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Regional Variations in Semen Quality of Community-Dwelling Young Men From Flanders Are Not Paralleled by Hormonal Indices of Testicular Function

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Abstract

Epidemiological studies of sperm quality are hampered by problems such as low participation rates and poor comparability of results due to methodological differences in semen analysis. More objective sperm quality-related serum markers would facilitate worldwide comparisons of male reproductive status. Our objectives were to investigate to what extent a set of hormonal indices of testicular function, previously established in a clinical setting, could predict regional variations in seminal parameters in men from the general population. We recruited 101 men aged 20–40 years from two regions in Flanders, and assessed their sperm parameters and levels of serum reproductive hormones. In one region compared to another, the participants had lower sperm counts (by 34%; $P = .06$), lower total sperm counts (by 41%; $P = .02$) and poorer sperm morphology (by 32%; $P < .001$), which were paralleled by significantly lower levels of free testosterone (by 11%; $P = .03$), while for total testosterone (T) and follicle-stimulating hormone (FSH), the differences were not significant (both $P = .09$) at 10% and 17%, respectively. There were no differences in inhibin B and the T to luteinizing hormone (LH) ratio, which are markers of testicular function. Receiver operating characteristic curve analysis revealed that T:LH, inhibin B, and the inhibin B/FSH ratio had significant discriminatory power between men with sperm concentrations below or above 13.5 x

10^6/mL. Regional variations in the semen quality of community-dwelling individuals are not necessarily reflected in altered hormonal indices of testicular function and thus, these markers, validated in clinical settings, are not valid substitutes for the traditional semen quality assessment used in epidemiological population studies.

Key words: FSH, inhibin B, spermatogenic arrest, sperm quality, T:LH ratio

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Male sexual differentiation and development, as well as male fertility and sexuality, are under stringent endocrine regulation by the hypothalamo-pituitary-testicular (HPT) axis. Any factor that disturbs this axis may cause male gonadal dysfunction and reduced fertility. Conversely, primary testicular dysfunction tends to be reflected in altered hormonal parameters of HPT function. The determination of the latter hormonal parameters is part of the standard clinical investigation into male infertility (Skakkebaek et al, 1994). Indeed, in nonobstructive azoospermia, high levels of follicle-stimulating hormone (FSH) reflect drastic testicular failure (de Kretser et al, 1972). Similarly, in subfertile (or oligospermic) men, higher FSH and, less distinctly, luteinizing hormone (LH) levels together with decreasing sperm counts indicate an adaptive gonadotropic reaction towards testicular impairment (Hunter et al, 1974; Purvis et al, 1975). Furthermore, the testosterone to LH ratio (T:LH), which is a measure of Leydig cell responsiveness, seems to correlate positively with sperm concentration and negatively with FSH level (Andersson et al, 2004a) and is suggested to be an independent parameter of infertility in infertile men with normal FSH levels (Giagulli and Vermeulen, 1988). The testicular polypeptide hormone inhibin B, which regulates the negative feedback of pituitary FSH secretion, reflects the functional state of the seminiferous epithelium, as shown in normal men and men with testicular failure (Anawalt et al, 1996). The inhibin B to FSH ratio (Inh:FSH) is suggested to be a better predictor of infertility than FSH or inhibin B alone (Andersson et al, 2004b). In contrast to the above-described markers of testicular health, low to low-normal FSH and LH levels in conjunction with low testosterone suggests secondary hypogonadism (Nieschlag et al, 1999).

The complex interplay between environment, lifestyle, and genetic factors during fetal life as well as in adulthood hampers the assessment of their respective roles in male reproductive failure. Furthermore, epidemiological studies of sperm quality are rendered difficult by practical problems, such as low participation rates and poor comparability of sperm analyses between laboratories (Cohn et al, 2002; Keel, 2004). In this respect, the question as to whether sperm quality has decreased worldwide during the recent era of explosive growth of the chemical industry remains a matter of debate (Carlsen et al, 1992; Sherins, 1995). In any case, there exist regional differences in semen parameters and evidence for their decline (Swan et al, 2000; Jørgensen et al, 2001, 2002). Whether this is paralleled by differences in reproductive endocrine status is a largely unanswered question (Jørgensen et al, 2002).

