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# AGE-RELATED DECLINE OF PLASMA BIOAVAILABLE TESTOSTERONE IN ADULT MEN

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**Summary**—Plasma bioavailable and total testosterone (T), gonadotropins (FSH, LH) and prolactin (PRL) were determined in 70 ambulatory men subdivided into 3 groups according to age: group I ($n = 22$; age 20–35 yr), group II ($n = 22$; age: 36–50 yr) and group III ($n = 26$; age 51–70 yr). Bioavailable T levels declined significantly with age ($r = -0.42$; $P < 0.01$) while those of total T decreased less significantly ($r = -0.28$; $P < 0.05$). In addition, the decrease of bioavailable T occurred earlier. FSH was shown to increase with age ($r = 0.41$; $P < 0.01$) whereas LH and PRL were not found to change significantly. Bioavailable T was correlated with total T ($r = 0.25$; $P < 0.05$) and inversely correlated with FSH ($r = -0.26$; $P < 0.05$). No correlation could be demonstrated between LH and either bioavailable or total T. In view of the age-related increase of sex hormone binding globulin, a fact generally observed in the literature, bioavailable T may be considered a more reliable index than total T for the evaluation of T production. Thus it may be concluded that the early decrease of bioavailable T in ambulatory men not known to have any pathology or any medication altering testicular function corresponds in fact to age-related decline of T secretion by the testes.

## INTRODUCTION

The influence of age on gonadal function in man has been extensively studied and currently available data suggest a progressive impairment of Leydig cell function with increasing age. Indeed, though the existence of a hyperplasia [1, 2] or an attrition [3] of Leydig cells in elderly men is still debated, the capacity of these cells to synthesise [4, 5] and to secrete androgens [6], and their responsiveness to choriogonadotropin (hCG) administration [7–13] were found to decline with advancing age. However, conflicting data were obtained regarding peripheral plasma levels of total testosterone (T), corresponding to protein-bound plus unbound fractions. Indeed, they were shown either to diminish [4, 5, 8–10, 12, 14–32] or to remain unchanged with advancing age [7, 11, 13, 33–37]. Conversely, the unbound fraction was demonstrated to decrease more constantly and even more significantly [9, 14, 16, 17, 20, 21, 24, 26–28, 30, 34, 38–40] yet some authors reported no age-related decrease of this fraction [11, 34]. Recently, it was found that the fraction of circulating T which may exert its effects in the target cells was not only confined to that not bound to plasma proteins but also to that bound to serum albumin [41]. Indeed the sum of free plus albumin-bound T, called non-SHBG bound or bioavailable T, was demonstrated to be the best index to evaluate androgen activity [42]. In addition, it was shown to decrease in elderly men in comparison with young men [20, 31, 43]. Since these studies concerned a limited number of subjects, particularly in the elderly group, it seemed worthy to evaluate plasma bioavailable T in large series of subjects aged between 20 and 70 yr. Total T as well as gonadotropins and prolactin were also determined and the interrelationships among these parameters were studied.

## EXPERIMENTAL

### *Subjects*

Seventy men with an age range of 20–70 yr were studied. All were ambulatory, non-obese, without any known endocrine, hepatic or renal disease and without any medication known to alter testicular function. Though most of the patients below 50-yr of age were complaining of infertility, their sperm analysis was normal. The subjects above 50-yr of age had potency problems.

According to age, the subjects were divided into three groups: group I (range: 20–35 yr; $n = 22$), group II (range: 36–50 yr; $n = 22$) and group III (range: 51–70 yr; $n = 26$).

Because of circannual and circadian rhythms of plasma T in men [44] blood samples were collected on EDTA between the end of September and the end of November at 8–10 a.m. Plasma was separated after centrifugation and kept frozen at −20°C until analyzed.

### *Methods*

#### *Determination of non-SHBG bound testosterone*

Bioavailable T was determined according to a modification of the technique of Tremblay and

Table 1. Evaluation of the precision

| | Inter-assay variability | | | | Inter-assay variability | | | |
|---|---|---|---|---|---|---|---|---|
| | n | Mean (ng/ml) | SD | CV (%) | n | Mean (ng/ml) | SD | CV (%) |
| Bioavailable testosterone | 11 | 0.95 | 0.07 | 7.4 | 7 | 0.55 | 0.05 | 9.8 |
| | 12 | 1.80 | 0.12 | 6.7 | — | — | — | — |
| | 9 | 2.20 | 0.12 | 5.5 | 7 | 2.45 | 0.14 | 5.7 |
| Total testosterone | 5 | 1.08 | 0.05 | 4.6 | 30 | 1.02 | 0.10 | 9.8 |
| | 5 | 2.18 | 0.18 | 8.3 | 30 | 2.10 | 0.20 | 9.5 |
| | 5 | 5.50 | 0.42 | 7.6 | 30 | 4.70 | 0.50 | 10.6 |
| | 5 | 10.20 | 0.88 | 8.7 | — | — | — | — |

