

Article

Light retardance by human oocyte spindle is positively related to pronuclear score after ICSI



After attaining a diploma in biology at the Ruhr-University, Bochum, Ursula Eichenlaub-Ritter has been Professor for Cell Biology at the University of Bielefeld, Department of Gene Technology/Microbiology since 1991. Her major interests are in the development of non-invasive methods to assess oocyte and embryo quality, and regulation of gene expression, spindle formation and cell cycle control at mammalian oogenesis, and in reproductive toxicology and stem cell research.

Dr Ursula Eichenlaub-Ritter

Y Shen^{1,2}, T Stalf¹, C Mehnert¹, L De Santis⁴, I Cino⁴, H-R Tinneberg^{1,2}, U Eichenlaub-Ritter^{3,5}

¹Centre of In-Vitro-Fertilization (CIF) in the Justus-Liebig-University, D-35392 Giessen; ²Department of Gynaecology and Obstetrics, Women's Hospital, Justus-Liebig-University Giessen, D-35392 Giessen; ³University of Bielefeld, Faculty of Biology, Gene Technology/Microbiology, D-33501 Bielefeld, Germany; ⁴Vita-Salute University, H S. Raffaele, Department of Obstetrics and Gynaecology, IVF Unit, Milan, Italy

⁵Correspondence: Tel: +49 521 1064832; e-mail: EiRi@uni-bielefeld.de

Abstract

Disturbed spindle assembly increases risks of chromosome mal-segregation. Non-invasive polarization microscopy (PolScope) was employed in two centres to assess spindle integrity for the first time quantitatively in human oocytes from consenting patients undergoing intracytoplasmic sperm injection (ICSI) with respect to pronuclear (PN) score after fertilization. In one centre oocytes were selected before ICSI, in another selection was after ICSI according to PN score. In both centres, mean retardance of light by birefringent spindles in oocytes forming a pre-embryo with good PN score after ICSI was significantly higher compared with spindles in oocytes developing into a lower PN score pre-embryo with limited developmental potential ($P < 0.001$). Transfers involving oocytes with high retardance and at least one good PN score embryo resulted more frequently in a conception than transfers from oocytes with spindles of lower mean retardance and lower PN score embryos. There was a trend for an inverse relationship between age and magnitude of retardance in a small oocyte cohort. The study suggests that quantitative evaluation of mean retardance of light by the oocyte spindle predicts oocyte health, is related to PN score of the embryo and may be especially useful to assess oocyte quality in countries with legal restrictions to select after fertilization.

Keywords: aneuploidy, ICSI, oocyte selection, oocyte spindle, polarization microscopy, pronuclear score

Introduction

Although overall pregnancy rates have improved over the years, birth rate in assisted reproduction programmes is still fairly low, in spite of the fact that more than one embryo is transferred in many centres. Due to transfer of several embryos, the rate of multiple pregnancies following IVF and ICSI is high (Pandian *et al.*, 2005). Transfer of only one embryo might reduce the incidence of multiple pregnancies, after accurate assessment of embryo quality, but apart from the fact that prolonged culture under potentially sub-optimal conditions could lead to abnormalities (Corcoran *et al.*, 2005), ethical considerations and legal regulations in several European and non-European countries prohibit discarding of embryos and selection for transfer after fertilization or syngamy. For instance, in Italy, selection has to occur even before fertilization. The present

retrospective study aimed at identifying new non-invasive markers in terms of having more parameters for culminate scoring in assessment of oocyte/embryo quality for fertilization or selection for transfer.

The spindle apparatus is an essential cellular organelle, which is crucial for the high fidelity of chromosome segregation at both meiotic divisions of oogenesis (for review, see Eichenlaub-Ritter, 1998; Eichenlaub-Ritter *et al.*, 2002, 2004). In addition, spindles act as sink for components needed for cell cycle progression of the zygote, and expression of a functional spindle may support the association and transfer of maternal proteins promoting early embryogenesis (Eichenlaub-Ritter and Peschke, 2002; Simerly *et al.*, 2003; Tang *et al.*, 2004; for discussion, see Eichenlaub-Ritter *et al.*, 2004). Spindle aberrations are a hallmark of aged oocytes (Battaglia *et al.*,

1996; Volarcik *et al.*, 1998; Eichenlaub-Ritter *et al.*, 2004), and may also indicate that the expression patterns for cytoskeletal proteins and maternal factors are disturbed in the oocyte, dependent or independent of age (Hamatani *et al.*, 2004). Certainly, exposures during maturation (Shen *et al.*, 2005b), freezing (Bianchi *et al.*, 2005; Rienzi *et al.*, 2005) and ageing processes (Eichenlaub-Ritter *et al.*, 2004) causing disturbances in the regulation of spindle formation are associated with a high risk of chromosome malsegregation, and, in consequence, may lead to a reduced reproductive potential and survival of embryos and live births in humans. Critical assessment of spindle integrity and function is therefore important.

In the past, data on spindle integrity and morphology were predominantly obtained from fixed oocytes using conventional immunofluorescence microscopy (Battaglia *et al.*, 1996; Volarcik *et al.*, 1998; Boiso *et al.*, 2002; Eichenlaub-Ritter *et al.*, 2002). Such methods provide only a static image of the spindle, do not account for the highly dynamic nature and order of the microtubular cytoskeleton, and are invasive and therefore not suitable for clinical use.

Orientation independent polarizing microscopy (PolScope) was a breakthrough (Oldenbourg 1996; Liu *et al.*, 2000a), since non-invasive analysis of spindles in human oocytes became possible (reviewed by Eichenlaub-Ritter *et al.*, 2002; Keefe *et al.*, 2003). Based on the intrinsic optical properties of highly ordered spindle microtubules to induce birefringence and retard polarized light, PolScope in combination with the respective software has been used to assess presence and localization of the spindle in oocytes qualitatively and analyse spindle morphology (e.g. Wang *et al.*, 2001a,b; Eichenlaub-Ritter *et al.*, 2002; Moon *et al.*, 2003; Rienzi *et al.*, 2003, 2005; De Santis *et al.*, 2005). Oocytes possessing birefringent spindles more frequently develop to the blastocyst compared with those without spindle, and absence of a spindle is an indicator for reduced oocyte quality (e.g. Wang *et al.*, 2001b). However, since most oocytes possess spindles, qualitative analysis of spindle expression is of limited value for selection.

In addition to providing qualitative information on expression and localization of the spindle, recently quantitative methodology was also developed. The relative magnitude of the retardance of the polarized light is an indicator for density, high order alignment, or thickness of a birefringent object (Schaap and Forer, 1984; Oldenbourg, 1996; LaFountain and Oldenbourg, 2004). Although there were reports correlating changes in the magnitude of retardance by the spindle with developmental stage (e.g. before and after fertilization; Liu *et al.*, 2000b) or maternal age (De Santis *et al.*, 2005), so far there were no representative quantitative studies on oocyte developmental potential with respect to possessing a highly birefringent spindle with high numbers of ordered spindle microtubules in the spindle as compared with those with less dense/ ordered microtubular fibres producing lower mean retardance by the spindle in PolScope microscopy. The present study was aimed at improving the non-invasive strategies for selection of high quality human oocytes prior to ICSI by analysing for the first time the optical properties of the spindle apparatus quantitatively by analysis of mean magnitude of retardance of light by the oocyte spindle by polarization microscopy. A quantitative analysis of birefringence retardation (termed retardance) was performed independently at two IVF

centres along a line scan through the long axis of the spindle of oocytes from ICSI cycles. In one centre (Giessen), all polar body oocytes were fertilized. Accordingly, all fertilized oocytes could be analysed for pronuclear score after ICSI. The selection for transfer was then done blindly with respect to PolScope data, by selecting two or three embryos with the best pronuclear (PN) scores. In the other centre (Milano), oocytes were selected by morphology before ICSI, and PN scores were obtained only for the fertilized oocytes. In order to assess the developmental potential of oocytes with spindles producing high or low mean retardance in PolScope microscopy, the fate of each oocyte was followed with respect to fertilization and formation of a pre-embryo with 'good' or rather mediocre pronuclear score by established scoring criteria by Scott and Smith (1998) (Giessen centre) or Tesarik and Greco (1999) (Milano centre) respectively. Furthermore, conception was analysed with respect to birefringence of the meiotic spindle of oocytes contributing to transfers after ICSI. The study was initially set up in the IVF centre at Giessen University, obtaining data from a large cohort of oocytes that were fertilized by ICSI. The cohort included oocytes selected for implantation as well as those that were not used for transfer. To obtain more information on the validity of observations, recently a small cohort of oocytes from an IVF centre in Milano was also studied in an independent approach. The cohort of oocytes from Milano was analysed for mean retardance of spindles with the same standard PolScope methodology employed also at the Giessen centre, but included only those oocytes that were selected for treatment prior to fertilization since only the embryos obtained after ICSI could be assessed for pronuclear score.

Materials and methods

Analysis by Giessen Centre

Oocytes

A total of 1369 oocytes obtained at the Giessen centre during a period of 2 years from patients with a mean maternal age of 32.5 ± 4.5 years and from 182 stimulation cycles for ICSI were initially examined non-invasively by PolScope after informed consent. This study is reporting exclusively the data from 103 cycles of these 182 cycles, with a total of 1140 oocytes, in which quantitative rather than only qualitative PolScope microscopy was performed (**Table 1**). For this, mean retardance of light by the oocyte spindle was determined along a line scan of the long spindle axis, apart from scoring for absence or presence of a spindle, and PN scores were analysed for each oocyte fertilized by ICSI with respect to mean retardance of light by the spindles of oocytes before ICSI. Fertilization with respect to positioning of the spindle in individual oocytes could also be analysed in 731 of 739 oocytes with a spindle from a total of 1140 polar body oocytes from patients with a mean maternal age of 32.5 ± 4.4 years (**Table 1**). Furthermore, 792 embryos from oocytes with ($n = 676$) and without ($n = 116$) spindle were analysed for pronuclear-score 18 h after ICSI (**Table 1**). A total of 676 oocytes developing into pre-embryos, in which pronuclear (PN) scores were determined individually, were included in the quantitative assessments of mean length and retardance of the spindle, and PN scores were compared with 116 oocytes without birefringent spindle obtained from the same patients (**Table 1**). From the 676 embryos with and 116 without spindles obtained

Table 1. Summary of data on patients from the Giessen cohort included in qualitative PolScope analysis of spindles in oocytes, numbers of oocytes with and without spindle, numbers of oocytes assessed for pronuclear scores and variables between patients whose oocytes contributed to a conception cycle (CC) or non-conception cycle (NCC).

