1 **Title**:

2 Comprehensive immune complexome analysis detects disease-specific immune complex antigens in seminal
3 plasma and follicular fluids derived from infertile men and women

4

5 **Running title**

6 Immune complex antigens in seminal and follicular fluid

7

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37 nano-LC-MS/MS, nano-liquid chromatography-tandem mass spectrometry; SP, seminal plasma

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54

Abstract

Background: Autoimmune reactions and subsequent inflammation may underlie spermatogenic dysfunction and endometriosis-related infertility. The aim of this study is to identify disease-specific antigens in immune complexes (ICs) in seminal plasma (SP) and in follicular fluid (FF).

Methods: Immune complexome analysis, in which nano-liquid chromatography-tandem mass spectrometry is employed to comprehensively identify antigens incorporated into ICs in biological fluids, was performed for specimens collected from infertile couples undergoing assisted reproduction. Forty-two male patients consisting of subjects with oligozoospermia (n=6), asthenozoospermia (n=8), and normal semen analysis (n=28). Fifty-eight female patients consisting of subjects with ovarian endometriosis (n=10) and control women without disease (n=48).

Results: Four disease-specific antigens were identified in subjects with oligozoospermia, while five disease-specific antigens were detected in subjects with asthenozoospermia, some of which are involved in sprematogenesis. Eight antigens were detected only in subjects with endometriosis.

Conclusion: Functional characteristics of disease-specific antigens were found to correspond to the pathogenesis of male and female infertility. The formation of ICs may contribute to spermatogenic dysfunction and endometriosis-related infertility via loss of function of the related proteins. Immune complexome analysis is expected to be a valuable tool for the investigation of novel diagnostic methods and treatment strategies for infertility.

74 1. **Introduction**

75 Local immunity may play an important role in human reproduction, and disorders in local immunity can
76 be the cause of male and female infertility [1, 2].

77 Spermatogenic dysfunction is a major cause of male infertility, though its pathogenesis is not fully
78 understood. Inflammation in the male reproductive tract may disrupt spermatogenesis and sperm function.
79 Destruction of testicular microstructures can induce immunity against sperm [3]. Although autoimmune
80 orchitis and epididymitis may be rare occurrences, these conditions may relate to immunological male
81 infertility [4]. However, even subclinical inflammation and local autoimmune reaction in the male
82 reproductive tract can be a cause of male infertility. To permit normal spermatogenesis and fertility, the
83 mammalian testis is maintained as an immune-privileged organ wherein immunogenic germ cells are
84 protected from immune surveillance [1]. The Sertoli cell barrier, also known as the blood–testis barrier (BTB),
85 plays an important role in the construction of this unique microenvironment [5]. When the BTB is impaired
86 due to infection, injury, or obstruction of genital ducts, a large amount of sperm antigen is exposed to immune
87 cells by leakage or infiltration, leading to sperm immunity [6]. Inflammation of the testis may affect male
88 reproductive function. Subacute and chronic inflammation of the testis and epididymis are asymptomatic in
89 the most patients, and there are no reliable clinical diagnostic measures [3]. As a local body fluid of the male
90 reproductive tract, seminal plasma (SP), mediates male fertility by supporting sperm metabolism, modulating
91 sperm function, and protecting sperm against the damage induced by the immune system via suppression of
92 immune activity [7, 8]. SP, which is formed by secretion from male reproductive organs including the
93 epididymis, seminal vesicles, prostate gland, and Cowper's gland, contains many kinds of tissue-specific
94 proteins, reflecting the afore-mentioned specific characteristics of male reproductive organs. Most of these

95 tissue-specific proteins are intracellular proteins, including those associated with the membrane, cytoplasm,
96 and nucleus [9-11]. The high concentration of intracellular proteins in SP may reflect the fact that most body
97 cells interact with plasma and release components into the plasma during cell damage or death. [12]. As a
98 result, these tissue- and cell-specific proteins are abundant in SP compared to serum or urine. Although only
99 10% of the protein components of SP originate from testis or epididymis, these proteins play an important role
100 in sperm quality, are associated with testicular function, and have been implicated in male reproductive
101 disorders [8-11, 13]. Thus, changes in composition of the SP proteome may reflect a pathological process in
102 male reproductive disorders [9].

