

## Forum Minireview

## Life Style-Related Diseases of the Digestive System: Gene Expression in Nonalcoholic Steatohepatitis Patients and Treatment Strategies

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**Abstract.** Nonalcoholic steatohepatitis (NASH) is a subset of nonalcoholic fatty liver disease (NAFLD) and sometimes progresses to cirrhosis and liver failure. We analyzed the expression profiles of approximately 50,000 genes and biological pathways in NASH patients in comparison with simple steatosis patients by using the analytical technique of GSEA (Gene Set Enrichment Analysis) by DNA microarrays. Although expressions of various genes were altered, GSEA showed clearly lower expression of nuclear receptors, including the peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) pathway. In a preliminary study we therefore investigated the therapeutic effect of low-dose pioglitazone (15 mg/day per body for 24 weeks), a synthetic ligand for PPAR $\gamma$ , in 12 NASH patients. A decrease in aminotransferase (ALT) values to within the normal range was observed in 7 (58.3%) of the patients, and because the dose of pioglitazone was lower than that ordinarily used, no side effects, such as fatigue, lower extremity edema, or weight gain, were observed. In conclusion, the results confirmed involvement of the PPAR $\gamma$  pathway in NASH and the therapeutic utility of a PPAR $\gamma$  ligand.

**Keywords:** life style-related disease, nonalcoholic steatohepatitis (NASH), nonalcoholic fatty liver disease (NAFLD), microarray, Gene Set Enrichment Analysis (GSEA), pioglitazone

### Nonalcoholic steatohepatitis (NASH): natural history and insulin resistance

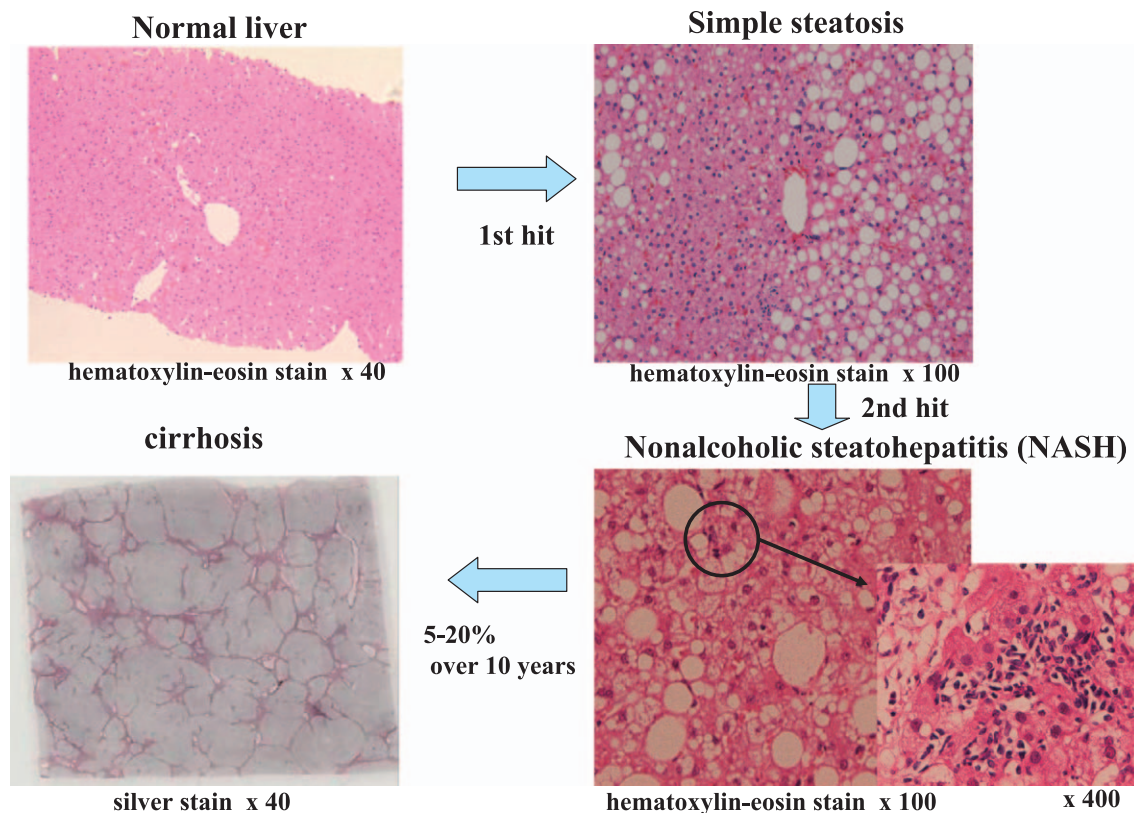
Nonalcoholic fatty liver disease (NAFLD) is now recognized as one of the most common liver diseases in the world. NAFLD is a general term that has been adopted to cover the full spectrum of metabolic fatty liver disorders (1). NASH is a subset of NAFLD and

sometimes progresses to cirrhosis and liver failure (1–3). NAFLD has a worldwide distribution, and population-based screening studies have shown that its prevalence ranges from 17%–33% of the general population. By contrast, the prevalence of NASH, the more serious form of NAFLD, is approximately 3% in general populations and is more prevalent among obese persons (4). NASH sometimes progresses to liver cirrhosis and liver failure, and even to hepatocellular carcinoma (1–3), and fatty livers are predisposed to forms of injury that involve oxidative stress. NASH has been hypothesized to be induced in two consecutive steps:

