

Nuclear Magnetic Resonance Spectroscopy-Based Metabolomics of the Fatty Pancreas: Implicating Fat in Pancreatic Pathology

Nicholas J. Zyromski^a Abhishek Mathur^a G.A. Nagana Gowda^b Carl Murphy^b
Deborah A. Swartz-Basile^a Terence E. Wade^a Henry A. Pitt^a Daniel Raftery^b

^aDepartment of Surgery, Indiana University, Indianapolis, Ind., and ^bDepartment of Chemistry, Purdue University, West Lafayette, Ind., USA

Key Words

Obesity · Inflammation · Metabolomics · Pancreatitis · Pancreatic cancer

Abstract

Background: Obesity is a worldwide epidemic and a significant risk factor for pancreatic diseases including pancreatitis and pancreatic cancer; the mechanisms underlying this association are unknown. Metabolomics is a powerful new analytical approach for describing the metabolome (complement of small molecules) of cells, tissue or biofluids at any given time. Our aim was to analyze pancreatic fat content in lean and congenitally obese mice using both metabolomic analysis and conventional chromatography. **Methods:** The pancreatic fat content of 12 lean (C57BL/6J), 12 obese leptin-deficient (Lep^{ob}) and 12 obese hyperleptinemic (Lep^{db}) mice was evaluated by metabolomic analysis, thin-layer and gas chromatography. **Results:** Pancreata of congenitally obese mice had significantly more total pancreatic fat, triglycerides and free fatty acids, but significantly less phospholipids and cholesterol than those of lean mice. Metabolomic analysis showed excellent correlation with thin-layer and gas chromatography in measuring total fat, triglycerides and phospholipids. **Conclusions:** Differences in pancreatic fat con-

tent and character may have important implications when considering the local pancreatic proinflammatory milieu in obesity. Metabolomic analysis is a valid, powerful tool with which to further define the mechanisms by which fat impacts pancreatic disease.

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Introduction

The burgeoning obesity epidemic in the United States has focused a spotlight on the role of adipose tissue in the multiple pathologic effects of obesity [1–4]. Adipose tissue secretes a number of proteins collectively referred to as adipokines that are important modulators of metabolism, inflammation and energy intake [5]. Obesity leads to dysfunctional production of adipokines. Specifically, circulating concentrations of the proinflammatory adipokine leptin are elevated, and those of the anti-inflammatory adipokine adiponectin are paradoxically de-

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creased. This imbalance results in a generalized proinflammatory milieu, which is manifest at the local level by increased tissue macrophage infiltration as well as by increased production of the proinflammatory cytokines tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6) and IL-1 β [5, 6]. Fat infiltration of an organ is a known trigger for initiating this inflammatory cascade, with nonalcoholic steatohepatitis being a well-established example of this phenomenon [1, 7].

Clinical evidence also suggests that fat plays an important role in the development of pancreatic disease. Obesity is clearly a risk factor for increased severity of acute pancreatitis [8–11], and numerous well-controlled epidemiological studies have linked obesity to an increased incidence of pancreatic cancer [12–15]. In addition, some experimental work has begun to address the mechanisms by which fat contributes to pancreatic disease. Recent data from our laboratory showed that obese Lep^{ob} mice have an elevated pancreatic fat content and increased baseline levels of the proinflammatory cytokines TNF- α and IL-1 β relative to lean wild-type mice [3]. In addition, obese leptin-deficient Lep^{ob} and obese hyperleptinemic Lep^{db} mice develop more severe pancreatitis than lean mice when subjected to cerulein hyperstimulation [16]. Continuing investigation will be aided greatly by employing novel analytical techniques.

Metabolomics is an exciting new analytical field in systems biology that uses sophisticated techniques such as mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy to define the complement of low molecular weight molecules present in cells, tissue or biofluids in a particular physiologic state [17]. The metabolome of a cell is the collection of downstream products of gene transcription, translation and post-translational protein modification. Thus, the metabolome may be considered a more accurate representation of the true cellular phenotype at any given time, and metabolomics represents a powerful analytical tool with which to identify cellular differences between discrete populations. NMR studies allow analysis of metabolites directly from the intact tissue. To date, no data exist regarding the pancreatic metabolome in lean or congenitally obese mice. Therefore, the aims of the current study were to describe the pancreatic lipid metabolome in lean and congenitally obese mice and to compare these results with those obtained by the conventional technique of lipid measurement using thin-layer and gas chromatography.

Materials and Methods

Animals and Diets

Twelve lean control (C57BL/6J), 12 obese leptin-deficient (Lep^{ob}) and 12 obese leptin-resistant (Lep^{db}) female mice were obtained from Jackson laboratory (Bar Harbor, Me., USA). At 8 weeks of age, all mice were fed a diet composed of 25% fat (soybean and corn oil), 55% carbohydrate (sucrose and cornstarch) and 20% protein (caesin; Dyets Inc., Bethlehem, Pa., USA) for 4 weeks. Our laboratory has accumulated extensive baseline data under these experimental conditions. Animals were weighed weekly. All protocols for these animal studies were approved by the Indiana University Institutional Animal Care and Use Committee.

Tissue Collection

At 12 weeks of age, after an overnight fast with water allowed ad libitum, the mice were sedated with isoflurane and anesthetized with an intraperitoneal injection of xylazine (15 mg/kg) and ketamine (50 mg/kg). The animals were weighed and then underwent laparotomy and total pancreatectomy. Pancreata were frozen immediately in liquid nitrogen and preserved at -80°C for subsequent analysis by NMR ($n = 6/\text{strain}$) as well as by thin-layer and gas chromatography ($n = 6/\text{strain}$). In addition, visceral fat was harvested from the left iliopsoas fat pad of each mouse and frozen at -80°C for subsequent analysis of lipid composition by thin-layer and gas chromatography.

