

University of Warwick institutional repository: <http://go.warwick.ac.uk/wrap>

This paper is made available online in accordance with publisher policies. Please scroll down to view the document itself. Please refer to the repository record for this item and our policy information available from the repository home page for further information.

To see the final version of this paper please visit the publisher's website. Access to the published version may require a subscription.

Author(s): Cavilla JL, Kennedy CR, Byskov AG, Hartshorne GM
Article Title: Immature oocytes grow during in vitro maturation culture
Year of publication: 2008
Link to published version:
<http://humrep.oxfordjournals.org/cgi/content/abstract/23/1/37>

1

2

3

Immature oocytes grow during in vitro maturation culture

4

5

6

Cavilla JL⁴, Kennedy CR², Byskov AG³, Hartshorne GM^{1,2}

7

8

¹Clinical Sciences Research Institute, Warwick Medical School, University of Warwick,

9

Clifford Bridge Road, Coventry, CV2 2DX, UK, ²Centre for Reproductive Medicine,

10

University Hospitals Coventry and Warwickshire NHS Trust, Coventry, CV2 2DX, UK

11

and ³Laboratory of Reproductive Biology, Juliane Marie Centre, Section 5712,

12

Rigshospitalet, Blegdamsvej 9, DK-2100 Copenhagen, Denmark.

13

⁴Current address: Assisted Conception Unit, Kings College Hospital, Denmark Hill,

14

London, SE5 9RS, UK.

15

16

¹To whom correspondence should be addressed at:

17

Clinical Sciences Research Institute, Warwick Medical School, University of Warwick,

18

Coventry, CV2 2DX, UK

19

Tel: 02476 968679/528382

20

Fax: 02476 968880

21

Email: Geraldine.Hartshorne@warwick.ac.uk

22

23

Running title: Oocyte growth during IVM

24

25 Abstract

26 **BACKGROUND.** Oocyte competence for maturation and embryogenesis is associated
27 with oocyte diameter in many mammals. This study aimed to test whether such a
28 relationship exists in humans and to quantify its impact upon in vitro maturation (IVM).
29 **METHODS.** We used computer-assisted image analysis daily to measure average
30 diameter, zona thickness and other parameters in oocytes. Immature oocytes originated
31 from unstimulated patients with polycystic ovaries, and from stimulated patients
32 undergoing ICSI. They were cultured with or without meiosis activating sterol (FF-
33 MAS). Oocytes maturing in vitro were inseminated using ICSI and embryo development
34 was monitored. A sample of freshly collected in vivo matured oocytes from ICSI patients
35 were also measured. **RESULTS.** Immature oocytes were usually smaller at collection
36 than in vivo matured oocytes. Capacity for maturation was related to oocyte diameter and
37 many oocytes grew in culture. FF-MAS stimulated growth in ICSI derived oocytes, but
38 only stimulated growth in PCO derived oocytes if they eventually matured in vitro.
39 Oocytes degenerating showed cytoplasmic shrinkage. Neither zona thickness,
40 perivitelline space, nor the total diameter of the oocyte including the zona were
41 informative regarding oocyte maturation capacity. **CONCLUSIONS.** Immature oocytes
42 continue growing during maturation culture. FF-MAS promotes oocyte growth in vitro.
43 Oocytes from different sources have different growth profiles in vitro. Measuring
44 diameters of oocytes used in clinical IVM may provide additional non-invasive
45 information that could potentially identify and avoid the use of oocytes that remain in the
46 growth phase.

47 **Key words:** diameter/growth/human/IVM/oocyte

48 **Introduction**

49

50 Studies in several species have highlighted the relationship between oocyte diameter and
51 competence for maturation and embryonic development. However, relatively little
52 information is available in humans despite the accessibility of oocytes during clinical in
53 vitro maturation (IVM). We measured oocytes during maturation culture in order to test
54 the hypothesis that maturation and developmental competence are dependent upon oocyte
55 growth beyond a threshold value. This would provide useful information on the potential
56 of oocyte diameter measurements as a non-invasive predictor of developmental
57 competence.

58

59 There is a substantial body of research on oocyte diameter and maturation in animals.
60 Eppig and Schroeder (1989) introduced the concept that competence to develop through
61 successive stages of meiosis and early embryogenesis in mice is dependent upon age and
62 oocyte size. They showed that isolated oocytes from mice ≤ 13 days of age, having mean
63 diameters $>60\mu\text{m}$, were able to undergo spontaneous breakdown of the germinal vesicle
64 (GVBD) in culture, but larger oocytes from mice ≥ 15 days of age were more likely to
65 mature completely to metaphase II (MII) in culture. Hirao *et al.* (1993) confirmed that
66 the threshold diameter of $60\mu\text{m}$ for GVBD remained the same even when mouse oocytes
67 were grown in vitro. Similar evidence of maturation competence relating to oocyte
68 growth was obtained in rats by Daniel *et al.* (1989) and in pigs by Hirao *et al.* (1994),
69 where the threshold diameters for GVBD were $55\mu\text{m}$ and $90\mu\text{m}$ respectively. Continuing
70 transcription in small bovine oocytes indicates that their growth is not complete (Fair *et*
71 *al.*, 1995) and hence, their complement of maternally derived mRNA, necessary for early
72 embryonic growth, might also be incomplete, providing a possible mechanism for these

73 observations. However, Canipari *et al.* (1984) observed mouse oocytes that became
74 GVBD competent after being cultured in conditions that did not promote significant
75 growth, suggesting that the events of meiotic resumption and oocyte growth may be
76 separable when non-physiological conditions are applied in vitro.

77

78 The capacity to cleave after maturation and insemination in vitro is also acquired with
79 increasing age and oocyte diameter. Bao *et al.* (2000) showed that the developmental
80 competence of mouse oocytes progresses in a stepwise manner as oocyte diameter
81 increases from 65-75 μ m and that developmental changes occurring during the final stages
82 of oocyte growth are critical for full developmental competence.

