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Low levels of IgG recognizing the alpha-1-antitrypsin (A1AT) [50-63] peptide and its association with Taiwanese women with primary Sjögren's syndrome

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Running Title: Low level of IgG anti-A1AT$^{50-63}$ in patients with pSS

Abbreviations:

pSS, primary Sjögren's syndrome; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; HNE, 4-Hydroxy-2-nonenal; 1-D SDS-PAGE, one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis; nano-LC–MS/MS, nano-liquid chromatography–tandem mass spectrometry; HC, healthy control; Ig, immunoglobulin; ACR, American College of Rheumatology; IP, immunoprecipitation; A1AG1, alpha-1-acid glycoprotein 1; A1AT, alpha-1-antitrypsin; RSD, relative standard deviation; SD, standard deviation; ROC, receiver operating characteristic; AUC, area under the ROC curve; CBB, Coomassie brilliant blue; RF, rheumatoid factor; ANA, antinuclear antibody ; anti-Ro (SSA), anti-Sjögren's-syndrome-related antigen A; anti-La (SSB), anti-Sjögren's-syndrome-related antigen B; CRP, C-

53 Reactive protein; ESR, erythrocyte sedimentation rate; OR, odds ratio; ESSDAI,
54 Sjögren's syndrome disease activity index; NSAIDs, nonsteroidal anti-inflammatory
55 drugs; DMARDs, Disease-modifying anti-rheumatic drugs.

56

57 Key words:
58 primary Sjögren's syndrome; alpha-1-antitrypsin; inhibitor; 4-hydroxy-2-nonenal;
59 autoantibody isotypes; serum

60

Abstract

The aim of this study was to examine oxidative stress and low level of alpha-1-antitrypsin (A1AT) in primary Sjögren's syndrome (pSS), and evaluate the associated autoreactivity against unmodified and their 4-hydroxy-2-nonenal (HNE)-modified peptides with pSS. Two differentially expressed proteins, alpha-1-acid glycoprotein 1 (A1AG1) and A1AT, exhibited 2-fold differences, and their HNE modifications were identified by depleted-albumin and immunoglobulin G (IgG) serum protein, in-solution digestion, in-gel digestion, and nano-LC-MS/MS from pSS patients and age-matched healthy controls (HCs). Furthermore, levels of proteins, confirmation of HNE modifications, HNE-protein adducts and autoreactivity against unmodified and their HNE-modified peptides were further validated. Levels of the HNE-protein adduct and A1AG1 were significantly higher in pSS patients than HCs, but levels of A1AT were significantly lower in pSS patients compared to HCs. Only the HNE modification of A1AT was confirmed. Further, concentrations of anti-$A1AT^{50-63}$ IgG and anti-$A1AT^{50-63}$ HNE IgA were significantly lower in pSS patients than HCs. Our study suggests that elevated HNE-protein adduct, oxidative stress, level [odds ratio (OR) 4.877, $p = 0.003$], lowered A1AT level (OR 3.910, $p = 0.010$) and a decreased level of anti-$A1AT^{50-63}$ IgG (OR 3.360, $p = 0.010$) showed an increased risk in pSS patients compared to HCs, respectively.

1. Introduction

Primary Sjögren's syndrome (pSS) is a chronic inflammatory autoimmune disease characterized by dysfunction of the exocrine glands leading to dryness of the mouth and eyes [1]. Patients with pSS feature the presence of autoantibodies mainly against the ribonucleoprotein complex SS-related antigen A (SSA, Ro) and SS-related antigen B (SSB, La) [1]. In 2000~2008, the prevalence of pSS was 16.0 (females, 28.8, males, 3.7; female: male ratio, 7.9) per 100,000 persons; the incidence rate of pSS was 10.6 (females, 18.5, males, 2.9; female: male ratio 6.3) per 100,000 person-years; and the mortality from pSS was 1304.7 (females 987.4, males 3444.2; age-adjusted female: male ratio 0.4) per 100,000 person-years in Taiwan [2]. The etiology and pathogenesis of Sjögren's syndrome are not clearly understood [3]. Jonsson and Brun proposed etiopathogenic events prior to a diagnosis of SS including a genetic predisposition, environmental triggers, autoantibodies, pathological injury, clinical disease, and clinical presentation [4].

Norheim *et al.* reported that patients with pSS have high levels of oxidative stress compared to healthy controls (HCs) [5]. Wakamatsu *et al.* found an increase in 4-hydroxy-2-nonenal (HNE)-protein adducts, marker of oxidative stress, in the conjunctiva of SS patients that may play a role in the pathogenesis of dry-eye disease [6,7]. HNE is one of the lipid peroxidation products that has an alkene bond and an aldehyde group which react with amino acid residues that form HNE-protein adducts via types of Michael addition and Schiff-base adducts, respectively [8]. Amino acid residues that can react with HNE include cysteine (C), histidine (H), lysine (K), arginine (R), glutamine (Q), alanine (A), and leucine (L) [8-10]. The HNE-protein adduct is an autoantigen and can elicit specific autoantibody formation [11,12].

Breit *et al.* indicated that several immune-mediated diseases were associated with an alpha-1-antitrypsin (A1AT) deficiency including rheumatoid arthritis (RA),

anterior uveitis, systemic lupus erythematosus (SLE), and asthma in which A1AT may play roles as an anti-inflammatory and immune regulator [13]. A1AT is a serine protease inhibitor [14]. Further, two cases were reported with an A1AT deficiency in patients with pSS in which the A1AT level of plasma declined by 1.28~2.10-fold [15,16].