In 1999, as part of a feasibility study on biomarkers of environmental exposure and of health in humans (Flemish Environment and Health Study) (Staessen et al, 2001), we recruited male adults of reproductive age living in a rural or urban region in Flanders. We observed regional differences in seminal parameters, and assessed whether these were paralleled by differences in reproductive endocrine status. Furthermore, we examined the potential use of the above-described hormonal indices of testicular function, previously validated mainly in cohorts of infertile or oligospermic men recruited through infertility clinics, to assess the reproductive status of a sample of men from the general population. We restricted participant age to 20–40 years, to exclude, as much as possible, age-related endocrine alterations in the hypothalamo-pituitary-gonadal axis (Kaufman and Vermeulen, 2005). Furthermore, we excluded men who might be occupationally exposed to testicular toxicants

(Auger et al, 2001) and those who commuted over long distances.

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Geographical Areas

We recruited male candidates from 2 regions in Flanders, the urban area of Antwerp and the rural area of Peer, during the Summer of 1999. In total, 2487 short questionnaires with an accompanying letter were mailed to young men, aged 20–40, who were selected randomly from the two municipal population registries. The response rate in Peer (28%) was slightly lower than that in Antwerp (33%). Of the 744 responders, 207 men were eligible after consideration of the following exclusion criteria: vasectomy, commuting over long distances, and having a job with risk of exposure to conditions harmful for male reproductive health (chemical industry, dry cleaning, industrial production, military airport). The candidates were contacted by telephone to check their willingness to participate and to provide further details regarding sperm sample collection and minimal hygienic conditions. The subjects were asked to report carefully the hour of semen collection and the abstinence period, with stress on the need to observe a 3-day abstinence period. Similar proportions of candidates in Antwerp (82%) and Peer (92%) were contacted before the goal of 50 participants within each area was reached, whereby preference was given to nonsmokers and lifelong residents of the area. Overall, 60% of the contacted persons agreed to participate in the study. Reasons for non-participation were vacation (n = 17), refusal (n = 29), and personal reasons (n = 20). Two currently smoking men were allowed to enter the study, 1 in each region. Due to a misclassification, an extra eligible person in Peer was included in the study (n = 51). In neither area were differences in age or having children noted between those that were contacted and those that were not or between those that entered the study and those that refused during the telephone conversation. A greater proportion of the eligible persons in Peer (72%) had proven fertility compared to those in Antwerp (44%), which was possibly related to the age difference in the 2 cohorts (34.5 and 31.6 years, respectively). All participants showed up at the investigation and gave their informed consent. The ethics committee of the Catholic University of Leuven approved the study.

Questionnaires, Clinical Measurements, Semen Collection and Analysis

Semen samples collected by masturbation were processed within 45 minutes at the site of investigation, whereby a seminal smear was prepared by trained nurses, 2 mL of Hayem anticoagulant and preservative solution was added, and the specimen was stored at 4 °C. All samples reached the andrology department within 4 days of semen collection, and the technicians were unaware of sample origin. Ejaculate volumes were estimated using a graduated pipette. A single technician scored the sperm concentrations in duplicate using disposable counting chambers (Cellvision, Heerhugowaard, The Netherlands) and assessed sperm morphology in air-dried Papanicolaou stained seminal smears. Using the morphology assessment method recommended by the World Health Organization (1992), only spermatozoa with absolutely no defects were classified as normal. The total sperm count (TSC) was derived by multiplying the individual's sperm concentration by the volume. The participants filled in a questionnaire to assess health, lifestyle, social class, use of tobacco and alcohol, and intake of medicines, as previously reported for the adolescent cohort of the FLEHS project (Staessen et al, 2001). For each participant, weight and length were measured at the site of investigation and the body mass index (BMI) was calculated as kg/m^2.

