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

4

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40 Running Title: Low level of IgG anti-A1AT$^{50-63}$ in patients with pSS
41
42 Abbreviations:
43 pSS, primary Sjögren's syndrome; RA, rheumatoid arthritis; SLE, systemic lupus
44 erythematosus; HNE, 4-Hydroxy-2-nonenal; 1-D SDS-PAGE, one-dimensional
45 sodium dodecyl sulfate polyacrylamide gel electrophoresis; nano-LC–MS/MS, nano-
46 liquid chromatography–tandem mass spectrometry; HC, healthy control; Ig,
47 immunoglobulin; ACR, American College of Rheumatology; IP, immunoprecipitation;
48 A1AG1, alpha-1-acid glycoprotein 1; A1AT, alpha-1-antitrypsin; RSD, relative
49 standard deviation; SD, standard deviation; ROC, receiver operating characteristic;
50 AUC, area under the ROC curve; CBB, Coomassie brilliant blue; RF, rheumatoid
51 factor; ANA, antinuclear antibody ; anti-Ro (SSA), anti-Sjögren's-syndrome-related
52 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

61 **Abstract**

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

80

81 **1. Introduction**

82 Primary Sjögren's syndrome (pSS) is a chronic inflammatory autoimmune
83 disease characterized by dysfunction of the exocrine glands leading to dryness of the
84 mouth and eyes [1]. Patients with pSS feature the presence of autoantibodies mainly
85 against the ribonucleoprotein complex SS-related antigen A (SSA, Ro) and SS-related
86 antigen B (SSB, La) [1]. In 2000~2008, the prevalence of pSS was 16.0 (females,
87 28.8, males, 3.7; female: male ratio, 7.9) per 100,000 persons; the incidence rate of
88 pSS was 10.6 (females, 18.5, males, 2.9; female: male ratio 6.3) per 100,000 person-
89 years; and the mortality from pSS was 1304.7 (females 987.4, males 3444.2; age-
90 adjusted female: male ratio 0.4) per 100,000 person-years in Taiwan [2]. The etiology
91 and pathogenesis of Sjögren's syndrome are not clearly understood [3]. Jonsson and
92 Brun proposed etiopathogenic events prior to a diagnosis of SS including a genetic
93 predisposition, environmental triggers, autoantibodies, pathological injury, clinical
94 disease, and clinical presentation [4].

95 Norheim *et al.* reported that patients with pSS have high levels of oxidative
96 stress compared to healthy controls (HCs) [5]. Wakamatsu *et al.* found an increase in
97 4-hydroxy-2-nonenal (HNE)-protein adducts, marker of oxidative stress, in the
98 conjunctiva of SS patients that may play a role in the pathogenesis of dry-eye disease
99 [6,7]. HNE is one of the lipid peroxidation products that has an alkene bond and an
100 aldehyde group which react with amino acid residues that form HNE-protein adducts
101 via types of Michael addition and Schiff-base adducts, respectively [8]. Amino acid
102 residues that can react with HNE include cysteine (C), histidine (H), lysine (K),
103 arginine (R), glutamine (Q), alanine (A), and leucine (L) [8-10]. The HNE-protein
104 adduct is an autoantigen and can elicit specific autoantibody formation [11,12].

105 Breit *et al.* indicated that several immune-mediated diseases were associated
106 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.

121 **2. Results**

122 2.1 Identification and validation of differentially expressed serum proteins by in-
123 solution digestion and LC-MS/MS

124 Enrichment of depleted-albumin and IgG serum protein samples from a single
125 pair of each of nine pooled serum samples (patients with pSS *vs.* HCs) was analyzed
126 in triplicate by in-solution digestion coupled to nano-LC-MS/MS (Table 1). In total,
127 255 proteins were detected, of which 28 differentially expressed proteins significantly
128 varied, as shown in Table 1 and Supplementary Table 2. There were seven
129 upregulated proteins and 21 downregulated proteins; relative to the HC serum pools,
130 two of the identified proteins, alpha-1-acid glycoprotein 1 (A1AG1) and A1AT,
131 differed by a 2-fold increase or decrease in patients with pSS serum pools, and 26
132 proteins differed by a 1.7~1.9-fold increase or decrease (Table 1).

