Investigation of metabolite alteration in dimethylnitrosamine-induced liver fibrosis by GC–MS

**Background:** A metabolomic study of biomarkers associated with dimethylnitrosamine (DMN)-induced hepatic fibrosis in Sprague-Dawley rats was performed using GC–MS. The clinical chemistry of the collected blood and the histopathology of excised liver samples were examined, and urine samples were prepared by solvent extraction.

**Results:** Through pattern analysis, the DMN-treated group was divided into two subgroups based on the aspartate aminotransferase (AST) levels compared with the control, a moderately higher group (DMN subgroup A) and a significantly higher group (DMN subgroup B). Uric acid, orotic acid, N-phenylacetylglucine and glutaric acid were biomarkers for DMN subgroup A, aminomalonic acid was a biomarker for DMN subgroup B, and arabitol level distinguished control versus DMN treatment regardless of AST level. **Conclusion:** This study suggests that the identification and profiling of AST level-related metabolites may be useful as a diagnostic tool and for the study of the mechanism of liver fibrosis induced by DMN.

Liver disease can be categorized by cause, which may include infection, injury, exposure to drugs or toxic compounds, autoimmune processes or a genetic defect that leads to the deposition and build-up of damaging substances, such as iron or copper [1-4]. To understand the many signs of hepatotoxicity, such as inflammation, scarring, obstructions, clotting abnormalities and liver failure, it is important to understand the role of the liver in the metabolism of xenobiotics. The liver is the key organ in the metabolism and detoxification of foreign compounds. By using metabolomics technology, metabolites and the end products of metabolism can be quantified, and, then provide diagnostic biomarkers and information to interpret the mechanisms of hepatotoxicity.

Hepatic injury by xenobiotics can result in morphologic changes and increases in serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, which are commonly used as clinical markers for liver damage [5,6]. AST and ALT are located in the cytoplasm of hepatocytes, and the levels of these enzymes increase with hepatocellular injury [7]. Morphologic changes are usually detected by liver biopsy, while AST and ALT levels are measured in the blood. A drop in aminotransferase level might indicate a depletion of viable hepatocytes and acute massive (fulminant) hepatitis, which often has a poor prognosis. Conversely, exceedingly high (>1000 IU/l) aminotransferase levels are typically associated with acute viral hepatitis, severe toxic or drug reactions or ischemic hepatitis (i.e., an insufficient flow of blood to the liver). Less dramatic aminotransferase elevation is observed in many types of hepatic injury [8].

However, these diagnostic tests have limits; both biopsy and blood tests are considered invasive, and they have the potential for sampling error. Although elevated ALT can reflect even minimal amounts of active hepatocyte damage, AST and ALT level tests often result in false-negative or -positive outcomes depending on injury time or the causative compound [9]. Moreover, there are no set guidelines currently linking particular drugs or toxic compounds to specific ranges of AST and ALT values.

According to Parkes et al., fibrosis stage in chronic hepatitis C could not be adequately determined using ten different panels of serum markers of hepatic fibrosis; therefore, more accurate biomarkers are needed to diagnose chronic liver damage [10].

Most recent metabolomic toxicology studies have been performed using several toxicants, and these experiments have generally aimed to identify biomarkers of severe liver damage induced by high doses of an acute toxicant [1,11,12]. However, a metabolomics-based biomarker study correlating enzyme values and endogenous metabolites has not been conducted, especially in the context of chronic toxicant exposure.
This metabolomics study was performed using GC–MS on urine samples from rats treated with \( N,N \)-dimethyl nitros amide, also known as dimethyl nitrosamine (DMN). DMN is a well-studied alkylating agent that causes liver disease by alkylating hepatocyte macromolecules [13–17]. DMN causes hemorrhagic zone 3 necrosis, steatosis, progressive fibrogenesis, cirrhosis and fatty changes when repeatedly administered at doses near the LD\(_{50}\) [14,18]. In contrast to the events of acute hepatotoxicity, the liver progresses from fibrotic to cirrhotic in chronic hepatic fibrosis; this may cause fluctuations in the serological and biochemical data in chronic toxicology studies [11,19]. Clayton et al. has shown that the liver toxicity induced by paracetamol was related with its metabolic fate, and laboratory animals with the same genetic backgrounds can be predicted with urine metabolite profile [20]. Additionally, in metabolomics studies, the laboratory animals could be classified as either responders or nonresponders due to the inter-subject variation induced by pharmacological or nutritional effect [21]. In the present study, the fluctuation of AST values was observed and the DMN-treated group was divided as two sub-groups, focusing on the correlations between AST level, ALT level and metabolite changes during liver damage. This study was expected to identify biomarkers altered by DMN, which could then be used to interpret the biochemistry of the disease.

