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ORIGINAL ARTICLE

Clinical experience with azoospermia: aetiology and chances for spermatozoa detection upon biopsy

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Summary

The clinical workup of the infertile male with azoospermia aims at determining the aetiology and estimating the chances of finding spermatozoa by testicular sperm extraction (TESE). To establish prognostic criteria, 1583 consecutive patients with azoospermia consulting the Centre of Reproductive Medicine and Andrology, Münster, a tertiary referral centre, between 1976 and 2009 comprising 9.8% of all patients providing a semen sample were included in this retrospective analysis. The frequencies of diagnoses were as follows: 21% genetic causes (14% Klinefelter syndrome, 1% other chromosomal aberrations, 2% Y-chromosomal microdeletions, 1% hypogonadotropic hypogonadism, 3% congenital bilateral absence of the vas deferens), 31% current or former maldescended testes, varicocele, urogenital infections, 15% malignancies, 11% obstructions, 7% endocrine or other chronic diseases and 12% idiopathic azoospermia. Receiver-operating characteristic curves for chances of finding spermatozoa by testicular biopsy were calculated for testicular volume, serum follicle-stimulating hormone (FSH) and the seminal markers α-glucosidase, fructose and zinc where these data were available ($N = 283$). Histograms of the seminal markers comparing data from men with obstructive azoospermia and normozoospermia visualize their discriminating power. Evidence-based threshold values for high chances of positive testicular biopsy serving as surrogate marker for TESE were derived from the subgroup of men with obstructive azoospermia for testicular volume (≥21 mL), FSH (≤10 U/L) and seminal α-glucosidase (≤18 mU/ejaculate). Fructose and zinc could not predict the chances of finding spermatozoa upon biopsy. Based on these three parameters, positive biopsy and presumably TESE success can be quickly and reliably estimated in everyday practice with the colour-coded figures constructed from these data. As a seminal α-glucosidase reference limit of 18 mU/ejaculate can also be used to diagnose congenital bilateral absence of the vas deferens, α-glucosidase (rather than seminal fructose) should be determined as part of the clinical routine when counselling patients before testicular biopsy.

Introduction

Azoospermia, defined as the complete absence of spermatozoa in the ejaculate [World Health Organization (WHO), 1987, 1992, 1999)], is found in 5–15% of infertile men (Jarow *et al.*, 1989; Thonneau *et al.*, 1991; Mazzilli *et al.*, 2000). Identifying the underlying aetiology of azoospermia and predicting the chances of finding spermatozoa by testicular sperm extraction (TESE) are essential for counselling patients desiring paternity. However, predictions are often unreliable and a final answer can frequently only be achieved by testicular biopsy (Schlegel, 2004). Based on a large group of azoospermic patients, the aims of this study were to provide an aetiological classification of azoospermia contributing to understanding the pathophysiology and to evaluate clinical, hormonal and

biochemical parameters to predict the chances for sperm retrieval from biopsies. We retrospectively investigated 1583 azoospermic patients of a period of over 30 years who underwent a complete clinical workup including medical history, physical examination and semen and hormone analysis. A subgroup of 453 of them underwent testicular biopsies. Here, we show that the measurement of seminal α-glucosidase may improve the prognostic value of testicular volume and follicle-stimulating hormone (FSH) for finding spermatozoa in testicular biopsies and consequently TESE success.

