Y-chromosome microdeletions are not associated with SHOX haploinsufficiency

C. Chianese1, D. Lo Giacco2,3, F. Tüttelmann4, A. Ferlin5, P. Ntostis1, S. Vinci1, G. Balercia6, E. Ars2, E. Ruiz-Castañé2,3, S. Giglio7, G. Forti8, S. Kliesch9, and C. Krausz1,3,*

1Andrology Unit, Department of Experimental and Clinical Biomedical Sciences, University of Florence, Florence 50139, Italy, 2Molecular Biology Laboratory, Fundació Puigvert, Universitat Autònoma de Barcelona, Barcelona 08025, Catalonia, Spain, 3Andrology Service, Fundació Puigvert, Barcelona 08025, Catalonia, Spain, 4Institute of Human Genetics, University of Münster, Münster 48149, Germany, 5Department of Molecular Medicine, Section of Clinical Pathology and Centre for Human Reproduction Pathology, University of Padova, Padova 35128, Italy, 6Division of Endocrinology, Department of Clinical and Molecular Sciences, Umberto I Hospital, Polytechnic University of Marche, Ancona 60121, Italy, 7Medical Genetics Unit, Meyer Children’s University Hospital, Florence 50139, Italy, 8Endocrinology Unit, Department of Experimental and Clinical Biomedical Sciences, University of Florence, Florence 50139, Italy, and 9Centre of Reproductive Medicine and Andrology, University Clinics, Münster 48149, Germany

*Correspondence address. E-mail: c.krausz@dfc.unifi.it

Submitted on May 24, 2013; resubmitted on July 5, 2013; accepted on July 16, 2013

STUDY QUESTION: Are Y-chromosome microdeletions associated with SHOX haploinsufficiency, thus representing a risk of skeletal anomalies for the carriers and their male descendents?

SUMMARY ANSWER: The present study shows that SHOX haploinsufficiency is unlikely to be associated with Y-chromosome microdeletions.

WHAT IS KNOWN ALREADY: Y-chromosome microdeletions are not commonly known as a major molecular genetic cause of any pathological condition except spermatogenic failure. However, it has been recently proposed that they are associated not only with infertility but also with anomalies in the pseudoautosomal regions (PAR), among which SHOX haploinsufficiency stands out with a frequency of 5.4% in microdeletion carriers bearing a normal karyotype. This finding implies that sons fathered by men with Y-chromosome defects will not only exhibit fertility problems, but might also suffer from SHOX-related conditions.

STUDY DESIGN: Five European laboratories (Florence, Münster, Barcelona, Padova and Ancona), routinely performing Y-chromosome microdeletion screening, were enrolled in a multicenter study.

PARTICIPANTS/MATERIALS, SETTING, METHODS: PAR-linked and SHOX copy number variations (CNVs) were analyzed in 224 patients carrying Y-chromosome microdeletions and 112 controls with an intact Y chromosome, using customized X-chromosome-specific array-CGH platforms and/or qPCR assays for SHOX and SRY genes.

MAIN RESULTS AND THE ROLE OF CHANCE: Our data show that 220 out of 224 (98.2%) microdeletion carriers had a normal SHOX copy number, as did all the controls. No SHOX deletions were found in any of the examined subjects (patients as well as controls), thus excluding an association with SHOX haploinsufficiency. SHOX duplications were detected in 1.78% of patients (n = 4), of whom two had an abnormal and two a normal karyotype. This might suggest that Y-chromosome microdeletions have a higher incidence for SHOX duplications, irrespective of the patient’s karyotype. However, the only clinical condition observed in our four SHOX-duplicated patients was infertility.

LIMITATIONS, REASONS FOR CAUTION: The number of controls analyzed is rather low to assess whether the SHOX duplications found in the two men with Y-chromosome microdeletions and a normal karyotype represent a neutral polymorphism or are actually associated with the presence of the microdeletion.

WIDER IMPLICATIONS OF THE FINDINGS: Men suffering from infertility due to the presence of Y-chromosome microdeletions can resort to artificial reproductive technology (ART) to father their biological children. However, infertile couples must be aware of the risks implied and this makes genetic counseling a crucial step in the patient’s management. This study does not confirm previous alarming data that showed an association between Y-chromosome microdeletions and SHOX haploinsufficiency. Our results imply that deletion carriers have no augmented
risk of SHOX-related pathologies (short stature and skeletal anomalies) and indicate that there is no need for radical changes in genetic counseling of Yq microdeletion carriers attempting ART, since the only risk established so far for their male offspring remains impaired spermatogenesis.

