The decline of serum testosterone levels in community-dwelling men over 70 years of age: descriptive data and predictors of longitudinal changes.

Short title: The decline of androgens in elderly men.

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Abstract.

**Objective:** This study was designed to assess longitudinal changes in serum T levels, explore relationships with aging, genetic-, health- and lifestyle-related factors and investigate predictors of changes in healthy elderly men.

**Design:** Population-based, longitudinal, 4-year observational study in 221 community-dwelling men aged 71 - 86 years at baseline.

**Methods:** Hormone levels assessed by immunoassay, anthropometry, questionnaires on general health, genetic polymorphisms. Predictors of changes in T levels explored using linear mixed-effects modeling for longitudinal analyses.

**Results:** TT, FT and BioT levels decreased with aging, decreases in BioT being most marked. No changes in SHBG or E_2, while LH and FSH levels increased during follow-up. Subjects who gained weight displayed a greater decline in TT levels, mainly due to decreasing SHBG levels. However, baseline body composition was not predictive for subsequent changes in T levels. Baseline E_2 (P=.023-.004), LH (P=.046-.005) and FSH (P<.002) levels were independently positively associated with a faster decline in T fractions, although only FSH remained significant when adjusting for baseline T (P=.041-.035). Carriers of a “TA”-haplotype of the ERα PvuII and XbaI polymorphisms displayed a slower decline of TT and BioT (P=.041-.007).

**Conclusions:** In elderly men with already low serum T levels, a further decline was observed, independent from baseline age. The identification of FSH levels as a predictor of this decline appears to reflect the testicular mechanisms of aging-related changes in T production, whereas associations with E_2 and ERα polymorphisms are suggestive for estrogen-related processes, possibly related to changes in the neuroendocrine regulation of T production.
Abbreviations: T, testosterone; TT, total testosterone; FT, free testosterone; BioT, bio-available testosterone; SHBG, sex hormone binding globulin; E₂, estradiol; LH, luteinizing hormone; FSH, follicle stimulating hormone; ERα, estrogen receptor alpha gene.
Introduction.

It is now widely accepted that normal chronological aging in men is associated with a progressive decline in androgen levels. More than 20% of healthy men over 60 years of age present with serum levels below the reference range for young men. In contrast to women, who experience a rather precipitous and profound reduction of endogenous estradiol (E2) production during menopausal transition, the decline of testosterone (T) production in aging men is much more gradual and individually highly variable. This decline results from both testicular changes and altered neuroendocrine regulation of luteinizing hormone (LH) secretion (1). Between the 3rd and the 8th decade of life, a 20 to 30% decrease in population mean serum T levels can be observed. As serum levels of sex hormone binding globulin (SHBG) increase with age, the decrease in serum free T (FT) and non-SHBG bound T levels, often referred to as bioavailable T (BioT), is of even greater magnitude reaching up to 50% over the same age range (2-5).

Understanding the physiology of this decline is important because T has actions in many tissues and low serum T levels have been associated with a variety of adverse conditions, which comprise increased risk of diabetes mellitus and impaired glucose tolerance, reduced bone and muscle mass, increased (abdominal) fat mass, impaired sexual function, decreased quality of life and even increased mortality risk (1, 6-8). These features resemble the symptoms observed in young hypogonadal men, and in accordance with this view, treatment of hypogonadism in both young and older men with low serum T may result in an improvement of some of these conditions (1, 9). However, a causal relationship between these clinical changes and the age-related decrease of T levels remains a matter of debate. Indeed, many of these conditions are
non-specific, multifactorially determined and often associated with normal aging *per se* (1). Up to now, the risk-benefit ratio for testosterone supplementation in elderly men has not been established (10).

While chronological aging *per se* seems to be associated with declining T production, co-morbid chronic illnesses and certain medications as well as the aging-related changes in body composition may accentuate and contribute to these changes (1, 4, 11, 12). Furthermore, lifestyle-related factors such as smoking may also influence T levels (2, 7, 11). In addition, the possibility of genetic polymorphisms influencing this decline cannot be excluded (13). Most information about changes in T levels with aging results from cross-sectional studies (2, 3, 5, 7, 14, 15), while information from longitudinal studies remains more limited (4, 16-19). Moreover, it should be noted that most studies report on T levels of middle-aged men and relatively few studies have focused specifically on men older than 70 yrs, whereas many of the adverse conditions potentially associated with the age-related decline of T levels occur relatively late in life.

In this population-based longitudinal study we followed a homogenous group of 221 community-dwelling elderly men, aged 71 - 86 years at baseline, for a period of 4 years. We report on the distribution and changes of gonadal steroid and gonadotropin concentrations. Using mixed-effects modeling, predictors of longitudinal changes in androgen levels were explored.

