1 **Running title:** Peritoneal macrophages correlate with pelvic pain and endometriosis

2 **Title:** Peritoneal macrophage phenotype correlates with pain scores in women with suspected

3 endometriosis

4

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14   Abstract

15   **Objective**: To characterise peritoneal macrophage populations in women with suspected

16   endometriosis and assess if they are correlated with severity of pelvic pain symptoms.

17   **Design**: Flow cytometry analysis of peritoneal fluid samples and clinical data.

18   **Setting**: University Research Institute.

19   **Patients**: Clinical questionnaires, surgical data and peritoneal fluid were collected with

20   informed consent from women undergoing diagnostic laparoscopy for suspected

21   endometriosis (n=54).

22   **Intervention(s):** None

23   **Main Outcome Measure(s):** Severity of pelvic pain symptoms was assessed by the EHP-30

24   questionnaire. Immune cells recovered from peritoneal fluid were analysed by flow cytometry.

25   **Results:** Pain scores (pain domain of EHP30) did not differ according to endometriosis

26   diagnosis, stage of endometriosis or whether or not women were receiving hormone

27   treatment. Analysis of immune cells in peritoneal fluid revealed two populations of peritoneal

28   macrophages: $CD14^{high}$ and $CD14^{low}$ which were not altered by menstrual cycle phase or

29   hormone treatment. $CD14^{high}$ peritoneal macrophages were increased in women with

30   endometriosis compared to those without but were not altered by coincident reproductive

31   health issues such as infertility or heavy menstrual bleeding. Peritoneal macrophage

32   phenotype correlated with pelvic pain symptoms in women with suspected endometriosis.

33   Notably, $CD14^{high}$ peritoneal macrophages negatively correlated with pain scores whereas

34    $CD14^{low}$ peritoneal macrophages were positively correlated. This association was independent

35   of endometriosis diagnosis.

36   **Conclusion:** Peritoneal macrophage phenotypes correlate with pelvic pain symptoms in

37   women with suspected endometriosis and are altered by presence of disease. These results

38   provide new insight into the association between endometriosis pathophysiology and pelvic

39   pain symptoms.

40   **Keywords**: Endometriosis, macrophage, peritoneal, pain.

41 Introduction

42 Endometriosis is a chronic neuroinflammatory pain condition affecting ~180 million women

43 worldwide and has a socioeconomic impact similar to diabetes (1). It is defined by the

44 presence of endometrial-like tissue outside the uterus ('lesions'), commonly within the pelvic

45 cavity. The most common symptoms of endometriosis are chronic pelvic pain, dyspareunia,

46 and dysmenorrhoea (1, 2). While the exact aetiology of endometriosis is unknown, changes

47 in inflammatory processes are thought to contribute to the pathogenesis of the disease.

48 Peritoneal macrophages are the most abundant immune cell in the peritoneal cavity (3) with

49 defined roles in clearance of apoptotic cells and immune surveillance. Studies in mouse

50 models suggest that peritoneal macrophages are required for the establishment and

51 persistence of endometriosis lesions. For example, the number of peritoneal macrophages is

52 increased in mice with experimentally-induced endometriosis compared to controls (4) and

53 macrophages expressing the angiopoietin receptor Tie-2 are reported to accumulate in lesions

54 and promote lesion vascularisation and growth (5). Depletion of peritoneal macrophages

55 prevents lesion formation and depleting peritoneal macrophages in mice with established

56 lesions reduces lesion vascularisation and prevents further growth (5).

57 These mechanistic studies in mice have been complemented by studies comparing peritoneal

58 fluid from women with and without endometriosis. Notably, increased concentrations of

59 macrophage growth factors and chemokines such as colony stimulating factor 1 (CSF1) and

60 monocyte chemoattractant protein 1 (CCL2) are detected in peritoneal fluid from women with

61 endometriosis (6-8) and this is associated with increased numbers of peritoneal macrophages

62 compared to women without disease (8-10). Furthermore, peritoneal macrophage function

63 appears to be dysregulated in endometriosis patients and this is characterised by increased

64 secretion of pro-inflammatory cytokines and reduced phagocytic capacity (8, 11-13). Thus,

65 peritoneal macrophage dysfunction may contribute to persistence of endometrial tissue and

66 establishment of lesions in the cavity.

67 Macrophage dysfunction may also affect pain symptoms via secretion of neurotrophic factors
68 that promote nerve growth (14). Notably, CD68-positive macrophages co-localise with nerve
69 fibres within peritoneal endometriotic lesions and it has been speculated that they can promote
70 endometriosis pain symptoms in women (15). It is not clear if peritoneal macrophages directly
71 contribute to pain symptoms in women but in a mouse model of endometriosis peritoneal
72 macrophages appear to promote endometriosis-associated hyperalgesia, putatively via
73 secretion of macrophage-derived insulin-like growth factor 1 (16).

74 To date, peritoneal macrophages have largely been assessed as a single population in women
75 with endometriosis and this has limited our understanding of their function. However, recent
76 studies in other disorders have reported there is heterogeneity within human peritoneal
77 macrophage populations based on expression of complement receptor of the immunoglobulin
78 superfamily (CRIg) (17) or canonical markers, such as CD14, CD16 and HLA-DR (18, 19).
79 Notably, peritoneal macrophage subsets appear to be altered in some diseases, such as liver
80 cirrhosis (17). It is not known whether different subtypes of peritoneal macrophage are present
81 in endometriosis, and if so, whether their phenotype is associated with disease severity or
82 pain symptoms.

