

1 **TNF α induced IKK β complex activation influences epithelial, but not**
2 **stromal cell survival in endometriosis**

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16 **Running title:** IKK β in endometriosis

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24 **Abstract:**

25

26 **Study Hypothesis:** Can the activity of the I κ B kinase (IKK β) complex in endometriotic cells
27 contribute to endometriotic lesion survival?

28 **Study answer:** There is a constitutive activity of the IKK β catalytic complex in peritoneal
29 and deeply infiltrating lesions that can influence epithelial, but not stromal cell viability.

30 **What is known already:** Endometriotic lesions exist in an inflammatory microenvironment
31 with higher local concentrations of cytokines, such as tumor necrosis factor α (TNF α). TNF α
32 stimulates the activation of the IKK β complex, an important nodal point in multiple signalling
33 pathways that influence gene transcription, proliferation and apoptosis. However, few data on
34 the regulation of IKK β in endometriotic tissue are currently available.

35 **Study Design, size, duration:** A retrospective analysis of endometriotic tissue from
36 peritoneal, ovarian and deeply infiltrating lesions from 37 women.

37 **Participants/materials, setting, methods:** Basal and activated (phosphorylated) IKK β
38 concentrations were analysed by Western blotting and immunohistochemistry. The
39 relationship between the expression and activation of these proteins and peritoneal fluid
40 (TNF α) concentrations, measured via ELISA, was examined. A subsequent *in vitro* analysis
41 of TNF α treatment on the activation of IKK β and the effect on epithelial and stromal cell
42 viability by its inhibition with PS1145 was also performed.

43 **Main results and role of chance;** Levels of the phosphorylated IKK β complex in
44 endometriotic lesions had a significant positive correlation with peritoneal fluid TNF α
45 concentrations. Phosphorylated IKK β complex was more prevalent in peritoneal and DIE
46 lesions compared to ovarian lesions. IKK β was present in both epithelial and stromal cells in
47 all lesions but active IKK β was limited to epithelial cells. TNF α stimulated an increased
48 expression of phosphorylated IKK β and the inhibition of this kinase with PS1145
49 significantly influenced ectopic epithelial cells viability but not eutopic epithelial cells, or
50 endometrial stromal cells.

51 **Limitations, reasons for caution;** *In vitro* analysis on epithelial cells was performed with
52 immortalized cell lines and not primary cell cultures and only low sample numbers were
53 available for the study.

54 **Wider implications of the findings;** The regulation of aberrant signalling pathways
55 represents a promising yet relatively unexplored area of endometriosis progression. The IKK β
56 complex is activated by inflammation and is critical nodal point of numerous downstream
57 kinase-signalling pathways, including NF κ B, mTOR and BAD. This study shows a
58 significant relationship between peritoneal fluid TNF α and IKK β activation in epithelial cells
59 that will have significant consequences for the continued survival of these cells at ectopic
60 locations through the regulation of downstream pathways.

61 **Large scale data:** none

62 **Study funding/competing interests;** The study was funded by the Swiss National Science

63 Foundation (Grant Number. 320030_140774). The authors have no conflict of interest to

64 declare.

65

66

67 **Key Words;** endometriosis, kinase, signalling, IKK, TNF, inflammation, transcription factor,

68 DIE, peritoneal, endometrioma

69 **Introduction**

70 Endometriosis is characterized by the growth of endometrial epithelial and stromal cells
71 outside the uterine cavity. It is an extremely prevalent disease occurring in 10-20 % of women
72 of reproductive age and is accompanied by chronic pelvic pain and subfertility. Although the
73 exact pathogenesis is not yet clear Sampson's theory of transplanted is commonly accepted
74 (Sampson, 1928). This theory proposes that viable endometrial epithelial and stromal cells are
75 refluxed back through the Fallopian tube into the peritoneal cavity during menstruation. Once
76 in this ectopic environment these cells avoid immune detection, invade the underlying
77 mesothelial layer and stimulate a chronic inflammatory response.

78

79 Numerous inflammatory cytokines and chemokines (Borrelli *et al.*, 2013; 2014) are increased
80 in the peritoneal fluid of women with endometriosis, which occurs through the coordinated
81 interaction of the refluxed endometrial and peritoneal immune cells. Refluxed endometrial
82 cells produce and secrete chemokines (Hornung *et al.*, 1997) that attract leukocytes and
83 activated peritoneal macrophages (Halme *et al.*, 1983). The activated macrophages produce
84 inflammatory cytokines, which in turn further stimulate cytokine production by the
85 endometrial cells, creating a feed forward regulatory loop (Lebovic *et al.*, 2001) and the
86 chronic inflammatory environment. This inflammatory environment has the potential to both
87 influence symptomology (McKinnon *et al.*, 2015) and disease progression (Bruner-Tran *et*
88 *al.*, 2013).

89

90 A chronic inflammatory environment can contribute to endometriotic lesion progression
91 through the activation of a series of intracellular kinase signalling pathways (McKinnon *et al.*,
92 2016). The I κ B kinase (IKK) complex represents a significant, early nodal point in many of
93 the kinase signalling pathways. In the nuclear factor (NF) κ B signalling pathway the IKK
94 complex removes the inhibitory I κ B protein from NF κ B allowing translocation into the
95 nucleus and gene transcription (Bonizzi and Karin, 2004) subsequently influencing the gene
96 expression of many cytokine and chemokines, immunoreceptors, cell adhesion molecules,
97 stress response genes, and growth factors (Pahl, 1999). IKK β also interacts with the tubular
98 sclerosis (TSC2) protein in the mammalian target of rapamycin (mTOR) pathway influencing
99 cellular proliferation (Lee *et al.*, 2007) and phosphorylates Bcl2-antagonist of cell death
100 (BAD) pathway suppressing apoptosis (Yan *et al.*, 2013).

101

102 The IKK β complex consists of two catalytic subunits, IKK α and IKK β and one regulatory
103 subunit (IKK γ) (Hinz and Scheidereit, 2014). The binding of extracellular tumor necrosis
104 factor (TNF) α to its cell membrane receptor TNFR (Haider and Knöfler, 2009) stimulates the
105 phosphorylation of both IKK α and IKK β and activation of the IKK β complex. TNF α

106 concentrations are increased in the peritoneal fluid of endometriosis (Harada *et al.*, 1999) and
107 are correlated with the severity of the disease (Bedaiwy *et al.*, 2002) and thus TNF α
108 stimulated IKK β activity may have a significant influence on the endometriotic lesions.