**STUDY FUNDING/COMPETING INTEREST(S):** This work was supported by the Italian Ministry of University (grant PRIN 2010-2012 to C.K.), Tuscan Regional Health Research Program (’Progetto Salute 2009’) to G.F., the Spanish Ministry of Health (grant FIS-11/02254) and the European Union ‘Reprotrain’ Marie Curie Network (project number: 289880 to C.K.). The authors declare that no conflicting interests exist.

**Key words:** Y chromosome / SHOX / male infertility / spermatogenesis / gr/gr deletion

**Introduction**

The Y chromosome displays a distinctive genomic landscape for most of its extent, rich in repetitive elements that provide a favorable environment for the generation of copy number variations (CNVs). There is ever-growing evidence that collocates CNVs among those genetic factors that deserve prime attention when dealing with complex human diseases. To date, the only CNVs unquestionably associated with male infertility are the Y-chromosome microdeletions, which involve the azoospermia factor (AZF) region on Yq and are thus termed AZFa, AZFb and AZFc microdeletions (Krausz et al., 2011). Each type of microdeletion is in a clear-cut cause–effect relationship with a distinct abnormal semen phenotype, but until recently microdeletion carriers with normal karyotype have not been proven to be at risk for any condition other than infertility.

However, one recent study (Jorgez et al., 2011) reported that microdeletion carriers also displayed aberrations in the pseudoautosomal regions (PAR1 and PAR2), short homologous regions sited at the extremities of the gonosomes. Therefore, the authors proposed that the mechanism underlying Y-chromosome microdeletions might also be associated with the occurrence of PAR rearrangements. The salient, and alarming, finding of this study resided in the detection of haploinsufficiency of the PAR1-located SHOX (Short stature HOmeoboX-containing) gene in 5.4% of men carrying Yq microdeletions and a normal karyotype. It is widely ascertained that SHOX haploinsufficiency leads to disproportionate short stature and diverse skeletal anomalies such as Leri–Weill dyschondrosteosis (LWD) (Helena Mangs and Morris, 2007). In contrast, the occurrence of SHOX duplications is apparently rare, with only few cases reported so far (Grigelioniene et al., 2001; Tachdjian et al., 2008; Roos et al., 2009; Thomas et al., 2009; D’Haene et al., 2010; Gervasini et al., 2010) and no direct relationship with any specific phenotype has yet been defined. The findings by Jorgez et al. (2011) raised the question whether microdeletion carriers might be at higher risk of incurring PAR-related pathologies. If this were true, genetic counseling would radically change, since infertile couples undergoing artificial reproductive technology (ART) would need to be informed that their sons not only will have fertility problems but also will be at risk of developing PAR-related disorders. Moreover, screening for SHOX-linked CNVs should then become compulsory for men carrying Yq microdeletions.

Given the relevance and the potential clinical impact of this issue, we performed a multicenter investigation on a large study population—almost doubling that of the above mentioned study (Jorgez et al., 2011)—in order to investigate whether the hypothesis of an association between Yq microdeletions and SHOX haploinsufficiency could be confirmed.

**Materials and Methods**

**Subjects**

The study population counted a total of 336 Caucasian men, comprising 224 patients carrying different types of Y-chromosome microdeletions (4 complete AZFa; 6 AZFb; c; 153 complete AZFc; 4 AZFa,b,c; 57 partial AZF deletions; 40 gr/gr, 7 gr/gr-b2/b4 duplication, 3 partial AZFa deletion, 5 b2/b3, 1 b1/b3, 1 b3/b4) and 112 men with an intact Y chromosome, referred to as controls. The detection of Y-chromosome microdeletions was achieved by screening patients according to the European Academy of Andrology (EAA) guidelines (Simoni et al., 2004), in the following participating laboratories: Florence (n = 66); Münster (n = 56); Barcelona (n = 43); Padova (n = 49); Ancona (n = 10). Karyotype was available for 300 subjects, including patients and controls.

Germline DNA deriving from peripheral blood lymphocytes was originally isolated in each participating center by standard methods. DNA quality was assessed using a Nanodrop ND-1000 UV-VIS spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and samples showing a A260/280 ratio > 1.8 were used. All individuals gave informed consent for the routine AZF analysis and further scrutiny.

