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Condensation and protamination of sperm chromatin affect ICSI outcomes when gametes from healthy individuals are used

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STUDY QUESTION: Do defects in sperm chromatin protamination and condensation have an impact on ICSI outcomes?

SUMMARY ANSWER: Sperm protamination is related to fertilization rates in healthy donors, and the *in vitro* capacity of sperm to condense their chromatin is linked to blastocyst rates, both associations being more apparent in women <33 years of age.

WHAT IS KNOWN ALREADY: Previous data on how sperm chromatin damage affects ICSI outcomes are inconsistent. Revealing which sperm factors influence embryo development is necessary to understand the male contribution to ICSI success and to develop novel sperm selection techniques or male-based treatments. Sperm chromatin is mainly condensed in protamines, which are cross-linked through disulphide bridges. This study aimed to determine whether sperm protamination and the integrity of disulphide bonds (condensation) are related to embryo development after ICSI.

STUDY DESIGN, SIZE, DURATION: The design was a retrospective study with a blind analysis of sperm chromatin. Gametes were divided into two groups: double donation (DD) cohort and single donation (SD) cohort. Samples from 45 semen donors used in 55 ICSI cycles with oocyte donors (age range 19–33 years), generating 491 embryos, were included in the DD cohort. The SD cohort consisted of samples from 34 semen donors used in 41 ICSI cycles with oocytes from healthy females (single-parent families or lesbian couples, age range 20–44 years), generating a total of 378 embryos.

PARTICIPANTS/MATERIALS, SETTINGS, METHODS: Donor sperm samples from DD and SD cohorts were used for standard ICSI, and embryo development was observed by time-lapse imaging. The incidence of thiol reduction (dibromobimane, DBB) and the degree of chromatin protamination (chromomycin A3, CMA_3, indicating non-protaminated regions) in sperm were determined by flow cytometry at 0 and 4 h post-thawing.

MAIN RESULTS AND THE ROLE OF CHANCE: Percentages ± standard deviation of CMA_3 were 21.08 ± 9.09 and 35.01 ± 14.68 at 0 and 4 h post-thawing, respectively, in the DD cohort and 22.57 ± 9.48 and 35.79 ± 12.58, at 0 and 4 h post-thawing, respectively, in the SD cohort. Percentages of DBB^+ were 16.57 ± 11.10 and 10.51 ± 8.40 at 0 and 4 h post-thawing ($P < 0.0001$), respectively, in the DD cohort and 17.98 ± 10.19 and 12.72 ± 8.76 at 0 and 4 h post-thawing ($P < 0.0001$), respectively, in the SD cohort. Female age correlated with fertilization rates, and the relation between sperm chromatin and embryo development was determined through multiple linear regression. While CMA_3 was associated with fertilization rates, with no influence of female age, in the DD cohort ($\beta_1 = -1.036$, $P < 0.001$ for CMA_3; $\beta_2 = 0.667$, $P = 0.304$ for female age), this was not observed in the SD cohort, where female age had a significant effect, masking the effects of CMA_3 ($\beta_1 = -0.066$, $P = 0.804$ for CMA_3; $\beta_2 = -1.451$, $P = 0.003$ for female age). The *in vitro* capacity of sperm to condense their chromatin after 4 h of incubation was associated with blastocyst rates, independent of female age (DD cohort: $\beta_1 = -0.238$, $P = 0.008$ for $\%DBB^+$ variation; $\beta_2 = 0.404$, $P = 0.638$ for female age; SD cohort: $\beta_1 = -0.278$, $P = 0.010$ for $\%DBB^+$ variation; $\beta_2 = -0.292$, $P = 0.594$ for female age). The *in vitro* capacity of sperm to condense their chromatin was also related to the time required

for the embryo to reach blastocyst stage in the DD cohort ($P = 0.007$). Finally, multiple logistic regression showed that both chromatin protamination and condensation, together with the age of the oocyte donors and the embryo recipients, had an impact on pregnancy achievement ($P < 0.01$) and on live birth rates ($P < 0.01$).

LIMITATIONS, REASONS FOR CAUTION: The main limitation was the restrictive selection of couples, which led to a relatively small sample size and could influence the observed outcomes. For this reason, and to reduce Type I error, the level of significance was set at $P \leq 0.01$. On the other hand, the use of cryopreserved samples could also be a limitation.

WIDER IMPLICATIONS OF THE FINDINGS: This research demonstrated that protamination and condensation of sperm chromatin are related to embryo development after ICSI, but female age could be a confounding factor when oocytes from older females are used.

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Key words: sperm chromatin / protamination / condensation / ICSI / gamete donors / chromatin damage / chromatin integrity

Introduction

ART has become the mainstay for the treatment of human infertility and has allowed the birth of >2 million of babies in Europe during the last decade (European IVF-Monitoring Consortium (EIM) for the European Society of Human Reproduction and Embryology (ESHRE) et al., 2021). The number of treatments has significantly increased over recent years, as nearly 1435 cycles per million inhabitants were conducted in Europe during 2017, compared to 474 cycles/year per million between 2008 and 2010 (Dyer et al., 2016; European IVF-Monitoring Consortium (EIM) for the European Society of Human Reproduction and Embryology (ESHRE) et al., 2021). While ICSI is the most used ART method in Europe (2.1 ICSI cycles per conventional IVF cycle), it does not offer an advantage over IVF in non-male factor infertility (Bhattacharya et al., 2001; Practice Committees of the American Society for Reproductive Medicine and Society for Assisted Reproductive Technology, 2012; Evers, 2016). As ICSI effectiveness has been stable at between 21% and 23% in the 2009–2017 period (European IVF-Monitoring Consortium (EIM) for the European Society of Human Reproduction and Embryology (ESHRE) et al., 2021), developing new methods to improve ART success and investigating the factors underlying implantation failures are warranted. The development of new diagnostic tools, medical devices or procedures, however, is not an easy task, as research into human (in)fertility involves many technical differences, heterogeneous populations and inter-individual variations (Setti et al., 2022; West et al., 2022; Yao et al., 2022).

While male factor infertility is present in half of the couples seeking medical assistance (Louis et al., 2013; Agarwal et al., 2015), more attention to female factors has been paid in previous investigations involving ART. In addition, although the conventional spermiogram (concentration, motility and morphology) may not be directly related to fertilization rates and embryo quality after ICSI, it is still unclear whether other sperm variables, such as chromatin defects, have an influence on embryo development and clinical outcomes after ART (Hervás et al., 2022; Repalle et al., 2022). A recent meta-analysis about the effects of DNA damage on IVF and ICSI outcomes showed that DNA integrity exerts a negative impact on implantation and pregnancy rates in conventional IVF but not in ICSI, as results did not reach statistical significance (Ribas-Maynou et al., 2021). For this reason, whether sperm chromatin negatively influences ICSI success is a question that remains open.

To protect the paternal genome from genotoxic agents, histones are replaced by protamines in the chromatin of mature sperm, in a process that is believed to involve binding sites for CTCF (CCTC-binding factor) (Torres-Flores and Hernández-Hernández, 2020). Protamines are cross-linked through oxidized disulphide bonds, which help stabilize sperm chromatin into toroidal structures (Hud et al., 1993; Ward, 2010). Upon fertilization, the formation of the male pronucleus requires the replacement of protamines by histones and the epigenetic reprogramming of the paternal genome. The injection of round spermatids, which do not contain protamines, leads to impaired embryo development; therefore, these proteins appear to play a vital role in post-fertilization events (Kimura and Yanagimachi, 1995; Yamaguchi et al., 2018). Furthermore, not only does sperm chromatin integrity suffer from DNA breaks, but also protamine deficiency and premature reduction of disulphide bridges can occur in mature sperm, having a detrimental impact on male fertility (Ribas-Maynou et al., 2020; Llavanera et al., 2021; Hologlu et al., 2022). These deficiencies, which result in chromatin regions with unprotected DNA, may impair the formation of the male pronucleus and have adverse effects on embryo development. To the best of the authors' knowledge, no study has previously assessed how the reduction of sperm disulphide bridges, and therefore, chromatin decondensation alters ICSI outcomes in humans. In addition, the relation between chromatin protamination and ICSI success is inconsistent in the literature as whereas some authors found an association between poor protamination and reduced fertilization rates (Tavalaee et al., 2009; Iranpour, 2014), others reported the opposite (Nijs et al., 2009; Gill et al., 2018). It is worth mentioning that no earlier research assessing sperm chromatin protamination was conducted in cycles where both sperm and oocytes came from healthy young donors. Hence, it could be that most of our current knowledge in this realm was biased by the confounding factors present in infertile cohorts; in which case, the use of healthy individuals would provide another approach to understanding what drives male infertility. The aim of the present study, therefore, was to determine

the relations of sperm chromatin protamination and condensation with ICSI outcomes in cycles including oocytes from healthy women.

