Y chromosome microdeletions, sperm DNA fragmentation and sperm oxidative stress as causes of recurrent spontaneous abortion of unknown etiology

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BACKGROUND: The aim of the present study was to evaluate the implication of male factor, in terms of sperm DNA oxidation and fragmentation, and Y chromosome microdeletions in recurrent spontaneous abortion (RSA) of unknown origin in a strictly selected cohort.

METHODS: A prospective cohort study was carried out in a private university-affiliated setting. Three groups, each comprised of 30 males, were compared. The first was formed by healthy and fertile sperm donors (SD) with normal sperm parameters (control group), the second by men presenting severe oligozoospermia (SO) without RSA history, and the third by men from couples who had experienced idiopathic RSA. Frequency of Y chromosome microdeletions and mean sperm DNA fragmentation and oxidation were determined.

RESULTS: Y chromosome microdeletions were not detected in any of the males enrolled in the study. Moreover, sperm DNA oxidation measurements were not demonstrated to be relevant to RSA. Interestingly, sperm DNA fragmentation was higher in the SO group than in the RSA and the SD groups, and also higher in the RSA group compared with the SD group, but lacked an adequate predictive power to be employed as a discriminative test of RSA condition.

CONCLUSIONS: Sperm DNA features and Y chromosome microdeletions do not seem to be related to RSA of unknown origin. Other molecular features of sperm should be studied to determine their possible influence on RSA.

Key words: recurrent spontaneous abortion / semen / Y chromosome microdeletions / sperm DNA fragmentation / sperm oxidative stress

Introduction

Recurrent spontaneous abortion (RSA), defined as ≥3 clinical pregnancy losses before the fetus has reached viability (Rai and Regan, 2006), is a frustrating condition that affects 1% of couples of childbearing age (Porter and Scott, 2005). Although some causes have been identified, others remain mere speculation. Thus, 50% of couples are still classified as having unexplained RSA since the underlying mechanism(s) is never found (Porter and Scott, 2005).

Known causes include paternal or ‘de novo’ chromosomal aberrations (Rai and Regan, 2006), antiphospholipid syndrome (Empson et al., 2005), some inherited thrombophilias, such as Factor V Leiden and prothrombin G20210A gene mutation (Rey et al., 2003; Kovalevsky et al., 2004; Krabbendam et al., 2005), congenital or acquired uterine anomalies (Deví Wold et al., 2006), endocrine, autoimmune or alloimmune disturbances (Arredondo and Noble, 2006; Christiansen et al., 2006) and perhaps unhealthy lifestyle habits (smoking, obesity, psychological stress; Lashen et al., 2004; Pandey et al., 2005). However, most of these conditions are related to the woman, with the male’s contribution remaining relatively underexplored.

The alteration of sperm parameters evaluated by classic criteria (concentration, motility, morphology; World Health Organization,
1999) is not clearly associated with the risk of either sporadic or recurrent abortion (Carrell et al., 2003b; Bhattacharya, 2008), although some of these sperm deficiencies could represent the manifestation of an underlying related cause. This is the case for embryos with chromosomally abnormalities originating in meiotic segregation errors in the spermatozoa of men with severe oligoasthenoteratozoospermia (Bernardini et al., 2005; Pang et al., 2005). In fact, some authors have proposed performing fluorescent in situ hybridization analysis in severely altered sperm samples to detect cases that may benefit from preimplantation genetic screening, thus reducing the abortion rate (Rubio et al., 2001). Nevertheless, there is no consensus in the current medical literature about this topic (Harper et al., 2008).

In addition, many reported cases of male partners of RSA subjects show normal parameters in their ejaculates (Al-Hassan et al., 2005).

Moreover, as sperm physiology is ascertained, an increasing number of molecular factors are implicated in male fertility (Garrido et al., 2009a, b), such as oxidative stress-related molecules (Meseguer et al., 2004). DNA oxidation and fragmentation (Aguilar et al., 2009) and mRNA expression profiles (Garrido et al., 2008a, b), but their implication in RSA has received little or no attention.