83

84 In rhesus monkey oocytes, meiotic competence occurs late during oocyte development,
85 however, oocyte diameter appeared relatively constant as competence for GVBD arose,
86 suggesting no close relationship with oocyte diameter (Schramm *et al.*, 1993). Durinzi *et*
87 *al.* (1995) examined the relationship between oocyte size and maturation in vitro in
88 unstimulated human oocytes from women aged 25-39yrs undergoing gynaecological
89 operations not associated with ovarian pathology. They observed a significant difference
90 in maturation capability of oocytes measuring 86-105 μ m at collection versus those
91 measuring 106-125 μ m, leading to the conclusion that, in common with other species, the
92 unstimulated human oocyte has a size-dependent ability to resume meiosis and complete
93 maturation.

94

95 During a study of human IVM, fertilization and embryo development (Cavilla *et al.*,
96 2001), we captured computerised micrographic images over culture periods of up to six
97 days. This afforded the opportunity to quantify human oocyte growth under the in vitro

98 conditions employed, and to explore the possibility of using a non-invasive measure of
99 oocyte development as a predictor for subsequent developmental competence. Our
100 findings confirm the size dependence of human oocyte maturation in vitro, however, they
101 have also highlighted unexpected and interesting growth patterns of maturing oocytes that
102 are novel and of potential importance in the clinical setting.

103 Methods

104 The methods of collection and culture of the human oocytes used in this study have been
105 previously described in detail, as have the maturation, fertilization and embryo
106 development results (Cavilla *et al.*, 2001). This manuscript presents additional results
107 obtained on the same source material using image analysis as a non-invasive means of
108 measuring oocyte parameters. The project was approved by Coventry Research Ethics
109 Committee and the Human Fertilisation and Embryology Authority. Briefly, immature
110 oocytes were collected from two sources: (1) 17 women (mean age 28.1 years, range 22-
111 35) with polycystic ovaries undergoing laparoscopic surgery for tubal patency assessment
112 and/or laser drilling of ovaries. These women donated 128 immature oocytes. (2) 28
113 women (mean age 32.4 years, range 27-40) receiving ovarian stimulation with
114 intracytoplasmic sperm injection (ICSI) treatment for infertility, who donated 72
115 immature oocytes. Oocytes from these two sources had distinctly different origins.
116 Those from PCO patients had been exposed to a prolonged abnormal endocrine and
117 intrafollicular environment, while those remaining immature in ICSI patients had done so
118 despite an ovulatory stimulus.

119 Immature oocytes (both GV and GVBD) were randomly allocated to culture with or
120 without meiosis activating sterol derived from human follicular fluid (FF-MAS: 0, 10 or
121 30µg/ml). Oocytes were checked for maturity at 16, 24, 40 and 48 hours. Those
122 observed to have a polar body were injected promptly with a sperm from a fertile donor.
123 Fertilization and embryo development were monitored.

124

125 Oocytes were considered to have reached metaphase II and therefore 'mature' if they
126 extruded a polar body. All oocytes lacking a polar body were considered immature (GV
127 and GVBD oocytes). Oocytes remaining immature after 48 hrs were considered

128 incompetent for maturation. Atretic oocytes were characterized by a dark appearance and
129 clearly shrunken or irregular ooplasmic outline.

130

131 A further group of 20 oocytes, that were mature at the time of their collection from ICSI
132 patients (in vivo matured), had ooplasmic diameter measured once only after cumulus
133 removal and before ICSI on the day of collection, for comparison with the IVM oocytes.

134

135 Light microscopic images of individual oocytes and embryos were collected daily using a
136 computerized image analysis system (Image pro-plus, Media Cybernetics) linked via a
137 video camera to an inverted microscope (Nikon) with Hoffman contrast optics. Images
138 were analysed to assess whether any measured parameter related to the culture conditions
139 employed or the subsequent development of the oocyte/embryo. The image analysis
140 package was used to measure the following parameters:

141

142 **Oocyte diameter:** calculated by measuring the mean length of diameters to the oolemma
143 at two-degree intervals passing through the oocyte's centroid. Control experiments,
144 measuring 10 oocytes 10 times each, established the variability of such measurements as
145 <1% (data not shown).

146

147 **Oocyte+zona diameter:** calculated as for oocyte diameter, but measured to the outer
148 circumference of the zona pellucida. It therefore included both the oocyte and its zona
149 pellucida, and incorporated differences in perivitelline space and zona thickness.

150

151 **Zona pellucida thickness:** calculated by averaging measurements of the zona thickness
152 at 2 μ m intervals around its circumference.

153

154 The perivitelline space (PVS) was also measured separately, but tended to vary according
155 to orientation. There were no significant findings in respect of this parameter (data not
156 shown).

157

158 **Statistics**

159 The measurements for each oocyte over the assessment period were analysed according to
160 the treatment that the oocyte received and the outcome of attempted maturation and
161 fertilization in vitro. Average and threshold values at collection and after IVM culture
162 were identified for various features of oocyte development.

163 For PCO oocytes, diameters were compared for those with dense cumulus at collection
164 (where measurable), versus those with less or no cumulus cover, using a contingency
165 table with χ^2 test. A one-tailed t-test was performed on oocyte diameters on day of
166 collection from the two patient groups.

167

168 Within both patient groups the following tests were performed: oocyte diameters on day 0
169 were compared according to the outcome of in vitro culture (mature, immature, atretic)
170 and tested for statistical significance using the Kruskal-Wallis test (Campbell, 1989). For
171 each patient group, parameters were compared between day of collection and day 0 of
172 oocytes that became atretic, within each culture condition using the Mann-Whitney U-test
173 (Campbell, 1989). Oocyte growth during culture, for those oocytes that matured, was
174 tested for statistical significance using the non-parametric sign test (Campbell, 1989),
175 according to the culture conditions. In Figure 3, data were 'normalised' to day 0 as the
176 day of insemination of mature oocytes. Thus, for the 18 oocytes that matured within 24

177 hours, day 0 was analysed as 1 day after collection, whereas for all other oocytes, day 0 is
178 2 days after collection.

