Age-dependent inhibin B concentration in relation to FSH and semen sample qualities: a study in 2448 men

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Abstract

Inhibin B is an important serum marker of spermatogenesis, whereas sensitivity and predicting power for the spermatogenic situation at several ages are under debate. We performed a retrospective analysis of data from 2448 men who attended our University-based male infertility clinic to evaluate inhibin B in relation to age and semen sample qualities in comparison with FSH. Moreover, the range of inhibin B in 82 nonobstructive azoospermic patients was correlated with the sperm retrieval in testicular sperm extraction procedures. Inhibin B correlated with FSH (Spearman rank correlation (R) = -0.50 ; $P < 0.00001$). Inhibin B and inhibin B/FSH ratio (IFR) showed an inverse U-shaped dependence on age, whereas FSH showed a U-shaped dependence on age (optimum 20–40 years). However, in men with normal spermiograms inhibin B concentrations did not differ between age groups. Their levels of inhibin B amounted to 130.5, 54.5–247 ng/l (median, 10th–90th percentile), and of IFR to 38.3, 12.5–104.8 (median, 10th–90th percentile), which might be taken as the reference range. Using the 10th percentile of IFR, correct classification in normal or pathological semen groups was achieved in 99.1%. The percentage of aniline blue-negative spermatozoa, i.e. mature spermatozoa with protamines, did not correlate with FSH ($P > 0.05$) but with inhibin B ($R = 0.15$, $P < 0.001$). The probability of retrieving testicular spermatozoa decreased with declining inhibin B: < 20 ng/l sperm could never be found. Our results from a large group of men with a wide spectrum of semen qualities allow estimating reference values for inhibin B and IFR. Inhibin B and especially the IFR are more sensitive markers of male infertility than FSH alone.

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Introduction

Reproductive hormones play an essential role in initiating and maintaining male reproductive function. FSH, LH, testosterone and inhibin B are associated with sufficient spermatogenesis and male fertilization potential (Uhler *et al.* 2003, Andersson *et al.* 2004, Meeker *et al.* 2007). Male reproductive processes demand complex regulation between endocrine systems, germ cell production and gonad maturation (de Kretser *et al.* 2002). During recent decades, the inhibin family was the hormone group considered to be relevant to reproduction and was therefore studied intensively and characterized (Meachem *et al.* 2001). The development of specific immunoassays for the biologically active, dimeric glycoprotein form of inhibin (Groome & O'Brien 1993) proved that inhibin B is the only inhibin protein present in male serum (Illingworth *et al.* 1996, Hayes *et al.* 2001). McCullagh (1932) postulated the existence of inhibin as a nonsteroidal substance from the testis capable of affecting pituitary function in 1932. Many years later, the substance was identified as a glycoprotein hormone and a

disulfide-linked dimer of two different subunits, a common α subunit and a β_B subunit denoted inhibin B. It is predominantly produced in the Sertoli cells in the testis and circulates to inhibit pituitary FSH secretion, serving as the testicular feedback signal for FSH (Hayes *et al.* 2001, Boepple *et al.* 2008). Thus, it reflects the level of testicular function as a marker of the functional state of seminiferous epithelium, especially Sertoli cells (Anawalt *et al.* 1996, Mahmoud *et al.* 2000, Anderson 2001, Meachem *et al.* 2001, Fujisawa *et al.* 2004). However, the function and regulation mechanisms with predictive power to competent spermatogenesis are not fully understood. In a group of idiopathic infertile men, FSH alone had slightly higher positive predictive value compared with inhibin B alone in identifying infertility (Andersson *et al.* 2004). On the other hand, inhibin B was more strongly correlated with semen parameters than FSH in another group of infertility patients (Kumanov *et al.* 2006). The heterogeneity of earlier results (Andersson *et al.* 2004, Kumanov *et al.* 2006, Goulis *et al.* 2009) and studies based on relatively small numbers of infertile men (Andersson *et al.* 2004) recommended further investigation of the relationship

among inhibin B, other fertility-related hormones, semen quality, and age in a large group of males. Bailly *et al.* (2003) reported better predictive power for inhibin B than FSH with regard to successful sperm retrieval. Although the results could not be confirmed (Halder *et al.* 2005), a combination of the two hormone parameters for a superior prediction was recommended (Bohring *et al.* 2002). Only one study investigated inhibin B concentrations in a larger but uniform cohort of young healthy men (Jorgensen *et al.* 2010). Therefore, further investigation is required. Our study of a large number of men might improve our understanding of the role of inhibin B in spermatogenesis and hormonal regulation. Moreover, we evaluated whether inhibin B in combination with FSH is a more useful predictor of semen sample quality than FSH alone. We examined: i) the relationship between hormone status, especially inhibin B, age and semen sample quality in 2448 men, ii) the inhibin B concentration in the subpopulation with normal sperm count and iii) the predictive power with regard to sperm retrieval in testicular sperm extraction (TESE).