103 Endometriosis is a chronic pelvic inflammatory disease and one of the major causes of female infertility.
104 Endometriosis is characterized by the presence of ectopic endometrial tissue outside of the uterine cavity, and
105 is manifested by chronic local inflammation in the pelvis. As with spermatogenic dysfunction, the
106 pathogenesis of endometriosis is enigmatic. However, local immunity in the female pelvis also may be
107 involved in the pathogenesis of endometriosis-related infertility. The mechanisms of endometriosis-related
108 infertility vary, and include peritoneal adhesion, dysfunctional uterotubal motility, disturbed folliculogensis,
109 and detrimental effects on spermatozoa [14, 15]. Ovarian endometriotic lesions (endometriomas) are one of
110 the main disease phenotypes, and local inflammatory reactions surrounding endometriomas may affect
111 folliculogenesis and the process of oocyte maturation [14]. In fact, the ovary affected by endometriomas may
112 show fibrosis and altered folliculogenesis, effects that may result from local chronic inflammation [16, 17].
113 These findings imply that the local follicular milieu may be disturbed by endometriosis. On the other hand,
114 several studies have reported a relationship between endometriosis and autoimmune disease [18, 19]. In these
115 reports, endometriosis shows symptoms similar to those of autoimmune disease. In addition, various

116 auto-antibodies are produced in subjects with endometriosis [20]. The contents of follicular fluid (FF) are
117 formed by osmotic pressure gradient generated by hyaluronan and versican recruits blood exudate into the
118 follicle. Antibodies can pass through the ovarian blood-follicle barriers and diffuse into FF [21, 22]. Moreover,
119 cytokines, reactive oxygen species, and antioxidants produced by inflammation may be elevated in the FF of
120 subjects with endometriosis [23]. Due to these facts, the FF generated by subjects with endometriosis may in
121 turn affect the growth of endometriomas [24].

122 As mentioned above, autoimmune reactions and subsequent inflammation may underlie spermatogenic
123 dysfunction and endometriosis-related infertility. However, while SP and FF are local body fluids of the
124 gonads and reproductive tract, it is unknown what component(s) of SP and FF are recognized as auto-antigens,
125 thereby triggering inflammation in the corresponding tissues. Immune complexes (ICs) are formed by
126 noncovalent interactions between foreign antigens or autoantigens and antibody molecules [25]. Enhanced
127 formation and defective clearance of ICs occurs in autoimmune diseases [26]. In order to comprehensively
128 identify and profile constituent antigens in ICs, we developed a proteomic strategy, designated immune
129 complexome analysis, in which ICs are separated from whole serum and then subjected to direct tryptic
130 digestion and nano-liquid chromatography-tandem mass spectrometry [27]. We have successfully used this
131 method to identify specific antigens in circulating ICs (CIC-antigens) in serum or cerebrospinal fluid
132 recovered from subjects with autoimmune diseases, infectious diseases, and cancers, as well as those who are
133 liver transplant recipients [27-31].

134 In the present study, we applied immune complexome analysis to SP and FF collected from infertile
135 couples undergoing assisted reproduction. The goal of this analysis was to comprehensively identify
136 IC-antigens, with the intent of identifying those IC-antigens specific for infertile males and for infertile

137 women with endometriosis. Formation of ICs between the specific antigens and their corresponding
138 autoantibodies might affect the physiological functions of the antigens, possibly leading to male and female
139 infertility. Additionally, IC formation and deposition on tissues is known to stimulate inflammatory processes
140 via the action of the complement system. It is hoped that the present study may lead to elucidation of the
141 pathogenesis of male infertility and endometriosis involving immune abnormality, and to the development of
142 new therapies to treat these fertility challenges.