In the present study, our aim was to investigate whether a low level of serum A1AT occurs in Taiwanese women with pSS and then identify the HNE modification on A1AT using depleted-albumin and immunoglobulin G (IgG) serum, in-solution digestion, one-dimensional sodium dodecylsulfate polyacrylamide gel electrophoresis (1-D SDS-PAGE), in-gel digestion, and label-free nano-liquid chromatography tandem mass spectrometry (nano-LC-MS/MS) from pSS patients *vs.* HCs. Further, we also assessed associations of autoantibody isotypes against $A1AT^{50-63}$ and their HNE-modified peptides with pSS patients compared to HCs.

2. Results

2.1 Identification and validation of differentially expressed serum proteins by in-solution digestion and LC-MS/MS

Enrichment of depleted-albumin and IgG serum protein samples from a single pair of each of nine pooled serum samples (patients with pSS *vs.* HCs) was analyzed in triplicate by in-solution digestion coupled to nano-LC-MS/MS (Table 1). In total, 255 proteins were detected, of which 28 differentially expressed proteins significantly varied, as shown in Table 1 and Supplementary Table 2. There were seven upregulated proteins and 21 downregulated proteins; relative to the HC serum pools, two of the identified proteins, alpha-1-acid glycoprotein 1 (A1AG1) and A1AT, differed by a 2-fold increase or decrease in patients with pSS serum pools, and 26 proteins differed by a 1.7~1.9-fold increase or decrease (Table 1).

Next, we validated the LC-MS/MS data of A1AG1 and A1AT, and protein levels of A1AG1 (~48 kDa) and A1AT (~55 kDa) were analyzed by Western blotting (Figure 1). A1AG1 expression levels were significantly higher in pSS samples by 1.53-fold ($p = 0.0001$) than in HCs, but A1AT levels in pSS samples were significantly lower than those in HCs by 1.84-fold ($p = 0.0071$, Figure 1A, B, right upper panel). Equal amounts of serum proteins in these experiments were examined (Figure 1A, B, right bottom panel). The AUC value, sensitivity, and specificity of serum A1AG1 and A1AT in pSS samples *vs.* HCs were calculated based on these results and plotted on an ROC curve. The Western blot results of A1AG1 showed that the AUC was 0.75, the sensitivity was 85.0%, and the specificity was 62.5% for pSS detection at an average densitometric cutoff of 19,994.82; the AUC was 0.67, the sensitivity was 77.5%, and the specificity was 60.0% for pSS detection by A1AT at an average densitometric cutoff of 104,087.25 (Figure 1C).

147 2.2 Novel HNE modification identification of serum proteins by in-gel digestion and
148 LC-MS/MS

149 In addition to serum protein levels, we further identified HNE modifications of
150 A1AG1 and A1AT. The average coverage of amino acid sequences in A1AG1 and
151 A1AT were estimated to be 52% and 70%, respectively. No HNE modification was
152 identified on A1AG1 (data not shown). Novel HNE modifications of A1AT were
153 identified by manual examination of the modified spectra using the PeaksPTM
154 module in PEAKS 7 software. Further, HNE modifications of A1AT were confirmed
155 in the two pooled serum samples (patients with pSS vs. HCs) through IP-Western
156 blotting, which detected signals of approximately 55 kDa (Figure 2). Because low
157 coverage of A1AG1 was identified, we also confirmed HNE modifications of A1AG1
158 using IP-Western blotting, but no signal was detected (data not shown).

159 MS/MS spectrum data of HNE-modified peptides on A1AT are presented in
160 Supplementary Figure 1B and Supplementary Table 3. The peptide ^{50}-
161 ITPNLAEFAFSLYR-^{63} was used to identify A1AT as pSS-specific and was found to
162 have an HNE modification at A58. Identification of the peptide moieties was based on
163 the presence of b- and y-series ions derived from the peptide, and HNE-modified
164 residues were confirmed by an unmodified b8 ion followed by a modified y6 ion that
165 corresponded to a mass increase of 156.11504 Da (Supplementary Figure 1C, upper
166 panel). The peptide ^{360}-AVLTIDEK-^{367} was used to identify A1AT as HC-specific
167 and was found to have an HNE modification at A360. Identification of the peptide
168 moieties was based on the presence of b- and y-series ions derived from the peptide,
169 and HNE-modified residues were confirmed by an unmodified y7 ion followed by a
170 modified b1 ion that corresponded to a mass increase of 138.10446 Da
171 (Supplementary Figure 1C, bottom panel).

172

173 2.3 Autoreactivity against A1AT$^{50-63}$ and A1AT$^{50-63}$ HNE peptides

174 Serum samples were assessed with autoantibody isotypes against A1AT$^{50-63}$
175 and A1AT$^{50-63}$ HNE peptides by an ELISA. The level of the anti-A1AT$^{50-63}$ IgG
176 antibody in RA was significantly higher than that of HCs by 1.80-fold (p = 0.0002),
177 that of SLE *vs.* HC was 2.41-fold higher (p < 0.0001), that of RA *vs.* pSS was 2.52-
178 fold higher (p < 0.0001), that of SLE *vs.* pSS was 3.38-fold higher (p < 0.0001), and
179 that of SLE *vs.* RA was 1.34-fold higher (p = 0.0321); however, that of pSS was
180 significantly lower than that of the HCs by 1.40-fold (p = 0.0488, Figure 3A, left
181 panel). Anti-A1AT$^{50-63}$ IgM expression levels did not significantly differ among
182 patients with pSS, RA, SLE, and HCs, except that of RA was significantly higher than
183 that of pSS by 1.57-fold (p = 0.0143, Figure 3A, middle panel). Levels of the anti-
184 A1AT$^{50-63}$ IgA antibody did not significantly differ among patients with pSS, RA,
185 SLE, and HCs (Figure 3A, right panel).