109

110 At present very little information is available on the expression and activity of this important
111 upstream nodal kinase in endometriotic cells. In this study we used a combination of clinical
112 and *in vitro* experiments to determine the presence and importance of IKK β in endometriosis.
113 We found that peritoneal fluid TNF α concentrations had a significant positive correlation
114 with the activated IKK β complex and that this was most likely due to epithelial cell
115 expression. Furthermore IKK β activity was important in regulating ectopic epithelial cell, but
116 not stromal cell survival. These results therefore suggest a significant role of IKK β in
117 endometriotic epithelial cells that deserves further attention.

118 **Methods:**

119 *Patient samples*

120 Prior to surgery the relevant institutional review board granted ethical approval and informed
121 consent was obtained from all patients. During surgery performed for suspected
122 endometriosis samples of endometrium, peritoneal fluid and endometriotic lesions were
123 collected. Endometrial biopsies were collected via soft curette (Pipelle de Cornier,
124 Laboratoire CCD, France) and stored in RNAlater (Thermo Fischer Scientific, USA) at -80
125 °C as described previously (Santi *et al.*, 2011). Peritoneal fluid was collected during the
126 laparoscopic procedure from the cul-de-sac and centrifuged to remove blood cells, aggregates
127 and debris. Samples were excluded if blood remained in the samples. The pelvic cavity was
128 examined and any endometriotic lesions were removed and the patient staged (no
129 endometriosis, or stage I-IV) according to the revised American Fertility Society staging
130 system (rAFS) ("Revised American Society for Reproductive Medicine classification of
131 endometriosis," 1997). The lesions were recorded as either peritoneal, ovarian or deeply
132 infiltrating endometriosis (DIE). All surgeries were performed during the proliferative phase
133 of the menstrual cycle and endometriosis was confirmed by histological analysis.

134

135 Endometrial biopsies were collected from both women with (n = 8) and without
136 endometriosis (n = 7) and used for the isolation of primary cells via collagenase digestion and
137 size exclusion, as described previously (McKinnon *et al.*, 2012). Strong yields were obtained
138 for the primary stromal cells (ESC) from all women except one without endometriosis,
139 however only limited amounts of epithelial cells could be successfully isolated. As
140 endometrial epithelial cells are terminally differentiated and do not propagate immortalized
141 epithelial cells were acquired. Matching peritoneal fluid of sufficient quality was not always
142 available for cytokine measurement therefore if peritoneal fluid was available the ectopic
143 lesions were immediately frozen and stored for stored fresh frozen for subsequent Western
144 blot analysis. If peritoneal fluid was not available, they were formalin fixed and paraffin
145 embedded for immunohistochemistry analysis.

146

147 *Cytokine measurement in peritoneal fluid*

148 TNF α was measured by an enzyme-linked immunoabsorbent assay (ELISA) kit (R&D
149 Systems, Abingdon, England) using a high-sensitivity NADH cascade amplified alkaline
150 phosphatase with antigen-antibody incubations at 28 °C in a dry incubator and at a dilution of
151 1:2 in the diluent provided. Peritoneal fluid progesterone concentrations were also measured
152 via a radioimmunoassay (Coata-count, DPC; Buhlmann Laboratories, Allschwil, Switzerland)
153 to confirm the patient cycle phase (McKinnon *et al.*, 2014).

154

155 *Protein isolation and Quantification in ectopic lesions*

156 Approximately 30mg of fresh frozen ectopic tissue was used to prepare whole cell extracts
157 via homogenization with the FastPrep 120 tissue homogenizer (30 seconds at 4.0 m/s) in
158 radioimmunoprecipitation assay buffer (RIPA; 50mM Tris-Cl, pH 7.4, 150mM NaCl, 1mM
159 EDTA, 1% v/v triton X-100, 1 % w/v sodium deoxycholate, 0.1% w/v sodium dodecyl sulfate
160 and 1% v/v protease and phosphatase inhibitor cocktail (Cell Signalling Technology,
161 Danvers, Massachusetts)). Final protein concentrations were determined by the bicinchoninic
162 acid assay (QuantiPro BCA; Sigma).

163

164 Proteins separation was performed by heating 20µg of total protein into LDS buffer
165 (Invitrogen) to 70 °C for 10 minutes and running it on a 4 -15 % Novex NuPAGE Bis/Tris gel
166 (Invitrogen). Proteins were transferred to a 0.45µm nitrocellulose membrane in 4-
167 morpholinepropanesulfonic acid buffer (MOPS; Invitrogen) pH 7.7 for 1 hour. Non-specific
168 binding was blocked by incubation with 5% bovine serum albumin (BSA) in phosphate
169 buffered saline (PBS) containing 0.1% Tween 20 (PBST). Membranes were probed with
170 rabbit anti-IKKβ antibody (1:1000) (Cell Signalling Technology), and with rabbit anti-
171 pIKKα/β(176/180)(1:1000) antibody (Cell Signaling Technology), or mouse anti-actinβ
172 antibody (Abcam, Cambridge, UK) 1:5000. Secondary anti-rabbit (GE Healthcare, Opfikon,
173 Switzerland) and anti-mouse (Sigma) antibodies conjugated to horseradish peroxidase were
174 diluted 1:50 000 and 1:200 000 respectively. Immuno-reactivity was determined with the
175 SuperSignal West Femto kit (Pierce; Thermo Scientific) using the Chemi-Doc XRS+ system
176 (Bio-Rad Laboratories, AG, Cressier, Swituzerland). Band densitometry was quantified with
177 the Quantity One software and in each Western blot a calibrator sample with strong IKKβ,
178 and pIKKα/β expression was included to normalize concentrations across gels and determine
179 protein concentrations relative to the calibrator sample. The relative expression of each
180 sample was expressed as a percentage of the calibrator. Actinβ was used as a loading control.

