Clinical success of IUI cycles with donor sperm is not affected by total inseminated volume: a RCT

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STUDY QUESTION: What is the impact on live birth rates (LBR) when a donor IUI (dIUI) cycle is performed with an insemination volume of 0.5 mL versus the usual 0.2 mL?

SUMMARY ANSWER: LBR after a dIUI cycle is no different when performed with 0.5 versus 0.2 mL.

WHAT IS ALREADY KNOWN: An IUI has an important role in the treatment of severe male infertility, and is often used in same-sex female couples and single parents. Different variables have been studied to determine factors correlated with clinical outcomes (IUI scheduling, ovarian stimulation, sperm parameters) but little is known about the inseminated volume. The use of conical bottom test tubes could contribute substantially to the loss of inseminated spermatozoa because it precludes the total recovery of the sample. Additionally, the insemination catheter could uphold this reduction causing sperm adhesion on the inner walls of the insemination catheter, decreasing even more the total inseminated volume. It is expected that utilizing an IUI approach that increases sperm volume in the fallopian tubes (0.5 mL rather than 0.2 mL) at the time of ovulation will lead to higher LBRs. To avoid bias related to sperm quality, the study population was restricted to dIUI cycles.

STUDY DESIGN, SIZE, AND DURATION: A parallel-group, double-blinded, RCT, including patients undergoing natural or stimulated dIUI, was performed between March 2013 and April 2015. dIUI cycles (n = 293) were randomized through a computer-generated list to undergo insemination with 0.2 mL (control group) or 0.5 mL (study group), of which 24 were excluded (protocol deviation) and 269 received the allocated intervention. Patients with the presence of tubal factor infertility, grades III–IV endometriosis, >3 previous dIUI cycles or with ≥3 follicles >14 mm were excluded. The study was designed with 80% power to detect a 5% difference in LBR with a reference of 15% and a two-tailed 5% significance level. The required sample size was 118 per group.

PARTICIPANTS/MATERIALS, SETTING AND METHOD: There were 143 cycles (0.2 mL group) and 126 cycles (0.5 mL group). The primary end-point of the trial was LBR per dIUI cycle in both treatment groups. Clinical pregnancy rate and miscarriage rate were evaluated as secondary outcomes.

MAIN RESULTS AND THE ROLE OF CHANCE: No adverse events were reported during the study trial. Study groups (0.2 versus 0.5 mL, respectively) were similar in age (35.8 ± 3.9 versus 35.4 ± 4.0 years, mean±SD), and had similar anti-Mullerian hormone levels (2.2 ± 2.0 ± 1.5 ng/mL), basal antral follicle count (13.2 ± 6.4 versus 13.6 ± 6.0), BMI (23.5 ± 3.9 versus 23.7 ± 4.1 kg/m²), number of follicles (17 mm (1.1 ± 0.5 versus 1.1 ± 0.5), total gonadotrophin dose (553.1 ± 366.3 versus 494.6 ± 237.1 IU), and total motile sperm count (8.22 ± 2.1 versus 7.7 ± 5.7 million). Similar clinical pregnancy rates (18.9% (27/143) versus 19.8% (25/126), NS), LBRs (15.4% (22/143) versus 19.0% (24/126), NS) and miscarriage rates (18.5% (5/27) versus 4.0% (1/25), NS) were observed between groups.
**Introduction**

IUI remains a common first-line treatment for subfertility or infertility (Velman-Verhulst et al., 2012). By passing through the vagina and cervix, an IUI allows for a highly concentrated sperm source to be placed in the uterus and Fallopian tubes during the periovulatory period (Velman-Verhulst et al., 2012). Furthermore, when combined with ovulation induction (OI), the use of IUI has demonstrated higher pregnancy rates (PRs) (10–15%) when compared to timed intercourse (Cohlen et al., 1999; Goverde et al., 2000). Overall, the IUI method is a frequently used ART, the latest European IVF-Monitoring Consortium forESHRE reports that 174390 IUI cycles were performed in 23 reporting countries in 2011 (Assisted reproductive technology in Europe, 2011, 2016). Many factors have influenced IUI cycle outcomes and include patient’s age (Campana et al., 1996), ovarian reserve testing (anti-Mullerian hormone (AMH)) (Li et al., 2010), the number of mature follicles (Dickey et al., 1991; van Rumste et al., 2008), scheduling timing (Blockeel et al., 2014), number of insemination procedures (Osuna et al., 2004), type of catheter used (Vermeylen, 2006), sperm count in the catheter (Van Voorhis et al., 2001), and the total volume inseminated (Do Amaral et al., 2001). Out of all of these influencers, there remains limited data to describe the appropriate inseminated volume during an IUI procedure.