<i>Qualitative analysis</i>	<i>Total</i>	<i>CC</i>	<i>NCC</i>
<i>Patients</i>			
Number of patients	103	42	61
Maternal age ^a	32.5 ± 4.4	32.5 ± 3.9	32.4 ± 4.3
Number of attempts ^a		2.1 ± 1.6	2.7 ± 1.4
Peak oestradiol concentrations (ng/ml) ^a		1643.7 ± 735.5	1553.6 ± 645.5
Number of follicles ^a		11.8 ± 3.7	12.2 ± 5.0
Number of polar body oocytes ^a		8.5 ± 3.6	8.8 ± 4.1
Fertilization rate (%) ^b		81.2	82.3
<i>Oocytes</i>			
Total number of oocytes	1140		
Number of mature oocytes	897		
Mature oocytes with spindle (%)	739 (82.4)		
Oocytes assessed for displacement of spindle before ICSI (without/with displacement)	731 (620/111)		
Oocytes assessed for PN-scoring after ICSI (with/without spindle)	792 (676/116)		
Oocytes assessed for mean retardance and PN score after ICSI (non-transferred/transferred)	676 (422/254)		
<i>Oocytes transferred after ICSI</i>			
Number of transferred oocytes after ICSI	268	105	163
Transfer of oocytes with birefringent spindles (%) ^b	254	100 (95.3)	154 (94.5)
Oocytes with displaced spindle (%) ^b		6/100 (6.0)	22/154 (14.3)
Oocytes stimulated by short protocol (%) ^b		21/105 (20.0)	35/163 (21.5)
Average number of transferred embryos/patient ^a		2.5 ± 0.6	2.7 ± 0.8
Transfers at day 2 (%) ^b		41/42 (97.6)	57/61 (93.4)
Mean retardance of light by spindle in transfer oocytes (nm) ^a		1.65 ± 0.43	1.67 ± 0.44
Mean length of spindle in transfer oocytes (µm) ^a		12.9 ± 2.0	12.8 ± 1.8

^at-test, $P > 0.1$, no significant difference between the CC and NCC group.

^bChi-squared test, $P > 0.1$, no significant difference between the CC and NCC group.

ICSI = intracytoplasmic sperm injection; PN = pronuclear.

after ICSI and pronuclear scoring 268 embryos were selected for transfer, 254 of which contained a birefringent spindle before fertilization (**Table 1**). Mean retardance of spindles of transfer oocytes in conception cycles (100 oocytes) was compared with that in non-conception cycles (154 oocytes) (**Table 1**). Also, maternal age and mean retardance was compared between all oocytes/embryos in conception ($n = 246$) and non-conception cycles ($n = 430$) (**Table 2**), including transfer (254) and non-transfer oocytes (422).

Stimulation and oocyte retrieval

For the Giessen cohort, ovulation induction was induced by gonadotrophin-releasing hormone (GnRH) agonist (Menogon®; Ferring, Kiel, Germany) in either a long-treatment (77 patients; with a mean maternal age of 30.5 ± 3.3 years) or a short-treatment protocol (26 patients, with a mean maternal age of 37.6 ± 1.9 years). Human chorionic gonadotrophin (10,000 IU HCG; Organon, Munich, Germany) was administered when dominant follicles reached a diameter of about 20 mm. Isolated cumulus–oocyte complexes (COC) were subsequently denuded by brief exposure to 80 IU/ml hyaluronidase (Sigma, Deisenhofen, Germany) and gentle aspiration by pipette. On average, 11.1 ± 5.5 oocytes were retrieved per cycle by transvaginal aspiration 36 h after HCG administration. For ethical and practical considerations, the study included only those cycles, in which 4–20 oocytes were obtained from a patient. Those cases with <4 fertilized oocytes were not all analysed for pronuclear score and were therefore generally excluded. Cycles with more than 20 oocytes were also excluded to avoid any delay of ICSI due to the longer time needed to save images of each individual oocyte of the patient by PolScope. Eight hundred and ninety seven metaphase II oocytes, of which 82.4% possessed a spindle, were in total included in the study (**Table 1**) and examined by PolScope within 2 h after retrieval, immediately prior to ICSI (38 h post-HCG). For ICSI, oocytes were transferred to a different microscopic stage for injection. ICSI was performed blindly with respect to expression and localization of a birefringent spindle. Oocytes arrested at germinal vesicle (GV) and metaphase I stage were excluded from ICSI. The study was approved by the ethics committee of the University Hospital of Giessen and with informed consent by patients.

Non-invasive PolScope imaging of the spindle of human oocytes

Enhanced polarizing microscopy (PolScope-microscopy) was employed to visualize the spindle apparatus in living human oocytes (De Santis *et al.*, 2005; Shen *et al.*, 2005a). In the Giessen centre, each oocyte was individually placed into 5 μ l of prewarmed HEPES-buffered human tubal fluid medium (HTF medium; Irvine Scientific, Santa Ana, CA, USA) covered by mineral oil (M-8410, Sigma) in a WillCo Wells BV dish (ref. no. GWSt-5040; Amsterdam, Netherlands), as described previously (Shen *et al.*, 2005a). In brief, oocytes were placed onto the heated object stage of a Nikon TE 2000 inverted microscope equipped with a strain-free 40 \times objective lens and examined after orienting oocytes by gentle manipulation such that the spindle became visible (**Figure 1a**). Oocytes were gently moved such that spindles were located perpendicular to the plane of view (**Figure 1a**), and the image could be saved. Positioning of the spindle relative to the polar body could be later assessed in

a large proportion of the oocytes. For quantitative analysis of spindle birefringence and mean retardance, a line was drawn through the middle of the spindle body, along its long axis (see below, **Figure 4**). To determine the pole-to-pole distance of the spindle and calculate the position of the meiotic spindle with respect to the first polar body, oocytes were aligned such that the first polar body was visible at the periphery of the oocyte and the long axis of the spindle was oriented perpendicularly to the light path. Setting of the microscope and calibration against background were done according to the manufacture's protocol (Shen *et al.*, 2005a). Data were saved for later analysis by the *SpindleView*™ image processing system (Shen *et al.*, 2005a). All oocytes were subsequently fertilized by ICSI within the next few minutes.

Quantitative assessment of light retardance of spindle apparatus

According to the expression of a birefringent spindle oocytes were divided into two groups in the Giessen cohort. One comprised oocytes expressing a birefringent spindle, while no spindle was detectable in the other group of oocytes (**Figure 1a,b**). Fertilization of oocytes with and without a spindle was scored retrospectively. The development of oocytes with and without spindle to pre-embryos was determined quantitatively with respect to PN score of the pre-embryos.

The localization of the metaphase II spindle relative to the first polar body was also analysed in 731 oocytes containing a birefringent spindle in the Giessen cohort. Spindle deviation angle was described by the line connecting the oocyte centre with the middle of the meiotic spindle and a line connecting the oocyte centre with the centre of the first polar body (**Figure 1c,d**). Fertilization rate was assessed and compared between those oocytes containing a spindle close to the first polar body ($\leq 40^\circ$ distance, **Figure 1c**) and those with the spindle located further away from first polar body (deviation angle of $>40^\circ$, **Figure 1d**). Furthermore, the proportion of oocytes developing to a pre-embryo with 2PN, with abnormal fertilization pattern (1PN and >2 PN) and the number of unfertilized oocytes was determined for the group of oocytes with and without spindle, and for oocytes with the spindle in proximity ($\leq 40^\circ$) or away from the first polar body ($>40^\circ$) (**Figure 2**).

In oocytes with spindles, irrespective of spindle localization, spindle length and retardance of light was quantitatively assessed by PolScope software along a line scan parallel to the spindle long axis from one to the other spindle pole in the centre of the spindle body (**Figure 4**). The mean retardance magnitude of the spindle in each oocyte was then calculated by averaging retardance of points spaced 0.5 μ m apart (in the Giessen laboratory) along the whole cross-section curve corresponding to spindle pole-to-pole length. In Giessen, the two outermost points were not included in assessment (as indicated by the arrows in **Figure 4**).

Assessment of pronuclear score

The evaluation of pre-embryo quality was performed at the pronuclear stage, 18 h post-insemination for oocytes with and without spindle in Giessen University. The criteria of pronuclear scoring (PN score) were according to standard

Table 2. Quantitative assessment of mean retardance of light and spindle length in all oocytes of the Giessen cohort selected or non-selected for transfer developing into pre-embryos with different pronuclear (PN) score after intracytoplasmic sperm injection: PN score of pre-embryos of all oocytes from patients of different age according to criteria by Scott and Smith (1998).