Dube[45] with the previously described products and reagents [46]. To 0.5 ml aliquot of plasma was added dropwise 0.5 ml of cold saturated solution of ammonium sulfate. The precipitated SHBG-bound T was separated by centrifugation and an aliquot of 0.2 ml of the supernatant was extracted with 2 ml hexane/chloroform (4:1, v/v). The aqueous phase was then frozen and the solvent transferred to a clean tube and evaporated to dryness under a stream of nitrogen. The residue was redissolved with 1 ml ethanol. Duplicate 0.1 ml aliquots were pipetted for RIA which was performed with an $^{125}$I-labelled tracer as already described for saliva T [46] except that the antiserum was used at 1/50,000 dilution. Separation of the bound and unbound fractions was performed with dextran-coated charcoal.

Concerning the specificity, the antiserum obtained in rabbits injected with testosterone-3(*O*-carboxymethyl)oxime-bovine serum albumin displayed significant cross-reaction particularly with 5-dihydrotestosterone (DHT) (51.4%) [46]. However, this interference in bioavailable T assay can be considered as negligible since in men approximately 60% of circulating DHT is bound to SHBG [47] and thus are precipitated simultaneously with the protein by ammonium sulfate at 50% saturation. in fact, when bioavailable T was assayed ($n = 20$) either directly or after Celite microcolumn chromatography [48], the calculated regression line between the levels obtained with the direct technique ($Y$) and those yielded by the chromatographic technique ($x$) was: $Y = (1.009 \pm 0.062)x + (0.063 \pm 0.164)$. The slope and the intercept with the ordinate axis were not significantly different from 1 and 0 respectively. Intra- and interassay variabilities were evaluated at different levels and the results shown on Table 1 proved that the technique was highly reproducible.

*Determination of total testosterone*

Plasma total T was radioimmunoassayed with a non-chromatographic technique including an extraction step only. Plasma was extracted with the same solvent mixture as that used for the extraction of bioavailable T.

Chromatography was omitted since the results obtained with the direct technique were comparable to those yielded when plasma extracts were submitted to chromatography on Celite microcolumns [48]. The equation of the calculated regression lines between the results obtained without chromatography ($Y$) and those with chromatography ($x$) was: $Y = (0.995 \pm 0.038)x + (0.283 \pm 0.192)$. Thus the slope and the intercept with the ordinate axis of the regression line were not significantly different from 1 and 0 respectively. Intra- and interassay variabilities at different levels are shown on Table 1.

*Determination of gonadotropins and prolactin*

FSH, LH and prolactin (PRL) were determined by immuno-radiometric assays (BioMérieux, Marcy-l'Etoile, France). Intra- and interassay variabilities were—for FSH: 2.8 and 7.5% respectively at the level 2.3 mIU/ml and 2.4 and 3.0% respectively at the level 10.4 mIU/ml; for LH: 6.3 and 7.7% respectively at the level 2.1 mIU/ml and 2.6 and 9.1% respectively at the level 10.7 mIU/ml; for PRL: 2.8 and 8.6% respectively at the level 6.7 ng/ml and 1.8 and 9.7% respectively at the level 20 ng/ml.

The normal range (5th and 95th percentiles) in adult men with normal sperm counts and aged between 20 and 50 yr is—FSH: 1.0–5.0 mIU/ml; LH: 0.8–4.0 mIU/ml; prolactin: 1–15 ng/ml.

*Statistical analysis*

Results were expressed as the arithmetic mean ± SD. After checking that the data were sampled from gaussian population, by the Kolmogorov–Smirnov test, the group means were compared, by analysis of variance and Student's $t$-test. When the variation could be hypothesized to be unidirectional, one-tailed tests were applied for group comparisons.

## RESULTS

*Variations of plasma levels with age*

Plasma total and bioavailable T, FSH and LH levels for each individual are shown in relation to age in Fig. 1. Table 2 shows the same data with means for the three age groups.

