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A comparison of the prevalence of chronic endometritis determined by the use of different diagnostic methods in women with and without reproductive failure

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1 **F & S _ style revision**

2 **Title Page**

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4 Prevalence of chronic endometritis

5 **Title**

6 A comparison of the prevalence of chronic endometritis determined by the use of different
7 diagnostic methods in women with and without reproductive failure

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40 **Conflict of interest**

41 The authors declare no conflict of interest.

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45 **Capsule**

46 Based on new methods of plasma cell assessment, the prevalence of chronic endometritis in
47 women with various categories of reproductive failure may have been over-estimated in
48 earlier studies.

49 **ABSTRACT**

50 **Objective:** To compare the prevalence of chronic endometritis (CE) when different
51 diagnostic methods are used.

52 **Design:** Prospective observational study.

53 **Setting:** University affiliated hospital.

54 **Patient(s):** Four groups of women were studied, including women with proven fertility
55 (Fertile, n=40), unexplained recurrent miscarriage (RM, n=93), recurrent implantation failure
56 (RIF, n=39) and infertile subjects undergoing endometrial scratch in a natural cycle preceding
57 frozen-thawed embryo transfer (Infertility, n=48).

58 **Intervention(s):** Endometrial biopsy was performed precisely seven days following LH
59 surge (LH+7). Plasma cells were identified by traditional H&E staining and
60 immunohistochemistry (IHC) for Syndecan-1(CD138).

61 **Main Outcome Measure(s):** The prevalence of chronic endometritis.

62 **Result(s):** The use of CD138 epitope is more sensitive than H&E staining in identifying
63 plasma cells**.** The use of plasma cell count per unit area had the lowest observer variability
64 than that of cell count per 10 randomly chosen HPF or cell count per section. Using this
65 method, the prevalence of CE in women with RM, RIF and Infertility were 10.8%, 7.7%, and
66 10.4%, respectively, not significantly higher than that of fertile subjects (5.0%).

67 **Conclusion(s):** Using what may be a new method of plasma cell assessment, it appears that
68 the prevalence rates of CE reported in many earlier studies may have been over-estimated.

69 **Chinese Clinical Trials Registry Number:** ChiCTR-IOC-16007882

70 **Key Words:** chronic endometritis; plasma cell; prevalence; reference range; reproductive
71 failure

72 **Text**

73 # INTRODUCTION

74 Chronic endometritis (CE) refers to local persistent inflammation of the endometrium. CE
75 has been reported to be associated with various subgroups of reproductive failure, including
76 infertility (1-3), recurrent miscarriage (4-8), or recurrent implantation failure (4, 9-11).

77 The presence of plasma cells in endometrial stroma has been accepted as the gold standard
78 method to establish a diagnosis of CE (12). Nevertheless, the reported prevalence of CE in
79 endometrial biopsy specimens varied considerably, ranging from 3 to 60% (Table 1). There
80 are several possible explanations to account for the wide variation reported. Firstly, there are
81 two different methods used to **identify** plasma cells. Traditionally, plasma cells are identified
82 in H&E stained specimens. However, the identification of plasma cells in H&E sections
83 requires experience coupled with diligent search, without which they can be easily missed. A
84 more recently introduced method is immunohistochemistry (IHC) staining for Syndecan-1
85 (CD138), a proteoglycan found on the cell surface of plasma cells and keratinocytes. This has
86 been found to improve the sensitivity and accuracy for identifying plasma cells, essential for
87 the diagnosis of CE (13-15).

88 Secondly, various investigators have used different approaches to **quantify** CD138+ cell
89 count (Table 1). In the first approach, the number of plasma cell per whole section was
90 measured. In the second approach, the plasma cell count per a defined number of randomly
91 chosen high power field (HPF, say 10) was measured. There are rationales behind each of
92 these two approaches. Some investigators advocated scrutinizing the entire specimen as they
93 believed that plasma cells are not normally present in the endometrium and the finding of one
94 or more plasma cells is indicative of a diagnosis of CE (12, 16, 17). One shortcoming of such

95 an approach is that it does not take into account the size of the specimen. One would expect
96 that, other things being equal, the larger is the specimen size, the more likely it is to find
97 plasma cells, and vice versa. Consequently, other authors introduced the concept of plasma
98 cell density to correct for the size of the specimen examined; they advocated examining 10 or
99 more chosen HPFs and expressed the number of plasma cell detected per HPF or per 10 HPF,
100 as each HPF is equivalent to a defined area (4, 6, 10, 11, 15, 18, 19). To avoid bias in
101 selecting the HPFs to be examined and to improve objectivity, it is desirable to have
102 randomly chosen fields. However, the potential disadvantage of such an approach is that
103 plasma cells are usually present in low numbers and so the inclusion of only 10 selected
104 HPFs may not be sufficient to produce a consistently reproducible result. We postulate that a
105 new method of plasma cell assessment which combines the positive attributes of the two
106 above-mentioned methods would be to count all CD138+ cells in the entire section, then
107 measure the area of the examined tissue section and express the result as plasma cell count
108 per unit area. In this way, it would overcome the problem of local fluctuation of plasma cell
109 count as well as correcting for the variation in results due to sample size difference.