83 The objective of this study was to phenotype and quantify peritoneal macrophage
84 subpopulations in the peritoneal fluid of women with suspected endometriosis and determine
85 if specific populations are associated with endometriosis stage, symptoms, or hormone
86 treatment.

87   Material and methods

88   *Study approval*

89   Written informed consent was obtained from all study participants prior to surgery; ethical

90   approval was granted by the Lothian Research Ethics Committee (LREC 11/AL/0376).

91   Methods were carried out in accordance with NHS Lothian Tissue Governance guidelines and

92   EPHect guidelines (https://endometriosisfoundation.org/ephect/).

93   *Clinical samples and patient data*

94   Peritoneal fluid was recovered from women undergoing diagnostic laparoscopy for suspected

95   endometriosis in NHS Lothian (n=54). Pain scores were determined using the pain domain of

96   the Endometriosis Health Profile Questionnaire (EHP-30) (a validated endometriosis-specific

97   pain questionnaire (20)). Age, BMI, menstrual cycle stage and hormone status were obtained

98   and recorded along with other key clinical data (Supplementary Table 1 and 2).

99   For women who were not on hormones and had regular menstrual cycles, the diagnosis of

100   endometriosis was confirmed macroscopically at laparoscopy in nineteen women, whereas no

101   evidence of endometriosis was found in ten women ('no endo'). Women with endometriosis

102   diagnosis were subsequently classified according to the revised American Fertility Society

103   guidelines (rAFS) as stage I (n=10), II (n=4) and III/IV (n=5). For some analyses, women who

104   were receiving hormone treatment were included (n=25). In women who met this criterion,

105   diagnosis of endometriosis was confirmed in fourteen women, while eleven were classified as

106   'no endo' (no obvious pelvic pathology at laparoscopy).

107   *Flow Cytometry*

108   Immune cells recovered from peritoneal fluid were analysed by flow cytometry using standard

109   methods (21). Briefly, cells were washed and subjected to red cell lysis before being counted

110   and resuspended in FACS buffer at a concentration of 0.5-1x10^6 cells/100µL. Antibodies were

111   used at manufacturer's recommended concentration (Supplementary table 3). Cells were

112   gated to select immune cells (live, CD45$^+$) and further sub-gated to select myeloid cells

113 (CD11b$^+$) and exclude T, B and NK cells (CD3$^-$, CD19$^-$, CD56$^-$). Gating of subpopulations was

114 performed based on fluorescence minus one (FMO) and population distribution (CD14). This

115 population gating was applied to all samples. Samples were assessed using a Becton

116 Dickinson FACS Aria II. Data were analysed using FlowJo™ Software.

117 *Statistics*

118 Statistical analysis was performed using Graphpad prism. T test was used to test the

119 difference in the means of two groups. Two-way ANOVA was used to determine significance

120 between treatments in grouped data. Non-parametric testing was utilised where sample sizes

121 were insufficient to confirm normality of data distribution; Kruskal-Wallis test was used to

122 assess differences between multiple groups or Mann Whitney test to assess variance between

123 two groups. Pearson correlation was used to quantify the degree to which two variables

124 (macrophages abundance and pain score) were related. Criterion for significance was $p < 0.05$.

125 All data are presented as mean ± SEM.

126

127   Results

128   *Pelvic pain does not correlate with endometriosis diagnosis or AFS stage*

129   Baseline characteristics of the patient cohort were assessed to identify potential factors which

130   may influence pelvic pain in women with suspected endometriosis. Pain scores were

131   determined using the pain domain of the EHP-30 questionnaire (20). For women who were

132   not on hormones, Age, BMI, and pain scores did not differ between those women categorised

133   as 'no endo' or those with surgically diagnosed endometriosis (Fig.1A and Supplementary

134   table 1). There was no statistically significant difference in pain score between AFS stages I,

135   II or III/IV, although pain scores were highly variable among some AFS stages (Fig. 1B). Oral

136   contraceptives and treatments which suppress hormone production are reported to decrease

137   endometriosis-associated pain in some women (22, 23). In this study, pain scores in women

138   receiving hormonal treatment (H) were not different to those women who were not on

139   hormones (NH) and this was similar in women with (Fig. 1C) and without (Fig. 1D) a confirmed

140   surgical diagnosis of endometriosis.

141   *Two populations of peritoneal macrophages are present in the peritoneal fluid of women with*

142   *suspected endometriosis and they are not altered by hormone status*

143   Cells were recovered from peritoneal fluid and analysed by flow cytometry. Cells were gated

144   to select immune cells and further sub-gated to select myeloid cells and exclude T, B and NK

145   cells (Fig. 2A &B). Two populations of peritoneal macrophages (PMφ) were identified which

146   were HLA-DR-positive and had either high or low intensity expression of CD14 (Fig. 2C).

147   In women with regular menstrual cycles, the phase of the menstrual cycle (categorised as

148   proliferative or secretory), had no significant impact on the abundance of CD14^{high} or CD14^{low}

149   PMφ populations (Fig. 2D). Although hormone treatment did not alter abundance of any of

150   these immune cell populations (Fig. 2E; supplementary figure 1), samples from patients on

151   hormones were excluded from subsequent analysis to avoid any confounding effects of this

152   variable on other disease parameters.