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**Fig. 1.** Natural history of nonalcoholic steatohepatitis (NASH), from steatosis to cirrhosis.

excess fat accumulation in the liver followed by necroinflammation in the liver, the so-called two-hit hypothesis (Fig. 1 and Ref. 5). Progression of fibrosis in NASH has been histologically demonstrated in 32%–37% of the patients (6, 7). Obesity, diabetes, and the initial severity of the fibrosis are the factors most conspicuously associated with fibrotic progression (6–8). Estimated rates of cirrhosis development over 10 years of 5%–20% have been reported by 3 independent studies (8–10) (Fig. 1). NASH patients with advanced fibrosis are at risk of developing liver complications, which is similar to chronic hepatitis C with cirrhosis among untreated patients or those who experience non-response to antiviral therapy (10).

Hepatocellular carcinoma has been detected in several NASH patients, most often at the time of diagnosis, and rarely, during follow up (8, 11, 12). In the larger Olmsted County Community Study (11), 2 of 420 NAFLD patients developed hepatocellular carcinoma during a 7-year follow-up period. The estimated rate of liver-related deaths over 10 years was 12% for NASH patients (9, 10).

Older age and impaired glucose tolerance were associated with higher risk of mortality. Mitochondrial dysfunction has been demonstrated in the livers of

NASH patients (13) and may have a genetic basis (14). It is likely to be worsened by aging and environmental factors such as consumption of highly saturated fats (15). Whether hepatic insulin resistance causes cellular injury and inflammation in the liver or results from both inflammation and steatosis is a key question in unraveling the pathogenesis of NASH (16). One molecular mechanism of insulin resistance is intracellular accumulation of fatty acids and their metabolites that activates protein kinase C (17, 18). Protein kinase C catalyzes serine/threonine phosphorylation of the insulin receptor substrate (IRS)-1 and 2, and hepatic over-expression of cytochrome P450 (CYP) 2E1 in NASH patients creates oxidative stress associated with impaired insulin receptor signaling (16). Tumor necrosis factor alpha (TNF $\alpha$ ), another candidate molecule in the transition from steatosis to steatohepatitis, is liberated by the adipose tissues of obese persons (19) and could worsen hepatic insulin resistance via activation of an inhibitor of kappaB kinase (IKK- $\beta$ ) or c-Jun N-terminal kinase. Furthermore, adiponectin facilitates transport of fatty acids into mitochondria where they undergo  $\beta$ -oxidation and suppresses hepatic fatty acid synthesis, thereby countering the effects of high serum insulin levels. Low serum adiponectin levels are a characteristic of NASH

that distinguishes it from simple steatosis (20).

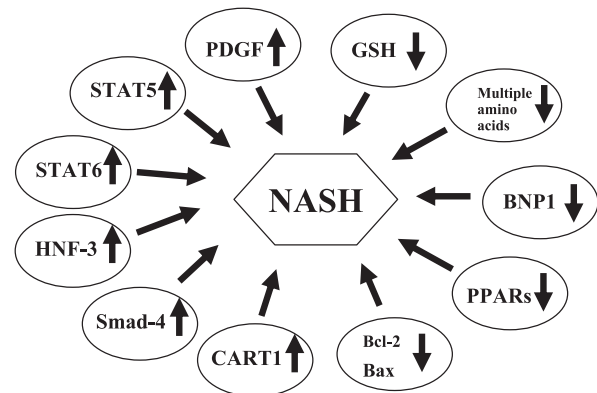
### Microarray technology to gain insight into the biochemical pathway responsible for the pathogenesis of NASH