179

180 Only for ICSI oocytes , non-parametric statistical analyses (Mann Whitney U tests) were
181 applied to detect any significant difference in oocyte diameter between oocytes that
182 matured within 24 hr and those that matured within 48 hr. This was performed for oocytes
183 within each culture group, and using pooled data (all culture groups combined) using
184 Kruskal Wallis test.

185 **Results**

186

187 The 20 in vivo matured oocytes from ICSI patients had a mean ooplasmic diameter of
188 116 μ m, ranging from 112-119 μ m.

189

190 A total of 128 oocytes were collected from PCO patients. On the day of collection, 86
191 (67%) of these oocytes could be measured while 42 could not, due mostly to dense
192 cumulus cells obscuring the oolemma.. In some cases, by enhancing the image contrast
193 and converting to grey scale it was possible to measure the oolemma through the attached
194 cumulus cells.

195

196 A total of 72 oocytes were donated by patients undergoing ICSI treatment, 48 oocyte
197 diameters were measured at collection and 24 were not. Eight oocytes were not measured
198 on either the day of collection or day 0 due to camera failure while the others were
199 omitted because of faint oolemmas and/or adherent cumulus cells. The numbers of
200 successful measurements increased between collection and day 0 as a result of improved
201 visibility due to cumulus expansion in vitro and the use of hyaluronidase to remove
202 cumulus cells in preparation for ICSI during the experiment.

203

204 Figures 1a and b present the mean diameter at collection and after culture of viable
205 oocytes collected from PCO patients or ICSI patients respectively. For PCO patients,
206 these results approximated a normal distribution with a mean and mode of 106-108 μ m at
207 the time of collection; whereas the distribution for oocytes from ICSI patients was
208 positively skewed with a mode of 109-111 μ m. The immature oocytes from ICSI patients
209 were significantly larger at collection than those from PCO patients ($p < 0.001$), and they

210 grew in culture, achieving a mode of 112-114 μ m in both mature oocytes and those that
211 remained immature (Figure 1b). In contrast, those from PCO patients showed minimal
212 evidence of growth in vitro as a cohort (Figure 1a), however, as shown in Figure 3,
213 individual oocytes either grew or shrank during culture. For oocytes from PCO patients,
214 the chances of atresia during culture reduced with increasing diameter on day 0 (Figure
215 2a).

216

217 At the time of collection, immature oocytes from both PCO and ICSI patients were
218 usually smaller than those that had undergone maturation in vivo, however, there was
219 some overlap with the largest immature oocytes and the smallest of the mature oocytes.
220 After culture, some ICSI derived immature oocytes had grown (see Figure 1B) to more
221 nearly approximate the size range of oocytes that were mature at collection (mean 116 μ m
222 range 112-119 μ m).

223

224 With the exception of one PCO oocyte (81 μ m), all oocytes that underwent GVBD in
225 culture had diameters on day 0 of at least 102 μ m. The threshold diameter for IVM to MII
226 in this study was 100 μ m at collection and 103 μ m on day 0. However, most oocytes that
227 matured (82% in PCO group and 100% in ICSI group) had diameters >106 μ m on day 0.
228 There was no relationship between mean oocyte diameter and the likelihood of
229 maturation in the oocytes from ICSI patients, in contrast to those from PCO patients
230 (Figure 2). The low number of small oocytes from ICSI patients precludes any comment
231 on a threshold size for maturation in oocytes from this source.

232

233 Data from the PCO group (Table I) shows that atresia was more likely when cumulus
234 cells were absent, however, maturation of surviving oocytes did not relate to cumulus

235 levels at collection. There was no relationship between cumulus cover and oocyte
236 diameter at collection or growth in vitro (data not shown). This analysis was not
237 performed for the ICSI group because cumulus cells had already been removed.

238

239 Table II shows the diameters of IVM oocytes in relation to fertilisation and cleavage. The
240 same fertile sperm donor was used throughout. The apparent difference in oocyte
241 diameter in the PCO group according to whether or not fertilization occurred was not
242 significant.

243

244 Figure 3 shows oocyte diameters during culture with and without FF-MAS. The
245 diameters of individual oocytes were plotted according to the culture conditions (0, 10, 30
246 $\mu\text{g/ml}$ FF-MAS) and oocyte outcome. In all groups, oocytes that became atretic tended to
247 shrink, while those maturing tended to enlarge, except in the PCO control group. In FF-
248 MAS (10 and 30 $\mu\text{g/ml}$) the mean diameters of mature, immature and atretic oocytes on
249 day 0 were significantly different ($p < 0.05$) despite their diameters at collection being
250 similar (Figs 3b and 3c). Interestingly, this difference did not occur in PCO oocytes
251 cultured in control conditions (Fig 3a) and was not significant in those collected from
252 ICSI cycles (Figs 3d-f).

253

254 In the ICSI group, 50% of oocytes maturing in vitro had done so by 24 hr, compared to
255 $< 5\%$ of PCO derived oocytes (Cavilla et al, 2001). There was no significant difference in
256 oocyte diameter on day 0 between those maturing in 24 hr and those in 48 hr, within each
257 culture group (control, 10 $\mu\text{g/ml}$ FF-MAS and 30 $\mu\text{g/ml}$ FF-MAS) or when pooling all
258 the culture groups (24 hr, median 113 μm , interquartile range 110-113.75, vs 48 hr,
259 median 112 μm , interquartile range 108.5-114.5).

260

261 Figure 4 shows the IVM oocytes fertilizing and cleaving according to oocyte diameter for
262 the ICSI group. For oocytes that matured within 24hr of culture, 2/6 (33%) of the
263 fertilized oocytes subsequently cleaved. However, of oocytes that matured within 48hr,
264 5/7 (71%) fertilized oocytes subsequently cleaved. While this may provide some
265 suggestion that prolonged maturation could be associated with improved cleavage
266 potential, the numbers of embryos were too few for meaningful analysis.