110 There is also no consensus on the **diagnostic criteria** used to define what constitutes CE. At
111 least seven criteria have been reported in the literature, which included ≥1 plasma cell/section
112 (20), ≥1 plasma cell/HPF (10), ≥1 plasma cell/10 HPF (3), ≥5 plasma cells /10 HPF (4), ≥5
113 plasma cells/20 HPF (22), and the presence of 1-5 plasma cells/HPF or discrete clusters <20
114 plasma cells (7), ≥0.25 endometrial stromal plasmacyte density index (ESPDI, the sum of the
115 stromal CD138+ cell counts divided by the number of the HPFs evaluated) (11) (see Table 1).
116 The proposed criteria all appeared rather arbitrarily chosen, not based on reference ranges
117 derived from normal fertile populations.

118 In this study, our aim was to establish a reference range of plasma cell count in the

119 endometrium of fertile subjects by using two different methods of identification and three
120 different methods of quantification, as discussed above, followed by a comparison of the
121 performance of these methods. The prevalence rates of CE so derived among women with
122 reproductive failure was then determined, using this methodology, with a view to
123 determining the optimal strategy to identify and quantify plasma cell and to diagnose CE.

124
MATERIALS AND METHODS

125
Participants

126 Subjects were recruited from women attending the Department of Obstetrics and Gynecology,
127 Prince of Wales Hospital, The Chinese University of Hong Kong. Women were recruited
128 from four groups: (1) fertile control group: healthy women with at least one live birth within
129 2 years (n=40); (2) unexplained RM group: women with recurrent miscarriage, which was
130 defined as the loss of three or more consecutive pregnancies before 24 weeks gestation (23)
131 (n=93); (3) RIF group: women with recurrent implantation failure which was defined as
132 failure to achieve a clinical pregnancy after transfer of at least four good quality embryos in
133 three or more transfer cycles in women under the age of 40 years (24) (n=39); and (4)
134 infertile group: women with infertility undergoing endometrial scratch in a natural cycle
135 preceding frozen-thawed embryo transfer using non donor oocytes (n=48).

136 The inclusion criteria were women who were between 20 to 40 years of age with regular
137 menstrual cycle (25-35 days), normal pelvic ultrasonography, and had not used any
138 antibiotics, oestrogen or progestogen hormonal therapy, steroid treatment or intrauterine
139 contraceptive device within 2 months of recruitment. The exclusion criteria included the
140 presence of hydrosalpinx, structural uterine abnormalities, parental chromosomal
141 abnormalities and significant medical conditions such as systemic lupus erythematosus.

142 **Endometrial Biopsy**

143 All subjects in this study had daily urine dipstick test from day 9 of the menstrual cycle
144 onwards to identify the LH surge (ovulation), which was used to precisely time the
145 endometrial biopsies to seven days after the LH surge (day LH+7). All biopsies were
146 obtained using a Pipelle sampler (Prodimed, France) or Pipet Curettage (Cooper Surgical,
147 USA). The specimens were immediately placed into 10% neutral buffered formalin for over-
148 night fixation at room temperature and then embedded into paraffin wax.

149 **Processing of Specimen**

150 The paraffin-embedded human endometrial tissues were cut into sections (4 μm) and then
151 dewaxed in xylene and then rehydrated through descending ethanol to phosphate-buffered
152 saline.

153 *Haematoxylin-Eosin (H&E) staining.* Paraffin-embedded human endometrial tissue sections
154 were examined with routine H&E staining.

155 *Immunohistochemistry (IHC) staining*. Slides were pre-treated with microwave heating for 20
156 minutes in sodium citrate buffer for antigen retrieval, and then quenched with 0.3%v/v
157 hydrogen peroxidase in methanol to block endogenous peroxidase activity. Then the sections
158 were blocked with rabbit serum to prevent non-specific binding and then incubated with a
159 1:50 dilution of mouse monoclonal antibody against human Syndecan-1 (clone B-A38; Cell
160 Marque, Rocklin, CA, USA) overnight at 4°C. After incubation, the sections were washed in
161 phosphate-buffered saline Tween 20 and incubated with secondary rabbit anti-mouse
162 horseradish peroxidase (HRP) labelled antibody (1:100, ab97046, Abcam, Cambridge, MA,
163 USA) for one hour, followed by colour development with 3, 3-diaminobenzidine (DAB;
164 DAKO, CA, USA), counterstained with haematoxylin, dehydrated with ethanol, cleared in

165 xylene and mounted with a cover slide.

