U.PORTO
INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

Dissertação
Mestrado Integrado em Medicina

FATORES PREDITIVOS DE RECÉM-NASCIDO EM CICLOS DE MICROINJEÇÃO INTRACITOPLASMÁTICA COM ESPERMATOZÓIDES DO EJACULADO

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Porto, 21 de Maio de 2017

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PUBLICAÇÕES

Comunicações Nacionais:

Posters:

21 Congresso de Obstetrícia e Ginecologia. Convento de S. Francisco, Coimbra. 1-4 June.

Submitted: January

Accepted: April

Comunicações orais:

I Simpósio de Diabesidade e Fertilidade 22-23 September.

Submitted: Mai

Accepted: July

Publicações Nacionais:

Acta Obstetrica e Ginecologica Portuguesa.

Submitted: January

Accepted: April

Comunicações Internacionais

Posters:

33 rd Annual Meeting, European Society of Human Reproduction and Embryology (ESHRE). Geneva, Switzerland. 2-5 July.

Submitted: January

Accepted: April

Publicações Internacionais:

Human Reproduction.

Submitted: January

Accepted: April

Published: June

Reproductive Sciences.

Submitted: May

Accepted: under revision

Journal of Basic and Clinical Reproductive Sciences.

Submitted: May

AGRADECIMENTOS

Deixo aqui o meu agradecimento a algumas das muitas pessoas que tornaram possível a realização deste trabalho da melhor forma possível.

Inicio por agradecer ao meu orientador, Prof. Dr. Mário Sousa, por toda a confiança que depositou em mim e neste trabalho. Foi uma honra e um prazer trabalhar nesta área e admiro e respeito toda a seriedade, rigor científico e entrega em cada bocado de ciência que produz. Foi uma inspiração.

Agradeço ao Prof. Dr. Alberto Barros, ao Centro de Genética da Reprodução Prof. Alberto Barros e ao seu corpo clínico e restantes funcionários, por toda a ajuda e pela oportunidade de trabalhar com esta base de dados tão representativa.

Queria agradecer ao Tiago Azevedo, que sempre se mostrou disponível para qualquer necessidade e que fez parte da revisão final do meu artigo. A ele um enorme obrigado pela seriedade e preocupação com o rigor do meu trabalho em termos estatísticos e linguísticos.

Não posso deixar de agradecer ao Ricardo Rodrigues, pelo apoio incondicional, por toda a ajuda e pelas inúmeras revisões finais deste artigo. Um enorme obrigado pela paciência e pelo pragmatismo, sempre tão essenciais em momentos destes.

Por último, mas não menos importante, queria agradecer à minha mãe, Ilídia Castro, e em especial ao meu pai, Paulo Pinto, pela revisão final do meu trabalho. A vossa enorme confiança em mim, disponibilidade e todo o apoio emocional foram indispensáveis para a realização deste artigo.

A todos aqueles não discriminados, amigos, familiares, colegas e profissionais de saúde um obrigado por terem feito parte da minha formação e da minha vida.

ARTIGO

Clinical outcome predictors in ICSI cycles with ejaculated sperm: report on 3844 consecutive treatment cycles

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Abstract

Background: Although numerous studies have been undertaken to establish predictive clinical factors associated with a higher likelihood of attaining a live birth, there is still no consensus, and its generally discussed only specific factors. The aim of the present study was to assess whether any clinical characteristics in intracytoplasmic sperm injection cycles are associated with an increased likelihood of live birth delivery.

Methods: Retrospective evaluation of 3844 treatment cycles with intracytoplasmic sperm injection using homologous ejaculated sperm. The study was conducted during 11 years (from 2003 to 2014). Cycles were divided in two groups according to live birth delivery outcomes: 993 cycles (25.8%) with newborn and 2851 cycles (74.2%) without newborn. Comparisons were performed regarding detailed demographic, stimulation, embryologic and clinical characteristics.

Results: A higher live birth delivery rate was associated to lower age and lower basal follicle stimulating hormone levels; lower total dose of gonadotropins, time of stimulation and dose of human chorionic gonadotropin; higher number of follicles and estradiol levels; higher number of aspirated oocytes and mature oocytes, higher rates of embryo cleavage, high quality embryos and blastocysts.

Conclusions: Several indicators appeared associated with an increase in the likelihood of live birth, eliciting the determination of cut-offs for female age, time of infertility, basal follicle stimulating hormone, estradiol levels, number of follicles, retrieved oocytes, mature oocytes, high quality embryos, embryos transferred and the day of embryo transfer. Antagonist and agonist protocols, as well as recombinant follicle stimulating hormone and human menopausal gonadotropin, gave similar outcomes, and thus the decision for one or another should be patient personalized. Spermiogram values did not affect live birth. These findings are thus considered relevant for clinical guidance and patient information regarding the likelihood of live birth.

Keywords: Intracytoplasmic sperm injection, Assisted reproductive treatment predictors, Clinical outcomes, Newborn outcomes

Background

The possibility of fathering for patients with compromised spermiogram values became a reality with the development of ICSI (Intracytoplasmic sperm injection) [Swain et al., 1992; van Steirteghem et al., 1993a,b; Tesarik and Sousa, 1995]. Although numerous studies have been undertaken to establish predictive clinical factors associated with a higher likelihood of attaining a live birth, there is still no consensus [van Loendersloot et al., 2010].

Regarding ovarian stimulation, the recent advances with gonadotropin releasing hormone (GnRH) antagonists and agonists elicited the development of personalized stimulation protocols [Huirne et al., 2007]. The subsequent appearance of ovarian hyperstimulation syndrome [Sousa et al., 2015] could later be reduced to near zero with GnRH agonist ovulation triggering, either associated with a strong luteal supplementation, or followed by embryo freezing for later embryo frozen thaw transfer [Humaidan et al., 2011]. In this context, several predictors have been raised, such as estradiol levels [Joo et al., 2010], progesterone levels [Kolibianakis et al., 2004], the number of follicles [Papanikolaou et al., 2006] and the number of oocytes aspirated [Steward et al., 2014]. Other success limiting factors appeared related to the embryo transfer technique [Schoolcraft, 2016], endometrial thickness [Kovaks et al., 2003], the number of embryos transferred [Pandian et al., 2013] and the day of embryo transfer [Glujosky et al., 2016].

Female infertility factors also limit assisted reproductive treatment (ART) outcomes, such as time of infertility [Niinimäki et al., 2015], karyotype anomalies, anatomic defects, tumors, tubar pathology, endocrinological disarrangements, polycystic ovarian syndrome, endometriosis, immunologic and trombophilic conditions and body mass index [Rizk et al., 2008]. Another female limiting factor is ovary aging. The ovarian reserve can be predicted based on basal FSH (follicle-stimulating hormone) levels [van Loendersloot et al., 2010], anti-Mullerian hormone levels [Reijnders et al., 2016] and antral follicle count [Fleming et al., 2015]. However, ovary aging also conditions oocyte quality as, after follicle retrieval, oocytes may be immature [Beall et al., 2010] or dimorphic [Rienzi et al., 2011; Sousa et al., 2016]. Morphological inspection of the morphological oocyte is also a critical predictive factor, and the use of zona pellucida birefringence and metaphase II plaque positioning, as determined by specific optics [Montag et al., 2011], can improve oocyte selection.