Hormone Measurements

Venous blood was obtained and centrifuged at the site. Serum was aliquoted for determinations of

markers of hormone status and was stored at -20 °C until analysis. Commercial immunoassays were used to determine the serum levels of total testosterone (T) (Medgenix, Fleurus, Belgium), LH, and FSH (Roche Diagnostics, Vilvoorde, Belgium), sex hormone-binding globulin (SHBG; Orion Diagnostica, Espoo, Finland), total 17ß-estradiol (E2) (Clinical Assay; DiaSorin s.r.l., Saluggia, Italy; adapted protocol to using double amount of serum), and inhibin B (Serotec, Oxford, United Kingdom). The free fractions of T (f T) and E2 (f E2) were calculated from the serum T, SHBG, assuming a fixed albumin concentration using a validated equation (Vermeulen et al, 1999; Szulc et al, 2004). The intraassay and interassay coefficients of variation for all assays were less than 12%. For each individual, the T:LH ratio was calculated as nmol:IU, the T to E2 (T:E2) ratio as pmol:pmol, and the Inh:FSH ratio as ng:IU.

Statistical Analyses and Exclusions

The hormone levels are reported as molar units or IU per liter of serum, except for inhibin B, which is expressed in ng/L. One participant from Peer had a hormone and sperm profile that was suggestive of Klinefelter syndrome, and was excluded from the dataset, reducing the number in Peer to 50 participants. Normal data are described by the average and standard deviation (SD). Data that are not normally distributed are described by the median and interquartile range (IQR) or are log-transformed and described by the geometric mean (GM) and 95% confidence intervals (95% CI) in case of correction for confounding variables. We compared the means and proportions across the two areas using the Mann-Whitney *U* test and Fisher's exact test, respectively. Next, applying analysis of covariance, potentially important covariates were forced into the models irrespective of statistical significance. We explored inter-hormone and hormone-sperm parameter relationships using scatter plots and Spearman's rank correlation (*r*). Subsequently, we built general linear models with inclusion of area of residence, an interaction term between the explanatory variable and the area of residence and known confounders. The odds ratio for a disorder was calculated using multiple logistic regression. Due to the non-linear relationship between sperm parameters and abstinence period, the latter was coded less than 2 days, between 2 and 4 days, and between 4 and 6 days or more, and corrected for in the multiple linear regression. Age was included as a confounder during sperm parameter analyses (Kidd et al, 2001). Receiver Operating Characteristic (ROC) analysis was performed using the Medcalc version 8.1 software (Mariakerke, Belgium). All other statistical analyses were performed with the SPSS version 12.0 software (SPSS Inc, Chicago, Ill).

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Subject Characteristics

The characteristics of the population are presented in Table 1. Participants from Peer were older and had a higher average BMI. On average, blood was collected 2 hours later in Peer than in Antwerp. The groups were not different in terms of education level. In both regions, a similar proportion of participants took medication, which had no influence on any hormone or sperm parameter, more than 80% of them felt "good" at the time of the investigation and none reported a "bad" health status. Participants with a history of serious illness or surgery had drastically lower total sperm counts, before and after correction for age, completeness of the semen sample and abstinence period (GM [95% CI]: 38.4 x 10^6/mL [20.3–73.0] vs 117.7 x 10^6/mL [95.1–145.8], P = .02).

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Table 1. *Characteristics of the study population*

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Sperm volume and total sperm count, but not sperm concentration, correlated positively with duration of abstinence (r for sperm volume = 0.35, $P < .001$ and TSC = 0.25, $P = .01$), which was reported to be more than 3 days by 90% of the participants. In this relatively young population, age was not related to sperm concentration, sperm volume, total sperm count or sperm morphology. The time (minutes) between reported semen sample collection and first processing by a nurse was identical at both sites (37 minutes). The number of days between semen processing and laboratory investigations was significantly higher in Antwerp but this did not negatively influence the measured sperm concentration, semen volume or TSC, as the respective correlation coefficients were all small and nonsignificant. Finally, there were no differences in the reported completeness of the semen samples between the sites. The serum levels of E2, f E2, LH, FSH, SHBG, and inhibin B were not related to age, in contrast to T and fT (r = -0.32, $P = .001$ and r = -0.38, $P < .001$, respectively). BMI correlated significantly with total testosterone (r = -0.38, $P < .001$) but not with f T, E2, LH, FSH or any semen parameter. In a combined general linear model, testosterone declined by 3.2% (95% CI: 1.6%–4.7%, $P < .001$), 3.4% (95% CI: 1.9%–4.9%, $P < .001$) and 1.5% (95% CI: 0.6%–2.4%, $P = .002$) with every hour later that the serum was sampled, every unit increase in BMI, and every year of age, respectively. The sperm and hormone values of the 2 current smokers were well within the normal range of the total population.