Class parameters of sperm evaluation cannot be employed to identify structural alterations in the sperm chromatin (Erenpreiss et al., 2006; Erenpreiss et al., 2008; Avendano et al., 2009; Cohen-Bacrie et al., 2009). Sperm DNA fragmentation has been related to male subfertility, sporadic abortion and poorer reproductive outcome, especially after assisted conception technologies ( Larson-Cook et al., 2003; Borini et al., 2006; Lewis et al., 2008; Lin et al., 2008; Zini et al., 2008), but with contradictory results (Collins et al., 2008; Nicopoullos et al., 2008; Tavalaee et al., 2009). In addition, different methods of analysis have been employed to gather data [Comet Assay, Sperm Chromatin Structure Assay (SCSA), TUNEL Assay; Practice Committee of American Society for Reproductive Medicine, 2008; Zini et al., 2008], and scarce information is currently available about their implication in RSA (Carrell et al., 2003a).

Similarly, some authors have reported increased DNA damage in sperm caused by oxidative stress in infertile men (Meseguer et al., 2008; Aguilar et al., 2009), but nothing is known about this in relation to RSA.

Two reports have also described a notably higher prevalence of Y chromosome microdeletions in the male partners of couples with RSA (Dewan et al., 2006; Kaaer et al., 2008), but this has not been confirmed (Kaaer et al., 2008; Lu et al., 2008).

The present study was designed to prospectively evaluate the relationship of Y chromosome microdeletions, sperm DNA fragmentation and sperm DNA oxidation caused by oxidative stress with RSA and to compare their values among healthy and fertile sperm donors (SD) with normal sperm parameters, men with severe oligozoospermia (SO) and men from couples with RSA.

Materials and Methods

Study design

This is a prospective case–control study, in which men were enrolled between 1 January 2007 and 1 February 2009. Subjects were assigned to one of the three groups according to the following inclusion criteria.

The control group included 30 healthy SD of 18–35 years of age, all Caucasian, with normal karyotype, no family history of diseases, normal sperm analysis result (more than 80 million total motile spermatozoa per ejaculate) and previous term pregnancies without complications conceived with their sperm samples.

The oligozoospermic group (SO) consisted of 30 Caucasian men with >1 year infertility, <45 years of age, absence of autoimmune or endocrine disorders, normal karyotype, no history of RSA, normal genetic examination, normal FSH, LH, testosterone and prolactin values and at least two sperm analysis results showing SQ (≤5 million spermatozoa/ml, but >1 million of total sperm per ejaculate in order to make feasible the sperm tests). These men were considered to represent cases of severe idiopathic oligozoospermia. No assisted reproduction treatment had been performed for these males at the time they entered in the study.

The RSA group was composed of the male partners of 30 Caucasian couples who had previously experienced ≥3 clinical first trimester (5–14 weeks) spontaneous abortions, with normal karyotypes of both male and female and no autoimmune or endocrine disorders. These couples had not attempted assisted reproduction treatments at the time they were accepted for the study. The female partners of these subjects were >38 years old, presented normal ovarian function, a normal uterus confirmed by vaginal ultrasound and/or hysterosalpingography/hysteroscopy and absence of acquired or inherited thrombophilia (anti-phospholipid syndrome, activated protein C resistance, serum fasting homocystine, protein C, protein S, antithrombin III, prothrombin G20210A gene mutation, factor V Leiden). All the men were under 45 years old and previous sperm analysis had given a normal or only a mildly altered result (>10 million/ml, >35% A + B motile spermatozoa, >10% of normal spermatozoa following Kruger’s strict criteria).

To ensure the reliability of the results obtained, subjects completed a clinical questionnaire about previous consumption of alcohol and/or drugs, recent episodes of fever, exposure to gonadotoxins (e.g. during chemotherapy or radiotherapy), pesticide or heavy metals (professionally) and vitamin intake in the 3 months prior to the enrolment in the study. Andrological examinations were performed to rule out the presence of varicoceles, testicular torsion, traumasisms and other alterations in the genital tract, such as genital tract inflammation or recurrent infections (Viloria et al., 2009). None of the men presented any of these conditions.

The study was approved by the Institutional Research Board and Ethics Committee. The study purposes and procedures were carefully explained, and the informed consent was obtained from those willing to participate. The study was also registered in ClinicalTrials.gov, with the reference NCT00447395.