166 **Image Acquisition and Analysis**

167 Image analysis was performed by one operator who firstly scanned the slides at lower
168 magnification, and then captured images (×400) of all the fields of CD138+ plasma cells
169 using the Leica DM6000B system (Leica Microsystems Ltd., Wetzlar, Germany). Then the
170 whole section of each sample was tile scanned under ×50 magnifications by the same system,
171 which was able to merge separate images into one image covering all the tissue. Cell counts
172 and section area were analysed using Image J (Version 1.51a, Wayne Rasband, NIH, USA).
173 The total number of plasma cells was determined by counting immune-positive cells in the
174 entire specimen. Cells were considered to be likely CD138+ plasma cells, if they exhibited
175 unambiguous complete brown staining with intact cell membrane, a clearly defined nucleus
176 typical of a plasma cell, and occurring singly or as small clusters of cells, excluding
177 background stroma, glands and other confounders. The identification and counting of the
178 CD138+ cells was performed manually under the microscope, whereas the measurement of
179 the specimen area was made on images captured in the computer by Image software entirely.

180 **Determination of Observer Variability**

181 The intra-observer variability of CD138+ cell count was determined by asking a single
182 observer to repeat the measurement of 20 randomly chosen specimens on two separate
183 occasions, without knowledge of the results of the first count. The observer utilised three
184 different **quantification methods** to do the measurement, namely method I: CD138+ cell
185 count per 10 randomly chosen HPF; method II: CD138+ cell count per whole section;
186 method III: CD138+ cell count per unit area (cell density). For the third method, Image J
187 software was used to measure the area of the tissue section.

188 The inter-observer variability was determined by asking two observers (Y.L. and X.C.) to
189 perform the measurement of CD138+ cell count on the same set of 20 randomly chosen
190 specimens, independently of each other, also using the three different quantification methods
191 as in the case of intra-observer variability study.

192 **Reference Range**

193 In this study, the reference range of plasma cell count or density was derived from the 40
194 fertile control subjects. Values below the 95th percentile were considered as normal, whereas
195 values above the 95th percentile were considered as abnormal and indicative of a diagnosis of
196 CE.

197 **Definition of CE**

198 Chronic endometritis was defined as the presence of CD138+ plasma cell count or density
199 above the established reference ranges (95th percentile), whatever the quantification method
200 was used.

201 **Ethical Considerations**

202 This study was approved by the local hospital ethics committee (CREC Ref. No.: 2015.477).
203 Written informed consents were obtained from all participants.

204 **Statistical Analysis**

205 We have previously analysed the distribution of our data and confirm that results in the
206 control population was not normally distributed which was the reason why we adopted non-
207 parametric method to analyse the data and used 95th centile as the cut-off instead of 2SD
208 above the mean (parametric method) as the cut-off. The maternal age and body mass index

209 (BMI) of the women in the four groups were compared by ANOVA analysis. Intra- and inter-
210 class correlation coefficients (intra- and inter-CC) with 95% confidence interval (95% CI)
211 were used to assess the intra- and inter-observer agreement in the calculation of plasma cell
212 count, area of tissue examined and plasma cell density (intra- and inter- CC values <0.40
213 were considered as poor agreement, between 0.40 and 0.75 as moderate, and >0.75 as
214 excellent agreement). Chi-Square test was used to compare the prevalence of CE between
215 subgroups. Statistical analysis was performed by using SPSS version 23.0 (SPSS Inc.,
216 Chicago, IL, USA) and P value with <0.05 was considered as a statistical significance.

217 # RESULTS

218 From December 2014 to June 2017, 229 subjects underwent endometrial biopsy. Nine
219 subjects were excluded because of insufficient tissue obtained. In total, 220 subjects were
220 included in the study.

221 **Demographics**

222 The demographic details of the subjects are summarized in Supplementary Table 1. The
223 mean age of all the subjects was 34.4 (range from 21 to 40) years. The mean age in
224 reproductive failure group (35.4 ± 3.1 years) was significantly (P<0.01) higher than that of
225 control group (29.6 ± 3.4 years), whilst there was no significant difference between RM, RIF
226 and Infertility subgroups. There was also no significant difference in body mass index
227 between groups.