Area Differences in Reproductive Parameters

With regard to the threshold values of subfertility suggested by Guzick et al (2001), 16% of the men in Peer and 10% of the men in Antwerp ($P = .6$, Fisher's exact test) had a sperm concentration of less than 13.5 x 10^6 cells/mL and 33% of the men in Peer vs 14% in Antwerp (Fisher's exact test $P = .03$) had a sperm morphology of less than 9% normal forms. The age-adjusted odds ratio for men living in Peer to have a sperm morphology value of less than 9% was 3.0 (95% CI: 1.1–8.7; $P = .04$). The differences in the average values for hormone and sperm parameters, after correction for confounding variables, are presented in Table 2. The uncorrected median (IQR) sperm concentration, sperm volume, total sperm count, and sperm morphology for Peer vs Antwerp were 45.1 (19.5–76.9) vs 49.2 (30.9–90.4) x 10^6/mL ($P = .18$), 2.8 (1.6–4.1) vs 3.1 (2.0–4.1) mL ($P = .67$), 116.0 (49.2–177.7) vs 131.2 (90.2–250.0) x 10^6 ($P = .12$), and 12.0 (6.5–16.0) vs 18.0 (11.5–24.5) ($P < .001$), respectively. Excluding those subjects who reported a previous serious illness or operation or including BMI as an extra confounding variable did not influence the statistics on regional differences in terms of free testosterone, sperm morphology or total sperm count.

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Table 2. *Cohort differences in semen parameters and hormone values, corrected for confounding variables*

None of the participants had LH or SHBG levels outside the laboratory reference ranges (1–12 IU/L and 11–71 nmol/L, respectively), while in Antwerp, 3 persons had FSH values above 12 IU/L. In Peer, 4 persons had free testosterone levels below 0.21 nmol/L, while 15 inhabitants of Peer and 5 of Antwerp had total testosterone levels below the reference value of 11.1 nmol/L (odds ratio (95% CI):

3.86 (1.28–11.6); *P* = .02). The latter area difference disappeared after correction for BMI, age, and time of day the blood was sampled (*P* = .5).

In the overall population (ie, two regions combined), sperm concentration and TSC were negatively correlated to FSH (*r* = -0.23, *P* = .02 and *r* = -0.20, *P* = .04, respectively) and positively correlated to inhibin B (*r* = 0.23, *P* = .02 and *r* = 0.28, *P* = .005, respectively) and the T:LH ratio (*r* = 0.25, *P* = .01 and *r* = 0.20, *P* = .05, respectively), whereas they did not correlate with T, E2 or the free fractions thereof. Furthermore, inhibin B correlated negatively with FSH (*r* = -0.32, *P* = .001) and positively with T (*r* = 0.24, *P* =.02), which together with f T correlated positively with LH (*r* = 0.25, *P* = .01 and *r* = 0.24, *P* = .02 respectively). Table 3 shows the correlation coefficients between the hormones and between the hormone and sperm parameters in the 2 populations. There was a general trend towards stronger correlations in Peer than in Antwerp. We further investigated a limited number of dose-effect relationships using multiple linear regression and including possible confounders (Table 4). Introducing the area of residence and an interaction term between the latter parameter and the main explanatory variable did not reveal any relevant area differences in the investigated relationships.