<i>Giessen data PN-score of pre-embryo</i>	<i>Numbers of pre-embryos derived by oocytes with spindle</i>	<i>Mean maternal age/oocyte</i>	<i>Mean retardance (nm)</i>	<i>Mean length of spindle (μm)</i>
Group 1: score A, B	180	31.9 ± 4.2	1.72 ± 0.43	12.7 ± 1.8
Group 2: score C	51	32.0 ± 3.8	1.53 ± 0.40^a	12.5 ± 1.6
Group 3: score D	324	32.2 ± 4.0	1.52 ± 0.44^b	12.6 ± 1.7
Group 4: score E and abnormal	121	31.2 ± 4.3	1.39 ± 0.46^b	$11.7 \pm 1.7^{c,d}$
Total	676	32.2 ± 4.2	1.55 ± 0.45	12.5 ± 1.8
Total in non-conception cycles	430	32.1 ± 3.7	1.56 ± 0.46	12.4 ± 1.8
Total in conception cycles	246	32.3 ± 4.5	1.53 ± 0.44	12.6 ± 1.8

Significant differences according to one-way analysis of variance: versus group 1, ^a $P < 0.05$; ^b $P < 0.001$; ^c $P = 0.001$; versus group 3, ^d $P = 0.005$.

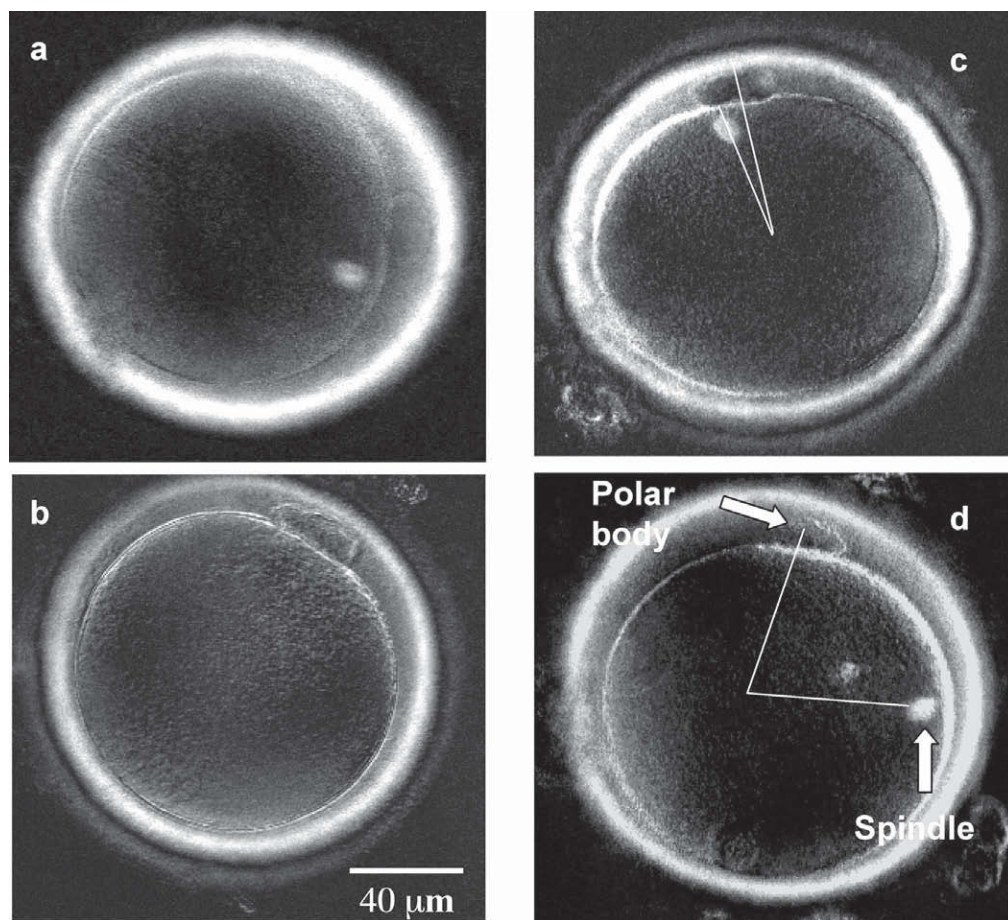


Figure 1. Expression of spindles in human oocytes viewed by non-invasive PolScope microscopy. (a) Spindle apparatus in living human oocyte in proximity to the first polar body. (b) Oocyte without birefringent spindle. (c) Typical oocyte containing the spindle close to the first polar body. (d). Characteristic example of an oocyte expressing a spindle with a deviation angle over 40° relative to the first polar body. Bar: $40 \mu\text{m}$.

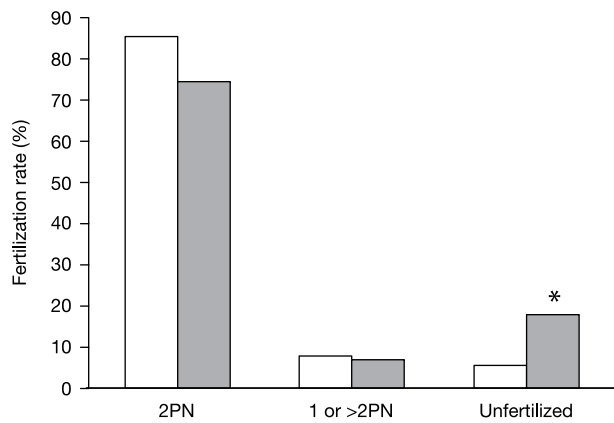


Figure 2. Comparison of the fertilization rate and development to two-pronuclear (PN) pre-embryos or pre-embryos with 1 or >2PN of cohorts of oocytes with a spindle close to the first polar body (PB) ($\leq 40^\circ$, open bars; $n = 620$) or with the spindle located at an angle of $> 40^\circ$ away from the spindle (grey bars; $n = 111$) in 731 oocytes from 103 stimulation cycles in the Giessen cohort. Asterisk (*) indicates significant difference from oocytes with the spindle located at an angle of $\leq 40^\circ$ away ($P < 0.001$).

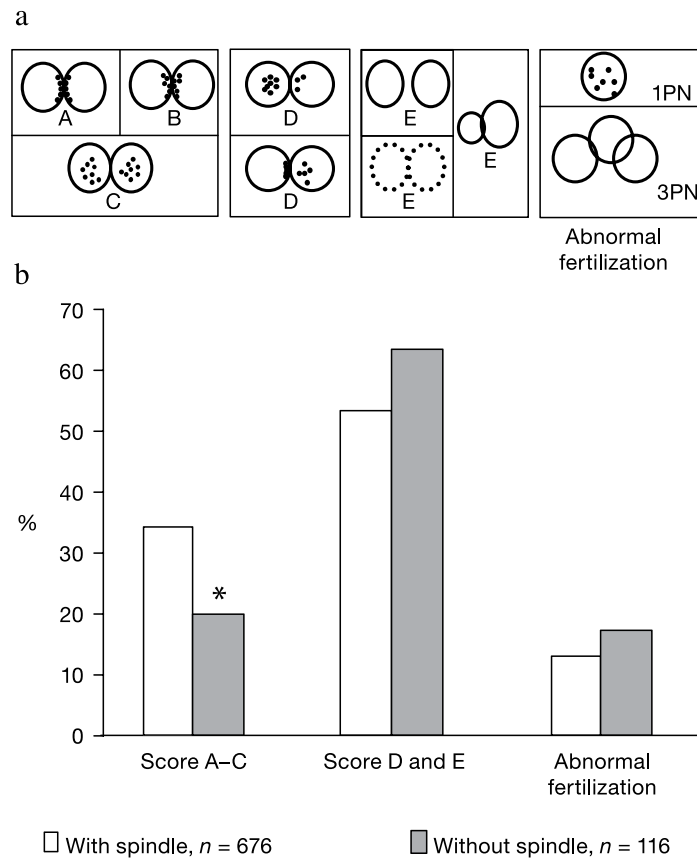


Figure 3. Development of oocytes with and without birefringent spindle with respect to pronuclear scores of pre-embryos from the Giessen cohort. (a) Characteristics of pronuclei (represented as circles) and nucleolar precursor bodies distribution (dark dots) in pre-embryos according to pronuclear (PN) score A, B, C, D, E or abnormal fertilization (for explanation, see materials and methods). (b) The proportion (per cent) of presumably high quality embryos with good PN score (A-C) and embryos with mediocre and low scores from oocytes possessing a birefringent spindle (open bars) and oocytes without a spindle (grey bars). Asterisk (*) indicates significant difference from oocytes with spindles ($P < 0.001$).

