The serum inhibin B concentration and reference ranges in normozoospermia

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Abstract

Objective: Although an inhibin B assay may be useful in the assessment of testicular function in a number of genital conditions, reliable reference ranges are still lacking. The present study sought to establish the reference range for serum inhibin B by applying the updated Gen II assay.

Design: This prospective study included 818 men referred for semen analysis: 377 were normozoospermic (reference group) and 441 presented at least one abnormal semen parameter (case group).

Methods: Semen parameters were interpreted according to the 2010 World Health Organization manual and David’s modified classification for normal morphology. The inhibin B concentration was determined with the current ELISA.

Results: In the reference group, the 2.5th percentile for inhibin B was 92 pg/ml and the 97.5th percentile for FSH was 7.8 IU/l. In the overall population, an inhibin B level <92 pg/ml was associated with increased odds ratio (OR; 95% CI) for oligozoospermia (16.93 (9.82–29.18), P<0.0001), asthenozoospermia (4.87 (2.88–8.10), P<0.0001), and teratozoospermia (2.20 (1.31–3.68), P=0.0026). The combination of a FSH >7.8 IU/l and an inhibin B <92 pg/ml was associated with greater OR for oligozoospermia (98.74 (23.99–406.35), P<0.0001) than for each hormone considered separately.

Conclusions: A new reference range for serum inhibin B was established by the use of updated immunoassay. The correlations between hormone levels and semen parameters highlighted the importance of establishing these values with respect to the spermogram. When combined with FSH assay, the inhibin B range may be of value in the evaluation of spermatogenesis in a number of male genital conditions.

Introduction

Inhibin B has enriched our knowledge about testicular function and regulation of the pituitary–gonadal axis in a number of genital conditions such as maturation and senescence, infertility, hypogonadism, gonadotropin administration, hormonal contraception, and testicular damage resulting from radiation or chemotherapy (1). Inhibin B is a heterodimeric glycoprotein composed of a common α subunit and a specific βB subunit (2). In men, the protein is produced exclusively by the testis (1). Inhibin B production is stimulated by the secretion of pituitary follicle-stimulating hormone (FSH). The resulting inhibin B exerts a negative feedback on FSH secretion. Inhibin B also exerts a paracrine intratesticular effect (2, 3, 4, 5, 6).
(7, 8). When measured, inhibin B levels correlate quite well with FSH concentrations in the vast majority of cases. However, the diagnostic accuracy of FSH is limited by the fact that some conditions do not lead to changes in FSH secretion (9). FSH and inhibin B are complementary tools in gonadal male conditions and andrological diagnostics (10, 11, 12, 13, 14, 15). In particular, FSH and inhibin B together are more sensitive than either alone in predicting the histological status of the testis and the presence of sperm in bioptic tissue (11, 15).

The specific inhibin B assay first developed in the mid-1990s (16, 17) is the template on which the only commercially available ELISA is based. It should be considered that the data published so far have been obtained using only one assay, which still has a rather poor interassay precision and cannot be properly evaluated in terms of accuracy. This ELISA was updated in 2010 (yielding the Gen II version). International recognized reference preparations are derived from cohorts of various conditions (15, 18, 19, 20, 21, 22). Moreover, most of these studies were based on an inhibin B ELISA assay that is no longer available. Those of mid-1990s (16, 17) is the template on which the only commercially available ELISA is based. It should be considered that the data published so far have been obtained using only one assay, which still has a rather poor interassay precision and cannot be properly evaluated in terms of accuracy. This ELISA was updated in 2010 (yielding the Gen II version). International recognized reference preparations are derived from cohorts of various conditions (15, 18, 19, 20, 21, 22). Moreover, most of these studies were based on an inhibin B ELISA assay that is no longer available. Those of

Hence, the present study evaluated the serum inhibin B concentration in men who delivered semen samples at the University Hospital’s Reproductive Biology Center (Lille, France), using the currently available Gen II assay. We determined the reference ranges and thresholds for serum inhibin B, FSH, and testosterone levels in normozoospermic men defined according to the updated version of the WHO published in 2010 (26). Lastly, we examined the association of hormone reference values with semen parameters.