267

268 **Oocyte + zona diameter**

269 The measurements of 'oocyte+zona' were positively skewed for PCO oocytes, and
270 approximately normal for ICSI derived oocytes (Figure 5), in contrast to the data for
271 oocyte diameter (Figures 1 and 2). The majority (79%) of viable PCO oocytes had mean
272 diameters (including zona) in the 146-163 μ m range at the time of collection, which
273 showed minimal change after 2 days of culture (Figure 5). As observed for oocyte
274 diameter, oocytes with larger measurements of 'oocyte + zona' in the PCO group
275 appeared more likely to mature in vitro (Figure 6a) however, this was not a significant
276 difference. The diameter of the oocyte/zona complex did not change in culture for oocytes
277 derived from ICSI patients, despite the extensive enlargement of ooplasm that occurred
278 over the same period (Fig 1b vs Fig 5b), and was not associated with maturation in vitro
279 (Fig 6b). There was no significant relationship between oocyte+zona measurements and
280 maturation, fertilisation or cleavage in vitro (data not shown).

281

282 **Zona pellucida thickness**

283 Frequency distributions were plotted of the mean zona thickness of viable oocytes from
284 both patient groups on the day of collection and for oocytes that did or did not mature in

285 vitro. There were no significant differences in zona thickness between the two groups,
286 and no relationship between zona thickness and FF-MAS (data not shown).

287

288 The zona thicknesses on day 1 and day 2 were compared in matured oocytes that did or
289 did not fertilise after ICSI. PCO oocytes that fertilized had significantly thicker zona
290 pellucidas on day 1 than those that did not (21.8 ± 1.9 vs $16.9 \pm 2.7 \mu\text{m}$, $p < 0.05$). No
291 significant differences were observed on day 1 or day 2 for in vitro matured oocytes from
292 ICSI patients (fertilized 20.5 ± 0.8 vs $20.3 \pm 0.6 \mu\text{m}$ unfertilized). The results on day 2 were
293 20.1 ± 1.9 (fertilized) vs 17.2 ± 3.4 (unfertilized); and 21.2 ± 0.9 (fertilized) vs $19.3 \pm 0.5 \mu\text{m}$
294 (unfertilized) for the PCO and ICSI groups respectively.

295 **Discussion**

296

297 Oocyte development in preparation for ovulation includes both increasing size (growth)
298 and maturation of oocyte constituents (ooplasm and genetic material). This report shows
299 that measurable growth of human oocytes may continue during the final hours of oocyte
300 development in vitro and may relate to the eventual outcome of maturation and
301 insemination. This is potentially important because incomplete growth has been linked to
302 reduced developmental capacity (Moor *et al.*, 1998). Moreover, imprinting of certain
303 genes occurs late in the growth phase in mouse oocytes (Lucifero *et al.*, 2004) and
304 imprinting may be disturbed by in vitro conditions in mice (Kerjean *et al.*, 2003). The
305 possibility of incomplete imprinting may therefore be relevant to the safety and clinical
306 outcome of IVM and insemination of oocytes that have not yet achieved their full size.

307

308 *Oocyte growth*

309 During its growth phase, the human oocyte increases in diameter from ~30 μ m to
310 >110 μ m, over a period of at least 8 weeks (Gougeon, 1986). During this time, its nucleus
311 remains arrested in first meiotic prophase. The diameter of the in vivo matured human
312 oocyte, excluding the zona pellucida, is normally approximately 110-120 μ m (which we
313 confirm here) while the zona pellucida is normally approximately 15-20 μ m thick (Veeck,
314 1999). Including the zona pellucida and perivitelline space, the pre-ovulatory oocyte
315 commonly has a diameter around 150 μ m (Veeck, 1999).

316

317 Measurements of oocyte diameter of immature oocytes at collection and after IVM
318 culture confirmed the size dependence of maturation, as has been extensively documented
319 in other species. However, it also resulted in unexpected observations of the relatively

320 small size of immature oocytes relative to those matured in vivo, as well as evidence of
321 growth of immature oocytes in vitro. An increase of 3 μm average diameter from 106 to
322 109 μm (Fig 1b) would result in $\sim 54461\mu\text{m}^3$ increase in cytoplasmic volume, constituting
323 an astonishing 8% increase in volume over two days. Hence, a relatively small change in
324 diameter that could easily pass unnoticed during routine clinical procedures is associated
325 with a relatively large change in volume. It therefore seems likely to us that growth of
326 human oocytes in vitro has been underestimated and may provide worthwhile information
327 about oocyte potential. Oocyte growth in vitro differed between the patient groups
328 studied, suggesting that endocrine or other patient factors may contribute to its control.
329 Further study is clearly indicated.

330

331 The oocytes we observed from patients undergoing ICSI achieved growth in the total
332 absence of somatic cellular support. To our knowledge, this is a novel observation.
333 Others have documented that oocyte growth in fetal ovary cultures does not depend
334 exclusively upon intimate follicular cell communication (McLaren and Buehr, 1990;
335 Zhang *et al.*, 1995), however, somatic cells were present in large numbers in these
336 systems. The nature of the oocyte growth observed in our cultures has not been
337 established, however, variables in the medium are not thought to be the cause since
338 oocytes from PCO patients were cultured under identical conditions and did not show the
339 same extent of growth. Control experiments demonstrated that the osmolarity of cultures
340 maintained in a humidified incubator (37°C, 5% CO₂ in air) varied by <1% after 24 hr.
341 Moreover, both increases and decreases in oocyte diameter were observed in the same
342 culture preparations, discounting alterations in media osmolarity as the mechanism by
343 which oocyte size changes occurred.

