Upregulation of Calprotectin and Downregulation of Retinol Binding Protein in the Serum of Workers with Trichloroethylene-induced Hypersensitivity Dermatitis

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Abstract: Upregulation of Calprotectin and Downregulation of Retinol Binding Protein in the Serum of Workers with Trichloroethylene-induced Hypersensitivity Dermatitis: Zhenlie HUANG, et al. Guangdong Prevention and Treatment Center for Occupational Diseases, China—Objectives: The aim of this study was to explore the involved pathophysiological processes and develop biomarkers of trichloroethylene-induced hypersensitivity dermatitis (THD). Methods: We examined the impact of THD on the serum proteome in 8 male patients by comparing the serum samples between acute and healed stages. Sample pooling and immunodepletion were applied for sample preparation. Two-dimensional gel electrophoresis coupled with matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-TOF/MS) was utilized to identify and quantitate differentially expressed proteins. Changes in selected proteins were further confirmed by an ELISA assay. Results: A total of 41 spots were quantitated with significant alteration (p<0.05; fold-change± 3.0) in the serum between the acute and healed stages. Of these proteins, 26 proteins were identified by MALDI-TOF-TOF/MS. The identified proteins could be categorized into diverse functional classes, e.g., immunity and defense response, vitamin and lipid transport, fatty acid biosynthesis, actin binding, proteolysis and glycolysis. The ELISA assay confirmed the relative upregulation of calprotectin (S100A8/A9) and downregulation of retinol binding protein (RBP4) in the serum of the acute stage. The alteration of calprotectin and RBP4 was found to be specific to THD rather than trichloroethylene exposure. Conclusions: The pathophysiological processes underlying THD may involve elevated inflammatory responses and oxidative stress, inhibition of vitamin transport, depression of fatty acid biosynthesis, loss of extracellular actin scavenger, increase in oxygen transport, dysfunction in lipid transport, proteolysis and glycolysis. The combination of higher calprotectin and lower RBP4 levels in the serum could be used as potential biomarkers of THD.

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Key words: Hypersensitivity dermatitis, Proteomics, Serum, Trichloroethylene

Trichloroethylene (TCE) is a chlorinated solvent that has been used extensively in industrial operations involving metal cleaning and degreasing. Occupational TCE exposure is declining in industrialized countries because of technological innovation and legislation1, but it is increasing in emerging industrialized countries, especially in Asia2. Recently, occupational exposure to TCE was associated with severe hypersensitive skin damage and impaired hepatic function3, 4, which has become one of the critical occupational health issues in Asia5, 6. In the past 10 yr, an increasing number of cases suffering from TCE-induced hypersensitivity dermatitis (THD) have been reported in Guangdong Province in southern China7. Concern that TCE may exert immunotoxic effects8 has driven epidemiological and experimental studies investigating TCE exposure and risk of THD. The genetic polymorphism of human leukocyte antigen HLA-B*1301 is strongly associated with THD among exposed workers and might be used as a biomarker to predict high-risk individuals exposed to TCE8. Overlap in terms of human herpesvirus 6 reactivation
with THD has also been discussed\(^5\). A guinea pig maximization test suggests TCE induces dermatitis with hepatic injury by a delayed-type hypersensitivity mechanism\(^5\). Serological proteome analysis exploring candidates associated with TCE-induced autoimmune disease revealed the existence of different autoantibodies between patients being treated and those who had already recovered\(^10\). Despite extensive research, questions remain regarding the exact mechanisms of THD, and valid biomarkers of THD are needed.

Proteomic analysis is currently considered to be a powerful tool for the global evaluation of protein expression and has played a central role in clinical diagnosis and monitoring, with a major resurgence of interest in the human serum proteome\(^11,12\). Evidence exists that many disease processes are associated with quantitative and functional changes in the proteins of body fluids\(^13\). Thus, in this study, to elucidate the pathophysiological processes underlying THD and develop biomarkers, we examined the effects of THD on the serum proteome in a population of workers with THD using two-dimensional gel electrophoresis (2-DE) coupled with matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-TOF/MS). Changes in selected proteins were further confirmed by an ELISA assay.

