Diet-induced mouse model of fatty liver disease and nonalcoholic steatohepatitis reflecting clinical disease progression and methods of assessment

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Clapper JR, Hendricks MD, Gu G, Wittmer C, Dolman CS, Herich J, Athanacio J, Villescaz C, Ghosh SS, Heilig JS, Lowe C, Roth JD. Diet-induced mouse model of fatty liver disease and nonalcoholic steatohepatitis reflecting clinical disease progression and methods of assessment. Am J Physiol Gastrointest Liver Physiol 305: G483–G495, 2013. First published July 25, 2013; doi:10.1152/ajpgi.00079.2013.—Shortcomings of previously reported preclinical models of nonalcoholic steatohepatitis (NASH) include inadequate methods used to induce disease and assess liver pathology. We have developed a dietary model of NASH displaying features observed clinically and methods for objectively assessing disease progression. Mice fed a diet containing 40% fat (of which ~18% was trans fat), 22% fructose, and 2% cholesterol developed three stages of nonalcoholic fatty liver disease (steatosis, steatohepatitis with fibrosis, and cirrhosis) as assessed by histological and biochemical methods. Using digital pathology to reconstruct the left lateral and right medial lobes of the liver, we made comparisons between and within lobes to determine the uniformity of collagen deposition, which in turn informed experimental sampling methods for histological, biochemical, and gene expression analyses. Gene expression analyses conducted with animals stratified by disease severity led to the identification of several genes for which expression was highly correlated with the histological assessment of fibrosis. Importantly, we have established a biopsy method allowing assessment of disease progression. Mice subjected to liver biopsy recovered well from the procedure compared with sham-operated controls with no apparent effect on liver function. Tissue obtained by biopsy was sufficient for gene and protein expression analyses, providing the opportunity to establish an objective method of assessing liver pathology before subjecting animals to treatment. The improved assessment techniques and the observation that mice fed the high-fat diet exhibit many clinically relevant characteristics of NASH establish a preclinical model for identifying pharmacological interventions with greater likelihood of translating to the clinic.

nonalcoholic fatty liver disease; steatosis; fibrosis; steatohepatitis; in vivo tissue sampling

NONALCOHOLIC FATTY LIVER DISEASE (NAFLD) is the most common cause of liver disease in the Western world (11). NAFLD is considered the hepatic manifestation of the metabolic syndrome and is strongly associated with dyslipidemia, central obesity, hypertension, and insulin resistance (12). NAFLD encompasses a spectrum of pathologies that range from simple hepatic steatosis (>55 mg triglyceride/g liver) (35) to nonalcoholic steatohepatitis (NASH), hepatic steatosis with inflammation, cellular ballooning, and varying degrees of fibrosis. Whereas steatosis in the absence of inflammation and fibrosis is generally considered benign, the more advanced state of NASH, and in particular fibrosing NASH, is a leading cause of cirrhosis and liver-related mortality (10, 13, 38).

Currently, no pharmacological agents are specifically approved for the treatment of NAFLD/NASH. The failure to identify effective therapies has been attributed, in part, to the failure of the available preclinical models to recapitulate essential features of the clinical condition (steatosis, inflammation, and fibrosis) while preserving the natural history and etiology of the disease. Genetic disruption or overexpression of genes involved in hepatic function and metabolism have been used to cause spontaneous development of NAFLD and/or NASH; however, compensatory and other mechanistic changes prevent these models from reproducing all pathophysiological features of the disease. For example, impairments in leptin (Lepr-null/Lepr−/−; Refs. 6, 16) or melanocortin (KK-A°; Ref. 36) signaling produce varying degrees of obesity, insulin resistance, and diabetes, and the development of fatty liver without further progression to more advanced forms of steatohepatitis with fibrosis in animals reared on standard diets. Other transgenic and knockout mouse models [e.g., sterol regulatory element binding protein 1c (SREBP-1c)-overexpressing mice or phosphatase and tensin homologue deleted on chromosome 10 (PTEN) mice] develop a more severe hepatic phenotype but do not display the concomitant metabolic dysfunction such as obesity and insulin resistance that normally accompanies the disease (36). Although a severe NASH-like phenotype can also be induced by toxic interventions (e.g., CCl4) or nutrient-deficient diets (methionine and choline deficiency), these models do not replicate the underlying mechanisms relevant to clinical disease progression and also fail to develop the characteristics of metabolic syndrome associated with NASH (34).

Emerging models that rely solely on ad libitum feeding of diets enriched in various combinations of fat, cholesterol, and fructose more closely mimic the causal factors associated with disease progression in humans. However, animals fed these diets typically develop only fatty liver and mild NASH with a minimally fibrotic phenotype (36, 37, 39). Thus there remains a clear need for preclinical models that reproduce both the disease phenotype and its etiology to support mechanistic and pharmacological studies of NASH.

Preclinical studies of NASH also face several technical challenges. Elegant histological scoring systems have been developed for clinical diagnostic use (5, 18, 29, 43) and applied...
to preclinical models; however, these assessments are subject to interobserver variability and lack the dynamic range to distinguish subtle changes in fibrosis. A rigorous standardized and quantitative staining analysis by microscopic observation for determining the degree of liver fibrosis in preclinical studies has not been reported. Image-based quantitative assessments of hepatic fibrosis from representative photomicrographs are widely reported; however, the criteria for determining how many lobes of the liver needed to be sampled and which subregions of the individual lobes should be analyzed to represent the status of the organ in its entirety have not been described.

An often-overlooked feature in the application of preclinical models is the failure to determine the disease stage prior to pharmacological treatment. As is seen clinically, disease progression in animal models of NAFLD is inherently variable. Animals maintained on an experimental diet will develop NAFLD at differing rates and of varying severity. Because of a lack of diagnostic biomarkers, the stage of disease in this heterogeneous population cannot easily be assessed in the live animal prior to initiation of pharmacological intervention. This severely limits the value of preclinical models for detection of treatment effects due to the confounding effects of heterogeneity in the study population. For example, a drug anticipated to prevent progression to fibrosis can be effective only in the prefibrotic subset of subjects. In contrast, agents expected to reverse fibrosis can only be effective in animals that already have developed fibrosis prior to treatment. In these cases, heterogeneity in the pretreatment population will lead to misinterpretation of the efficacy of either potential therapeutic.