Subjects and methods

Patients

The study population comprised healthy men referred for semen analysis to the Reproductive Biology Institute at the Lille University Hospital between January 2011 and December 2012. All the men were members of subfertile couples. For a given individual, the semen and blood samples were provided on the same day. Men gave their written informed consent to participation.

Semen collection and analysis

Semen samples were provided in the laboratory by masturbation into a sterile plastic specimen cup, following a requested period of 2–7 days of sexual abstinence. The actual abstinence period was recorded based on the information given by men at the time of the semen collection. After 30 min of liquefaction at 37°C, a manual semen analysis included routine measurements of the seminal volume, pH, sperm concentration, sperm motility, and morphology according to standard methodologies. Semen volume was measured directly in a graduated glass (Recisperme, JCD SA, La Mulatière, France). Sperm concentration was assessed by counting two sides of a Thoma hemocytometer in duplicate based on the same diluted drop of the semen sample. Sperm progressive motility (WHO grades a+b combined) was assessed in duplicate in 10 µl of well-mixed semen placed on a glass slide and covered with a 22 × 22 mm coverslip. Sperm vitality was measured using the eosin–nigrosin method. Sperm morphology was assessed by light microscopy of Shorr-stained smears at a final magnification of 1000. The semen volume, total sperm number, sperm concentration, motility, and vitality were interpreted according to the 2010 WHO guidelines (26). Normal forms were analyzed and interpreted according to the standardized David’s modified classification (27).

Hormone assays

All blood samples for hormone assays were collected from the cubital vein between 0800 and 1000 h, and then centrifuged at +4°C to recover the serum. Serum samples were distributed into several aliquots, which were immediately frozen at −80°C and stored until required for assays. The serum inhibin B was determined in duplicate using the Inhibin B Gen II ELISA Kit (launched in 2010 by Beckman Coulter, Villepinte, France). This kit does not require sample pretreatment (i.e. no boiling is required) and features a total incubation time of <4 h. The kit’s capture antibody is specific for the βB subunit of inhibin B, whereas the peroxidase-labeled antibody binds to the α-subunit. The degree of enzymatic activity was determined by measuring absorbance at a wavelength of 450 nm for the primary test filter and 630 nm for the main filter reference. The measured absorbance was directly proportional to the inhibin B concentration in the sample. The dynamic range of the standard curve is 10–1000 pg/ml. The intra- and interassay coefficients of variation (CV) were <12 and <17% respectively. Since serum inhibin B concentrations were relatively constant from 1 day to another (28) and appear to decrease during the daytime (29, 30), we collected a single serum sample per patient in the morning.
Serum FSH levels were measured with a chemiluminescence assay on a multiparameter analyzer (Architect, Abbott Laboratories). The assay's limit of detection was 0.05 mIU/ml. The intra- and interassay CV for FSH were <3.5 and <5% respectively. Total testosterone was measured by RIA using the Coat-A-Count Kit (Siemens Diagnostics, Inc., Los Angeles, CA, USA). The assay's limit of quantification was 0.14 nmol/l (0.04 ng/ml) and the interassay CV was between 5.9 and 11% for a range of concentrations between 0.76 and 13 ng/ml.

**Statistical analysis**

All statistical analyses were performed with SAS Software (version 9.1, SAS Institute, Inc., Cary, NC, USA). Semen parameters and hormone levels were expressed as the median (2.5th–97.5th percentiles). The Mann–Whitney U test was used to compare the reference and case groups. Relationships between hormone levels and semen parameters were analyzed using Spearman's correlation test. As the semen and hormone variables did not follow a normal distribution or log-normal distribution, non-parametric tests were performed and no transformation was applied.

Smoothed graphs showing relationships between FSH levels, inhibin B levels, and the sperm count were assessed in a univariate linear regression model using least-squared, locally weighted scatterplot smoothing (LOESS).

