methods

# Pancreatic contamination of mesenteric adipose tissue samples can be avoided by adjusted dissection procedures

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**Abstract** **Mesenteric adipose tissue, located in the mesenterium of the intestines, is believed to play a central role in the development of obesity-related diseases. We have found that mesenteric fat samples harvested from rodents are frequently of poor quality, exhibiting partly degraded RNA. To investigate the background for this observation, we screened adipose tissue samples from two independent studies on rodents for markers of different tissues and cell types. We found that mesenteric adipose tissue samples of low quality are "contaminated" by pancreatic tissue. To locate the affected area, we dissected the mesenteric fat depots from 14 mice and measured abundance of pancreas-specific gene expression and amylase activity. As expected, we observed that the proximal section of the mesenterium, located near the pancreas, expressed pancreatic markers, whereas the distal sections did not. Approximately one-third of the mesenteric adipose tissue depots contained pancreatic tissue. Because the boundary between pancreas and mesenteric fat cannot be easily distinguished during dissection, we conclude that investigators should routinely exclude the proximal section of the mesenteric adipose tissue depot to avoid pancreatic contamination.—**Caesar, R., and C. A. Drevon. **Pancreatic contamination of mesenteric adipose tissue samples can be avoided by adjusted dissection procedures.** *J. Lipid Res.* **2008.** 49: **1588–1594.**

**Supplementary key words** visceral • rodent • RNA integrity • obesity • abdominal • metabolic syndrome • yield • mouse • rat

The prevalence of obesity and obesity-related health problems such as insulin resistance, hypertension, low HDL level and hypertriglyceridemia are increasing worldwide. The development of these conditions often represents complex processes in which many tissues are involved and interact. Adipose tissue depots are important contributors, not only as the major determinant of fatty acid storage and metabolism, but also as an important endocrine organ (1).

It has been shown that the distribution of body fat is of crucial importance for the development of obesity-related diseases. Individuals with intra-abdominal fat accumulation generally run increased risk of developing metabolic syndrome and myocardial infarction (2), whereas others with peripheral obesity tend to have fewer metabolic abnormalities and less risk. The background for this observation is still controversial, but intra-abdominal adipose tissue depots have anatomical and metabolic characteristics that are of interest for understanding disease development related to obesity (3). Secretory molecules of the mesenteric and omental adipose tissue depots located close to the intestines are drained via the portal vein and reach the liver at higher concentrations than observed in the systemic circulation. Some secreted products from these depots might therefore have more-profound effects than expected by their systemic concentration (4). Abdominal adipose depots also exhibit distinct patterns of secreted adipokines and cytokines, which may be crucial for disease development (5). Finally, differences in metabolic activity and responsiveness to adrenergic agents between intra-abdominal and peripheral depots have been observed, although the reports are rather contradictive and difficult to interpret (6–9).

The most important model organisms for studying adipose tissue, obesity, and metabolic syndrome are mice and rats. These animals possess all the major adipose tissue depots found in humans, but the size proportions may differ greatly. For example the omental depot may be of massive size in obese humans, whereas it is generally small in rodents (10).

Compared with other tissues, the anatomy and metabolism of the adipose tissue is rather poorly characterized, and there is no generally accepted definition or nomencla-

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Abbreviations: RIN, RNA integrity number; TTA, tetradecylthioacetic acid.

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ture of the depots. This makes sampling somewhat difficult to standardize. In addition, adipose tissue has a homogenous appearance, making it difficult to take samples from exactly the same site to avoid within-depot variations (11). Thus, there are probably great variations in routines for harvesting adipose tissue samples among scientists and laboratories.

The mesenteric adipose tissue depot is located in the thin connective tissue that supplies the intestines with blood and lymph vessels and autonomous nerves. At the proximal end, the mesenteric tissue attaches to the peritoneum beneath the stomach. We have experienced great variation in sample quality when analyzing mesenteric adipose tissue samples from rodents, in samples harvested in our own laboratory and by collaborators in other laboratories. Whereas adipose tissue samples from other depots are very consistent in quality and yield, we have observed that mesenteric adipose tissue samples frequently exhibit highly increased yield and decreased stability of RNA.