Metabolomic Analysis

Six pancreata from each group of mice (lean, Lep^{ob} and Lep^{db}) were analyzed by proton and phosphorus NMR using a CMX 400 MHz wide-bore solid spectrometer (Chemagnetics-Varian, Palo Alto, Calif., USA). The spectra of the intact tissue were obtained at 10°C by magic angle sample spinning at 3,000 Hz to remove broadening of NMR signals caused by dipolar coupling or chemical shift anisotropy. ^1H NMR spectra were obtained using either a single pulse sequence without water presaturation, the 1D-NOESY sequence with water presaturation during recycle delay and mixing time, or the Carr-Purcell-Meiboom-Gill pulse sequence with water presaturation. ^{31}P NMR spectra were obtained using a single pulse sequence. The resulting spectra were subjected to principal component analysis (PCA) to identify the metabolites with the highest variance among the different samples.

Lipid Analysis by Thin-Layer and Gas Chromatography

Pancreatic lipid content was determined by thin-layer and gas chromatography as previously described [2]. Briefly, lipids were extracted by the method of Folch-Lees. Individual lipid classes were separated by thin-layer chromatography using Silica Gel 60 A plates and visualized by rhodamine 6G. In addition, total cholesterol was analyzed by the method of Goldblatt et al. [2]. An aliquot of the Folch extract was saponified with 1 N KOH in 90% methanol. The nonsaponifiable sterol was extracted using hexane, and total cholesterol was determined using gas chromatography. The sodium salt of trimethylsilylpropionic acid, of known concentration, was used as a chemical shift as well as quantitative reference.

Lipid Quantitation by NMR

Concentrations of the total fat, triglycerides and phospholipids were determined by comparing the peak intensities of lipids,

triglycerides and choline signals, respectively, with the internal reference peak of trimethylsilylpropionic acid. The number of protons representing each peak was taken into account for the determination of the concentrations.

Statistical Analysis

Statistical analysis was performed using Sigma Stat Statistical Software (Jandel Corp., San Rafael, Calif., USA). All data are expressed as the mean \pm SEM. Differences in animal body weight, pancreatic lipids and visceral lipids were tested for statistical significance by ANOVA and the Tukey test where appropriate. For analysis of the metabolome, both ^1H and ^{31}P NMR data were Fourier transformed using 4,096 data points, and the resulting spectral data were subjected to principal component multivariate statistical analysis [18] using Pirouette software version 3.11 (Infometrix Inc., Pa., USA). A p value <0.05 was considered statistically significant for all data analysis.

Results

Animal Weight

As expected, both the Lep^{ob} and Lep^{db} obese mice weighed significantly more than the lean mice (47 ± 1 and 40 ± 1 vs. 17 ± 1 g; $p < 0.001$). In addition, the Lep^{ob} mice weighed significantly more than the Lep^{db} mice (47 ± 1 vs. 40 ± 1 ; $p < 0.001$).

Metabolomic Analysis of Pancreatic Fat

^1H NMR Data and PCA. Typical ^1H NMR spectra of the pancreas of lean, Lep^{ob} and Lep^{db} mice are shown in figure 1. Dramatic biochemical differences are evident among the 3 groups. ^1H NMR spectra of all tissues were dominated primarily by lipids. Specifically, much smaller intensity phosphocholine $-\text{N}-\text{CH}_3$ signals (3.2 ppm) compared with lipid tail $-\text{CH}_3$ clearly indicate the predominance of triglycerides over phospholipids in both obese groups of mice. Not surprisingly, the water content was significantly lower in both obese groups compared with lean mice ($p < 0.001$), though no differences existed in water content between the 2 groups of obese mice. As shown in figure 1b, PCA distinctly separates lean and obese (Lep^{ob} and Lep^{db}) mice based on metabolic changes. The PCA loading plot is consistent with the results that lipids and triglycerides increase in obese mice whereas phospholipids are higher in the lean mice.

^{31}P NMR Data and PCA. ^{31}P NMR spectra and PCA of these data are shown in figure 2. ^{31}P NMR spectra in all tissues were dominated by resonances from inorganic phosphate (Pi), phosphomonoesters (PME) and phosphodiester; these signals showed distinct differences among the 3 groups of pancreata. Specifically, both PME and Pi were higher in lean than in Lep^{ob} and Lep^{db} mice.

The PCA analysis of these data showed a discrete clustering pattern in each strain, and the loading plot clearly indicates increased PME and Pi signals in lean mice compared with the obese groups (fig. 2b).

Total Pancreatic Fat

Chromatography. The pancreatic total fat was elevated more than 2 fold in both obese groups of mice compared with lean mice ($p < 0.05$). Although Lep^{db} mice had less total fat than Lep^{ob} mice, this difference was not statistically significant. Pancreatic total fat data quantitated by chromatography and metabolomics are shown in figure 3.

Metabolomics (^1H NMR). Total pancreatic fat analysis by metabolomics demonstrated a pattern similar to chromatographic analysis with total fat being significantly elevated in both the obese strains of mice versus the lean mice ($p < 0.05$). No significant differences existed between Lep^{ob} and Lep^{db} mice in total pancreatic fat, although the pancreata of Lep^{db} mice had less total fat than those of Lep^{ob} mice.

Triglyceride Analysis

Chromatography. Pancreatic triglyceride content had a pattern similar to that of total pancreatic fat, showing a 3- to 4-fold elevation in the pancreata of both obese groups versus those of lean mice ($p < 0.05$). No significant differences existed between Lep^{ob} and Lep^{db} mice in pancreatic triglycerides, although the Lep^{db} mice had lower triglyceride levels than the Lep^{ob} mice. Triglyceride data quantified by both chromatography and metabolomics are shown in figure 4.

Metabolomics (^1H NMR). The ^1H NMR analysis also showed that the pancreatic triglycerides were elevated in both obese groups of mice compared with lean animals ($p < 0.05$). In addition, no significant differences in pancreatic triglyceride content were detected between Lep^{ob} and Lep^{db} mice. Importantly, metabolomic analysis again corroborated the findings of conventional chromatography.