143

144 **2. Materials and Methods**

145 *2.1. Patients*

146 All samples were collected from infertile couples undergoing assisted reproduction technology (ART) at
147 Nagasaki University Hospital; written consent was obtained from all participating patients. Infertility is
148 defined as the couple who suffer from the failure to achieve a clinical pregnancy after 12 months or more of
149 regular unprotected sexual intercourse. Before ART, male subjects received semen analysis and female
150 subjects received the test for tubal patency by hysterosalpingography or laparoscopy, ovarian function by
151 hormonal analysis and serial transvaginal ultrasonography, pelvic pathology, such as uterine and ovarian
152 tumors, by ultrasonography and/or MRI. The couples with azoospermia or primary ovarian insufficiency were
153 excluded. This study was performed according to Helsinki Declaration and was approved by the Nagasaki
154 University Hospital Ethics Committee (Research Ethics Committee Approval No. 16020804).

155

156 *2.2. SP*

157 Forty-two semen samples were collected. After complete liquefaction, an aliquot of the ejaculate was

158 employed for semen analysis using a Makler counting chamber (Irvine Scientific, Santa Ana, CA). Semen
159 volume (mL), sperm concentration (10^6/mL), and motility (%) were recorded and classified according to the
160 WHO guidelines [32]. Subjects were divided into three groups: the oligozoospermia group (n=6), which
161 consisted of male subjects with sperm concentrations lower than 15×10^6/mL without regard for sperm
162 motility; the asthenozoospermia group (n=8), which consisted of male subjects with sperm motility less than
163 40% but with normal sperm concentrations (>15 $\times10^6$/mL); and the normal control group (n=28), which
164 consisted of males for whom both sperm concentration and motility exceeded the lower reference limits of the
165 WHO criteria (>15 $\times10^6$/mL, >40%). After semen analysis, the remaining semen was washed using Sperm
166 Washing Medium® (Irvine Scientific) and then was centrifuged at 2.0×10^4 x *g* for 10 min to separate the
167 pellet and supernatant. The resulting supernatant was frozen at -40 °C; an aliquot (10 μL) was used for the
168 immune complexome analysis. In this study, SP samples were derived from not only men with definitive male
169 factor infertility but also the couple with female factor infertility. In oligozoospemia group, all couple were
170 primary infertility and there were no female partners with infertility factors. In asthenozoospermia group, six
171 subjects were primary infertility and two subjects were secondary infertility, and there were three couples with
172 female factor infertility, such as endometriosis (n=2) and ovulatory dysfunction (n=1). In normal control
173 group, 24 subjects were primary infertility and four subjects were secondary infertility, and there were 15
174 couples with female factor infertility, such as tubal factor (n=6), ovulatory dysfunction (n=5), endometriosis
175 (n=3), and woman with anti-centromere antibody (n=1).
176
177 *2.3. FF*
178 Fifty-eight samples were collected; the subjects were divided into two groups. The endometriosis group

179 consisted of 10 women who had been diagnosed as having one or more endometriomas at a previous operation.
180 At the time of surgery, cystectomy was performed in six subjects, drainage and cystic wall ablation were
181 performed in three subjects and hemilateral cystectomy and contralateral ablation were performed in one
182 woman with bilateral lesions. The average period from surgery to oocyte retrieval was 2.8 ± 4.1 years (mean ±
183 standard deviation). The other women lacked subjective and objective clinical symptoms of endometriosis
184 and were designated as a control group (n=48). Although all subjects received pelvic examination and
185 transvaginal ultrasonography before ART to rule out the pelvic pathology, 15 (31%) of them had been
186 undergone pelvic surgery for indications other than endometriosis, and they were confirmed not to have the
187 disease. At the time of transvaginal oocyte retrieval, FF was collected from the first punctured follicle. After
188 transferring the egg to the culture medium, FF was centrifuged at 2.8×10^4 x *g* for 5 min and an aliquot (10
189 μL) of the supernatant was used for immune complexome analysis.
190
191 **3. Experimental**
192 *3.1. Immune complexome analysis*
193 ICs in SP or FF were collected using Proceptor™-sepharose beads. An aliquot (40 μL) of each bead
194 type was incubated with 10 μL of pooled human serum diluted with 90 μL phosphate-buffered saline (PBS)
195 for 30 min with gentle mixing. The beads were pelleted by 1 min of centrifugation and the supernatant was
196 removed with a pipette. The beads were washed three times with 500 μL PBS/wash. Washed beads were
197 suspended in 50 μL of 10 μg/mL papain solution (0.04 M EDTA, 0.04 M L-cysteine) and incubated at 37 °C
198 for 30 min. Then, 50 μL of 0.06 M iodoacetamide dissolved in PBS was added to quench the papain digestion.
199 Next, we added 100 μL of 10 mM dithiothreitol and further incubated the sample at 56 °C for 45 min. Then,