**Subjects and methods.**
Study subjects.

Subjects were recruited from the population registry of the semi-rural community of Merelbeke (Belgium). A sample of 352 community-dwelling men, aged 71 - 86 years, agreed to participate (initial participation rate was 47.1%). This longitudinal, population-based study was specifically designed to investigate the process of aging, focusing on hormonal changes, bone metabolism and body composition in elderly men at yearly intervals over a period of 4 years. The study was approved by the ethics review board of the Ghent University Hospital (Belgium). All participants gave written informed consent for participation in this study and completed questionnaires pertaining to general health (Geriatric Depression Scale, Short Form-36 and Rapid Disability Rating Scale-2 (20)), dietary habits and physical activity. Baseline characteristics, exclusion and inclusion criteria have been described extensively in previous publications (20-22). Following exclusions because of past or current history of disorders or treatments known to affect androgen status, 221 eligible subjects were included in the longitudinal analyses, for a total of 912 observations. Five serial measurements were available in 125 subjects, for the remaining subjects 2 to 4 data points were available. Reasons for loss to follow-up were institutionalization, withdrawal of consent, death and occurrence of diseases or treatments known to affect androgen levels. All exclusions were censored before hormonal analysis. Longitudinal changes in BioT over 4yrs in 214 men of this longitudinal cohort have previously been shown in figure 1 of the review article under reference 1.

Hormonal assays.
Each consecutive year, venous blood was obtained between 0800 h and 1000 h a.m. after overnight fasting and serum was stored at -80°C until batch analysis. Blood collection was completed over a period of 2 months during summer, except for the first year when blood collection was performed from March till June. Commercial immunoassays were used to determine serum levels of total T, FSH and LH (Medgenix®, Fleurus, Belgium), estradiol (E2) (Clinical Assay®, DiaSorin s.r.l., Saluggia, Italy; according to a modified protocol that doubles the serum amount (22)), SHBG (Orion Diagnostica, Espoo, Finland), insulin (Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden), insulin-like growth factor-1 (IGF-1), IGF-binding protein 3 (IGF-BP3) (Diagnostic Laboratory Systems Inc., Webster, TX, USA) and leptin (Linco Research Inc., St. Louis, MO, USA). T and SHBG levels were determined at all visits, whereas E2, LH and FSH were assessed at the first and last study visit. All samples from the same subject were assayed in a single assay run using duplicate measurements. The intra- and interassay coefficients of variation (CV) were 4.8% and 5.1% for T and 3.6% and 6.6% for SHBG, respectively. Intra- and inter-CV for all other measurements were below 10% and 15%, respectively. Serum non-SHBG bound T (“bioavailable T”; BioT), free testosterone (FT), non-SHBG bound E2 (“bioavailable E2”; BioE2) and free E2 (FE2) were calculated from serum total T, total E2, SHBG and albumin concentrations using a previously validated equation derived from the mass action law (23, 24).

_Determination of gene polymorphisms._
Genomic DNA was extracted from EDTA-treated blood using a commercial kit (Qiagen Midi Kit®, QiagenInc, CA, USA).

**Androgen receptor gene (AR) CAG-repeat length.** As previously described (21), PCR to amplify exon 1 of the AR gene with primers 5’AGCCTGTTGAACCTTCTTGAGC3’ (sense) and 5’CTGCCTTTACACACTCCTTGGC3’ (antisense). After ethanol precipitation, the amplified fragment was directly sequenced on a ABI Prism 310 sequencer (ABI Prism®, Perkin-Elmer Applied Biosystems, CA, USA), using BigDye Terminator Cycle Sequencing Reaction Kit (ABI Prism®, Perkin-Elmer Applied Biosystems, CA, USA). Fragment length size was determined running GeneScan-400HD Analysis Software (ABI Prism®, Perkin-Elmer Applied Biosystems, CA, USA).

**CYP19 gene (TTTA)ₙ-repeat length.** As previously described (22), (TTTA)ₙ-repeat length was assessed by fragment analysis of the PCR products using primers published by Haiman et al.(25). The forward primers was 5’-labeled with a fluorescent dye for automated fragment analysis on a ABI Prism 310 sequencer (ABI Prism®, Perkin-Elmer Applied Biosystems, CA, USA), using BigDye Terminator Cycle Sequencing Reaction Kit (ABI Prism®, Perkin-Elmer Applied Biosystems, CA, USA). To confirm repeat length, homozygotic representatives of the different observed allele lengths (7, 8, 9, 10, 11, 12 and 13) were sequenced. The allelic variant consisting of (TTTA)₇-repeats was the most frequently observed in this population of healthy elderly men. Subjects were classified in three genotype groups according to the presence of the shortest allele length.

**Estrogen receptor alpha gene (ERα).** Both XbaI and PvuII ERα single nucleotide polymorphisms (SNP’s) were determined by a single polymerase chain reaction fragment in accordance with a study by Yamada et al. (26). The amplified products were then digested with
the XbaI and PvuII restriction enzymes (New England Biolabs Inc., Beverly, MA, USA). Haplogenotypes were constructed using Phase software (27). Subjects were then defined by the presence of a “TA”-haplotype.

Body composition.

