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Serum Testosterone, Dihydrotestosterone and Estradiol Concentrations in Older Men Self-Reporting Very Good Health: The Healthy Man Study

Short title: The Healthy Man Study

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Abstract

Objective: To determine serum concentrations, intra-individual variability and impact of age-related co-morbidities on serum testosterone (T), dihydrotestosterone (DHT), estradiol (E_2) and estrone (E_1) in older men.

Design: Observational, repeated measures study

Participants: Men (n=325) 40 years and older self-reporting very good or excellent health.

Measurements: Standardized history and physical examination, 9 blood samples at fixed time intervals over 3 months (three at 20 min intervals on days 1 (fasting) and 2 (non-fasting), one at days 7, 30 and 90). Serum T, DHT, E_2 and E_1 (n=2900, >99% of scheduled samples) measured by liquid chromatography-tandem mass spectrometry (LC-MS) were analyzed by linear mixed model analysis with fasting, age and obesity as covariables.

Results: Mean serum T did not vary with age (p=0.76) but obesity (-0.35 nmol/L per body mass index (BMI) unit, p<0.0001) and ex-smoker status (-1.6 nmol/L, p<0.001) had significant effects. Serum DHT was increased with age (+0.011 nmol/L per year, p=0.001) but decreased with obesity (-0.05 nmol/L per BMI unit, p<0.0001). Serum E_2 did not vary with age (p=0.31) or obesity (p=0.12). Overnight fasting increased (by 9-16%, all p<0.001) and reduced variability in morning serum T, DHT, E_2 and E_1. Non-fasting serum T and DHT were stable over time (day, week, month or 3 month; p>0.28).

Conclusions: Serum T, DHT and E_2 displayed no decrease associated with age among men over 40 years of age who self-report very good or excellent health although obesity and ex-smoking status were associated with decreased serum androgens (T, DHT) but not E_2. These findings support the interpretation that the age-related decline in blood T accompanying non-specific symptoms in older men may be due accumulating age-related co-morbidities rather than a symptomatic androgen deficiency state.

Introduction

The decline in blood T during male ageing is well known from numerous observational studies since the advent of T immunoassay in the early 1970's [1]. Such studies are typically cross-sectional and obtain only a single blood sample for T measurement. More recent longitudinal studies involving serial blood sampling have appeared [2-5] although still relying on single blood samples per timepoint which frequently are inconsistent [6]. Although causality cannot be ascribed from observational studies, the coincidence of declining blood T concentrations with non-specific symptoms that are consistent with, but not diagnostic of, androgen deficiency (AD) [7-9] has led to the 'andropause hypothesis', which postulates that these symptoms may be due to age-related AD [10, 11]. Using the analogy of unequivocal hormone deficiency states, such as pathologically-based AD in young men and menopause, this proposes that symptomatic age-related AD may contribute a reversible component to deterioration in somatic and reproductive health of older men. Definitive testing of this hypothesis requires a placebo-controlled, randomized clinical trial which remains a prospect for the future [12]. The alternative view is that the decline in blood T levels in older men is a consequence of the accumulation of co-morbidities of ageing which can depress blood T levels unrelated to the coincidental non-specific symptoms of chronic disease which resemble those of AD. This alternative hypothesis, whereby blood T is effectively a non-specific barometer of (ill)-health, cannot be proved directly but would be a plausible interpretation if the andropause hypothesis is refuted. In the interim, while the andropause hypothesis remains untested, consensus clinical guidelines [10, 13] and analysis of large scale observational studies [11] tentatively define age-related AD ("andropause", "late-onset hypogonadism") providing tacit support for unproven T treatment of older men according to criteria which combine non-specific symptoms and blood T thresholds into a putative diagnosis of age-related AD.