Clinical trials are typically designed to determine whether an agent can improve fibrosis or inflammation in subjects with biopsy-confirmed disease; a population that is generally at a more advanced stage of disease than that induced by the reported animal models (18, 31).

To ultimately enable a systematic evaluation of candidate drugs and their ability to reverse or delay progression of NAFLD in a physiologically relevant context, we further refined the characterization of our mouse model in which disease was induced by a nutritional regime resembling that of the typical Western diet (39). In addition, to facilitate pharmacological studies, and more closely parallel clinical practice, we developed a biopsy procedure to allow the assessment of disease in each animal before treatment and have developed comprehensive histological sampling and scoring techniques that ensure pathological findings represent the status of the organ in its entirety. Finally, to identify potential molecular signatures of disease progression, array-based mRNA analyses were conducted from animals at defined stages of disease.

**MATERIALS AND METHODS**

**Animals.** All studies were approved by the Institutional Animal Care and Use Committee at Amylin Pharmaceuticals, LLC in accordance with Animal Welfare Act guidelines. Animals were housed individually in standard cages at 22°C in a 12:12-light-dark cycle. Male C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were allowed ad libitum access to a diet enriched in fat (40% kcal; Primex partially hydrogenated vegetable oil shortening), fructose (22% by wt), and cholesterol (2% by wt) (Research Diets, New Brunswick, NJ, cat. no. D09100301). We previously reported that this diet induced varying degrees of NAFLD in leptin-deficient (Lep<sup>−/−</sup>/Lep<sup>−/−</sup>) and C57BL/6 mice (39). Mice maintained on this diet are referred to as the Amylin liver NASH model (AMLN). A low-fat diet (10% kcal; hereafter referred to as LFD) with no fructose or cholesterol was used as a control diet (Research Diets, cat. no. D09100304). The use of this validated LFD allowed us to establish a group of control mice that maintain a “normal” hepatic phenotype for comparison with animals fed the AMLN diet. A total of 35 LFD and 73 AMLN mice were used for these experiments. Total animal numbers for each experiment are indicated under RESULTS.

**Histology and digital image analysis.** At termination, right medial and/or left lateral lobes of the liver (≥50% of each lobe harvested) were excised and fixed in 10% neutral-buffered formalin (at least 7 days at room temperature). Liver tissue was paraffin embedded, sectioned (5 μM), and mounted taking care to select similarly sized sections representative of both the tissue edge and center. Hematoxylin and eosin stains were used for morphological analyses, and Masson’s trichrome and Sirius red stains were used for assessment of hepatic fibrosis. Histopathological analysis was performed by a pathologist (Pathology Associates, Frederick, MD) blinded to the study. NAFLD and NASH were scored by use of criteria outlined by Kleiner and colleagues (18).

For quantitative assessment of fibrosis, whole Sirius red-stained sections were scanned by use of the ScanScope CS whole slide scanning system (Aperio, Vista, CA) at ×20 magnification. Images were extracted and Sirius red-stained collagen profiles from entire tissues were measured by the color cube-based method with ImageJ.

**Table 2. Phenotypic and biochemical profiles of mice maintained on AMLN or LFD diets for 30 wk**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>LFD</th>
<th>AMLN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>32.7 ± 0.9</td>
<td>41.2 ± 0.2*</td>
</tr>
<tr>
<td>Liver weight, g</td>
<td>1.36 ± 0.04</td>
<td>4.64 ± 0.15*</td>
</tr>
<tr>
<td>Liver weight, % of body wt</td>
<td>4.2 ± 0.1</td>
<td>11.2 ± 0.3*</td>
</tr>
<tr>
<td>Intrahepatic lipids, mg/g</td>
<td>66.0 ± 4.1</td>
<td>321.4 ± 14.9*</td>
</tr>
<tr>
<td>ALT, U/l</td>
<td>20.0 ± 5.2</td>
<td>339.6 ± 25.8*</td>
</tr>
<tr>
<td>AST, U/l</td>
<td>74.8 ± 27.3</td>
<td>260.7 ± 20.4*</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>124.5 ± 6.4</td>
<td>315.8 ± 11.5*</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>68.7 ± 5.0</td>
<td>55.7 ± 2.2*</td>
</tr>
<tr>
<td>Fasting glucose, mg/dl</td>
<td>159.3 ± 6.2</td>
<td>165.5 ± 5.0</td>
</tr>
<tr>
<td>Fasting insulin, pg/ml</td>
<td>369.7 ± 73</td>
<td>761.8 ± 82.6*</td>
</tr>
<tr>
<td>HOMA-IR, mU·μU·ml&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>4.2 ± 0.9</td>
<td>9.1 ± 1.1*</td>
</tr>
<tr>
<td>Adiponectin, μg/ml</td>
<td>32.0 ± 1.7</td>
<td>21.0 ± 0.7*</td>
</tr>
</tbody>
</table>

Results are means ± SE. Low-fat diet (LFD), N = 6–8; Amylin liver NASH model (AMLN), N = 12. ALT, alanine aminotransferase; AST, aspartate aminotransferase; HOMA-IR, homeostasis model assessment of insulin resistance. *P < 0.05 vs. LFD.
Pro Analyzer software (MediaCybernetics v.6.2, Bethesda, MD). Total collagen staining (reported as % of total area) was assessed from three to four representative sections from each animal (except for the comprehensive liver fibrosis assessment experiment where additional sections were evaluated). All histological analyses were performed blinded.