Expression profiling by DNA microarray makes it possible to assess the relative levels of expression of a broad range of genes in small tissue samples (21, 22). A large number of studies have used microarrays to discern changes in gene expression patterns in well-defined cellular populations responding to a specific stimulus in vitro (23, 24) or in complex clinical settings, such as cancer (25), where large changes in expression of individual genes have often been observed. Microarray technology has also been used to study NAFLD, and to date, there have been two high-throughput microarray studies of this disease (26, 27). In the first, Sreekumar et al. (26) examined gene expression levels in three distinct groups of cirrhotic patients: a group with cirrhosis secondary to NASH, a group with HCV-related cirrhosis, and a group with cirrhosis secondary to primary biliary cirrhosis (PBC). The results highlighted the suppression of the genes involved in scavenging and reducing reactive oxygen species (ROS), this being especially prominent for superoxide dismutase 1 (SOD1); and other gene groups repressed in the NASH patients were those involved in fatty acid metabolism and glucose metabolism. In the second study, Younossi et al. (27) examined the cDNA array of 5220 genes in three distinct groups: NASH patients, obese patients, and non-obese patients. The important findings were a decreased level of expression of fibrinogen-like 1 (FGL1), which is involved in liver regeneration; decreased expression of adrenomedullin, which protects the liver against organ damage via oxidative stress; and an increase in alfa-fetoprotein (AFP) production, all in the NASH patients.

However, cellular processes often affect sets of genes acting in concert. If all genes encoding components of a metabolic pathway showed a 20% increase in expression, this may dramatically alter the flux through the pathway and may have a greater impact than a 20-fold increase in the expression of a single gene (28). Traditional strategies for gene expression analysis have focused on identifying individual genes that exhibit differences in expression between two states of interest. Although such strategies are useful, they have failed to detect biological processes such as metabolic pathways, transcriptional programs, and stress responses that are distributed across an entire network of genes and subtle at the level of individual genes. In the present study we therefore investigated the mechanisms of the patho-

genesis of NASH by using a robust technique for analyzing molecular profiling data, Gene Set Enrichment Analysis (GSEA), that was developed by Mootha et al. in 2003 (29) as a means of analyzing gene expression by using pathway or ontology information. The gene sets are defined based on prior biological knowledge, for example, published information about biochemical pathways or coexpression in previous experiments.

We analyzed the gene expression profiles of approximately 54,675 genes and biological pathways in NASH patients ( $n=9$ ) in comparison with simple steatosis patients ( $n=9$ ) by the GSEA analytical technique with high density oligonucleotide microarrays using an analytical technique of GSEA by using a high density oligonucleotide microarrays. The analysis highlighted upregulated gene sets of the overexpression of the platelet-derived growth factor (PDGF) receptor pathway, the signal transducer and activator of transcription (STAT)5 pathway, the STAT6 pathway, the hepatocyte factor (HNF)-3 pathway, the Smad 4 pathway, the cartilage homeoprotein-1 (CART1) pathway, organic cation transporter (OCT)1 pathway, and the mammary activating factor (MAF) pathway. In addition, we analyzed the downregulated gene sets of the pathway in NASH patients. The analysis indicated that there were downregulated gene sets in many pathways of glutathione; in the pathways for the degradation and metabolism of the several amino acids, including lysine, glycine, serine, threonine, arginine, and proline; and in the brain natriuretic peptide (BNP)1 pathway. Furthermore, it is noteworthy that the pathway of nuclear receptors, including those of peroxisome proliferators activated receptors (PPARs), was downregulated in NASH patients (Fig. 2). Our results providing insight into the mechanisms responsible for the progression of NASH was considered to be very helpful in designing therapeutic strategies for this disease entity.



**Fig. 2.** Schematic illustration of the possible involvement of up-regulated or down-regulated genes identified in the present study in the pathogenesis of NASH.

**Table 1.** Drugs used to treat NASH

Drugs	(Ref.)	Year	Type of study	Effect
<b>Insulin-sensitizing agents</b>				
Metformin	(38)	2005	Open-label, randomized trial (n = 55)	Improved aminotransferase
Pioglitazone	(33)	2004	Open-label (n = 18)	Improved aminotransferase + liver histology
Pioglitazone	(39)	2006	Double-blind, randomized controlled trial (n = 55)	Improved aminotransferase + liver histology
Pioglitazone + Vitamin E	(40)	2004	Open-label, randomized (n = 21)	Improved aminotransferase + liver histology
Rosiglitazone	(36)	2003	Open-label (n = 30)	Improved aminotransferase + liver histology
<b>Antioxidants</b>				
Vitamin E	(41)	2000	Open-label (n = 11)	Improved aminotransferase
Vitamin E	(42)	2001	Open-label (n = 22)	Improved aminotransferase
Vitamin E + C	(43)	2003	Random control vs placebo (n = 45)	Improved histology
Betaine	(44)	2000	Random control vs placebo (n = 191)	Improved aminotransferase
Betaine	(45)	2001	Open-label (n = 8)	Improved aminotransferase
<b>Hepatoprotectants</b>				
UDCA	(46)	2004	Random control trial vs placebo (n = 100)	No difference
UDCA + Vitamin E	(47)	2006	Double-blind, random controlled trial (n = 48)	Improved aminotransferase + liver histology
<b>Others</b>				
Orlistat	(48)	2004	Open-label (n = 10)	Improved aminotransferase + liver histology
Pentoxifylline	(49)	2004	Open-label (n = 20)	Improved aminotransferase