Current sperm washing methods in IUI procedures, such as swim-up or density gradients preparation, remove debris in order to maximize the amount of normal motile spermatozoa (Boomsma et al., 2007), thereby concentrating the total sample in ~0.2 mL containing 1–10 million total motile sperm. Nevertheless, total sperm gradually decline in count along the length of the female reproductive tract. It has been reported that a maximum of roughly 200 spermatozoa are eventually present in the ampulla (Mamas, 1996), thus, total IUI volume is suspected to be an important contributor to achieving high PRs. Variability in total sperm volume led Kahn et al. (1993) to introduce an alternative method of IUI using robust volumes of semen (≥4 mL), which, once inseminated, equated to a fallopian perfusion (FP). This technique enhanced pregnancy rates (25%) as compared to traditional IUIs (8–10%). Subsequent RCTs comparing FP and traditional IUI have shown varying results (Kahn et al., 1993; Fanchin et al., 1995; Gregoriou et al., 1995; Karande et al., 1995; Mamas, 1996; Nuojua-Huttunen et al., 1997), some of them arguing that the use of a high volume of sperm at insemination during a FP could cause overflow and flushing, a circumstance that may push the oocyte back to the pouch of Douglas.

To date, no RCT has been carried out to determine the ideal volume of sperm at IUI to maximize PRs per cycle. This study postulates that the use of conical bottom test tubes could contribute substantially to the loss of inseminated spermatozoa because it precludes total recovery of the sample. Moreover, the insemination catheter could sustain this reduction by establishing hydrogen bonds between the surface and the H2O molecules present in the insemination sample, causing sperm adhesion on the inner walls of the insemination catheter.

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**WHAT DOES THIS MEAN FOR PATIENTS?**

This study looks at whether the amount of sperm used in a donor IUI (intrauterine insemination) cycle makes a difference to the chances of a successful outcome.

IUI is used to treat same-sex couples, single women and for some fertility problems, but although research has looked at a number of different factors which may influence outcome, little is known about whether the volume of sperm used makes a difference.

In this study, people were randomized into two groups. One group was given the usual volume of sperm (0.2 mL) and the other group was given a higher volume (0.5 mL) to see whether this affected outcomes. The live birth rates for both groups were similar, and it was concluded that the volume used for insemination makes no difference to the outcome.
The goal of this study is to prospectively determine the most appropriate insemination volume in IUI cycles. To further avoid bias related to sperm quality (Ombrelet et al., 2003; Wainer et al., 2004), the study population was restricted to IUI cycles in which donor sperm was utilized. It is expected that utilizing donor IUI (dIUI), which increases the volume of inseminated sperm in the fallopian tubes (0.5 mL rather than 0.2 mL) at the time of ovulation, will lead to higher live birth rates (LBR).

Materials and Methods

Study design

A parallel-group, double blind (clinician- and patient-blinded) RCT was performed, including patients undergoing dIUI with ovulation induction or a natural cycle. The trial was established to compare two inseminated volumes, 0.2 mL (control group) and 0.5 mL (study group), and conducted between March 2013 and April 2016. The Institutional Review Board of Institut Universitari Dexeus approved the research project and written informed consent was obtained from all patients participating to the study. The study was registered at the Clinical Trials website (www.clinicaltrials.gov, trial number NCT03006523).

Eligibility criteria

Eligible patients were 18–40 years old and had regular menstrual cycles (21–35 days). All patients underwent a full infertility evaluation, including hormonal assessment between Days 2 and 5 of the cycle, and confirmation of fallopian tubes patency. Indications for the use of donor sperm were characterized by severe male factor infertility requiring donor sperm use, the presence of a heritable genetic disorder in the male partner, a single- or same-sex couples’ partners undergoing dIUI. Exclusion criteria were the presence of tubal factor infertility, a grades III–IV endometriosis, ≥3 previous dIUI cycles or ≥3 follicles >14 mm observed during the dIUI cycle. Patients were allowed to participate in the study only once, and only the first cycle of any given patient was included in the study. The randomization was performed by dIUI cycle. The group allocation took place the day of the dIUI procedure and the biologist randomized all included patients into one of the two groups using an open computer-generated list (allocation ratio 1:1).