Liver biopsy. Mice were anesthetized with isoflurane (2–3%) in 100% oxygen. A small abdominal incision, ~0.5 cm left of midline, was made and the left lateral lobe of the liver was exposed. A wedge of liver tissue (~50 mg) was excised from the distal portion of the lobe, immediately placed in a vial, and snap frozen in liquid nitrogen. A wedge of absorbable gelatin sponge (GelFoam, Pfizer, NY) was inserted into the cut edges of the liver. Once hemostasis was achieved (typically within 1 min) and the gelatin sponge was well-adhered to the biopsy site, the liver was returned to the abdominal cavity, the abdominal wall sutured, and the skin stapled. Mice received a single injection of buprenorphine (0.05 mg/kg, subcutaneous) at the time of the surgery to control postoperative pain. Sham-operated mice underwent an identical procedure except no incision was made in the liver.

Plasma and serum analysis. Plasma glucose, triglycerides, total cholesterol, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) levels were measured by using an Olympus AU400e Bioanalyzer (Olympus America Diagnostics, Center Valley, PA). Plasma samples were diluted 1:10 with PBS for measurement of ALT and AST. Total plasma adiponectin and fasting serum insulin were measured according to the manufacturer’s instructions with commercially available electrochemiluminescence kits (Meso Scale Discovery, Gaithersburg, MD).

Quantification of total hepatic lipid and collagen content. Total hepatic lipid was extracted from the liver using a protocol adapted from Folch et al. (14). Frozen liver tissue (~0.3 g) was homogenized

Table 3. Hepatic pathology scores

<table>
<thead>
<tr>
<th>Parameters</th>
<th>LFD</th>
<th>AMLN</th>
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<tbody>
<tr>
<td>Microvesicular steatosis</td>
<td>0.1 ± 0.1</td>
<td>3.4 ± 0.1*</td>
</tr>
<tr>
<td>Macrovesicular steatosis</td>
<td>0.0 ± 0.0</td>
<td>3.2 ± 0.4*</td>
</tr>
<tr>
<td>Ballooning degeneration</td>
<td>0.0 ± 0.0</td>
<td>2.0 ± 0.2*</td>
</tr>
<tr>
<td>Inflammation</td>
<td>0.3 ± 0.2</td>
<td>2.1 ± 0.1*</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>0.1 ± 0.1</td>
<td>2.0 ± 0.2*</td>
</tr>
<tr>
<td>NAFLD score</td>
<td>0.0 ± 0.0</td>
<td>3.0 ± 0.0*</td>
</tr>
<tr>
<td>NASH score</td>
<td>0.0 ± 0.0</td>
<td>2.2 ± 0.1*</td>
</tr>
</tbody>
</table>

Blinded pathology scores from mice fed the AMLN diet for 30 wk. Results are means ± SE. NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis. LFD, N = 7; AMLN, N = 12. *P < 0.05 vs. LFD.
in 10 ml of 2:1 chloroform-methanol solution. The homogenate was filtered using fat-free filter paper and funneled into a preweighed 15-ml glass vial. An additional 5 ml of 2:1 chloroform-methanol was added followed by 2.5 ml of 0.9% NaCl. The lipids were separated by centrifugation at 1,800 g, 10°C for 5 min, the aqueous layer was discarded, and the tube was flushed with nitrogen until the lipid pellet was dry. The tube containing the lipid pellet was reweighed, and total lipid extracted per gram of total liver was calculated.

Total collagen content in the liver was measured by colorimetric determination of hydroxyproline residues by acid hydrolysis of collagen (Quickzyme, Leiden, Netherlands).

**Determination of extractable collagen-1α1 protein by protein blot.** Tissue cores (~50–100 mg) were collected from the left lateral lobe of the liver, snap frozen in liquid nitrogen, and stored at −80°C until processed. The tissue was homogenized in lysis buffer containing protease inhibitors. Protein concentration of the cleared supernatant was measured with a BCA protein assay kit (Pierce, Rockford, IL). Liver tissue lysates (~50 μg) were separated on reducing 4–12% Nupage gels (Life Technologies, Carlsbad, CA) and transferred to nitrocellulose membranes. Membranes were cut between the 50- and 60-kDa markers and blocked with 5% Blotto. The upper half was probed with anti-collagen-1α1 (1:1,000; cat. no. NBPI–30054; Novus Biologicals, Littleton, CO), which detects the COOH-terminal telopeptide portion of the collagen-1α1 protein. For normalization, the lower half was probed with anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:7,500; cat. no. 3683; Cell Signaling Technologies, Danvers, MA). Following incubation with horseradish peroxidase anti-rabbit antibody, protein expression was detected with enhanced chemiluminescence (Thermo Scientific, Rockford, IL), and densitometry was performed with a FluorChem System (Cell Biosciences, Santa Clara, CA). Densitometry analysis of collagen-1α1 included both the 140-kDa mature protein as well as a slightly larger band, corresponding to a glycosylated form or a partially processed collagen-1α1 protein.

![Fig. 2. Heterogeneity of collagen deposition.](http://ajpgi.physiology.org/)