181

182 *Immunohistochemistry*

183 Immunohistochemistry was performed using serial sections of 4µm mounted onto glass slides
184 (Superfrost, Braunschweig, Germany), dewaxed in xylene and rehydrated through a series of
185 decreasing ethanol concentrations. Epitope retrieval was performed with 10mM citrate buffer,
186 pH 5.5 for 5 minutes in a 450W microwave. Endogenous peroxidase activity was blocked
187 with 3% hydrogen peroxide (H₂O₂) and a blocking step performed with 3% BSA for 30
188 minutes in Tris buffered saline (TBS; Tris 100mM, NaCl 0.15 M; pH 7.4). Rabbit anti-IKKβ
189 antibody (1:100) and rabbit anti-pIKKα/β (176/180) antibody (1:100) were diluted in 3%
190 BSA in TBS and incubated at 4 °C overnight in a humidified chamber. Slides were washed
191 with TBS and 0.1% Tween 20 (TBST) prior to incubation with an affinity purified, biotin

192 conjugated goat anti-rabbit antibody (Dako, Glostrup, Denmark) for 90 minutes at room
193 temperature. After a final wash slides were incubated with an avidin-biotin HRP complex
194 (Vectastain, ABC Kit, Vector Laboratories, Burlingame, CA, USA) for 45 minutes. The
195 antigen-antibody complex was detected by incubation with 3,3' diaminobenzidine substrate
196 and slides were counterstained with hematoxylin and mounted in Aquatex (Merck). The
197 primary antibodies were excluded for the negative controls. Images were photographed with a
198 Nikon Eclipse E800 microscope (Nikon, Japan). Semi-quantitative analysis of antibody
199 staining in the epithelial and stromal cells of the endometriotic lesions was determined by the
200 allocation of scores between 0-3; 0 (negative), 1 (weak), 2 (moderate) and 3 (strong), based
201 on the intensity of staining. The percentage of cells with positive immuno-reactivity was also
202 determined and allocated a score between 0-6 as described 0% = 0, 1-10% = 1, 11-30% = 2,
203 31-50% = 3, 51-70% = 4, 71-90% = 5; > 91% = 6 in each cell type of the endometriotic
204 lesions. For the final immuno-reactive score the staining intensity and percentage of positive
205 cells was multiplied, as described previously (Samartzis *et al.*, 2012).

206

207 *Cell culture and TNF α treatment*

208 Isolated primary endometrial stromal cells (ESC) were maintained in Iscoves's modified
209 Eagle medium (IMEM) (Invitrogen) supplemented with 10% fetal calf serum (FCS)
210 (Invitrogen) and 1% antibiotic/antimycotic (Invitrogen). The immortalized epithelial cell
211 lines, EM E6/E7 and EM'osis, were provided by Professor Kyo, Kanazawa, Japan and were
212 isolated from eutopic endometrium (Kyo *et al.*, 2003) and an ectopic endometrioma (Bono *et al.*,
213 2012) respectively. These cells were maintained in Dulbecco's modified Eagles medium
214 (DMEM) (Invitrogen) with 10% FCS and 1% antibiotic/antimycotic. The 12Z cells were
215 provided by Professor Starzinski-Powitz, Goethe University and were originally isolated from
216 a peritoneal endometriotic lesion (Zeitvogel *et al.*, 2001). These cells were also maintained in
217 complete DMEM media.

218 To determine the influence of TNF α on pIKK α/β activity in all cell types the cells were
219 seeded into 6 well plates at approximately 3×10^5 cells/ well. After reaching approximately
220 80% confluence the media was changed to 0.5% FCS for overnight incubation prior to
221 treatment. Cells were treated either with control media (0.5% FCS in normal media) or
222 control media plus 10ng/ml and 100ng/ml recombinant human TNF α (R&D systems, United
223 Kingdom) for 6 hours. At the end of the treatment period the cells were rinsed and collected
224 in RIPA buffer.

225

226 *MTS assay and treatment with PS1145*

227 PS1145 is a small molecular weight compound that is a specific inhibitor of IKK β activity
228 (Lam *et al.*, 2005). Inhibition of IKK β activity with PS1145 was performed in 96 well plates

229 seeded at a density of 6×10^3 / well. Twenty-four hours prior to treatment the cells were
230 changed into serum free media and treatment media prepared by diluting PS1145 into either
231 serum free media at a final concentration of $1 \mu\text{M}$. Subsequent concentrations were prepared
232 by a 1:3 serial dilution (333.33nM, 111.11nM, 37.04nM, 12.3nM & 4.12nM). Cell viability
233 was measured after 72 hours by the CellTiter 96 Aqueous One Solution Cell Proliferation
234 Assay (Promega). Triplicate wells were used for each cell type and experiment. For the
235 immortalized epithelial cell lines the experiment was repeated three times and for the primary
236 ESC an experiment on each of the eight endometriosis and seven non-endometriosis
237 preparations was performed separately. A control (without PS1145) was included for each
238 experiment and designated as 100% viability and subsequent values expressed as a percent of
239 control.

240

241 *Statistical Analysis;*

242 All statistical analyses were performed with Graphpad Prism version 6.0. The correlation
243 between the peritoneal fluid cytokines and IKK β and pIKK α/β expression was performed
244 using the non-parametric Spearman's rank correlation coefficient. Two groups comparisons
245 were performed with a non-parametric Mann-Whitney U test and the comparison of three or
246 more groups with the non-parametric Kruskal-Wallis One-way analysis (ANOVA) and
247 Dunn's multiple comparison *Post hoc* test. The interaction between two variables was
248 determined via a two-way ANOVA with a *post-hoc* Tukey's multiple comparison test to
249 determine the difference between individual groups or conditions.

250

251 **Results:**

252 **Patient data and characteristics**

253 In total, endometriotic tissue was removed from 37 endometriosis patients and endometrial
254 biopsies from eight women without endometriosis. Of these 37 women we collected
255 accompanying peritoneal fluid from 21 in order to compare peritoneal fluid TNF α and ectopic
256 IKK β . Of the 21 samples 14 were collected from women without any hormonal treatment,
257 four were using combined oral contraceptives (COC) and three were using GnRH analogues.
258 No significant variation in TNF α , IKK β or pIKK α/β based on hormonal use (**Table 1**) was
259 identified. Five of the lesions were peritoneal, eleven ovarian and five DIE.