344

345 In this study, oocytes from patients with PCO were retrieved laparoscopically from antral
346 follicles ~10mm diameter or less, whilst oocytes donated by patients undergoing ICSI
347 were retrieved transvaginally from larger follicles >10mm diameter. Other important
348 differences exist between the groups. The endocrine environments in PCO patients and
349 those receiving ovarian stimulation in preparation for ICSI are distinctly different.
350 Moreover, oocytes that remain immature despite an ovulatory stimulus may be defective
351 and harbour cytogenetic abnormalities, even if maturation occurs (Magli et al, 2006).
352 Immature oocytes exposed to an ovulatory stimulus are known to undergo IVM more
353 quickly than those without a stimulus (Chian et al, 2000), as has been documented as a
354 difference between the patient groups in this study (Cavilla et al, 2001). Dubey *et al.*
355 (1995) suggested that competence in human oocytes may normally be conferred relatively
356 late, perhaps only when follicles have reached diameters of >10mm, although occasional
357 pregnancies have resulted from IVM of oocytes from smaller follicles (Trounson *et al.*,
358 1994). Oocytes retrieved from ICSI patients were significantly larger at collection than
359 those retrieved from PCO patients (mean diameter 111µm vs 106µm), which may have
360 been partially due to the larger size of follicles in patients undergoing ICSI.

361

362 Based upon data from unstimulated gynaecology patients, Durinzi *et al.*, (1995) deduced
363 that an oocyte diameter of 105µm at the time of collection was the threshold for GVBD,
364 while oocytes of >115µm would mature to MII. Our data for oocytes retrieved from
365 patients with PCO produced lower thresholds for GVBD (81µm) and MII (103µm), and
366 most of the oocytes reaching MII in our study had a diameter <115µm.

367

368 *Effect of FF-MAS on oocyte growth*

369 Mature, immature and atretic oocytes cultured with FF-MAS (10 or 30 μ g/ml), but not
370 those in control conditions, had significantly different diameters on day 0 ($p < 0.05$) in the
371 PCO group. For oocytes from ICSI patients, the differences in diameter between mature,
372 immature and atretic oocytes on day 0 were not significant. Interestingly, in the oocytes
373 from ICSI patients, there was significant growth between collection and day 0. Growth
374 was greater in oocytes that became mature than in those that remained immature.
375 Oocytes becoming atretic tended to shrink. The observation of large oocytes from ICSI
376 patients undergoing atresia upon exposure to FF-MAS is intriguing. This could perhaps
377 reflect either an adverse effect of FF-MAS on fully grown oocytes, or that large immature
378 oocytes have a reduced quality and developmental potential. However, the result was
379 non-significant.

380

381 The mechanism of action of FF-MAS is not yet known, and its potential as an adjunct to
382 oocyte and embryo cultures is controversial (Downs *et al.*, 2001; Vaknin *et al.*, 2001;
383 Tsafiriri *et al.*, 2002, 2005; Bergh *et al.*, 2004; Loft *et al.*, 2004; Marín Bivens *et al.*,
384 2004). One possibility arising from our data is that FF-MAS may influence oocyte
385 growth. FF-MAS is a steroid related to lanosterol and cholesterol (Byskov *et al.*, 1995,
386 2002). Cholesterol is known to influence membrane fluidity and the function of
387 membrane proteins (McIntosh and Simon, 2006) and relative levels of cholesterol and
388 MAS change in follicular fluid during maturation (Bokal *et al.*, 2006). While no direct
389 effects of FF-MAS upon membrane fluidity have been reported, oocyte growth from
390 diameters of 106 to 109 μ m, as exemplified above, would result in an associated increased
391 surface area of 2026 μ m² (5.4%) (assuming the oocyte to be spherical – in fact, if the
392 number of microvilli also increased, the overall surface area could increase more), so
393 membrane elasticity and/or synthetic capacity may be a crucial factor for oocyte growth

394 and subsequent embryo cleavage. We therefore hypothesise that FF-MAS may be
395 involved in membrane biochemistry, in addition to any role in local communication.
396 There is some evidence in amphibians to support membrane fluidity having a role in
397 meiotic arrest, controlled by progesterone and cAMP, so this idea warrants further study
398 (Morrill *et al.*, 1989; 1993). An alternative perspective, if our hypothesis is correct, is
399 that the ooplasm could become less rigid and oocytes more likely to flatten slightly under
400 their own weight. This could explain the increased diameters of a focal plane observed
401 through the oocyte's centre. Three dimensional imaging will be required to test this idea.

402

403 As oocytes from both our patient groups have grown in vitro, it is clear that either the
404 growth phase of these immature oocytes has not been completed in vivo, or that it may be
405 resumed under certain conditions. IVM oocytes are smaller than their in vivo counterparts
406 in mice, however, 87% were capable of emitting a polar body and undergoing normal
407 nuclear maturation (Sun *et al.*, 2005). In 1998, Moor *et al.* suggested that the reduced
408 developmental potential observed in human oocytes matured in vitro might be attributable
409 to incomplete oocyte growth, however, no data were presented on human oocytes to
410 illustrate the point. In the present study, our data provide evidence that in vivo matured
411 oocytes from ICSI patients are larger than immature oocytes, showing that the immature
412 oocytes were not fully grown at collection. Moreover, the prospect that crucial events
413 such as genetic imprinting may be incomplete in such oocytes (Lucifero *et al.*, 2004;
414 Borghol *et al.*, 2006) should promote re-evaluation of IVM protocols to avoid the
415 collection of growing oocytes, or to accommodate their need for further growth.

416

417 *Zona pellucida*

418 The zona pellucida, synthesized by the oocyte, is crucial to fertilization and early
419 development. According to Bertrand *et al.* (1995), human zona thickness varies from 10-
420 31 μm , with a mean of 17.5 μm . In the present study, on day 0 all mature oocytes had a
421 zona thickness of 15-24 μm . This was within the expected range and was unrelated to
422 maturity.

423

424 The oocyte + zona measurements at collection for ICSI patients relative to the PCO group
425 is consistent with their larger oocyte diameter at collection. The oocyte + zona
426 measurement did not offer any additional information over that of oocyte diameter, and
427 may reduce the discriminatory potential of oolemma measurements.

