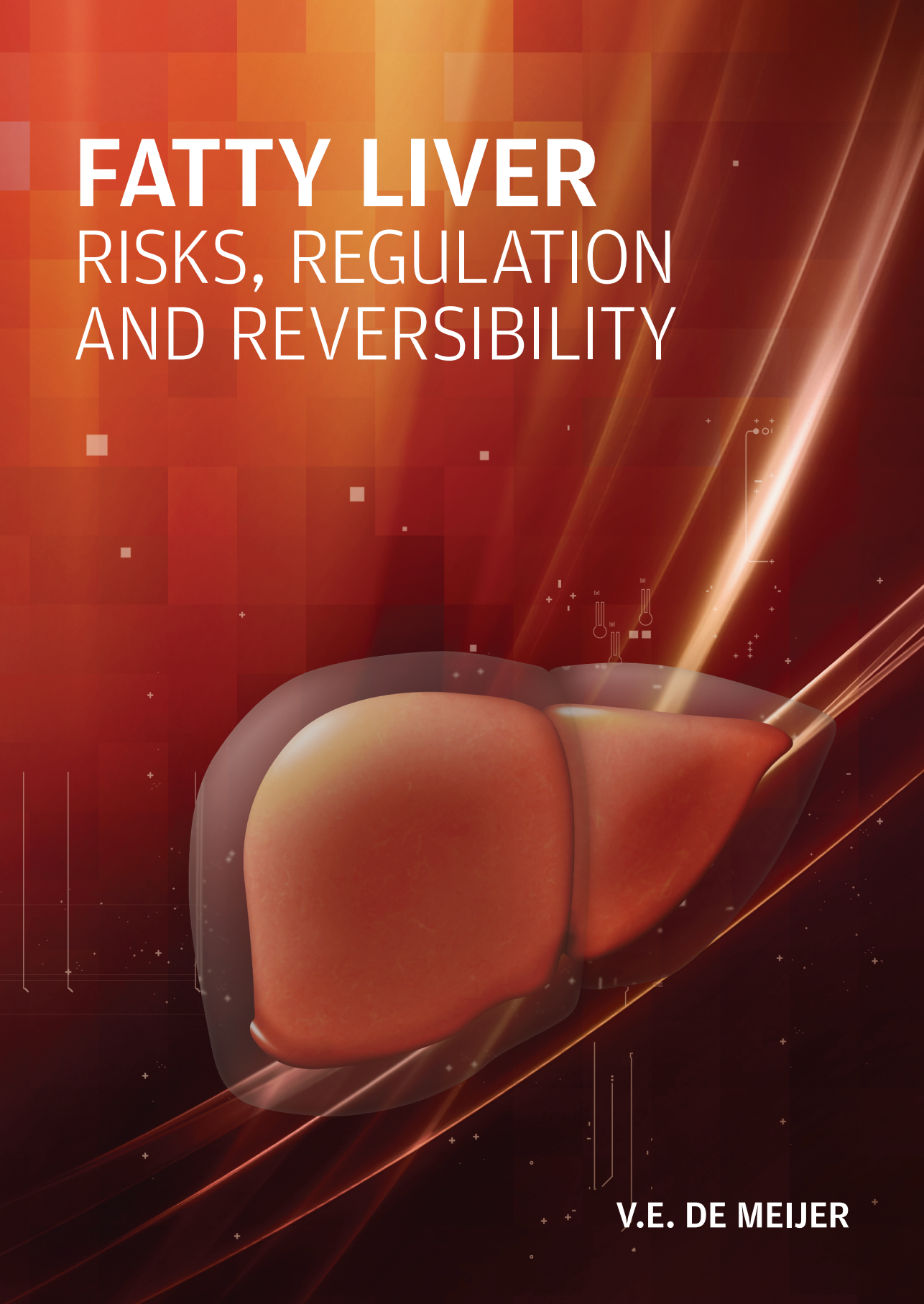
FATTY LIVER
RISKS, REGULATION AND REVERSIBILITY



V.E. DE MEIJER

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RISKS, REGULATION AND REVERSIBILITY

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Fatty Liver

Risks, Regulation and Reversibility

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Risico's, Regulering en Omkeerbaarheid

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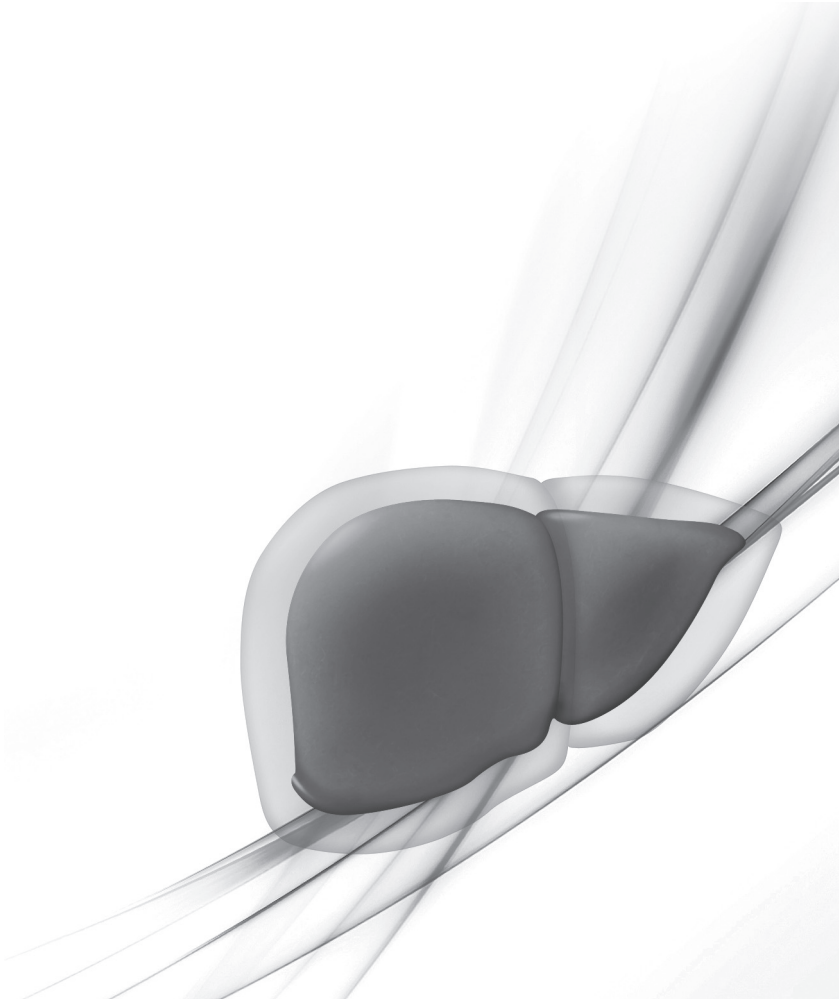
Chapter 1

General introduction and outline of the thesis



INTRODUCTION

As a consequence of sustained over-nutrition, obesity has become epidemic in industrialized countries and is increasingly common in developing countries worldwide, occurring in all ethnic groups and at younger ages. The World Health Organization estimates that globally approximately 1.6 billion adults were overweight in 2005, of whom at least 400 million were obese. These rates are predicted to be doubled by 2015.[1] Obesity is accompanied by dyslipidemia and ectopic deposition of excess triglyceride in adipose tissue as well as in the liver, known as non-alcoholic fatty liver disease (NAFLD). NAFLD is now the leading cause of chronic liver disease in the Western world with a prevalence of up to 30% in the general population and up to 74% in obese persons, mirroring the increasing epidemic of obesity and the metabolic syndrome.[2,3] The prevalence in non-Western countries is also expected to increase, mainly as a result of globalization of the Western diet. Mounting scientific interest in this topic is reflected in the number of publications that have appeared in the medical literature over the last 15 years (**Figure 1**); however, the mechanisms underlying the pathogenesis of NAFLD remain elusive. With obesity being an important risk factor universally, NAFLD is now receiving greater attention and is regarded as a public health issue. In addition, as a result of an aging population and the improving control of other major causes of chronic liver disease,

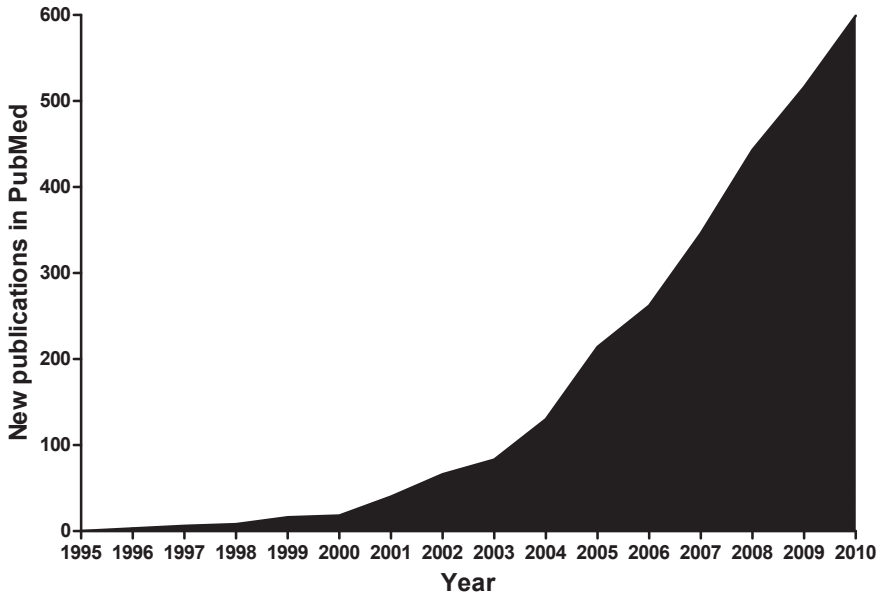


Figure 1. Number of original publications per year, using the search term "NAFLD AND liver OR NASH AND liver" (limited to Title/Abstract) in the PubMed database.

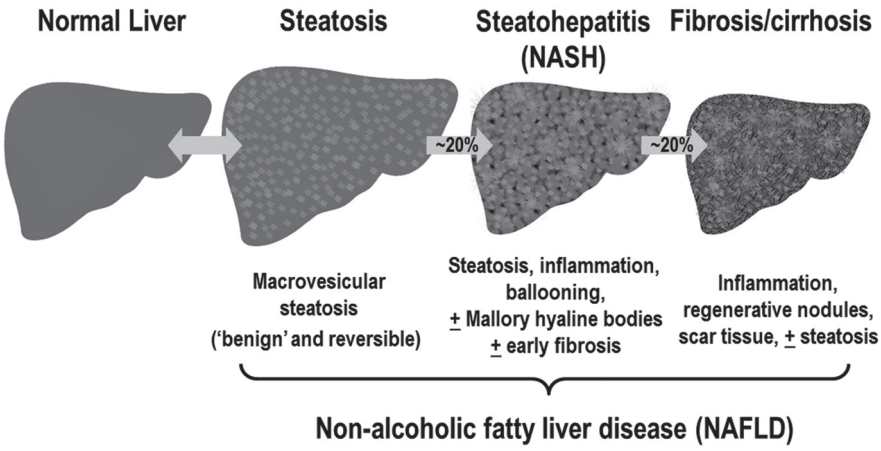


Figure 2. Natural history of non-alcoholic fatty liver disease (NAFLD) and associated pathologic changes.

such as hepatitis B and C, NAFLD has become a major indication for liver transplantation while its burden is expected to increase in years to come.[4]

NAFLD consists of a variety of pathologic states ranging from the simple buildup of fat in the liver (hepatic steatosis) to nonalcoholic steatohepatitis, cirrhosis, and ultimately liver failure (**Figure 2**).[5,6] The vast majority of obese individuals with NAFLD will have 'simple' hepatic steatosis, which is a stable and benign condition for decades. In others, however, hepatic necroinflammation evolves, cumulative liver damage ensues and cirrhosis ultimately results.[5,6] Currently, the only definitive treatment for advanced fibrosis and cirrhosis is liver transplantation. The demand for organ grafts, however, outweighs their availability,[4] stressing the need for effective prevention strategies and development of interventions to treat and reverse NAFLD.[7,8]

Although the majority of individuals with NAFLD will never require hepatic surgery, liver resection is the only curative treatment option for those who develop primary and secondary hepatobiliary malignancies.[9,10] The risk of morbidity and mortality associated with hepatic surgery is closely related to the volume and function of the remnant liver[11] and steatosis is an established risk factor for primary non-function of hepatic allografts.[12] Approximately 20% of patients undergoing liver resection and up to 25% of liver donors have some degree of steatosis, indicating the need for pharmacological intervention.[12] Currently, the cornerstone of clinical management is weight loss through diet and exercise.[13] In reality, however, surgical management usually does not allow time for such dramatic life style changes, justifying the search for a pharmacologic approach. Novel evidence suggests that vitamin E therapy and pioglitazone may be of use in patients

with non-alcoholic steatohepatitis.[14] Pharmacological options to reverse steatosis prior to surgery in order to optimize the liver, however, are lacking.

AIM AND OUTLINE OF THE THESIS

The aim of the studies described in this thesis is to examine the risk of morbidity and mortality following hepatic resection in patients with steatosis; to explore the natural history of NAFLD and obesity followed by dietary intervention using a mouse model; and to identify and test novel therapeutic modalities for the treatment of NAFLD. Using experimental, murine models of diet-induced obesity, this thesis aims to provide new insights regarding the risks, regulation and reversibility of NAFLD.

While extensive surgery can be performed safely on healthy livers, the risk of major hepatic resection in patients with steatosis remains unclear. **Chapter 2** comprises a systematic review and meta-analysis of observational studies to establish the best estimate of the impact of hepatic steatosis on patient outcome following major hepatic surgery.

Although NAFLD results from overconsumption, the type of diet that is conducive to the development of this disease has not been established. In **Chapter 3** we hypothesized that the onset of hepatic steatosis in mice is linked to the consumption of a diet with a high fat content, rather than related to excess caloric consumption or decreased levels of physical activity. In addition, we also hypothesized that fully manifested hepatic steatosis could be reversed by reducing the fat percentage in the diet of obese mice.

Pharmacologic treatment options for NAFLD are scarce at best and signaling involved in the transition from diet-induced hepatic steatosis to more severe forms of liver pathology is poorly understood. Elucidating the mechanisms involved in the pathogenesis of NAFLD is therefore essential to identify potential new targets for intervention. In **Chapter 4**, using a mouse model of diet-induced obesity we investigated whether development of NAFLD and obesity might be associated with alterations in gene expression of matrix metalloproteinases (MMPs).

Chapter 5 describes the results of a study using a pharmacologic inhibitor of MMPs and the tumor necrosis factor-α converting enzyme (TACE), which belongs to the disintegrin and metalloproteinase family of MMPs.[15,16] We hypothesized that treatment with this drug would improve insulin resistance and reverse established experimentally-induced steatosis in mice, leading to improved outcome following hepatectomy.

In **Chapter 6** we investigate a potential pitfall in the methodology of drug delivery when experimental compounds are tested in animal models. In a murine model of diet-induced obesity, we hypothesized that long-term orogastric gavage would negatively influence the development of hepatic steatosis and associated metabolic syndrome.

The progression from steatosis to steatohepatitis and fibrosis is believed to result from superimposition of secondary 'hits' that overwhelm cell survival mechanisms and provoke hepatocyte injury and death. Inflammation is considered a prerequisite and important co-contributor to fibrosis and is, in part, mediated by TACE. In **Chapter 7**, we hypothesized that treatment with a broad-spectrum MMP and TACE-inhibitor would ameliorate hepatic injury and inflammation, leading to decreased fibrogenesis in a murine model of repeated hepatotoxin-induced liver injury.

Finally, in **Chapter 8**, the findings of this thesis and their implications in the context of ongoing research are summarized and discussed, and directions for future research are highlighted.

REFERENCES

1. World Health Organization. Fact sheet 311: Obesity and overweight. *http://www.who.int/mediacentre/ factsheets/fs311/en/* [accessed January 28, 2011].
2. Browning JD, Szczepaniak LS, Dobbins R, *et al*. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology* 2004; 40: 1387-1395.
3. Lazo M, Clark JM. The epidemiology of nonalcoholic fatty liver disease: a global perspective. *Semin Liver Dis* 2008; 28: 339-350.
4. Adam R, Hoti E. Liver transplantation: the current situation. *Semin Liver Dis* 2009; 29: 3-18.
5. Angulo P. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; 346: 1221-1231.
6. Adams LA, Lymp JF, St Sauver J, *et al*. The natural history of nonalcoholic fatty liver disease: a population-based cohort study. *Gastroenterology* 2005; 129: 113-121.
7. Musso G, Gambino R, Cassader M, *et al*. A meta-analysis of randomized trials for the treatment of nonalcoholic fatty liver disease. *Hepatology* 2010; 52: 79-104.
8. Popov Y, Schuppan D. Targeting liver fibrosis: strategies for development and validation of antifibrotic therapies. *Hepatology* 2009; 50: 1294-1306.
9. Al-Asfoor A, Fedorowicz Z, Lodge M. Resection versus no intervention or other surgical interventions for colorectal cancer liver metastases. *Cochrane Database Syst Rev* 2008: CD006039.
10. Verslype C, Van Cutsem E, Dicato M, *et al*. The management of hepatocellular carcinoma. Current expert opinion and recommendations derived from the 10th World Congress on Gastrointestinal Cancer, Barcelona, 2008. *Ann Oncol* 2009; 20 Suppl 7: vii1-vii6.
11. Yamanaka N, Okamoto E, Oriyama T, *et al*. A prediction scoring system to select the surgical treatment of liver cancer. Further refinement based on 10 years of use. *Ann Surg* 1994; 219: 342-346.
12. Selzner M, Clavien PA. Fatty liver in liver transplantation and surgery. *Semin Liver Dis* 2001; 21: 105-113.
13. Vuppalanchi R, Chalasani N. Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis: Selected practical issues in their evaluation and management. *Hepatology* 2009; 49: 306-317.
14. Sanyal AJ, Chalasani N, Kowdley KV, *et al*. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *N Engl J Med* 2010; 362: 1675-1685.
15. Black RA, Rauch CT, Kozlosky CJ, *et al*. A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. *Nature* 1997; 385: 729-733.
16. Moss ML, Jin SL, Milla ME, *et al*. Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-alpha. *Nature* 1997; 385: 733–736.

Chapter 2

Steatosis is a risk factor for complications and mortality following major hepatic resection: a systematic review and meta-analysis

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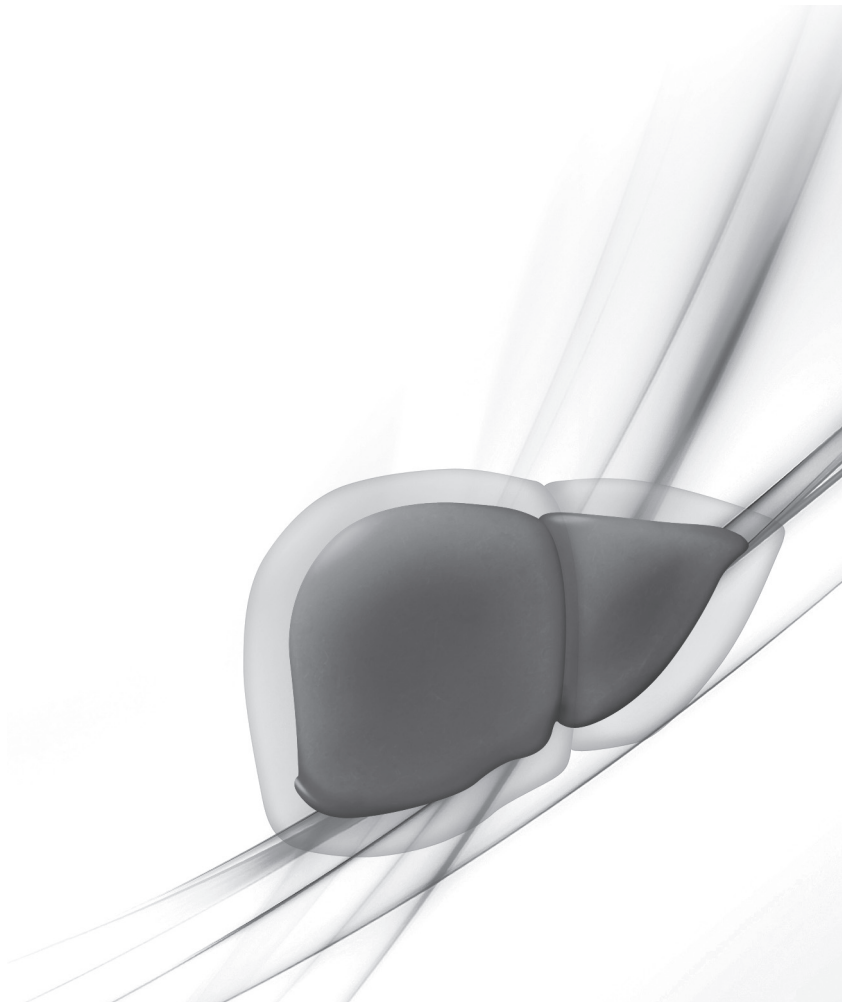
Br J Surg 2010;97(9):1331-9



ABSTRACT

Background: The risk of major hepatic resection in patients with hepatic steatosis remains controversial. A meta-analysis was performed to establish the best estimate of the impact of steatosis on patient outcome following major hepatic surgery.

Methods: A systematic search was performed following MOOSE guidelines. Relative risks (RR) for complication and mortality rates were calculated for patients with no, <30% and ≥30% steatosis, and a meta-analysis was performed.

Results: Of six observational studies that were identified, four studies including a total of 1000 patients were subjected to meta-analysis; two others were tabulated separately. Patients with <30% and ≥30% steatosis had a significantly increased risk of post-operative complications, with a RR of 1.53 [95% confidence interval (CI) 1.27, 1.85] and 2.01 (95% CI 1.66, 2.44), respectively. Patients with ≥30% steatosis had an increased risk of postoperative mortality (RR 2.79; 95% CI 1.19, 6.51).

Conclusions: Patients with steatosis had an up to 2-fold increased risk of postoperative complications, and those with excessive steatosis had an up to 2.8-fold increased risk of mortality.

INTRODUCTION

Non-alcoholic fatty liver disease consists of a variety of pathologic states ranging from hepatic steatosis to non-alcoholic steatohepatitis, cirrhosis, and ultimately liver failure.[1] Mostly unrecognized before 1980, it is now estimated that up to 30% of the Western adult population has some degree of steatosis.[2] Today, non-alcoholic fatty liver disease is the most common chronic liver disease in Western countries and its prevalence has mirrored the increasing epidemic of obesity and the metabolic syndrome.[2,3] Prevalence in non-Western countries is expected to increase as well, mainly due to the globalization of the Western diet.

Hepatic surgery is the only curative treatment option for patients with primary and secondary hepatobiliary malignancies[4-6], and hepatic resection is increasingly performed for living donor liver transplantation.[7] Advances in surgical technique and improvements in preoperative evaluation of liver function in selected patients has resulted in a decline in perioperative mortality to as low as 0%.[8,9] The risk of morbidity and mortality associated with hepatic surgery, however, is closely related to volume and function of the remnant liver.[10] It is estimated that about 20% of patients undergoing liver resection and up to 25% of donors for liver transplantation have some degree of steatosis.[11] While extensive surgery can be safely performed on healthy livers, the risk of major hepatic resection in patients with steatosis remains unclear.[12,13]

Although steatosis is an established risk factor for primary non-function of hepatic allografts[11,14,15], the literature with respect to surgical outcome following a major hepatectomy has not been systematically reviewed. The aim of this systematic review was to assess the impact of hepatic steatosis on complications and mortality following major hepatic surgery for either hepatic neoplasms or living liver donation.

METHODS

Literature search strategy

A systematic search of PubMed, Embase, Science Citation Index, CINAHL, and the Cochrane Library was performed for articles published between January 1994 and May 2009 (cut-off date May 1, 2009) relevant to steatosis as a risk factor in major hepatobiliary surgery for either hepatic neoplasms or living liver donation. Prior to 1994, the literature offered little to no clinical data on the impact of steatosis on surgical outcome. The MeSH headings "surgical procedures, operative" and "fatty liver" were used in PubMed. Keywords used in other databases included "steatosis" and "surgery". Manual reference

checks of accepted papers in recent reviews as well as included papers were performed to supplement the above electronic searches.

Literature screening

Studies were evaluated for inclusion by two independent reviewers (VDM and BTK) for relevance to the subject. Study selection was accomplished through three levels of study screening (**Figure 1**). At level 1, studies were excluded for the following reasons: reviews, letters, case reports, editorials and comments; animal or *in vitro* studies; fewer than 10 patients in the study; and languages other than English. At level 2, abstracts of all studies accepted at level 1 were reviewed for relevance. The full text was obtained for relevant papers and for any citations for which a decision could not be made from the abstract. For level 3 screening, inclusion required that studies (1) described patients who underwent major hepatobiliary surgery, defined as a resection of ≥3 liver segments, for either hepatic neoplasmatic disease or living liver donation; (2) measured steatosis by histology and assessed the degree of steatosis independently; (3) described surgical outcomes (morbidity and/or mortality) and stratified outcomes by degree of steatosis; and (4) described the outcomes of the donor, not the recipient, in studies of living liver donation. These selection criteria were independently assessed by the reviewers and scored on a standardized form. Any discrepancies in inclusion were resolved by discussion between reviewers.

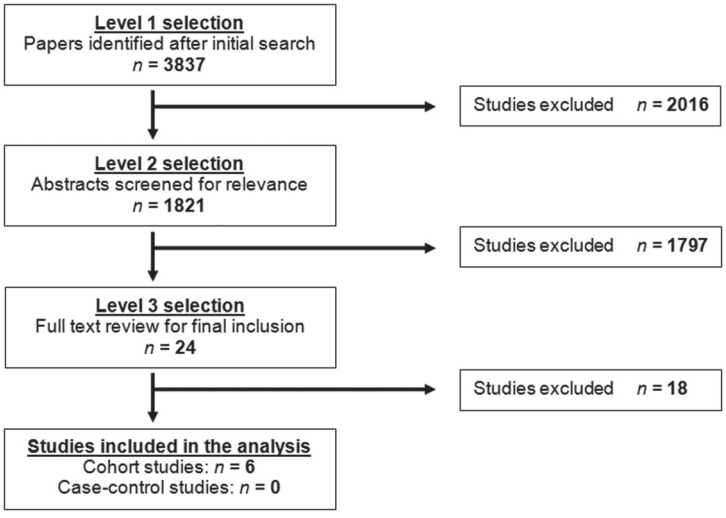


Figure 1. Flow diagram showing selection of articles.

Data extraction and Critical Appraisal

Data, including study design, study population, exposure, and outcomes, were extracted in duplicate from each included article using a predetermined form. Because differences in underlying parenchymal disease and associated comorbidities could affect homogeneity, articles that described outcomes for patients who underwent major hepatic resection for either hepatic neoplasms or living liver donation were tabulated separately to allow for more accurate risk assessment. Kin relationships, defined as multiple publications describing the same or overlapping series of patients, were identified and included only once to avoid double counting of patients. Level of evidence of each article was scored using the Oxford Centre for Evidence-Based Medicine Level of Evidence scale and the quality of articles was assessed according to the Newcastle-Ottawa Scale for observational and case-control studies, which scores selection, comparability and outcome.[16]

Statistical analysis

Relative risks (RR) and 95% confidence intervals (CI) were calculated from raw data using patients without steatosis as the reference group. Where possible, outcome data resulting from multivariable analysis were extracted from the identified manuscripts. For univariable analysis, statistical significance between groups was assessed using Fisher's Exact test.

A meta-analysis was performed on complications and mortality as outcome measures using Review Manager (RevMan) software (version 5.0.21, Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2008). Each study was weighted by means of sample size. Studies were not weighed for study quality (quality of allocation concealment). The complications and mortality of the different degrees of steatosis (no steatosis vs. <30% steatosis, and no steatosis vs. ≥30% steatosis) was estimated as a pooled relative risk with 95% CI using the random effects model of DerSimonian and Laird.[17] Overall effects were determined using the Z test. A two-sided P-value < 0.05 was considered statistically significant. Statistical heterogeneity was explored by inspecting the forest plot, and χ^2 and inconsistency (I^2) statistics, with $I^2 \geq 50\%$ defining substantial heterogeneity.[18]

Sensitivity analysis showed that the overall estimates using the fixed effects model were virtually identical, indicating relatively little variation between included studies. Other sensitivity analyses included removal of one study at a time to determine whether the conclusion was driven by any single study, cumulative meta-analysis to determine sensitivity to publication date, and assessment of the width of the confidence interval around a summary effect size to determine the robustness of a quantitative estimate. The meta-

analysis was carried out in accordance with the Meta-analysis Of Observational Studies in Epidemiology (MOOSE) guidelines.[19]

RESULTS

Quantity and quality of evidence

After screening 3837 articles 6 articles were identified that fell within the scope of the study (**Figure 1**).[13,20-24] Four articles described outcomes for patients who underwent major hepatic resection for hepatic neoplasms (**Table 1**)[20-23], whereas two articles described patients who underwent major surgery for living liver donation (**Table 2**).[13,24] All identified studies followed an observational design, scored 7 or more (out of 9) on the Newcastle-Ottawa Scale and provided level 2b evidence on the Oxford Level of Evidence scale (**Table 1**).[25]

Complications as an outcome

Upon univariable analysis, Gomez *et al.*[22], Kooby *et al.*[21], and McCormack *et al.*[23] found that patients with any degree of steatosis had a significantly (P all ≤ 0.01) increased risk of post-operative complications following hepatic surgery. Multivariable analysis revealed that when adjusted for potential confounders such as presence of co-morbidity, extent

Table 1. Characteristics, outcome measures and major findings of studies describing patients who underwent major hepatic surgery for benign or malignant neoplasms.

Study	Study type	Steatosis	n	NOS	Evidence	Primary outcome	Major findings
Behrns[20]	retrospective	No steatosis	72	7	2b	Complications/death	Patients with steatosis:
1998	cohort study	Total steatosis:	63			1. <30 days, or	1. ↑ complication rate;
USA		<30%	56			2. prior to discharge	2. ↑ bilirubin leak
		≥30%	7				
Kooby[21]	prospective	No steatosis	160	9	2b	Complications/death	Patients with steatosis:
2003	cohort study	Total steatosis:	325			1. <60-days, or	1. ↑ complication rate;
USA		<30%	223			2. prior to discharge	2. not ↑ mortality
		≥30%	102				
Gomez[22]	prospective	No steatosis	192	8	2b	Complications/death	Patients with steatosis:
2007	cohort study	Total steatosis:	194			prior to discharge;	1. ↑ complication rate;
UK		<30%	122			blood loss;	2. not ↑ mortality;
		30-60%	60			length of ICU stay;	3. ↑ blood loss;
		>60%	12			liver failure	4. ↑ length of ICU stay
McCormack[23]	prospective	No steatosis	58	9	2b	Death/complications	Patients with steatosis:
2007	cohort study	Total steatosis:	58			<90-days;	1. ↑ complication rate;
USA	(matched)	10-30%	44			blood loss;	2. not ↑ mortality;
		>30%	14			length of ICU stay	3. ↑ blood loss;
							4. ↑ length of ICU stay

NOS, Newcastle–Ottawa Scale; ICU, intensive care unit; ↑, increased.

Table 2. Characteristics, outcome measures and major findings of studies describing patients who underwent major hepatic surgery for living related liver donation.

Study	Study type	Steatosis	*n*	NOS	Evidence	Primary outcome	Major findings
Cho[13] 2006 Korea	prospective cohort study	No steatosis Total steatosis: 5-30%	36 18 18	7	2b	Complications/ death	Patients with steatosis: 1. not ↑ complication rate; 2. not ↑ mortality; 3. early regeneration impaired; 4. long-term regeneration not impaired
Nagai[24] 2009 Japan	prospective cohort study	No steatosis Total steatosis: 5-20%	31 10 10	8	2b	Death/ complications; liver regeneration; liver function	Patients with steatosis: 1. not ↑ complication rate; 2. long-term regeneration not impaired; 3. ↑ hyperbilirubinemia

NOS, Newcastle–Ottawa Scale; ICU, intensive care unit; ↑, increased.

of hepatic resection and amount of intra-operative blood loss, any degree of steatosis remained associated with total post-operative complications, with RR ranging from 1.24 to 3.84.[21-23] In addition, Kooby *et al.* found that patients with any degree of steatosis were more likely to suffer from infective, wound-related, hepatobiliary, and gastrointestinal post-operative complications.[21] Gomez *et al.* also found that patients with any degree of steatosis had an increased risk for infective complications.[22]

In contrast, Behrns *et al.*[20], Cho *et al.*[13] and Nagai *et al.*[24] did not find a significant association between overall complications and steatosis in patients who underwent hepatic resection. Behrns *et al.*[20] described patients who underwent hepatic resection for hepatic neoplasms, whereas Cho *et al.*[13] and Nagai *et al.*[24] described the outcome of living liver donation. On univariable analysis, Behrns *et al.* found that patients with steatosis were more likely to develop hyperbilirubinemia and increased aspartate aminotransferase levels following hepatic surgery.[20] Logistic regression analysis by Nagai *et al.* corroborated this result by demonstrating that steatosis was associated with the development of hyperbilirubinemia in living liver donors with mild steatosis (5 to 20%) following hepatectomy (odds ratio 7.94; 95% CI 1.17, 54.03; $P = 0.03$).[24] In contrast, Cho *et al.* found that biliary leakage was the most common major complication among living liver donors following hepatectomy, although rates for patients with and without steatosis were not significantly different (RR 1.27; 95% CI 0.94, 1.72; $P = 0.10$).[13]

A meta-analysis was subsequently conducted investigating steatosis as a risk factor for complications following major (≥3 segments) hepatic resection. Four studies were included in the meta-analysis (Berhns *et al.*[20], Kooby *et al.*[21], Gomez *et al.*[22] and McCormack

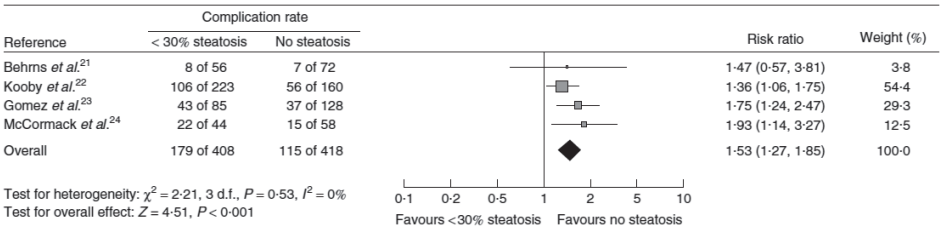


Figure 2. Forest plot of complication rates in patients with less than 30% steatosis versus those with no steatosis. Risk ratio estimates, shown with 95% confidence intervals, were calculated using the random-effects model. The diamond represents the overall treatment effect from the pooled studies spanning the 95% confidence interval. No steatosis was defined as no hepatocytes containing fat infiltration[21-23] and <10% of hepatocytes with fat droplets.[24]

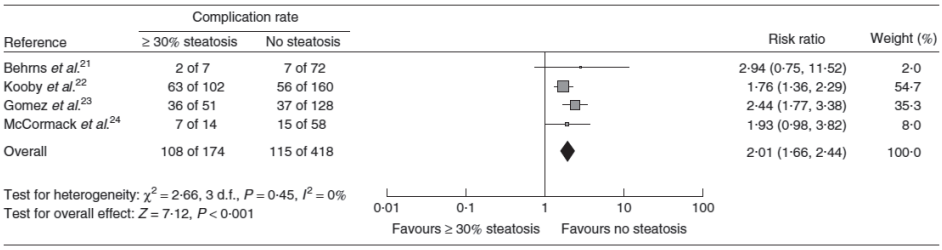


Figure 3. Forest plot of complication rates in patients with at least 30% steatosis versus those with no steatosis. Risk ratio estimates, shown with 95% confidence intervals, were calculated using the random-effects model. The diamond represents the overall treatment effect from the pooled studies spanning the 95% confidence interval. No steatosis was defined as no hepatocytes containing fat infiltration[21-23] and <10% of hepatocytes with fat droplets.[24]

et al.[23]), all of which examined patients who underwent hepatic resection for primary or secondary malignancies. For those studies also including patients undergoing resection of <3 segments, only data for patients who underwent resection of ≥3 segments was included in the meta-analysis. A total of 1000 patients was analyzed, including 418 patients with no steatosis, 408 patients with <30% steatosis and 174 patients with ≥30% steatosis. Two studies (Cho *et al.*[13] and Nagai *et al.*[24]) were excluded to avoid introduction of heterogeneity because the authors studied living liver donors who, by definition, did not have underlying liver pathology. There was no substantial heterogeneity in comparing complication rate in patients with either <30% or ≥30% steatosis to patients without steatosis ($I^2 = 0\%$).

The summary RR of patients with <30% steatosis compared to patients without steatosis was 1.53 (95% CI 1.27, 1.85; $P < 0.01$; **Figure 2**). The summary RR of patients with more severe steatosis (≥30%) compared to patients without steatosis was 2.01 (95% CI 1.66, 2.44; $P < 0.01$; **Figure 3**).

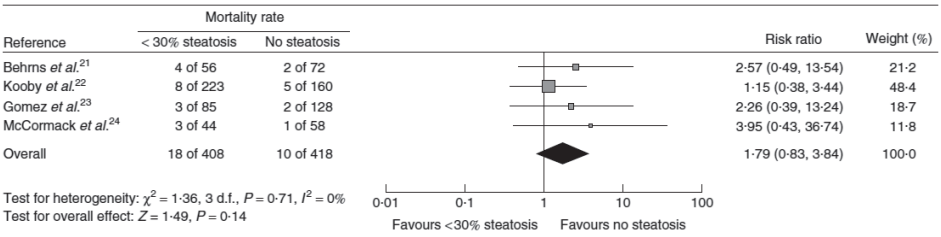


Figure 4. Forest plot of mortality rates in patients with less than 30% steatosis versus those with no steatosis. Risk ratio estimates, shown with 95% confidence intervals, were calculated using the random-effects model. The diamond represents the overall treatment effect from the pooled studies spanning the 95% confidence interval. No steatosis was defined as no hepatocytes containing fat infiltration[21-23] and <10% of hepatocytes with fat droplets.[24]

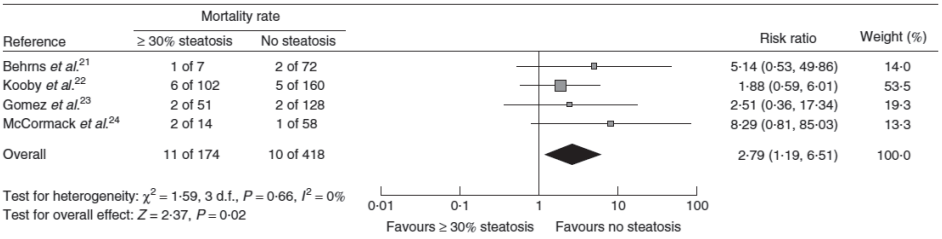


Figure 5. Forest plot of mortality rates in patients with at least 30% steatosis versus those with no steatosis. Risk ratio estimates, shown with 95% confidence intervals, were calculated using the random-effects model. The diamond represents the overall treatment effect from the pooled studies spanning the 95% confidence interval. No steatosis was defined as no hepatocytes containing fat infiltration[21-23] and <10% of hepatocytes with fat droplets.[24]

Mortality as an outcome

None of the six studies in the systematic review found a significant difference in mortality rates between patients with or without any degree of steatosis on univariable analysis, with RR ranging from 1.00 to 1.08.[13,20-24]

The meta-analysis of steatosis as a risk factor for mortality following major hepatic resection (≥3 segments) was limited to four studies (Behrns *et al.*[20], Kooby *et al.*[21], Gomez *et al.*[22], and McCormack *et al.*[23]). There was no substantial heterogeneity in comparing mortality rate in patients with either <30% or ≥30% steatosis to patients without steatosis ($I^2 = 0\%$).

The summary RR for mortality for patients with <30% steatosis compared to patients without steatosis was not significantly increased to 1.79 (95% 0.83, 3.84; $P = 0.71$; **Figure 4**). The summary RR, however, significantly increased when patients with more severe steatosis (≥30%) were compared to patients without steatosis (RR 2.79; 95% CI 1.19, 6.51; P = 0.02; **Figure 5**).

DISCUSSION

While obesity, diabetes mellitus, the metabolic syndrome and associated non-alcoholic fatty liver disease are reaching epidemic proportions throughout the world, the full impact of hepatic steatosis on postoperative outcome is only beginning to be understood. Although the scientific evidence is relatively scarce, steatosis is commonly considered as a significant risk factor in hepatic surgery.[1,12,26,27] The results of the present systematic review and meta-analysis revealed a significant association between degree of steatosis and increased risk of postoperative complications and mortality.

In the systematic review, four publications reporting on patients undergoing hepatic resection for benign or malignant neoplastic disease, and two reporting outcome of living liver donors following resection were identified.[13,20-24] Since differences in underlying parenchymal disease and associated comorbidities could possibly affect homogeneity, studies of living liver donors were excluded from the meta-analysis. Interestingly, Cho *et al.* demonstrated that living liver donors with <30% steatosis did not have increased postoperative complication and mortality rates, and more importantly, long-term regeneration was not impaired.[13] Nagai *et al.* also demonstrated that liver regeneration was not impaired; however, mild steatosis (5 to 20%) was associated with hyperbilirubinemia.[24]

Since the severity of steatosis and extent of resection appeared to be important predictors for postoperative complications in patients undergoing hepatic resection for benign or malignant neoplastic disease[20-23], data for patients who underwent a resection of ≥3 segments was extracted for meta-analysis. It was demonstrated that the risk of developing postoperative complications as well as mortality increased in parallel with the severity of steatosis. Several potential limitations of this study, however, warrant discussion.

This meta-analysis was limited by the availability of only observational studies, which are more prone to confounding factors and bias.[28] Therefore, explicit inclusion and exclusion criteria were applied and study quality was strictly analyzed, focusing on extracting the best available evidence. In the systematic review process, many papers were excluded because of limitations in study design, most commonly because of poor definition of complications or degree of steatosis. Due to these rigorous selection criteria, the review was limited to a disappointing number of six studies with 2b level of evidence. However, all studies scored at least 7 out of 9 points on the Newcastle-Ottawa Scale, indicating good quality.[16] This resulted in not incorporating study quality as a weighing factor in the meta-analysis; furthermore, such weighing by study quality remains controversial.[29]

Although only papers assessing degree of steatosis according to the current gold standard were included, recent evidence suggests that the histological evaluation of steatosis, even performed on large wedge biopsies and assessed by expert pathologists, may be unreliable.[30] This inconsistent assessment of hepatic steatosis may be worrisome; however, until alternatives such as computerized analysis are validated to assess degree of steatosis, liver biopsy and subsequent histological evaluation remain the gold standard.

In the analysis, all postoperative complications were included and no attempt was undertaken to classify outcomes according to previously described validated complication classification systems, such as the "Clavien-Dindo" classification or the "Accordion" severity grading system.[31,32] There was a lack of uniformity in presentation and definition of complications among the studies. Furthermore, the format of a scoring system to classify surgical complications is still under debate.[33]

Selective use of Pringle's manoeuvre[34] (inflow clamping of the porta hepatis) may predispose the remnant liver to an additional ischemic insult that by itself may be a factor for complications.[26] It has been proposed that steatotic liver may be more vulnerable to temporary interruption of blood flow[26,27]; however, a recent Cochrane meta-analysis concluded that vascular occlusion did not significantly affect morbidity and mortality following major hepatic resection.[35] Behrns *et al.*[20] reported that inflow occlusion time was shorter in patients with ≥30% steatosis, whereas inflow occlusion time was similar between groups in the reports by Kooby *et al.*[21] and McCormack *et al.*[23] The use of inflow occlusion was not related to postoperative outcome in any of these papers. Cho *et al.*[13], Gomez *et al.*[22] and Nagai *et al.*[24] did not specifically mention the use of vascular occlusion in their reports.

Comorbidities like the presence of diabetes mellitus may have potentially confounded the results, since this risk factor is independently related with poor surgical outcome.[36] Studies examining the impact of obesity on surgical outcomes have yielded conflicting results, although the two largest studies concluded that obesity is not a risk factor for death or complications in patients undergoing elective general surgery.[37,38] Gomez *et al.*[22] and McCormack *et al.*[23] both demonstrated significant correlations between presence of steatosis and diabetes and obesity, whereas in the studies by Behrns *et al.*[20], Kooby *et al.*[21] and Cho *et al.*[13], the presence of steatosis was only related to obesity. Multivariable analysis in the studies by Kooby *et al.*[21] and McCormack *et al.*[23], however, did not show an association between body mass index and presence of diabetes and surgery-related morbidity and mortality. Further, pre-operative chemotherapy is linked to the development of hepatic steatosis, and translates into increased postoperative

infection rates.[39,40] Of the four studies in which patients were treated for hepatic malignancies, only Kooby *et al.* reported that patients with steatosis were more likely to have received preoperative chemotherapy.[21] Since the other studies did not find a significant association between preoperative chemotherapy treatment and presence of steatosis, the effects of preoperative chemotherapy as a potential confounder may be negligible, but cannot be ruled out.

Substantial heterogeneity between studies may preclude a pooled comparison. The pre-formulated hypothesis and comprehensive search of multiple biomedical databases minimized the presence of publication bias.[41] In addition, a clinically meaningful patient group was selected and the degrees of steatosis were clearly defined. In exploring heterogeneity using funnel plots and the χ^2 and inconsistency (I^2) statistics, significant heterogeneity was not observed.

This is the first systematic review and meta-analysis investigating steatosis as a risk factor following hepatic resection. In a previous narrative review, data was summarized from experimental and clinical studies regarding steatosis as a risk factor in liver surgery.[12] The authors concluded that steatosis was a major determinant of patient outcome after hepatic surgery. After applying rigorous selection criteria in the current analysis, however, only two out of the five clinical studies that were included by the authors remained.[20,21] The other three papers were excluded because of poor definition of steatosis and because the papers used overlapping patient populations (kin relationships). In the current paper, four other articles that have been published after publication of the above review were identified and included. Another narrative review summarizes the literature regarding liver surgery in the presence of cirrhosis and steatosis; however, no attempt was made to establish an effect estimate of steatosis as a risk factor following hepatic surgery.[27] With the rising prevalence of steatosis in patients undergoing liver resection, surgeons should be aware of the potential risks, inform their patients and, if feasible, intervene prior to surgery.