153   *Peritoneal macrophages are not altered by infertility or heavy menstrual bleeding*

154   We next assessed whether the co-morbidities infertility or heavy menstrual bleeding (HMB),

155   recorded as part of patient cohort data collection, could affect the abundance of PMφ

156   subpopulations in women with endometriosis. Infertility was more common in women with

157   endometriosis than women without endometriosis (42% vs 20%; supplementary table 1),

158   however, infertility did not alter the abundance of $CD14^{high}$ or $CD14^{low}$ macrophages in women

159   with endometriosis (Fig. 2F). HMB was less common in women with endometriosis than in

160   women without endometriosis (42% vs 60%; supplementary table 1) but PMφ populations

161   were not altered by presence of HMB (Fig. 2G).

162   *$CD14^{high}$ peritoneal macrophages are increased in women with endometriosis*

163   PMφ were assessed in women with and without endometriosis who had regular cycles and

164   were not receiving hormone treatment ('No endo' vs 'AFSI-IV'). $CD14^{high}$ PMφ were

165   significantly higher in the women with endometriosis (AFS I-IV) compared to women without

166   disease (Fig. 3A, p<0.05). No difference was observed for $CD14^{low}$ PMφ, although the average

167   was lower for women with endometriosis (Fig. 3B). To assess the relationship between the

168   two populations we next assessed the ratio of $CD14^{high}$ to $CD14^{low}$ macrophages. $CD14^{high}$

169   PMφ were the predominant population in the peritoneal cavity and this difference was more

170   pronounced in women with endometriosis (Fig. 3C) and with increasing AFS stage (Fig. 3D).

171   Analysis according to AFS stage (Figure 3C) identified a significant difference between

172   subpopulations of $CD14^{high}$ and $CD14^{low}$ PMφ in women classified as AFS stage I (p<0.01), II

173   (p<0.0001) or III/IV (p<0.001). No significant difference was found between $CD14^{high}$ and

174    $CD14^{low}$ PMφ in women without endometriosis.

175   *Peritoneal macrophages correlate with pelvic pain scores in women with suspected*

176   *endometriosis*

177   The abundance of either $CD14^{high}$ or $CD14^{low}$ PMφ was compared to pain scores in women

178   with suspected endometriosis and Pearson correlation calculated. No correlation was

8

179   observed in women without endometriosis but this subgroup had the fewest patients (n=8;

180   supplementary figure 2). When only women with diagnosed endometriosis (AFSI-IV) were

181   assessed, neither CD14^{high} PMφ (Fig. A, p=0.058) nor CD14^{low} PMφ significantly correlated

182   with pain scores (Fig. 4B, p=0.067), although a trend for negative and positive correlation,

183   respectively, was evident. In women with suspected endometriosis (no endo and AFSI-IV

184   combined), who were not receiving hormone treatment, CD14^{high} PMφ negatively correlated

185   with pain scores (Fig. 4C, p=0.031) and CD14^{low} PMφ positively correlated with pain scores

186   (Fig. 4D, p=0.032).

187   Discussion

188   Peritoneal macrophages are believed to play a fundamental role in the pathophysiology of

189   endometriosis (1, 2, 4, 24) but to date, information regarding different phenotypes of

190   macrophage and how they relate to pain symptoms has been limited. In this study, we

191   performed multi-parameter flow cytometry analysis of peritoneal fluid immune cells form

192   women with suspected endometriosis and identified two populations of PMφ, CD14^{high} and

193   CD14^{low}. Most notably, we believe our data are the first to demonstrate that CD14^{high} PMφ are

194   increased in women with laparoscopically-diagnosed endometriosis and that both CD14^{high}

195   and CD14^{low} PMφ populations correlate with pain symptoms independent of endometriosis

196   diagnosis.

197   We assessed pain scores in 54 women undergoing diagnostic laparoscopy for suspected

198   endometriosis. Pelvic pain symptoms were recorded using the pain dimension of the EHP-30

199   questionnaire, an extensively validated endometriosis-specific health-related quality of life

200   measurement (25, 26). We found that pain scores in women with endometriosis (mean and

201   SEM, 45.5±5.54) were comparable to previous reports using the EHP-30 questionnaire in

202   large cohorts of women from the UK (mean pain score 43.3, n=594 women), USA (mean pain

203   score 49.3, n=225 women) and Australia (mean pain score 40.7, n=189 women) (27-29).

204   These studies only assessed pain scores in women with surgically diagnosed endometriosis

205   but in the current study we also assessed pain scores in women who did not receive a

206   diagnosis of endometriosis at laparoscopy. Interestingly, mean pain score in the 'no endo'

207   group was actually higher (mean and SEM, 57.3±4.78) than women with endometriosis but

208   we found no significant difference between pain scores in women with or without

209   endometriosis. Pain scores were not significantly different in patients graded as AFS stage I

210   (47.3), II (38.3) or III/IV (47.8). Thus, in the current patient cohort, neither diagnosis of

211   endometriosis nor AFS stage affected pain scores.

