Is the lack of prior exposure to sperm antigens associated with worse neonatal and maternal outcomes? A 10-year single-center experience comparing ICSI–TESE pregnancies to ICSI pregnancies

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Abstract
Background: Nowadays, pathogenesis of preeclampsia (PE) is still unknown. Among the different etiological hypotheses, some authors proposed that it might be because of an abnormal immunologic response to a foreign fetal antigen derived from the father’s spermatozoa. Indeed, the fetus is considered a semi-allograft, being one half paternally derived in its antigenicity, and the first pathogenic insult of PE may be an abnormal maternal immune response toward this semi-allogenic implant. In the context of artificial reproductive techniques, it has been shown that the use of donor and surgically retrieved spermatozoa (e.g., testicular sperm extraction [TESE]) increases the risk of PE, confirming the protective effect of sperm exposure on maternal complications.

Objective: Determining whether the lack of exposure to sperm antigens is associated with worse maternal and neonatal outcomes in pregnancies obtained through intracytoplasmic sperm injection after TESE (ICSI–TESE) for obstructive azoospermia (OA).

Materials and methods: This is a single-center case–control retrospective study, focusing on all first pregnancies obtained through ICSI–TESE for OA at Humanitas Fertility Center between January 1, 2010 and December 31, 2019. Controls included patients that achieved their first pregnancy with ICSI and ejaculated spermatozoa, for a diagnosis other than azoospermia, in the same time period. Cases were matched with controls in a 1:2 ratio, considering female age, female BMI, and year of controlled ovarian stimulation. The primary outcome measure was the delivery rate, defined as the number of deliveries divided by the total number of clinical pregnancies. Secondary outcome measures focused on maternal and neonatal complications, such as miscarriage rate, rate of main obstetric complications, prematurity rate, and rate of congenital malformations.

Results: By analyzing overall 113 pregnancies among cases and 214 pregnancies among controls, this study showed that the delivery rate was higher in controls with respect to cases (92.06% vs. 84.07%, \( p = 0.026 \)); among deliveries, live births were
98.95% and 100%, respectively, whereas only one stillbirth occurred in cases. The first trimester miscarriage rate was higher in the cases than controls (13.27% vs. 6.07%, \( p = 0.027 \)), whereas no difference was found among the rate of second trimester miscarriages, therapeutic abortions, and ectopic pregnancies. There was no difference regarding the rate of maternal complications, including gestational hypertension, PE, HELLP syndrome, gestational diabetes, placenta previa, placental abruption, and premature rupture of the membranes. Considering neonatal complications, it was shown that twins belonging to controls had a higher prematurity rate with respect to cases (65.79% vs. 50.00%) but without a statistical relevance. Lastly, the rate of congenital malformations did not differ among the two groups.

**Discussion:** This study showed that, once couples diagnosed with OA achieve a pregnancy, they have a much higher risk of miscarriage in the first trimester in respect to non-azoospermic patients. Moreover, controls had a higher delivery rate in respect to cases; however, when the fetal status at birth was compared, no difference was found between live births and stillbirths.

**Conclusions:** Differently from the findings in the literature, no association with PE was found. This might be related to a collider bias/left truncation bias: As azoospermic patients are at higher risk of early termination of pregnancy, it results that they do not have the possibility to develop PE and other adverse outcomes.

**Keywords**
ICSI, neonatal outcomes, obstructive azoospermia, preeclampsia, pregnancies, TESE

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**1 INTRODUCTION**

Even if most pregnancies have favorable outcomes, it is possible to face severe obstetric pathologies that lead to serious maternal and neonatal complications.\(^1\) The great obstetrical syndromes include preeclampsia (PE), hypertensive disorders, intrauterine growth restriction (IUGR), gestational diabetes mellitus, and premature delivery.\(^2\) Although in the last two conditions the pathophysiologic mechanism and possible interventions are clearer with respect to some decades ago, the real pathogenesis of PE and IUGR is still unknown, and therefore their only definitive cure is delivery, with the resolution of symptoms upon termination of pregnancy.\(^3\)

Despite vast research in the past years, the definition of PE is still based on a clinical diagnosis\(^4\): According to the American College of Obstetricians and Gynecologists, PE is diagnosed in the presence of new-onset hypertension accompanied by proteinuria. If proteinuria criteria are not met, the new-onset hypertension must be associated with signs of organ failure such as thrombocytopenia, renal or hepatic insufficiency, acute pulmonary edema, or new-onset headache.\(^5\)

Nowadays, PE is a major cause of morbidity and mortality in both mothers and neonates and complicates about 2%–8% of pregnancies worldwide.\(^6\)

Pregnant women affected by PE may develop potentially lethal complications, such as placental abruption, disseminated intravascular coagulation, hemorrhagic stroke, liver failure, acute kidney insufficiency, and, eventually, long-term cardiovascular morbidity.\(^7\)

Several risk factors are associated with an increased risk of developing PE, including the previous history of PE, maternal age > 40 years old, BMI > 35 kg/m\(^2\), nulliparity, multifetal gestations, pre-existing diabetes, pre-existing hypertension, chronic autoimmune diseases, and the presence of antiphospholipid autoantibodies.\(^8\)

The exact etiology of PE is not completely known. Some authors have proposed that PE has an immunological component. This is related to other risk factors such as limited sperm exposure\(^9\) (in the case of barrier contraception and surgically retrieved spermatozoa), pregnancies achieved with donor insemination, and oocyte and embryo donation.\(^10\)

The fetus is considered a semi-allograft, being one half paternally derived in its antigenicity.\(^11\) The first pathogenic insult of PE may be an abnormal maternal immune response toward the semi-allogenic implant.\(^12\)\(^13\)

Several studies support the hypothesis that PE might be because of an abnormal immunologic response to a foreign fetal antigen derived from the father’s spermatozoa.\(^14\) With continuous and prolonged maternal exposure to the paternal antigens both during sexual intercourse and pregnancy, memory T cells eventually expand and induce a paternal antigen-specific tolerance.\(^15\)

In the context of artificial reproductive techniques (ART), the use of spermatozoa from a donor increases the risk of PE, because of the negative exposure history to those paternal antigens.\(^16\) As for sperm
donors, also the use of spermatozoa surgically retrieved from the testis (e.g., testicular sperm extraction—TESE) increases the risk of PE, confirming moreover the protective effect of sperm exposure on maternal complications.