Y chromosome microdeletions: isolation of DNA and sequence-tagged site analysis

Genomic DNA was extracted from the peripheral blood leukocytes using the MagNA Pure Compact instrument (Roche Diagnostics Corporation) and the MagNA Pure Compact Nucleic Acid Isolation I method (Roche Applied Science, Indianapolis, Ind,) according to the manufacturer’s instructions. Screening for Yq microdeletions was carried out in controls and patients using polymerase chain reaction (PCR) techniques by amplifying 20 different sequence-tagged sites (STSs) on the long arm of the Y chromosome, corresponding to four AZF loci spread over intervals 5 and 6. These included SY14 from SRY, SY81, SY84s and SY86 from AZFa; SY182 from KALY, SY121, SYPR3, SY124, SY127, SY128, SY130, SY133 and SY134 from AZFb; SY145 and SY152 from proximal AZFc/d locus; SY242, SY208, SY254 and SY255 from AZFc; and SY157 (heterochromatic distal Yq region).

Multiplex PCR was performed using the Y chromosome AZF Analysis System (Promega, USA) under the PCR conditions recommended by the
manufacturer. For analysis, the microdeletions PCR products were run by electrophoresis on a 2% agarose gel impregnated with ethidium bromide for visualization under UV light. Failure of amplification for a given STS was confirmed twice. DNA from a fertile male and water served as negative Y chromosome microdeletion controls, whereas DNA from man with a Y chromosome microdeletion served as a positive control for multiplex reactions.

Sperm analysis

Semen parameters of every ejaculate were evaluated by two independent observers. After 10 min liquefaction of the semen at 37°C and 5% CO₂, the samples were examined for concentration and motility in a Mäckler chamber (Sefi Laboratories, Tel Aviv, Israel), according to the WHO guidelines (12). Results were only accepted when the differences between two observers were less than 5%.

Two semen aliquots were taken from each semen sample to be processed for flow cytometry and sperm chromatin dispersion (SCD) tests. The samples were directly frozen by immersion in liquid nitrogen (SCD) or in cold ethanol 70% (for flow cytometry experiments). These protocols have been validated previously and have been demonstrated to be safe in human semen (13). The samples were subsequently stored in sealed tubes with abundant distilled water, the slides were dehydrated in increasing concentrations of alcohol, and finally the slides were removed, dried, and immediately immersed in an acid solution (1:1) for 5 min, and then incubated overnight with 10 μl FITC-Conjugate at 4°C. Samples were washed twice (Wash Solution) and resuspended in 2 ml PBS. Cells were maintained in the dark on ice until their resuspension in FACS fluid and were read in a flow cytometer with FITC filters.

Flow cytometry analysis was performed on an Epics Elite Flow Cytometer (Coulter Cytometry, Hialeah, FL, USA) using an argon-ion laser tuned at 488 nm and 15 mW. Debris was excluded by the analysis of the scatter properties. At least 10 000 events per sample were stored in list-mode files. Data were expressed as percentage of stained cells (compared with negative controls) and fluorescence intensity measured as fluorescence arbitrary units.

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Science version 17.0 (SPSS Inc., Chicago, IL, USA). Categorical data were expressed as number and percentage, and numerical data as means with 95% confidence intervals (95% CI). When a quantitative analysis of the data was performed, groups were compared by analysis of variance, with Bonferroni and Scheffe’s post hoc analysis if more than two groups were compared and a Student’s t-test when only two groups were compared. Crosstabs and chi-square tests were applied for the qualitative analysis of the data. The predictive power of sperm DNA oxidation and fragmentation to forecast miscarriage were determined by receiver operator curve (ROC) analysis. Significance was defined as P < 0.05.

Results

Demographics

The baseline comparisons of the three groups are presented in Table I. In brief, age was significantly lower among SD males (ranging from 20 to 34 years) than among men in the SO (range 26–44 years) and RSA (range 30–42 years) groups. Sperm quality was also significantly higher in the first group, in which the values of sperm concentration, motility, and post-hoc analysis if more than two groups were compared and a Student’s t-test when only two groups were compared. Crosstabs and chi-square tests were applied for the qualitative analysis of the data. The predictive power of sperm DNA oxidation and fragmentation to forecast miscarriage were determined by receiver operator curve (ROC) analysis. Significance was defined as P < 0.05.