Concerning total T, the slope of the regression line: $[y = (0.028 \pm 0.011)x + (7.508 \pm 0.539)]$ was statistically significant but total T decreases mildly with age ($r = -0.28$; $P < 0.05$) (Table 3). In comparison with group I, the means of groups II and III were significantly lower ($P < 0.05$ and $P < 0.01$ respectively). No

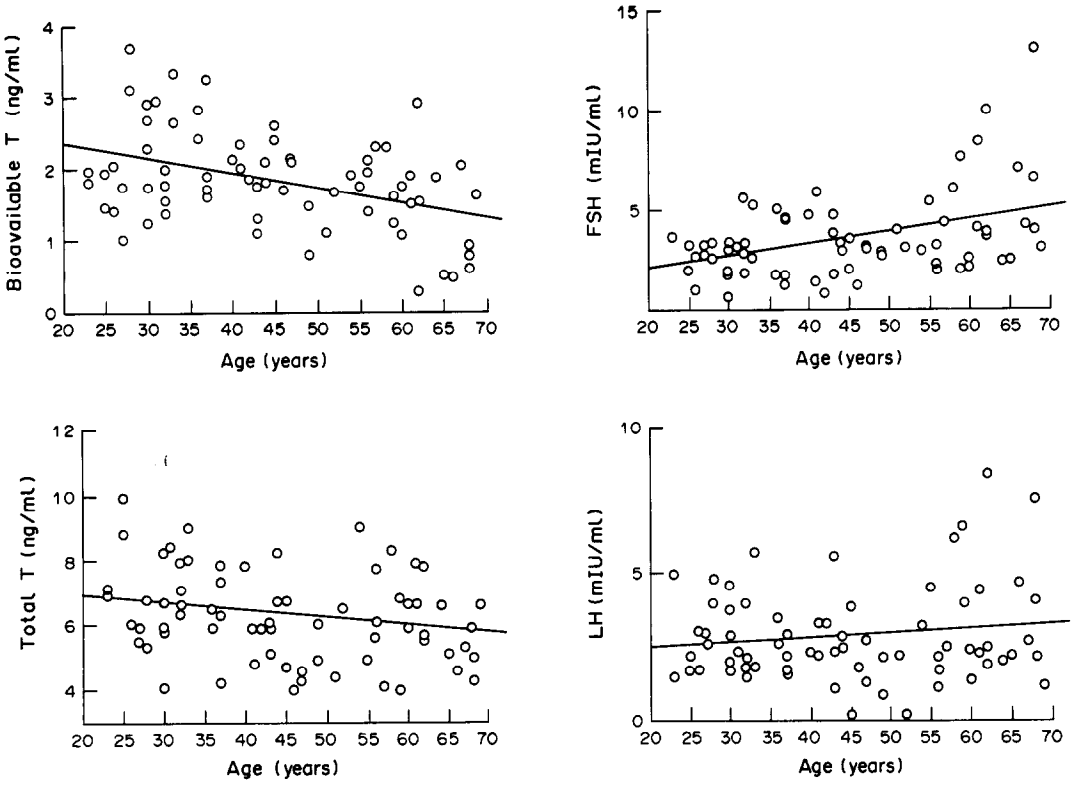


Fig. 1. Plasma levels of bioavailable testosterone, total testosterone, FSH and LH in men of various ages. The solid line is drawn from least squares linear regression.

significant difference could be demonstrated between groups II and III.

By contrast, bioavailable T declines more significantly with age ($r = -0.42$; $P < 0.01$) (Table 3) and the slope of the regression line, $[y = (-0.021 \pm 0.005)x + (2.781 \pm 0.255)]$, was significantly different from 0 ($P < 0.001$). As shown in Table 3, the mean level of group I was not different from that of group II but significantly higher than that of group III ($P < 0.01$). Similarly, the mean of group II was higher than that of group III ($P < 0.01$).

Concerning FSH levels a significant increase with age was demonstrated ($r = 0.41$; $P < 0.01$) and the slope of the regression line, $[y = (0.061 \pm 0.017)x + (0.832 \pm 0.783)]$, was significantly different from 0 ($P < 0.001$). No difference could be demonstrated between the mean levels of groups I and II while that of group III was significantly higher than those of the groups I and II ($P < 0.01$ and $P < 0.001$ respectively) (Table 3).

Conversely, the slope of the regression line calculated between LH levels and age was not signi-

Table 2. Plasma levels (mean ± SD) of total testosterone (Total T), bioavailable testosterone (Bio. T), FSH, LH and prolactin (PRL) in three groups of men of different ages

| Group | Age (yr) | $n$ | Total T (ng/ml) | Bio. T (ng/ml) | FSH (mIU/ml) | LH (mIU/ml) | PRL (ng/ml) |
|---|---|---|---|---|---|---|---|
| I | 20–35 | 22 | 6.9 ± 1.4[a] (4.1–9.9)[b] | 2.1 ± 0.7 (1.0–3.7) | 2.9 ± 1.2 (0.6–3.6) | 2.9 ± 1.3 (1.5–5.0) | 5.6 ± 3.0 (2.0–11.0) |
| II | 36–50 | 22 | 5.9 ± 1.2 (4.2–8.2) | 2.0 ± 0.6 (0.8–3.2) | 3.0 ± 1.5 (0.8–5.0) | 2.4 ± 1.1 (0.2–5.6) | 5.7 ± 2.6 (2.0–11.0) |
| III | 51–70 | 26 | 6.0 ± 1.3 (4.0–9.0) | 1.5 ± 0.6 (0.3–2.9) | 4.6 ± 2.5 (2.1–10.0) | 3.2 ± 2.1 (0.2–8.4) | 4.8 ± 2.0 (1.0–10.0) |

[a]Mean ± SD; [b]range.