Consistently with the previously stated hypotheses, the aim of our study is to determine if the lack of exposure to sperm antigens is associated with worse maternal and neonatal outcomes in pregnancies obtained after intracytoplasmic sperm injection (ICSI)-TESE for obstructive azoospermia (OA).

2 | MATERIALS AND METHODS

2.1 | Study design and population

This was a single-center case–control retrospective study, focusing on all the first pregnancies obtained through ICSI–TESE for OA at Humanitas Fertility Center between January 1, 2010 and December 31, 2019. Those pregnancies were achieved with both fresh and frozen embryo transfer.

The cases were matched with controls in a 1:2 ratio and the inclusion and exclusion criteria were as follows:

• The case group included women that did not have any previous exposure to the spermatozoa of their current partner, because of the male obstructive etiology. Therefore, all couples that had a previous pregnancy or miscarriage, either spontaneous or through ART in our or a secondary center, were excluded.

• In addition, patients with either pre-gestational hypertension or diabetes mellitus type 1 were ruled out in order to limit potential confounders. No age limitations were taken into consideration.

• The control group included patients that achieved their first pregnancy through cycles of ICSI using ejaculated spermatozoa, for a diagnosis of infertility other than azoospermia, in the same time frame at Humanitas Fertility Center. ICSI was performed either on fresh or cryopreserved oocytes and was followed by fresh embryo transfer.

The matching was performed taking into consideration:

1. maternal age;
2. BMI classes: < 20, 20–25, and > 25 kg/m²;
3. year of controlled ovarian stimulation (COS).

2.2 | Data collection and follow-up

The information collected for the database was retrieved from the Fertility Center internal web-based registry, in which patients’ data are safeguarded by advanced threat prevention, enterprise-class encryption, and any user needs to periodically renew their password.

Routinely, pregnant women were followed up during the gestation by psychologists of Fertility Center team, in order to monitor those patients who decided to turn to other centers for further obstetric controls. Psychologists were in charge of patients’ follow-up for reducing biases during the collection of data. The internal registry was updated routinely during the pregnancy, at delivery, and at 1-month post-partum, in order to gain accurate information on neonates’ well-being.

Moreover, all data from ART cycles, including pregnancy outcomes, were collected by the Istituto Superiore di Sanità, Rome, Italy, and stored on the internet website www.iss.it/rpma, set up by the ART National Registry at the National Center for Epidemiology, Surveillance and Health Promotion. In order to reduce recall bias, data from the Fertility Center registry were implemented and, in case, corrected with information gathered from hospital discharge letters of patients’ delivery. Each patient was contacted via phone call or email and was asked to send the hospital discharge letters summarizing patients’ hospitalization for labor and delivery, and their offspring in the pediatric ward.

2.3 | Interventions

2.3.1 | Obstructive azoospermia and TESE

According to the WHO, azoospermia is defined as the absence of spermatozoa in at least two different ejaculate samples and can be classified as obstructive and non-obstructive (NOA).

OA is “the absence of both spermatozoa and spermatogenetic cells in semen and post-ejaculate urine because of the bilateral obstruction of the epididymis or the seminal or ejaculatory ducts.” The classification of OA can be based on the onset of the obstruction (congenital or acquired), or on the level of the obstruction (epididymal, vassal, or ductal).

Patients were diagnosed with OA, and TESE was performed for both diagnostic and therapeutic purposes.

TESE was performed through open testicular biopsy as an outpatient surgery, under local anesthesia or deep sedation with propofol. A single incision of 4–6 mm was performed on the tunica albuginea and two testicular specimens of testicular parenchyma were collected: The first one was immediately transferred to Bouin’s solution for histological evaluation, whereas the second specimen (50 ± 200 mg) was collected in a tube containing Quinn’s HTF medium and HEPES (Biocare Europe). Later, in the IVF laboratory, testicular parenchyma was transferred into Petri dishes with Quinn’s sperm washing medium (SAGE BioPharma, Bedminster, NJ, USA), dissected into small pieces under a stereomicroscope using two coverslides and then centrifuged at 600/800 g for 10 min in a sterile conic tube (Becton Dickinson Labware Europe, Le Pont De Claux, France). The amount of retrieved spermatozoa was estimated using the five-degree scale described by Hauser et al.
This procedure was carried out before initiating the COS on the female partner, in order to avoid the risk of not finding any spermatooza at the time of oocytes retrieval, and the testicular spermatooza was cryopreserved following a rapid two-phase protocol until the time thawing and using for ICSI.

2.3.2 | Controlled ovarian stimulation

According to the anti-Müllerian hormone (AMH) levels and antral follicular count, patients were stratified into different categories for the prediction of ovarian response to the ovarian stimulation and, in accordance with the Poseidon criteria, a specific protocol was initiated: GnRH antagonist protocol, GnRH agonist long protocol, GnRH agonist short protocol, and flare-up GnRH agonist protocol.

During COS, patients were monitored daily or every other day, to evaluate the number and diameter of ovarian follicles and endometrial thickness. Once the targeted measures were reached, final oocyte maturation was triggered by administering subcutaneously recombinant hCG-Ovitrelle (Merck Serono). After 36 h, oocyte pick up was performed in the operating room and retrieved oocytes were then fertilized with ICSI and, eventually, supernumerary oocytes were cryopreserved.

2.3.3 | Intracytoplasmic sperm injection

Both fresh and cryopreserved oocytes were used for ICSI. Once aspirated from the ovarian follicles, fresh oocyte-cumulus complexes were then treated by the biologist in several media, in order to prepare the oocyte for the injection. Cumulus cells and corona radiata were removed by transferring oocytes into an M2 medium with hyaluronidase 1 mg/mL for 1 min.

On the other hand, cryopreserved oocytes were properly warmed and treated, and then transferred into an injection dish for the procedure.

A morphologically normal and motile spermatozoon was isolated, either from cryopreserved testicular spermatozoa for the case group or from fresh semen for the control group, immobilized and then aspirated from the tail into the tip of the microinjection pipette. The oocyte was held by the holding pipette, and the spermatozoon was injected into the oocyte cytoplasm with the microinjection needle. The injection pipette was withdrawn gently and the oocyte was released from the holding pipette.

2.3.4 | Embryo transfer and frozen embryo transfer

In fresh embryo transfers, after 18–20 h from ICSI, oocytes were reexamined, and their fertilization status was evaluated. The embryo transfer took place either on day 2–3 or day 5 after ICSI, carrying on embryo maturation up to cleavage or blastocyst stage, based on the prognosis of each patient. In our center, supernumerary embryos are cryopreserved at blastocyst stage, whereas fresh embryos are usually transferred at cleavage stage.