DNA oxidation (8-oxoguanine DNA sperm measurement by flow cytometry)

The OxyDNA Assay (OxyDNA assay kit, Calbiochem, Barcelona, Spain) is based on the direct binding of a fluorescent probe to 8-oxoguanine moieties in the DNA of fixed cells (Aguilar et al., 2009). Fluorescence can then be quantified using flow cytometry (the OxyDNA Assay is performed and observed directly in the cells). Sperm cells were washed twice in PBS and fixed-permeabilized with ice-cold ethanol 70% at −20°C. Ethanol-treated cells were pelleted at 230 × g for 5 min, and the supernatants were removed and pellets were washed twice with PBS. Sperm cells were resuspended in 3 ml of Wash Solution [Tris-buffered Saline/TWEEN® 20 Detergent (TBST), containing Thimerosal], and centrifuged at 230g for 5 min, after which the supernatant was removed and non-specific binding sites were blocked with 50 μl freshly prepared Blocking Solution for 1 h at 37°C. Then 3 ml of Wash Solution was added and washes were repeated twice, and then incubated overnight with 100 μl FITC-Conjugate at 4°C. Samples were washed twice (Wash Solution) and resuspended in 2 ml PBS. Cells were maintained in the dark on ice until their resuspension in FACS fluid and were read in a flow cytometer with FITC filters.

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a trend toward a lower mean number of cigarettes smoked per day was observed in the SD group (ranging from 0 to 3 cigarettes/day) in comparison with the SO (ranging from 0 to 20 cigarettes/day) and RSA (ranging from 0 to 30 cigarettes/day) groups.

The mean number and mean gestational age of miscarriages are also presented in Table I. The number of pregnancy losses among the couples of the RSA group ranged from 3 to 7, whereas the mean gestational week in which pregnancy was lost ranged from 5 to 14.

Male’s genetic contribution to RSA

We were unable to find any Y chromosome microdeletions in any of the males included in the SD, SO or RSA groups.

Regarding sperm DNA quality, two parameters were assessed: sperm DNA fragmentation and sperm DNA oxidation. The results can be found in Figs 1 and 2.

In short, the mean number of sperm cells with fragmented DNA was 24.06% (95% CI 20.87–27.23) in the SD group, in comparison with 46.01% (95% CI 40.40–51.62) in the SO group and 33.48% (95% CI 28.24–38.72) in the RSA group, with statistically significant differences between the SD and RSA and between the SD and SO groups.

The results of the analysis of sperm DNA oxidation in the three groups were comparable in terms of percentage of oxidized cells, with values of 32.14% (95% CI 28.40–35.88) in the SD group, 24.01% (95% CI 16.85–31.17) in the SO group and 23.34% (95% CI 24.62–40.24) in the RSA group.

When the DNA oxidation staining intensity was compared, the mean sperm cell DNA oxidation was 87.25 (95% CI 61.61–113.34) relative fluorescence units (RFU) in the SD group, 87.58 RFU (95% CI 63.69–111.48) in the SO group and 68.04 RFU (95% CI 53.55–82.53) in the RSA group. The differences between the groups were not significant.

The predictive value of sperm DNA fragmentation and DNA oxidation for forecasting recurrent miscarriage are shown in Fig. 3. The low values of the areas under the curve indicate the absence of a predictive power of sperm DNA fragmentation or oxidation analysis in spermatozoa with respect to RSA.

If only males with non-severe sperm alterations (i.e. members of the SD and RSA groups) are considered, the capacity of the sperm
DNA fragmentation and oxidation measurements to discriminate between males from couples with or without recurrent miscarriages can be seen in Fig. 4. Only the percentage of oxidized cells presented a mild capacity to adequately predict RSA occurrence, though it was not sufficient for it to be applied as a diagnostic tool (where an optimal area under the curve should be 0.8).

**Discussion**

In the present study, Y chromosome microdeletions and sperm DNA fragmentation and oxidation did not prove to be related to RSA of unknown origin, at least in men with normal or mildly altered sperm samples. Therefore, the assessment of these parameters in men affected by this condition is not advisable since their alterations are not currently demonstrated to present a causative role.

DNA oxidative damage is indicative of a number of conditions involving the generation of oxygen-free radicals. This damage could be the primary source of DNA fragmentation (Aguilar et al., 2009). In fact, a defective antioxidant system activity could be related to an impairment of chromatin packaging (Tarozzi et al., 2009). However, this is not the only the mechanism of DNA fragmentation (Erenpreiss et al., 2006; Practice Committee of American Society for Reproductive Medicine, 2008). This is why we considered DNA fragmentation and DNA oxidation independently because, despite the association that exists between the two, they are not synonymous.