**Subjects and Methods**

**Subjects**

The study protocols were conducted according to the principles of the Declaration of Helsinki and were approved by the Scientific and Medical Ethics Committee of the Guangdong Prevention and Treatment Center for Occupational Diseases (GDPTCOD) and Medical Science Foundation, Guangdong, China. All the subjects gave their written informed consent before their inclusion in the study. Patients who met the Chinese National Diagnostic Criteria of Occupational Disease (GBZ38-2002) (Ministry of Health, China, 2002) and were diagnosed with various degrees of occupational THD by a panel of occupational physicians of the GDPTCOD were recruited in this study. The principles of diagnosis and four clinical categories of cases, including exfoliative dermatitis (ED), multiform erythema (ME), Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN), were well described in our previous study\(^8\). The patients received treatment starting with a high dose of corticosteroid, and the dose was then tapered over several weeks to less than 10 mg/d of prednisone. For proteomic analysis, 8 male cases who received a diagnosis of ED were enrolled (Table 1).

All cases characterized by erythematous maculopapular rash and multorgan involvement (e.g., hepatitis, nephritis) were accompanied by systemic manifestations (e.g., fever, eosinophilia, lymphadenopathy, hepatomegaly, and splenomegaly). The reasons for choosing these cases for proteomic analysis were their typical symptoms, clear diagnoses, the availability of exposure level and their integrated clinical data. For the validation experiment (ELISA assay), the 8 cases used in the proteomic analysis and another 22 cases (17 males and 5 females) with a diagnosis of ED were included. These 30 cases were selected from 36 patients officially reported in the study area between June 2006 and July 2008. As TCE-exposed controls, 30 age- and gender-matched workers without THD who were recruited in December 2007 were included in this study. In addition, 30 habitual and controlled blood donors in the community of Guangzhou voluntarily donated healthy control blood samples. These volunteers were interviewed, further experimentally

**Table 1. Characteristics of THD cases included in the proteomic profiles**

<table>
<thead>
<tr>
<th>No.</th>
<th>Age (yr)</th>
<th>TCE exposure duration (day)</th>
<th>Urinary TCA (mg/l)</th>
<th>ALT (IU/l)</th>
<th>AST (IU/l)</th>
<th>GGT (IU/l)</th>
<th>T-Bil (µmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18</td>
<td>39</td>
<td>25.1±0.12</td>
<td>2,553/40</td>
<td>523/40</td>
<td>364/109</td>
<td>139.1/13.9</td>
</tr>
<tr>
<td>2</td>
<td>19</td>
<td>28</td>
<td>23.0/0.20</td>
<td>149/36</td>
<td>383/33</td>
<td>222/36</td>
<td>9.9/8.6</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>15</td>
<td>29.2/0.37</td>
<td>500/24</td>
<td>93/15</td>
<td>290/33</td>
<td>19.6/6.2</td>
</tr>
<tr>
<td>4</td>
<td>17</td>
<td>15</td>
<td>10.7/0.34</td>
<td>118/49</td>
<td>91/19</td>
<td>114/41</td>
<td>66.9/7.4</td>
</tr>
<tr>
<td>5</td>
<td>23</td>
<td>31</td>
<td>21.9/0.18</td>
<td>3,516/29</td>
<td>2,954/21</td>
<td>235/38</td>
<td>161.7/11.1</td>
</tr>
<tr>
<td>6</td>
<td>36</td>
<td>24</td>
<td>32.8/0.12</td>
<td>1,269/32</td>
<td>454/16</td>
<td>747/65</td>
<td>81.7/9.1</td>
</tr>
<tr>
<td>7</td>
<td>20</td>
<td>54</td>
<td>25.0/1.90</td>
<td>4,552/56</td>
<td>1,304/63</td>
<td>463/76</td>
<td>191.4/5.4</td>
</tr>
<tr>
<td>8</td>
<td>51</td>
<td>50</td>
<td>14.2/0.23</td>
<td>666/16</td>
<td>1,292/22</td>
<td>263/18</td>
<td>16.4/6.9</td>
</tr>
<tr>
<td>Mean</td>
<td>25</td>
<td>32</td>
<td>22.7/0.43</td>
<td>1,665/35</td>
<td>887/29</td>
<td>337/52</td>
<td>85.8/8.6</td>
</tr>
<tr>
<td>SD</td>
<td>12</td>
<td>15</td>
<td>7.3/0.56</td>
<td>1,680/12</td>
<td>960/16</td>
<td>193/30</td>
<td>70.8/2.8</td>
</tr>
</tbody>
</table>

\(^a\)Parameters in acute-stage THD cases. \(^b\)Parameters in healed-stage THD cases. \(^c\)Statistically significant differences were observed (p<0.05) in the acute stage compared with the healed stage (n=8, paired Student t-test).
confirmed to be free from histories of occupational TCE exposure and matched with cases according to age, gender and history of smoking and alcohol consumption.

