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Ana Isabel Correia de Pinho

Clinical and molecular characterization of Y microdeletions and X-linked CNV67 implications in male fertility: a 20-year experience

Caracterização das microdeleções do cromossoma Y e CNV67 na fertilidade masculina: a experiência de 20 anos

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TÍTULO DISSERTAÇÃO

Clinical and molecular characterization of Y microdeletions and X-linked CNV67 implications in male fertility: a 20-year experience

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Faculdade de Medicina da Universidade do Porto, 11/03/2019

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Dedicatória

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Title: Clinical and molecular characterization of Y microdeletions and X-linked CNV67 implications in male fertility: a 20-year experience

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Short title: Y microdeletions and CNV67 in male infertility

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Abstract

Background: Approximately 15% of couples worldwide are affected with infertility, attributed to a male co-factor in about half of the cases. Y chromosome microdeletions are the second most common genetic cause for male infertility, with a global prevalence of 2-10% in infertile men. Recently, CNV67, localized in X chromosome, have emerged as potential contributor to male infertility, with a described frequency of 1,1% in the oligo/azoospermic men.

Objectives: To investigate the prevalence of Y-linked CNVs in a cohort of Portuguese infertile men and correlate the patients' phenotypes with a genetic alteration; to investigate the CNV67 deletion in a subset of patients and corroborate the role of this CNV in male infertility.

Materials and Methods: We retrospectively analysed a database of 4000 Portuguese infertile men for karyotype anomalies and Y-microdeletions and selected a cohort of 200 for CNV67 screening analysis by quantitative PCR.

Result(s): Karyotype anomalies were present in 263 patients (6.6%), with Klinefelter syndrome representing the most frequent karyotype anomaly (2.8%). Among the 4000 patients, the prevalence of Yq microdeletions was 4.6%. Ninety microdeletions (10.1%) were found in the azoospermic group, 44 deletions (4.5%) in the severe oligozoospermic group, 1 AZFc partial deletion (0.3%) in the mild-moderate oligozoospermic group and 2 partial AZFc deletions (0.4%) in the normozoospermic group. AZFc deletions represented 80.4% of the Yq microdeletions. In CNV67 analysis, 2 individuals had this deletion.

Conclusion(s): This study presents one of the largest samples of infertile men worldwide with the main purpose of correlating the Yq-microdeletions with sperm count. Our findings are supported by previous reviews with large data and provide a reliable estimation of the prevalence of these anomalies in a Portuguese population. CNV67 was exclusively deleted in patients with spermatogenic impairment (1%), showing a consistent genotype-phenotype correlation and a significant prevalence.

Introduction

Approximately 15% of couples worldwide are affected with infertility, attributed to a male co-factor in about half of the cases (de Kretser 1997, Tuttelmann, *et al.* 2011). Although many genetic mutations and polymorphisms linked to spermatogenesis have been identified during the last years and despite full clinical workup (including karyotype, Y chromosome microdeletion testing, cystic fibrosis transmembrane conductance regulator (CFTR) gene screening and hypogonadotropic hypogonadism mutation screening in some cases) (Wosnitzer 2014), the etiopathogenesis of male infertility remains unclear in around 50% of cases (Jungwirth *et al.* 2012, Krausz *et al.* 2018). The aetiology of most of these "idiopathic infertility" cases is likely to be related to genetic abnormalities (Krausz *et al.* 2018) since more than one thousand genes are now thought to be functionally required to spermatogenesis, some of them specifically expressed on the male gonads and consequently strong candidates to explain spermatogenic failure (Krausz 2011, Matzuk & Lamb 2008).

Numerous studies have been conducted across the years and it is now accepted that genetic defects account for an important part of testicular dysfunction (Colaco & Modi 2018, Krausz 2011, Tournaye *et al.* 2017), the major cause of male infertility, clinically manifested as azoospermia (absence of sperm in the ejaculate) or oligozoospermia (sperm concentration <15x 10^6/ ml according to WHO guidelines 2010), asthenozoospermia (<32% progressively motile spermatozoa) and teratozoospermia (<4% morphologically normal spermatozoa), often denominated oligoasthenoteratozoospermia (Krausz 2011, WHO 2010). Besides acquired diseases, testicular infertility may result from congenital abnormalities such as numerical chromosomal anomalies (Klinefelter's syndrome is the most common karyotype abnormality), structural rearrangements (autosomal translocations and inversions) and Copy Number Variations (CNVs) namely the Yq11 chromosomal microdeletions, between others of low prevalence and rare assessment (Chandley 1998, Huang & Yen 2008, Krausz *et al.* 2014).