Materials and methods

Inclusion criteria for participants, ethics committee and semen collection

The present retrospective study was conducted in two human cohorts both using semen from healthy donors. The analysis of the chromatin status was blinded from the reproductive results of each sample. The first cohort (double donation (DD) cohort) included 45 semen donors used in 55 ICSI cycles (491 total embryos obtained); oocytes were obtained from healthy female donors and each set of embryos was transferred to a single couple. High-quality blastocysts (HQB) from these 55 ICSI cycles were transferred to infertile women. The second cohort (single donation (SD) cohort) consisted of 34 semen donors used in 41 ICSI cycles (378 total embryos obtained); oocytes were obtained from single females requiring a donor for reproduction (35 from single-parent family and six from same-sex couples). HQB were transferred to the same woman who provided the oocytes in the case of single-parent family cycles, and to the partner in the case of same-sex couples. Out of the couples included in the SD cohort, 14.63% previously had natural pregnancies. The inclusion of the individuals into the two study cohorts (DD and SD) aimed to represent the different healthy individuals that one can find in the general population. The DD cohort included highly selected oocyte donors, whose age was <35 years old. The SD cohort included unselected woman who did not report a previous history of reproductive problems, could be of advanced maternal age and could even be in their first attempt to get pregnant. This SD cohort would be more representative of the general healthy population attempting to get pregnant.

For semen donors, the inclusion criteria were being in overall good health, not having a urinary tract infection, not having a previous history of hereditary diseases, not suffering from a sexually transmitted disease, and having normal seminal quality according to the reference values of the World Health Organization (WHO, 2010). For oocyte donors, the inclusion criteria were being <34 years old, having a normal karyotype, not having anatomical or endocrine alterations, not suffering from a sexually transmitted disease and not having a known hereditary disease. A comprehensive list of the inclusion and exclusion criteria for donors is shown in Supplementary Table SI. As couples included in the SD cohort were not selected on the basis of these criteria, but only required not to have a history of infertility, their available reproductive and clinical health data are displayed in Supplementary Table SII.

While cryopreserved straws from the same ejaculate intended for ICSI were utilized to evaluate sperm variables and for ICSI procedures, oocytes were not subjected to other analyses. ICSI for the treatment of patients followed the standard technique. The present study was approved by the Ethics Committee, Hospital Doctor Josep Trueta (Girona, Spain), under the reference *PTI-HUMA1001201 8*. All procedures complied with the Spanish legislation and followed the Helsinki Declaration, and all participants provided written informed consent.

Sperm samples were collected through ejaculation into a sterile cup, after 2–5 days of ejaculatory abstinence. Because semen was collected in the fertility clinics, samples were immediately brought to the laboratory, where they were allowed to liquefy for 30 min at 37°C. Thereafter, the sample volume was recorded, a small aliquot was kept at 37°C for basic semen analysis, and the rest of the ejaculate was cryopreserved. Cryopreserved straws were used for ICSI and for the evaluation of protamination and condensation of chromatin, as described below.

Macroscopic and microscopic semen analysis

After liquefaction, ejaculates were assessed and diagnosed following the criteria established by the 5th Edition of WHO Laboratory Manual for the Examination and Processing of Human Semen (WHO, 2010). First, a macroscopic evaluation was conducted, and sperm concentration, motility and morphology were subsequently determined.

Macroscopic evaluation comprised the assessment of volume, viscosity and pH. Volume was recorded in a volumetric tube. Viscosity (viscous versus non-viscous) was assessed using a Pasteur pipette. pH was evaluated by spreading a drop onto pH paper (range 6.0–10.0), and the resulting colour was compared to the calibration strip after 30 s.

Sperm concentration and motility were analysed using a commercial sperm analysis system (LensHooke® X1 PRO [X1 PRO], Bonraybio, Taichung, Taiwan), as per the manufacturer's protocol. Concentration was expressed in number of sperm cells per millilitre. For motility, the percentage of sperm with progressive motility (%Type A + %Type B), the percentage of sperm with non-progressive motility (%Type C) and the percentage of immotile sperm (%Type D) were recorded. Sperm morphology was evaluated using Diff-Quik staining kit (RAL Diagnostics, Martillac, France) according to the standard protocol and under the microscope at 1000× magnification. Percentages of morphologically normal and abnormal sperm were recorded, and alterations in sperm head and midpiece and tail were counted.

Cryopreservation and thawing of sperm samples

Liquefied sperm samples were slowly mixed at room temperature with Freezing Medium containing Test Yolk Buffer (Fujifilm Irvine Scientific; Santa Ana, CA, USA) at a ratio of 1:1 (v:v). Labelled straws (0.5 ml) were loaded with the final mixture and exposed to nitrogen vapours for 20 min. After that, straws were transferred into a liquid nitrogen tank and stored. Different straws from the same semen sample were used for the analysis of sperm chromatin and for fertilization of different oocyte sets.

For thawing, all straws were incubated at room temperature for 40 s, and then in a water bath at 37°C for further 40 s. Following this, the content of each straw was transferred into a sterile tube and kept at room temperature for 2 min. In the case of sperm evaluation, thawed sperm samples were centrifuged at 300×*g* at room temperature for 5 min; pellets were resuspended in PureSperm Wash at 37°C (PureSperm, Nidacon, Sweden) to a final concentration of 20×10^6 sperm/ml. Samples at this point were assessed as the 0-h timepoint and then incubated for a further 4 h for a dynamic evaluation of sperm chromatin.

For the straw used for ICSI, the aforesaid thawing protocol was applied, followed by centrifugation through a density gradient (90%-75%; PureSperm, Nidacon, Mölndal, Sweden) at 200×*g* at room temperature for 20 min for the selection of motile sperm. The resulting pellet was washed twice; first, with 3 ml and then with 2 ml of gamete medium (Sequential Fert.; Origio, Måløv, Denmark), by centrifuging at 200×*g* at room temperature for 5 min. The final pellet was diluted in gamete medium to a final concentration of 0.1–0.5 × 10^6 progressively motile sperm/ml. The concentration and percentage of progressively motile sperm after recovery was determined.

Analysis of the oxidation-reduction status of disulphide bridges (chromatin condensation)

The disruption of disulphide bridges was evaluated in unprocessed samples at 0 h post-thawing and after 4 h of incubation at 37°C in PureSperm Wash. The reduction of disulphide bridges was evaluated through cross-linking with dibromobimane (DBB) (Sigma-Aldrich, St. Louis, MO, USA), a cell-permeant bifunctional fluorogenic thiol-specific reagent that alkylates thiol pairs within ≈6Å (Sinz and Wang, 2001; Cox and Cardozo-Pelaez, 2007). Upon alkylation, DBB has an emission peak at 490 nm and an excitation peak at 394 nm. The alkylation of DBB occurs when a disulphide bond is reduced (R-SH HS-R); therefore, increased values of DBB^+ represent a decrease in chromatin condensation (Fig. 1). A stock solution of 4 mmol/l DBB was prepared by dilution in 100% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA).

Sperm were diluted 1:20 (v:v) in PBS to a final concentration of 1 × 10^6 sperm/ml. Samples were subsequently incubated with 20 µmol/l DBB at room temperature for 20 min. Following this, the fluorescence emitted by DBB was examined with a flow cytometer following the protocol described below. For each sample, a negative control without DBB was included. Positive controls were used to set up the method and consisted of incubation of sperm with 5 mmol/l dithiothreitol (DTT) for 45 min. Percentages of sperm with positive DBB labelling at 0 h (DBB^+ 0 h) and 4 h (DBB^+ 4 h) were recorded as variables assessing chromatin condensation. The relative variation of DBB^+-sperm between 0 and 4 h of incubation at 37°C (DBB^+ 4 h/DBB^+ 0 h) was recorded as an indicator of the resilience of sperm chromatin to decondensation (i.e. formation of disulphide bonds). Reduced values of this parameter thus indicated that sperm chromatin became more condensed; conversely, greater chromatin decondensation was assumed when they increased (Fig. 1).