Normozoospermia (reference group) corresponded to a total sperm count ≥39 million/ejaculate, progressive motility ≥32% (as defined in the 2010 WHO manual), and normal forms ≥15% (as defined in our laboratory). In the reference group, the thresholds for inhibin B and FSH were defined as the 2.5th and 97.5th percentiles. These thresholds were then applied to all 818 men for examining the associations between inhibin B and FSH with semen parameters. Associations between these thresholds and oligozoospermia (total sperm count <39 million/ejaculate), asthenozoospermia (progressive motility <32%), and teratozoospermia (normal forms <15%) were examined using logistic regression and calculation of the odds ratio (OR; 95% CI). The threshold for statistical significance was set to \( P<0.05 \). Both age and sexual abstinence period were tested as confounders, and adjusted ORs were calculated.

**Results**

**Semen characteristics and hormone levels of the study populations**

The overall population comprised 818 men (Table 1). Of these, 377 presented semen parameters within the reference limits and thus constituted the reference group. In contrast, 441 men presented at least one abnormal semen parameter (as defined above) and thus constituted the case group. In the reference group, the median (2.5th–97.5th percentiles) age was 34 (25–48) years and the median sperm concentration was 70 million/ml (Table 1). The 2.5th percentile value for inhibin B was 24 pg/ml in the overall population and 92 pg/ml in the reference group. The 97.5th percentile for inhibin B was 310 pg/ml in the overall population and 316 pg/ml in the reference group. The 2.5th percentile for FSH was 1.2 IU/l in both the overall population and the

<table>
<thead>
<tr>
<th></th>
<th>Overall population (n=818)</th>
<th>Reference group (n=377)</th>
<th>Case group (n=441)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>34 (24–49)</td>
<td>34 (25–48)</td>
<td>33 (23–49)</td>
<td>0.86</td>
</tr>
<tr>
<td>Abstinence (days)</td>
<td>3 (2–7)</td>
<td>3 (2–7)</td>
<td>3 (2–7)</td>
<td>0.71</td>
</tr>
<tr>
<td>Semen volume (ml)</td>
<td>3.1 (0.9–6.7)</td>
<td>3.3 (1.6–6.8)</td>
<td>3.0 (0.6–6.5)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Sperm concentration (ml/ml)</td>
<td>32 (0–220)</td>
<td>70 (15–236)</td>
<td>9 (0–160)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Total sperm count (ml/ml)</td>
<td>97 (0–747)</td>
<td>221 (53–897)</td>
<td>25 (0–369)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Progressive motility (%)</td>
<td>45 (3–65)</td>
<td>55 (35–70)</td>
<td>25 (1–60)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Vitality (%)</td>
<td>75 (25–92)</td>
<td>80 (62–92)</td>
<td>68 (25–90)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Normal forms (%)</td>
<td>25 (2–58)</td>
<td>33 (16–59)</td>
<td>14 (2–50)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Inhibin B (pg/ml)</td>
<td>157 (24–310)</td>
<td>174 (92–316)</td>
<td>133 (16–296)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>FSH (IU/l)</td>
<td>3.8 (1.2–18.8)</td>
<td>3.2 (1.2–7.8)</td>
<td>4.5 (1.4–25.9)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Testosterone (nmol/l)</td>
<td>15.7 (8.0–27.3)</td>
<td>15.7 (8.0–26.0)</td>
<td>15.7 (8.0–30.1)</td>
<td>0.78</td>
</tr>
</tbody>
</table>