Table 3. Correlation matrix of plasma total testosterone (Total T), bioavailable testosterone (Bio T), FSH, LH and prolactin (PRL) levels

| | Total T | Bio. T | FSH | LH | PRL |
|---|---|---|---|---|---|
| Age | −0.28** | −0.42* | 0.41* | 0.14[a] | −0.16[a] |
| Total T | | 0.25** | −0.08[a] | 0.04[a] | 0.13[a] |
| Bio. T | | | −0.26** | −0.09[a] | 0.20[a] |
| FSH | | | | 0.70* | −0.08[a] |
| LH | | | | | 0.04[a] |

*$P < 0.01$; **$P < 0.05$; [a]Not significant.

ficantly different from 0: [$y = (0.016 \pm 0.014)x + (2.175 \pm 0.646)$] and the means of the 3 groups were not statistically different.

Similarly, PRL levels were not found to change throughout the period studied. The regression line was: $y = (-0.028 \pm 0.021)x + (6.598 \pm 1.013)$ and the slope was not significantly different from 0. The means of the 3 groups were similar.

*Interrelationships between the studied parameters*

Plasma bioavailable T levels were correlated with those of total T ($r = 0.25$; $P < 0.05$) and inversely correlated with FSH concentrations ($r = -0.26$; $P < 0.05$) (Table 3). Besides, a positive and highly significant correlation was found between FSH and LH levels ($r = 0.70$, $P < 0.01$). It is noteworthy that LH was correlated neither with total nor with bioavailable T. Prolactin was not correlated with any of the parameters studied.

## DISCUSSION

As already shown by O'Connor *et al.*[49], the supernatant obtained after precipitation of SHBG with a 50% saturated ammonium sulfate solution corresponds to the fraction of T bound to transcortin, that bound to albumin and the free fraction. The technique described here does not include a preliminary equilibration of the plasma sample with labelled T [41, 42, 45, 49–51]. In this step which may last up to 2 h [51], highly pure tritiated T has to be used so that regular chromatographic purification with high resolution systems has to be performed [51]. In fact, traces of non-binding impurities in the tracer may induce important errors [38]. Moreover, in these methods using tritiated T a percentage is calculated and the amount of non-SHBG bound T is obtained by referring to total T whereas in the present highly sensitive technique bioavailable T could be determined directly in the supernatant obtained after selective precipitation of the SHBG-bound fraction and centrifugation.

Because SHBG affinity is higher at low temperatures Loric *et al.*[51] performed the ammonium sulfate precipitation step at 37°C. In other studies, the temperature at which this step was carried out was not reported [41, 45, 49, 50] yet Cumming and Wall[42] added cold ammonium sulfate to the plasma sample refrigerated by crushed ice. It is noteworthy that the data reported by Loric *et al.*[51] in normal women did not seem to be different from those found by Cumming and Wall[42]. In any case, SHBG precipitation as well as the subsequent centrifugation are much more controllable at low temperature.

In this study single blood samples were collected because the result of T determination obtained from a single sample was shown to be as good as the mean obtained from three samples taken at 15- [52] or 30-min intervals [21]. Moreover, the application of the multiple-sampling procedure [53], which was recommended because of the pulsatile pattern of plasma T in man, is unpractical and uncomfortable for the patient.

The mean plasma levels of bioavailable T found in the men of group I and II (below 50-yr of age) are similar to those observed by other authors in the same age-matched healthy subjects [20, 41, 43, 49]. This finding is consistent with the data of Corker *et al.*[54] concerning plasma total T levels in men attending a sub-fertility clinic.

However, the present results found in men below 50 years of age are 3-fold higher than Tenover *et al.*[31] data. In fact, while the same methodology was generally used, selective precipitation of SHBG with ammonium sulfate at a final concentration of 50% [41, 43, 49], Tenover *et al.*[31] calculated non-SHBG bound T from the molar concentration of total T and SHBG. Bartsch[20] has also obtained non-SHBG bound T fraction by calculation, yet his data agreed with the present results.

In the subjects of the third group (above 50-yr of age) bioavailable T levels are lower than the data of Bartsch[20] in age-matched men yet they are rather similar to the findings observed in above 65-yr old subjects [20, 43].