Analysis of chromatin protamination

Protamination of sperm chromatin was assessed in unprocessed samples at 0 h post-thawing and after 4 h of incubation at 37°C in PureSperm Wash medium. Deprotaminated sites were detected through incubation with chromomycin A3 (CMA_3), an antibiotic that binds DNA in the presence of Mg^{2+}. As, in sperm cells, this binding is only possible when protamines are not bound to DNA, CMA_3 is a marker of regions with poor protamination. When attached to DNA, CMA_3 presents an excitation peak at 430 nm and an emission peak at 590 nm. A stock solution for CMA_3 was prepared at 5 mg/ml in ethanol.

Samples at 20 × 10^6 sperm/ml were diluted 1:1 (v:v) in 2× McIlvine solution (60 mmol/l citric acid, 280 mmol/l Na_2HPO_4 and 20 mmol/l $MgCl_2$) containing 12.5 µg/ml CMA_3 and incubated at room temperature for 30 min. Afterwards, samples were diluted 1:10 (v:v) in PBS, and the fluorescence emitted by CMA_3 was analysed using a flow cytometer following the protocol described below. For each sample, a negative control without CMA_3 was included. Positive controls were also included to set up the method and involved incubation of sperm with 5 mmol/l DTT and 1 mol/l NaCl for 8 min. Percentages of sperm with positive CMA_3-staining at 0 h (CMA_3^+ 0 h) and 4 h (CMA_3^+ 4 h) were recorded as variables evaluating chromatin protamination. The relative variation in CMA_3^+-sperm between 0 and 4 h of incubation (CMA_3^+ 4 h/CMA_3^+ 0 h) was recorded as an indicator of the resilience of sperm chromatin to deprotamination. Increased values of this parameter indicated that sperm chromatin became more deprotaminated (Fig. 1).

Flow cytometry

Fluorescence emitted by DBB and CMA_3 was analysed using a CytoFLEX flow cytometer (Beckman Coulter, Fullerton, CA, USA), equipped with red, blue and violet lasers (637, 488 and 405 nm, respectively). Each sample was evaluated on the basis of 10 000 sperm, at a flow rate between 10 and 60 µl/s. The flow cytometer was calibrated daily using CytoFLEX Daily QC Fluorospheres (Beckman Coulter, Fullerton, CA, USA). Analysis of dot-plots was carried out through CytoExpert Software (Beckman Coulter, Fullerton, CA, USA).

For both DBB and CMA_3, forward scatter and side scatter were used to gate the sperm cell population depicting the characteristic flame shape. For DBB, samples were excited with the violet laser, and the emitted fluorescence was collected through the KO525 channel (525/40 band pass). The average fluorescence intensity (arbitrary units) and the percentage of sperm with reduced disulphide bonds were recorded, taking into account the thresholds established from the positive control. For CMA_3, samples were excited with the violet laser and the emitted fluorescence was collected through the Violet610 channel (610/20 band pass). As for DBB, the average fluorescence intensity and the percentage of sperm with increased deprotamination were recorded.

Ovarian stimulation, oocyte retrieval and denudation

The protocol of ovarian stimulation and the doses of administered hormones were selected on the basis of donor anamnesis. Pituitary down-regulation was achieved using either a GnRH agonist or an antagonist. Ovarian stimulation was conducted through urine-derived or recombinant FSH. Final follicular maturation was triggered with hCG and/or a GnRH agonist when at least two leading follicles were found to measure 17 mm in diameter.

Thirty-six hours after triggering, ultrasound-guided collection of oocyte cumulus complexes (COCs) was performed transvaginally under sedation. Collected COCs were cultured in specific media for fertilization (Sequential Fert, Origio) covered with LifeGuard oil (LifeGlobal, Cooper-Surgical, Måløv, Denmark). After at least 2 h of culture, oocytes were prepared for ICSI using enzymatic hyaluronidase (FertiPro, Beernem, Belgium) and mechanical denudation.

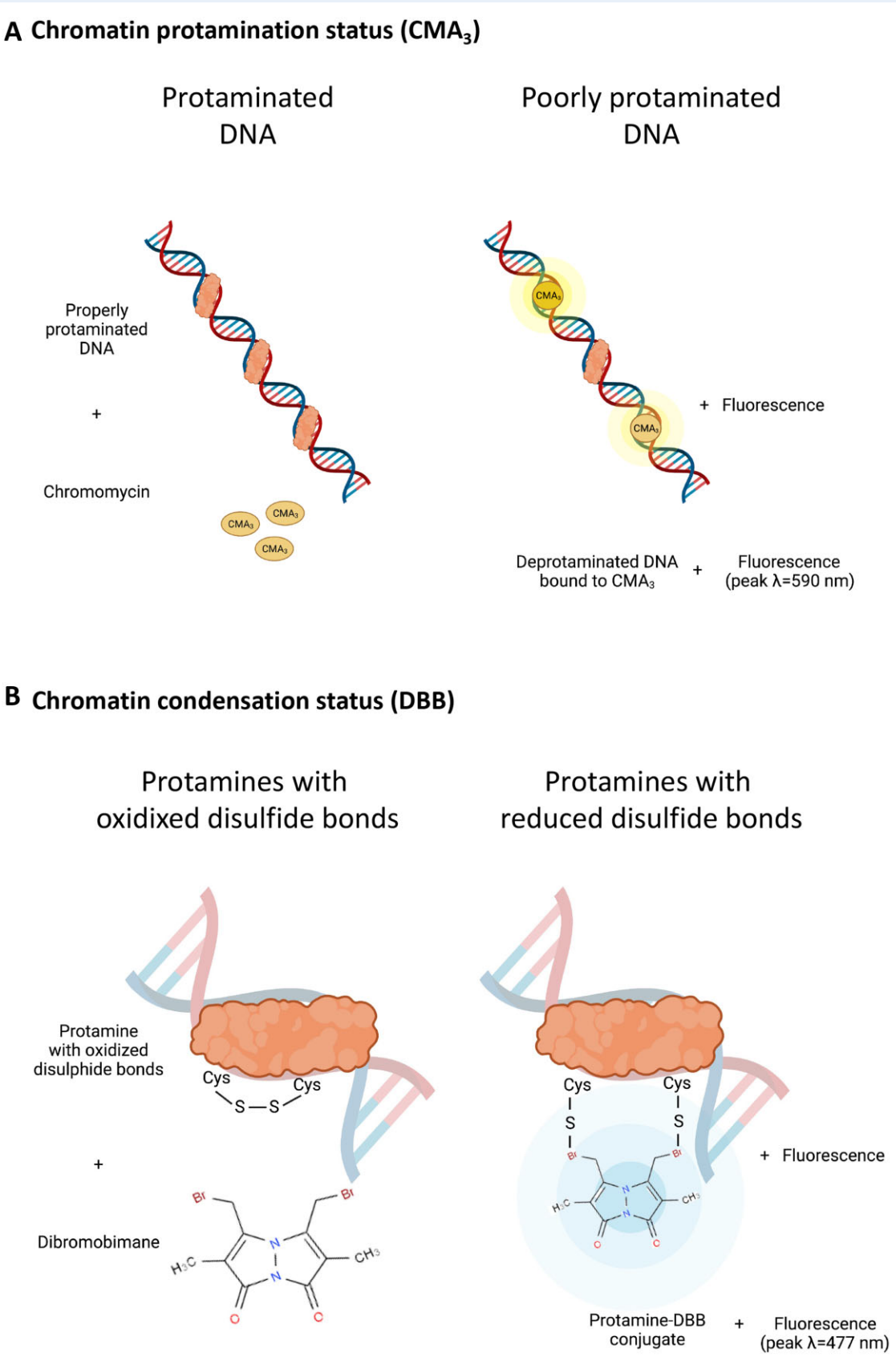

Figure 1. Schematic representation of human sperm chromatin protamination and condensation. (**A**) Analysis of sperm protamination using chromomycin A3 (CMA_3). In normal conditions, chromatin is properly protaminated in the minor groove of DNA; as CMA_3 is unable to

(continued)

ICSI and embryo culture

Sperm immobilization and micromanipulation were conducted in 10% polyvinylpyrrolidone in FertiCult Flushing medium (FertiPro). ICSI was performed using metaphase II oocytes (MII) between 39 and 41 h after hCG administration (3–5 h after COCs' collection). After ICSI, putative embryos were individually placed into EmbryoSlide culture dish microwells (Vitrolife, Denmark). Uninterrupted culture was conducted using a single culture medium (SAGE 1-Step with Human Albumin Solution; Origio) in an EmbryoScope+ time-lapse incubator (Vitrolife; Västra Frölunda, Denmark) at 37°C, 7% O_2, balanced N_2 and ~6% CO_2. The pH of the medium was previously adjusted to 7.2–7.3.