\( P<0.05 \) was considered to be statistically significant.
Semen parameters | Population | Inhibin B | FSH | Testosterone |
<table>
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</tr>
</thead>
<tbody>
<tr>
<td>Sperm concentration</td>
<td>Overall population (n=818)</td>
<td>0.44 (&lt;0.0001)</td>
<td>-0.45 (&lt;0.0001)</td>
<td>-0.01 (0.66)</td>
</tr>
<tr>
<td></td>
<td>Reference group (n=377)</td>
<td>0.21 (&lt;0.0001)</td>
<td>-0.24 (&lt;0.0001)</td>
<td>-0.05 (0.37)</td>
</tr>
<tr>
<td></td>
<td>Case group (n=441)</td>
<td>0.45 (&lt;0.0001)</td>
<td>-0.46 (&lt;0.0001)</td>
<td>0.03 (0.52)</td>
</tr>
<tr>
<td>Total sperm count</td>
<td>Overall population (n=818)</td>
<td>0.45 (&lt;0.0001)</td>
<td>-0.44 (&lt;0.0001)</td>
<td>-0.01 (0.75)</td>
</tr>
<tr>
<td></td>
<td>Reference group (n=377)</td>
<td>0.21 (&lt;0.0001)</td>
<td>-0.20 (&lt;0.0001)</td>
<td>-0.03 (0.55)</td>
</tr>
<tr>
<td></td>
<td>Case group (n=441)</td>
<td>0.47 (&lt;0.0001)</td>
<td>-0.46 (&lt;0.0001)</td>
<td>0.03 (0.56)</td>
</tr>
<tr>
<td>Progressive motility</td>
<td>Overall population (n=818)</td>
<td>0.26 (&lt;0.0001)</td>
<td>-0.22 (&lt;0.0001)</td>
<td>-0.02 (0.65)</td>
</tr>
<tr>
<td></td>
<td>Reference group (n=377)</td>
<td>0.003 (0.95)</td>
<td>0.03 (0.54)</td>
<td>-0.01 (0.78)</td>
</tr>
<tr>
<td></td>
<td>Case group (n=441)</td>
<td>0.15 (0.002)</td>
<td>-0.11 (0.03)</td>
<td>0.002 (0.96)</td>
</tr>
<tr>
<td>Normal forms</td>
<td>Overall population (n=818)</td>
<td>0.18 (&lt;0.0001)</td>
<td>-0.23 (&lt;0.0001)</td>
<td>-0.07 (0.06)</td>
</tr>
<tr>
<td></td>
<td>Reference group (n=377)</td>
<td>-0.07 (0.14)</td>
<td>-0.04 (0.42)</td>
<td>-0.04 (0.41)</td>
</tr>
<tr>
<td></td>
<td>Case group (n=441)</td>
<td>0.09 (0.08)</td>
<td>-0.16 (0.002)</td>
<td>-0.09 (0.08)</td>
</tr>
</tbody>
</table>

The quoted values are the correlation coefficient $r$ ($P$). $P<0.05$ (Spearman’s test) was considered to be statistically significant.
below 15 million/ml (15.16 (8.70–26.39), \( P < 0.0001 \)), a total sperm count <39 million/ejaculate (16.93 (9.82–29.18), \( P < 0.0001 \)), asthenozoospermia (4.87 (2.88–8.10), \( P < 0.0001 \)), and teratozoospermia (2.20 (1.31–3.68), \( P = 0.0026 \)) (Table 3). An FSH level >7.8 IU/l was associated with increased ORs for oligozoospermia, asthenozoospermia, and teratozoospermia (Table 3).

The combination of an FSH level >7.8 IU/l and an inhibin B level <92 pg/ml (\( n = 73 \)) was associated with a greater OR for a total sperm count <39 million/ejaculate (98.74 (23.99–406.35), \( P < 0.0001 \)) than each hormone considered separately. Inclusion of age and the period of sexual abstinence in the model did not modify the adjusted OR.

**Discussion**

In the present study, we analyzed blood levels of reproductive hormones and their relationships with semen parameters. To the best of our knowledge, the present study is the first to report a normative reference range for inhibin B using the updated Gen II assay. In fact, previous studies relied on a now unavailable inhibin B assay that also required sample pretreatment.

The WHO manual for the examination of human semen is widely used as a source of reference values by laboratories engaged in semen analyses. In the determinations of normative inhibin B values reported in the literature (18, 21, 22, 23), semen parameters were usually analyzed in accordance with the now obsolete 1999 WHO manual (25). The current 2010 WHO manual has much wider reference limits than the previously presented normal or reference values and is based on data generated in various regions of the world. Furthermore, the literature data on hormone thresholds were derived from variously defined reference populations and were obtained in several laboratories that may well have used different analytical methods.