Materials and methods

Study population

The Centre of Reproductive Medicine and Andrology (former Institute of Reproductive Medicine) is a tertiary referral centre for male infertility and hypogonadism. From the electronic database and patient record Androbase (Tüttelmann *et al.*, 2006), of all patients consecutively consulting from November 1976 to May 2009 who provided at least one semen sample ($N = 16\,161$), those with azoospermia ($N = 1583$, 9.8%) were selected retrospectively. Infertile patients with normozoospermia according to WHO (1987, 1992, 1999) consulting for couple infertility ($N = 212$) where all clinical parameters were available were chosen for comparison. All subjects gave written informed consent for scientific evaluation of their clinical data. Scrotal contents had been investigated by ultrasonography since 1988 (available for 86% of patients) by applying a high-frequency 7.5-MHz convex scanner. Testicular volumes were calculated for each side using the ellipsoid method (Behre *et al.*, 1989) and totalled as bi-testicular volume. Seven men with primary hypogonadism (19%), 29 with Klinefelter syndrome (13%) and 2 with hypogonadotropic hypogonadism (6%) were on testosterone replacement therapy and 2 of the latter received gonadotropins (6%) upon first evaluation. Diagnoses were assigned after complete workup by trained andrologists.

Semen and hormone analyses and testicular histology

Azoospermia was defined as no spermatozoa in both replicates of 1 : 10-diluted semen in the central grid of a Neubauer chamber, followed by centrifugation of undiluted semen at 1000 ***g*** for 10 min and finding no spermatozoa in two 10-μL aliquots of the pellet. Seminal plasma markers were analysed in sperm-free fluid obtained by centrifuging semen at 1000 ***g*** for 15 min. Semen volume was measured in a graduated collection vessel to 0.1 mL accuracy. Neutral α-glucosidase, fructose and zinc were measured by multi-well spectrophotometric assays (WHO, 1987, 1992, 1999; Cooper *et al.*, 1990a,b).

Serum concentrations of luteinizing hormone (LH), FSH and sex hormone-binding globulin (SHBG) were included after March 1985 (available for 95% of patients), when the method for gonadotropin determination changed from radioimmunoassay (RIA) to immunofluorometric assays (Autodelfia; Perkin Elmer, Freiburg, Germany). Normal ranges for LH and FSH in our laboratory are 2–10 and 1–7 U/L, respectively. Serum testosterone (T) was measured over this period with different assays (RIA, luminescence immunoassay, enzyme-linked immunoassay) without changes in normal ranges (>12 nmol/L). Free testosterone was calculated from total testosterone and SHBG concentrations (Vermeulen *et al.*, 1999).

Bilateral and multilocular (at least two sites per testis) testicular biopsies were performed in 453 patients after scrotal incision and preparation of the tunica albuginea of the testis (Bergmann & Kliesch, 2009). Each biopsy sample (about 5 mm in length and 2 mm in diameter) was immediately fixed in Bouin's solution for 10–24 h and then washed in 70% alcohol. Paraffin sections of 4–5 μm were stained with haematoxylin–eosin and periodic acid-Schiff. Histological analysis included the evaluation of all individual tubules of each side, noting the number of tubules with elongated spermatids. Indication for testicular biopsy was infertility and thus the biopsy was routinely combined with TESE procedure ($N = 284$, 63%) and cryopreservation of testicular samples. In some cases, merely diagnostic biopsies ($N = 169$, 37%) were performed, for example, because of altered ultrasound findings such as microlithiasis or former contralateral cancer. Histology was considered positive if at least one tubule contained elongated spermatids and TESE was taken as successful if at least one spermatozoon was found.

Genetic analyses

Karyotype analysis was performed when Klinefelter syndrome was suspected clinically ($N = 644$). The cystic fibrosis (CF) transmembrane conductance regulator (*CFTR*) gene was analysed if congenital bilateral absence of the vas deferens (CBAVD) was assumed ($N = 151$). Y-chromosome microdeletion screening was performed in all patients visiting after 1996 who provided a DNA sample ($N = 1039$).