**Array-comparative genomic hybridization**

Customized X-chromosome-specific array-comparative genomic hybridization (CGH) platforms (8X60 K, Agilent Technologies, Santa Clara, CA, USA) were generated using the e-Array software (http://earray.chem.agilent.com/); 53 069 probes (60-mer oligonucleotides) were selected from the Xq PARs, with a mean resolution of 4 kb. Four replicate probe groups, with each probe present in two copies on the platform, were designed in regions containing mouse infertility-associated genes, i.e. sperm protein associated with the nucleus, X-linked family members (SPANX); testis expressed 11 (TEX11), TAF7-like RNA polymerase II (TAF7L), TATA box binding protein (TBP). In these regions, the medium resolution is 2 kb. For the normalization of copy number changes, the array also included Agilent control clones spread along all autosomes (6842 probes). As a reference DNA, the same male subject with no Yq microdeletions and normal karyotype was used. This control DNA was already characterized for CNV content in previous array-CGH experiments against eight different normozoospermic controls and presented one private gain of 27 kb mapping to Xcen tr which was not considered for the frequency analyses. Three hundred nanograms of test DNA and control DNA were double-digested with RsaI and Alul (Promega, Madison, WI, USA) for 1 h at 37°C. After digestion, samples were incubated at 65°C for 20 min to inactivate the enzymes, and then labeled by random priming (Agilent Technologies, Santa Clara, CA, USA) for 2 h using Cy5-dUTP for the test DNA and Cy3-dUTP (Agilent Technologies, Santa Clara, CA, USA) for the control DNA. Labeled DNAs were incubated at 65°C for 10 min and then purified with Microcon YM-30 filter units (Millipore, Billerica, MA, USA). Every purified sample
was brought to a total volume of 9.5 ml in 1xTE (pH 8.0, Promega, Madison, WI, USA), and yield and specific activity were determined for each sample using a NanoDrop ND-1000 UV-VIS Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The appropriate cyanine 5- and cyanine 3-labeled samples were combined in a total volume of 16 ml. After sample denaturation and pre-annealing with 5 μl of Human Cot-1 DNA (Invitrogen, Carlsbad, CA, USA), hybridization was performed at 65 °C with shaking for 24 h. After two washing steps, the array was analyzed through the Agilent scanner and the Feature Extraction software (v10.1.1). Graphical overview was obtained using the DNA Analytics (v4.0.73). All the array experiments were analyzed using the ADM-2 algorithm at threshold 5. Aberrant signals including three or more adjacent probes were considered as genomic CNVs. The positions of oligomers refer to the Human Genome March 2006 assembly (hg18). Array-CGH calls for the detected CNVs are provided in Supplementary data, Table SI and raw data are depicted in Supplementary data, Figs A–D, referring to samples presented in Table I.

Quantitative real-time PCR
To estimate SHOX copy number (CN), a quantitative real-time PCR (qPCR) assay targeting exon 2 of the gene was designed (Applied Biosystem). Primers and probes were designed with the Primer Express v2.0 software (Applied Biosystems, Foster City, CA, USA) and tested for specificity using the NCBI’s BLAST software. To estimate SRY CN, we used a commercially available qPCR assay (Roche, Mannheim, Germany). Reactions were performed in triplicate in a 10 μl final volume. The target gene (FAM-labeled) and the reference gene RNaseP (VIC-labeled) were co-amplified in a duplex qPCR. Four internal controls were always included in each experiment: (i) a subject with normal karyotype (calibrator); (ii) a subject with SRY CN, (iii) a 47,XXY subject and (iv) a no-template negative control. All runs were performed using 7900HT Fast System. Target genes CN was determined by relative quantification using the Copy-Caller-Software, v1.0 (Applied Biosystems, Foster City, CA, USA), based on the comparative ddCt method (Fig. 1). qPCR profiles are shown in Supplementary data, Figs E–G. Assay reproducibility was validated by calculating the coefficient of variation (Cv) between five different experiments, in which CN was estimated for the three positive controls (normal man, deleted man and 47,XXY man). Standard deviations (S) and mean values (M) were calculated and Cv was computed as: \( Cv = \frac{S}{M} \times 100 \), denoting a very low inter-assay variability ranging between 0.4 and 2%.