At each visit study subjects had their body weight measured to the nearest 0.1 kg on a calibrated balance scale in light indoor clothing without shoes. Height was measured to the nearest 0.1 cm after removal of shoes. Body mass index (BMI) was calculated as the weight (kg) divided by the height in m squared (m²). Bioelectrical impedance analysis (Bodystat 1500®, Bodystat Ltd., Isle of Man, UK) was used to estimate fat and lean mass percentage. The CV was 1.3% and 0.5% for fat mass and lean mass percentage, respectively, as calculated from duplicate measurements in 15 study subjects.

Statistical analysis.

Cross-sectional analyses. Continuous variables were described in terms of mean ± standard deviation (SD) if their distribution was normal according to the Kolmogorov-Smirnov test, and in terms of median, first and third quartile otherwise. Bivariater partial correlations, adjusting for age and BMI or fat mass percentage, were used to show associations between hormonal and various clinical parameters. SPSS 12.0 software package (SPSS Inc., Chicago, IL, USA) was used for the descriptive analyses and bivariate correlations; a P value of <0.05 was considered to indicate statistical significance; all P values were two-tailed.
Longitudinal analyses. Linear mixed-effects models with random intercepts and autoregressive residual correlation structure were used for longitudinal analysis. Using likelihood ratio tests, no evidence of random time slopes was found. Parameters for baseline predictors were estimated via restricted maximum likelihood estimation and model-based confidence intervals and $P$-values are reported. No adjustments for baseline T levels were performed when it was anticipated that these levels could be affected by the considered exposure (e.g. genetic polymorphism, smoking,...) (28). All analyses were performed in the software package SAS 9.1.3, Service Pack 4 (SAS Institute, Inc., Cary, NC, USA).

Results.

Baseline clinical and hormonal characteristics.

Baseline clinical characteristics of the study population are described in Table 1 (n= 218; 3 subjects excluded from baseline data because of temporary use of medication). Mean age at baseline was 74 yr (1\textsuperscript{st} and 3\textsuperscript{rd} quartile: 73-78 yr), 40 out of 218 (18.3\%) were current smokers, 32 (20.1\%) consumed > 2 alcoholic beverages per day, 36 (16.5\%) were obese (BMI $\geq$ 30 kg/m$^2$), 80 (36.7\%) reported the use of at least 3 prescription medications and 71 (32.6\%) reported to be physically active for > 2 hours per week. After 4 years of follow-up, 20 out of 152 (13.2\%) were current smokers, 16 (10.5\%) consumed > 2 alcoholic beverages per day, 23 (15.1\%) were obese and 78 (51.3\%) reported the use of at least 3 prescription medications. More men than at baseline, i.e. 95 (62.5\%), reported to be physically active for > 2 hours per week.
Median length of the AR CAG-repeat polymorphism was 21 (1st - 3rd quartile: 20-24). Sixty (28.8%) subjects had 7 (TTTA)-repeats of the CYP19 on both alleles, 97 (46.6%) had only one allele with 7 (TTTA)-repeats and in 51 (24.5%) subjects the number of (TTTA)-repeats exceeded 7 on both alleles. Regarding the ERα polymorphisms, 72 (33.0%) subjects were homozygous for the “TA”-haplotype, 105 (48.5%) had only one allele with the “TA”-haplotype and 41 (18.8%) had no “TA”-haplotype.

Age at baseline was negatively associated with FT, BioT ($\rho = -0.20$ to -0.22; $P < 0.01$), and positively with SHBG, LH and FSH levels ($\rho = 0.13$ to 0.27; $P < 0.05$). In the age range of our study participants (71 - 86 yrs), mean cross-sectional changes of -0.66% [95% C.I. -1.77; 0.44%], 1.15% [95% C.I. -2.11; -0.41%], and 1.76% [95% C.I. -2.75; -0.77%] per year were observed for TT, FT and BioT, respectively. After controlling for age, BMI was negatively associated with TT, FT, BioT and SHBG ($\rho = -0.14$ to -0.30; $P < 0.05$), and positively with FE2 and BioE2 levels ($\rho = 0.15; P < 0.05$). Both absolute (kg) and relative (%) fat mass displayed a negative association with all three T fractions ($\rho = -0.23$ to -0.33; $P < 0.001$). Absolute fat mass was negatively associated with SHBG levels ($\rho = -0.24; P < 0.001$). After controlling for absolute fat mass, insulin levels were negatively associated with SHBG levels ($\rho = -0.15; P = 0.016$) and positively with BioE2 levels ($\rho = 0.15; P = 0.025$). Controlling for total body weight, both IGF-1 and IGF-BP3 were negatively associated with SHBG levels ($\rho = -0.21$ to -0.29; $P < 0.01$), IGF-BP3 displayed a positive association with FT and BioT levels ($\rho = 0.14$ to 0.16; $P < 0.05$). As reported previously, serum leptin levels were negatively correlated with all three T fractions after adjusting for absolute fat mass (29).
No significant differences in sex steroid or SHBG levels between current smokers, past smokers or subjects who never smoked were found (all $P > 0.129$). Among current or past smokers, the number of pack-year smoked was inversely correlated with both FT ($\rho = -0.20; P = 0.011$) and BioT ($\rho = -0.20; P = 0.009$) after controlling for age and BMI. As reported previously, no associations between hormone or SHBG levels and AR CAG-repeat length (21) or CYP19 TTTA-repeat length (22) were observed in this elderly population. Neither for the ERa XbaI and PvuII SNP’s, nor for the combined haplogenotypes were differences in hormone levels detected (all $P > 0.32$; data not shown).