Male infertility factors can also limit ART outcomes, such as karyotype anomalies, Y chromosome microdeletions, anatomic defects, tumors, endocrinological disarrangements, epididymis and vasa deferent occlusion, and spermatogenesis failure [Nieschlag et al., 2010]. In this context, spermiogram parameters have critical predictive importance [Mahat et al., 2016; Pereira et al., 2016], and several methods were developed to determine sperm aneuploidy [Nicopoullos et al., 2008], apoptosis [Almeida et al., 2005] and DNA fragmentation rates [Sá et al., 2015] to guide clinical decisions. Additionally, several methods were introduced to selectively isolate the best sperm for microinjection, such as the use of specific optics [Perdrix and Rives, 2013] and magnetic cell sorting [Bucar et al, 2015].

Other critical aspects that can influence ART outcomes include the optimization of laboratory ART rooms and personnel qualification [Magli et al., 2008; De los Santos et al., 2016], ART technical aspects [Rubino et al., 2016], the number of metaphase II oocytes, fertilization, embryo cleavage and the number of high quality embryos at day 3 [Sousa and Tesarik, 1994; Stoop et al., 2012]. Other important factors have been consistently improved, such as those related to embryo cryopreservation, amelioration of embryo culture media, extended embryo culture up to the blastocyst stage [Gardner and Lane, 2000; Swain and Smith, 2011; Sousa et al., 2012] and embryo dynamics screening [Rubio et al., 2014]. Finally, the use of preimplantation genetic diagnosis [Cooper and Jungheim, 2010; Mastenbroek et al., 2011] enabled couples not only to have their children free of diseases but also enabled to find and counteract the causes of implantation failures.

In order to give patients more reliable information and to clinicians better guidelines on the likelihood of attaining a live birth, we analysed 3844 ICSI cycles to uncover critical factors, with suggested cut-offs, using detailed demographic, stimulation and embryological data.

Methods

Ethics approval

According to the National Law on Medically Assisted Procreation and the National Council on Medically Assisted Procreation guidelines, databases were used after patient informed and written consent.

Patients

Retrospective evaluation of 3844 treatment cycles using homologous ejaculated sperm and ICSI. Seropositive, anejaculation, retrograde ejaculation, preimplantation genetic diagnosis and Y chromosome microdeletions cases were excluded. The period of analysis was between 25-09-2003 and 17-01-2014 (11 years).

Karyotype analysis

Karyotypes were evaluated using G-banding with analysis of at least 30 metaphases from peripheral blood lymphocytes, according to general protocols [Rooney and Czepulkowski, 1997]. There were 10 Klinefelter cases (3 NB from 3 distinct cases).

Stimulation Protocol

Women underwent controlled ovarian hyperstimulation with a GnRH antagonist protocol in 92.2% of the cases (Merck Serono, Geneve, Switzerland; Organon, Oss, Netherlands) and with an agonist protocol in 7.8% of the cases (Sanofi Aventis, Frankfurt, Germany). For stimulation, recombinant follicle stimulating hormone (rFSH) was used in 56.4% of the cases (Organon; Merck Serono); human menopausal gonadotropin (HMG) was added to rFSH in 42.9% of the cases (Ferring, Kiel, Germany); and only HMG in 0.7% of the cases. Ovulation trigger was performed with human chorionic gonadotropin (HCG) in 79.8% of the cases (Organon); with recombinant HCG in 18.9% of the cases (Merck-Serono); and with a GnRH agonist in 1.3% of the cases (Sanofis, Aventis, Frankfurt, Germany; Ipsen Pharma Biotech, Signes France). Estradiol serum levels were assayed at the day of HCG or one day before [Huirne et al., 2007; Pinto et al., 2009].

Gamete and Embryo Handling

Media from Medicult (Jyllinge, Denmark) or Vitrolife (Kungsbacka, Sweden) were used. Microinjection was performed in a Hoffman optics inverted microscope (Nikon, Tokyo, Japan) equipped with Narishige micromanipulators (Tokyo, Japan), using Swemed micropipettes (Goteborg, Sweden) and under a described technique [Tesarik and Sousa, 1995]. Embryo quality was evaluated according to described methods [Vandervorst et al., 1998; Gardner et al., 2000]. Ultrasound-guided embryo transfer used a Sure View Wallace Embryo Replacement Catheter or Wallace malleable stylet (Smiths Medical Int, Kent, UK).

Luteal Supplementation

All patients had luteal supplementation with intravaginal administration of natural-micronized progesterone (Jaba, Besins Int, Montrouge, France; Effik, Bievres, France), from the day of oocyte retrieval. Implantation was confirmed by a rise in β-HCG serum twelve days after embryo transfer. Clinical pregnancy was established by ultrasound at seven weeks of gestation. When a GnRH agonist was used for triggering final oocyte maturation, luteal support was associated with oral estradiol (Novo Nordisk, Bagsvaerd, Denmark) and HCG (1500 IU) at the day of oocyte pick-up [Radesic and Tremellen, 2011].

Statistical Analysis

For statistical analysis the *IBM SPSS Statistics 22* program for Windows was used. Means were compared using t-Test for independent samples. Categorical variables were analysed using descriptive statistics and Chi square test, with continuity correction. All statistical tests were two-tailed, with significance level of 0.05 ($p < 0.05$).

Results

Patients and study groups

The 3844 ICSI cycles were divided in two groups according to their outcomes and compared regarding demographic, stimulation, embryologic, clinical and newborn (NB) characteristics: 993 cycles (25.8%) with NB and 2851 cycles (74.2%) without NB.

Demographic characteristics

Regarding demographic characteristics (Table 1), the NB group showed a significant lower mean female and male ages, a similar time of infertility, a significant higher number of cases with pure male factor and a significant lower number of cases with pure female and mixed infertility factors, and a significant lower mean level of basal FSH (bFSH). There were no significant differences regarding female and male karyotypes. The prevalence of chromosome abnormalities in the general population is about 0.5-1% [Turnpenny and Ellard, 2012]. The present results thus confirm that the rate of abnormal karyotypes is higher in infertile individuals [Sousa et al., 2016; Gekas et al., 2001].

Stimulation characteristics

Regarding stimulation characteristics (Table 1), the NB group manifested a significant lower mean total dose of gonadotropins and HCG used; a lower mean time of stimulation; a higher mean number of developed ovarian follicles; and higher mean serum estradiol (E2) levels.