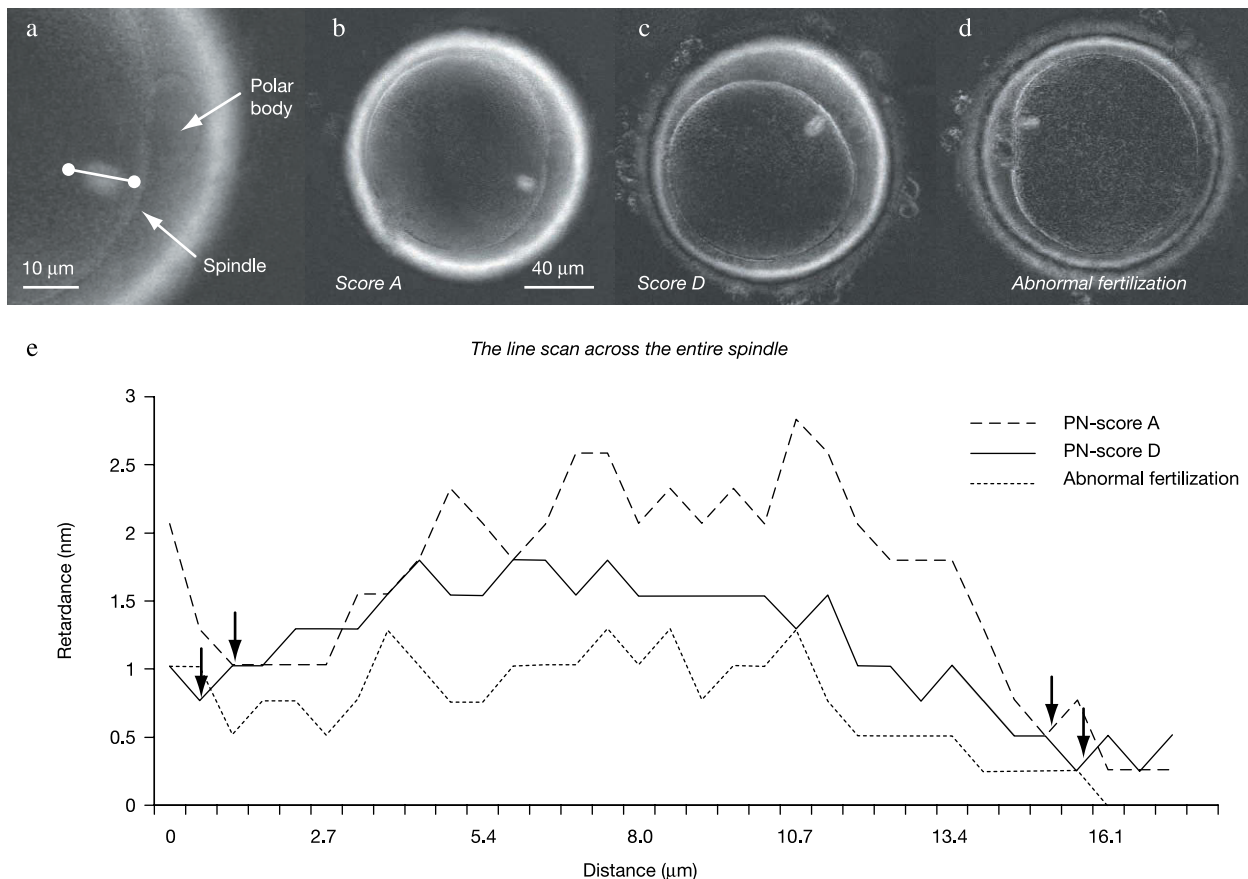


Figure 4. Examples for line scans through the long axis of the meiotic metaphase II spindle from pole-to-pole from the Giessen centre for analysis of mean magnitude of retardance (a) and characteristic images of oocytes forming a pre-embryo with pronuclear (PN) score A (b) and D (c), or abnormal fertilization (d). Characteristic examples of retardance curve of a line scan (e) through the spindle apparatus of an oocyte forming a pre-embryo with score A (dashed line), with a score D (solid line) or with abnormal fertilization (dotted line). Arrows show the outer boundary and the two measurement points excluded from the calculation of the average magnitude of retardance of light by the spindle. Y-axis shows retardance of polarized light by the spindle in nm; x-axis depicts distance in μm along the line scan corresponding to measurement points included in calculation of mean retardance along the whole spindle length. Bar in a: 10 μm. Bar in b for b–d: 40 μm.

criteria used in the IVF centre at Giessen University as previously described (Shen *et al.*, 2005a). Selection was based primarily on the position of both pronuclei and the alignment of nucleolar precursor bodies (NPB) in the pronuclei (according to the slightly modified scoring criteria from Scott and Smith, 1998) as well as the overall morphology of the pre-embryo. A schematic drawing of position of PN relative to each other and of NPB is provided in **Figure 3a**.

In Giessen PN score was assessed in a total of 676 oocytes with a spindle and 116 oocytes without a spindle (**Table 1**). Retrospectively, oocytes with a spindle were grouped into three or four subgroups, according to their PN score after insemination to compare with mean retardance magnitude of spindles before ICSI (Shen *et al.*, 2005a).

In embryos with a score of A and B, there was close nuclear apposition (indicated by touching of the two open spheres representing maternal and paternal pronuclei in schematic

drawing of pronuclear characteristics in **Figure 3a**) and NPB (indicated as dark dots in **Figure 3a**) were symmetrically distributed in the nucleoplasm, either in close apposition to the nuclear periphery where pronuclei face each other (score A), or were similar in number and still in close proximity to each other in the half of the nucleoplasm where pronuclei were apposed to each other (score B) (as indicated in **Figure 3a**). Embryos with a score of C contained NPB of similar number distributed more randomly within the nucleoplasm of both pronuclei. They were assigned to group 2 for assessment of average retardance (**Table 2**). Embryos with a score of D had uneven numbers, and/or asymmetric distribution of nucleoli (NPB, dark dots) within the nucleoplasm of the paternal and maternal pronucleus (**Figure 3a**). They were assigned to group 3 in assessment of mean retardance. Finally, embryos with a score of E, possessing nuclei and/or NPB not clearly distinct or with unevenly sized nuclei or with nuclei positioned apart from each other (score E in **Figure 3a**), together with abnormally fertilized embryos possessing one or >2PN (**Figure 3a**), were all assigned to group 4 for assessment

of mean retardance (**Table 2**). Regarding selection for transfer, the presence of a halo and other morphological parameters were considered as additional indicators of higher quality of the embryo in the Giessen cohort (Stalf *et al.*, 2002), and was therefore used in embryo selection for transfer, especially between embryos of otherwise similar PN score.

The mean retardance magnitudes for spindles in oocytes giving rise to an embryo in each of the four groups were compared. Furthermore, in the Giessen cohort average retardance of spindles of oocytes selected for transfer after ICSI was also compared with mean retardance of spindles in oocytes not used for transfer (**Table 3**).

Two to three embryos with the presumably best quality were selected for embryo transfer in the Giessen centre, blindly with respect to spindle data. Embryos were usually transferred to the uterus on day 2 (in 98 cycles). In a few cases (five cycles), embryos were transferred only on day 3 after oocyte retrieval, due to practical considerations (**Table 1**). In total, spindles in 254 oocytes selected for embryo transfer after fertilization were analysed by PolScope microscopy (**Table 1**) and compared with 422 oocytes excluded from transfer due to lower PN score (**Table 3**). A pregnancy was considered, when the patient had positive results in the pregnancy test three times, 2 weeks after embryo transfer. To assess correlations between mean retardance of the oocyte spindle, transfer of high or lower PN-score embryo and conception, embryos were grouped according to the numbers of high PN score embryos in individual transfers (**Table 4**). One group referred to transfers with two or three embryos of highest PN-score (A–C). Data were compared with transfers with only one good PN score embryo (A–C) together with embryos of score D–E in group 2, and transfers comprising exclusively lower PN-score embryos in group 3 (**Table 4**). Relative contribution to conception was calculated per cycle and per oocytes/embryos in each group (**Table 4**). Mean retardance and mean spindle length of all oocytes with spindles included in the groups was compared. Mean retardance was also analysed for all oocytes, for oocytes used for transfer and for non-transfer oocytes with spindles with respect to maternal age (**Table 3**).

Analysis by Milano centre

Oocytes

In the Milano centre only a small cohort of oocytes retrieved during a period of 2 months was analysed quantitatively by PolScope. The number of fertilized eggs at the pronuclear stage enrolled in this study was limited by the prescription of the Italian IVF law that prohibits the generation of more than three embryos during each cycle. Therefore, no more than three oocytes per patient were assessed with respect to spindle retardance. In total, 59 oocytes from 28 patients with a mean maternal age of 33.7 ± 4.3 years were included in the study (**Table 5**).

Stimulation and oocyte retrieval

In the Milano cohort ovarian stimulation was conducted according to a long protocol using GnRH agonist (Enantone®; Takeda, Osaka, Japan) and recombinant FSH (Puregon®; Organon or Gonal F®, Serono, Geneva, Switzerland). Ovulation

was triggered with HCG (Ovitrelle® or Gonasi®, Serono). Oocytes were collected transvaginally under ultrasound guidance at 36 h after HCG administration. Cumulus and corona radiata cells were immediately removed after retrieval by a short exposure to HEPES-buffered medium (Sage IVF Inc., Trumbull, CT, USA) containing 20 IU/ml hyaluronidase (Sage) and gentle aspiration in and out of a pipette (Flexi-Pet™; Cook, Queensland, Australia) and mechanically cleaned from the remaining surrounding cumulus cells by aspiration using a denuding pipette with a 170–130 µl diameter (Denuding Flexi-Pet™; Cook). The denuded oocytes were then assessed with respect to their meiotic maturation status. In preparation for ICSI, oocytes with an extruded first polar body (PBI) (presumably at the metaphase II stage) were selected for meiotic spindle detection and PBI morphology assessment, while oocytes without a polar body or presenting a germinal vesicle (GV stage) were excluded from the study.

The study was approved by the local Institutional Ethical Committee and with informed consent by patients.

Non-invasive PolScope imaging of the spindle of human oocytes

For meiotic spindle observation in the Milano IVF Unit, oocytes were placed in 5 µl drops of HEPES-buffered medium (Sage IVF Inc) covered with mineral oil (Sage) on a glass-bottomed culture dish (Will-Co dish, Intracel, Royston, Herts, UK), which was maintained at 37°C on a heated stage (Labotech, Gottingen, Germany).

Meiotic spindle visualization in Milano was performed at 20× magnification on a specially equipped Olympus 1X70 Inverted Microscope with LC PolScope optics and controller (SpindleView; CRI, Woburn, MA, USA), combined with a computerized image analysis system (SpindleView software). The mean retardance magnitude of the spindle in each oocyte was then calculated by averaging retardance of points spaced 1 µm apart along the whole cross section curve, as done at 0.5 µm points by the Giessen centre. Since only few oocytes were selected for fertilization in the Milano centre, polar body positioning was not further analysed with respect to outcomes.