Sample collection

The THD cases’ blood (6 ml) and urine (50 ml) were taken on the 2nd (before starting corticosteroid therapy) and the last day of hospitalization, which was at least 1–2 wk after stopping corticosteroid therapy. The samples of the TCE-exposed workers without THD and healthy controls were taken on the day of their interviews. Paired samples from the same THD patients were used to reduce subject-to-subject variation in proteomic analysis. The healed status of THD cases was judged by the disappearance of involved symptoms and recovery in laboratory examinations (Fig. 1 and Table 1). Peripheral blood from each subject was processed into serum within 6 h, aliquoted and stored with urine at –80°C until use. Samples collected at the beginning of hospitalization were marked as the acute stage of THD, and those collected at the end of hospitalization were used as the control (healed stage; per subject). Hemolysis was not observed during the processes of blood collection.

Serological assay

Serological assays were performed with a CL-8000 Clinical Chemistry Analyzer (Shimadzu Corporation, Kyoto, Japan) in each subject and included serum alanine transaminase (ALT), serum aspartate aminotransferase (AST), serum γ-glutamyl transpeptidase (GGT) and serum total bilirubin (T-Bil).

Urinary trichloroacetic acid (TCA) concentration

The TCA concentrations of urine samples from acute- and healed-stage THD cases, TCE-exposed workers without THD and healthy controls were analyzed by gas chromatography-mass spectrometry (GC-MS) (6890N gas chromatograph, 5957 Mass Selective Detector, 7683 Automatic Liquid Sampler; Agilent Technologies, Santa Clara, CA, USA) as described previously3). The detection limit of TCA was 0.10 mg/l.

Immunodepletion

Stored serum was thawed to room temperature, and two pooled samples were made of an equal amount of protein (100 µg) from each individual serum (n=8) in each of the acute and healed stages. Two pooled samples were then individually processed for depletion of high-abundance serum proteins using an Albumin and IgG Removal Kit (QIAGEN, Hilden, Germany) according to the instructions provided by the manufacturer. Protein concentration was determined with a Bradford Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as a standard.

2-DE

Before the first-dimension isoelectric focusing (IEF), each immunodepleted pool sample (150 µg) was subjected to precipitation using 10% TCA in acetone (−20°C, overnight) followed by an acetone wash (−20°C, 15 min). Then the mixture was centrifuged at 12,000 × g at 4°C for 30 min, and the protein pellet was combined with 300 µl of the rehydration...
buffer containing 7 M urea, 2 M thiourea, 4% w/v 3-[(3-cholamidopropyl) dimethylammonio]-1 propane sulfonate (CHAPS), 65 mM dithiothreitol (DTT), 1% immobilized pH gradient (IPG) buffer (pH 3–10) and a trace of bromophenol blue. In-gel rehydration of the IPG strips (Immobiline DryStrips, 17 cm, nonlinear pH 3–10; Bio-Rad Laboratories) with the samples was performed for 12 h under 50 V at 17°C. IEF was run using a Protean IEF cell system (Bio-Rad Laboratories) at 250 V for 250 V h, at 1 kV for 1 kV h and at 10 kV for 50 kV h. After reduction and alkylation with 10 mg/ml DTT and 25 mg/ml iodoacetamide, respectively, the second-dimension 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (17 cm × 20 cm) was run on a Protean xi cell electrophoresis unit (Bio-Rad Laboratories) at 50 V/gel for 30 min, followed by 150 V/gel till the bromophenol blue reached the bottom of the gels. Triplicate experiments were performed for each pooled sample under the same conditions.

Silver staining and image analysis
Silver staining of gel was performed using the standard protocol provided in the PlusOne Silver Staining Kit (GE Healthcare, Piscataway, NJ, USA). The stained gels were scanned with a fluorescence image scanning GS-800 densitometer (Bio-Rad Laboratories). Intra-gel matching was performed using PDQuest software (version 7.4.0, Bio-Rad Laboratories). Image analysis included the following procedures: spot detection, spot editing, background subtraction and spot matching. Protein levels were considered to have changed when the following two criteria were both met: 1) statistically significant change in the protein level (two-tailed Student’s t-test, p<0.05, relative to the control) and 2) ≥3-fold change in protein level.