228 **Identification of Plasma Cell**

229 No classical plasma cells were identified in any of the routing H&E stained sections (n=220)
230 examined in this study while the use of CD138 IHC staining identified presence of one or

231 more plasma cells in 95/220 (43.2%) of the specimens examined (Figure 1). In most cases,
232 the distribution of plasma cells within the endometrium was not uniform, being localized
233 focally or widely dispersed in the stroma.

234 **Intra-observer Variability of Quantification Methods**

235 The results of the intra-observer variability of the three different quantification methods of
236 measurement, namely method I: CD138+ cell count per 10 randomly chosen HPF; method II:
237 CD138+ cell count per whole section; method III: CD138+ cell count per unit area (cell
238 density) are compared in Supplementary Table 2. The intra-class correlation coefficient
239 (intra-CC) value of cell count per 10 randomly chosen HPF was 0.46, which was considered
240 as moderate. The intra-CC of cell count per whole section and cell count per unit area of
241 whole section were 0.90 and 0.84, respectively, both considered as excellent.

242 **Inter-observer Variability of Quantification Methods**

243 The results of the inter-observer variability of the three different methods of measurement are
244 compared in Supplementary Table 3. The intra-CC of plasma cell count per 10 randomly
245 chosen HPF was 0.39, which was considered as poor. The intra-CC of cell count per whole
246 section and cell count per unit area of whole section were 0.88 and 0.83, respectively, both
247 considered as excellent.

248 **Reference Range**

249 In establishing the reference range of the plasma cell count or density, the specimens from
250 fertile control subjects were examined and the 95th percentile of the results was used to define
251 the upper limit of the reference range. The reference range for three different methods of

252 quantification were: (I) 1.95 CD138+ cells per 10 randomly chosen HPF; (II) 2.95 CD138+
253 cells per section; and (III) 5.15 CD138+ cells per $0.1mm^2$ (Table 2).

254 **Prevalence of CE**

255 The prevalence of CE in the various subgroups in this study as determined by the three
256 different methods of CD138+ cell quantification, in conjunction with two different diagnostic
257 criteria selected (one based on a previously literature report and the other based on reference
258 range derived from fertile women) was analysed and compared in Table 2. Quantification
259 using method I and method II consistently produced higher prevalence rates than
260 quantification using method III. The application of previously published criteria consistently
261 produced higher prevalence rates than those produced by the application of criteria based on
262 reference range derived from fertile population. The prevalence of CE in women with
263 reproductive failure as determined by quantification methods I or II, regardless of the
264 diagnostic criteria used, was significantly higher than fertile subjects in three out of the four
265 chosen criteria used (Table 2). However, the prevalence of CE in women with reproductive
266 failure and its subgroups, determined by using quantification method III and diagnostic
267 criteria based on established reference range, was not significantly ($p>0.05$) higher than that
268 of fertile subjects.

269 **Confounding Variable**

270 The possible impact of age as a confounding on the results of the expression of CD138 was
271 examined with the use of regression analysis. There was no significant association between
272 CD138+ cell density and age.

273 **DISCUSSION**

274 In this prospective observational study, we have used different methods to identify and
275 quantify plasma cell count and apply different criteria to diagnose CE; using our proposed
276 new methods of plasma cell assessment, we found that the prevalence rates of CE reported in
277 in earlier studies of women with reproductive failure may have been over-estimated.

278 **Identification of Plasma Cells**

279 The identification of plasma cells in endometrial biopsy specimen continues to be considered
280 as the gold standard method for the diagnosis of CE (12, 13, 25). Typical plasma cells have a
281 large cell body, high nuclei/cytoplasm ratio, basophilic cytoplasm, and nuclei with
282 heterochromatin in a unique arrangement called "spoke-wheel" or "clock-face" pattern (13,
283 17). However, plasma cells may from time to time be missed in routine histological
284 examination. The results from this study agree with earlier reports (1, 13-15) on the
285 usefulness of CD138 immunostaining in the identification of plasma cells, which has been
286 shown to be a more sensitive and accurate method to identify plasma cells compared with the
287 conventional H&E staining method (13-15). In accordance with previous publications, we
288 could not find any classic plasma cells in any of the H&E stained specimens, whilst the use
289 of CD138 staining led to the detection of one or more plasma cells in 46.1% (83/180) of
290 samples from women with reproductive failure, and 95/220 (43.2%) for all the subjects in
291 this study.