212   We profiled PMφ subpopulations in women with suspected endometriosis to determine if

213   specific populations are altered by endometriosis stage, symptoms, or treatment. We identified

214 two populations of peritoneal macrophage, CD14^{high} and CD14^{low}, which, based on relative

215 abundance and comparative transcriptional profiling in other studies (17), we believe to be

216 equivalent to large (LpM) or small (SpM) peritoneal macrophage populations described in mice

217 (30). Injection of endometrial tissue to induce lesion formation is associated with an increase

218 in total peritoneal macrophages (4) and is associated with decreased LpM and increase SpM

219 in mouse models of endometriosis (24). In our study, we detected an increase in CD14^{high}

220 PMφ in women with endometriosis, consistent with previous reports assessing PMφ as single

221 population in mouse and human (4, 8). However, the increase we detected was specific to the

222 CD14^{high} PMφ subpopulation and is contrary to the decrease in the equivalent LpM population

223 reported in the mouse model of endometriosis described by Yuan et al. (24). In response to

224 peritoneal cavity inflammation in mice, LpM decrease and this is associated with infiltration of

225 monocytes that replenish the LpM pool. Notably, it is not possible to distinguish between

226 'resident' PMφ and infiltrating monocytes in women as they both express CD14. Thus, the

227 increase in CD14^{high} cells we detected in women with endometriosis may in part be accounted

228 for by monocyte infiltration. This may also be reflected by the increase in the proportion of

229 CD14^{high} relative to CD14^{low} we detected with AFS stages I, II and III/IV which may be

230 associated with increased peritoneal inflammation in response to disease burden. Future

231 studies will need to profile paired monocyte and PMφ samples from women with endometriosis

232 in order to distinguish their respective contribution to the peritoneal environment in

233 endometriosis.

234 Having identified two populations of peritoneal macrophage that were altered by presence of

235 disease we next assessed whether these populations could offer insight into pain symptoms

236 in endometriosis. A positive correlation was found between CD14^{low} PMφ and pelvic pain

237 scores and a negative correlation between CD14^{high} PMφ and pelvic pain scores. These

238 correlations were significant when all patients were assessed and were independent of

239 endometriosis diagnosis. This may indicate that pelvic pain is more strongly associated with

240 peritoneal macrophage dysfunction than the presence of active endometriosis lesions *per se*.

11

241 Peritoneal macrophages change throughout the inflammatory response, resulting in a state of

242 adaptive homeostasis. Thus, although some patients may have no 'active' or visible disease

243 at time of laparoscopy they may exhibit detectable changes in macrophages which correlate

244 to pelvic pain. A previous study reported a correlation between concentrations of IL6 in

245 peritoneal fluid and pelvic pain symptoms in women with endometriosis (31). Notably, PMφ

246 are reported to be the principle source of IL-6 and this cytokine is increased in peritoneal fluid

247 in women with endometriosis (32). Pain symptoms may therefore be affected by secretion of

248 pro-inflammatory cytokines from PMφ in response to 'active' disease or as a legacy of previous

249 inflammation from 'historic' disease. Thus, further profiling of peritoneal macrophages to

250 account for both their phenotype and function could provide new insights into the relationship

251 between endometriosis and pelvic pain symptoms.

252 Current treatment approaches for endometriosis focus on either surgical removal of disease

253 or hormonal suppression of lesion growth but this does not improve pain symptoms in all

254 women (22, 33, 34). Our new data suggest that divergence in pain responses may be

255 influenced by the direct response of peritoneal macrophages to a given treatment. For

256 example, GnRH agonists are reported to reduce pelvic pain in women with endometriosis (35)

257 and, notably, this treatment has been shown to increase PMφ cytotoxicity (36) and reduce

258 peritoneal fluid concentrations of the pain-correlated cytokine IL6 in women with endometriosis

259 (37). Large-scale clinical trials have shown that the non-peptide GnRH antagonist Elagolix has

260 a clinically meaningful reduction in the pain domain of EHP30 but only in around half of the

261 women tested (22, 34). To the best of our knowledge, the impact of Elagolix on peritoneal

262 macrophage function has not been investigated but baseline differences in peritoneal

263 macrophage phenotype could account for some of the reported differences in pain response.

264 We therefore propose that PMφ may have future utility for predicting response to treatment.

265 Conclusions

266 The novel findings in this study provide new evidence for a link between abundance of

267 peritoneal macrophage subpopulations and the experience of pelvic pain symptoms in women

268 with suspected endometriosis. Collectively, our data suggest that the inflammatory profile of

269 the peritoneal environment has a greater impact on pain symptoms than the presence/volume

270 of visible disease. Reframing this perspective opens a path to future targeted therapies that

271 aim to alter peritoneal macrophage function and reduce pelvic pain in women with

272 endometriosis.

273 Capsule

274 Peritoneal macrophage subpopulations are correlated with pelvic pain symptoms in women

275 with suspected endometriosis and altered by presence of disease.

276 Acknowledgements

288 **Author contributions**

289 Experimental design; DAG, AWH, PTKS, experimental procedures; DAG, BD, FC, manuscript

290 preparation; DAG, FC, AWH, PTKS.

291   Figure legends

292   **Figure 1. Pain score does not differ by endometriosis diagnosis, stage of disease or**

293   **hormone treatment**

294   **A** Presence/absence of endometriosis did not affect pain score (pain domain of EHP30) and

295   **B** did not differ according to AFS stage. Hormone treatment did affect pain score in women

296   without (**C**) or with endometriosis diagnosis **(D).**

297   **Figure 2. Analysis of peritoneal fluid immune cells from women with diagnosed**

298   **endometriosis reveals two populations of peritoneal macrophages that are not altered**

299   **by hormones or other reproductive health disorders**

300   Representative flow cytometry plots from pelvic peritoneal fluid (PF) immune cells. Cells were

301   gated to select; **A** Live, CD45$^+$ (all immune cells) and **B** CD11b$^+$ (myeloid cells), CD3$^-$CD19$^-$

302   CD56$^-$ ('DUMP'; T, B and NK cells). **C** Two peritoneal macrophage (PMφ) subpopulations

303   were identified that were HLA-DR$^+$ and CD14$^+$ (High vs low). **D** Phase of menstrual cycle (Pro:

304   proliferative; Sec: secretory) did not affect the abundance of CD14^{high} (*t* test, p=0.5035) or

305   CD14^{low} cells (Mann Whitney test, p=0.7664) thus samples were thereafter not stratified on

306   this basis. **E** The abundance of CD14^{high} (Mann Whitney test, p=0.3723) or CD14^{low} cells

307   (Mann Whitney test, p=0.3345) did not differ if women were not on hormones (NH) or on a

308   hormonal treatment (H). **F** The abundance of CD14^{high} or CD14^{low} peritoneal macrophages

309   (PMφ) did not differ between women reporting infertility (True) and those who did not (False).