CNVs are thought to contribute to the high complexity of testicular failure due to submicroscopic rearrangements of 1kb or larger, leading to deletions and duplications of individual genes which results in numeric and genomic instability with defective cell

cycle and modifications at candidate genes function and protein expression levels (Feuk *et al.* 2006, Redon *et al.* 2006).

Y chromosome microdeletions are the only proven CNVs that interfere with spermatogenesis (Krausz *et al.* 2014) and are the second most common genetic cause for male infertility, with a global prevalence of 2-10% in infertile men (Hofherr *et al.* 2011, Johnson *et al.* 2018, Lo Giacco *et al.* 2013, Mascarenhas *et al.* 2016, Tahmasbpour *et al.* 2014, Wosnitzer 2014) and a lower frequency estimated in Europe (around 3%) (Colaco & Modi 2018). According to the current knowledge, the Y microdeletions can be complete, partial or affect different regions simultaneously and are divided in AZFa (0,5-4%), AZFb (1-5%), AZFb+c (1-3%) and AZFc (60-80%) (Colaco & Modi 2018, Krausz *et al.* 2014). The clinical phenotype varies with the type of microdeletion (Vogt *et al.* 1996): AZFa deletions are typically associated with azoospermia with germinative cells aplasia (Sertoli Cell Only Syndrome - SCOS) (Goncalves *et al.* 2017, Kleiman *et al.* 2012). AZFb deletions are mainly associated with germ cell maturation arrest. The most common deletion, in the AZFc region, encompassing DAZ (Deleted in AZoospermia) gene, displays a larger spectrum of clinical and histological manifestations, with variable sperm production capacity, therefore most of these men show residual spermatogenesis which allows spermatozoa retrieval for in-vitro fertilization/ Intracytoplasmic sperm injection (ICSI) (Goncalves *et al.* 2017, Hopps 2003, Krausz & Casamonti 2017, Krausz *et al.* 2014, Pastuszak & Lamb 2012, Vogt *et al.* 1996, Vogt & Fernandes 2003). Furthermore, the AZFc region may be partially deleted (b1/b3, b2/b3 and gr/gr) leading to copy number variations of some genes that play a role in spermatogenesis which results in different amounts of proteins produced and a wide heterogeneity in sperm counts and fertile status depending on the Y genetic background (Colaco & Modi 2018, Fernandes *et al.* 2002, Krausz *et al.* 2014, Navarro-Costa *et al.* 2010).

Recently, other CNVs described on autosomes and X chromosome have emerged as potential determinants of male infertility (Eggers *et al.* 2015, Krausz *et al.* 2012, Stouffs *et al.* 2012, Tuttelmann *et al.* 2011).

High resolution X chromosome-specific approaches identified CNV67, localized in Xq28 and maternally inherited, as a recurrent patient-specific CNV, with a frequency of 1,1% in the studied population (oligo/azoospermic men) (Krausz *et al.* 2012). A recent study in the Portuguese population found a higher frequency (2%) of the CNV67 deletion in azoospermic and severe oligozoospermic men (Costa *et al.* 2017). These data

hypothesize the potential value of CNV67 on the fertility workup as an additional contribution to explain the currently classified as idiopathic cases. The clinical implications of this X-linked deletion seem to be related with the loss of *MAGEA9B* gene, from the X-Cancer Testis Antigens gene family, which is involved in the spermatogenesis regulation, among others presenting specific or highest expression in the male germ cells (Krausz *et al.* 2012, Lo Giacco *et al.* 2014). Other study support that CNV67 could be a risk factor for spermatogenic failure across different populations (population-independent effects on sperm production) since males with CNV67, CNV69 and CNV64 deletions presented lower sperm counts than the non-carriers (Shen *et al.* 2017).

With this study, we would like to evaluate the prevalence of Y-linked CNVs in our cohort of 4000 infertile men with abnormal sperm counts and correlate the patients' phenotypes with a genetic alteration. Additionally, we will test CNV67 in a subset of these patients with normal karyotype and spermatogenic impairment and corroborate the role of this CNV in male infertility.