The decision on how many embryos to transfer was taken according to the 2017 American Society of Reproductive Medicine guidelines. The procedure was performed in the operating room in sterile conditions without the need of any anesthesia or analgesia. Accordingly, one or two embryos were released using a set of catheters of 1–1.5 cm from the uterine fundus under pelvic ultrasound visualization.

In frozen embryo transfers, endometrial synchronization was established before the transfer procedure through three different protocols: natural cycles, modified natural cycles with urinary hCG triggering, and artificial replacement cycles, as already described by De Cesare et al.

2.4 | Outcomes’ definitions

The primary outcome measure was the delivery rate, which was defined as the number of deliveries divided by the total number of clinical pregnancies.

The secondary outcomes focused on obstetric and neonatal complications.

The obstetric outcomes were the miscarriages (both first and second trimesters) rate, the frequencies of live birth and stillbirth, and the rate of the main obstetric complications such as pre-eclampsia, gestational hypertension, gestational diabetes, placenta previa, placental abruption, and premature rupture of membranes (PROM). The miscarriage rate was described as the number of clinical pregnancies who failed to continue beyond 12 weeks of gestation divided by the total number of clinical pregnancies. A live birth was defined as the delivery of a living baby after at least 22 weeks of gestation, whereas stillbirth was described as the death of a fetus prior to delivery after 28 weeks of gestation.

The neonatal outcomes are gestational age, prematurity rate, birth weight and its subcategories (appropriate for gestational age [AGA], small for gestational age [SGA], and large for gestational age [LGA]), sex ratio, 1- and 5-min APGAR, and the rate of congenital defects. A birth was defined at term if delivery occurred between 37 and 42 weeks of gestation; a preterm birth is defined as a delivery occurring before 37 weeks of gestation and, more specifically, it is defined as moderate-to-late preterm when it occurs between 32 and 37 weeks; very preterm between 28 and 32 weeks; and extremely preterm less than 28 weeks of gestation. According to the birth weight, growth percentiles were calculated using Medicine Fetal Barcelona Calculator and newborns were divided into three categories: AGA (between 10th and 90th percentile), SGA (<10th percentile), and LGA (>90th percentile). Macrosomia was defined as birth weight exceeding 4000 g. The APGAR score was included in order to objectively report the overall status of the neonates immediately after birth and their response to resuscitation, if performed. An APGAR score between 7 and 10 indicates an excellent status, with a score of 4–6 moderately depressed and a score of 0–3 severely depressed.
2.5 | Ethical approval

Patients had previously signed a written informed consent regarding the use of their medical records for research purposes, as long as their anonymity was preserved and confidentiality of the medical record was guaranteed.

The study was approved on March 23, 2021 by the Independent Ethical Committee of the Humanitas Institutional Clinic (Milan, Italy) (approval number 14/21), and it was registered on clinicalTrials.gov before full variables extraction and statistical analysis were conducted (registration number NCT04852237).

2.6 | Statistical analysis

Data were described as number and percentage or as median and interquartile range, as appropriate.

Continuous variables were analyzed with the Wilcoxon rank-sum test, whereas dichotomous variables were with a chi-squared test. All variables were analyzed by univariable logistic regression, and variables with a $p$ value lower than 0.2 were submitted to multivariable regression analysis, in order to identify the possible factors associated with the outcomes. Logistic regression results were reported as an odds ratio (OR) and 95% confidence interval.

A $p$ value lower than 0.05 was considered significant. All analyses were carried out using Stata 15.0 (StataCorp. 2017. Stata Statistical Software: Release 15. College Station, TX: StataCorp LP).

3 | RESULTS

3.1 | Population description

A total of 113 pregnancies occurring between January 1, 2010 and December 31, 2019, at Humanitas Fertility Center fulfilled the inclusion and exclusion criteria and were considered cases. These were matched with a ratio of almost 1:2 to a control group, based on female age at cycle induction, BMI classes, and year of COS; a total of 214 patients were included in controls.

As it is possible to see from Table 1, the baseline characteristics between the two groups were well distributed regarding basal characteristics and ovarian reserve. No $p$ values regarding female age and BMI were reported, as they were used as matching variables.

Considering the ovarian reserve, the median AMH level (2.4 vs. 2.1) and basal FSH level (7 vs. 6.8) do not diverge between cases and controls ($p = 0.483$ and $0.836$, respectively).

Considering the diagnosis of infertility, among cases 110 patients had a diagnosis of primary infertility and were nulliparous, whereas three patients were diagnosed with secondary infertility, because of a previous pregnancy achieved with a different partner; among those, one carried on a full-term pregnancy, whereas two had decided for the voluntary termination of pregnancy.

Among controls, 212 patients had a diagnosis of primary infertility with their current partner and were nulliparous, whereas 2 patients were diagnosed with secondary infertility, because of a previous pregnancy achieved with a different partner; both of them performed a voluntary interruption.

The indication to infertility treatment differed among the two groups: The malefactor (OA) was the main indication to treatment in cases (84.07%), whereas in controls the main indications were malefactors (45.79%), along with reduced ovarian reserve (13.08%), tubal factors (9.35%), idiopathic cause (5.14%), and endometriosis (4.21%).

Male FSH level was compared between the two groups: 4.4 mUI/mL in cases and 6.8 mUI/mL in controls, being statistically significant ($p$ value <0.001). However, there was a large portion of missing data (25.66% in cases vs. 81.31% in controls); this is because of the fact that FSH level was not investigated in all patients, because once there were clinical (e.g., cystic fibrosis transmembrane regulator [CTFR] mutation) or diagnostic imaging (e.g., ultrasound) evidence of male genital tract obstruction, no hormonal tests were carried out.

Among the 113 patients affected by OA, 61% were diagnosed with congenital OA, whereas 39% had an acquired OA.

Among 69 patients with congenital causes of OA, the two most common were of genetic origin: congenital bilateral agenesis of the vas deferens (CBAVD) (88.4%), mainly related to a mutation of CTFR in either healthy carriers or affected, and unilateral renal agenesis and congenital absence of the vas (7.3%). Other least common causes were Mullerian duct cysts (2.15%) and congenital malformation of the verumontanum (2.15%).

On the other hand, the etiologies of acquired causes of OA most likely arose before initiating any sexual relationship with their current partners.