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REFERENCES

1. Angulo P. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; 346: 1221-1231.
2. Browning JD, Szczepaniak LS, Dobbins R, *et al.* Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology* 2004; 40: 1387-1395.
3. Bellentani S, Saccoccio G, Masutti F, *et al.* Prevalence of and risk factors for hepatic steatosis in Northern Italy. *Ann Intern Med* 2000;132:112-117.
4. Al-Asfoor A, Fedorowicz Z, Lodge M. Resection versus no intervention or other surgical interventions for colorectal cancer liver metastases. *Cochrane Database Syst Rev* 2008:CD006039.
5. Verslype C, Van Cutsem E, Dicato M, *et al*. The management of hepatocellular carcinoma. Current expert opinion and recommendations derived from the 10th World Congress on Gastrointestinal Cancer, Barcelona, 2008. *Ann Oncol* 2009; 20 Suppl 7: vii1-vii6.
6. Aljiffry M, Abdulelah A, Walsh M, *et al*. Evidence-based approach to cholangiocarcinoma: a systematic review of the current literature. *J Am Coll Surg* 2009; 208: 134-147.
7. Petrowsky H, Busuttil RW. Evolving surgical approaches in liver transplantation. *Semin Liver Dis* 2009; 29: 121-133.
8. Imamura H, Seyama Y, Kokudo N, *et al*. One thousand fifty-six hepatectomies without mortality in 8 years. *Arch Surg* 2003; 138: 1198-1206.
9. Fan ST, Lo CM, Liu CL, *et al*. Hepatectomy for hepatocellular carcinoma: toward zero hospital deaths. *Ann Surg* 1999; 229: 322-330.
10. Yamanaka N, Okamoto E, Oriyama T, *et al.* A prediction scoring system to select the surgical treatment of liver cancer. Further refinement based on 10 years of use. *Ann Surg* 1994; 219: 342-346.
11. Selzner M, Clavien PA. Fatty liver in liver transplantation and surgery. *Semin Liver Dis* 2001; 21: 105-113.
12. Vetelainen R, van Vliet A, Gouma DJ, *et al.* Steatosis as a risk factor in liver surgery. *Ann Surg* 2007; 245: 20-30.
13. Cho JY, Suh KS, Kwon CH, *et al*. Mild hepatic steatosis is not a major risk factor for hepatectomy and regenerative power is not impaired. *Surgery* 2006; 139: 508-515.
14. Angulo P. Nonalcoholic fatty liver disease and liver transplantation. *Liver Transpl* 2006; 12: 523-534.
15. Adam R, Hoti E. Liver transplantation: the current situation. *Semin Liver Dis* 2009; 29: 3-18.
16. Wells GA, Shea B, O'Connell D, *et al*. The Newcastle-Ottawa Scale (NOS) for assessing the quality of non-randomized studies in meta-analysis. *Third Symposium on Systematic Reviews: Beyond the Basics* 2000.
17. DerSimonian R, Laird N. Meta-analysis in clinical trials. *Control Clin Trials* 1986; 7: 177-188.
18. Higgins JP, Thompson SG. Quantifying heterogeneity in a meta-analysis. *Stat Med* 2002; 21: 1539-1558.
19. Stroup DF, Berlin JA, Morton SC, *et al*. Meta-analysis of observational studies in epidemiology: a proposal for reporting. Meta-analysis Of Observational Studies in Epidemiology (MOOSE) group. *Jama* 2000; 283: 2008-2012.
20. Behrns KE, Tsiotos GG, DeSouza NF, *et al*. Hepatic steatosis as a potential risk factor for major hepatic resection. *J Gastrointest Surg* 1998; 2: 292-298.
21. Kooby DA, Fong Y, Suriawinata A, *et al*. Impact of steatosis on perioperative outcome following hepatic resection. *J Gastrointest Surg* 2003; 7: 1034-1044.
22. Gomez D, Malik HZ, Bonney GK, et al. Steatosis predicts postoperative morbidity following hepatic resection for colorectal metastasis. *Br J Surg* 2007; 94: 1395-1402.

23. McCormack L, Petrowsky H, Jochum W, *et al*. Hepatic steatosis is a risk factor for postoperative complications after major hepatectomy: a matched case-control study. *Ann Surg* 2007; 245: 923-930.
24. Nagai S, Fujimoto Y, Kamei H, *et al*. Mild hepatic macrovesicular steatosis may be a risk factor for hyperbilirubinaemia in living liver donors following right hepatectomy. *Br J Surg* 2009; 96: 437-444.
25. Phillips B, Ball C, Sackett D, *et al*. Levels of Evidence (March 2009). *Oxford Centre for Evidence-Based Medicine Levels of Evidence*. http://www.cebm.net (Accessed November 21, 2009).
26. Clavien PA, Petrowsky H, DeOliveira ML, *et al*. Strategies for safer liver surgery and partial liver transplantation. *N Engl J Med* 2007; 356: 1545-1559.
27. McCormack L, Capitanich P, Quinonez E. Liver surgery in the presence of cirrhosis or steatosis: Is morbidity increased? *Patient Saf Surg* 2008; 2: 8.
28. Egger M, Schneider M, Davey Smith G. Spurious precision? Meta-analysis of observational studies. *Bmj* 1998; 316: 140-144.
29. Detsky AS, Naylor CD, O'Rourke K, *et al*. Incorporating variations in the quality of individual randomized trials into meta-analysis. *J Clin Epidemiol* 1992; 45: 255-265.
30. El-Badry AM, Breitenstein S, Jochum W, *et al.* Assessment of hepatic steatosis by expert pathologists: the end of a gold standard. *Ann Surg* 2009; 250:691-697.
31. Clavien PA, Barkun J, de Oliveira ML, *et al*. The Clavien-Dindo classification of surgical complications: five-year experience. *Ann Surg* 2009; 250:187-196.
32. Strasberg SM, Linehan DC, Hawkins WG. The accordion severity grading system of surgical complications. *Ann Surg* 2009; 250: 177-186.
33. Clavien PA, Strasberg SM. Severity grading of surgical complications. *Ann Surg* 2009; 250: 197-198.
34. Pringle J. Notes on the arrest of hepatic hemorrhage due to trauma. *Ann Surg* 1908; 48: 541-549.
35. Gurusamy KS, Kumar Y, Ramamoorthy R, *et al*. Vascular occlusion for elective liver resections. *Cochrane Database Syst Rev* 2009: CD007530.
36. Little SA, Jarnagin WR, DeMatteo RP, *et al*. Diabetes is associated with increased perioperative mortality but equivalent long-term outcome after hepatic resection for colorectal cancer. *J Gastrointest Surg* 2002; 6: 88-94.
37. Mullen JT, Moorman DW, Davenport DL. The obesity paradox: body mass index and outcomes in patients undergoing nonbariatric general surgery. *Ann Surg* 2009; 250: 166-172.
38. Dindo D, Muller MK, Weber M, *et al*. Obesity in general elective surgery. *Lancet* 2003; 361: 2032-2035.
39. Aloia T, Sebagh M, Plasse M, *et al.* Liver histology and surgical outcomes after preoperative chemotherapy with fluorouracil plus oxaliplatin in colorectal cancer liver metastases. *J Clin Oncol* 2006; 24: 4983-4990.
40. Zorzi D, Laurent A, Pawlik TM, *et al*. Chemotherapy-associated hepatotoxicity and surgery for colorectal liver metastases. *Br J Surg* 2007; 94: 274-286.
41. Dickersin K, Scherer R, Lefebvre C. Identifying relevant studies for systematic reviews. *Bmj* 1994; 309: 1286-1291.

Chapter 3

Dietary fat intake promotes the development of hepatic steatosis independently from excess caloric consumption in a murine model

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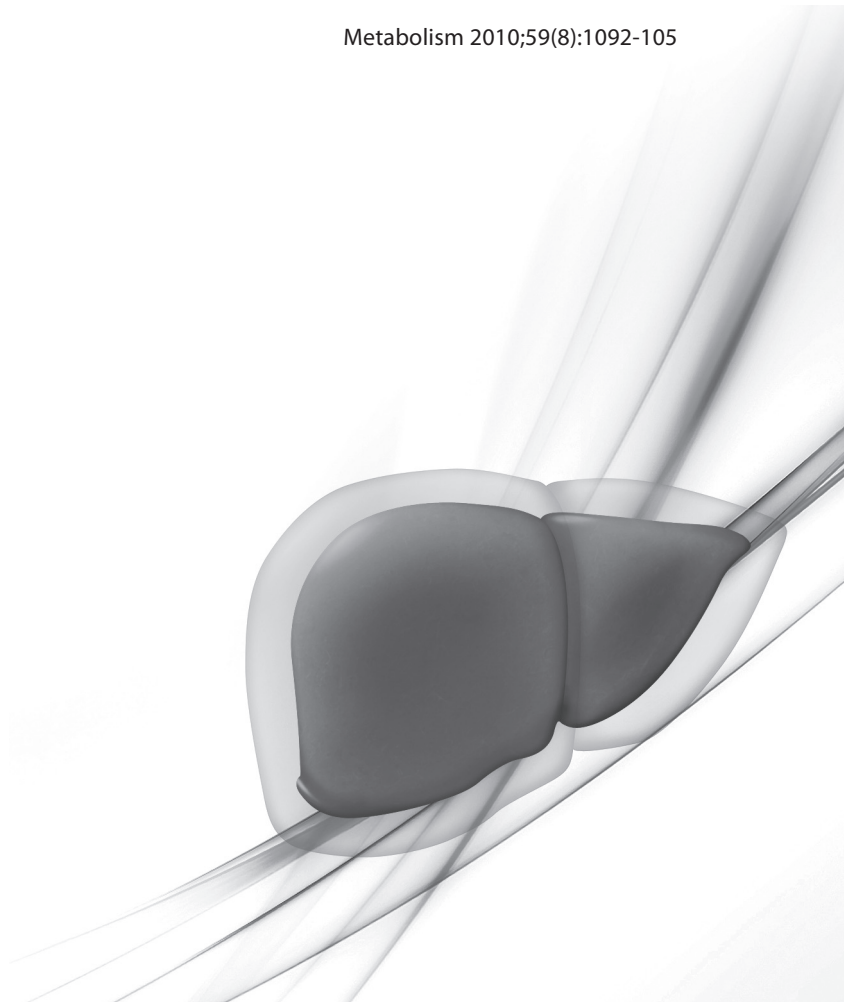

ABSTRACT

Background: Non-alcoholic fatty liver disease (NAFLD) results from over-consumption and is a significant and increasing cause of liver failure. The type of diet that is conducive to the development of this disease has not been established and evidence-based treatment options are currently lacking. We hypothesized that the onset of hepatic steatosis is linked to the consumption of a diet with a high fat content, rather than related to excess caloric intake. In addition, we also hypothesized that fully manifested hepatic steatosis could be reversed by reducing the fat percentage in the diet of obese mice.

Methods: C57Bl/6J male mice were either fed a purified rodent diet containing 10% fat or a diet with 60% of calories derived from fat. A pair-feeding design was used to distinguish the effects of dietary fat content and caloric intake on dietary-induced hepatic lipid accumulation and associated injury. Livers were analyzed by quantitative RT-PCR for lipid metabolism-related gene expression.

Results: After 9 weeks, mice on the 60% fat diet exhibited more weight gain, insulin resistance and hepatic steatosis, compared to mice on a 10% fat diet with equal caloric intake. Furthermore, mice with established metabolic syndrome at 9 weeks showed reversal of hepatic steatosis, insulin resistance and obesity when switched to a 10% fat diet for an additional 9 weeks, independent of caloric intake. Quantitative RT-PCR revealed that transcripts related to both *de novo* lipogenesis and increased uptake of free fatty acids were significantly upregulated in mice pair-fed a 60% fat diet, compared to 10% fat-fed animals.

Conclusion: Dietary fat content, independent from caloric intake, is a crucial factor in the development of hepatic steatosis, obesity and insulin resistance in the C57Bl/6J diet-induced obesity model caused by increased uptake of free fatty acids and *de novo* lipogenesis. In addition, once established, all these features of the metabolic syndrome can be successfully reversed after switching obese mice to a diet low in fat. Low fat diets deserve attention in the investigation of a potential treatment of patients with NAFLD.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) consists of a variety of pathologic states ranging from the simple build-up of fat in the liver (hepatic steatosis) to non-alcoholic steatohepatitis, cirrhosis, and ultimately liver failure.[1-3] Mostly unrecognized before 1980, today NAFLD is the most common chronic liver disease in Western countries with a prevalence up to 30%.[4,5] Its prevalence in non-Western countries is also increasing, mainly due to globalization of the Western diet .[6,7] NAFLD is strongly associated with metabolic disorders such as obesity[8,9] and diabetes mellitus[10], and is considered to be the hepatic manifestation of the metabolic syndrome.[11]

C57Bl/6J male mice fed a high fat (HF) diet is the most commonly used model of diet-induced obesity (DIO) sharing many of the same obesity phenotypes as humans. When allowed *ad libitum* access to a HF diet, the animals gradually develop visceral adiposity, hyperglycemia, insulin and leptin resistance, as well as hepatic steatosis.[12] In contrast, C57Bl/6J mice raised on regular, low fat (LF) chow are lean, euglycemic, have normal insulin and leptin levels and do not develop hepatic steatosis.

Although high fat feeding is associated with hyperphagia, the onset of obesity and insulin resistance in the DIO model is associated with an increased dietary fat content, rather than being simply a consequence of excess caloric consumption, or from decreased levels of physical activity.[13,14] Furthermore, in C57Bl/6J mice receiving a HF diet for 16 weeks, complete reversal of both diabetes and obesity has been demonstrated after switching to a LF diet.[15] In human studies, reduction of dietary fat content produces weight loss for periods up to 7 years and is considered to be one of the cornerstones of obesity management.[16,17] It must be stated, however, that this remains controversial since other studies emphasize the importance of reduced caloric intake, regardless of diet macronutrient composition.[18] The combination of reducing dietary fat consumption and increasing physical activity has been shown to reduce the incidence of diabetes by 58% in subjects with impaired glucose tolerance.[19] The effect of dietary fat content reduction on the prevention and reversal of hepatic steatosis, however, remains to be established.

We hypothesized that the onset of hepatic steatosis in C57Bl/6J mice results primarily from consumption of a diet with a high fat content, rather than from excess caloric intake. In addition, we also hypothesized that fully manifested hepatic steatosis could be reversed by reducing the fat percentage in the diet of obese mice. To test these hypotheses, a pair-feeding design was used to distinguish the effects of dietary fat content and caloric intake on dietary-induced hepatic lipid accumulation and associated injury.

METHODS

Animals

Male 5-week-old C57BL6/J mice (Jackson Laboratories, Bar Harbor, ME) were housed five per cage on paper chip bedding in a barrier room with regulated temperature (21°C ± 1.6°C), humidity (45% ± 10%), and an alternating 12-hour light and dark cycle. The animals had free access to tap water and standard rodent chow pellets (Prolab Isopro, RMH 3000 #25; containing 14% fat, 26% protein, and 60% carbohydrate by calories; energy density 4.1 kcal/g; Prolabs Purina, Richmond, IN) for an acclimation period of 1 week prior to study initiation. During the study period, the animals were weighed twice a week and food intake was measured daily. Intake was measured per cage to avoid the stress of individual housing. Animal protocols complied with the National Institutes of Health Animal Research Advisory Committee guidelines and were approved by the Children's Hospital Boston Animal Care and Use Committee (protocol no. A06-08-065R).

Study diets

Study diets were stored at -4°C and provided fresh each day. The LF diet is the standard American Institute of Nutrition (AIN) 93M (TD.94048; Harlan Teklad, Madison, WI) purified diet with 10.2% of calories derived from fat, and 13.8% and 76% calories from protein and carbohydrates, respectively (energy density of 3.6 kcal/g).[20] All fat in the LF purified rodent diet is comprised of polyunsaturated fatty acids (100% soybean oil). The HF diet (TD.97070; Harlan Teklad, Madison, WI) consists of 59.9% of fat calories, and has 18.8% and 21.3% of calories derived from protein and carbohydrates, respectively (energy density of 5.1 kcal/g).[21] The HF diet is comprised of 45% saturated, 24% trans, 24% monounsaturated, and 7% polyunsaturated fatty acids.

Study design

After the one week acclimation period, fifteen mice were randomized into three groups. The first group (LF) had *ad libitum* access to the LF diet, and a second group (HF) had the same *ad libitum* access to a HF diet. The third group (HF-P) was pair-fed with the LF group, and received a HF diet restricted to the amount of calories that the LF group had consumed the day before. When present, residual food in each cage was weighed, discarded, and replaced with fresh diet every 24 hours for the complete study period of 9 weeks.

In a separate study, fifteen mice were acclimatized for one week, and were fed a HF diet *ad libitum* in order to develop obesity, insulin resistance and hepatic steatosis. After 9 weeks, mice were anesthetized and blood was collected for baseline parameters. After allowing the animals to recover for an additional week, mice were randomized equally

to remain on an *ad libitum* HF diet (RHF), an *ad libitum* LF purified rodent chow diet (RLF), or a HF diet restricted to the amount of calories that the LF group had consumed the day prior (RHF-P). Mice remained on the diets for another 9 weeks until sacrifice.

Chemistry

At the end of the feeding experiments, mice were fasted for 6 hours. Glucose concentration was determined from tail vein blood using an OneTouch UltraSmart Blood Glucose Monitoring System (LifeScan, Milpitas, CA). Mice were then anesthetized and blood was collected via retro-orbital sinus puncture and centrifuged at 14000 rpm at 4°C for 10 min to obtain plasma. Plasma was delivered to the Clinical Laboratory at Children's Hospital Boston for analysis of alanine aminotransferase (ALT), total cholesterol and triglyceride levels. Insulin levels were measured using a rat/mouse insulin ELISA kit (Linco Research, St. Charles, MO).

Surrogate indexes of insulin sensitivity and resistance

Surrogate indexes for insulin sensitivity were calculated, including quantitative insulin-sensitivity check index (QUICKI), homeostasis model assessment (HOMA) and log(HOMA).[22] The calculations were performed as follows: $QUICKI=1/[\log(I_0)+\log(G_0)]$, where I_0 is the fasting insulin (μU/mL) and G_0 is the fasting glucose (mg/dL); $HOMA=(G_0 \times I_0)/22.5$ (with glucose expressed as mmol/L and insulin expressed as μU/mL); and log(HOMA).

Histology

Paraffin-embedded sections of the liver were stained by hematoxylin and eosin and periodic acid Schiff's/diastase to examine cellular architecture, glycogen deposition and lipid accumulation. Frozen tissue sections were stained with Oil Red-O to detect fat. A pathologist blinded to the treatment groups conducted a histological analysis of the liver sections.

Fat pad collection

White adipose tissue was dissected according to previously defined anatomic landmarks, weighed and snap-frozen in liquid nitrogen.[15] Inguinal (all subcutaneous fat between the lower part of the rib cage and mid-thigh), mesenteric (all fat along the mesentery from the lesser curvature of the stomach to the sigmoid colon), retroperitoneal and epididymal fat pads were weighed and expressed relative to eviscerated body weight. A white adipose tissue fat-index was calculated using the sum of the individual fat pads as a percentage of the eviscerated body weight.[15]

Magnetic resonance imaging

MR imaging was performed by the MR Laboratory at Beth Israel Deaconess Medical Center as described previously.[23] Briefly, samples were thawed at room temperature for 1 hour prior to analysis, blotted free of excess water and connective tissue, and placed in 5 mm diameter glass tubes for MR spectroscopy. An 8.5 T vertical bore magnet (DRX system, Bruker Instruments, Billerica, MA) was used for spectroscopic measurements of fat and water resonances. Specifically, a point resolved echo spectroscopic acquisition was applied to homogenous regions of liver, as identified from fast low angle shot images of the liver specimen. Voxel volumes interrogated spectroscopically with the point resolved echo spectroscopic sequence were 2 mm^3. The repetition and echo times were 8 s and 12 ms, respectively, and 16 signal averages were acquired per spectrum. The water resonance and the methylene/methyl resonances were numerically integrated using the manufacturer supplied Paravision 4.0 software (Bruker Instruments, Billerica, MA). The methylene/methyl area was divided by the sum of the methylene/methyl area plus the water area to obtain the MR spectroscopy parameter representing hepatic fat fraction used for group comparisons.

Analysis of mRNA expression

Isolation of mRNA, reverse transcription and quantitative real-time RT-PCR was performed as described previously.[24] Briefly, 200-300 mg snap-frozen liver tissue was homogenized and total RNA was extracted using Tri Reagent (Molecular Research Center, Cincinnati, OH). cDNA was obtained by reverse transcription of 1 mg of total RNA using Superscript II Reverse Transcriptase (Invitrogen, Karlsruhe, Germany), using 50 pmol random hexamer and 100 pmol oligo-dT primers (Promega, Mannheim, Germany). Relative transcript levels of sterol regulatory element-binding protein (SREBP)-1c, SREBP-2, elongation of long chain fatty acids family member(Elovl)-6, fatty acid synthase (Fasn), stearoyl-CoA desaturase (SCD)-1, and peroxisome proliferator activated receptor (PPAR)-α were quantified by real-time RT-PCR on a LightCycler 1.5 instrument (Roche, Mannheim, Germany) using the TaqMan methodology. TaqMan probes (dual-labeled with 5'-FAM and 3'-TAMRA) and primers were designed using the Primer Express software (Perkin Elmer, Waltham, MA), synthesized at MWG Biotech AG (Ebersberg, Germany), and are outlined in **Table 1**. The housekeeping gene beta-2 microglobulin (β2MG) was amplified in parallel reactions for normalization.

Statistical analysis

Data are expressed as means ± standard error of the mean (SEM). Data sets involving more than two groups were assessed by analysis of variance (ANOVA). Repeated measures ANOVA was used to analyze body weight gain. Differences between two groups were assessed using the unpaired two-tailed Student's *t* test, or if nonparamet-

Table 1. Primers and probes used in quantitative real-time RT-PCR.

Target gene	5′-Primer	TaqMan probe	3′-Primer
β2MG	CTGATACATACGCCTGCAGAGTTAA	GACCGTCTACTGGGATCGAGACATGTG	ATGAATCTTCAGAGCATCATGAT
SREBP1c	CCAGAGGGTGAGCCTGACAA	CAATCAGGACCATGCCGACCTCT	AGCCTCTGCAATTTCCAGATCT
SREBP2	GCGGACAACACACAATATCATTG	AGCGCTACCGGTCCTCCATCA	TGACTAAGTCCTTCAACTCTATGATTTTG
Fasn	CCTGGATAGCATTCCGAACCT	CCTGAGGGACCCTACCGCATAGC	AGCACATCTCGAAGGCTACACA
SCD-1	CAACACCATGGCGTTCCA	TGACGTGTACGAATGGGCCCGA	GGTGGGCGCGGTGAT
Elovl-6	GCGCTGTACGCTGCCTTTAT	TCGGCATCTGATGAACAAGCGAGC	GCGGCTTCCGAAGTTCAAA
PPAR-α	TGGTTCCTGGTGCCGATTTA	TGGTGGTAGATGCCTGCAACCCCA	ACTAGCATCCCACTTAATTATGTATCTGAA

ric, by using the Mann-Whitney *U* test. A paired *t* test was used when the difference was calculated between two paired observations. $P<0.05$ was considered statistically significant. All data were collected in a computerized Microsoft Excel database (Microsoft Inc., Redmond, WA). The analysis was performed with SPSS version 16.0 (SPSS Inc., Chicago, IL) statistical software, and figures were created using GraphPad Prism version 5.0 (GraphPad Software Inc., La Jolla, CA) software.

RESULTS

Feeding C57Bl/6J mice a HF diet ad libitum for 9 weeks induces hyperphagia and increases body weight gain, liver weight and white adipose tissue, compared to mice fed a LF diet

Mice fed a diet containing 60% of calories from fat for 9 weeks *ad libitum* (group HF) consumed more calories than did mice receiving a diet containing 10% of calories from fat *ad libitum* (group LF) (**Table 2**, **Figure 1A**). As a consequence of this hyperphagia, HF mice gained more weight and had larger livers compared to the mice with *ad libitum* access to the LF (**Table 2**; **Figure 1B**). HF mice showed an increased caloric efficiency compared to LF mice, determined by dividing the total weight gain per group by their total intake in kilocalories (**Table 2**).The white adipose tissue fat index, as a measure of obesity, was increased in HF mice (21.88±0.80 %) compared to LF mice (4.63±0.11 %, $P=0.0079$) (**Table 2**).

Pair-feeding C57Bl/6J mice a HF diet increases body weight gain, liver and white adipose tissue, compared to mice on a LF diet with the same caloric intake

Mice receiving the HF diet restricted to the same amount of calories as the LF mice to rule out the effects of hyperphagia (group HF-P) gained 53% more weight ($P=0.0097$) and had 27% larger livers ($P=0.0052$) than did the pair-fed LF mice (**Table 2**; **Figure**

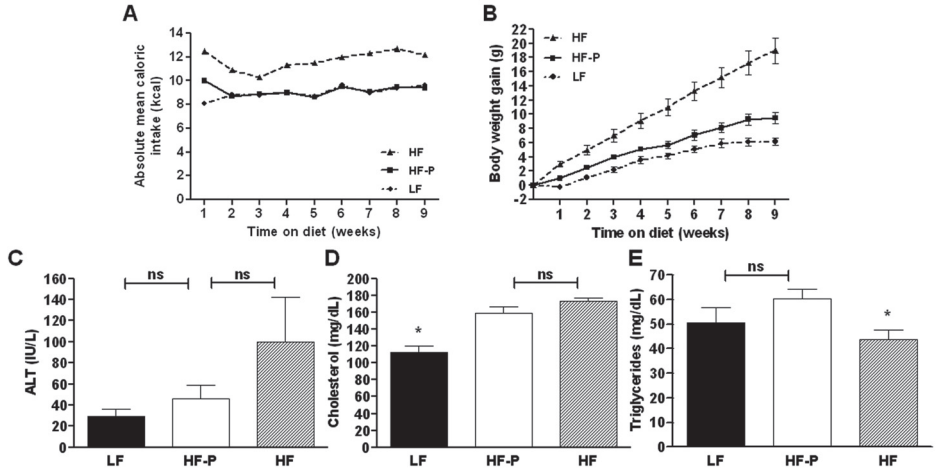

Figure 1. Absolute mean caloric intake per mouse per week (A) was calculated per group on a daily base. Body weight gain (B) was calculated relative to the weight of each individual animal before initiation of the experiment. Plasma ALT (C), total cholesterol (D), and triglyceride (E) levels in the different groups. Values represent the mean ± SEM. Statistical significance is calculated between the HF-P animals and the difference between LF animals and HF animals (*P < 0.05). NS indicates not significant.

Table 2. Body weights, tissue weights, caloric intake and surrogate markers for insulin resistance in mice assigned to the different dietary interventions after 9 weeks.

		LF (*n*=5) 9 weeks		HF-P (*n*=5) 9 weeks	HF (*n*=5) 9 weeks	
Body weight gain	(g)	6.2 ± 0.5	**	9.5 ± 0.8	19.0 ± 1.8	**
Liver weight	(g)	0.89 ± 0.03	**	1.13 ± 0.05	1.72 ± 0.17	**
Liver/body weight ratio		0.034 ± 0.00	**	0.039 ± 0.00	0.045 ± 0.00	
Total caloric intake / mouse	(kcal)	559		569	727	
Caloric efficiency	(g/kcal)	0.0111		0.0167	0.0261	
Relative Inguinal fat mass	(%)	1.19 ± 0.17	**	2.60 ± 0.33	6.92 ± 0.38	***
Mesenteric fat pad	(%)	0.58 ± 0.04	***	2.02 ± 0.12	3.55 ± 0.14	***
Retroperitoneal fat pad	(%)	0.57 ± 0.08	**	1.34 ± 0.13	2.33 ± 0.16	**
Epididymal fat pad	(%)	2.29 ± 0.17	***	5.42 ± 0.31	9.09 ± 0.38	***
White adipose tissue fat index	(%)	4.63 ± 0.11	***	11.39 ± 0.54	21.88 ± 0.80	***
Fasting glucose	(mg/dL)	168 ± 32		227 ± 24	406 ± 40	**
Fasting insulin	(µU/mL)	11.2 ± 1.3		30.1 ± 10.2	42.8 ± 3.2	
QUICKI		0.31 ± 0.01	*	0.27 ± 0.01	0.24 ± 0.00	*
HOMA		4.3 ± 0.6	*	18.1 ± 7.4	43.0 ± 6.8	*
Log(HOMA)		0.62 ± 0.06	*	1.12 ± 0.17	1.62 ± 0.06	*

Values given are means ± SEM; HF-P group was used as reference; *P<0.05; **P<0.01; ***P<0.001. LF indicates low fat; HF-P, high fat pair-fed; HF, high fat; QUICKI, quantitative insulin-sensitivity check index; HOMA, homeostasis model assessment.

1A). When determining caloric efficiency, HF-P mice demonstrated more weight gain per consumed calorie compared to the LF control mice (**Table 2**). Although their caloric intake was similar, relative inguinal, mesenteric, retroperitoneal, and epididymal fat masses, as well as the white adipose tissue fat index were significantly increased in HF-P mice, compared to the LF mice (**Table 2**).

Insulin sensitivity is impaired in C57Bl/6J mice fed a HF diet restricted to the caloric intake of mice on a LF diet

Insulin sensitivity was determined by calculating surrogate indexes for insulin sensitivity, including QUICKI, HOMA and log(HOMA). Although fasting plasma levels of glucose and insulin in HF-P and LF mice were not significantly different, HF-P mice had significantly impaired insulin sensitivity as determined by all surrogate indexes, compared to LF mice (**Table 2**).

Pair-feeding C57Bl/6J mice a HF diet aggravates plasma liver function tests, compared to mice on a LF diet with the same caloric intake

ALT is used as a marker for evaluation of hepatic injury and is increased in mice with hepatic steatosis (**Figure 1**). HF-P animals did not exhibit a significant elevation of plasma values for ALT (46±12 IU/L) than did LF animals (29±7 IU/L; P=0.2631). Compared to LF mice, HF mice demonstrated an elevated mean ALT level of 100±42 IU/L (P=0.0317), which was not statistically different to HF-P mice (P=0.2222). Compared to LF mice, pair-fed HF-P mice had increased mean plasma values for cholesterol (P=0.0027). Plasma triglyceride levels were not significantly elevated in HF-P mice, compared to LF mice (P=0.2202). These data suggest that the liver injury associated with the HF diet was not completely prevented by restricting the animals to the caloric intake of LF diet fed animals, but that cholesterol levels showed a similar increase as mice fed a HF diet *ad libitum*.

High dietary fat content, apart from excess caloric intake, stimulates hepatic steatosis in C57Bl/6J mice on histology

Hematoxylin and eosin and Oil Red-O stained liver sections from LF-fed mice exhibited hepatic architecture with no evidence of hepatic steatosis (**Figure 2**). In contrast, liver sections from HF-fed mice showed fat throughout the liver parenchyma, including both macro- and microvesicular steatosis. Microvesicular and macrovesicular steatosis was present predominantly in the periportal and midzone areas, whereas occasional ballooned hepatocytes and macrovesicular steatosis were present in the central vein area. HF-P mice that received the HF diet restricted to the caloric intake of the LF group, however, showed moderate steatosis, predominantly microvesicular. Occasional macrovesicular hepatocytes were observed in the periportal zone. Analysis of periodic

acid Schiff's/diastase-stained liver sections excluded glycogen deposition as a cause of microvesicular changes in hepatocytes (data not shown). Steatohepatitis and acute inflammation were not observed in any of the experimental groups.

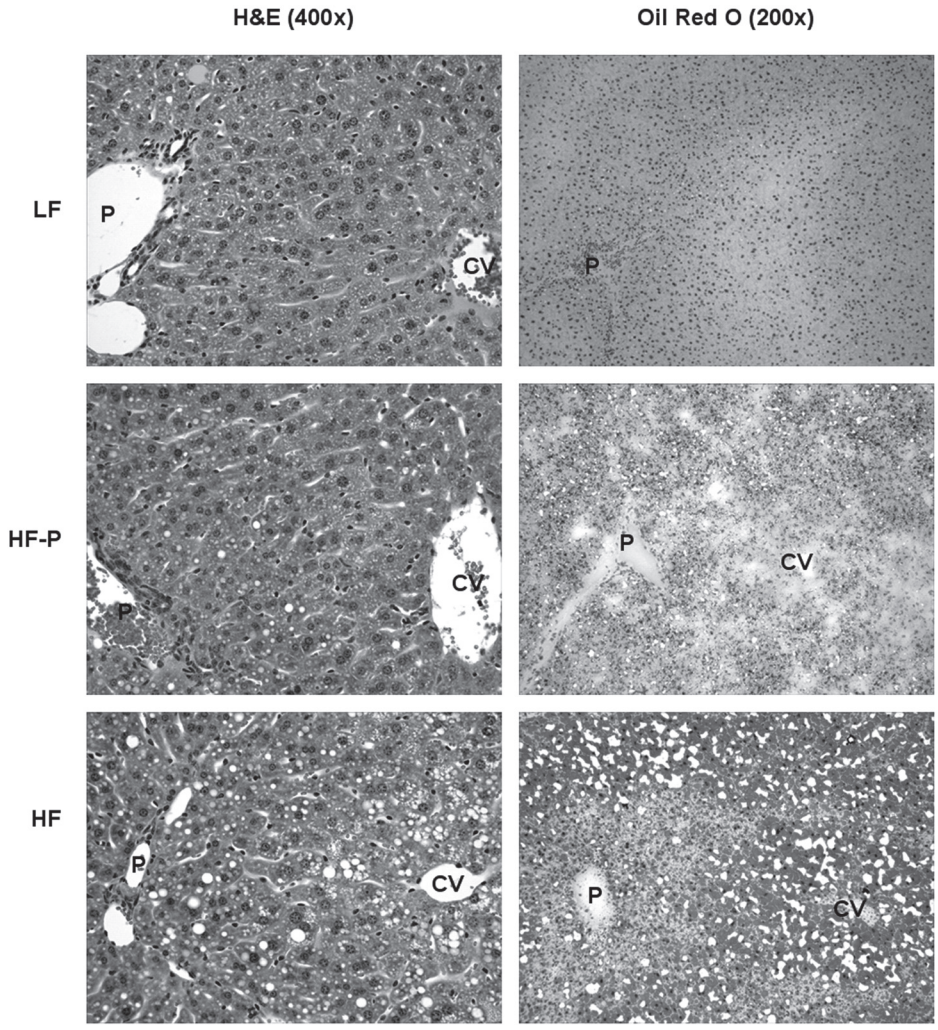

Figure 2. Representative liver sections stained with hematoxylin and eosin (left panels; original magnification 400×), and oil red O (right panels; original magnification 200×). The LF livers exhibited normal hepatic architecture, whereas the HF-P livers revealed moderate microvesicular steatosis, and HF livers revealed extensive microvesicular and macrovesicular steatosis. H&E indicates hematoxylin and eosin; P, portal tract; CV, central vein.

High dietary fat content, apart from excess caloric intake, stimulates hepatic fat accumulation in C57Bl/6J mice determined by MR spectroscopy

Hepatic fat content was quantified by using MR spectroscopy. Representative spectra of the different groups are shown (**Figure 3**). HF mice fed a 60% fat diet *ad libitum* exhibited an increase in liver fat content to 34.5±8.2% (*P*=0.0159) when compared to HF-P mice, demonstrating the aggravating effect of hyperphagia on hepatic fat accumulation (Figure 3D). Although both groups had a similar caloric intake, LF animals were found to have a liver fat content of 3.2±0.8%, whereas HF-P animals demonstrated increased fat content of 12.3±2.6% (*P*=0.0079). These data corroborated our histological findings that a HF diet, apart from hyperphagia-induced excess caloric intake, leads to the onset of hepatic steatosis.

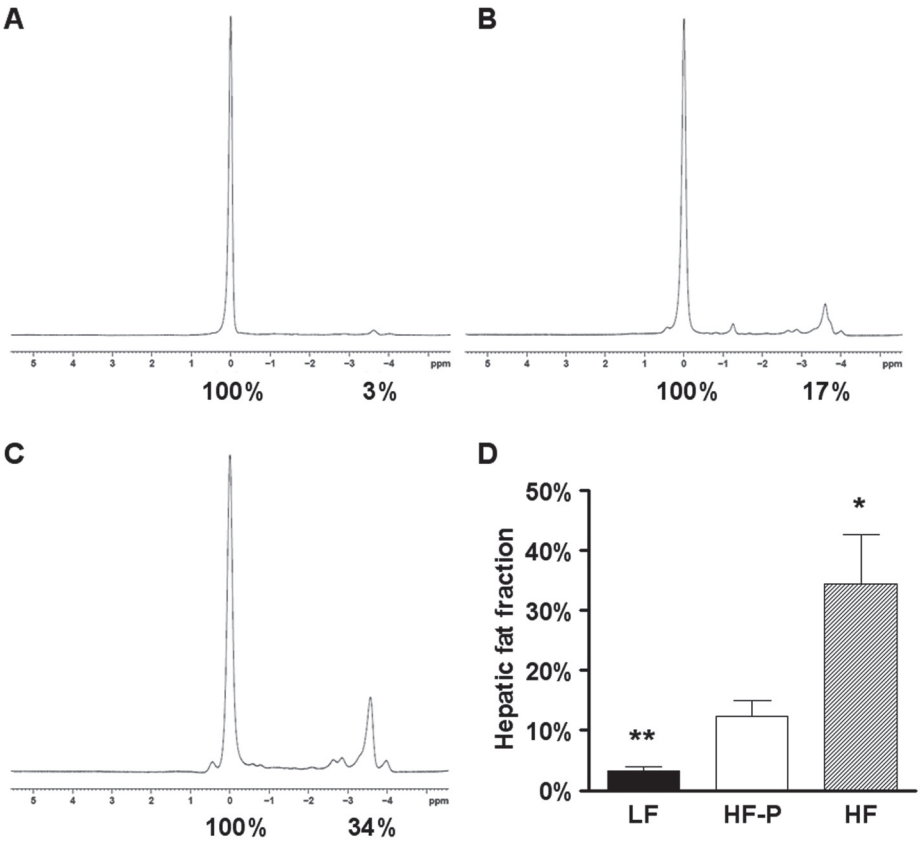

Figure 3. Magnetic resonance spectra for LF (A), HF-P (B), and HF (C) livers. Percentage fat content was determined relative to water (100%) by numerical integration of the areas under the lipid and water peaks. Mean hepatic fat fraction as measured by MR spectroscopy (D). Statistical significance is calculated between the HF-P animals and the difference between LF animals and HF animals (*P < 0.05 and **P < 0.01). Variance statistic is SEM.

Reduction of fat percentage, but not caloric intake alone, decreased body, liver and white adipose tissue weights in C57Bl/6J mice with established metabolic syndrome

In mice that had been fed a HF diet *ad libitum* for 9 weeks, we investigated whether previously established obesity, insulin resistance and hepatic steatosis could be reversed by either switching to a LF diet (group RLF), or by pair-feeding the HF diet to reduce caloric intake (group RHF-P). A control group that had remained on the HF diet *ad libitum* for another 9 weeks (group RHF) demonstrated a significant increase in body, liver and white adipose tissue weights (**Table 3**). After switching mice from a HF diet to a LF diet *ad libitum*, we initially observed a decrease in mean caloric intake (**Figure 4A**). After 3 weeks, however, the mice on the LF diet consumed the same mean caloric intake as we had demonstrated before (**Figure 1A**). Consistent with a previous report, reducing the amount of fat in the diet significantly reversed obesity in the RLF mice that had been switched to an *ad libitum* LF diet (**Figure 4B**).[15] Although pair-feeding RHF-P animals to the caloric intake of the RLF mice resulted in significant less weight gain, smaller livers and less white adipose tissue compared to RHF mice, they gained significantly more weight, had larger livers and more adipose tissue than did the RLF group (**Table 3**). This indicates that even though both RLF and RHF-P groups consumed an equal amount of calories, the higher fat content in the diet of the RHF-P mice resulted in maintenance of the obese phenotype.

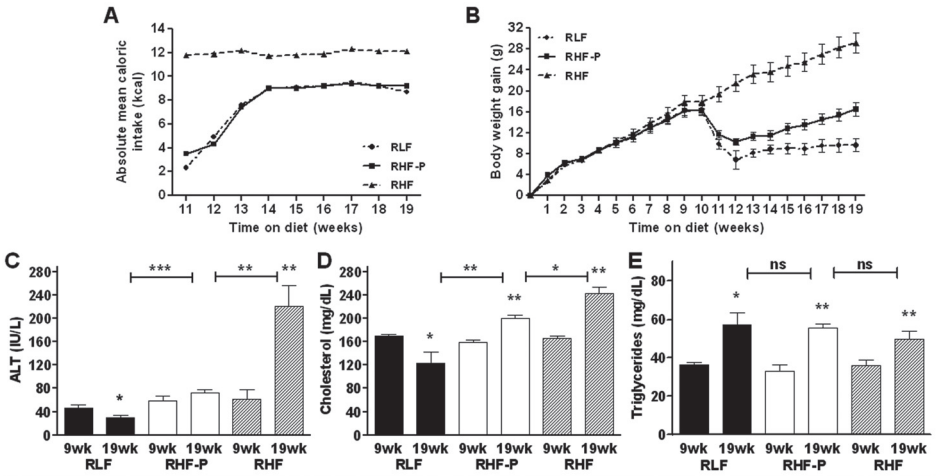

Figure 4. Body weight gain (A) was calculated relative to the weight of each individual animal before initiation of the experiment. After 9 weeks, blood was collected via retro-orbital puncture. Mice were allowed 1 week to recover before groups were randomly assigned to the study diets. Absolute mean caloric intake per mouse per week (B) was calculated per group on a daily base. Plasma ALT (C), total cholesterol (D), and triglyceride (E) levels in the different groups. Values represent the mean ± SEM. Statistical significance is calculated between the RHF-P animals and the difference between RLF animals and RHF animals, as well as repeated measurements within groups (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$). NS indicates not significant.

Table 3. Body weights, tissue weights, caloric intake and surrogate markers for insulin resistance in mice that had been fed an ad libitum high fat diet for 9 weeks, and after 1 week recovery from phlebotomy had been switched to the intervention diets for another 9 weeks.

		RLF (*n*=5) 19 weeks		RHF-P (*n*=5) 19 weeks	RHF (*n*=5) 19 weeks	
Body weight gain	(g)	-6.8 ± 0.9	**	0.3 ± 1.1	11.3 ± 0.8	***
Liver weight	(g)	0.98 ± 0.06	***	1.63 ± 0.07	3.52 ± 0.40	**
Liver/body weight ratio		0.034 ± 0.00	***	0.044 ± 0.00	0.072 ± 0.01	**
Total caloric intake / mouse	(kcal)	468		492	754	
Caloric efficiency	(g/kcal)	-0.0145		0.0006	0.0150	
Relative Inguinal fat mass	(%)	3.73 ± 0.72	**	7.37 ± 0.58	9.36 ± 0.48	*
Mesenteric fat pad	(%)	1.91 ± 0.29	**	3.50 ± 0.23	4.38 ± 0.14	*
Retroperitoneal fat pad	(%)	1.67 ± 0.35	*	2.74 ± 0.26	4.32 ± 0.24	**
Epidydimal fat pad	(%)	4.56 ± 0.61	***	8.44 ± 0.45	12.28 ± 0.70	**
White adipose tissue fat index	(%)	11.87 ± 1.92	**	22.05 ± 1.40	29.33 ± 1.36	**
Fasting glucose	(mg/dL)	204 ± 8		250 ± 15	252 ± 11	
Fasting insulin	(µU/mL)	16.9 ± 2.2	**	72.6 ± 7.1	90.4 ± 13.8	
QUICKI		0.28 ± 0.01	***	0.24 ± 0.00	0.23 ± 0.00	
HOMA		8.6 ± 1.3	**	44.5 ± 5.6	57.2 ± 11.2	
Log(HOMA)		0.91 ± 0.07	***	1.63 ± 0.06	1.72 ± 0.09	

Values given are means ± SEM; RHF-P group was used as reference; *P<0.05; **P<0.01; ***P<0.001. RLF indicates reversal low fat; RHF-P, reversal high fat pair-fed; RHF, reversal high fat; QUICKI, quantitative insulin-sensitivity check index; HOMA, homeostasis model assessment.

Pair-feeding obese C57Bl/6J mice a HF diet aggravates insulin sensitivity, compared to mice switched to a LF diet with the same caloric intake

In order to compare the effects of our dietary interventions on insulin sensitivity we measured fasting plasma values for glucose and insulin and calculated surrogate markers for insulin sensitivity. Values after 9 weeks on a HF diet were compared to their corresponding values at the end of the study. Mice that remained on a HF diet for another 9 weeks experienced decreased insulin sensitivity, as calculated by QUICKI (0.24±0.00 to 0.23±0.00, *P*=0.0185), HOMA (32.5±7.2 to 57.2±11.2, *P*=0.0359) and log(HOMA) (1.48±0.08 to 1.72±0.09, *P*=0.0187). Switching mice from a HF diet to a LF diet decreased fasting glucose levels from 316±28 mg/dL to 204±8 mg/dL (*P*=0.0134) and decreased fasting insulin levels from 40.8±5.0 µU/mL to 16.9±2.2 µU/mL (*P*=0.0061). QUICK, HOMA, and log(HOMA) all improved from 0.24±0.00 to 0.28±0.01 (*P*=0.0026), 30.6±2.9 to 8.6±1.3 (*P*=0.0026) and 1.48±0.04 to 0.91±0.07 (*P*=0.0020), respectively. More interestingly, restricting mice in the RHF-P group to the lower amount of calories of the RLF group but maintaining their HF diet did not improve their fasting glucose levels (289±12 mg/dL to 250±15 mg/dL, *P*=0.2021), and increased fasting insulin levels from 43.7±8.2 µU/mL to

72.6±7.1 µU/mL (*P*=0.0202). In contrast to the RLF mice, these mice did not improve their insulin sensitivity as calculated by QUICKI (0.25±0.00 to 0.24±0.00, *P*=0.0876), HOMA (30.4±5.2 to 44.5±15.6, *P*=0.0880) and log(HOMA) (1.46±0.07 to 1.63±0.06, *P*=0.0903).

Plasma liver function tests improved in C57Bl/6J mice switched to a LF diet, but not in mice that had been fed a HF diet restricted to the caloric intake of mice on a LF diet

To detect the presence of hepatic steatosis, liver enzymes were measured on all experimental groups (**Figure 4**). When compared to their corresponding base-line parameters, RHF mice exhibited an increase in mean ALT values (*P*=0.0054), suggesting progression of hepatic steatosis. RLF animals exhibited a decrease in mean ALT values (*P*=0.0476), whereas RHF-P mice showed no improvement (*P*=0.1562). Cholesterol levels decreased in the RLF group (*P*=0.0470), whereas both RHF-P and RHF animals showed elevations (*P*=0.0018 and *P*=0.0017, respectively). Triglyceride levels significantly increased in all three groups. This indicates that even though both RLF and RHF-P groups consumed an equal amount of calories, the RLF mice showed resolution of both their liver injury and elevated cholesterol, whereas the higher fat content in the diet of the RHF-P mice resulted in maintenance of the elevated ALT levels and progression of cholesterol elevation.

Switching to a LF diet resolved hepatic steatosis, but obese C57Bl/6J mice pair-fed a HF diet maintained their hepatic steatosis as determined by histology

Figure 5 shows liver histopathological features of all groups studied. Liver sections from RHF mice showed extensive macrovesicular steatosis diffusely affecting the liver parenchyma, with ballooning of hepatocytes present mostly in the central vein area. In contrast, liver sections from RLF mice exhibited hepatic architecture with only rare microvesicular steatosis in midzone hepatocytes with an occasional hepatocyte showing macrovesicular steatosis. Liver sections from the RHF-P group showed diffuse macro- and microvesicular steatosis. Macrovesicular changes were most marked in the periportal and midzone hepatocytes, whereas ballooned hepatocytes were noted in the central vein area. Inflammatory changes suggestive for steatohepatitis were not observed in any of the groups.