Statistical analysis

Comparisons among groups were carried out using the non-parametric Kruskal–Wallis test followed by Dunn's multiple comparison post hoc test. As multiple comparisons for the clinical parameters were performed, p-level (two-sided) was set as 0.01. Frequencies were compared by the Chi-squared test and p-values < 0.05 were

considered statistically significant. All calculations including receiver-operating characteristic (ROC) curves were performed with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

Results

Cohort description

The distribution of main diagnoses of the 1583 azoospermic patients is shown in Table 1. The diagnosis 'idiopathic azoospermia' was applied when no explanation for azoospermia could be found (12.3%). Maldescended testes (16.7%), varicoceles (10.3%) and infections of the genital tract (10.3%) were found frequently. A total of 225 patients with Klinefelter syndrome (14.2%) and 9 XX males, together with some rare structural chromosomal anomalies, constituted the group with chromosomal aberrations (15.4%) as a frequent cause of azoospermia. The diagnosis of primary hypogonadism was provided when low testosterone (<8 nmol/L) in conjunction with specific clinical symptoms of androgen deficiency was found. Oncological diseases were present in 15.0%, with non-testicular tumours slightly more frequent than testicular tumours (8.1% and 6.8%, respectively). Of the azoospermic patients with malignancies, 40.1% (95/237) consulted our clinic for cryopreservation of spermatozoa before treatment. Altogether, 179 men (11.3%) suffered from obstruction of their excurrent ducts with 94 (5.9%) resulting from vasectomy; 53 men (3.3%) had proven mutations of the *CFTR* gene and either CF with CBAVD or CBAVD alone; 32 additional men had other (e.g. surgically) proven obstructions; 7.3% had chronic diseases (e.g. gynaecomastia, diabetes) and regularly took medication which might have been cofactors for their infertility. Secondary (hypogonadotropic) hypogonadism (2.0%) – including Kallmann syndrome and isolated hypogonadotropic hypogonadism (IHH) – and Y-chromosomal deletions (1.7%) were the least frequent causes of azoospermia. Details of patients with deletions of the azoospermia factors (*AZF*) have been reported recently (Simoni *et al.*, 2008). Patients with a definite genetic cause of azoospermia (chromosomal, CF/CBAVD, *AZF* deletion or Kallmann syndrome/IHH) constituted 21.4% (339) of the cohort.

Table 1 Distribution of diagnoses of 1583 men with azoospermia with genetic diagnoses highlighted (in grey)

Clinical diagnosis	Percentage[a] (total number/histology available)
General andrological diagnosis[b]	31.0 (491/160)
Maldescended testis/testes	16.7 (264/97)
Varicocele	10.3 (163/33)
Urogenital infections	10.3 (163/42)
Chromosomal aberration	15.4 (244/45)
Klinefelter syndrome (47,XXY)	14.2 (225/42)
XX male	0.6 (9/1)
Translocation	0.3 (5/1)
Other	0.3 (5/1)
Malignancy	15.0 (237/44)
Non-testicular (lymphoma, leukaemia, sarcoma)	8.1 (129/13)
Before gonadotoxic treatment	2.0 (31/2)
After treatment	6.2 (98/11)
Testicular tumour	6.8 (108/31)
Before gonadotoxic treatment	4.0 (64/22)
After treatment	2.8 (44/9)
Idiopathic azoospermia (no cause identifiable)	12.3 (195/81)
Obstruction	11.3 (179/58)
Vasectomy	5.9 (94/27)
CF, CBAVD	3.3 (53/27)
Other	2.0 (32/4)
Endocrine or other chronic disease (e.g. diabetes)	7.3 (115/31)
Primary hypogonadism	2.3 (36/11)
Secondary (hypogonadotropic) hypogonadism	2.0 (32/2)
Kallmann syndrome	0.5 (8/0)
Isolated hypogonadotropic hypogonadism	0.4 (7/1)
Pituitary insufficiency after surgery	0.4 (6/0)
Other	0.7 (11/1)
Y-chromosomal deletion	1.7 (27/17)
AZFa	0.1 (2/2)
AZFb	0.1 (2/2)
AZFc	1.2 (19/11)
Other	0.3 (4/2)
Other	1.7 (27/4)

[a]All frequencies in relation to complete cohort; [b]combinations of diagnoses possible.