Fig. 2. Heterogeneity of collagen deposition. Sirius red-stained sections from the center of the lobe (after discard of at least 1 mm of surface tissue) reflect the different distribution of collagen throughout the left lateral lobe of LFD (A–C) or AMLN diet-fed (D–F) mice exhibiting Low Fibrosis. Staining is evident near the tissue edge around vessels and in the perisinusoidal spaces, consistent with normal collagen deposition in LFD mice (A and B). In more central regions of the liver (>1 mm from the edge), collagen is limited to the area directly surrounding vessels (A and C). In AMLN mice, collagen is most densely deposited at the tissue margins (D and E). Although collagen is present more medially, it is heterogeneously distributed (D and F). Scale bars: A and D = 500 μM; B, C, E, and F = 100 μM.
**Hepatic gene expression changes.** Tissue samples from the left lateral lobe of the liver were harvested with a 6-mm tissue coring tool or by the biopsy method, snap frozen in liquid nitrogen, and stored at −80°C until processed. Total RNA from liver samples (20 μg) was extracted by use of TRI Reagent (Life Technologies) and then further purified with a Qiagen RNeasy Plus Mini kit (Qiagen, Valencia, CA). RNA integrity was determined by using the Agilent 6000 nano kit on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). cDNA was prepared by using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). Changes in gene expression were calculated by the ABI Prism 7900HT Fast Real-Time PCR System (Applied Biosystems/Life Technologies). Among the five housekeeping genes systems). Changes in gene expression on the array were calculated by the ABI Prism 7900HT Fast Real-Time PCR System (Applied Biosystems/Life Technologies). Changes in gene expression were calculated by the comparative threshold cycle (C_{T}) method with peptidylprolyl isomerase A (PpiA) and Gapdh for normalization. For gene arrays, cDNA samples were run on Mouse Fibrosis RT² Profiler PCR Arrays (PAMM-120C, RT² SYBR Green/ROX qPCR Master Mix; SABiosciences) by using the ABI Prism 7900HT Fast Real-Time PCR System (Applied Biosystems/Life Technologies). Changes in gene expression on the array were calculated by the comparative C_{T} method using DataAssist v3.0 software (Applied Biosystems/Life Technologies). Among the five housekeeping genes included in the Mouse Fibrosis RT² Profiler PCR Array, hypoxanthine phosphoribosyltransferase 1 (Hprt) and Gapdh had the most stable expression according to the stability scores calculated by DataAssist v3.0 software. The mean of the chosen endogenous control genes was used as the normalization factor to calculate the relative expression of each gene. To confirm the results obtained by using the fibrosis array, TaqMan Gene expression assays were conducted for a selection of genes determined by the array to be upregulated, downregulated, or unchanged. For the LFD control group, average threshold cycle results for Mmp8, Timp1, and Timp4 exceeded 35 cycles and, therefore, results of these analyses were not considered reliable.

**Cluster analysis and heat map of gene expression data.** The graphical heat map display was generated by use of DataAssist v3.0 software. Distances between samples and assays were calculated for hierarchical clustering on the basis of the ΔC_{T} values by using Pearson’s correlation and average linkage in an Assay Centric map view. Mip1a and Grem1 were excluded from analysis because of inconsistent results.

**Statistical analysis.** Results are expressed as means ± SE. Statistical significance was evaluated by one- and two-way ANOVA with Dunnett’s or Newman-Keuls post hoc tests where appropriate. For nonparametric comparisons a Kruskal-Wallis test with a Dunn’s post hoc test was performed. P ≤ 0.05 was considered significant. The P values for the array data were adjusted by using the Benjamini-Hochberg false discovery rate correction to correct for multiple comparisons.

### RESULTS

**Biochemical and histological features of AMLN mice.** Male C57BL/6 mice were maintained on the AMLN diet for 30 wk. A cohort of AMLN diet-fed mice (n = 12) of similar body weight was selected and compared with a group of mice fed the LFD (n = 6–8). AMLN-fed mice weighed more and presented with clear hepatomegaly and significantly higher liver weight and intrahepatic lipid content. Plasma ALT and AST concentrations were elevated by ~17- and ~4-fold, respectively. Total plasma cholesterol was increased in AMLN mice whereas plasma total triglycerides were decreased. Fasting glucose was not elevated.

**Cluster analysis and heat map of gene expression data.**
in AMLN mice; however, a significant ~2-fold increase in fasting insulin and a similar increase in the homeostasis model assessment of insulin resistance (HOMA-IR) were observed, suggesting that AMLN mice developed insulin resistance. Consistent with an obese and insulin-resistant state, circulating levels of plasma adiponectin were significantly decreased in the AMLN mice (Table 2).

Histological examination of hematoxylin and eosin and Masson’s trichrome-stained liver sections revealed the development of severe steatosis with necroinflammatory changes and fibrosis in AMLN mice. Moderate to marked macro- and microvesicular steatosis was present without pattern throughout the hepatic lobule, whereas animals maintained on the LFD displayed minimal evidence of hepatic fat accumulation. Periportal inflammation and ballooning degeneration were observed, indicative of a progression from simple steatosis to steatohepatitis. Visualization of Masson’s trichrome-stained liver sections demonstrated significant fibrosis in the AMLN mice, consistent with an advanced NASH phenotype. Fibrosis was most evident in the tissue margins but also penetrated into the center of the tissue, affecting nearly all portal triads, and in many cases was present in a “bridging” pattern throughout the hepatic lobule (Fig. 1; Table 3).

Quantification of hepatic fibrosis in mice maintained on the AMLN diet. Given the observed heterogeneous pattern of hepatic collagen deposition during NAFLD progression, we sought to optimize methods for the future histological assessment of fibrosis throughout the liver. Two major lobes (the left lateral and the right medial) comprising >50% of the total liver mass were collected from three mice maintained on the AMLN diet and evaluated for collagen accumulation. Sections from the entire left lateral and right medial lobes (as many as 136 sections/lobe) were collected from the dorsal to ventral surface. Every fourth section distributed evenly across approximately the top two-thirds of the entire lobe was assessed for collagen content by staining with Sirius red. The entire section was scanned at ×20 magnification and captured as a digital image. Sirius red staining was quantified to determine total collagen in each whole section (expressed as % of total tissue area). Parallel analysis of livers from LFD-fed animals revealed minimal collagen accumulation. Staining was most evident at the tissue edge and the vessel walls, indicative of normal