The most common acquired causes were idiopathic (proximal or distal obstruction without specified etiology) (54.6%), iatrogenic (related to urological or abdominal surgeries, mainly bilateral hernia repair, vasectomy, and varicocelectomy) (31.8%), and post-infective (related to male accessory glans inflammation) (13.6%).

Table 2 illustrates the different interventions to which patients underwent: Fresh embryo transfer was performed in 84 cases (74.34%) and in 206 controls (96.26%), whereas frozen embryo transfer was performed in 29 cases (25.66%). ICSI was performed with fresh oocytes and testicular retrieved spermatozoa in all cases, whereas in controls it was performed using fresh oocytes in 206 patients and thawed oocytes in 8 patients, along with spermatozoa from fresh semen.

Generally, a mean of 1.80 ± 0.55 embryos per ET was transferred in cases and 1.90 ± 0.51 in controls ($p = 0.091$). Regarding the stage at which embryos were transferred, the blastocysts were transferred at a higher percentage in cases (30.09% vs. 16.82%, $p = 0.006$), whereas cleavage stage embryos were mostly used in controls (69.91 vs. 83.18%). This was mainly related to the fact that in our center, supernumerary embryos are cryopreserved at blastocyst stage and, therefore, frozen embryo transfer is performed mainly with blastocysts; instead, fresh embryo transfer is performed mainly with embryos at cleavage stage.
### Table 1: Patients’ baseline characteristics

| Characteristics | Cases | Controls | \( p \) value |
|-----------------|-------|----------|---------------|
| Patients (n)    | 113   | 214      |               |
| Female age (years) | 33 (29–36) | 34 (31–36) |               |
| Male age (years) | 36 (32–40) | 37 (33–40) | 0.299         |
| Infertility duration (years) | 3.1 (2.2–4.8) | 3.3 (2.2–4.5) | 0.793         |
| Female BMI (kg/m²) | 21.5 (19.9–24.3) | 21.5 (19.8–23.7) |               |
| Active smoking  | 28 (24.78%) | 44 (20.56%) | 0.381         |
| Basal FSH level (mUI/mL) | 7 (5.8–8.2) | 6.8 (5.6–8.6) | 0.778         |
| Male FSH level (mUI/mL) | 4.4 (2.8–5.6) | 6.8 (4.3–13.6) | <0.001 |
| Missing male FSH | 29 (25.66%) | 174 (81.31%) | <0.001 |
| AMH level       | 2.4 (1.2–3.5) | 2.1 (1.1–3.7) | 0.542         |

**Normal female karyotype**

**Normal male karyotype**

**Indication for treatment**

| Indication for treatment | Cases | Controls | \( p \) value |
|--------------------------|-------|----------|---------------|
| Male factor alone        | 95 (84.07%) | 98 (45.79%) | <0.001 |
| Male and female factors  | 18 (15.93%) | 37 (17.29%) | 0.754 |
| Female factors alone tubal | –       | 20 (9.35%) |       |
| Unexplained              | –       | 11 (5.14%) |       |
| Endometriosis            | –       | 9 (4.21%)  |       |
| Anovulation              | –       | 4 (1.87%)  |       |
| Reduced ovarian reserve  | –       | 28 (13.08%)|       |
| Multiple female factors  | –       | 6 (2.80%)  |       |

Note: Results shown as number and percentage or as median and IQR, as appropriate.

Abbreviations: AMH, anti-Müllerian hormone; IQR, interquartile range. FSH (Follicle-Stimulating Hormone).

### Table 2: Interventions

| Interventions | Cases | Controls | \( p \) value |
|---------------|-------|----------|---------------|
| Fresh embryo transfer | 84 (74.34%) | 206 (96.26%) |               |
| Frozen embryo transfer | 29 (25.66%) | – |               |
| ICSI on cryopreserved oocytes and fresh ET | – | 8 (3.74%) |               |
| Transferred embryos per ET (n) | 2 (1–2) | 2 (2–2) | 0.091 |

**Stage of transfer**

| Stage of transfer | Cases | Controls | \( p \) value |
|-------------------|-------|----------|---------------|
| Cleavage stage    | 79 (69.91%) | 178 (83.18%) |               |
| Blastocyst stage  | 34 (30.09%) | 36 (16.82%) | 0.005         |

Note: Results shown as number and percentage or as median and IQR, as appropriate.

Abbreviation: ICSI, intracytoplasmic sperm injection.

### Table 3: Pregnancy outcomes

| Characteristics | Cases | Controls | \( p \) value |
|-----------------|-------|----------|---------------|
| Outcomes        |       |          |               |
| Total clinical pregnancies | 113 | 214 |               |
| Ectopic pregnancy | 2 (1.77%) | 2 (0.96%) | 0.611         |
| First trimester miscarriage | 15 (13.27%) | 13 (6.07%) | 0.027 |
| Second trimester miscarriage | 1 (0.88%) | 1 (0.47%) |       |
| Therapeutic abortion | – | 1 (0.47%) |       |
| Delivery         |       |          |               |
| Live birth (%)   | 94 (98.95%) | 197 (100%) |       |
| Stillbirth (%)   | 1 (1.05%) | – |       |

**Types of delivery**

| Types of delivery | Cases | Controls | \( p \) value |
|-------------------|-------|----------|---------------|
| Spontaneous       | 44 (45.32%) | 99 (50.25%) |               |
| Operative         | 5 (5.26%) | 12 (6.09%) |               |
| Cesarean section  | 46 (48.42%) | 86 (43.65%) | 0.789         |

Note: Results shown as number and percentage.

3.2 Pregnancy outcomes

Table 3 depicts the pregnancy outcomes in the two populations.

The first trimester miscarriage rate was definitely higher in the cases (13.27%) than in controls (6.07%), and this difference was statistically significant (\( p = 0.027 \)). There was no difference between the rate of ectopic pregnancies (1.77% vs. 0.93%, \( p = 0.611 \)) of the two groups.

The rate of second trimester miscarriages (0.88% vs. 0.47%) and therapeutic abortions (0% vs. 0.47%) was similar among the two groups. Two second trimester miscarriages occurred at 13 and...
21.6 weeks of gestation. The therapeutic abortion was performed at 12.3 weeks because of a body stalk anomaly, a severe abdominal wall defect.

The delivery rate was 84.07% for cases and 92.06% for controls ($p = 0.037$); when comparing the fetal status at birth, no difference was found: Live births were 98.95% and 100%, whereas only one stillbirth occurred in cases at 32 weeks of gestation.