428

429 Various studies of zona pellucida thickness, or thickness variation, as an indicator of
430 oocyte function have resulted in conflicting results (Bertrand *et al.*, 1995, 1996; Garside
431 *et al.*, 1997; Gabrielsen *et al.*, 2001; Pelletier *et al.*, 2004; Shiloh *et al.*, 2004; Shen *et al.*,
432 2005; Sun *et al.*, 2005; Kilani *et al.*, 2006). Both thickening and thinning of the zona have
433 been reported in cultured embryos, however, our study has not identified changes in zona
434 thickness with time, nor was zona pellucida thickness a useful measure related to oocyte
435 maturation.

436

437 The zona thickness measurements obtained for fertilized oocytes matured in vitro in this
438 study were larger than measurements of in vivo matured oocytes obtained by others using
439 differential interference optics (eg day 1, $16.4 \pm 3.1\mu\text{m}$, Bertrand *et al.*, 1996; $17.7 \pm$
440 $0.14\mu\text{m}$, Garside *et al.*, 1997) or computer assisted methods (eg, day 1, 19.9 ± 1.92 in
441 conception cycles and $18.6 \pm 1.8\mu\text{m}$ in non-conception cycles, Shen *et al.*, 2005). This

442 could indicate an effect of culture or differences in the source of oocytes and their
443 developmental potential.

444

445 *Conclusion*

446 In conclusion, we have extended previous observations on human oocyte maturation in
447 relation to the oocyte's dimensions and origins. Moreover, we have provided the first
448 quantitative non-invasive analysis of oocyte growth during maturation in vitro,
449 highlighting differences from in vivo matured oocytes and demonstrating effects of FF-
450 MAS upon oocyte growth. This work has raised prospects for a non-invasive assessment
451 of oocyte growth in vitro as well as indicating the risks inherent in using oocytes that are
452 not fully grown for clinical application.

453

454 **Acknowledgements**

455 All staff at the Centre for Reproductive Medicine, University Hospitals Coventry and
456 Warwickshire NHS Trust, are warmly thanked for their support.

457 **References**

458

- 459 Bao S, Obata Y, Carroll J, Domeki I and Kono T (2000). Epigenetic modifications
460 necessary for normal development are established during oocyte growth in mice.
461 Biol Reprod 62, 616-621.
- 462 Bergh C, Loft A, Lundin K, Ziebe S, Nilsson L, Wikland M, Grondahl C, Arce JC:
463 CEMAS II Study Group (2004) Chromosomal abnormality rate in human pre-
464 embryos derived from in vitro fertilization cycles cultured in the presence of
465 Follicular Fluid Meiosis Activating Sterol (FF-MAS). Hum Reprod 19, 2109-2117
- 466 Bertrand E, Van den Bergh M and Englert Y (1995) Does zona pellucida thickness
467 influence the fertilization rate? Hum Reprod 10, 1189-1193.
- 468 Bertrand E, Van den Bergh M and Englert Y (1996) Clinical parameters influencing
469 human zona pellucida thickness Fertil. Steril 66, 408-411.
- 470 Bokal EV, Tacer KF, Vrbnjak M, Leposa S, Klun IV, Verdenik I and Rozman D (2006)
471 Follicular sterol composition in gonadotrophin stimulated women with polycystic
472 ovarian syndrome. Mol Cell Endocrinol 249, 92-98
- 473 Borghol N, Lornage J, Blachere T, Sophie Garret A, Lefevre A (2006) Epigenetic status
474 of the H19 locus in human oocytes following in vitro maturation. Genomics 87:
475 417-426.
- 476 Byskov AG, Andersen CY, Nordholm L, Thogersen H, Guoliang X, Wassman O et al.
477 (1995) Chemical structure of sterols that activate oocyte meiosis. Nature 374, 559-
478 562
- 479 Byskov AG, Andersen CY and Leonardsen L (2002) Role of meiosis activating sterols,
480 MAS, in induced oocyte maturation. Mol Cell Endocrinol 187, 189-196.
- 481 Campbell RC (1989) Statistics for Biologists. 3rd edition. Cambridge, UK: Cambridge
482 University Press.
- 483 Canipari R, Palombi F, Riminucci M and Mangia F (1984) Early programming of
484 maturation competence in mouse oogenesis. Dev Biol 102, 519-524.
- 485 Cavilla JL, Kennedy CR, Baltzen M, Klentzeris LD, Byskov AG and Hartshorne GM
486 (2001) The effects of meiosis activating sterol on in vitro maturation and
487 fertilization of human oocytes from stimulated and unstimulated ovaries. Hum
488 Reprod 16, 547-555.