Evolution of hormonal parameters over time.

Table 2 presents descriptive statistics for number of participants, age and BMI at all visits, as well as for T and SHBG levels. During the 4-year follow-up, 69 subjects left the study. Dropout analysis using logistic regression showed that dropout was associated with lower FT levels and a lower score of functional capacity and mental status at their last visit, lower physical activity scores at baseline and the “TA,X” haplogenotype of the ERa (data not shown).

The evolution of TT and BioT levels is displayed in Figure 1. Mean TT levels declined from 444 ± 148 to 414 ± 157 ng/dL over the 4-year period. The greatest decrease was observed between visit 1 and 2, which was reflected in SHBG levels with generally higher levels at visit 1 compared to visit 2. The evolution of FT and BioT showed a more gradual decline (mean levels at visit 1 were 7.4 ± 2.1 and 173 ± 51 ng/dL vs. 6.9 ± 2.2 and 153 ± 50 ng/dL at visit 5 for FT and BioT, respectively). Longitudinal data analysis, adjusting for baseline age and BMI, revealed a yearly mean decrease of -9.5 ng/dL [95% C.I. -12.7; -6.2 ng/dL], -0.16 ng/dL [95% C.I. -0.21; -0.11] for FT and BioT, respectively.
-0.10 ng/dL] and -6.1 ng/dL [95% C.I. -7.3; -4.9 ng/dL] for TT, FT and BioT, respectively. This corresponds to yearly mean percentage decreases of -1.26% [95% C.I. -2.58; -0.01], -1.33% [95% C.I. -2.61; -0.01] and -2.43% [95% C.I. -3.78; -1.08] for TT, FT and BioT, respectively. No significant overall changes in SHBG levels were detected (data not shown).

Percentages of participants with T levels below the reference range for young men (320 ng/dL for TT, 6.5 ng/dL for FT and 140 ng/dL for BioT, respectively (1)) are displayed for all visits in Figure 2. At baseline, 19.3%, 32.1% and 25.7% of participants presented with levels below this reference range for TT, FT and BioT, respectively. At visit 5, this rose to 30.3%, 43.4% and 38.8%, respectively.

Over the 4-year follow-up period, a modest decrease in BMI was noted (mean Δ = -0.10 ± 1.37 kg/m²). After adjusting for baseline age, subjects presenting with an increase in BMI (> 0 kg/m²: n = 70 or 42.2%) presented a greater decline in TT levels compared to those with a decrease in BMI (< 0 kg/m²: n = 96 or 57.8%): -58.4 ng/dL vs. -17.9 ng/dL, respectively (P = 0.019). No differences in changes of FT or BioT levels were noted between these groups (P > 0.16). This reflected the mean decrease of SHBG levels in subjects presenting with an increase in BMI, compared to increasing SHBG levels in subjects with a decrease in BMI: -1.7 nmol/L vs. +2.8 nmol/L, respectively (P = 0.015). Additional adjustment for baseline T or SHBG levels did not alter these findings (data not shown).

Baseline predictors of changes in T and SHBG levels (Table 3).

Lifestyle- and health-related factors, body composition, insulin and IGF-1. Age at baseline was not associated with the evolution of T or SHBG levels over time (all P > 0.50; data not
shown), neither were indicators of quality of life, psychological and functional well-being, medical history or number of medications (either as a continuous variable or as more or less than three prescribed medications), physical activity or frequency of alcoholic consumptions at baseline (all $P > 0.20$; data not shown). Adjusting for baseline age and BMI, current or former smoking at baseline was associated with a faster yearly decline of FT levels with 0.13 ng/dL ($P = 0.037$) compared to subjects who never smoked; similar trends for TT ($P = 0.12$) and BioT ($P = 0.09$) did not reach statistical significance. These observations held true when additionally adjusting for baseline T levels (data not shown). Amongst smokers, the number of pack year smoked was not associated with changes in T levels ($P > 0.50$).

BMI at baseline was not associated with changes in T or SHBG levels, neither was absolute nor relative fat or lean mass. There was no difference in rate of change of T levels between subjects who were obese (BMI $> 30$ kg/m$^2$) and/or overweight (BMI $> 25$ kg/m$^2$) at baseline compared to those who were not. Baseline glucose, insulin, leptin, IGF-1 or IGF-BP3 levels were not associated with changes in T or SHBG levels (all $P > 0.33$; data not shown).