The type of delivery was also reported: spontaneous delivery (46.32% vs. 50.25%), operative delivery (5.26% vs. 6.09%), and cesarean section (48.42% vs. 43.65%). No significant difference was found between the two populations.

Univariate logistic regression analysis was performed to determine the relationship between the investigated variables and birth as outcome, shown in Table 4: A statistical significance was observed in having a male factor as an indication to treatment (OR 2.09, $p = 0.043$) and belonging to the cases (OR 0.46, $p = 0.029$).

The two variables were then submitted to multivariable logistic regression analysis, which further confirmed the significant impact of male factor and belonging to the cases (OR 0.25, $p = 0.001$; OR 3.84, $p = 0.002$, respectively). Maternal age did not show a significant impact ($p = 0.338$).

Univariate logistic regression analysis was additionally performed to determine the relationship between the investigated variables and miscarriage as outcome, as seen in Table 5: A statistical significance was observed in belonging to cases (OR 2.39, $p = 0.029$); of interest was female age at induction, which was close to the statistical significance ($p = 0.055$).

The two variables were then submitted to multivariable logistic regression analysis, which further confirmed the significant impact of maternal age and belonging to the cases (OR 1.11, $p = 0.038$; OR 2.57, $p = 0.019$, respectively).

### Table 4

Univariate and multivariable logistic regression analyses considering delivery

| Variable                      | Univariate OR (95% CI) | $p$  | Multivariable OR (95% CI) | $p$  |
|-------------------------------|------------------------|------|--------------------------|------|
| Female age (years)            | 0.93 (0.85–1.01)       | 0.092| –                        | 0.338|
| Male age (years)              | 1.00 (0.94–1.07)       | 0.978|                          |      |
| Female BMI (kg/m²)            | 1.00 (0.90–1.12)       | 0.947|                          |      |
| Infertility duration (years)  | 0.96 (0.82–1.13)       | 0.648|                          |      |
| Active smoking                | 1.15 (0.48–2.74)       | 0.761|                          |      |
| Basal FSH level (mUI/mL)      | 0.92 (0.82–1.10)       | 0.174|                          |      |
| AMH level                     | 1.09 (0.89–1.35)       | 0.403|                          |      |
| Indication for treatment      |                        |      |                          |      |
| Male                          | 2.08 (1.02–4.23)       | 0.043| 0.25 (0.11–0.57)         | 0.001|
| Male and female factors       | 0.46 (0.20–1.10)       | 0.054|                          | 0.711|
| Transferred embryos per ET    | 1.02 (0.53–1.99)       | 0.950|                          |      |
| Group (cases)                 | 0.46 (0.22–0.92)       | 0.029| 3.84 (4.39–12.71)        | 0.002|

Note: Results reported as odds ratio (OR) and confidence interval (CI).

Abbreviation: AMH, anti-Müllerian hormone.

### 3.3 Maternal complications

The maternal complications can be appreciated in Table 6. Overall, 22 complications were recorded in cases and 45 complications in controls; no statistically significant difference was observed ($p = 0.989$).

Univariate logistic regression analysis was performed to determine the relationship between the investigated variables and maternal complications, but no significant impact was underlined, as reported in Table 7.

### 3.4 Neonatal complications

The neonatal population was differentiated into singleton pregnancies and multiple pregnancies: Overall, the singleton gestations were 77 vs. 159, whereas twin gestations (all bicornic-biamniotic) were 18 vs. 38.

Table 8 shows the neonatal population of singleton pregnancies. All newborns were alive at birth, but two shortly died within 48 hours from delivery: One among cases had a transient intrapartum cardiac arrest, whereas one among controls was affected by a severe form of neonatal respiratory distress syndrome, because of prematurity (29 weeks) related to HELLP syndrome.

In cases, the median gestational age was 39.2 weeks and the prematurity rate was 10.39%; among the premature newborns, four were moderate-to-late preterms, three very preterms, and one extremely preterm. Controls had a median gestational age of 39.3 weeks and a prematurity rate of 6.29%; also in this group, the majority of premature newborns were moderate-to-late preterm. There were no statistically significant differences between the two groups when gestational age and prematurity rate were considered ($p = 0.383$ and 0.266, respectively).
TABLE 5  Univariate and multivariable logistic regression analyses considering miscarriage

| Variable             | Univariate OR (95% CI) | p     | Multivariable OR (95% CI) | p     |
|----------------------|------------------------|-------|--------------------------|-------|
| Female age (years)   | 1.10 (1.00–1.22)       | 0.055 | 1.11 (1.01–1.23)         | 0.038 |
| Male age (years)     | 1.00 (0.93–1.07)       | 0.991 |                          |       |
| Female BMI (kg/m²)   | 1.01 (0.89–1.14)       | 0.867 |                          |       |
| Infertility duration (years) | 1.05 (0.88–1.24)     | 0.597 |                          |       |
| Active smoking       | 0.97 (0.38–2.48)       | 0.941 |                          |       |
| Basal FSH level (mUI/mL) | 1.10 (0.97–1.24)   | 0.147 |                          | 0.293 |
| AMH level            | 0.79 (0.59–1.04)       | 0.094 |                          |       |
| Indication for treatment |                      |       |                          |       |
| Male                 | 0.76 (0.35–1.65)       | 0.486 |                          |       |
| Male and female factors | 1.15 (0.42–3.17)    | 0.791 |                          |       |
| Transferred embryos per ET | 1.13 (0.54–2.36) | 0.737 |                          |       |
| Group (cases)        | 2.39 (1.09–5.23)       | 0.029 | 2.57 (1.16–5.67)         | 0.019 |

Note: Results reported as odds ratio (OR) and confidence interval (CI).
Abbreviation: AMH, anti-Müllerian hormone.

TABLE 6  Maternal complications

| Characteristics          | Cases  | Controls | p value |
|--------------------------|--------|----------|---------|
| Overall maternal complications | 22 (22.92%) | 45 (22.84%) | 0.989   |
| Gestational hypertension | 3 (3.13%) | 10 (5.08%) | 0.557   |
| Preeclampsia              | 1 (1.04%) | 7 (3.55%)  | 0.280   |
| HEELP syndrome            | 1 (1.04%) | 1 (0.51%)  | 0.547   |
| Gestational diabetes      | 3 (3.13%) | 9 (4.57%)  | 0.757   |
| Placenta previa           | 2 (2.08%) | 4 (2.03%)  | 1.000   |
| PROM/pPROM                | 16 (16.67%) | 18 (9.14%) | 0.059   |
| Placental abruption       | 1 (1.04%) | 3 (1.52%)  | 1.000   |

Note: Results shown as number and percentage.