Plasma bioavailable T levels were shown to decline with advancing age and this is consistent with the data displaying a significant decrease between elderly and young men [20, 31, 43]. However, the decrease was found to be significant only after 66-yr of age by Bartsch[20] while in this study it was found to occur much earlier, namely in the fifties. A similar progressive decline of free T was also reported [9, 16, 17, 21, 24, 26–28, 30, 35, 38–40] yet the correlation coefficient reported by Vermeulen *et al.*[38] between apparent free T index and age, $r = -0.55$, appears to be a little higher than that, $r = -0.42$, found here between bioavailable T and age. This variance might be due to the large age range (up to 85 yr) studied by these authors.

Such a decline of plasma free T was not, however, always observed [11, 34]. Similarly, salivary T which is considered as a good reflection of plasma unbound T fraction [55] was comparable in young and old subjects [36]. Thus it seems that there is no consensus concerning age-related changes of free T and this has also been observed for total T. These discrepancies were thought to be related to the time of blood sampling and indeed loss or alteration of the circadian rhythm of serum T was demonstrated to occur in elderly men [27, 30, 56–59] so that the difference between young and old men was significant in the morning and not in the afternoon. Physical fitness, sexual activity, health status as well as environmental factors were also claimed to account for these conflicting results [37]. Other factors such as the high inter-individual variability and the age range of the studied subjects might also be implicated. In fact, subjects over 70-yr old were either not included or represented a limited number in several studies

[11, 13, 20] while the decrease of T becomes significant after the seventh decade [16, 27, 30]. In this study where men over 70 yr were not included mean total T levels were found to decrease significantly between group I and II and to remain rather unchanged thereafter. This finding might be accounted for by the rather elevated levels observed in group I in comparison with our previous data [46, 60]. The decrease with age was rather slight and the value of the correlation coefficient between total T and age was lower than that observed between age and bioavailable T. It was very similar to that reported by others [21, 22].

In any case, total T plasma levels cannot reflect reliably Leydig cell secretion capacity in elderly subjects since there is a concomitant age-related reduction of the production rate and of the metabolic clearance rate of testosterone [14, 17]. The decrease of the clearance rate might be related to SHBG increase [15, 17, 25, 26, 28, 34, 35, 61] but whether the latter is the consequence of increased estrogen production is still debated since plasma estradiol was reported either to increase [9, 15, 17, 22, 40, 61] or to remain unchanged [11, 12, 16, 24–27, 30, 34, 63] or even to decrease [7] with age.

In contrast, the decrease of plasma free and bioavailable T reflects not only the decrease of testicular production but also the increase of SHBG. Indeed, when both total and free T were determined concomitantly, the decline of the latter was more significant than that of the former [16, 26–28, 30, 40] and occurred earlier [16, 27, 30]. This is consistent with our findings.

Bioavailable as well as total T were significantly correlated with FSH and this agrees with Rubens *et al.*[9] and Davidson *et al.*[26] data. Moreover, it is noteworthy that the correlation coefficient demonstrated here was similar to that reported by Davidson *et al.*[26]. Conversely the fact that neither bioavailable nor total T were correlated with LH does not agree with literature data [9, 16, 26]. This variance might be due to the non-significant change of LH levels in relation to age at least until 70 yr while 42 out of 220 subjects studied by Davidson *et al.*[26] were above 70-yr old. Indeed according to their data, LH levels disclose a steep rise only after 70-yr of age and this is consistent with other findings [16, 17, 64]. Conversely, no age-dependent change of LH was observed according to either single plasma levels [33] or 24-hr mean plasma concentrations [24].

Finally the finding of a weak correlation between bioavailable and total T is in variance with the data showing a close parallelism between free and total T levels [21, 24]. The reason of this discrepancy does not appear to be clear.

In conclusion, though there is no consensus concerning the age-related change of either total or free plasma T levels, the increase of SHBG with advancing age seems to be generally accepted. In these conditions the decrease of T production in senescence would be blunted if it is evaluated through total T determination. This drawback is circumvented with bioavailable T determination which appears to be the best index to evaluate androgenicity. Indeed this study has shown that bioavailable T declined more significantly with age than did mean total T levels and that this decrease occurred earlier.