Materials and Methods

Y microdeletions analysis

Subjects

We retrospectively analysed a database of 4000 Portuguese infertile men over the age of 18 screened for Y chromosome microdeletions between 1998 and 2018.

Before recurring to genetic diagnosis, all patients underwent full clinical work-up, with a complete medical history, physical examination, semen and hormonal analysis and scrotal ultrasound to exclude other causes of infertility. Semen analysis was performed according to the WHO guidelines (WHO 2010). Overall, 109 males were classified as obstructive azoospermic, 1035 as non-obstructive azoospermic, 1016 as severe oligozoospermic (sperm concentration $\leq 5x10^6$/mL), 308 as oligozoospermic (5-$20x10^6$/mL). 501 subjects had normal sperm count with low motility, <4% normal morphology or both (asthenoteratozoospermia). For 1031 patients, semen parameters were not available (Table 1). Peripheral blood samples from infertile men with unexplained oligozoospermia, azoospermia and, less often, asthenoteratozoospermia were collected for genetic diagnosis' purposes. Karyotype was performed in all individuals.

Cytogenetic analysis

Chromosome analysis was performed in the cytogenetics laboratory on peripheral blood samples using standard culture protocols and the karyotype was obtained after analysing at least thirty metaphases with G-banding techniques (Rooney & Czepulkowski 1997).

Molecular analysis

Genomic DNA was obtained from peripheral blood lymphocytes using a salting out method. Yq11.2-AZF microdeletions were detected through routine molecular diagnosis following the EAA/EMQN guidelines (Krausz *et al.* 2014). Y-microdeletion screening was done by multiplex PCR using the following sequence-tagged sites (STS): AZFa: sY84, USP9Y (DFFRY) and DDX3Y (DBY), AZFb: sY134, EIFIAY, sY135 and sY142; AZFc: sY1197, sY1192, BPY2, sY152, sY254, DAZ1, sY1291, CDY1, sY157, sY1201 and sY1206. The markers used were combined in four different multiplex-PCR

and amplicons were separated and detected by capillary electrophoresis (QIAxcel, Qiagen, Hilden, Germany). According to the most updated nomenclature, the Y-microdeletions were denominated as AZFA, AZFb (P5/proximal P1), AZFb+c (P5/distal P1 or P4/distal P1), AZFc (b2/b4) and Partial AZFc (DAZ1/2 or gr/gr) (Figure 1). Genomic DNA samples of a fertile man and a woman were used, respectively, as positive and negative controls in each multiplex PCR reaction. Multiplex PCR conditions were as follows: 35 cycles with a pre-soak for 3 min at 94°C, denaturation for 1 min at 94°C, annealing for 1 min (specific annealing temperature for each multiplex primer mix), polymerization for 1 min at 72°C, and final extension for 30 min at 60°C.

CNV67 screening analysis

Patients Selection

In 2017, a selected sample of 100 Portuguese idiopathic infertile men, with different sperm counts (Costa *et al.* 2017), from our 4000 cases database, was screened for CNV67 deletion. From the most recent cases of our 4000 patients, another subset of 100 strictly idiopathic infertile men with severe oligozoospermia ($<5x10^6$/ml) or azoospermia were randomly selected to be submitted to a CNV67 screening analysis. A woman and a healthy fertile man were used as normal controls. The exclusion criteria were abnormal karyotype and known obstructive cause for azoospermia.

Overall, 200 idiopathic infertile male patients, with different grades of spermatogenic failure, were screened for CNV67 deletion (Table 1).

Quantitative PCR (qPCR)

qPCR assays were performed to quantify the number of CNV67 copies, using a TaqMan probe (Hs03323870_cn, Applied Biosystems). Reactions were performed in triplicate under controlled conditions recommended by the manufacturer.

Data analysis

The number of CNV67 copies was determined using Applied Biosystems Copy Caller Software v2.0. In male samples, a predicted copy number higher than 1 was regarded as a copy number gain, while a predicted copy number of 0 represented a copy number loss.

This study was approved by the local Ethical Committees of the Faculty of Medicine of University of Porto/ Centro Hospitalar S. João. Informed consent was obtained from all patients involved in the study during their reproductive medical work-up.