- 489 Chian RC, Buckett WM, Tulandi T, Tan SL. (2000) Prospective randomized study of
490 human chorionic gonadotrophin priming before immature oocyte retrieval from
491 unstimulated women with polycystic ovarian syndrome. *Hum Reprod* 15, 165-70
- 492 Daniel SAJ, Armstrong DT and Gore-Langton RE (1989) Growth and development of rat
493 oocytes in vitro. *Gamete Res* 24, 109-121.
- 494 Downs SM, Ruan B and Schroepfer GJJr (2001) Meiosis-activating sterol and the
495 maturation of isolated mouse oocytes. *Biol Reprod* 54, 197-207.
- 496 Dubey AK, Wang HA, Duffy P and Penzias AS (1995) The correlation between follicular
497 measurements, oocyte morphology, and fertilization rates in an in vitro fertilization
498 program. *Fertil Steril* 64, 787-790.
- 499 Durinzi KL, Saniga EM and Lanzendorf SE (1995) The relationship between size and
500 maturation in vitro in the unstimulated human oocyte. *Fertil Steril* 63, 404-406.
- 501 Eppig JJ and Schroeder AC (1989) Capacity of mouse oocytes from preantral follicles to
502 undergo embryogenesis and development to live young after growth, maturation
503 and fertilization in vitro. *Biol Reprod* 41, 268-276.
- 504 Fair T, Hyttel P and Greve T (1995) Bovine oocyte diameter in relation to maturational
505 competence and transcriptional activity. *Mol Reprod Dev* 42, 437-442.
- 506 Gabrielsen A, Lindenberg S and Petersen K (2001) The impact of the zona pellucida
507 thickness variation of human embryos on pregnancy outcome in relation to
508 suboptimal embryo development. A prospective randomized controlled study.
509 *Hum Reprod* 16, 2166-2170
- 510 Garside WT, Loret de Mola JR, Bucci JA, Tureck RW and Heyner S (1997) Sequential
511 analysis of zona thickness during in vitro culture of human zygotes: correlation with
512 embryo quality, age and implantation. *Mol Reprod Dev* 47, 99-104.
- 513 Gougeon A (1986) Dynamics of follicular growth in the human: a model from
514 preliminary results. *Hum Reprod* 2, 81-87.
- 515 Hirao Y, Miyano T and Kato S (1993) Acquisition of maturational competence in in vitro
516 grown mouse oocytes. *J Exp Zool* 267, 543-547.
- 517 Hirao Y, Nagai T, Kubo M, Miyano T, Miyake M, Kato S (1994) In vitro growth and
518 maturation of pig oocytes. *J Reprod Fertil* 100, 333-339.
- 519 Kerjean A, Couvert P, Heams T, Chalas C, Poirier K, Chelly J, Jouannet P, Paldi A and
520 Poirot C (2003) In vitro follicular growth affects oocyte imprinting establishment
521 in mice. *Eur J Hum Genet* 11, 493-496.

- 522 Kilani SS, Cooke S, Kan AK, Chapman MG (2006) Do age and extended culture affect
523 the architecture of the zona pellucida of human oocytes and embryos? *Zygote* 14,
524 39-44
- 525 Loft A, Bergh C, Ziebe S, Lundin K, Andersen AN, Wikland M, Kim H and Arce JC.
526 (2004) A randomized, double-blind, controlled trial of the effect of adding
527 follicular fluid meiosis activating sterol in an ethanol formulation to donated human
528 cumulus-enclosed oocytes before fertilization. *Fertil Steril* 81, 42-50
- 529 Lucifero D, Mann MRW, Bartolomei MS, Trasler JM (2004) Gene-specific timing and
530 epigenetic memory in oocyte imprinting. *Hum Mol Genet* 13, 839-849
- 531 Magli MC, Ferraretti AP, Crippa A, Cappi M, Feliciani E, Gianaroli L. (2006) First
532 meiosis errors in immature oocytes generated by stimulated cycles. *Fertil Steril* 86,
533 629-635
- 534 Marin Bivens CL, Lindenthal B, O'Brien MJ, Wigglesworth K, Blume T, Grøndahl C and
535 Eppig JJ (2004) A synthetic analogue of meiosis-activating sterol (FF-MAS) is a
536 potent agonist promoting meiotic maturation and preimplantation development of
537 mouse oocytes maturing in vitro. *Hum Reprod* 19, 2340-2344
- 538 McIntosh TJ and Simon SA. (2006) Roles of bilayer material properties in function and
539 distribution of membrane proteins. *Annu Rev Biophys Biomol Struct* 35, 177-98.
- 540 McLaren A and Buehr M. (1990) Development of mouse germ cells in cultures of fetal
541 gonads. *Cell Differ Dev* 31, 185-95.
- 542 Moor RM, Dai Y, Lee C and Fulka JJr (1998) Oocyte maturation and embryonic failure.
543 *Hum Reprod Update* 4, 223-236.
- 544 Morrill GA, Doi K, Erlichman J, Kostellow AB. (1993) Cyclic AMP binding to the
545 amphibian oocyte plasma membrane: possible interrelationship between meiotic
546 arrest and membrane fluidity. *Biochim Biophys Acta* 1158, 146-154
- 547 Morrill GA, Doi K and Kostellow AB (1989) Progesterone induced transient changes in
548 plasma membrane fluidity of amphibian oocytes during the first meiotic division.
549 *Arch Biochem Biophys* 269, 690-694.
- 550 Pelletier C, Keefe DL, Trimarchi JR (2004) Noninvasive polarized light microscopy
551 quantitatively distinguishes the multilaminar structure of the zona pellucida of
552 living human eggs and embryos. *Fertil Steril* 81, suppl 1, 850-856
- 553 Schramm RD, Tennier MT, Boatman DE and Bavister BD (1993) Chromatin
554 configurations and meiotic competence of oocytes are related to follicular diameter
555 in non-stimulated rhesus monkeys. *Biol Reprod* 48, 349-356.