Phospholipid Analysis

Chromatography. Interestingly, pancreatic phospholipids were significantly lower in both obese strains of mice compared with the lean mice ($p < 0.05$). Moreover, gas chromatography showed that the pancreata of Lep^{db} mice contained significantly more phospholipids compared with Lep^{ob} mice ($p < 0.05$). Phospholipid analysis by both chromatography and metabolomics is shown in figure 5.

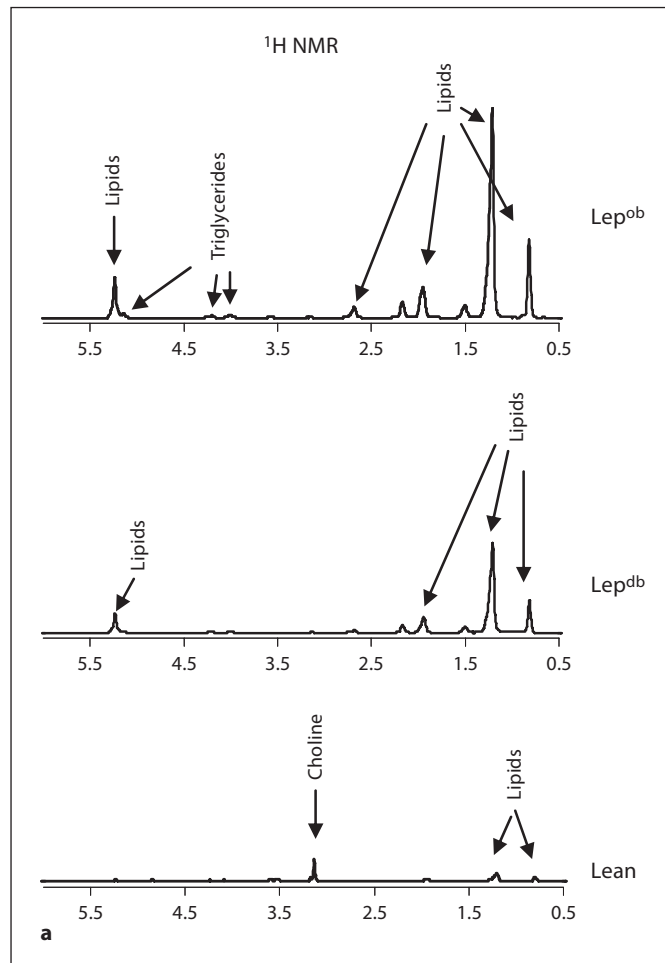
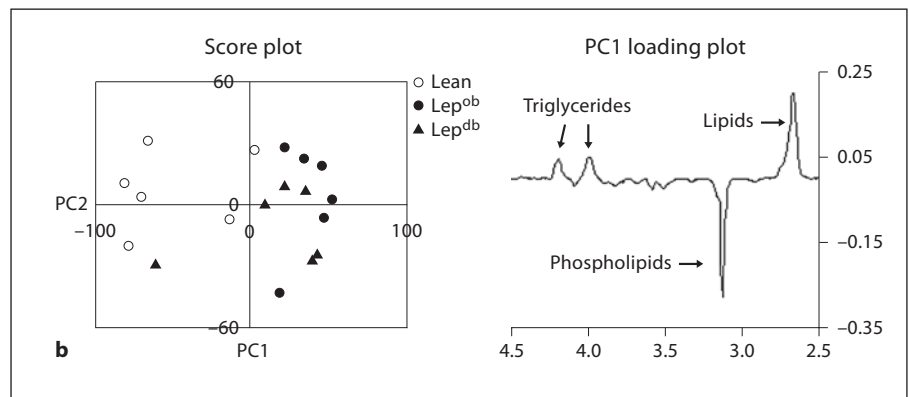


Fig. 1. a Typical ^1H spectra of intact pancreas tissue samples from lean, Lep^{ob} and Lep^{db} mice. All the spectra were obtained using magic angle sample spinning NMR techniques and plotted with identical scales for direct comparison of various lipids in different animal models. As can be seen from the spectra, triglycerides are higher in both Lep^{db} and Lep^{ob} compared with the lean mice by nearly an order of magnitude. On the other hand, phospholipids are higher in lean mice compared with Lep^{db} and Lep^{ob} mice (peak labeled as choline is a marker of phosphatidylcholine). **b** Score plot obtained from the PCA of the ^1H NMR (Carr-Purcell-Meiboom-Gill) data of Lep^{db} , Lep^{ob} and lean mice. Both Lep^{db} and Lep^{ob} show distinctly separate clusters from the lean, indicating significant metabolic differences between obese and lean mice. The loading plot along PC1 direction indicates that lipids and triglycerides increase in obese mice (upward peaks), whereas the phospholipid content is higher in lean mice (downward peak).