Time-lapse monitoring and embryo scoring

Time-lapse imaging of embryos was carried out through seven focal planes at intervals of 15 min. Uninterrupted monitoring lasted the entire period of culture (from immediately after ICSI and until Day 5 or 6), and the resulting sequences of images were evaluated by an experienced embryologist. Morphology and development kinetics of each embryo were recorded and scored through the KIDScore D5 tool (Vitrolife, Viby J, Denmark). Also, on Day 5 or 6, all blastocysts were graded following Gardner score criteria (Gardner *et al.*, 2000).

Blastocyst vitrification/warming and embryo transfer

High-quality blastocysts (HQB) corresponded to embryos with equal or >3BB in the Gardner score. HQB embryos from freeze-all cycles and those supernumerary from cycles with fresh embryo transfer were vitrified in Kitazato vitrification medium (Kitazato, Tokyo, Japan) using Cryotop® (Kitazato), following the manufacturer's standard procedure. In all patients, one single blastocyst (Day 5 or 6) was transferred into the uterine cavity, on a freeze-all basis. Blastocysts were warmed at least 2 h before transfer utilizing Kitazato warming medium (Kitazato).

Between 12 and 15 days after embryo transfer, all patients were asked to undergo a β-hCG pregnancy test. After confirmation, pregnancies were followed up by an ultrasound scan, to distinguish both the gestational sac and heartbeat. All pregnancies were traced until the final outcome (i.e. birth or miscarriage).

Statistical analyses

Although each semen sample from a donor could be used in separate ICSI cycles, it was treated as an independent statistical case. Hence, all reproductive outcomes obtained from a given sperm sample, which was used to fertilize different oocytes, were aggregated with the aim to increase the robustness of the male effect assessment.

Statistical analysis and graph preparation were performed using IBM SPSS for Windows 27.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism ver. 8 (GraphPad Software, La Jolla, CA, USA). First, normal distribution and homogeneity of variances were assessed through Shapiro–Wilk and Levene tests, respectively. For those parameters that did not fit with parametric assumptions, non-parametric tests were run. Comparisons between three or more groups were made through Kruskal–Wallis, followed by the Mann–Whitney test for pair-wise comparisons. The Wilcoxon test was used for comparisons between related samples, and correlations were evaluated through the Spearman test. The effects of sperm variables (independent variables) on fertilization and blastocyst rates (quantitative dependent variables) were determined through multiple linear regression using the least squares method. The effects of sperm variables (independent variables) on clinical outcomes (qualitative dependent variables: β-hCG, pregnancy rate and live birth rate) were determined through multiple logistic regression, where the dependent variables were dichotomic (positive or negative).

To reduce the chances of obtaining biased significant values owing to the low sample size and thus reduce the Type I error, the level of significance was set at $P \leq 0.01$.

Results

The two cohorts studied differed in the age of women providing oocytes

Descriptive data about age, BMI, conventional spermiogram, ICSI outcomes and clinical variables for DD and SD cohorts are shown in Table I. While no differences in semen donor age, BMI, sperm quality parameters or ICSI outcomes were observed between SD and DD cohorts ($P > 0.01$), the age of women providing oocytes was significantly higher in the SD than in the DD cohort ($P < 0.001$). Remarkably, the age of women receiving transferrable embryos was lower in the SD than in the DD cohort ($P < 0.001$). Because of these differences, the potential bias of female age on the effects of sperm chromatin was interrogated.

Chromatin protamination, but not condensation, is related to sperm quality

Chromatin condensation showed no correlation to sperm count, motility or morphology ($P > 0.01$). In contrast, poor chromatin protamination was negatively correlated to the percentage of sperm with normal morphology ($Rs = -0.391$; $P = 0.008$) and progressive motility after density gradient washing ($Rs = -0.423$; $P = 0.003$) (Supplementary Table SIII), indicating that sperm chromatin protamination was lower in samples with lower sperm quality.

Figure 1. Continued
bind DNA, fluorescence is not emitted. When chromatin is poorly protaminated, the DNA is more accessible and is bound by CMA_3, which emits fluorescence with a peak at 590 nm. (**B**) Analysis of chromatin condensation with dibromobimane (DBB). When protamine disulphide bridges are oxidized, DBB is unable to bind free thiols and does not emit fluorescence. When disulphide bridges are reduced, free Br covalently binds thiols and DBB emits fluorescence with a peak at 477 nm.

Table I Descriptive variables of double and single donation cohorts.

	Double donation cohort (DD cohort)		Single donation cohort (SD cohort)		
	Mean ± SD	*95% CI*	*Mean ± SD*	*95% CI*	*P*-value
Physiological parameters					
Age of women providing oocytes (years)	25.20 ± 3.72	23.00–26.00	34.02 ± 5.59	32.00–37.00	**<0.0001**
Age of women receiving embryos (years)	43.56 ± 3.80	43.00–45.00	38.25 ± 5.77	36.00–41.00	**<0.0001**
Age of men providing sperm (years)	24.20 ± 5.29	21.00–26.00	26.06 ± 6.21	22.00–28.00	0.188
BMI of women providing oocytes (kg/m^2)	22.16 ± 3.06	20.31–22.44	23.31 ± 3.55	20.66–24.74	0.187
BMI of women receiving embryos (kg/m^2)	22.95 ± 3.44	21.92–23.98	22.30 ± 5.51	20.38–24.22	0.935
Basic sperm quality parameters					
Volume (ml)	3.76 ± 1.62	3.00–4.50	3.34 ± 1.58	2.50–3.50	>0.999
Sperm concentration (sperm/ml)	80.62 ± 31.23	65.00–96.00	69.24 ± 25.39	58.00–81.00	>0.999
Sperm count (total sperm)	296.60 ± 158.50	216.00–330.00	236.40 ± 163.80	146.00–260.00	>0.999
Progressive motile sperm (%)	59.62 ± 12.72	56.00–66.00	60.44 ± 12.20	56.00–66.00	>0.999
Non-progressive motile sperm (%)	7.73 ± 4.76	6.00–9.00	7.65 ± 4.53	4.00–11.00	>0.999
Immotile sperm (%)	32.64 ± 11.25	27.00–35.00	31.91 ± 11.94	25.00–33.00	>0.999
Sperm with normal morphology (%)	15.58 ± 4.10	15.00–16.00	16.32 ± 4.68	84.00–86.00	>0.999
Head abnormalities (%)	55.38 ± 9.58	49.00–59.00	55.18 ± 10.79	49.00–61.00	>0.999
Midpiece and tail abnormalities (%)	1.76 ± 0.98	2.00–2.00	1.68 ± 1.07	1.00–2.00	>0.999
Sperm concentration after motile sperm recovery (sperm/ml)	10.12 ± 11.73	3.43–8.50	12.07 ± 11.99	5.80–13.00	>0.999
Progressive motile sperm after motile sperm recovery (%)	72.02 ± 19.44	72.00–84.00	75.98 ± 15.73	74.00–85.00	>0.999
Total motile sperm after motile sperm recovery (sperm/ml)	6.99 ± 7.33	2.60–6.88	9.17 ± 8.68	3.96–10.12	>0.999
ICSI outcomes					
Metaphase II oocytes retrieved (n)	10.80 ± 7.29	8.00–11.00	11.41 ± 5.59	8.00–13.00	0.244
Metaphase II oocytes injected (n)	10.89 ± 7.33	8.00–11.00	11.53 ± 5.51	8.00–14.00	0.237
Fertilized embryos obtained (n)	7.96 ± 5.08	5.00–8.00	8.56 ± 4.63	6.00–10.00	0.308
Blastocysts obtained (n)	5.49 ± 4.00	3.00–6.00	5.62 ± 3.77	3.00–6.00	0.756
Transferred embryos per cycle	1.40 ± 0.81	1.00–1.00	1.53 ± 1.13	1.00–2.00	0.950
Fertilization Rate (% 2PN)	75.88 ± 18.30	70.00–84.62	73.82 ± 16.21	64.29–84.21	0.507
Blastocyst Rate (% Blastocysts)	67.03 ± 22.68	60.00–75.00	64.92 ± 18.82	54.55–75.00	0.491
%Blastocysts with grade A for trophectoderm	22.58 ± 22.55	15.64–29.52	19.31 ± 18.45	12.76–25.85	0.679
%Blastocysts with grade A for inner cell mass	28.21 ± 26.36	20.10–36.32	24.14 ± 26.98	14.58–33.71	0.298
%Blastocysts with grade A + B for trophectoderm	70.12 ± 23.96	62.74–77.49	76.12 ± 21.60	68.47–83.78	0.275
%Blastocysts with grade A + B for inner cell mass	80.16 ± 17.37	74.82–85.51	80.63 ± 18.30	74.15–87.12	0.883
Clinical outcomes					
Implantation rate (%, implanted embryos/transferred embryos)	68.25 % (43/63)		63.46 % (33/52)		#
Clinical pregnancy rate (%, number pregnancies/number cycles)	60.31 % (38/63)		51.92% (25/52)		#
Live birth rate (%, number live birth/number cycles)	53.96 % (35/63)		40.38 % (21/52)		#
Miscarriage rate (%, number miscarriages/number cycles)	4.76 % (3/63)		9.61 % (5/52)		#