Embryological and clinical outcomes

As expected, regarding embryological and clinical outcomes, the NB group (Table 2) showed significant higher mean values for all parameters analysed, which included the number of oocytes retrieved (COC), mature metaphase II oocytes (MII), fertilization rate (FR), embryo cleavage rate, high grade embryos at day 3, blastocyst rate, implantation rate (IR), biochemical (BP), clinical (CP) and ongoing (OP) pregnancy rates, without any cases of ectopic pregnancy or abortion.

Newborn outcomes

The characteristics of NB are given in Table 2 and Table 3. The rate of NB malformations was 1.3%, 1% major and 0.3% minor (Table 2). These rates are quite lower regarding population studies that revealed 2-5% of major and 10% of minor NB malformations [Turnpenny and Ellard, 2012]. About 80% of the NB had a term gestation and 65% a normal weight (Table 3).

Subgroup analysis

To uncover some thresholds on the likelihood of achieving a live birth deliver (LBD), several characteristics were analysed: female age, time of infertility, stimulation protocol, gonadotropins, follicles, number of retrieved COC, mature MII, high quality embryos, number of embryos transferred, day of embryo transfer, and sperm concentration, rapid progressive motility and normal morphology (Figs. 1-3).

The female age was divided into six stages (Fig. 1). The NB group showed a significant lower number of cases with ≥ 40 years, and no significant differences were observed in the younger stages. Most of the successful cases were found in women with < 35 years (61%) and < 39 years (89%) (Fig. 1 left). Results evidenced a continuous decrease in the likelihood of LBD with advancing female age, with the higher likelihood of LBD belonging to the age stages of < 30 years and 30-35 years (Fig. 1 right).

The time of infertility was divided into four stages (Fig. 1). No significant differences were observed except when the time of infertility was < 5 years (Fig. 1 left). Results evidenced a continuous decrease in the likelihood of getting a successful pregnancy with advancing time of infertility, with the higher likelihood of achieving a LBD being observed with < 5 years of infertility (Fig. 1 right).

Regarding the stimulation protocol the differences found were significant. The antagonist protocol was used in 90% of the cycles in the NB group and in 93% of the cycles in the no-NB group; whereas the agonist protocol was used in 10% of the cycles in the NB group and in 7% of the cycles in the no-NB group 187 (Fig. 1 left). Results evidenced a higher likelihood of attaining a LBD with the agonist protocol (Fig. 1 right).

In relation to gonadotropins, the NB group evidenced a significant higher number of cases that used only rFSH and a significantly lower number of cases where both rFSH and HMG were used, with no significant differences regarding the sole use of HMG (Fig. 1 left). There was a decrease in the likelihood of achieving a LBD from the use of rFSH, to rFSH+HMG and then to HMG, with the highest likelihood of LBD being observed in cases where only rFSH was used (Fig. 1 right).

For the analysis of follicles we divided cycles in 6 intervals (Fig. 1). Most of the successful cases presented >4 follicles (Fig. 1 left). The likelihood of a successful LBD increased with the number of follicles, being significantly lower when <4 follicles and between 4-9 follicles, and higher between 10-13 and 14-18 follicles (Fig. 1 right).

The number of retrieved COC was divided in six intervals (Fig. 2). The NB group showed a significant higher number of cases with 4-9 COC and 4-15 COC, a significant lower number of cases with ≤3 COC, and no significant differences when there were >10 COC retrieved, with most of the cases with NB presenting 4-15 COC (Fig. 2 left). There was a lower likelihood of LBD with ≤3 COC and a progressive higher likelihood of LBD up to 15 COC retrieved (Fig. 2 right).

The number of MII was divided in six intervals (Fig. 2). The NB group presented 4-9 MII in the majority of the cases, a significant lower number of cases with ≤3 MII and a significant higher number of cases with 4-15 MII, with no significant differences in the other intervals (Fig. 2 left). The likelihood of LBD was lower with ≤3 MII and progressively higher up to 15 MII (Fig. 2 right).

The number of high quality embryos at day 3 (AB) was divided in two groups (Fig. 2). The NB group showed a significant lower number of cases with <3 AB and a significant higher number of cases with ≥3 AB (Fig. 2 left). The likelihood of LBD was lower when <3 high quality embryos were obtained and higher in cases with ≥ 3AB (Fig. 2 right).

The number of embryos transferred was divided in four groups (Fig. 2). The NB group evidenced a significant lower number of cases with transfer of 1 or 3 embryos, and a significant higher number of cases with transfer of 2 embryos (Fig. 2 left). The likelihood of LBD decreased with the number of embryos transferred, being higher with transfer of 2 embryos (Fig. 2 right).

The day of embryo transfer was divided in five groups (Fig. 2). The NB group showed a significant higher number of cases with embryo transfer performed at day 5, and a significant lower number of cases with embryo transfer performed at day 2 and day 3 (Fig. 2 left). The likelihood of LBD increased with the day of embryo transfer, and was higher at day 5 (Fig. 2 right).

Sperm concentration, rapid progressive motility and normal morphology were divided in five intervals (Fig. 3). For normal morphology, although the NB group presented ≥ 20 M sperm/ml in the majority of the cases, with significance attained with ≥ 5M sperm/ml (Fig. 3 left), the likelihood of LBD decreased with sperm concentration (Fig. 3 right). For rapid progressive motility, although in the NB group the majority of cases presented ≥ 10%, with significance attained when ≥ 5% (Fig. 3 left), the likelihood of LBD decreased with sperm rapid progressive motility (Fig. 3 right). For normal morphology, although the NB presented ≥5% in the majority of the cases, with significance observed when <5% (Fig. 3 left), the likelihood of LBD was independent of sperm normal morphology (Fig. 3 right). Regarding clinical subgroups, although the NB group showed a significant higher number of teratozoospermia cases (Fig. 3 left), the likelihood of LBD was independent of clinical spermiogram classifications (Fig. 3 right).

Discussion

Several factors can influence pregnancy outcomes in ICSI cycles with ejaculated sperm. In this report of 3,844 cycles the LBD was associated with lower couple ages, time of infertility, bFSH levels, time of stimulation, total dose of gonadotropins and HCG dose, and with a higher mean number of follicles and E2 levels. Regarding embryological and clinical outcomes, LBD was associated with a higher mean number of COC, MII, cleaved embryos, high quality embryos and blastocysts. Bellow, the relative predictive value of the main factors analysed in the present report are discussed.