- 556 Shen Y, Stalf T, Mehnert C, Eichenlaub-Ritter U, Tinneberg H-R (2005) High magnitude
557 of light retardation by the zona pellucida is associated with conception cycles. *Hum*
558 *Reprod* 20, 1596-1606
- 559 Shiloh H, Lahav-Baratz S, Koifman M, Ishai D, Bidder D, Weiner-Meganz Z and
560 Dirnfeld M (2004) The impact of cigarette smoking on zona pellucida thickness of
561 oocytes and embryos prior to transfer into the uterine cavity. *Hum Reprod* 19, 157-
562 159
- 563 Sun F, Betzendahl I, Shen Y, Cortvrindt R, Smitz J, Eichenlaub-Ritter U. (2004) Preantral
564 follicle culture as a novel in vitro assay in reproductive toxicology testing in
565 mammalian oocytes. *Mutagenesis* 19, 13-25
- 566 Sun YP, Xu Y, Cao T, Su YC and Guo YH (2005) Zona pellucida thickness and clinical
567 pregnancy outcome following in vitro fertilization. *Int J Gynaecol Obstet* 89, 258-
568 262
- 569 Trounson AO, Wood C and Kausche A (1994) In vitro oocyte maturation and the
570 fertilization and developmental competence of oocytes recovered from untreated
571 polycystic ovarian patients. *Fertil Steril* 62, 353-362.
- 572 Tsafiriri A, Cao XM, Vaknin KM and Popliker M (2002) Is meiosis-activating sterol
573 (MAS) an obligatory mediator of meiotic resumption in mammals. *Mol Cell*
574 *Endocrinol* 187, 197-204.
- 575 Tsafiriri A, Cao X, Ashkenazi H, Motola S, Popliker M and Pomerantz SH. (2005)
576 Resumption of oocyte meiosis in mammals: on models, meiosis activating sterols,
577 steroids and EGF-like factors. *Mol Cell Endocrinol* 234, 37-45
- 578 Vaknin KM, Lazar S, Popliker M and Tsafiriri A (2001) Role of meiosis-activating sterols
579 in rat oocyte maturation: effects of specific inhibitors and changes in the expression
580 of lanosterol 14 alpha-demethylase during the preovulatory period. *Biol Reprod* 64,
581 299-309
- 582 Veeck L (1999) Abnormal morphology of the human oocyte and conceptus. In *An Atlas*
583 *of Human Gametes and Conceptuses*. Lancs, UK: The Parthenon Publishing Group
584 Ltd.
- 585 Zhang J, Liu J, Xu KP, Liu B and DiMattina M. (1995) Extracorporeal development and
586 ultrarapid freezing of human fetal ova. *J Assist Reprod Genet* 12, 361-8.
- 587
588

589

590

591 **Table I Outcome of oocyte culture according to levels of cumulus on immature**
 592 **oocytes (n=128) at collection from patients with PCO.**

593

594

595

Cumulus grade	Oocyte after culture		
	Mature	Immature	Atretic
0 (n=65)	13 (20.0%)	23 (35.4%)	29 (44.6%)
1 (n=17)	10 (58.8%)	6 (35.3%)	1 (5.9%)
2 (n=5)	2 (40%)	3 (60%)	0
3 (n=41)	13 (31.7%)	20 (48.8%)	8 (19.5%)

596

597 **Key:** 0 = devoid of cumulus/no more than 10 scattered cells; 1 = partial cover; 2 =
 598 complete cover; 3 = substantial multilayered cover.

599 **Table II.**600 **Oocyte diameters on day of maturation according to origin of oocyte and**
601 **developmental competence in vitro.**

602

		Oocyte diameter (μm) on day 0			
		Surviving but not maturing in vitro	Maturing in vitro	2PN fertilisation by ICSI	Cleavage having fertilized with 2PN
PCO	Median	107	108	112.5	112.5
	Interquartile range	(105-108)	(106-113)	(107-116)	(108-116)
	Range	(81-140)	(103-121)	(105-121)	(105-121)
	n	32	28	10	8
ICSI	Median	115	114	113	113
	Interquartile range	(112-118)	(110-116)	(109-114)	(111-117)
	Range	103-126	(106-131)	(106-125)	(107-125)
	n	25	35	13	7

603

604 PCO = polycystic ovaries. These patients underwent laparoscopic retrieval of oocytes
605 without ovarian stimulation.606 ICSI = intracytoplasmic sperm injection. These patients underwent transvaginal oocyte
607 collection after ovarian stimulation for a clinical cycle of ICSI as a treatment for
608 infertility.

609

610

611

612

613

614 **FIGURE LEGENDS**

615

616 **Figure 1**

617 **Frequency histograms of mean oocyte diameter at the time of oocyte collection and**
618 **after IVM culture.**

619 Only oocytes viable at the time of collection were measured.

620 A: Oocytes from unstimulated PCO patients (86 measurements at collection, 90 after
621 culture)

622 B: Oocytes from stimulated ICSI patients (48 measurements at collection, 61 after
623 culture).

624 Notice that ICSI patient-derived oocytes have grown during the culture while those from
625 PCO patients have not.

626 Similar results were obtained when only those oocytes having measurements available
627 both at collection and after culture were plotted.

628

629 **Figure 2**

630 **Frequency histograms of mean oocyte diameter after culture for oocytes that either**
631 **matured in vitro, remained immature or became atretic in culture.**

632 A: Oocytes from unstimulated PCO patients

633 B: Oocytes from stimulated ICSI patients.

634

635 **Figure 3**

636 **Oocyte diameters during culture in control conditions or with FF-MAS for oocytes**
637 **from PCO patients or patients undergoing ICSI treatment. Results are presented**
638 **according to the outcome of in vitro maturation culture.**

639 Oocytes in a-c were collected from unstimulated PCO patients with and those in d-f were
640 collected from patients undergoing ICSI treatment.

641 The control, 10 μ g/ml FF-MAS and 30 μ g/ml FF-MAS results are shown in the top,
642 middle and bottom panels respectively. Mean \pm SEM.

643 Points with similar symbols are significantly different ($p < 0.05$).

644

645 **Figure 4**

646 **Numbers of oocytes donated by patients undergoing ICSI treatment, that fertilized**
647 **and cleaved after maturation in vitro, according to oocyte diameter**

648 a) Oocytes that matured within 24 hr

649 b) Oocytes that matured within 48 hr

650

651 **Figure 5**

652 **Frequency histograms of mean oocyte+zona diameter at the time of oocyte collection**
653 **and after IVM culture.**

654 Only oocytes viable at the time of collection were measured.

655 A: Oocytes from unstimulated PCO patients (48 measurements at collection and after
656 culture)

657 B: Oocytes from stimulated ICSI patients (46 measurements at collection and 60 after
658 culture).

659 Similar results were obtained when only those oocytes having measurements available
660 both at collection and after culture were plotted.

661

662 **Figure 6**

663 **Frequency histograms of mean oocyte+zona diameter after culture for oocytes that**
664 **either matured in vitro, remained immature or became atretic in culture.**

665 A: Oocytes from unstimulated PCO patients

666 B: Oocytes from stimulated ICSI patients.

667

668