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## REFERENCES

1. Kothari L. K. and Gupta A. S.: Effects of ageing on the volume, structure and total Leydig cell content of the human testis. *Int. J. Fertil.* **19** (1974) 140–146.
2. Honoré L. H.: Ageing changes in the human testis: a light-microscopic study. *Gerontology* **24** (1978) 58–65.
3. Kaler L. W. and Neaves W. B.: Attrition of the human Leydig cell population with advancing age. *Anat. Rec.* **192** (1978) 513–518.
4. Takahashi J., Higashi Y., LaNasa J. A., Yoshida K.-I., Winters S. J., Oshima H. and Troen P.: Studies of the human testis. XVIII. Simultaneous measurement of nine intratesticular steroids: evidence for reduced mitochondrial function in testis of elderly men. *J. Clin. Endocr. Metab.* **56** (1983) 1178–1187.
5. Hammar M.: Impaired *in vitro* testicular endocrine function in elderly men. *Andrologia* **17** (1985) 444–449.
6. Serio M., Gonnelli P., Borrelli D., Pampaloni A., Fiorelli G., Calabresi E., Forti G., Pazzagli M., Mannelli M., Baroni A., Giannotti P. and Giusti G.: Human testicular secretion with increasing age. *J. Steroid Biochem.* **11** (1979) 893–897.
7. Longcope C.: The effect of human chorionic gonadotropin on plasma steroid levels in young and old men. *Steroids* **21** (1973) 583–592.
8. Nieschlag E., Kley H. K., Wiegelmann W., Solbach H. G. and Krüskemper H. L.: Lebensalter und endokrine funktion der testes des erwachsenen mannes. *Dtsch. Med. Wschr.* **98** (1973) 1281–1284.
9. Rubens R., Dhont M. and Vermeulen A.: Further studies on Leydig cell function in old age. *J. Clin. Endocr. Metab.* **39** (1974) 40–45.
10. Nawata H., Kato K.-I. and Ibayashi H.: Age-dependent change of serum 5α-dihydrotestosterone and its relation to testosterone in man. *Endocr. Jap.* **24** (1977) 41–45.
11. Harman S. M. and Tsitouras P. D.: Reproductive hormones in aging men. I. Measurement of sex steroids, basal luteinizing hormone, and Leydig cell response to human chorionic gonadotropin. *J. Clin. Endocr. Metab.* **51** (1980) 35–40.
12. Nankin H. R., Lin T., Murano E. P. and Osterman J.: The aging Leydig cell: III. Gonadotropin stimulation in men. *J. Androl.* **2** (1981) 181–189.
13. Nieschlag E., Lammers U., Freischem C. W., Langer K. and Wickings E. J.: Reproductive functions in young fathers and grandfathers. *J. Clin. Endocr. Metab.* **55** (1982) 676–681.
14. Vermeulen A., Rubens R. and Verdonck L.: Testosterone secretion and metabolism in male senescence. *J. Clin. Endocr. Metab.* **34** (1972) 730–735.
15. Pirke K. M. and Doerr P.: Age related changes and interrelationships between plasma testosterone, oestradiol and testosterone-binding globulin in normal adult males. *Acta Endocr. (Copenh.)* **74** (1973) 792–800.
16. Stearns E. L., MacDonnell J. A., Kaufman B. J., Padua R., Lucman T. S., Winter J. S. D. and Faiman C.: Declining testicular function with age. *Am. J. Med.* **57** (1974) 761–766.
17. Baker H. W. G., Burger H. G., De Kretser D. M., Hudson B., O'Connor S., Wang C., Mirovics A.,