Results

Array-CGH indicates that PAR-linked CNVs are mainly related to the individual’s karyotype
Array-CGH was performed for twenty men with Yq microdeletions and twelve controls with an intact Y chromosome. Karyotype was available for 18 of the Yq microdeletion carriers, 13 with normal and 5 with abnormal karyotypes. All controls displayed a normal karyotype. This preliminary array-CGH analysis revealed that four of twenty carriers (20%) displayed CNVs, both losses and gains, at the PAR level. Of these four subjects, three carried an AZFb,c microdeletion and one carried a complete AZFc microdeletion. However, the array-CGH results were not unexpected since all the detected CNVs were explainable by the associated abnormal karyotype (Table IA). None of the twelve controls displayed CNVs in the PARs. Validation of array-CGH-detected CNVs was performed using predesigned SHOX and SRY TaqMan CN Assays.

SHOX CN evaluation by qPCR
We screened another set of 204 men carrying various types of Yq microdeletions (including partial AZFc deletions and duplications) with SHOX qPCR alone, considering SHOX CN as a proxy of PAR1 rearrangements. Karyotype was available for 170 men, of which 164 had a normal karyotype and 6 had karyotype anomalies. We found that almost all subjects analyzed (201/204; 98.5%) had a normal SHOX CN, except for three men that displayed an extra copy of the SHOX gene (1.47%): samples D1056, P7806 (both carrying a complete AZFc microdeletion) and Mmp1000 (carrying an AZFb,c microdeletion) (Table IB). These patients were then screened for the SRY gene, as well. We found that one also had an extra copy of the SRY gene, whereas the other two had a normal SRY CN (Table IB). Although Mmp1000’s case history reported a 46,XYq-karyotype, the presence of two copies of the SRY gene clearly indicates that this is a 46,X,idic(Yp) with potential breakpoint in P6/P7 (Lange et al., 2009). No SHOX deletions were found in any of the Yq microdeletion carriers. If we only consider men with complete AZFc microdeletions,

<table>
<thead>
<tr>
<th>Table I</th>
<th>Patients with PAR-linked CNVs detected by qPCR and/or array-CGH.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Array-CGH analysis</td>
<td></td>
</tr>
<tr>
<td>Patient</td>
<td>Center</td>
</tr>
<tr>
<td>A146</td>
<td>Florence</td>
</tr>
<tr>
<td>A372</td>
<td>Florence</td>
</tr>
<tr>
<td>A1389</td>
<td>Florence</td>
</tr>
<tr>
<td>A116b</td>
<td>Florence</td>
</tr>
<tr>
<td>B. qPCR screening</td>
<td></td>
</tr>
<tr>
<td>Patient</td>
<td>Center</td>
</tr>
<tr>
<td>Mmp1000</td>
<td>Ancona</td>
</tr>
<tr>
<td>P7806</td>
<td>Padova</td>
</tr>
<tr>
<td>D1056</td>
<td>Muenster</td>
</tr>
</tbody>
</table>

CGH: comparative genomic hybridization; CN: copy number; CNV: copy number variation.

For patient A116, qPCR was not performed due to DNA sample extinction.

Each qPCR was performed in triplicate and each assay was repeated three times.

SHOX CN, except for three men that displayed an extra copy of the SHOX gene (1.47%): samples D1056, P7806 (both carrying a complete AZFc microdeletion) and Mmp1000 (carrying an AZFb,c microdeletion) (Table IB). These patients were then screened for the SRY gene, as well. We found that one also had an extra copy of the SRY gene, whereas the other two had a normal SRY CN (Table IB). Although Mmp1000’s case history reported a 46,XYq-karyotype, the presence of two copies of the SRY gene clearly indicates that this is a 46,X,idic(Yp) with potential breakpoint in P6/P7 (Lange et al., 2009). No SHOX deletions were found in any of the Yq microdeletion carriers. If we only consider men with complete AZFc microdeletions,
we observe that samples D1056 and P7806 are the only ones having SHOX CNVs, accounting for 1.31% (2/153) of carriers.

**SHOX CNVs in microdeletions carriers and normal karyotype**

If we solely consider men with Yq microdeletions and a normal karyotype, we observe that, on a total of 177 subjects (15 analyzed by array-CGH/qPCR and 164 analyzed by qPCR only), SHOX deletions were never found, whereas only SHOX duplications were detected in samples D1056 and P7806 (1.1%). Hence, 98.9% (175/177) of men with microdeletions and a normal karyotype did not display any SHOX CNVs. As a counterpart, we finally screened an extra set of 100 men, of two different nationalities (Italian and Spanish), displaying an intact Y chromosome and normal stature, and all resulted with a normal SHOX CN.