Results

Relationship of hormones to age and spermiogram parameters

A total of 2448 men were included in this study with a total sperm count (TSC) of 88.0 (4–407) million and a 3.5 (0–33.5) million TSC with normal morphology (TSN (median; 10th and 90th percentiles)). Inhibin B was strongly correlated with FSH ($R = -0.50$; $P < 0.00001$; Fig. 1A). TSC and TSN decreased significantly in subjects < 20 and > 40 years of age ($P < 0.05$; Table 1);

sperm motility did not differ significantly in the computer-assisted motion analysis. The serum concentration of inhibin B and the inhibin B:FSH ratio (IFR) showed an inverse U-shaped dependence on age. The serum concentration of FSH displayed a U-shaped dependence on age with an optimum between 20 and 40 years (Table 1). Possibly late adolescence might have been one factor leading to slightly higher FSH values in men < 20 years. At ages > 30 years, inhibin B was negatively correlated with age ($R = -0.14$; $P < 0.000001$; Fig. 1B). Testosterone showed a similar association with age ($R = -0.17$; $P < 0.00001$).

In the subgroup of men with normal spermiogram parameters ($n = 846$), FSH was significantly elevated in men > 40 -year-old. Concordantly, in the same group of men, inhibin B tended to be lower, but it failed to reach statistical significance (Table 2). It might have been a problem of the relative low number of men > 40 years with normal spermiogram parameter ($n = 69$). However, the correlation with age is less pronounced in inhibin B than in FSH. As expected testosterone declined with age.

Men with normal spermiogram parameters (World Health Organization 1999) allowed us to determine the reference values for inhibin B (130.5; 54.5–247 ng/l) and the IFR (38.3; 12.5–104.8; Table 2). Analysis of the relationships of TSC and TSN with inhibin B, FSH and IFR revealed the strongest correlation between sperm count and IFR, followed by that between inhibin B and FSH (Table 3). The velocity straight line (VSL) and velocity average path (VAP) were significantly correlated with inhibin B only. Interestingly the correlation of TSC and TSN with inhibin B and FSH became stronger with decreasing sperm counts per ejaculate (Table 3).

Figure 1 Relationship between inhibin B and parameters relevant for male fertility (A, B, C and D). (A) Correlation between inhibin B and FSH, $n = 2448$ men, $R = -0.50$; $P < 0.00001$. (B) Correlation between inhibin B and age, $n = 1480$ men at ages > 30 years, $R = -0.14$; $P < 0.000001$. (C) Relationship between inhibin B and mean bilateral Johnsen score, $n = 164$ testicular biopsates of 82 nonobstructive azoospermic patients, $r = 0.53$; $P < 0.0001$. (D) Probability of retrieving spermatozoa in relation to inhibin B concentration, no sperm retrieval at inhibin B below 20 ng/l.

Table 1 Semen sample parameters and serum hormone concentrations (mean $\pm$ s.e.m.; median (10th and 90th percentiles)) of the 2448 men at different periods of age.