DD cohort: 45 semen donors used in 55 ICSI cycles; SD cohort: 34 semen donors used in 41 ICSI cycles. Pair-wise comparisons were assessed using the Mann–Whitney test.
Values were not compared because while the embryos resulting from oocytes from SD cohort were transferred to the same woman, the embryos resulting from oocytes from DD cohort were transferred to a different woman.
Bold values indicate statistically significant differences.

Although post-thawing incubation decreases chromatin protamination, it increases condensation through oxidation of close free thiol groups

Table II shows mean and standard deviation for sperm chromatin condensation and protamination of sperm chromatin at 0 and 4 h post-thawing. Incubation of frozen-thawed sperm for 4 h led to a reduction of chromatin protamination, since the values of CMA_3^+ cells increased in both cohorts (relative increase over 4 h for DD: 82.69 ± 77.29%; relative increase for SD: 79.66 ± 77.51; $P < 0.001$). Despite this observed loss of protamines, sperm chromatin condensation was augmented, as the presence of close free thiol groups (DBB^+) was reduced over the 4-h period ($P < 0.001$). The relative

Table II Levels of protamination and condensation of human sperm chromatin at different time points after thawing.

	T = 0 h		T = 4 h		% Variation (T4–T0)/T0		
	Mean ± SD	95% CI	Mean ± SD	95% CI	Mean ± SD	95% CI	P-value
Double donation cohort							
Sperm with poor chromatin protamination (%CMA_3^+)	21.08 ± 9.09	17.04–24.3	35.01 ± 14.68	29.3–36.68	82.69 ± 77.29	45.93 to 95.01	**<0.0001**
Sperm with poor chromatin condensation (% DBB^+)	16.57 ± 11.1	9.54–19.26	10.51 ± 8.401	5.5–12.02	−30.49 ± 38.19	−42.22 to −11.52	**<0.0001**
Single donation cohort							
Sperm with poor chromatin protamination (% CMA_3^+)	22.57 ± 9.476	15.89–27.29	35.79 ± 12.58	30.11–36.85	79.66 ± 77.51	42.53 to 92.17	**<0.0001**
Sperm with poor chromatin condensation (% DBB^+)	17.98 ± 10.19	11.26–19.26	12.72 ± 8.765	7.02–17.6	−26.25 ± 34.04	−29.17 to −11.95	**<0.0001**

DD cohort: 45 semen donors used in 55 ICSI cycles; SD cohort: 34 semen donors used in 41 ICSI cycles. Pair-wise comparisons were assessed using the Mann–Whitney test.
P-value indicates differences between 0 h and 4 h of incubation.
CMA_3: chromomycin A3; DBB: dibromobimane.
Bold values indicate statistically significant differences.

decrease of DBB^+ sperm, which was −30.50 ± 38.19% in DD and −26.30 ± 34.04% in SD, can be understood as the *in vitro* capacity of sperm to condense their chromatin, a notion that is referred to below.

The age of females providing oocytes correlates to fertilization and blastocyst rates only in the SD cohort

Female age is widely known as a relevant parameter to define oocyte quality indirectly, therefore we assessed whether age had an influence on ICSI outcomes. In the DD cohort, the age of females providing oocytes was not correlated to fertilization or blastocyst rates ($P > 0.01$; Supplementary Table SIV); these females, nevertheless, were young and their age ranged between 18 and 33 years old. In the SD cohort, female age was negatively correlated to fertilization rates ($Rs = -0.475$; $P = 0.004$; Supplementary Table SIV); it is worth mentioning that age in this cohort ranged between 20 and 44 years old.

Poor chromatin protamination is related to fertilization rates, and the in vitro capacity of sperm to condense their chromatin is associated with blastocyst rates

As fertilization or blastocyst rates were not found to be correlated to sperm count, motility or morphology ($P > 0.01$; Supplementary Table SIV), whether condensation and protamination of sperm chromatin were correlated to ICSI outcomes was tested (Supplementary Table SIV). While fertilization rate was found to be negatively correlated to the percentage of poor chromatin protamination in the DD cohort ($Rs = -0.527$; $P < 0.001$; Fig. 2A, upper-left graph), no correlation in the case of the SD cohort was observed ($Rs = -0.053$; $P = 0.764$; Fig. 2A, bottom-left graph). In addition, the relative decrease of protamination over the 4-h incubation period was not related to fertilization rate ($P > 0.01$; Fig. 2A, graphs on right). Regarding chromatin condensation measured through the DBB test, it was not found to be correlated to fertilization rates ($P > 0.01$; Fig. 3A).

Blastocyst rates were not correlated to sperm protamination status measured through the CMA_3 test ($P > 0.01$; Fig. 2B). The *in vitro* capacity of sperm to condense their chromatin (measured as the relative variation of DBB^+ between 0 and 4 h of incubation), however, was correlated to blastocyst rates in both DD ($Rs = -0.393$; $P = 0.008$; Fig. 3B, upper-right graph) and SD cohorts ($Rs = -0.473$, $P = 0.005$; Fig. 3B, bottom-right graph). This correlation suggests that sperm that are more capable of condensing their chromatin *in vitro* (i.e. after incubation at 37°C for 4 h) give rise to higher blastocyst rates.

As the age of women providing oocytes was found to influence fertilization rates, the aforementioned relations were tested through multiple linear regression. CMA_3 was found to be associated with fertilization rate independently from female age in the DD cohort ($\beta_1 = -1.036$, $P < 0.001$ for CMA_3; $\beta_2 = 0.667$ $P = 0.304$ for female age); in the SD cohort, nevertheless, female age had a greater impact than CMA_3 on fertilization rate ($\beta_1 = -0.066$, $P = 0.804$ for CMA_3; $\beta_2 = -1.451$, $P = 0.003$ for female age). Moreover, the *in vitro* capacity of sperm to condense their chromatin was found to be associated with blastocyst rates independently from female age, in both DD ($\beta_1 = -0.238$, $P = 0.008$ for %DBB^+ variation; $\beta_2 = 0.404$, $P = 0.638$ for female age) and SD cohorts ($\beta_1 = -0.278$, $P = 0.010$ for %DBB^+ variation; $\beta_2 = -0.292$, $P = 0.594$ for female age).