Given the anticipated central importance of mesenteric adipose tissue for disease development, the inconsistency in sample quality is troublesome. We tested the hypothesis that the observed variations could be due to pancreatic tissue contamination during harvest of samples. By sectioning the mesenteric adipose tissue in rodents and examining expression of mRNA and proteins specific for cells/tissues other than adipose tissue, we locate the area affected by pancreas infiltration and suggest guidelines for standardized, fast, and reliable harvesting and processing of mesenteric adipose tissue from rodents.

## MATERIALS AND METHODS

### Animals and housing

Male ApoE3Leiden mice (12) were bred at Leiden University Medical Center (Leiden, The Netherlands) and kept on chow (RM3, Special Diet Services; Witham, Essex, UK) up to 14 weeks of age. The mice were then fed either a high-fat diet (D12451, Research Diets, New Brunswick, NJ) supplemented with 45% palm oil or continued on chow diet for 4 or 16 weeks before euthanization. Diet and water were given ad libitum.

Male rats of the Wistar strain SPF, Mol (Møllegaard Breeding Centre, Ejby, Denmark) were fed a low-fat reference diet (chow) for 1 week. The rats were then divided into three feeding groups and offered lard (19.5% lard), omega-3 PUFA (9% lard and 10.5% Triomar; EPAX5500, Pronova Biocare, Oslo, Norway), or a diet containing the synthetic fatty acid tetradecylthioacetic acid (TTA) (19.5% lard and 0.29% TTA) for 49 days. Soybean oil (1.5%) was provided to all dietary groups to prevent essential fatty acid deficiency. The animals were fed 20 g diet/day and had free access to water.

Male and female mice of C57BL/6J background were fed a chow diet (RM3; Special Diet Services) for 14 to 40 weeks. All animals were kept under standard housing conditions at a temperature of 21°C, air humidity of 55%, a 12 h light/dark cycle, and conventional cages.

ApoE3Leiden mouse and rat samples were harvested from animal feeding experiments intended for nutritional "omics" studies in several tissues, whereas the C57BL/6J mice were used exclusively for the present study.

The study was performed in accordance with Public Health Service Policy on Human Care and Use of Laboratory Animals.

### Harvesting adipose tissue

Animals were euthanized by cervical dislocation and opened via ventral abdominal incision. Mesenteric adipose tissue was removed by lifting the intestines and cutting the intermediate fat free, starting at the distal end close to the appendix. When adipose tissue was divided into smaller parts, these parts were dissected piece by piece from the animal, starting at the proximal end of the depot. Harvested samples were immediately snap-frozen on liquid nitrogen and stored at −80°C or on liquid nitrogen.

### RNA extraction

Frozen adipose tissue was crushed and powderized under liquid nitrogen and subsequently homogenized for 1 min using an Ultratorax homogenizer (IKA Labortechnik, Staufen, Germany). Total RNA was isolated from 100 mg tissue using the RNeasy Lipid Tissue Mini Kit (Qiagen, The Netherlands). RNA was quantified spectrophotometrically (NanoDrop 1000; NanoDrop Technologies, Boston, MA), and the quality was evaluated by capillary electrophoresis (Agilent 2100 Bioanalyzer; Agilent Technologies, Palo Alto, CA). RNA integrity was assessed by calculation of RNA integrity number (RIN) value. In RIN analysis, RNA is separated by electrophoresis on microfabricated chips and subsequently monitored by laser-induced fluorescence detection. The RIN value is calculated from the entire electrophoretic trace of the RNA sample, including presence or absence of degradation products, to determine total RNA integrity. This method has proven more reliable than conventional methods such as ultraviolet spectroscopy and 28S:18S area ratios (13). Although a high RIN value indicates intact RNA and good quality for further analysis, the RIN value cannot predict the usefulness of gene expression data without validation of RNA quality in relation to results from other experimental data (14).