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Fig. 4. AMLN diet-fed mice develop progressive NAFLD/NASH. Mice fed the AMLN diet for 20 or more wk exhibit a distribution of liver pathologies including significant steatosis without evidence of steatohepatitis or fibrosis (A and B) (Fatty Liver), frank NASH with inflammatory infiltrate (arrowheads) and fibrosis (C and D) (Moderate Fibrosis), to evidence of extensive scar tissue and regenerative micronodules (*) in mice with hepatic cirrhosis (E and F). A, C, and E: hematoxylin and eosin. B, D, and F: Masson’s trichrome. Scale bar = 100 µM.
extracellular matrix (ECM) deposition (Fig. 2, A–C). In AMLN mice, collagen density was more pronounced at the perimeter of the liver, within the first ~1 mm of the margin, affecting the portal triads and perisinusoidal space throughout the hepatic lobule (Sirius red staining, 3.10 ± 0.29%; Fig. 2, D–F). In deeper regions of the hepatic parenchyma (>3 mm from organ perimeter) staining for collagen was reduced and generally isolated to the periportal regions with only minimal perisinusoidal fibrosis (Sirius red staining, 0.47 ± 0.04%). The degree of fibrosis in the left lateral lobe and right medial lobe was noted to be similar in pattern and magnitude (data not shown). These histological findings enabled us to implement sampling methods that would only require examination of a limited set of tissue sections to represent the disease state of the entire organ. These sampling methods were employed on all subsequent analyses and are described in the Discussion.

Using our optimized histological analysis on mice maintained on the AMLN diet for 30 wk demonstrated an approximately threefold increase in hepatic collagen deposition relative to mice maintained on a LFD (Sirius red staining density expressed as % of total area, LFD: 1.45 ± 0.07, n = 4; AMLN: 4.56 ± 0.46, n = 12; P < 0.05). Consistent with the morphological findings, biochemical analyses revealed that hepatic collagen-I protein and total collagen content were elevated ~24- and 4-fold, respectively, in AMLN-fed mice (Fig. 3, A–C). Furthermore, mRNA levels of ECM proteins, Col1a2 and Lamc1, and the chemokine marker of inflammation, Ccl2, were increased ~11-, 2-, and 8-fold, respectively (Fig. 3, D–F).

**AMLN mice exhibit all stages of NAFLD.** On the basis of these histological assessments, we categorized mice by three stages of disease severity: simple steatosis, NASH with fibrosis, and cirrhosis. Mice with simple steatosis showed severe macro- and microvesicular lipid accumulation in the liver tissue with minimal evidence of necroinflammatory changes and fibrosis (Fig. 4, A and B). Mice with progressive NASH were identified by the presence of inflammatory infiltrates and significant periportal and perisinusoidal fibrosis (Fig. 4, C and D). Cirrhotic mice were identified by extensive scar tissue, inflammatory infiltrate, and the presence of regenerative micronodules (Fig. 4, E and F). Whereas overall time on diet was associated with disease severity, individual mice progressed through the stages of NAFLD at different rates. All stages of the disease were represented in a single cohort of animals reared on the AMLN diet for any period greater than ~20 wk. As is likely the case with other models, staging animals simply by duration of exposure to the NAFLD/NASH-inducing conditions introduces unacceptable heterogeneity to a study population, requiring the development of better methods for determining disease severity at initiation of treatment.

**Liver biopsy allows accurate staging of liver pathology.** Given the inherent variability in the rate of disease progression, we developed a liver biopsy technique to assess the stage of NAFLD (e.g., fatty liver vs. steatohepatitis with fibrosis) in live mice to establish a baseline status prior to pharmacological interventions. A group of AMLN mice underwent liver biopsy or sham operation (n = 10/group). Excessive blood loss during the procedure was not observed and all biopsied and sham-operated animals survived the 14-day duration of the study. Food intake was identical in both groups and concentrations of liver enzymes were similar between the biopsy and sham-operated mice 14 days after surgery (ALT, sham: 81.2 ± 9.3 U/l; ALT, biopsy: 109.1 ± 12.1 U/l; AST, sham: 101.7 ± 12.6 U/l; AST, biopsy: 125.9 ± 9.9 U/l, P > 0.05). Liver appearance was normal on postmortem observation (14 days after biopsy), and histological evaluation of liver tissue revealed that the biopsy site had been replaced by scar tissue that stained positively for

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**Fig. 5. Biopsy does not cause damage beyond the site of incision.** Low (A and B) and high (C and D) magnification of biopsy site 14 days after the procedure. The sampled region of the liver appears to have been replaced with regenerative scar tissue that stained positively for Sirius red and was isolated to the site of the lesion (A and B). Magnified observations (C and D) show a clear separation of the lesioned and nonlesioned portions of the liver (boxes in A and B, respectively). A and C: H&E. B and D: Sirius red. Scale bars: A and B = 400 μM; C and D = 100 μM.
collagen but remained isolated to the site of the lesion and did not alter the morphology of the intact liver (Fig. 5, A–D). Sufficient sample (~50 mg) was collected to perform histological analysis and mRNA and protein analyses. Quantification of Col1a2 obtained from biopsy samples was found to correlate significantly with histological assessment of fibrosis (Pearson $r = 0.81$, $P < 0.001; n = 15$).

**Hepatic gene expression changes in AMLN mice staged by level of fibrosis.** A targeted array of 84 genes (see METHODS) encoding proteins involved in the regulation of tissue remodeling, fibrosis, and inflammation was used to evaluate tissue from animals categorized by histopathological criteria to represent defined stages of NAFLD progression. Livers were removed from animals fed either the AMLN ($n = 18$) or LFD diet ($n = 10$), apportioned, and prepared appropriately for histopathology and for gene expression analysis. Tissues were categorized as Fatty Liver, Low Fibrosis, or High Fibrosis on the basis of morphology and the extent of collagen deposition. Mice maintained on the LFD had $1.34 \pm 0.10\%$ Sirius red staining for collagen (Fig. 6, A and F). The Fatty Liver mice were characterized by highly steatotic livers but little to no evidence of fibrosis (Sirius red staining density, $0.64 \pm 0.07\%$ of total area). Mice categorized as Low Fibrosis were highly steatotic and also exhibited low-grade periportal fibrosis (Sirius red staining density, $2.55 \pm 0.30\%$ of total area). High-Fibrosis mice were those observed to have significant steatosis and bridging fibrosis with significant collagen accumulation (Sirius red staining density, $5.56 \pm 0.43\%$ of total area; Fig. 6, B–D and F). Protein blot analysis of collagen-1 and total collagen content by hydroxyproline assay revealed a similar graded

![Fig. 6](http://ajpgi.physiology.org/)
increase in collagen protein accumulation in the four groups, confirming their fibrotic phenotype (Fig. 6, E, G, and H). Other phenotypic and serum analyses were consistent with our previous findings (Table 4).