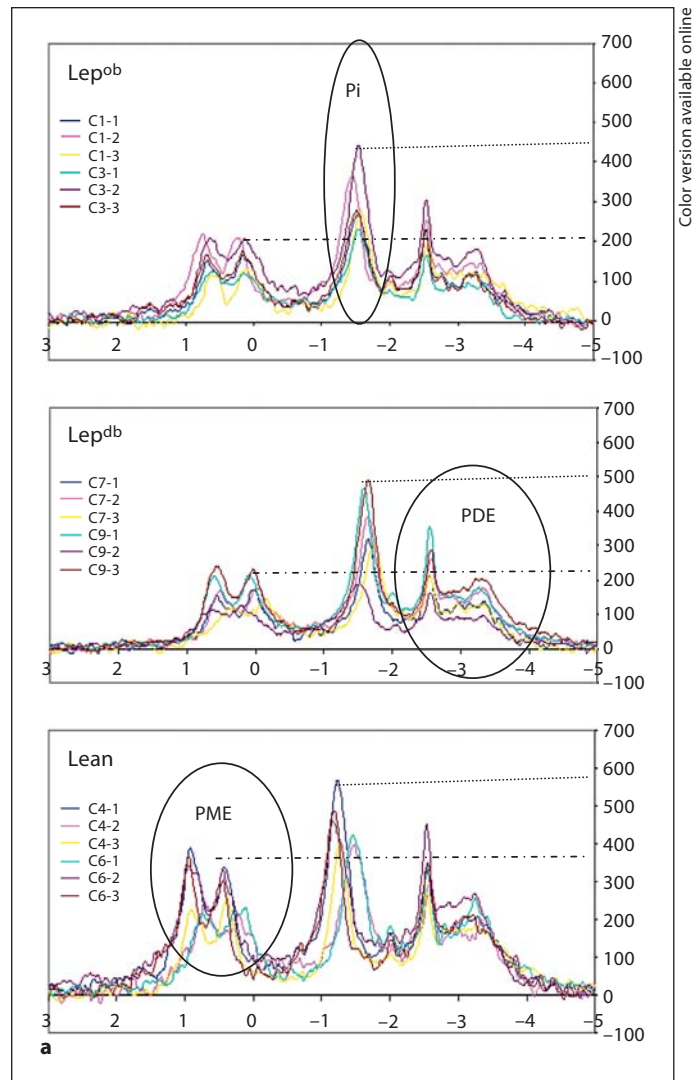


Metabolomics (^1H NMR). The observation by chromatography that the pancreatic phospholipids were significantly decreased in both obese strains of mice compared with the lean mice ($p < 0.05$) was confirmed by metabolomic analysis. However, the chromatography finding of elevated phospholipids in Lep^{db} mice compared with

Lep^{ob} mice was not observed in the metabolomic analysis.

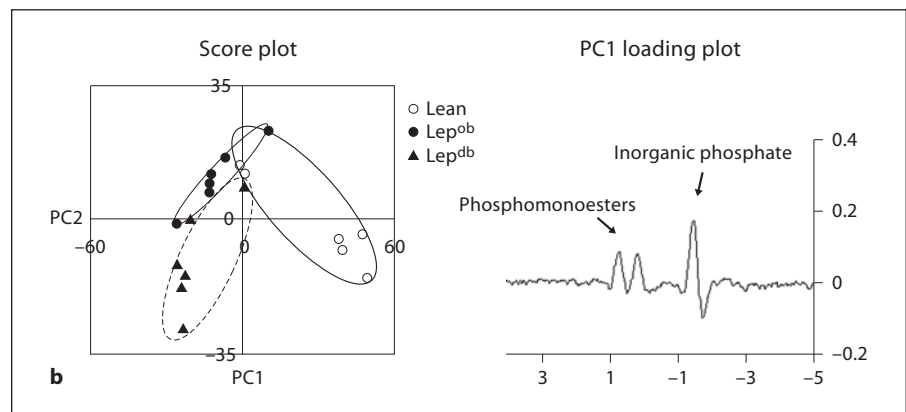
Free Fatty Acid Analysis

Chromatography. Total free fatty acids and individual free fatty acid chains are shown in table 1. Both saturated



Color version available online

Fig. 2. a ^{31}P NMR spectra of pancreas tissue from lean, Lep^{ob} and Lep^{db} mice. All spectra were obtained using magic angle sample spinning NMR techniques and plotted with identical scales for direct comparison of various phosphorus metabolites in different animal models. From the comparison of peak intensities, it can be seen that PME, phosphodiester (PDE) and Pi are higher in the lean than in Lep^{ob} and Lep^{db} . **b** Score plot obtained from the PCA of the ^{31}P NMR data of Lep^{db} , Lep^{ob} and lean mice. Both Lep^{db} and Lep^{ob} show distinctly separate clusters from the lean, indicating significant metabolic differences between obese and lean mice. The loading plot along the PC1 direction indicates that the phosphorus metabolites are higher in lean compared with obese mice (upward peaks).



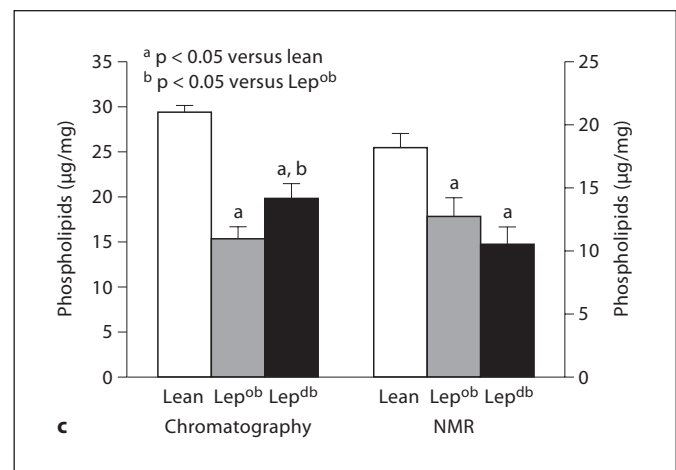
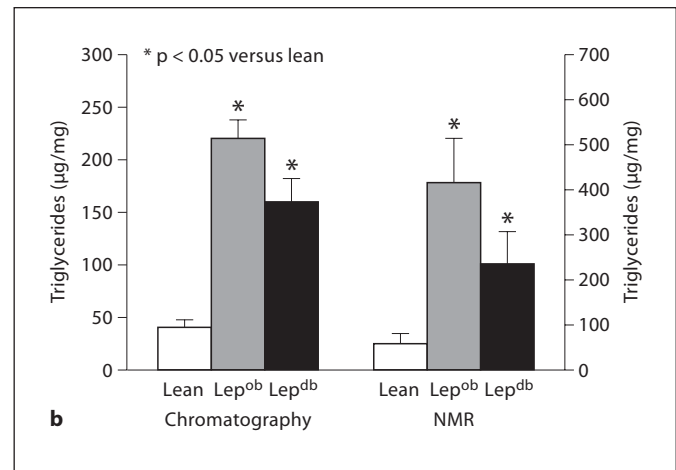
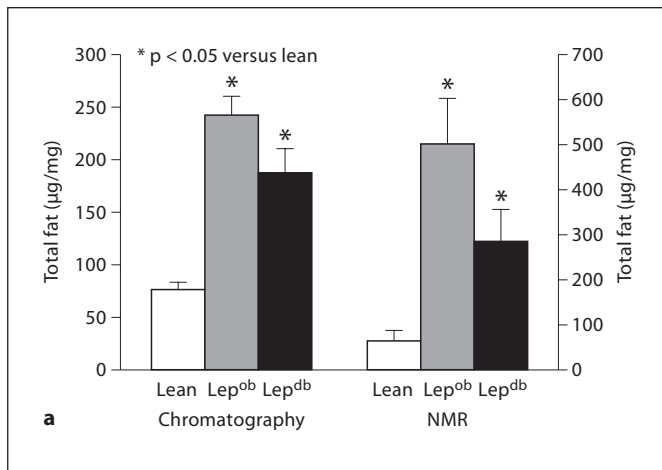