CF, cystic fibrosis; CBAVD, congenital bilateral absence of the vas deferens; *AZF*, azoospermia factor.

Prediction of spermatozoa detection upon biopsy

When positive histology (at least one tubule with elongated spermatids) and successful TESE were compared, 84% of cases with both information available ($N = 284$) were in agreement (both negative in 78 and both positive in 161 cases); 9% and 7% had discrepant findings with either only positive histology or only successful TESE. As the majority of cases were in agreement, testicular histology was further evaluated, of which a larger amount of cases were available, and TESE success is used synonymously.

Testicular histology, testicular volumes, FSH and the seminal markers α-glucosidase, fructose and zinc of 283 patients without genetic and oncological causes of azoospermia were available and used to construct ROC curves. The predictive power of testicular volume [area under curve (AUC): 0.68, $p < 0.0001$] was comparable with that of FSH (AUC: 0.71, $p < 0.0001$) for finding spermatozoa by testicular biopsy (Fig. 1A). Of the seminal markers

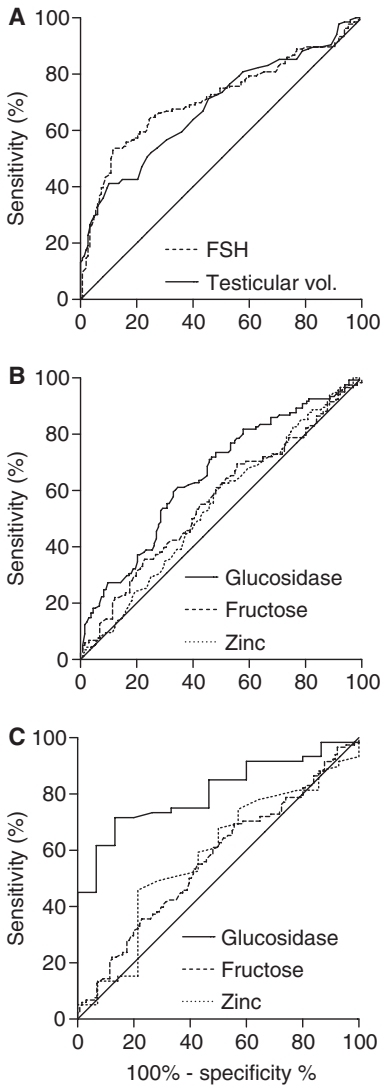

Figure 1 Receiver-operating characteristic curves of serum follicle-stimulating hormone (FSH) and testicular volume (A), seminal α-glucosidase, fructose and zinc (B) in azoospermic men with testicular histology available ($N = 283$) and seminal markers only in men with serum FSH ≤ 10 U/L ($N = 90$; C).

(Fig. 1B), only α-glucosidase could significantly predict spermatozoa (AUC: 0.65, $p < 0.0001$), in contrast to fructose (AUC: 0.56, $p = 0.08$) and zinc (AUC: 0.54, $p = 0.22$). In addition, the frequency distributions (histograms) of the seminal markers comparing data from men with obstructive azoospermia (vasectomized men and men with CBAVD) and normozoospermia show that only α-glucosidase has discriminative power with only small overlap of the cohorts (Fig. 2), whereas low seminal fructose is specific for CBAVD (Fig. 2B).

As recommended for setting one-sided reference limits in clinical chemistry (Solberg, 2004), the 5th percentile of testicular volume, corresponding to 21 mL, and the 95th percentile of FSH (10 U/L) in men with proven obstructive azoospermia ($N = 179$) were chosen as thresholds for a high likelihood of spermatogenesis present in the testis. When seminal markers were re-evaluated in men whose parameters were within these thresholds ($N = 90$; Fig. 1C), the predictive value of α-glucosidase increased significantly (AUC: 0.81, $p < 0.001$), whereas fructose and zinc still had no predictive power (AUC: 0.59, $p = 0.31$; AUC: 0.58, $p = 0.36$, respectively). In this subgroup, α-glucosidase had a 100% specificity to predict spermatozoa up to a threshold of 18 mU/ejaculate derived from the ROC curve, which was close to the 95th percentile calculated from men with obstructive azoospermia (22 mU/ejaculate).