Switching to a LF diet resolved hepatic steatosis, but obese C57Bl/6J mice pair-fed a HF diet maintained their hepatic steatosis as determined by MR spectroscopy

In order to objectively quantify the effects of the dietary interventions, MR spectroscopy was performed on livers. Representative spectra of the different groups are shown in **Figure 6**. RHF mice that had been fed a 60% HF diet *ad libitum* for 19 weeks increased their liver fat content to a value of 67.1±2.5%, compared to the hepatic fat fraction of HF mice at 9 weeks (34.5±8.2%; *P*=0.0079). RHF-P mice, in contrast, exhibited a similar

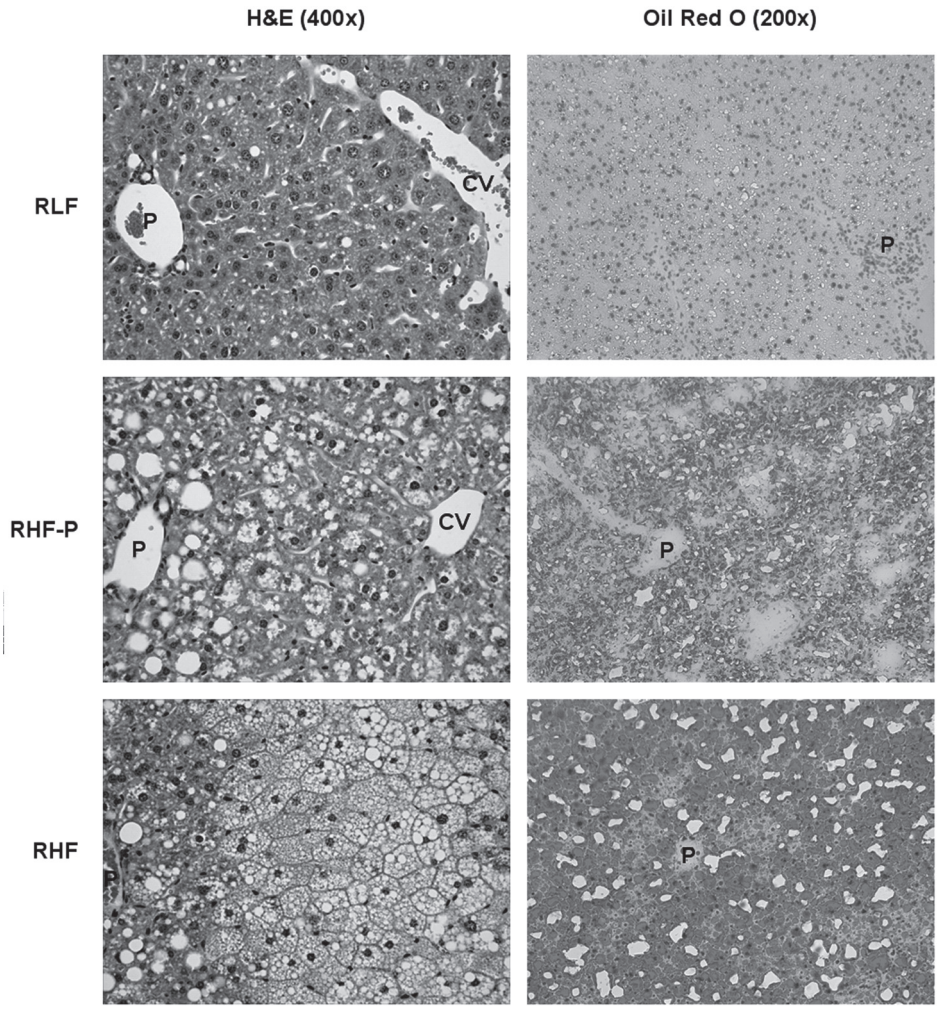

Figure 5. Representative liver sections stained with hematoxylin and eosin (left panels) and oil red O (right panels). The RLF livers exhibited normal hepatic architecture with occasional fat droplets. The RHF-P livers revealed moderate macrovesicular steatosis around the portal tract and moderate microvesicular steatosis around the central vein. The RHF livers revealed extensive microvesicular steatosis around the central vein and extensive macrovesicular steatosis around the portal tract. Original magnification 200×.

hepatic fat fraction of 37.7±3.7%, when compared to HF mice (*P*=0.7389). This indicates that a profound reduction of caloric intake, but not dietary fat content, halted progression of hepatic steatosis. RLF animals that had a similar caloric intake to RHF-P mice but were switched to a 10% LF diet were found to have their liver fat content reduced to 14.2±2.3%, when compared to HF mice at 9 weeks (*P*=0.0317). These data corroborated our finding on histology that switching to a LF diet was efficacious in reducing hepatic steatosis, whereas mice that had been pair-fed a diet with a 60% fat content showed no resolution.

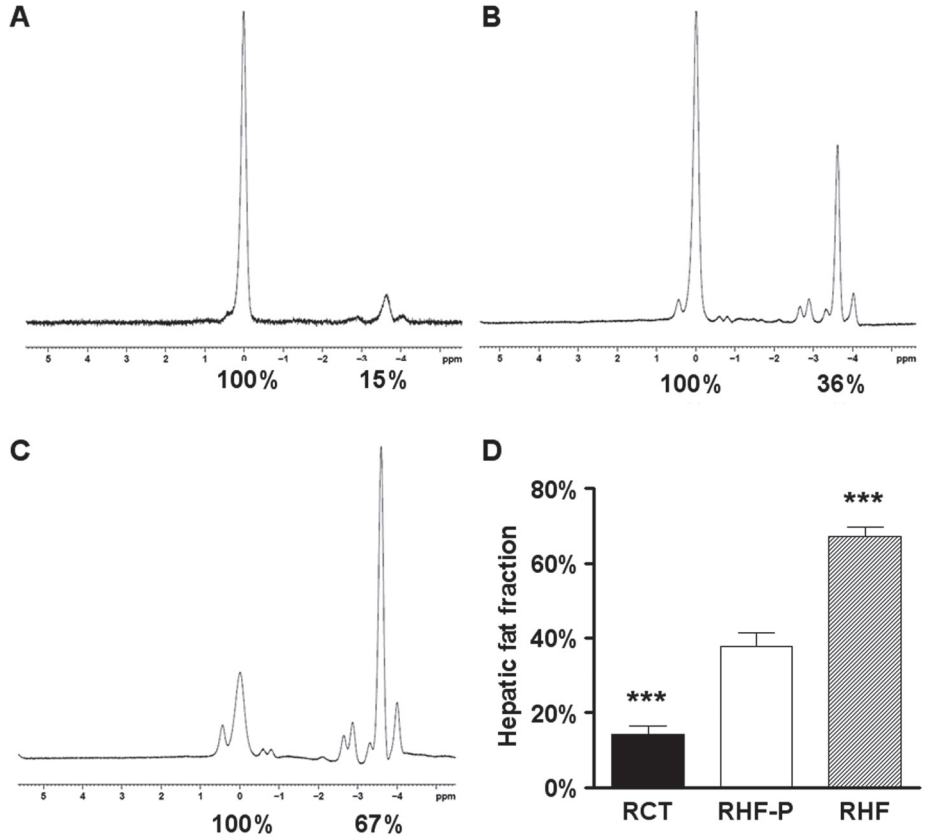

Figure 6. Magnetic resonance spectra for RLF (A), RHF-P (B), and RHF (C) livers. Percentage fat content was determined relative to water (100%) by numerical integration of the areas under the lipid and water peaks. Mean hepatic fat fraction as measured by MR spectroscopy (D). Statistical significance is calculated between the RHF-P animals and the difference between RLF animals and RHF animals (***$P < 0.001$). Variance statistic is SEM.

HF feeding affected hepatic gene expression levels in both the 9 weeks and 19 weeks experiments

To explore the mechanism why mice on a 60% HF diet that were pair-fed to mice on a 10% LF diet developed hepatic steatosis (HF-P) and did not reverse their fatty liver in the reversal experiment (RHF-P), we examined hepatic transcript levels of genes involved in lipid metabolism (**Figure 7**). In the 9 weeks experiment, feeding mice a HF diet *ad libitum* resulted in significantly reduced Elovl6, SREBP1c and Fasn levels, whereas SREBP2 levels remained unchanged, suggesting a decrease in *de novo* lipogenesis compared to LF controls. SCD1 gene expression levels, however, were significantly increased. Being a downstream target of SREBP1c activation, increased SCD1 gene expression suggests that the increased dietary free fatty acid influx was mainly responsible for the net build-

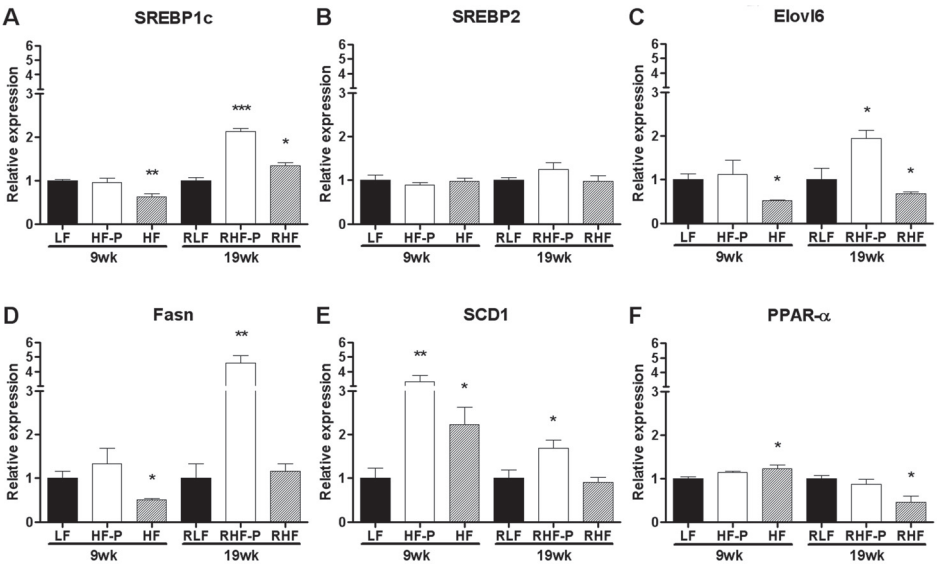

Figure 7. Up-regulation of genes related to lipogenesis in livers of mice pair-fed an HF diet and in mice that were fed an HF diet ad libitum compared with LF-fed controls. Hepatic expression of SREBP1c (A), SREBP2 (B), Elovl6 (C), Fasn (D), SCD1 (E), and PPAR-α (F). Relative transcript levels were quantified by TaqMan quantitative RT-PCR, normalized by β2MG, and expressed in arbitrary units vs LF (9-week experiment) or RLF (19-week experiment) controls (*P <0.05, **P < 0.01, and ***P < 0.001). Variance statistic is SEM.

up of fat in the liver, followed by *de novo* lipogenesis. PPAR-α levels were somewhat increased illustrating the liver's response to oxidize the accumulated fat. In HF-P mice, however, none of the genes related to *de novo* lipogenesis were upregulated in the 9 weeks experiment. In contrast, SCD1 transcript levels were upregulated threefold compared to LF mice, suggesting that in this 9 weeks experiment the development of hepatic steatosis in the HF pair-fed group was mainly attributed to increased delivery and uptake of long chain fatty acids into the hepatocytes, followed by early *de novo* lipogenesis.

In the 19 weeks reversal experiment, the gene transcript levels of SREBP1c, Elovl6, Fasn as well as SCD1 in RHF-P mice were all markedly upregulated, which is compatible with increased *de novo* lipogenesis combined with increased uptake of free fatty acids. In contrast, SREBP2 and PPAR-α levels remained unchanged. This suggests that in the 19 weeks reversal experiment, both *de novo* lipogenesis and increased uptake of free fatty acids were responsible for maintaining the hepatic steatosis. This is in sharp contrast with the 10% LF pair-fed RLF animals that reversed their steatosis markedly.

DISCUSSION

Hepatic steatosis in patients with NAFLD is a consequence of increased free fatty acid delivery to the liver combined with increased *de novo* lipogenesis.[25] Although the pathogenesis of this common disorder remains poorly understood, it is generally accepted that over-consumption and insulin resistance may play an important role in the development of NAFLD. Unlike the other components of the metabolic syndrome, the association between dietary macronutrient intake and NALFD has not been established.[26] In this study, we sought to determine the effects of dietary fat content on the development of NAFLD and other components of the metabolic syndrome, corrected for caloric intake using a pair-feeding design. Our results indicate that the development of hepatic steatosis in a murine DIO-model can be prevented and reversed when switching to a standard diet low in fat. Moreover, we demonstrate that a diet composed of 60% fat calories, when corrected for caloric intake, contributed to the development of hepatic steatosis by activating genes related to both *de novo* lipogenesis and increased uptake of free fatty acids.

Different dietary manipulations have been shown to induce obesity, insulin resistance and NAFLD in different strains and species of rodents, thereby reproducing the human condition. This suggests that over-consumption with either carbohydrates, fats, or both may play a role in the development of hepatic steatosis associated with obesity and insulin resistance.[27] Our experimental HF diet contains predominantly saturated fat and has small amount of polyunsaturated fat, quite similar to the diets consumed by obese and NAFLD patients.[28] Likewise, saturated fat intake positively correlates with insulin sensitivity and transaminase levels in NALFD patients.[29] In accordance with these reports, our results show that mice fed a 60% fat diet consistently developed hyperglycemia, hyperinsulinemia, insulin resistance, visceral adiposity and hepatic steatosis. Since HF feeding is associated with hyperphagia, one of our aims was to elucidate whether the effects observed in the HF groups resulted from increased caloric or fat intake. A previous report showed that reducing the number of calories consumed from a HF diet attenuated but did not prevent the development of insulin resistance and obesity in mice, thus indicating a role of HF diet independent of caloric intake.[13] In addition to corroborating these findings, we now demonstrate that the development of hepatic steatosis directly results from an increased fat intake that is independent from the amount of calories consumed.

SCD1 is induced by dietary carbohydrates, saturated fat, and cholesterol through activation of SREBP-1c and liver X receptor.[30] Increased delivery and uptake of the long chain saturated fatty acids palmitate (C16:0) and stearate (C18:0) by hepatocytes is associated

with an increase in SCD1 expression and activity, resulting in a net formation of the monounsaturated fatty acids palmitoleate (C16:1) and oleate (C18:1), triglyceride storage and development of hepatic steatosis.[31,32] In our 9 weeks study, the development of hepatic steatosis was mainly a result from an overflow of free long chain fatty acids, as demonstrated by increased transcript levels of SCD1, but absence of SREBP1c, Elovl6 or Fasn upregulation. Even when mice were pair-fed to LF animals to correct for HF-diet associated hyperphagia the effects remained, dissecting the pathway of overfeeding from diet composition.

In our reversal experiment, hepatic transcript levels of SREBP1c, Elovl6 and Fasn were increased. SREBP1c is independently activated by insulin, glucose and fructose, and is a key transcriptional activator of hepatic lipogenic genes such as Fasn and SCD1.[33] SREBP1c inhibits insulin receptor substrate-2 signaling[34], and overexpression of SREBP1c in livers of transgenic mice leads to marked increases in *de novo* lipogenesis and development of hepatic steatosis.[35] Elovl6 is an enzyme catalyzing the conversion of the long chain saturated fatty acid palmitate (C16:0) to stearate (C18:0). Mice deficient in the gene for Elevl6 that were fed a HF diet had markedly reduced SREBP1c expression levels and were able to maintain insulin receptor substrate-2 signaling, despite the development of hepatic steatosis and obesity.[36] Elevated transcript levels of Elovl6 as seen in our experiments may have further activated SREBP1c expression. Fasn catalyzes the synthesis of long chain fatty acids from acetyl-CoA and malonyl-CoA and is one of the rate limiting steps in *de novo* lipogenesis.[37] The increased transcript levels of Fasn in the RHF-P mice may have contributed to the increased synthesis of fatty acids, ultimately resulting in hepatic steatosis.

Weight loss and increased physical activity are generally recommended as treatments for patients with NAFLD, despite the lack of scientific evidence regarding diet composition and its effect on liver histology and long-term outcomes of NAFLD.[38,39] This paucity of data makes it difficult to make evidence-based recommendations about dietary modification to treat NAFLD. In mice receiving a HF diet for 16 weeks, complete reversal of both insulin resistance and obesity has been demonstrated after switching to a diet low in fat.[15] Our findings show that reducing the amount of fat in the diet of obese mice with established insulin resistance and NAFLD not only resulted in weight loss and improved insulin sensitivity but also in complete reversal of hepatic steatosis, independent from caloric intake. Although these results can not immediately be extrapolated to humans, our findings justify further investigation regarding the role of diets low in total saturated, and trans-fat in the treatment of patients with NAFLD.

Three study limitations may warrant consideration. First, it can be hypothesized that a HF diet results in a reduction in the metabolic rate, shifting the energy equation toward energy storage. It may be possible that despite pair-feeding, in our study the HF fed animals were in greater positive energy balance. This may be a consequence of some decrease in activity and/or energy expenditure. Although this was not assessed in our study, previous reports show that the development of obesity was unrelated to motor activity in mice, and that rats fed a 59% fat diet demonstrated a transient decrease in energy expenditure at 30 days, but no difference at 70 days compared to LF fed controls.[14,40] Likewise, humans fed a 40% fat diet demonstrated weight gain without decreased energy expenditure.[41] Data that HF diet induced obesity is affected by metabolic rate is lacking, however, it remains possible that the metabolic rate in the animals used in our study under our particular experimental conditions was decreased.

Second, the observed results may be a consequence of fat source, rather than fat content. Indeed, our HF diet is mainly derived from saturated fat (45% saturated, 24% trans, 24% monounsaturated, and 7% polyunsaturated fatty acids), whereas the LF purified rodent diet is comprised of polyunsaturated fatty acids (100% soybean oil). Saturated fatty acids may promote endoplasmatic reticulum stress and hepatocyte injury resulting in hepatic dysfunction in rodents.[42] Also, a saturated fat intake exceeding 10% of total energy may promote insulin resistance in humans and therefore may not be suitable for NAFLD patients.[28] Polyunsaturated fatty acids, in contrast, decrease cardiovascular disease when consumed as an alternative to saturated fats.[43] A recent study showed that mice fed a saturated LF diet gained significantly more weight compared to mice fed an unsaturated LF diet, whereas HF diets resulted in significant weight gain regardless of fat composition.[44] In this study, however, liver fatty acid content was not examined. It may therefore be hypothesized that weight gain and the development of hepatic steatosis may at least in part be a result of dietary saturated fat. This hypothesis is beyond the scope of the current study, but remains under investigation in our ongoing research.

Finally, the degree to which these findings apply to humans remains speculative. Humans are biologically different from rodents as they tend to have lower rates of *de novo* lipogenesis, which might attenuate the effects of macronutrient dietary content on hepatic lipid accumulation. The DIO model in male C57Bl/6J mice fed a HF diet, however, is the most widely referenced model in diabetes and obesity research, and closely resembles the development of the metabolic syndrome in response to the increasing sedentary lifestyle in humans. In contrast, many other experimental models utilized to investigate the pathophysiology of NAFLD are either based on genetic leptin defects or the methionine and choline deficiency diets. Although scientific evidence is lacking, a low glycemic diet with decreased saturated and trans-fat intake, but increased

mono and polyunsaturated fatty acid consumption, is currently recommended to treat patients with NAFLD.[39] The results from our study showing reversal of hepatic steatosis when obese mice had been switched to a diet low in saturated and trans-fats, but high in polyunsaturated fats, in part, support these recommendations in terms of dietary fat composition but suggest that total fat provided is a prominent factor as well.

In conclusion, this study recapitulates the effects of high dietary fat content on the development and reversal of visceral obesity and insulin resistance independent of caloric intake. Onset of hepatic steatosis in this model resulted from an increased dietary intake of fat, in addition to excess caloric intake. Moreover, we demonstrated that hepatic steatosis was successfully reversed after switching obese mice to a diet with an appropriate fat composition and content. These results may serve to further investigate the role of diets low in total saturated, and trans fat in the treatment of patients with NAFLD.

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REFERENCES

1. Angulo P. Nonalcoholic fatty liver disease. *N Engl J Med* 2002;346:1221-1231.
2. Powell EE, Cooksley WG, Hanson R, *et al*. The natural history of nonalcoholic steatohepatitis: a follow-up study of forty-two patients for up to 21 years. *Hepatology* 1990;11:74-80.
3. Teli MR, James OF, Burt AD, *et al*. The natural history of nonalcoholic fatty liver: a follow-up study. *Hepatology* 1995;22:1714-1719.
4. Browning JD, Szczepaniak LS, Dobbins R, *et al*. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology* 2004;40:1387-1395.
5. Bellentani S, Saccoccio G, Masutti F, *et al*. Prevalence of and risk factors for hepatic steatosis in Northern Italy. *Ann Intern Med* 2000;132:112-117.
6. Fan CY, Pan J, Usuda N, *et al*. Steatohepatitis, spontaneous peroxisome proliferation and liver tumors in mice lacking peroxisomal fatty acyl-CoA oxidase. Implications for peroxisome proliferator-activated receptor alpha natural ligand metabolism. *J Biol Chem* 1998;273:15639-15645.
7. Nomura H, Kashiwagi S, Hayashi J, *et al*. Prevalence of fatty liver in a general population of Okinawa, Japan. *Jpn J Med* 1988;27:142-149.
8. Ratziu V, Giral P, Charlotte F, *et al*. Liver fibrosis in overweight patients. *Gastroenterology* 2000;118:1117-1123.
9. Wanless IR, Lentz JS. Fatty liver hepatitis (steatohepatitis) and obesity: an autopsy study with analysis of risk factors. *Hepatology* 1990;12:1106-1110.
10. Clark JM, Diehl AM. Hepatic steatosis and type 2 diabetes mellitus. *Curr Diab Rep* 2002;2:210-215.
11. Marchesini G, Brizi M, Bianchi G, *et al*. Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. *Diabetes* 2001;50:1844-1850.
12. Collins S, Martin TL, Surwit RS, *et al*. Genetic vulnerability to diet-induced obesity in the C57BL/6J mouse: physiological and molecular characteristics. *Physiol Behav* 2004;81:243-248.
13. Petro AE, Cotter J, Cooper DA, *et al*. Fat, carbohydrate, and calories in the development of diabetes and obesity in the C57BL/6J mouse. *Metabolism* 2004;53:454-457.
14. Brownlow BS, Petro A, Feinglos MN, *et al*. The role of motor activity in diet-induced obesity in C57BL/6J mice. *Physiol Behav* 1996;60:37-41.
15. Parekh PI, Petro AE, Tiller JM, *et al*. Reversal of diet-induced obesity and diabetes in C57BL/6J mice. *Metabolism* 1998;47:1089-1096.
16. Howard BV, Manson JE, Stefanick ML, *et al*. Low-fat dietary pattern and weight change over 7 years: the Women's Health Initiative Dietary Modification Trial. *Jama* 2006;295:39-49.
17. Astrup A. Dietary management of obesity. *JPEN J Parenter Enteral Nutr* 2008;32:575-577.
18. Sacks FM, Bray GA, Carey VJ, *et al*. Comparison of weight-loss diets with different compositions of fat, protein, and carbohydrates. *N Engl J Med* 2009;360:859-873.
19. Tuomilehto J, Lindstrom J, Eriksson JG, *et al*. Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance. *N Engl J Med* 2001;344:1343-1350.
20. Reeves PG, Nielsen FH, Fahey GC, Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr* 1993;123:1939-1951.
21. Hermanowski-Vosatka A, Balkovec JM, Cheng K, *et al*. 11beta-HSD1 inhibition ameliorates metabolic syndrome and prevents progression of atherosclerosis in mice. *J Exp Med* 2005;202:517-527.
22. Muniyappa R, Lee S, Chen H, *et al*. Current approaches for assessing insulin sensitivity and resistance in vivo: advantages, limitations, and appropriate usage. *Am J Physiol Endocrinol Metab* 2008;294:E15-26.

23. Alwayn IP, Andersson C, Lee S, *et al*. Inhibition of matrix metalloproteinases increases PPAR-alpha and IL-6 and prevents dietary-induced hepatic steatosis and injury in a murine model. *Am J Physiol Gastrointest Liver Physiol* 2006;291:G1011-1019.
24. Popov Y, Patsenker E, Fickert P, *et al*. Mdr2 (Abcb4)-/- mice spontaneously develop severe biliary fibrosis via massive dysregulation of pro- and antifibrogenic genes. *J Hepatol* 2005;43:1045-1054.
25. Donnelly KL, Smith CI, Schwarzenberg SJ, *et al*. Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. *J Clin Invest* 2005;115:1343-1351.
26. Harrison SA, Day CP. Benefits of lifestyle modification in NAFLD. *Gut* 2007;56:1760-1769.
27. Koteish A, Diehl AM. Animal models of steatosis. *Semin Liver Dis* 2001;21:89-104.
28. Musso G, Gambino R, De Michieli F, *et al*. Dietary habits and their relations to insulin resistance and postprandial lipemia in nonalcoholic steatohepatitis. *Hepatology* 2003;37:909-916.
29. Tiikkainen M, Bergholm R, Vehkavaara S, *et al*. Effects of identical weight loss on body composition and features of insulin resistance in obese women with high and low liver fat content. *Diabetes* 2003;52:701-707.
30. Flowers MT, Ntambi JM. Role of stearoyl-coenzyme A desaturase in regulating lipid metabolism. *Curr Opin Lipidol* 2008;19:248-256
31. Sampath H, Miyazaki M, Dobrzyn A, *et al*. Stearoyl-CoA desaturase-1 mediates the pro-lipogenic effects of dietary saturated fat. *J Biol Chem* 2007; 282:2483-2493.
32. Li ZZ, Berk M, McIntyre TM, *et al*. Hepatic lipid partitioning and liver damage in nonalcoholic fatty liver disease: role of stearoyl-CoA desaturase. *J Biol Chem* 2009;284:5637-5644.
33. Horton JD, Goldstein JL, Brown MS. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest* 2002;109:1125-1131.
34. Hotamisligil GS. Inflammation and metabolic disorders. *Nature* 2006;444:860-867.
35. Shimomura I, Bashmakov Y, Horton JD. Increased levels of nuclear SREBP-1c associated with fatty livers in two mouse models of diabetes mellitus. *J Biol Chem* 1999;274:30028-30032.
36. Matsuzaka T, Shimano H, Yahagi N, *et al*. Crucial role of a long-chain fatty acid elongase, Elovl6, in obesity-induced insulin resistance. *Nat Med* 2007;13:1193-1202.
37. Hillgartner FB, Salati LM, Goodridge AG. Physiological and molecular mechanisms involved in nutritional regulation of fatty acid synthesis. *Physiol Rev* 1995;75:47-76.
38. Bellentani S, Dalle Grave R, Suppini A, *et al*. Behavior therapy for nonalcoholic fatty liver disease: The need for a multidisciplinary approach. *Hepatology* 2008;47:746-754.
39. Zivkovic AM, German JB, Sanyal AJ. Comparative review of diets for the metabolic syndrome: implications for nonalcoholic fatty liver disease. *Am J Clin Nutr* 2007;86:285-300.
40. Wilsey J, Zolotukhin S, Prima V, *et al*. Central leptin gene therapy fails to overcome leptin resistance associated with diet-induced obesity. *Am J Physiol Regul Integr Comp Physiol* 2003;285:R1011-1020.
41. Piers LS, Walker KZ, Stoney RM, *et al*. The influence of the type of dietary fat on postprandial fat oxidation rates: monounsaturated (olive oil) vs saturated fat (cream). *Int J Obes Relat Metab Disord* 2002;26:814-821.
42. Wang D, Wei Y, Pagliassotti MJ. Saturated fatty acids promote endoplasmic reticulum stress and liver injury in rats with hepatic steatosis. *Endocrinology* 2006;147:943-951.
43. Laaksonen DE, Nyyssonen K, Niskanen L, *et al*. Prediction of cardiovascular mortality in middle-aged men by dietary and serum linoleic and polyunsaturated fatty acids. *Arch Intern Med* 2005;165:193-199.
44. Townsend KL, Lorenzi MM, Widmaier EP. High-fat diet-induced changes in body mass and hypothalamic gene expression in wild-type and leptin-deficient mice. *Endocrine* 2008;33:176-188.

Chapter 4

Matrix metalloproteinases are differentially expressed in liver and adipose tissue during development of non-alcoholic fatty liver disease and obesity

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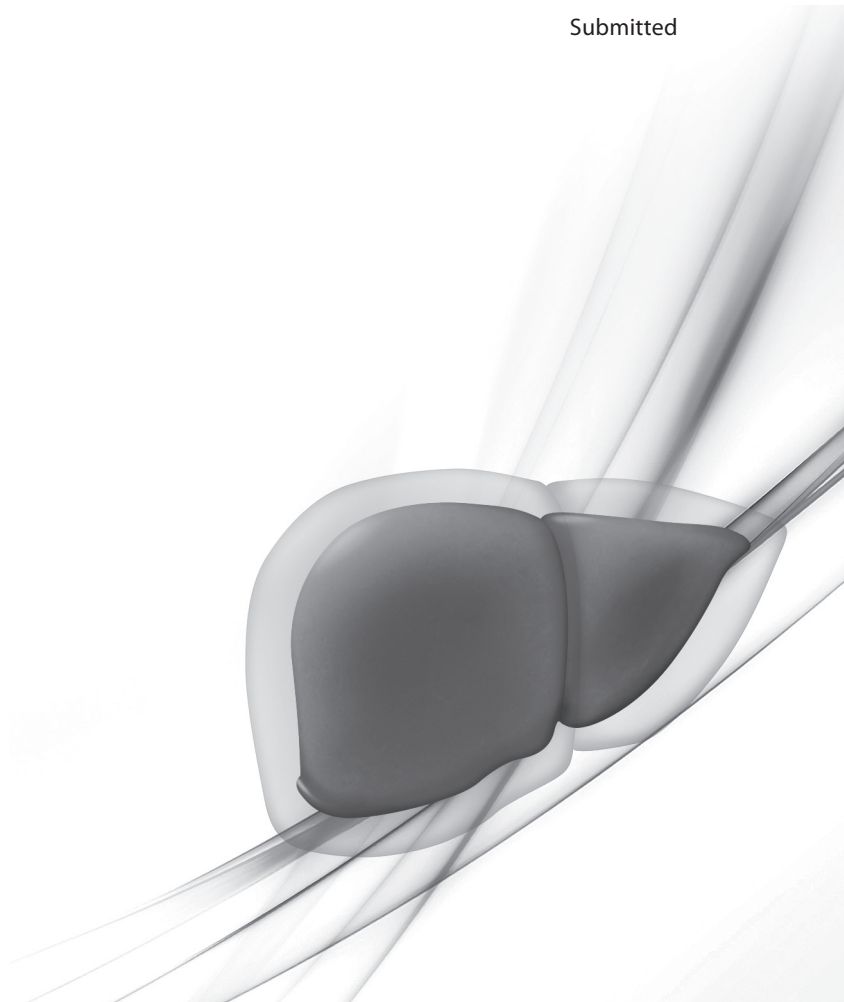
Submitted



ABSTRACT

Background: Development of obesity is associated with extensive remodeling of the extracellular matrix of adipocytes, regulated by matrix metalloproteinases (MMPs). In the liver, changes in patterns of matrix degradation mediated by MMPs are critical for fibrogenesis. While low grade inflammation is found in both visceral fat and hepatic tissue in obesity, these changes result in progressive disease and fibrosis only in liver and not in fat tissue. We hypothesized that tissue-specific differential expression of MMPs are responsible for such difference.

Methods: Mice were fed either standard chow or a high fat diet over 19 weeks. Steatosis was assessed by histology and quantified via magnetic resonance spectroscopy. Paired liver tissues, epididymal fat tissues and sera were analyzed by quantitative RT-PCR for MMP- and fibrosis-related gene expression. Correlation analysis to obesity and hepatic steatosis parameters was performed.

Results: Mice on a high fat diet developed severe adiposity, insulin resistance, steatosis and liver injury. Quantitative RT-PCR revealed that following high fat feeding in adipose tissue, almost all MMPs were upregulated, whereas in liver only *Mmp12, Mmp13* and *Timp1* expression was increased, and to a lesser extent. Hepatic and epididymal fat content correlated with corresponding *Mmp12, Mmp13* and *Timp1* expressions.

Conclusion: This data may suggest that changes in MMP expression in livers and adipose tissue of obese mice may contribute to profibrogenic signaling, potentially increasing their susceptibility to develop fibrosis.

INTRODUCTION

As a consequence of sustained over-nutrition, obesity has become epidemic in industrialized countries and is increasingly common in developing countries worldwide, occurring in all groups at younger ages. The World Health Organization estimates that in 2005 globally approximately 1.6 billion adults were overweight, of whom at least 400 million were obese. These rates are predicted to be doubled by 2015.[1] Obesity is accompanied by dyslipidemia and ectopic deposition of excess triglyceride in adipose tissue as well as in the liver, known as non-alcoholic fatty liver disease (NAFLD). NAFLD is a significant and increasing cause of liver failure[2], with a clinical spectrum ranging from the simple build-up of fat in the liver (hepatic steatosis) to non-alcoholic steatohepatitis (NASH), cirrhosis, and ultimately liver failure.[3,4] The progression from simple steatosis to non-alcoholic steatohepatitis and fibrosis, however, requires additional insults which may be induced by hepatocellular injury and death.[4,5]

Development of obesity is associated with extensive remodeling of the extracellular matrix of adipocytes, regulated by matrix metalloproteinases (MMPs) along with their endogenous tissue inhibitors (TIMPs).[6] In the liver, changes in patterns of matrix degradation mediated by MMPs are critical for fibrogenesis.[7] Hepatocellular injury usually leads to inflammation and activation of the innate immune system, resulting in release of growth factors, cytokines and small molecular mediators that can stimulate extracellular matrix synthesis by activation of quiescent hepatic stellate cells.[8,9] In the presence of chronic hepatic injury such as NAFLD, an imbalance between fibrogenesis and fibrolysis may eventually lead to excess extracellular matrix deposition and scar formation.[7,10]

While low grade inflammation is found in both visceral fat and hepatic tissue in obesity, these changes result in progressive disease (NAFLD-NASH) and fibrosis only in liver and not in fat tissue. We hypothesized that tissue-specific differential expression of MMPs, primary proteases capable of extracellulary degradation, are responsible for such difference.

METHODS

Animals

Male 5-week-old C57BL6/J mice (Jackson Laboratories, Bar Harbor, ME) were housed five per cage on paper chip bedding in a barrier room with regulated temperature (21°C ± 1.6°C), humidity (45% ± 10%), and an alternating 12-hour light and dark cycle. The animals had free access to water and study diets. Animal protocols complied with the

National Institutes of Health Animal Research Advisory Committee guidelines and were approved by the Children's Hospital Boston Animal Care and Use Committee (protocol no. A06-08-065R).

Experimental protocol

After an acclimation period of one week, twenty C57BL/6J male mice were randomized into two groups and placed on either a standard purified rodent diet containing 10% of calories from fat (D12450B; Research Diets, New Brunswick, NJ) or a high fat diet with 60% of calories derived from fat (D12492; Research Diets, New Brunswick, NJ) for either 9 or 19 weeks. Study diets were stored at -80°C and provided fresh each day to avoid lipid peroxidation.

Tissue collection

At the end of the feeding experiments, mice were fasted for 6 hours. Mice were anesthetized with 2.5% Avertin (2,2,2-Tribromoethanol, Sigma-Aldrich, St. Louis, MO) by intraperitoneal injection and blood was collected via retro-orbital sinus puncture and centrifuged at 14000 rpm at 4°C for 10 min to obtain serum. A midline laparotomy was performed to observe, excise, and weigh the liver. White adipose tissue was dissected according to previously defined anatomic landmarks.[11,12] A white adipose tissue fat-index was calculated using the sum of the individual fat pads as a percentage of the eviscerated body weight.[11,12]

Chemistry

Serum was delivered to the Clinical Laboratory at Children's Hospital Boston for analysis of alanine aminotransferase (ALT) and total cholesterol levels.

Histology

Paraffin-embedded sections of visceral fat and liver tissue were stained with hematoxylin and eosin and periodic acid Schiff's/diastase to examine cellular architecture, glycogen deposition and lipid accumulation. Masson trichrome stains of paraffin-embedded liver sections were used to evaluate collagen architecture and presence of fibrosis. Frozen tissue sections were stained with Oil Red-O to detect fat. A pathologist blinded to the treatment groups conducted a histological analysis of the liver sections.

Magnetic resonance imaging

The left lateral lobe of the liver was excised and collected for magnetic resonance (MR) spectroscopy analysis to objectively quantify hepatic fat fraction as described previously.[11,12]

Analysis of mRNA expression

Isolation of mRNA, reverse transcription and quantitative real-time RT-PCR was performed as described previously.[7,11] Relative transcript levels of genes of interest were quantified by real-time RT-PCR on a LightCycler 1.5 instrument (Roche, Mannheim, Germany) using the TaqMan methodology. TaqMan probes (dual-labeled with 5'-FAM and 3'-TAMRA) and primers were designed using the Primer Express software (Perkin Elmer, Waltham, MA), synthesized at MWG Biotech AG (Ebersberg, Germany), and are published elsewhere.[7,11] The housekeeping gene beta-2 microglobulin (β2MG) was amplified in parallel reactions for normalization.

Statistical analysis

Data are expressed as means ± standard error of the mean (SEM). Differences between two groups were assessed using the unpaired two-tailed Student's *t* test, or if nonparametric, by using the Mann-Whitney *U* test. Data sets involving more than two groups were assessed by analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant. Correlations were calculated using linear regression analysis. All data were collected in a computerized Microsoft Excel database (Microsoft Inc., Redmond, WA). The analysis was performed with SPSS version 16.0 (SPSS Inc., Chicago, IL) statistical software, and figures were created using GraphPad Prism version 5.0 (GraphPad Software Inc., La Jolla, CA) software.

RESULTS

Feeding mice a 60% fat diet progressively induced obesity, insulin resistance and hepatic steatosis over time, compared to control mice fed a 10% fat diet

As expected, mice fed a diet containing 60% of calories from fat for 19 weeks gained more weight than did mice receiving a diet containing 10% of calories from fat (**Figure 1A**). White adipose tissue fat index, as a measure of obesity, was increased in 60% fat fed mice (21.9±0.8% after 9 weeks, P=0.008; and 26.0±1.0% after 19 weeks, P<0.001) compared to control mice (4.6±0.1% and 10.2±1.0% after 9 and 19 weeks, respectively) (**Figure 1B**). Insulin sensitivity, determined by calculating log(HOMA) as a surrogate index was significantly impaired in mice fed a 60% fat diet (**Figure 1C**). Mice fed a 60% fat diet developed enlarged, fatty livers. In control mice, mean liver to body weight ratios were 0.034±0.001 and 0.037±0.001 after 9 and 19 weeks, respectively. In contrast, mean liver to body weight ratios in 60% fat fed mice were increased to 0.045±0.003 (P=0.011) and 0.052±0.005 (P=0.012) after 9 and 19 weeks, respectively (**Figure 1D**). Hepatic fat content was quantified by using MR spectroscopy. Mice fed a 60% fat diet for 9 and 19 weeks exhibited an increase in liver fat content to 38.6±9.4% (P=0.016) and 44.8±4.7%

(*P*<0.001), when compared to 10% fat fed control mice (3.5±0.8% and 8.6±3.8%, respectively) (**Figure 1E**).

High dietary fat content progressively induced hepatic steatosis over time, as demonstrated by chemistry and histology

ALT is used as a marker for evaluation of hepatic injury and was increased in mice with hepatic steatosis after 9 and 19 weeks (**Figure 1F**). After 9 weeks, 60% fat fed animals exhibited a significant elevation of mean serum values for ALT (100±42 IU/L) than did 10% fat fed animals (29±7 IU/L; *P*=0.032). After 19 weeks, 60% fat fed mice demonstrated an elevated mean ALT level of 239±72 IU/L, compared to 10% fat fed mice (48±12 IU/L; *P*=0.008). Compared to 10% fat fed mice, 60% fat fed mice had progressively increased mean serum values for cholesterol after 9 and 19 weeks (*P*<0.001 and *P*=0.008, respectively, **Figure 1G**).

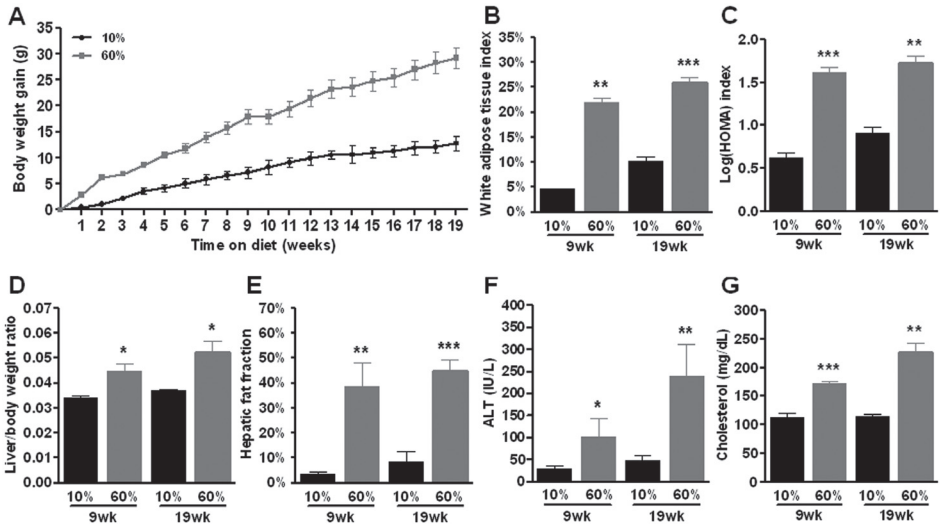


Figure 1. High fat feeding was associated with the development of obesity and hepatic steatosis, growth of fat mass and liver size and development of insulin resistance. Body weight gain, calculated relative to the weight of each individual animal before initiation of the experiment, increased significantly following feeding mice a 60% fat diet (A). Compared to controls, obesity adipose tissue fat mass (expressed as white adipose tissue index; B), insulin resistance (expressed as log(HOMA) index; C), liver size (expressed as liver to body weight ratio; D), hepatic steatosis (expressed as hepatic fat fraction, determined by magnetic resonance spectroscopy; E) were significantly increased in 60% high fat-fed animals after both 9 and 19 weeks. Serum levels for alanine aminotransferase (ALT; F) and total cholesterol (G) differed significantly in the experimental groups at both 9 and 19 weeks. Values represent the mean (n=5 in each group). Variance statistic is SEM. Statistical significance is calculated between the 10% and 60% animals at both time points (*, P<0.05; **, P<0.01; ***, P<0.001).

Macroscopically, livers from 60% fat fed animals were enlarged and pale yellow, suggesting marked fatty liver changes. In contrast, livers from control animals appeared normal (**Figure 2**, top panels). Hematoxylin and eosin and Oil Red-O stained liver sections from 10% fat fed mice exhibited normal hepatic architecture with no evidence of hepatic steatosis over time (**Figure 2**). In contrast, liver sections from mice fed a 60% fat diet for 9 weeks showed fatty changes throughout the liver parenchyma, including both macro- and microvesicular steatosis. These histological changes progressed over time and after 19 weeks liver sections from 60% fat fed mice demonstrated severe steatosis, predominantly macrovesicular, with almost complete loss of normal hepatic architecture (**Figure 2**). Analysis of periodic acid Schiff's/diastase-stained liver sections excluded glycogen deposition as a cause of microvesicular changes in hepatocytes (data not shown). Fibrosis was not observed when examining liver sections stained with Masson trichrome in any of the experimental groups (data not shown). These data corroborated the findings that a 60% fat diet progressively led to the onset of hepatic steatosis, but that fibrotic changes were not seen.

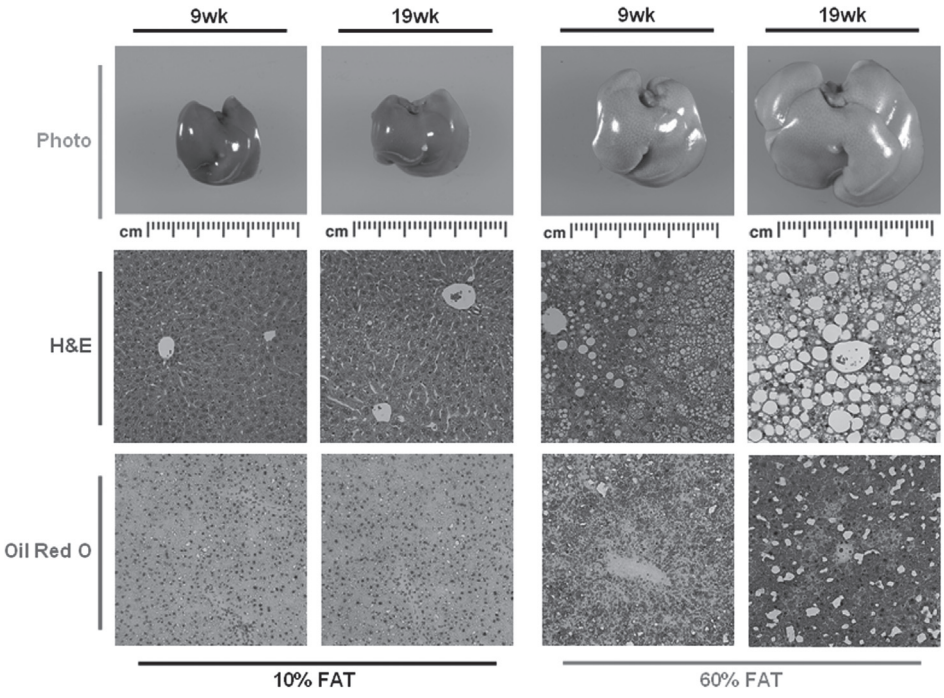


Figure 2. High fat diet-induced obesity progressively led to development of hepatic steatosis, both macroscopically, as well as microscopically. Representative photos of gross liver specimen (top panels), representative liver sections stained with hematoxylin and eosin (H&E; middle panels; original magnification 400x), and Oil Red O (lower panels; original magnification 200x). At both 9 and 19 weeks, livers from 10% fat-fed animals (10% FAT) exhibited normal hepatic architecture, whereas livers from 60% fat-fed mice (60% FAT) revealed extensive, microvesicular and macrovesicular steatosis.