**Sex steroids and gonadotropins.** After controlling for age and BMI, baseline E$_2$, LH and FSH levels were associated with a faster decline in T levels over time. However, for E$_2$ and LH this association was no longer significant after adjusting for the predictive effects of baseline T levels, whereas a significant trend was still observed for higher baseline FSH levels being associated with a faster decline in T levels (of 0.40 ng/dL, 0.007 ng/dL and 0.15 ng/dL per IU/L increase in FSH levels for TT, FT and BioT, respectively). The evolution of BioT levels associated with median, upper and lower quartiles of baseline FSH levels is illustrated in *Figure 3*. No associations with the changes in SHBG levels were found (data not shown).
Genetic polymorphisms. Neither the CAG-repeat length of the AR nor the TTTA-repeat length of the CYP19 gene were associated with subsequent changes in TT, FT, BioT or SHBG levels (data not shown). The polymorphisms of the ERα were significantly associated with the evolution of T levels over time. Specifically, after controlling for baseline age and BMI, a slower yearly average decline of TT levels was observed with 19.0 ng/dL ($P = 0.009$) for carriers of the (“TA,TA”) haplogenotype and with 15.0 ng/dL ($P = 0.024$) for carriers of the (“TA,X”) haplogenotype as compared to those carrying the (“X,X”) haplogenotype. Similarly, a slower yearly average decline of BioT levels was observed with 7.6 ng/dL ($P = 0.007$) for carriers of the (“TA,TA”) haplogenotype and with 5.2 ng/dL ($P = 0.041$) for carriers of the (“TA,X”) haplogenotype, as compared to those carrying the (“X,X”) haplogenotype. For FT, a significantly slower decline was found for carriers of the (“TA,TA”) haplogenotype (of 0.26 ng/dL per year; $P = 0.026$) as compared to those carrying the (“X,X”) haplogenotype. This indicates a slower decline in T levels in carriers of a “TA”-allele compared to subjects without a “TA”-allele. None of the genetic polymorphisms were associated with changes in SHBG levels (data not shown). Adjusting for the predictive effects of baseline T or E₂ levels did not alter these observations (data not shown).

Discussion.

In this study we present 4-year longitudinal data on testosterone levels in community-dwelling elderly men and discuss predictors of the observed changes. We demonstrated that even in elderly subjects with already decreased T levels, a further decline in T levels can still be
observed. Furthermore, in accordance with previous reports (4-7, 18) the decrease is least apparent for TT and most accentuated for calculated BioT levels, due to the relatively stable SHBG levels. Consistent with the results of the Massachusetts Male Aging Study (MMAS), the observed longitudinal changes in T levels were of greater magnitude than the baseline cross-sectional trends (4). In addition, in these elderly men the magnitude of these longitudinal changes was comparable with three previous longitudinal studies covering a broader age range (4, 16, 19), and slightly higher than the results obtained by the Baltimore Longitudinal Study on Aging (BLSA) and by Zmuda et al. (17, 18).

In our cross-sectional analyses, age was negatively associated with FT and BioT and positively with SHBG, LH and FSH levels. These results are consistent with those reported in other studies (7, 15). However, within the considered older age group, age at baseline was not predictive for subsequent changes in T or SHBG levels, indicating a decrease in T production over time which is independent of age. This is in agreement with results of the Multiple Risk Factor Intervention Trial (MRFIT study) (17) and with the previously discussed observation of a similar magnitude of decline among different populations and age ranges, but at variance with the results of the BLSA, where age at baseline was negatively associated with TT levels over time (18). Further, health status at baseline neither was predictive for longitudinal changes in T or SHBG levels. However, the inclusion criteria we applied yielded a population of healthy elderly men, and it can therefore not be concluded that co-morbidity is not an important determinant of T levels (1). Consistent with previous findings (3, 7, 12, 15), BMI at baseline was negatively associated with TT, FT, BioT and SHBG levels. Moreover, those who gained weight over the 4-year period presented a greater decline in TT, explained by a decrease in SHBG levels, compared to those with a decrease in BMI. These results suggest that changes in BMI over time modulate age-
related changes in TT and SHBG, but in our study population neither FT nor BioT levels were affected. This is in agreement with the findings of the BLSA and MMAS in elderly men (18, 30) and the results of the CARDIA Male Hormone Study in a younger population (31).

However, in this study no baseline body composition parameters (BMI, fat or lean mass as assessed by bio-electrical impedance) were predictive for subsequent changes in T or SHBG levels. This is consistent with the results of the MRFIT study (17), but in contrast with the results from the larger BLSA- and MMAS-cohorts, where obesity at baseline predicted a greater decline over time in TT and SHBG, and even in FT levels in the MMAS, (12, 18). It remains unclear whether body composition influences sex steroid hormone concentrations over time or whether, conversely, sex steroid hormone concentrations alter body composition; most likely this relationship is bidirectional.