133 Next, we validated the LC-MS/MS data of A1AG1 and A1AT, and protein
134 levels of A1AG1 (~48 kDa) and A1AT (~55 kDa) were analyzed by Western blotting
135 (Figure 1). A1AG1 expression levels were significantly higher in pSS samples by
136 1.53-fold ($p = 0.0001$) than in HCs, but A1AT levels in pSS samples were
137 significantly lower than those in HCs by 1.84-fold ($p = 0.0071$, Figure 1A, B, right
138 upper panel). Equal amounts of serum proteins in these experiments were examined
139 (Figure 1A, B, right bottom panel). The AUC value, sensitivity, and specificity of
140 serum A1AG1 and A1AT in pSS samples *vs.* HCs were calculated based on these
141 results and plotted on an ROC curve. The Western blot results of A1AG1 showed that
142 the AUC was 0.75, the sensitivity was 85.0%, and the specificity was 62.5% for pSS
143 detection at an average densitometric cutoff of 19,994.82; the AUC was 0.67, the
144 sensitivity was 77.5%, and the specificity was 60.0% for pSS detection by A1AT at
145 an average densitometric cutoff of 104,087.25 (Figure 1C).

146

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$^{50\text{-}63}$ and A1AT$^{50\text{-}63}$ HNE peptides with pSS patients

209 In Table 2, HNE-protein adduct, serum A1AT and anti-A1AT$^{50\text{-}63}$ 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).

216

217 **3. Discussion**

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

230 A1AG is an acute-phase protein, and its serum levels are elevated in response
231 to a local inflammatory stimulus in several diseases including depression, cancer, and
232 acquired autoimmune deficiency syndrome [25]. A1AG may have anti-inflammatory
233 and immunomodulatory properties [26]. In the macrophage deactivation process,
234 A1AG1 may act as a signaling molecule in the maintenance of tissue homeostasis and
235 remodeling [27]. Rantapaa-Dahlqvist *et al.* reported that pSS patients with pericarditis
236 had significantly higher levels of A1AG than did pSS patients without pericarditis;
237 however, no information on A1AG1's involvement in the development of pSS has
238 been reported. In this study, serum protein levels of A1AG1 in pSS were significantly
239 higher than those of HCs by 1.53-fold (Figure 1A). Saroha et *al.* indicated that altered
240 glycosylation and expression of plasma A1AG may play a role in RA progression
241 [28].

242 A1AT is also an acute-phase protein that has anti-inflammatory and tissue-
243 protective properties and is an immune regulator [12, 29]. Human A1AT protein
244 levels can increase to inhibit elastase and serine-type proteinase during inflammation
245 [13]. Serum protein levels of A1AT in patients with pSS were significantly lower than
246 those of HCs by 1.84-fold (Figure 1B); these results are consistent with previous
247 studies [14, 15]. In this study, patients with pSS showed a feature of low A1AT level
248 (Figure 1B). Thus, low serum level of A1AT is a risk factor for the development of
249 pSS (Table 2). Further, serum levels of anti-A1AT$^{50\text{-}63}$ IgG and anti-A1AT$^{50\text{-}63}$ HNE
250 IgA were significantly lower in pSS patients (Figure 3). However, Elshikha *et al.*
251 demonstrated that the human A1AT protein has protective effects through inhibition
252 of dendritic cell (DC) activation and function to attenuate autoimmunity in RA mouse
253 models [30]. Ciobanu *et al.* indicated that significantly lower levels of A1AT in
254 rheumatoid synovial fluid can decrease the anti-protease activity in RA [31].
255 Stefanescu *et al.* showed that levels of anti-A1AT antibodies were significantly
256 elevated in RA [32]. In several previous studies, elevated IgA-A1AT complex levels
257 were reported in RA, SLE, mixed connective tissue disease, and ankylosing
258 spondylitis compared to HCs [33-35]. Further, Lacki *et al.* suggested that a high level
259 of the IgA-A1AT complex may cause worsening of bone erosion in RA [35]. In this
260 study, levels of IgA-A1AT$^{50\text{-}63}$ and their HNE-modified peptide complexes did not
261 significantly differ among patients with RA and SLE compared to HCs (Figure 3A, B,
262 right panel). Levels of IgG-A1AT$^{50\text{-}63}$ and their HNE-modified peptide complexes
263 were significantly higher among patients with RA and SLE compared to HCs (Figure
264 3A, B, left panel). Further, we observed that low levels of the anti-A1AT$^{50\text{-}63}$ IgG
265 antibody obviously increased the risk against pSS, but the anti-A1AT$^{50\text{-}63}$ HNE IgG
266 antibody reduced the risk against pSS (Table 2). The presence of self-reactive IgG
267 autoantibodies in human serum is thought to represent as pathogenic antibodies in

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268 patients with pSS [29]. Further, the HNE-modified epitope belong to oxidation-
269 specific epitopes (OSEs) [30]. OSEs are present on damaged proteins and induce
270 specific autoantibodies formation [11,12]. Anti-OSEs autoantibodies have conveyed
271 protection from autoimmune pathogenesis [29,31,32]. Importantly, oxidative stress
272 remained in patients with pSS (Supplementary Table 1).
273
274