### RT-PCR

Total RNA was reverse transcribed in 20 μl reactions using a high-capacity cDNA reverse transcription kit, including an RNase inhibitor (Applied Biosystems; Foster City, CA) according to the manufacturer's protocol. Real-time PCR was performed using TaqMan probes on a 7900HT Fast Real-Time PCR System (Applied Biosystems). The following genes were analyzed (product code in parenthesis). Mouse: Cuzd1 (Mm00492748_m1), Ela3b (Mm00840378_m1), Mac-2 (Mm00802901_m1), CD3e (Mm00599683_m1), Pref1 (Mm00494477_m1), CD19 (Mm00515420_m1), Pcam1 (Mm00476702_m1), gapdh (Mm99999915_g1); rat: Ela1 (Rn00561140_m1), Prss1 (Rn00754931_m1) and gapdh (Rn99999916_s1). Pancreatic markers were selected based on Unigene expression profile data (http://www.ncbi.nlm.nih.gov/sites/entrez?db=unigene). Relative expression was calculated by the ΔΔCt method (15) using gapdh as the endogenous control housekeeping gene in both species.

### Amylase assay

Amylase activity was measured by EnzChek® Ultra Amylase Assay Kit (E33651; Molecular Probes, Eugene, OR) following the manufacturer's protocol.

## RESULTS

### Mesenteric adipose tissue samples exhibit high RNA yield and unstable RNA

Mesenteric and subcutaneous adipose tissues from males were harvested from mice fed a high-fat diet for

16 weeks and from animals fed chow. Total RNA yield varied markedly between the depots and feeding groups. The yield of RNA from mesenteric adipose tissue was 2.1 μg/mg tissue for animals fed chow and 0.6 μg/mg tissue for animals fed a high-fat diet. For subcutaneous adipose tissue, the RNA yield was 0.24 μg/mg tissue for both feeding groups. Thus, there were large and significant ($P < 0.05$) differences in total RNA yield, not only between the depots, but also between mesenteric adipose tissue depots from the two investigated feeding groups. We also observed that RNA integrity and yield were positively correlated. Samples clustered in two groups, with all subcutaneous samples and the mesenteric samples from animals fed a high-fat diet in one group exhibiting RIN values between 7 and 8, and mesenteric samples from animals fed chow exhibiting RIN values between 2 and 5 (**Table 1**).

In another independent experiment, mesenteric, subcutaneous, perirenal, epididymal, and interscapular adipose tissue samples were harvested from rats after a 7 week feeding experiment in which the animals were fed either a relatively high-fat lard diet (40% of total energy supply as fat), a lard diet supplemented with marine omega-3 fatty acids, or a lard diet supplemented with the synthetic fatty acid TTA. In this study, we also found diet- and depot-dependent differences in RNA yield and integrity. The average RNA yield from mesenteric adipose tissue from rats fed lard and TTA was 1.3 μg/mg and 2.0 μg/mg, respectively, whereas the corresponding figure was 0.24 μg/mg for omega-3 fatty acid-fed animals. The difference in RNA yield between the omega-3 fatty acid-fed group and the other dietary groups was significant on a $P < 0.05$ level. The other adipose tissue depots displayed average RNA recoveries of 0.07 μg/mg for perirenal and epididymal adipose tissue, 0.35 μg/mg for subcutaneous tissue, and 0.59 μg/mg for interscapulary adipose tissue. These latter adipose depots exhibited insignificant differences in yield between the dietary groups. As in the case of the mouse study described above, high yield of RNA was associated with low RNA integrity, but the degradation in the rat study was not as extensive as in the mouse study. The lowest RIN values (excluding a single outlier) were in the range 6.5–7 (Table 1).

In summary, diet affects RNA stability and yield in mesenteric adipose tissue samples from both mice and rats. In mice, the chow diet causes higher yield and lower stability of RNA as compared with the high-fat diet. In rats, lard and TTA diets cause higher yield and lower stability of RNA, as