Variables	Total (n=2448)	Age $\leq$ 20 years (n=92)	Age >20– $\leq$ 30 years (n=876)	Age >30– $\leq$ 40 years (n=1257)	Age >40– $\leq$ 70 years (n=223)
Inhibin B (ng/l)	117.8 $\pm$ 1.59 105.0 (27.6–219.0)	91.9 $\pm$ 7.33 80.4 (15–188)* [†]	123.6 $\pm$ 2.80 109.5 (30.5–228)* [†]	119.2 $\pm$ 2.19 107.0 (32.3–219)* [†] , [§]	98.6 $\pm$ 4.58 82.2 (17.1–187)* [§] , [§]
FSH (IU/l)	6.4 $\pm$ 0.13 4.6 (2.1–13.0)	9.9 $\pm$ 0.98 6.1 (2.1–23.8)* [†]	6.2 $\pm$ 0.23 4.3 (1.9–12.3)* [†]	6.2 $\pm$ 0.15 4.5 (2.2–11.9)* [†] , [†]	7.5 $\pm$ 0.47 5.4 (2.6–14.6)
Inhibin ratio	18.2 $\pm$ 1.24 22.2 (3.1–81.9)	9.5 $\pm$ 13.0 13.1 (0.7–75.9)* [†]	19.8 $\pm$ 2.6 25.7 (3.7–93.8)* [†]	19.9 $\pm$ 1.2 25.3 (3.5–78.1)* [†]	14.9 $\pm$ 2.33 15.5 (2.1–60.0)* [†]
LH (IU/l)	4.6 $\pm$ 0.12 4.0 (2.0–7.7)	6.0 $\pm$ 0.63 4.7 (2.0–9.8)*	4.9 $\pm$ 0.20 4.2 (2.0–8.3)	4.1 $\pm$ 0.13 3.7 (2.0–6.8)*	4.5 $\pm$ 0.26 3.7 (1.8–7.8)
Testosterone (nmol/l)	15.9 $\pm$ 0.13 15.3 (9.2–23.3)	17.7 $\pm$ 0.72 17.4 (9.5–26.5)* [†]	16.9 $\pm$ 0.23 16.4 (10.2–24.7)* [†]	15.3 $\pm$ 0.17 14.8 (9.0–22.1)* [†] , [†]	15.0 $\pm$ 0.40 14.2 (8.7–22.1)* [†]
Total sperm count (millions)	158.6 $\pm$ 4.0 88.0 (4–407)	96.0 $\pm$ 14.9 30.0 (0–264)* [†] , [†]	165.1 $\pm$ 6.6 96.0 (3.0–434.0)* [†] , [†]	161.9 $\pm$ 5.67 93.3 (4.5–409.5)	140.5 $\pm$ 12.9 78.0 (3.0–350)* [†] , [†]
Total sperm count with normal morphology (millions)	12.1 $\pm$ 0.51 3.5 (0.0–33.5)	6.5 $\pm$ 1.3 1.6 (0–21.5)* [†] , [§]	12.6 $\pm$ 0.78 4.1 (0.0–34.2)* [†] , [†]	12.6 $\pm$ 0.79 3.6 (0–35.9)* [†] , [§]	9.2 $\pm$ 1.2 2.4 (0–28.6)* [†] , [†]
Motile sperm (%)	37.2 $\pm$ 0.55 35.6 (9.9–69.0)	36.3 $\pm$ 2.42 36.6 (11.2–67.6)	36.9 $\pm$ 0.90 34.9 (10.0–69.0)	37.5 $\pm$ 0.79 35.6 (9.9–69.0)	37.5 $\pm$ 2.1 35.6 (8.9–71.2)
VSL (μ m/s)	27.2 $\pm$ 0.32 24.5 (13.3–44.5)	27.1 $\pm$ 1.56 24.4 (12.8–47.9)	27.2 $\pm$ 0.53 24.7 (13.3–43.0)	27.1 $\pm$ 0.44 24.7 (13.6–44.4)	28.1 $\pm$ 1.3 24.9 (13.1–48.5)
VAP (μ m/s)	38.8 $\pm$ 0.40 35.2 (21.1–61.5)	40.2 $\pm$ 2.01 36.0 (23.3–63.5)	38.7 $\pm$ 0.65 35.3 (21.0–60.8)	38.4 $\pm$ 0.54 35.1 (21.1–60.1)	40.4 $\pm$ 1.57 34.8 (21–65.5)

Statistical test: Mann–Whitney *U* test; values with an identical symbol in each line are significantly different (*, [†], [§] $P < 0.001$, [†], [§] $P < 0.05$). VAP, velocity average path; VSL, the velocity straight line.

In all sperm count groups, inhibin B was more strongly correlated with sperm counts than FSH was. Inhibin B ($R = 0.13$), FSH ($R = -0.10$), and especially IFR ($R = 0.15$) correlated significantly with testicular volume ($P < 0.05$).