Assessment of pronuclear score

In the Milano group, pronuclear scores were acquired 16–18 h after oocyte injection. Only the few oocytes fertilized by ICSI could be characterized for PN score. PN scoring was performed according to criteria defined by Tesarik and Greco (1999) that is based on positioning of pronuclei and NPB, similar to scoring by Scott and Smith (1998) but also assesses size of pronuclei relative to each other. Pre-embryos of presumably best quality (score 0 characterized by polarized NPB and a difference between both pronuclei not higher than three) were combined in group 1, the one with presumably highest quality. Pre-embryos with PN score 1 (characterized by a size difference >3 in both pronuclei) were combined in group 2; pre-embryos with PN score 2 [characterized by small number (<7) of NPB without polarization in at least one pronucleus] were combined in group 3, and all embryos of presumably lower quality with PN score of 3, 4 and 5 were combined in group 4, for quantitative assessment of average mean retardance of spindles as assessed

Table 3. Mean retardance of spindles of human oocytes from younger or aged patients of the Giessen cohort that were selected for transfer or non-transferred after intracytoplasmic sperm injection and pronuclear-scoring.

Age (years)	Total			Transferred		Non-transferred	
	Cycles	Oocytes	Mean retardance (nm)	Oocytes	Mean retardance (nm)	Oocytes	Mean retardance (nm)
≤30	40	271	1.58 ± 0.49	100	1.70 ± 0.47	171	1.51 ± 0.49
31–35	37	273	1.54 ± 0.42	94	1.69 ± 0.41	179	1.46 ± 0.41
≥36	26	132	1.53 ± 0.44	60	1.58 ± 0.42	72	1.50 ± 0.46
Total	103	676	1.55 ± 0.45	254	1.66 ± 0.43	422	1.49 ± 0.44 ^a

One-way analysis of variance: significant difference to transferred, ^a $P < 0.001$.

Table 4. Conception (biochemical pregnancy) in patients of the Giessen cohort with transfer of two or more pre-embryos with good pronuclear (PN) score (A–C), with only one good PN score embryo and one or two lower score embryos (D–E), or transfer with two to three embryos with no good PN score in relation to average mean retardance and mean spindle length of oocytes used for transfer after intracytoplasmic sperm injection in the cycle.

Good PN score embryos in transfers	Numbers of patients	Mean maternal age (years)	Average number of transferred embryos	Conception (% in group)	Total number of transferred pre-embryos (without spindle)	Pre-embryos in conception (% of group)	Mean retardance of the spindle (nm)	Mean spindle length (μm)
≥2 with PN score A–C	32	31.6 ± 4.6	2.6 ± 0.5	14 (43.8)	82 (3)	33 (40.2)	1.78 ± 0.47 ^b	12.9 ± 1.8
One with PN score A–C	36	32.6 ± 4.3	2.6 ± 0.6	17 (47.2)	92 (4)	38 (41.3)	1.63 ± 0.45 ^b	13.0 ± 2.0
None with PN score A–C	35	33.0 ± 3.6	2.7 ± 0.7	11 (31.4)	94 (7)	29 (30.9) ^a	1.55 ± 0.36 ^b	12.5 ± 2.0
Transferred embryos	103	32.4 ± 4.2	2.6 ± 0.7	42 (40.8)	268 (14)	100 (37.3)	1.66 ± 0.43	12.8 ± 1.9

^aChi-squared: asymptotic significance; $P > 0.05$.

^bOne-way analysis of variance: significant difference between all groups, $P < 0.05$.

Table 5. Quantitative assessment of spindle retardance in oocytes developing into pre-embryos with different pronuclear (PN) score in the Milano cohort, mean maternal age, mean retardance of spindle in oocytes developing into pre-embryos that contribute to a conception cycle (CC; gestational sac) or non-conception cycle (NCC); PN-score of pre-embryos from selected oocytes from patients of different age according to criteria by Tesarik and Greco (1999).

Milano data PN score (Tesarik and Greco, 1999)	Numbers of pre-embryos	Mean maternal age	Mean retardance of all (nm)	Implantation (sacs) (%)	Mean retardance in CC (nm)
Group 1: score 0	15	32.5 ± 5.2	2.15 ± 0.86	4 (26.7)	3.02 ± 1.05
Group 2: score 1	16	34.9 ± 3.6	1.96 ± 0.39	3 (18.8)	2.46 ± 0.48
Group 3: score 2	20	34.8 ± 3.2	1.86 ± 0.67	2 (10.0)	2.68 ± 0.14
Group 4: score 3–5	8	33.4 ± 4.8	1.59 ± 0.35 ^{a, b}	0 (0)	
Total	59	33.7 ± 4.3	1.93 ± 0.64	9 (15.3)	2.76 ± 0.73
Total in NCC (from group 1–5)	50	33.9 ± 4.1	1.78 ± 0.50		
Total in CC (from group 1–3)	9	32.6 ± 5.2	2.76 ± 0.73 ^c		

Chi-squared: significant difference to group 1: ^a $P < 0.05$; significant difference to group 2: ^b $P < 0.05$.

t -test: significant difference to NCC, ^c $P = 0.002$.

by PolScope. Average age of patients from whom oocytes were retrieved was obtained by calculating mean age of patients per oocyte in each group. Conception was assessed by presence of a gestational sac. Conception was analysed with respect to PN-score of embryos, and mean retardance was compared between oocytes in conception cycles and non-conception cycles.

Statistical analysis

Chi-squared test was performed to compare the fertilization rate of oocytes with and without a birefringent spindle, and the proportion of 2 PN formation in the oocytes containing a spindle nearby the polar body and far away from the polar body. Two-tailed Student's *t*-test or one-way analysis of variance (ANOVA) was used for the quantitative analysis of the retardance of the spindle apparatus. A significant difference was considered at $P < 0.05$. All the analysis was performed with Statistics Package for Social Sciences 12.0.

Results

Giessen laboratory data

Expression of birefringent oocyte spindle and localization of spindle with respect to outcomes

From a total of 1140 oocytes retrieved from 103 stimulated cycles 897 were mature, and of these 82.4% expressed a typical barrel shaped metaphase II spindle (**Figure 1a,c,d**), whereas the spindle apparatus was not detected by PolScope in 158 mature oocytes (17.6%) (**Table 1; Figure 1b**). Fertilization occurred in 91.5% (676 of 739) of the oocytes possessing a spindle versus 73.4% (116 of 158) in the group without a spindle, significantly different from each other ($P < 0.001$).

In 731 of 739 oocytes possessing a spindle, its position could be analysed with respect to the first polar body. The majority of the 731 oocytes contained a spindle close to the first polar body with a deviation angle below 40° (**Figure 1c**). A spindle displacement over 40° relative to the first polar body (**Figure 1d**) was found in only 15.2% oocytes (111 of 731; **Table 1**). The oocytes without pronounced spindle displacement had a relatively higher fertilization rate with low failure of formation of a pronuclear pre-embryo (5.6%) compared with the oocytes with displacement of the spindle from the first polar body (18.0%) ($P < 0.01$) (**Figure 2**). In contrast, the rate of oocytes showing abnormal fertilization patterns (one pronucleus or more than two pronuclei) was similar in both groups (**Figure 2**).

The fate of 792 fertilized oocytes with (676) and without spindle (116; **Table 1**) was assessed further for development into a high or low quality pre-embryo according to criteria defined by Scott and Smith (1998) (**Figure 3**). The proportion of presumably high quality embryos with PN score A, B and C was significantly higher for oocytes possessing a birefringent spindle compared with oocytes without a spindle (34.2% versus 19.9%; $P < 0.001$) (**Figure 3**). Conversely, the percentage of oocytes forming a pre-embryo with a PN score D or E or with abnormal fertilization tended to be higher in the oocytes without a birefringent spindle, although differences did not reach statistical significance.

Retardance magnitude and pole-to-pole distance of the meiotic spindle of human oocytes

Figure 4e shows three characteristic examples of cross-sections along a line scan of the long axis of the meiotic spindle in human oocytes obtained in the Giessen cohort in correlation to light retardance measured at $0.5 \mu\text{m}$ intervals across the entire spindle. The striped line is from an oocyte developing into a pre-embryo with PN score A (**Figure 4b**), characteristic for oocytes in subgroup 1 (**Table 2**). The solid line is from an oocyte forming a pre-embryo with a PN score D after fertilization, characteristic for subgroup 3 (**Figure 4c**). The dotted line marked by triangles corresponds to the retardance curve of an oocyte developing into a pre-embryo with lowest quality (abnormal fertilization) (**Figure 4d**).

Based on the PN scores of the pre-embryos after fertilization of oocytes, the mean retardance magnitudes of meiotic spindles were calculated for all oocytes, including oocytes that were later used for transfer and those non-transferred, in each subgroup analysed in the Giessen cohort. The mean age of patients was 32.5 ± 4.4 years (**Table 1**) and was similar between groups giving rise to pre-embryos of good or mediocre quality (**Table 2**). In contrast, the mean retardance magnitude of meiotic spindles was positively related to pronuclear scores of the pre-embryos (**Table 2**). Oocytes of group 1 forming embryos with a PN score of A or B contained a highly birefringent spindle with a mean retardance magnitude of 1.72 nm, while mean retardance magnitude was 1.53 nm and 1.52 nm for subgroups 2 and 3 respectively, not different from each other but significantly lower ($P < 0.05$ and $P < 0.001$ respectively) compared with subgroup 1. The oocytes developing into embryos with poor quality (subgroup 4) had a spindle with an even lower retardance magnitude of only 1.39 nm (**Table 2**), significantly lower compared with subgroup 1 and 3 ($P < 0.001$). The retardance magnitude of subgroup 4 was nearly 20% lower compared with group 1.