TABLE 1. Yield and stability of RNA in different adipose tissue depots harvested from mice and rats

| | | | Yield | | RIN | |
|---|---|---|---|---|---|---|
| Animal | Adipose Tissue Depot | Diet (N) | Average | SEM | Average | SEM |
| | | | *μg RNA/mg tissue* | | | |
| Mice | Mesenteric | Chow (9) | 2.12 | 0.12 | 3.18 | 0.37 |
| | | High-fat (5) | 0.56 | 0.06 | 7.36 | 0.31 |
| | Subcutaneous | Chow (5) | 0.24 | 0.03 | 7.03 | 0.38 |
| | | High-fat (5) | 0.24 | 0.07 | 7.83 | 0.12 |
| Rats | Mesenteric | Lard (3) | 1.33 | 0.47 | 7.63 | 0.53 |
| | | Omega-3 (5) | 0.24 | 0.10 | 8.34 | 0.34 |
| | | TTA (4) | 1.98 | 0.43 | 7.13 | 0.22 |
| | Subcutaneous | Lard (5) | 0.21 | 0.05 | 8.60 | 0.23 |
| | | Omega-3 (5) | 0.55 | 0.18 | 7.80 | 0.25 |
| | | TTA (5) | 0.31 | 0.06 | 7.38 | 0.70 |
| | Interscapulary | Lard (5) | 0.59 | 0.03 | 8.50 | 0.49 |
| | | Omega-3 (5) | 0.36 | 0.06 | 8.38 | 0.16 |
| | | TTA (4) | 0.82 | 0.20 | 8.20 | 0.12 |
| | Perirenal | Lard (4) | 0.07 | 0.01 | 9.25 | 0.17 |
| | | Omega-3 (5) | 0.09 | 0.01 | 8.94 | 0.20 |
| | | TTA (5) | 0.06 | 0.01 | 9.12 | 0.08 |
| | Epididymal | Lard (5) | 0.07 | 0.01 | 9.02 | 0.21 |
| | | Omega-3 (5) | 0.08 | 0.01 | 8.86 | 0.12 |
| | | TTA (5) | 0.06 | 0.01 | 9.26 | 0.17 |

N represents the number of individuals in each group. RIN, RNA integrity number; SEM, standard error of the mean; TTA, tetradecylthioacetic acid.

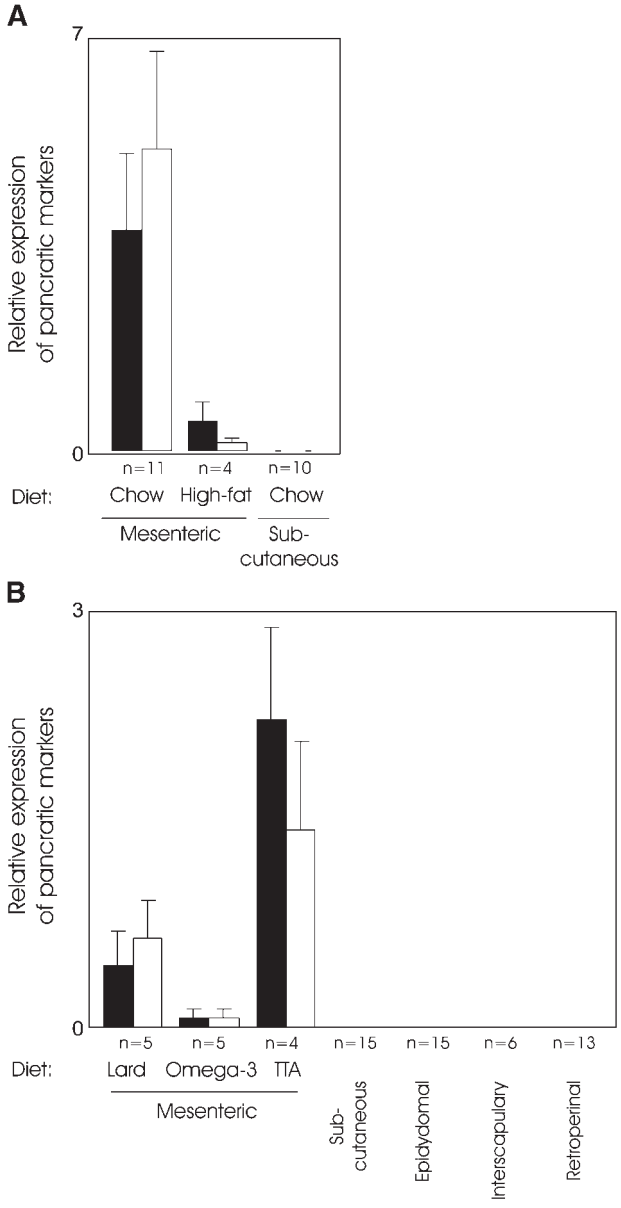


**Fig. 1.** Relative expression of pancreatic markers in adipose tissue. Expression of Cuzd1 (black bars) and Ela3 (white bars) in mouse adipose tissue samples (A), and expression of Prss1 (black bars) and Ela1 (white bars) in rat adipose tissue samples (B). Error bars represent SEM.