### Experimental

#### Chemicals

All organic solvents, DMN, and \( N,O \)-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA 1% TMCS) were purchased from Sigma-Aldrich (MO, USA).

#### Animals & treatments

Male Sprague-Dawley rats were purchased from Orient Bio Laboratory Animal Research Center Co. Ltd (Gyeonggi-do, Korea). Animals were kept in a humidity- and temperature-controlled facility under 12-h day–night cycles, and standard rat chow and free access to drinking water was provided. Rats were divided into two treatment arms, the DMN and control arms. The two groups were treated as described in our previous report [22]. At the end of the fourth week, urine samples were collected in bottles that had been pre-cooled for 24 h and contained 0.5 ml of sodium azide solution (100 mg/ml), and all rats were euthanized. Blood samples and liver tissue were collected for biomedical and histochemical analyses, and the collected samples were stored at -80°C until further analysis. All experiments were approved and reviewed by Inha University (Approval ID: 090601–9).

#### Sample preparation

The collected urine samples were centrifuged at 14,000 g for 5 min to remove macroparticles, and 200 µl of the supernatant was diluted in 800 µl of cold methanol. After vortexing for 10 s, the extracts were centrifuged at 14,000 g for 5 min and the supernatant obtained from centrifugation was transferred to a new glass vial. For derivatization of the sample extracts, samples were dried completely under nitrogen and then agitated with a mixture of BSTFA 1% TMCS and pyridine (1:2) at 60°C for 15 min. The reaction mixture was transferred to a 2-ml glass vial equipped with glass micro-inserts (Agilent Technologies, CA, USA) and capped immediately.

#### Histopathological data

Liver specimens were sliced into 5-µm thick sections and fixed in formaldehyde for hematoxylin and eosin, and Masson’s trichrome staining. Liver section images were analyzed under a light microscope. The degree of hepatic fibrosis was measured as previously described [22].

#### Biomedical analysis of serum

The liver damage indicators, serum AST and ALT, were analyzed using the standard methods (7600 Analyzer, Hitachi, Tokyo, Japan Wako Diagnostics reagents, Wako Pure Chemical Industries Ltd, Osaka, Japan) by the Inha University Hospital (Incheon, Korea).

#### GC–MS

GC–MS analysis was performed using a 6890 gas chromatograph (Agilent Technologies) coupled with a JMS-GC mate (Jeol Ltd, Tokyo, Japan). Chromatographic separation was performed on a DB-5 column (60 m × 0.25 mm ID, 0.25 µm film thickness, Hewlett-Packard, CA, USA). The GC oven temperature was held at 60°C for 5 min, increased to 320°C at a rate of 10°C/min and held at the final temperature for 10 min. A 1-µl sample was injected in split mode (10:1) and helium (99.9999% He) was used as a carrier gas at a constant flow of 1 ml/min. The transfer line and ion source temperatures were set at 280°C and 250°C, respectively. After a 300-s solvent delay, mass spectra were obtained.
at 20-scans per second with a mass range of m/z 55–500. The ionization energy was 70 eV in electron impact mode.

Data processing & statistics
All obtained GC–MS data were converted to ASCII format and reduced into 0.1 min buckets, which were then normalized by creatinine levels and imported into SIMCA-P 12.0 (Umetrics, Umeå, Sweden). Additionally, all variables were scaled using a Pareto scaling function to enhance the weight of medium features without inflating baseline noise. Principal component analysis was performed with all variables, and two principal components (PCs), PC1 and PC2, were plotted to identify trends between groups. The missing values tolerance was set at 60%, and outliers within the group were identified by Hotelling’s T2 distribution analysis, which is a multivariate generalization of the Student’s t-distribution. Orthogonal partial least squares-discriminant analysis (orthogonal PLS-DA) and PLS-DA were employed for the supervised pattern analysis. The S-plot and variable importance in projection (VIP) list were obtained from these analyses, respectively. The S-plot was utilized to filter out putative biomarkers from the data, and VIPs were used to indicate key buckets for interpretation of the relationship between groups of variables. The combined compounds in the key buckets were divided manually by mass to charge ratio (m/z) and retention time, and their differences were evaluated by one-way ANOVA (student t-test p-values <0.05). Metabolites with a p-value <0.05 were classified as potential biomarkers to discriminate between the disease and control group. The compounds were identified using authentic standards and the NIST library.