No statistically significant difference was found neither between the birth weight (3230 vs. 3160 g, \(p = 0.485\)) nor growth percentiles (50% vs. 37%, \(p = 0.116\)) of the two groups; when considering SGA, LGA, and macrosomia subgroups, no statistically significant difference was underlined.

In addition, newborns belonging to both groups had an overall excellent status at delivery, as indicated by median 1 and 5-min APGAR scores.

Table 9 shows the neonatal population of twin pregnancies. In total, 38 newborns were alive at birth in cases, whereas one stillbirth occurred at 32 weeks of gestation; in controls, all twins were alive at birth, but one neonate shortly died after delivery because of Potter’s syndrome, a rare fatal condition related to anidramnios.

The median gestational age of the two groups was, respectively, 37 vs. 36.3 weeks (\(p = 0.366\)). No difference was found between the prematurity rates of the two groups (50.00% vs. 65.79%, \(p = 0.259\)); however, the majority of premature newborns in both groups were moderate-to-late preterm. There was no statistically significant difference between the median birth weight (2300 vs. 2290 g) and growth percentiles (12% vs. 11%).

TABLE 7  Univariate logistic regression analyses considering delivery

| Variable             | Univariate OR (95% CI) | p     |
|----------------------|------------------------|-------|
| Female age (years)   | 1.02 (0.96–1.09)       | 0.478 |
| Female BMI (kg/m²)   | 1.00 (0.92–1.08)       | 0.969 |
| Infertility duration (years) | 0.96 (0.85–1.09) | 0.527 |
| Smoking               | 1.56 (0.87–2.81)       | 0.137 |
| FSH level (mUI/mL)   | 1.02 (0.93–1.12)       | 0.647 |
| AMH level             | 0.93 (0.80–1.08)       | 0.313 |
| Indication for treatment |                      |       |
| Male                 | 1.14 (0.67–1.91)       | 0.632 |
| Tubal                | 0.16 (0.02–1.24)       | 0.079 |
| Unexplained           | 0.96 (0.25–3.71)       | 0.954 |
| Male and female factors | 0.86 (0.42–1.75)   | 0.677 |
| Endometriosis        | 2.65 (0.65–10.83)      | 0.176 |
| Anovulation          | 5.23 (0.47–58.52)      | 0.179 |
| Reduced ovarian reserve | 1.16 (0.48–2.77)  | 0.746 |
| Multiple female factors | 0.64 (0.07–5.79)    | 0.689 |
| Transferred embryos per ET | 0.87 (0.53–1.42) | 0.576 |
| Group (cases)        | 1.06 (0.62–1.82)       | 0.825 |
| Twin pregnancies      | 1.01 (0.53–1.91)       | 0.987 |

Note: Results reported as odds ratio (OR) and confidence interval (CI).
Abbreviation: AMH, anti-Müllerian hormone.
### TABLE 8 Description of singleton neonatal population

| Characteristics            | Cases                   | Controls                 | p value |
|----------------------------|-------------------------|--------------------------|---------|
| Singleton pregnancies (%)  | 77 (81.05%)             | 159 (80.71%)             | 0.945   |
| Live born (n)              | 77 (100%)               | 159 (100%)               |         |
| Stillborn (n)              | 1 (1.30%)               | 1 (0.63%)                | 0.547   |
| Neonatal death             | 1 (1.30%)               | 1 (0.63%)                |         |
| Male/female ratio          | 0.71                    | 1.04                     | 0.176   |
| Gestational age (weeks)    | 39.2 (38.2–40.3)        | 39.3 (38.3–40.5)         | 0.382   |
| Prematurity                |                         |                          |         |
| Moderate-to-late preterm   | 8 (10.39%)              | 10 (6.29%)               | 0.266   |
| Very preterm               | 3 (37.5%)               | 2 (20%)                  |         |
| Extremely preterm          | 1 (12.5%)               | 1 (10%)                  |         |
| Birth weight (g)           | 3230 (2780–3550)        | 3160 (2850–3520)         | 0.485   |
| Growth percentiles         |                         |                          |         |
| AGA (10–90th percentile)   | 50 (19–78)              | 37 (14–61)               | 0.116   |
| SGA (<10th percentile)     | 14 (18.18%)             | 32 (20.13%)              | 0.724   |
| LGA (>90th percentile)     | 10 (12.99%)             | 12 (7.55%)               | 0.178   |
| Macrosomia                 | 3 (3.90%)               | 3 (1.89%)                | 0.395   |

#### APGAR score

|       | Cases       | Controls     | p value |
|-------|-------------|--------------|---------|
| 1 min | 9 (9–9)     | 9 (9–9)      | 0.632   |
| 5 min | 10 (9–10)   | 10 (10–10)   | 0.136   |

**Note:** Results shown as number and percentage.

**Abbreviations:** AGA, appropriate for gestational age; LGA, large for gestational age; SGA, small for gestational age.

### TABLE 9 Description of twin neonatal population

| Characteristics            | Cases                   | Controls                 | p value |
|----------------------------|-------------------------|--------------------------|---------|
| Twin pregnancies (%)       | 18 (19.95%)             | 38 (19.29%)              | 0.945   |
| Live born (n)              | 35 (97.22%)             | 76 (100%)                |         |
| Stillbirth (n)             | 1 (2.78%)               | –                        |         |
| Neonatal death             | –                       | 1 (2.63%)                | 1.000   |
| Male/Female ratio          | 0.64                    | 1                        | 0.271   |
| Gestational age (weeks)    | 37 (34–37.4)            | 36.3 (35.2–37)           | 0.366   |
| Prematurity                |                         |                          |         |
| Moderate-to-late preterm   | 9 (50.00%)              | 25 (65.79%)              | 0.259   |
| Very preterm               | 7 (77.78%)              | 22 (88.00%)              |         |
| Extremely preterm          | 1 (11.11%)              | 3 (12.00%)               |         |
| Birth weight (g)           | 2300 (1943–2610)        | 2290 (1900–2555)         | 0.568   |
| Growth percentiles         |                         |                          |         |
| AGA (10–90th percentile)   | 12 (4–32)               | 11 (1–33)                | 0.379   |
| SGA (<10th percentile)     | 17 (48.57%)             | 39 (51.32%)              | 0.788   |
| LGA (>90th percentile)     | 16 (45.71%)             | 36 (47.37%)              | 0.871   |
| Macrosomia                 | –                       | –                        |         |