compared with the omega-3 fatty acid-supplemented diet. Diet does not change yield or stability in other adipose tissue depots.

### Mesenteric adipose tissue samples contaminated by pancreatic tissue

To investigate why mesenteric adipose tissue samples from certain dietary groups display increased RNA yield, we screened mesenteric adipose samples for markers of different types of cells and tissues. Strikingly, high levels of pancreatic markers were detected in mesenteric adipose tissue from both mice and rats.

In mice, we observed expression of the pancreas markers Cuzd1 and Ela3b encoding CUB and zona pellucida-like domains 1 and pancreatic elastase 3, respectively. Cuzd1 transcripts were 7-fold and Ela3b transcripts 37-fold more abundant in samples from mesenteric fat in mice fed chow as compared with samples from mice fed the high-fat diet. The differences were significant on a $P < 0.05$ level. Pancreatic marker expression in subcutaneous samples was negligible (**Fig. 1A**).

In rats, we measured expression of the genes Prss1 and Ela1 encoding pancreatic trypsin 1 and pancreatic elastase 1 (Fig. 1B). The expression of Prss1 in TTA-fed animals was 5-fold higher than in lard-fed rats and 32-fold higher than in omega-3 fatty acid-fed animals. For Ela1, the expression was 2.3-fold higher in TTA-fed than in lard-fed animals, and 22-fold higher in TTA-fed than in omega-3 fatty acid-fed animals. Despite the large differences in mean marker expression levels between dietary groups, only the difference between Prss1 expression in TTA-fed and omega-3 fatty acid-fed animals was significant on a $P < 0.05$ level. As in the mouse experiment, pancreatic marker expression in other adipose tissue depots was negligible.

In addition to pancreatic markers, mouse adipose tissue was also screened for expression of markers for several other cell types. The abundance of Mac-2 (macrophages), Pref1 (preadipocytes), Pecam1 (vascularization), CD3