292 **Quantification of Plasma Cell**

293 In this study, we have based the diagnosis of CE on the stromal plasma cell count only; there
294 are a number of morphological features which have been reported to be associated with
295 chronic endometritis, namely superficial stroma edema, stromal inflammatory infiltrate,
296 increased stromal density, focal stromal haemorrhage and spindling of stroma, most notably

297 in the upper half of the mucosa (12). Greenwood & Morgan (12) argued for the inclusion of
298 these additional morphologic features in the definition of chronic endometritis, which was
299 supported by Bayer-Garner and Korourian (13), and Cicinelli et al. (32), but some
300 investigators based the diagnosis on plasma cell count only (19, 27). We have not included
301 the additional morphological features proposed by Greenwood & Morgan in our analysis
302 partly because there is as yet no consensus on the diagnostic value of these features nor are
303 they easily quantifiable.

304 It should be noted that the proposed "plasma cell density" measurement in this study referred
305 to plasma cell count per unit area, calculated from the entire area of the specimen, consisting
306 of all fields whether complete or not, which is different from the endometrial stromal
307 plasmacyte density index (ESPDI) which was calculated as the sum of the stromal CD138+
308 cell counts divided by the number of the high power fields evaluated (11), which is in
309 essence plasma count per selected number of complete HPFs.

310 **Observer Variability**

311 Observer variability is a measure of how reproducible the results are. In this study we directly
312 compare the observer variability of three different quantification methods and found that the
313 coefficient of variation of results obtained from the cell count per HPF method was
314 considerably higher than that of the cell count per section method and the cell density method.
315 It is likely that the source of variation of the cell count per HPF method comes from the
316 random nature of selection of the HPF, primarily because the (plasma) cell count is low.
317 Missing one or two positively stained cells could make a significant difference to the results.
318 The other two methods, both had intra-observer and inter-observer coefficient of correlation
319 in the excellent category. The intra and inter-observer variability for cell density appeared
320 slighter higher than that of cell count per section, which is to be expected, as the

321 measurement of cell density requires an additional measurement of area and so introduces an
322 additional source of variation.

323 **Reference Range**

324 In this study we have chosen to establish the normal ranges derived from a fertile population
325 and define results above the 95th centile as abnormal (basis for the diagnosis of chronic
326 endometritis). This approach is commonly used for establishing clinical laboratory values.
327 The sample size of 40 in the control group is somewhat small and may well be a limitation of
328 our study. On the other hand, a particularly strength of our study is the special efforts made to
329 ensure the homogeneity of the specimens (including control group) by collecting them
330 precisely on day LH+7, which would have reduced the variance of results.

331 Our finding agrees with that of Achilles and co-workers (26) who found plasma cells were
332 commonly present in the endometrium of asymptomatic, fertile and healthy women and their
333 presence alone, in small numbers, may not signify upper genital tract inflammation. Together,
334 these studies suggest that the hitherto held view that the finding of one or more plasma cell in
335 the endometrium is abnormal and diagnostic of CE may lead to over diagnosis of the
336 condition. Some previous studies used hysterectomy specimen as control (21, 22) which
337 called into question their validity as many of these subjects could well have uterine pathology.

338 **Prevalence of CE in Women with Reproductive Failure**

339 Using the reference range established and based on the CD138+ cell density quantification
340 method, the prevalence of CE in each of the three subgroups of women with reproductive
341 failure (10.8% for women with recurrent miscarriage, 7.7% in women with RIF and 10.4% in
342 women with infertility) was not significantly different from that of the fertile group. In
343 addition, the prevalence of CE among the entire group of reproductive failure (18/180, 10.0%)

344 was also not significantly different from the fertile group. This finding agrees with the earlier
345 reports of Kasius et al. (27, 28) who observed in a randomized controlled trial that the
346 prevalence of CE in a population of symptomatic infertile patients was low (2.8%) and that
347 the contribution of CE to reproductive failure could have been over-estimated in earlier
348 studies. Further analysis of data in Table 2 showed that the use of cell count per randomly
349 chosen HPF quantification method or the cell count per section quantification methods both
350 resulted in over-estimation of prevalence rates. Moreover, the application of arbitrarily
351 chosen diagnostic criteria used in the literature also led to over-estimation of the prevalence
352 rates. Our observation provided an explanation for the rather high prevalence rates (up to
353 60%) of CE reported in earlier studies (Table 1).

354 **Precise Timing of Specimen**

355 A notable feature of our study was that we obtained all our specimens on a precise
356 chronological date, that is exactly 7 days after the LH surge. Whilst is considered acceptable
357 to have specimens collected on LH+7 +/- day, we have preferred to include only samples on
358 a precise chronological date to reduce possible variance in results due to cyclic changes in the
359 cell count. Several studies suggested that the prevalence of CE appeared to be higher when
360 the biopsy was obtained in the proliferative phase than the secretory phase (29-31). It remains
361 possible that timing of the biopsy in different stages of the cycle could be a confounding
362 variable.