Results

Routine diagnostic analysis

Karyotype anomalies were present in 263 patients (6.6%). Klinefelter syndrome (47,XXY) represented the most frequent karyotype anomaly, present in a total of 113 cases (2.8%), 92 of which were azoospermic, 6 severe oligozoospermic, 2 normozoospermic and 13 had no phenotype classification (details on Table 2). Abnormal karyotype was found in 13.9% of azoospermic males (144 out of 1035) and in 4.2% of oligozoospermic males (55/1324). Klinefelter's syndrome and 47,XXY mosaics were observed in 9.6% (98 cases) of our azoospermic population (n=1035).

Among the 4000 patients, the total prevalence of Yq microdeletions was 4.6% (185 cases).

In the selected group with normal karyotype (n=3737), Yq microdeletions were present in 4.0% (148 cases). Using AZF specific STS analysis, we identified 7 cases of AZFa deletions (rate of per type of microdeletion - 4.7%), 6 cases of AZFb deletions (4.0% - 1 complete AZFb deletion and 5 partial AZFb + DAZ1/2 deletion), 3 cases of AZFa+b deletions (2.0%), 13 cases of AZFb+c deletions (8.8%), 84 cases of complete AZFc deletions (56.8%) and 35 cases of partial AZFc deletions (23.6%).

The remaining 37 Y microdeletion cases were found in men with abnormal karyotypes (14.1%). In the Klinefelter syndrome subset, we identified 1 partial AZFc deletion (0.8%).

Genotype-phenotype correlation in Y microdeletions

In the selected group with normal karyotype (n=3737), 108 men had obstructive azoospermia, 893 men presented with non-obstructive azoospermia, 971 men had severe oligozoospermia, 299 men had moderate or mild oligozoospermia, 489 men were normozoospermic and 977 cases had no phenotype classification.

All patients with AZFa+b and AZFa deletion were azoospermic. Half of the patients with AZFb deletion presented azoospermia, the other half was severe oligozoospermic (partial AZFb+DAZ1/2). All patients with AZFb+c deletion presented azoospermia.

In the complete AZFc deletion group (n=84), 48 subjects were azoospermic (57.1%), 31 subjects were severe oligozoospermic (36.9%) and the remaining had no sperm count information. A partial AZFc deletion (DAZ1/2 or gr/gr) was found in 35 patients, of

which 16 were azoospermic, 10 severe oligozoospermic, 1 oligozoospermic, 2 normozoospermic and 6 with no phenotype classification (Table 3).

Summarizing, a total of 90 deletions (10.1%) were found in the azoospermic group (n=893), 44 deletions (4.5%) were found in the severe oligozoospermic group (n=971), 1 AZFc partial deletion (0.3%) was found in the mild-moderate oligozoospermic group (n=299) and 2 AZFc partial deletions (0.4%) were found in the normozoospermic group (n=489).

Complete and partial AZFc deletions represented 80.4% of the Yq microdeletions, with phenotypes ranging from azoospermia (the majority of complete deletions) to normozoospermia.

CNV67 Screening Analysis

From the 200 male samples screened for CNV67 deletion with different sperm count phenotypes, as described in table 2, 197 individuals had 1 copy (no deletion), 1 individual had 1.5 copies and 2 individuals had 0 copies (CNV67 deletion) when compared to the healthy male and female controls. Of the 2 individuals who were found to carry this deletion, 1 presented with azoospermia in a clinical context of SCOS (diagnosed after testicular biopsy) and 1 with severe oligozoospermia ($2x10^6$ sperm/mL).

Discussion

This study presents one of the largest samples of infertile men worldwide with the main purpose of correlating the Yq microdeletions with sperm count phenotypes.

Karyotype analysis and Yq microdeletion screening became the two routine genetic tests performed during the infertility workup of severe oligozoospermic and azoospermic males (Krausz 2011).