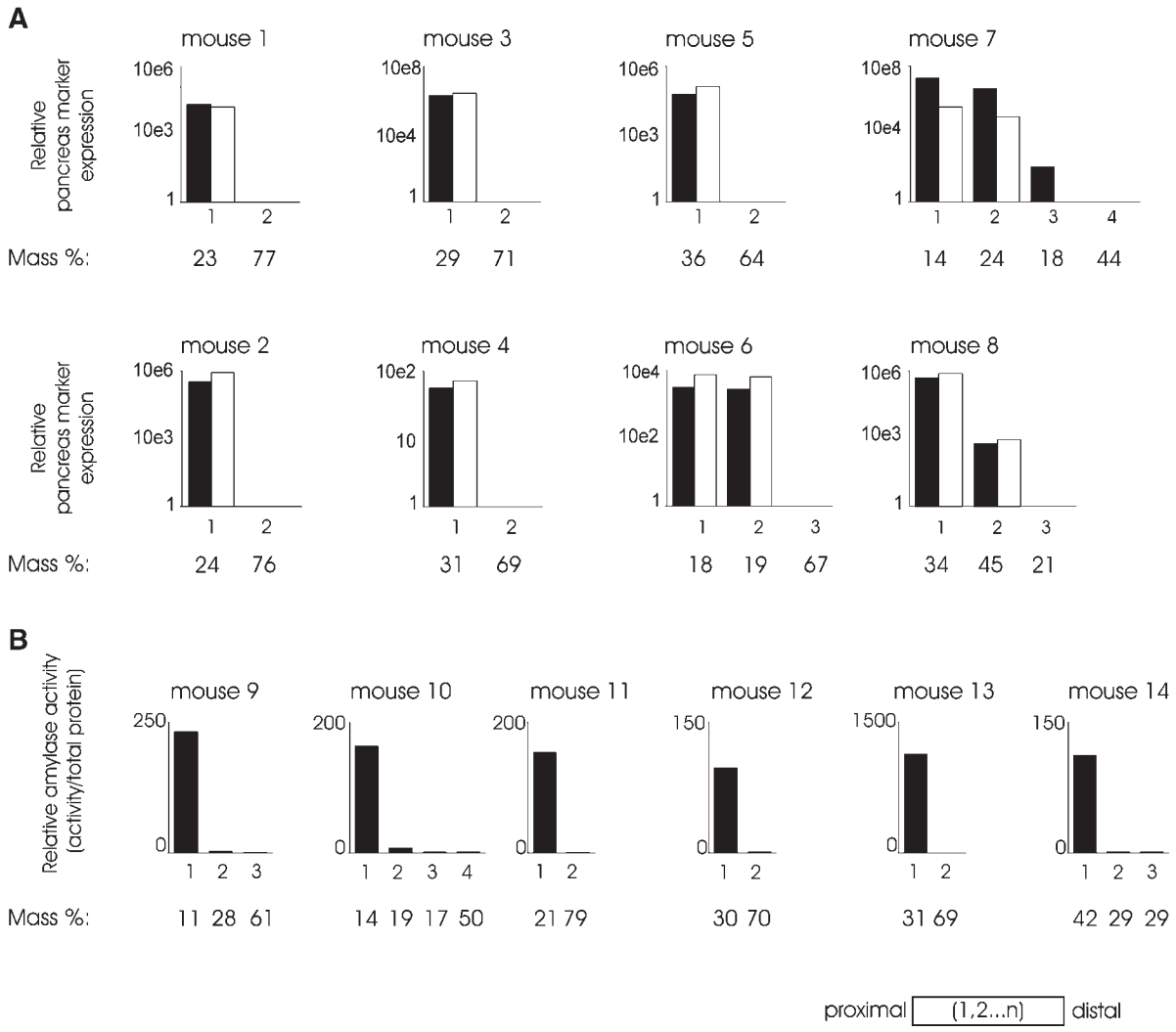


**Fig. 2.** Expression of pancreatic mRNA markers and amylase enzyme activity in sections of the mesenteric adipose tissue depot. Samples are numbered from the proximal end (left on the *x* axis) of the depot toward the distal end. The size (given as percent; w/w) of the total mesenteric adipose tissue depot is given under the graphs. Relative expression of the pancreatic markers Cuzd1 (black bars) and Ela3b (white bars) (A). Relative amylase activity per mg protein (B).

(T-cells), and CD19 (B-cells) transcripts exhibited little or no variation, and the expression level was not correlated to RNA yield (data not shown).

### Expression of pancreatic markers and increased amylase activity in proximal mesenteric adipose tissue

To examine what part of the mesenteric adipose tissue depot is affected by pancreatic tissue contamination, mice were euthanized and the mesenteric fat was sampled in small sections. Starting from the proximal end and continuing along the small intestine toward the appendix, the mesenteric adipose tissue depot was divided into two to four parts.

The proximal section (close to the pancreas) of the mesenteric adipose depot exhibited the highest level of pancreas-specific gene expression (**Fig. 2A**; note the logarithmic *y* axis). Most samples exhibited a distinct all-or-none expression pattern in which the abundance of marker gene transcripts varied many orders of magnitude between affected and nonaffected samples. From 23% (mouse 1) to 38% (mouse 7, sections 1 and 2) of the depot was affected. In mice 6 and 7, the second section displayed pancreas marker expression as high as that in the proximal section, illustrating that pancreatic tissue may reach far into the mesenteric adipose tissue depot and is not confined to the proximal part. In mouse 7, even section 3 exhibited some Cuzd1 expression, but no Ela3b expression. In mouse 8, pancreas marker expression in the mid-section was three orders of magnitude lower than in the proximal section but three orders of magnitude higher than in the distal section. This probably illustrates that the mid-section contained a very small amount of pancreatic tissue.