310   **G** The abundance of CD14^{high} or CD14^{low} PMφ in women with endometriosis did not differ

311   between women reporting HMB (True) and those who did not (False).

312   **Figure 3. CD14^{high} peritoneal macrophages are increased in women with endometriosis**

313   **but not altered by other**

314   **A** Frequency of CD14^{high} peritoneal macrophages (PMφ) was greater in women with

315   endometriosis (AFS stage I-IV, n=19, t test, p<0.05) than those without endometriosis ('No

316   endo'; n=10). **B** Frequency of CD14^{low} PMφ was not significantly different in women with

317   endometriosis compared to those without disease **C** The ratio of CD14^{high} to CD14^{low} PMφ was

318   greater in women with endometriosis (AFSI-IV) than those without endometriosis (no endo).

319   **D** The ratio of CD14^{high} to CD14^{low} PMφ was greater in women from each AFS stage than

320   those without endometriosis (no endo). **E** CD14^{high} PMφ were significantly higher than CD14^{low}

321   PMφ in women with endometriosis stratified according to AFS I (n=10, P<0.01), AFS II (n=4,

322   P>0.0001) and AFS III/IV (n=5 P>0.001) but not in women without endometriosis (n=9; Two-

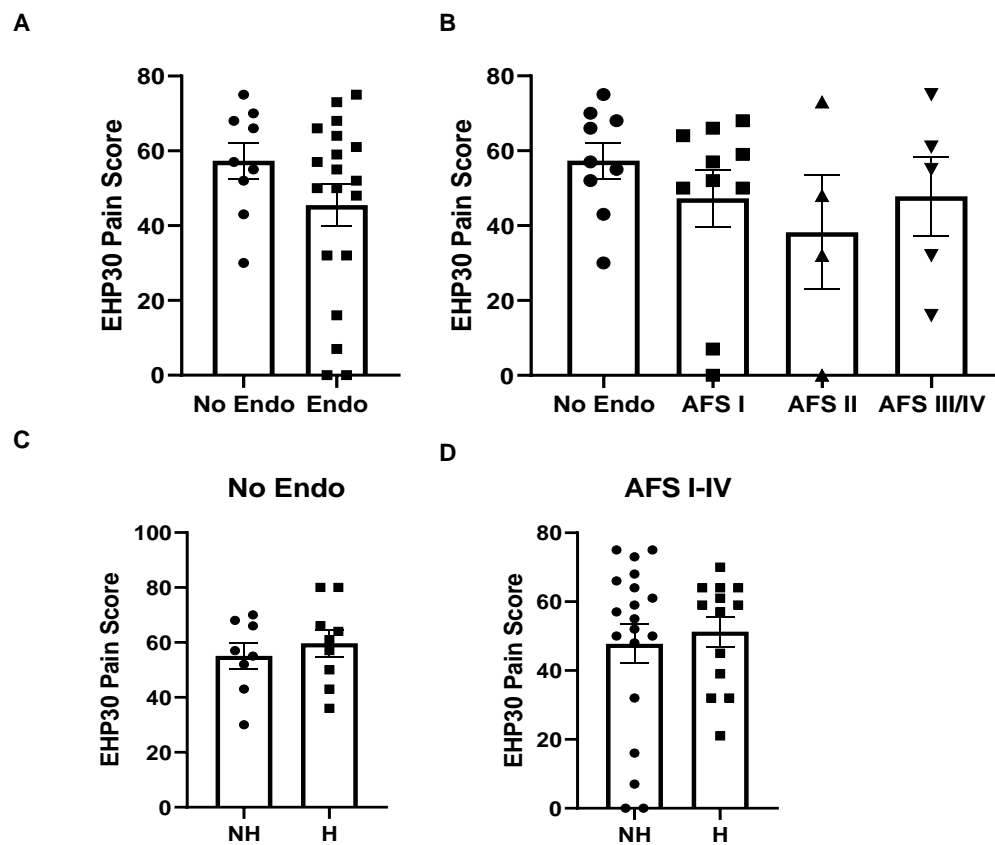323   way ANOVA). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 .

324   **Figure 4. Peritoneal macrophages correlate with severity of pelvic pain in women with**

325   **suspected endometriosis.**

326   **A** Frequency of CD14^{high} PMφ did not significantly correlate with pain scores in endometriosis

327   patients not on hormones (n=21). **B** Frequency of CD14^{low} PMφ did not significantly correlate

328   with pain scores in endometriosis patients not on hormones (n=21). **C** Frequency of CD14^{high}

329   PMφ negatively correlated with pain scores in all patients with suspected endometriosis and

330   not on hormones (p<0.05; n=28). **D** Frequency of CD14^{low} PMφ positively correlated with pain

331   scores in all patients with suspected endometriosis and not on hormones (p<0.05; n=28). P

332   value, correlation coefficient (r) and goodness of fit (R^2) calculated by Pearson correlation.