The karyotype analysis is recommended in men with quantitative spermatogenic disturbances. In 2002, Vincent *et al.* estimated 4% of moderate oligozoospermia ($<10x10^6$ spermatozoa/ml), 9-10% of severe oligozoospermia and 15-16% of non-obstrutive azoospermia cases to be associated with some chromosomal abnormality (Vincent *et al.* 2002). A previous review of pooled data from 11 surveys of 9766 infertile men with sperm count alterations found a 5.8% incidence of chromosomal abnormalities, in which sex chromosome anomalies were predominant with an incidence of 4.2% and autosome anomalies had a frequency of 1.5% (Johnson 1998). In our study, we found a 6.6% incidence of chromosome abnormalities, of which Klinefelter syndrome (47,XXY) represented the most frequent karyotype anomaly, present in 2.8% of cases. If the analyses are limited to samples with known sperm count alteration, we have a 8.1% percentage of chromosome anomalies (199 cases in 2468 known oligo/azoospermic patients) and a 4.0% percentage of Klinefelter's syndrome (98 cases). In our sample, 13.9% of azoospermic males and 4.2% of oligozoospermic males have an abnormal karyotype. These findings are supported by other review of the cumulative data from cytogenetic studies where 13.7% of azoospermic males and 4.6% of oligozoospermic males have an abnormal karyotype (Van Assche *et al.* 1996). The percentage of chromosomal abnormalities increases as the sperm count decreases. Klinefelter's syndrome and 47,XXY mosaics were previously estimated in 10.8% of the azoospermic males (Van Assche *et al.* 1996). In our study, Klinefelter's syndrome and 47,XXY mosaics were observed in 9.6% of our azoospermic population. The differences between our study and previous ones may lay in the different population origins and sizes and selection criteria. In the American Urological Association and the European Academy of Andrology guidelines, the karyotype analysis is recommended in all men with a total motile sperm count $<5x10^6$ spermatozoa and non-obstructive azoospermia (AUA/ASRM 2006). Other guidelines suggest karyotype analysis in men

with a total motile sperm count <$10x10^6$ spermatozoa and in couples who have not succeed in achieving a pregnancy after one year of unprotected sexual relations (Foresta *et al.* 2002). Our study could be further improved if we have had complete clinical data and more information about all the genetic tests performed during the diagnostic workup in each patient.

Indications for Yq microdeletion analysis are usually based on sperm count and include azoospermia and severe oligozoospermia (Krausz 2011, Krausz *et al.* 2014). The literature reveals most of the clinical relevant deletions are found in patients with azoospermia or severe oligozoospermia with less than $2x10^6$ spermatozoa/ml. Rarely, deletions can be found in patients with a sperm concentration of $2\text{-}5x10^6$ spermatozoa/ml (Johnson *et al.* 2018). Deletions in patients with a sperm concentration of $5\text{-}20x10^6$ spermatozoa/ml are extremely rare (<1%) (Krausz 2011, Lo Giacco *et al.* 2013). According to EAA/EMQN best practice guidelines (Krausz *et al.* 2014), the main reasons for AZF deletion screening are infertile males without chromosomal abnormalities, obstructive azoospermia, hypogonadotropic hypogonadism nor any other known cause for their impaired sperm production; patients with azoospermia or severe oligozoospermia and diagnosed with varicocele, testicular tumour and after chemotherapy/radiotherapy (because these diagnosis may not fully explain the phenotype or the presence of the deletion may affect the subsequent procedures); azoospermic men who will be submitted to Testicular/Epididymal Sperm Extraction (TESE/ICSI) because this is not recommended in cases of complete AZFa and AZFb deletions. Then, testing for Y microdeletions has not only a diagnostic but also a prognostic value and influences the therapeutic options.

The prevalence of Yq microdeletions in our population was 4.6% (4.0% if the analysis was limited to individuals with normal karyotype), which supports the values described in literature (second cause for male infertility, with a global prevalence of 2-10% in infertile men (Hofherr *et al.* 2011, Johnson *et al.* 2018, Lo Giacco *et al.* 2013, Mascarenhas *et al.* 2016, Tahmasbpour *et al.* 2014, Wosnitzer 2014) and a lower frequency estimated in Europe (around 3%) (Colaco & Modi 2018). Country and population variations seem to be responsible for the different values reported in different studies. The frequency of Yq microdeletions in the normal karyotype group is higher in the azoospermic group (10.1%), followed by the severe oligozoospermic subset (4.5%).