186 The level of the anti-A1AT$^{50-63}$ HNE IgG antibody in RA was significantly
187 higher than that of HCs by 2.10-fold (p < 0.0001), that of SLE *vs.* HC was 2.70-fold
188 higher (p < 0.0001), that of RA *vs.* pSS was 2.69-fold higher (p < 0.0001), that of SLE
189 *vs.* pSS was 3.48-fold higher (p < 0.0001), and that of SLE *vs.* RA was 1.29-fold
190 higher (p = 0.0469); however, that of pSS did not significantly differ from that of the
191 HCs (Figure 3B, left panel). Anti-A1AT$^{50-63}$ HNE IgM expression levels did not
192 significantly differ among patients with pSS, RA, SLE, and HCs, except that of SLE
193 was significantly higher than that of HCs by 1.36-fold (p = 0.0433) and that of SLE *vs.*
194 pSS was 1.47-fold higher (p = 0.0088) (Figure 3B, middle panel). Levels of the anti-
195 A1AT$^{50-63}$ HNE IgA antibody did not significantly differ among patients with pSS,
196 RA, SLE, and HCs, except that of pSS was significantly lower than that of the HCs by
197 1.15-fold (p = 0.0484) and that of RA *vs.* pSS was 1.17-fold higher (p = 0.0346)
198 (Figure 3B, right panel).

199

200 2.4 Determination of HNE-protein adducts

201 The level of the HNE-protein adduct can present the oxidative stress status and
202 plays important pathogenic roles in several diseases including cancer, and
203 neurodegenerative, chronic inflammatory, and autoimmune diseases [28]. As shown
204 in Supplementary Table 1, serum levels of the HNE-protein adduct in pSS patients
205 were significantly higher compared to those of the HCs (1.27-fold, $p = 0.0004$).

206

207 2.5 Association of elevated HNE-protein adduct, lowed A1AT level or decreased
208 autoreactivity against $A1AT^{50-63}$ and $A1AT^{50-63}$ HNE peptides with pSS patients

209 In Table 2, HNE-protein adduct, serum A1AT and anti-$A1AT^{50-63}$ IgG of pSS
210 patients carried a 4.887-fold risk ($p = 0.003$, power = 0.708), 3.910-fold risk ($p =$
211 0.010, power = 0.726) and a 3.360-fold risk ($p = 0.010$, power = 0.802) showed a
212 significant difference compared to HCs after adjusting for age, in the logistic
213 regression analyses, respectively. Risks did not significantly differ after the age-
214 adjusted logistic regression, suggesting that they were associated with other low levels
215 of autoantibodies (Table 2).

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3. Discussion

This is the first study to investigate the association between decreased serum levels of autoantibody isotypes against A1AT$^{50-63}$ and A1AT$^{50-63}$ HNE peptides and the risk of low A1AT levels in pSS patients. In the present study, two differentially expressed proteins, A1AG1 and A1AT, had 2-fold differences in depleted-albumin and IgG serum protein pools of nine pSS patients *vs.* nine HCs, identified in triplicate from in-solution digestion coupled to LC-MS/MS (Table 1). However, A1AG1 (1.53-fold increase, AUC = 0.75) and A1AT (1.84-fold decline, AUC = 0.67) showed acceptable diagnostic values for discriminating between pSS patients and HCs according to a Western blot analysis (Figure 1C). In this study, significantly higher serum levels of HNE-protein adducts indicated increments in the oxidative stress status of pSS patients (Supplementary Table 1); these results are consistent with those of previous studies [5, 6].

A1AG is an acute-phase protein, and its serum levels are elevated in response to a local inflammatory stimulus in several diseases including depression, cancer, and acquired autoimmune deficiency syndrome [25]. A1AG may have anti-inflammatory and immunomodulatory properties [26]. In the macrophage deactivation process, A1AG1 may act as a signaling molecule in the maintenance of tissue homeostasis and remodeling [27]. Rantapaa-Dahlqvist *et al.* reported that pSS patients with pericarditis had significantly higher levels of A1AG than did pSS patients without pericarditis; however, no information on A1AG1's involvement in the development of pSS has been reported. In this study, serum protein levels of A1AG1 in pSS were significantly higher than those of HCs by 1.53-fold (Figure 1A). Saroha et *al.* indicated that altered glycosylation and expression of plasma A1AG may play a role in RA progression [28].