Of the 84 genes surveyed, expression levels of 39 were significantly elevated and 10 were significantly reduced (≥2-fold change vs. LFD, P ≤ 0.05, Supplementary Table S1; supplemental material for this article is available online at the Journal website). Changes in expression of a subset of the candidate regulated genes identified from the array were confirmed by individual TaqMan quantitative real-time PCR (qRT-PCR) assays (data not shown). Aggregate analysis of the genes by unsupervised hierarchical clustering of expression patterns from each animal was used to visualize the results in a heat map (Fig. 7). The different gene expression profiles generated groupings of animals that accurately correlated with the disease stage of each animal as determined histologically prior to analysis. Thus these findings corroborated our histological methods and indicate that each disease stage may be characterized by a distinct molecular signature.

Genes encoding proteins known to be implicated in the pathophysiology of NAFLD and commonly evaluated in animal models of NAFLD such as matrix metalloproteinases (Mmp) types 2, 3, 8, and 13; tissue inhibitor of metalloproteinases (Timp) types 2, 3, 8, and 13; and tissue inhibitor of metalloproteinases (TIMP) types 2, 3, 8, and 13; collagen-3 alpha 1 (Col3a1), and collagen-4 alpha 2 (Col4a2) were all significantly elevated (Supplementary Table S1). Of note, expression levels of Timp1 determined by the fibrosis array were at the lower limit of assay detection in the LFD mice with threshold cycles exceeding 35. Timp1 expression was reliably quantified by TaqMan-based qRT-PCR and was found to be substantially elevated in all three experimental groups (LFD, 1.00 ± 0.20; Fatty Liver, 5.56 ± 0.76; Low Fibrosis, 21.41 ± 1.95; High Fibrosis, 34.25 ± 1.42; all groups P < 0.05 vs. LFD). Genes associated with inflammation [tumor necrosis factor-alpha (Tnf), e-Jun (Jun), and interleukin-1 beta (IL1b)] and fibrosis [α-smooth muscle actin (Act2), transforming-growth-factor-B1 (Tgfb1), and platelet-derived growth factor types α and b (Pdgfa/b)] were also moderately but significantly increased (1.4- to 3.6-fold) relative to the LFD mice (Supplementary Table S1). Of particular interest is a subset of genes found to be dramatically upregulated (≥10-fold in the High-Fibrosis group), with mRNA expression patterns correlating positively with hepatic collagen density (Supplementary Table S1 and Fig. 8). These include tissue plasminogen activator (Plat), lysyl oxidase (Lox), integrin-β6 (Itgb6), Collα2, and Timp1 (Fig. 8, A–E). In contrast, α1 antitrypsin (Serpinα1a) was the most highly downregulated gene of the subset evaluated (expression was reduced 4.3-fold in the High-Fibrosis group) and expression correlated negatively with degree of fibrosis (Fig. 8F). Interestingly, bone morphogenic protein-7 (Bmp7) was selectively increased in the Fatty Liver group (1.95-fold, Supplementary Table S1) without change in the other groups, suggesting a possible transient elevation in expression of this gene in the early stages of NAFLD.

**Discussion**

Preclinical models of NASH have yielded numerous insights into mechanisms of disease etiology and progression. The model and methods described here offer several improvements to the understanding of NASH and to the search for effective means of intervention. First, this dietary model of NASH more closely mimics the etiology of the disease in humans and replicates many of the biochemical and histopathological hallmarks of clinical NAFLD progression. Second, our comprehensive histological analyses reveal that hepatic collagen accumulation is heterogeneous distributed, revealing that the methods used for sample collection and histological quantification can affect study outcome. Our development of a biopsy technique allows characterization of pretreatment hepatic pathology. Initial results indicate that the AMLN diet appears to influence expression of numerous genes that are highly regulated and associated with the development of hepatic fibrosis.

Our dietary model induces NASH without reliance on genetic mutations (e.g., leptin deficiency), the use of toxins (e.g., CCL4), or nutrient deficiency (e.g., methionine and choline deficiency). The AMLN diet (39) was modeled after the American Lifestyle-Induced Obesity Syndrome (ALIOS) model developed by Tetri and colleagues (37) with a few important differences. As in the ALIOS diet, the source of the trans fats in the AMLN diet is Primex partially hydrogenated vegetable oil shortening. However, the cholesterol content in the AMLN diet is greater (2% by wt), and fructose (22% by wt) was provided in the food pellets rather than the drinking water.

We have shown previously that Lepob/Lepob mice maintained on the AMLN diet for at least 12 wk develop features of NASH including fibrosis, whereas the development of NASH in C57BL/6 mice was more variable and less pronounced even after 16 wk (39). We reasoned that prolonged exposure to the AMLN diet might result in more frequent and possibly a more severe NASH phenotype. Indeed, histological assessment confirmed the development of NASH in a majority of mice.