Regarding **female age**, the present results showed a decline in the likelihood of LBD after 30 years, with significant differences found in women < 35 years, < 39 years and ≥ 40 years. Results confirm previous studies, albeit with a lower number of cycles. One study showed that the likelihood of CP decreased progressively with female age [Ottosen et al., 2007]. Another work evidenced that the chance of achieving a CP in women < 30 years is 3.2 higher, between 30-34 years is 2.8 higher and with < 35 years is 2.5 higher [Sabatini et al., 2008]. Another study showed that the CP rate increases up to 30 years, then slowly decreases and finally sharply decreases when ≥ 40 years. In that report, women of 30 years were 1.5 times more likely to become pregnant, women with 35 years had a similar chance, and 40 years old women had 4 times lower expectancy [Pinto et al., 2009]. Finally, a meta-analysis indicated that increasing female age was associated with lower CP rates [van Loendersloot et al., 2010]. Taking into account those observations and results of our large series, female age can be used as a predictive factor for LBD, with an upper cut-off of 35 years.

In relation to the **time of infertility**, results showed a decline in the likelihood of LBD after 2 years of infertility. In accordance, though with a lower number of cycles, other authors observed that the likelihood of CP decreased after 1 year of infertility [Ottosen et al., 2007]. A meta-analysis also confirmed that OP rates decreased with the time of infertility [van Loendersloot et al., 2010]. Based on results from our large series and on those cited above, the time of infertility can be used as predictive factor for LBD, with a positive cut-off of < 2 years and a negative cut-off of > 5 years. The levels of **bFSH** were significantly lower in the NB group, which reflects the higher capacity of the ovary for follicle growth in younger women. Several previous studies, regardless of a lower number of cycles, showed that when bFSH levels are > 10 IU/L, the embryological, clinical and NB outcomes are significantly decreased [Ottosen et al., 2007; Sabatini et al., 2008; Abdalla and Thum., 2004; Thum et al, 2009]. Based on the present results, on those studies cited above and on a recent meta-analysis [van Loendersloot et al., 2010], bFSH levels can be used as a predictive factor of LBD, with an upper cut-off of 10 IU/L.

The **GnRH antagonist protocol** was used in the large majority of the cases, but the likelihood of LBD significantly favoured the agonist protocol. Previous studies showed that the antagonist protocol was associated with lower time of stimulation, higher number of COC, MII, high quality embryos and embryo cleavage rate, whereas the agonist protocol exhibited a higher FR, with no significant differences between both protocols regarding the rates of BP, CP, implantation and LBD [Pinto et al., 2009]. Although there is a general consensus that the antagonist protocol is devoid of side effects, enables lower time of stimulation, gonadotrophin dose and risk of ovarian hyperstimulation syndrome (OHSS), other reports debate that the agonist protocol is associated with better embryological and clinical outcomes [Kolibianakis et al., 2006; Al-Inany et al, 2016]. Taking into account these previous studies, differences between both protocols should be considered marginal and clinically irrelevant, and the decision of using one or another stimulation protocol should rely on individual patient characteristics.

Regarding **gonadotropin stimulation**, results showed that the use of rFSH is significantly associated with higher LBD than the combination of rFSH with HMG or the use of HMG. Previous studies revealed distinct results. Cochrane reviews indicated that HMG was associated with higher rates of CP and LBD than rFSH [van Wely et al., 2011]. Another report showed that although HMG was associated with higher female age, bFSH levels and total dose of gonadotropins, and lower E2 levels, COC, MII, cleaved embryos and high quality embryos, it exhibited similar rates of implantation, CP and LBD to rFSH [Shavit et al., 2016]. Based on results and those of the above reports, differences between rFSH and HMH should be considered marginal and clinically irrelevant, and the decision of using one or another stimulation protocol should rely on individual patient characteristics.

The **levels of E2** were significantly higher in the NB group, which was associated with a higher number of follicles. Previous studies showed that although the CP rate was not affected by E2 levels of normal responders (<2500 pg/ml) or high responders (>2500 pg/ml) [Papageorgiou et al., 2002], the rates of LBD increased with E2 levels, with the better LBD rates being achieved with 3000-4000 pg/ml E2 for women <38 years and 279 2000-3000 pg/ml E2 for women >38 years [Joo et al., 2010]. This is in line with the defined upper cut-off for an increased risk of OHSS, which is 4500 pg/ml [Sousa et al., 2015]. Thus, E2 levels can be used as a predictive factor for LBD, with a cut-off of 2000-4000 pg/ml. In relation to the **mean number of follicles**, results suggested a lower likelihood of LBD with ≤4 follicles and a progressive higher likelihood of LBD from 4 to 18 follicles, with the maximized LBD attained in the interval 14-18 follicles. Although unable to find similar studies for comparisons, an upper limit of 14 follicles and a lower cut-off of 4-9 follicles are suggested. The number of follicles is related to the development of OHSS. In the NB group, of the 4 cases (0.4%) where an agonist was used for oocyte trigger due to risk of OHSS, 2 cases had > 18 follicles. However, in the no-NB group, of the 43 cases (1.5%) that used an agonist, 32 (74.4%) had >18 follicles, and with ≤14 follicles this risk decreased to 7 cases (16%). These results are reinforced by previous studies that showed an increased risk of OHSS when the number of follicles was >18 [Sousa et al., 2015; Papanikolaou et al., 2006]. The **mean number of retrieved cumulus-oocyte complexes** has been used to define women as poor (≤3 COC), normal (4-9 COC) or high (≥15 COC) responders [Polyzos et al., 2014; Polyzos and Sunkara, 2015], with the observance of an increased risk of OHSS when ≥15 COC were retrieved [Sousa et al., 2015]. The present results confirmed the low likelihood of LBD when ≤3 COC were retrieved and a higher likelihood of LBD from 4 to 15 COC, with the maximized LBD attained in the interval 10-15 COC. This is in accordance with cut-off suggested by other authors: 15 COC [Sunkara et al., 2011], 8-15 COC [Cai et al, 2013] and 10-15 COC [Steward et al., 2014]. Results suggest that the mean number of follicles can be used as a predictive factor for LBD, with a cut-off of 4-15 COC.

In relation to the **number of MII**, the present results confirmed the low likelihood of LBD when ≤ 3 MII were obtained, and a progressive higher likelihood of LBD from 4 to 15 MII, with the maximized LBD attained in the interval 10-15 MII. Although unable to find similar studies for comparison, previous studies evidenced that the number of MII is positively correlated with LBD [Cai et al., 2011]. Results suggest that the mean number of MII can be used as a predictive factor for LBD, with a cut-off of 4-15 MII. Previous studies revealed that the **number of high quality embryos** at day 3 is a critical predictive factor, but a relevant cut-off was not offered. Those authors observed that the transfer of high quality embryos increased the CP rate [Ottosen et al., 2007; Cai et al., 2011], the rates of CP and LBD [Dennis et al., 2006], or the LBD rate [Niinimäki et al., 2015]. The present results showed that the likelihood of LBD increased with the number of high quality embryos, being higher when ≥ 3, which indicates that the number of high quality embryos can be used as a predictive factor for LBD, with a cut-off of ≥ 3 high quality embryos.