275 **4. Materials and methods**

276 4.1 Patient samples

277 This study was approved by the institutional review board of the study
278 hospital, and all volunteers provided informed consent before being allowed to
279 participate. Serum samples from 168 female patients [49 with pSS (55.50 ± 12.85
280 years old), 40 with RA (54.30 ± 11.30 years old), and 30 with SLE (40.60 ±11.18
281 years old)] and 49 age-matched female HCs (55.40 ± 11.67 years old) were obtained
282 from the Division of Allergy, Immunology and Rheumatology, Department of
283 Internal Medicine and Department of Laboratory Medicine, Shuang-Ho Hospital
284 (New Taipei City, Taiwan). Patients with pSS, RA, or SLE were diagnosed by a
285 rheumatologist and had satisfied appropriate classification criteria. RA patients had
286 received a diagnosis from a rheumatologist and had fulfilled appropriate classification
287 criteria—either the 2010 American College of Rheumatology (ACR)/European
288 League Against Rheumatism classification criteria [33] or the 1987 ACR
289 classification criteria [34]. pSS patients were diagnosed according to the American-
290 European Consensus Group (AECG) classification criteria [35]. SLE patients fulfilled
291 the 1997 ACR SLE classification criteria [36]. Differentially expressed serum
292 proteins were identified through in-solution digestion and nano-LC-MS/MS using
293 pooled depleted-albumin and IgG serum protein samples randomly selected from nine
294 RA patients and nine age-matched HCs. Two differentially expressed proteins,
295 A1AG1 and A1AT, exhibited 2-fold differences in patients with pSS compared to
296 HCs, and these were selected to examine protein levels through Western blotting
297 using individual serum samples randomly selected from another 40 pSS patients and
298 40 age-matched HCs. HNE modifications of A1AT and A1AG1 were identified by in-
299 gel digestion and nano-LC-MS/MS. HNE modifications of proteins were evaluated
300 through immunoprecipitation (IP) and Western blotting using the aforementioned 40

301 pairs of pooled serum samples. Next, autoantibody isotypes against unmodified and
302 their HNE-modified peptides were assessed among 49 pSS, 40 RA, and 30 SLE
303 patients, and 49 HCs. Serum was stored at -20 °C until being analyzed. Clinical and
304 demographic characteristics of pSS, RA, and SLE patients, and HCs are presented in
305 Supplementary Table 1. However, the age of patients with SLE was significantly
306 lower compared to those of the pSS, RA, and HC cohorts (Supplementary Table 1).
307

308 4.2 Depleted-albumin and IgG serum proteins, in-solution digestion, and protein
309 identification by LC-MS/MS

310 Protein concentrations of serum were determined using a Coomassie Plus
311 (Bradford) Assay Kit according to the manufacturer's protocol. Albumin and IgG of
312 serum samples were removed using an Albumin and IgG Depletion SpinTrap column
313 according to the protocol of Uen *et al.* [37]. Three micrograms of depleted-albumin
314 and IgG serum proteins was used to perform in-solution digestion using an In-
315 Solution Tryptic Digestion and Guanidination Kit according to the manufacturer's
316 instructions. Tryptic peptide mixtures were analyzed in triplicate using NanoLC-
317 nanoESi-MS/MS that was performed on a nanoAcquity system (Waters, Milford,
318 MA, USA) connected to an LTQ-Orbitrap XL^{TM} hybrid mass spectrometer (Thermo
319 Fisher Scientific, Bremen, Germany) equipped with a nanospray interface (Proxeon,
320 Odense, Denmark). Differentially expressed proteins were quantified using label-free
321 peptide quantification by the Peaks Q module of the PEAKS 7 software
322 (Bioinformatics Solutions, Waterloo, Canada) [38]. Details are provided in
323 "Supplementary information".
324