To further investigate what part of the mesenteric adipose tissue depot is infiltrated by pancreatic tissue, samples from six other mice were used to measure amylase activity (Fig. 2B). In all six animals, the proximal sample of mesenteric adipose tissue exhibited by far the highest activity, with two to three orders of magnitude higher activity than the more distal sections. The affected part accounted for 11–42% (w/w) of the total mesenteric adipose depot size.

### Pancreatic contamination is negatively correlated with RNA yield

In our experience, the yield of total RNA from adipose tissue is low, compared with most other tissues. Mesenteric adipose tissue without pancreatic contamination exhibits a yield of approximately 0.1 μg total RNA/mg tissue (**Table 2**, Fig. 2). Total RNA yield from pure mouse pancreatic tissue has previously been measured as roughly 3.5–8 μg total RNA/mg tissue (16). To investigate the relationship between RNA yield in mesenteric adipose samples and the level of pancreatic tissue contamination, RNA yield was plotted against pancreatic marker expression (**Fig. 3**). The linear correlation coefficients were 0.84 and 0.90 from Prss1 and Ela1, respectively. Both correlations were significant on a $P < 0.0001$ level. The linear relationship between pancreas marker expression and RNA yield indicates that increased RNA yield in mesenteric adipose tissue samples is caused mainly by pancreas tissue contamination.

TABLE 2. RNA yield from mesenteric adipose tissue

| Mouse | Section[a] | Yield |
|---|---|---|
| | | *μg RNA/mg tissue* |
| 1 | 1 | 1.07 |
| | 2 | 0.065 |
| 2 | 1 | 1.22 |
| | 2 | 0.08 |
| 3 | 1 | 4.23 |
| | 2 | 0.11 |
| 4 | 1 | 1.67 |
| | 2 | 0.06 |
| 5 | 1 | 0.65 |
| | 2 | 0.05 |
| 6 | 1 | 3.59 |
| | 2 | 4.53 |
| | 3 | 0.11 |
| 7 | 1 | 1.23 |
| | 2 | 0.85 |
| | 3 | 0.058 |
| | 4 | 0.09 |
| 8 | 1 | 0.58 |
| | 2 | 0.05 |
| | 3 | 0.10 |

[a] Sections are numbered from the proximal toward the distal end. Mouse and section numbers correspond to mouse and section numbers in Fig. 2.

## DISCUSSION

Pancreatic tissue may be difficult to distinguish from the surrounding tissues in rodents. Adipose tissue morphologists perform dissection under magnification and use tools adapted for fine surgery to be able to separate adipose depots from pancreas (S. Cinti, personal communication). In many experiments, these careful procedures are not applicable, because speed is often critical to avoid degradation of nucleic acids, proteins, and other bioactive molecules. We have analyzed mesenteric adipose tissue samples from two independent experiments performed in two different laboratories, and have found that the samples are often

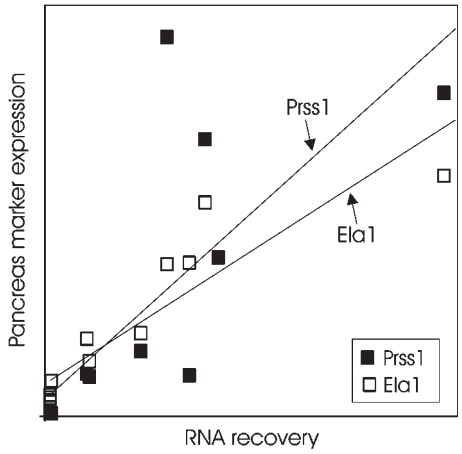


**Fig. 3.** Correlation between RNA yield and pancreatic marker expression in rat mesenteric adipose tissue. The regression lines from Prss1 and Ela1, calculated by the least squares method, are indicated in the figure. The correlation coefficients were 0.84 and 0.90 from Prss1 and Ela1, respectively ($P < 0.0001$).