Use of FSH and inhibin B to diagnose pathological semen characteristics

When using the 10th percentile of inhibin B concentration (54.5 ng/l), the 10th percentile of IFR (12.5), and the 90th percentile of FSH concentration among men with normal spermiograms (>40 million sperm and >6 million sperm with normal morphology per ejaculate), correct classification of men in normal and pathological semen groups was achieved in 94.1% of men for inhibin B, in 99.1% for IFR and in 90.5% for FSH. We found azoospermia in 4.7% of men with inhibin B above our cutoff level of 54.5 ng/l, but in

11.4% of men with FSH below our cutoff level of 6.8 IU/l. These findings indicate a false prediction.

Inhibin B, FSH, IFR and percentage of spermatozoa with mature nuclear protein structure

The nuclear protein structure was examined by aniline blue staining (Dadoune *et al.* 1988). The percentage of aniline blue-negative spermatozoa, i.e. mature spermatozoa with protamines, did not correlate with FSH ($P > 0.05$) but it did correlate significantly with inhibin B ($R = 0.15$, $P < 0.001$; $n = 1314$).

Multiple regression analysis of inhibin B, FSH and IFR to sperm variables

The stepwise multiple regression analyses of the relationship of TSC with the three endocrine parameters inhibin B, FSH, and IFR revealed that the closest

Table 2 Serum hormone concentrations (mean $\pm$ s.e.m.; median (10th and 90th percentiles)) of men with normal routine spermiogram parameters (>40 million sperm and >6 million sperm with normal morphology per ejaculate) at different periods of age.

Variables	Total (n=846)	Age $\leq$ 20 years (n=19)	Age >20– $\leq$ 30 years (n=317)	Age >30– $\leq$ 40 years (n=441)	Age >40– $\leq$ 70 years (n=69)
Inhibin B (ng/l)	143.7 $\pm$ 2.72 130.5 (54.5–247)	142.7 $\pm$ 17.56 146.7 (34.2–195)	143.3 $\pm$ 4.71 127.0 (54.4–248)	145.8 $\pm$ 3.64 132.0 (56.9–247)	132.5 $\pm$ 8.92 123.5 (52.3–231.5)
FSH (IU/l)	4.2 $\pm$ 0.09 3.7 (1.8–6.8)	5.1 $\pm$ 1.15 4.1 (1.8–6.6)	4.3 $\pm$ 0.16 3.6 (1.7–7.4)*	4.1 $\pm$ 0.10 3.7 (1.9–6.6)* [†]	4.8 $\pm$ 0.28 4.4 (2.4–7.6)* [†]
Inhibin B/FSH ratio	36.4 $\pm$ 2.20 38.3 (12.5–104.8)	30.0 $\pm$ 4.8 38.8 (8.6–86.4)	38.6 $\pm$ 3.9 39.2 (12.7–115.7)*	39.6 $\pm$ 2.11 38.9 (13.7–101.9)* [†]	28.4 $\pm$ 3.39 30.3 (6.5–68.8)* [†]
LH (IU/l)	3.8 $\pm$ 0.11 3.5 (1.9–6.2)	3.8 $\pm$ 0.36 3.3 (2.0–5.4)	4.3 $\pm$ 0.21 3.9 (2.1–7.3)*	3.4 $\pm$ 0.12 3.3 (1.9–5.0)*	4.2 $\pm$ 0.48 3.6 (1.8–6.6)
Testosterone (nmol/l)	18.5 $\pm$ 2.23 15.8 (9.5–24.2)	15.2 $\pm$ 1.63 13.6 (6.6–26.1)	17.3 $\pm$ 0.39 17.0 (10.4–25.9)* [†]	15.8 $\pm$ 0.30 15.4 (9.5–22.5)* [†]	14.8 $\pm$ 0.68 14.2 (9.2–22.8)*

Statistical test: Mann–Whitney *U* test; values with an identical symbol in each line are significantly different (*, [†] $P < 0.05$).

Table 3 Relationship of inhibin B and FSH on semen sample parameters at different sperm counts, $P<0.0001$.