Genes involved in regulation of extracellular matrix turnover in epididymal fat and liver tissue are differentially expressed in diet-induced obesity

To investigate the contribution of MMPs and TIMPs to high fat diet-induced obesity and NAFLD in mice, epididymal fat and liver tissue were examined. Transcript levels of several MMPs (**Figure 3A**) and TIMPs (**Figure 3B**) were measured and compared. In adipose tissue, *Mmp2, Mmp3, Mmp12* and *Mmp13* levels were about 9-fold, 26-fold, 60-fold and up to 45-fold increased, respectively, compared to controls, whereas *Mmp8* and *Mmp9* levels remained largely unchanged (**Figure 3A**). Interestingly, in livers from 60% fat fed animals only an 8-fold increase in transcript levels of *Mmp12* and *Mmp13* was seen, compared to 10% fat fed controls; whereas *Mmp2*, *Mmp3*, *Mmp8* and *Mmp9* levels remained largely unchanged (**Figure 3D**). In epididymal fat tissue from animals fed a 60% high fat diet, mRNA expression of the endogenous inhibitors of MMPs *Timp1*, *Timp2* and *Adam17* was up to 12-fold, 4-fold and 3-fold increased, respectively, compared to control animals. *Timp3* expression, however, was not affected by the diet (**Figure 3B**). In liver tissue, only *Timp1* expression increased up to 9 to 12-fold in animals on a 60% fat

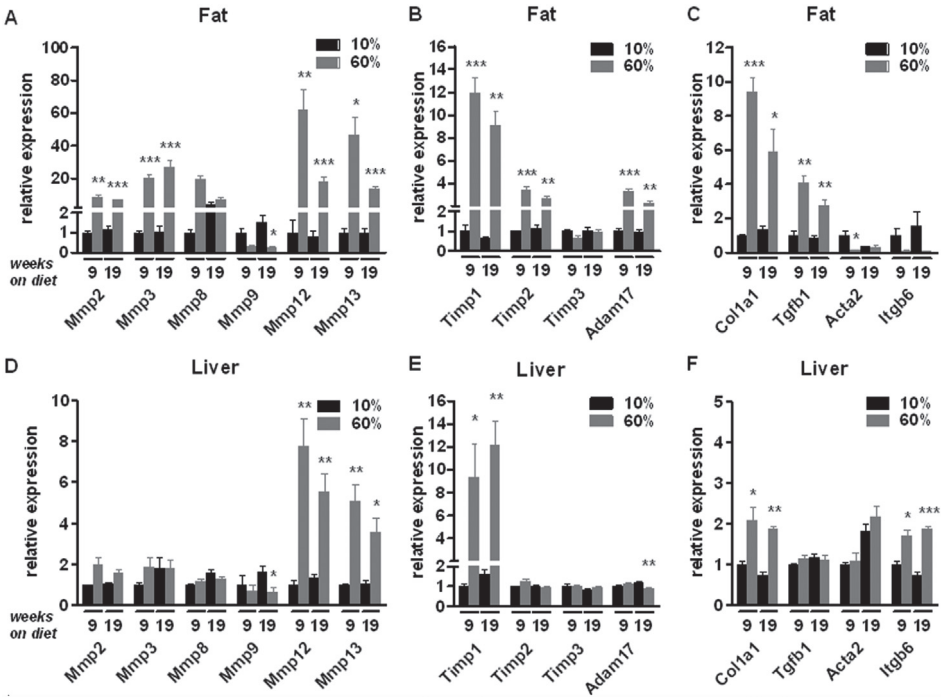


Figure 3. Relative gene expression of MMPs and TIMPs, as well as genes related to fibrogenesis in adipose tissue and livers of mice fed a 60% high fat diet, compared to 10% fat-fed controls, at both 9 and 19 weeks. Relative transcript levels were quantified by TaqMan quantitative RT-PCR, normalized by β2MG and expressed in arbitrary units versus 10% (9 weeks experiment) controls. Values represent the mean (n=5 in each group). Variance statistic is SEM. (*, P<0.05; **, P<0.01; ***, P<0.001).

diet for 9 and 19 weeks, whereas *Timp2*, *Timp3* and *Adam17* mRNA expression remained unchanged in all experimental groups (**Figure 3E**).

Expression of classic fibrogenic genes in epididymal fat and liver tissue remained largely unchanged following high fat feeding

In epididymal fat tissue transcript from animals fed a 60% fat diet for 9 and 19 weeks levels of the pro-fibrogenic genes *Col1a1* and *Tgfb1* showed an up to 9-fold and 4-fold increase, respectively, compared to animals fed a 10% fat diet (**Figure 3C**). Transcript levels of α-SMA (*Acta2*) and *Itgb6* remained largely unchanged in the experimental groups. In the liver, among other pro-fibrogenic genes such as *Tgfb1* and α-SMA only *Col1a1* and *Itgb6* both showed a moderate 2-fold increase in hepatic transcript levels of animals fed a 60% fat diet for 9 and 19 weeks, compared to control animals (**Figure 3F**). These findings indicate that in diet-induced obesity mRNA expression of classic markers for fibrosis progression remained largely unchanged in both adipose and liver tissue, suggesting a potential role for MMPs in steatosis progression.

Expression of Mmp12, Mmp13 and Timp1 in adipose tissue increasingly correlated with corresponding white adipose tissue index over time

Using linear regression analysis, gene expression of *Mmp12, Mmp13* and *Timp1* in adipose tissue was correlated with white adipose tissue index of the corresponding animal. R^2 values at 9 weeks were 0.6495, 0.5305 and 0.7977 for *Mmp12, Mmp13* and *Timp1*, respectively (**Figure 4A**). After 19 weeks, R^2 values were increased to 0.8586, 0.9184 and 0.8245, respectively (**Figure 4B**).

Expression of Mmp12, Mmp13 and Timp1 in liver tissue highly correlated with corresponding hepatic fat content

Using a similar approach, gene expression of *Mmp12, Mmp13* and *Timp1* in liver tissue was correlated with hepatic fat content of the corresponding animal. R^2 values for *Mmp12, Mmp13* and *Timp1* were 0.8810, 0.9584 and 0.77893 at 9 weeks, respectively (**Figure 4C**), whereas after 19 weeks, R^2 values somewhat decreased to 0.8135, 0.6063 and 0.8174, respectively (**Figure 4D**).

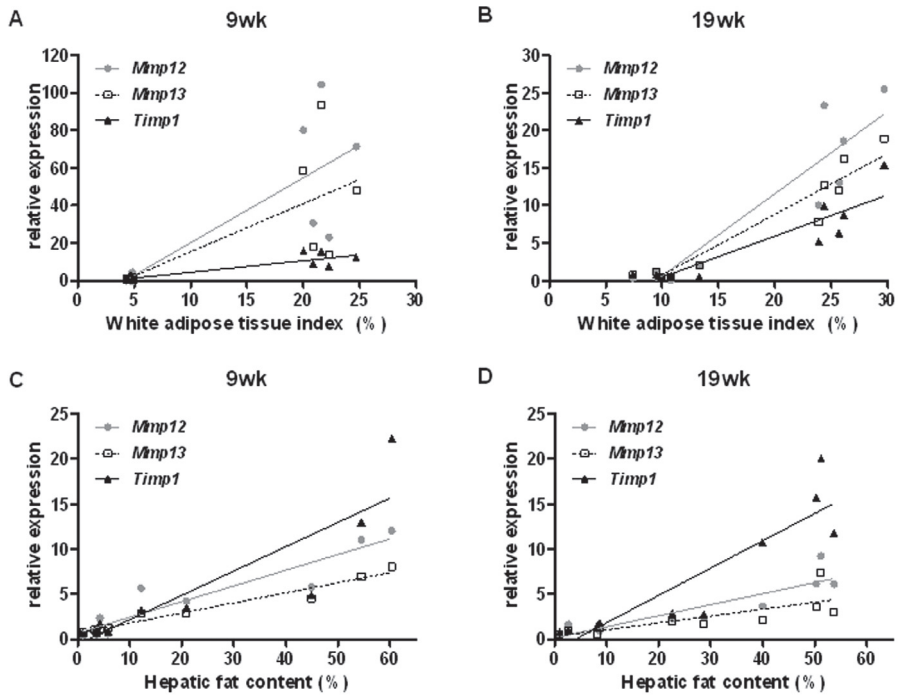


Figure 4. Expression of Mmp12, Mmp13 and Timp1 in adipose tissue and liver correlated with corresponding white adipose tissue index and hepatic fat fraction over time. Using linear regression, a correlation coefficient was calculated at both (A, C) and 19 weeks (B, D).

DISCUSSION

Hepatic steatosis in patients with obesity is increasingly recognized as a major cause of liver-related morbidity and mortality.[13] Although the pathogenesis of this common disorder has still not been fully elucidated, it is generally accepted that a subset of patients with steatosis may progress to steatohepatitis and fibrosis-induced chronic, low grade inflammation, injury and hepatocyte death.[2-5] Unlike well-defined pathways such as inflammatory signaling and activation of the innate immune system[2-5], the association between extracellular matrix turnover and development of NAFLD has not been established. In this study, we sought to determine the potential involvement of MMPs in the pathophysiology of NAFLD.

Both visceral fat and hepatic tissue have low grade inflammation associated with obesity; however, only one (the liver) develops the disease. In animal studies we usually cannot detect diet-induced fibrosis yet, but may observe early changes on mRNA level predisposing inflamed liver to a pro-fibrotic state. Classic pro-fibrogenic genes, such as *Tgfb1*,

α-SMA, *Col1a1* and *Itgb6* have been investigated in this setting previously. Although MMPs, primary proteases capable of ECM degradation, were studied both in liver and fat tissue, there has never been a direct comparison between tissues in the same organism. We therefore hypothesized that tissue-specific expression of MMPs may be responsible for the differential effects of diet-induced obesity on adipose and fat tissue.

Our results indicate that the development of diet-induced obesity in a murine model is associated with overexpression of many MMPs in adipose tissue including *Mmp2, Mmp3*, *Mmp12*, *Mmp13, Timp1, Timp2, ADAM17*, which concurs with studies by Maquoi *et al.*[14] and Chavey *et al.*[15]. All studies taken together, the most robust finding was an increase in gene expression for *Mmp12, Mmp13* and *Timp1*. In addition, gene expression of these MMPs in adipose tissue showed an increasing correlation with corresponding white adipose tissue index over time. In steatotic liver tissue, however, MMP gene expression was much less pronounced showing a significant increase only in gene expression for *Mmp12*, *Mmp13* and *Timp1,* compared to lean controls. Using linear regression analysis, we found that *expression of Mmp12, Mmp13* and *Timp1* in liver tissue highly correlated with corresponding hepatic fat content. In addition, expression of classic fibrogenic genes in epididymal fat and liver tissue remained largely unchanged following high fat feeding. This may suggest that changes in MMP expression in livers and adipose tissue of obese mice may contribute to pro-fibrogenic signaling, potentially increasing their susceptibility to develop fibrosis.

MMP12, also known as macrophage metalloelastase, is an enzyme predominantly expressed in mature tissue macrophages[16] and has recently been implicated in the regulation of IL-13–dependent hepatic fibrosis following schistosomiasis infection.[17] In addition, MMP12 may promote hepatic fibrosis by limiting the expression of extracellulary matrix-degrading MMPs such as MMP2 and MMP13.[17] We have demonstrated that progressive high fat feeding induced hepatic steatosis and obesity, leading to a markedly increased expression of *Mmp12* in liver as well as in epididymal fat tissue. TIMP1 is the most relevant physiological MMP-inhibitor in fibrotic diseases, including hepatotoxic and cholestatic injury, whose expression is upregulated by various inflammatory cytokines.[18] Transgenic mice overexpressing human *Timp1* developed more liver fibrosis when subjected to chronic hepatotoxic injury[19], and demonstrated attenuated spontaneous fibrosis resolution.[20] We have shown an increase in Timp1 gene expression in both liver and fat tissue following high fat feeding, compared to control animals. This suggests that with the development of obesity and steatosis, a simultaneous increase in *Mmp12* and *Timp1* expression may lead to increased profibrogenic signaling, rendering the liver more susceptible for the development of fibrosis.

In contrast, MMP13, also known as collagenase 3, has been identified as the main collagenase responsible for the remodeling of fibrillar collagen in rodents. It has been considered the rodent equivalent of MMP1, the major interstitial collagenase in humans.[21] Macrophage-derived MMP13 plays an important role in mediating the resolution of hepatic fibrosis.[22] We have found increased gene expression of *Mmp13*, suggesting successful initiation of scar tissue remodeling, thereby potentially counterbalancing profibrogenic stimuli such as MMP12 and TIMP1.

In conclusion, up to 19 weeks of high fat feeding led to the development of obesity and hepatic steatosis, as well as increased gene expression of *Mmp12, Mmp13* and *Timp1* in both adipose and liver tissue. Although animals did not develop hepatic fibrosis this data may suggest that changes in MMP expression in livers and adipose tissue of obese mice may contribute to profibrogenic signaling, potentially increasing their susceptibility to develop fibrosis.

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REFERENCES

1. World Health Organization. Fact sheet 311: Obesity and overweight. *http://www.who.int/mediacentre/ factsheets/fs311/en/* [accessed January 28, 2011].
2. Lazo M, Clark JM. The epidemiology of nonalcoholic fatty liver disease: a global perspective. *Semin Liver Dis* 2008;28:339-350.
3. Angulo P. Nonalcoholic fatty liver disease. *N Engl J Med* 2002;346:1221-1231.
4. Argo CK, Northup PG, Al-Osaimi AM, *et al.* Systematic review of risk factors for fibrosis progression in non-alcoholic steatohepatitis. *J Hepatol* 2009;51:371-379.
5. Sanyal AJ. Mechanisms of Disease: pathogenesis of nonalcoholic fatty liver disease. *Nat Clin Pract Gastroenterol Hepatol* 2005;2:46-53.
6. Manicone AM, McGuire JK. Matrix metalloproteinases as modulators of inflammation. *Semin Cell Dev Biol* 2008;19: 34-41.
7. De Meijer VE, Sverdlov DY, Popov Y, *et al.* Broad-spectrum matrix metalloproteinase inhibition curbs inflammation and liver injury but aggravates experimental liver fibrosis in mice. *PLoS One* 2010;5;e11256.
8. Friedman SL. Mechanisms of hepatic fibrogenesis. *Gastroenterology* 2008;134:1655-1669.
9. Bataller R, Brenner DA. Liver fibrosis. *J Clin Invest* 2005;115:209-218.
10. Schuppan D, Afdhal NH. Liver cirrhosis. *Lancet* 2008;371:838-851.
11. De Meijer VE, Le HD, Meisel JA, *et al.* Dietary fat intake promotes the development of hepatic steatosis independently from excess caloric consumption in a murine model. *Metabolism* 2010;59:1092-1105.
12. De Meijer VE, Le HD, Meisel JA, *et al.* Repetitive orogastric gavage affects the phenotype of diet-induced obese mice. *Physiol Behav* 2010;100:387-393.
13. De Meijer VE, Kalish BT, Puder M, *et al.* Systematic review and meta-analysis of steatosis as a risk factor in major hepatic resection. *Br J Surg* 2010;97:1331-1339.
14. Maquoi E, Munaut C, Colige A, *et al.* Modulation of adipose tissue expression of murine matrix metalloproteinases and their tissue inhibitors with obesity. Diabetes 2002;51:1093-1101.
15. Chavey C, Mari B, Monthouel MN, *et al.* Matrix metalloproteinases are differentially expressed in adipose tissue during obesity and modulate adipocyte differentiation. *J Biol Chem* 2003;278:11888-11896.
16. Shipley JM, Wesselschmidt RL, Kobayashi DK, *et al.* Metalloelastase is required for macrophage-mediated proteolysis and matrix invasion in mice. *Proc Natl Acad Sci U S A* 1996;93:3942-3946.
17. Madala SK, Pesce JT, Ramalingam TR, *et al.* Matrix metalloproteinase 12-deficiency augments extracellular matrix degrading metalloproteinases and attenuates IL-13-dependent fibrosis. *J Immunol* 2010;184: 3955-3963.
18. Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res* 2003;92:827-839.
19. Yoshiji H, Kuriyama S, Miyamoto Y, *et al.* Tissue inhibitor of metalloproteinases-1 promotes liver fibrosis development in a transgenic mouse model. *Hepatology* 2000;32:1248-1254.
20. Yoshiji H, Kuriyama S, Yoshii J, *et al.* Tissue inhibitor of metalloproteinases-1 attenuates spontaneous liver fibrosis resolution in the transgenic mouse. *Hepatology* 2002;36:850-860.
21. Knauper V, Lopez-Otin C, Smith B, *et al.* Biochemical characterization of human collagenase-3. *J Biol Chem* 1996;271:1544-1550.

22. Fallowfield JA, Mizuno M, Kendall TJ, *et al*. Scar-associated macrophages are a major source of hepatic matrix metalloproteinase-13 and facilitate the resolution of murine hepatic fibrosis. *J Immunol* 2007;178:5288-5295.

Chapter 5

Tumor necrosis factor α-converting enzyme inhibition reverses steatosis and insulin resistance and improves surgical outcome in mice

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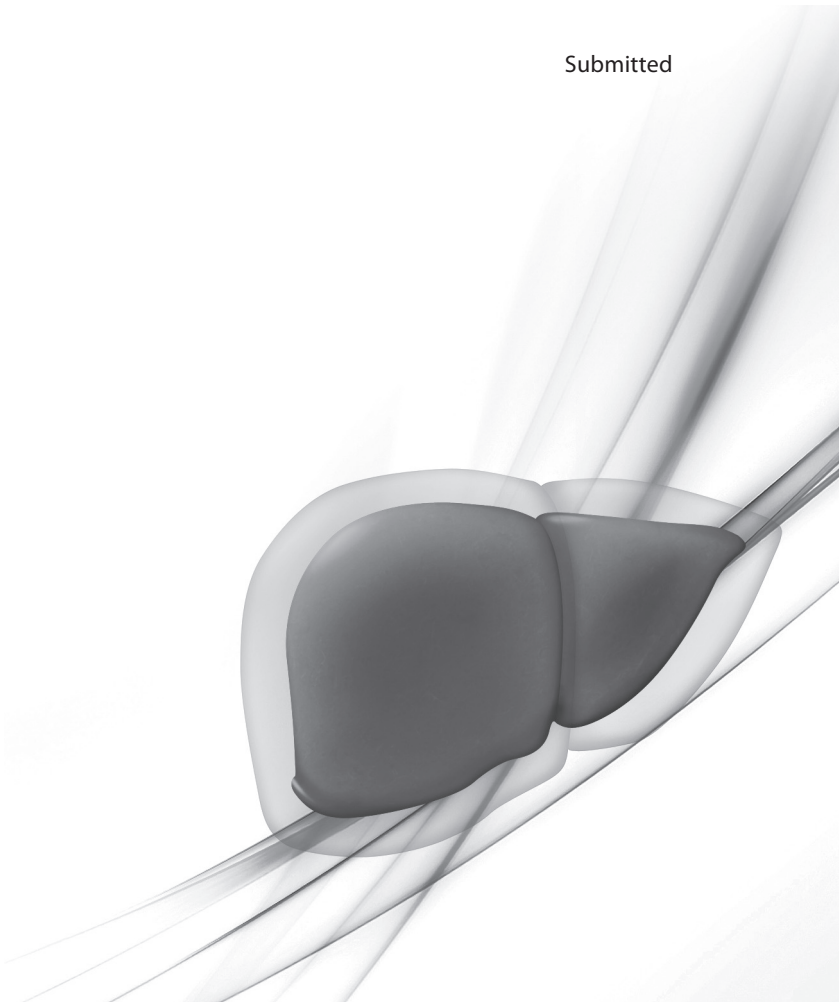
Submitted



ABSTRACT

Objectives: We hypothesized that treatment with the pharmacologic tumor necrosis factor-α converting enzyme (TACE)-inhibitor Marimastat would improve insulin resistance and reverse established steatosis, leading to improved outcome following hepatectomy.

Methods: C57BL/6 male mice were fed a high fat diet for 9 weeks to establish obesity, hepatic steatosis and insulin resistance, and were administered either Marimastat or vehicle for an additional 2 or 4 weeks. Leptin deficient *ob/ob* mice were treated with Marimastat for 4 weeks. Hepatic steatosis was quantified by magnetic resonance spectroscopy and confirmed by histology. Following pre-treatment with Marimastat or vehicle for two weeks, high fat fed C57BL/6 mice were subjected to two-thirds hepatectomy.

Results: After two weeks, Marimastat-treated animals significantly improved insulin sensitivity and liver histology, and experienced a 66% decrease in steatosis (P=0.010). These findings were confirmed in *ob/ob* mice. Transcripts related to fatty acid synthesis were significantly downregulated in Marimastat-treated animals. Post-operative liver injury as quantified by serum aspartate aminotransferase levels and alanine aminotransferase levels was significantly decreased by 57% (P=0.020) and 44% (P=0.032), compared to controls.

Conclusion: Treatment with the TACE-inhibitor Marimastat improved insulin sensitivity and reversed steatosis in mouse models of diet-induced obesity and leptin deficiency, thereby attenuating post-operative injury following hepatectomy. This may suggest a potential therapeutic role in patients with fatty liver disease; especially those who need to undergo surgery.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is one of the hallmarks of the metabolic syndrome and is a significant and increasingly more common cause of liver failure.[1,2] NAFLD is characterized by an accumulation of triglycerides in hepatocytes (hepatic steatosis), and, together with lipotoxic injury, may lead to hepatocellular injury and inflammation (non-alcoholic steatohepatitis), advanced fibrosis, and ultimately liver failure.[3-5] Although its prevalence in both children and adults is increasing worldwide[1,2], the mechanisms underlying the pathogenesis of NAFLD remain elusive.

Steatosis is an established risk factor for primary non-function of hepatic allografts[6] and for complications following major hepatic resection.[7] Patients with steatosis have up to a twofold increased risk of postoperative complications, and those with excessive steatosis (>30%) have an almost threefold increased risk of death.[7] Approximately 20% of patients undergoing liver resection and up to 25% of liver donors have some degree of steatosis, indicating the need for pharmacological intervention.[8] Currently, the cornerstone of clinical management is weight loss through diet and exercise.[9] In reality, however, surgical management usually does not allow time for such dramatic life style changes, justifying the search for a pharmacologic approach. Novel evidence suggests that vitamin E therapy and pioglitazone may be of use in patients with non-alcoholic steatohepatitis.[10] Pharmacological options to reverse steatosis prior to surgery, however, are lacking.

Insulin resistance and the resultant hyperinsulinemia, commonly associated with obesity and the metabolic syndrome, are involved in the imbalance of formation and utilization of free fatty acids leading to steatosis.[11,12] In addition, visceral and hepatic lipid accumulation may activate inflammatory gene networks, suggesting a causal function of inflammation in the pathogenesis of the metabolic syndrome.[13] Tumor necrosis factor (TNF)-α is an inflammatory cytokine involved in linking nutrient availability to innate immune activation and is the major negative regulator of the insulin receptor pathway.[14,15] TNF-α release from the plasma membrane through its ectodomain shedding is mediated by TNF-α converting enzyme (TACE, also known as ADAM17 and CD156b), which belongs to the disintegrin and metalloproteinase family of zinc-metalloproteinases.[16,17] TACE is endogenously inhibited by tissue inhibitor of metalloproteinase (TIMP)-3[18], and may be activated by metabolic stimuli such as hyperinsulinemia.[19] Mice deficient in TIMP-3 demonstrate elevated levels of TNF-α and develop insulin resistance and hepatic steatosis, mediated by increased TACE activity.[20,21] In contrast, pharmacologic TACE-inhibition abrogates the inflammatory response and has been demonstrated to improve insulin resistance.[22,23]

The TACE/TIMP-3 system has recently emerged as a novel mediator between metabolic stimuli and innate immunity; however, the effects of pharmacologic TACE-inhibition on insulin resistance and hepatic steatosis remain to be established. We hypothesized that treatment with Marimastat would ameliorate insulin resistance and reverse established hepatic steatosis, potentially resulting in improved surgical outcome following hepatectomy in murine models of diet-induced obesity, hepatic steatosis and insulin resistance.

METHODS

Animal experiments

Animal protocols complied with the National Institutes of Health Animal Research Advisory Committee guidelines and were approved by the Children's Hospital Boston Animal Care and Use Committee (protocol #A06-08-065R). Mice were housed 4 or 5 animals per cage on paper chip bedding in a barrier room with regulated temperature (21°C ± 1.6°C), humidity (45% ± 10%), and an alternating 12-hour light and dark cycle. The animals had free access to water and study diets, but a pair-feeding design was used after animals were assigned to the treatments to avoid bias resulting from a potential decreased caloric intake.[24,25]

After an acclimation period of one week, twenty-five C57BL/6 male mice (#000664; Jackson Laboratories, Bar Harbor, ME) were placed on a high fat diet with 60% of calories derived from fat (#D12492; Research Diets, New Brunswick, NJ), whereas another group of five animals that were fed a standard purified rodent diet containing 10% of calories from fat (#D12450B; Research Diets) served as controls. Study diets were stored at -80°C and provided fresh each day to avoid lipid peroxidation. After 9 weeks, five animals from each group were sacrificed and tissues were analyzed to serve as baseline controls. The remaining twenty animals on the high fat diet were randomized to receive either 100 mg/kg of Marimastat (BB-2516, British Biotech, UK) in 0.45% methylcellulose (Sigma-Aldrich, St. Louis, MO) vehicle twice daily via orogastric gavage (MAR), or vehicle alone (VEH) for an additional two or four weeks (n=5 per group). At the end of the treatment period, animals were sacrificed to evaluate hepatic steatosis and related parameters (**Figure 1A**).

In a separate experiment, fifteen B6.V-*Lep*^{ob}/J male mice (#000632; Jackson Laboratories), commonly referred to as *ob/ob* mice[26,27], were acclimatized for one week and placed on a standard purified rodent diet containing 10% of calories from fat. After two weeks, five animals were sacrificed to serve as base-line controls and the remaining animals were randomized to receive either Marimastat twice daily (MAR) or vehicle alone (VEH) for an additional four weeks (**Figure 1B**).

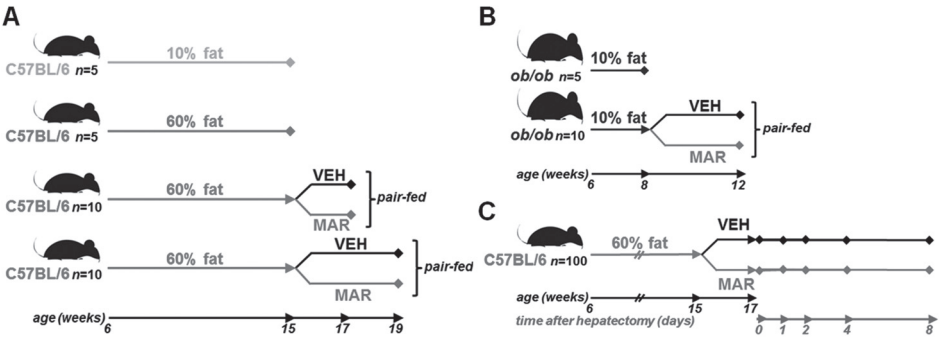


Figure 1. Design of the studies.

The efficacy of Marimastat to reverse steatosis and improve surgical outcome was studied using fifty C57BL/6 male mice placed on a similar high fat diet for 9 weeks, followed by two weeks of treatment with either Marimastat twice daily (MAR) or vehicle alone (VEH) while remaining on the high fat diet. After thirteen days, animals were withheld treatment for 24 hours to avoid potential inhibitory effects on liver regeneration.[28] The following morning, mice underwent the surgical removal of two-thirds of the liver under isoflurane anesthesia. The left upper, right upper, and left lower lobes of the liver were ligated with 5-0 silk ties (Ethicon, Somerville, NJ) and then excised, as previously described.[29] Animals were sacrificed either immediately to serve as controls, or after one, two, four and eight days to evaluate liver regeneration and related parameters (**Figure 1C**).

Sample collection and serum biochemistry

At the end of the feeding experiments, mice were fasted for 6 hours. Glucose concentration was determined from tail vein blood using the OneTouch UltraSmart Blood Glucose Monitoring System (LifeScan, Milpitas, CA). Serum was delivered to the Clinical Laboratory at Children's Hospital Boston for analysis. The left lateral lobe was excised and collected for magnetic resonance (MR) spectroscopy analysis. White adipose tissue was dissected according to previously defined anatomic landmarks.[24,25] A white adipose tissue fat-index was calculated using the sum of the individual fat pads as a percentage of the eviscerated body weight.[24,25]

Surrogate index of insulin sensitivity and resistance

Insulin levels were measured using a rat/mouse insulin enzyme-linked immunosorbent assay kit (#EZRMI-13K; Millipore, St. Charles, MO). A surrogate index for insulin sensitivity was calculated using the log(HOMA) formula.[24,25,30]

Histology

Paraffin-embedded sections of the liver were stained by hematoxylin and eosin and periodic acid Schiff's/diastase to examine cellular architecture, glycogen deposition and lipid accumulation. Frozen tissue sections were stained with Oil Red-O to detect fat. A pathologist blinded to the treatment groups conducted a histological analysis of the liver sections.

Magnetic resonance imaging

Magnetic resonance (MR) spectroscopy analysis was used to objectively quantify hepatic fat fraction as described previously.[24,25] Briefly, samples were thawed at room temperature for 1 hour prior to analysis, blotted free of excess water and connective tissue, and placed in 5 mm diameter glass tubes for MR spectroscopy. An 8.5 T vertical bore magnet (DRX system, Bruker Instruments, Billerica, MA) was used for spectroscopic measurements of fat and water resonances. Specifically, a point resolved echo spectroscopic acquisition was applied to homogenous regions of liver, as identified from fast low angle shot images of the liver specimen. Voxel volumes interrogated spectroscopically with the point resolved echo spectroscopic sequence were 2 mm^3. The repetition and echo times were 8 s and 12 ms, respectively, and 16 signal averages were acquired per spectrum. The water resonance and the methylene/methyl resonances were numerically integrated using the manufacturer supplied Paravision 4.0 software (Bruker Instruments). The methylene/methyl area was divided by the sum of the methylene/methyl area plus the water area to obtain the MR spectroscopy parameter representing hepatic fat fraction used for group comparisons.

Cytokine and adipokine analysis

Serum levels of Adiponectin (#EZMADP-60K; Millipore), Leptin (#EZML-82K; Millipore), soluble TNF-α receptor II (p75) (#MRT20; Quantikine, R&D Systems, Minneapolis, MN) and IL-6 (#M6000B; Quantikine) were determined using commercial enzyme-linked immunosorbent assay kits. Optical density was read at 450 nm and analyzed with Softmax PRO Software (Molecular Devices, Sunnyvale, CA).

Very low density lipoprotein secretion assay

After being fasted for 6 hours, mice were injected with 500 mg/kg tyloxapol (Triton-1339; Sigma-Aldrich) dissolved in saline at a concentration of 150 mg/mL via the tail vein. Both lipolysis and tissue uptake of lipoproteins are completely inhibited in mice under these conditions, and the accumulation of triglyceride in serum after injection of tyloxapol can be used to estimate rates of secretion of very low density lipoprotein.[31] Tail bleeds were done just before injection (t_0) and 45 and 90 min following injection. Serum was deliv-

ered to the Clinical Laboratory at Children's Hospital Boston for analysis of triglyceride levels. The slope of the line denotes the rate of VLDL production.

TACE activity assay

Determination of TACE activity was performed using a commercially available α-secretase activity assay according to manufacturer's instructions (#FP001; R&D Systems, Minneapolis, MN). Briefly, 10 µL of recombinant human (#903-ADB; R&D Systems) and mouse (#2978-AD; R&D Systems) TACE were used as standards on a 96-well plate in triplicates, and increasing doses of Marimastat dissolved in H_2O were added. After 1 hour, a TACE-specific peptide conjugated to the reporter molecules EDANS and DABCYL was added. In the uncleaved form, fluorescent emissions from EDANS were quenched by the physical proximity of the DABCYL moiety, which exhibits maximal absorption at the same wavelength (495 nm). Cleavage of the peptide by TACE physically separates the EDANS and DABCYL reporter molecules, allowing for the release of a fluorescent signal. The level of TACE enzymatic activity was proportional to the fluorometric reaction, measured at room temperature using the Wallac Victor2 Multilabel Counter (Perkin Elmer, Waltham, MA); with excitation at 355 nm, and emission at 510 nm.

Analysis of mRNA expression

Isolation of mRNA, reverse transcription and quantitative real-time RT-PCR were performed as described previously.[29,34] Relative transcript levels of genes of interest were quantified by real-time RT-PCR on a LightCycler 1.5 instrument (Roche, Mannheim, Germany) using the TaqMan methodology. TaqMan probes (dual-labelled with 5'-FAM and 3'-TAMRA) and primers were designed based on published sequences (**Table 1**) using the Primer Express software (Perkin Elmer), synthesized at MWG Biotech AG (Ebersberg, Germany), or acquired commercially (Life Technologies Corporation, Carlsbad, CA).[29,34] The housekeeping gene beta-2 microglobulin (β2MG) was amplified in parallel reactions for normalization.

Statistical analysis

Data are expressed as means ± standard error of the mean (SEM). Differences between two groups were assessed using the unpaired two-tailed Student's *t* test, or if nonparametric, by using the Mann-Whitney *U* test. Data sets involving more than two groups were assessed by analysis of variance. $P<0.05$ was considered statistically significant. The analysis was performed with SPSS version 16.0 (SPSS Inc., Chicago, IL) statistical software, and figures were created using GraphPad Prism version 5.0 (GraphPad Software Inc., La Jolla, CA) software.

Table 1. Primers and probes used in quantitative real-time RT-PCR.

Target gene	5´-Primer	TaqMan probe	3´-Primer
Acc1	TGTCCGCACTGACTGTAACCA	TCTTCCTCAACTTTGTGCCCACGGTC	TGCTCCGCACAGATTCTTCA
Fasn	CCTGGATAGCATTCCGAACCT	CCTGAGGGACCCTACCGCATAGC	AGCACATCTCGAAGGCTACACA
Scd1	CAACACCATGGCGTTCCA	TGACGTGTACGAATGGGCCCGA	GGTGGGCGCGGTGAT
Elovl6	GCGCTGTACGCTGCCTTTAT	TCGGCATCTGATGAACAAGCGAGC	GCGGCTTCCGAAGTTCAAA
Srebp1	CCAGAGGGTGAGCCTGACAA	CAATCAGGACCATGCCGACCTCT	AGCCTCTGCAATTTCCAGATCT
Srebp2	GCGGACAACACACAATATCATTG	AGCGCTACCGGTCCTCCATCA	TGACTAAGTCCTTCAACTCTATGATTTTG
Ppara	TGGTTCCTGGTGCCGATTTA	TGGTGGTAGATGCCTGCAACCCCA	ACTAGCATCCCACTTAATTATGTATCTGAA
LXRa	CGACAGAGCTTCGTCCACAA	CGGAAAAAGGGCCCAGCCCC	GCTCGTTCCCCAGCATTTT
FXR	CACGAAGATCAGATTGCTTTGC	CAAAGGGTCCGCAGTGGAGGCC	CCGCCGAACGAAGAAACA
Mttp	*	*	*
Apob	*	*	*
Timp3	*	*	*
TACE	*	*	*
β2MG	CTGATACATACGCCTGCAGAGTTAA	GACCGTCTACTGGGATCGAGACATGTG	ATGAATCTTCAGAGCATCATGAT

*, Not given; commercially acquired from Life Technologies Corporation, Carlsbad, CA.

RESULTS

Marimastat treatment reduced diet-induced hepatomegaly and normalized gross macroscopic appearance of livers from diet-induced obese mice

C57BL/6 animals fed a high fat diet for 9 weeks, followed by two or four weeks of treatment with Marimastat demonstrated subtle body weight loss, which was only significant after four weeks (**Figure 2A**). Body weights of *ob/ob* animals fed a normal fat diet followed by a four week course of Marimastat treatment remained unchanged throughout the study (**Figure 2A**). Macroscopic appearance of livers from C57BL/6 mice fed a 10% fat purified rodent diet for 9 weeks demonstrated normal macroscopic liver appearance (**Figure 2B**; left upper pane). Baseline animals that were switched to a 60% fat diet for 9 weeks developed severe steatosis, indicated by liver enlargement and pale yellow discoloring (**Figure 2B**; left lower panel).

Two and four weeks of Marimastat treatment resulted in significant improvement of macroscopic appearance (**Figure 2B**; middle and right lower panels), similar to the baseline control liver. Livers from vehicle treated mice remained pale yellow, indicative of fatty changes (**Figure 2B**; middle and right upper panels). Because of the marked differences already after two weeks, we present the data from this earlier time point throughout the rest of the manuscript. In both animal models, Marimastat treatment decreased liver to body weight ratios suggesting attenuation of hepatic steatosis (**Figure 2C**).

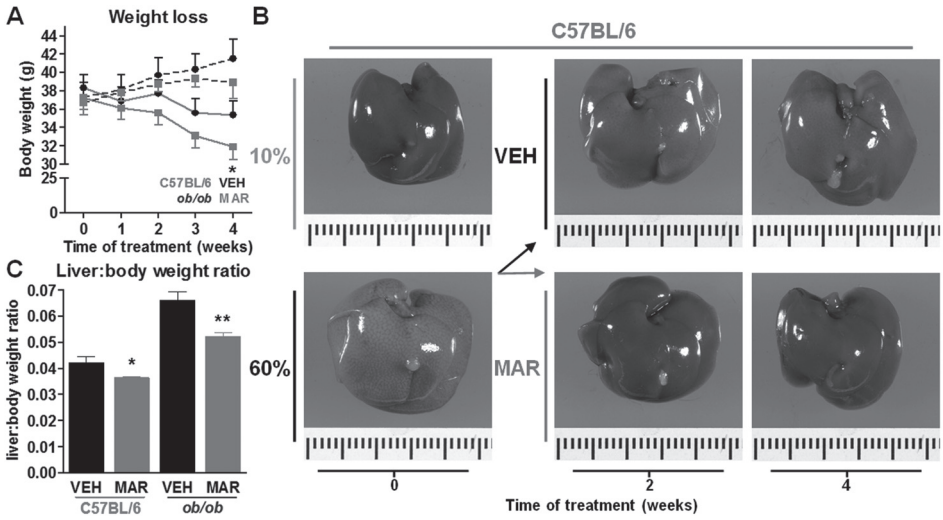


Figure 2. Marimastat treatment resulted in decreased liver weights and normalized macroscopic appearance. C57BL/6 animals were fed a high fat diet for 9 weeks, followed by two or four weeks of treatment with either Marimastat (MAR) or vehicle (VEH) control. Ob/ob animals were fed a normal fat diet and received 4 weeks of MAR or VEH control. Body weight loss was calculated relative to the weight of each individual animal before initiation of the experiment (A). Macroscopic appearance of livers from C57BL/6 mice at time of sacrifice (B). Baseline animals that were fed a 10% fat purified rodent diet for 9 weeks demonstrated normal macroscopic liver appearance (left upper panel, B), whereas baseline animals that were switched to a 60% fat diet for 9 weeks developed severe steatosis, indicated by liver enlargement and pale yellow discoloring (left lower panel, B). Two and four weeks of Marimastat treatment resulted in significant improvement of macroscopic appearance (middle and right lower panels, B), similar to the baseline control liver. Livers from vehicle treated mice remained pale yellow, indicative for fatty changes (middle and right upper panels, B). Liver to body weight ratios were calculated (C). *, $P<0.05$; **, $P<0.01$.

Marimastat reversed hepatic steatosis and improved insulin sensitivity in diet-induced obese mice as well as in leptin-deficient mice

Liver sections from diet-induced obese animals treated for two weeks with Marimastat showed a significant reduction in steatosis, as demonstrated by H&E stains, and confirmed with Oil Red O staining (**Figure 3A**). Livers from Marimastat treated diet-induced obese mice exhibited almost normal hepatic architecture, whereas livers from control animals revealed micro- and macrovesicular steatosis. In *ob/ob* mice, Marimastat treatment led to a decrease in predominantly macrovesicular lipid droplets mainly around the portal tract, compared to livers from control animals that exhibited severe macrovesicular steatosis and ballooning (**Figure 3B**). To objectively confirm the histological findings, hepatic fat content was quantified by using MR spectroscopy. Two weeks of Marimastat treatment of diet-induced obese mice nearly normalized hepatic fat fraction to 9.0±4.3%, compared to control animals (26.1±2.6%, P=0.010). In *ob/ob* animals, Marimastat treatment decreased hepatic fat fraction from 48.8±2.6% to 34.3±3.7% (P=0.019) (**Figure 4A**). White adipose tissue fat index, as a measure of obesity, significantly decreased following

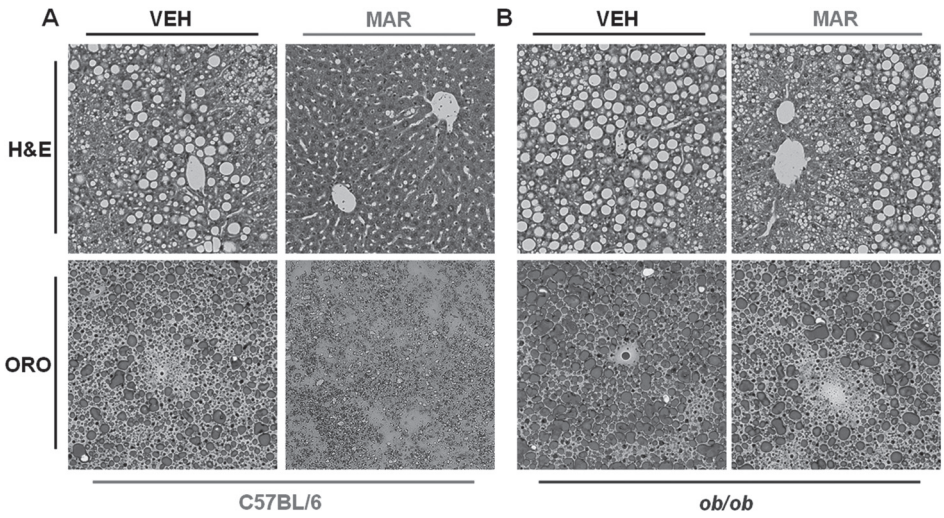


Figure 3. Marimastat reversed hepatic steatosis in diet-induced obese mice as well as in ob/ob animals, as demonstrated by histology. C57BL/6 animals were fed a high fat diet for 9 weeks, followed by two weeks of treatment with either Marimastat (MAR) or vehicle (VEH) control. Ob/ob animals were fed a normal fat diet and received 4 weeks of MAR or VEH control. Representative liver sections stained with hematoxylin and eosin (H&E; top panels; original magnification 200x), and Oil Red O (bottom panels; original magnification 200x). Livers from MAR treated diet-induced obese animals exhibited almost normal hepatic architecture, whereas VEH livers revealed micro- and macrovesicular steatosis (A). Livers from VEH treated ob/ob mice revealed extensive, predominantly macrovesicular steatosis (B). MAR treated livers from ob/ob mice, in contrast, showed a decrease in macrovesicular lipid droplets mainly around the portal tract (B).

Marimastat treatment in diet-induced obese mice as well as in *ob/ob* mice with 45% and 13%, respectively (P=0.001 and P=0.034; **Figure 4B**). ALT was used as a marker for evaluation of hepatic injury associated with steatosis (**Figure 4C**). After two weeks of treatment with Marimastat, diet-induced obese animals exhibited a significant decrease of mean serum values for ALT (54±12 IU/L) than did control animals (89±7 IU/L; P=0.035). Following four weeks of Marimastat treatment, ob/ob mice demonstrated a decreased mean ALT level of 166±20 IU/L, compared to control mice (480±87IU/L; P=0.008).

In addition, in the diet-induced obesity model as well as in *ob/ob* mice Marimastat treatment significantly lowered fasting glucose (P=0.004 and P=0.041, respectively; **Figure 4D**) and insulin (P=0.013 and P=0.039, respectively; **Figure 4E**) levels, resulting in improved insulin sensitivity, as measured using the log(HOMA) formula as a surrogate marker (P<0.001 and P=0.026, respectively; **Figure 4F**). These data indicate that Marimastat treatment decreased liver injury and improved insulin sensitivity in parallel with reversal of steatosis.

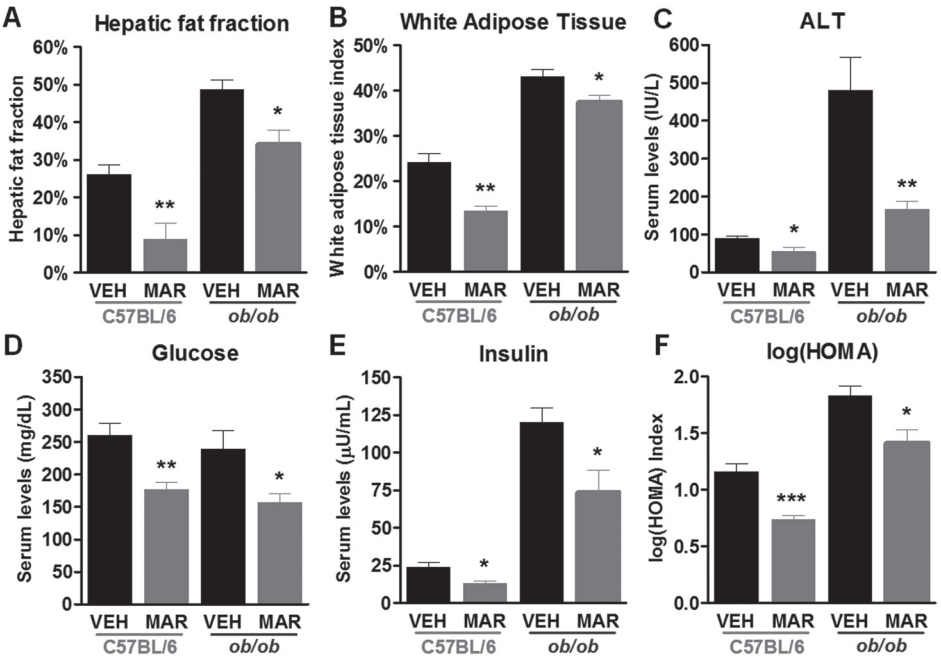


Figure 4. Marimastat reversed hepatic steatosis and improved insulin sensitivity in diet-induced obese mice as well as in ob/ob animals. C57BL/6 animals were fed a high fat diet for 9 weeks, followed by two weeks of treatment with either Marimastat (MAR) or vehicle (VEH) control. Ob/ob animals were fed a normal fat diet and received 4 weeks of MAR or VEH control. Mean hepatic fat fraction as measured by MR spectroscopy was decreased following Marimastat treatment (A), as well as the white adipose tissue fat-index, as calculated using the sum of the individual fat pads as a percentage of the eviscerated body weight (B). Marimastat treatment resulted in a decrease of plasma alanine aminotransferase levels (C), indicating decreased hepatic injury. Glucose (D) and insulin (E) levels in the fasted state, as well as log of homeostasis assessment (log(HOMA)) as a surrogate marker for insulin resistance (F) were improved following treatment with Marimastat. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ns, not significant.

Effects of Marimastat on serum adipokine levels and hepatic fatty acid export

To further explore the metabolic changes after Marimastat treatment, enzyme-linked immunosorbent assays were performed to detect serum levels of several adipokines. In both animal models, Marimastat treatment did not affect serum levels of adiponectin (**Figure 5A**), leptin (**Figure 5B**) or TNF-α receptor II (p75) (**Figure 5C**); however, serum levels of IL-6 (**Figure 5D**) were significantly increased. Because IL-6 is involved in hepatic export of triglyceride and cholesterol[32], hepatic lipid export rates were quantified by injection of tyloxapol, an inhibitor of very low-density lipoprotein hydrolysis. Marimastat treatment did not affect serum triglyceride levels, indicating that Marimastat did not contribute to reversal of steatosis by increasing fatty acid export (**Figure 5E**).