333   Line of best fit (black line) with 95% confidence intervals (dashed line). NH – not on hormones.

334

335   Figures

336   Figure 1

337   Figure 2

338

339

340   Figure 3

343   Figure 4

A Endometriosis - NH

$p = 0.058$
$r = -0.432$
$R^2 = 0.186$

B Endometriosis - NH

$p = 0.067$
$r = 0.408$
$R^2 = 0.184$

C All patients - NH

$p = 0.031$
$r = -0.408$
$R^2 = 0.166$

D All patients - NH

$p = 0.032$
$r = 0.406$
$R^2 = 0.166$

344
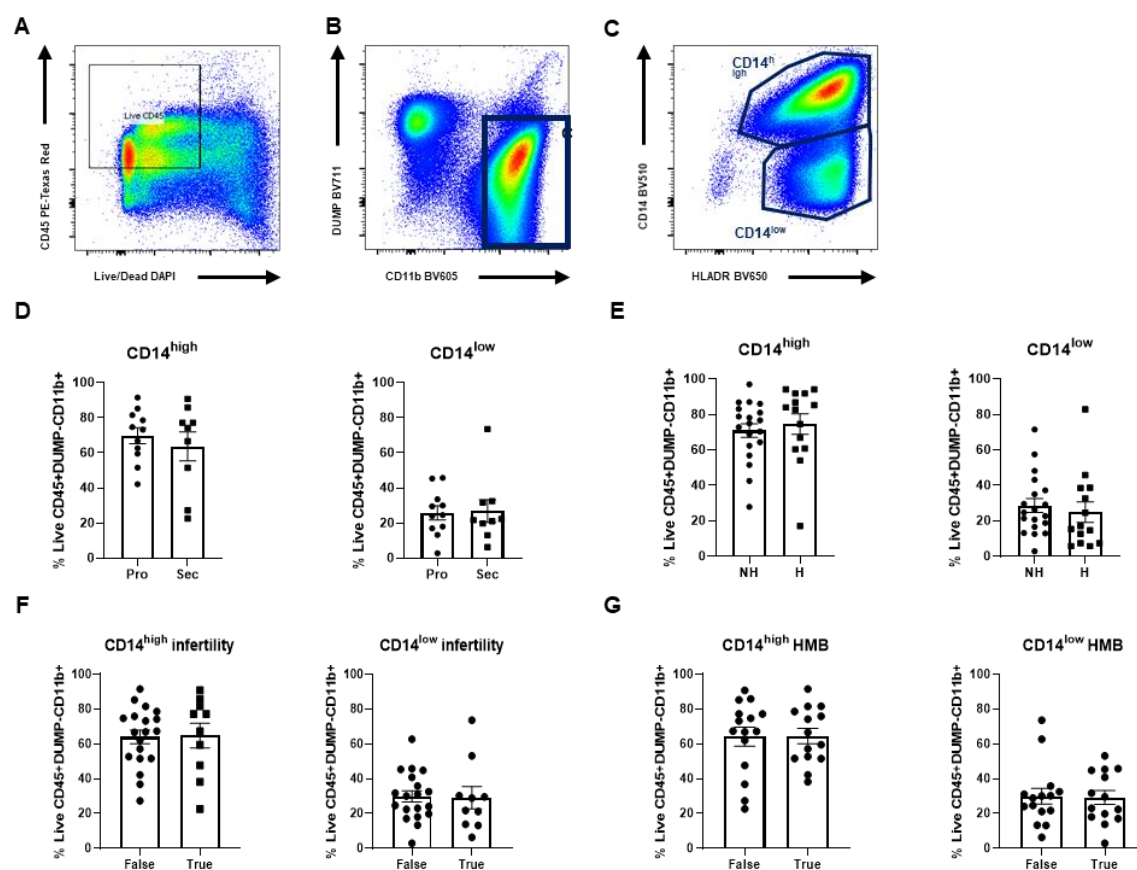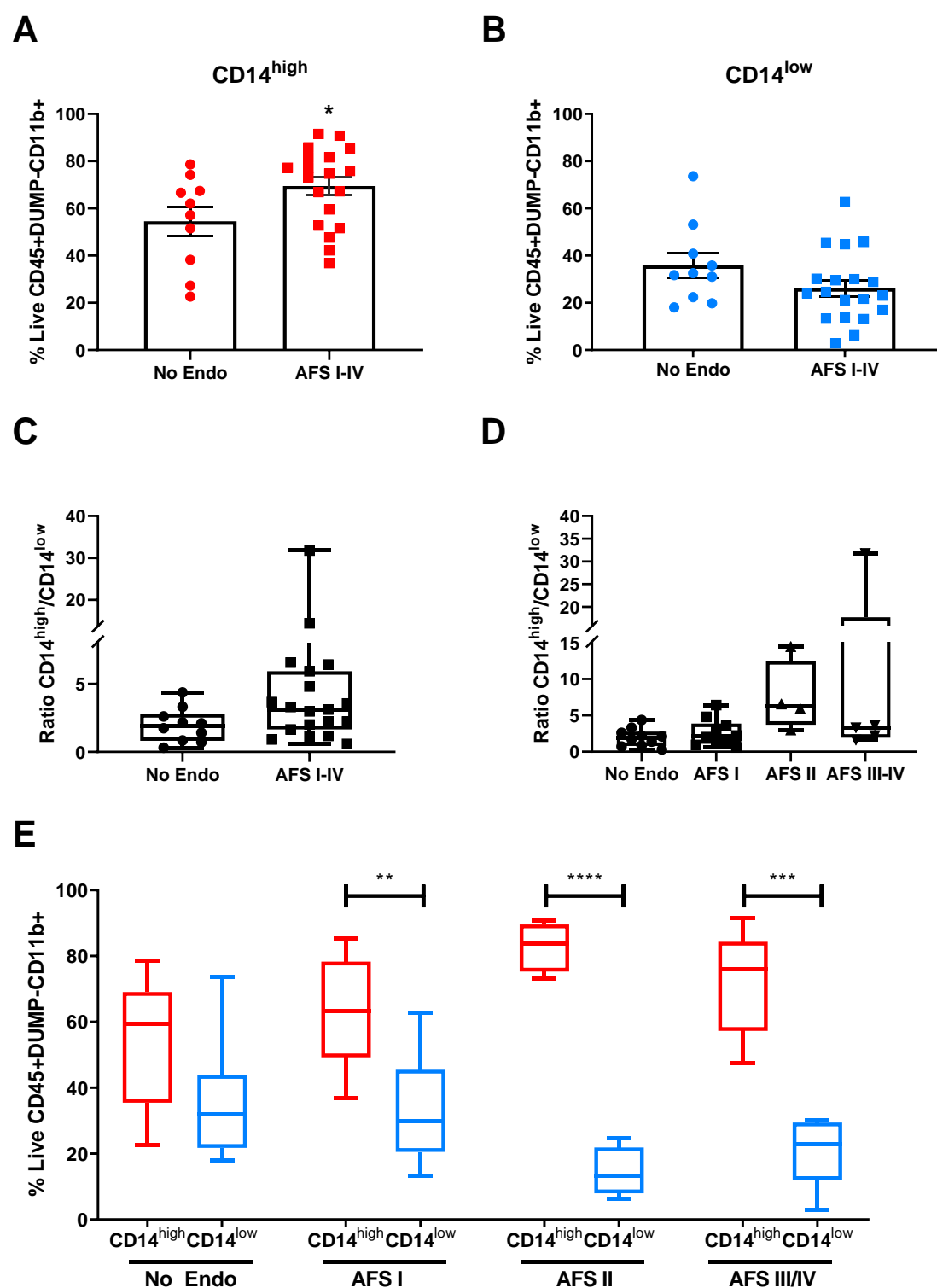
345   Supplementary data

