Human immature oocytes grow during culture for IVM

J.L. Cavilla1,4, C.R. Kennedy1, A.G. Byskov2 and G.M. Hartshorne1,3,5

1Centre for Reproductive Medicine, University Hospitals Coventry and Warwickshire NHS Trust, Coventry, CV2 2DX, UK; 2Laboratory of Reproductive Biology, Juliane Marie Centre, Section 5712, Rigshospitalet, Blegdamsvej 9, DK-2100 Copenhagen, Denmark; 3Clinical Sciences Research Institute, Warwick Medical School, University of Warwick, Clifford Bridge Road, Coventry, CV2 2DX, UK; 4Present address: Assisted Conception Unit, Kings College Hospital, Denmark Hill, London, SE5 9RS, UK
5Correspondence address. Tel: +44-2476-968697/528382; Fax: +44-2476-968653; E-mail: geraldine.hartshorne@warwick.ac.uk

BACKGROUND: Oocyte competence for maturation and embryogenesis is associated with diameter in many mammals. We aimed to test whether this relationship exists in humans and to quantify its impact upon in vitro maturation (IVM). METHODS: We used computer-assisted image analysis daily to measure average diameter, zona thickness and other parameters in oocytes. Immature oocytes originated from unstimulated patients with polycystic ovaries, and from stimulated patients undergoing intracytoplasmic sperm injection (ICSI). Some were cultured with meiosis activating sterol (FF-MAS). Matured oocytes were inseminated using ICSI and embryo development was monitored. In vivo matured oocytes were also measured. RESULTS: Immature oocytes were smaller at collection than in vivo matured oocytes. Maturation was related to oocyte diameter and many oocytes grew in culture. FF-MAS stimulated growth in oocytes derived from ICSI patients, but only stimulated growth in PCO derived oocytes if they matured in vitro. Degenerating oocytes showed cytoplasmic shrinkage. Neither zona thickness, perivitelline space, nor the total diameter of the oocyte plus zona were informative regarding maturation capacity. CONCLUSIONS: Immature oocytes grow during maturation culture. FF-MAS promotes oocyte growth in vitro. Oocytes from different sources have different growth profiles in vitro. Measuring oocytes in clinical IVM may provide additional non-invasive information that could potentially avoid the use of growing oocytes.

Keywords: diameter; growth; human; IVM; oocyte

Introduction

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

There is a substantial body of research on oocyte diameter and maturation in animals. Eppig and Schroeder (1989) introduced the concept that competence to develop through successive stages of meiosis and early embryogenesis in mice is dependent upon age and oocyte size. They showed that isolated oocytes from mice <13 days of age, having mean diameters >60 μm, were able to undergo spontaneous breakdown of the germinal vesicle (GVBD) in culture, but larger oocytes from mice ≥15 days of age were more likely to mature completely to metaphase II (MII) in culture. Hirao et al. (1993) confirmed that the threshold diameter of 60 μm for GVBD remained the same even when mouse oocytes were grown in vitro. Similar evidence of maturation competence relating to oocyte growth was obtained in rats by Daniel et al. (1989) and in pigs by Hirao et al. (1994), where the threshold diameters for GVBD were 55 μm and 90 μm, respectively. Continuing transcription in small bovine oocytes indicates that their growth is not complete (Fair et al., 1995) and hence, their complement of maternally derived mRNA, necessary for early embryonic growth, might also be incomplete, providing a possible mechanism for these observations. However, Canipari et al. (1984) observed mouse oocytes that became GVBD competent after being cultured in conditions that did not promote significant growth, suggesting that the events of meiotic resumption and oocyte growth may be separable when non-physiological conditions are applied in vitro.

The capacity to cleave after maturation and insemination in vitro is also acquired with increasing age and oocyte diameter. Bao et al. (2000) showed that the developmental competence of mouse oocytes progresses in a stepwise manner as oocyte diameter increases from 65 to 75 μm and that developmental changes occurring during the final stages of oocyte growth are critical for full developmental competence.
In rhesus monkey oocytes, meiotic competence occurs late during oocyte development, however, oocyte diameter appeared relatively constant as competence for GVBD arose, suggesting no close relationship with oocyte diameter (Schramm et al., 1993). Durini et al. (1995) examined the relationship between oocyte size and maturation in vitro in unstimulated human oocytes from women aged 25–39 years undergoing gynaecological operations not associated with ovarian pathology. They observed a significant difference in maturation capability of oocytes measuring 86–105 μm at collection versus those measuring 106–125 μm, leading to the conclusion that, in common with other species, the unstimulated human oocyte has a size-dependent ability to resume meiosis and complete maturation.