	Parameter	Coefficient of correlation (Spearman)		
		Inhibin B (ng/l)	FSH (IU/l)	Inhibin B: FSH ratio
All semen samples $n=2448$	TSC	$r=0.51$	$r=-0.47$	$r=0.54$
	TSN	$r=0.47$	$r=-0.42$	$r=0.49$
Subgroup (sperm count):				
>40 millions sperm $n=1571$	TSC	$r=0.26$	$r=-0.24$	$r=0.30$
	TSN	$r=0.27$	$r=-0.19$	$r=0.27$
<20 millions sperm $n=479$	TSC	$r=0.55$	$r=-0.41$	$r=0.51$
	TSN	$r=0.38$	$r=-0.27$	$r=0.33$
<10 millions sperm $n=361$	TSC	$r=0.55$	$r=-0.40$	$r=0.50$
	TSN	$r=0.40$	$r=-0.28$	$r=0.35$

TSC, total sperm count; TSN, total count of sperm with normal morphology.

relationship was between sperm variables and IFR. Less powerful but significant relationships to inhibin B and FSH were observed (Table 4). Inhibin B and IFR showed closer correlations with TSN than FSH. The percentage of motile spermatozoa did not show any correlation with the inhibin B-FSH system, in contrast to VSL and VAP ($P<0.05$), which were significantly correlated with inhibin B and, to a lesser extent, with FSH. A low but significant correlation ($P<0.05$) between inhibin B and testosterone was detected.

Predicting power of inhibin B and FSH for the results of testicular biopsy and success of TESE

We studied the relationship of 164 testicular biopsates of 82 nonobstructive azoospermic patients with inhibin B, FSH and IFR. The 164 biopsates displayed a mean Johnsen score of 4.7 ± 0.27 and a maximum Johnsen score of 6.0 ± 0.32 (mean $\pm$ S.E.M.). The IFR was most strongly correlated with histological results and sperm retrieval (Table 5). There was a significant relationship between inhibin B and mean bilateral Johnsen score ($R=0.53$; $P<0.0001$; Fig. 1C). In 32 of the 82 patients (39%), we found sperm that could be used for TESE. Figure 1D shows a decreasing probability of retrieving spermatozoa with declining inhibin B; sperm could never be retrieved at concentrations below 20 ng/l. Eighteen patients with Sertoli cell-only syndrome

(SCO) showed undetectable inhibin B; FSH was 19.8 (10.2–34.9) IU/l (median; 10th and 90th percentiles). In our patients, no sperm could be retrieved in 16 of 81 (19.7%) cases with FSH below our cutoff level of 6.8 IU/l or in nine of 81 (11.1%) of patients with inhibin B above our cutoff value of 54.5 ng/l.

Discussion

The current study is the first detecting serum inhibin B concentrations in such a large group of more than 2400 men with a wide spectrum of semen sample qualities with the aim to further define the relationship to spermatogenesis, to investigate age-related effects, and to determine cutoff values, e.g. for sperm detection in TESE procedures.

Our results suggest that inhibin B and especially the IFR are slightly more sensitive markers of male factor infertility than FSH. These findings support earlier reports with limited numbers of patients (Bohring *et al.* 2002, Kumanov *et al.* 2006). Our study confirmed the direct relation of inhibin B serum concentrations to testicular volume (Pierik *et al.* 1998, Bohring & Krause 1999, Anderson 2001, Andersson *et al.* 2004) as a consequence of the number of Sertoli cells in the testis (Sharpe *et al.* 1999). Serum inhibin B was also correlated to serum total testosterone (Mahmoud *et al.* 2000). Rat Leydig cells showed increased testosterone production after stimulation with inhibin B (Hsueh *et al.* 1987). We found in our large group of men a weak but significantly positive correlation of inhibin B with testosterone and a strong and negative correlation with FSH. Furthermore, inhibin B showed a highly significantly negative correlation with age in subjects older than 30 years. Another study that included only a smaller group of men detected a weak but significant correlation of serum inhibin B with age (Mahmoud *et al.* 2000).

The correlation of inhibin B and FSH to the spermogram parameters highlights their role as markers of spermatogenesis. We found a slightly higher correlation of TSC with inhibin B than with FSH, in accordance with earlier reports on small number of subjects (Kumanov *et al.* 2006). This result is reasonable because inhibin B is more directly related to testicular

Table 4 Comparison of the relationship of inhibin B, inhibin B/FSH ratio, and FSH on semen sample parameters by stepwise multiple regression (β) analysis ($n=2448$; $P<0.05$).