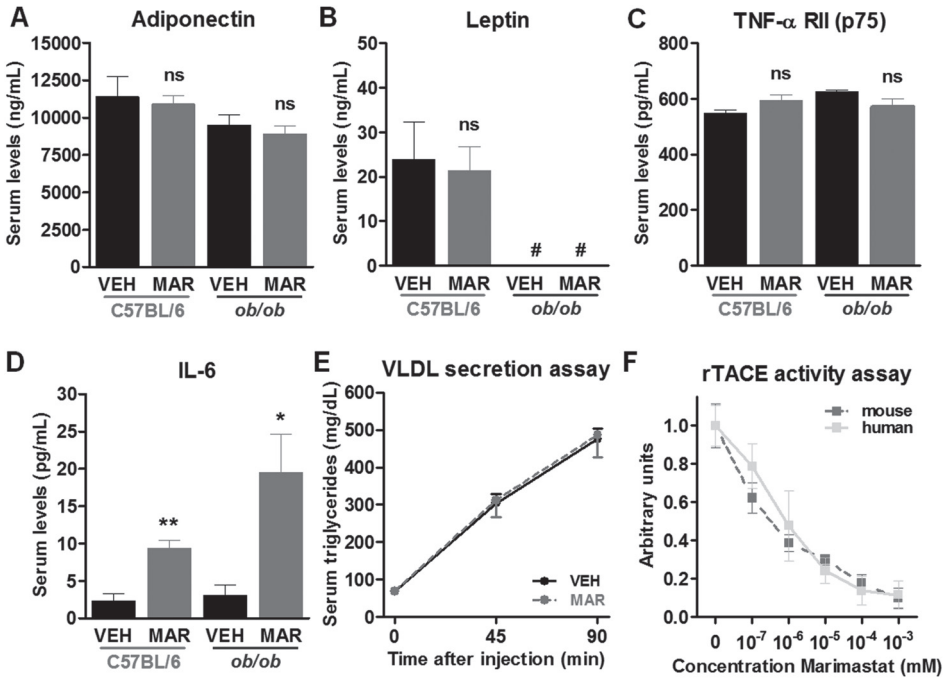


Figure 5. Effects of Marimastat on serum adipokine levels, fatty acid export from the liver and TACE activity. C57BL/6 animals were fed a high fat diet for 9 weeks, followed by two weeks of treatment with either Marimastat (MAR) or vehicle (VEH) control. Ob/ob animals were fed a normal fat diet and received 4 weeks of MAR or VEH control. MAR did not affect serum levels of adiponectin (A), leptin (B) and tumour necrosis factor-α receptor II (p75) (C); however, serum levels of IL-6 (D) were significantly increased in MAR animals in both studies. MAR did not affect hepatic lipid export rates, as quantified by injection of tyloxapol (E). MAR inhibited both human and mouse recombinant TACE in a dose-dependent matter. *, $P<0.05$; **, $P<0.01$; ns, not significant.

Marimastat treatment decreased TACE activity in vitro and decreased genetic feedback by Timp3

To confirm inhibition of TACE by Marimastat, an activity assay was performed. This assay demonstrated efficient inhibition by Marimastat of both human and mouse recombinant TACE in a dose-dependent matter (**Figure 5F**). Quantitative real-time RT-PCR revealed that hepatic mRNA expression of TACE remained unchanged following Marimastat treatment; however, transcript levels of *Timp3*, the endogenous inhibitor of TACE, were decreased by 58% (**Figure 6A**). This suggests that Marimastat does not inhibit TACE at the gene level, but that inhibition of TACE activity seizes the need for endogenous inhibition, resulting in decreased mRNA expression of *Timp3*.

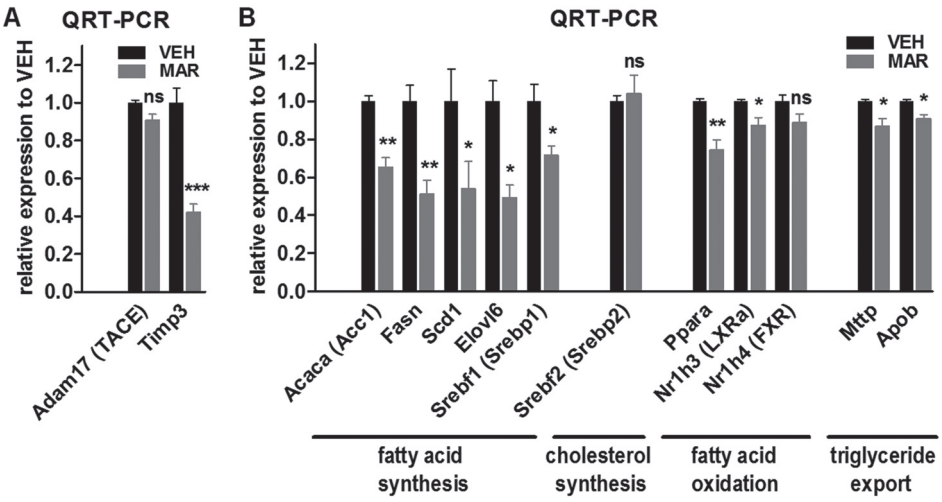


Figure 6. Analysis of hepatic mRNA expression of metabolic genes, as quantified by real-time RT-PCR. C57BL/6 animals were fed a high fat diet for 9 weeks, followed by two weeks of treatment with either Marimastat (MAR) or vehicle (VEH) control. Following Marimastat treatment, mRNA expression of Timp3, the endogenous inhibitor of TACE, was 58% decreased, whereas transcript levels of TACE remained unchanged (A). Genes involved in fatty acid synthesis were significantly downregulated following Marimastat treatment, whereas cholesterol synthesis remained unaffected. Marimastat treatment slightly decreased hepatic transcript levels of genes related to fatty acid oxidation and triglyceride export (B). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ns, not significant.

Hepatic mRNA expression analysis of metabolic genes, as quantified by real-time RT-PCR

To investigate the changes in metabolic gene signalling induced by Marimastat treatment, hepatic transcript levels of metabolic genes were examined using real-time RT-PCR (**Figure 6B**). Marimastat treatment significantly downregulated genes involved in fatty acid synthesis such as acetyl-coenzyme a carboxylase alpha (*Acaca*), fatty acid synthase (*Fasn*), stearoyl-coenzyme a desaturase 1 (*Scd1*), elongation of long chain fatty acids family member 6 (*Elovl6*) and sterol regulatory element binding factor 1 (*Srefp1*), whereas levels of *Srebf2*, key transcription factors involved in cholesterol synthesis, remained unaffected. Marimastat treatment slightly decreased hepatic transcript levels of genes related to fatty acid oxidation such as peroxisome proliferator activated receptor alpha (*Ppara*), liver X receptor alpha (*Nr1h3*) and farnesoid X receptor (*Nr1h4*), as well as genes involved in triglyceride export such as microsomal triglyceride transfer protein (*Mttp*) and apolipoprotein b (*Apob*). These findings indicate that the improvement in insulin resistance, steatosis and inflammation by Marimastat treatment is associated with inhibition of genes related to fatty acid synthesis, rather than genes involved in cholesterol synthesis, fatty acid oxidation or fatty acid export.

Short-term reversal of established steatosis using Marimastat prior to hepatic resection ameliorated post-operative liver injury, without affecting regeneration

To further explore the potential clinical benefits of Marimastat, we studied whether pre-treatment with a short course of Marimastat to reverse hepatic steatosis and insulin resistance prior to surgery (as described above) would improve the outcome of liver resection in the context of fatty liver. To address this question, we utilized a model of two-thirds hepatectomy, which was performed in diet-induced obese animals with hepatic steatosis, pre-treated with Marimastat or not as described above. Indices of liver regeneration and injury were evaluated as surrogate predictors of post-operative outcomes.

As expected, two weeks of treatment with Marimastat successfully reversed steatosis, whereas livers of vehicle-treated animals remained fatty (**Figure 7A**). Importantly, speed

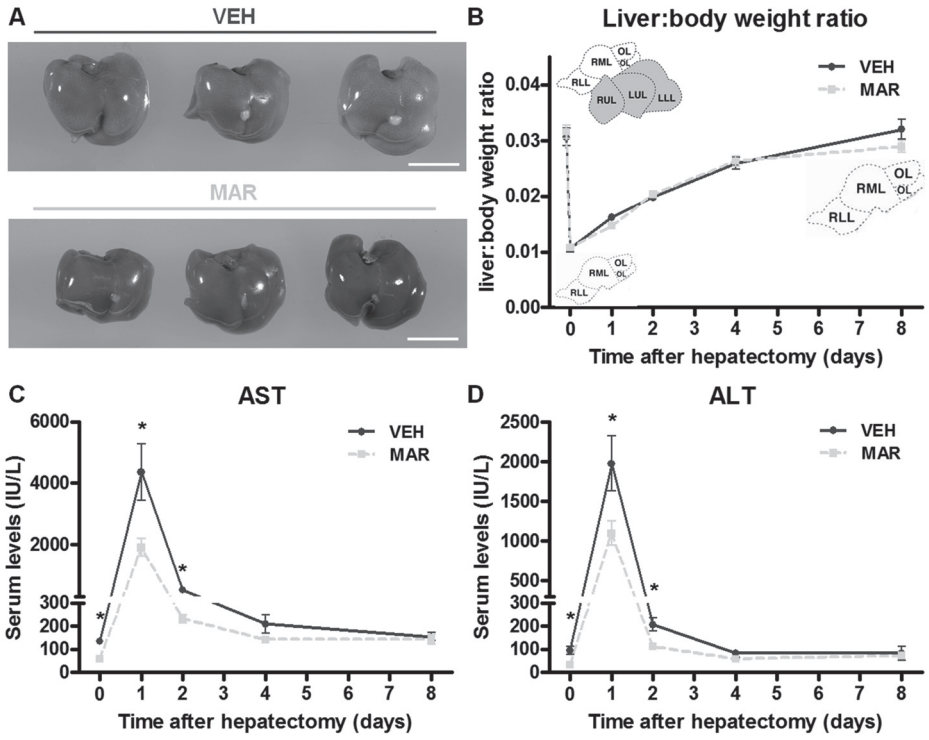


Figure 7. Reversal of established steatosis using Marimastat ameliorated post-operative liver injury, without affecting regeneration. C57BL/6 animals were fed a high fat diet for 9 weeks, followed by two weeks of treatment with either Marimastat (MAR) or vehicle (VEH) control. After reversal of steatosis (A), animals were subjected to a two-thirds hepatectomy. Marimastat did not affect speed of liver regeneration, as measured by liver to body weight ratios over time (B). Post-operative liver injury was significantly decreased in diet-induced obese animals that had their steatosis reversed following Marimastat treatment, as quantified by aspartate aminotransferase (AST; C) and alanine aminotransferase (ALT; D). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

of liver regeneration was similar in both groups, as determined by the restoration of liver to body weight ratios in the eight days following surgery (**Figure 7B**). More strikingly, post-operative liver injury as quantified by AST (**Figure 7C**) and ALT (**Figure 7D**) was significantly decreased by 57% (P=0.020) and 44% (P=0.032), respectively, after 24 hours, and by 55% (P=0.036) and 45% (P=0.022), respectively, after 48 hours in the group of diet-induced obese animals that had their steatosis reversed following Marimastat treatment, compared to controls. In both groups, AST and ALT levels normalized after four to eight days. Post-operative mortality was limited to one death in each group related to technical failure. These data indicate that the reversal of steatosis with Marimastat significantly attenuated post-operative injury following hepatectomy.

DISCUSSION

The present study aimed to determine the effects of the broad-spectrum matrix metalloproteinase and TACE-inhibitor Marimastat on hepatic steatosis and insulin resistance in murine models of diet-induced obesity and leptin deficiency. In addition, we studied the surgical outcome of treated versus untreated animals following hepatectomy. We demonstrate that Marimastat treatment improves insulin sensitivity and reverses steatosis in both animal models by interfering, in all likelihood, with TACE activation and signaling. The beneficial effects of steatosis reversal on post-operative outcome by pharmacologic TACE-inhibition are encouraging, and provide a rationale for current investigations in the search for a pharmacologic agent to optimize the steatotic liver prior to surgery. For this indication, a drug such as Marimastat with its known safety profiles and limited side-effects would allow rapid transfer to clinical practice.

Previous reports have implicated the TACE/TIMP-3 system as a mediator between metabolic stimuli and innate immunity. Animals double-heterozygous for the *insulin receptor* and *Timp3* developed spontaneous mild hyperglycemia and hyperinsulinemia at three months, and overt glucose intolerance and hyperinsulinemia at six months.[33] Mice heterozygous for *Tace* are protected from diet-induced insulin resistance and diabetes[34], whereas pharmacologic TACE-inhibition reduced hyperglycemia and vascular inflammation in diabetic mice heterozygous for the insulin receptor[33], as well as in fructose-fed rats.[23] In contrast, *Timp3* deficient animals fed a high fat diet for five months experienced increased TACE activity and became glucose-intolerant and insulin-resistant.[20] These data indicate that the interplay between reduced insulin action and increased TACE activity promotes diabetes and vascular inflammation. Therefore, we aimed to investigate the effects of TACE-inhibition in two commonly used models of obesity and hepatic steatosis. In the diet-induced obesity model, we show that two weeks of Marimastat

treatment almost completely reverses the metabolic phenotype, compared to vehicle-treated obese controls. These findings were validated in a second, mechanistically different animal model of obesity and hepatic steatosis using the murine model of leptin deficiency.

The role of the TACE/TIMP-3 system in the pathogenesis of diet-induced hepatic steatosis has only recently been elucidated. Mice deficient in *Timp3* that were fed a high fat diet for five months developed severe macrovesicular steatosis compared to wild-type mice.[20] In another study, mice double-heterozygous for the *insulin receptor* and *Timp3* that were placed on a high fat diet developed macrovesicular steatosis and showed a higher degree of hepatic and adipose tissue inflammation compared to wild-type mice.[21] In contrast, mice double-heterozygous for the *insulin receptor* and *Tace* had a nearly opposite phenotype.[21] Until now, the effects of pharmacologic TACE-inhibition on hepatic steatosis remained to be established. In the diet-induced obesity model, we demonstrated that two weeks of Marimastat treatment almost completely reversed hepatic steatosis, compared to vehicle-treated obese controls. Moreover, even in leptin deficient *ob/ob* mice, Marimastat treatment significantly improved hepatic fat content.

Adipokines, a group of polypeptide molecules primarily secreted by adipose tissue exert local, peripheral and/or central actions and are involved in the pathogenesis of NAFLD.[35] The adipokines TNF-α and IL-6, for example, are involved in induction of insulin and leptin resistance and can be stimulated by the adipokine leptin (regulated by the *ob* gene), or inhibited by the adipokine adiponectin.[35] TACE, in part, regulates the production of TNF-α and IL-6, and may indirectly affect release of adiponectin and leptin. Our data demonstrates that despite efficient inhibition of TACE activity by Marimastat, serum levels of the adipokines, adiponectin, leptin and TNF-α receptor II remained unchanged. In contrast, serum levels of IL-6 were significantly elevated following Marimastat treatment. Because IL-6 is also involved in hepatic triglyceride secretion[32], we hypothesized that the increase in IL-6 could have contributed to the improvement of hepatic steatosis by regulating hepatic fatty acid export by the liver. To address this, we quantified hepatic lipid export by injecting tyloxapol, an inhibitor of very low-density lipoprotein hydrolysis, and measured serum triglyceride levels. The rate of very low-density lipoprotein production was similar in both groups, indicating that Marimastat treatment did not affect hepatic fatty acid export.

Pathways and mechanisms linking the TACE/TIMP-3 axis to insulin resistance and hepatic steatosis are not completely elucidated, but involve expression of proteins playing a role in fatty acid uptake, triglyceride synthesis and methionine metabolism[20]. Hepatic gene expression analysis in our study indicates that Marimastat treatment significantly

decreased transcript levels of genes related to fatty acid synthesis, such as *Acaca*, *Fasn*, *Scd1*, *Elovl6* and *Srebf1*, while *Srepf2*, a gene involved in cholesterol synthesis, remained unchanged. Hepatic transcript levels of genes involved in fatty acid oxidation such as *Ppara*, *Nr1h3* and *Nr1h4* were slightly, but significantly, decreased following Marimastat treatment. This was similar for hepatic gene expression of *Mttp* and *Apob*, both involved in triglyceride export. This data suggests that a significant inhibition of hepatic fatty acid synthesis, in combination with relatively unchanged rates of fatty acid oxidation and triglyceride export, may be, at least in part, responsible for the beneficial effects of Marimastat in the reversal of hepatic steatosis.

The link between obesity-triggered inflammation and insulin resistance, and the importance of the TACE/TIMP-3 system in liver inflammation and regeneration has been studied previously. Mice deficient in *Timp3* had increased activity of TACE and elevated levels of TNF, and developed spontaneous, severe inflammation in the liver.[36] In addition, following partial hepatectomy these mice succumbed to liver failure, indicating the importance of TNF signaling in liver regeneration[36]. In line with this data, we have previously demonstrated that matrix metalloproteinases and TACE-inhibition by Marimastat significantly attenuated hepatic injury and inflammation following carbon tetrachloride intoxication[37], but may impair hepatic regeneration when administered concomitantly.[28] Also, in a murine model of fat free diet-induced essential fatty acid deficiency, we have demonstrated that Marimastat treatment markedly prevented hepatic triglyceride accumulation, despite elevated rates of *de novo* lipogenesis and onset of essential fatty acid deficiency.[38] In the current paper, Marimastat treatment ameliorated hepatic injury in both diet-induced obese mice as well as in leptin deficient *ob/ob* animals, corroborating our previous findings. More importantly, in the first post-operative days following two-thirds hepatectomy in diet-induced obese mice, Marimastat pre-treatment reversed hepatic steatosis and decreased hepatic injury by about 50%. Liver growth rate was not impaired, because Marimastat treatment was halted 24 hours prior to hepatic resection to avoid any potential effects of TACE-inhibition on hepatic regeneration.[28] These findings may deserve further experimental investigation in the search for a pharmacologic treatment for the reversal of steatosis.

Although we cannot extrapolate our experimental *in vivo* results with Marimastat to human clinical trials directly, it is possible that Marimastat as well as other drugs with anti-TACE activity may have similar effects on hepatic steatosis. Clinically, Marimastat has been used in multiple oncologic clinical trials up to phase III; however, its overall therapeutic benefit was limited and the trials were halted.[39-41] A final note of caution is warranted when patients with chronic, active liver diseases such as hepatitis or non-alcoholic steatohepatitis receive long-term treatment with TACE and matrix metallo-

proteinase-inhibitors, since inhibition of fibrolytic matrix metalloproteinases will likely accelerate hepatic fibrogenesis formation.[37] However, this is unlikely to cause a problem in a short term treatment mode prior to surgery. Nonetheless, the use of these drugs could still be used in those with simple steatosis or for short periods of time, since the effects on fibrogenesis may only apply to long-term use.[37]

In conclusion, we demonstrated that TACE-activity inhibition by Marimastat in two murine models of hepatic steatosis resulted in reversal of steatosis, coupled with improvement of insulin sensitivity. Short-term use of Marimastat decreased hepatic fat content of high fat-fed obese mice, and subsequently attenuated hepatic injury following major hepatic resection. Since effective inhibition of TACE activity improved surgical outcome, our data suggests a potential use in patients with steatosis who need to undergo major hepatic resection. Based on these findings in animal models, we advocate further investigation of pharmacologic TACE-inhibitors in human patients affected by NAFLD.

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REFERENCES

1. Angulo P. Nonalcoholic fatty liver disease. *N Engl J Med* 2002;346:1221-1231.
2. Lazo M, Clark JM. The epidemiology of nonalcoholic fatty liver disease: a global perspective. *Semin Liver Dis* 2008;28:339-350.
3. Argo CK, Northup PG, Al-Osaimi AM, *et al.* Systematic review of risk factors for fibrosis progression in non-alcoholic steatohepatitis. *J Hepatol* 2009;51:371-379.
4. Jou J, Choi SS, Diehl AM. Mechanisms of disease progression in nonalcoholic fatty liver disease. *Semin Liver Dis* 2008;28:370-379.
5. Neuschwander-Tetri BA. Hepatic lipotoxicity and the pathogenesis of nonalcoholic steatohepatitis: The central role of nontriglyceride fatty acid metabolites. *Hepatology* 2010;52:774-788.
6. Adam R, Hoti E. Liver transplantation: the current situation. *Semin Liver Dis* 2009;29:3-18.
7. De Meijer VE, Kalish BT, Puder M, *et al.* Systematic review and meta-analysis of steatosis as a risk factor in major hepatic resection. *Br J Surg* 2010;97:1331-1339.
8. Selzner M, Clavien PA. Fatty liver in liver transplantation and surgery. *Semin Liver Dis* 2001;21:105-113.
9. Vuppalanchi R, Chalasani N. Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis: Selected practical issues in their evaluation and management. *Hepatology* 2009;49:306-317.
10. Sanyal AJ, Chalasani N, Kowdley KV, *et al.*; NASH CRN. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *N Engl J Med* 2010;362:1675-1685.
11. Sanyal AJ. Mechanisms of Disease: pathogenesis of nonalcoholic fatty liver disease. *Nat Clin Pract Gastroenterol Hepatol* 2005;2:46-53.
12. Tilg H, Moschen AR. Insulin resistance, inflammation, and non-alcoholic fatty liver disease. *Trends Endocrinol Metab* 2008;19:371-379.
13. Hotamisligil GS. Inflammation and metabolic disorders. *Nature* 2006;444:860-867.
14. Murphy G, Murthy A, Khokha R. Clipping, shedding and RIPping keep immunity on cue. *Trends Immunol* 2008;29:75-82.
15. Uysal KT, Wiesbrock SM, Marino MW, *et al.* Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. *Nature* 1997;389:610-614.
16. Black RA, Rauch CT, Kozlosky CJ, *et al.* A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. *Nature* 1997;385:729-733.
17. Moss ML, Jin SL, Milla ME, *et al.* Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-alpha. *Nature* 1997;385:733-736.
18. Amour A, Slocombe PM, Webster A, *et al.* TNF-alpha converting enzyme (TACE) is inhibited by TIMP-3. *FEBS Lett* 1998;435:39-44.
19. Chen CD, Podvin S, Gillespie E, *et al.* Insulin stimulates the cleavage and release of the extracellular domain of Klotho by ADAM10 and ADAM17. *Proc Natl Acad Sci USA* 2007;104:19796-19801.
20. Fiorentino L, Vivanti A, Cavalera M, *et al.* Increased tumor necrosis factor alpha-converting enzyme activity induces insulin resistance and hepatosteatosis in mice. *Hepatology* 2010;51:103-110.
21. Menghini R, Menini S, Amoruso R, et al. Tissue inhibitor of metalloproteinase 3 deficiency causes hepatic steatosis and adipose tissue inflammation in mice. *Gastroenterology* 2009;136:663-672.
22. Doggrell SA. TACE inhibition: a new approach to treating inflammation. *Expert Opin Investig Drugs* 2002;11:1003-1006.
23. Togashi N, Ura N, Higashiura K, *et al.* Effect of TNF-alpha--converting enzyme inhibitor on insulin resistance in fructose-fed rats. *Hypertension* 2002;39:578-580.

24. De Meijer VE, Le HD, Meisel JA, *et al.* Dietary fat intake promotes the development of hepatic steatosis independently from excess caloric consumption in a murine model. *Metabolism* 2010;59:1092-1105.
25. De Meijer VE, Le HD, Meisel JA, *et al.* Repetitive orogastric gavage affects the phenotype of diet-induced obese mice. *Physiol Behav* 2010;100:387-393.
26. Halaas JL, Gajiwala KS, Maffei M, *et al.* Weight-reducing effects of the plasma protein encoded by the obese gene. *Science* 1995;269:543-546.
27. Pelleymounter MA, Cullen MJ, Baker MB, *et al.* Effects of the obese gene product on body weight regulation in ob/ob mice. *Science* 1995;269:540-543.
28. Alwayn IP, Verbesey JE, Kim S, *et al.* A critical role for matrix metalloproteinases in liver regeneration. *J Surg Res* 2008;145:192-198.
29. Greene AK, Puder M. Partial hepatectomy in the mouse: technique and perioperative management. *J Invest Surg* 2003;16:99-102.
30. Muniyappa R, Lee S, Chen H, *et al.* Current approaches for assessing insulin sensitivity and resistance in vivo: advantages, limitations, and appropriate usage. *Am J Physiol Endocrinol Metab* 2008;294:E15-26.
31. Otway S, Robinson DS. The use of a non-ionic detergent (Triton WR 1339) to determine rates of triglyceride entry into the circulation of the rat under different physiological conditions. *J Physiol* 1967;190:321-332.
32. Hong F, Radaeva S, Pan HN, *et al.* Interleukin 6 alleviates hepatic steatosis and ischemia/reperfusion injury in mice with fatty liver disease. *Hepatology* 2004;40:933-941.
33. Federici M, Hribal ML, Menghini R, *et al.* Timp3 deficiency in insulin receptor-haploinsufficient mice promotes diabetes and vascular inflammation via increased TNF-alpha. *J Clin Invest* 2005;115:3494-3505.
34. Serino M, Menghini R, Fiorentino L, *et al.* Mice heterozygous for tumor necrosis factor-alpha converting enzyme are protected from obesity-induced insulin resistance and diabetes. *Diabetes* 2007;56:2541-2546.
35. Marra F, Bertolani C. Adipokines in liver diseases. *Hepatology* 2009;50:957-969.
36. Mohammed FF, Smookler DS, Taylor SE, *et al.* Abnormal TNF activity in Timp3-/- mice leads to chronic hepatic inflammation and failure of liver regeneration. *Nat Genet* 2004;36:969-977.
37. De Meijer VE, Sverdlov DY, Popov Y, *et al.* Broad-spectrum matrix metalloproteinase inhibition curbs inflammation and liver injury but aggravates experimental liver fibrosis in mice. *PLoS One* 2010;5:e11256.
38. Alwayn IP, Andersson C, Lee S, *et al.* Inhibition of matrix metalloproteinases increases PPAR-alpha and IL-6 and prevents dietary-induced hepatic steatosis and injury in a murine model. *Am J Physiol Gastrointest Liver Physiol* 2006;291:G1011-1019.
39. Bramhall SR, Hallissey MT, Whiting J, *et al.* Marimastat as maintenance therapy for patients with advanced gastric cancer: a randomised trial. *Br J Cancer* 2002;86:1864-1870.
40. Coussens LM, Fingleton B, Matrisian LM. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. *Science* 2002;295:2387-2392.
41. Rosenbaum E, Zahurak M, Sinibaldi V, *et al.* Marimastat in the treatment of patients with biochemically relapsed prostate cancer: a prospective randomized, double-blind, phase I/II trial. *Clin Cancer Res* 2005;11:4437-4443.

Chapter 6

Repetitive orogastric gavage affects the phenotype of diet-induced obese mice

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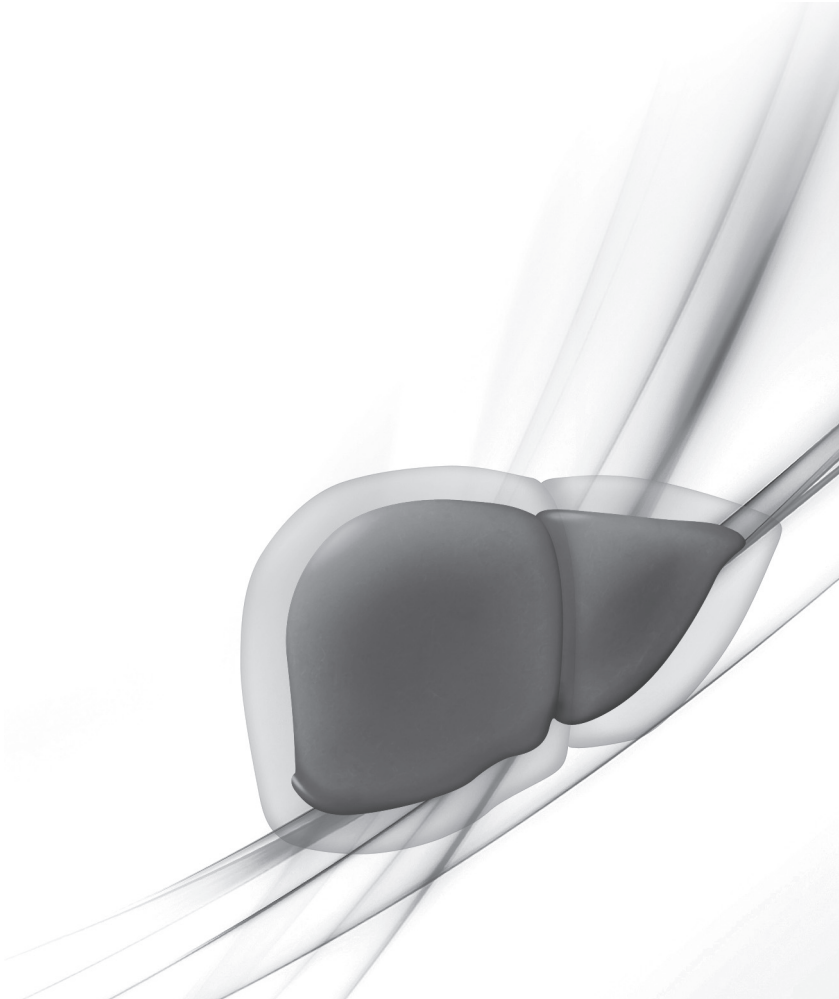

ABSTRACT

Background: Interest in pharmacological intervention to combat metabolic syndrome and its complications is increasing as the prevalence of obesity is reaching epidemic proportions. The potential efficacy of drugs is often tested in animal models; however, the method of drug delivery is frequently overlooked and may act as a confounder due to stress. We hypothesized that long-term orogastric gavage would negatively influence the development of hepatic steatosis and the metabolic syndrome in a murine model of diet-induced obesity.

Methods: C57BL/6J male mice were fed a high fat diet and were gavaged with a vehicle once or twice daily for 9 weeks. A group without orogastric gavaging served as control. A similar experiment was performed using leptin deficient *ob/ob* mice that were fed a standard diet for 4 weeks. Food intake was monitored, insulin resistance determined, and steatosis was assessed by histology and quantified via magnetic resonance spectroscopy.

Results: After 9 weeks, control C57BL/6J mice exhibited significantly more weight gain, insulin resistance and hepatic steatosis, compared to mice that were gavaged daily, or twice daily. This effect was likely due to decreased food consumption associated with gavage-induced stress. In contrast, the phenotype of leptin deficient *ob/ob* mice was not affected by orogastric gavage.

Conclusion: Orogastric gavage may lead to increased stress, thereby affecting food intake and the development of diet-induced obesity in a murine model. The effects of what may seem to be trivial laboratory routines, such as orogastric gavage, should be taken into account when designing animal studies for drug development.

INTRODUCTION

Metabolic syndrome is a cluster of interrelated risk factors for diabetes and cardiovascular disease, including obesity, hyperglycemia, dyslipidemia and elevated blood pressure.[1] The prevalence of the metabolic syndrome is increasing worldwide, estimated to be 35-40% both in the US[1] and in the Middle East[2], depending on the criteria used. Risk factors for the metabolic syndrome are mainly related to a sedentary lifestyle and obesity. The prevalence of non-alcoholic fatty liver disease, which is considered to be the hepatic manifestation of the metabolic syndrome[3], is increasing as well, affecting up to 30% of individuals with the syndrome.[4] Non-alcoholic fatty liver disease is a spectrum of disease ranging from the simple build-up of fat in the liver (hepatic steatosis) to non-alcoholic steatohepatitis, cirrhosis, and ultimately liver failure.[5] The main goal of treating metabolic syndrome is to reduce obesity, especially abdominal girth, through a multimodality approach that includes diet, excise, and pharmacologic intervention.[6] Pharmacologic agents can be used to target both energy intake and energy expenditure, reduce food consumption, and to improve markers of insulin sensitivity, hypercholesterolemia and hepatic steatosis. Despite the availability of several classes of drugs such as antihyperglycemics, antihypertensives, and lipid lowering agents, further drug development is warranted to overcome current limitations, potentially leading to improved clinical management of the metabolic syndrome and its associated complications.

In order to test the efficacy of drugs on treating the metabolic syndrome, animal models are essential to investigate the relative influence of various treatments on energy intake, energy expenditure, and body weight. In animal models, drugs can be administered intravenously, intraperitoneally or via orogastric gavage, the last considered to be the least invasive route. However, even non-invasive laboratory routines such as personnel entering the animal housing room, cage maintenance, and body weight measurements may elicit an acute stress response.[7] More stressful procedures such as restraining the animal, may induce catabolism by activating the corticotrophin-releasing hormone system.[8,9] Moreover, chronic activation of the stress response has been associated with altered energy homeostasis and reduction of body fat content.[10] Other reports, however, support a link between exposure to chronic stress and the development of obesity.[11,12] To this day, it remains to be demonstrated whether an acute stressor such as repetitive orogastric gavage may by itself influence the metabolic phenotype of the model studied.

The aim of this study was to investigate the degree of stress induced by different frequencies of orogastric gavage on food intake, fat deposition, insulin resistance and hepatic steatosis in obesity-prone C57BL/6J and *ob/ob* mice. Using the classic model of diet-induced obesity as well as the transgenic model of leptin deficiency, we hypothesized

that long-term, repetitive orogastric gavaging of mice would alter the development of the metabolic syndrome, and in particular, hepatic steatosis.

METHODS

Animals and general housing conditions

C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) fed a high fat diet *ad libitum* gradually develop visceral adiposity, hyperglycemia, insulin and leptin resistance, as well as hepatic steatosis, sharing many of the same obesity related phenotypes as humans.[13] B6.V-*Lep*^{ob}/J mice (Jackson Laboratories, Bar Harbor, ME), commonly referred to as *ob/ob* mice, are homozygous for the spontaneous mutation of the *Lep*^{ob} gene and exhibit obesity, hyperphagia, diabetes, and hepatic steatosis. They are both hypometabolic and hypothermic.[14,15] Male 5-week-old mice were housed 4 (*ob/ob*) or 5 (C57BL/6J) animals per cage on paper chip bedding in a barrier room with regulated temperature (21°C ± 1.6°C), humidity (45% ± 10%), and an alternating 12-hour light and dark cycle. All animals had free access to water and standard rodent chow pellets (Prolab Isopro, RMH 3000 #25; containing 14% fat, 26% protein, and 60% carbohydrate by calories; energy density 4.1 kcal/g; Prolabs Purina, Richmond, IN) for an acclimation period of 1 week prior to study initiation. During the study period, the animals were weighed twice a week and food intake was measured daily. Intake was measured per cage to avoid potential additional stress of individual housing. Animal protocols complied with the National Institutes of Health Animal Research Advisory Committee guidelines, and were approved by the Children's Hospital Boston Animal Care and Use Committee (protocol no. A06-08-065R).

Study diets

Study diets were stored at -80°C and provided fresh each day to avoid lipid peroxidation. In the high fat purified rodent diet (D12492; Research Diets, New Brunswick, NJ), the amount of calories derived from fat, protein, and carbohydrates were 60%, 20%, and 20%, respectively (energy density of 5.24 kcal/g). In the standard purified rodent diet (D12450B; Research Diets, New Brunswick, NJ), 10% of calories were derived from fat, 20% from protein, and 70% from carbohydrates (energy density of 3.85 kcal/g).

Intervention

Animals received 0.2 mL of a common vehicle, 0.45% (w/v) methylcellulose (Sigma-Aldrich, St. Louis, MO) in double distilled H_2O, via orogastric gavage through a 30 mm 20 Gauge feeding needle (Fine Sciences Tools Inc., Foster City, CA). Mice were gavaged daily between 07:00 and 09:00 h, or if twice, again between 19:00 and 21:00 h. Control mice were disturbed and manually handled daily without gavaging. Orogastric gavage

was routinely performed by one experienced investigator (VM) throughout the study, with 0% mortality.

Study design

After the one week acclimation period, fifteen C57BL/6J mice were placed on the 60% high fat diet and randomized into three groups. In a separate study, twelve leptin deficient *ob/ob* mice were acclimatized for one week, were fed a standard purified rodent diet and randomized into three groups. In both experiments, animals in the control group (0x/day) were not gavaged and served as controls, whereas animals in the second (1x/day) and third group (2x/day) were gavaged daily, or twice daily, respectively, throughout the study period.

Specimen collection

At the end of the feeding experiments, mice were fasted for 6 hours. Glucose concentration was determined from tail vein blood using the OneTouch UltraSmart Blood Glucose Monitoring System (LifeScan, Milpitas, CA). Mice were then anesthetized with 2.5% Avertin (2,2,2-Tribromoethanol, Sigma-Aldrich, St. Louis, MO) by intraperitoneal injection. Blood was then collected via retro-orbital sinus puncture and centrifuged at 14000 rpm at 4°C for 10 min to obtain serum. Serum was delivered to the Clinical Laboratory at Children's Hospital Boston for analysis of alanine aminotransferase (ALT), total cholesterol and triglyceride levels.

We then performed a midline laparotomy to observe, excise, and weigh the liver. The frontal lobe of the liver was fixed in 10% formalin at 4°C overnight, washed with phosphate-buffered saline, and then embedded in paraffin. The left lateral lobe was excised and collected for magnetic resonance (MR) spectroscopy analysis. The remaining liver was immediately snap-frozen in liquid nitrogen and stored at -80°C.

White adipose tissue was dissected according to previously defined anatomic landmarks.[16] It was then weighed, snap-frozen in liquid nitrogen, and stored at -80°C. Inguinal (all subcutaneous fat between the lower part of the rib cage and mid-thigh), mesenteric (all fat along the mesentery from the lesser curvature of the stomach to the sigmoid colon), retroperitoneal and epididymal fat pads were weighed and expressed relative to eviscerated body weight. A white adipose tissue fat-index was calculated using the sum of the individual fat pads as a percentage of the eviscerated body weight.[16]

Surrogate indexes of insulin sensitivity and resistance

Insulin levels were measured using a rat/mouse insulin ELISA kit (Linco Research, St. Charles, MO). Surrogate indexes for insulin sensitivity were calculated, including quantitative insulin-sensitivity check index (QUICKI), homeostasis model assessment (HOMA) and log(HOMA).[17] The calculations were performed as follows: $QUICKI=1/[\log(I_0)+\log(G_0)]$, where

I_0 is the fasting insulin (μU/mL) and G_0 is the fasting glucose (mg/dL); HOMA=(G_0xI_0)/22.5 (with glucose expressed as mmol/L and insulin expressed as μU/mL); and log(HOMA).

Histology

Paraffin-embedded sections of the liver were stained with hematoxylin and eosin and periodic acid Schiff's/diastase to examine cellular architecture, glycogen deposition and lipid accumulation. Frozen tissue sections embedded in tissue medium (Optimal Cutting Temperature OCT, Sakura Fenetek, Torrance, CA) were stained with Oil Red-O to detect fat droplets. A pathologist blinded to the treatment groups conducted a histological analysis of the liver sections.

MR spectroscopy

MR spectroscopy was performed by the MR Laboratory at Beth Israel Deaconess Medical Center.[18] Samples were thawed at room temperature for 1 hour prior to analysis, blotted free of excess water and connective tissue, and placed in 5 mm diameter glass tubes for MR spectroscopy. An 8.5 T vertical bore magnet (DRX system, Bruker Instruments, Billerica, MA) was used for spectroscopic measurements of fat and water resonances. Specifically, a point resolved echo spectroscopic acquisition was applied to homogenous regions of liver, as identified from fast low angle shot images of the liver specimen. Voxel volumes interrogated spectroscopically with the point resolved echo spectroscopic sequence were 2 mm^3. The repetition and echo times were 8 s and 12 ms, respectively, and 16 signal averages were acquired per spectrum. The water resonance and the methylene/methyl resonances were numerically integrated using the manufacturer supplied Paravision 4.0 software (Bruker Instruments, Billerica, MA). The methylene/methyl area was divided by the sum of the methylene/methyl area plus the water area to obtain the MR spectroscopy parameter representing hepatic fat fraction used for group comparisons.

Statistical analysis

Continuous data are expressed as means ± standard error of the mean (SEM). The Kolmogorov-Smirnov one sample test was used to check Gaussianity of the continuous data. Data sets involving more than two groups were assessed by analysis of variance (ANOVA), or if nonparametric, using the Kruskal-Wallis test. Repeated measures ANOVA was used to analyze body weight gain. If the ANOVA showed significant effects, group means were further compared using the unpaired two-tailed Student's *t* test, or if nonparametric, by using the Mann-Whitney *U* test. $P \leq 0.05$ was considered statistically significant. All data were collected in a computerized Microsoft Excel database (Microsoft Inc., Redmond, WA). The analysis was performed with SPSS version 16.0 (SPSS Inc., Chicago, IL) statistical software, and figures were created using GraphPad Prism version 5.0 (GraphPad Software Inc., La Jolla, CA) software.

RESULTS

Repetitive orogastric gavage of C57BL/6J mice on a high fat diet decreased body weight gain, energy intake, liver weights and adiposity, compared to non-gavaged animals

After 9 weeks of high fat feeding and a varying frequency of orogastric gavaging among groups, control (0x/day) animals gained significantly more weight compared to 1x/day and 2x/day animals ($F(2,12)=17.87$, $P=0.0003$) (**Table 1**; **Figure 1**). Compared to once (1x/day) or twice daily (2x/day) gavaged animals, the control group consumed more food throughout the study resulting in a higher caloric efficiency (**Table 1**; **Figure 1**). Liver weights, liver/body weight ratios and individual fat pad weights were all significantly higher in the non-gavaged, control animals (**Table 1**). After 9 weeks, control animals had a significantly higher white adipose tissue fat index compared to both 1x/day and 2x/day animals (Kruskal-Wallis statistic=10.50, $P=0.0052$) (**Table 1**). Repetitive orogastric gavage thus negatively affected the development of obesity in C57BL/6J mice fed a high fed diet, likely due to decreased energy consumption.

Table 1. Body weights, tissue weights, caloric intake and surrogate markers for insulin resistance in C57BL/6J mice that were gavaged with different frequencies for 9 weeks.

		Gavage frequency				
		0x/day	1x/day		2x/day	
Body weight gain	(g)	19.0 ± 1.8	9.8 ± 1.3	**	8.6 ± 0.8	***
Liver weight	(g)	1.72 ± 0.17	1.25 ± 0.09	*	1.12 ± 0.04	**
Liver/body weight ratio		0.045 ± 0.00	0.040 ± 0.00		0.040 ± 0.00	
Total caloric intake / mouse	(kcal)	740	636		600	
Caloric efficiency	(g/kcal)	0.0257	0.0154		0.0143	
Relative Inguinal fat mass	(%)	6.92 ± 0.38	4.39 ± 0.70	*	2.91 ± 0.07	***
Mesenteric fat pad	(%)	3.55 ± 0.14	2.39 ± 0.28	**	1.89 ± 0.16	***
Retroperitoneal fat pad	(%)	2.33 ± 0.16	1.61 ± 0.33		1.48 ± 0.04	**
Epididymal fat pad	(%)	9.09 ± 0.38	7.20 ± 1.31		6.08 ± 0.14	***
White adipose tissue fat index	(%)	21.88 ± 0.80	15.58 ± 2.32	*	12.36 ± 0.22	***
Fasting glucose	(mg/dL)	406 ± 40	322 ± 18		351 ± 37	
Fasting insulin	(µU/mL)	42.8 ± 3.2	28.0 ± 8.7		29.6 ± 3.2	
QUICKI		0.24 ± 0.00	0.27 ± 0.01		0.26 ± 0.01	
HOMA		43.0 ± 6.8	22.6 ± 7.4		28.0 ± 11.1	
Log(HOMA)		1.62 ± 0.06	1.19 ± 0.23		1.29 ± 0.19	

Values given are means ± SEM; 0x/day group was used as reference; *P<0.05; **P<0.01; ***P<0.001. QUICKI indicates quantitative insulin-sensitivity check index; HOMA, homeostasis model assessment.

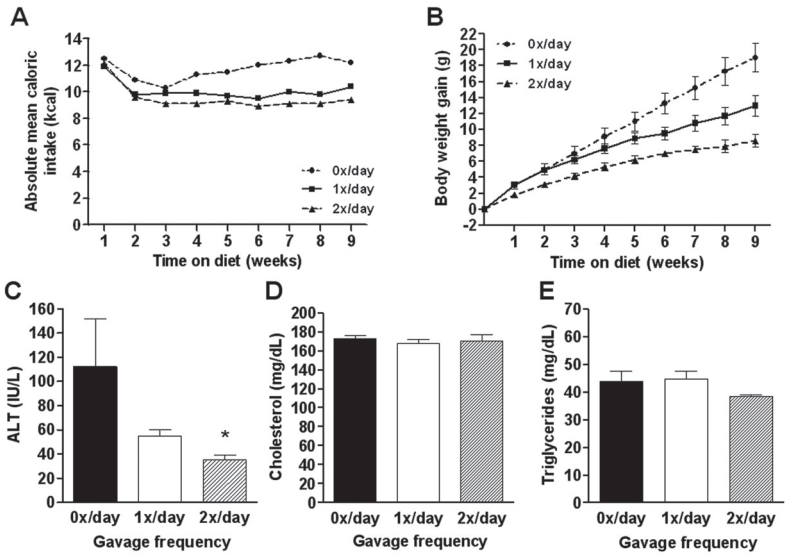

Figure 1. Absolute mean caloric intake per C57BL/6J mouse per week (A) was calculated per group on a daily base. Body weight gain (B) was calculated relative to the weight of each individual animal before initiation of the experiment. Plasma alanine aminotransferase (ALT; C), total cholesterol (D) and triglyceride (E) levels in the different groups. Values represent the mean±SEM. Statistical significance is calculated between the control animals (0×/day) and the difference between 1×/day animals and 2×/day animals (*, $P < 0.05$).

Repetitive orogastric gavage of C57BL/6J mice on a high fat diet does not impair insulin sensitivity, compared to non-gavaged animals

In order to compare the effects of a varying frequency of orogastric gavage on insulin sensitivity we measured serum values for glucose and insulin and calculated surrogate markers (QUICKI, HOMA and log(HOMA)) for insulin sensitivity. Values of mice that were gavaged 1x/day and 2x/day were compared to those of control animals. Fasting blood glucose ($F(2,12)=1.63$, $P=0.2365$), insulin levels ($F(2,12)=1.08$, $P=0.3692$), and surrogate markers of insulin sensitivity (QUICKI: Kruskal-Wallis statistic=2.24, $P=0.3263$); HOMA: $F(2,12)=1.50$, $P=0.2628$; log(HOMA): $F(2,12)=1.62$, $P=0.2384$) were not significantly affected by repetitive orogastric gavage (**Table 1**).

Repetitive orogastric gavage of C57BL/6J mice on a high fat diet decreased ALT values, but did not affect triglyceride and cholesterol levels, compared to non-gavaged animals

ALT is used as a marker for evaluation of hepatic injury and is elevated in mice with hepatic steatosis. Liver enzymes were measured in all experimental groups (**Figure 1**). When compared to controls, 1x/day and 2x/day mice exhibited lower mean ALT values (Kruskal-Wallis statistic=7.75, $P=0.0207$), suggesting impaired progression of hepatic steatosis. Cholesterol ($F(2,12)=0.22$, $P=0.8050$) and triglyceride levels (Kruskal-Wallis

statistic=1.83, *P*=0.3999) were not significantly affected by repetitive orogastric gavage (**Figure 1**). These data suggest that the liver injury associated with high fat feeding in the model of diet-induced obesity was significantly impaired by repetitive orogastric gavage.