Chromatin protamination and condensation are not related to blastocyst quality

The quality of trophectoderm and inner cell mass was assessed in embryos reaching the blastocyst stage (Table I). Neither chromatin protamination nor condensation evaluated at 0 and 4 h post-thawing were found to be correlated to the percentage of blastocysts with top quality (grade A) or high quality (A + B) for trophectoderm ($P > 0.01$) and for inner cell mass ($P > 0.01$) (Supplementary Table SIV). Similarly, blastocyst quality was not found to be correlated to sperm quality, female age or BMI ($P > 0.01$, Supplementary Table SIV).

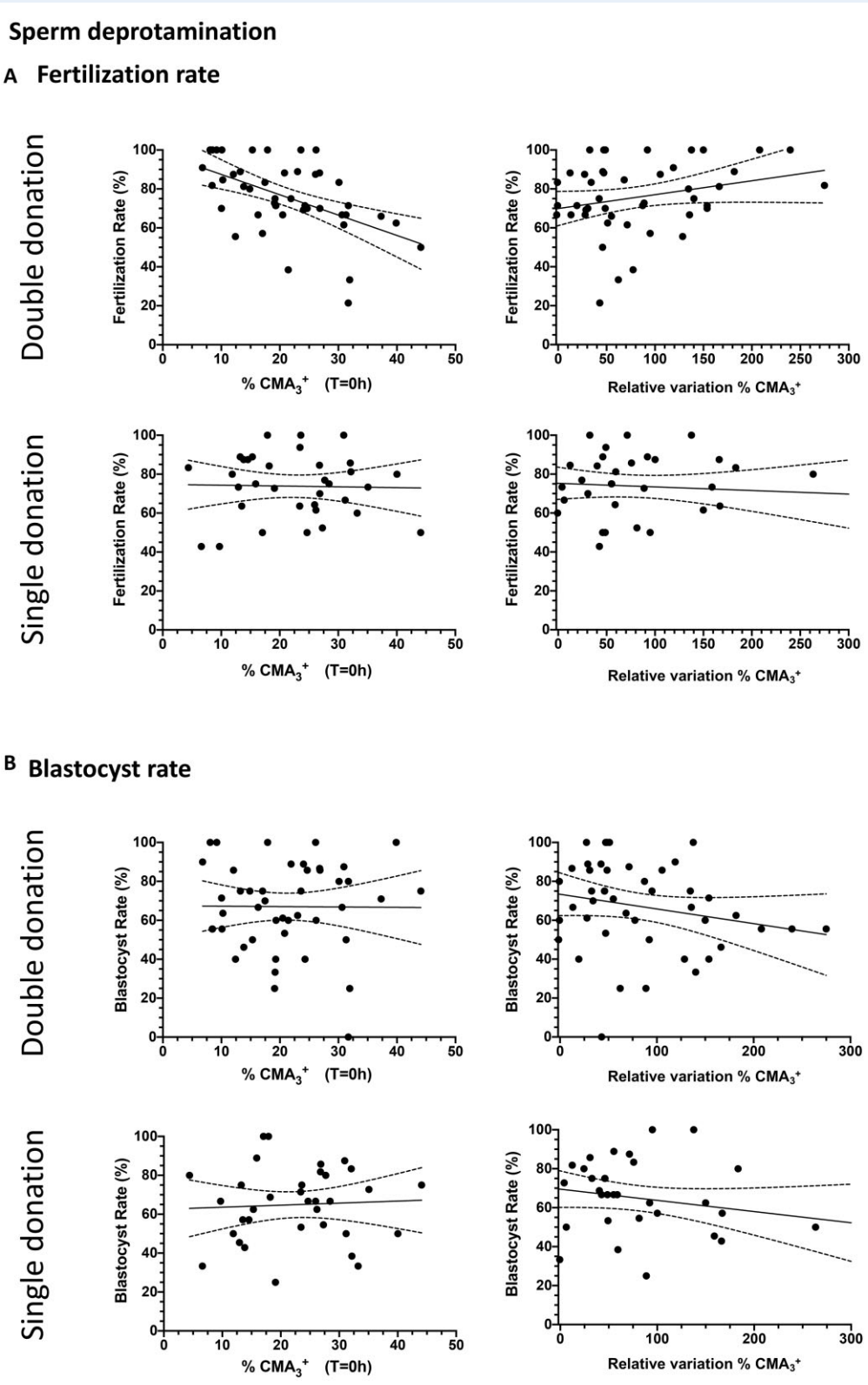

Figure 2. Panel representing correlations with human sperm chromatin protamination. (**A**) Correlations of sperm chromatin protamination with fertilization rates after ICSI and (**B**) correlations of sperm chromatin protamination with blastocyst rates. chromomycin A3 (CMA_3). Double donation cohort: 45 semen donors used in 55 ICSI cycles; single donation cohort: 34 semen donors used in 41 ICSI cycles. Correlations were calculated with the Spearman test.

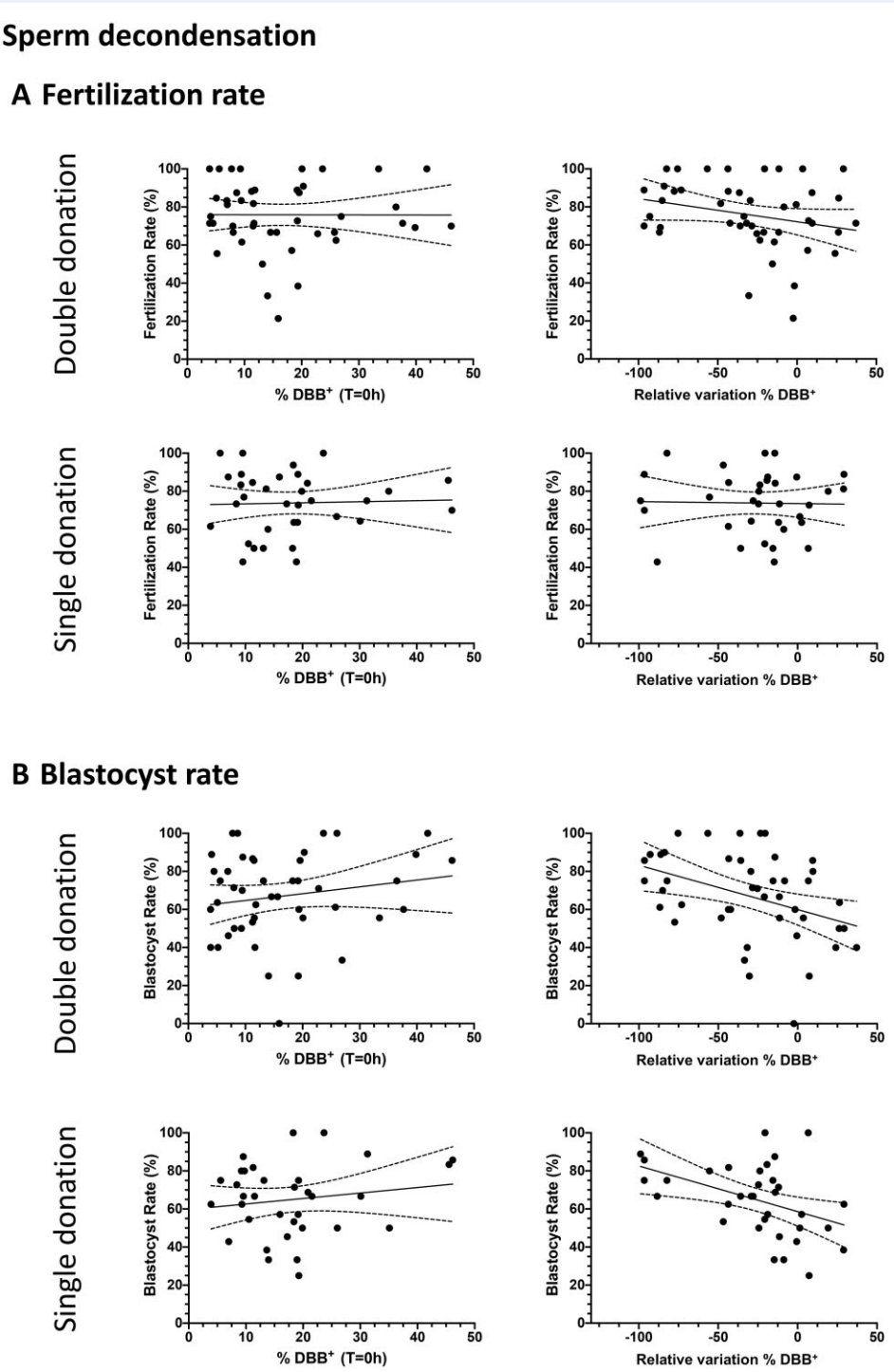

Figure 3. Panel representing correlations with human sperm chromatin condensation. (**A**) Correlations of sperm chromatin condensation with fertilization rates after ICSI and (**B**) correlations of sperm chromatin condensation with blastocyst rates. dibromobimane (DBB). Double donation cohort: 45 semen donors used in 55 ICSI cycles; single donation cohort: 34 semen donors used in 41 ICSI cycles. Correlations were calculated with the Spearman test.