The thresholds for testicular volume, FSH and α-glucosidase were also tested for patients with other diagnoses, but only one patient with a translocation, one with Klinefelter syndrome, three after chemotherapeutic treatment of a testicular tumour and two of other malignancies, four with secondary hypogonadism and two with *AZFc* deletions fulfilled these criteria. Histology was only available for the Klinefelter man with spermatozoa in his biopsy and who was actually diagnosed as having low-grade mosaicism (47,XXY[1]/46,XY[29]). Of 79 patients, 77 could be correctly predicted as having spermatozoa in their biopsy (specificity 99%) independent of their diagnoses, albeit with a low sensitivity of 38% (129 of 342 men not fulfilling the criteria also had spermatozoa). Interestingly, 95% of patients with CBAVD (36/38) also had α-glucosidase levels ≤18 mU/ejaculate.

For practical use, when counselling patients, colour-coded tables depicting the chances of finding spermatozoa in testicular biopsies were constructed from the same cohort described before with testicular histology available ($N = 283$), using the established threshold values and adding some thresholds for testicular volume and FSH. According to Fig. 3A, the chances for a single patient can be well classified as high (>95% chance), medium or low (<5%) if testicular volume and FSH are known. If the α-glucosidase cut-off value of 18 mU/ejaculate is applied in addition (Fig. 3B), the probability of finding spermatozoa increases for patients with higher testicular volume and lower FSH levels, and decreases for those with lower testicular volume and higher FSH levels, demonstrating that the predictive value of these two parameters is sharpened by α-glucosidase measurement. Therefore, adding α-glucosidase increases the accuracy of prediction, providing an additional, evidence-based tool useful in patient counselling.

Discussion

This study, based on the largest cohort of azoospermic patients reported so far, showed that azoospermia is