Fig. 3. a Pancreatic total fat ($\mu\text{g}/\text{mg}$ pancreatic tissue) as measured by conventional chromatography and metabolomic (NMR) analysis. Both congenitally obese strains of mice have significantly more fat than lean mice; good concordance between chromatographic and metabolomic analysis is apparent. **b** Pancreatic triglycerides ($\mu\text{g}/\text{mg}$ pancreatic tissue) as measured by conventional chromatography and metabolomic (NMR) analysis. Both congenitally obese strains of mice have significantly more triglycerides than lean mice; again, good concordance between chromatographic and metabolomic analysis is apparent. **c** Pancreatic phospholipids ($\mu\text{g}/\text{mg}$ pancreatic tissue) as measured by conventional chromatography and metabolomic (NMR) analysis. Both congenitally obese strains of mice have significantly lower phospholipid concentrations than lean mice. Again, chromatographic and metabolomic results are similar.

Table 1. Total and individual free fatty acid chains by chromatography ($\mu\text{g}/\text{mg}$ pancreatic tissue)

FFA chains	Lean	Lep ^{ob}	Lep ^{db}
Total FFA	2.8 ± 0.2	4.3 ± 0.1 ^a	5.2 ± 0.3 ^a
Saturated FFA	1.2 ± 0.1	1.4 ± 0.2	1.8 ± 0.2 ^a
Unsaturated FFA	1.6 ± 0.1	2.9 ± 0.4 ^a	3.5 ± 0.2 ^a
Myristic (14:00)	0.05 ± 0.01	0.1 ± 0.01 ^a	0.1 ± 0.08 ^a
Palmitic (16:00)	0.9 ± 0.1	1.1 ± 0.1	1.4 ± 0.1 ^a
Palmitoleic (16:01)	0.2 ± 0.02	0.6 ± 0.07 ^a	0.7 ± 0.05 ^a
Stearic (18:00)	0.3 ± 0.02	1.2 ± 0.03 ^a	0.3 ± 0.02 ^b
Oleic (18:01)	0.8 ± 0.08	1.3 ± 0.15 ^a	1.6 ± 0.1 ^a
Linoleic (18:02)	0.5 ± 0.0	0.8 ± 0.1 ^a	0.8 ± 0.0 ^a
Arachadonic acid	0.1 ± 0.01	0.1 ± 0.02	0.2 ± 0.01 ^b

Values are the mean ± SEM. FFA = Free fatty acid.
^a $p < 0.05$ versus lean; ^b $p < 0.05$ versus Lep^{ob}.

and unsaturated free fatty acids were significantly elevated in pancreata of obese groups compared with their lean counterparts ($p < 0.05$). However, saturated fatty acids were significantly elevated only in Lep^{db} mice ($p < 0.05$). In addition, palmitic acid was the most abundant saturated free fatty acid chain in all 3 strains. Palmitic acid followed the same pattern as saturated free fatty acids being maximally elevated in the pancreata of Lep^{db} mice ($p < 0.05$). The most abundant unsaturated free fatty acid was oleic acid which was elevated in both obese strains versus the lean mice ($p < 0.05$); no significant difference in oleic acid concentration existed between the obese strains.

Total Cholesterol and Cholesterol Ester Analysis

Chromatography. Pancreatic total cholesterol was significantly elevated in lean mice ($2.2 \pm 0.1 \mu\text{g}/\text{mg}$) versus

both Lep^{ob} (1.3 ± 0.1 µg/mg; p < 0.05) and Lep^{db} (1.8 ± 0.2 µg/mg; p < 0.05) mice. No significant difference existed between the obese strains. Additionally, no significant differences existed across the strains for cholesterol esters (lean mice = 0.6 ± 0.1, Lep^{ob} = 0.8 ± 0.2 and Lep^{db} = 1.0 ± 0.3 µg/mg; p = 0.6).

Total Cholesterol/Phospholipid Ratio

Across all 3 groups, no significant differences were observed in the total pancreatic cholesterol/phospholipid ratio (lean mice = 0.1 ± 0.0; Lep^{ob} = 0.1 ± 0.0; Lep^{db} = 0.1 ± 0.0; p = 0.19).

Visceral Fat Analysis by Chromatography

Visceral fat composition is shown in table 2.

Visceral Triglycerides

As expected, visceral fat composition was dominated by triglycerides. The visceral fat triglyceride content was significantly elevated in both obese strains of mice compared with their lean counterparts (p < 0.001). However, no significant differences were seen in triglyceride levels between Lep^{ob} and Lep^{db} mice. The total triglyceride content (in µg/mg tissue) was significantly greater in visceral fat compared with pancreatic fat.

Visceral Free Fatty Acids

Counterintuitively, total free fatty acids were decreased in the visceral fat of both obese Lep^{ob} (0.7 ± 0.1 µg/mg) and Lep^{db} (1.1 ± 0.1 µg/mg) strains compared with the lean mice (2.2 ± 0.1 µg/mg; p < 0.001). Saturated free fatty acids (lean mice = 0.7 ± 0.1, Lep^{ob} = 0.2 ± 0.0 and Lep^{db} = 0.4 ± 0.1 µg/mg) and unsaturated free fatty acids (lean mice = 1.5 ± 0.1, Lep^{ob} = 0.5 ± 0.1 and Lep^{db} = 0.7 ± 0.0 µg/mg) were similarly decreased in both obese strains of mice versus the lean mice (p < 0.001).