#### APGAR score

|       | Cases       | Controls     | p value |
|-------|-------------|--------------|---------|
| 1 min | 9 (9–9)     | 9 (8–9)      | 0.691   |
| 5 min | 10 (9–10)   | 10 (9–10)    | 0.779   |

**Results shown as number and percentage or as median and IQR, as appropriate.**

**Abbreviations:** AGA, appropriate for gestational age; LGA, large for gestational age; SGA, small for gestational age.
TABLE 10 Congenital malformations

| Characteristics                | Cases       | Controls    | p value |
|--------------------------------|-------------|-------------|---------|
| Total newborns                 | 113         | 235         |         |
| Newborns with congenital defects | 9 (7.96%)   | 11 (4.68%)  | 0.226   |
| • Uro-genital defects          | 3           | 3           |         |
| • Mandibular defects           | 1           | –           |         |
| • Ear defects                  | 1           | 1           |         |
| • Pharynx defects              | 1           | –           |         |
| • Thymic hyperplasia           | 1           | –           |         |
| • Cardiovascular defects       | –           | 1           |         |
| • Gastrointestinal defects     | –           | 1           |         |
| • Musculoskeletal defects      | 1           | 3           |         |
| • Skin pathology               | 1           | 1           |         |
| • Body stalk anomaly           | –           | 1           |         |

Note: Results shown as number and percentage.

Despite the overall higher prematurity rate, newborns of both groups had an excellent APGAR score at 1 min (9 vs. 9, \(p = 0.691\)) and 5 min (10 vs. 10, \(p = 0.779\)).

There was no significant difference in the rate of congenital malformations (7.96% vs. 4.68%, \(p = 0.226\)) between the 113 newborns of cases and 235 of controls, as shown in Table 10.

Newborns of the case group had congenital defects regarding the following districts: face (asymmetry of the mandible and palate); ears, nose, and throat (congenital bilateral deafness and pharyngeal immaturity); thorax (thymic hyperplasia); urinary tract (bilateral pyelectasis); genital tract (testicular atrophy and congenital anomalies of the vas deferens); musculoskeletal (clubfoot); and skin (angioma on the tight).

Newborns of the control group presented congenital malformations affecting the following: skull (turricephaly and plagiocephaly); ears (preauricular appendix); heart (unspecified anomalies of the valves); gastrointestinal tract (anorectal malformations); urinary tract (bilateral enlarged kidneys = bilateral pyelectasis and ureteral anomalies); genital tract (congenital urachal anomaly and unilateral cryptorchidism); musculoskeletal (clubfoot and congenital hip dysplasia); and skin (angioma). In addition, a neonate had Potter’s syndrome and shortly died after birth. One had an umbilical cord with a single umbilical artery.

4 | DISCUSSION

The objective of this retrospective case–control study was to determine whether the lack of exposure to sperm antigens was associated with worse maternal and neonatal outcomes in pregnancies obtained after ICSI–TESE for OA.

The results showed that, by comparing two different populations of patients with similar basal characteristics, there was a higher miscarriage rate in cases with respect to controls. Once the pregnancy was established and evolved beyond the 12 weeks of gestation, the delivery rate was higher in controls with respect to cases. Regarding the maternal and neonatal complications, no substantial differences were found.

To the best of our knowledge, this is the first study underlying a higher risk of miscarriage in patients diagnosed with OA.

This finding is in contrast with other studies that investigated the abortion rate in other populations: Oldereid et al. compared pregnancies obtained with ICSI and ejaculated spermatozoa in patients with oligozoospermia to pregnancies obtained with ICSI and surgically retrieved spermatozoa, either testicular or epididymal, in patients with OA, NOA, aspermia, or unclassifiable etiology. The spontaneous abortion rate did not show substantial differences according to male diagnosis or sperm origin. Tehraninejad et al. conducted a prospective study evaluating pregnancy outcomes in three different groups (oligozoospermic patients, obstructive azoospermic patients, and non-obstructive azoospermic patients): There was no significant difference in the early abortion rate among the three populations.

Other studies mainly focused on the comparison between the type of azoospermia (OA vs. NOA): According to Pasqualotto et al., NOA patients have an increased risk of abortion in respect to OA patients; instead, Bocca et al. and Cellikten et al. did not find any significant difference in miscarriage rate between the two groups. Ghazzawi et al. found a significant increase in abortion rate in the testicular NOA group in respect to ejaculate and epididymal OA groups.

Taking into consideration the site of sperm extraction in OA (testicular vs. epididymal), Kamal et al. did not find any significant difference between the two; also, when analyzing the specific etiology of obstruction, congenital vs. acquired, there was no difference in the site of sperm aspiration/biopsy. Instead, other studies found that miscarriage rates were higher in cases that used spermatozoa retrieved by TESE, in comparison with retrievals by percutaneous epididymal sperm aspiration.

After investigating the different causes of OA (CBAVD, vasectomy, non-infective, infective/inflammatory and ejaculatory), Nicopoulos et al. found a negligible disparity in early abortion rates across the five groups.

The ectopic pregnancy rate was comparable among our groups, in line with one study found in the literature.

Despite the ambiguity surrounding the pathogenesis of PE, one hypothesis proposes the maternal immune intolerance toward the foreign fetal antigen derived from the father’s spermatozoa. Therefore, among infertile couples, patients whose male partners are diagnosed with azoospermia are thought to be at a greater risk of gestational hypertension and PE.

Nulliparous women are at a higher risk of developing PE in respect to multiparous women, but, in the case of new partner, the risk of the latter group returns similar to the risk during a first pregnancy. The protective effect of pregnancy is seen also with both spontaneous and
voluntary abortions.\textsuperscript{44} In addition, multiparous women who become pregnant after at least 10 years from their previous pregnancy are as likely to have PE as nulliparous women.\textsuperscript{45} Prolonged sexual cohabitation (≥ 12 months) and vaginal sperm exposure from the same partner reduce the risk of PE\textsuperscript{46}; moreover, the use of barrier contraceptive methods, such as condoms, increases the risk of PE.\textsuperscript{47} In addition, it has been shown that pre-conceptual oral exposure to semen decreases the incidence of PE, thanks to the induction of oral tolerance.\textsuperscript{48}

The results of the present study showed that cases did not have a higher risk of developing gestational hypertension and PE with respect to controls. In addition, also the rate of other obstetrical complications (gestational diabetes, placenta previa, placental abruption, and PROM) was analyzed and no difference between the two groups was found. When maternal complications were studied through univariate regression analysis, none of the considered variables seemed to have a statistically significant impact.