260

261 The remaining 16 samples without accompanying peritoneal fluid were kept for
262 immunohistochemistry. Of these, six women had no history of hormonal treatment, five were
263 using COC and five were using GnRH analogues. Three lesions were peritoneal, seven
264 ovarian and six DIE. No significant variation was observed between either pIKK α/β , or IKK β
265 expression in both the epithelial and stromal cells based on hormonal treatment (**Table II**).

266

267 **IKK β expression and activation in endometriotic tissue and its relationship to peritoneal**
268 **fluid TNF α concentrations;**

269 Comparison of peritoneal fluid TNF α and endometriotic lesion IKK β and pIKK α/β showed a
270 significant positive correlation between TNF α and pIKK α/β ($r = 0.6268$, $n = 21$, $p = 0.0024$)
271 (**Figure 1A**), but not IKK β ($r = 0.4216$, $n = 21$, $p = 0.0570$) (**Figure 1B**), as determined by semi
272 quantitative Western blot (**Figure 1C**). A significant variation in pIKK α/β concentrations
273 between lesions from different locations ($p < 0.05$) was observed with a *post-hoc* analysis
274 confirming a significantly lower expression in ovarian lesions (50 ± 8.7 , $n = 11$) compared to
275 the peritoneal lesions (99 ± 9.7 , $n = 5$) ($p = 0.041$) (**Figure 1D**). No significant difference was
276 observed with the DIE lesions (91 ± 21.6 , $n = 5$). In contrast, IKK β expression showed no
277 variation between lesion types ($p = 0.4905$) with similar expression in the peritoneal ($138 \pm$
278 29.3 , $n = 5$), ovarian (128 ± 26.7 , $n = 11$) and DIE lesions (80 ± 26.9 , $n = 5$) (**Figure 1E**). The
279 active to inactive ratio of IKK β (pIKK α/β :IKK β) varied significantly between lesions ($p =$
280 0.0123) with both peritoneal (1.0 ± 0.39 , $n = 5$) and DIE (1.7 ± 0.54 , $n = 5$) higher than
281 ovarian lesions (0.5 ± 0.09 , $n = 11$) with a *post-hoc* analysis showing a significant difference
282 between DIE and ovarian lesions ($p = 0.0168$).

283

284 **Cell specific activation of IKK in endometriotic lesions**

285 Immunohistochemistry identified a low but uniform expression of IKK β in peritoneal (**Figure**
286 **2A**), ovarian (**Figure 2B**) and DIE lesions (**Figure 2C**) in both stromal (*red arrows*) and
287 epithelial cells (*black arrows*). In contrast, pIKK α/β in peritoneal (**Figure 2D**), ovarian

Brett McKinnon 28.7.2016 22:48

Kommentar [1]: The value in the table
has been corrected

288 (Figure 2E) and DIE (Figure 2F) lesions was predominantly epithelial (*black arrows*), with
289 significantly less stromal cells expression (*red arrows*). Negative controls showed no
290 expression in peritoneal (Figure 2G), ovarian (Figure 2H), or DIE (Figure 2I) lesions. No
291 statistically significant difference was observed in IKK β expression between either lesion
292 location ($p = 0.2420$) or cell type ($p = 0.1972$) (Figure 2J), although this could be due to a
293 lack of power. pIKK α/β expression was significantly different in cell types ($p = 0.0198$), but
294 no statistically significant difference could be observed between lesion type ($p = 0.3402$)
295 (Figure 2K) possibly again due to lack of power.

296

297 *IKK β activity after TNF α treatment*

298

299 Western blot analysis of pIKK α/β after TNF α treatment of epithelial cell cultures confirmed a
300 low but positive expression in all cell lines examined (Figure 3A). Semi-quantitation of band
301 densitometry indicated that the 12Z cells showed the strongest basal expression (no TNF α),
302 but this did not vary after TNF α treatment ($p = 0.2320$). The EM'E6/E7 cells showed lower
303 basal expression and also no significant variation after TNF α treatment ($p = 0.4475$). In
304 contrast, TNF α treatment of EM'osis cells significantly increased pIKK α/β above the no
305 treatment control (46 ± 5.0 , $n = 3$) at concentrations of both 10ng/ml (141 ± 34.2 , $n = 3$, $p =$
306 0.0173) and 100ng/ml (197 ± 23.3 , $n = 3$, $p = 0.0085$) (Figure 3B). Similar Western blots
307 were performed on protein lysate isolated from stromal cells however no protein expression
308 could be observed in these preparations.

309

310 *Cell specific influence of IKK β inhibition on viability*

311

312 In the epithelial cell cultures there was a significant influence of PS1145 on cell viability
313 based on cell type ($p < 0.0001$) (Figure 4A). No significant effect of PS1145 was observed on
314 the EM E6/E7 at any concentration. For EM'osis cells there was a significant increase in cell
315 viability at the lowest concentrations (PS1145 2.43nM; 123 ± 0.2 , $n = 3$, $p < 0.001$) that was
316 gradually diminished as concentrations increased (PS1145 1 μ m; 106 ± 6.3 $p > 0.05$), whereas
317 12Z cell viability was significantly decreased at the lowest concentrations (PS1145 2.43nM;
318 70 ± 4.6 , $n = 3$, $p < 0.0001$) and remained significantly reduced through to the highest
319 concentration (PS1145 1 μ m; 73 ± 2.6 , $n = 3$, $P < 0.0001$). In contrast primary ESC isolated
320 from women with and without endometriosis showed no significant variation based on either
321 PS1145 ($p = 0.8868$) or cell type ($p = 0.3516$) (Figure 4B).

322

323

324

Chris Ford 27.7.2016 11:38

Kommentar [2]: Please be wary of interpreting this to mean that the values are similar.