A1AT is also an acute-phase protein that has anti-inflammatory and tissue-protective properties and is an immune regulator [12, 29]. Human A1AT protein levels can increase to inhibit elastase and serine-type proteinase during inflammation [13]. Serum protein levels of A1AT in patients with pSS were significantly lower than those of HCs by 1.84-fold (Figure 1B); these results are consistent with previous studies [14, 15]. In this study, patients with pSS showed a feature of low A1AT level (Figure 1B). Thus, low serum level of A1AT is a risk factor for the development of pSS (Table 2). Further, serum levels of anti-A1AT$^{50\text{-}63}$ IgG and anti-A1AT$^{50\text{-}63}$ HNE IgA were significantly lower in pSS patients (Figure 3). However, Elshikha *et al.* demonstrated that the human A1AT protein has protective effects through inhibition of dendritic cell (DC) activation and function to attenuate autoimmunity in RA mouse models [30]. Ciobanu *et al.* indicated that significantly lower levels of A1AT in rheumatoid synovial fluid can decrease the anti-protease activity in RA [31]. Stefanescu *et al.* showed that levels of anti-A1AT antibodies were significantly elevated in RA [32]. In several previous studies, elevated IgA-A1AT complex levels were reported in RA, SLE, mixed connective tissue disease, and ankylosing spondylitis compared to HCs [33-35]. Further, Lacki *et al.* suggested that a high level of the IgA-A1AT complex may cause worsening of bone erosion in RA [35]. In this study, levels of IgA-A1AT$^{50\text{-}63}$ and their HNE-modified peptide complexes did not significantly differ among patients with RA and SLE compared to HCs (Figure 3A, B, right panel). Levels of IgG-A1AT$^{50\text{-}63}$ and their HNE-modified peptide complexes were significantly higher among patients with RA and SLE compared to HCs (Figure 3A, B, left panel). Further, we observed that low levels of the anti-A1AT$^{50\text{-}63}$ IgG antibody obviously increased the risk against pSS, but the anti-A1AT$^{50\text{-}63}$ HNE IgG antibody reduced the risk against pSS (Table 2). The presence of self-reactive IgG autoantibodies in human serum is thought to represent as pathogenic antibodies in

patients with pSS [29]. Further, the HNE-modified epitope belong to oxidation-specific epitopes (OSEs) [30]. OSEs are present on damaged proteins and induce specific autoantibodies formation [11,12]. Anti-OSEs autoantibodies have conveyed protection from autoimmune pathogenesis [29,31,32]. Importantly, oxidative stress remained in patients with pSS (Supplementary Table 1).

4. Materials and methods

4.1 Patient samples

This study was approved by the institutional review board of the study hospital, and all volunteers provided informed consent before being allowed to participate. Serum samples from 168 female patients [49 with pSS (55.50 ± 12.85 years old), 40 with RA (54.30 ± 11.30 years old), and 30 with SLE (40.60 ±11.18 years old)] and 49 age-matched female HCs (55.40 ± 11.67 years old) were obtained from the Division of Allergy, Immunology and Rheumatology, Department of Internal Medicine and Department of Laboratory Medicine, Shuang-Ho Hospital (New Taipei City, Taiwan). Patients with pSS, RA, or SLE were diagnosed by a rheumatologist and had satisfied appropriate classification criteria. RA patients had received a diagnosis from a rheumatologist and had fulfilled appropriate classification criteria—either the 2010 American College of Rheumatology (ACR)/European League Against Rheumatism classification criteria [33] or the 1987 ACR classification criteria [34]. pSS patients were diagnosed according to the American-European Consensus Group (AECG) classification criteria [35]. SLE patients fulfilled the 1997 ACR SLE classification criteria [36]. Differentially expressed serum proteins were identified through in-solution digestion and nano-LC-MS/MS using pooled depleted-albumin and IgG serum protein samples randomly selected from nine RA patients and nine age-matched HCs. Two differentially expressed proteins, A1AG1 and A1AT, exhibited 2-fold differences in patients with pSS compared to HCs, and these were selected to examine protein levels through Western blotting using individual serum samples randomly selected from another 40 pSS patients and 40 age-matched HCs. HNE modifications of A1AT and A1AG1 were identified by in-gel digestion and nano-LC-MS/MS. HNE modifications of proteins were evaluated through immunoprecipitation (IP) and Western blotting using the aforementioned 40

pairs of pooled serum samples. Next, autoantibody isotypes against unmodified and their HNE-modified peptides were assessed among 49 pSS, 40 RA, and 30 SLE patients, and 49 HCs. Serum was stored at -20 °C until being analyzed. Clinical and demographic characteristics of pSS, RA, and SLE patients, and HCs are presented in Supplementary Table 1. However, the age of patients with SLE was significantly lower compared to those of the pSS, RA, and HC cohorts (Supplementary Table 1).

4.2 Depleted-albumin and IgG serum proteins, in-solution digestion, and protein identification by LC-MS/MS

Protein concentrations of serum were determined using a Coomassie Plus (Bradford) Assay Kit according to the manufacturer's protocol. Albumin and IgG of serum samples were removed using an Albumin and IgG Depletion SpinTrap column according to the protocol of Uen *et al.* [37]. Three micrograms of depleted-albumin and IgG serum proteins was used to perform in-solution digestion using an In-Solution Tryptic Digestion and Guanidination Kit according to the manufacturer's instructions. Tryptic peptide mixtures were analyzed in triplicate using NanoLC-nanoESi-MS/MS that was performed on a nanoAcquity system (Waters, Milford, MA, USA) connected to an LTQ-Orbitrap XL^{TM} hybrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a nanospray interface (Proxeon, Odense, Denmark). Differentially expressed proteins were quantified using label-free peptide quantification by the Peaks Q module of the PEAKS 7 software (Bioinformatics Solutions, Waterloo, Canada) [38]. Details are provided in "Supplementary information".