Sperm parameter	Stepwise multiple regression					
	Inhibin B (ng/l)	FSH (IU/l)	Inhibin B (ng/l)	Inhibin B/FSH ratio	FSH (IU/l)	Inhibin B/FSH ratio
Total sperm count (millions)	$\beta=0.39$	$\beta=-0.24$	$\beta=0.14$	$\beta=-0.42$	$\beta=0.13$	$\beta=0.65$
Total count of normomorph sperm (millions)	$\beta=0.22$	$\beta=-0.12$	$\beta=0.12$	$\beta=0.38$	$\beta=0.15$	$\beta=0.64$
Motile sperm (%)	NS	NS	NS	NS	NS	NS
VSL (μ m/s)	$\beta=-0.16$	$\beta=-0.11$	$\beta=-0.23$	$\beta=0.25$	$\beta=-0.21$	$\beta=-0.25$
VAP (μ m/s)	$\beta=-0.18$	$\beta=-0.12$	$\beta=-0.26$	$\beta=0.28$	$\beta=-0.24$	$\beta=-0.28$

The highest level of relationship is given in bold in each line. VAP, velocity average path; VSL, the velocity straight line.

Table 5 Relationships of inhibin B, FSH and inhibin B/FSH ratio with the mean and maximum Johnson score as well as to sperm recovery in testicular biopsies of nonobstructive azoospermic men; $n=164$; Spearman's correlation test ($P<0.0001$).

Variables	Inhibin B (ng/l)	FSH (IU/l)	Inhibin B/FSH ratio
Maximum Johnson score	$R=0.51$	$R=-0.63$	$R=0.65$
Mean Johnson score	$R=0.53$	$R=-0.68$	$R=0.69$
Sperm recovery (yes/no)	$R=0.42$	$R=-0.39$	$R=0.58$

function than FSH. However, another study (Meeker *et al.* 2007) measured a lower sensitivity of inhibin B concentration than FSH for predicting below-threshold sperm concentrations. Our findings for highly significant associations of inhibin B, FSH and sperm concentrations supported several previous studies that included distinctly lower numbers of subjects (Pierik *et al.* 1998, Mahmoud *et al.* 2000, Mabeck *et al.* 2005, Jorgensen *et al.* 2010). Moreover, although some studies have reported associations of inhibin B and FSH with sperm motility (Uhler *et al.* 2003), this was not in concordance with earlier studies (Anderson *et al.* 1998). For FSH, we have been able to demonstrate enhancement of VAP and VCL under FSH supplementation in infertile men (Glander & Kratzsch 1997) previously. Again, most previous studies for this relationship have been based on a relatively small number of infertile men. In the stepwise multiple regression we detected in our large group of men a significant association of inhibin B and FSH to VSL and VAP but not to the percentage of motile spermatozoa. Both VSL and VAP are markers of quality and supply of energy for sperm motility. Perhaps, the FSH–inhibin B system exerts an influence on the sperm motility organelles.

The correlation of inhibin B and FSH with sperm concentration raises questions related to the predictive power of both hormones and their cutoff levels in discriminating competent from impaired spermatogenesis. Pierik *et al.* (1998) considered inhibin B concentrations at 139 ng/l and FSH at 4.9 IU/l as the appropriate cutoff levels. These values correspond to the lower limits of inhibin B concentrations in a fertile control population. Jensen *et al.* (1997) reported that inhibin B concentrations <80 ng/l and FSH >10 IU/l had the predictive power of 100% in detecting sperm counts below 20×10^6 /ml. Andersson *et al.* (2004) reported cutoff levels in proven fertile men to be 119 pg/ml for inhibin B, 6.86 IU/l for FSH and 23.5 for the IFR. Applying these findings to our group of 2448 men allowed correct classification of men with total normal sperm count of >40 million and total count of normomorph spermatozoa (TCN) of >6 million in 61.4% of men for inhibin B, in 71.7% for IFR, and in 90.6% for FSH. Thus, our results confirm the cutoff value reported by Andersson *et al.* (2004) for FSH only. Our cutoff level suggested for inhibin B is lower than that

suggested by other authors (Pierik *et al.* 1998, Andersson *et al.* 2004). This finding supports the results of Meeker *et al.* (2007), who also recommended lower cutoff level.

The producer of inhibin B, the Sertoli cells, is also responsible for the maturation of sperm nucleoproteins, which can be monitored by aniline blue staining. The percentage of mature, aniline blue-negative spermatozoa correlated positively and significantly with inhibin B concentration. Sertoli cells might influence not only the quantity of spermatogenesis but also its quality. A direct influence of Sertoli cells on sperm nucleoproteins as well as an indirect effect via proteins secreted by Sertoli cells and subsequent maturation of spermatozoa during epididymal passage was described in previous papers (Xing *et al.* 2003).