In relation to the **number of embryos transferred**, the present results showed the transfer of 1 or 3 embryos was associated with a significant lower LBD, with the likelihood of LBD being higher when 2 embryos were transferred. Results also revealed that single blastocyst transfer was associated with no multiple pregnancies and 35% of LBD, whereas double blastocyst transfer presented 20% of twin pregnancies and 44% of LBD. On the other hand, single embryo transfer at day 3 was associated with no multiple pregnancies and 17% of LBD, whereas double embryo transfer at day 3 presented 9% of twin pregnancies and 30% of LBDR. This confirms previous studies, which indicated that although single embryo transfers decreased the LBD rate it also reduced the risk of multiple pregnancies, and that with the subsequent replacement of a single frozen embryo the LBD rate became comparable to double embryo transfer [Pandian et al., 2013; Baruffi et al., 2009; Martikainen et al., 2001; Pandian et al., 2005]. The present results and those of previous studies evidence that the number of embryos transferred can be used as a predictive factor for LBD, with a cut-off of 1-2 embryos. Results also indicate that in cases of blastocyst transfer a single embryo transfer should be the first choice. The clinical decision on transfer of two embryos at day 2 or 3 is frequently dependent on the lower LBD, as 2-3 subsequent replacements of a single frozen embryo would be needed to give patients an acceptable LBD per cycle.

Regarding the **day of embryo transfer**, the present results showed a significant increase in the likelihood of LBD with the day of embryo transfer, with the maximum being attained at day 5. This confirms previous reports of randomized controlled clinical trials [Papanikolaou et al., 2008] and Cochrane reviews [Glujosky et al., 2016], which showed higher LBD with blastocyst transfer. The present results and those of previous studies evidence

that the day of embryo transfer can be used as a predictive factor for LBD, and suggest that the preferable day for embryo transfer is day 5, as blastocysts elicit a significant higher LBD rate.

Evaluation of **sperm parameters** revealed that sperm concentration, rapid progressive motility and morphology had no clinical impact in the likelihood of achieving a LBD. These observations confirm previous studies, which showed that severe teratozoospermia [French et al., 2010] or decreased sperm concentration and progressive motility [Zheng et al., 2016] had no negative impact on clinical outcomes. Those studies suggested that these apparently strange observations might be due to the strict sperm preparation method and selection for microinjection. Nevertheless, from the present results and those of previous studies it is indicated that sperm parameters have no negative impact on clinical outcomes and cannot be used as a predictive factor for LBD.

Conclusions

The present results, using a large number of cases (3844 cycles) with microinjection of ejaculated sperm, indicated that LBD is associated with several demographic and stimulation variables such as younger age, more pure male infertility factors, lower bFSH, total gonadotropin dose, time of stimulation and HCG dose, and higher number of follicles and estradiol levels. Regarding embryological outcomes, the LBD was associated with significantly higher mean number of COC, MII, cleaved embryos, high quality embryos and blastocysts. Data from the present results and that of previous studies suggest the following indicators (cut-off) that appeared associated with an increased likelihood of LBD: female age $\leq$ 35 years; time of infertility $\leq$ 5 years (ideal < 2 years); bFSH $\leq$ 10 IU/L; both the antagonist and agonist protocols, as well as rFSH and HMG, gave similar outcomes, and thus the choice should be individualised; E2 levels 2000-4000pg/ml; number of follicles $\geq$ 4 (ideal maximum of 14 follicles); number of COC $\geq$ 4 (ideal maximum of 15 COC); number of MII $\geq$ 4 (ideal maximum of 15 MII); number of high quality embryos $\geq$ 3; double embryo transfer at day 3, single embryo transfer at day 5; and no relation between spermiogram values and LBD. These findings are thus considered relevant for clinical guidance and patient information regarding the likelihood of LBD.

Acknowledgements:

-For oocyte retrieval to Jorge Beires, MD, PhD, Gynecology & Obstetrics, Dept. of Gynecology & Obstetrics, Director of the Unit of Gynecology and Reproductive Medicine, São João Hospital, E.P.E, Porto, Portugal) and José Manuel Teixeira da Silva, MD, Gynecology & Obstetrics;

-For anesthesiology to José Correia, MD, Anesthetist, Dept. of Anesthesiology, São João Hospital, E.P.E, Porto, Portugal;

-For Andrology laboratorial work to: Ana Gonçalves, MSc, and Cláudia Osório, MSc (CGR);

-We also would like to thank to all Colleagues from the many other research articles, reviews, Book chapters and Books that we did not cited, but whose lecture was fundamental to the writing of the present manuscript.

Funding:

Authors have their own salaries paid by their respective institutions. MS by ICBAS-UP; JS, PV, MC, NB, AB by CGR; CO, JTS, PX, AC by their own private medical offices. UMIB is funded by National Funds through FCT-Foundation for Science and Technology, under the Pest-OE/SAU/UI0215/2014.

Availability of data and materials:

Data for this study has been extracted from the Centre for Reproductive Genetics A. Barros, a private IVF Clinic. The contents are under strict confidentiality and thus not available to any other person. However, if the Editor suspects of incorrect Author behaviour on data processing, the Clinic data-base can be supplied to the Editor after permission from the Clinic Director.

Ethics approval and consent to participate:

Ethical guidelines were followed in the conduct of research, with written informed consent having been obtained before the beginning of the present work. This work did not involve human or animal experiments. An approval by an Ethics Committee and the provisions of the Declaration of Helsinki as revised in Tokyo 2004 on human experimentation does not apply to this kind of work. According to the National Law on Medically Assisted Procreation (Law 32/2006..\..\..\IVF\Leis\Lei PMA 2016.pdf) and the National Council on Medically Assisted Procreation guidelines (CNPMA, 2015), patient databases were used after patient informed and written consent from cases enrolled in infertility treatments.

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ANEXOS

Legend to Figures

Left panel. Percentage of cycles with NB (dark box) and without NB (light box). This percentage was calculated within each group independently using the total of 993 cycles for the NB group and the total of 2,851 cycles for the no-NB group.

Right panel. Percentage of cycles with NB (dark box) and without NB (light box). This percentage was calculated using the total number of cycles in each grade (with NB + without NB). The linear tendency line and the linear regression (R2) indicate the likelihood of achieving a live birth.

Female age, Time of infertility (years);
Ag: Agonist protocol;
Antag: Antagonist protocol;
COC: Cumulus-oocyte complexes (retrieved oocytes);
MII: Mature metaphase II oocytes;
AB: High quality embryos at day 3;
Nº ET: Number of embryos transferred;
ET day: day of embryo transfer;
Conc: sperm concentration (M/ml);
RPM: sperm rapid progressive motility (%);
NM: sperm normal morphology (%);
O: oligozoospermia;
A: asthenozoospermia;
T: teratozoospermia.