contaminated with pancreatic tissue. By dissection of the mesenteric adipose tissue depot, we then located the area of pancreatic infiltration to the proximal part of the depot. To investigate whether there are any published data on pancreas-contaminated mesenteric adipose tissue samples, we screened the database GEO (http://www.ncbi.nlm.nih.gov/geo/) for array sets using the search terms "mesenteric" or "visceral" in combination with "*Mus musculus*" or "*Rattus Norvegicus*." The result lists were inspected manually, and we found two data sets that met our criteria: mesenteric adipose tissue samples from mouse or rat, in which RNA had been isolated from whole-tissue samples. In the first data set, gene expression in mesenteric adipose tissue from rats given oleoyl-estrone was compared with control rats (17). Four arrays were analyzed, and none of these exhibited expression of pancreatic genes. In the second data set, mesenteric adipose tissue from rats whose mothers had been subjected to a protein-restricted diet during pregnancy was compared with controls (18). One of the four samples showed moderate expression of the pancreatic markers Ela1 and Prss1, and we could not tell whether pancreas infiltration affected the analysis significantly. The observation does, however, show that mesenteric adipose tissue samples contaminated by pancreatic tissue are found in published works, and because raw data are rarely published, this finding may be only the tip of the iceberg.

Because of the high content of triglycerides, the concentration of RNA, DNA, and protein in adipose tissue is generally low. This makes adipose tissue samples vulnerable to infiltration with other tissues, because even minor contaminations may represent a high proportion of foreign molecules. In our dissection of mouse mesenteric adipose tissue depots, we found RNA yields ranging between 0.06 and 4.2 μg/ml (Table 2). The highest values were found in samples contaminated markedly with pancreatic tissue. Assuming that the sample with the highest RNA yield consists solely of pancreatic tissue, whereas the sample with the lowest yield contains only adipose tissue, we will get a 70-fold difference in yield. In the adipose tissue sample, 1.4% (w/w) pancreas tissue would then give a stoicheiometric relationship of 1:1 between adipose and pancreatic RNA. The difference in yield is probably an underestimation, because even samples with high RNA yield probably contain a portion of adipose tissue.

Another problem with pancreatic contamination is the high content of hydrolytic enzymes. Our experience shows that it is very difficult to isolate intact RNA from pancreas-containing samples. Because RNA molecules vary greatly in stability, even a moderate level of degradation causes severe degradation of some gene products (13). In large-scale analysis such as microarray, this might give rise to numerous artifacts.

Interestingly, the degree of pancreatic contamination of mesenteric adipose tissue samples seems to differ between intervention groups in both mice and rats. The investigation of the background for these diet-dependent differences falls outside the scope of this paper, but one may speculate that the anatomy of either adipose tissue or pancreas changes in response to diet.

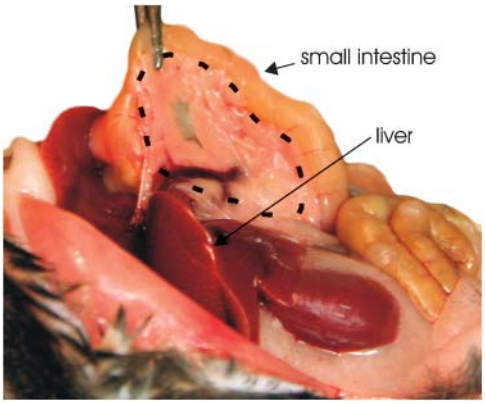


**Fig. 4.** The proximal section of the mesenteric adipose tissue. The dashed line indicates the area of the mesenteric adipose tissue depot in which pancreatic tissue is detected.

Based on our dissection of the mesenteric adipose tissue depot in rodents, we recommend that the proximal mesenteric adipose depot be excluded when harvesting the tissue for RNA or protein analysis. We estimate that approximately 30–40% (w/w) of the fat attached to the small intestines should be removed to avoid contaminating samples with pancreatic tissue. In practice, this represents the fat situated under the first loop of the small intestine extending from the stomach. This area can easily be identified by lifting the intestines as illustrated in **Fig. 4**.

When analyzing mesenteric adipose tissue, one should measure the yield and stability of RNA and/or protein; these could be an indication of the presence of foreign tissue. By following our suggested directions, investigators will avoid expensive and time-consuming mistakes when harvesting mesenteric adipose tissue.

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