325 4.3 Western blotting

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326 Serum protein levels of differentially expressed proteins showing 2-fold
327 differences in pSS patients *vs.* HCs were examined using a Western blot analysis.
328 A1AG1 (2 μg of protein in 10% SDS-PAGE) or A1AT (2 μg of protein in 8% SDS-
329 PAGE) was evaluated using a mouse anti-A1AG1 monoclonal antibody (sc-69753,
330 Santa Cruz Biotechnology, Dallas, TX, USA) or a mouse anti-A1AT monoclonal
331 antibody (sc-69752, Santa Cruz Biotechnology). Details are provided in
332 "Supplementary information".
333
334 4.4 1-D SDS-PAGE, in-gel digestion, and HNE identification by LC-MS/MS
335 Fifty-microgram protein samples (pooled serum proteins of A1AG1 or A1AT)
336 were run on 10% SDS-PAGE with in-gel digestion according to a previously
337 described method with minor modifications **(**Supplementary Figure 1A**)** [39]. HNE
338 modifications were identified in triplicate using tryptic peptide mixtures of gel slices
339 by the aforementioned nano-LC-MS/MS (nanoAcquity system and LTQ-Orbitrap
340 XL^{TM} hybrid mass spectrometer). HNE-modified peptide sequences and sites of
341 serum A1AG1 and A1AT were identified using the PeaksPTM module of the PEAKS
342 7 software (Bioinformatics Solutions). Details are provided in "Supplementary
343 information".
344
345 4.5 Immunoprecipitation (IP)
346 An IP experiment for A1AG1 or A1AT was performed using a mouse anti-
347 A1AG1 monoclonal antibody (sc-69753, Santa Cruz Biotechnology) or a mouse
348 monoclonal antibody (sc-69752, Santa Cruz Biotechnology). HNE modifications of
349 A1AG1 or A1AT were evaluated through a Western blot analysis with a goat
350 polyclonal anti-HNE antibody (MBS536107, MyBioSource, San Diego, CA, USA).
351 Details are provided in "Supplementary information".

352

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

354 Polypeptides corresponding to the 50~63 amino acid sequence of human
355 A1AT, i.e., ITPNLAEFAFSLYR (named A1AT$^{50\text{-}63}$) were synthesized (Yao-Hong
356 Biotechnology, New Taipei City, Taiwan) and their HNE-modified A1AT$^{50\text{-}63}$ (named
357 A1AT$^{50\text{-}63}$ 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$^{50\text{-}63}$ and anti-A1AT$^{50\text{-}63}$ 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$^{50\text{-}63}$ and A1AT$^{50\text{-}63}$ 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).

378 The threshold for up- or downregulated proteins was a 1.0-fold change in expression.
379 Comparisons of pSS *vs.* HC serum samples were performed. Proteins that had a 2-fold
380 difference were selected for validation by a Western blot analysis. Univariate and
381 multiple logistic regression models were further used to estimate the adjusted odds
382 ratios (ORs) and their 95% confidence intervals (CIs) for the pSS risk. Power
383 estimations were determined using SAS (vers. 9.3; SAS Institute, Cary, NC, USA).
384 Receiver operating characteristic (ROC) curves were generated to evaluate the
385 diagnostic performance of differentially expressed proteins using MedCalc Statistical
386 Software (vers. 15.4; MedCalc Software, Ostend, Belgium). The area under the ROC
387 curve (AUC), sensitivity, and specificity were estimated at a 95% confidence level.
388 For all statistical tests, the significance level was set to $p < 0.05$.
389
390

391

392 **5. Conclusions**

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

398

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407

408 **Author Contributions:**

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

414

415 **Competing interests**

416 The authors declare that there are no competing interests.

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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

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443 Sjögren's syndrome (pSS), rheumatoid arthritis (RA), and systemic lupus
444 erythematosus (SLE) with an ELISA. $OD_{450/620}$, optical density at 450/620 nm.
445
446 Table 1. Differentially expressed serum proteins identified by in-solution digestion
447 and LC-MS/MS analysis in patients with primary Sjögren's syndrome (pSS) and
448 healthy controls (HCs).
449
450 Table 2. Association among HNE-protein adduct, A1AT, anti-A1AT$^{50-63}$ and their
451 HNE-modified peptides antibodies and pSS patients, in patients with pSS vs. healthy
452 controls.
453
454 Supplementary Figure 1. The gel was rapidly stained with Coomassie brilliant blue,
455 and gel bands were cut into slices according to the molecular weight of A1AG1 (48
456 kDa) and A1AT (55 kDa), respectively (A). Identification of 4-hydroxy-2-nonenal
457 (HNE) modifications of A1AT (B). A representative MS/MS spectrum of the peptide
458 sequence of ^{50}-ITPNLAEFAFSLYR-^{63} and the modified peptide bearing the HNE
459 modification at alanine 58 in primary Sjögren's syndrome (pSS) (C, upper panel). The
460 MS/MS spectrum of ^{360}-AVLTIDEK-^{367} and the modified peptide bearing the HNE
461 modification at alanine 360 in healthy controls (HCs) (C, bottom panel).
462
463 Supplementary Table 1. Demographic and clinical characteristics of individual
464 subjects contributing to serum for the healthy controls (HCs), and patients with
465 primary Sjögren’s syndrome (pSS), rheumatoid arthritis (RA), and systemic lupus
466 erythematosus (SLE).
467

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468 Supplementary Table 2. A list of 255 identified proteins and peptides of depleted-
469 albumin and IgG serum protein.
470
471 Supplementary Table 3. Post-translational modifications (PTMs), identified proteins,
472 and peptides of serum A1AT.
473

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