Repetitive orogastric gavage of C57BL/6J mice on a high fat diet diminished hepatic steatosis as assessed by histology, compared to non-gavaged animals

To further explore the potential effects of orogastric gavage on the development of hepatic steatosis in the diet-induced obesity model, we analyzed hematoxylin and eosin, and Oil Red-O stained liver sections (**Figure 2**). Liver sections from control mice showed fat throughout the liver parenchyma, including both macro- and microvesicular steatosis. Microvesicular and macrovesicular steatosis was present predominantly in the periportal and midzone areas, whereas occasional ballooned hepatocytes and macrovesicular steatosis were present in the central vein area. In contrast, liver sections from 1x/day and 2x/day animals showed moderate steatosis, predominantly microvesicular. Occasional macrovesicular hepatocytes were observed in the periportal zone. Analysis of periodic acid Schiff's/diastase-stained liver sections excluded glycogen deposition as a cause of microvesicular changes in hepatocytes (data not shown). Steatohepatitis and acute inflammation were not observed in any of the experimental groups.

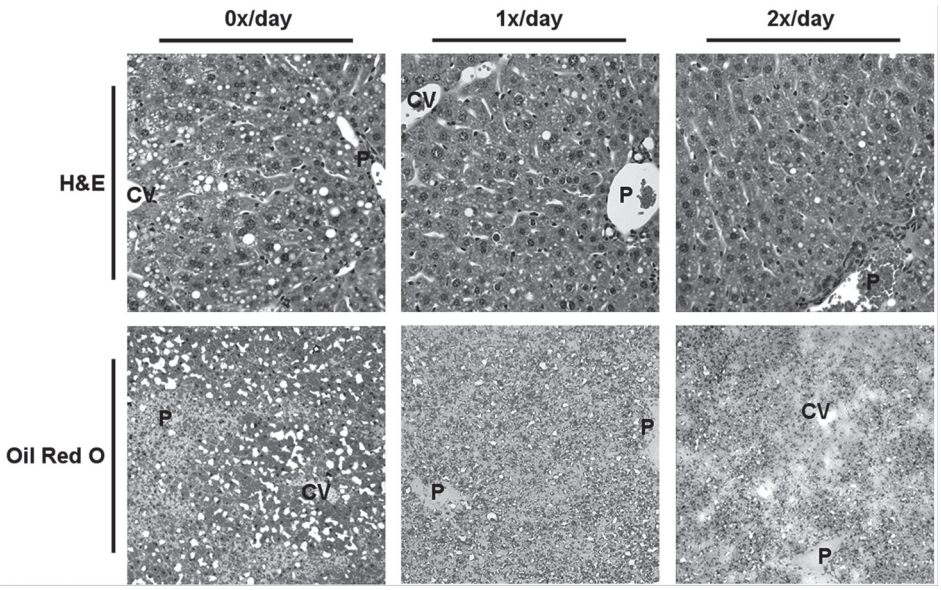

Figure 2. Representative liver sections stained with hematoxylin and eosin (H&E; top panels; original magnification 400×), and Oil Red-O (lower panels; original magnification 200×). Livers from C57BL/6J animals that had not been gavaged revealed extensive, microvesicular and macrovesicular steatosis after 9 weeks (left panels). Livers from animals that had been gavaged once (middle panels), or twice daily (right panels), exhibited moderate, predominantly microvesicular steatosis. P indicates portal tract; CV, central vein.

Repetitive orogastric gavage of C57BL/6J mice on a high fat diet diminished hepatic steatosis as assessed by MR spectroscopy, compared to non-gavaged animals

Hepatic fat content was quantified by using MR spectroscopy. Representative spectra of the different groups are shown (**Figure 3**). Mice that were gavaged once (1x/day) or twice daily (2x/day) exhibited a decreased liver fat content of 14.0±3.4% and 8.9±2.4%, respectively ($F(2,12)=6.50$, $P=0.0122$), when compared to non-gavaged mice (0x/day; 34.5±8.2%), demonstrating the effect of orogastric gavage on hepatic fat accumulation (**Figure 3D**). These data corroborate our histological findings that repetitive orogastric gavage of animals fed a high fat diet significantly affected the development of hepatic steatosis.

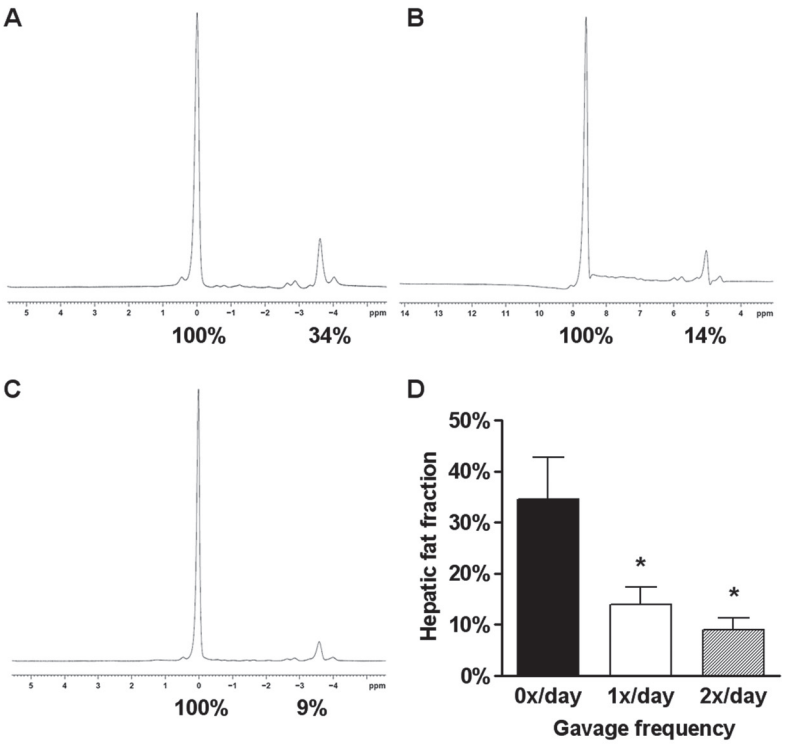

Figure 3. Magnetic resonance spectra for 0×/day (A), 1×/day (B) and 2×/day (C) livers from C57BL/6J mice. Percent fat content was determined relative to water (100%) by numerical integration of the areas under the lipid and water peaks. Mean hepatic fat fraction as measured by magnetic resonance spectroscopy (D). Statistical significance is calculated between the 0×/day animals and the difference between 1×/day animals and 2×/day animals, respectively (*, $P < 0.05$). Variance statistic is SEM.

Repetitive orogastric gavage of ob/ob mice on a standard diet did not influence body weight gain, energy intake, liver and fat pad weights, and fasting glucose levels, compared to non-gavaged animals

Since the confounding effects of orogastric gavage in the diet-induced obesity model appeared, at least in part, to be mediated by decreased food intake, we next investigated whether previously established obesity, insulin resistance and hepatic steatosis could be affected by orogastric gavage of leptin deficient *ob/ob* mice. *Ob/ob* mice exhibit profound hyperphagia, even on low fat diets, and may thus be less susceptible to gavage-induced stress. After 4 weeks, there were no significant differences in body weight gain ($F(2,9)=0.38$, $P=0.6924$), liver weights ($F(2,9)=0.13$, $P=0.8769$), adipose tissue weights ($F(2,9)=0.18$, $P=0.8361$) or fasting glucose levels ($F(2,9)=1.15$, $P=0.3633$) between control *ob/ob* mice and *ob/ob* mice that were gavaged once daily (1x/day) or twice daily (2x/day) (**Table 2**; **Figure 4**). More importantly, total caloric intake and caloric efficiency were similar among groups, indicating that repetitive orogastric gavage did not inhibit food intake in leptin deficient *ob/ob* mice (**Table 2**; **Figure 4**).

Repetitive orogastric gavage of ob/ob mice on a standard diet did not affect the severity of hepatic steatosis, compared to non-gavaged animals

Liver enzymes were measured on all experimental groups to evaluate the effects of repetitive orogastric gavage on *ob/ob* mice (**Figure 4**). ALT ($F(2,9)=1.12$, $P=0.3716$), cholesterol ($F(2,9)=0.77$, $P=0.4949$) and triglyceride levels ($F(2,12)=0.34$, $P=0.7231$) were not significantly different among groups. Liver sections stained with hematoxylin and eosin and Oil Red-O were analyzed and demonstrated severe, predominantly macrove-

Table 2. Body weights, tissue weights and caloric intake in ob/ob mice that were gavaged with different frequencies for 4 weeks.

		Gavage frequency		
		0x/day	**1x/day**	**2x/day**
Body weight gain	(g)	4.7 ± 0.2	4.1 ± 0.3	4.3 ± 0.5
Liver weight	(g)	2.47 ± 0.12	2.41 ± 0.20	2.31 ± 0.26
Liver/body weight ratio		0.059 ± 0.00	0.056 ± 0.00	0.055 ± 0.00
Total caloric intake / mouse	(kcal)	322	323	297
Caloric efficiency	(g/kcal)	0.0146	0.0127	0.0145
Relative Inguinal fat mass	(%)	16.32 ± 0.15	15.67 ± 0.53	16.31 ± 0.50
Mesenteric fat pad	(%)	5.07 ± 0.16	4.85 ± 0.10	4.78 ± 0.51
Retroperitoneal fat pad	(%)	9.60 ± 0.17	9.98 ± 0.24	9.10 ± 0.59
Epidydimal fat pad	(%)	12.96 ± 0.45	13.02 ± 0.33	12.76 ± 0.25
White adipose tissue fat index	(%)	43.96 ± 0.33	43.52 ± 0.56	42.95 ± 1.70
Fasting glucose	(mg/dL)	206 ± 10	200 ± 12	239 ± 29

Values given are means ± SEM; 0x/day group was used as reference.

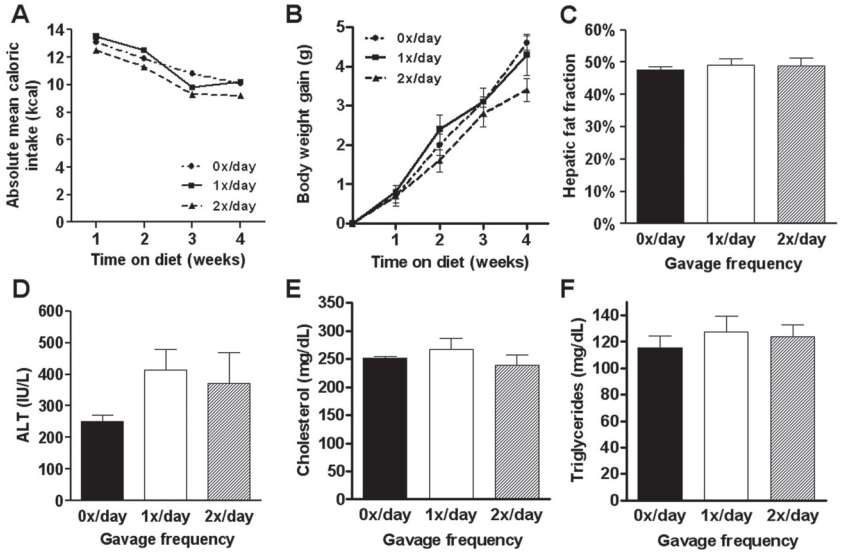

Figure 4. Absolute mean caloric intake per ob/ob mouse per week (A) was calculated per group on a daily base. Body weight gain (B) was calculated relative to the weight of each individual animal before initiation of the experiment, showing no difference. After 4 weeks, mean hepatic fat fraction as measured by magnetic resonance spectroscopy revealed no significant difference between groups (C). Plasma alanine aminotransferase (ALT; C), total cholesterol (D) and triglyceride (E) levels were not statistically different between groups after 4 weeks. Values represent the mean±SEM. Statistical significance is calculated between the control animals (0×/day) and the difference between 1×/day animals and 2×/day animals (*, $P < 0.05$).

sicular steatosis throughout the parenchyma, with extensive hepatocyte ballooning but without inflammation, in all groups (data not shown). No differences were observed between control *ob/ob* mice and *ob/ob* mice that were gavaged once daily (1x/day) or twice daily (2x/day). MR spectroscopy was used to quantify the effect of orogastric gavage on hepatic fat accumulation (**Figure 4**). Again, no significant differences were seen among groups ($F(2,9)=0.14$, $P=0.8689$) thereby corroborating our histological findings that repetitive orogastric gavage of *ob/ob* mice did not affect the severity of hepatic steatosis.

DISCUSSION

The prevalence of the metabolic syndrome and its associated complications, including hepatic steatosis, is reaching epidemic proportions. In addition to lifestyle modifications, pharmacologic interventions are being increasingly explored. Murine models of diet-induced obesity as well as transgenic models are widely used to investigate the potential efficacy of drugs on controlling the metabolic syndrome. However, methods of

drug administration, even those that are perceived to be the least invasive, may induce stress. This can potentially introduce confounders that will affect outcomes. In this study we aimed to investigate the effect of various frequencies of orogastric gavaging on food intake, fat deposition, insulin resistance and hepatic steatosis in mice. Our results indicated that an acute stressor such as repetitive orogastric gavage influenced the development of the metabolic syndrome, in particular, hepatic steatosis, in diet-induced obese C57BL/6J mice, but not in transgenic *ob/ob* mice.

Orogastric gavage is a common laboratory procedure in toxicology, pharmacology and drug-development studies, used to deliver the experimental compounds. Gavaging involves the physical stress of handling and restraining the animal, followed by insertion of a rigid metal feeding needle from mouth to stomach. Orogastric gavage may lead to respiratory interference and stomach distension. Besides possible toxic effects of the agent studied, complications of orogastric gavage may include inadvertent tracheal cannulation/administration, reflux, aspiration pneumonia, esophageal perforation, hemothorax and death.[7,19] Many of these complications are associated with restraint and incorrect placement of the feeding needle. In our study, however, orogastric gavage was routinely performed by one experienced investigator resulting in neither morbidity nor mortality.

In this study we demonstrated that orogastric gavage led to decreased food intake in the C57BL/6J mice, thereby altering the development of the metabolic syndrome. In particular, the severity of hepatic steatosis, which is increasingly recognized as the hepatic manifestation of the metabolic syndrome, was significantly reduced in gavaged C57BL/6J animals, as demonstrated by histology and confirmed by MR spectroscopy. In contrast, *ob/ob* mice which exhibit obesity, hyperphagia, diabetes and hepatic steatosis due to a spontaneous mutation in the Lep^{ob} gene, were not affected by daily or twice daily orogastric gavage. The difference might be due to their distorted leptin signal leading to hyperphagia, thereby maintaining their food intake level. We believe that this difference, combined with their hypometabolic and hypothermic physiology, may have counterbalanced the impact of orogastric gavage induced stress.[14,15]

Handling and restraining during repetitive orogastric gavage has been associated with an increased stress response, which may have led to the observed decreased food intake in the C57BL/6J mice.[7] In addition, although we did not study damage of the oropharynx upon necropsy, development of granulation tissue in the oropharynx following repeated gavage of rats has been reported.[20] This may indicate that repetitive orogastric gavage can lead to recurrent abrasion of the oropharynx, potentially leading to scarring and aversion to oral intake. However, the reported effects might have been strain-specific.[21]

Although this study is the first of its kind to demonstrate the effects of orogastric gavage in mouse models of the metabolic syndrome, an important limitation warrants consideration. The acute stress response can be analyzed by measuring the plasma concentrations of corticosterone, the principal glucocorticoid produced by mice in the stressed state.[22,23] Acute stress, such as restraining the animal, may also activate the corticotrophin-releasing hormone system, potentially inducing catabolism.[8,9] In this study we did not directly measure plasma concentrations of stress related hormones. Although blood sampling to analyze hormone levels may be the most sensitive and accurate method of continual stress analysis, blood sampling is itself an invasive technique which has been shown to increase corticosterone levels.[23] An alternative method would have been the measurement of fecal corticosterone concentrations[24]; however, because the stressful event only lasted for a short time but was repeated over a longer period, the exact timing of sampling would have been difficult to assess.

In conclusion, this study demonstrates that long-term, repetitive orogastric gavage induced stress may affect food intake and, subsequently, the development of the metabolic syndrome and hepatic steatosis in diet-induced obese C57BL/6J mice, but not in leptin deficient *ob/ob* mice. The effects of laboratory routines such as orogastric gavage on the diet-induced obesity model should be taken into account when designing animal studies for drug development. Alternative, minimally invasive methods of drug administration, such as implantation of an mini-osmotic pump[25], may be more suitable for long-term drug administration in diet-induced obesity models by avoiding the recurrent gavage-related stress response and its associated complications.

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REFERENCES

1. Alberti KG, Eckel RH, Grundy SM, *et al.* Harmonizing the Metabolic Syndrome. A Joint Interim Statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. *Circulation* 2009;120:1640-1645.
2. Delavari A, Forouzanfar MH, Alikhani S, *et al*. First nationwide study of the prevalence of the metabolic syndrome and optimal cutoff points of waist circumference in the Middle East: the national survey of risk factors for noncommunicable diseases of Iran. *Diabetes care* 2009; 32: 1092-1097.
3. Marchesini G, Brizi M, Bianchi G, et al. Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. *Diabetes* 2001; 50: 1844-1850.
4. Browning JD, Szczepaniak LS, Dobbins R, et al. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology* 2004; 40: 1387-1395.
5. Angulo P. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; 346: 1221-1231.
6. Grundy SM, Cleeman JI, Daniels SR, *et al*. Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. *Circulation* 2005; 112: 2735-2752.
7. Balcombe JP, Barnard ND, Sandusky C. Laboratory routines cause animal stress. *Contemp Top Lab Anim Sci* 2004; 43: 42-51.
8. Krahn DD, Gosnell BA, Majchrzak MJ. The anorectic effects of CRH and restraint stress decrease with repeated exposures. *Biol Psychiatry* 1990; 27: 1094-1102.
9. Weninger SC, Dunn AJ, Muglia LJ, *et al.* Stress-induced behaviors require the corticotropin-releasing hormone (CRH) receptor, but not CRH. *Proc Natl Acad Sci U S A* 1999; 96: 8283-8288.
10. Michel C, Duclos M, Cabanac M, *et al*. Chronic stress reduces body fat content in both obesity-prone and obesity-resistant strains of mice. *Horm Behav* 2005; 48: 172-179.
11. Tamashiro KL, Hegeman MA, Nguyen MM, *et al*. Dynamic body weight and body composition changes in response to subordination stress. *Physiol Behav* 2007; 91: 440-448.
12. Bartolomucci A, Cabassi A, Govoni P, *et al.* Metabolic consequences and vulnerability to diet-induced obesity in male mice under chronic social stress. *PloS One* 2009; 4: e4331.
13. Collins S, Martin TL, Surwit RS, *et al*. Genetic vulnerability to diet-induced obesity in the C57BL/6J mouse: physiological and molecular characteristics. *Physiol Behav* 2004; 81: 243-248.
14. Pelleymounter MA, Cullen MJ, Baker MB, *et al*. Effects of the obese gene product on body weight regulation in ob/ob mice. *Science* 1995; 269: 540-543.
15. Halaas JL, Gajiwala KS, Maffei M, *et al*. Weight-reducing effects of the plasma protein encoded by the obese gene. *Science* 1995; 269: 543-546.
16. Parekh PI, Petro AE, Tiller JM, *et al*. Reversal of diet-induced obesity and diabetes in C57BL/6J mice. *Metabolism* 1998; 47: 1089-1096.
17. Muniyappa R, Lee S, Chen H, *et al*. Current approaches for assessing insulin sensitivity and resistance in vivo: advantages, limitations, and appropriate usage. *Am J Physiol Endocrinol Metab* 2008; 29: E15-26.
18. Alwayn IP, Andersson C, Lee S, *et al.* Inhibition of matrix metalloproteinases increases PPAR-alpha and IL-6 and prevents dietary-induced hepatic steatosis and injury in a murine model. *Am J Physiol Gastrointest Liver Physiol* 2006; 291: G1011-1019.
19. Brown AP, Dinger N, Levine BS. Stress produced by gavage administration in the rat. *Contemp Top Lab Anim Sci* 2000; 39: 17-21.

20. Germann PG, Ockert D. Granulomatous inflammation of the oropharyngeal cavity as a possible cause for unexpected high mortality in a Fischer 344 rat carcinogenicity study. Lab Anim Sci 1994; 44: 338-343.
21. Germann PG, Ockert D, Heinrichs M. Pathology of the oropharyngeal cavity in six strains of rats: predisposition of Fischer 344 rats for inflammatory and degenerative changes. *Toxicol Pathol* 1998; 26: 283-289.
22. Hunt C, Hambly C. Faecal corticosterone concentrations indicate that separately housed male mice are not more stressed than group housed males. *Physiol Behav* 2006; 87: 519-526.
23. Mostl E, Palme R. Hormones as indicators of stress. *Domest Anim Endocrinol* 2002; 23: 67-74.
24. Good T, Khan MZ, Lynch JW. Biochemical and physiological validation of a corticosteroid radioimmunoassay for plasma and fecal samples in oldfield mice (Peromyscus polionotus). *Physiol Behav* 2003; 80: 405-411.
25. Rowland RR, Reyes E, Chukwuocha R, *et al*. Corticosteroid and immune responses of mice following mini-osmotic pump implantation. *Immunopharmacology* 1990; 20: 187-190.

Chapter 7

Broad-spectrum matrix metalloproteinase inhibition curbs inflammation and liver injury but aggravates experimental liver fibrosis in mice

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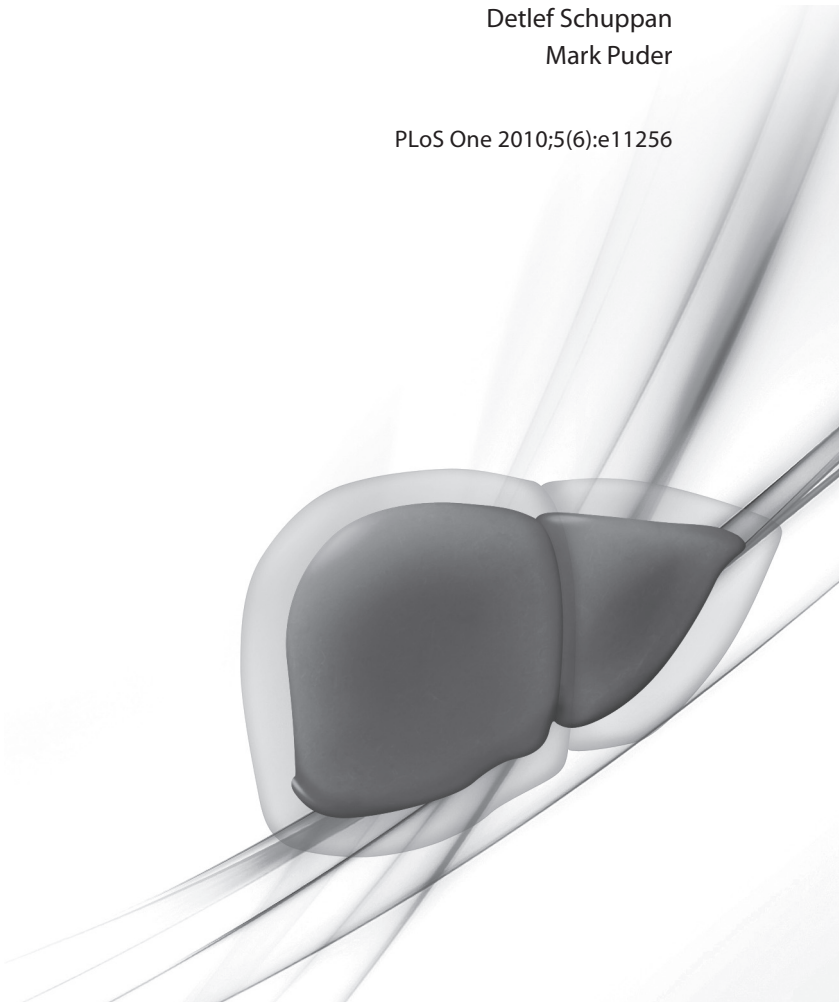

ABSTRACT

Background: Liver fibrosis is characterized by excessive synthesis of extracellular matrix proteins which prevails over their enzymatic degradation, primarily by matrix metalloproteinases (MMPs). The effect of pharmacological MMP inhibition on fibrogenesis, however, is largely unexplored. Inflammation is considered a prerequisite and important co-contributor to fibrosis and is in part mediated by tumor necrosis factor (TNF)-α-converting enzyme (TACE). We hypothesized that treatment with a broad-spectrum MMP and TACE-inhibitor (Marimastat) would ameliorate injury and inflammation, leading to decreased fibrogenesis during repeated hepatotoxin-induced liver injury.

Methods: Liver fibrosis was induced in mice by repeated carbon tetrachloride (CCl_4) administration, during which the mice received either Marimastat or vehicle twice daily. A single dose of CCl_4 was administered to investigate acute liver injury in mice pretreated with Marimastat, mice deficient in *Mmp9*, or mice deficient in both TNF-α receptors. Liver injury was quantified by alanine aminotransferase (ALT) levels and confirmed by histology. Hepatic collagen was determined as hydroxyproline, and expression of fibrogenesis and fibrolysis-related transcripts was determined by quantitative reverse-transcription polymerase chain reaction.

Results: Marimastat-treated animals demonstrated significantly attenuated liver injury and inflammation, but a 25% increase in collagen deposition. Transcripts related to fibrogenesis were significantly less upregulated compared to vehicle treated animals, while MMP expression and activity analysis revealed efficient pharmacologic MMP-inhibition and decreased fibrolysis following Marimastat treatment. Marimastat pre-treatment significantly attenuated liver injury following acute CCl_4-administration, whereas *Mmp9* deficient animals demonstrated no protection. Mice deficient in both TNF-α receptors exhibited an 80% reduction of serum ALT, confirming the hepatoprotective effects of Marimastat via the TNF-signaling pathway.

Conclusions: Inhibition of MMP and TACE activity with Marimastat during chronic CCl_4 administration counterbalanced any beneficial anti-inflammatory effect, resulting in a positive balance of collagen deposition. Since effective inhibition of MMPs accelerates fibrosis progression, MMP inhibitors should be used with caution in patients with chronic liver diseases.

INTRODUCTION

Hepatic fibrosis represents the wound healing response to chronic insult and is the final common pathway for most chronic liver diseases, regardless of their mechanism.[1-3] Progressive fibrosis ultimately leads to increased mortality and morbidity from portal hypertension, end-stage liver failure and ultimately cirrhosis, and is associated with an increased risk of hepatic malignancies.[4] Currently, the only definitive treatment for advanced fibrosis and cirrhosis is liver transplantation; however, the demand for organ grafts outweighs their availability[5], stressing the need for effective antifibrotic approaches.[6,7]

Hepatocellular injury usually leads to inflammation and activation of the innate immune system, leading to release of growth factors, cytokines and small molecular mediators that can stimulate extracellular matrix (ECM) synthesis by activation of quiescent hepatic stellate cells and fibroblasts/myofibroblasts (collectively named HSCs).[1,2]. Upon fibrogenic activation, HSCs as well as inflammatory cells release and respond to the cytokine transforming growth factor (TGF)-β.[8] TGF-β strongly upregulates production and deposition of the major ECM constituents, while it downregulates fibrolytic matrix metalloproteinases (MMPs).[8,9] In the presence of chronic hepatic injury, an imbalance between fibrogenesis and fibrolysis may lead to excess ECM deposition and scar formation.

Cell surface-bound and soluble MMPs along with their endogenous tissue inhibitors (TIMPs) constitute an important system for regulating ECM turnover; however, MMPs also regulate inflammatory processe.[10] Chronic inflammation is an important driver in fibrogenesis, serving both as a trigger and perpetuator of fibrosis progression.[11] A critical mediator of the inflammatory response is tumor necrosis factor (TNF)-α, which exists in a biologically active, soluble form and as an inactive, membrane-anchored precursor.[12] Cleavage of the TNF-α proform into its soluble form is mediated by TNF-α-converting enzyme (TACE, also known as ADAM17 and CD156b), which belongs to the disintegrin and metalloproteinase (ADAM) family of zinc-metalloproteinases.[13,14] Mice deficient in TIMP3, the endogenous physiological inhibitor of TACE[15], demonstrate elevated levels of TNF-α and develop severe inflammation of the liver, presumably due to depressed TACE activity.[16] In contrast, pharmacologic TACE-inhibition abrogates the inflammatory response and has been demonstrated to have therapeutic potential in a variety of pathological conditions.[17,18] Many TACE-inhibitors, however, are relatively non-specific and also inhibit various MMPs.

MMPs are widely believed to be important players in fibrosis due to their collagen-cleaving activity.[19-21] Identification of novel MMP substrates, however, revealed their involvement in highly complex processes such as the regulation of cell behavior, cell-cell communication, and tumor progression.[22,23] Hence, these insights indicate that MMPs have a much more complex function in fibrosis than merely ECM degradation. Effects of MMP-inhibition on fibrogenesis, however, remain to be established. We hypothesized that treatment with a broad-spectrum MMP and TACE-inhibitor would ameliorate both injury and inflammation, resulting in decreased fibrosis formation in a murine model of repeated carbon tetrachloride (CCl_4) administration.

METHODS

Animals

Male 6-week-old C57BL6/J mice (Jackson Laboratories, Bar Harbor, ME) were housed five per cage on paper chip bedding in a barrier room with regulated temperature (21°C ± 1.6°C), humidity (45% ± 10%), and an alternating 12-hour light and dark cycle. The animals had free access to water and standard American Institute of Nutrition (AIN) 93M (TD.94048; Harlan Teklad, Madison, WI) purified rodent diet throughout the study. Animal protocols complied with the National Institutes of Health Animal Research Advisory Committee guidelines and were approved by the Children's Hospital Boston Animal Care and Use Committee (protocol no. A06-08-065R).

Animal experiments

After one week of acclimation, 40 C57BL6/J mice were randomized into 4 groups (10 mice each). The first week, two groups received 100 mg/kg of Marimastat (BB-2516, British Biotech, UK) in 0.45% methylcellulose (Sigma-Aldrich, St. Louis, MO) vehicle twice daily via orogastric gavage (MAR), and two groups received vehicle alone (VEH). These treatments were continued until study completion. Marimastat efficiently inhibits MMP-2, MMP-3, MMP-7, MMP-9, MMP-13 and TACE activity, with IC_{50}s in the nM range.[26,54] After one week of treatment with either Marimastat or vehicle, animals received three intragastric doses via oral gavage (Monday, Wednesday and Friday) of CCl_4 (anhydrous, ≥99.5%, Sigma-Aldrich, St. Louis, MO) dissolved in mineral oil (Sigma-Aldrich, St. Louis, MO) or vehicle alone each week for another six weeks. The initial dose was 0.875 mL/kg, followed by 8 doses of 1.75 mL/kg and 9 doses of 2.5 mL/kg, respectively. At the end of the seven week treatment period and three days after the last dose of CCl_4, animals were sacrificed to evaluate hepatic fibrosis and related parameters (**Figure 1A**).

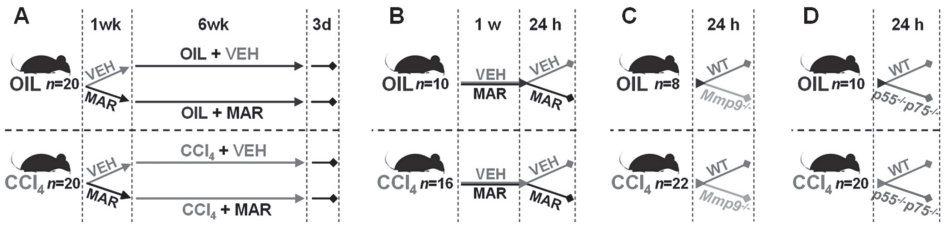

Figure 1. Design of the studies. Two groups of C57Bl/6J mice were randomized to two subgroups to receive either Marimastat or methylcellulose vehicle twice daily (A). Liver fibrosis was induced by repeated carbon tetrachloride (CCl4) administration for 6 weeks in one group of animals, whereas a second group of control animals received the mineral oil vehicle alone (A). The protective effects of Marimastat were further evaluated in a model of acute CCl_4-induced hepatotoxicity (B). C57Bl/6J mice received either Marimastat or methylcellulose vehicle twice daily for 1 week, after which they were subjected to a single dose of CCl_4 or mineral oil as control (B). The mechanism was further elucidated by subjecting $Mmp9^{-/-}$ mice and their wild type (WT) littermates (C), or TNF $55^{-/-}$ $p75^{-/-}$ mice and their WT littermates (D) to either a single dose of CCl_4, or mineral oil. In all acute CCl4-experiments, animals were sacrificed after 24 hours. Oil, non-fibrotic control group; CCl_4, fibrotic mice; VEH, vehicle treated control group; MAR, Marimastat treated experimental group; WT, wild type.

In separate experiments, the effect of MMP inhibition on acute liver injury was studied in 64 C57BL6/J mice after orogastric administration of a single dose of 1.5 mL/kg CCl_4. Starting one week prior to the hepatotoxic challenge, 32 mice received 100 mg/kg of Marimastat twice daily via orogastric gavage (MAR), and 32 mice received vehicle alone (VEH). Treatment was continued until sacrifice. Animals were sacrificed 12 h, 24 h, 48 h and 96 h after CCl_4 administration (n=8 per treatment and time point) to evaluate hepatic injury (**Figure 1B**). Since the peak of hepatic injury occurred after 24 h, this time point was chosen as surrogate endpoint in subsequent experiments.

The contribution of MMP and TACE-dependent pathways was studied using mice deficient in MMP-9, as well as mice deficient in both TNF-α receptors. Thirty mice homozygous null for the *Mmp9* gene (B6.FVB(Cg)-*Mmp9*^{tm1Tvu}/J mice; Jackson Laboratories, Bar Harbor, ME) and their wild type (WT) littermates, and 30 mice homozygous for both TNF-α receptor I (*Tnfrsf1a*^{tm1Imx}; p55) and TNF-α receptor II (*Tnfrsf1b*^{tm1Imx}; p75) null mutations (B6;129S-*Tnfrsf1a*^{tm1Imx} *Tnfrsf1b*^{tm1Imx}/J; Jackson Laboratories, Bar Harbor, ME) and their WT littermates were subjected to either a single dose of 1.5 mL/kg CCl_4 via orogastric gavage, or mineral oil (**Figures 1C, D**). Animals were sacrificed after 24 h to evaluate hepatic injury.

Sample collection and serum biochemistry

Animals in all groups were euthanized, and pieces of their livers fixed in 10% formalin for histology or immediately frozen and stored for RNA extraction and hydroxyproline measurements. Blood was collected via retro-orbital sinus puncture and plasma was obtained via centrifugation. Serum was frozen at -80°C for analysis of alanine amino-

transferase (ALT) and alkaline phosphatase (AP) activities at the Clinical Laboratory of Children's Hospital Boston. Serum levels of IL-6 and the soluble TNF-α receptor II (p75) were determined using a commercial ELISA kit (Quantikine, R&D Systems, Minneapolis, MN). Optical density was read at 450 nm and analyzed with Softmax PRO Software (Molecular Devices, Sunnyvale, CA).

Histology

Paraffin-embedded sections from the frontal lobes of the liver were stained by hematoxylin and eosin (H&E) to examine cellular architecture and lipid accumulation. A pathologist (VN) blinded to the treatment groups conducted a histological analysis of the liver sections [55]. Lobular inflammation was quantified by assessing the number of inflammatory foci per microscopic field. Five fields were checked at 200x magnification as follows: 0 (absent), 1 (<2 foci), 2 (2-4 foci), and 3 (>4 foci). Steatosis was scored by the percentage (%) of liver cells containing fat: 0 (<5%), 1 (5-33%), 2 (>33-66%) and 3 (>66%). Necrosis was scored as 0 (absent) or 1 (pericentral area occupied by necrosis). Masson trichrome (MT) and Sirius Red stains of paraffin-embedded sections were used to qualitatively assess collagen architecture and extent of fibrosis. Morphometric analysis for fibrosis quantification was performed using ten random high power fields (HPF) per animal at 200x magnification. These images were quantified using NIH ImageJ software (http://rsb.info.nih.gov/ij/).

Immunohistochemistry

Immunohistochemistry was performed using 4 μm thick formalin-fixed, paraffin-embedded tissue sections. Briefly, slides were soaked in xylene, passed through graded alcohols and put in distilled water. Slides were then pre-treated with 1.0-mM EDTA, pH 8.0 (Zymed, South San Francisco, CA) for anti-CD3 or Citrate buffer for anti-alpha smooth muscle actin (α-SMA) in a steam pressure cooker (Decloaking Chamber, BioCare Medical, Walnut Creek, CA) as per manufacturer's instructions followed by washing in distilled water. All further steps were performed at room temperature in a hydrated chamber. Slides were pre-treated with Peroxidase Block (DAKO USA, Carpinteria, CA) for 5 minutes to quench endogenous peroxidase activity. For CD3, polyclonal rabbit anti-murine CD3 antibody (Cell Marque, Rocklin, CA. Cat #CMC363) was applied 1:1500, and for α-SMA, rabbit anti-murine α-SMA (Abcam, Cambridge, MA, Cat #ab5694) was applied 1:200 in diluent (DAKO) for 1 hour. Slides were washed in 50-mM Tris-Cl, pH 7.4, and antigens detected with anti-rabbit Envision+ kit (DAKO) as per manufacturer's instructions. After further washing, immunoperoxidase staining was developed using a DAB chromogen (DAKO) and counterstained with hematoxylin. For F4/80 staining, slides were incubated with proteinase K applied 1:5 in DAKO diluent for 10 minutes and then washed in 50-mM Tris-Cl, pH 7.4, followed by incubation with rat F4/80 antibody (Serotec, Raleigh, NC,

cat# MCA497GA) applied 1:10,000 in DAKO diluent for 1 hour. Slides were then washed and incubated with rabbit anti-rat secondary (DAKO) diluted 1:750 for 30 minutes, washed, and detected with anti-rabbit Envision+ kit (DAKO) as described above. For quantification purposes, positive cells were counted in ten random HPF per animal at 200x magnification and expressed as mean positive cells / 10 HPF.

Liver hydroxyproline determination

Hepatic collagen content was quantified biochemically by determining liver hydroxyproline using an established method with minor modifications.[56-58] Briefly, snap-frozen liver tissue from two different lobes (50-60 mg each) was hydrolyzed at 110°C for 16h in 5mL 6N HCl. The hydrolysate was filtered, 50-µL aliquots were evaporated under vacuum, and the sediment was dissolved in 1.2 mL of 50% isopropanol and incubated with 0.2 mL of 0.84% chloramine-T in 42 mmol/L sodium acetate, 2.6 mmol/L citric acid, and 39.5% (vol/vol) isopropanol (pH 6.0), followed by incubation for 10 minutes at room temperature. Next, 0.248 g *p*-dimethylaminobenzaldehyde, dissolved in 0.27 mL of 60% perchloric acid, and 0.73 mL isopropanol were added and incubated at 50°C for 90 minutes. Relative hydroxyproline (µg/g liver) was then quantified photometrically at 558 nm and total hydroxyproline (mg/whole liver) was calculated based on individual liver weights and the corresponding relative hydroxyproline content from representative liver samples, as established previously.[57,58]

Quantitative Real-Time RT-PCR

200-300mg snap-frozen liver tissue from two lobes was homogenized; total RNA was extracted using Tri Reagent (Molecular Research Center, Cincinnati, OH) and reverse transcribed as described .[58,59] Relative transcript levels were quantified by real-time RT-PCR on a LightCycler 1.5 instrument (Roche, Mannheim, Germany) using the TaqMan methodology as described in detail.[57-59] TaqMan probes (dual-labeled with 5'-FAM and 3'-TAMRA) and primers were designed based on published sequences (**Table 1**) using the Primer Express software (Perkin Elmer, Wellesley, USA), synthesized at MWG Biotech AG (Ebersberg, Germany), and are published elsewhere.[57,59] The housekeeping gene beta-2 microglobulin (β2MG) was amplified in parallel reactions for normalization.

Gelatinase and Interstitial Collagenase Activity Assays

Determination of gelatinase and interstitial collagenase activity was performed as described previously with assays based on degradation of DQ-gelatin and DQ-collagen type I, respectively (Molecular Probes Inc., Eugene, OR).[58,59] DQ-substrates are heavily labeled with FITC which quenches their fluorescence, but fluorescent peptides are generated upon proteolytic cleavage. 20µL of 10% liver homogenates prepared as described previously[57] were diluted in a total volume of 200µL of MMP-activity buffer (50mM Tris-

Table 1. Primers and probes used in quantitative real-time RT-PCR.

Target gene	5´-Primer	TaqMan probe	3´-Primer
Procoll. α1(I)	TCCGGCTCCTGCTCCTCTTA	TTCTTGGCCATGCGTCAGGAGGG	GTATGCAGCTGACTTCAGGGATGT
β6 Integrin	GCAGAACGCTCTAAGGCCAA	TGGCAAACGGGAACCAATCCTCTGT	AAAGTGCTGGTGGAACCTCG
TGF-β1	AGAGGTCACCCGCGTGCTAA	ACCGCAACAACGCCATCTATGAGAAAACCA	TCCCGAATGTCTGACGTATTGA
TGF-β2	GTCCAGCCGGCGGAA	CGCTTTGGATGCTGCCTACTGCTTTAGAAAT	GCGAAGGCAGCAATTATCCT
α-SMA	ACAGCCCTCGCACCCA	CAAGATCATTGCCCCTCCAGAACGC	GCCACCGATCCAGACAGAGT
TIMP-1	TCCTCTTGTTGCTATCACTGATAGCTT	TTCTGCAACTCGGACCTGGTCATAAGG	CGCTGGTATAAGGTGGTCTCGTT
TNF-α	GGGCCACCACGCTCTTC	ATGAGAAGTTCCCAAATGGCCTCCCTC	GGTCTGGGCCATAGAACTGATG
MMP-2	CCGAGGACTATGACCGGGATAA	TCTGCCCCGAGACCGCTATGTCCA	CTTGTTGCCCAGGAAAGTGAAG
MMP-3	GATGAACGATGGACAGAGGATG	TGGTACCAACCTATTCCTGGTTGCTGC	AGGGAGTGGCCAAGTTCATG
MMP-8	CAGGGAGAAGCAGACATCAACA	TGCTTTCGTCTCAAGAGACCATGGTGAC	GATTCCATTGGGTCCATCAAA
MMP-9	CAGGATAAACTGTATGGCTTCTGC	CTACCCGAGTGGACGCGACCGT	GCCGAGTTGCCCCCA
MMP-13	GGAAGACCCTCTTCTTCTCT	TCTGGTTAACATCATCATAACTCCACACGT	TCATAGACAGCATCTACTTTGTT
β2MG	CTGATACATACGCCTGCAGAGTTAA	GACCGTCTACTGGGATCGAGACATGTG	ATGAATCTTCAGAGCATCATGAT

HCl, 150mM NaCl, 5mM CaCl2, 0.025% Brij 35™, pH 7.5, supplemented with EDTA-free protease inhibitor cocktail (Complete™, Roche Applied Science, Mannheim, Germany)) and incubated with DQ-substrates (25μg/mL) and increasing doses of Marimastat dissolved in H_2O at room temperature for 1-20h in 96-well plates in triplicates. Fluorescence was measured using the Wallac Victor2 Multilabel Counter (PerkinElmer, Inc., Waltham, MA); with excitation at 485nm, and emission at 535nm. Human recombinant activated MMP-1 (collagenase) and MMP-2 (gelatinase) were used as positive controls. Since all procedures including extraction were performed in the presence of protease inhibitors to prevent *ex vivo* MMP activation, data obtained represent the net endogenous gelatinolytic and collagenolytic activities.

Statistical analysis

Data are expressed as means ± standard error of the mean (SEM). Data sets involving more than two groups were assessed by analysis of variance (ANOVA). Differences between two groups were assessed using the unpaired two-tailed Student's *t* test, or if nonparametric, by using the Mann-Whitney *U* test. $P \leq 0.05$ was considered statistically significant. All data were collected in a computerized Microsoft Excel database (Microsoft Inc., Redmond, WA). The analysis was performed with SPSS version 16.0 (SPSS Inc., Chicago, IL) statistical software, and figures were created using GraphPad Prism version 5.0 (GraphPad Software Inc., La Jolla, CA) software.

RESULTS

Chronic broad-spectrum MMP-inhibition dramatically reduces histological liver injury in mice subjected to chronic CCL_4-intoxication

Chronic CCl_4-administration resulted in liver enlargement and fibrosis (**Figure 2A**). Liver sections of vehicle treated controls exhibited areas of necrosis, steatosis, and inflamma-

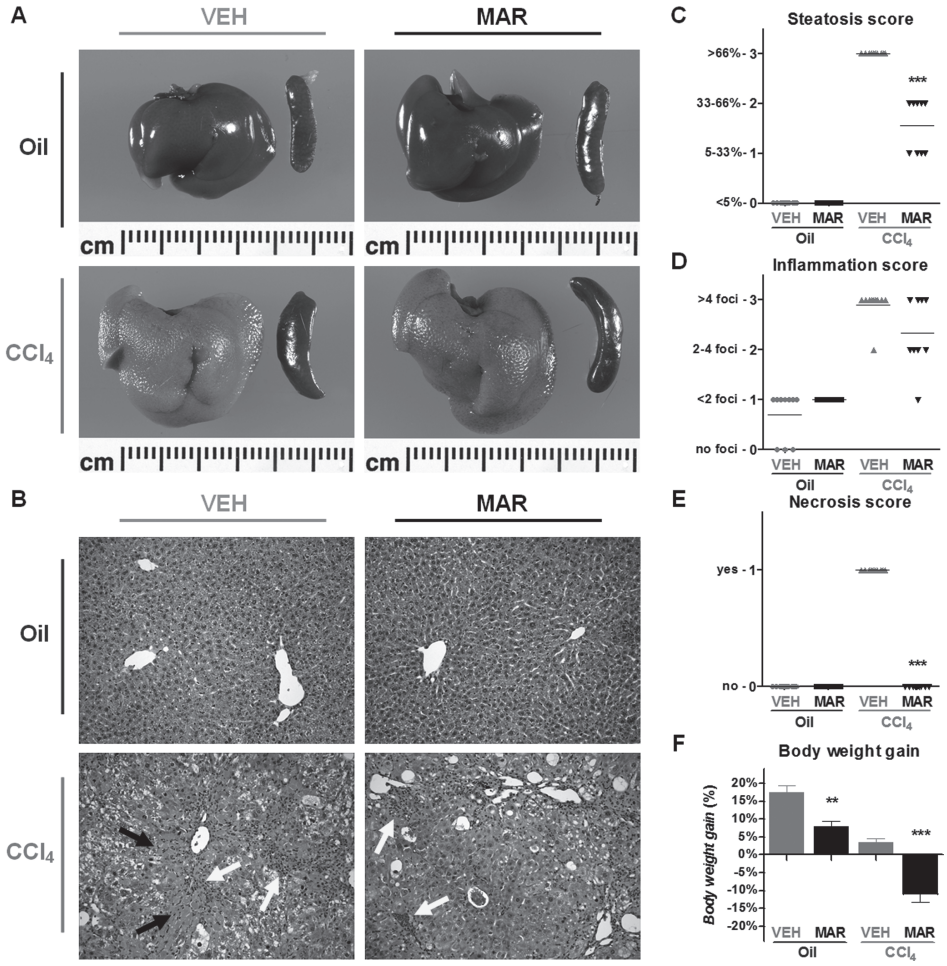

Figure 2. Marimastat treatment reduced liver injury, necrosis, and inflammation following repeated carbon tetrachloride (CCl_4) administration. Chronic CCl_4 administration resulted in liver enlargement and fibrosis (A). Hematoxylin and eosin staining of liver sections revealed decreased steatosis and inflammation (yellow arrows), and no evidence of necrosis (black arrows) in the Marimastat treated mice (B). On liver sections scored by a blinded pathologist and compared to vehicle treated controls, Marimastat treated animals showed a significantly lower steatosis score (C), less inflammatory foci per 200x field (D) and essentially no evidence of necrosis was observed (E); despite body weight loss (F). Oil, non-fibrotic control group; CCl_4, fibrotic mice; VEH, vehicle treated control group; MAR, Marimastat treated experimental group; ***, $P<0.001$ vs. vehicle alone. Data are expressed as means ± standard error. Original magnification: 200x.

tory lymphocytic infiltrates –hallmarks of severe chronic hepatic injury (**Figure 2B**). Liver sections from Marimastat treated animals, however, showed a significant reduction in steatosis (**Figure 2C**), inflammation (**Figure 2D**) and necrosis (**Figure 2E**), suggesting attenuation of hepatic injury and inflammation, despite a loss of body weight (**Figure 2F**).