Patient monitoring

Patients undergoing a dIUI cycle under a natural menstrual cycle or with OI were included. All patients were instructed to notify the researchers of the first day of menses. Patients undergoing a natural cycle started ultrasound monitoring on cycle Day 12 after a spontaneous menses. When the leading follicle was >17 mm, daily ultrasounds and urine LH measurements were carried out until spontaneous ovulation was confirmed, and dIUI was scheduled for roughly 24 h later. dIUI cycles with OI were performed with clomiphene citrate (Omnifin®, Laboratorios Effik, Madrid, Spain) or with recombinant FSH (r-FSH) (Gonal®, Merck Serono Europe Ltd, Madrid, Spain; or Puregon®, Merck Sharp & Dohme de España, Spain) administered starting cycle day three to cycle day seven of a spontaneous cycle. A starting dosage of 100 mg was used with clomiphene citrate, and of 50-75 IU for r-FSH, until ovarian response was observed. Monitoring by transvaginal ultrasound was performed starting on cycle Day 8 for r-FSH-stimulated cycles and cycle Day 12 for clomiphene citrate-stimulated cycles, until a dominant follicle (≥20 mm) was observed. Ovulation was triggered with recombinant hCG (Ovitrelle®, Merck Serono Europe Ltd, Madrid, Spain), and 36 h thereafter patients were scheduled for a dIUI.

Luteal phase support was administered vaginally with micronized progesterone (either Utrogestan®, SEID Laboratories, Barcelona, Spain; or Progeffik®, Laboratorios Effik, SA, Alcobendas, Spain) once a day for 10 days, starting the first day after the dIUI.

Sperm preparation

The method of sperm processing remained the same throughout the course of this study. Because samples are chosen according to the patient’s phenotype and blood type, donor samples were obtained from two different sperm bank sources in order to meet the legal requirements of the country’s study center and sample availability. All samples were IUI-ready upon arrival at the study center. Frozen semen was prepared for IUI by incubating it at 37°C for 5 min until thawing was complete. The specimen was then washed for 10 min at 150 g with 5 mL of PureSperm Wash® medium (Nidacon International AB, Göteborg, Sweden) in a 10 mL conical tube test (Nunc®, Thermofisher Scientific Europe, Madrid, Spain) to remove the cryoprotector. The supernatant was removed to lower the final volume to 0.2 mL (control group) or 0.5 mL (study group). A Makler chamber was used to assess the percentage of motile sperm, concentration (million/mL), and motility of the processed sample.

Insemination procedure

The dIUI was performed with a Soft-Pass™ Coaxial Insemination Catheter (Cook Ireland Ltd, Limerick, Ireland). The sperm sample was deposited at the uterine fundus under abdominal ultrasound guidance. All dIUIs were performed between 12 and 4 p.m. After the procedure, 10 min of bed rest was prescribed. Inseminations were performed every day of the week.

Outcome measures

The primary end-point of the trial was LBR per dIUI cycle in both treatment groups. Clinical PR and miscarriage rate were evaluated as secondary outcomes. A live birth was considered as any birth after 24 weeks of gestation. A clinical pregnancy was determined by the presence of a gestational sac >7–10 days following a positive pregnancy test by measuring serum β-hCG. Miscarriage was defined as a pregnancy loss following a positive pregnancy test and/or a detectable gestational sac. LBR, clinical PR and miscarriage rate were defined as the percentage of cycles that led to a live birth, a clinical pregnancy or to a miscarriage, respectively.

Statistical analysis

According to our sample size calculation, 232 dIUI cycles in total (116 in each arm) were essential in order to detect an increase in LBR from 15 to 20% between both groups with a power of 80% and two-sided 5% significance level. Although previous reports suggest a PR of 11% in dIUI (Besselinik et al., 2008), the authors consider a 5% increase a more practical effect with an intervention such as volume increase. With a predicted dropout level of 5%, we aimed to recruit 244 cases (122 in each arm). To recruit this number of dIUI cycles, a 2-year inclusion period was anticipated.