Regarding the influence of genetic polymorphisms considered in this study, we observed an association of $ER\alpha$ polymorphisms with changes in T levels, indicating a slower decline in T levels in carriers of a “TA”-allele compared to non-carriers. At variance with the longitudinal results from Krithivas et al. (13), no effect of $AR$ CAG-repeat length on the evolution in T levels was found. However, it should be noted that their study population was clearly younger with a time interval of 8 years between visits, which could result in a greater absolute decline in T levels.

In this study, baseline $E_2$, LH and FSH levels were predictive for a subsequent decline in T levels. However, when controlling for baseline T levels, only FSH levels remained significantly associated with a decline in T levels. This observation could be explained by the fact that both in case of primary hypogonadism and of aging in healthy men, the rise of FSH levels is steeper than
that of LH levels (4, 16, 32), and by the greater ultradian variability of LH levels. Moreover, we have already reported on the negative association between testicular volume and FSH levels in this elderly population, with FSH levels being an independent determinant of total testicular volume (33). As a consequence, in elderly patients presenting with relatively high FSH levels despite having low-normal serum T levels, biochemical follow-up in the next years may be considered.

As a whole, these findings point towards a substantial testicular factor in the observed decline in T production, whereas the association with E2 levels and the \( ER\alpha \) polymorphisms is suggestive for estrogen-related processes and might be related to changes in the neuroendocrine regulation of T production in elderly men that are characterized by relative deficiency of LH secretion (1). Indeed, E2 levels did not change in our study population, despite the overall decline in T levels, which suggests increased aromatase activity (34), and circulating E2 is known to be a major player in LH negative feedback regulation in men (35). Nevertheless, a comparative study in young and older men of the effects of aromatase inhibition has failed to disclose a greater restraining action of E2 on LH and T levels in the elderly as compared to young men (36).

Notwithstanding the observation of a clear age-related decline in serum T levels, this does not necessarily mean that the same holds true at tissue level. Bélanger et al. already suggested that the observed decline in androgen levels could be partially compensated by an increase in steroid-converting enzymes in peripheral tissues, especially the 5\(\alpha\)-reductase activity resulting in tissue dihydrotestosterone levels unaffected by aging (14). On the other hand, there is also evidence of decreased androgen tissue concentrations (1) and of reduced androgen sensitivity, with reports of decreased numbers of androgen receptor in various tissues in elderly men (37, 38). However, up
till now no practical and clinically useful biological marker of androgen activity at tissue level is available. Therefore, one has to further rely on serum T levels as an indirect and less than optimal parameter of androgen activity at tissue level.

This is the first study to report on yearly-based, longitudinal changes in T levels in a fairly homogenous population of community-dwelling men over 70 years of age at baseline, with 20% of subjects over 80 years. Notably, this age group is often underrepresented in the available literature but is steadily increasing in the general population. The strength of this study is the yearly time interval for longitudinal assessment of serum T levels and it is a population- rather than clinic- or complaint-based study. Moreover, most men were in relatively good state of health, suggesting that the studied longitudinal changes largely reflect effects related to aging per se; adjusting for illnesses and medication produced little, if any, change in the observed overall effects of aging. Mixed-effects modeling analysis for longitudinal data treats every measurement as a separate outcome, but accounts for the interdependence of repeated measurements from the same individual. Finally, all samples from the same subject were analyzed in a single assay run, eliminating the possibility of within-subject variation due to inter-assay variability.

A limitation of our study is that it is based on single time-point hormonal measurements for each visit. Nevertheless, it has been shown previously that single time-point estimates are a valid approach for population studies (39). Further, bio-electrical impedance analysis has been shown to have a suboptimal accuracy and is limited by various factors for assessing body composition, which could possibly mask associations of baseline body composition with subsequent changes in T levels. Another possible concern are the clearly higher TT and SHBG levels at visit 1.
compared to the subsequent visits. This could point towards a selection bias, with dropout between visit 1 and 2 of subjects with higher T levels. However, dropout analysis showed that dropout was associated with lower T levels at the previous visit. While mixed-effects model analyses naturally allow for dropout to be associated with the outcome history (40), and thus with lower T levels at the previous visit, we could not account for the possibility that drop-out was associated with higher/lower T levels at the current (or future) visits. Another hypothesis could be a seasonal effect on T levels, since blood sampling at visit 1 was mainly performed from March till June, whereas for the subsequent visits this was done from July till August. However, from the study by Brambilla et al., it appeared that seasonal variation is unlikely to be an important source of variation in serum sex steroid levels in men (41). Finally, the present study was performed in a well-characterized and specific subset of the population consisting of generally healthy men of high age with a relatively short follow-up period, and the results should therefore not be extrapolated to the general population. In the general population, factors related to co-morbidity are likely to accentuate the observed age-related hormonal changes.