The pole-to-pole distance of the metaphase II spindle in living human oocytes was also analysed by PolScope microscopy in the four sub-groups. Mean spindle length was similar in subgroups 1, 2 and 3. However, a significantly shorter spindle was characteristic for oocytes developing to embryos with poor quality (subgroup 4) compared with the other three groups (**Table 2**, $P \leq 0.001$). Overall, mean retardance was 1.55 nm for all oocytes analysed. The mean retardance of oocyte spindles in oocytes selected for transfer after ICSI was 1.66 nm, significantly higher compared with all non-transfer oocytes/embryos (1.49 nm; **Table 3**).

Selection of embryos for transfer according to PN score enriched for oocytes with comparatively longer spindles since pole-to-pole distance was significantly shorter in non-transfer oocytes compared with transfer oocytes ($12.3 \pm 1.8 \mu\text{m}$ versus $12.8 \pm 1.9 \mu\text{m}$; one-way ANOVA: significantly different, $P < 0.005$).

Maternal age and spindle organization

For the Giessen cohort, mean maternal age of patients having oocytes with a highly birefringent spindle (mean retardance over 1.55 nm) was compared with that giving

rise to oocytes with a spindle with low birefringence (mean retardance below 1.55 nm). Mean age of patients with highly birefringent spindle was 32.3 ± 4.1 (found in 324 of the total of 676 oocytes from the cohort), while that with low birefringence was 31.5 ± 4.1 (in 352 oocytes); there was no significant difference for this group of patients, in which 4–20 oocytes were available for analysis. Mean retardance of spindles was also compared between all oocytes from patients older than 36 years (26 from 103 cycles), those aged 31–35 years (in 37 cycles) and those aged ≤ 30 years (in 40 cycles) (**Table 3**). Mean retardance of spindles in all transfer and non-transfer oocytes of patients older than 36 years (132 oocytes) was 1.53 ± 0.44 nm, not much different from that in intermediate age (31–35 years; 273 oocytes, 1.54 ± 0.42 nm) or younger patients (≤ 30 years; 271 oocytes, 1.58 ± 0.49 nm). Comparing retardance of transfer oocytes showed that there was a tendency for a lower mean retardance of the spindle in oocytes from aged women (>36 years) selected for transfer (60 oocytes; 1.58 nm; **Table 3**) compared with those in the two younger age groups (1.69 nm and 1.70 nm, for 94 and 100 oocytes respectively), but this did not reach statistical significance. Mean retardance was very similar between non-transfer oocytes from all three age groups implying that the average retardance and spindle quality of the most superior quality oocyte in a stimulated cycle was possibly influenced by maternal age but that the overall quality was not much affected by maternal age in the Giessen cohort, excluding patient groups who were poor responders with four or fewer oocytes, and with only few patients aged ≥ 38 years in the study group (8 of 103; 7.8% of all).

Clinical pregnancy in oocytes with lower or higher mean retardance of spindle

In the Giessen cohort, the mean retardance of spindles in oocytes, which were selected at pronuclear stage for transfer after ICSI (254 oocytes) was significantly higher, compared with oocytes giving rise to pre-embryos, which were not selected for transfer according to their comparatively poorer PN score (422 oocytes) ($1.66 \text{ nm} \pm 0.43$ versus $1.49 \text{ nm} \pm 0.44$, $P < 0.001$; **Table 3**). Of the total of 268 transfer oocytes, 254 possessed a spindle (**Table 1**). There was no significant difference in mean retardance of spindles between transfer oocytes giving rise to conception cycles versus those contributing to a non-conception cycle ($1.65 \text{ nm} \pm 0.43$ versus $1.67 \text{ nm} \pm 0.44$; **Table 1**). There was also no significant difference in mean retardance magnitude of spindles in non-transfer oocytes between conception cycles (CC) versus non-conception cycles (NCC) cycles ($1.46 \text{ nm} \pm 0.43$ versus $1.50 \text{ nm} \pm 0.46$) or in the total cohort of oocytes obtained by individual patients, including transfer and non-transfer oocytes between CC and NCC oocytes ($1.53 \text{ nm} \pm 0.44$ versus $1.56 \text{ nm} \pm 0.46$; **Table 2**). There was also no other parameter, such as number of follicles or number of polar body oocytes, or fertilization rate, which could be identified to be distinct between the CC and NCC group (**Table 1**).

However, more than one embryo of the same or a different PN score (2–3 embryos) was actually transferred in each cycle. Therefore, grouping was carried out according to transfers, in which either two to three good PN-score embryos (PN

score A–C, corresponding to group 1 and 2 in **Table 2**) were present or only one good pre-embryo together with embryos of PN score D–E (corresponding to group 3 and 4 in **Table 2**), or transfers in which only two to three embryos of lowest PN scores (D and E) were present. About 43–47% of those cycles with at least one good PN score embryo of PN score A–C led to a conception (**Table 4**). In contrast, only 31.4% of cycles involving low PN score embryos resulted in a conception. The percentage of pre-embryos contributing to conceptions with transfer of only two to three low PN score embryos was significantly lower compared with those with at least one embryo with PN score A–C (**Table 4**). Furthermore, mean retardance of the spindle was lowest in the group with no good PN score embryo at transfer (1.55 ± 0.36 nm), and was significantly ($P < 0.05$) lower compared with the groups with one good embryo (1.63 ± 0.45 nm) and with two or three good pre-embryos with score A–C in transfers (1.78 ± 0.47 nm; **Table 4**). Mean spindle length was similar in all three groups. Thus, oocytes with low birefringent spindles appeared to develop frequently into low PN score pre-embryos after ICSI and contributed significantly less frequently ($P < 0.05$) to a conception compared with those with highly birefringent spindles.

Milano laboratory data

Retardance magnitude and pole-to-pole distance of meiotic spindle of human oocytes

The mean retardance magnitude of meiotic spindles was also calculated for a small sample of oocytes, which were obtained from stimulated cycles in the Milano laboratory. According to Italian law, a maximum of three oocytes were fertilized by ICSI and were later analysed for pronuclear score according to criteria described by Tesarik and Greco (1999). Mean retardance of spindles was analysed for those oocytes producing a pre-embryo with highest quality (score 0) and compared with average retardance of oocytes giving rise to lower embryo quality (score 1, score 2 and score 3–5) (**Table 5**). As in the Giessen study, there was a tendency that more oocytes producing pre-embryos of highest quality (score 0) had high spindle retardance (2.15 ± 0.86 nm) and those developing into embryos with mediocre quality (group 2, mean retardance of 1.96 ± 0.39 nm; group 3, mean retardance of 1.86 ± 0.67 nm) or poor quality (e.g. score 3–5; group 4) had lower mean retardance (1.59 ± 0.35 nm; **Table 5**). When arbitrarily divided into oocytes with a mean retardance of <1.6 nm only 16.67% of oocytes developed into a pre-embryo with highest score 0 while 29.27% of oocytes with retardance ≥ 1.6 nm developed into pre-embryos with highest PN score.

Maternal age and spindle organization

In the Milano cohort, when grouped according to age, there was a more pronounced tendency for oocytes from younger patients (≤ 30 years, $n = 7$) to possess spindles of higher mean retardance (2.19 ± 1.06 nm) compared with those of intermediate age (31–35 years; $n = 10$; 2.01 ± 0.6 nm, and 36–39 years; $n = 9$; 1.77 ± 0.37 nm) and those of the most advanced age (≥ 40 years; $n = 2$; 1.65 ± 0.39 nm). However, due to the very limited sample size, statistical analysis was not possible.

Clinical pregnancy in oocytes with lower or higher mean retardance of the spindle

With respect to implantation, nine of 59 pre-embryos contributed to a conception assessed as gestational sac (**Table 5**). Four of 15 oocytes/embryos in the Milano cohort (26.7%) that had a PN score of 0 with the highest mean retardance by the spindle exhibited a gestational sac (**Table 5**), while three and two of the 16 and 20 pre-embryos of intermediate PN score 1 and 2 that were transferred contributed to a conception (18.8 and 10.0% of all in the group respectively). None of the group 4 pre-embryos with poorest quality and lowest mean retardance of the spindle contributed to a conception. Mean retardance of oocytes contributing to an embryo with best PN score that were in conception cycles was 3.02 ± 1.05 nm, higher compared with those in group 2 or 3 that had a lower PN score (2.46 ± 0.48 nm and 2.68 ± 0.14 nm, respectively, **Table 5**). Most importantly, there was a significant difference between mean retardance of the spindle in oocytes contributing to a conception cycle (2.76 ± 0.73 nm) compared with those in a non-conception cycle (1.78 ± 0.50 nm; $P = 0.002$; **Table 5**).

Discussion

Presence of a spindle as predictive parameter in oocyte selection

Several previous studies analysed the significance of expression of a spindle or absence of a birefringent spindle on oocyte developmental potential and conception. However, it is so far unknown whether oocytes with a large spindle or one with especially dense and highly ordered microtubule fibres have a higher chance of contributing to a conception cycle compared with ones with a smaller and/or less organized spindle. The main purpose of this study was therefore to assess spindle quality of PB oocytes from ICSI cycles non-invasively in a quantitative rather than mere qualitative approach with respect to pre-embryo quality employing PolScope microscopy and PN scoring. From the larger cohort in the Giessen laboratory, it appears that most of the in-vivo matured oocytes expressed a birefringent spindle (82.4%) comparable with observations by other groups (e.g. Wang *et al.*, 2001a,b; Moon *et al.*, 2003; Rienzi *et al.*, 2003). In agreement with previous studies (Wang *et al.*, 2001a,b; Moon *et al.*, 2003), oocytes without spindle had an overall lower capacity to develop into a normal embryo, and absence of a birefringent spindle was associated with a significantly higher risk for fertilization failure compared with oocytes with spindle ($P < 0.001$). Moreover, fertilization rate and percentage of oocytes forming a pre-embryo with 'good' PN score was higher in the oocytes with spindle compared with those without spindles. The present analysis confirmed thus that mere study of spindle expression provided information on oocyte quality but was still of limited value for oocyte selection in IVF since the majority of oocytes possessed a birefringent spindle apparatus.