We analyzed DNA oxidation by ruling out all the external factors that have been related to increased free radical damage (see the ‘Materials and Methods’ section; Practice Committee of American Society for Reproductive Medicine, 2008; Viloria et al., 2009). Similarly, a smoking habit, which is also associated with increased DNA damage in animal models and humans (Kunzle et al., 2001; Pasqualotto et al., 2008), was identified in a similar proportion in the three study groups. Despite the fact that almost 27% of women included in the RSA group were smokers (Table I), the mean number of cigarettes smoked per day was low (3.2). Still, the number of cigarettes smoked daily in the RSA and the SO groups was significantly higher than in the SD group and this may contribute to the higher rate of DNA fragmentation. However, a smoking has not been clearly related to unexplained recurrent miscarriage in recent studies (Zhang et al., 2010). The correlation between male age and sperm DNA fragmentation is controversial, with some reports showing an increase in sperm DNA damage with age (Plastira et al., 2007; Vagnini et al., 2007) and others finding no relationship (Winkle et al., 2009), even after 40 years. In our study, we aimed to avoid this possible confounding factor by limiting the age of the study population to 45 years old, although the SD group was significantly younger compared with the SD and the RSA groups. This also may have contributed to the higher DNA fragmentation rates in the latter two groups.

According to our results, sperm DNA quality, in terms of DNA fragmentation and oxidation, two of the main symptoms of reactive oxygen species attack (Garrido et al., 2004; Aguilar et al., 2009), is not related to recurrent idiopathic spontaneous abortions. Although statistical differences were confirmed among the groups, these do not point to an increase in sperm DNA fragmentation or oxidation in men from RSA couples with respect to the other groups of patients. Interestingly, the group with the highest sperm DNA fragmentation was that formed by males yielding severely oligospermic samples. Increased levels of impairment to DNA integrity in severe oligospermic males have been reported previously in the literature and could explain the poor results described in assisted reproduction treatments by some authors (Meseguer et al., 2008). However, in a recent meta-analysis of 13 studies involving infertile couples, despite a small but statistically significant association between sperm DNA integrity test results and pregnancy outcome in IVF and ICSI cycles, sperm DNA integrity did not prove to be predictive of pregnancy outcome and provided no clinical usefulness in the routine practice (Collins et al., 2008).

The influence of sperm DNA damage on sporadic pregnancy loss has been mainly evaluated after assisted reproductive technology.
Though controversial, a recent systematic review and meta-analysis of 11 studies concluded that the risk of pregnancy loss after IVF and ICSI was 2.5 higher in men with DNA damage, but differed depending on the assay (SCSA or TUNEL) employed (Zini et al., 2008). However, only two previous reports have considered couples with unexplained RSA. In both studies (Carrell et al., 2003a; Qiu et al., 2008), DNA fragmentation was significantly higher in the RSA group than among donors of known fertility and men from the general population. Classic parameters of sperm quality correlated with sperm DNA fragmentation in one of the studies (Qiu et al., 2008), but not in the other (Carrell et al., 2003b). Our results are similar, since sperm samples of the RSA group were normal or only mildly altered, and DNA fragmentation in the same group was significantly higher than among fertile donor controls. However, when we assessed the clinical predictive value of this finding by means of ROC curves, it was absent due to the overlap of values between men belonging to both groups (Fig. 4). The findings were the same when oligozoospermic men were taken into account in the ROC curves (Fig. 3). Therefore, despite the fact that DNA fragmentation seems to be increased in the sperm of men with unexplained RSA, the clinical value of sperm DNA integrity analysis as a predictive tool is negligible. Similarly, the causative role of DNA fragmentation in RSA cannot be demonstrated.

To the best of our knowledge, this is the first study in which sperm oxidative stress has been evaluated in relation to idiopathic RSA. Similar levels of DNA oxidation, in terms of both the percentage of cells with oxidized DNA and mean intensity of oxidation, were found in all the three groups assessed (SD, SO and males from couples with RSA). Only the percentage of oxidized cells exhibited a mild capacity to adequately predict RSA occurrence (Fig. 4), although this predictive power does not appear to be sufficient for its implementation as a diagnostic tool (where an optimal area under the curve should be >0.8).