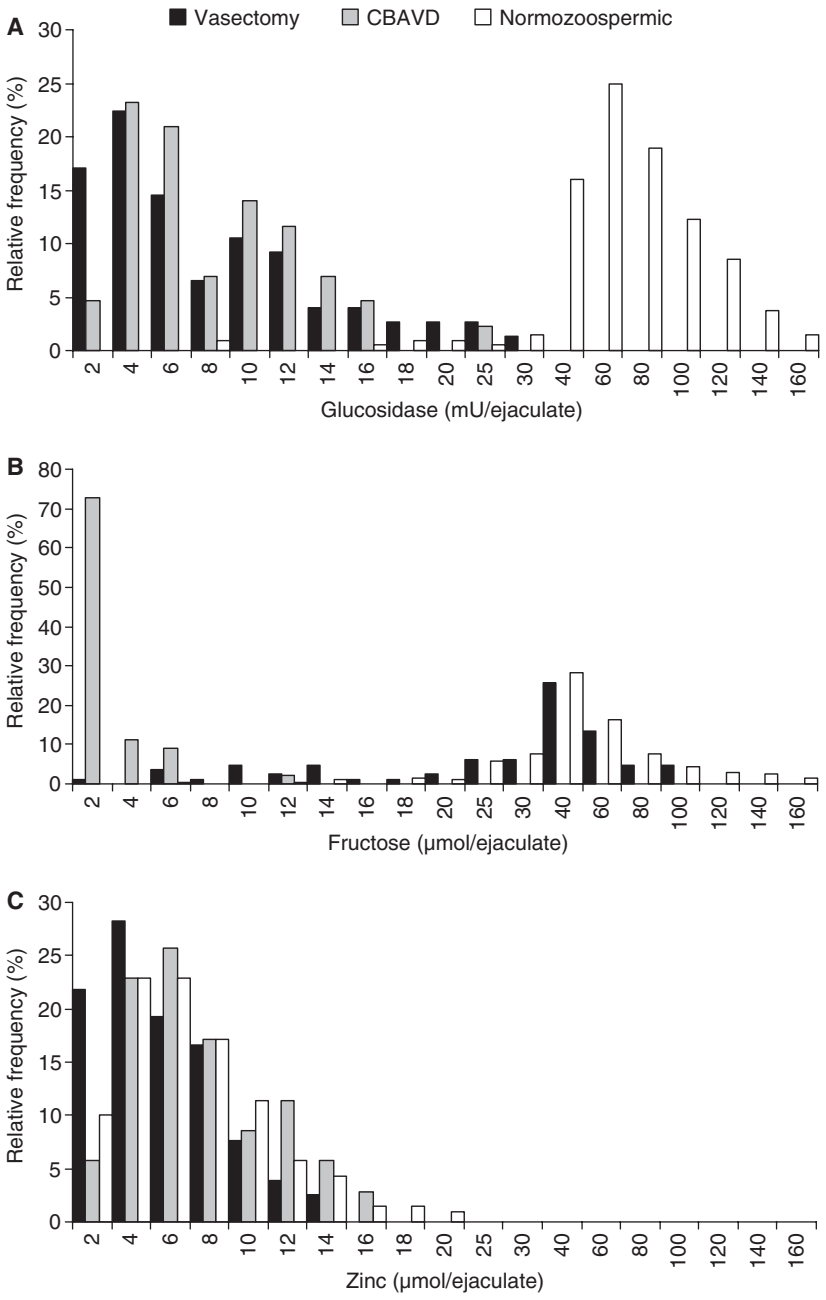

Figure 2 Histograms of seminal α-glucosidase (A), fructose (B) and zinc (C) of men after vasectomy (N = 94), with congenital bilateral absence of the vas deferens (N = 53) and with normozoospermia (N = 212).

present in about 10% of men consulting for infertility, a value in concordance with previous reports (Jarow *et al.*, 1989). The aetiology of azoospermia in this cohort is essentially comparable with that reported earlier by two smaller studies based on 100 and 55 azoospermic men (Fedder *et al.*, 2004; Fogle *et al.*, 2006) and also reflects the specific scope of our centre with a high percentage of Klinefelter patients and endocrine diseases. Klinefelter syndrome was highly prevalent (14.2%), whereas 1.2% of patients had other chromosomal aberrations, consistent with the literature (Van Assche *et al.*, 1996; Vincent *et al.*, 2002). Details of the majority of the Klinefelter patients and XX males have been given elsewhere (Kamischke *et al.*, 2003; Lanfranco *et al.*, 2004; Vorona *et al.*, 2007). With 0.6% compared with >1% in other studies (Van Assche *et al.*, 1996; Vincent *et al.*, 2002), autosomal aberrations may be underrepresented because of selection bias, as karyotyping was mainly performed if Klinefelter syndrome was suspected.

One cause of obstructive azoospermia is CF which results in CBAVD (Popli & Stewart, 2007) and minor mutations of the *CFTR* gene may cause isolated CBAVD in otherwise