AZF complete deletions are not present in normozoospermic infertile men, however gr/gr deletion, that removes half of AZFc region (Repping *et al.* 2003), can be found in both oligo/azoospermic and normozoospermic carriers (Ferlin *et al.* 2005, Giachini *et al.* 2008). The frequency of partial AZFc deletion in the normal karyotype group was 0.9% (35 cases in 3737 infertile individuals) and 2 AZFc partial deletions (0.4%) were found in the normozoospermic group (2 cases in 489 individuals). The clinical effect of this partial deletion is still controversial because carriers show a wide range of spermatogenic phenotypes. Nevertheless, recent studies present gr/gr deletion with an Y background-dependent effect that significantly varies across different ethnic and geographical populations (Giachini *et al.* 2008, Krausz *et al.* 2014). Furthermore, the loss of *DAZ1*/2 and *CDY1* is common in gr/gr deletion carriers with sperm count abnormalities (Fernandes *et al.* 2002, Giachini *et al.* 2005) and normozoospermic carriers exhibit a lower sperm count compared with non-carriers (Visser *et al.* 2009), even though partial AZFc deletions are not specific for spermatogenic failure. This supports gr/gr deletion as a genetic risk factor for spermatogenic impairment, although the effects on final phenotype are not yet clear (Giachini *et al.* 2005, Navarro-Costa *et al.* 2010, Tuttelmann *et al.* 2007, Visser *et al.* 2009). Although carriers of this partial deletion may naturally father children or be candidates for assisted reproductive techniques, they will transmit it to their male descendants increasing the susceptibility to expand to a complete AZFc deletion through the generations (Zhang *et al.* 2007). To date, no general agreement has been achieved for including this deletion in a routine screening. However, partial deletions may deserve further examination, especially in the populations where partial AZFc deletions are common and prior to assisted reproductive techniques (Giachini *et al.* 2005, Krausz *et al.* 2014).

A high incidence of AZF deletions in Klinefelter patients had been reported in two small studies (Hadjkacem-Loukil *et al.* 2009, Mitra *et al.* 2006), in contrast with other larger studies that have not found AZF deletions in this set of patients (Choe *et al.* 2007, Johnson *et al.* 2018, Rajpert-De Meyts *et al.* 2011, Simoni *et al.* 2008). We just found 1 partial AZFc deletion in the Klinefelter's syndrome subset which supports that there is no association between microdeletions and 47,XXY karyotype and the previous findings reporting a positive association are likely to be methodological artefacts.

In 2017, our group found a 2% prevalence of CNV67 deletion in a sample of 100 Portuguese idiopathic infertile men, with different sperm counts (Costa *et al.* 2017), selected from our 4000 cases database. The percentage found in this Portuguese

population was significantly higher than the 1,1% percentage reported by larger surveys in other populations (Krausz *et al.* 2012, Lo Giacco *et al.* 2014). Our initial aim was to duplicate the number of infertile patients screened for CNV67 to obtain a wider sample and corroborate the findings of Costa *et al* in the Portuguese population and the role of this CNV in male infertility.

After screening the 200 cases, we found a CNV67 deletion frequency of 1% in the studied sample. Even though our sample size was still small, we found a prevalence of this deletion in our population similar to the previous reported by other authors (Krausz *et al.* 2012, Lo Giacco *et al.* 2014). This finding supports the CNV67 deletion percentage found in the international studies and draws our Portuguese population nearer the others, which hypothesises that the higher frequency found in the first study (Costa *et al.* 2017) was due to the small sample size.

CNV67 was exclusively deleted in patients with spermatogenic impairment in our and in the previous studies, showing a consistent genotype-phenotype correlation and a significant prevalence (Costa *et al.* 2017, Krausz *et al.* 2012, Lo Giacco *et al.* 2014). Furthermore, this deletion may remove *MAGEA9B* (melanoma antigen family A, 9B) gene, belonging to the Cancer Testis Antigen (CTA) X-linked family specifically expressed on the male gonads and some tumours (Shen *et al.* 2017), and affects X chromosome reading frame (CXorf40A) and regulatory elements of Heat Shock Transcription Factor Family, X-linked 1/2 (*HSFX1/2*) (Krausz *et al.* 2012, Lo Giacco *et al.* 2014). Bearing this in mind, the question arises whether this deletion is the main responsible for the impaired sperm production or is solely a contributor because of increased genomic instability. Further investigation, with larger sample sizes and focus on CNV67 deletion consequences, is needed in order to clarify how and why CNV67 may affect spermatogenesis and its potential value as an additional genetic test for infertility workup.

This study has some limitations because the clinical data was collected retrospectively and some information was missing.