The predictive power of inhibin B and FSH is especially relevant in the case of azoospermic patients to assess the success of TESE. We found azoospermia in 4.7% of men with inhibin B above our cutoff level but in 11.4% of men with FSH below our cutoff level, indicating a higher frequency of incorrect predictions of azoospermia when based upon FSH compared with inhibin B measurements. Spermatogenic arrest at a late spermatocyte stage could be an explanation for normal inhibin B concentration in this state (Andersson *et al.* 1998). The association between inhibin B and sperm count is primarily based on gonadotrophin-independent inhibin B secretion (Anderson 2001). Inhibin B regulation is dependent not only on Sertoli cell number but also on spermatogenesis. Inhibin B in adult men is a product of Sertoli cells contacting germ cells, particularly those ranging from the pachytene spermatocyte to the early spermatid stages of development (Fujisawa *et al.* 2004). This explains why serum inhibin B concentrations in postpubertal men are closely related to the presence of germinal cells. Patients with spermatidic arrest in the testicular biopsy have inhibin B concentrations similar to those in normozoospermic subjects, while concentrations are subnormal in patients with spermatogonial or spermatocytic arrest. Undetectable concentrations of inhibin B reflect the functional state of the Sertoli cells in the absence of germ cells; this condition is denoted as primary or secondary SCO (Fujisawa *et al.* 2004). Primary SCO is caused by a prenatal defect in migration of germ cells into the seminiferous tubules, and secondary SCO is a result of postnatal damage to healthy testicular tissue that may result in a focal histological SCO pattern (Weller *et al.* 2005). Our patients showed a decreasing probability of retrieving spermatozoa from testicular tissue with declining inhibin B concentrations, whereas mature testicular spermatozoa were never found when concentrations dropped below 20 ng/l. Ballesca *et al.* (2000) have suggested a cutoff value of 40 ng/l for inhibin B to be fully predictive of sperm count upon TESE. In our patients, no sperm could be retrieved from testicular tissue in 19.7% of cases with FSH below our cutoff level

and in 11.1% of patients with inhibin B above our cutoff value. This percentage was lower than that reported by Meachem *et al.* (2001), who were unable to retrieve spermatozoa in 25% of the cases with normal FSH and normal inhibin B values. The clinical decision as to whether TESE should be performed in a given patient cannot be based on inhibin B, either alone or in combination with FSH and other parameters (von Eckardstein *et al.* 1999). However, the probability of success can be assessed. The combination of FSH and inhibin B is currently the best predictor for the presence of spermatozoa which may be found upon TESE. But this prediction is not absolutely reliable. TESE can also be successful when both hormone concentrations are beyond the threshold level (Halder *et al.* 2005).

The protocol for collection of serum samples may influence the determination of inhibin B concentrations. Higher inhibin B concentrations were observed in samples collected in the morning compared with those collected in the afternoon (Meeker *et al.* 2007, Jorgensen *et al.* 2010). Therefore, we always collected one sample per patient in the morning. A single blood sample can provide a reliable measure of reproductive hormones over both short and long time-periods in population studies. Day-to-day variations of inhibin B concentrations were relatively low in men and do not seem to be influenced by seasonal factors (Andersson *et al.* 2003).

Our large population group confirmed several of the associations of inhibin B which were described in previous papers, while others have not been reported elsewhere, such as the relationship to aniline blue staining. Moreover we found a stronger correlation of semen parameters to inhibin B and FSH with decreasing sperm counts. Another interesting point concerned the influence of age on inhibin B concentration in context with semen sample quality. Consistent with previous studies, our results showed strong associations between semen quality and FSH as well as inhibin B. The evidence of a threshold supports the use of FSH and/or inhibin B as complementary tools in the context of semen analysis based upon andrological diagnostics.

Materials and Methods

Patient group

In this study, we studied the first ejaculate of 2448 men who attended our andrological clinic, with the aim of characterizing semen quality in a 7-year period. The men were characterized by the following facts: i) attending a semen analysis, ii) allowance for determination of hormones, iii) absence of genital inflammations, iv) no application of hormones, and v) no previous or current infertility treatment.