Trypsin digestion and protein identification
Spots were excised from the gel and destained with 60 µl of 50 mM NH₄HCO₃, and 50% acetonitrile (ACN) twice and then dried with 60 µl of ACN twice. The dried gel pieces were digested in 12.5 mg/ml sequencing-grade trypsin (Promega, Mannheim, Germany) overnight. The peptides were extracted twice by 60 µl of 50% ACN/0.1% trifluoroacetic acid (TFA) for 20 min each and dried with N₂. The peptide eluate (0.8 µl) was mixed with 5 mg/ml α-cyano-4-hydroxycinnamic acid (CHCA) matrix (Sigma–Aldrich, Steinheim, Germany) in 50% ACN/0.1% TFA and spotted onto a MALDI target. MALDI-TOF MS and tandem TOF/TOF MS were performed on a 4700 MALDI-TOF/TOF Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA). Trypsin-digested peptides of horse myoglobin were used as a mass standard to calibrate the instrument, and then the default calibration was applied for the sample peptides. After MS acquisition, the six strongest peptides per spot were selected automatically for the MS/MS analysis. Data were searched with MASCOT (Matrix Science, London, UK) against the NCBI nonredundant protein sequence (NCBIInr) database. MS/MS data were investigated only against the Homo sapiens (human) subset of the sequences. The mass tolerance was set as 0.3 Da, and the MS/MS tolerance was 0.4 Da. The automatic data analysis and database searching were performed by the GPS Explore software (Applied Biosystems). Protein scores>63 were considered statistically significant (p<0.05) under the above parameters.

ELISA assay
To confirm the altered expressions of S100A8/A9 (calprotectin) and retinol binding protein (RBP4) in the serum, ELISA kits for human calprotectin (Hycult® Biotech, Uden, Netherlands, HK325) and RBP4 (CircuLex™ Co., Ltd., Nagano, Japan, CY-8072) were respectively utilized to investigate their levels in each group of 30 samples of healthy controls, TCE-exposed workers without THD and THD cases both in the acute and healed stages, according to the standard procedures supplied by the manufacturers. Absorbance was determined using a Powerscan 4 multiplex plate reader (DS Pharma Biomedical, Osaka, Japan), and analysis of results was conducted using CurveExpert 1.30 (http://www.curve-expert.net/).

PANTHER analysis
Protein ontology classification was performed by importing proteins into the protein analysis through evolutionary relationships (PANTHER) classification system (http://www.pantherdb.org/). S100A8/A9 and RBP4 were respectively designated the TCA level as an independent variable and the calprotectin and RBP4 levels as dependent variables. Statistical analyses were performed using the JMP 8.0 software (SAS Institute Inc., Cary, NC, USA). A probability (p) value <0.05 with Bonferroni adjustment was considered statistically significant.
Results

Characteristics of THD cases used in the proteomic analysis

The 8 male cases included in the proteomic analysis were 25 ± 12 yr of age, and the duration of TCE exposure was 32 ± 15 days (Table 1). The urinary TCA level decreased significantly (p<0.05) from 22.7 ± 7.3 mg/l in the acute stage to 0.43 ± 0.56 mg/l in the healed stage (Table 1). The levels of serological parameters of ALT, AST, GGT and T-Bil all decreased significantly (p<0.05) from 1,665 ± 1,680 IU/l, 887 ± 960 IU/l, 337 ± 195 IU/l and 85.8 ± 70.8 µmol/l in the acute stage to 35 ± 12 IU/l, 29 ± 16 IU/l, 52 ± 30 IU/l and 8.6 ± 2.8 µmol/l in the healed stage, respectively (Table 1). Representative images of skin symptoms of THD cases are shown in Fig. 1. The results showed that all 8 cases were exposed to TCE and suffered from severe hepatic damage simultaneous to the occurrence of THD.

Characteristics of controls and TCE-exposed workers without THD used in the ELISA assay

The 30 healthy controls and 30 TCE-exposed workers without THD included in the ELISA assay were 26 ± 10 yr of age and 27 ± 12 yr of age, respectively. The urinary TCA level was significantly higher (p<0.001) at 60.0 ± 88.5 mg/l in the TCE-exposed workers compared with the level in the healthy controls, which was below the detection limit.