200 100 μL of 55 mM iodoacetamide was added, and the mixture was incubated in the dark at room temperature
201 for another 30 min. Trypsin in 0.05% acetic acid was added to yield a final concentration of 0.5 g of trypsin/L,
202 and the mixture was incubated overnight at 37 °C. An aliquot (12 μL) of 10% TFA in water was added to the
203 mixture to quench the digestion. The beads were pelleted by 1 min of centrifugation; the resulting supernatant
204 (approximately 400 μL) was recovered, vacuum-reduced to a volume of approximately 80 μL, and stored at
205 4 °C pending subsequent analysis by nano-LC-MS/MS. The peptide mixture (1 μL) was injected into an
206 LC–electrospray ionization (ESI)–MS/MS instrument (Q-Exactive, Thermo Fisher Scientific, Waltham, MA,
207 USA) was equipped with EASY-nLC^{TM} 1200 system consisting of a nano LC pump) and an autosampler was
208 used for anlaysis. Peptides were deionized and were concentrated on pre-column (Acclaim PepMap^{TM} 100, 75
209 μm x 2 cm, nano Viper, C18, 3 μm, 100 Å, Thermo Fisher Scientific), and were subsequently separated on a
210 nano-LC column (C18, 75 μm i.d. x 125 mm, 3 μM particle, 100 Å pore size, Nikkyo Technos, Tokyo, Japan)
211 and ion-sprayed into MS with a spray voltage of 1.5 kV. The separation was performed by using the mobile
212 phase A (0.1% formic acid) and mobile phase B (0.1% formic acid in 90% acetonitrile), employing a gradient
213 elution from 5% to 33% mobile phase B in 100 min, and 100% mobile phase B held for 10 min. MS/MS data
214 were extracted using Proteome Discoverer 1.3.1.339 (Thermo Fisher Scientific). Spectra were searched
215 against sub-databases from the public nonredundant protein database of UniProt Knowledgebase (human,
216 2015.01.29 download) with the following search parameters: mass type, monoisotopic precursor and
217 fragments; enzyme, trypsin (KR); enzyme limits, full enzymatic cleavage allowing up to two missed
218 cleavages; peptide tolerance, 10 ppm; fragment ion tolerance, 0.8 Da; ion and ion series calculated, B and Y
219 ions; static modification, C (carbamidomethylation); and differential modifications, M (oxidation), N, and Q
220 (deamidation). All the results were obtained by triplicate analyses. All the peptides and proteins found in the

221 first, second and/or third analysis were counted in the numbers of identified peptides and proteins. The

222 procedure has been described in detail in our previous publication [33].

223

224 *3.2. Statistical analysis*

225 Statistical analysis was performed using Stat Mate V software (ATMS, Tokyo, Japan); the significance

226 level was defined as a p value <0.05. An F test was used to test the normality of distributions. Continuous

227 variables that exhibited skewed distributions by the F test were analyzed using a two-tailed Kruskal-Wallis H

228 test with post-hoc Dunnett's test. Continuous variables that exhibited normal distributions by the F test were

229 analyzed using a two-tailed one-way analysis of variance (ANOVA) with post-hoc Tukey-Kramer test. For

230 female patients, age, serum anti-Müllerian hormone (AMH), and the number of retrieved oocytes were

231 analyzed using a Mann-Whitney U-test. Fisher's exact test for parity was used for comparisons between two

232 groups.