Court J., Dunlop M. and Rennie G. C.: Changes in the pituitary-testicular system with age. *Clin. Endocr.* **5** (1976) 349–372.
18. Lewis J. G., Ghanadian R. and Chisholm G. D.: Serum 5-dihydrotestosterone and testosterone changes with age in man. *Acta Endocr. (Copenh.)* **82** (1976) 444–448.
19. Vermeulen A. and Verdonck L.: Radioimmunoassay of 17β-hydroxy-5α-androstan-3-one, 4-androstene-3,17-dione, dehydroepiandrosterone, 17-hydroxyprogesterone and progesterone and its application to human male plasma. *J. Steroid Biochem.* 7 (1976) 1–10.
20. Bartsch W.: Interrelationships between sex hormone-binding globulin and testosterone, 5α-dihydro-testosterone and oestradiol-17β in blood of normal men. *Maturitas* **2** (1980) 109–118.
21. Dai W. S., Kuller L. H., LaPorte R. E., Gutai J. P., Falvo-Gerard L. and Caggiula A.: The epidemiology of plasma testosterone levels in middle-aged men. *Am. J. Epidemiol.* **114** (1981) 804–816.
22. Drafta D., Schindler A. E., Stroe E. and Neacsu E.: Age-related changes of plasma steroids in normal adult males. *J. Steroid Biochem.* **17** (1982) 683–687.
23. Murono E. P., Nankin H. R., Lin T. and Osterman J.: The aging Leydig cell. V. Diurnal rhythms in aged men. *Acta Endocr. (Copenh.)* **99** (1982) 619–623.
24. Zumoff B., Strain G. W., Kream J., O'Connor J., Rosenfeld R. S., Levin J. and Fukushima D. K.: Age variation of the 24-hour mean plasma concentrations of androgens, estrogens, and gonadotropins in normal adult men. *J. Clin. Endocr. Metab.* **54** (1982) 534–538.
25. Winters S. J. and Troen P.: Episodic luteinizing hormone (LH) secretion and the response of LH and Follicle-stimulating hormone to LH-releasing hormone in aged men: evidence for coexistent primary testicular insufficiency and an impairment in gonadotropin secretion. *J. Clin. Endocr. Metab.* **55** (1982) 560–565.
26. Davidson J. M., Chen J. J., Crapo L., Gray G. D., Greenleaf W. J. and Catania J. A.: Hormonal changes and sexual function in aging men. *J. Clin. Endocr. Metab.* **57** (1983) 71–77.
27. Deslypere J. P. and Vermeulen A.: Leydig cell function in normal men: Effect of age, life-style, residence, diet, and activity. *J. Clin. Endocr. Metab.* **59** (1984) 955–962.
28. Royer G. L., Seckman C. E., Schwartz J. H., Bennett K. P. and Hendrix J. W.: Relationship between age and levels of total, free, and bound testosterone in healthy subjects. *Curr. Ther. Res.* **35** (1984) 345–353.
29. Moroz E. V. and Verkhratsky N. S.: Hypophyseal-gonadal system during male aging. *Arch. Geront. Geriat.* **4** (1985) 13–19.
30. Vermeulen A. and Deslypere J. P.: Testicular endocrine function in the ageing male. *Maturitas* 7 (1985) 273–279.
31. Tenover J. S., Matsumoto A. M., Plymate S. R. and Bremner W. J.: The effects of aging in normal men on bioavailable testosterone and luteinizing hormone secretion: Response to clomiphene citrate. *J. Clin. Endocr. Metab.* **65** (1987) 1118–1126.
32. Blackman M. B., Weintraub B. D., Rosen S. W. and Harman S. M.: Comparison of the effects of lung cancer, benign lung disease, and normal aging on pituitary-gonadal function in men. *J. Clin. Endocr. Metab.* **66** (1988) 88–95.
33. Sköldefors H., Carlström K. and Eneroth P.: Influence of aging upon the serum hormone levels in the male. *Int. J. Androl.* 1 (1978) 308–316.
34. Sparrow D., Bosse R. and Rowe J. W.: The influence of age, alcohol consumption, and body build on gonadal function in men. *J. Clin. Endocr. Metab.* **51** (1980) 508–512.
35. Purifoy F., Koopmans L. H. and Mayes D. M.: Age differences in serum androgen levels in normal adult males. *Hum. Biol.* **53** (1981) 499–511.
36. Nieschlag E., Freischem C. W., Wickings E. J. and Schürmeyer Th.: A new look at testicular functions in old age. In *Recent Advances in Male Reproduction: Molecular Basis and Clinical Implications* (Edited by R. D'Agata, M. B. Lipsett, P. Polosa and H. J. van der Molen). Raven Press, New York (1983) pp. 145–149.
37. Tsitouras P. D.: Effects of age on testicular function. *Endocr. Metab. Clin.* **16** (1987) 1045–1059.
38. Vermeulen A., Stoïca T. and Verdonck L.: The apparent free testosterone concentration, an index of androgenicity. *J. Clin. Endocr. Metab.* **33** (1971) 759–767.
39. Vermeulen A. and Verdonck L.: Some studies on the biological significance of free testosterone. *J. Steroid Biochem.* **3** (1972) 421–426.
40. Pirke K. M. and Doerr P.: Age related changes in free plasma testosterone, dihydrotestosterone and oestradiol. *Acta Endocr. (Copenh.)* **80** (1975) 171–178.
41. Manni A., Pardridge W. M., Cefalu W., Nisula B. C., Bardin C. W., Santner S. J. and Santen R. J.: Bioavailability of albumin-bound testosterone. *J. Clin. Endocr. Metab.* **61** (1985) 705–710.
42. Cumming D. C. and Wall S. R.: Non-sex hormone-binding globulin-bound testosterone as a marker for hyperandrogenism. *J. Clin. Endocr. Metab.* **61** (1985) 873–876.
43. Nankin H. R. and Calkins J. H.: Decreased bioavailable testosterone in aging normal and impotent men. *J. Clin. Endocr. Metab.* **63** (1986) 1418–1420.
44. Reinberg A., Lagoguey M., Chauffournier J.-M. and Cesselin F.: Circannual and circadian rhythms in plasma testosterone in five healthy young parisian males. *Acta Endocr. (Copenh.)* **80** (1975) 732–743.
45. Tremblay R. R. and Dube J. Y.: Plasma concentrations of free and non-TeBG bound testosterone in women on oral contraceptives. *Contraception* **10** (1974) 599–605.
46. Nahoul K., Rao L. V. and Scholler B.: Saliva testosterone time-course response to hCG in adult normal men. Comparison with plasma levels. *J. Steroid Biochem.* **24** (1986) 1011–1015.
47. Dunn J. F., Nisula B. C. and Rodbard D.: Transport of steroid hormones: Binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma. *J. Clin. Endocr. Metab.* **53** (1981) 58–68.
48. Nahoul K., Bournique B., Adeline J. and Scholler R.: Radioimmunoassay of 5-androstene-3β,17β-diol in plasma and in breast cyst fluid. *J. Steroid Biochem.* **24** (1986) 835–842.
49. O'Connor S., Baker H. W. G., Dulmanis A. and Hudson B.: The measurement of sex steroid binding globulin by differential ammonium sulphate precipitation. *J. Steroid Biochem.* **4** (1973) 331–339.
50. Stumpf P. G., Nakamura R. M. and Mishell D. R.: Changes in physiologically free circulating estradiol and testosterone during exposure to levonorgestrel. *J. Clin. Endocr. Metab.* **52** (1981) 138–143.
51. Loric S., Guéchot J., Duron F., Aubert P. and Giboudeau J.: Determination of testosterone in serum not bound by sex-hormone-binding globulin: diagnostic value in hirsute women. *Clin. Chem.* **34** (1988) 1826–1829.
52. Brain J., Langevin R., D'Costa M., Sanders R. M. and Hucker S.: Serum pituitary and steroid hormone levels in the adult male: one value is as good as the mean of three. *Fert. Steril.* **49** (1988) 123–126.
53. Goldzieher J. W., Dozier T. S., Smith K. D. and Steinberger E.: Improving the diagnostic reliability of rapidly fluctuating plasma levels by optimized multiple-sampling techniques. *J. Clin. Endocr. Metab.* **43** (1976) 824–830.
54. Corker C. S., Davidson D. W. and Edmond P.: Plasma testosterone levels in men attending a sub-fertility clinic. *J. Steroid Biochem.* **9** (1978) 375–376.
55. Wang C., Plymate S., Nieschlag E. and Paulsen C. A.:

Salivary testosterone in men: further evidence of a direct correlation with free serum testosterone. *J. Clin. Endocr. Metab.* **53** (1981) 1021–1024.
56. Marrama P., Carani C., Baraghini G. F., Zini D., Celani M. F. and Montanini V.: Circadian rhythm of testosterone and prolactin in the ageing. *Maturitas* **4** (1982) 131–138.
57. Bremner W. J., Vitiello M. V. and Prinz P. N.: Loss of circadian rhythmicity in blood testosterone levels with aging in normal men. *J. Clin. Endocr. Metab.* **56** (1983) 1278–1281.
58. Montanini V., Simoni M., Chiossi G., Baraghini G. F., Velardo A., Baraldi E. and Marrama P.: Age-related changes in plasma dehydroepiandrosterone sulphate, cortisol, testosterone and free testosterone circadian rhythms in adult men. *Horm. Res.* **29** (1988) 1–6.
59. Tenover J. S., Matsumoto A. M., Clifton D. K. and Bremner W. J.: Age-related alterations in the circadian rhythms of pulsatile luteinizing hormone and testosterone secretion in healthy men. *J. Geront. Med. Sci.* **43** (1988) M163–M169.
60. Scholler R., Nahoul K., Castanier M., Rotman J. and Salat-Baroux J.: Testicular secretion of conjugated and unconjugated steroids in normal adults and in patients with varicocele. *J. Steroid Biochem.* **20** (1984) 203–215.
61. Kley H. K., Nieschlag E., Bidlingmaier F. and Krüskemper H. L.: Possible age-dependent influence of estrogens on the binding of testosterone in plasma of adult men. *Horm. Metab. Res.* **6** (1974) 213–215.
62. Maruyama Y., Aoki N., Suzuki Y., Sinohara H. and Yamamoto Y.: Variation with age in the levels of sex-steroid-binding plasma protein as determined by radioimmunoassay. *Acta Endocr. (Copenh.)* **106** (1984) 428–432.
63. Scholler R., Roger M., Grenier J., Castanier M., Nahoul K. and Tea N. T.: Les oestrogènes dans l'exploration de la fonction testiculaire. In *Les Oestrogènes, Rapports Présentés à la XIVe Réunion Des Endocrinologistes de Langue Française*, Hôpital Cochin, Masson, Paris (1977) pp. 181–196.
64. Blackman M. R.: Pituitary hormones and aging. *Endocr. Metab. Clin.* **16** (1987) 981–994.