Analysis and identification of differentially expressed proteins

In order to detect low-abundance proteins, albumin and IgG were specifically depleted using affinity column chromatography. The efficiency of the immunoaffinity was evaluated by protein quantitation and silver staining and was in accordance with the manufacturer’s results (data not shown). On average, approximately 10% of the starting total protein sample was recovered. To evaluate the reproducibility of 2-DE, which is one of its possible limitations, triplicate experiments of each pooled sample were performed. A total of 483 ± 29 spots (451, 476 and 522) in 3 gels of the acute stage of THD were detected, resulting in a matching rate (the number of common spots among 3 gels divided by the number of total spots in a standard “Master” gel defined by the PDQuest software) of 82.9%. Similarly, 491 ± 27 spots (469, 474 and 529) in 3 gels in the healed stage of THD were detected, and the matching rate was 82.5%. These results emphasized the reproducibility of the 2-DE applied in the present study.

The protein expression levels for a given spot were compared between the acute-stage and the healed-stage groups, and the results showed that 41 spots were differentially expressed (p<0.05, fold change≥3-fold) (Fig. 2). Twenty-one of these spots showed upregulation in the acute stage, whereas 20 spots showed downregulation compared with the healed stage. MALDI-TOF-TOF/MS identified 26 protein spots (Table 2). Of these identified proteins, expressions of 13 proteins were upregulated in the acute stage, whereas expressions of 13 proteins were downregulated, compared with the healed stage. The remaining protein spots could not be identified, probably because the amount of protein was too low, as revealed by a retrospective analysis of the spot volumes.

Functional categories of identified proteins

To understand the biological impact of THD on the human serum proteome, the 26 differentially expressed proteins identified by 2-DE analyses combined with MALDI-TOF-TOF/MS were imported into the PANTHER database. The PANTHER classification system revealed that the proteins can be classified into eight groups according to their functional properties (Table 3): (1) immunity and defense response, (2) vitamin transport, (3) fatty acid biosynthesis, (4) actin binding, (5) oxygen transport, (6) lipid transport, (7) proteolysis and (8) glycolysis.

Validation of altered calprotectin and RBP4 protein

The levels of the calprotectin and RBP4 proteins in the serum were quantified by an ELISA assay in order to confirm their altered expressions revealed by proteomic analysis and validate their potential as biomarkers of THD. These proteins were chosen because of the commercial availability of antibodies and their greatest inhibition (RBP4, change-fold = −5.30) or activation (calprotectin, change-fold = +14.43) over the clinical course compared with other altered proteins. The relative calprotectin level in the serum of the acute stage was detected to be significantly increased (p<0.01) in 30 THD cases (Fig. 3A), whereas the RBP4 level was downregulated significantly (p<0.01) (Fig. 3B) compared with the samples in the healed-stage THD cases or healthy controls. No significant differences (p>0.05) were observed in calprotectin and RBP4 levels between the samples of healthy controls and healed-stage THD cases, healthy controls and TCE-exposed workers without THD (Fig. 3A and B). Regression analysis found no significant (p>0.05) relationship between urinary TCA level and alterations of calprotectin and RBP4 in the serum of TCE-exposed workers without THD. These results indicated that the altered expressions of calprotectin and RBP4 were consistent with the proteomic results, and their alterations may be specific to THD but not TCE exposure.
To obtain insight into the involved pathophysiological processes underlying THD and develop biomarkers of THD for clinical diagnosis and drug treatment, we employed the gel-based proteomic approach coupled with MALDI-TOF-TOF/MS to compare the serum proteome of THD between the acute stage and healed stage. The 26 altered proteins were involved in a variety of biological processes, e.g., inflammatory response and oxidative stress, vitamin transport, fatty acid biosynthesis, actin binding, oxygen transport, proteolysis and glycolysis. The relative upregulation of calprotectin and downregulation of RBP4 in the serum of the acute stage of THD cases were confirmed by ELISA assay and were found to be specific to THD but not TCE exposure.