**SHOX duplications and the associated phenotype**

A total of four patients, carrying both altered and normal karyotypes, displayed an extra copy of the SHOX gene. At the moment of consultation, all these patients presented with neither stature abnormalities nor any medical condition other than azoospermia (Table II). In the case of patient P7806, testicular sperm extraction upon bilateral TESE allowed the isolation of 13 fully maturated spermatozoa from the right side and 0.025 × 10^6 from the left side. ICSI performed with the patient’s cryopreserved spermatozoa resulted in the delivery of a healthy male child.

**Figure I** SHOX qPCR results. The figure represents data derived from a single experiment and elaborated by CopyCaller™ software v1.0. Deleted = 1 copy of SHOX; Control = 2 copies of SHOX; Klinefelter = 3 copies of SHOX.

**Discussion**

Y-chromosome microdeletions represent the most frequent genetic cause of spermatogenic failure in infertile men, second only to Klinefelter syndrome (Krausz et al., 2011). In order to overcome their condition and father biological children, some carriers opt for treatment with ART. However, Yq genetic defects will be inevitably transmitted to their male offspring, who will predictably suffer from fertility problems. Therefore, genetic counseling for these couples is of inestimable importance.

Twenty years of interest in Y-chromosome microdeletions has produced a collection of numerous articles, but only a minority of these studies aimed to define whether Yq deletions might lead to other pathological conditions, beside spermatogenic failure. Microdeletions were reported in association with 45,X/46,XY mosaic karyotype and ambiguous genitalia (Papadimas et al., 2001; Patsalis et al., 2002, 2005; Papankolau et al., 2003; Tian et al., 2012); consistently, our data provide further evidence that Yq microdeletions do associate with mosaicism (as four samples presented 45,X cell lines), supporting the hypothesis that Y chromosomes bearing AZF deletions are more instable and thus predispose to the formation of Y-chromosome nullisomic cell lines.

Recently, a paper by Jorgez et al. (2011) reported that Y-chromosome microdeletions might not only cause spermatogenic failure but also increase the risk for PAR-related pathologies, especially emphasizing SHOX gene involvement. On a total of 87 men with Yq microdeletions, they found SHOX haploinsufficiency in five samples, four carrying a

**Table II** Phenotype characterization of Y-chromosome microdeletion carriers with an extra copy of SHOX.

<table>
<thead>
<tr>
<th>Patient</th>
<th>D1056</th>
<th>P7806</th>
<th>A416</th>
<th>Mmp1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>32</td>
<td>39</td>
<td>44</td>
<td>38</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>180</td>
<td>188</td>
<td>n.a.</td>
<td>174</td>
</tr>
<tr>
<td>Nationality</td>
<td>German</td>
<td>Italian</td>
<td>Italian</td>
<td>12</td>
</tr>
<tr>
<td>Mean testis (cc)</td>
<td>7</td>
<td>6</td>
<td>n.a.</td>
<td>0</td>
</tr>
<tr>
<td>Tot sperm count (x10^6)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Karyotype</td>
<td>46,XY</td>
<td>46,XY</td>
<td>46,XY,idic(Y)</td>
<td>46,XYq-</td>
</tr>
<tr>
<td>FSH (UI/L)</td>
<td>29.9</td>
<td>21.9</td>
<td>17.9</td>
<td>13.9</td>
</tr>
<tr>
<td>LH (UI/L)</td>
<td>11.2</td>
<td>9.2</td>
<td>6.7</td>
<td>8.6</td>
</tr>
<tr>
<td>Testosterone (ng/ml)</td>
<td>3.77</td>
<td>3.54</td>
<td>5.75</td>
<td>4.61</td>
</tr>
<tr>
<td>Testis histology</td>
<td>Bilateral SCOS</td>
<td>Unilateral SCOS and hypospermatogenesis*</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Other medical conditions</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

SCOS, Sertoli cell only syndrome; n.a., not available.

*Quantitative alteration in which a very small portion of seminiferous tubules containing fully maturated spermatozoa (13 in the right testis and 0.025 × 10^6 in the left testis) was found.
normal karyotype and one carrying an idic(Y); however, information is missing about these patients’ phenotype. Moreover, they found nine men with SHOX duplications, all displaying abnormal karyotypes; still, stature was available only for six patients and clinical data only for four subjects, of whom only two displayed other medical conditions (congenital urological defects, diabetes and cataracts).