Displacement of spindle in scoring for oocyte quality

Another approach to identify high and/or low quality oocytes

is based on analysis of spindle position relative to the first polar body. There is an ongoing debate on the significance and origin of spindle displacement from the first polar body with respect to developmental potential. In the present study, a larger deviation angle of $>40^\circ$ of the metaphase II spindle positively related to the rate of fertilization failure but not to the rate of multipronuclear formation as might be expected by damage of the spindle apparatus during sperm microinjection. The spindle was essentially always located close to the PB in denuded in-vitro matured human oocytes (data not shown). It has been suggested that displacement may be mainly caused by the mechanical stress by manipulation at or after oocyte retrieval rather than related to reduced oocyte quality (Rienzi *et al.*, 2003). In the present study, a lower number of oocytes had spindles displaced for $>40^\circ$ away from the PB compared with other reports (e.g. Cooke *et al.*, 2003), and a fair proportion of the fertilized oocytes with displaced spindles (34.2% of all 111 oocytes with displaced spindle and 45.8% of the ones developing to 2PN zygotes) still developed into pre-embryos with high or mediocre PN score (PN score A–C). In conclusion, the assessment of spindle positioning was not very predictive of oocyte quality in the Giessen cohort, and it is therefore believed that analysis of displacement may only be useful in cases where the creation of supernumerary embryos is to be avoided (Rienzi *et al.*, 2003).

Quantitative assessment of spindle birefringence in oocytes

By analysis of birefringence of kinetochore microtubules in images through optical planes through spindles of fairly flat crane fly spermatocytes by PolScope, LaFountain and Oldenbourg (2004) recently succeeded in calculating the numbers of microtubules attached to each individual homologous chromosome in bivalents with respect to chromosome alignment. Such optical sectioning of spindles was not possible in the large and spherical human oocyte with the standard microscope equipment and 40× or 20× objective lenses used in this study. Calculation of mean retardance of the spindle does not provide an absolute quantitative assessment of numbers of microtubule fibres in spindles and density of fibres at each point of measurement. One has to keep in mind that the oocyte spindle is not a homogenous organelle and is highly dynamic. Previous quantitative measurements assessed major rather than average retardance, e.g. upon thawing after freezing of human oocytes (Bianchi *et al.*, 2005) or zona major retardance before and after fertilization (Pelletier *et al.*, 2004). The line scan performed in the present study was in the middle of the spindle body, along the long axis of the spindle, and thus should give information on mean retardance throughout the spindle body. However, longer or shorter segments of chromosome arms might be located in this region and might have contributed to background or to a lower value in the assessment of average retardance. Nevertheless, overall density of fibres is not affected and values obtained should be representative. Because there are differences in optics and culture conditions it is difficult to standardize measurements and compare absolute values between different laboratories. Liu *et al.* (2000b) calculated mean retardance in oocyte spindles of the mouse after parthenogenetic activation or fertilization either in the whole spindle area or in line scans through the spindle equator, basically associated with the same problems.

They found for both methods of calculation different absolute values of mean retardance, but concordantly and reproducibly observed significant increases in mean retardance values upon activation (Liu *et al.*, 2000b; Navarro *et al.*, 2005). From experience, it appears that calculation of mean retardance of the spindle along a line scan under standard conditions in a unit helps to minimize the experimental variables and provides a good semi-quantitative estimate of the overall order and density of tubules within the spindle apparatus in assessment of oocyte quality.

Relationship between mean retardance and conception

The data from the large cohort of oocytes from the Giessen IVF unit as well as those from the much smaller cohort of the Milano group suggest that oocytes with low mean retardance magnitude of light by the spindle have a lower developmental potential in terms of forming a low PN score pre-embryo and failing to contribute to a conception cycle more often compared with the ones with spindles producing high mean retardance of light. There was a significantly higher retardance in oocytes contributing to a conception compared with those failing to induce pregnancy ($P = 0.002$ in the Milano cohort). Legal restriction prohibited both laboratories from further verifying the finding by following the embryo fate from oocytes that were fertilized but excluded from transfer that might or might not have developed to blastocysts. Embryo quality could only be assessed by PN scoring that was performed on all embryos obtained by ICSI of all mature oocytes in the Giessen centre. Further fate could be analysed only in those used in selection of pre-embryos for transfer due to the strict legal regulations in Germany. Selection for best PN scores was positively related to mean retardance in the Giessen cohort, while, *vice versa*, when selection was by oocyte morphology in the Milano centre high retardance of spindles in oocytes was also positively related to PN scores. It is feasible that the difference in patient age, which was greater in the Milano compared with the Giessen group, may be responsible for the more pronounced maternal age dependent differences in mean retardance values in the Milano compared with the Giessen cohort, and the failure to detect relationships between mean retardance of the spindle in all oocytes between CC and NCC cycles in the Giessen cohort.

PN scoring and assessment of spindle birefringence

The pronuclear scoring systems used presently account for pronuclear size and position, and number and alignment of NPB (nucleolar precursor bodies) as originally defined by Scott and Smith (1998) and in a modified form by Tesarik and Greco (1999), including morphological features such as clear cytoplasmic halo, and have been employed in a modified form routinely in both IVF centres. Both scoring systems are commonly and successfully used to assess embryo quality (Scott, 2003; Payne *et al.*, 2005), and select pre-embryos for transfer in countries like Germany (Zollner *et al.*, 2002). Several recent studies suggest that pre-embryos with low PN score are more likely to develop into chromosomally aberrant embryos compared with those with high PN score (Kahraman *et al.*, 2002; Chen *et al.*, 2003; Gianaroli *et al.*, 2003; Balaban *et al.*, 2004; Edirisinghe *et al.*, 2005). In accordance, oocytes with spindles

with low birefringence that develop to pre-embryos with lower PN scores may be more frequently abnormal compared with healthy oocytes with robust spindles that presumably have highly organized microtubular fibres and normal chromosomal constitution. The observations on conception involving transfers with high and low PN score embryos confirmed that PN scoring was positively related to conception cycles and that transfers with embryos of lower scores were associated with failures to conceive much more frequently than those involving better PN score embryos, irrespective of the scoring systems. Concomitantly, mean magnitude of retardance of light by the spindle was positively related to conception cycles. The limited size of the samples in both centres and the relative heterogeneity of patients, e.g. more patients of advanced age in the Milano group compared with the Giessen group, precluded comparison of effectiveness between PN scoring according to the Scott and Smith (1998) or the Tesarik and Greco (1998) criteria. From the present data, it appears that effectiveness of scoring according to mean retardance rather than PN scoring may overcome limitations of the PN scoring systems and thus contribute to identification of high quality oocytes before ICSI.

Birefringence of the meiotic spindle and maternal age

From the large cohort of oocytes in Giessen that had between 4 and 20 oocytes at retrieval, there was no evidence for a significant relationship between length or mean retardance by the spindle and maternal age when comparing age groups of patients ≤ 30 years, 31–35 years and ≥ 36 years (Tables 2 and 3). There was a minor age-related reduction in mean retardance value of the transfer oocytes from aged patients but it did not reach statistical significance. Mean retardance of spindles was also calculated in all oocytes of individual patients with respect to high and low numbers of retrieved oocytes or cycles that had or had no immature GV stage at retrieval in the Giessen cohort (data not shown), and did not detect any relationships. This suggests that overall oocyte quality in terms of spindle retardance is not directly influenced by stimulation response resulting in maturation of few or large numbers of oocytes. The opportunity may have been missed to detect age-associated correlations in the Giessen cohort since cycles with very low or high numbers of oocytes were excluded from the analysis and there were very few patients aged ≥ 38 years (8 in 103 cycles, only 7.8% of all patients). For instance, it is feasible that predominantly poor responders with four or even fewer oocytes may have short spindles with low mean retardance, or that stimulation of maturation of more than 20 oocytes has adverse effects on the overall quality of the retrieved oocytes. The short protocol used in stimulation of aged patients in the Giessen cohort may also optimize follicular and oocyte maturation *in vivo*, such that some age-related effects are reduced.

The small cohort of patients in the Milano centre included more patients of advanced age (11 of 28 of ≥ 36 years, corresponding to 39.3%) compared with the Giessen cohort (25.2% ≥ 36 years), and cycles with few oocytes. The Milano data therefore may be better suited to assess age-effects, and data support the notion that overall spindle quality may decline with advancing age, in accordance with immunofluorescent studies and previous reports (Battaglia *et al.*, 1996; De Santis *et al.*, 2005). Further work is required to analyse correlations between age and spindle morphology, and treatment and handling protocols,

in particular, mean retardance in correlation to chromosomal constitution of human oocytes.