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Table 3: *Spearman's rank correlation coefficients (P-value) between the semen parameters and hormone values of subjects from Peer and Antwerp*

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Table 4. *Dose-effect relationships between hormone levels or between hormone and sperm quality measures in the whole population (Peer plus Antwerp)*

Using ROC curve analysis, we determined the efficiency of FSH, inhibin B, and the Inh:FSH and T:LH ratios to discriminate between fertile and subfertile men according to the above-described threshold values of subfertility. An area under the ROC curve (AUC) significantly greater than 0.5 (and ideally close to 1.0) indicates that the marker has the potential to discriminate between 2 groups. In categorizing men with sperm concentrations lower or higher than 13.5 x 10^6 cells/mL, the AUC (95% CI) for FSH, inhibin B, Inh:FSH, and T:LH were: 0.65 (0.55–0.74), *P* = .09; 0.69 (0.59–0.78), *P* = .007; 0.69 (0.59–0.78), *P* = .006; and 0.72 (0.62–0.80), *P* = .001, respectively. None of these hormone markers of testicular function had any significant power to discriminate between men with sperm morphology lower or higher than 9% normal forms. In addition, none of these markers could discriminate between men living in Peer or Antwerp, in contrast to both testosterone and (more importantly) free testosterone (AUC, 95% CI: 0.71 (0.61–0.80), *P* < .001 and 0.74 (0.64–0.82), *P* < .001, respectively).

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We investigated the reproductive capacity of a sample of community-dwelling young men at 2 sites in Flanders that were 80 km apart, with alleged differences in patterns of lifestyle and environmental factors. We found significantly lower total sperm counts and sperm morphologies, which were paralleled by lower free testosterone levels in the rural area of Peer than in the industrialized city of Antwerp. However, the levels of FSH and inhibin B and the Inh:FSH and T:LH ratios, all of which are considered to be markers of testicular function, were not different in the 2 populations.

The total sperm counts and sperm concentrations were more than 30% lower in Peer than in Antwerp. The regional sperm quality measures were uniformly distributed and the observed differences could not be explained by outlying observations (data not shown). Although, in accordance with the literature (Andersson et al, 2004b), inhibin B correlated significantly with both total sperm count and sperm concentration, its distribution in Peer overlapped completely with that in Antwerp (data not shown and Table 2). The testicular polypeptidic heterodimer inhibin B is the physiologically relevant circulating form of inhibin in men, constituting the afferent arm of the feedback loop to the pituitary and inhibiting the release of FSH (Hayes et al, 2001). In adulthood, normal inhibin B secretion is dependent upon both Sertoli cell function and the presence of germ cells. It has been proposed that inhibin B is in fact a joint product of Sertoli cells and germ cells of the perimeiotic pachytene to round spermatid stage, both in testes with normal spermatogenesis and in testes with spermatogenic arrest (Andersson et al, 1998; Marchetti et al, 2003). Partial spermatogenic arrest at or beyond the stage of meiosis would thus lead to decreased sperm output, without significantly affecting the inhibin B levels, as has been argued previously (Andersson et al, 1998; von Eckardstein et al, 1999).

High T levels in the interstitial and seminal tubules of the testes are indispensable for successful spermatogenesis (Sharpe, 1994). The levels of total and free testosterone were slightly lower in Peer than in Antwerp. Although peripheral testosterone levels cannot be used to infer intratesticular levels (Maddocks et al, 1993), it seems improbable that the slight decrease in serum testosterone levels in Peer is reflective of a drastically lower intratesticular testosterone concentration that would be needed to, by itself, cause a greater than 30% reduction in semen quality.

The importance of FSH action on sperm production has long been a matter of debate. The clinical and experimental data support a theoretical paradigm in which FSH exerts stimulatory effects on both the quantitative and qualitative aspects of testosterone-driven spermatogenesis (Sharpe, 1994; Moudgal and Sairam, 1998; Nieschlag et al, 1999; Plant and Marshall, 2001). This hormonal synergism implies that relatively moderate alterations in testosterone and FSH can have profound effects on the spermatogenetic cycle, as has been suggested previously for testosterone (Maddocks et al, 1993). In this respect, it is important to note that FSH was 17% lower in Peer than in Antwerp, although this did not reach the $P = .05$ level of significance. FSH has been described to affect spermiogenesis (Moudgal and Sairam, 1998; Tesarik et al, 1998) but how this relates to a possible effect on the traditional sperm morphology parameters remains unknown. Irrespective of the exact physiological mechanism, the observations of Skakkebaek et al (1973) of an increased level of spermatogenic arrest at the meiotic stage in infertile men presenting with both low sperm counts and poor morphology compared to controls with good semen quality suggest that in this disorder, both sperm quality measures go hand-in-hand, as is the case in the present study.