Other Lipid Groups

The preponderance of triglycerides in visceral fat samples interfered with the chromatographic analysis of the other lipid groups including phospholipids, cholesterol and cholesterol esters. Therefore, no data are available regarding the relative quantity of these lipid groups.

Discussion

The major finding of these experiments was that pancreata of congenitally obese mice have significantly more fat and a different composition of fat (i.e. more triglycer-

Table 2. Visceral fat analysis by chromatography (µg/mg pancreatic tissue)

	Lean	Lep ^{ob}	Lep ^{db}
Triglycerides	459 ± 32	613 ± 21 ^a	663 ± 16 ^a
Total FFA	2.2 ± 0.1	0.7 ± 0.1 ^a	1.1 ± 0.1 ^a
Saturated FFA	0.7 ± 0.1	0.2 ± 0 ^a	0.4 ± 0.1 ^a
Unsaturated FFA	1.5 ± 0.1	0.5 ± 0.2 ^a	0.7 ± 0 ^a

Values are the mean ± SEM. FFA = Free fatty acid.

^a p < 0.001 versus lean.

ides and free fatty acids, and less phospholipids and cholesterol) relative to lean wild-type mice. An equally significant primary aim of this study was to validate the utility of the powerful new technique of metabolomics in this type of tissue analysis. Importantly, metabolomic analysis (by ¹H and ³¹P NMR with PCA) correlated closely with conventional gas chromatographic analysis in identifying pancreatic total fat, triglyceride and phospholipid concentrations.

Fatty infiltration into visceral organs leads to an increased proinflammatory milieu with subsequent organ dysfunction. This process has been well documented in the cardiovascular system [19], kidney [20], gallbladder [2] and liver [1, 7]. The molecular mechanisms by which this process occurs are beginning to emerge, exemplified by the progression from steatosis to steatohepatitis to fibrosis/cirrhosis and malignancy observed in the liver [1, 7]. It is intuitive that obesity will lead to a similar situation in the pancreas; however, documentation of this phenomenon is less clear.

The association between obesity and pancreatic fat content has been shown both in human autopsy [21, 22] and recent radiology studies [23, 24]. Clinical studies have clearly shown that obesity leads to increased severity of acute pancreatitis [8–11], and obesity appears to confer an increased risk of developing pancreatic cancer [12–15]. Our group has recently shown that increased pancreatic fat is associated with an increased risk of complications after pancreatic surgery [25], and that congenitally obese (Lep^{ob} and Lep^{db}) mice develop more severe acute pancreatitis relative to lean wild-type mice [16]. Interestingly, in the latter experiments, the volume of fat per se did not correlate with severity of pancreatitis, suggesting that other factors such as alteration of the adipokine milieu or differential fat composition play a significant role in this pathological process.

The current experiments showed that both congenitally obese groups of mice (Lep^{ob} and Lep^{db}) had significantly more total pancreatic fat, triglycerides and free fatty acids than lean wild-type mice. This finding is important, as triglycerides and free fatty acids represent the 'toxic' components of adipose tissue.

Triglycerides compose the largest proportion of fat in the adipocyte, and triglyceride accumulation represents the necessary 'first hit' towards organ dysfunction in the well-described context of nonalcoholic fatty liver disease [7]. As triglycerides accumulate, adipocyte size increases, leading to insulin resistance. Insulin resistance, in turn, promotes further triglyceride accumulation, as well as increased production of the proinflammatory cytokine TNF- α [26, 27]. Thus, this vicious cycle serves to augment the local inflammatory milieu.

Free fatty acids represent a much smaller proportion of the total lipid pool. Despite their relative paucity, free fatty acids (and particularly saturated free fatty acids) are critically important mediators of the proinflammatory milieu [28–30]. Circulating free fatty acids, and specifically the saturated free fatty acid palmitate, correlate positively with concentrations of the proinflammatory cytokine IL-6 [31]. In addition, animal models have shown that saturated free fatty acids preferentially upregulate the production of proinflammatory cytokines (including TNF- α and IL-6) via mechanisms mediated in part through the Toll-like receptor 4/nuclear factor- κ B pathway [29, 31–35]. Furthermore, free fatty acids are an important source of reactive oxygen species through increased lipid peroxidation [36] and correlate positively with increased plasma concentrations of the proinflammatory adipokine leptin [30, 37]. Thus, our finding of increased triglycerides and free fatty acids in the pancreata of congenitally obese mice suggests that these components may play a significant role in enhancing the local proinflammatory milieu.

In contrast to triglycerides and free fatty acids, both congenitally obese strains of mice had significantly less pancreatic phospholipids and cholesterol than lean animals. As both phospholipids and cholesterol are integral constituents of cell membranes, it seems likely that this observation is related to the overall increased fat volume and/or adipocyte size, with a relatively smaller proportion of cell membrane material. It was interesting to note that there were no strain differences in the total cholesterol/phospholipid ratio, suggesting that despite an overall increase in adiposity, membrane stability/fluidity remained constant.

As would be expected, visceral fat composition was dominated by triglycerides, which were significantly in-

creased in both obese strains of mice relative to lean mice. Interestingly, the total free fatty acid volume was significantly lower in visceral fat of obese mice compared with lean animals. Further study will be necessary to see if this observation correlates with circulating free fatty acid content.

The field of metabolomics is emerging as a powerful new analytical approach in systems biology. Metabolomic analysis has been applied to toxicology studies, drug safety tests and disease detection (biomarkers). The current study represents an early effort in applying metabolomic analysis to the study of pancreatic disease, outside of 2 recent studies on pancreatic cancer [38, 39]. Our findings validate the metabolomic methodology, as patterns of total pancreatic fat, triglycerides and phospholipid concentration showed excellent correlation with those obtained by conventional thin-layer and gas chromatographic analysis. However, the real power of metabolomic analysis in evaluating pancreatic disease is yet to be recognized. Advances such as ¹³C NMR derivative analysis [40] promise to allow more accurate identification of metabolites unique to a specific study population (i.e. the obese mouse that develops more severe pancreatitis than a lean animal), and therefore, direct investigative focus toward specific metabolic pathways that are altered in the disease state of interest. The use of mass spectrometry especially in combination with NMR is highly promising in this direction [41–43].