		No Endo	**Endo**	**Endo by AFS stage**		
	AFS stage	N/A	I-IV	I	II	III/IV
	N	10	19	10	4	5
Age	*Mean*	29.1	31.83	29.67	33.5	34.4
	Std. Deviation	*6.28*	*7.09*	*7.583*	*5.447*	*7.403*
	Std. Error of Mean	*1.986*	*1.671*	*2.528*	*2.723*	*3.311*
	P value	*0.5308*		*0.4782*		
BMI	*Mean*	25.1	24.26	25.1	22	24.4
	Std. Deviation	*4.771*	*3.016*	*2.998*	*2.309*	*3.13*
	Std. Error of Mean	*1.509*	*0.6918*	*0.9481*	*1.155*	*1.4*
	P value	*0.6914*		*0.1876*		
EHP30 pain	*Mean*	57.33	45.53	47.3	38.25	47.8
	Std. Deviation	*14.35*	*24.14*	*24*	*30.58*	*23.59*
	Std. Error of Mean	*4.784*	*5.537*	*7.591*	*15.29*	*10.55*
	P value	*0.2576*		*0.741*		
Infertility	*(% True)*	20.0	42.1	30.0	75.0	40.0
HMB	*(% True)*	60.0	42.1	50.0	25.0	40.0

346   Supplementary Table 1. Age, BMI and EHP30 pain score of patients not on hormones did not

347   significantly differ by presence/absence of disease or according to AFS stage – Variance

348   between Endo and No Endo – Mann Whitney U test. Variance between AFS - Kruskal-Wallis

349   test.

350

		No Endo	**Endo**	**Endo by AFS stage**		
AFS stage		N/A	I-IV	I	II	III/IV
N		10	10	8	0	2
Age	*Mean*	25.7	32.2	31.75		34
	Std. Deviation	3.268	7.54	7.324		11.31
	Std. Error of Mean	1.033	2.384	2.589		8
	P value	0.049		0.6667		
BMI	*Mean*	24	23.75	24.33		22
	Std. Deviation	3.78	3.919	4.274		2.828
	Std. Error of Mean	1.336	1.386	1.745		2
	P value	0.8218		0.4286		
EHP30 pain	*Mean*	57.13	53.89	50.14		67
	Std. Deviation	13.94	15.85	16.07		4.243
	Std. Error of Mean	4.93	5.282	6.073		3
	P value	0.9074		0.1389		
Infertility	*(% True)*	28.6	0.0	0.0		0.0
HMB	*(% True)*	33.3	55.6	42.9		100.0

351 <u>Supplementary Table 2.</u> Age, BMI and EHP30 pain score of patients on hormones did not

352 significantly differ according to AFS stage. When compared by presence/absence of disease

353 'No Endo' patients were younger. Variance between Endo and No Endo or AFS I vs AFS III/IV

354 by Mann Whitney test.