Brett McKinnon 28.7.2016 23:00

Kommentar [3]: I have included the phrase 'statistically significant'

325 **Discussion**

326
327 In this study we examined the expression of the IKK β protein kinase complex and the
328 activation of its catalytic subunits pIKK α/β in both endometriotic lesions, as well as its
329 influence on cell survival in *in vitro* models. The results show a significant relationship
330 between the phosphorylation of the IKK β complex and peritoneal fluid TNF α . Subsequent
331 immunohistochemistry staining showed that although no statistically significant difference in
332 IKK β expression was observed across all cells and lesion types the phosphorylated IKK β
333 complex was predominantly epithelial. This data was supported by the *in vitro* studies that
334 confirmed pIKK α/β expression in epithelial cell culture models, but not primary stromal cells
335 and that inhibition of IKK β activity significantly influenced endometriotic epithelial cell
336 viability, but not eutopic epithelial cell viability, nor the viability of endometrial stromal cells
337 from women with and without endometriosis. These results therefore suggest that a
338 dysregulation of the IKK β kinase occurs in ectopic epithelial cells that may be related to the
339 inflammatory microenvironment. Given the role of IKK β in transmitting extracellular signals
340 into cell survival via kinase signalling pathways it may represent a significant molecule in
341 endometriosis pathogenesis.

342
343 At present there is very little known about the role of IKK β in endometriosis. The results
344 from our clinical samples suggest that the constitutive IKK β activity is significantly different
345 between peritoneal and DIE lesions compared to ovarian lesions. This difference of
346 expression was supported by our *in vitro* results that showed TNF α stimulated an increase in
347 pIKK α/β expression and that inhibition of IKK β activity increased the Em'osis cell viability.
348 In contrast in the peritoneal derived epithelia cells TNF α had a limited influence of pIKK α/β
349 expression and inhibition of IKK β activity decreased cell viability. Unfortunately a DIE
350 derived cell line was not available.

351
352 Endometriosis is a significantly heterogeneous condition, although whether these lesions have
353 different pathologies (Nisolle and Donnez, 1997), or represent a continuum of the same
354 disease (Somigliana *et al.*, 2004) is still debated. These data suggest a varied cellular response
355 to inflammation may occur in different lesions. It has previously been shown that rectovaginal
356 septum lesions have a distinctly inflammatory phenotype (Bertschi *et al.*, 2013) and that
357 concentrations of inflammatory mediators are stronger in the peritoneal fluid of DIE
358 compared to lesions from other locations (Santulli *et al.*, 2012). As IKK β can be associated
359 with TNF α both in ours and other studies (Lee *et al.*, 2007) it is possible that the increased
360 production of inflammation associated with DIE lesions is related to the higher IKK β activity.

Chris Ford 27.7.2016 11:50

Kommentar [4]: Please consider if this conclusion is justified. Some groups are small so statistical power is limited.

Brett McKinnon 28.7.2016 23:00

Kommentar [5]: I have changed the wording to temper the conclusion

361 The identification of a TNF α influenced activation of the IKK β in endometriotic tissue is
362 significant because of the multiple downstream pathways it regulates (**Figure 5**) and the
363 influence this can have on gene transcription, protein translation and both cellular
364 proliferation and apoptosis. Activation of IKK β stimulates NF κ B gene transcription and a
365 constitutive activation of NF κ B has been observed in peritoneal endometriosis (González-
366 Ramos *et al.*, 2007). Multiple factors present in the peritoneal fluid of women with
367 endometriosis including cytokines and iron overload (Alvarado-Díaz *et al.*, 2015) may lead to
368 this constitutive activation. Furthermore an increased NF κ B activity has been linked to
369 recurrence of ovarian endometrioma (Shen *et al.*, 2008). Neither of these studies however
370 examined IKK β expression directly. In the immortalized epithelial 12Z cells IKK β inhibition
371 attenuated inflammatory cytokine secretion (Grund *et al.*, 2008) and in ectopic endometrial
372 stromal cells miR200a suppresses IKK β (Dai *et al.*, 2012), raising the possibility of
373 suppressed IKK β activity in stromal cells occurs via an epigenetic regulation. In contrast to its
374 role in inflammation via the NF κ B pathway, IKK β can also regulate cellular proliferation and
375 apoptosis through the mTOR and BAD pathways (Dunlop and Tee, 2014) (Yan *et al.*, 2013).
376 A dysregulation of mTOR has previously been implicated in endometriosis pathogenesis of
377 DIE lesions leading to increased proliferation (Leconte *et al.*, 2011), as has a role for mTOR
378 mediated autophagy (Choi *et al.*, 2014) and BAD activation in ovarian endometriomas
379 (Stickles *et al.*, 2015). Together this suggests that TNF α has the potential to modulate all of
380 these activities via IKK β activation.

381

382 We found that the constitutive activation and influence on cell survival was largely restricted
383 to epithelial cells. Endometriotic lesions are a combination of epithelial and stromal cells and
384 an interdependency between the cells types is required for endometriotic lesions to continue
385 proliferating as tissue integrity of refluxed endometrial matter is essential to endometrial
386 tissue implantation (Nap *et al.*, 2003). We have also previously shown that the stromal cells
387 produce significantly more inflammatory cytokines than epithelial cells in response to
388 stimulation (Bersinger *et al.*, 2008). It could therefore be postulated that a paracrine
389 regulation occurs in the lesions through the stromal cells production of cytokines stimulating
390 a constitutive activation of the IKK β complex in epithelial cells, which ultimately contributes
391 to cell survival. More research however is required to explore this hypothesis.

392

393 Furthermore, the activity of IKK β in other cell types other than endometriotic cells was not
394 directly addressed in this study, but may give further insight into this mechanism in normal
395 tissue. For endometriosis, however whether this mechanism also happens in healthy eutopic
396 epithelial cells may be of less consequence. This is because epithelial cells will only be
397 present in the peritoneal cavity when endometriosis is present, and when endometriosis is

398 present there is a constant inflammatory environment. We believe it is this confluence of
399 ectopic epithelial cells and constant inflammation that makes the contribution of TNF α
400 stimulated IKK β activity significant. It may be such that this is a characteristic not inherent in
401 the endometrium, but rather acquired during the life of the lesion and contribute more to
402 progression through a constant stimulation of the inflammatory cascade. Further study on
403 whether there is a significant difference between the activation of IKK β in the eutopic
404 endometrium of women with and without endometriosis would be an interesting follow-up.