One of the most significant findings of this work is that no differences in the prevalence of Y chromosome microdeletions were found between the groups. In fact, no presence of Y microdeletions was detected in any of the 90 subjects included. These results are in complete contrast to what was initially published by Dewan et al. (2006). In their work, 14 out of 17 males (82%) from RSA couples presented microdeletions in one or more of the four segments of the proximal AZFa region studied, whereas this percentage dropped to 20% in infertile males with severely impaired spermatogenesis and was non-existent among men with proven fertility. Recently, Karaer et al. (2008) also detected Y chromosome microdeletions in one or more of the four STSs assessed in the AZFb and AZFd regions in 16% of men from couples with RSA and reported no case among fertile men. However, the percentage of microdeletions detected differed hugely between these two studies (82 versus 16%) and the regions affected were mostly different.

Figure 3 ROC curve analysis of the predictive value of sperm DNA fragmentation and oxidation to forecast RSA.
In our work, the increased sample size should have allowed clarification of the considerable differences reported previously by Dewan et al. (2006). At least, some Y chromosome microdeletions should have been observed given that we have considered similar populations. In addition, we assessed 20 Y-linked STSs belonging to four AZF loci regions (AZFa, AZFb, AZFc and AZFd) rather than only four STSs in one or two AZF regions (Dewan et al., 2006; Karaer et al., 2008).

Furthermore, Dewan et al. (2006) argue that their patients were selected from a tertiary referral center that receives a disproportionate number of RSA patients and that these patients were likely to have failed evaluations and treatments prior to referral, thus skewing the results. In our study, patients were selected following strict inclusion criteria. The couples described as infertile in the Dewan et al. report exhibited similar conditions of sperm quality as those in our study, with the exception of those males presenting azoospermia, who were not included in our population due to the impossibility of performing the described DNA fragmentation and oxidation tests with their samples. This fact may partly explain the lack of Y chromosome microdeletions in our SO group, since these alterations have been described in higher proportions in men with very low sperm counts (<1 million/ml; Martinez et al., 2000). In addition, despite the strict inclusion criteria employed, the sample size considered in the study (90 men) may also represent a limitation for the results obtained.

Similarly to our study, other recent reports have not identified Y chromosome microdeletions in male partners of RSA couples. Kaare et al. (2008) evaluated 40 men from couples with at least three miscarriages and analyzed 33 STS loci spanning the whole Y chromosome plus the 4 STS loci used by Dewan et al. (2006). No microdeletion was found in any case. A study of a Chinese population by Lu et al. (2008) also failed to detect any case of Y chromosome microdeletions in 26 chorionic villous samples of abortive male embryos or in 51 blood samples of men whose wives had experienced RSA. They studied 12 STSs in the AZFa, AZFb and AZFc regions of Yq11.2.

Differences in the presence of Y chromosome microdeletions could be due to variations in the study populations (race and ethnicity), subject selection criteria, the STSs considered and methodological...
aspects of the analysis. Nevertheless, no clear evidence currently exists regarding the relationship between idiopathic RSA and Y chromosome microdeletions.

To conclude, there seems to be no clinical use for the assessment of Y chromosome microdeletions and DNA fragmentation and oxidation when dealing with couples with idiopathic RSA, at least when the sperm samples of the male partner are not severely impaired. Until such a clinical use is demonstrated, this assessment can only serve research purposes. Given the continuous description of sperm molecular factors related to male infertility, further research into RSA using the newly discovered molecules as markers should be conducted. Recent advances in microarray technology applied to sperm will undoubtedly constitute powerful tools in the characterization of these factors.

Authors’ roles

J.B. designed the study, selected the patients and wrote the paper; M.M. helped in the design of the study and performed the DNA oxidation studies; L.M. performed the DNA fragmentation studies; S.G.-H. collaborated in the DNA oxidation studies and in the writing of the paper; M.A.M.B. included many of the patients analyzed and helped in the design of the paper; A.L.G. performed the Y chromosome microdeletion analysis; J.R. helped in the design and monitored the study; A.P. helped in the statistical approach and monitored the study and the paper; N.G. designed the study, performed the statistical analysis, coordinate the sperm and blood analysis and helped in the writing of the paper.

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