The data for the men ranging in age from 17 to 72 (32.2 ± 6.7 years; mean $\pm$ s.d.) years were retrieved from our computerized database Winsperm (Paasch *et al.* 2004). The inhibin

concentration in comparison with FSH and in relation to age and the results of routine semen analysis were emphasized in this study independent of proven fertility.

Semen analysis

The semen samples were collected after a period of 3–5 days of sexual abstinence and after the individuals provided written informed consent, in accordance with the standard ethical guidelines of the University of Leipzig. The approval for this study was obtained from the ethics committee of the University of Leipzig. Semen sample parameters were determined according to the World Health Organization guidelines (World Health Organization 1999). As data over a 7-year period were used, the WHO guideline from 1999 was applied. Sperm morphology was evaluated according to Tygerberg's strict criteria (Menkveld & Kruger 1995). Semen samples were collected by masturbation into sterile plastic Petri dishes. The computer-aided sperm motion analysis was performed by Mika cell motion analysis (Version 2.0 for Windows NT 4.0, Mika Medical GmbH, Montreux, Switzerland). Aliquots of semen samples (5 μ l) were placed in 10 μ m deep disposable counting chambers (Stroemberg-Mika) on a 36 °C microscope stage warmer. A minimum of 100 spermatozoa from at least four different fields were analyzed from each specimen (Paasch & Glander 1998). In our study, the TSC, the TCN, the percentage of motile spermatozoa with a VAP > 15 μ m/s, VSL (μ m/s) and VAP (μ m/s) of the spermatozoa, and the basic serum concentrations of inhibin B, testosterone, FSH and LH were considered.

Aniline blue staining

Aniline blue stains nucleoproteins rich in arginine (histones), which are replaced by protamines during the terminal stages of spermatogenesis under the control of Sertoli cells (Francavilla *et al.* 2001), the producer of inhibin B. Thus, aniline blue reveals the replacement of histones by protamines as well as chromatin condensation in spermatozoa. Air-dried spermatozoa on glass slides were fixed in 3% glutaraldehyde in 0.2 M PBS for 30 min. After careful rinsing of the slides, they were stained with 5% aniline blue at pH 3.5 for 3 min (Dadoue *et al.* 1988). The slides were examined using light microscopy (microscope BX41, Olympus, Hamburg, Germany) at a magnification of 1000 $\times$ under oil immersion to detect the percentage of spermatozoa with unstained heads.

Hormone assays

Hormones were quantitatively determined in serum by commercial immunoassay kits: LH, FSH and testosterone were measured by the Elecsys fully mechanized platform (Roche). Serum concentrations of inhibin B were determined by the IRMA from Beckman Coulter (Sinsheim, Germany). For each subject, an IFR was calculated as inhibin B (ng/l)/FSH (IU/l), which was regarded as a marker of Sertoli cell function and Sertoli cell response to FSH. The samples were collected between 0900 and 1100 h (Jorgensen *et al.* 2010).

Testicular biopsies

In 82 nonobstructive azoospermic patients, bilateral testicular biopsies were performed as described previously (Glander *et al.* 2000). Briefly, samples of about 200–300 mg were placed in a Petri dish containing about 2 ml Ham's F-10 medium with 2% BSA. After the specimen was divided, one part was shredded with a microscopic glass slide and the supernatant was checked for the presence of spermatozoa. The other specimen was fixed and embedded to yield sections with a thickness of 3–5 µm. These were stained with hematoxylin–eosin and evaluated by light microscopy (microscope BX41, Olympus). The germinal epithelium was assessed according to a modified Johnsen score (Johnsen 1970).

Statistical analysis

After retrieval from our Winsperm computerized database system, the data were analyzed using Statistica 7.0 software for Windows from StatSoft, Inc. (Tulsa, OK 74104, USA). Only the first semen sample from each man was included in the statistical analysis. Results are expressed as mean ± s.e.m. as well as median plus 10th and 90th percentiles. The Shapiro–Wilks *W* test for large samples excluded a normal distribution for the following parameters: TSC, TCN, concentration of FSH in serum and IFR. The unpaired Mann–Whitney *U* test and the Wilcoxon-matched pairs test were used to calculate the differences between groups. The Spearman rank correlation test (*R*) was used to study the association between different parameters. All tests were two tailed, and significance was indicated by *P* < 0.05. Moreover, the results were analyzed by stepwise multiple linear regression after transforming the parameters into a log-normal distribution.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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