## Therapeutic strategy for NASH patients

NASH is a condition that sometimes progresses to cirrhosis, and its prevalence is thought to be increasing because of incidences of its typical features, fatty liver disease, obesity, and type 2 diabetes mellitus, have been increasing (30). Evidence shows that modest and sustained weight reduction, particularly in association with exercise, not only improves aminotransferase levels and reduces steatosis, but also causes steatohepatitis to resolve and reverses hepatic fibrosis (18, 31). Weight loss remains the only standard of care because no pharmacologic therapy has been conclusively demonstrated to be effective against NASH. Therefore pharmacological therapy of NASH has elicited considerable interest (Table 1).

The mechanism involved in the pathogenesis of NASH was elucidated by the data obtained in our pathway analysis by using a DNA microarray. Notably, the results indicated that the downregulation of PPARs is one of the major mechanisms for the pathogenesis of NASH. We therefore decided to use a peroxisome proliferators-activated receptor gamma (PPAR $\gamma$ ) agonist to treat NASH patients. The thiazolidinedione-derivative PPAR $\gamma$ -agonist pioglitazone has been reported to ameliorate insulin resistance and improve glucose and lipid metabolism in type 2 diabetes (32). Insulin resistance in NASH is frequently associated with chronic hyperinsulinemia, hyperglycemia, and an excessive

supply of plasma free fatty acids to the liver; and the thiazolidinediones reverse these abnormalities by ameliorating insulin resistance in adipose tissue (33, 34), the liver (32 – 34), and muscle (32, 35). The thiazolidinediones may reverse many of the abnormalities associated with NASH, but the patients who take pioglitazone often experience side effects such as fatigue and mild lower extremity edema. Weight gain is another unfavorable effect that has been found to occur in at least two thirds of patients in rosiglitazone and pioglitazone NASH treatment trials (33, 36). We therefore used low-dose pioglitazone (15 mg/day per body) for 24 weeks to treat 12 NASH patients while preventing side effects. In this preliminary study, the ALT values of 7 (58.3%) of the patients decreased to normal in response to treatment with pioglitazone. Both steatosis, measured as the ratio of the computed tomography (CT) attenuation value of the liver to that of the spleen (L/S ratio) (37), and insulin resistance uniformly improved. The patients treated with low-dose pioglitazone had no side effects such as fatigue, lower extremity edema, or weight gain (Table 2).

## Conclusion

Application of microarray technology to NASH patients clearly advanced our understanding of this disease and may lead to effective and targeted interventions without the toxicity of many conventional treat-

**Table 2.** Low-dose pioglitazone (15 mg/day per body) treatment of 12 NASH patients for 24 weeks (preliminary data)

	Before pioglitazone treatment	After pioglitazone treatment	<i>P</i> value
Weight (kg)	70.4 ± 5.2	71.2 ± 7.2	0.1679
AST (U/ml)	49.3 ± 4.1	35.2 ± 5.2	0.0045
ALT (U/ml)	75.7 ± 9.6	46.1 ± 9.6	0.0002
HDL cholesterol (mg/l)	52.9 ± 4.6	57.3 ± 5.1	0.0326
LDL cholesterol (mg/l)	118.5 ± 9.3	107.7 ± 9.6	0.0642
Triglyceride (mg/l)	154.8 ± 28.0	129.2 ± 14.3	0.2308
FBS (mg/dl)	137.4 ± 9.9	123.3 ± 7.8	0.0317
IRI ( $\mu$ l/ml)	13.5 ± 2.9	9.8 ± 1.6	0.0187
HOMA-IR	4.6 ± 2.9	3.0 ± 1.6	0.0679
HbA <sub>1c</sub> (%)	6.6 ± 0.3	6.1 ± 0.3	0.0014
VFA (cm <sup>2</sup> )	127.4 ± 16.9	117.78 ± 14.3	0.9773
SFA (cm <sup>2</sup> )	211.5 ± 33.4	195.3 ± 22.9	0.9415
L/S ratio	0.753 ± 1.062	0.964 ± 0.057	0.0340

Data are expressed as means ± S.E.M. AST: aspartate aminotransferase, ALT: alanine aminotransferase, FBS: fasting blood sugar, IRI: immunoreactive insulin, HOMA-IR: homeostasis model assessment of insulin resistance, VFA: visceral fat area, SFA: subcutaneous fat area, L/S ratio: liver to spleen ratio.

ments. Our data revealed the alterations in expression of multiple gene sets that are essential to the pathogenesis of NASH, and our molecular profiling data should enable remarkable progress in research on the mechanism and treatment of NASH. Based on our microarray data and clinical trial, treatment with low-dose pioglitazone can improve NASH without side effects.

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