Jørgensen et al (2002) have reported on an east-west gradient in better sperm quality in the Nordic

Baltic region, which was not paralleled by differences in FSH, LH or testicular hormones, including inhibin B, and could not be explained by differences in confounding factors. However, the population that was recruited in these 4 countries was comprised of relatively young men, aged 18–19 years, which is an age category that may be less appropriate for the detection of potentially subtle hormonal differences. In a group of men aged 20–35 years, Jensen et al (1997) found a significant regional difference in sperm concentration, which was accompanied by higher inhibin B and lower FSH levels in the men with better sperm quality, suggestive of differences in testicular functioning in the 2 cohorts. Our data do not indicate that a testicular factor causes the observed lower semen quality status in the population in Peer. The T:LH ratio, which is a marker of Leydig cell sensitivity (Andersson et al, 2004a), was similar in both cohorts. Furthermore, an undefined testicular threat leading to impaired testosterone secretion logically would have resulted in increased compensatory LH levels, which was not seen in the present study. Together with the decreased FSH levels in Peer compared to those in Antwerp, these findings suggest the possibility of partial spermatogenic arrest at the spermatid level related to altered pituitary function (Morrow et al, 1986; Martin-du Pan and Campana, 1993). It might be suggested that an interfering factor at the pituitary level would logically also have resulted in lower LH levels in Peer. However, differences in LH between groups are generally more difficult to assess due to the high intraindividual variation of LH secretion.

We found a tendency towards stronger correlations (Table 3) in the region with the poorer sperm characteristics. However, in further investigations of a number of relevant relationships between the hormone and semen parameters (Table 4), no significant regional differences in the dose-effect curves were seen. Nevertheless, our data are in line with those of Andersson et al (2004a), who reported similar relationships to be stronger in 357 idiopathic infertile men compared to 318 men with proven fertility. Uhler et al (2003) have presented graphs from which strong associations of FSH with sperm concentrations of less than 40 x 10^6 cells/mL and sperm counts of less than 100 x 10^6/ejaculate can be deduced, with apparently less-pronounced relationships at higher semen values in normal couples. Finally, our data are in accordance with those of Morrow et al (1986), who have reported a positive relationship between testosterone and LH in subjects with mild hypospermatogenesis and germ cell arrest, which was different from those subjects with severe forms of seminiferous tubule failure, a finding that has been corroborated by other studies (Aafjes et al, 1977; Giagulli and Vermeulen, 1988).

Jensen et al (1997) found in two samples from the general population that an FSH level of more than 10 IU/L combined with an inhibin B of less than 80 pg/mL had 100% predictive power for having a sperm concentration of less than 20 x 10^6/mL. Only 8 persons in our population had FSH levels above 10 IU/L, and 3 of these subjects were oligospermic, while none of the participants had an inhibin B level less than 80 pg/mL. However, Vernaeve et al (2002) have concluded that inhibin B alone or in combination with serum FSH fails to predict the presence of sperm in men with non-obstructive azoospermia undergoing testicular sperm extraction. The Inh:FSH ratio has been described to be a better prognostic factor for infertility than either inhibin or FSH alone (Andersson et al, 2004b). Using ROC curve analysis, we found that inhibin B and the Inh:FSH ratio indeed had some power to discriminate between men with sperm concentrations less than 13.5 x 10^6/mL, which is the threshold of subfertility suggested by Guzick et al (2001). In addition, in our population, T:LH was a better discriminator of subfertility than Inh:FSH. Although the existence of an association of lesions of the seminiferous tubules with Leydig cell dysfunction has been questioned (Ruder et al, 1974), the T:LH ratio seems to correlate positively with sperm concentration and negatively with FSH (Andersson et al, 2004a), as was also found in the present study. Furthermore, the T:LH ratio has been suggested to be an independent parameter of infertility in infertile men with normal FSH (Giagulli and Vermeulen, 1988). However, none of these markers of testicular function could discriminate

between men living in Peer or Antwerp, in contrast to testosterone, and more importantly, free testosterone.