During a study of human IVM, fertilization and embryo development (Cavilla et al., 2001), we captured computerized micrographic images over culture periods of up to 6 days. This afforded the opportunity to quantify human oocyte growth under the in vitro conditions employed, and to explore the possibility of using a non-invasive measure of oocyte development as a predictor for subsequent developmental competence. Our findings confirm the size dependence of human oocyte maturation in vitro; however, they have also highlighted unexplored and interesting growth patterns of maturing oocytes that are novel and of potential importance in the clinical setting.

### Materials and Methods

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

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

Oocytes were considered to have reached metaphase II and therefore ‘mature’ if they extruded a polar body. All oocytes lacking a polar body were considered immature (GV and GVBD oocytes). Oocytes remaining immature after 48 h were considered incompetent for maturation. Atretic oocytes were characterized by a dark appearance and clearly shrunken or irregular ooplasmic outline.

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

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

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

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

- **Zona pellucida thickness**: calculated by averaging measurements of the zona thickness at 2 μm intervals around its circumference. The PVS was also measured separately, but tended to vary according to orientation. There were no significant findings in respect of this parameter (data not shown).

### Statistics

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

For PCO oocytes, diameters were compared for those with dense cumulus at collection (where measurable), versus those with less or no cumulus cover, using χ² contingency table with χ² test. A one-tailed t-test was performed on oocyte diameters on day of collection from the two patient groups.

Within both patient groups, the following tests were performed: oocyte diameters on day 0 were compared according to the outcome of in vitro culture (mature, immature and atretic) and tested for statistical significance using the Kruskall–Wallis test (Campbell, 1989). For each patient group, parameters were compared between day of collection and day 0 of oocytes that became atretic, within each culture condition using the Mann–Whitney U-test (Campbell, 1989). Oocyte growth during culture, for those oocytes that matured, was tested for statistical significance using the non-parametric sign test (Campbell, 1989), according to the culture conditions. In Fig. 1, data were ‘normalized’ to day 0 as the day of insemination of mature oocytes. Thus, for the 18 oocytes that matured within 24 h, day 0 was analysed as 1 day after collection, whereas for all other oocytes, day 0 is 2 days after collection. In other words, for mature oocytes, day 0 is the same as the day of collection. For immature oocytes that matured within 24 h, day 0 is one day after collection. For immature oocytes that did not mature within 24 h, day 0 is 2 days after collection.

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

### Results

The 20 in vivo matured oocytes from ICSI patients had a mean ooplasmic diameter of 116 μm, ranging from 112–119 μm.
A total of 128 oocytes were collected from PCO patients. On the day of collection, 86 (67%) of these oocytes could be measured whereas 42 could not, due mostly to dense cumulus cells obscuring the oolemma. In some cases, by enhancing the image contrast and converting to grey scale, it was possible to measure the oolemma through the attached cumulus cells.

A total of 72 oocytes were donated by patients undergoing ICSI treatment, 48 oocyte diameters were measured at collection and 24 were not. Eight oocytes were not measured on either the day of collection or day 0 due to camera failure whereas those from PCO patients have not. Similar results were obtained when only those oocytes having measurements available both at collection and after culture were plotted.

Figure 1: Frequency histograms of mean oocyte diameter at the time of oocyte collection and after IVM culture
Only oocytes viable at the time of collection were measured. (a) Oocytes from unstimulated PCO patients (86 measurements at collection, 90 after culture). (b) Oocytes from stimulated ICSI patients (48 measurements at collection, 61 after culture). Notice that ICSI patient-derived oocytes have grown during the culture whereas those from PCO patients have not. Similar results were obtained when only those oocytes having measurements available both at collection and after culture were plotted.

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

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

Figure 1a and b present the mean diameter at collection and after culture of viable oocytes collected from PCO patients or ICSI patients, respectively. For PCO patients, these results approximated a normal distribution with a mean and mode of 106–108 μm at the time of collection, whereas the distribution for oocytes from ICSI patients was positively skewed with a mode of 109–111 μm. The immature oocytes from ICSI patients were significantly larger at collection than those from PCO patients (P < 0.001), and they grew in culture, achieving a mode of 112–114 μm in both mature oocytes and those that remained immature (Fig. 1b). In contrast, those from PCO patients showed minimal evidence of growth in vitro as a cohort (Fig. 1a), however, as shown in Fig. 3, individual oocytes either grew or shrank during culture. For oocytes from PCO patients, the chances of atresia during culture were reduced with increasing diameter on day 0 (Fig. 2a).