Prospects of quantitative assessment of spindle birefringence

Low retardance due to spindle abnormality and chromosomal imbalance may be just the tip of the iceberg indicating deficiencies in the oocytes causing development of low quality embryos. Other problems, for instance, changes in expression pattern (Hamatani *et al.*, 2004) or failures to mediate activation of zygotic gene expression may all contribute to failures in conception from such oocytes. Thus, overall mean retardance by spindles in all oocytes or in transfer oocytes that led to a conception and those in a non-conception cycle was not different in the Giessen cohort supporting the notion of additional factors that are important in supporting conception. Especially in the younger patient group, deficiencies other than spindle malfunction might be prevalent to affect developmental potential. However, from observations, it appears that oocytes chosen for transfer after ICSI by PN score were, in fact, the best ones from the individual patient, since mean retardance of spindles of transfer oocytes was significantly higher ($P < 0.001$) compared with those of the non-transfer oocytes. According to observations, the oocytes with the most robust spindles were therefore selected for transfer by employing the PN scoring. The data from the two centres based on either selection by PN scoring (Giessen) or rather selection by oocyte morphology before ICSI (Milano) suggest that both methods for selection appear equivalent, and that selection by spindle analysis may be as powerful as or even better than PN scoring to identify healthy oocytes in a patient cohort. This notion was supported by the analysis of conceptions involving transfers with at least one high PN score embryo in Giessen and those with low PN score embryos that came from oocytes with spindles of significantly lower mean retardance ($P < 0.05$). Combined, the limited data of the study in the two centres agree to suggest that mean retardance of the spindle positively correlates with conception cycles. A comparison of outcomes with PN scoring or spindle analysis has now to be confirmed by a prospective study to evaluate the effectiveness of each method. The majority of the oocytes analysed in this study of spindle retardance was also previously assessed for thickness and retardance of the zona pellucida (Shen *et al.*, 2005a). In this cohort of oocytes, those with a high retardance of the inner layer of the zona pellucida were significantly more often contributing to a conception cycle compared with those with low retardance of the zona inner layer. Attempts are currently being made to improve methodologies for quick and efficient quantitative assessment of both, mean retardance by the spindle and the zona inner layer. Combined data obtained from both measurements should provide a straightforward approach to score oocytes in ICSI cycles non-invasively before fertilization. Such screening might present an option to improve selection, especially in conditions where ethical and legal regulations restrain suitability of post-fertilization screening.

References

- Balaban B, Yakin K, Urman B *et al.* 2004 Pronuclear morphology predicts embryo development and chromosome constitution. *Reproductive BioMedicine Online* **8**, 695–700.
- Battaglia DE, Klein NA, Soules MR 1996 Changes in centrosomal domains during meiotic maturation in the human oocyte. *Molecular Human Reproduction* **2**, 2217–2222.
- Bianchi V, Coticchio G, Fava L *et al.* 2005 Meiotic spindle imaging in human oocytes frozen with a slow freezing procedure involving high sucrose concentration. *Human Reproduction* **20**, 1078–1083.
- Boiso I, Marti M, Santalo J *et al.* 2002 A confocal microscopy analysis of the spindle and chromosome configurations of human oocytes cryopreserved at the germinal vesicle and metaphase II stage. *Human Reproduction* **17**, 1885–1891.
- Chen CK, Shen GY, Horng SG *et al.* 2003 The relationship of pronuclear stage morphology and chromosome status at cleavage stage. *Journal of Assisted Reproduction and Genetics* **20**, 413–420.
- Cooke S, Tyler JP, Driscoll GL 2003 Meiotic spindle location and identification and its effect on embryonic cleavage plane and early development. *Human Reproduction* **18**, 2397–2405.
- Corcoran D, Fair T, Loneragan P 2005 Predicting embryo quality, mRNA expression and the preimplantation embryo. *Reproductive BioMedicine Online* **11**, 340–348.
- De Santis L, Cino I, Rabellotti E 2005 Polar body morphology and spindle imaging as predictors of oocyte quality. *Reproductive BioMedicine Online* **11**, 36–42.
- Edirisinghe WR, Jemmott R, Smith C *et al.* 2005 Association of pronuclear Z score with rates of aneuploidy in *in vitro*-fertilised embryos. *Reproduction, Fertility and Development* **17**, 529–534.
- Eichenlaub-Ritter U 1998 Genetics of oocyte ageing. *Maturitas* **30**, 143–169.
- Eichenlaub-Ritter U, Peschke M 2002 Expression in *in-vivo* and *in-vitro* growing and maturing oocytes, focus on regulation of expression at the translational level. *Human Reproduction Update* **8**, 21–41.
- Eichenlaub-Ritter U, Vogt E, Yin H *et al.* 2004 Spindles, mitochondria and redox potential in ageing oocytes. *Reproductive BioMedicine Online* **8**, 45–58.
- Eichenlaub-Ritter U, Shen Y, Tinneberg HR 2002 Manipulation of the oocyte, possible damage to the spindle apparatus. *Reproductive BioMedicine Online* **5**, 117–124.
- Gianaroli L, Magli MC, Ferraretti AP *et al.* 2003 Pronuclear morphology and chromosomal abnormalities as scoring criteria for embryo selection. *Fertility and Sterility* **80**, 341–349.
- Hamatani T, Falco G, Carter MG 2004 Age-associated alteration of gene expression patterns in mouse oocytes. *Human Molecular Genetics* **13**, 2263–2278.
- Kahraman S, Kumtepe Y, Sertyel S *et al.* 2002 Pronuclear morphology scoring and chromosomal status of embryos in severe male infertility. *Human Reproduction* **17**, 3193–3200.
- Keefe D, Liu L, Wang W *et al.* 2003 Imaging meiotic spindles by polarization light microscopy, principles and applications to IVF. *Reproductive BioMedicine Online* **7**, 24–29.
- LaFountain JR Jr, Oldenbourg R 2004 Maloriented bivalents have metaphase positions at the spindle equator with more kinetochore microtubules to one pole than to the other. *Molecular Biology of the Cell* **15**, 5346–5355.
- Liu L, Oldenbourg R, Trimarchi JR *et al.* 2000a A reliable, noninvasive technique for spindle imaging and enucleation of mammalian oocytes. *Nature Biotechnology* **18**, 223–225.
- Liu L, Trimarchi JR, Oldenbourg R *et al.* 2000b Increased birefringence in the meiotic spindle provides a new marker for the onset of activation in living oocytes. *Biology of Reproduction* **63**, 251–258.
- Moon JH, Hyun CS, Lee SW *et al.* 2003 Visualization of the metaphase II meiotic spindle in living human oocytes using the PolScope enables the prediction of embryonic developmental competence after ICSI. *Human Reproduction* **18**, 817–820.
- Navarro PA, Liu L, Trimarchi JR *et al.* (2005) Noninvasive imaging of spindle dynamics during mammalian oocyte activation. *Fertility and Sterility* **83** (Suppl. 1), 1197–1205.
- Oldenbourg R 1996 A new view on polarization microscopy. *Nature* **381**, 811–812.
- Pandian Z, Templeton A, Serour G *et al.* 2005 Number of embryos for transfer after IVF and ICSI, a Cochrane review. *Human*

- Reproduction* **20**, 2681–2687.
- Payne JF, Raburn DJ, Couchman GM *et al.* 2005 Relationship between pre-embryo pronuclear morphology (zygote score) and standard day 2 or 3 embryo morphology with regard to assisted reproductive technique outcomes. *Fertility and Sterility* **84**, 900–909.
- Pelletier C, Keefe DL, Trimarchi JR 2004 Noninvasive polarized light microscopy quantitatively distinguishes the multilaminar structure of the zona pellucida of living human eggs and embryos. *Fertility and Sterility* **81**, 850–856.
- Rienzi L, Ubaldi F, Iacobelli M *et al.* 2005 Meiotic spindle visualization in living human oocytes. *Reproductive BioMedicine Online* **10**, 192–198.
- Rienzi L, Ubaldi F, Martínez F *et al.* 2003 Relationship between meiotic spindle location with regard to the polar body position and oocyte developmental potential after ICSI. *Human Reproduction* **18**, 1289–1293.
- Schaap CJ, Forer A 1984 Video digitizer analysis of birefringence along the lengths of single chromosomal spindle fibres. I. Description of the system and general results. *Journal of Cell Science* **65**, 21–40.
- Scott L 2003 Pronuclear scoring as a predictor of embryo development. *Reproductive BioMedicine Online* **6**, 201–214.
- Scott LA, Smith S 1998 The successful use of pronuclear embryo transfers the day following oocyte retrieval. *Human Reproduction* **13**, 1003–1013.
- Shen Y, Stalf T, Mehnert C *et al.* 2005a High magnitude of light retardation by the zona pellucida is associated with conception cycles. *Human Reproduction* **20**, 1596–1606.
- Shen Y, Betzendahl I, Sun F *et al.* 2005b Non-invasive method to assess genotoxicity of nocodazole interfering with spindle formation in mammalian oocytes. *Reproductive Toxicology* **19**, 459–471.
- Simerly C, Dominko T, Navara C *et al.* 2003 Molecular correlates of primate nuclear transfer failures. *Science* **300**, 297.
- Stalf T, Herrero J, Mehnert C *et al.* 2002 Influence of polarization effects in ooplasm and pronuclei on embryo quality and implantation in an IVF program. *Journal of Assisted Reproduction and Genetics* **19**, 355–362.
- Tang CJ, Hu HM, Tang TK 2004 NuMA expression and function in mouse oocytes and early embryos. *Journal of Biomedical Science* **11**, 370–376.
- Tesarik J, Greco E 1999 The probability of abnormal preimplantation development can be predicted by a single static observation on pronuclear stage morphology. *Human Reproduction* **14**, 1318–1323.
- Volarek K, Sheehan L, Goldfarb J *et al.* 1998 The meiotic competence of in-vitro matured human oocytes is influenced by donor age, evidence that folliculogenesis is compromised in the reproductively aged ovary. *Human Reproduction* **13**, 154–160.
- Wang WH, Meng L, Hackett RJ *et al.* 2001a Limited recovery of meiotic spindles in living human oocytes after cooling–rewarming observed using polarized light microscopy. *Human Reproduction* **16**, 2374–2378.
- Wang WH, Meng L, Hackett RJ *et al.* 2001b Developmental ability of human oocytes with or without birefringent spindles imaged by PolScope before insemination. *Human Reproduction* **16**, 1464–1468.
- Zollner U, Zollner KP, Hartl G *et al.* 2002b The use of a detailed zygote score after IVF/ICSI to obtain good quality blastocysts, the German experience. *Human Reproduction* **17**, 1327–1333.

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