4.3 Western blotting

Serum protein levels of differentially expressed proteins showing 2-fold differences in pSS patients *vs.* HCs were examined using a Western blot analysis. A1AG1 (2 μg of protein in 10% SDS-PAGE) or A1AT (2 μg of protein in 8% SDS-PAGE) was evaluated using a mouse anti-A1AG1 monoclonal antibody (sc-69753, Santa Cruz Biotechnology, Dallas, TX, USA) or a mouse anti-A1AT monoclonal antibody (sc-69752, Santa Cruz Biotechnology). Details are provided in "Supplementary information".

4.4 1-D SDS-PAGE, in-gel digestion, and HNE identification by LC-MS/MS

Fifty-microgram protein samples (pooled serum proteins of A1AG1 or A1AT) were run on 10% SDS-PAGE with in-gel digestion according to a previously described method with minor modifications **(**Supplementary Figure 1A**)** [39]. HNE modifications were identified in triplicate using tryptic peptide mixtures of gel slices by the aforementioned nano-LC-MS/MS (nanoAcquity system and LTQ-Orbitrap XL^{TM} hybrid mass spectrometer). HNE-modified peptide sequences and sites of serum A1AG1 and A1AT were identified using the PeaksPTM module of the PEAKS 7 software (Bioinformatics Solutions). Details are provided in "Supplementary information".

4.5 Immunoprecipitation (IP)

An IP experiment for A1AG1 or A1AT was performed using a mouse anti-A1AG1 monoclonal antibody (sc-69753, Santa Cruz Biotechnology) or a mouse monoclonal antibody (sc-69752, Santa Cruz Biotechnology). HNE modifications of A1AG1 or A1AT were evaluated through a Western blot analysis with a goat polyclonal anti-HNE antibody (MBS536107, MyBioSource, San Diego, CA, USA). Details are provided in "Supplementary information".

352

353 4.6 Detection of autoreactivity against A1AT$^{50-63}$ and their HNE-modified peptides

354 Polypeptides corresponding to the 50~63 amino acid sequence of human
355 A1AT, i.e., ITPNLAEFAFSLYR (named A1AT$^{50-63}$) were synthesized (Yao-Hong
356 Biotechnology, New Taipei City, Taiwan) and their HNE-modified A1AT$^{50-63}$ (named
357 A1AT$^{50-63}$ HNE) used in an enzyme-linked immunosorbent assay (ELISA). In total,
358 168 serum samples were assessed for the presence of IgG, IgM, and IgA isotypes of
359 anti-A1AT$^{50-63}$ and anti-A1AT$^{50-63}$ HNE peptide antibodies. The absorbance was
360 measured at 450 nm with the reference filter set to 620 nm. All samples were treated
361 in duplicate. Details are provided in "Supplementary information".

362

363 4.7 Detection of serum HNE-protein adducts

364 Levels of HNE-protein adducts were quantified using 168 serum samples for
365 the ELISA protocol of Weber *et al.* [40]. All samples were analyzed in duplicate.
366 Details are provided in "Supplementary Information".

367

368 4.8 Statistical analyses

369 Student's *t*-test was used to determine the significance of differences in blot
370 densitometry, levels of serum proteins, and levels of HNE-protein adducts, and levels
371 of autoantibody isotypes against A1AT$^{50-63}$ and A1AT$^{50-63}$ HNE peptides. GraphPad
372 Prism (vers. 5.0; Graphpad Software, San Diego, CA, USA) was used to assess
373 differences in Student's *t*-tests between groups, and a dot plot was drawn. Ages and
374 clinical test results are presented as the mean ± standard deviation (SD). Spectral
375 count data are presented as the mean ± relative SD (RSD). The RSD is a coefficient of
376 variation (CV) and is calculated as a percentage. Multiples of change were defined as
377 (mean of pSS-normalized spectral counts) / (mean of HC-normalized spectral counts).

The threshold for up- or downregulated proteins was a 1.0-fold change in expression. Comparisons of pSS *vs.* HC serum samples were performed. Proteins that had a 2-fold difference were selected for validation by a Western blot analysis. Univariate and multiple logistic regression models were further used to estimate the adjusted odds ratios (ORs) and their 95% confidence intervals (CIs) for the pSS risk. Power estimations were determined using SAS (vers. 9.3; SAS Institute, Cary, NC, USA). Receiver operating characteristic (ROC) curves were generated to evaluate the diagnostic performance of differentially expressed proteins using MedCalc Statistical Software (vers. 15.4; MedCalc Software, Ostend, Belgium). The area under the ROC curve (AUC), sensitivity, and specificity were estimated at a 95% confidence level. For all statistical tests, the significance level was set to $p < 0.05$.

5. Conclusions

We identified HNE modifications on the human serum A1AT protein *in vivo* to investigate autoantibody isotypes against $A1AT^{50-63}$ and $A1AT^{50-63}$ HNE peptides associated with pSS patients. Our results showed that low levels of the anti-$A1AT^{50-63}$ IgG antibody had an increased risk in pSS patients. However, this possibility needs to be confirmed in larger studies.

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Author Contributions:

Ching-Yu Lin, Che-Chang Chang, Yu-Sheng Chang, Kai-Leun Tsai and Sheng-Hong Lin conceived and designed the experiments; Han-Wen Chou, Chih-Chun Tai and Yi-Fang Lin performed the experiments; Jin-Hua Chen, Chih-Hong Pan, Yi-Ying Lu and Han-Wen Chou analyzed the data; Ching-Yu Lin and Che-Chang Chang wrote the paper.