In vitro capacity of sperm to condense their chromatin is related to delayed embryo development in DD cycles

After determining that protamination and condensation of sperm chromatin have an effect on fertilization and blastocysts rates, respectively, time-lapse monitoring was used to address whether these variables also affected embryo development kinetics (Table III).

For DD cycles, the time needed for the embryo to reach the blastocyst stage was found to be correlated to the percentage of sperm with poor protamination after 4 h of incubation ($Rs = 0.457$; $P = 0.002$) and to the *in vitro* capacity of sperm to condense their chromatin ($Rs = 0.392$; $P = 0.009$) (Supplementary Table SV). As female age could influence these correlations, multiple linear regression analysis was run. In the case of chromatin protamination, female age was a cofactor influencing the aforesaid correlation; thus, chromatin protamination did not show a statistically significant correlation with the time required for the embryo to reach the blastocyst stage ($\beta_1 = 0.665$, $P = 0.014$ for CMA_3; $\beta_2 = 3.586$, $P < 0.001$ for female age). On the other hand, both the *in vitro* capacity of sperm to condense their chromatin and female age influenced the time needed for the embryo to reach the blastocyst stage ($\beta_1 = 0.189$, $P = 0.007$ for $\%DBB^+$ variation; $\beta_2 = 4.365$, $P < 0.001$ for female age).

For SD cycles, neither chromatin protamination nor condensation or female age were found to be correlated to embryo development kinetics ($P > 0.01$) (Supplementary Table SV).

Protamination and condensation of sperm chromatin have an impact on clinical outcomes when HQB are transferred

After testing the effects of chromatin protamination and condensation on embryo development, whether these parameters influenced clinical outcomes was interrogated. For this purpose, multiple logistic regression including clinical outcomes as dependent variables was run; CMA_3^+, DBB^+, their variation between 0 and 4 h and the ages of females providing oocytes and of those receiving embryos were considered as independent variables. In the case of the DD cohort, statistically significant models were found for the achievement of clinical pregnancy, with an AUC of 0.747 (95% CI: 0.596–0.904) ($P = 0.01$), and for live birth, with an AUC of 0.756 (95% CI: 0.614–0.899) ($P = 0.005$). The estimate parameters and odds ratios for these models are shown in Supplementary Table SVI. For the SD cohort, statistically significant regression models were set for positive β-hCG, with an AUC of 0.961 (95% CI: 0.899–1.000) ($P < 0.001$); for clinical pregnancy, with an AUC of 0.904 (95% CI: 0.792–1.000) ($P < 0.001$); and for live birth, with an AUC of 0.864 (95% CI: 0.738–0.990) ($P < 0.001$). Values of parameters and odds ratios for each significant model are shown in Supplementary Table SVI; ROC curves are depicted in Supplementary Fig. S1.

Discussion

Under natural conditions, sperm genotoxic defects are known to detrimentally affect male fertility (Esteves *et al.*, 2021). In spite of this, it has not been fully elucidated whether these alterations have an influence on ICSI cycles, where the injection of a single sperm cell into the ooplasm bypasses the natural gamete fusion process (Ribas-Maynou *et al.*, 2021). Although different molecular biomarkers, including sperm DNA damage, have hitherto been tested to predict ICSI outcomes, less attention has been paid to other sperm chromatin alterations, such as chromatin condensation or protamination. For this reason, the present work evaluates, for the first time, the effects of chromatin condensation and protamination on ICSI embryos produced using gametes from healthy individuals. In particular, the sperm and oocytes utilized here were obtained from cycles of double gamete donation (DD cohort) and from cycles involving a semen donor and a single-parent family or a lesbian couple providing the oocytes without history

Table III Human embryo kinetics for both double and single donation cohorts.

	Double donation cohort (DD cohort)		Single Donation cohort (SD cohort)	
	Mean ± SD	*95% CI*	*Mean ± SD*	*95% CI*
tP	11.03 ± 4.14	9.78–12.27	11.80 ± 3.97	10.39–13.21
tPNf	22.30 ± 2.02	21.70–22.91	22.75 ± 2.24	21.96–23.55
t2	25.25 ± 2.41	24.52–25.97	25.43 ± 2.30	24.62–26.25
t4	37.76 ± 4.30	36.47–39.05	37.66 ± 3.34	36.47–38.84
t6	50.59 ± 4.92	49.11–52.07	51.08 ± 5.18	49.24–52.92
t8	56.58 ± 5.60	54.90–58.27	58.63 ± 6.15	56.45–60.81
tSC	76.97 ± 6.23	75.10–78.84	78.91 ± 6.29	76.68–81.14
tM	86.19 ± 5.71	84.45–87.93	87.84 ± 7.29	85.25–90.42
tSB	97.02 ± 4.52	95.65–98.40	98.94 ± 5.25	97.08–100.80
tB	107.00 ± 5.38	105.30–108.60	109.20 ± 7.77	106.50–112.00
tEB	115.40 ± 6.26	113.40–117.30	117.50 ± 6.57	115.00–120.00

t2: time to 2 cells; t4: time to 4 cells; T6: time to 6 cells; T8: time to 8 cells; tEB: time to expanded blastocyst; tB: time to blastocyst; tM: time to Morula; tP: time to pronuclear appearance; tPNf: time to pronuclear fading; tSB: time to starting blastocyst; tSC: time to starting compaction.

of infertility (SD cohort). The evaluation and comparison of sperm parameters in these cohorts is of great importance to help segregate the influence of sperm chromatin from other confounding factors that may bias the results (Gat et al., 2017; Esbert et al., 2018; Borges et al., 2019; Green et al., 2020). Such a bias is well identified in clinical studies involving infertile individuals and is usually attributed to the female or other unknown factors causing infertility. As a result, investigations unequivocally assessing the utility of paternal biomarkers when ICSI is conducted have been repeatedly requested by different fertility societies (Barratt et al., 2010; Jarow et al., 2010). For this purpose, the use of gametes from healthy individuals is of great importance.

In the present study, poor chromatin protamination was shown to be correlated to lower fertilization rates independently from female age in DD cycles. Such a relation, however, was not observed in the SD cohort, where the age of the female providing oocytes appeared to have a stronger impact than sperm chromatin protamination. In addition to that, the *in vitro* capacity of sperm to condense their chromatin, defined as the decrease of DBB^+ after 4 h of incubation at 37°C, was found to be related to blastocyst rates in both cohorts, regardless of female age. Regarding embryo development kinetics, sperm chromatin condensation had an influence on the time needed for the embryo to reach the blastocyst stage in the DD, but not in the SD cohort. On the other hand, chromatin protamination was not related to embryo development kinetics, as female age was found to be a confounding factor masking this association. Finally, condensation and protamination of sperm chromatin were not correlated to the morphological quality of blastocysts when inner cell mass or trophectoderm were evaluated.

Previous systematic reviews and meta-analyses about the effects of sperm DNA damage on fertilization and embryo development were not conclusive in the case of ICSI (Simon et al., 2017; Deng et al., 2019; Ribas-Maynou et al., 2021); therefore, the present work focused on other aspects of sperm chromatin, such as condensation and protamination, that have been less well investigated. Remarkably, previous research indicated that features of sperm chromatin other than DNA fragmentation could be relevant to male (in)fertility. For instance, an altered protamine 1:protamine 2 ratio is known to cause male infertility (Mengual et al., 2003; Torregrosa et al., 2006), and regions retaining histones may contain genes necessary for early embryo development (Hammoud et al., 2009; Yoshida et al., 2018). While chromatin condensation is known to protect sperm DNA from damaging agents, including reactive oxygen species (Sakkas and Alvarez, 2010; Ward, 2010), no study has previously addressed if chromatin protamination and condensation are related to ICSI outcomes in healthy individuals.