233

234 **4. Results**

235 *4.1.1 Clinical backgrounds of the male subjects provided seminal plasma*

236 Age and the results of semen analysis in the male subjects are summarized in Table 1. There was no

237 statistically significant difference in age or semen volume among the three groups. Consistent with the

238 group-assignment criteria, sperm concentrations in the oligozoospermia group were significantly lower than

239 those in the other groups ($p < 0.05$ versus asthenozoospermia, $p < 0.001$ versus normal semen analysis).

240 Similarly, there was a statistically significant difference in sperm motility, between the asthenozoospermia

241 group and the normal group ($p < 0.001$). We also detected a significant difference in sperm motility between

242 the oligozoospermia and normal groups ($p < 0.01$).

243 *4.1.2 Clinical backgrounds of the female subjects provided follicular fluid*

244 Table 2 shows the clinical backgrounds of the female subjects, including: age, serum AMH levels, the
245 number of retrieved oocytes, the number of subjects with nulligravida, the number of subjects with nullipara,
246 the stage of endometriosis according to the revised American Society for Reproductive Medicine system, the
247 number of subjects from whom FF was collected from the affected side, and the number of subjects with
248 recurrent cyst(s) at the time of oocyte retrieval [34]. There were no statistically significant differences between
249 the two groups for any of these variables.

250 *4.2. Immune complexome analysis*

251 *4.2.1. SP*

252 Here, we present for the first time (to our knowledge) a comprehensive identification of the
253 constituent antigens assembled into CICs in SP from male infertility patients with spermatogenesis
254 dysfunction (oligozoospermia or asthenozoospermia), or into CICs in FF from female infertility patients with
255 ovarian endometriosis. Among the studied subjects, we identified 391 and 327 human antigens in SP of male
256 subjects and FF of female subjects (respectively) via immune complexome analysis.

257 While the majority of antigens detected in SP were observed in both SP derived from patients with
258 spermatogenesis dysfunction (oligo- and astheno-zoospermia) and SP derived from normal controls, four
259 antigens were found only in SP derived from subjects with oligozoospermia; these antigens were not detected
260 in SP from the other two groups (Table 3). Among the four disease-specific antigens identified in SP of
261 oligozoospermia, sperm protein associated with the nucleus on the X chromosome D (also known as
262 SPANX-D) was found in three of six oligozoospermia subjects (50%). The other three

263 oligozoospermia-specific antigens included zyxin, fasciculation and elongation protein zeta-2, and a probable
264 asparagine-tRNA ligase; these three antigens were found in two of six subjects, without a specific pattern. SP
265 from two subjects exhibited three of these oligozoospermia-specific antigens; SP from one subject retained
266 two of these antigens; SP from one subject retained one of these antigens; and SP from two subjects did not
267 retain any of these antigens. There was no specific pattern regarding the number of antigens present per
268 subject, nor did the presence of various antigens appear to relate to the severity of oligozoospemia (data not
269 shown).

270 Five asthenozoospermia-specific antigens (dual specificity testis-specific protein kinase 2 (also
271 known as TESK2), probable E3 ubiquitin-protein ligase HERC1, uncharacterized protein KIAA1109, protein
272 arginine N-methyltransferase 7, and ATP-binding cassette sub-family F member 1) were detected in in SP
273 from six of eight subjects with asthenozoospermia; these antigens were not detected in SP from the other two
274 groups (Table 4). Each antigen was detected at a frequency of 25% within subjects with asthenozoospermia
275 without a specific pattern. SP from two subjects retained three disease-specific antigens; SP from four patients
276 retained one specific antigen; and SP from two patients did not retain any of these specific antigens. There was
277 no specific pattern regarding the number of antigens present per subject, nor did the presence of various
278 antigens appear to relate to the severity of asthenozoospermia (data not shown).