Competing interests

The authors declare that there are no competing interests.

417

418 **Legends of Figures and Tables**

419 Figure 1.Protein levels of A1AG1 and A1AT in serum were examined using anti-
420 A1AG1 (A), and anti-A1AT (B) antibodies through Western blotting. Average blot
421 densitometric values were calculated from duplicate data. Percentages of SDS-PAGE
422 gel and loading amounts of serum proteins used in Western blotting were 10% and 2
423 μg for A1AG1, and 8% and 2 μg for A1AT, respectively. A duplicate gel was stained
424 with Coomassie brilliant blue (CBB) as a loading control (right, bottom panel). The
425 red arrow indicates the A1AG1 or A1AT protein. Receiver operating characteristic
426 (ROC) curves were generated according to blot densitometry of A1AG1 and A1AT.
427 The area under the ROC curve (AUC), sensitivity, and specificity were further
428 estimated (C).

429

430 Figure 2. 4-Hydroxy-2-nonenal (HNE) modification of the serum A1AT protein was
431 validated using IP and Western blotting. A1AT was immunoprecipitated from pooled
432 serum samples [40 patients with primary Sjögren's syndrome (pSS) and 40 healthy
433 controls (HCs)] using anti-A1AT antibodies and then subjected to Western blotting
434 with anti-HNE antibodies (upper panel). Individually selected random serum samples
435 (patient with pSS and HC) were used as controls; these were simultaneously used for
436 Western blotting with anti-HNE antibodies. A duplicate gel was stained with
437 Coomassie brilliant blue as a loading control (bottom panel). The red arrow indicates
438 the A1AT protein.

439

440 Figure 3. Dot plot of serum concentrations (absorbance units at 450/620 nm) of IgG,
441 IgM, and IgA autoantibody isotypes recognizing $A1AT^{50-63}$ (A) and $A1AT^{50-63}$ 4-
442 hydroxy-2-nonenal (HNE) (B) in healthy controls (HCs), patients with primary

Sjögren's syndrome (pSS), rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE) with an ELISA. $OD_{450/620}$, optical density at 450/620 nm.

Table 1. Differentially expressed serum proteins identified by in-solution digestion and LC-MS/MS analysis in patients with primary Sjögren's syndrome (pSS) and healthy controls (HCs).

Table 2. Association among HNE-protein adduct, A1AT, anti-$A1AT^{50-63}$ and their HNE-modified peptides antibodies and pSS patients, in patients with pSS vs. healthy controls.

Supplementary Figure 1. The gel was rapidly stained with Coomassie brilliant blue, and gel bands were cut into slices according to the molecular weight of A1AG1 (48 kDa) and A1AT (55 kDa), respectively (A). Identification of 4-hydroxy-2-nonenal (HNE) modifications of A1AT (B). A representative MS/MS spectrum of the peptide sequence of ^{50}-ITPNLAEFAFSLYR-^{63} and the modified peptide bearing the HNE modification at alanine 58 in primary Sjögren's syndrome (pSS) (C, upper panel). The MS/MS spectrum of ^{360}-AVLTIDEK-^{367} and the modified peptide bearing the HNE modification at alanine 360 in healthy controls (HCs) (C, bottom panel).

Supplementary Table 1. Demographic and clinical characteristics of individual subjects contributing to serum for the healthy controls (HCs), and patients with primary Sjögren's syndrome (pSS), rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE).

468 Supplementary Table 2. A list of 255 identified proteins and peptides of depleted-
469 albumin and IgG serum protein.

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471 Supplementary Table 3. Post-translational modifications (PTMs), identified proteins,
472 and peptides of serum A1AT.

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References

1. Jonsson, R.; Vogelsang, P.; Volchenkov, R.; Espinosa, A.; Wahren-Herlenius, M.; Appel, S. The complexity of sjogren's syndrome: Novel aspects on pathogenesis. *Immunol Lett* **2011**, *141*, 1-9.
2. Yu, K.H.; See, L.C.; Kuo, C.F.; Chou, I.J.; Chou, M.J. Prevalence and incidence in patients with autoimmune rheumatic diseases: A nationwide population-based study in taiwan. *Arthritis Care Res (Hoboken)* **2013**, *65*, 244-250.
3. Maslinska, M.; Przygodzka, M.; Kwiatkowska, B.; Sikorska-Siudek, K. Sjogren's syndrome: Still not fully understood disease. *Rheumatology international* **2015**, *35*, 233-241.
4. Jonsson, R.; Brun, J.G. Sjögren's syndrome. In *Els*, John Wiley & Sons, Ltd: , 2010.
5. Norheim, K.B.; Jonsson, G.; Harboe, E.; Hanasand, M.; Goransson, L.; Omdal, R. Oxidative stress, as measured by protein oxidation, is increased in primary sjogren's syndrome. *Free radical research* **2012**, *46*, 141-146.
6. Wakamatsu, T.H.; Dogru, M.; Matsumoto, Y.; Kojima, T.; Kaido, M.; Ibrahim, O.M.; Sato, E.A.; Igarashi, A.; Ichihashi, Y.; Satake, Y.*, et al.* Evaluation of lipid oxidative stress status in sjogren syndrome patients. *Investigative ophthalmology & visual science* **2013**, *54*, 201-210.
7. Uchida, K. Protein-bound 4-hydroxy-2-nonenal as a marker of oxidative stress. *Journal of Clinical Biochemistry and Nutrition* **2005**, *36*, P1-10.
8. Zhao, J.; Chen, J.; Zhu, H.; Xiong, Y.L. Mass spectrometric evidence of malonaldehyde and 4-hydroxynonenal adductions to radical-scavenging soy peptides. *Journal of agricultural and food chemistry* **2012**, *60*, 9727-9736.
9. Lv, L.; Lin, H.; Li, Z.; Yuan, F.; Gao, Q.; Ma, J. Effect of 4-hydroxy-2-nonenal treatment on the ige binding capacity and structure of shrimp (metapenaeus ensis) tropomyosin. *Food Chem* **2016**, *212*, 313-322.
10. Uchida, K. 4-hydroxy-2-nonenal: A product and mediator of oxidative stress. *Prog Lipid Res* **2003**, *42*, 318-343.
11. Eggleton, P.; Nissim, A.; Ryan, B.J.; Whiteman, M.; Winyard, P.G. Detection and isolation of human serum autoantibodies that recognize oxidatively modified autoantigens. *Free radical biology & medicine* **2013**, *57*, 79-91.
12. Alzolibani, A.A.; Al Robaee, A.A.; Al-Shobaili, H.A.; Rasheed, Z. 4-hydroxy-2-nonenal modified histone-h2a: A possible antigenic stimulus for systemic lupus erythematosus autoantibodies. *Cellular immunology* **2013**, *284*, 154-162.
13. Breit, S.N.; Wakefield, D.; Robinson, J.P.; Luckhurst, E.; Clark, P.; Penny, R. The role of alpha 1-antitrypsin deficiency in the pathogenesis of immune disorders. *Clinical immunology and immunopathology* **1985**, *35*, 363-380.