**Table 4. Phenotypic and biochemical profiles of mice staged by severity of fibrosis**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>LFD</th>
<th>Fatty Liver</th>
<th>Low Fibrosis</th>
<th>High Fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>31.2 ± 1.0</td>
<td>35.2 ± 0.4*</td>
<td>38.9 ± 0.3*</td>
<td>41.5 ± 0.2*</td>
</tr>
<tr>
<td>Liver weight, g</td>
<td>1.29 ± 0.05</td>
<td>2.88 ± 0.09*</td>
<td>3.98 ± 0.11*</td>
<td>4.90 ± 0.11*</td>
</tr>
<tr>
<td>Liver weight, % of body wt</td>
<td>4.1 ± 0.3</td>
<td>8.2 ± 0.3*</td>
<td>10.2 ± 0.3*</td>
<td>11.8 ± 0.3*</td>
</tr>
<tr>
<td>Intrahepatic lipids, mg/g</td>
<td>81.9 ± 9.5</td>
<td>320.2 ± 12.9*</td>
<td>377.8 ± 31.3*</td>
<td>324.6 ± 24.0*</td>
</tr>
<tr>
<td>ALT, U/I</td>
<td>24.6 ± 5.8</td>
<td>182.2 ± 7.3*</td>
<td>354.9 ± 26.0*</td>
<td>396.0 ± 18.4*</td>
</tr>
<tr>
<td>AST, U/I</td>
<td>132.9 ± 49.2</td>
<td>242.0 ± 32.1</td>
<td>356.6 ± 56.3*</td>
<td>298.9 ± 14.5*</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>129.7 ± 5.0</td>
<td>228.0 ± 5.1*</td>
<td>286.1 ± 10.0*</td>
<td>336.7 ± 11.9*</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>72.8 ± 8.0</td>
<td>72.0 ± 5.6</td>
<td>67.8 ± 5.2</td>
<td>55.7 ± 2.5</td>
</tr>
<tr>
<td>Adiponectin, μg/ml</td>
<td>35.2 ± 2.4</td>
<td>27.9 ± 1.1*</td>
<td>26.24 ± 1.5*</td>
<td>19.3 ± 0.3*</td>
</tr>
</tbody>
</table>

Results are means ± SE. LFD, N = 7–10; Fatty Liver, Low Fibrosis, and High Fibrosis, N = 6. *P < 0.05 vs. LFD.
maintained on the AMLN diet for 30 wk. Affected mice displayed severe panlobular macro- and microvesicular steatosis, perportal inflammation, and ballooning degeneration. Importantly, portal and bridging fibrosis, requisite features of the clinical diagnosis of severe NASH, was also evident. Plasma ALT and AST were markedly elevated and tissue indexes of fibrosis (Col1a2 mRNA and extractable collagen-1a1 protein expression as well as total collagen content) and inflammation (Cc12 mRNA) were increased, consistent with the development of fibrosing steatohepatitis in the AMLN mice. Importantly, the AMLN mice also became overweight and insulin resistant, paralleling the natural history of clinical disease. Lipidemic profiles in plasma revealed an increase in total circulating cholesterol and a significant reduction in triglycerides. Because of the high cholesterol content of the food it is not surprising that the plasma cholesterol levels were increased; however, it is noteworthy that despite a substantial increase in intrahepatic lipid content in the AMLN mice (32%), triglycerides were reduced, possibly reflecting impairment in very low-density lipoprotein secretion from the liver. Similar findings were reported with the ALIOS model, in which plasma triglycerides were not affected by the diet but found to be increased when trans fats were replaced with lard (37).

In humans, hepatic fibrosis is known to develop heterogeneously and, as a consequence, biopsy-based diagnoses of NASH are estimated to be incorrect in 10–30% of individuals (23). The distribution of collagen accumulation in the rodent liver during the development of NASH has not been reported. Our analyses revealed that the development of fibrosis originates at the liver periphery, where collagen content of the first 1 mm is ~6.5-fold greater than the region >3 mm from the edge. After 30 wk of the AMLN diet, collagen deposition was detected in deeper regions of the tissue parenchyma. The findings that fibrosis develops heterogeneously suggest that traditional methods for quantification of hepatic collagen content that rely on highly magnified observations of limited areas of tissue may not accurately represent disease severity. To account for heterogeneity in collagen distribution we propose several refinements to a liver sampling protocol. To eliminate overestimation of liver fibrosis, the first 1 mm of peripheral tissue should be excluded, and large sections of liver tissue (>25% of the lobe on a single x-y plane), including both the tissue margin and center of the organ should be collected for quantification of collagen. We suggest that collagen should be quantified on the entire section of a lobe and that determinations from three sections of equivalent area that are separated by at least 100 μm in a single lobe are sufficient to provide representative estimates of collagen density. These guidelines will improve the reliability and reproducibility of liver collagen quantification in experimental animals.

The progression from simple steatosis to more severe steatohepatitis and cirrhosis was found to occur unpredictably and at varying rates in the AMLN mice. Individual members of a cohort of mice maintained on the AMLN diet for 20 wk could be categorized into three general stages of disease: 1) steatosis without fibrosis, 2) NASH with fibrosis, and 3) cirrhosis. We suspect similar heterogeneity would be evident in mice subject to other methods of induction of liver fibrosis. In humans, simple fatty liver disease without evidence of inflammation or fibrosis is common among obese and diabetic patients; however, the transition to NASH and ultimately to cirrhosis is less

**Fig. 7.** Changes in gene expression associated with hepatic fibrosis. Heat map depicting the pattern of gene expression in AMLN and LFD mice categorized histopathologically by degree of hepatic fibrosis. Rows represent individual genes (labeled on the right) and each column represents a single animal. In the graphical display, the assay-centric view is used where the ΔC_T value of the middle expression level for that primer set is set as the median for all the ΔC_T values for that primer set among all the samples. Green indicates a decrease in expression with ΔC_T values above the median and red indicates an increase in expression with ΔC_T values below the median. Pearson’s correlation was used to measure the relationship between variables, and average linkage was selected for the clustering method.
frequent (1, 9, 11). Although it is hypothesized that various insults to the liver (e.g., lipotoxicity, reactive oxygen species, endoplasmic reticulum stress, endotoxemia) secondary to insulin resistance and hepatic fat accumulation might contribute to this transition, it is unclear what causes a subset of NAFLD patients to progress to NASH (26, 38). In addition to a myriad of genetic determinants of disease, there is also evidence of environmental and epigenetic effects on pathogenesis of chronic metabolic disorders (19, 33). Because inbred mice are considered to be essentially isogenic, we speculate that epigenetic influences may contribute to the observed phenotypic diversity.