405

406 Whether other cell types also show a constitutive activity of IKK β in the presence of
407 inflammation would also be interesting. In this study the images in Figure 2 indicate cells
408 proximal to the endometriotic lesion are largely negative for pIKK α/β expression, providing
409 circumstantial evidence for the preferential activation of pIKK α/β in endometriotic epithelial
410 cells. Previous studies suggest that cells proximal to the lesion may have different
411 characteristics to cells distal to the lesions (Young *et al.*, 2014) and these cells thus may also
412 be interesting to study, however we were unable to collect this tissue due to our current
413 ethical approvals. Future studies on this topic may however be warranted.

414

415 Limitations of this study were the inclusion of women with hormonal treatment. Previous
416 research however has suggested there was no significant influence on NF κ B activation by
417 oral contraceptives (González-Ramos *et al.*, 2007). We also observed no statistically
418 significant difference for IKK β in this study, although the power of this analysis was limited
419 by the small sample size. It is possible that this may introduce a variability in peritoneal fluid
420 cytokine concentrations in endometriotic women, as GnRHa analogues have been shown to
421 have an influence on the inflammatory environment (Nirgianakis *et al.*, 2013), however we
422 postulate that a reduction in inflammatory cytokines by hormonal treatment would also be
423 reflected by a subsequent reduction in IKK β activation, maintaining any correlation between
424 the extra and intracellular environment. A further limitation of this study is the small sample
425 size. An expansion of the sample number would provide more definitive information on both
426 the influence of TNF α , as well as hormonal treatments, on IKK β activity. It is difficult to
427 draw direct conclusions on the contribution of IKK β to cell survival in different lesions types
428 as immortalized cell lines were used, however given the similarity observed in the *in vitro*
429 results to the clinical samples we can be confident that IKK β has a role in epithelial
430 endometriotic cells.

431

432 In conclusion we have observed a significant relationship between TNF α and the activation of
433 IKK β complex in the endometriotic microenvironment and that this activation occurs
434 predominantly in the epithelial cells of peritoneal and DIE lesions. Furthermore IKK β

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Kommentar [6]: Given the number of observations and the variance, please consider whether hormonal; effects can be excluded with such confidence.

435 inhibition *in vitro* significantly influenced epithelial cell, but not stromal cell behaviour. The
436 regulation of kinase signalling pathways is a significant, but under explored area of
437 endometriosis pathogenesis and progression and may represent potential non-hormonal
438 treatment targets for endometriosis (McKinnon *et al.*, 2016). Given the ability of the
439 extracellular inflammatory environment to influence IKK β activity and its subsequent affect
440 on downstream pathways this kinase may be significant interest in endometriosis.

441

442

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447
448 **Authors Roles;** **VK** performed experiments, cell culture and assisted with sample collection.
449 **CW** performed immunohistochemistry and analysis. **GG** assisted with cell culture. **NAB**
450 assisted with sample collection and intellectual development of the project. **MDM** assisted
451 with sample collection and intellectual development of the project. **BDM** conceived project,
452 performed experiments and prepared the manuscript.

453
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456
457 **Conflict of Interest:**
458 None
459

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589 **Table 1; Comparison of endometriotic lesion and peritoneal fluid protein expression in**
 590 **samples removed from women using different hormonal treatment. Data are Mean \pm**
 591 **SEM**

	Hormone treatment				p
	No hormone (n=14)	Combined oral contraceptive (n = 4)	GnRH analogue (n=3)	Total (n =21)	
pIKK α / β (%)	66 \pm 9.8	77 \pm 27.8	92 \pm 17.5	72 \pm 8.5	0.4732
IKK β (%)	120 \pm 23.5	90 \pm 27.7	151 \pm 27.7	119 \pm 16.9	0.3819
TNF α (pg/ml)	2.1 \pm 0.61	1.2 \pm 0.53	1.5 \pm 0.54	1.9 \pm 0.43	0.7721

592 -IKK β and pIKK α / β protein values were determined via Semi-quantitative Western blot
 593 densitometry analysis and expressed as a percentage of a standardised control sample
 594 included in each Western blot, as described in detail in the methods section.

595 -TNF α values represent peritoneal fluid TNF α concentrations determined by ELISA and
 596 expressed as pg/ml.

597 -Analysis of significance was performed via the non-parametric One-way ANOVA test
 598 (Kruskal-Wallis) and a post hoc comparison of all groups (Dunn's multiple comparison test)
 599 significance $p < .05$

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602 **Table II: Comparison of protein expression in formalin fixed paraffin embedded**
 603 **endometriotic tissue according to hormonal treatment. Data are mean ± SEM**

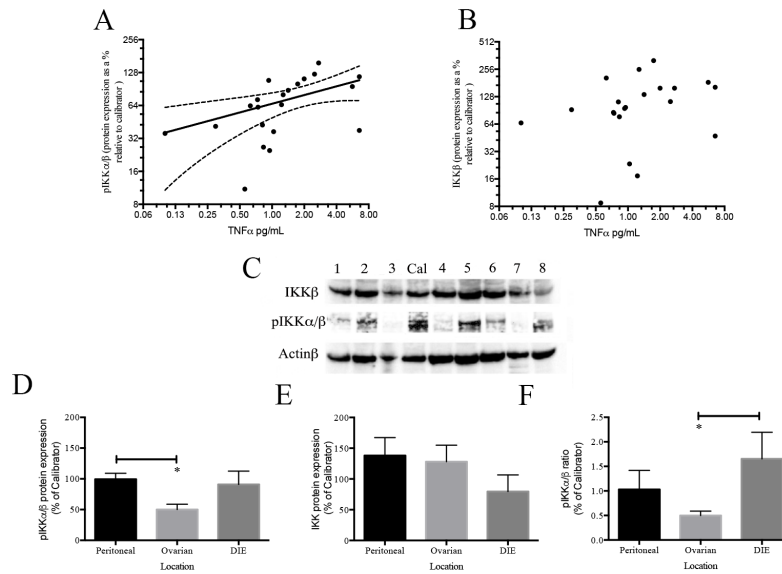
Hormonal use	No hormone (n= 6)	Combined oral contraceptive (n = 5)	GnRH analogue (n=5)	Total (n =16)	*P
pIKKα/β					
Epithelial	1.7 ± 0.56	3.4 ± 1.78	6.2 ± 2.27	3.6 ± 0.98	0.108
Stromal	0.3 ± 0.33	0.8 ± 0.37	0.6 ± 0.60	0.5 ± 0.24	0.448
IKKβ					
Epithelial	2.3 ± 0.95	2.2 ± 1.11	3.0 ± 1.76	2.5 ± 0.70	0.949
Stromal	0.7 ± 0.42	12.4 ± 1.75	0.0 ± 0.00	1.00 ± 0.58	0.327