S100A8/A9 (calprotectin) and S100A8 were upregulated to a greater extent than other altered proteins in the sera of THD cases and showed increased amyloid-related serum protein (SAA) and plasma glutathione peroxidase (GPX3), suggesting elevated inflammatory responses and oxidative stress in THD. S100A8 and S100A8/A9 are involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation and have been identified as biomarkers for acute and chronic inflammation\textsuperscript{15}, chronic bronchitis and cystic fibrosis\textsuperscript{16}, chronic inflammatory bowel disease\textsuperscript{17} and systemic lupus erythematosus\textsuperscript{18}. Recently, S100A8/A9 has

**Fig. 2.** 2-DE gels from the serum samples of acute-stage (left) and healed-stage (right) THD cases (A) and representative spots showing different expression ($p<0.05$, fold change$\geq$3-fold; paired Student’s t-test) (B).
Table 2. Differentially expressed proteins in the acute- and healed-stage THD cases identified by MALDI-TOF-TOF/MS

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Accession no.</th>
<th>Protein identified</th>
<th>Theoretical Mr/pI</th>
<th>Measured Mr/pI</th>
<th>Sequence coverage</th>
<th>Protein score</th>
<th>Average ratio</th>
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</thead>
<tbody>
<tr>
<td>Upregulation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>gi51594277</td>
<td>Hemoglobin Lepore-Baltimore</td>
<td>11.4/6.2</td>
<td>11.3/7.5</td>
<td>62%</td>
<td>66</td>
<td>+4.45</td>
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<tr>
<td>3</td>
<td>gi21614544</td>
<td>S100 calcium-binding protein A8 (S100A8)</td>
<td>10.8/6.5</td>
<td>10.7/6.6</td>
<td>60%</td>
<td>96</td>
<td>+12.20</td>
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<td>6</td>
<td>gi2225986</td>
<td>Amyloid-related serum protein (SAA)</td>
<td>11.7/5.9</td>
<td>11.9/5.9</td>
<td>84%</td>
<td>367</td>
<td>+10.80</td>
</tr>
<tr>
<td>7</td>
<td>gi2225986</td>
<td>Amyloid-related serum protein (SAA)</td>
<td>11.7/5.9</td>
<td>11.8/5.6</td>
<td>82%</td>
<td>410</td>
<td>+10.78</td>
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<td>8</td>
<td>gi82407444</td>
<td>Human calprotectin (S100A8/A9)</td>
<td>13.1/5.7</td>
<td>14.1/5.6</td>
<td>87%</td>
<td>208</td>
<td>+14.43</td>
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<tr>
<td>11</td>
<td>gi182516</td>
<td>Ferritin light subunit (FTL)</td>
<td>16.4/5.6</td>
<td>18.2/5.6</td>
<td>67%</td>
<td>342</td>
<td>+9.21</td>
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<tr>
<td>12</td>
<td>gi224917</td>
<td>Apolipoprotein CIII (APOCIII)</td>
<td>87.6/4.7</td>
<td>15.7/5.2</td>
<td>64%</td>
<td>133</td>
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<tr>
<td>17</td>
<td>gi404108</td>
<td>Plasma glutathione peroxidase (GPX3)</td>
<td>16.6/8.9</td>
<td>25.7/5.5</td>
<td>57%</td>
<td>83</td>
<td>+5.80</td>
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<tr>
<td>21</td>
<td>gi4557325</td>
<td>Apolipoprotein E (APOE)</td>
<td>36.1/5.6</td>
<td>36.2/5.5</td>
<td>74%</td>
<td>437</td>
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<tr>
<td>22</td>
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<td>36.1/5.6</td>
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<td>23</td>
<td>gi4557325</td>
<td>Apolipoprotein E (APOE)</td>
<td>36.1/5.6</td>
<td>35.8/5.5</td>
<td>71%</td>
<td>404</td>
<td>+4.24</td>
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<tr>
<td>29</td>
<td>gi4335941</td>
<td>Leucine aminopeptidase (LAP)</td>
<td>56.0/4.6</td>
<td>62.4/6.4</td>
<td>39%</td>
<td>120</td>
<td>+9.80</td>
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<tr>
<td>36</td>
<td>gi11514029</td>
<td>Fructose 1,6-bisphosphate aldolase (ALDOB)</td>
<td>39.3/8.0</td>
<td>38.9/8.0</td>
<td>38%</td>
<td>74</td>
<td>+8.60</td>
</tr>
</tbody>
</table>