Conventional chromatography methods often involve very tedious sample preparation and analysis procedures. Moreover, chromatographic methods are often selective to a certain class of metabolites, and hence, different procedures need to be employed for the analysis of a broad range of metabolites in the biological samples. On the other hand, NMR spectroscopy detects all the metabolites present in the samples, quantitatively and reproducibly. It is a high throughput analytical tool requiring less than 5 min per sample for the acquisition of the NMR data. Currently, NMR is the only technique available for profiling metabolites from intact tissue samples. Therefore, NMR spectroscopy promises immense utility in the pathophysiology of the pancreas.

In summary, these experiments demonstrate discrete differences in total fat volume and character in the pancreata of lean and congenitally obese mice. Obese mice had significantly greater pancreatic triglyceride and free fatty acid content, which may have important implications when considering the local proinflammatory milieu of the pancreas in obesity. In addition, this study highlights the utility of metabolomics-based analysis as

a powerful new analytic tool with which to define the precise mechanisms by which fat impacts pancreatic disease. Logical follow-up experiments include defining the pancreatic metabolome under various dietary conditions (i.e. low fat, high fat) as well as during pathological conditions such as pancreatitis and malignancy.

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References

- Farrell GC, Larter CZ: Nonalcoholic fatty liver disease: from steatosis to cirrhosis. *Hepatology* 2006;43:S99–S112.
- Goldblatt MI, Swartz-Basile DA, Al-Azzawi HH, Tran KQ, Nakeeb A, Pitt HA: Nonalcoholic fatty gallbladder disease: the influence of diet in lean and obese mice. *J Gastrointest Surg* 2006;10:193–201.
- Mathur A, Murine M, Lu D, Swartz-Basile DA, Saxena R, Zyromski NJ, Pitt HA: Nonalcoholic fatty pancreas disease. *HPB* 2007;9:312–318.
- Schaffer JE: Lipotoxicity: when tissues overeat. *Curr Opin Lipidol* 2003;14:281–287.
- Greenberg AS, Obin MS: Obesity and the role of adipose tissue in inflammation and metabolism. *Am J Clin Nutr* 2006;83:461S–465S.
- Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr: Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 2003;112:1796–1808.
- McCullough AJ: Pathophysiology of nonalcoholic steatohepatitis. *J Clin Gastroenterol* 2006;40:S17–S29.
- DeWaele B, Vanmierlo B, Van Nieuwenhove Y, Delvaux G: Impact of body overweight and class I, II and III obesity on the outcome of acute biliary pancreatitis. *Pancreas* 2006;32:343–345.
- Martinez J, Johnson CD, Sanchez-Paya J, de Madaria E, Robles-Diaz G, Perez-Mateo M: Obesity is a definitive risk factor of severity and mortality in acute pancreatitis: an updated meta-analysis. *Pancreatol* 2006;6:206–209.
- Papachristou GI, Papachristou DJ, Avula H, Slivka A, Whitcomb DC: Obesity increases the severity of acute pancreatitis: performance of APACHE-O score and correlation with the inflammatory response. *Pancreatol* 2006;6:279–285.
- Suazo-Barahona J, Carmona-Sanchez R, Robles-Diaz G, Milke-Garcia P, Vargas-Vorackova F, Uscanga-Dominguez L, Pelaez-Luna M: Obesity: a risk factor for severe acute biliary and alcoholic pancreatitis. *Am J Gastroenterol* 1998;93:1324–1328.
- Calle EE, Rodriguez C, Walker-Thurmond K, Thun MJ: Overweight, obesity, and mortality from cancer in a prospectively studied cohort of US adults. *N Engl J Med* 2003;348:1625–1638.
- Giovannucci E, Michaud D: The role of obesity and related metabolic disturbances in cancers of the colon, prostate, and pancreas. *Gastroenterology* 2007;132:2208–2225.
- Michaud DS, Giovannucci E, Willet WC, Colditz GA, Stampfer MJ, Fuchs CS: Physical activity, obesity, height and the risk of pancreatic cancer. *JAMA* 2001;286:921–929.
- Patel AV, Rodriguez C, Bernstein L, Chao A, Thun MJ, Calle EE: Obesity, recreational physical activity, and the risk of pancreatic cancer in a large US cohort. *Cancer Epidemiol Biomarkers Prev* 2005;14:459–466.
- Zyromski NJ, Mathur A, Yancey K, Gripe JT, Walker JJ, Lu D, Swartz-Basile DA, Lillemoe KD, Pitt HA: A murine model of obesity implicates the adipokine milieu in the pathogenesis of severe acute pancreatitis. *Am J Physiol Gastrointest Liver Physiol* 2008;295:G552–G558.
- Griffin JL, Nicholls AW: Metabolomics as a functional genomic tool for understanding lipid dysfunction in diabetes, obesity and related disorders. *Pharmacogenomics* 2006;7:1095–1107.
- Lindon JC, Holmes E, Nicholson JK: Metabolomics techniques and applications to pharmaceutical research and development. *Pharm Res* 2006;23:1075–1088.
- Kumada M, Kihara S, Ouchi N, Kobayashi H, Okamoto Y, Ohashi K, Maeda K, Nagaretani H, Kishida K, Maeda N, Nagasawa A, Funahashi T, Matsuzawa Y: Adiponectin specifically increased tissue inhibitor of metalloproteinase-1 through interleukin-10 expression in human macrophages. *Circulation* 2004;109:2046–2049.
- Lee JE, Choi SY, Huh W, Kim YG, Kim DJ, Oh HY: Metabolic syndrome, C-reactive protein, and chronic kidney disease in nondiabetic, nonhypertensive adults. *Am J Hypertens* 2007;20:1189–1194.
- Ogilvie RF: The islands of Langerhans in 19 cases of obesity. *J Pathol Bacteriol* 1933;37:473–481.
- Olsen TS: Lipomatosis of the pancreas in autopsy material and its relation to age and overweight. *Acta Microbiol Scand Sect A* 1978;86:367–373.
- Kovanlikaya A, Mittelman SD, Ward A, Geffner ME, Dorey F, Gilsanz V: Obesity and fat quantification in lean tissues using three-point Dixon MR imaging. *Pediatr Radiol* 2005;35:601–607.
- Matsumoto S, Mori H, Miyake H, Takaki H, Maeda T, Yamada Y, Oga M: Uneven fatty replacement of the pancreas: evaluation with CT. *Radiology* 1995;194:453–458.
- Mathur A, Pitt HA, Maxine M, Saxena R, Schmidt CM, Howard TJ, Nakeeb A, Zyromski NJ, Lillemoe KD: Fatty pancreas: a factor in postoperative pancreatic fistula. *Ann Surg* 2007;246:1058–1064.
- Hotamisligil GS, Shargill NS, Spiegelman BM: Adipose expression of tumor necrosis factor- α : direct role in obesity-linked insulin resistance. *Science* 1993;259:87–91.
- Hirata T, Unoki H, Bujo H, Ueno K, Saito Y: Activation of diacylglycerol O-acyltransferase 1 gene results in increased tumor necrosis factor- α gene expression in 3T3-L1 adipocytes. *FEBS Lett* 2006;580:5117–5121.
- Cha MC, Chou CJ, Boozer CN: High-fat diet feeding reduces the diurnal variation of plasma leptin concentration in rats. *Metabolism* 2000;49:503–507.
- Fernandez-Real JM, Broch M, Vendrell J, Ricard W: Insulin resistance, inflammation, and serum fatty acid composition. *Diabetes Care* 2003;26:1362–1368.
- Lovejoy JC, Windhauser MM, Rood JC, de la Bretonne JA: Effect of a controlled high-fat versus low-fat diet on insulin sensitivity and leptin levels in African-American and Caucasian women. *Metabolism* 1998;47:1520–1524.
- Nguyen MT, Satoh H, Favelyukis S, Babendure JL, Imamura T, Sbodio JI, Zalevsky J, Dahiyat BI, Chi NW, Olefsky JM: JNK and tumor necrosis factor- α mediate free fatty acid-induced insulin resistance in 3T3-L2 adipocytes. *J Biol Chem* 2005;280:35361–35371.
- Bastard JP, Maachi M, Lagathu C, Kim MJ, Caron M, Vidal H, Capeau J, Feve B: Recent advances in the relationship between obesity, inflammation, and insulin resistance. *Eur Cytokine Netw* 2006;17:4–12.