Conclusion

Although the genetic diagnosis is worldwide recognized and accepted as an essential part of the diagnostic workup of infertile men, this analysis is still limited to a few genetic tests. Many genetic alterations have been identified, some with putative direct or indirect implications in fertility and others with unknown precise effects (Wosnitzer 2014). With tests such as cytogenetic and Yq microdeletions analyses, cystic fibrosis transmembrane conductance regulator (CFTR) gene and congenital hypogonadotropic hypogonadism mutation screenings, half of the cases of male infertility remains idiopathic (Krausz *et al.* 2018). Additional investigations on still unknown factors involved are required to identify the potential contribution of genome alterations in this disorder (Benkhalifa *et al.* 2014), and its short and long-term consequences on the offspring in those who have assisted reproductive techniques as a therapeutic option (Krausz *et al.* 2012).

Genetic alterations like CNV67 deletion are now emerging as potential clinical factors with relevance in male infertility cases previously classified as idiopathic. CNV67 has a significant frequency in oligo-azoospermic population and presents a reliable phenotype correlation, which merits further investigation to clarify its implications on male spermatogenesis.

Karyotype analysis and Y-microdeletions analysis are the first line studies in the genetic workup of infertile men and are of primordial importance because together they clarify more than 10% of the male infertility causes.

Our study analysed a database with 4000 cases of male infertility, studied over 20 years for diagnostic proposes in the Genetic Unit of Faculty of Medicine of University of Porto. The present study has one of the largest samples worldwide for this type of analysis and is the first Portuguese study investigating Y-microdeletions and CNV67 frequencies.

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Authors' Contribution

Ana Pinho, Medical student

Responsible for CNV67 screening and Y patients database organization and analysis.

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Responsible for critical revision and final text approval.

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Responsible for Y chromosome microdeletions screening, AP masterwork supervising, data interpretation, critical discussion and final text approval.

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Legends

Table 1 - Clinical phenotype of the 4000 infertile men and of the CNV67 study population

Table 2 - Genotype-phenotype correlation in patients with abnormal karyotype (n=263)

Table 3 - Summary of Yq microdeletions found (n=3737 for normal karyotype).

Figure 1 – Graphic representation of actual Y-microdeletions denominations on AZFb (P5/proximal-P1), AZFb partial+c (P4/distal P1), AZFb+c (P5/distal-P1), AZFc (b2/b4) and Partial AZFc (DAZ1/2 or gr/gr)

Table 1

Patient's semen phenotype	**n=4000**
Obstructive Azoospermia	109
Non-obstructive Azoospermia	1035
Severe Oligozoospermia (<5x10^6/ mL)	1016
Oligozoospermic (5-20x10^6/mL)	308
Asthenoteratozoospermia	501
Semen parameters not available	1031
CNV67 Patient's semen phenotype	**n=200**
Non-obstructive Azoospermia	82
Severe Oligozoospermia (<5x10^6/ mL)	102
Oligozoospermic (5-20x10^6/mL)	11
Normozoospermic	5

Table 2

	AZS	SOZ	OZ	AT	NA	Sub-total
47,XXY	92	6	0	2	13	113
47,XXY mosaics	6	2	0	0	2	10
Other Karyotype anomalies	46	38	9	9	38	140
Total	**144**	**46**	**9**	**11**	**53**	**263**