Table 1.

TABLE 1 - Demographic and stimulation characteristics. ICSI cycles using ejaculated sperm				
Variable	**Total**	**with NB**	**without NB**	**p**
Cycles (n)	3844	993	2851	
Female age (y) (mean ± SD)	35.41 ± 4.49	33.46 ± 3.76	36.09 ± 4.52	a-b-c
Male age (y) (mean ± SD)	36.92 ± 5.73	35.46 ± 5.24	37.43 ± 5.81	a-b-c
Time infertility (y) (mean ± SD)	3.25 ± 2.70	3.18 ± 2.52	3.27 ± 2.76	NS
Male factor (rate)	72.4	77.6	70.5	a-c
Female factor (rate)	4.5	2.5	5.2	a-c
Mixed factors (rate)	23.1	19.9	24.3	a-c
Abnormal female karyotype (rate)	2.5	2.7	2.5	NS
Abnormal male karyotype (rate)	2.4	1.7	2.8	NS
bFSH (IU) (mean ± SD)	7.42 ± 3.71	6.59 ± 2.52	7.71 ± 4.01	a-b-c
Follicles (mean ± SD)	10.93 ± 6.45	12.99 ± 5.84	10.22 ± 6.50	a-b-c
Total dose (IU) (mean ± SD)	2195.30 ± 1132.29	1779.37 ± 824.01	2340.27 ± 1187.97	a-b-c
Time stimulation (days) (mean ± SD)	8.53 ± 1.86	8.37 ± 1.50	8.58 ± 1.97	a-c
Estradiol (pg) (mean ± SD)	1281.10 ± 955.29	1365.17 ± 740.14	1250.43 ± 1020.99	a-c
HCG dose (IU) (mean ± SD)	9025.79 ± 2070.44	8552.56 ± 2134.67	9201.69 ± 2018.38	a-b-c
Busereline (IU) (mean ± SD)	0.80 ± 0.00 (n=47)	0.80 ± 0.00 (n=4)	0.80 ± 0.00 (n=43)	b

Significance ($p < 0.05$).
Comparisons as:
a = Total cycles vs cycles with NB.
b = Total cycles vs cycles without NB.
c = cycles with NB vs cycles without NB.
NS: not significant.

NB newborn, *bFSH* basal follicle stimulating hormone, *Total dose* total gonadotropin dose, *HCG* human chorionic gonadotropin.

Table 2.

TABLE 2 - Embryological and clinical outcomes. ICSI cycles using ejaculated sperm

Parameters	**Total**	**with NB**	**without NB**	**p**
Cycles (n)	3844	993	2851	
Embryo transfer cycles (n)	3334	993	2341	
COC (n, mean ± SD)	26581 (6.91 ± 4.83)	8554 (8.61 ± 4.42)	18027 (6.32 ± 4.83)	a-b-c
MII (n, rate)	20818 (5.9)	6948 (7.0)	13870 (5.4)	
Maturation rate (MII/COC) (rate)	78.3	81.2	76.9	a-b-c
2PN/2PB (n, rate)	16068 (4.7)	5569 (5.6)	10499 (4.3)	
Fertilization rate (2PN/MII) (rate)	77.2	80.2	75.7	a-b-c
Embryos cleaved-day 2 (n, rate)	15938 (4.6)	5555 (5.6)	10383 (4.2)	
Embryo cleavage rate (d2/2PN) (rate)	99.2	99.7	98.9	a-b-c
Day 3 embryos (n, rate) (n, rate)	11409 (4.2)	4304 (4.8)	7105 (3.9)	
Day 3 grade A/B embryos (n, rate)	10891 (4.0)	4146 (4.7)	6745 (3.7)	
Day 3 grade A/B rate (rate)	95.5	96.3	94.9	a-c
Day-4 embryos (n, rate)	5695 (3.9)	2469 (4.3)	3226 (3.7)	
Day-5 embryos (n, rate)	4355 (3.5)	1917 (3.9)	2438 (3.3)	
Blastocyst rate (rate)	47.6	52.4	44.4	a-b-c
nº of transferred embryos (n, mean ± SD)	6211 (1.86 ± 0.51)	1896 (1.90 ± 0.39)	4315 (1.84 ± 0.55)	a-c
Biochemical pregnancy (/ETC) (n, rate)	1354 (40.6)	990 (99.7)	364 (15.5)	
Sacs (n)	1534	1304	230	
Implantation rate (nº sacs/nº ET) (rate)	24.7	68.8	5.3	
Clinical pregnancy (/ETC) (n, rate)	1221 (36.6)	993 (100)	228 (9.7)	
Singletons (/CP) (n, rate)	863 (70.7)	685 (69)	178 (78.1)	
Twins (/CP) (n, rate)	331 (27.1)	305 (30.7)	26 (11.4)	
Triplets (/CP) (n, rate)	3 (0.2)	3 (0.8)	0	
Ectopic pregnancy (/CP) (n, rate)	24 (2.0)	0	24 (10.5)	
Abortion (/CP) (n, rate)	204 (16.7)	0	204 (89.5)	
OP (/ETC)-(CP-Ab-Ect) (n, rate)	993 (29.8)	993 (100)	0	
Delivery (/ETC) (n, rate)	984 (29.5)	984 (99.1)	0	
Stillborn (n)	3	3	0	
LBDR (/ETC)-(Delivery-Stillborn) (n, rate)	981 (29.4)	981 (98.8)	0	
Newborn (/ETC): (n, rate):	1234 (37.0)	1234 (100)	0	
Male (/NB)	638 (52.0)	638 (52)		
Female (/NB)	588 (48.0)	588 (48.0)		
M/F ratio	1.2	1.2		
NB malformations (/NB): (n, rate):	16 (1.3)	16 (1.3)		
Major (/NB)	12 (1.0)	12 (1.0)		
Minor (/NB)	4 (0.3)	4 (0.3)		
ENdeath (/ETC) (n)	0	0		

Significance ($p < 0.05$).
Comparisons as:
a = Total cycles vs cycles with NB.
b = Total cycles vs cycles without NB.
c = cycles with NB vs cycles without NB.
NS = not significant.

NB newborn, *COC* cumulus-oocyte complexes (aspirated oocytes), *MII* mature oocytes at metaphase II of meiosis, *2PN-2PB* normal fertilized oocytes (with 2 pronuclei and 2 polar bodies), *OP* ongoing pregnancy, *LBDR* live-birth delivery rate, *EN death* early neonatal death.

Table 3.