279

280 *4.2.2. FF*

281 Although the majority of 327 antigens identified in FF were present both in FF from subjects with
282 endometriosis and in FF from those without endometriosis, eight antigens were detected only in the
283 endometriosis group; these eight antigens were not detected in FF from the control group (Table 5). These

284 specific antigens (fibroblast growth factor receptor 1 (also known as FGFR1), probable ubiquitin
285 carboxyl-terminal hydrolase FAF-Y (also known as Deubiquitinating enzyme FAF-Y), interleukin-6 receptor
286 subunit beta (also known as gp130), sentrin-specific protease 1, centlein, Neuralized-like protein 4,
287 apolipoprotein B receptor, and WSC domain-containing protein 1) in FF were detected in six of ten subjects.
288 Each antigen was detected at a frequency of 20% among subjects with endometriosis; no specific pattern was
289 observed. FF from one subject each retained five specific antigens, four specific antigens, and three specific
290 antigens; FF from three subjects retained one of these antigens, while those from the remaining four subjects
291 did not harbor any of these specific antigens. There was no specific pattern regarding the number of antigens
292 present per subject, nor did the presence of various antigens appear to relate to the severity of endometriosis
293 (data not shown).

294

295 **5. Discussion**

296 In this study, we demonstrated for the first time (to our knowledge) that disease-specific ICs are formed
297 in the local body fluids of the reproductive tracts, such as SP and FF, obtained from infertile males and
298 females, when assessed by proteomic immune complexome analysis. ICs are formed by the binding of
299 immunoglobulins to self and non-self antigens to promptly recognize autoantigens or prevent the spread of
300 non-self antigens. When excessive numbers of ICs are produced, the complexes may induce inflammation and
301 tissue damage via activation of complement. In addition, the deposition of ICs in tissues can cause fibrosis,
302 atrophy, and dysfunction due to type-III hypersensitivity, endothelial dysfunction, and tissue remodeling [35].
303 Moreover, IC-associated antigens (proteins) may lose their function due to IC formation. Thus, comprehensive
304 analysis of ICs and their antigens may facilitate the definition of the pathogenesis of disorders relevant to

305 immune responses and inflammation.

306 In this study, we demonstrated for the first time (to our knowledge) that disease-specific ICs are formed
307 in the local body fluids of the reproductive tracts, such as SP and FF, obtained from infertile males with
308 spermatogenesis dysfunction and females with endometriosis, when assessed by proteomic immune
309 complexome analysis. Immune response and inflammation in the local environment may be involved in the
310 pathogenesis of both disorders. To our knowledge, most of the disease-specific antigens identified in this
311 study had not previously been reported to be associated with the respective disease; nonetheless, some of these
312 antigens may correspond to the disease pathology. The specific antigens detected in SP may function as
313 components of BTB, DNA repair machinery, or sperm nuclear envelopes. In contrast, the proteins specifically
314 detected in FF are known to be related to inflammation. Interestingly, none of the detected IC-antigens were
315 shared among the oligozoospermia, asthenozoospermia, and endometriosis groups. These results may reflect
316 the specific role of ICs in each disorder, implying that the analysis of ICs may contribute to expanding our
317 knowledge of specific diseases.

318 Several papers have suggested an association between spermatogenic dysfunction and autoimmune
319 response against sperm [1, 3, 5, 6]. For instance, the BTB is formed by Sertoli cells in the seminiferous
320 epithelium and plays an important role in maintaining a microenvironment (notably, an immunoprivileged
321 compartment) that is suitable for spermatogenesis [1]. However, the BTB can be disrupted by inflammation;
322 therefore, identification of specific BTB autoantigens may be crucial for understanding the pathological
323 processes of BTB disruption [36]. In this context, among the nine disease-specific antigens identified in the SP
324 of the oligozoospermia and asthenozoospermia groups, zyxin and TESK2 are known components of the BTB
325 [37, 38]. Production of antibodies against these proteins, as suggested by the presence of corresponding ICs in

326 the present study, may lead to antigen-specific inflammation and BTB disruption.

327 SPANX-D, which was detected in half of SP specimens derived from oligozoospermia subjects, and the 328 probable E3 ubiquitin-protein ligase HERC1 (HECT-type E3 ubiquitin transferase HERC1), which was 329 specifically detected in the asthenozoospermia group, are proteins involved in DNA damage repair [39-41]. IC 330 formation may result in disorganized sperm production due to accumulation of DNA damage and genome 331 instability by loss of the functions of these proteins.