In the present work, the chromatin of human sperm was shown to become deprotaminated upon post-thawing incubation for 4 h. Yet, in this process, free thiols in sperm chromatin were found to be oxidized, thus leading to greater condensation. The *in vitro* loss of protamines observed in the current study agrees with previous works conducted in bovine sperm, where deprotamination was also observed over post-thawing incubation (Llavanera et al., 2021), and with other authors suggesting that this removal could be related to an increase in DNA damage (Tvrdá et al., 2018). In fact, regions with poor protamination may be more susceptible to enzymatic DNA damage (Ribas-Maynou et al., 2015). In the present study, while sperm were found to be deprotaminated after incubation at 37°C for 4 h, the degree of condensation, measured as the oxidized status of protamines and the number of disulphide bonds formed, increased. While, at first glance, this observation could seem to be inconsistent, one could hypothesize that the greater DNA condensation observed could result from a physiological mechanism providing some DNA regions with a higher protection during the transport of sperm towards the oocyte. This mechanism could be especially important during sperm capacitation, as this event is associated with a certain increase of reactive oxygen species, which are known to impair sperm DNA integrity (Aitken, 2017; Betarelli et al., 2018). Thus, increased chromatin condensation could serve to protect important regions of the sperm genome that bear genes involved in early embryo development. This conjecture would be supported by the sperm chromatin condensation observed, which was ~26–30%, and would agree with a certain plasticity of the sperm genome. Future studies should address whether sperm chromatin can condense further during capacitation, as this would confirm or dismiss the mechanism hypothesized here.

To understand how protamination and condensation of sperm chromatin may affect human embryo development and to address if these parameters could be prognostic of ICSI outcomes, their relation with fertilization and blastocyst rates in both SD and DD cohorts was tested here. Female age was correlated to blastocyst rates in the SD cohort; therefore, it was added as a parameter in the multiple linear regression analyses assessing sperm chromatin. Chromatin protamination was found to be related to fertilization rates in the DD cohort, independently from female age. In the SD cohort, however, this relationship was not observed; in fact, female age had a stronger impact on fertilization rates and this could mask the effects of sperm protamination. While no previous ICSI research has been conducted in fertile donors, the relation between sperm chromatin protamination and fertilization rates in infertile couples undergoing ICSI is conflicting among studies. Indeed, some authors reported a significant correlation (Nasr-Esfahani et al., 2005, 2008; Ahmed et al., 2018), and others indicated a lack of association between these two variables (Tarozzi et al., 2009; Zeyad et al., 2018; Bichara et al., 2019). Moreover, other studies found that sperm chromatin protamination has an effect on embryo quality parameters (Sadeghi et al., 2009; Marchiani et al., 2017; Mangoli et al., 2018).

The inclusion of both SD and DD cohorts is a strength of the current study, as it allows a determination of the impact of sperm chromatin protamination on fertilization rates when using oocytes from young healthy donors compared to older healthy women. This approach also helps understand the potential bias caused by female age when male-factor infertility is assessed. The correlation between chromatin protamination and fertilization rates observed in the DD cohort could also be explained by the fact that round spermatids, which lack protamines, have limited capacity to activate the oocyte (Tesarik et al., 1996; Yanagimachi, 2022). While this decreased ability has been attributed to the failure of round spermatids to trigger Ca^{2+} oscillations and to the lack of some sperm-borne RNAs (Yazawa et al., 2000), one should not exclude the instrumental role played by nucleoproteins, as protaminated regions drive the inheritance of histones (including those that are epigenetically modified) at certain regions of the genome that are potentially involved in embryo development (Lambrot et al., 2021). Examples of these regions are, for instance, CTCF-binding sites, which are present in genes involved in early embryo development and have been suggested to regulate the replacement of histones by protamines (Torres-Flores and Hernández-Hernández, 2020).

On the other hand, the *in vitro* capacity of sperm to condense their DNA was found to be related to blastocyst rates, but not to fertilization rates, in the two cohorts. Thus, sperm that could better react by condensing their chromatin in response to an incubation period of 4 h were those that would lead to greater blastocyst rates. Previous experiments conducted in animal models indicated that treating sperm with DTT to reduce disulphide bridges does not affect fertilization rates or embryo development to blastocyst (Ahmadi and Ng, 1999; Shirazi *et al.*, 2011; Arias *et al.*, 2014). In this study, the capacity of sperm to condense their chromatin during post-thawing incubation, rather than the degree of condensation itself, was found to be correlated to blastocyst rates. These results suggest that sperm may need to provide an extra protection to those DNA regions that are important for preimplantation development; at the time of fertilization, this could be more relevant for embryogenesis than the degree of chromatin condensation. In fact, improper condensation of specific regions could represent an advantage for genotoxic compounds to damage the sperm genome, thus affecting embryo development at later stages. To shed light into this hypothesis, whether chromatin protamination and condensation could affect embryo morphology and embryo kinetics was also investigated. Results showed no correlation between embryo morphology and sperm chromatin condensation or protamination. Yet, the time needed for the embryo to reach the blastocyst stage correlated to the *in vitro* capacity of sperm to condense their chromatin in the DD cohort, but not in the SD cohort, where female age had a stronger impact. Embryo development kinetics were previously associated with the presence of sperm genotoxic damage in infertile couples (Wdowiak *et al.*, 2015; Casanovas *et al.*, 2019; Setti *et al.*, 2021) and fertile sperm donors (Esbert *et al.*, 2018). Based on the current data, one may suggest that, in addition to the aforementioned, the evaluation of sperm condensation may help to predict embryo development following ICSI when young oocytes are used and, thus, the maternal effect is null.

The assessment of clinical outcomes through multiple logistic regression revealed that condensation and protamination of sperm chromatin interact with the age of oocyte donors and of the embryo recipients; all these factors are related to fertility outcomes. While odds ratios indicated a similar effect of sperm chromatin and female age on clinical outcomes in the DD cohort, the impact of female age was higher than that of sperm chromatin in the SD cohort. As mentioned above, it is worth highlighting that no previous study has investigated the joint effects of sperm chromatin alterations and female age on fertilization rates and embryo development in ICSI cycles using gametes from healthy individuals. Taking all these findings into consideration, it appears that assessing sperm chromatin parameters corrected by female age could be useful to predict ICSI outcomes.

Finally, it is worth noting that the restrictive couple selection conducted in the present study led to a relatively small sample size, which could affect the observed outcomes. For this reason, and to reduce Type I error, the level of significance was set at $P \leq 0.01$. Furthermore, the use of cryopreserved samples could also be a limitation; thus, further research addressing whether freeze-thawing has an impact on the protamination and condensation of sperm chromatin is much needed.

In summary, three main conclusions could be reached from the present study. First, while poor sperm chromatin protamination negatively affects fertilization rates in ICSI cycles involving gametes from healthy donors, advanced female age has a noticeable impact and masks the potential effect of sperm chromatin alterations when older females are tested. Second, the *in vitro* capacity of sperm to condense their chromatin after 4 h of incubation is correlated to blastocyst rates and embryo development, and this association is independent from the age of females providing oocytes. Finally, in healthy individuals, sperm chromatin status (protamination and condensation), the age of females providing oocytes and that of those receiving embryos are associated with pregnancy rates and successful live birth following ICSI.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

Databases obtained during the current study are available from the corresponding author on reasonable request.

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Figure 1 was created with BioRender.com.

Authors' roles

Conceptualization: J.R.-M., A.S.-H. and M.Y.; methodology: J.R.-M., S.N., A.S.-H., S.R. and M.A.; writing—original draft preparation: J.R.-M.; writing—review and editing: J.R.-M. and M.Y.; supervision: M.A. and M.Y. All authors have read and agreed to the published version of the article.

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Conflict of interest

The authors declare that they have no competing interests.

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