Downregulation | | | | | | | |
| 9 | gi73535906 | Transferrin (TR) | 13.9/5.4 | 17.6/5.4 | 70% | 251 | –3.23 |
| 14 | gi2030284 | Retinol binding protein (RBP4) | 20.9/5.3 | 21.8/5.4 | 50% | 65 | –5.30 |
| 15 | gi542750 | Acetyl-CoA carboxylase (ACC) | 264.4/5.6 | 20.9/5.3 | 15% | 74 | –3.12 |
| 16 | gi59961583 | Apolipoprotein M (APOM) | 13.0/7.7 | 25.3/5.4 | 55% | 152 | –3.33 |
| 18 | gi296653 | Hsp2-alpha (H2P) | 41.5/6.2 | 20.9/5.8 | 13% | 66 | –3.60 |
| 19 | gi4502067 | Protein AMBP (AMBP) | 39.0/6.0 | 32.2/5.3 | 32% | 81 | –4.20 |
| 24 | gi1212947 | Haptoglobin (HP1) | 38.4/6.3 | 38.5/5.6 | 32% | 123 | –4.30 |
| 27 | gi386789 | Hemopexin (HPX) | 51.5/6.6 | 60.8/6.0 | 23% | 79 | –3.05 |
| 28 | gi386789 | Hemopexin (HPX) | 51.5/6.6 | 60.8/6.1 | 30% | 94 | –3.12 |
| 31 | gi4503011 | Carboxypeptidase N (CPN1) | 52.2/6.9 | 52.2/6.9 | 27% | 112 | –4.68 |
| 32 | gi78101268 | Complement component C3 (C3) | 112.9/5.6 | 51.1/6.8 | 26% | 338 | –3.16 |
| 33 | gi78101268 | Complement component C3 (C3) | 112.9/5.6 | 51.1/7.0 | 25% | 203 | –3.28 |
| 34 | gi55664491 | Nebulin-related anchoring protein (NRAP) | 197.8/9.2 | 68.8/8.9 | 19% | 64 | –3.96 |

Notes: Spot no. was defined according to spot positions in 2-DE gels. Accession no. was recorded as a reference for identification in the NCBInr database. Protein was named referring to each matched protein in the NCBInr database. Theoretical Mr/pI: theoretical molecular weight of matched protein in kDa and theoretical isoelectric point of matched protein. Measured Mr/pI: molecular weight of matched protein calculated in the PDQuest software. Sequence coverage: percentage of identified sequence to the complete sequence of the known protein. Protein score: spots identified by MS/MS analysis; the MASCOT score is indicated. Average ratio calculated by normalization of protein expression in the acute stage to the healed stage; the p-value is <0.05 (paired Student’s t-test). Upregulation (+)/downregulation (–).

been identified as an important endogenous damage-associated molecular pattern that binds to Toll-like receptor 4 and plays a pivotal role during amplification of inflammation and represents a promising new therapeutic target\(^{19}\). Elevated serum S100A8/A9 is reported in different chronic immune and immunopathological reactions, especially in acute inflammation or Th1-mediated responses\(^{15}\). We speculated that activated monocytes and macrophages in THD may contribute to the overproduction of S100 proteins. SAAs are multifunctional apolipoproteins that are involved in cholesterol transport and metabolism and in modulating numerous immunological responses during inflammation\(^{20}\). Hepatic SAAs are mainly induced by IL-1, TNF-\(\alpha\) and IL-6\(^{20}\). Considering that the serum TNF-\(\alpha\) level was upregulated among THD cases\(^{21}\), we may understand the positive correlation between upregulated TNF-\(\alpha\) with elevated serum SAA. GPX3 reduces hydrogen and lipid peroxides to less toxic hydroxy fatty acids using glutathione as a reducing agent and plays a major role in defending the body against reactive oxygen species\(^{22}\). Upregulated serum GPX3 may indicate its involvement in the defense response to increased oxidative stress in THD.