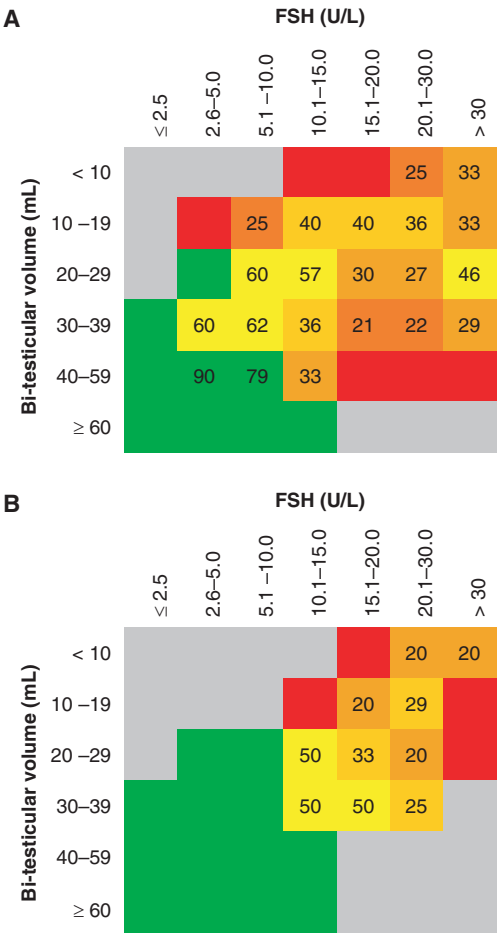

Figure 3 Chance of finding elongated spermatids upon testicular biopsy in all patients (A; *N* = 283) and only in men with seminal α-glucosidase levels ≤18 mU/ejaculate (B; *N* = 75) colour coded and given as percentage. Green squares without number have a calculated chance of 100%, red squares of 0%; for practical reasons, these should be considered as >95% and <5% chances, respectively. Grey squares depict data not available.

healthy men. CBAVD is characterized by obstructive azoospermia and low ejaculate volume, seminal plasma pH, α-glucosidase and fructose (von Eckardstein *et al.*, 2000). As confirmed by this study, low values of these parameters should lead to the suspicion of CBAVD and screening for mutations of the *CFTR* gene in such men. Given the good prognosis for TESE/ICSI (intracytoplasmic sperm injection), *CFTR* sequencing is essential because the offspring is at high risk of developing the severe form of CF if the female partner is also a mutation carrier.

Microdeletions of the Y-chromosome are found in up to, or more than, 10%, depending on the study populations and methods (Simoni *et al.*, 2008). The prevalence in our complete cohort was below 2%, but would be calculated as 4–5% if other causes of azoospermia had been excluded, as in other studies. Details of these men have been given elsewhere (Simoni *et al.*, 2008).

In total, a genetic cause for azoospermia was found in about 21% of cases, which is a high proportion but still probably an underestimation, as genetic analyses were not performed in all azoospermic men, mainly because tests were unavailable at earlier times. The lack of genetic analyses in several cases is a limitation for the evaluations concerning the biopsies as some patients considered 'idiopathic' may well carry genetic causes making this group heterogeneous. However, with our retrospective design, additional genetic analyses were not possible, but, in contrast, even the group of patients analysed genetically remains heterogeneous until more – probably genetic – causes are established in the future.

Being informed about the diagnosis of azoospermia is regularly very frustrating for infertile patients, who will then be counselled about the possibility of a testicular biopsy, usually in conjunction with TESE, as the only tool to try to achieve paternity. These men, confronted with the decision whether to undergo surgery or not, regularly ask for their chances of success. When the chances are high as, for example, in a man with CBAVD, the patient will be more comfortable to undergo the procedure and most probably a conventional biopsy is sufficient. However, when his chances are low, he might be referred to undergo microsurgical TESE (see next). Currently, no evidence-based algorithm exists to estimate TESE success and to decide when to opt for microsurgical TESE. As the major outcome of this study, parameters predicting the chance of finding spermatozoa by testicular biopsy, which is a good surrogate marker for TESE success, were identified. A subgroup of patients presented herein has been previously analysed, but selection and scope were different (Zitzmann *et al.*, 2006). Testicular volume or FSH alone is not sufficient as predictors for finding spermatozoa upon TESE and, as in this study, the concordance of positive histology and successful TESE is high (Zitzmann *et al.*, 2006). However, the diagnostic value of α-glucosidase in infertility workup remains controversial (Krause & Bohring, 1999). This study predicts high TESE success when using evidence-based thresholds of testicular volume (20 mL), FSH (10 U/L) and seminal α-glucosidase (18 mU/ejaculate) derived from a group of vasectomized men and confirmed by ROC curves calculated from other patients with biopsies. The analyses show a clear chemical-organ system correlation of α-glucosidase with obstructions, that is, high chances of sperm retrieval. Albeit low, the predictive value of fructose might stem from distal obstructions and zinc may be a marker of spermatogenesis (Yamaguchi *et al.*, 2009). The α-glucosidase activity of 18 mU/ejaculate is almost identical to the lower reference