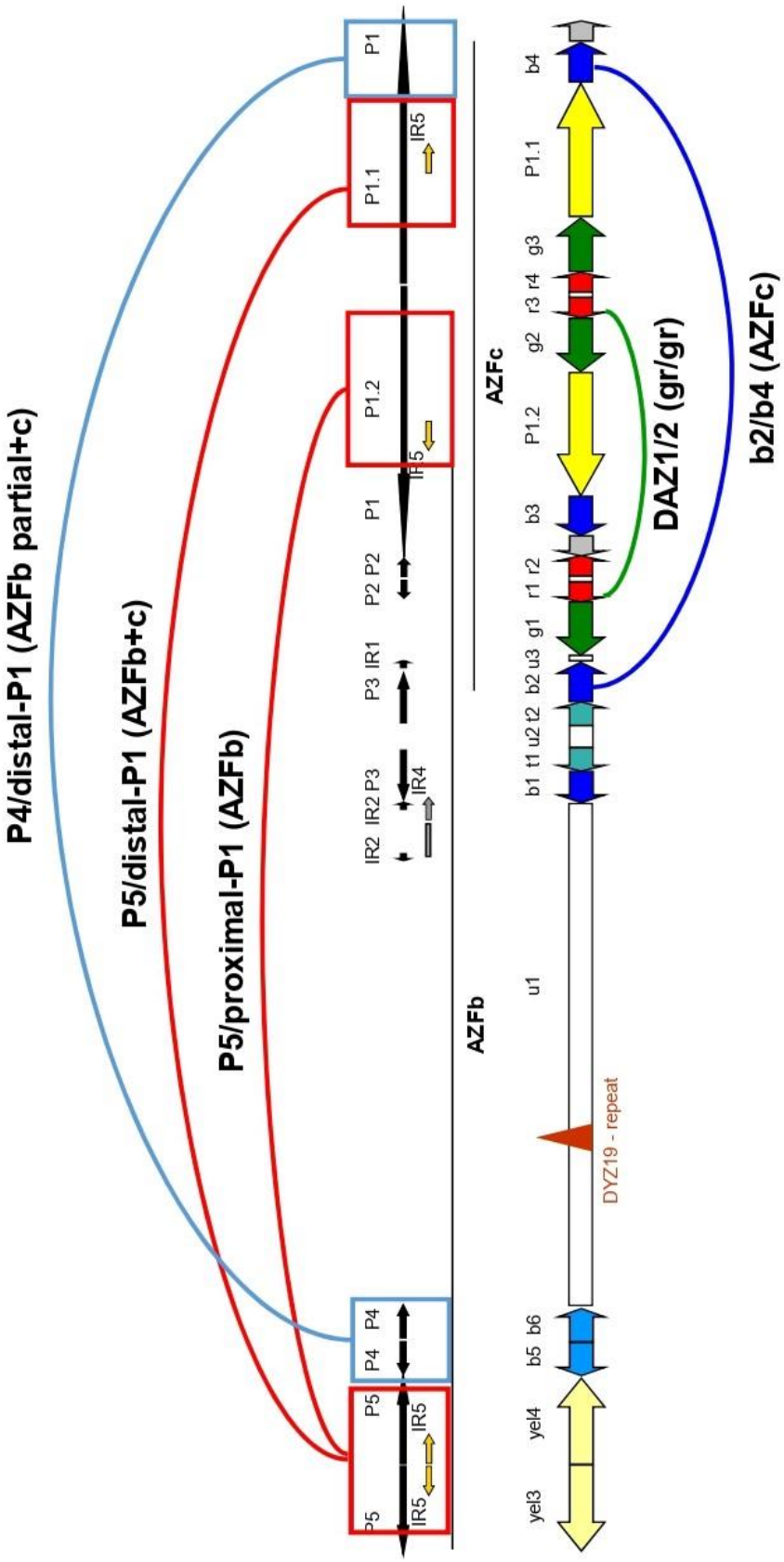
AZS-Azoospermia; SOZ-Severe Oligozoospermia; OZ-Oligozoospermia; AT-Asthenoteratozoospermia; NA-No semen parameters available

Table 3

Y microdeletions	**n=148 (100%)**	**Genotype-phenotype correlation**
AZFa	**7** (4,7%)	7 AZS
AZFa+b	**3** (2.0%)	3 AZS
AZFb	**6** (4.0%)	3 AZS and 3 SOZ
AZFb+c	**13** (8.8%)	13 AZS
AZFc	**84** (56.8%)	48 AZS, 31 SOZ, 5 NA
DAZ1/2	**35** (23.6%)	16 AZS, 10 SOZ, 1 OZ, 2 N, 6 NA

AZS-Azoospermia; SOZ-Severe Oligozoospermia; OZ-Oligozoospermia; N-Normozoospermia;
NA-no semen parameters available

Figure 1


P4/distal-P1 (AZFb partial+c)
P5/distal-P1 (AZFb+c)
P5/proximal-P1 (AZFb)
AZFb
AZFc
DAZ1/2 (gr/gr)
b2/b4 (AZFc)
DYZ19 - repeat
P1
P1.1
P1.2
P2
P3
IR1
IR2
IR4
IR5
P4
P5
b1 t1 u2 t2 b2 u3 g1 r1 r2 b3 g2 r3 r4 g3 P1.2 P1.1 b4
u1
b5 b6 yel4 yel3

Annex

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