14. Macen, J.L.; Upton, C.; Nation, N.; McFadden, G. Serp1, a serine proteinase inhibitor encoded by myxoma virus, is a secreted glycoprotein that interferes with inflammation. *Virology* **1993**, *195*, 348-363.
15. Lindstrom, F.D.; Skogh, T.; Lundstrom, I.M. Alpha(1) antitrypsin deficiency in a patient with systemic vasculitis and primary sjogren's syndrome. *Annals of the rheumatic diseases* **2002**, *61*, 945-946.
16. Malfait, R.; Gorus, F.; Sevens, C. Electrophoresis of serum protein to detect alpha 1-antitrypsin deficiency: Five illustrative cases. *Clinical chemistry* **1985**, *31*, 1397-1399.
17. Fournier, T.; Medjoubi, N.N.; Porquet, D. Alpha-1-acid glycoprotein. *Biochimica et biophysica acta* **2000**, *1482*, 157-171.
18. Hochepied, T.; Berger, F.G.; Baumann, H.; Libert, C. Alpha(1)-acid glycoprotein: An acute phase protein with inflammatory and immunomodulating properties. *Cytokine & growth factor reviews* **2003**, *14*, 25-34.
19. Gemelli, C.; Martello, A.; Montanari, M.; Zanocco Marani, T.; Salsi, V.; Zappavigna, V.; Parenti, S.; Vignudelli, T.; Selmi, T.; Ferrari, S.*, et al.* The orosomucoid 1 protein is involved in the vitamin d - mediated macrophage de-activation process. *Experimental cell research* **2013**, *319*, 3201-3213.
20. Saroha, A.; Biswas, S.; Chatterjee, B.P.; Das, H.R. Altered glycosylation and expression of plasma alpha-1-acid glycoprotein and haptoglobin in rheumatoid arthritis. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences* **2011**, *879*, 1839-1843.
21. Grimstein, C.; Choi, Y.K.; Wasserfall, C.H.; Satoh, M.; Atkinson, M.A.; Brantly, M.L.; Campbell-Thompson, M.; Song, S. Alpha-1 antitrypsin protein and gene therapies decrease autoimmunity and delay arthritis development in mouse model. *Journal of translational medicine* **2011**, *9*, 21.
22. Elshikha, A.S.; Lu, Y.; Chen, M.J.; Akbar, M.; Zeumer, L.; Ritter, A.; Elghamry, H.; Mahdi, M.A.; Morel, L.; Song, S. Alpha 1 antitrypsin inhibits dendritic cell activation and attenuates nephritis in a mouse model of lupus. *PloS one* **2016**, *11*, e0156583.
23. Ciobanu, A.; Ciobanu, I.R.; Ionita, A.; Stoicescu, M.; Stroescu, I.; Caralicea, M.; Curut, O. Some humoral immunological aspects of the rheumatoid arthritis correlated with the ultrastructural changes of the rheumatoid synovial fluid. *Romanian journal of morphology and embryology = Revue roumaine de morphologie et embryologie* **1992**, *38*, 143-154.
24. Stefanescu, M.; Szegli, G.; Cremer, L.; Zarma, L.; Mazilu, E.; Naghiu, M.; Niculescu, D.; Gaches, A.; Onu, A. The presence and significance of some anti-enzyme antibodies (anti-plasminogen, anti-trypsin, anti-phospholipase c) in rheumatoid arthritis (ra) and reactive arthritis (ra). *Archives roumaines de pathologie experimentales et de microbiologie* **1989**, *48*, 47-53.

25. Struthers, G.R.; Lewin, I.V.; Stanworth, D.R. Iga-alpha 1 antitrypsin complexes in ankylosing spondylitis. *Annals of the rheumatic diseases* **1989**, *48*, 30-34.