Statistical analyses were performed using the SPSS statistical package (IBM, Armonk, NY, USA). Continuous variables were assessed by Student’s t-tests, or by Wilcoxon rank sum tests if the data did not appear normally distributed. Results are expressed as mean and SD with 95% CI. Categorical variables were assessed by Chi-square tests or two-tailed Fisher’s exact tests in cases of small cell frequencies. A P value of <0.05 was considered statistically significant. The Clopper–Pearson interval was used to calculate binomial CI for all reported proportions. Adjusted odds ratios (ORs) and their 95% CI for all outcomes were calculated to evaluate the relative odds of each event compared with the control group.
## Results

A total of 293 cycles were recruited and randomized during the study period, of which 24 were excluded due to protocol deviation (5 patients in the 0.2 mL group and 19 patients in the 0.5 mL group). Of the 269 cycles evaluated, 143 received an inseminated volume of 0.2 mL, from which 65.7% \((n = 94)\) were performed with OI and 34.3% \((n = 49)\) under a natural cycle. In the study group, 126 patients received an inseminated volume of 0.5 mL, with 73.8% \((n = 93)\) undergoing OI and 26.2% \((n = 33)\) a natural cycle (Fig. 1). Regarding the 24 excluded cycles, 14 patients did not return for a pregnancy test and were lost from follow up (all of them were international patients), and 10 patients asked their treating physician not to be included in the analysis.

All baseline demographic characteristics were comparable between both groups (Table I). Indications for treatment (single women, male factor or same-sex couples) are reported in Table I.

In terms of clinical outcomes, statistically similar clinical PR (18.9 versus 19.8%), miscarriage rate (18.5 versus 4.0%) and LBR (15.4 versus 1.8 ng/mL) and a higher average basal antral follicle count were observed between groups (Table II).

### Live birth versus no live birth—all patients

In order to identify factors associated with clinical success, a comparison of all patients (0.2 and 0.5 mL together) who achieved a live birth was carried out versus those who did not. Only the average basal AMH level (Table III) was found to be higher in those patients that did deliver (3.3 versus 2.0 ng/mL, \(P < 0.05\)). The total inseminated volume \((0.34 \pm 0.15 \text{ versus } 0.34 \pm 0.15 \text{ mL})\) and the total motile sperm inseminated \((7.7 \pm 6.1 \text{ versus } 8.0 \pm 6.4 \text{ million})\) were similar between patients that delivered and those that did not (Table III).

### Live birth versus no live birth—by allocated group

Additionally, patients who achieved a live birth were compared to those who did not, by allocated group. In the 0.2 mL group, a higher average number of total follicles in the subset of patients that achieved a live birth was observed (Table IV). For the 0.5 mL group, a lower average age \((32.8 \text{ versus } 36.0 \text{ years})\), a higher average AMH level \((3.5 \text{ versus } 1.8 \text{ ng/mL})\) and a higher average basal antral follicle count \((16.9 \text{ versus } 13.0)\) were observed in patients who did achieve a live birth (Table IV).

## Discussion

Roughly one out of every nine dIUI cycles results in a live birth (Besselink et al., 2008). Several treatment strategies have been studied with the intention of improving outcomes (Dickey et al., 1991; Campana et al., 1996; Do Amaral et al., 2001; Van Voorhis et al., 2001; Osuna et al., 2004; Vermeylen, 2006; van Rumst et al., 2008; Blockeel et al., 2014), but understanding the optimal volume of sperm to be inseminated remains elusive.
Table II: Clinical outcomes by study group.

|                      | IUI with 0.2 mL | IUI with 0.5 mL | OR   | P value |
|----------------------|----------------|----------------|------|---------|
| Clinical pregnancy rate | 18.9% (27/143) (95% CI 12.8–26.3) | 19.8% (25/126) (95% CI 13.3–27.9) | 0.9 (95% CI 0.5–1.7) | 0.8 |
| Live birth rate       | 15.4% (22/143) (95% CI 9.9–22.4)  | 19.0% (24/126) (95% CI 12.6–27.0) | 0.8 (95% CI 0.4–1.5) | 0.4 |
| Miscarriage rate      | 18.5% (5/27) (95% CI 6.3–38.1)    | 4.0% (1/25) (95% CI 0.1–20.4)  | 5.5 (95% CI 0.6–50.4) | 0.1 |

mL, milliliters; OR, odds ratio; NS, not significant.