604 -Values represent the scores derived from the semi-quantitative IHC analysis, described in
 605 detail in the methods.
 606 -Analysis of significance was performed via the non-parametric One-way ANOVA test
 607 (Kruskal-Wallis) and a post hoc comparison of all groups (Dunn's multiple comparison test)
 608 significance p < .05
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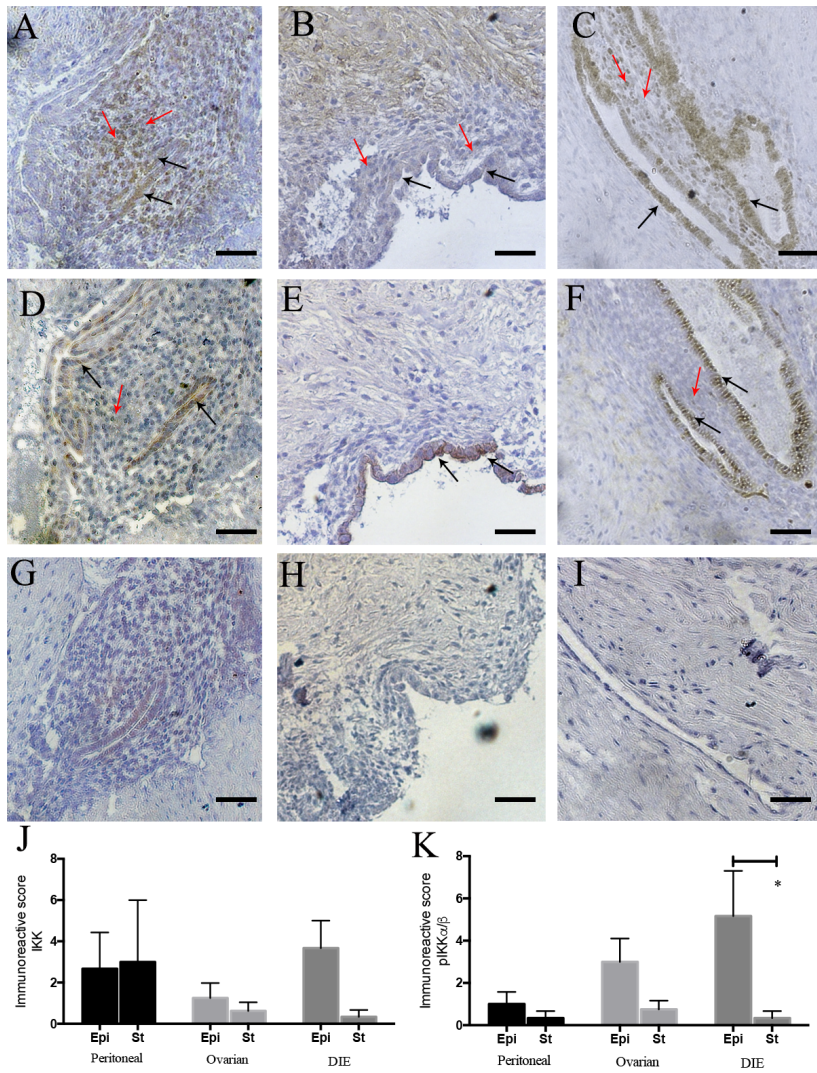
Kommentar [7]: A typo resulted in an additional 1 at the start of the number. The correct value is 2.4

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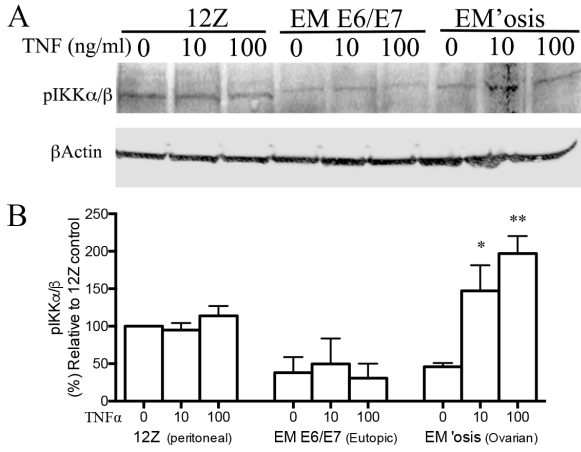


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Figure 1; IKKβ activation in endometriosis and its relationship to tumor necrosis factor (TNF)α. (A) A significant correlation ($r = 0.6268$, $n = 21$, $p = 0.0024$) was present between the peritoneal fluid TNFα expression and the pIKKα/β(176/180) protein complex in endometriotic lesions. (B) No significant association was observed between the expression of endometriotic lesion IKKβ and peritoneal fluid TNFα expression. (C) Western blot analysis of endometriotic tissue samples confirmed a consistent presence of both IKKβ. In contrast the expression of pIKKα/β varied significantly amongst samples. Actinβ was used as a loading control. (D) Analysis of pIKKα/β expression indicated that high concentrations were identified in the peritoneal and DIE lesions with lower concentrations observed in the ovarian lesions. (E) Basal IKK was more uniform amongst all samples, although with a slightly lower, but non-significant expression in the DIE lesions. (F) Analysis of the pIKKα/β: IKKβ ratio confirmed a lower ratio of activation in the ovarian lesions that was significantly lower than that observed in DIE lesions. Protein concentration in all components was calculated as relative to the calibrator sample and expressed as a percentage. Correlation was determined performed by Spearman's Rank correlation coefficient and comparison between lesion location performed by a non-parametric Kruskal-Wallis One-Way analysis (ANOVA) test with a *post-hoc* Dunn's multiple comparison. * $p < 0.05$. (P) peritoneal, (O) ovarian and DIE (deeply infiltrating endometriosis).