These observations prompted us to reconsider the inhibin B concentrations and reference values. First, we used the currently available, updated Gen II assay

### Table 3

Associations between inhibin B and FSH thresholds with abnormal semen parameters in the overall population (\( n = 818 \)).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Inhibin B &lt; 92 pg/ml (( n = 111 ))</th>
<th>FSH &gt; 7.8 IU/l (( n = 121 ))</th>
<th>FSH &gt; 7.8 IU/l and inhibin B &lt; 92 pg/ml (( n = 73 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm concentration &lt; 15 million/ml (( n = 294 ))</td>
<td>15.16 (8.70–26.39), ( P &lt; 0.0001 )</td>
<td>14.12 (8.42–23.68), ( P &lt; 0.0001 )</td>
<td>54.27 (16.90–174.19), ( P &lt; 0.0001 )</td>
</tr>
<tr>
<td>Total sperm count &lt; 39 million/ejaculate (( n = 268 ))</td>
<td>16.93 (9.82–29.18), ( P &lt; 0.0001 )</td>
<td>16.03 (9.62–26.69), ( P &lt; 0.0001 )</td>
<td>98.74 (23.99–406.35), ( P &lt; 0.0001 )</td>
</tr>
<tr>
<td>Progressive motility &lt; 32% (( n = 265 ))</td>
<td>4.87 (2.88–8.10), ( P &lt; 0.0001 )</td>
<td>4.51 (2.76–7.35), ( P &lt; 0.0001 )</td>
<td>4.91 (2.46–9.80), ( P &lt; 0.0001 )</td>
</tr>
<tr>
<td>Normal forms &lt; 15% (( n = 188 ))</td>
<td>2.20 (1.31–3.68), ( P = 0.0026 )</td>
<td>4.31 (2.33–7.07), ( P &lt; 0.0001 )</td>
<td>2.90 (1.46–5.76), ( P = 0.0023 )</td>
</tr>
</tbody>
</table>

\( P < 0.05 \) was considered to be statistically significant.
launched in 2010. Second, we studied men whose semen parameters had been scored according to the current 2010 WHO manual (26) except for sperm morphology.

Depending on studies, the lower reference limit for inhibin B level varied from 48 pg/ml (22) to 105 pg/ml (18). This broad dispersion might be due to differences in the inclusion criteria and the study populations (in terms of ethnic origin and geography). These findings emphasized the importance of defining the inclusion criteria for participants and the hormone reference ranges according to the center of recruitment. Interestingly, our range for inhibin B (92–316 pg/ml) is closed to that reported for proven fertile men (94–327 pg/ml, n=84) (15). Nevertheless, our cut-off value of 92 pg/ml is close to that proposed by Andersson et al. (18) (105 pg/ml) for 303 fertile men with normal sperm concentrations.

Considering the usual reference values of the FSH level in normozoospermic men, our results are in agreement with most studies of men with normal sperm concentrations and/or fertile men (18, 22, 23). When the reference group consisted of men from the general population, reference values have been very dispersed (with an upper limit varying from 7.73 IU/l (19) to 18.66 IU/l (31)). Therefore, our result was lower than the laboratory reference values usually suggested for FSH (32).

At present, there is no consensus on which individuals are suitable for providing the most plausible reference hormone values. The accuracy of the present study may have been limited by the inclusion of samples from a single reproductive center. However, studies performed in different centers will generate between-laboratory variations in methodology and outputs, which might represent real biological differences between men or laboratory-dependent measurement biases. Our present data were based on individuals with normal semen parameters but partners of infertile couples. These men represented men with various semen qualities. However, semen quality is a surrogate of male fecundity, and a high percentage of fertile men would be classified as subnormal if sperm morphology, concentration, and motility are considered (33). Our rationale is supported by the frequent documentation of significant correlations between FSH or inhibin B levels and normal spermatogenesis (18, 19, 29, 30, 34).