Marimastat treatment markedly blunts the increase of serum ALT and levels of TNF-α receptor II in CCl_4-induced chronic hepatic injury

Marimastat treatment resulted in a 1.4-fold reduction of alkaline phosphatase levels ($P \leq 0.05$, **Figure 3A**) and a 14-fold decrease in serum ALT levels ($P \leq 0.05$, **Figure 3B**), indicating markedly decreased hepatic injury following repeated CCl_4-administration. Serum levels of soluble TNF-α receptor II (p75) were 1.2-fold decreased in Marimastat treated animals ($P \leq 0.05$), likely reflecting pharmacologic inhibition of TACE and an ameliorated inflammatory response (**Figure 3C**).[22,24] Serum IL-6 levels were increased 2.9-fold ($P \leq 0.05$), suggesting hepatoprotection and stimulated liver regeneration (**Figure 3D**).[25]

Marimastat treatment leads to downregulation of major pro-fibrogenic genes

Hepatic expression of procollagen α1(I), β6 Integrin, TGF-β1, TGF-β2, alpha-smooth muscle actin (α-SMA) and TIMP-1 mRNA were strongly upregulated following repeated CCl_4-administration (**Figure 4**). Concomitant treatment with Marimastat, however, significantly decreased hepatic transcript levels of procollagen α1(I) (**Figure 4A**), TGF-β2 (**Figure 4D**), α-SMA (**Figure 4E**) and TIMP-1 (**Figure 4F**) compared to vehicle treated controls, whereas β6 Integrin (**Figure 4B**) and TGF-β1 (**Figure 4C**) mRNA levels remained unchanged.

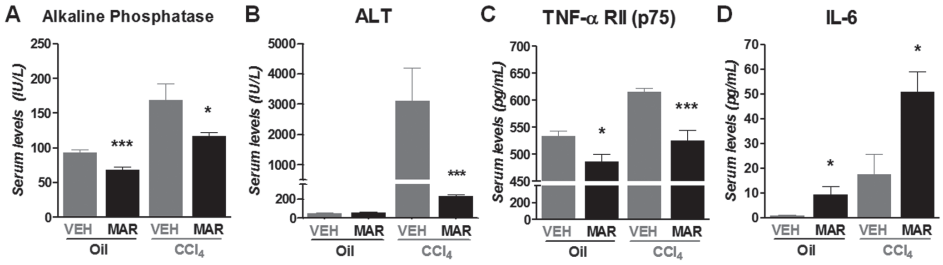

Figure 3. Marimastat treatment ameliorated hepatic injury and the inflammatory response following repeated carbon tetrachloride (CCl_4) administration. Marimastat treatment significantly reduced serum alkaline phosphatase levels (A), and resulted in a 14-fold decrease of serum ALT (B), indicating decreased hepatic injury. Serum TNF-α receptor II (p75) levels as measured by ELISA decreased following Marimastat treatment, suggesting successful inhibition of TNF-α converting enzyme (TACE) and an ameliorated inflammatory response. IL-6 serum levels as measured by ELISA increased following Marimastat treatment, suggesting hepatoprotection and stimulated liver regeneration (D). Oil, non-fibrotic control group; CCl_4, fibrotic mice; VEH, vehicle treated control group; MAR, Marimastat treated experimental group; ALT, alanine aminotransferase; TNF, tumor necrosis factor; IL, interleukin; *, $P<0.05$; ***, $P<0.001$ vs. vehicle alone. Data are expressed as means ± standard error.

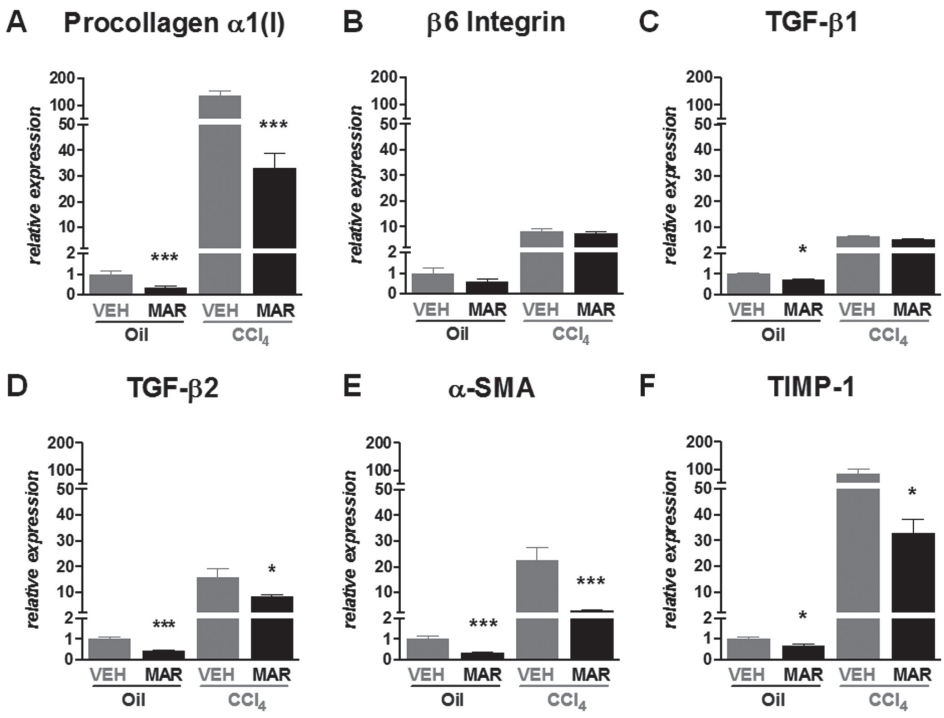

Figure 4. Marimastat altered the hepatic fibrosis-related gene expression profile by downregulation of multiple pro-fibrogenic transcripts following repeated carbon tetrachloride (CCl_4) administration. Hepatic procollagen α1(I) (A), β6 integrin (B), TGF-β1 (C), TGF-β2 (D), α-SMA (E) and TIMP-1 (F) expression as quantified by real-time RT-PCR in total liver RNA. Oil, non-fibrotic control group; CCl_4, fibrotic mice; VEH, vehicle treated control group; MAR, Marimastat treated experimental group; TGF, transforming growth factor; SMA, smooth muscle actin; TIMP, tissue inhibitor of metalloproteinases; *, $P<0.05$; ***, $P<0.001$ vs. vehicle alone. Data are expressed as means ± standard error and in arbitrary units relative to non-fibrotic vehicle treated control.

Marimastat does not diminish net collagen deposition and fibrosis formation

Treatment of mice with Marimastat during chronic CCl_4-administration significantly increased the liver and the spleen (a putative marker of portal hypertension) to body weight ratios, compared to controls (**Figure 5A, B**). Liver sections of the vehicle treated controls exhibited centrilobular fibrosis with areas of necrosis, whereas liver sections from Marimastat treated animals showed enhanced centrilobular collagen deposition indicating increased fibrosis formation (**Figure 5C**). To directly quantify the degree of fibrosis, we measured both relative (per g of liver) and total (per whole liver) collagen content biochemically via hepatic hydroxyproline determination. Marimastat treatment resulted in a significant increase in relative and total collagen (hydroxyproline) content (25% and 14%, respectively) compared to the controls (**Figures 5D, E**). This was corroborated using morphometric analysis of Sirius Red stained liver sections (**Figure 5F**), demonstrating that the relative fibrotic area was significantly increased in livers from mice treated with

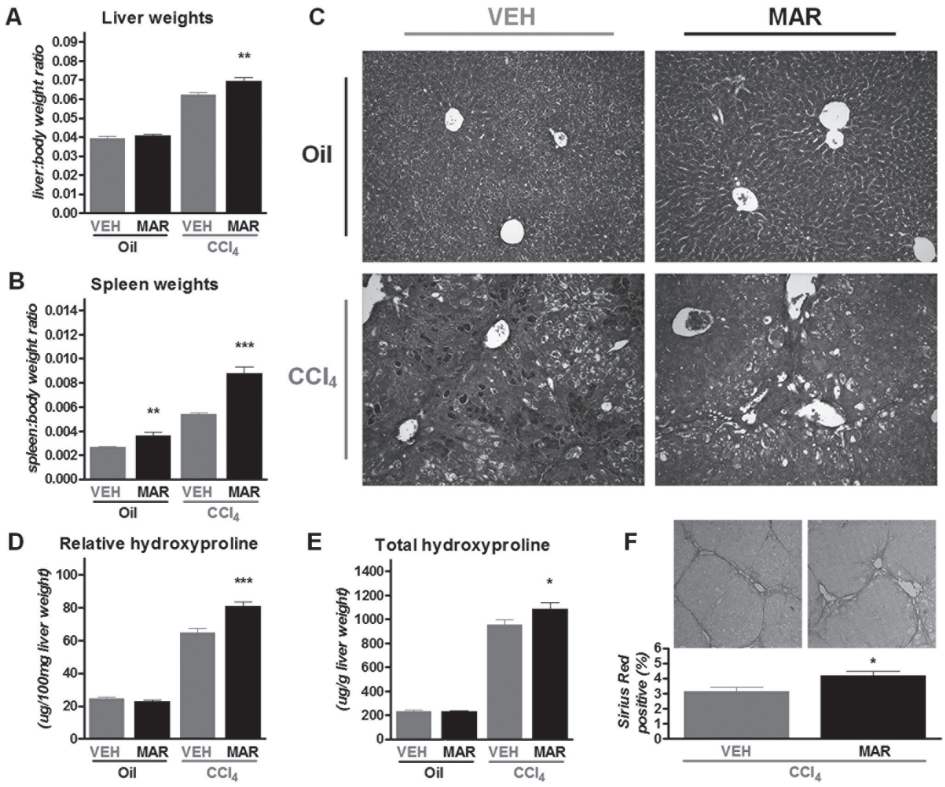

Figure 5. Marimastat treatment increased hepatic fibrosis following repeated carbon tetrachloride (CCl_4) administration. In mice treated with Marimastat, the liver to body weight ratio (A), as well as the spleen to body weight ratio (B) were increased. Masson trichrome staining of liver sections for collagen (blue; C) revealed bridging portal fibrosis. Livers from Marimastat treated animals showed occasional focal cirrhosis; however, advanced fibrosis was predominant (C). Livers from Marimastat treated mice exhibited increased collagen deposition as determined biochemically as relative hydroxyproline content and total hydroxyproline content in liver samples from two different lobes (D, E). Relative fibrotic area was increased in livers from mice treated with Marimastat, as quantified using morphometric analysis of Sirius Red stained liver sections (F). Oil, non-fibrotic control group; CCl_4, fibrotic mice; VEH, vehicle treated control group; MAR, Marimastat treated experimental group; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ vs. vehicle alone. Data are expressed as means ± standard error.

Marimastat, compared to controls (4.2% versus 3.1%, respectively; $P<0.05$). These results suggest dissociation between hepatic injury and inflammation on the one hand, and the degree of fibrosis on the other hand, upon pharmacological MMP inhibition.

Marimastat decreases hepatic stellate cell activation, but increases recruitment of inflammatory cells

Immunohistochemical staining for α-SMA (**Figure 6A**), as a marker for activated HSCs, was performed to confirm the observed decrease in α-SMA mRNA (**Figure 4E**). α-SMA positive cells were significantly increased following chronic CCl_4-administration;

however, concomitant treatment with Marimastat significantly decreased this number indicating decreased activation of a subset of activated HSCs (**Figure 6A,B**). This again suggests that Marimastat treatment led to a decrease in fibrogenic activity, which is in dissociation with the observed, net result of increased fibrosis (**Figure 5**). To further explore this finding, immunohistochemical staining for T-cells and macrophages (i.e., Kupffer cells) was performed. Chronic administration of CCl_4 induces an inflammatory response, elicited by accumulation of T-cells and macrophages that remove injured hepatocytes and stimulate fibroblast function.[26]. Immunohistochemical staining for

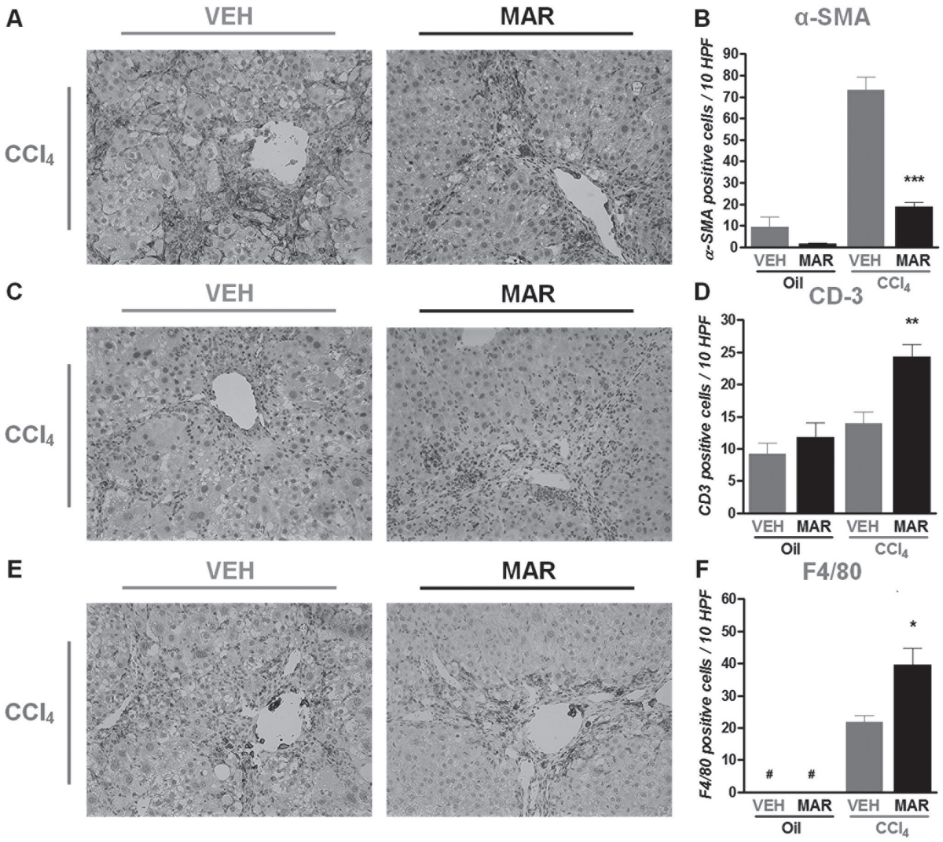

Figure 6. Marimastat decreases hepatic stellate cell (HSC) activation, but increases recruitment of inflammatory cells. Chronic CCl_4 administration in animals treated with Marimastat resulted in a decreased activation of HSCs, as identified by alpha-smooth muscle actin (α-SMA) staining (A). Quantification revealed that following chronic CCl_4 administration, Marimastat treated animals had a 74% decrease of activated HSCs, compared to controls (B). Liver sections from animals that were chronically challenged with CCl_4 showed that resident T cells (CD3, C,D) and macrophages (F4/80, E,F) counts increased up to 2-fold upon Marimastat treatment. Oil, non-fibrotic control group; CCl_4, fibrotic mice; VEH, vehicle treated control group; MAR, Marimastat treated experimental group; α-SMA, alpha-smooth muscle actin; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ vs. vehicle alone. Data are expressed as means ± standard error. Original magnification: 200x.

CD3 (**Figure 6C**), as a marker for T-cells, revealed a 1.8-fold increase in cell counts in Marimastat-treated animals that were subjected to chronic CCl_4-administration (**Figure 6D**). In addition, cell counts from F4/80 positive cells, as a marker for macrophages, revealed a 1.8-fold increase following treatment of mice with Marimastat during chronic CCl_4-administration (**Figure 6E,F**).

Marimastat treatment downregulates MMP gene expression and MMP-activities

To better understand the increased ECM deposition in animals treated with Marimastat, we analyzed hepatic MMP expression levels. In fibrotic animals, hepatic expression of MMP-2, MMP-3, MMP-8, MMP-9 and MMP-13 mRNA were all significantly upregulated, compared to non-fibrotic controls (**Figure 7**). Marimastat treatment during chronic CCl_4 administration, however, did not affect hepatic transcript levels of MMP-2 (**Figure 7A**), or MMP-3 (**Figure 7B**), compared to vehicle treated animals. Hepatic transcript levels

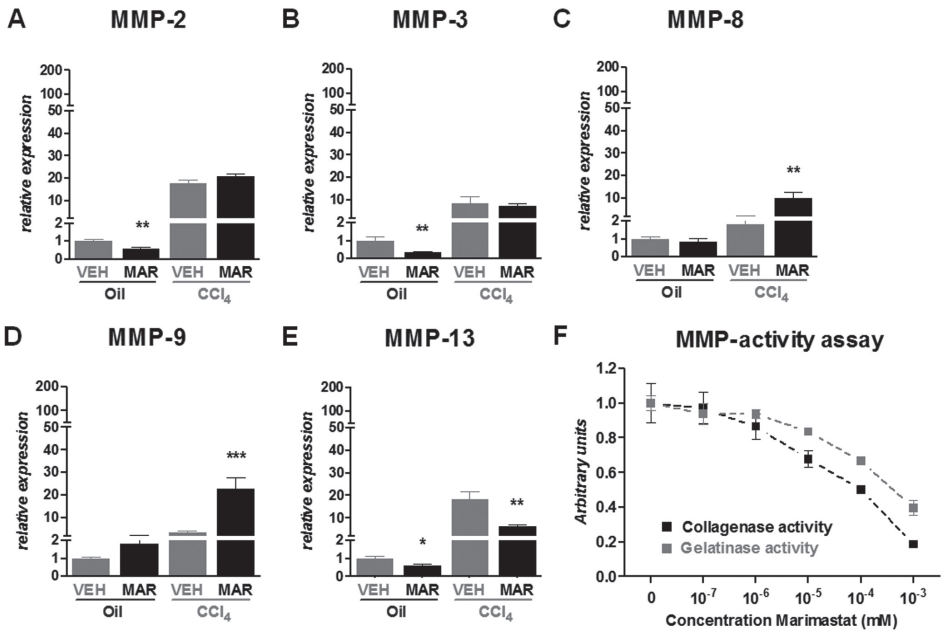

Figure 7. Marimastat treatment downregulates MMP gene expression and MMP-activities. Following repeated carbon tetrachloride (CCl_4) administration, Marimastat did not affect hepatic transcript levels of MMP-2 (A), or MMP-3 (B) as quantified by real-time RT-PCR. Hepatic MMP-8 (C) and MMP-9 (D) transcripts were significantly upregulated in livers from Marimastat treated animals, whereas hepatic MMP-13 mRNA was significantly higher in the vehicle treated animals (E). Marimastat inhibits interstitial collagenolytic and gelatinolytic activity in a dose-dependent matter (F). Relative gelatinase and interstitial collagenase activities after 4 hours in liver homogenates supplemented with increasing concentrations of Marimastat, as determined by degradation of DQ-gelatin and collagen (black and grey squares, respectively). Oil, non-fibrotic control group; CCl_4, fibrotic mice; VEH, vehicle treated control group; MAR, Marimastat treated experimental group; TNF, tumor necrosis factor; MMP, matrix metalloproteinases; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ vs. vehicle alone. Data are expressed as means ± standard error and in arbitrary units.

of MMP-8 (**Figure 7C**) and MMP-9 (**Figure 7D**) were 5.6-fold and 6.6-fold increased, respectively, in livers from Marimastat treated animals, whereas hepatic MMP-13 transcripts were 3.0-fold higher in the vehicle treated animals (**Figure 7E**). Increased MMP expression levels may have resulted from a positive feedback mechanism resulting from efficient pharmacologic inhibition of MMP-activity, although this would not explain downregulation of MMP-13.[22] We therefore performed an assay to investigate the ability of Marimastat to inhibit gelatinase (MMP-2, MMP-9) and interstitial collagenase (MMP-1) activities. Relative collagenase activity as well as relative interstitial gelatinase activity in liver homogenates supplemented with increasing concentrations of Marimastat showed a dose-dependent inhibition of both these MMP-activities (**Figure 7F**). These findings indicate efficient inhibition of putatively fibrolytic MMPs by Marimastat, potentially leading to increased fibrosis in the presence of continuous hepatic injury.

Broad spectrum MMP-inhibition with Marimastat using a model of acute administration of a single dose of CCl_4 predicts the evolution of the chronic CCl_4 model

To further explore the anti-inflammatory and hepatoprotective potential of pharmacologic broad-spectrum MMP and TACE inhibition, C57Bl/6J mice were pretreated for 7 days with Marimastat and subsequently challenged with a single dose of CCl_4. Analysis of liver sections at 12 h, 24 h, 48 h and 96 h time points revealed that acute CCl_4 intoxication resulted in severe necroinflammatory injury around the central vein areas with a peak at 24 h, regeneration and repair at 48 h characterized by influx of macrophages and inflammatory lymphocytic infiltrates, and impressive resolution of necrotic injury after 96 h (data not shown). After 24 h, Marimastat pre-treatment attenuated necroinflammatory hepatic injury as determined by histology (**Figure 8A**), and resulted in a 57% reduction in serum ALT levels ($P \leq 0.05$, **Figure 8B**). Since the peak of hepatic injury occurred after 24 h, this time point was chosen as surrogate endpoint in subsequent experiments.

Marimastat reduces necroinflammatory injury following a single dose of CCl_4 via a TNF-dependent pathway.

Next, we studied if the anti-inflammatory and hepatoprotective effects of Marimastat could be attributed to either an MMP- or a TACE- dependent mechanism by using gene deleted animals. MMP-9 is the major and most studied and abundant MMP in inflammation[10,27], playing a critical role in fulminant TNF-mediated hepatitis[28], mediating hepatic ischemia/reperfusion injury[29] and being involved in liver regeneration.[30] Given that Marimastat efficiently inhibits MMP-9 activity (**Figure 7**), *Mmp9* homozygous null mice (*Mmp9*$^{-/-}$) and their WT littermates were subjected to a single dose of CCl_4, or vehicle (mineral oil) to investigate the involvement of a single MMP, rather than multiple MMPs by pharmacologic broad-spectrum MMP-inhibition on CCl_4-induced hepatotoxicity. After 24 h liver sections from WT animals and *Mmp9*$^{-/-}$ animals exhibited extensive cen-

trilobular necroinflammatory changes (**Figure 8C**). Serum ALT levels were 28,410 IU/L in WT animals compared to 28,100 IU/L in *Mmp9*$^{-/-}$ animals (*P*=0.89; **Figure 8D**). Taken together, these findings do not indicate that MMP-9 plays a significant role in the acute phase of CCl_4-induced hepatic injury.

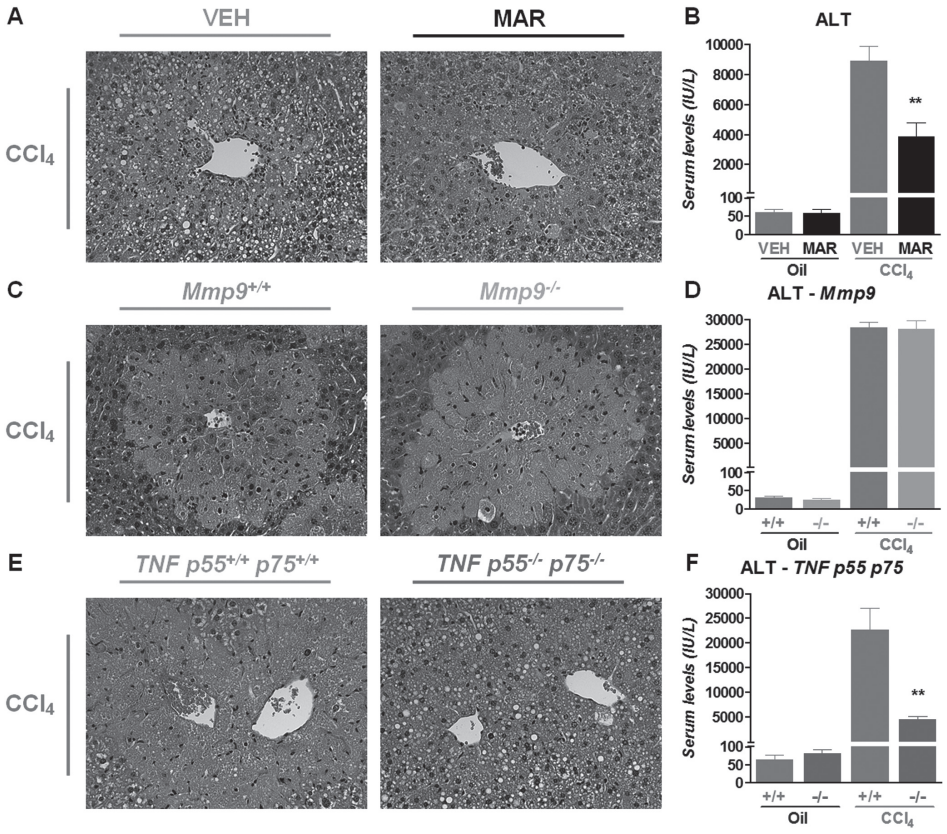

Figure 8. Marimastat reduces necroinflammatory injury following a single dose of carbon tetrachloride (CCl_4) via a TNF-dependent pathway. Liver sections from Marimastat-treated animals that had been administered a single dose of CCl_4 revealed decreased necroinflammatory injury after 24 h (A). Marimastat treatment resulted in a 57% decrease of serum ALT levels (B). Liver sections from *Mmp9*$^{-/-}$ and wild type (*Mmp9*$^{+/+}$) animals that were challenged with a single dose of CCl_4 showed comparable, extensive necroinflammatory changes around the central veins after 24 h (C). Serum ALT levels of *Mmp9*$^{-/-}$ animals were similar to those of wild type controls (D). 24 h after a single dose of CCl_4, liver sections from wild type (*TNF p55*$^{+/+}$ *p75*$^{+/+}$) animals revealed extensive necrosis and inflammation around the central veins, whereas *TNF p55*$^{-/-}$ *p75*$^{-/-}$ animals showed markedly reduced hepatic injury (E). Serum ALT levels in *TNF p55*$^{-/-}$ *p75*$^{-/-}$ animals were 5-fold lower compared to wild type controls, corroborating the findings on histology and indicating decreased hepatic injury in animals lacking both TNF-receptors (F).

The ability of Marimastat to inhibit TACE activity prompted us to study the role of TNF-α signaling in CCl_4-induced acute hepatotoxicity. Since TACE is one of the major activators of TNF-α, TACE-inhibition has been implicated as a promising anti-inflammatory approach.[17,24] Because homozygous TACE deletion is embryologically lethal[31], mice deficient in both TNF-α receptors (*TNF p55*$^{-/-}$ *p75*$^{-/-}$) and their WT littermates were used and subjected to either a single dose of CCl_4 or mineral oil, and sacrificed after 24 h to evaluate hepatic injury. Liver sections from *TNF p55*$^{-/-}$ *p75*$^{-/-}$ mice revealed a marked reduction of necroinflammatory injury, compared to WT controls (**Figure 8E**). These findings were corroborated by a marked, 80% reduction of serum ALT levels in *TNF p55*$^{-/-}$ *p75*$^{-/-}$ mice, compared to CCl_4-injected WT controls (4,581 IU/L and 22,660 IU/L, respectively, $P \leq 0.05$; **Figure 8F**). This implicates the involvement of TNF-signaling in CCl_4-induced hepatic injury and provides a mechanistic explanation for the hepatoprotective effects of Marimastat in both acute, and chronic CCl_4-induced hepatic injury.

DISCUSSION

The present study was aimed to determine the effects of the broad spectrum MMP-inhibitor Marimastat on fibrosis formation in a murine model of repeated, chronic CCl_4-induced hepatic injury. We demonstrate that pharmacologic pan-MMP inhibition very efficiently decreased hepatic injury by amelioration of the inflammatory response and by downregulation of pro-fibrogenic mRNA expression, through interfering, at least in part, with TNF-α activation and signaling. The detrimental effects of MMP-inhibition on scar formation, however, were unexpected. We initially hypothesized that inhibition of MMP activity would lead to a further decrease in hepatic injury, rather than impacting on collagen accumulation, resulting in decreased fibrosis formation. Obviously, broad spectrum MMP-inhibition, as determined by MMP transcript levels and activity assays, counterbalanced the potential beneficial anti-fibrogenic and anti-inflammatory effects by efficient inhibition of fibrolysis (**Figure 9**). This dissociation between inflammation and liver injury on the one hand, and fibrolysis on the other hand has to our knowledge not been described previously and may provide novel insights in the dual role of MMPs in fibrogenesis, and fibrolysis.

MMPs are secreted as zymogens and become activated by cleavage of their propeptide.[32,33] Marimastat is a synthetic, low molecular weight succinyl hydroxamate that inhibits MMPs via its hydroxamate group that complexes the zinc ion needed in the active site of MMPs.[27] Marimastat also inhibits TACE, with a suggested benefit in diseases that involve both inflammation and extracellular matrix remodeling.[34] Although MMPs were traditionally viewed as molecules that were only involved in degradation and

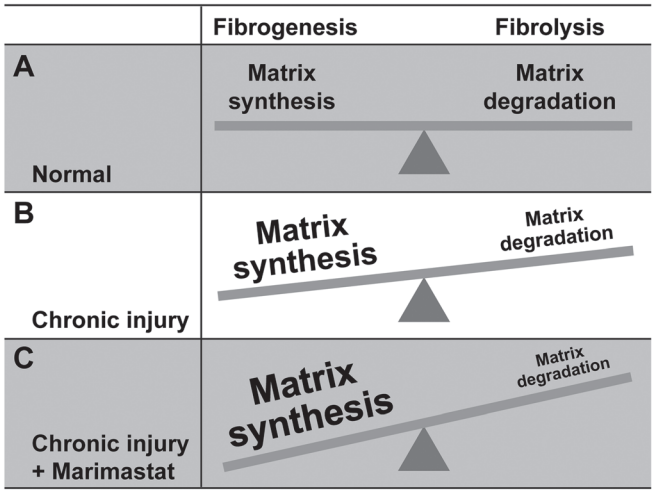

Figure 9. Broad-spectrum MMP-inhibition results in increased hepatic fibrosis following chronic hepatic injury. In the absence of liver injury a physiological balance exists between extracellular matrix synthesis and its degradation (A). Chronic hepatic injury causes excessive synthesis of extracellular matrix proteins including collagen, which prevails over their enzymatic degradation resulting in liver fibrosis (B). Despite a significant attenuation of fibrogenesis and inflammation, efficient inhibition of fibrolytic matrix metalloproteinases by a broad spectrum MMP-inhibitor has profound effects on collagen degradation, tilting the balance towards net extracellular matrix deposition and scar tissue formation (C).

turnover of the extracellular matrix, novel insights overturned this dogma and revealed that MMPs modulate the activities of a wide range of extracellular and intracellular signaling pathways.[22,23] By regulating cell proliferation, migration, adhesion, growth factor bioavailability, chemotaxis, and cell signaling, MMPs are crucial for physiological and pathophysiological processes such as inflammation, immunity, angiogenesis, tumorigenesis, metastasis, and wound healing. As a consequence, a broad spectrum MMP inhibitor such as Marimastat was expected to have both anti-inflammatory and potential antifibrotic properties.

It has been generally accepted that changes in patterns of matrix degradation are critical for fibrogenesis[35]; however, the role of MMP activity in the liver during fibrogenesis is not yet fully understood. In rodents, MMP-2, MMP-3, MMP-9, MMP-13, MMP-14, as well as TIMP-1 and TIMP-2 are expressed in early stages of HSC activation and have been implicated in fibrogenesis as well as fibrolysis (reviewed in [20]). We confirmed that the broad-spectrum MMP-inhibitor Marimastat efficiently inhibited gelatinolytic and collagenolytic MMP, as well as TACE activities. Among other pro-fibrogenic genes, hepatic transcript levels of α-SMA were significantly decreased in the Marimastat treated animals suggesting decreased activation of a subset of HSCs and ameliorated fibrogenesis. This was confirmed by immunohistochemical staining for α-SMA followed by quantification

of positive cell counts. It must be noted, however, that there exists heterogeneity of gene expression in myofibroblastic cells during fibrogenesis which reduces the usefulness of α-SMA as a marker for collagen production [36]. MMPs such as MMP-8, MMP-9, and MMP-13 possess the ability to degrade the extracellular matrix by breakdown of fibrillar collagen type I.[9,19,37] MMP-9 may indirectly contribute to fibrolysis by accelerating HSC apoptosis, whereas MMP-2 may rather drive hepatic stellate activation and unfavorable basement membrane remodeling resulting in more fibrosis.[20,38] We demonstrated efficient inhibition of gelatinolytic and collagenolytic MMP-activities which as a whole has shifted the balance towards a net accumulation of hepatic fibrosis.

TIMP-1 is the most relevant physiological MMP-inhibitor in fibrotic diseases, including hepatotoxic and cholestatic injury, whose expression is upregulated by various inflammatory cytokines.[32,33] Antagonizing TIMP-1 using both neutralizing antibodies and gene therapy, as well as indirect TIMP-1 mRNA reduction by antagonizing inflammatory cytokines improved experimental fibrosis in rodents.[20] In addition, transgenic mice overexpressing human TIMP-1 developed more liver fibrosis when subjected to chronic CCl_4 administration[39], and demonstrated attenuated spontaneous fibrosis resolution.[40] Reduced TIMP-1 levels may also accelerate hepatocyte proliferation following partial hepatectomy, illustrating its inhibitory role in hepatocyte regeneration.[41] In our study, inflammation was significantly decreased by TACE inhibition while downregulation of TIMP-1 may have further improved hepatic regeneration, as reflected by the marked decrease in serum levels of alkaline phosphatase and ALT.

To further elucidate the hepatoprotective effects of broad spectrum MMP-inhibition, we dosed Marimastat-pretreated C57Bl/6J mice with a single injection of CCl_4, to demonstrate reduced centrilobular necrosis and a marked (57%) reduction in serum ALT levels corroborating the hepatoprotective effects of Marimastat also in acute liver failure. These data are in line with a previous report describing the use of a similar broad-spectrum MMP-inhibitor (Batimastat; BB-94, British Biotech) in the prevention of acute, fulminant hepatitis induced by TNF-α combined with D-(+)-galactosamine.[28] In this study, mice treated with BB-94 as well as mice deficient in *Mmp2*, *Mmp3* or *Mmp9* had lower levels of apoptosis and necrosis of hepatocytes, and better survival. Although TACE inhibitors are efficient in protecting against lipopolysaccharide/D-(+)-galactosamine-induced lethal hepatitis by inhibition of TNF-α release, inhibition of TACE by BB-94 was deemed irrelevant in their model; however, data was not shown.[28] The authors speculated that the absence or presence of endogenous TNF-α does not influence the outcome after TNF-α / D-(+)-galactosamine challenge.[28]

Observations that soluble TNF-α receptor treatment improved the outcome following acute CCl_4-intoxication [42], and that monoclonal antibodies against TNF-α improved experimental CCl_4-induced fibrosis [43] led us to explore the involvement of TNF-α and TACE in CCl_4-mediated hepatotoxicity. We show that after a single dose of CCl_4, animals pretreated with the broad-spectrum MMP- and TACE-inhibitor Marimastat, as well as animals deficient in both TNF-α receptors (p55 and p75) were markedly protected, as demonstrated by attenuated necroinflammatory injury on histology and 5-fold lower serum ALT levels, compared to their wild type controls. These findings indicate the pivotal role of TNF-α signaling in CCl_4 mediated hepatotoxicity. A previous study failed to demonstrate a significantly ameliorated response of *TNF p55*$^{-/-}$ *p75*$^{-/-}$ mice to acute CCl_4 intoxication[44], which is in contrast with our results. But these authors and others found reduced fibrogenesis following repeated CCl_4-administration in animals lacking the TNF-α p55 receptor supporting the involvement of TNF-signaling in CCl_4-mediated hepatotoxicity.[44,45] These data suggest that specific inhibition of TACE may be an attractive approach to manipulate the inflammatory cascade following a hepatic insult.

We also explored the possible involvement of MMP-9 in the protection against acute CCl_4-induced injury. A previous report showed that acute CCl_4-induced liver injury in rats increased both active and latent MMP-9 to maximum levels at 24 h and remained elevated 3 days following the injection, suggesting its involvement in early hepatic injury [46]. We show that after a single dose of CCl_4 animals deficient in *Mmp9* exhibited similar hepatic injury compared to their wild type controls, as assessed by histology and serum ALT levels after 24 h. The interpretation of results with MMP knockout mice, however, is difficult since the net proteolytic activity of MMPs relies upon complex, direct interactions between the different protease and protease inhibitor families. In addition, adaptive upregulation of gelatinolytic/collagenolytic activities can have occurred, which would necessitate a conditional knockout. Nevertheless, the almost identical results of both *Mmp9*$^{-/-}$ animals and their wild type controls largely rules out a major role of MMP-9 in the protection against acute CCl_4 intoxication.

More recently, another paper described attenuation of liver injury following treatment with the MMP-inhibitor CTS-1027 (Conatus Pharmaceuticals, San Diego, CA, USA).[47] Using the bile duct ligation model, a decrease in hepatocyte apoptosis and a reduction in markers for HSC activation and fibrogenesis was demonstrated, which is in line with our results that Marimastat attenuated hepatic inflammation and necrosis coupled with downregulation of genes related to fibrogenesis. CTS-1027 is actually now being further evaluated as a potential drug in patients with Hepatitis C Virus[48]; however, we believe that in light of our results caution is warranted. Although it was demonstrated that two weeks of treatment with CTS-1027 resulted in attenuation of collagen deposition in

the bile duct ligation model, we found that inhibition of MMP- and TACE-activity with Marimastat during chronic CCl_4 administration for 6 weeks resulted in increased net fibrosis. It is well possible that two weeks of MMP-inhibition may attenuate injury without aggravating fibrosis, while longer-term treatment in the presence of ongoing liver injury may shut down matrix degradation, eventually leading to impaired fibrolysis. Although we cannot extrapolate our results with Marimastat to all MMP inhibitors and potential off-target effects of Marimastat may have contributed to our findings, it is likely that long-term use of all such inhibitors with anti-TACE activity will have similar divergent effects on liver injury and fibrosis.

Clinically, Marimastat has been used in multiple oncologic clinical trials up to phase III; however, its overall therapeutic benefit was limited and the trials were halted.[49-51] The most consistently reported side effect was musculoskeletal pain, likely due to inhibition of adaptive ECM remodeling; however, spontaneous fibrosis formation has never been reported. This suggests that in the absence of chronic hepatic injury such as hepatitis, or non-alcoholic steatohepatitis, broad-spectrum MMP-inhibition is unlikely to be harmful to the liver. This may also apply to other MMP-inhibitors such as the commonly prescribed antibiotic doxycycline.[52,53] Nonetheless, we believe that caution is warranted when patients with chronic, active liver diseases receive MMP-inhibitors, since inhibition of fibrolytic MMPs will likely accelerate hepatic fibrogenesis formation.

In conclusion, we demonstrate that broad-spectrum MMP- and TACE-activity inhibition with Marimastat during chronic CCl_4 administration resulted in significantly attenuated hepatic inflammation and necrosis coupled with downregulation of genes related to fibrogenesis, but resulted in increased liver fibrosis. Inhibition of MMPs and collagen degradation by Marimastat, however, counterbalanced the beneficial anti-inflammatory effect, resulting in a positive balance of collagen deposition. Since effective inhibition of fibrolytic activity by MMPs accelerates fibrosis progression, our data suggests a note of caution for the use of broad-spectrum MMP inhibitors in patients with chronic, ongoing liver diseases, or for the treatment of liver fibrosis itself. Specific inhibition of TACE, however, may still be an attractive approach to manipulate the inflammatory cascade following a hepatic insult.

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REFERENCES

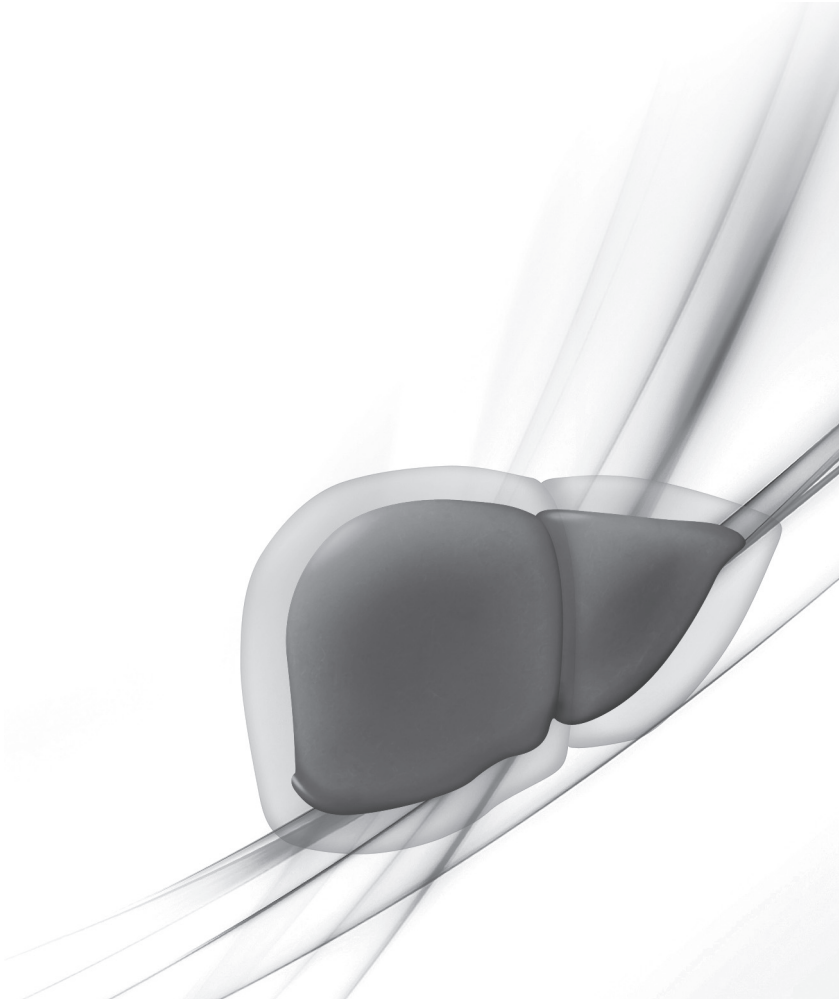
1. Friedman SL. Mechanisms of hepatic fibrogenesis. *Gastroenterology* 2008; 134: 1655-1669.
2. Bataller R, Brenner DA. Liver fibrosis. *J Clin Invest* 2005; 115: 209-218.
3. Schuppan D, Afdhal NH. Liver cirrhosis. *Lancet* 2008; 371: 838-851.
4. Gines P, Cardenas A, Arroyo V, *et al.* Management of cirrhosis and ascites. *N Engl J Med* 2004; 350: 1646-1654.
5. Adam R, Hoti E. Liver transplantation: the current situation. *Semin Liver Dis* 2009; 29: 3-18.
6. Albanis E, Friedman SL. Antifibrotic agents for liver disease. *Am J Transplant* 2006; 6: 12-19.
7. Popov Y, Schuppan D. Targeting liver fibrosis: strategies for development and validation of antifibrotic therapies. *Hepatology* 2009; 50: 1294-1306.
8. Wells RG. Fibrogenesis. V. TGF-beta signaling pathways. *Am J Physiol Gastrointest Liver Physiol* 2000; 279: G845-850.
9. Arthur MJ. Fibrogenesis II. Metalloproteinases and their inhibitors in liver fibrosis. *Am J Physiol Gastrointest Liver Physiol* 2000; 279: G245-249.
10. Manicone AM, McGuire JK. Matrix metalloproteinases as modulators of inflammation. *Semin Cell Dev Biol* 2008; 19: 34-41.
11. Iredale JP. Models of liver fibrosis: exploring the dynamic nature of inflammation and repair in a solid organ. *J Clin Invest* 2007; 117: 539-548.
12. Bazzoni F, Beutler B. The tumor necrosis factor ligand and receptor families. *N Engl J Med* 1996; 334: 1717-1725.
13. Black RA, Rauch CT, Kozlosky CJ, *et al*. A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. *Nature* 1997; 385: 729-733.
14. Moss ML, Jin SL, Milla ME, *et al*. Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-alpha. *Nature* 1997; 385: 733-736.
15. Amour A, Slocombe PM, Webster A, *et al.* TNF-alpha converting enzyme (TACE) is inhibited by TIMP-3. *FEBS Lett* 1998; 435: 39-44.
16. Mohammed FF, Smookler DS, Taylor SE, *et al.* Abnormal TNF activity in Timp3-/- mice leads to chronic hepatic inflammation and failure of liver regeneration. *Nat Genet* 2004; 36: 969-977.
17. Doggrell SA. TACE inhibition: a new approach to treating inflammation. *Expert Opin Investig Drugs* 2002; 11: 1003-1006.
18. Moss ML, Sklair-Tavron L, Nudelman R. Drug insight: tumor necrosis factor-converting enzyme as a pharmaceutical target for rheumatoid arthritis. *Nat Clin Pract Rheumatol* 2008; 4: 300-309.
19. Benyon RC, Arthur MJ. Extracellular matrix degradation and the role of hepatic stellate cells. *Semin Liver Dis* 2001; 21: 373-384.
20. Hemmann S, Graf J, Roderfeld M, *et al.* Expression of MMPs and TIMPs in liver fibrosis - a systematic review with special emphasis on anti-fibrotic strategies. *J Hepatol* 2007; 46: 955-975.
21. Uchinami H, Seki E, Brenner DA, *et al*. Loss of MMP 13 attenuates murine hepatic injury and fibrosis during cholestasis. *Hepatology* 2006; 44: 420-429.
22. Cauwe B, Van den Steen PE, Opdenakker G. The biochemical, biological, and pathological kaleidoscope of cell surface substrates processed by matrix metalloproteinases. *Crit Rev Biochem Mol Biol* 2007; 42: 113-185.
23. Morrison CJ, Butler GS, Rodriguez D, *et al*. Matrix metalloproteinase proteomics: substrates, targets, and therapy. *Curr Opin Cell Biol* 2009; 21: 645-653.
24. Murphy G, Murthy A, Khokha R. Clipping, shedding and RIPping keep immunity on cue. *Trends Immunol* 2008; 29: 75-82.