TABLE 3 - Newborn outcomes. ICSI cycles using ejaculated sperm

Parameters	Total	with NB	without NB	p
Cycles (n)	3844	993	2851	NA
Embryo transfer cycles (n)	3334	993	2341	
Newborn (n)	1234	1234	-	
Gestation age (weeks) (mean ± SD) (n)	37.7 ± 2.8	993		
Term (weeks) (mean ± SD) (rate, n)	38.7 ± 1.1 (79.7)	791		
Preterm (PT) (weeks) (mean ± SD) (rate, n)	33.5 ± 3.3 (20.0)	198		
Very PT (weeks) (mean ± SD) (rate, n)	28.0 ± 3.2 (4.1)	41		
Extremely PT (weeks) (mean ± SD) (rate, n)	23.80 ± 2.3 (1.2)	12		
Weight (g) (mean ± SD) (n)	2739.7 ± 703.3	1204		
Normal weight (g) (mean ± SD) (rate, n)	3111.4 ± 357.5 (65.4)	788		
Low weight (LW) (g) (mean ± SD) (rate, n)	1943.6 ± 490.0 (33.1)	399		
Very LW (g) (mean ± SD) (rate, n)	1102.4 ± 269.0 (6.3)	76		
Extremely LW (g) (mean ± SD) (rate, n)	790.6 ± 172.2 (2.2)	26		

NA not applicable.

Fig. 1

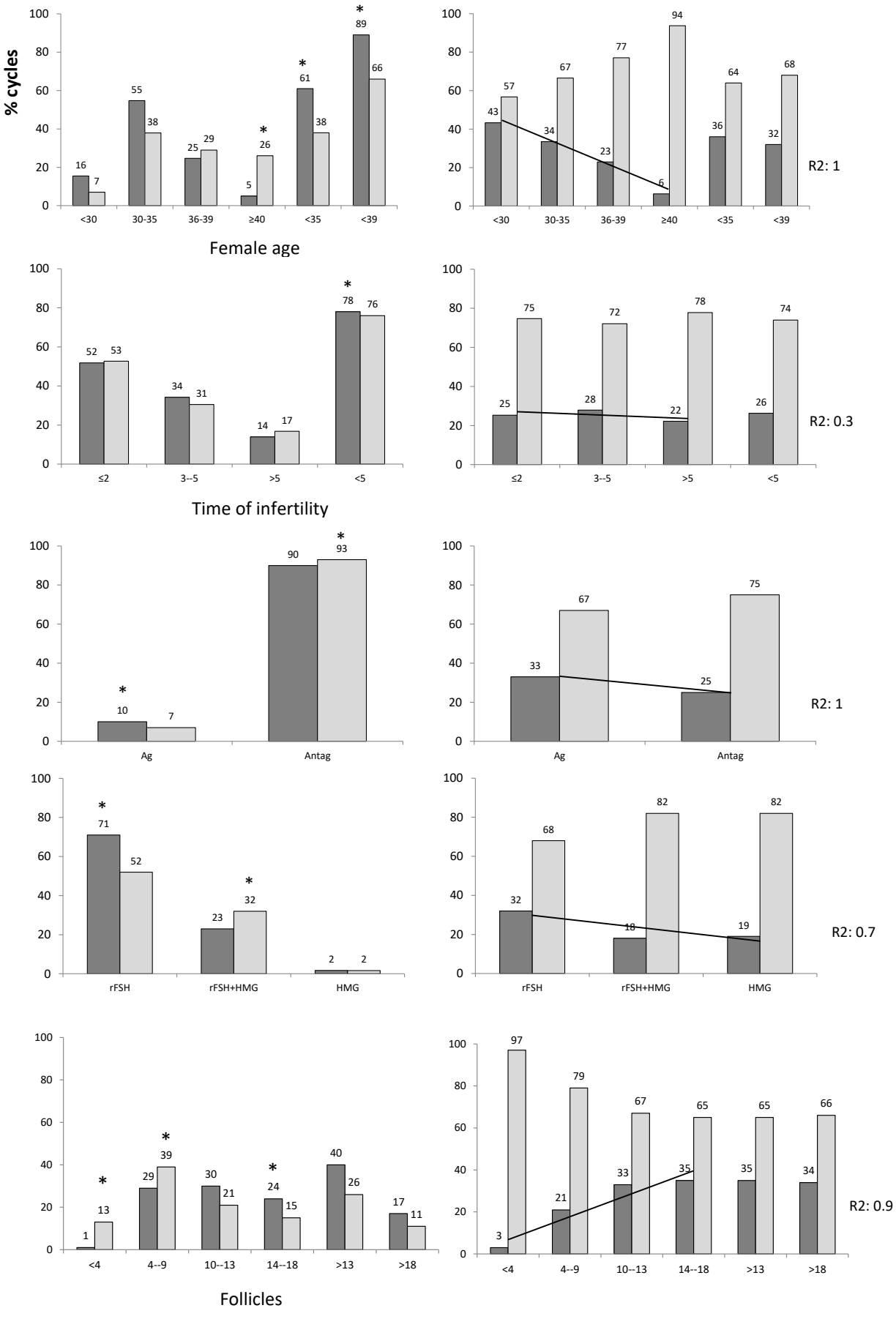

% cycles
<30
30-35
36-39
≥40
<35
<39
Female age
R2: 1
≤2
3--5
>5
<5
Time of infertility
R2: 0.3
Ag
Antag
R2: 1
rFSH
rFSH+HMG
HMG
R2: 0.7
<4
4--9
10--13
14--18
>13
>18
Follicles
R2: 0.9