Our results confirmed that inhibin B levels were significantly and positively correlated with the sperm count and that FSH levels were negatively correlated with the sperm count (20, 21, 23, 24, 29, 30, 31). We also confirmed a strong, negative correlation between inhibin B and FSH levels ($r = -0.55; \ P < 0.0001$) in normozoospermic men. However, the correlation coefficients with the total sperm count were higher for inhibin B than for FSH in both the overall population and the reference group. In line with previous studies (19, 20, 23, 31, 34), this observation suggests that inhibin B is a better marker of spermatogenesis (and Sertoli cell function, in particular) than FSH. We observed that these correlation coefficients were lower in normozoospermic men than in the case group. Associations between hormone levels and sperm count are stronger in studies of the general population (19, 31) or infertile men (20, 21, 30, 34) than in studies of fertile men (21, 29). This might be due to the marked inter-individual differences in sperm count seen in populations of primarily normozoospermic and/or fertile men. In total, categories of men (fertile, infertile, and normozoospermic) represented overlap in semen qualities, suggesting that inhibin B and FSH were not mainly markers of fertility but rather markers of semen quality and thereby fecundity.

Our data suggested that inhibin B represented sperm concentration and total sperm counts better than sperm motility and morphology. Indeed, Sertoli cells, considered as the main source of inhibin B, did not fulfill their function in the absence of germ cells (1, 3). Thus, the spermatogenic status seemed to be the prime regulator of inhibin B levels. Moreover, we showed that the associations between inhibin B or FSH levels and the sperm count were nonlinear. These correlations were stronger at low sperm counts. In the literature, higher correlations between inhibin B and semen parameters have been found in subjects with elevated FSH levels (20). Correlations between inhibin B levels and the total sperm count appear to be less significant as the total sperm count increases (29). The same observation has been reported for FSH levels in the general population (23, 31). Furthermore, we observed a relationship between sperm motility and inhibin B and FSH levels in the overall population but not in normozoospermic men. These relationships have been previously observed in infertile men (20, 21, 31). Correlations between inhibin B and FSH with absolute sperm motility (but not with the percent motile sperm) have been reported in the literature (23, 31). These correlations could be due to the known association between sperm motility and sperm concentration (21). Further studies will be required to determine the threshold value of inhibin B beyond which a low sperm count can be observed.

One of the strengths of our study relates to the inclusion of a large panel of normozoospermic individuals, which enabled us to perform a thorough assessment of serum inhibin B and FSH ranges. Our main finding was that men who have an inhibin B level below 92 pg/ml have higher risk of semen abnormalities (with a 84.7%
chance of oligozoospermia, in particular). We found that inhibin B alone and FSH alone were equally able to discriminate between oligozoospermia and a normal sperm count (with an 83.5% risk of oligozoospermia when FSH is above 7.8 IU/l). Since thresholds for inhibin B and FSH together were associated with a greater OR than the threshold for the individual hormones alone, the measurement of inhibin B might substantially increase the predictive power of FSH. In fact, the combined use of FSH and inhibin B has proved to be of particular value in azoospermic conditions (13, 35).

The clinical value of the inhibin B assay is emphasized by the fact that in contrast to FSH, inhibin B is produced inside the testis and might reflect close interactions between Sertoli and germ cells (36, 37). Thus, inhibin B levels correlate directly with spermatogenesis and reflect testicular sperm production.

In conclusion, we have generated new serum inhibin B and FSH reference values by using an updated immunoassay (for inhibin B) and the latest 2010 WHO sperm reference manual. The observed correlations between hormone levels and semen parameters highlighted the importance of establishing these values with respect to the spermogram–spermocytogram. It may well be of interest to apply these updated hormone reference values when examining the success rate for the routine management of various male genital conditions.

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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Author contribution statement
A-L Barbotin conceived the study, performed the experiments, and wrote the manuscript. C Ballot and J Sigala supplied data for patients recruited. A Duhamel and N Ramdane performed the statistical analysis. F Marcelli, J-M Rigot, and D Dewailly revised the manuscript for critical content. P Pigny and V Mitchell supervised the experiments, interpreted the data, and drafted the manuscript. All authors approved the final manuscript.

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