The greatest depression of serum RBP (RBP4), together with increased serum leucine aminopeptidase (LAP) and decreased carboxypeptidase N (CPN1), provided potential mechanisms of inhibition of retinol synthesis and abnormal proteolysis to better understand the hepatic damage in THD cases. RBP, mainly synthesized and stored in hepatocytes, is a specific carrier of retinol, transporting it from the liver to various target tissues. Decreased RBP4 plays a possible role in the pathogenesis of hepatic fibrosis and serves as a potential biomarker reflecting disease severity in postoperative biliary atresia patients\(^{23}\). Additionally, both animal and human studies have shown that RBP4 might be involved in the development of insulin resis-
Furthermore, serum RBP4 levels have been shown to be associated with the degree of steatosis and fibrosis in liver diseases and chronic hepatitis C. Considering the association between depressed serum RBP4 and hepatic damage, and the liver pathology of perivascular infiltrates and fibrosis in TCE-induced autoimmune hepatitis in MRL+/+ mice, further studies are needed to investigate the precise role of RBP4 in the hepatotoxicity in THD. Serum LAP is known to be increased almost exclusively in the presence of hepatobiliary obstruction and pancreatic disease. T-cell mitogen was reported to increase the intracellular LAP level, but B-cell mitogen did not. These results suggest that LAP originates from T cells that are active or destroyed during the active phase of THD, although no direct evidence has as yet been obtained.

This study also showed a variety of other biological responses to THD. Alteration of Hemoglobin Lepore-Baltimore suggested an increase in oxygen transport in THD, while changes in apolipoproteins (APOCIII, APOE and APOM) implicated dysfunction in lipid transport. Downregulation of ACC suggested inhibition of fatty acid biosynthesis, and upregulation of...
ALDOB pointed to the acceleration of metabolism in glycolysis. Finally, loss of NRAP suggested depression of extracellular actin scavenger.

Interestingly, another study compared serum proteomes of TCE-exposed workers with acute- and healed-stage cases and TCE-exposed workers without THD (exposed workers). Their results show alterations in apolipoprotein C-II, apolipoprotein A-I, apolipoprotein C-III and transthyretin in the acute-stage THD cases and complement component C4b in the healed-stage THD cases compared with the TCE-exposed workers. However, the above study design remains controversial. First, the mode of TCE-induced biological effects might be quite different in TCE-exposed workers (cumulative effects) and THD cases (hypersensitive effects). Second, the healed-stage THD cases or healthy controls were suggested to be the control group rather than the TCE-exposed workers. Third, the results have not been validated in a large-scale epidemiological investigation. Taken together, the results produced in this study do not sufficiently to explain clearly and directly the underlying mechanisms of THD, highlighting the need for further well-designed studies.

A sample pooling approach was applied in the present study to elucidate a common protein expression pattern of THD. Underlying sample pooling strategies is a biological averaging assumption that the measures taken for the pool are equal to the average of the measures taken for individuals\(^{33}\). Based on the above assumption, pooling reduces variability by minimizing individual variation, but this is only suitable for experiments when the focus is not on the individual but rather on characteristics of the population\(^{34, 35}\). The pooling approach in proteomics has been advocated and widely used in studies \textit{in vitro}\(^{33–37}\) and \textit{in vivo}\(^{38}\).

Considering the corticosteroid therapy given the THD patients, although there was only a trace dose of corticosteroid in the last application and a 1–2 wk interval between the last application and the second collection of blood samples, doubts might arise about the specificity of the altered proteins to THD. Our ongoing study in which serum proteomes are being compared between THD cases (acute stage) and healthy controls using iTRAQ-based methods confirms most of the altered proteins in the current study, e.g., HPX, SAA, LAP and ALDOB (data not shown), suggesting that this was not the case. Additionally, a minimally invasive approach to sampling of serum was used, and the most abundant proteins, e.g., albumin and IgG, were removed via affinity-based depletion to detect lower abundance biomarkers. However, it is likely that important binding proteins and/or antibodies connected with albumin and IgG were also depleted, resulting in the loss of some potential molecules associated with THD. Furthermore, 15 altered proteins that failed to be identified might also have important roles in the involved pathophysiological processes in THD.

**Conclusion**

The identified proteins probably mediate the effects on elevated inflammatory responses and oxidative stress, inhibition of vitamin transport, depression of fatty acid biosynthesis, loss of extracellular actin scavenger, increase in oxygen transport, dysfunction in lipid transport, proteolysis and glycolysis. Taking into account possible associations between increased calprotectin and systemic lupus erythematosus, as well as decreased RBP4 and hepatic pathology, the combination of higher calprotectin and lower RBP4 levels in the serum could be used as potential biomarkers of THD for clinical diagnosis and drug treatment. Proteins identified in this study also provide a valuable reference resource for future hypothesis-driven studies on TCE-induced immunotoxicity and hepatotoxicity.
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**References**


24) Graham TE, Yang Q, Bluher M, et al. Retinol-