In conclusion, a decline in serum T levels, especially in the non SHBG-bound and free T fractions, was shown to occur in generally healthy community-dwelling men aged 71 - 86 years with already decreased T levels compared to healthy young men. Moreover, this decline appeared to be independent of age at baseline, indicating a rather constant decline in T production with aging. Further, subjects who gained weight during follow-up displayed a greater decline in TT and SHBG levels, whereas baseline body composition was not predictive for subsequent changes in T levels. The predictive value of FSH levels appears to confirm the involvement of testicular changes as an important factor for the decline in T production and
might warrant future follow-up in case of low-normal T levels. In addition, the predictive effects of E\textsubscript{2} and the $ER\alpha$ polymorphisms suggest a contribution of estrogen-related processes in the decline of serum T levels, which might relate to the known involvement of changes in the neuroendocrine regulation of T production in elderly men.

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**References**


35. Raven G, de Jong FH, Kaufman JM & de Ronde W. In men peripheral estradiol levels directly reflect the action of estrogens at hypothalamo-pituitary level to inhibit gonadotropin secretion. *Journal of Clinical Endocrinology and Metabolism* 2006 jc-.


Table Legend

Table 1: Clinical characteristics of the study population (n = 218) at baseline.

footnote:

* = non-Gaussian distribution; data presented as median [1st - 3rd quartile]. At baseline, 3 subjects were additionally excluded for temporary use of medications affecting androgen status.
Table 1:

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)*</td>
<td>74.0 [73 - 78]</td>
<td>71 - 86</td>
</tr>
<tr>
<td>Heighth (m)</td>
<td>1.679 ± 0.063</td>
<td>1.500 - 1.880</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>74.4 ± 11.4</td>
<td>49.5 - 113.0</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.3 ± 3.4</td>
<td>18.4 - 35.2</td>
</tr>
<tr>
<td>Fat mass (%)</td>
<td>29.2 ± 3.6</td>
<td>20.7 - 40.9</td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>21.8 ± 5.0</td>
<td>12.7 - 41.6</td>
</tr>
<tr>
<td>Lean mass (kg)</td>
<td>52.5 ± 7.8</td>
<td>31.7 - 77.4</td>
</tr>
<tr>
<td>Insulin (IU/L)*</td>
<td>6.1 [4.0 - 9.3]</td>
<td>0.4 - 40.0</td>
</tr>
<tr>
<td>Leptin (ng/mL)*</td>
<td>6.0 [3.8 - 9.5]</td>
<td>1.0 - 37.3</td>
</tr>
<tr>
<td>IGF-1 (ng/dL)</td>
<td>200 ± 83</td>
<td>20 - 480</td>
</tr>
<tr>
<td>IGFBP-3 (ng/mL)</td>
<td>2500 ± 530</td>
<td>750 - 3260</td>
</tr>
</tbody>
</table>
Table Legend:

Table 2: Evolution of age, BMI, number of participants and hormonal levels over time.

footnote:

Data presented as estimated mean ± SD, unless * = non-Gaussian distribution; data presented as median [1st - 3rd quartile]. ng/dL may be converted to nmol/L by multiplying by 0.0347 for testosterone and to pmol/L by multiplying by 36.76 for estradiol. NA = not available.
Table 2:

<table>
<thead>
<tr>
<th>Visit</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>218</td>
<td>207</td>
<td>178</td>
<td>157</td>
<td>152</td>
</tr>
<tr>
<td>Age (yrs)*</td>
<td>74 [73 - 78]</td>
<td>75 [73 - 79]</td>
<td>76 [74 - 79]</td>
<td>77 [75 - 80]</td>
<td>78 [76 - 81]</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.3 ± 3.4</td>
<td>26.4 ± 3.5</td>
<td>26.4 ± 3.5</td>
<td>26.4 ± 3.5</td>
<td>26.4 ± 3.5</td>
</tr>
<tr>
<td>SHBG (nmol/L)*</td>
<td>43 [34 - 56]</td>
<td>42 [33 - 54]</td>
<td>43 [34 - 56]</td>
<td>43 [33 - 57]</td>
<td>44 [34 - 58]</td>
</tr>
<tr>
<td>Testosteron (ng/dL)</td>
<td>444 ± 143</td>
<td>409 ± 135</td>
<td>410 ± 145</td>
<td>414 ± 141</td>
<td>414 ± 148</td>
</tr>
<tr>
<td>Free T (ng/dL)</td>
<td>7.4 ± 2.0</td>
<td>6.9 ± 1.9</td>
<td>6.7 ± 1.9</td>
<td>7.0 ± 2.1</td>
<td>6.9 ± 2.1</td>
</tr>
<tr>
<td>Bio T (ng/dL)</td>
<td>173 ± 50</td>
<td>162 ± 46</td>
<td>158 ± 47</td>
<td>155 ± 48</td>
<td>153 ± 49</td>
</tr>
<tr>
<td>Estradiol (ng/dL)</td>
<td>2.05 ± 0.55</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>2.13 ± 0.58</td>
</tr>
<tr>
<td>Free E₂ (ng/dL)</td>
<td>0.034 ± 0.008</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0.036 ± 0.009</td>
</tr>
<tr>
<td>Bio E₂ (ng/dL)</td>
<td>1.31 ± 0.34</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>1.31 ± 0.36</td>
</tr>
<tr>
<td>LH (IU/L)</td>
<td>6.2 [4.3 - 8.7]</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>8.3 [4.8 - 9.2]</td>
</tr>
<tr>
<td>FSH (IU/L)</td>
<td>9.0 [6.3 - 16.2]</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>10.3 [6.9 - 17.8]</td>
</tr>
</tbody>
</table>
Table Legend:

Table 3: Baseline predictors of subsequent changes in T levels, using linear mixed-effects modeling for longitudinal data analysis.

footnote:

$\beta^*$ = estimates for the interaction terms (baseline variables x year), indicating whether the time evolution depends on baseline variables. Values of $\beta^*$ indicate how much the yearly average evolution in T levels changes per unit increase in the baseline predictor value. All models are adjusted for baseline age and BMI. $^*$ Models additionally adjusted for baseline testosterone levels. * Models with baseline TE2, FE2 and BioE2 for TT, FT and BioT, respectively.
Table 3:

<table>
<thead>
<tr>
<th>Baseline predictors</th>
<th>TT</th>
<th>FT</th>
<th>BioT</th>
<th>TT</th>
<th>FT</th>
<th>BioT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \beta^* )</td>
<td>95%CI</td>
<td>( P )</td>
<td>( \beta^* )</td>
<td>95%CI</td>
<td>( P )</td>
</tr>
<tr>
<td>Yearly average change</td>
<td>-9.5</td>
<td>[-12.7; -6.2]</td>
<td>&lt;0.001</td>
<td>-0.16</td>
<td>[-0.21; -0.10]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ER haplogenotypes$^*: )</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-(“TA,TA”)</td>
<td>+19.0</td>
<td>[4.9; 33.2]</td>
<td>0.009</td>
<td>+0.26</td>
<td>[0.03; 0.50]</td>
<td>0.026</td>
</tr>
<tr>
<td>-(“TA,X”)</td>
<td>+15.0</td>
<td>[2.0; 28.0]</td>
<td>0.024</td>
<td>+0.18</td>
<td>[-0.04; 0.39]</td>
<td>0.102</td>
</tr>
<tr>
<td>-(“X,X”)</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Current &amp; former smokers</td>
<td>-6.26</td>
<td>[-14.11; 1.60]</td>
<td>0.118</td>
<td>-0.13</td>
<td>[-0.26; -0.01]</td>
<td>0.037</td>
</tr>
<tr>
<td>Never smokers</td>
<td>0</td>
<td>-</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Calcium intake</td>
<td>+0.013</td>
<td>[0.0004; 0.025]</td>
<td>0.044</td>
<td>+0.0003</td>
<td>[0.00009; 0.0005]</td>
<td>0.005</td>
</tr>
<tr>
<td>( E_2^* )</td>
<td>-7.13</td>
<td>[-13.34; -1.12]</td>
<td>0.021</td>
<td>-7.23</td>
<td>[-13.47; -0.99]</td>
<td>0.023</td>
</tr>
<tr>
<td>( E_2^# )</td>
<td>+4.58</td>
<td>[-4.34; 13.50]</td>
<td>0.314</td>
<td>+0.07</td>
<td>[-0.07; 0.22]</td>
<td>0.321</td>
</tr>
<tr>
<td>LH</td>
<td>-0.78</td>
<td>[-1.32; -0.24]</td>
<td>0.005</td>
<td>-0.010</td>
<td>[-0.019; -0.002]</td>
<td>0.019</td>
</tr>
<tr>
<td>LH#</td>
<td>-0.39</td>
<td>[-1.23; 0.46]</td>
<td>0.369</td>
<td>-0.007</td>
<td>[-0.021; 0.007]</td>
<td>0.330</td>
</tr>
<tr>
<td>FSH</td>
<td>-0.44</td>
<td>[-0.68; -0.20]</td>
<td>0.001</td>
<td>-0.007</td>
<td>[-0.010; -0.003]</td>
<td>0.001</td>
</tr>
<tr>
<td>FSH#</td>
<td>-0.40</td>
<td>[-0.78; -0.03]</td>
<td>0.035</td>
<td>-0.007</td>
<td>[-0.013; -0.0005]</td>
<td>0.035</td>
</tr>
</tbody>
</table>
Figure 1:
Figure 2:
Figure 3:
**Figure Legend:**

**Figure 1:** Evolution of mean TT and BioT levels over time (profile plots with local regression smoother and 95% confidence intervals are depicted).

**Figure 2:** Percentage of participants with T levels below reference range for young men (320 ng/dL for TT, 6.5 ng/dL for FT and 140 ng/dL for BioT, respectively) at each visit.

**Figure 3:** Mean evolution of BioT levels over time between median, upper and lower quartile according to baseline FSH levels.