25. Taub R. Hepatoprotection via the IL-6/Stat3 pathway. *J Clin Invest* 2003; 112: 978-980.
26. Duffield JS, Forbes SJ, Constandinou CM, *et al*. Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. *J Clin Invest* 2005; 115: 56-65.
27. Hu J, Van den Steen PE, Sang QX, *et al.* Matrix metalloproteinase inhibitors as therapy for inflammatory and vascular diseases. *Nat Rev Drug Discov* 2007; 6: 480-498.
28. Wielockx B, Lannoy K, Shapiro SD, *et al.* Inhibition of matrix metalloproteinases blocks lethal hepatitis and apoptosis induced by tumor necrosis factor and allows safe antitumor therapy. *Nat Med* 2001; 7: 1202-1208.
29. Hamada T, Fondevila C, Busuttil RW, *et al*. Metalloproteinase-9 deficiency protects against hepatic ischemia/reperfusion injury. *Hepatology* 2008; 47: 186-198.
30. Olle EW, Ren X, McClintock SD, *et al*. Matrix metalloproteinase-9 is an important factor in hepatic regeneration after partial hepatectomy in mice. *Hepatology* 2006; 44: 540-549.
31. Peschon JJ, Slack JL, Reddy P, *et al*. An essential role for ectodomain shedding in mammalian development. *Science* 1998; 282: 1281-1284.
32. Chakraborti S, Mandal M, Das S, *et al*. Regulation of matrix metalloproteinases: an overview. *Mol Cell Biochem* 2003; 253: 269-285.
33. Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res* 2003; 92: 827-839.
34. Clements JM, Cossins JA, Wells GM, *et al.* Matrix metalloproteinase expression during experimental autoimmune encephalomyelitis and effects of a combined matrix metalloproteinase and tumour necrosis factor-alpha inhibitor. *J Neuroimmunol* 1997; 74: 85-94.
35. Arthur MJ, Friedman SL, Roll FJ, *et al*. Lipocytes from normal rat liver release a neutral metalloproteinase that degrades basement membrane (type IV) collagen. *J Clin Invest* 1989; 84: 1076-1085.
36. Magness ST, Bataller R, Yang L, *et al*. A dual reporter gene transgenic mouse demonstrates heterogeneity in hepatic fibrogenic cell populations. *Hepatology* 2004; 40: 1151-1159.
37. Winwood PJ, Schuppan D, Iredale JP, *et al*. Kupffer cell-derived 95-kd type IV collagenase/gelatinase B: characterization and expression in cultured cells. *Hepatology* 1995; 22: 304-315.
38. Olaso E, Ikeda K, Eng FJ, *et al*. DDR2 receptor promotes MMP-2-mediated proliferation and invasion by hepatic stellate cells. *J Clin Invest* 2001; 108: 1369-1378.
39. Yoshiji H, Kuriyama S, Miyamoto Y, *et al.* Tissue inhibitor of metalloproteinases-1 promotes liver fibrosis development in a transgenic mouse model. *Hepatology* 2000; 32:1248-1254.
40. Yoshiji H, Kuriyama S, Yoshii J, *et al*. Tissue inhibitor of metalloproteinases-1 attenuates spontaneous liver fibrosis resolution in the transgenic mouse. *Hepatology* 2002; 36: 850-860.
41. Mohammed FF, Pennington CJ, Kassiri Z, *et al*. Metalloproteinase inhibitor TIMP-1 affects hepatocyte cell cycle via HGF activation in murine liver regeneration. *Hepatology* 2005; 41: 857-867.
42. Czaja MJ, Xu J, Alt E. Prevention of carbon tetrachloride-induced rat liver injury by soluble tumor necrosis factor receptor. *Gastroenterology* 1995; 108: 1849-1854.
43. Bahcecioglu IH, Koca SS, Poyrazoglu OK, *et al*. Hepatoprotective effect of infliximab, an anti-TNF-alpha agent, on carbon tetrachloride-induced hepatic fibrosis. *Inflammation* 2008; 31: 215-221.
44. Simeonova PP, Gallucci RM, Hulderman T, *et al.* The role of tumor necrosis factor-alpha in liver toxicity, inflammation, and fibrosis induced by carbon tetrachloride. *Toxicol Appl Pharmacol* 2001; 177: 112-120.
45. Sudo K, Yamada Y, Moriwaki H, *et al*. Lack of tumor necrosis factor receptor type 1 inhibits liver fibrosis induced by carbon tetrachloride in mice. *Cytokine* 2005; 29: 236-244.
46. Knittel T, Mehde M, Grundmann A, *et al.* Expression of matrix metalloproteinases and their inhibitors during hepatic tissue repair in the rat. *Histochem Cell Biol* 2000; 113: 443-453.

47. Kahraman A, Bronk SF, Cazanave S, *et al.* Matrix metalloproteinase inhibitor, CTS-1027, attenuates liver injury and fibrosis in the bile duct-ligated mouse. *Hepatol Res* 2009; 39: 805-813.
48. ClinicalTrials.gov Identifier: NCT01051921. *Online: www.clinicaltrials.gov* (Accessed May 21, 2010).
49. Bramhall SR, Hallissey MT, Whiting J, *et al.* Marimastat as maintenance therapy for patients with advanced gastric cancer: a randomised trial. *Br J Cancer* 2002; 86: 1864-1870.
50. Rosenbaum E, Zahurak M, Sinibaldi V, *et al*. Marimastat in the treatment of patients with biochemically relapsed prostate cancer: a prospective randomized, double-blind, phase I/II trial. *Clin Cancer Res* 2005; 11: 4437-4443.
51. Coussens LM, Fingleton B, Matrisian LM. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. *Science* 2002; 295: 2387-2392.
52. Schneider BS, Maimon J, Golub LM, *et al*. Tetracyclines inhibit intracellular muscle proteolysis in vitro. *Biochem Biophys Res Commun* 1992; 188: 767-772.
53. Moses MA, Harper J, Folkman J. Doxycycline treatment for lymphangioleiomyomatosis with urinary monitoring for MMPs. *N Engl J Med* 2006; 354: 2621-2622.
54. Rasmussen HS, McCann PP. Matrix metalloproteinase inhibition as a novel anticancer strategy: a review with special focus on batimastat and marimastat. *Pharmacol Ther* 1997; 75: 69-75.
55. Kleiner DE, Brunt EM, Van Natta M, *et al.* Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005; 41: 1313-1321.
56. Jamall IS, Finelli VN, Que Hee SS. A simple method to determine nanogram levels of 4-hydroxyproline in biological tissues. *Anal Biochem* 1981; 112: 70-75.
57. Popov Y, Patsenker E, Fickert P, *et al*. Mdr2 (Abcb4)-/- mice spontaneously develop severe biliary fibrosis via massive dysregulation of pro- and antifibrogenic genes. *J Hepatol* 2005; 43: 1045-1054.
58. Popov Y, Patsenker E, Bauer M, *et al*. Halofuginone induces matrix metalloproteinases in rat hepatic stellate cells via activation of p38 and NFkappaB. *J Biol Chem* 2006; 281: 15090-15098.
59. Popov Y, Patsenker E, Stickel F, *et al.* Integrin alphavbeta6 is a marker of the progression of biliary and portal liver fibrosis and a novel target for antifibrotic therapies. *J Hepatol* 2008; 48: 453-464.

Chapter 8

Summary and discussion I.
Nederlandse samenvatting en discussie II.



I. SUMMARY AND DISCUSSION

Introduction

Non-alcoholic fatty liver disease (NAFLD) is one of the hallmarks of the metabolic syndrome and is a significant and increasingly more common cause of liver failure.[1,2] NAFLD is characterized by an accumulation of triglycerides in hepatocytes (hepatic steatosis), and, together with lipotoxic injury, may lead to hepatocellular injury and inflammation (non-alcoholic steatohepatitis), advanced fibrosis, and ultimately liver failure.[3-5] Steatosis is an established risk factor for primary non-function of hepatic allografts[6]; however, the risk of major hepatic resection in patients with hepatic steatosis remains controversial. In addition, although its prevalence in both children and adults is increasing worldwide[1,2], the mechanisms underlying the pathogenesis of NAFLD and its progression remain elusive. Currently, the cornerstone of clinical management is weight loss through diet and exercise.[7] In reality, however, management of hepatic steatosis prior to hepatic surgery usually does not allow time for such dramatic life style changes, justifying the search for a pharmacologic approach. Pharmacological options to reverse steatosis prior to surgery, however, are lacking.

Aim of this thesis

The aim of the studies described in this thesis is to examine the risk of morbidity and mortality following hepatic resection in patients with steatosis; to explore the natural history of NAFLD and obesity followed by dietary intervention using a mouse model; and to identify and test novel therapeutic modalities for the treatment of NAFLD. Using experimental, murine models of diet-induced obesity, this thesis aims to provide new insights regarding the risks, regulation and reversibility of NAFLD.

Main findings

While extensive surgery can be performed safely on healthy livers, the risk of major hepatic resection in patients with steatosis remains unclear. **Chapter 2** comprises a systematic review and meta-analysis of observational studies to establish the best estimate of the impact of hepatic steatosis on patient outcome following major hepatic surgery. Six high quality observational studies were identified, of which four including a total of 1000 patients were subjected to meta-analysis. Compared with patients without steatosis, those with less than 30% and at least 30% steatosis had a significantly increased risk of postoperative complications, with a relative risk (RR) of 1.53 (95% confidence interval (CI) 1.27 to 1.85) and 2.01 (1.66 to 2.44) respectively. Patients with at least 30% steatosis had an increased risk of postoperative death (RR 2.79, 95% CI 1.19 to 6.51). We concluded that given the increased risk of complications and death in patients who underwent a major hepatic resection in parallel to the severity of steatosis, and with the

rising prevalence of steatosis in patients undergoing liver resection, surgeons should be aware of the potential risks, inform their patients and, if feasible, intervene before surgery.

NAFLD results from overconsumption; however, the type of diet that is conducive to the development of this disease has not been established. In **Chapter 3** we hypothesized that the onset of hepatic steatosis in mice is linked to the consumption of a diet with a high fat content, rather than related to excess caloric consumption or decreased levels of physical activity. In addition, we also hypothesized that fully manifested hepatic steatosis could be reversed by reducing the fat percentage in the diet of obese mice. Using a pair-feeding design to distinguish the effects of dietary fat content and caloric intake on dietary-induced hepatic lipid accumulation and associated injury, groups of mice were fed either a standard, low diet containing 10% fat or a high fat diet with 60% of calories derived from fat. We found that dietary fat content, independent from caloric intake, is a crucial factor in the development of hepatic steatosis, obesity, and insulin resistance in the murine model of diet-induced obesity, caused by increased uptake of free fatty acids and *de novo* lipogenesis. In addition, once established, all these features of the metabolic syndrome could be successfully reversed after switching obese mice to a diet low in fat. We concluded that low-fat diets deserve attention in the investigation of a potential treatment of patients with NAFLD.

Although the pathogenesis of NAFLD has still not been fully elucidated, it is generally accepted that a subset of patients with steatosis will eventually progress to steatohepatitis and fibrosis induced chronic, low-grade inflammation, injury and hepatocyte death. Unlike well-defined pathways such as inflammatory signaling and activation of the innate immune system, the association between extracellular matrix turnover by matrix metalloproteinases (MMPs) and development of NAFLD has not been established. MMPs and their specific inhibitors play an important role in fibrogenesis and fibrolysis; therefore, in **Chapter 4** we sought to determine the potential involvement of MMPs in the pathogenesis of NAFLD using a mouse model of diet-induced obesity. We found that already after 9 weeks, mice on a high fat diet developed severe steatosis, liver injury and adiposity. Gene expression levels of *Mmp12* and *Mmp13*, as well as *Timp1* were significantly upregulated in livers as well as adipose tissue of mice on a high fat diet, compared to lean controls. This suggests that with the development of steatosis, a simultaneous increase in *Mmp12* and *Timp1* expression may lead to increased profibrogenic signaling, rendering the liver more susceptible to the development of fibrosis. On the other hand, increased gene expression of *Mmp13* may suggest successful initiation of scar tissue remodeling, thereby potentially counterbalancing profibrogenic stimuli. We concluded that obesity-induced alteration in the expression level of MMPs in liver

and adipose tissue may contribute to a pro-fibrogenic state, potentially leading to an increased susceptibility to develop fibrosis.

Chapter 5 describes the results of a study using a pharmacologic inhibitor of MMPs and tumor necrosis factor (TNF)-α converting enzyme (TACE), mediating TNF-α release from the plasma membrane. TNF-α is an inflammatory cytokine involved in linking nutrient availability to innate immune activation and the major negative regulator of the insulin receptor pathway. TACE-inhibition abrogates the inflammatory response and improves insulin resistance. We hypothesized that treatment with this drug would improve insulin resistance and reverse established experimentally-induced steatosis in mice, leading to improved outcome following hepatectomy. Using a mouse model of diet-induced obesity, we found that treatment with the MMP and TACE inhibitor significantly improved insulin sensitivity and liver histology, and resulted in a 66% decrease in steatosis (P=0.010). These findings were confirmed in transgenic, leptin deficient mice. Moreover, following hepatectomy, post-operative liver injury as quantified by serum aspartate aminotransferase levels and alanine aminotransferase levels was significantly decreased by 57% (P=0.020) and 44% (P=0.032) in treated animals, compared to controls. We concluded that this may suggest a potential therapeutic role in patients with fatty liver disease; especially those who need to undergo surgery.

In **Chapter 6** we investigate a potential pitfall in the methodology of drug delivery while testing experimental compounds in animal models. In mice, drugs can be administered intravenously, intraperitoneally or via orogastric gavage, the last considered to be the least invasive route. Because even non-invasive laboratory routines may elicit an acute stress response, we hypothesized that long-term orogastric gavage would negatively influence the development of hepatic steatosis and associated metabolic syndrome. We found that control mice exhibited significantly more weight gain, insulin resistance and hepatic steatosis, compared to mice that underwent orogastric gavage daily, or twice daily. This effect was likely due to decreased food consumption associated with gavage-induced stress. In contrast, the phenotype of leptin deficient mice was not affected by orogastric gavage. Therefore, we concluded that orogastric gavage may lead to increased stress, thereby affecting food intake and the development of diet-induced obesity in a murine model. The effects of what may seem to be trivial laboratory routines, such as orogastric gavage, should be taken into account when designing animal studies for drug development.

The progression from steatosis to steatohepatitis and fibrosis is believed to result from superimposition of secondary 'hits' that overwhelm cell survival mechanisms and provoke hepatocyte injury and death. Inflammation is considered a prerequisite and

important co-contributor to fibrosis and is, in part, mediated by TACE. In **Chapter 7**, we hypothesized that treatment with a broad-spectrum MMP and TACE-inhibitor would ameliorate hepatic injury and inflammation, leading to decreased fibrogenesis in a murine model of repeated hepatotoxin-induced liver injury. In this model, liver fibrosis was induced by repeated injections of carbon tetrachloride, during which the animals received either the drug or vehicle twice daily. Animals treated with the MMP and TACE-inhibitor indeed demonstrated significantly attenuated liver injury and inflammation, but a remarkable 25% increase in collagen deposition. Gene transcripts related to fibrogenesis were significantly less upregulated in drug-treated animals compared to vehicle-treated animals, while MMP expression and activity analysis revealed efficient pharmacologic MMP-inhibition and decreased fibrolysis. We concluded that pharmacologic inhibition of MMP and TACE activity during chronic hepatotoxic injury apparently counterbalanced any beneficial anti-inflammatory effect, resulting in a positive balance of collagen deposition. Since effective inhibition of MMPs accelerates fibrosis progression, MMP inhibitors should be used with caution in patients with chronic liver diseases.

CONCLUSIONS

Chapter 2: Following major hepatic resection, patients with steatosis had an up to two-fold increased risk of postoperative complications, and those with excessive steatosis had an almost threefold increased risk of death.

Chapter 3: Dietary fat content, independent from caloric intake, is a crucial factor in the development of hepatic steatosis, obesity, and insulin resistance in a murine model of diet-induced obesity model by increased uptake of free fatty acids and *de novo* lipogenesis. In addition, once established, all these features of the metabolic syndrome can be successfully reversed after switching obese mice to a diet low in fat.

Chapter 4: Obesity-induced alteration in the expression level of MMPs in liver and adipose tissue of mice may contribute to a pro-fibrogenic state, potentially leading to an increased susceptibility to develop fibrosis.

Chapter 5: Treatment with a pharmacologic MMP and TACE-inhibitor improved insulin sensitivity and reversed steatosis in mouse models of diet-induced obesity and leptin deficiency, thereby attenuating post-operative injury following hepatectomy.

Chapter 6: Orogastric gavage of experimental compounds may lead to increased stress, thereby affecting food intake and the development of diet-induced obesity in a murine model.

Chapter 7: Effective inhibition of MMP and TACE activity during chronic hepatotoxic injury counterbalanced any beneficial anti-inflammatory effect, resulting in accelerated progression of fibrosis.

FUTURE DIRECTIONS

It is clear that patients who have some degree of steatosis are at increased risk for morbidity and mortality following major hepatic resection, indicating the need for pharmacological intervention prior to surgery. We found that MMP and TACE-inhibition improved insulin sensitivity and reversed steatosis in mouse models of diet-induced obesity and leptin deficiency, thereby attenuating post-operative injury following hepatectomy. This may deserve further experimental investigation in the search for a pharmacologic treatment for the reversal of steatosis. Moreover, these promising results allow designing a pilot study to investigate the efficacy of MMP and TACE-inhibitors to reverse hepatic steatosis in humans.

Future investigations may include:

- Testing the efficacy of other, more specific TACE-inhibitors in the reversal of hepatic steatosis *in vivo*.
- Dissecting and elucidating the specific mechanism of action of the investigated MMP and TACE-inhibitor using advanced gene array, proteomics and lipidomics analyses.
- Designing a clinical trial to investigate the MMP and TACE-inhibitor in the treatment of hepatic steatosis in a selected patient group.

REFERENCES

1. Angulo P. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; 346: 1221-1231.
2. Lazo M, Clark JM. The epidemiology of nonalcoholic fatty liver disease: a global perspective. *Semin Liver Dis* 2008; 28: 339-350.
3. Argo CK, Northup PG, Al-Osaimi AM, *et al*. Systematic review of risk factors for fibrosis progression in non-alcoholic steatohepatitis. *J Hepatol* 2009; 51: 371-379.
4. Jou J, Choi SS, Diehl AM. Mechanisms of disease progression in nonalcoholic fatty liver disease. *Semin Liver Dis* 2008; 28: 370-379.
5. Neuschwander-Tetri BA. Hepatic lipotoxicity and the pathogenesis of nonalcoholic steatohepatitis: The central role of nontriglyceride fatty acid metabolites. *Hepatology* 2010; 52: 774-788.
6. Adam R, Hoti E. Liver transplantation: the current situation. *Semin Liver Dis* 2009; 29: 3-18.
7. Vuppalanchi R, Chalasani N. Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis: Selected practical issues in their evaluation and management. *Hepatology* 2009; 49: 306-317.
8. Coussens LM, Fingleton B, Matrisian LM. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. *Science* 2002; 295: 2387-2392.

II. NEDERLANDSE SAMENVATTING EN DISCUSSIE

Introductie

Niet-alcoholische leververvetting ("Non-alcoholic fatty liver disease"; NAFLD) is één van de kenmerken van het metabole syndroom en een belangrijke, toenemend voorkomende oorzaak van leverfalen.[1,2] Histopathologisch kenmerkend voor NAFLD is een opeenhoping van triglyceriden in de hepatocyten (zogenaamde leversteatose), wat tesamen met lipotoxicitiet kan leiden tot hepatocellulaire schade en ontsteking (niet-alcoholische steatohepatitis), gevorderde fibrose en uiteindelijk leverfalen.[3-5]

Leversteatose is een bewezen risicofactor voor primair falen van levertransplantaten[6]; echter, de invloed van leversteatose op het risico van postoperatieve complicaties na leverchirurgie is onduidelijk en controversieel. Ondanks dat de prevalentie van NAFLD in zowel kinderen als volwassenen wereldwijd toeneemt blijven de pathogenese en onderliggende mechanismen die hieraan ten grondslag liggen nog onduidelijk.[1,2] De hoeksteen van klinische behandeling is gewichtsverlies door dieet en beweging.[7] Echter, in werkelijkheid laat de behandeling van leversteatose vlak voor leverchirurgie weinig tijd voor dergelijke dramatische veranderingen in levensstijl. Een farmacologische aanpak zou derhalve ideaal zijn, maar medicijnen die leversteatose kunnen doen verminderen om de lever te optimaliseren voor chirurgie bestaan nog niet.

Doel van dit proefschrift

Het doel van de studies die uitmaken van dit proefschrift is om de morbiditeits- en mortaliteitsrisico's te onderzoeken van patienten met steatose die leverchirurgie ondergaan; om het natuurlijk beloop van NAFLD en obesitas (zwaarlijvigheid) en de effecten van een voedingsinterventie te onderzoeken in een diermodel; en om nieuwe farmacologische behandelingen voor NAFLD te identificeren en te testen. Op experimentele wijze door middel van het gebruik van modellen van dieet-geïnduceerde obesitas en leversteatose in de muis geeft dit proefschrift nieuwe inzichten in de risico's, regulering en omkeerbaarheid van NAFLD.

Belangrijkste bevindingen

Terwijl chirurgie op een gezonde lever relatief veilig blijkt, blijft het risico van een grote leverresectie bij patiënten met leversteatose onduidelijk. **Hoofdstuk 2** beschrijft een systematisch literatuuronderzoek en meta-analyse van observationele studies om een inschatting te maken van deze risico's. Zes kwalitatief goede observationele studies werden gevonden, waarvan er uiteindelijk vier met in totaal 1000 patiënten werden geïncludeerd voor meta-analyse. In vergelijking met patiënten zonder leversteatose was het hebben van minder dan 30% en minstens 30% leversteatose geassocieerd met een

significant verhoogd risico op postoperatieve complicaties, met een relatief risico (RR) van respectievelijk 1.53 (95% betrouwbaarheids interval (BI) 1.27 tot 1.85) en 2.01 (1.66 tot 2.44). Patiënten met minstens 30% leversteatose hadden een verhoogd risico op postoperatief overlijden (RR 2.79, 95% BI 1.19 tot 6.51). Wij concludeerden dat de kans op complicaties en overlijden bij patiënten die een grote leverresectie ondergingen parallel liep aan de ernst van steatose. Met de stijgende prevalentie van leversteatose bij patiënten die leverchirurgie ondergaan in het achterhoofd dienen chirurgen zich bewust te zijn van de potentiële risico's, hun patiënten hiervan op de hoogte te brengen en indien mogelijk te interveniëren voorafgaand aan de operatie.

NAFLD is het resultaat van overconsumptie, maar het type dieet dat verantwoordelijk is voor het ontwikkelen van deze ziekte is nog niet vastgesteld. In **Hoofdstuk 3** hebben we de hypothese getoetst dat het ontstaan van leversteatose bij muizen niet zozeer gerelateerd is aan consumptie van overtollige calorieën of verminderde lichamelijke activiteit, maar gekoppeld is aan consumptie van een dieet met een hoog vetgehalte. Tevens toetsten we de hypothese dat manifeste leversteatose omgekeerd kon worden door het vetpercentage in het dieet van reeds obese muizen te reduceren. Groepen muizen werden gepaard gevoed met een standaard dieet met een 10% vetgehalte of met een "hoog vet dieet" waarbij het vetgehalte 60% bedroeg. Wij toonden in dit muismodel aan dat het vetgehalte in het dieet een belangrijke factor is in de ontwikkeling van leversteatose, obesitas en insuline resistentie, onafhankelijk van calorische consumptie. Dit werd onder andere veroorzaakt door een verhoogde opname van vrije vetzuren door de lever en een verhoogde *de novo* lipogenese. Tevens konden al deze kenmerken van het metabole syndroom succesvol worden omgekeerd wanneer obese muizen een standaard, laag vet dieet werden gevoerd. Wij concludeerden dat vetarme diëten aandacht verdienen in het onderzoek naar een mogelijke behandeling van patienten met NAFLD.

Hoewel de pathogenese van NAFLD nog steeds niet volledig is opgehelderd is het algemeen aanvaard dat een subgroep van patiënten met steatose uiteindelijk steatohepatitis en fibrose ontwikkelen. In tegenstelling tot goed gedefinieerde routes zoals inflammatoire signalering en activering van het natieve immuunsysteem, is de associatie tussen het omzetten van extracellulaire matrix door matrix metalloproteinasen (MMP's) en het ontwikkelen van NAFLD onduidelijk. Omdat MMP's en hun specifieke remmers een belangrijke rol spelen in fibrogenese en fibrolyse, hebben we in **Hoofdstuk 4** getracht de rol van MMP's in de pathogenese van NAFLD te bepalen met behulp van een muismodel van dieet-geïnduceerde obesitas. We vonden dat muizen die een dieet met een hoog vetgehalte gevoerd werden al na 9 weken ernstige leversteatose, leverbeschadiging en adipositas ontwikkelden. Genexpressieniveaus van *Mmp12*, *Mmp13* en

Timp1 waren significant verhoogd in levers en vetweefsel van muizen op een hoog vet dieet, vergeleken met controle muizen. Dit suggereert dat tijdens de ontwikkeling van leversteatose een verhoging van *Mmp12* en *Timp1* expressie kan leiden tot verhoogde profibrogene signalering, waardoor de lever meer vatbaar is voor de ontwikkeling van fibrose.

Hoofdstuk 5 beschrijft de resultaten van een studie waarin we een farmacologische remmer van MMPs en het tumor necrose factor (TNF)-α converting enzym (TACE) onderzoeken. TNF-α is een cytokine dat onder andere betrokken is bij ontstekingsprocessen, maar ook bij de activering van het immuunsysteem en is een belangrijke negatieve regulator van de insulinesignalering. TACE-inhibitie remt de ontstekingsreactie en verbetert de insulineresistentie. Derhalve toetsten wij de hypothese dat behandeling met dit geneesmiddel insulineresistentie zou verbeteren en dieet-geïnduceerde leversteatose in muizen om zou kunnen keren, resulterend in een betere uitkomst na leverchirurgie. Gebruikmakend van een muismodel van dieet-geïnduceerde obesitas vonden we dat de behandeling met de MMP en TACE-remmer zowel de insulinegevoeligheid als de leverhistologie sterk verbeterde, resulterend in een 66% afname van leversteatose (P=0.010). Deze bevindingen werden bevestigd in transgene, leptine-deficiënte muizen. Bovendien waren behandelde muizen postoperatief na een leverresectie relatief beschermd tegen leverschade, gekwantificeerd door een 57% daling in postoperatieve serum aspartaataminotransferase niveaus (P=0.020) en een 44% daling in alanineaminotransferase niveaus (P=0.032). We concludeerden dat dit medicijn een therapeutische rol zou kunnen hebben bij patiënten met NAFLD, en met name voor degenen die leverchirurgie moeten ondergaan.

In **Hoofdstuk 6** onderzoeken we een mogelijke valkuil in de methodologie van het toedienen van experimentele stoffen bij muizen. Bij muizen kunnen medicijnen namelijk intraveneus, intraperitoneaal of via een maagknopsonde orofaryngeaal worden toegediend, waarbij de laatste als de minst invasieve route geldt. Omdat zelfs niet-invasieve laboratoriumhandelingen een acute stressrespons op kunnen wekken, onderzochten we de hypothese dat langdurig gebruik van een orofaryngeale maagknopsonde de ontwikkeling van leververvetting en het bijbehorende metabool syndroom negatief zou beïnvloeden. We vonden dat controle muizen aanzienlijk meer gewichtstoename hadden en meer insulineresistentie en leversteatose ontwikkelden in vergelijking met muizen die éénmaal daags of tweemaal daags met een orofaryngeale maagknopsonde een placebo gevoerd werden. Dit effect is waarschijnlijk te wijten aan verminderde voedselconsumptie als gevolg van geïnduceerde handeling-gerelateerde stress. Daarentegen werd het fenotype van transgene, leptine-deficiënte muizen niet beïnvloed door het voeren met een orofaryngeale maagknopsonde. We concludeerden dat het

gebruik van een orofaryngeale maagknopsonde kan leiden tot verhoogde stress, waardoor voedselconsumptie en de ontwikkeling van dieet-geïnduceerde obesitas negatief beïnvloed kunnen worden. Bij het ontwerpen van dierproeven voor de ontwikkeling van geneesmiddelen zal rekening gehouden moeten worden met de negatieve effecten van ogenschijnlijk triviale laboratoriumhandelingen, zoals bijvoorbeeld het gebruik van de orofaryngeale maagknopsonde.

De progressie van leversteatose tot steatohepatitis en fibrose is vermoedelijk het resultaat van een opstapeling van secundaire 'hits' die leiden tot schade aan en uiteindelijk dood van hepatocyten. Ontstekingsprocessen dragen in belangrijke mate bij aan het ontstaan van leverfibrose, deels gemedieerd door TACE. In **Hoofdstuk 7** hebben we de hypothese getoetst dat behandeling met een breed-spectrum MMP en TACE-remmer ontsteking en leverbeschadiging zou kunnen te verbeteren, leidend tot minder fibrogenese. In een muismodel van hepatotoxine-geïnduceerde leverbeschadiging werd leverfibrose geïnduceerd door herhaalde injecties van tetrachloorkoolstof ('carbon tetrachloride'). Muizen die behandeld werden met de MMP en TACE-remmer ontwikkelden inderdaad significant minder leverschade en ontsteking, maar toonden een opmerkelijke stijging van 25% in collageendepositie. Genexpressieniveaus gerelateerd aan fibrogenese waren beduidend minder verhoogd in experimenteel-behandelde muizen vergeleken met placebo-behandelde controles, terwijl MMP expressie- en activiteitsanalyse zeer efficiënte farmacologische MMP-inhibitie en een verminderde fibrolyse toonden. We concludeerden dat de farmacologische remming van MMP en TACE activiteit tijdens het induceren van chronische, toxische leverschade de kennelijk gunstige anti-inflammatoire werking antagoneert, wat netto resulteert in collageendepositie. Aangezien een effectieve remming van MMP's de fibroseprogressie versnelt zal voorzichtigheid geboden moeten zijn bij het gebruik van MMP-remmers bij patiënten met chronische leverziekten.

CONCLUSIES

Hoofdstuk 2: Na leverchirurgie hebben patiënten met leversteatose een ruim tweemaal verhoogd risico op postoperatieve complicaties en hebben mensen met overmatige leversteatose een bijna driemaal verhoogd risico om te overlijden.

Hoofdstuk 3: Het vetgehalte in het dieet is een belangrijke factor in de ontwikkeling van leversteatose, obesitas en insulineresistentie in de muis, onafhankelijk van calorische consumptie. Dit werd onder andere veroorzaakt door een verhoogde opname van vrije vetzuren door de lever en een verhoogde *de novo* lipogenese. Tevens konden al deze kenmerken van het metabole syndroom succesvol worden omgekeerd wanneer obese muizen een standaard, laag vet dieet werden gevoerd.

Hoofdstuk 4: Obesitas-geïnduceerde veranderingen in genexpressieniveaus van MMP's in lever- en vetweefsel van obese muizen kunnen bijdragen aan het ontstaan van veranderingen in de extracellulaire matrix, potentieel leidend tot een verhoogde kans op het ontwikkelen van fibrose.

Hoofdstuk 5: Behandeling met een farmacologische MMP and TACE-remmer verbeterde insulinegevoeligheid en keerde leversteatose om in muismodellen van zowel dieet-geïnduceerde obesitas als leptine deficiëntie, resulterend in een afname van postoperatieve leverenzymstoornissen na leverchirurgie.

Hoofdstuk 6: Het gebruik van een orofaryngeale maagknopsonde als methode van medicijntoediening leidt tot stress, waardoor de ontwikkeling van leversteatose en het bijbehorende metabool syndroom negatief werd beïnvloed in een muismodel van dieet-geïnduceerde obesitas.

Hoofdstuk 7: Effectieve remming van MMP en TACE activiteit tijdens het toedienen van chronische, toxische leverschade antagoneert de gunstige, anti-inflammatoire werking resulterend in een versnelde ontwikkeling van fibrose.

SUGGESTIES VOOR TOEKOMSTIG ONDERZOEK

Het is duidelijk dat patiënten met leversteatose een verhoogd risico hebben op morbiditeit en mortaliteit na een leverresectie, wat de noodzaak illustreert voor farmacologische interventie voorafgaand aan de operatie. Onze resultaten toonden dat het toedienen van een MMP en TACE-remmer aan obese muizen insulineresistentie en leversteatose omkeerden en dat postoperatieve leverenzymverhogingen na leverresectie gehalveerd werden. Deze resultaten rechtvaardigen toekomstig dierexperimenteel onderzoek naar een farmacologische behandeling van leversteatose. Tevens staat de deur open voor het opzetten van pilot studies om de effectiviteit van reeds bestaande geneesmiddelen met anti-MMP en TACE activiteit te testen op de mens om te onderzoeken wat de effecten zijn op leversteatose.

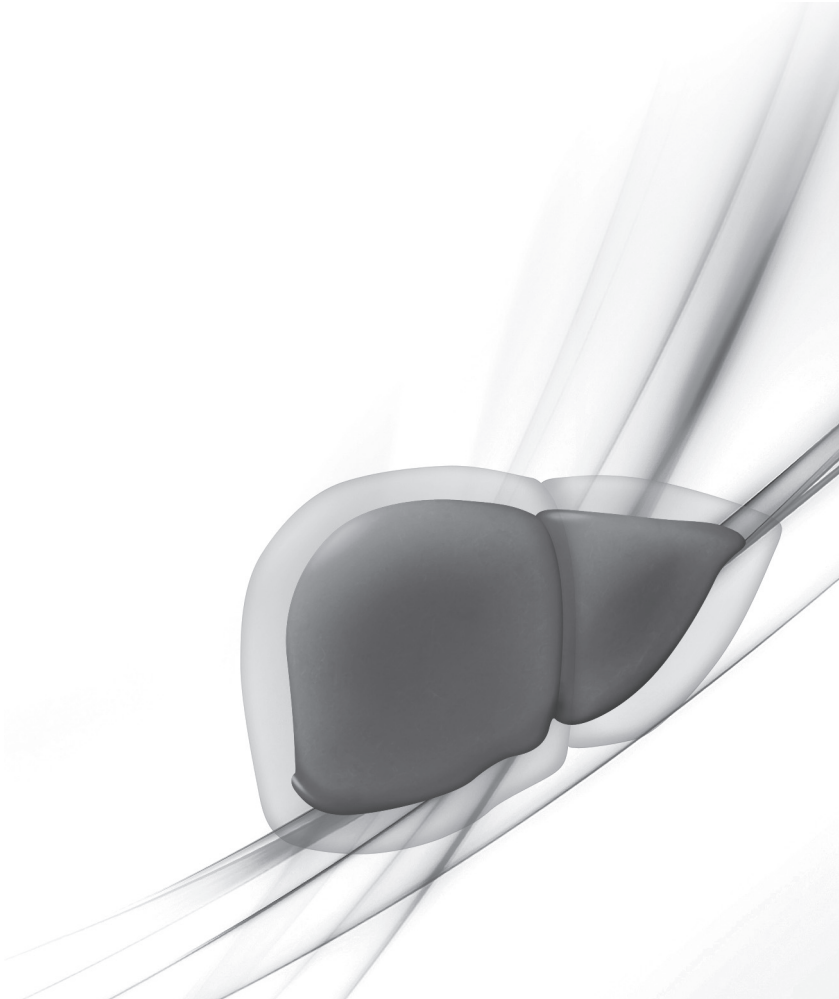
Toekomstig onderzoeksrichtingen zijn:

- Het testen van meer specifiekere TACE-remmers in het omkeren van leversteatose;
- Het ophelderen van de specifieke werkingsmechanismen van de onderzochte MMP en TACE-remmer door middel van 'state-of-the-art' 'gene array', 'proteomics' en 'lipidomics' analyses.
- Het ontwerpen en opzetten van een klinische studie om de onderzochte MMP en TACE remmer te testen in een specifiek gedefinieerde patiëntenpopulatie.

REFERENTIES

1. Angulo P. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; 346: 1221-1231.
2. Lazo M, Clark JM. The epidemiology of nonalcoholic fatty liver disease: a global perspective. *Semin Liver Dis* 2008; 28: 339-350.
3. Argo CK, Northup PG, Al-Osaimi AM, *et al*. Systematic review of risk factors for fibrosis progression in non-alcoholic steatohepatitis. *J Hepatol* 2009; 51: 371-379.
4. Jou J, Choi SS, Diehl AM. Mechanisms of disease progression in nonalcoholic fatty liver disease. *Semin Liver Dis* 2008; 28: 370-379.
5. Neuschwander-Tetri BA. Hepatic lipotoxicity and the pathogenesis of nonalcoholic steatohepatitis: The central role of nontriglyceride fatty acid metabolites. *Hepatology* 2010; 52: 774-788.
6. Adam R, Hoti E. Liver transplantation: the current situation. *Semin Liver Dis* 2009; 29: 3-18.
7. Vuppalanchi R, Chalasani N. Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis: Selected practical issues in their evaluation and management. *Hepatology* 2009; 49: 306-317.
8. Coussens LM, Fingleton B, Matrisian LM. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. *Science* 2002; 295: 2387-2392.

Appendices



APPENDIX I. LIST OF PUBLICATIONS

Publications related to the thesis

1. **De Meijer VE**, Kalish BT, Puder M, IJzermans JNM. Steatosis is a risk factor for complications and mortality following major hepatic resection: a systematic review and meta-Analysis. *Br J Surg* 2010; 97: 1331-1339 (**Chapter 2**).
2. **De Meijer VE**, Le HD, Meisel JA, Akhavan Sharif MR, Pan A, Nosé V, Puder M. Dietary fat intake promotes the development of hepatic steatosis independently from excess caloric consumption in a murine model. *Metabolism* 2010; 59: 1092-1105 (**Chapter 3**).
3. **De Meijer VE**, Sverdlov DY, Sharma AK, Le HD, Popov Y, Puder M. Matrix metalloproteinases are differentially expressed in liver and adipose tissue during development of non-alcoholic fatty liver disease and obesity. *Submitted* (**Chapter 4**).
4. **De Meijer VE**, Le HD, Meisel JA, Sharma AK, Popov Y, Puder M. Tumor necrosis factor α-converting enzyme inhibition reverses steatosis and insulin resistance and improves surgical outcome in mice. *Submitted* (**Chapter 5**).
5. **De Meijer VE**, Le HD, Meisel JA, Puder M. Repetitive orogastric gavage affects the phenotype of diet-induced obese mice. *Pysiol Behav* 2010; 100: 387-393 (**Chapter 6**).
6. **De Meijer VE**, Sverdlov DY, Popov Y, Le HD, Meisel JA, Nosé V, Schuppan D, Puder M. Broad-spectrum matrix metalloproteinase inhibition curbs inflammation and liver injury but aggravates experimental liver fibrosis in mice. *PLoS One* 2010; 5: e11256 (**Chapter 7**).

Other publications

7. **De Meijer VE**, Verhoef C, Kuiper JW, Alwayn IP, Kazemier G, IJzermans JNM. Radiofrequency ablation in patients with primary and secondary hepatic malignancies. *J Gastrointest Surg* 2006; 10: 960-973.
8. **De Meijer VE**, Van de Ven AWH, Den Hoed PT. FDG-PET and FDG-PET/CT for diagnosing infection in patients with multiple vascular bypass grafts - A report of two cases. *EJVES Extra* 2007; 13: 40-43.
9. **De Meijer VE**, Boelhouwer RU. [*Een vrouw met krampende pijn in de onderbuik*.] Diagnostic image: A woman with low abdominal pain spasms. *Ned Tijdschr Heelk* 2007; 16: 328.
10. **De Meijer VE**, IJzermans JNM, Verhoef C. A place for radiofrequency ablation in the treatment of resectable colorectal liver metastases? *Ann Surg Oncol* 2008; 15: 2063.
11. **De Meijer VE**, Van 't Sant HP, Spronk S, Kusters FJ, Den Hoed PT. Reference value of transcutaneous oxygen measurement in diabetic patients compared with nondiabetic patients. *J Vasc Surg* 2008; 48: 382-388.
12. **De Meijer VE**, Vles WJ, Kats E, Den Hoed PT. Iatrogenic diaphragmatic hernia complicating left nephrectomy: top down or bottom-up? *Hernia* 2008; 12: 655-658.

13. **De Meijer VE**, Gura KM, Meisel JA, Le HD, Puder M. Fish Parenteral fish oil as monotherapy for patients with parenteral nutrition-associated liver disease. *Pediatr Surg Int* 2009; 25: 123-124.
14. **De Meijer VE**, Verhoef C, IJzermans JNM. Radiofrequency Ablation in the Treatment of Hepatocellular Carcinoma – The Need for Centralization. *Ann Surg* 2009; 250: 497-498.
15. Puder M, Valim C, Meisel JA, Le HD, **De Meijer VE**, Robinson EM, Zhou J, Duggan C, Gura KM. Parenteral Fish Oil Improves Outcomes in Patients With Parenteral Nutrition-Associated Liver Injury. *Ann Surg* 2009; 250: 395-402.
16. **De Meijer VE**, Gura KM, Le HD, Meisel JA, Puder M. Fish oil-based lipid emulsions prevent and reverse parenteral nutrition-associated liver disease: The Boston Experience. *JPEN J Parenter Enteral Nutr* 2009; 33: 541-547.
17. Le HD, Meisel JA, **De Meijer VE**, Gura KM, Puder M. The essentiality of arachidonic acid and docosahexanoic acid. *Prostaglandins Leukot Essent Fatty Acids* 2009; 81: 165-170.
18. **De Meijer VE**, Gura KM, Puder M. Parenteral Nutrition in the Critically Ill Patient: Potential Benefit of Fish Oil-Based Lipid Emulsions. *N Engl J Med* 2010; 362: 81-82.
19. Le HD, Fallon EM, **De Meijer VE**, Malkan AD, Puder M, Gura KM. Innovative parenteral and enteral nutrition therapy for intestinal failure. *Semin Ped Surg* 2010; 19: 27-34.
20. **De Meijer VE**, Le HD, Meisel JA, Gura KM, Puder M. Parenteral fish oil as monotherapy prevents essential fatty acid deficiency in parenteral nutrition dependent patients. *J Pediatr Gastroenterol Nutr* 2010; 50: 212-218.
21. **De Meijer VE**, Le HD, Zurakowski D, Meisel JA, Puder M. Parenteral fish oil as monotherapy improves lipid profiles in patients with parenteral nutrition-associated cholestasis. *JPEN J Parenter Enteral Nutr* 2010; 34: 477-484.
22. **De Meijer VE**, Gura KM, Meisel JA, Le HD, Puder M. Parenteral Fish Oil Monotherapy in the management of patients with parenteral nutrition-associated liver disease. *Arch Surg* 2010; 145: 547-551.
23. Arsenault DA, Potemkin AK, Robinson EM, Fallon EM, Ozonoff A, **De Meijer VE**, Puder M. Surgical intervention in the setting of parenteral nutrition-associated cholestasis may exacerbate liver injury. *J Pediatr Surg* 2011; 46: 122-127.

Book chapters

1. Meisel JA, Le HD, **De Meijer VE**, Gura KM, Puder M. Using parenteral fish oil in NASH. *Nutrition, Diet Therapy, and the Liver.* Preedy VT, Lakshman R, Srirajaskanthan R, Watson RR (eds). Taylor & Francis Group: Boca Raton, Florida, USA, 2010; 81-95.

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APPENDIX IV. PORTFOLIO

Name PhD Student:	Vincent E. de Meijer
Erasmus MC Department:	Hepatobiliary and Transplant Surgery
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PhD Period:	December 2007 – December 2010
Promotors:	Prof.dr. J.N.M. IJzermans, Erasmus University Rotterdam
	Prof.dr. M. Puder, Harvard Medical School

General courses	**Year**	**Workload (ECTS)**
• Master of science in Clinical Epidemiology	2005	60.0
• Laboratory and environmental safety	2007	5.0
• Laboratory small and large animal training	2008	5.0
• Statistics and data analysis	2008	1.0
• Surgical Grand Rounds	2008	8.0
• Morbidity & Mortality	2008	8.0
• Surgical Grand Rounds	2009	8.0
• Morbidity & Mortality	2009	8.0
Presentations at conferences		
• National conferences	2005	1.0
• International conferences	2008	3.0
• International conferences	2009	8.0
• International conferences	2010	2.0

APPENDIX V. CURRICULUM VITAE AUCTORIS

Vincent Erwin de Meijer was born on February 8, 1983 in Meerkerk, the Netherlands. In 2001, he graduated *cum laude* from high school at the Titus Brandsma College in Dordrecht and attended medical school at the Erasmus University Rotterdam. During medical school, he worked as a student assistant in the operating room at the Department of Surgery for two years (2003-2004). After finishing his first year in 2002, he started his study in Clinical Epidemiology at the Netherlands Institute for Health Sciences at the Erasmus University Rotterdam and performed his research elective at the Department of Surgery, Erasmus MC – University Medical Center Rotterdam (Prof. dr. J.N.M. IJzermans). During this study, he spent electives at Cambridge School of Public Health (Cambridge, UK; March 2004) and at Harvard School of Public Health (Boston, MA, USA; July 2005). In 2005, he obtained his Master of Science degree in Clinical Epidemiology as well as his Masters of Science degree in Medicine, after which he started with his clinical rotations. During his clinical rotations, he actively acquired funds to finance an independent position as a post-doctoral research fellow, resulting in several grants and fellowships. In 2007, he graduated from medical school *cum laude*.

After medical school he was offered a post-doctoral research position as a PhD student at the Vascular Laboratory and Department of Surgery at Children's Hospital, Boston, MA, USA. Under daily supervision by Prof. dr. M. Puder in Boston and long-distance guidance by Prof. dr. J.N.M. IJzermans from Rotterdam, he focused on various aspects of hepatic steatosis, including parenteral nutrition associated liver disease as well as dietary-induced fatty liver disease. In 2009 he received an award at the Massachusetts Chapter of the American College of Surgeons Resident Research Competition, and in 2010 he received an award for the Top-5 Most Valuable Nutrition Papers of 2009 from the Journal of Parenteral and Enteral Nutrition and the American Society of Parenteral and Enteral Nutrition.

In January 2010, he started an internship in General Surgery at the Erasmus MC – University Medical Center, Rotterdam (Prof. dr. J.N.M. IJzermans and Prof. dr. J.J.B. van Lanschot), after which he started his training in General Surgery at the Ikazia Hospital in Rotterdam (Dr. P.T. den Hoed) in July 2010. He will complete the final two years of his residency (2014-2016) at the Erasmus MC – University Medical Center, Rotterdam (Prof.dr. J.N.M. IJzermans and Prof.Dr. J.J.B. van Lanschot). He remains closely involved with the ongoing projects at the laboratory of Dr. Puder and is passionate about research.