Fig. 2

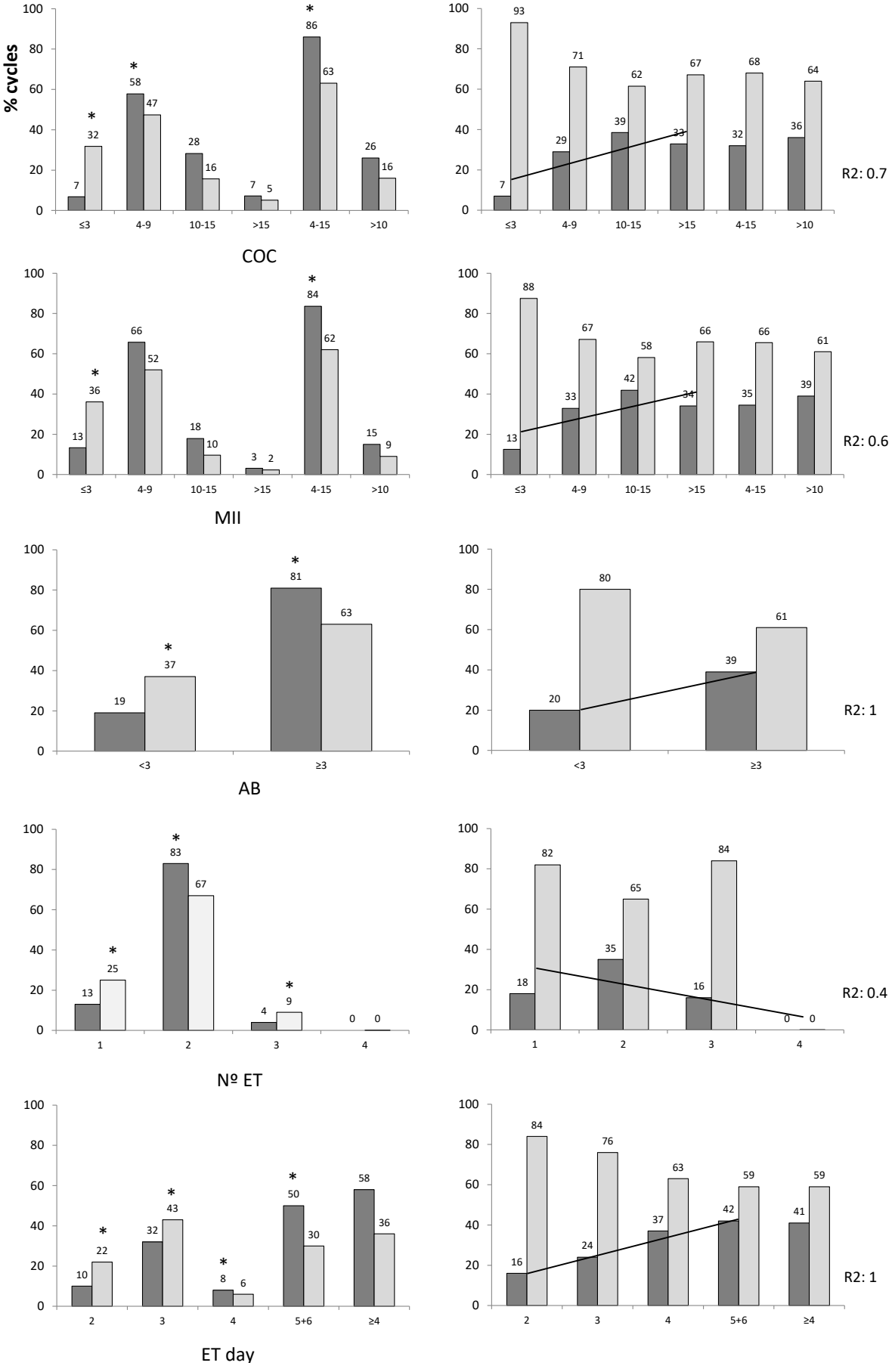
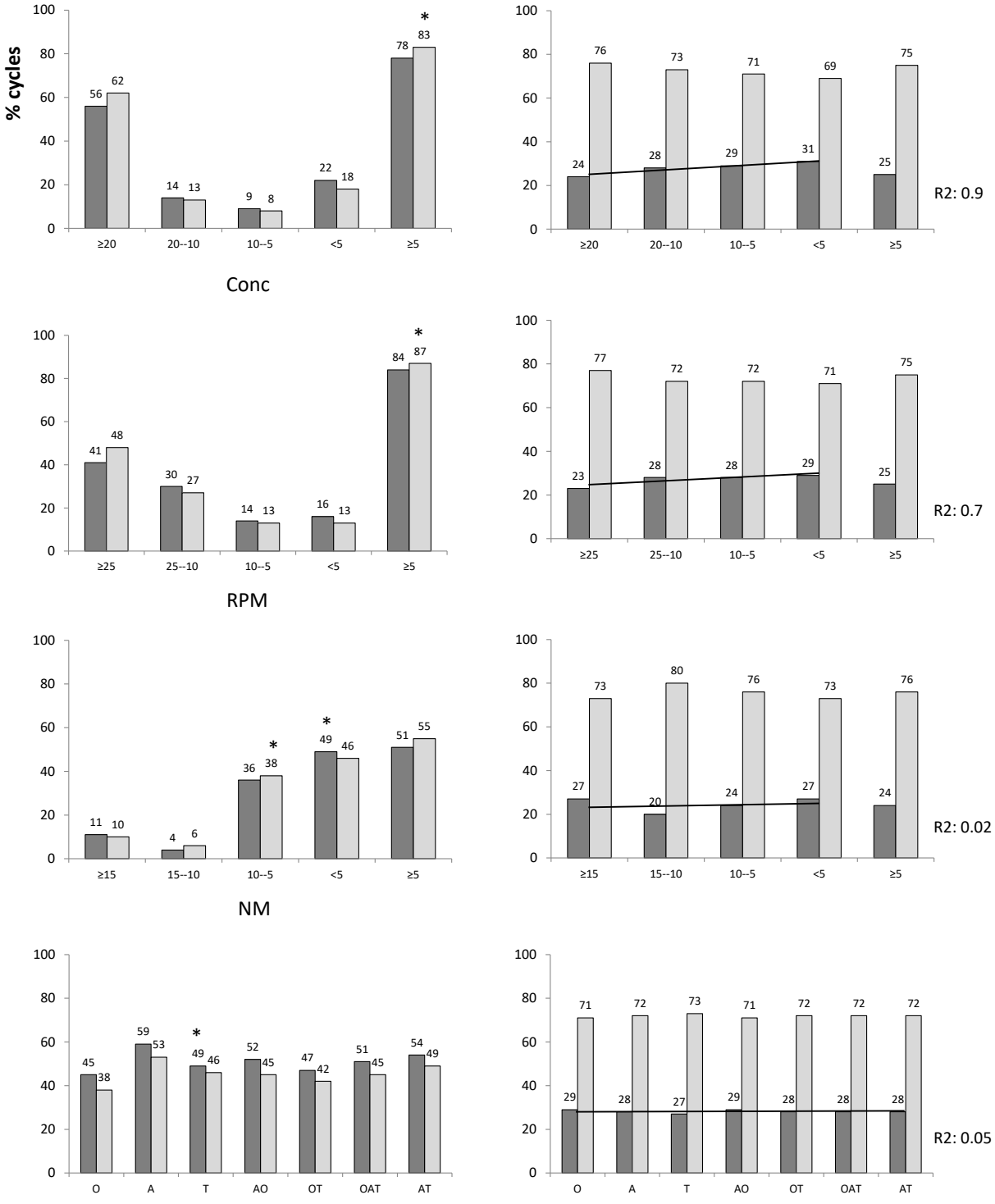

% cycles
COC
MII
AB
Nº ET
ET day
R2: 0.7
R2: 0.6
R2: 1
R2: 0.4
R2: 1
≤3
4-9
10-15
>15
4-15
>10
<3
≥3
1
2
3
4
2
3
4
5+6
≥4

Fig. 3


% cycles
Conc
RPM
NM
R2: 0.9
R2: 0.7
R2: 0.02
R2: 0.05
≥20
20--10
10--5
<5
≥5
≥25
25--10
≥15
15--10
O
A
T
AO
OT
OAT
AT