332 Among the eight specific antigens found in FF of subjects with endometriosis, several proteins might be 333 involved in the pathogenesis of endometriosis, such as gp130, Deubiquitinating enzyme FAF-Y, and FGFR1. 334 These proteins are known to regulate local inflammation, inflammasome formation, or the 335 epithelial-to-mesenchymal transition (EMT) [42-45]. FF may be involved in the growth and maintenance of 336 superficial ovarian endometriomas and peritoneal lesions [24]. Loss of function of specific antigens (which 337 would result from excess formation of ICs) may render the local pelvic micro-environment favorable to the 338 progression of endometriosis. IL-6 is a pro-inflammatory cytokine and may be involved in 339 endometriosis-associated infertility [46]. The formation of ICs that include the IL-6R subunit beta (also 340 known as gp130), which inhibits the pro-inflammatory *trans*-signaling cascade of IL-6 by binding to the 341 complex formed by IL-6 and sIL-6R [42, 47], might lead to activation of *trans*-signaling and exacerbation of 342 inflammation.

343 On the other hand, altered folliculogenesis caused by destruction of normal ovarian cortical structures 344 may be one of the causes of endometriosis-related infertility. Fibrotic changes in the ovarian cortex are 345 associated with decreased follicular density and enhanced follicular recruitment and atresia [17, 18]. 346 Dysregulation of the inflammasome and EMT caused by the formation of ICs including Deubiquitinating

347 enzyme FAF-Y and FGFR1, respectively, may be associated with exacerbation of inflammation and fibrosis

348 [48, 49]. Deubiquitinating enzyme FAF-Y may function as a polyubiquitin hydrolase that counteracts the

349 activity of TRIM33, an E3 ubiquitin-protein ligase [43]. TRIM33 is essential for activation of the NLRP3

350 inflammasome [44]. FGFR1 has been shown to affect myofibroblast differentiation by inhibiting signaling by

351 TGF-β1 and the FGF-1 ligand, events that lead to reversion of the EMT [45]. In terms of inflammation and

352 fibrosis, IC formation would result in activation of the complement system, a process that is known to be

353 involved in the pathogenesis of endometriosis [51].

354

355 **6. Conclusions**

356 We comprehensively identified the constituent antigens of ICs in SP and FF via immune complexome

357 analysis. Among the 391 and 327 human antigens detected in SP and FF, nine and eight antigens were found

358 to be specific to subjects with spermatogenic dysfunction (four antigens for oligozoospemia and five antigens

359 for asthenozoospermia) and ovarian endometriosis, respectively. Several antigens and the corresponding

360 proteins coincide with known disease characteristics and may be involved in the pathogenesis of male and

361 female infertility. Other specific antigens that lack known functions but were detected in SP and FF may have

362 unknown roles in infertility. Immune complexome analysis may be a useful technique for revealing disease

363 pathogenesis and may contribute to the development of new treatment strategies for reproductive dysfunction.

364 However, our results, which were derived from a relatively limited number of subjects in the present work,

365 will need to be confirmed in large-scale studies. Additionally, the exact relationship between specific antigens

366 and male and female infertility related to spermatogenesis and endometriosis will need to be examined. We

367 expect that further analysis of these disease-specific antigens may provide a better understanding of the

368 pathogenesis of both conditions.

369

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373

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Table 1

Results of semen analysis

	Oligozoospermia (n=6)	Asthenozoospermia (n=8)	Normal (n=28)
Age (years)	41 ±6	39 ±3	38 ±6
Semen volume (mL)	2.8 ±1.8	2.8 ±1.6	3.4 ±1.4
Sperm concentration (10^6/mL)	4 ±1 [a, b]	85 ±48	104 ±47
Motility (%)	37 ±12 [a]	28 ±8 [a]	58 ±13

Footnote: Data are presented as mean ± SD (standard deviation). Age, semen volume, sperm concentration, and motility were compared among the three groups. After an F test was used to check the variance among the groups, age, semen volume, and motility were analyzed using a two-tailed Kruskal-Wallis H test with a post-hoc Dunnett's test, while sperm concentration was analyzed using a two-tailed one-way Analysis of Variance (ANOVA) with a post-hoc Tukey-Kramer test.

[a] $p < 0.001$ versus normal semen.