Results & discussion

AST, ALT & histological analysis
To score the degree of liver fibrosis, AST and ALT activities were assessed based on the histological analysis of livers stained with hematoxylin and eosin and Masson’s trichrome. As shown in Figure 1, hematoxylin and eosin, and Masson’s trichrome staining reflected the degree of liver fibrosis in each group. Liver fibrosis, which is defined as the disruption of cell architecture and collagen growth, was increased by DMN treatment. Portal widening with occasional bridging fibrosis and mild fatty changes was observed from the eighth to tenth DMN samples. In the seventh DMN sample, macrovesicular fatty changes without fibrosis were noted (Figure 2). A comparison of biochemical and histological analyses indicated that less elevated AST and ALT seemed to be related to occasional bridging fibrosis accompanied by mild fatty changes (steatohepatitis), which are usually present with abdominal fullness or pain as their only complaints.

Mean AST levels were increased, albeit not significantly, from 109.6 ± 8.1 IU/l in the control group (n = 5) to 498.6 ± 409.2 IU/l in the DMN-treated group (n = 10) (p > 0.05), while the mean ALT levels increased significantly from 46.6 ± 6.9 IU/l in the control group to 287.4 ± 221.9 IU/l in the DMN-treated group (0.01 < p < 0.05; Table 1). These results demonstrate the change in AST levels after DMN treatment.

According to this observation, AST seems to be correlated with the degree of liver fibrosis in the context of chronic damage, while ALT indicates acute liver damage. ALT was not a specific marker for hepatic fibrosis (chronic liver damage), even though ALT and AST both indicate liver damage.

Pattern analysis
First, to identify the metabolites that are altered by treatment, the chromatograms of
all analyzed samples were compared in a 1D projection. However, it was difficult to interpret the changes between samples because the chromatograms reflected several variables, including retention time, mass fragment pattern and intensity (Figure 3). To simplify the variables and improve the comparison, principal component analysis, which is often used in metabolomics studies, was performed. Principal components were computed by the SIMCA-P+ program. The two major principal components, PC1 and PC2, are plotted in Figure 4A & B, which shows the trends and outliers within each group. The outliers, which were identified by drawing a tolerance ellipse based on the Hotelling’s T2 distribution (0.95), did not appear within the groups. The principal component analysis score plot performed using two groups show that the control and DMN groups were not discriminated by PC1 or PC2 (Figure 4C). In the supervised multivariate pattern analysis, the control versus DMN group was separated by PC1, and DMN samples were distinguished within group by PC1 and PC3, as shown in Figure 4D. Interestingly, this result seems to be affected by the levels of AST. Additionally, with respect to PC1, the pattern separated the control group, DMN one to six and DMN seven to ten. Samples seven to ten had lower AST levels than other DMN samples, and these samples are relatively similar to the control group with respect to PC3. This result suggests that the metabolites altered by changes in AST contribute to the PLS-DA model.

Compared with all pattern analysis data, the maximized group separation was well designed by the orthogonal PLS-DA model (Figure 5). The results of goodness of fit and the predictability of the pattern analysis models were calculated as R² and Q², respectively, and the results are shown in Table 2. The high R² and Q² values demonstrate that the model was clearly classified and predictable.

Biomarkers of liver fibrosis induced by DMN

Metabolites were analyzed by GC–MS, and the first annotation of each metabolite was performed using the NIST search library. To confirm endogenous metabolites detected by GC–MS, a built-in library consisting of the retention time and mass fragments produced at 70 eV energy was developed with authentic standards of amino acids, fatty acids and sugars (Table 3). All identified metabolites were integrated according to their specific m/z, which were normalized to the creatinine levels in each sample. The creatinine levels were determined with manual peak integration of creatinine peak, which was confirmed by creatinine standard. The first step of the biomarker search was to find metabolites that were altered between the control and treatment groups. In the present study, to identify biomarkers that might indicate DMN exposure, key buckets with small jackknife uncertainty bars were first selected in the VIPs list. Among the selected metabolites, arabitol was identified as a discriminator between the control and DMN-treated groups. Uric acid, orotic acid, N-phenylacetylglycine (PAG) and glutaric acid were identified as metabolites that were significantly different
The level of aminomalonic acid decreased when AST and ALT values were significantly elevated compared with controls, and it was slightly (but not significantly) increased at low levels of AST, even though the histopathology demonstrated liver fibrosis. Meanwhile, 5-oxoproline was identified as the biomarker that best
separated subgroups A and B. These metabolites were shown in the coefficient plot of X-variables with Y. Figure 7 shows that the retention times of glutaric acid, 5-oxoproline, arabitol, orotic acid, PAG and uric acid were strongly correlated with intensity (Y) in the established PLS-DA model. Aminomalonic acid is generated when errors in protein synthesis or oxidative damage to amino acid residues in proteins occur; therefore, an elevated aminomalonic acid level after DMN treatment was expected because DMN induces oxidative damage and impairs protein synthesis [23]. Contrary to this expectation, a decreased level of aminomalonic was observed after DMN treatment. In a previous study, Xue et al. showed that aminomalonic acid is a biomarker of hepatocellular carcinoma due to the degradation and ubiquitinylation of IRP2, which is related to the conversion of cysteine into aminomalonic acid [24]. Additionally, iron deposition accompanied with fibrosis induced by DMN was reported by He et al. [25]. These reports suggest that an increase in aminomalonic acid may be related to iron metabolism. However, while further study of this relationship is needed, our results suggest that aminomalonic acid could be a biomarker.