- 33 Boden G, She P, Mozzoli M, Cheung P, Gumireddy K, Reddy P, Xiang X, Luo Z, Ruderan N: Free fatty acids produce insulin resistance and activate the proinflammatory nuclear factor-kappaB pathway in rat liver. *Diabetes* 2005;54:3458–3465.
- 34 Ghanim H, Aljada A, Hofmeyer D, Syed T, Mohanty P, Dandona P: Circulating mononuclear cells in the obese are in a proinflammatory state. *Circulation* 2004;110:1564–1571.
- 35 Suganami T, Nishida J, Ogawa Y: A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: role of free fatty acids and tumor necrosis factor alpha. *Arterioscler Thromb Vasc Biol* 2005;25:2062–2068.
- 36 Inoguchi T, Li P, Umeda F, Yu HY, Kakimoto M, Imamura M, Aoki T, Etoh T, Hashimoto T, Naruse M, Sano H, Utsumi H, Nawata H: High glucose level and free fatty acids stimulate reactive oxygen species production through protein kinase C-dependent activation of NAD(P)H oxidase in cultured vascular cells. *Diabetes* 2000;49:1939–1945.
- 37 Yannakoulia M, Yiannakouris N, Bluher S, Matalas AL, Klimis-Zacas D, Mantzoros CS: Body fat mass and macronutrient intake in relation to circulating soluble leptin receptor, free leptin index, adiponectin, and resistin concentrations in healthy humans. *J Clin Endocrinol Metab* 2003;88:1730–1736.
- 38 Beger RD, Schnackenberg LK, Holland RD, Li D, Dragan Y: Metabolomic models of human pancreatic cancer using 1D proton NMR spectra of lipids in plasma. *Metabolomics* 2006;2:125–134.
- 39 Fang F, He X, Deng H, Chen Q, Lu J, Spraul M, Yu Y: Discrimination of metabolic profiles of pancreatic cancer from chronic pancreatitis by high-resolution magic angle spinning ¹H nuclear magnetic resonance and principal components analysis. *Cancer Sci* 2007;98:1678–1682.
- 40 Shanaiah N, Desilva MA, Nagana Gowda GA, Raftery MA, Hainline BE, Raftery D: Class selection of amino acid metabolites in body fluids using chemical derivatization and their enhanced ¹³C NMR. *Proc Natl Acad Sci USA* 2007;104:11540–11544.
- 41 Chen HW, Pan Z, Talaty N, Cooks RG, Raftery D: Combining desorption electrospray ionization mass spectrometry and nuclear magnetic resonance for differential metabolomics without sample preparation. *Rapid Commun Mass Spectrom* 2006;20:1577–1584.
- 42 Gu H, Chen H, Pan Z, Jackson AU, Talaty N, Xi B, Kissinger C, Duda C, Mann D, Raftery D, Cooks RG: Monitoring diet effects from biofluids and their implications for metabolomics studies. *Anal Chem* 2007;79:89–97.
- 43 Pan Z, Raftery D: Combining NMR spectroscopy and mass spectrometry in metabolomics. *Anal Bioanal Chem* 2007;387:525–527.