363 **Other Diagnostic Method**

364 In addition to the identification of plasma cell in endometrial biopsy specimen, hysteroscopy
365 has been proposed as an alternative method of diagnosis for CE (32-34). Although
366 hysteroscopy has an accuracy rate of 92.7% in the diagnosis of CE (35), histological

367 identification of plasma cell remains the gold standard of diagnosis of CE (12, 16, 17).

368 Although it is believed that CE is due to an underlying infection, routine microbial culture of
369 endometrial secretion in women with CE is often negative and so precludes its use in clinical
370 diagnosis (36). However, it is now possible to examine the entire microflora present in the
371 endometrium by genomic testing (microbiome study). A recent study by Moreno and co-
372 workers (37) demonstrated the existence of an endometrial microbiota that is highly stable
373 around the time of implantation and changes in microbiota profile appeared to be associated
374 with adverse reproductive outcomes. This finding adds a novel microbiological dimension to
375 our understanding of CE. It would be of interest to establish what specific changes in
376 microbiome induce the emergence of plasma cells in the endometrium.

377 **Consensus and Clinical Significance**

378 A review of the literature on the prevalence of CE, revealed that many investigators used
379 different methods of quantification and applied different diagnostic criteria (see Table 1),
380 often without justification. There was a lack of consensus in the diagnostic approach to define
381 CE. In order to make progress in the field and to provide effective and appropriate treatment
382 to women with reproductive failure a consensus on the quantification method and diagnostic
383 criteria is essential. We hope that the findings in this study serve to highlight the importance
384 of such a development. Whilst we have put forward data and argument to support that
385 "plasma cell density" measurement is a more reliable method of plasma cell assessment for
386 diagnosis of CE, ultimately, the proof of such a concept requires clinical studies to confirm
387 that the measurement is of useful prognostic value and leads to effective treatment.

388 To conclude, we have found that plasma cells may be present in small number in the
389 endometrium of fertile subjects, the quantification of plasma cell density improves the

390 accuracy of the diagnosis of CE and the prevalence of CE in women with reproductive failure
391 was only 10%, lower than previously reported.

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507 **Figure Legends**

508 **Figure 1.** Expression of plasma cells using (**A**) H&E staining, and (**B**)
509 immunohistochemistry staining for syndecan-1(CD138) in the same field of endometrial
510 tissue from the same woman. Plasma cells identified by black solid arrow. GE: glandular
511 epithelium; SC: stromal cell; LE: luminal epithelium. Magnification: ×400; Scale bar: 50 μm.

512 **Figure 2.** Scatter plot of CD138+ cell count per unit area (cell density) in four subgroups of
513 women (Fertile: fertile control women; RM: unexplained recurrent miscarriage; RIF:
514 recurrent implantation failure; Infertility: infertile women undergoing endometrial scratch in
515 a natural cycle preceding frozen-thawed embryo transfer). Reference range of CD138+ cell
516 count per unit area is up to 5.15 CD138+ cells/0.1 mm^2 showing on the Y axis. There was no
517 statistically significant difference between subgroups by chi-square test.

518 **Table 1** Prevalence of chronic endometritis reported in the literature among three groups of
519 women (I) infertility, (II) recurrent miscarriage, and (III) recurrent implantation failure, in
520 relation to inclusion criteria, diagnostic criteria and timing of endometrial biopsy.

References	Inclusion criteria	Diagnostic criteria (plasma cell count)	Timing of endometrial biopsy	Prevalence (n/N)
I. Infertility				
Cicinelli et al., 2005	Unexplained infertility	≥1/section	Follicular phase	30% (45/150)
Kitaya and Yasuo, 2010	Unexplained infertility	≥1/10 HPF	LH+6~8	29% (22/76)
Kasius et al., 2011	Infertility	≥1/section	Follicular phase	3% (17/606)
Kitaya et al., 2012	Infertility	≥5/20 HPF	Follicular phase	44% (23/52)
II. Recurrent miscarriage, RM				
Kitaya, 2011	≥3 miscarriages	≥1/10 HPF	LH+6~8	9% (5/54)
Zolghadri et al., 2011	≥3 miscarriages	≥1/section	Follicular phase	43% (61/142)
Cicinelli et al., 2014	≥3 miscarriages	≥1/section	Follicular phase	53% (190/360)
McQueen et al., 2015	≥2 miscarriages	1-5/HPF or discrete clusters <20	Not mentioned	56% (60/107)
Bouet et al., 2016	≥2 unexplained miscarriages	≥5/10 HPF	Follicular phase	27% (14/51)
III. Recurrent implantation failure, RIF				
Johnston-MacAnanny et al., 2010	≥2 failed ET cycles or >10 failed embryos transfer	≥1/HPF	Not mentioned	30% (10/33)
Kitaya et al., 2017	≥3 failed embryos transfer	≥0.25 ESPDI	Follicular phase	34% (142/421)
Cicinelli et al., 2015	≥3 failed ET cycles	≥1/section	Follicular phase	57% (61/106)
Bouet et al., 2016	≥3 failed embryos transfer	≥5/10 HPF	Follicular phase	14% (6/43)

521 *Note:* HPF = high power field, ×400 magnification. LH+6~8 = six to eight days after luteal hormonal surge.
522 ET = embryo transfer. ESPDI = The endometrial stromal plasmacyte density index.