Figure 4. Multivariate analysis by principal component analysis of control and dimethylnitrosamine treatment samples and the partial least-squares discriminant analysis. (A) Control, (B) dimethylnitrosamine group, (C) control and dimethylnitrosamine and (D) partial least-squares discriminant analysis. The scatter plots were plotted by t[1], and t[2], for principal component analysis, and t[1] and t[3] for partial least-squares discriminant analysis. The tolerance ellipses of all models were based on Hotelling’s T2 and drawn as circles. t[1]: 1st component; t[2]: 2nd component; t[3]: 3rd component.
of hepatic fibrosis when serum AST and ALT levels are high.

Uric acid, orotic acid, glutaric acid and PAG increased in subgroup A but were not changed in subgroup B. Uric acid is a final product of purine metabolism and is produced after hypoxanthine is oxidized to xanthine; there was a significant increase in uric acid in DMN subgroup A compared with the control group. This result is consistent with a previous report and the observation of elevated hypoxanthine guanine phosphoribosyl transferase levels after DMN injury [26,27]. However, as shown in Figure 6, uric acid was increased when the AST level is low, not high. Abnormalities in AST, that is, during liver dysfunction, may promote aspartate accumulation and induce nucleic acid synthesis in the presence of 5-phosphoribosyl 1-pyrophosphate, limiting the use of uric acid for recycling purposes [101]. Additionally, significantly elevated uric acid seems to be correlated with fatty changes rather than liver fibrosis. This suggests that an increase or decrease of uric acid after chronic DMN treatments due to nucleic acid metabolism; uric acid can reflect the AST level, but it cannot be used as a biomarker of hepatic fibrosis.

The increased level of orotic acid is well documented in relation to ammonia detoxification and arginine-deficient diets [28]. Moreover, according to a study of gene regulation changes promoted by DMN, arginosuccinate synthetase, which is involved in the urea cycle, was decreased by DMN treatment, resulting in increased urinary orotic acid levels [27]. However, in subgroup B, the expected result was not observed. Orotic acid is generated by pyrimidine synthesis, in which carbamyl phosphate and aspartate are condensed in a reaction catalyzed by AST [29]. Therefore, the level of orotic acid could be affected by AST activities, and high levels of AST in the serum indicate that neither pyrimidine synthesis nor the urea cycle is activated. Such as orotic acid and uric acid, metabolites affected by AST levels should be considered as biomarkers, but these were not identified as discriminators of hepatic fibrosis.

5-oxoproline, an uncommon amino acid derivative in which the free amino group of glutamic acid is cyclized to form a lactam, plays a role in glutathione synthesis and has been used as an indicator for glutathione synthetase deficiency [30]. Based on the results of this study, 5-oxoproline is a discriminator between subgroup A and B (Figures 6 & 7) even though the level of 5-oxoproline was not altered significantly after DMN treatment. This result contrasts with a previous report that glutathione synthetase gene expression was induced by DMN; liver regeneration induced by 4 weeks of DMN treatment might have caused this fluctuation, even though glutathione synthetase participates in the liver fibrosis process [27].