[b] $p < 0.05$ versus asthenozoospermia.

Table 2

Clinical background of female subjects who provided follicular fluid

		Endometriosis (n=10)	Control (n=48)
Age (years)		36 ±4	37 ±4
Serum AMH (ng/mL)		2.4 ±1.6	3.1 ±2.7
Number of retrieved oocytes		6 ±7	8 ±7
Nulligravida		7 (70%)	27 (56%)
Nullipara		10 (100%)	40 (83%)
Stage of endometriosis	III	5 (50%)	-
	IV	5 (50%)	-
Follicular fluid collected from ovary with endometriomas		6 (60%)	-
Recurrent cyst at OPU		5 (50%)	-

Foot note: Age, serum AMH (anti-Müllerian hormone), and the number of retrieved oocytes are presented as mean ± SD (standard deviation). To compare between groups, a Mann-Whitney U test was used to analyze age, serum AMH, and the number of retrieved oocytes; a Fisher's exact test was used to analyze gravidity and parity. There were no statistically significant differences between the two groups for any of these variables.

All women in the Endometriosis group has been diagnosed histologically as having uni- or bilateral endometriotic cyst(s).

OPU, oocyte pick-up

Table 3

Specific antigens found only in the oligozoospermia group

Accession		Q9BXN6	Q15942	Q9UHY8	Q96I59
Description		Sperm protein associated with the nucleus on the X chromosome D (SPANX-D)	Zyxin	Fasciculation and elongation protein zeta-2	Probable asparagine--tRNA ligase, mitochondrial
Case	1	●	●	●	
	2		●	●	●
	3	●			●
	4	●			
	5				
	6				
Frequency (%)[a]		50	33.3	33.3	33.3

Footnote: Four specific antigens were found in four of six subjects in the oligozoospermia group.

[a] Frequency (%) of each antigen was calculated based on the number of oligozoospermia subjects (n=6).

The table shows how many antigens were detected in each patient. No specific antigens were found in cases No. 5 and No. 6.

●: Presence of specific antigen

Table 4

Specific antigens found only in the asthenozoospermia group

Accession		Q96S53	Q15751	Q2LD37	Q9NVM4	Q8NE71
Description		Dual specificity testis-specific protein kinase 2 (TESK2)	Probable E3 ubiquitin-protein ligase HERC1	Uncharacterized protein KIAA1109	Protein arginine N-methyltransferase 7	ATP-binding cassette sub-family F member 1
Case	1	●		●		●
	2		●	●		●
	3	●				
	4		●			
	5				●	
	6				●	
	7					
	8					
Frequency (%)[a]		25	25	25	25	25

Footnote: Five specific antigens were found in six of eight subjects in the asthenozoospermia group.

[a]Frequency (%) of each antigen was calculated based on the number of asthenozoospermia subjects (n=8).

The table shows how many antigens were detected in each patient. No specific antigens were found in cases No. 7 and No. 8.

●: Presence of specific antigen

Table 5

Specific antigens found only in patients with endometriosis

Accession		P11362	O00507	P40189	Q9P0U3	Q9NXG0	Q96JN8	Q0VD83	Q658N2
Description		**Fibroblast growth factor receptor 1 (FGFR1)**	**Probable ubiquitin carboxyl-terminal hydrolase FAF-Y (Deubiquitinating enzyme FAF-Y)**	**Interleukin-6 receptor subunit beta (gp130)**	**Sentrin-specific protease 1**	**Centlein**	**Neuralized-like protein 4**	**Apolipoprotein B receptor**	**WSC domain-containing protein 1**
Case	1	●		●		●		●	●
	2		●		●	●			●
	3			●	●		●		
	4		●				●		
	5	●							
	6							●	
	7								
	8								
	9								
	10								
Frequency (%)[a]		20	20	20	20	20	20	20	20

Footnote: Eight specific antigens were found in six of ten subjects in the endometriosis group.

[a]Frequency (%) of each antigen was calculated based on the number of subjects with endometriosis (n=10).

The table shows how many antigens were detected in each patient. No specific antigens were found in cases No. 7 to No. 10.

●: Presence of specific antigen