Table 2 The prevalence of chronic endometritis using three different quantification methods of CD138+ cell (I) CD138+ cell count per 10 randomly chosen HPF; (II) CD138+ cell count per whole section; and (III) CD138+ cell count per unit area, in conjunction with selected diagnostic criteria.

Population	Methods used to quantify CD138+ cell							
	(I) CD138+ cell count per 10 randomly chosen HPF			**(II) CD138+ cell count per whole section**			**(III) CD138+ cell count per unit area**	P value[d]
	(I-a) CE+ if **≥1 cell/10HPF**	(I-b) CE+ if **≥1.95 cells/10HPF**	P value[c]	(II-a) CE+ if ≥ **1 cell/section**	(II-b) CE+ if **≥2.95 cells/section**	P value[c]	(III-b) CE+ if **≥5.15 cells/0.1mm^2**	
Fertile (n=40)	17.5%	5%	0.16	30.0%	5%	**<0.01**	5%	I-b vs II-b: 1.00 I-b vs III-b: 1.00 II-b vs III-b: 1.00
Reproductive Failure (n=180)	28.9%	18.9%	**0.02**	46.1%	22.2%	**<0.01**	10.0%	I-b vs II-b: 0.26 I-b vs III-b: **0.01** II-b vs III-b: **<0.01**
P value[e]	0.10	**0.02**		**<0.05**	**<0.01**		0.49	
RM (n=93)	19.4%	12.9%	0.16	38.7%	17.2%	**<0.01**	10.8%	I-b vs II-b: 0.27 I-b vs III-b: 0.41 II-b vs III-b: 0.15
P value[e]	0.51	0.29		0.22	**<0.05**		0.46	
RIF (n=39)	23.1%	15.4%	0.28	51.3%	20.5%	**<0.01**	7.7%	I-b vs II-b: 0.38 I-b vs II-b: 0.24 II-b vs III-b: <1.00
P value[e]	0.37	0.25		**<0.05**	**0.04**		0.98	
Infertility (n=48)	37.5%	29.2%	0.26	56.3%	33.3%	**0.02**	10.4%	I-b vs II-b: 0.41 I-b vs II-b: **0.02** II-b vs III-b: **<0.01**
P value[e]	**0.03**	**<0.01**		**0.01**	**<0.01**		0.59	

Note: RM = recurrent miscarriage. RIF = recurrent implantation failure.
Chi-Square test was used to compare the difference between subgroups.
a, an arbitrary criteria used in literature.
b, criteria based on reference range (95th percentile) derived from fertile subjects.
Three types of comparison are made:
[c] using the same quantification method, the prevalence rate based on two different diagnostic criteria, a and b, are compared (I-a vs I-b; II-a vs II-b); and
[d] using the same diagnostic criteria based on reference range derived from fertile subjects, i.e. b, the prevalence rate determined by three different quantification methods (I-b, II-b, III-b) are compared; and
[e] using the same quantification method and diagnostic criteria, the prevalence rate between fertile subjects and women with reproductive failure and subgroups are compared.

535 **Supplementary Table 1** A comparison of demographics between different groups of
536 subjects.
537

Characteristics	Fertile (n=40)	RM (n=93)	RIF (n=39)	Infertility (n=48)
Maternal age (y) [a,c]	29.6 ± 3.4	35.0 ± 3.4	36.1 ± 2.7	35.7 ± 2.8
BMI (kg/m^2) [a]	21.6 ± 2.0	22.1 ± 2.9	21.1 ± 2.4	21.7 ± 3.4
Gravidity [b]	2 (1 - 3)	3 (3 - 7)	0 (0 - 3)	1 (0 - 6)
Parity [b]	1 (1 - 3)	0 (0 - 1)	0 (0 - 1)	0 (0 - 2)
Miscarriages [b]	0 (0 - 1)	3 (3 - 6)	0 (0 - 1)	0 (0 - 2)

538 *Note*: RM = Recurrent miscarriage. RIF = Recurrent implantation failure. BMI = Body mass index.
539 ANOVA analysis was used to compare the difference between subgroups.
540 [a] mean ± SD
541 [b] median (range)
542 [c] P<0.01: Fertile vs RM; Fertile vs RIF; Fertile vs Infertility.