We could not avoid the presence of area differences in a number of possibly important confounding variables in the present study, so we corrected for these in the regression analysis, where appropriate. The men from Peer were slightly older than those from Antwerp. Older age is associated with decreased levels of free testosterone that are not adequately compensated for by a rise in LH secretion, although such age-related changes are usually seen after the age of 50 years (Kaufman and Vermeulen, 2005). Moreover, the neuroendocrine regulation of FSH is better preserved, meaning that the level of FSH generally increases with aging (Kaufman and Vermeulen, 2005), which contrasts with the lower FSH values in Peer.

It is thought that BMI lowers testosterone levels through an insulin-mediated decrease in SHBG production (Kaufman and Vermeulen, 2005). However, although the BMI values were significantly higher in Peer than in Antwerp, the SHBG levels were similar. Fat tissue possesses aromatase activity, which is usually measured by the T:E2 ratio, and obese men have been described to suffer from a mild hypogonadotropic hypogonadism, possibly related to increased estrogen-related negative pituitary feedback (Strain et al, 1982; Vermeulen et al, 1993). The T:E2 ratio was lower in Peer, which may be attributed to the decreased testosterone levels since the estrogen levels did not differ. Furthermore, BMI did not affect free testosterone or any sperm parameter, precluding BMI as a major explanatory factor for the observed differences in testosterone and semen.

In Peer, the men were investigated on average 2 hours later than in Antwerp. We accounted for differences in sampling hour using linear regression analyses, which is justifiable as for all hormones with diurnal secretory pattern, the decline during the time frame in which we operated is generally monotonic linear (Ahokoski et al, 1998; Carlsen et al, 1999). Moreover, the decline in testosterone concentration of 3.2% per hour is identical to the value reported by others (Andersson et al, 2004a).

Although not unusual for this type of study, an initial overall response rate of 30% may seem low, and thus, our study is at risk of selection bias, reducing the representative value of these sperm and hormone results for the total Belgian rural or urban population (Cohn et al, 2002). In particular, one cannot exclude the possibility that the observed differences are due to differential recruitment in both areas. If so, one would expect, in view of our present results, to see an overrepresentation of infertile men in Peer (Larsen et al, 1998). Whereas this contrasts with the higher percentage of people from this rural area with proven fertility compared to Antwerp, it does not exclude that the responders in Peer had an increased awareness of impaired fertility and thus were more willing to participate in a study without incentive (Larsen et al, 1998; Muller et al, 2004). In any case, even if confounding factors contributed to the observed regional semen quality differences, this does not alter the main conclusion that these differences are poorly reflected in the hormonal markers of testicular function.

It could be argued that the small sample size of the present study compromises the development of relationships between semen parameters and hormonal indices. The 28% regional difference in sperm concentration of 349 Danish men that was reported by Jensen et al (1997) was accompanied by a 28% lower average FSH level and a9% higher inhibin B level in the 187 men with better sperm quality. In view of the greater than 30% difference in semen quality found in the present study, post hoc analyses revealed that the present sample size of 50 men in each region yielded a statistical power of greater than 80% to detect between-cohort differences of 10% in inhibin B and 30% in all other hormones measured, including the T:LH ratio. Therefore, it would seem that in the present study,

meaningful differences in hormone values, if present, would most likely have been detected, which further calls into question the reliability of the investigated markers of testicular functioning as general predictors of spermatogenesis.

In conclusion, we found a greater than 30% regional difference in sperm quality measures, which was accompanied by lower testosterone and FSH levels, but which was not paralleled by altered inhibin B levels or T:LH and Inh:FSH ratios, which are considered to reflect testicular health. These results indicate that serum indices of testicular function or spermatogenesis that have been validated for use in clinical settings do not seem to be reliable surrogates for semen analysis in population studies.

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