355

Antigen	Fluorophore	Cross- reactivity	Clone	Cat number	Supplier
CD3	BV 711	Human	OKT3	317327	Biolegend
CD56	BV 711	Human	HCD56	318335	Biolegend
CD19	BV 711	Human	HIB19	302245	Biolegend
CD45	PE-Texas red	Human	HI30	MHCD4517	Invitrogen
CD11b	BV 605	Human	ICRF44	301331	Biolegend
CD14	BV 510	Human	M5E2	301841	Biolegend
HLA-DR	BV650	Human	L243	307649	Biolegend

356 Supplementary Table 3. Antibodies used in Flow cytometry analysis

357

358 **Supplementary Figure 1. Peritoneal macrophages are not altered by hormone**

359 **treatment.**
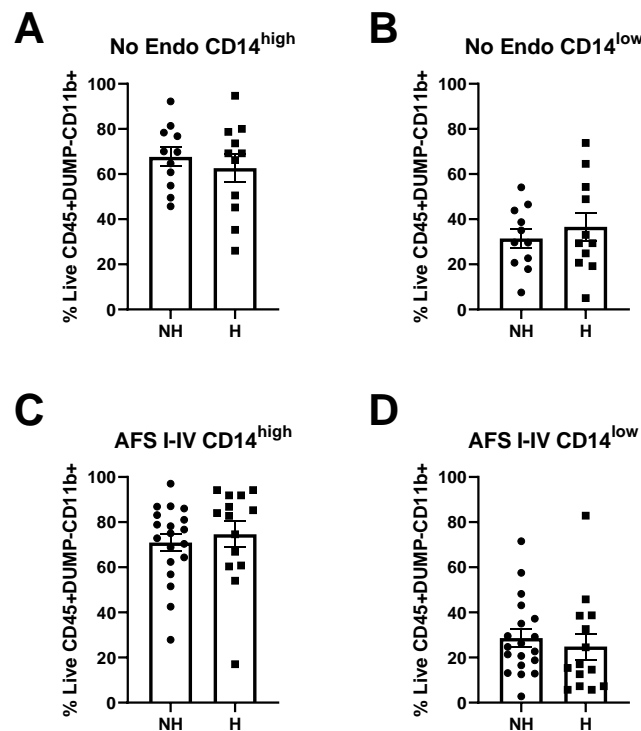
360

361 **A** Abundance of CD14^{high} peritoneal macrophages (PMφ) or **B** CD14^{low} PMφ in women without

362 endometriosis was not altered by hormone treatment. **C** CD14^{high} and **D** CD14^{low} PMφ in

363 women with endometriosis were not altered by hormone treatment. NH- No hormones, H -

364 Hormones.

Supplementary Figure 2. Peritoneal macrophages do not correlate with severity of pelvic pain in women without endometriosis.
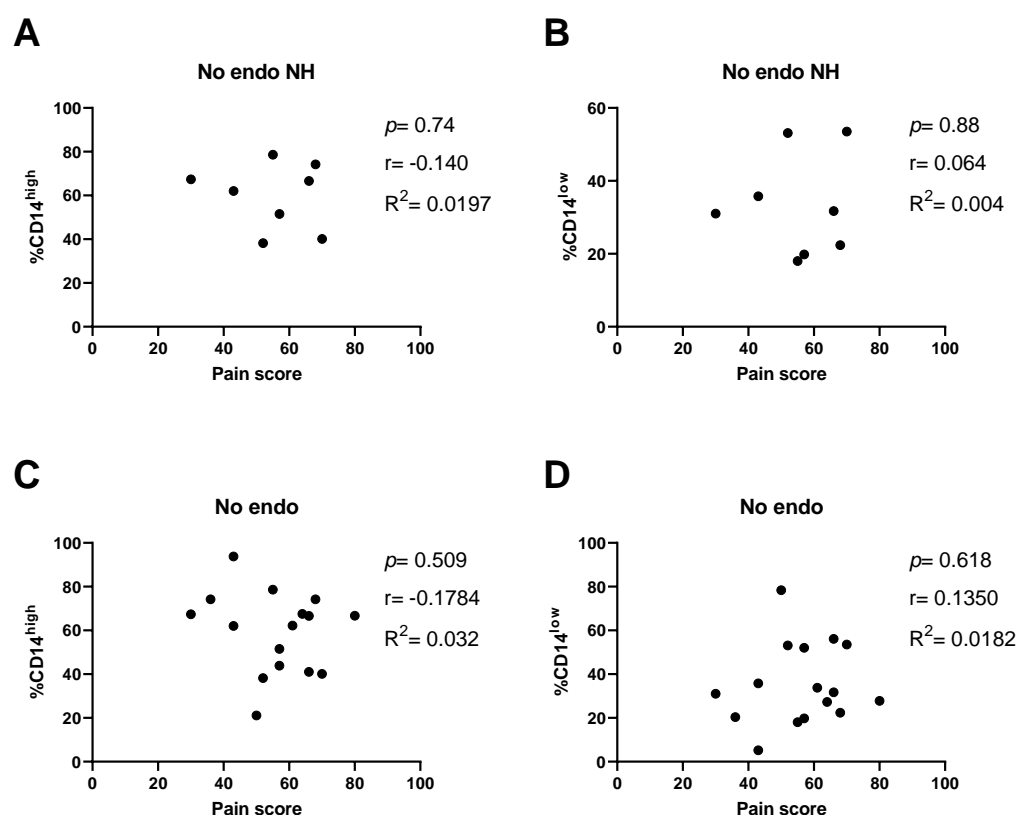
A Neither CD14^{high} nor **B** CD14^{low} PMφ correlated with pain scores in women without endometriosis and not on hormones (NH; n=8). **C** CD14^{high} or **D** CD14^{low} PMφ did not correlate with pain scores in women without endometriosis irrespective of hormone status (n=16).

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