543 **Supplementary Table 2** A comparison of the intra-observer variability (intraclass
544 correlation coefficient, Observer A) among three quantification methods of CD138+ cell (I)
545 CD138+ cell count per 10 randomly chosen HPF; (II) CD138+ cell count per whole section;
546 (III) CD138+ cell count per unit area.

Participant ID	(I) CD138+ cell count per 10 randomly chosen HPF		(II) CD138+ cell count per whole section		(III) CD138+ cell count per unit area			
	1st Observation	2nd Observation	1st Observation	2nd Observation	Specimen area (mm^2) 1st Observation	Specimen area (mm^2) 2nd Observation	1st Observation	2nd Observation
1	0	0	0	0	9.789	11.297	0	0
2	0	0	0	0	6.767	6.264	0	0
3	0	0	0	0	4.763	4.705	0	0
4	1	1	2	1	4.476	4.349	4.47	2.30
5	0	0	0	0	5.057	4.887	0	0
6	0	0	0	0	5.592	5.225	0	0
7	0	1	1	1	4.943	4.655	2.02	2.15
8	0	0	1	1	12.029	11.200	0.83	0.89
9	1	1	2	1	13.673	14.225	1.46	0.70
10	0	1	1	1	2.841	2.169	3.52	4.61
11	1	0	2	2	10.249	9.874	1.95	2.03
12	0	1	0	1	7.561	6.249	0	0
13	0	0	0	0	8.078	6.500	0	0
14	1	2	3	3	17.676	17.065	1.70	1.76
15	0	0	0	0	12.620	9.675	0	0
16	0	0	1	1	8.089	7.923	1.24	1.26
17	0	0	0	0	3.225	3.178	0	0
18	0	0	1	1	7.260	6.976	1.38	1.43
19	0	0	0	0	2.067	2.268	0	0
20	0	1	1	0	5.138	6.702	1.95	0
Intra-CC (95% CI)	0.46 (0.06 to 0.74)		0.90 (0.76 to 0.96)		0.97 (0.92 to 0.99)		0.84 (0.64 to 0.93)	

547 *Note:* HPF = high power field, ×400 magnification. Intra-CC = Intraclass Correlation Coefficient. 95% CI = 95%
548 Confidence Interval.

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553 **Supplementary Table 3** A comparison of the inter-observer variability (intraclass
554 correlation coefficient, Observer A and B) among three quantification methods of CD138+
555 cell (I) CD138+ cell count per 10 randomly chosen HPF; (II) CD138+ cell count per whole
556 section; (III) CD138+ cell count per unit area.

Participant ID	(I) CD138+ cell count per 10 randomly chosen HPF		(II) CD138+ cell count per whole section		(III) CD138+ cell count per unit area			
					Specimen area (mm^2)			
	Observer A	Observer B	Observer A	Observer B	Observer A	Observer B	Observer A	Observer B
1	0	0	0	0	9.789	12.184	0	0
2	0	0	0	0	6.767	6.771	0	0
3	0	0	0	0	4.763	4.737	0	0
4	1	1	2	2	4.476	5.793	4.47	3.45
5	0	0	0	0	5.057	4.890	0	0
6	0	0	0	0	5.592	6.071	0	0
7	0	1	1	1	4.943	5.173	2.02	1.93
8	0	0	1	1	12.029	11.803	0.83	0.85
9	1	0	2	1	13.673	11.727	1.46	0.85
10	0	1	1	1	2.841	2.923	3.52	3.42
11	1	0	2	2	10.249	9.248	1.95	2.16
12	0	1	0	1	7.561	5.487	0	1.82
13	0	0	0	0	8.078	8.552	0	0
14	1	2	3	3	17.676	18.555	1.70	1.62
15	0	0	0	0	12.620	10.188	0	0
16	0	0	1	1	8.089	6.859	1.24	1.46
17	0	0	0	0	3.225	3.293	0	0
18	0	0	1	0	7.260	6.328	1.38	0
19	0	0	0	0	2.067	2.011	0	0
20	0	0	1	0	5.138	5.077	1.95	0
Intra-CC	0.39		0.88		0.96		0.83	
(95% CI)	(-0.06 to 0.70)		(0.72 to 0.95)		(0.90 to 0.98)		(0.62 to 0.93)	

557 *Note*: HPF = high power field, ×400 magnification; Intra-CC = Intraclass Correlation Coefficient. 95% CI = 95%
558 Confidence Interval.