postmenopausal women who are interested in lifestyle intervention research on breast cancer biomarkers. We found that not only did participants agree to the procedure but they also preferred the method which we initially viewed as more invasive (method A), and overall reported few side effects. In addition, method A is advantageous from the standpoint of providing greater amounts of tissue for analyses. The skill and experience of the individual obtaining the biopsy could play a role in both participant preference and sample yield. All of our samples were collected by one individual and we did not observe a trend across time for preference and sample yield. We acknowledge that there are a number of other possible technique comparisons that could have been done, such as additional biopsies at other sites, use of a larger cannulae, or use of a larger incision with a punch biopsy to gather more tissue. However, our main goal was to develop a technique that was acceptable to participants in future intervention trials that otherwise do not include invasive procedures to maximize participant retention.

We chose to examine genes that have relevance for both energy balance and cancer risk, specifically the role of adipose tissue in energy signaling (i.e., leptin and adiponectin), aromatization to form estrogens (i.e., aromatase), and systemic inflammation (i.e., IL-6 and TNF-\( \alpha \)). Gene expression was detectable in adipose tissue samples for some (i.e., ADIPOQ, LEP, and CYP 19A1) but not all genes of interest (i.e., IL6 and TNF). There was good agreement in gene expression from two different subcutaneous abdominal sites within individuals for ADIPOQ and LEP, but not for CYP 19A1, which suggests that examining relative gene expression for certain genes using mRNA may be a feasible way to monitor changes over time in adipose tissue biology in an intervention study. However, our findings are inconsistent with several studies investigating adipose tissue gene expression, where mRNA for both IL-6 and TNF-\( \alpha \) has been quantified (17, 18, 29, 30) using similar methods for both collection (which yielded similar amounts of adipose tissue) and analysis. Further optimization of our detection methods may improve sensitivity, allowing measurement of these cytokines. For example, the selection of appropriate housekeeping genes for quantification of RNA in human adipose tissue is an area of ongoing study (31). Further, Dolinkova et al. (28) recently examined the differences in gene expression profiles of obese versus normal weight subjects for both visceral versus subcutaneous adipose tissue and whole adipose tissue versus isolated adipocytes. Their study provides a list of candidate gene targets for future studies examining the role of body composition and lifestyle interventions in adipose tissue biology.

Several other groups have used adipose tissue collected by needle aspiration biopsy to analyze the concentration of secreted proteins such as leptin, adiponectin or IL-6 (32–35). It has been suggested that more representative data are collected

### Table 1. Gene and protein expression from tissue culture (mean ± SD), including agreement in gene expression between the two methods and percent CVs for tissue culture supernatant protein expression

<table>
<thead>
<tr>
<th>Gene expression (n = 9) Relative to GAPDH</th>
<th>Protein secretion for method A only (n = 10) pg/mL</th>
<th>Intra-assay</th>
<th>Intra-individual</th>
<th>Reproducibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method A</td>
<td>Method B</td>
<td>Method A</td>
<td>Method B</td>
<td></td>
</tr>
<tr>
<td>Adiponectin</td>
<td>1.15 ± 0.5</td>
<td>1.28 ± 0.6</td>
<td>12,670 ± 9,472</td>
<td>3</td>
</tr>
<tr>
<td>Leptin</td>
<td>1.38 ± 0.6</td>
<td>1.23 ± 0.5</td>
<td>201 ± 85</td>
<td>1</td>
</tr>
<tr>
<td>IL-6</td>
<td>NA</td>
<td>NA</td>
<td>14.3 ± 15.2</td>
<td>10</td>
</tr>
<tr>
<td>Aromatase</td>
<td>3.48 ± 2.7</td>
<td>5.29 ± 7.1</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

*Intra-assay—duplicated samples in a single assay run (variability due to multiplex assay); intra-individual—splitting one tissue sample into two equal amounts for culture and comparing samples in a single assay run (variability between two tissue culture wells from the same biopsy sample); reproducibility—repeating samples following one freeze-thaw cycle (variability due to freeze-thaw cycle).
with a short incubation time and minimal manipulation of the adipose sample, especially for IL-6 and TNF-α (32). We sought to assess whether this is a feasible approach that could yield meaningful results. There was good agreement in the protein levels of duplicate supernatant samples run in the same assay; however, TNF-α was not at detectable levels in the majority of samples using a multiplex assay. When we split the sample into equal amounts for tissue culture, the intra-individual CV was high, suggesting that we may have not divided the tissue evenly between the two replicate cultures. This could have resulted from inaccurate weighing or heterogeneity of the tissue, where protein-secreting cells were not evenly distributed between the two samples. These findings suggest that measuring the concentration of proteins secreted from explanted tissue is highly variable, and that such data should be interpreted with great caution. In addition, variation in our measure of reproducibility was high when sample analysis was repeated following one freeze-thaw cycle, which suggests that sample handling is an important variable to consider when examining cytokines and adipokines. Comparison of results obtained from samples run at different times should be done with caution, and ideally, samples to be directly compared should be run in parallel.

There was no agreement between relative gene expression and protein levels for leptin and adiponectin, the two biomarkers where both levels were available. This indicates that the protein levels in tissue culture are not closely correlated with the mRNA levels present at the time of tissue harvest. This is not necessarily surprising because the former reflects protein secretion accumulated over time whereas the latter represents a steady-state measurement. In addition, there is evidence for posttranscriptional regulation of both of these genes (36–38), suggesting that measuring both mRNA and protein may provide nonredundant information about the expression and regulation of these genes.

Examining whole-tissue protein levels and mRNA expression does not account for the heterogeneous cellular composition of adipose tissue. The number of macrophages increases in adipose tissue with overall body fat mass (39), which seems to be the main source of TNF-α and IL-6 (7). Therefore, quantification of macrophage content may be a proxy measure of altered adipose biology in intervention studies. Further processing (i.e., collagenase digestion) to separate out the different constituents of adipose tissue has also been used (32), which, however, is time and labor intensive.

In the future, we plan to examine RNA expression following 6 months of lifestyle change (either dietary weight loss, aerobic exercise, or combined diet and exercise and control) following exploratory microarray analysis. Currently, we have refined our sample collection process slightly to reduce possible contamination of blood in the adipose sample by first placing the sample on a sterile absorbent nonstick Telfa pad. The serous fluid is trapped in the pad and the adipose sample is then processed.

In summary, our pilot study suggests that subcutaneous adipose tissue biopsies from healthy, overweight individuals who were recruited to cancer prevention studies provide adequate tissue for RNA extraction, gene expression analysis, and other possible uses. However, at this time we question the utility of examining protein expression derived from tissue culture due to the high variability observed.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
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