To the best of our knowledge, only Wang et al.\textsuperscript{10} addressed this issue in 2002; in their study, they compared three different groups (IVF with ejaculated spermatozoa, ICSI with ejaculated spermatozao, and ICSI with surgically obtained spermatozoa), finding that the risk of gestational hypertension was doubled and the risk of pre-eclampsia was tripled in women undergoing ICSI with surgically obtained spermatozoa. Those data suggest that the protective effect of semen exposure on the later development of gestational hypertension and pre-eclampsia is associated with exposure to sperm cells, or a factor closely linked with sperm, in the ejaculate. Differently from our study, the populations were not matched; however, when multivariate regression analysis was carried out, age and BMI did not seem to have any confounding effects on the results. Also, the demographic information of our study was similar to theirs. However, in their study neither the indication for surgical sperm retrieval (e.g., OA, non-OA, and aspermia) nor the site of aspiration or biopsy (testicular vs. epididymal) was specified.

In the same way, another study has reported that there is a higher incidence of PE in women conceiving by intrauterine insemination with washed donor spermatozoa compared with intrauterine insemination with washed partner spermatozoa. This supports, indirectly, an immunologic basis for PE. This retrospective analysis, however, includes a small number of cases, and it registered a higher incidence of PE in women conceiving by intrauterine insemination with washed donor spermatozoa compared with intrauterine insemination with washed partner spermatozoa.\textsuperscript{30}

Probably the sexual intercourse in our couple during a spontaneous search of pregnancy with the same partner allowed sensitization to the partner’s sperm antigens as well as in couples who performed ICSI from ejaculated semen. This hypothesis could be on the basis of the absence of increased risk of preclampsia in couples who perform ICSI by TESE.

The contrast of findings between this study and the aforementioned one could be explained by a selection bias/collider bias. Indeed, as the cases had a higher miscarriage rate and a lower delivery rate with respect to controls, the cases who experienced early termination of their pregnancy did not have the possibility to develop PE and other adverse outcomes. This was on the basis of the “smoking preeclampsia paradox”, explained by Luque-Fernandez et al.\textsuperscript{49}: After that several studies reported a reduced association between cigarette smoking and PE, this study showed how, by considering gestational age as a collider, the association between PE and smoking was biased. Indeed, smoking was associated with miscarriage, fetal death, and preterm delivery and, therefore, pregnant women who actively smoked and experienced early pregnancy termination or premature birth did not actually had the possibility to develop PE.

In relation to the neonatal outcomes, our study showed that singletons and twins born after ICSI with testicular spermatozoa from patients with OA had similar perinatal outcomes in comparison to the ones born with ICSI and ejaculated spermatozoa. As it is well-recognized that singletons and twins have different perinatal outcomes, separate analyses were carried out between these two subgroups. Overall, no differences were found among basal characteristics and complication rates between the two groups, with the exception of a higher prematurity rate in twins of the control group.

Our finding was in-line with the current literature findings; indeed, studies showed that neonatal outcomes of children conceived with non-ejaculated spermatozoa are equivalent to those of newborns conceived with ejaculated spermatozoa. Belva et al.\textsuperscript{50} compared pregnancies obtained from non-ejaculated spermatozoa (both testicular and epididymal, for NOA and OA, respectively) with those obtained with ejaculated spermatozoa, and the only significant finding was those twins from non-ejaculated spermatozoa had a greater risk of perinatal death, but not of stillbirth.

In our case–control study, the male-to-female ratio was similar, and also the rate of congenital defects did not vary between neonates born through ICSI–TESE and those born through ICSI with ejaculated spermatozoa. In 2007, Fedder et al. underlined an increased risk of hypospadias in male offsprings\textsuperscript{51}; in another paper from 2013, they compared newborns from four different groups (non-ejaculated sperm ICSI, ejaculated sperm ICSI, conventional IVF, and natural conception) and found that the overall rate of congenital malformation was comparable among populations, but the rate of congenital heart defects was greater in singleton boys conceived with non-ejaculated sperm, and the risk of undescended testicles increased in both singleton and twin boys according to the severity of male infertility.\textsuperscript{52}

The primary strength of this study is that, to the best of our knowledge, it is one of the first studies focusing on miscarriage and maternal complications rates in couples diagnosed with OA. In addition, the randomization of age and BMI controls allowed to reduce confounding factors. Furthermore, as the study was limited to a single center, all the interventions were performed by the same andrologists, reproductive gynecologists, and embryologists. At the same time, if a multicenter investigation was conducted, a larger sample size could have been studied.

Regarding possible study limitations, worth mentioning is the retrospective design of the study: Some data could have erroneously been recorded or reported by the patients, although the data retrieved were from the discharge letters obtained from the patients and our center’s internal web-based database.
5 | CONCLUSIONS

Generally, patients with surgically retrieved spermatozoa have lower chances to obtain a pregnancy. This study showed that, once couples diagnosed with OA achieve a pregnancy, they have a much higher risk of miscarriage in the first trimester in respect to non-azoospermic patients. Moreover, controls had a higher delivery rate in respect to cases; however, when the fetal status at birth was compared, no difference was found between live births and stillbirths.

Differently from the findings in the literature, no association with PE was found. This might be related to a collider bias/left truncation bias: As azoospermic patients are at higher risk of early termination of pregnancy, it results that they do not have the possibility to develop PE and other adverse outcomes.

CONFLICT OF INTEREST

No potential conflict of interest was reported by the authors.

AUTHOR CONTRIBUTION

Federico Cirillo: conceptualization, formal analysis, funding acquisition, investigation, methodology, project administration, supervision, visualization, writing—original draft, and writing—review & editing; Paola Costa: conceptualization, formal analysis, funding acquisition, investigation, methodology, project administration, supervision, visualization, writing—original draft, and writing—review & editing; Massimo Romano: project administration, supervision, visualization, writing—original draft, and writing—review & editing; Luciano Negri: project administration, supervision, visualization, writing—original draft, and writing—review & editing.

Emanuela Morenghi: formal analysis, funding acquisition, investigation, methodology, and supervision. Elena Albani: methodology, project administration, and supervision. Paolo Emanuele Levi Setti: conceptualization, formal analysis, funding acquisition, investigation, methodology, project administration, supervision, visualization, writing—original draft, and writing—review & editing.

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