In this study, we provide the largest collection of men carrying Y-chromosome microdeletions analyzed so far in association with SHOX CN. A novelty of our study consisted in the inclusion of additional Y-chromosome rearrangements, such as gr/gr deletions and gr/gr deletion-duplications. Moreover, we included a very large number of men with complete AZFc microdeletion (4-fold higher than the previous study) because they represent the more plausible candidates for ART attempts. Our data seemingly indicate that SHOX CNVs are mainly linked to the individual’s karyotype rather than the mere presence of microdeletions. As a matter of fact, of 177 deletion carriers with a normal karyotype only two (1.1%) displayed an abnormal SHOX CN; both patients bore duplications of the SHOX gene, but apart from a relatively high stature (though still within the normal range) the men’s clinical work-up revealed no other pathological conditions than azospermia. Literature is poor and unclear about SHOX over-dosage, which has been reported in association with normal to tall stature (Ogata et al., 2001; Adamson et al., 2002) as well as to more severe conditions such as LWd and idiopathic short stature (ISS) (Roos et al., 2009; Thomas et al., 2009; D’Haene et al., 2010; Benito-Sanz et al., 2012). In our study, the relatively low number of controls analyzed makes it difficult to define whether the SHOX duplications found in the two men with Yq microdeletions and a normal karyotype represent a polymorphism commonly found in the general population or are actually associated with the presence of the microdeletion. However, although we did not find SHOX over-dosage in controls, a recent study (Lopes et al., 2013) reported the presence of a duplication burden on the Y chromosome, including PAR1, in men with no Yq microdeletions, suggesting that the ‘duplication events’ that we observed are unlikely to be related to the presence of microdeletions.

Conversely, a clear picture exists concerning SHOX haploinsufficiency and its deleterious effects on stature (Helena Mangs and Morris, 2007). In our screening, none of the men with microdeletions had SHOX deletions, contrasting the aforementioned hypothesis that microdeletion carriers are at a higher risk of developing pathologies caused by SHOX haploinsufficiency.

The reasons underlying the discrepancy between our study and the previous publication might be related to methodological issues, although the extremely succinct description of the method in the paper by Jorgez et al. (2011) renders it difficult to make any meaningful comparison. In our study, much care was taken to validate every step: template quality, inter-assay variability and all technical aspects were thoroughly addressed to avoid artifacts. Another potential bias might be ethnicity; but again, the ethnic background was not specified in the previous publication and thus comparison with our study cannot be done.

In summary, our study confirms the previously reported association between complete AZF microdeletions and 45,X/46,XY mosaicism. Moreover, we show that both partial and complete microdeletions in men with 46,XY karyotype are unlikely to be associated with SHOX haploinsufficiency, providing reassurance that the only established risk for ART offspring born from men with Yq microdeletions remains spermatogenic impairment.

Supplementary data
Supplementary data are available at http://humrep.oxfordjournals.org/.

Acknowledgements
We thank Marilena Pantaleo (Meyer Hospital, Florence, Italy), Elena Rossi (University of Pavia, Italy) and Albrecht Röpke (University of Münster, Germany) for technical support and helpful discussions. Special thanks to Esperança Martí, President of the Fundacio Puigvert for her continuous support. We thank all the clinicians from the Andrology Unit of the University of Florence (M. Maggi, A. Magini and F. Lotti) and from the Fundacio Puigvert (L. Bassas, O. Rajmil, J. Sarquella, A. Vives and J.Sanchez-Curbelo) who helped in providing samples for this study.

Authors’ roles
All authors are justifiably credited with authorship, according to the authorship criteria. In detail, C.C.: design, a-CGH and qPCR analyses, acquisition of data, analysis and interpretation of data, drafting and revision of the manuscript; D.L.: partial AZFc deletion analysis; P.N.: qPCR in the 100 controls; S.V.: qPCR design; F.T., A.F., G.B., E.A., E.R.-C., G.F.: clinical definition of patients and DNA samples collection; S.G.: a-CGH analysis supervision; C.K.: conception and supervision of the project, funding, interpretation of data, drafting and revision of the manuscript and final approval given.

Funding
This work was supported by the Italian Ministry of University (grant PRIN 2010-2012 to C.K.), Tuscan Regional Health Research Program (‘Progetto Salute 2009’) to G.F., the Spanish Ministry of Health (grant FIS-11/02254) and the European Union ‘Reprotrain’ Marie Curie Network (project number: 289880 to C.K.).

Conflict of interest
None declared.

References