### Table 3. Altered metabolites in urine samples using databases based on NIST and built-in libraries.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>TMS m/z</th>
<th>Retention time (min)</th>
<th>Representative m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutaric acid†</td>
<td>276 (2-TMS)</td>
<td>18.46</td>
<td>261</td>
</tr>
<tr>
<td>Aminomalonic acid‡</td>
<td>335 (3-TMS)</td>
<td>19.46</td>
<td>320</td>
</tr>
<tr>
<td>5-oxoproline†</td>
<td>273 (2-TMS)</td>
<td>20.28</td>
<td>258</td>
</tr>
<tr>
<td>Arabitol†</td>
<td>512 (5-TMS)</td>
<td>22.42</td>
<td>319</td>
</tr>
<tr>
<td>Orotic acid†</td>
<td>357 (3-TMS)</td>
<td>22.58</td>
<td>357</td>
</tr>
<tr>
<td>N-phenylacetlyglycine‡</td>
<td>265 (1-TMS)</td>
<td>24.30</td>
<td>265</td>
</tr>
<tr>
<td>Uric acid‡</td>
<td>456 (4-TMS)</td>
<td>26.39</td>
<td>456</td>
</tr>
</tbody>
</table>

†Biomarkers to subdivide the dimethylnitrosamine-treated group based on the aspartate aminotransferase levels. Metabolites with a p-value <0.05 were identified using authentic standards; others were identified by NIST library search.

‡Biomarkers to distinguish between control and dimethylnitrosamine-treated groups. TMS: Trimethylsilylation.
Figure 6. Box-whisker plots of endogenous metabolites and biomarkers altered by dimethylnitrosamine treatment. (A) Glutaric acid, (B) N-phenylacetylglycine, (C) orotic acid, (D) uric acid, (E) arabitol, (F) 5-oxoproline, (G) aminomalonic acid. The y-axis plot represents the intensity ratio normalized to creatinine level, and box plots were generated with a stacked column chart using bottom (quartile 1), 2Q (median - quartile 1), and 3Q (quartile 3 - median) values. Control: n = 5; dimethylnitrosamine subgroup A: n = 4; dimethylnitrosamine subgroup B: n = 6. Error bars on the box plot are ± whisker value. *p < 0.05.
Glutaric acid is an intermediate breakdown product of lysine in collagen synthesis, and PAG, as an acyl glycine, is a well-known biomarker for liver phospholipidosis [31]. The pattern of these two biomarkers was also dependent on the change in AST level, as shown in Figure 6, indicating their correlation. Therefore, glutaric acid and PAG were designated as biomarkers related to mitochondrial fatty acid β-oxidation; fatty changes caused by DMN exposure were observed in DMN subgroup A.

Arabitol, which has not previously been reported to be related to DMN, was identified as a discriminator between control and DMN-treated groups (both subgroup A and subgroup B). Although it was recently reported that congenital liver cirrhosis was correlated with elevated urine levels of arabitol, this study is the first to demonstrate that arabitol is a biomarker of DMN exposure [32]. Arabitol could be used as a potential discriminator of hepatic damage induced by DMN rather than other hepatotoxins.

**Conclusion**

A metabolomics study using GC–MS was performed to identify biomarkers of the diverse pattern of liver fibrosis induced by DMN. After DMN pretreatment, arabitol, 5-oxoproline, aminomalonic acid, uric acid, orotic acid, PAG and glutaric acid levels were altered, and arabitol was confirmed as a biomarker of DMN-related damage, independent of elevated AST and ALT. Based on the results of the multivariate analysis, the DMN groups were categorized into two groups, and uric acid, orotic acid, PAG and glutaric acid showed a significant increase when both levels of AST and ALT were significantly raised. Aminomalonic acid was decreased when AST level was slightly elevated. Overall, the pattern analysis of the data from this study, including numerous variables, showed that aminomalonic acid accurately reflects the status of hepatic damage, and it may be a very useful tool when accompanied with biochemical and histological data. Consequently, liver damage induced by DMN can be represented using biomarkers obtained from a metabolomics study and metabolic biomarkers will be useful for studying the mechanism and diagnosis of liver disease with serum biomarkers such as AST and ALT.
Future perspective

Metabolomics has been an emerging technology in the identification of the metabolites induced by xenobiotics or environmental factors. In particular, it has many advantages over the traditional approach for the discovery of the biomarkers linked with diverse diseases. As with the study presented here, the biomarkers interrelated with the status of targeted organism will be useful for early diagnosis of disease as well as status of disease.

Financial & competing interests disclosure

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Executive summary

- Endogenous metabolites profiling was performed by GC–MS, which is known as a very stable and robust instrument.
- Based on the pattern analysis of the data, the dimethylnitrosamine (DMN) groups were categorized into two groups and biomarkers related with the diverse pattern of liver fibrosis were identified.
- Aminomalonic acid in DMN subgroup A, uric acid, orotic acid, phenylacetylglycine and glutaric acid in DMN subgroup B, and arabitol for both of the subgroups, were determined as the biomarkers correlated with aspartate aminotransferase levels.

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**Website**

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