At the time of collection, immature oocytes from both PCO and ICSI patients were usually smaller than those that had undergone maturation in vivo, however, there was some overlap with the largest immature oocytes and the smallest of the mature oocytes. After culture, some ICSI derived immature oocytes had grown (Fig. 1b) to more nearly approximate the size range of oocytes that were mature at collection (mean 116 μm; range 112–119 μm).

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

Data from the PCO group (Table I) shows that atresia was more likely when cumulus cells were absent, however, maturation of surviving oocytes did not relate to cumulus levels at collection. There was no relationship between cumulus cover and oocyte diameter at collection or growth in vitro (data not shown). This analysis was not performed for the ICSI group because cumulus cells had already been removed.

Table II shows the diameters of IVM oocytes in relation to fertilization and cleavage. The same fertile sperm donor was used throughout. The apparent difference in oocyte diameter

any comment on a threshold size for maturation in oocytes from this source.

Data from the PCO group (Table I) shows that atresia was more likely when cumulus cells were absent, however, maturation of surviving oocytes did not relate to cumulus levels at collection. There was no relationship between cumulus cover and oocyte diameter at collection or growth in vitro (data not shown). This analysis was not performed for the ICSI group because cumulus cells had already been removed.

Table II shows the diameters of IVM oocytes in relation to fertilization and cleavage. The same fertile sperm donor was used throughout. The apparent difference in oocyte diameter

<table>
<thead>
<tr>
<th>Cumulus grade</th>
<th>Oocyte after culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mature</td>
</tr>
<tr>
<td>0 (n = 65)</td>
<td>13 (20.0%)</td>
</tr>
<tr>
<td>1 (n = 17)</td>
<td>10 (58.8%)</td>
</tr>
<tr>
<td>2 (n = 5)</td>
<td>2 (40%)</td>
</tr>
<tr>
<td>3 (n = 41)</td>
<td>13 (31.7%)</td>
</tr>
</tbody>
</table>

0, devoid of cumulus/no more than 10 scattered cells; 1, partial cover; 2, complete cover; 3, substantial multilayered cover.
in the PCO group according to whether or not fertilization occurred was not significant.

Figure 3 shows oocyte diameters during culture with and without FF-MAS. The diameters of individual oocytes were plotted according to the culture conditions (0, 10 and 30 μg/ml FF-MAS) and oocyte outcome. In all groups, oocytes that became atretic tended to shrink, whereas those maturing tended to enlarge, except in the PCO control group. In FF-MAS (10 and 30 μg/ml), the mean diameters of mature, immature and atretic oocytes on day 0 were significantly different (P, 0.05) despite their diameters at collection being similar (Fig. 3b and c). Interestingly, this difference did not occur in PCO oocytes cultured in control conditions (Fig. 3a) and was not significant in those collected from ICSI cycles (Fig. 3d–f).

In the ICSI group, 50% of oocytes maturing in vitro had done so by 24 h, compared with <5% of PCO derived oocytes (Cavilla et al, 2001). There was no significant difference in oocyte diameter on day 0 between those maturing in 24 h and those in 48 h, within each culture group (control, 10 μg/ml FF-MAS and 30 μg/ml FF-MAS) or when pooling all the culture groups (24 h, median 113 μm, interquartile range 110–113.75 versus 48 h, median 112 μm, interquartile range 108.5–114.5).

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

Oocyte diameter, oocytes with larger measurements of ‘oocyte + zona’ in the PCO group appeared more likely to mature in vitro (Fig. 6a), however, this was not a significant difference. The diameter of the oocyte/zona complex did not change in culture for oocytes derived from ICSI patients, despite the extensive enlargement of ooplasm that occurred over the same period (Fig. 1b versus Fig. 5b), and was not associated with maturation in vitro (Fig. 6b). There was no significant relationship between oocyte + zona measurements and maturation, fertilization or cleavage in vitro (data not shown).

### Zona pellucida thickness

Frequency distributions were plotted of the mean zona thickness of viable oocytes from both patient groups on the day of

<table>
<thead>
<tr>
<th>Table II. Oocyte diameters on day of maturation according to origin of oocyte and developmental competence in vitro.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oocyte diameter (μm) on day 0</strong></td>
</tr>
<tr>
<td>--------------------------------------------------</td>
</tr>
</tbody>
</table>

PCO, polycystic ovaries. These patients underwent laparoscopic retrieval of oocytes without ovarian stimulation. ICSI, intracytoplasmic sperm injection. These patients underwent transvaginal oocyte collection after ovarian stimulation for a clinical cycle of ICSI as a treatment for infertility.
collection and for oocytes that did or did not mature in vitro.
There were no significant differences in zona thickness
between the two groups, and no relationship between zona
thickness and FF-MAS (data not shown).