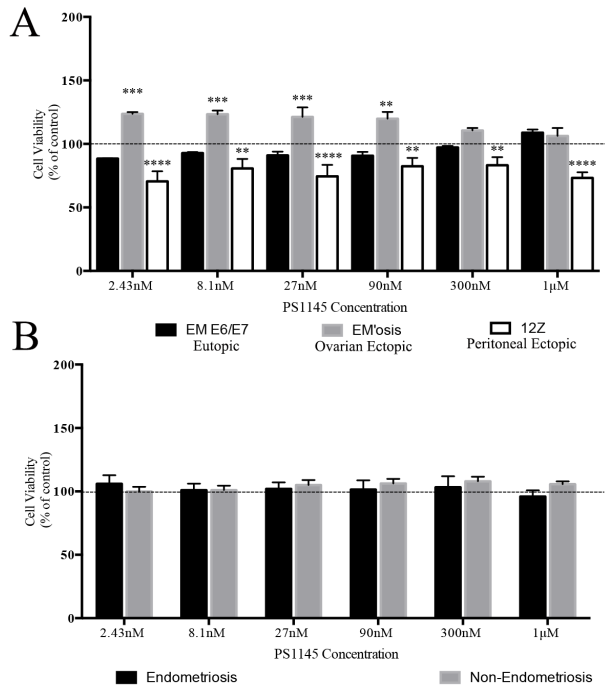


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 633 **Figure 2; Cell specific expression and activation of IKKβ in endometriotic lesions.** Basal
 634 IKKβ expression was observed in both epithelial (*black arrows*) and stromal cells (*red*
 635 *arrows*) of endometriotic lesions removed from the (A) peritoneal, (B) ovarian, and (C)
 636 DIE regions. The expression of the activated IKK complex (pIKKα/β) was limited predominantly
 637 to the epithelial cells (*black arrows*), although some stromal cell expression was observed
 638 (*red arrows*). This was consistent across lesions from the (D) peritoneal, (E) ovarian and (F)
 639 and DIE lesions. Negative controls showed no expression in lesions from the (G) peritoneal
 640 (H) ovarian, or (I) DIE region. A semi-quantitative analysis of the cell specific expression
 641 indicated that no statistically significant variation in (J) IKKβ expression was observed
 642 between epithelial and stromal cells, however pIKKα/β was significantly stronger in the
 643 epithelial cells for all lesions with a largest difference observed in the DIE lesions. (K)
 644 Analysis of protein activation and expression between cell type and lesion location was
 645 performed with a Two-way analysis of variance (ANOVA) test with a *post-hoc* Tukey's
 646 multiple comparison. Scale bars = 50μm. * p < 0.05
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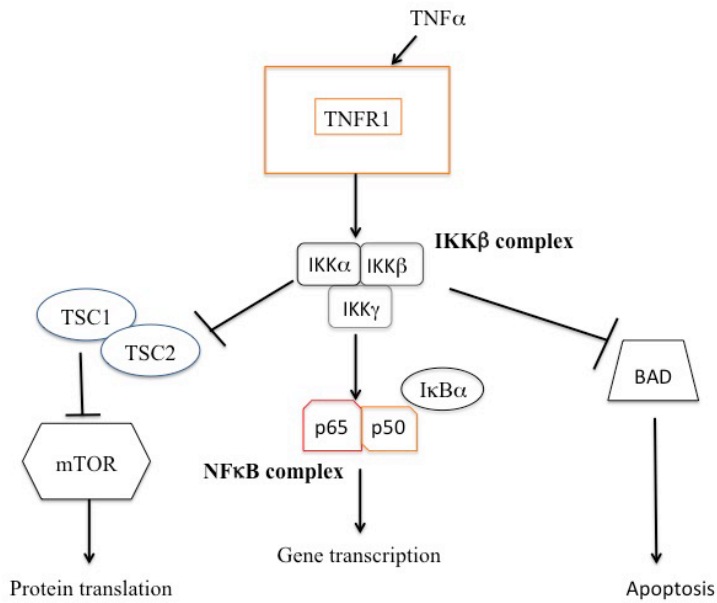
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Figure 3; pIKKα/β after tumor necrosis factor (TNF)α treatment in endometriotic epithelial cells. (A) Western blot analysis confirmed the expression of pIKKα/β in the 12Z, EM E6/E7 and EM 'osis cell lines both with and without TNFα treatment. **(B)** Semi-quantitative analysis indicated TNFα did not significantly influence either 12Z, or EM E6/E7 expression. There was however a significant increase in pIKKα/β after both 10ng/ml and 100ng/ml TNFα in the EM'osis cell line.



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Figure 4; Influence of IKKβ inhibition on epithelial and stromal cell viability. (A) Inhibition of IKKβ activity with increasing concentrations of PS1145 had no influence on the eutopic derived epithelial EM E6'E7 cells, significantly increased the cell viability of the ovarian derived EM'osis cells, significantly reduced the viability of the peritoneal derived 12Z cells. **(B)** PS1145 had no influence on stromal cells from women with and without endometriosis. Analysis on the influence between cell type and PS1145 concentrations on cell viability performed with a Two-way analysis of variance (ANOVA) test with a post-hoc Tukey's multiple comparison test. ** p < 0.01, *** p < 0.001, **** p < 0.0001.



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Figure 5; The IKK complex and its influence on downstream signalling pathways. Binding of extracellular tumor necrosis factor (TNF) α to TNF receptor 1 (TNFR1) stimulates the phosphorylation and activation of IKK α and IKK β that exist as a complex along with IKK γ . Activation of IKK β leads to the phosphorylation of I κ B α , which under basal conditions is bound to the p65 subunit of the NF κ B complex. Phosphorylation of I κ B α removes it from the NF κ B complex and initiates proteasomal degradation allowing NF κ B translocation into the nucleus and gene transcription. Activation of the IKK complex can also lead to an interaction with the tuberous sclerosis (TSC)2 protein that exists in a heterodimer with TSC1. Inhibition of TSC2 activity increases the activity of the mammalian target of rapamycin (mTOR) complex stimulating both protein translation and cellular proliferation. Activation of the IKK complex also leads to an inactivation of the BH3 only BAD protein inactivating TNF α stimulated apoptosis.