Objective and ideally minimally invasive methods for identifying and staging NAFLD in rodents and humans are lacking. Those that have been reported lack the predictive power to appropriately categorize individuals and to distinguish steatosis, fibrosis, and cirrhosis (2). To address the need for better diagnosis in preclinical models and to provide a method by which animals can be staged by disease severity prior to initiating pharmacological interventions, we developed a technique for in vivo sampling of liver tissue. This categorization of experimental animals according to disease severity prior to initiating interventions eliminates the noise introduced by assessing the median response. The biopsy method also permits evaluation of the effect of intervention on individual experimental animals, allowing each to be its own control, and thereby allowing determination of the distribution of effects in an experimental population. Furthermore, the ability to sample hepatic tissue from the live animal facilitates studies to identify objective diagnostic and prognostic markers of disease obtained by less invasive techniques. Methods incorporating clinical biopsy guns, diathermy, or freeze-clamping to control blood loss in rats (3, 7, 8) have not been widely adopted. To reduce the risk of tissue injury that can result from biopsy, we chose Gelfoam absorbable gelatin sponge to control bleeding (17). The procedure was rapid and two surgeons were able to choose Gelfoam absorbable gelatin sponge to control bleeding (17). The procedure was rapid and two surgeons were able to biopsy >50 mice in a single day with a mortality rate of <1%. Mice recover well following the biopsy; however, the procedure is considered stressful and must be performed under anesthesia and with postoperative analgesics; thus it is not recommended for short-term/acute experiments. Although the biopsy site appears to be replaced by collagenous scar tissue, the remaining liver tissue is not affected by the procedure, allowing evaluation of pharmacological treatment on the intact liver. Importantly, analysis of biopsied tissue for Col1a2 expression indicated significant correlation with the histological assessment of fibrosis, demonstrating the utility of tissue samples obtained by biopsy to appropriately stage the animals.

Initial studies were conducted to identify changes in gene expression coincident with progression from simple steatosis to NASH in AMLN mice in which the degree of hepatic fibrosis had been histologically assessed by collagen content. Unsupervised hierarchical clustering analysis of the aggregate gene expression profiles grouped the mice into the same categories.
as determined by histological analyses, thereby reinforcing the utility of these different methods for evaluating severity of liver disease. Interestingly, a number of genes not previously evaluated in dietary models of NAFLD and NASH were found to correlate with the degree of fibrosis in the AMLN mice. The potential significance of some of these genes is discussed below.

Plat (upregulated 18-fold in the High-Fibrosis group) encodes tissue plasinogen activator (tPA), which catalyzes the cleavage of plasinogen to the fibrinolytic enzyme plasin (41). In addition to its fibrinolytic activity, plasin degrades components of the ECM such as laminin, proteoglycans, and collagen and is an activator of matrix metalloproteinases, thus potentially resulting in reduced tissue fibrosis (4, 20, 22, 25, 28). The 15-fold increase of Lox in the AMLN mice also correlated with collagen accumulation, suggesting a role for this protein in the development of liver fibrosis. LOX was initially believed to stabilize ECM components by initiating the cross-linking of collagen and elastin. Recent reports point to a broader role in chemotaxis and proliferation (21). Although increased expression of Lox has been reported in CCl4-treated rat models of liver fibrosis (24, 32, 40), its role in diet-induced liver disease has not been revealed. The role of integrins in NAFLD has not been reported; however, our finding of elevated ITGβ6 expression in AMLN mice indicates the possibility that this integrin receptor protein may play a role in regulating disease progression, perhaps through the direct interaction of integrin receptors with the ECM (27). Other intriguing observations were the negative correlation of Serpin1ala expression (encoding α1 antitrypsin protein, AAT) with fibrosis and the selective elevation of Bmp7 in the Fatty Liver group. AAT is a regulator of the innate immune system, and in humans its deficiency is associated with cirrhosis and hepatocellular carcinoma. In the pediatric population, AAT deficiency is the most frequent reason for metabolic liver disease-related liver transplantation in the US (30). Bone morphogenetic protein 7 (BMP-7) is a member of the TGF-β superfamily and antagonizes the effects of the profibrotic cytokine TGF-β1. Exogenous delivery of BMP-7 has been shown to suppress TGF-β1 mRNA expression and attenuate liver fibrosis in CCl4-treated mice (15, 42). The role of BMP-7-mediated regulation of TGF-β1 in the development of hepatic fibrosis is supported by our dietary model of NASH, in which Bmp7 expression was significantly elevated in the Fatty Liver (without fibrosis) group whereas, Tgfb1 expression was only elevated in the Low- and High-Fibrosis groups and remained unchanged in the Fatty Liver group. Hepatic gene expression profiling of the AMLN mice has identified potential molecular markers of disease and disease progression and may also provide insight into the development of liver fibrosis.

Collectively, the findings from this dietary model highlight the refinement of our previously reported model of NAFLD. Continued exploration of changes in gene expression potentially associated with the progression of NASH may provide an objective and minimally invasive method of staging liver disease in preclinical studies and may ultimately lead to the identification of clinically useful prognostic and diagnostic biomarkers. The experimental design and refined methods may have broad utility in preclinical NASH models, allowing for a more precise pharmacological characterization of novel agents for the treatment of NASH.

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DISCLOSURES

All authors are employed by Amylin Pharmaceuticals, LLC and have held equity in Amylin Pharmaceuticals.

AUTHOR CONTRIBUTIONS


REFERENCES

IMPROVED MODEL AND METHODS FOR CHARACTERIZING NASH IN MICE