The zona thicknesses on day 1 and day 2 were compared in
matured oocytes that did or did not fertilize after ICSI. PCO
oocytes that fertilized had significantly thicker zona pellucidas
on day 1 than those that did not (21.8 ± 1.9 versus 16.9 ±
2.7 μm, P < 0.05). No significant differences were observed
on day 1 or day 2 for IVM oocytes from ICSI patients (ferti-
лизed 20.5 ± 0.8 versus 20.3 ± 0.6 μm unfertilized). The
results on day 2 were 20.1 ± 1.9 (fertilized) versus 17.2 ±
3.4 (unfertilized); and 21.2 ± 0.9 (fertilized) versus 19.3 ±
0.5 μm (unfertilized) for the PCO and ICSI groups,
respectively.

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

Oocyte growth
During its growth phase, the human oocyte increases in diam-
eter from ~30 to >110 μm, over a period of at least 8 weeks
(Gougeon, 1986). During this time, its nucleus remains arrested
in first meiotic prophase. The diameter of the in vivo matured
human oocyte, excluding the zona pellucida, is normally
~110–120 μm (which we confirm here), whereas the zona
pellucida is normally ~15–20 μm thick (Veeck, 1999).
Including the zona pellucida and PVS, the pre-ovulatory oocyte commonly has a diameter ~150 μm (Veeck, 1999). Measurements of oocyte diameter of immature oocytes at collection and after IVM culture confirmed the size dependence of maturation, as has been extensively documented in other species. However, it also resulted in unexpected observations of the relatively small size of immature oocytes relative to those matured in vivo, as well as evidence of growth of immature oocytes in vitro. An increase of 3 μm average diameter from 106 to 109 μm (Fig. 2b) would result in ~54 461 μm³ increase in cytoplasmic volume, constituting an astonishing 8% increase in volume over 2 days. Hence, a relatively small change in diameter that could easily pass unnoticed during routine clinical procedures is associated with a relatively large change in volume. It therefore seems likely to us that growth of human oocytes in vitro has been underestimated and may provide worthwhile information about oocyte potential. Oocyte growth in vitro differed between the patient groups studied, suggesting that endocrine or other patient factors may contribute to its control. Further study is clearly indicated.

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

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

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

**Effect of FF-MAS on oocyte growth**

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

The mechanism of action of FF-MAS is not yet known, and its potential as an adjunct to oocyte and embryo cultures is controversial (Downs et al., 2001; Vaknin et al., 2001; Tsafiriti et al., 2002, 2005; Bergh et al., 2004; Loft et al., 2004; Marin Bivens et al., 2004). One possibility arising from our data is that FF-MAS may influence oocyte growth. FF-MAS is a steroid related to lanosterol and cholesterol (Byskov et al., 1995, 2002). Cholesterol is known to influence membrane fluidity and the function of membrane proteins (McIntosh and Simon, 2006) and relative levels of cholesterol and MAS change in follicular fluid during maturation (Bokal et al., 2006). Although no direct effects of FF-MAS on membrane fluidity have been reported, oocyte growth from diameters of 106 to 109 μm, as exemplified above, would result in an associated increased surface area of 2026 μm² (5.4%) (assuming the oocyte to be spherical—in fact, if the number of microvilli also increased, the overall surface area could increase more), so membrane elasticity and/or synthetic capacity may be a crucial factor for oocyte growth and subsequent embryo cleavage. We therefore hypothesise that FF-MAS may be involved in membrane biochemistry, in addition to any role in local communication. There is some evidence in amphibians to support membrane fluidity having a role in meiotic arrest, controlled by progesterone and cAMP, so this idea warrants further study (Morrill et al., 1989, 1993). Such changes in lipids and increasing membrane fluidity also occur during sperm maturation (e.g. Rejraji et al., 2006). An alternative perspective, if our hypothesis is correct, is that
the ooplasm could become less rigid and oocytes more likely to flatten slightly under their own weight. This might explain the increased diameters of a focal plan observed through the oocyte’s centre. Three-dimensional imaging will be required to test this idea.

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

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

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

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

The zona thickness measurements obtained for fertilized oocytes matured in vitro in this study were larger than measurements of in vivo matured oocytes obtained by others using differential interference optics (eg day 1, 16.4 ± 3.1 μm, Bertrand et al., 1996; 17.7 ± 0.14 μm, Garside et al., 1997) or computer assisted methods (e.g. day 1, 19.9 ± 1.92 in conception cycles and 18.6 ± 1.8 μm in non-conception cycles, Shen et al., 2005). This could indicate an effect of culture or differences in the source of oocytes and their developmental potential.

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

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

References