26. Lacki, J.K.; Schochat, T.; Leszczynski, P.; Mackiewicz, S.H.; Muller, W. Iga-alpha-1-antitrypsin complex in systemic lupus erythematosus: Preliminary report. *Lupus* **1995**, *4*, 221-224.

27. Lacki, J.K.; Schochat, T.; Porawaska, W.; Mackiewicz, S.H.; Muller, W. Does the serum level of iga-alpha-1-antitrypsin complex correlate with radiological progression in early rheumatoid arthritis? A 3-year follow-up study. *Rheumatology international* **1995**, *14*, 227-230.

28. Barrera, G.; Pizzimenti, S.; Ciamporcero, E.S.; Daga, M.; Ullio, C.; Arcaro, A.; Cetrangolo, G.P.; Ferretti, C.; Dianzani, C.; Lepore, A.*, et al.* Role of 4-hydroxynonenal-protein adducts in human diseases. *Antioxidants & redox signaling* **2015**, *22*, 1681-1702.

29. Silosi, I.; Silosi, C.A.; Boldeanu, M.V.; Cojocaru, M.; Biciusca, V.; Avramescu, C.S.; Cojocaru, I.M.; Bogdan, M.; FolcuTi, R.M. The role of autoantibodies in health and disease. *Romanian journal of morphology and embryology = Revue roumaine de morphologie et embryologie* **2016**, *57*, 633-638.

30. Binder, C.J.; Papac-Milicevic, N.; Witztum, J.L. Innate sensing of oxidation-specific epitopes in health and disease. *Nature reviews. Immunology* **2016**, *16*, 485-497.

31. Schwartz-Albiez, R.; Monteiro, R.C.; Rodriguez, M.; Binder, C.J.; Shoenfeld, Y. Natural antibodies, intravenous immunoglobulin and their role in autoimmunity, cancer and inflammation. *Clinical and experimental immunology* **2009**, *158 Suppl 1*, 43-50.

32. Sutti, S.; Jindal, A.; Locatelli, I.; Vacchiano, M.; Gigliotti, L.; Bozzola, C.; Albano, E. Adaptive immune responses triggered by oxidative stress contribute to hepatic inflammation in nash. *Hepatology* **2014**, *59*, 886-897.

33. Aletaha, D.; Neogi, T.; Silman, A.J.; Funovits, J.; Felson, D.T.; Bingham, C.O., 3rd; Birnbaum, N.S.; Burmester, G.R.; Bykerk, V.P.; Cohen, M.D.*, et al.* 2010 rheumatoid arthritis classification criteria: An american college of rheumatology/european league against rheumatism collaborative initiative. *Arthritis and rheumatism* **2010**, *62*, 2569-2581.

34. Arnett, F.C.; Edworthy, S.M.; Bloch, D.A.; McShane, D.J.; Fries, J.F.; Cooper, N.S.; Healey, L.A.; Kaplan, S.R.; Liang, M.H.; Luthra, H.S.*, et al.* The american rheumatism association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis and rheumatism* **1988**, *31*, 315-324.

35. Vitali, C.; Bombardieri, S.; Jonsson, R.; Moutsopoulos, H.M.; Alexander, E.L.; Carsons, S.E.; Daniels, T.E.; Fox, P.C.; Fox, R.I.; Kassan, S.S.*, et al.* Classification criteria for sjogren's syndrome: A revised version of the european criteria proposed by the

american-european consensus group. *Annals of the rheumatic diseases* **2002**, *61*, 554-558.

36. Hochberg, M.C. Updating the american college of rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis and rheumatism* **1997**, *40*, 1725.

37. Uen, Y.H.; Lin, K.Y.; Sun, D.P.; Liao, C.C.; Hsieh, M.S.; Huang, Y.K.; Chen, Y.W.; Huang, P.H.; Chen, W.J.; Tai, C.C.*, et al.* Comparative proteomics, network analysis and post-translational modification identification reveal differential profiles of plasma con a-bound glycoprotein biomarkers in gastric cancer. *Journal of proteomics* **2013**, *83*, 197-213.

38. Huo, X.; Wu, B.; Feng, M.; Han, B.; Fang, Y.; Hao, Y.; Meng, L.; Wubie, A.J.; Fan, P.; Hu, H.*, et al.* Proteomic analysis reveals the molecular underpinnings of mandibular gland development and lipid metabolism in two lines of honeybees (apis mellifera ligustica). *Journal of proteome research* **2016**, *15*, 3342-3357.

39. Liao, C.C.; Chou, P.L.; Cheng, C.W.; Chang, Y.S.; Chi, W.M.; Tsai, K.L.; Chen, W.J.; Kung, T.S.; Tai, C.C.; Lee, K.W.*, et al.* Comparative analysis of novel autoantibody isotypes against citrullinated-inter-alpha-trypsin inhibitor heavy chain 3 (itih3)(542-556) peptide in serum from taiwanese females with rheumatoid arthritis, primary sjogren's syndrome and secondary sjogren's syndrome in rheumatoid arthritis. *Journal of proteomics* **2016**, *141*, 1-11.

40. Weber, D.; Milkovic, L.; Bennett, S.J.; Griffiths, H.R.; Zarkovic, N.; Grune, T. Measurement of hne-protein adducts in human plasma and serum by elisa-comparison of two primary antibodies. *Redox biology* **2013**, *1*, 226-233.