value from fertile men (WHO, 1987, 1992, 1999; Cooper *et al.*, 1991). If these cut-off values are applied to azoospermic patients, these patients are clinically indistinguishable from proven obstructive azoospermic men and can be considered as having a high chance of positive histology/TESE. The chance of finding spermatozoa by testicular biopsy can be predicted almost perfectly (positive predictive value 97%). The sensitivity, however, is low and men not falling into these categories may also have a fair chance of retrieving spermatozoa.

With the advance of microsurgical TESE (Schlegel, 1999), spermatozoa may be found even in Klinefelter patients in up to 50% of cases (Schiff *et al.*, 2005; Yarali *et al.*, 2009). To date, it remains unclear whether microsurgical TESE is favourable in any or only selected cases (Donoso *et al.*, 2007; Wald *et al.*, 2007), but has definitely been shown to yield better sperm retrieval rates when FSH is high (Colpi *et al.*, 2009; Ramasamy *et al.*, 2009). So far, no reliable marker(s) has (have) been described that are able to predict TESE success in these primarily 'low chance' patients (small testes, high FSH): for example, inhibin B is not established for clinical routine (von Eckardstein *et al.*, 1999), which is also the reason why it is not currently measured in our patients and not available for evaluation in this study. Applying fine needle aspiration mapping (Turek *et al.*, 2000), flow cytometry (Yeung *et al.*, 2007) or measuring serum antisperm antibodies (Lee *et al.*, 2009) and new seminal markers (Heshmat *et al.*, 2008; Roshdy & Mostafa, 2009) offer new approaches to solve this problem.

Our goal was to establish evidence-based criteria to be used when counselling patients about their chances of positive testicular biopsy and TESE. As microsurgical TESE has only been practised in recent years, it could not be evaluated in this retrospective study. The colour-coded figures presented here (Fig. 3) give an accurate estimate and require only parameters which are regularly measured in the workup of male infertility, that is, testicular volume and serum FSH. We show that the accuracy of the prediction can be further improved if seminal α-glucosidase is included. A prospective study applying these thresholds is now warranted to prove their usefulness under clinical conditions. Especially in men without an established cause for their azoospermia (patients with genetic and oncologic causes were excluded from this analysis), the three parameters in combination may serve as an accurate tool to predict TESE success. Patients with primarily low chances should, however, not be counselled against testicular biopsy, but instead be referred to a specialized centre performing microdissection (mTESE) increasing their chances for sperm retrieval (see before). Aside from this, the α-glucosidase cut-off of 18 mU/ejaculate predicted CBAVD in over 95% of cases, making it an almost perfect diagnostic criterion and rendering fructose determination superfluous for this purpose.

Conclusions

The prevalence of azoospermia in patients consulting our tertiary referral centre is high, around 10%. Genetic causes can be found in 21% of these patients, underlining the need for thorough genetic workup depending on the clinical parameters. The evidence-based thresholds of testicular volume and serum FSH in conjunction with seminal α-glucosidase discriminate between high and low chances of finding spermatozoa by testicular biopsy. Patients with predicted low chances should not be counselled against a biopsy/TESE, but about the new techniques available for sperm retrieval possibly increasing their chances by using microsurgical TESE procedures. α-glucosidase can also be used to diagnose CBAVD and should be applied in clinical routine in favour of seminal fructose before referring patients to TESE.

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