Table III: Baseline demographic and clinical characteristics of patients that achieved a live birth versus those that did not.

|                      | Live birth n = 46 | No live birth n = 223 | P value* |
|----------------------|------------------|-----------------------|---------|
| Age (years)          | 34.4 ± 4.6       | 35.9 ± 3.8            | 0.07    |
| BMI (kg/m²)          | 22.9 ± 3.4       | 23.7 ± 4.1            | 0.32    |
| Anti-Mulleran hormone (ng/mL) | 3.3 ± 1.6   | 2.0 ± 1.7             | 0.04    |
| Antral follicle count (n) | 14.9 ± 6.4   | 13.2 ± 6.2            | 0.12    |
| Follicles > 17 mm (n) | 1.1 ± 0.5        | 1.1 ± 0.4             | 0.68    |
| Total follicles (n)  | 2.3 ± 1.7        | 1.9 ± 1.1             | 0.32    |
| Total GND dose (IU)  | 494.4 ± 310.3    | 530.7 ± 314.7         | 0.31    |
| Total motile sperm (millions) | 7.7 ± 6.1   | 8.0 ± 6.4             | 0.72    |
| Inseminated volume (mL) | 0.34 ± 0.15   | 0.34 ± 0.15           | 0.81    |
| Indication for treatment |                  |                       |         |
| Male factor           | 39.1% (n = 18)   | 42.6% (n = 95)        |         |
| Single women          | 47.8% (n = 22)   | 55.6% (n = 124)       |         |
| Same-sex couple       | 13.0% (n = 6)    | 19.7% (n = 44)        |         |
| Stimulation protocol  |                  |                       |         |
| Natural cycle         | 30.4% (n = 14)   | 30.9% (n = 69)        |         |
| Ovulation induction   | 69.6% (n = 32)   | 69.1% (n = 154)       |         |

Results are expressed as mean ± SD. *Student’s t test.

Current standard practice involves the insemination of 0.2 mL of prepared sperm into the uterine cavity. This study investigated whether an increase to 0.5 mL would enhance dIUI outcomes. Taking into account that samples are concentrated with around 8 million sperm cells, losing 5–10% of 0.5 mL would result in the loss of less sperm cells that 5–10% of 0.2 mL. The findings presented here show that dIUI with 0.5 mL resulted in a statistically similar LBR when compared to dIUI with 0.2 mL (Table I). It is worth mentioning that, one might think that if a yet larger sample size would have been planned, significance could have been reached. The authors consider that, taking into consideration the confidence limits observed in the study population (Table II), a larger sample size would have not have detected statistical significance in LBR. Having said this, the authors consider that the 23% higher LBR, from 15.4 to 19.0%, could be clinically relevant and potentially alter everyday practice.

Interestingly, although it also did not reach statistical significance, a higher miscarriage proportion was observed when patients were inseminated with 0.2 mL. Even though we acknowledge there is no statistical difference, an 18.2% miscarriage rate is relatively high and it is worth paying attention to it. The authors speculate that it this outcome could be related to the presence of uterine contractions similar of those generated during sexual intercourse, which may be implicated in the inception of early biochemical embryo–endometrium communication. In ART, uterine contractions during embryo transfer in IVF cycles have a negative effect on pregnancy rates (Fanchin et al., 1998; Lesny et al., 1998; Lan et al., 2012), whereas their impact on IUI is unknown. There is only one study to our knowledge that has analyzed the association between uterine contractility after an IUI procedure and the outcome of the cycle (Blasco et al., 2014), showing which found that when the frequency of contractions was higher, the clinical pregnancy and LBRs were also increased significantly. Unfortunately, the authors did not include miscarriage rate as an outcome in their study. Myometrial contractions have been proposed to be necessary to achieve fertilization, since they seem to facilitate the upward transport of sperm cells towards the Fallopian tubes, where fertilization takes place (Kunz et al., 1997; Suarez and Pacey, 2006). One could argue that this could also be important after fertilization has occurred, helping to transport the embryo back to the uterus and promoting implantation.

Although the amount of sperm cells is physiologically important for eventual fertilization and thus pregnancy, limited data are available regarding the total inseminated volume necessary to achieve fertilization. Franco Júnior et al. (1992) evaluated the volume of material deposited during an IUI and the site involved by hysterosalpingography and noted that, starting from 0.4 mL, the contrast dye reached the uterus, isthmus and tube ampulla, whereas a volume of 0.2 mL did not reach the tube ampulla. Previous reports have compared FP (with around 4 mL of sperm suspension) to traditional IUI and failed to demonstrate higher outcomes (Cantineau et al., 2013), most probably secondary to the fact that the large volume of inseminate may flush the ova out of the tubes, resulting in expulsion of the ova from the tube and subsequent failure of fertilization (Nuojua-Huttunen et al., 1997). Instead, investigated, in a retrospective manner, if different low insemination volume related to patients and their impact on IUI is unknown. There is only one study to our knowledge that has analyzed the association between uterine contractility after an IUI procedure and the outcome of the cycle (Blasco et al., 2014), showing which found that when the frequency of contractions was higher, the clinical pregnancy and LBRs were also increased significantly. Unfortunately, the authors did not include miscarriage rate as an outcome in their study. Myometrial contractions have been proposed to be necessary to achieve fertilization, since they seem to facilitate the upward transport of sperm cells towards the Fallopian tubes, where fertilization takes place (Kunz et al., 1997; Suarez and Pacey, 2006). One could argue that this could also be important after fertilization has occurred, helping to transport the embryo back to the uterus and promoting implantation.

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patients that achieved a live birth. The authors consider the difference is most probably not clinically relevant, as in both groups AMH is within the normal limits (3.3 in live birth group versus 2.0 ng/mL, Table III); furthermore, the current study was not powered to detect a difference in AMH levels. When patients were compared by allocated group, in the 0.2 mL group patients who achieved a live birth had a higher average number of total follicles (Table IV). For the 0.5 mL group, patients who achieved a live birth had a lower average age (32.8 versus 36.0 years), a higher average basal AMH level (3.5 versus 1.8 ng/mL) and a higher average basal AFC (16.9 versus 13.0) (Table IV). The authors acknowledge that these factors, which were statistically significantly different between groups, are known to be correlated with implantation (van Loendersloot et al., 2010).

The restriction of the study population to women requiring insemination with donor sperm allowed the exclusion of potential confounding male or female fertility problems that could influence the outcome of an IUI and would present a bias to the results and the conclusion. Therefore, sperm quality, which has a significant impact on the success rate of IUI (Ombelet et al., 2003; Wainer et al., 2004), was not a confounding factor in our study. Nonetheless, the optimization of volume used in dIUI should be equally important in patients who undergo IUI because of mild male factor infertility or other indications. Hence, we believe the results also apply to this group of patients.

One limitation of our study is that the randomization was performed at the dIUI cycle level. This means that the results are reported as the success rate per dIUI cycle rather than per treated patient. However, the per IUI cycle approach was employed in previous trials investigating the success of IUI (Vermeulen, 2006; Blockeel et al., 2014), and when analyzing the results per patient, including only women who underwent their first treatment cycle of insemination, the outcome was in line with the per cycle analysis. The authors of the abovementioned studies considered that if more than one cycle was included, variables of patients included more than once would have had more statistical weight than those that achieved a live birth within the first cycle. A second limitation of our study was the fact that the treatment protocol included both natural and stimulated cycles. The authors consider this not clinically relevant. When cycles that achieved a live birth were compared to those that did not, both arms had the same proportion of patients with either treatment protocol. Furthermore, if only patients undergoing a dIUI cycle with OI were included, the n value (0.2 mL=94; 0.5 mL=93) would still be above the recommended sample size to detect a difference (72 per arm).

In conclusion, this trial did not demonstrate a benefit in performing donor insemination with a higher volume, as results show no significant relationship between post-wash inseminated semen volume (0.2 or 0.5 mL) and LBR. Concerns of handling, with possible sperm loss, during processing can be alleviated, as an increase in volume does not show considerable benefit to outcome. Thus, standard practice can be applied and patients can be informed that their clinical care is optimized with current dIUI standards.

Authors’ roles
J.R.P. contributed to the design of the study, the analysis of the data and drafted the article. L.L., C.G.-L. made substantial contribution to the concept and design of the study. L.L., M.B. and C.G.-L. contributed to acquisition of the data. J.R.P., I.R., I.G.-F. contributed to the analysis and interpretation of data. All authors made contributions to drafting and revising the article critically for important intellectual content. All authors approved the final version of the article to be published.

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Conflict of interest
All authors declare no conflict of interest with regard to this trial.

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