Yet the relationship of non-specific symptoms of AD to blood T levels remains poorly defined. Unequivocal AD, such as after bilateral orchidectomy or severe defects in Leydig cell T secretion in otherwise healthy young men, produces varied but characteristic symptoms accompanying low blood T levels [7-9]. With the exception of flushing, which is rare apart from acute androgen deprivation, the non-specific symptoms of chronic AD also feature in other hormonal deficiency states as well as chronic diseases. This creates intractable confusion in evaluating individual older men where the background accumulation of age-related co-morbidities provides multiple potential etiologies for the non-specific symptoms. In younger men with organic, pathologically-based disorders causing AD, the symptoms of AD display highly reproducible blood T thresholds within each person [7], although the leading symptoms

vary widely in type and threshold between people. This distinctive individuality of thresholds means that when symptoms are grouped together, the apparent thresholds of blood T are attenuated [9] while among populations of older men without pathologically-based AD [11], such blood T thresholds for the same non-specific symptoms display minimal or no cutpoints [14].

This study aimed to measure age-specific serum concentrations of the major sex steroids (T, DHT and E_2) among healthy older men as well as estimating the within-individual variability and the impact of common age-related co-morbidities (obesity, smoking). Based on the focus to define the nature of non-specific symptoms associated with AD [7], we therefore planned to recruit men who reported being in very good or excellent health, an elite cohort (rather than a community representative study population) from which to deduce the relationships between blood sex steroid levels and age and its related co-morbidities free from influence of non-specific symptoms of AD.

Methods

Study design

The study had an observational design recruiting men over the age of 40 years to undergo multiple blood sampling at fixed intervals over 3 months. The study inclusion criterion was based on the question "In general, would you say your health was excellent, very good, good, fair or poor?" (question 1 from SF-36 questionnaire) at screening, usually by phone. Only men reporting very good or excellent health were eligible for entry. The only exclusion criterion was the use of medications (eg 5α reductase inhibitors) interfering with blood sex steroid levels. Participants were offered no reimbursement for participation. Recruitment aimed to fill each age decade with 100 participants.

The participants provided written, informed consent and the study was approved by the Sydney South West Area Health Service Ethics Committee (Concord Hospital) and the Southern Health Human Research Ethics Committee within NHMRC guidelines on ethical conduct in human research.

Study Procedures

Men were recruited by local advertising on noticeboards in and around the hospital and in local newspapers or radio throughout the recruitment period. The advertisements sought men who considered themselves in excellent health ("to help us determine why you are so healthy") to make phone contact with the centre to determine their eligibility for the study. Eligible men attending a study centre for standardized medical history and physical examination and to provide 9 morning (8-11 am) blood samples during 5 visits over 3 months. Three blood samples were obtained at 20 min intervals on the first (day 1) and second (day 2) visit and then single blood samples on the third (day 7), fourth (day 30) and fifth (day 90) visits. Men were fasting overnight on the first but non-fasting on subsequent visits. Serum was stored frozen at – 20 C until analysis in batch at the end of the study. Height (nearest 0.1cm) and weight (nearest 0.1 kg) were measured with participants wearing a light gown and no shoes. Body composition was measured by multi-electrode bioelectrical impedance (IMP5 meter, ImpediMed, Brisbane, Australia) and by whole body densitometry [15]. Smoking status was classified by the participants into never, ex-smoker and current smokers. For those who had ever smoked, their pack-years of smoking were calculated and for ex-smokers the number of years since cessation was recorded. Obesity was defined as a body mass index (kg/m^2) over 30 and hypertension as a blood pressure (single measure at rest) with systolic >140 and diastolic >90 mm mercury.

Assays

Serum T, DHT, E_2 and estrone (E_1) were quantified within a single run without derivatization as described [16] and calibrated directly against the National Measurement Institute certified reference standard utilized by the Centers for Disease Control T standardization [17]. The assay limits of detection, limits of quantification and within-run and between-run coefficients of variation (%) were T (35 pmol/L, 90 pmol/L, 2.0%, 3.9-6.5%), DHT (10 pmol/L, 0.69 nmol/L, 8.1%, 6.7-13.4%), E_2(4 pmol/L, 18 pmol/L, 6.6%, 4.8-8.6%) and estrone (2 pmol/L, 9 pmol/L, 4.7%, 4.6-7.5%). Serum LH, FSH and sex hormone binding globulin (SHBG) levels in baseline blood samples were measured in batch by automated immunoassays (Roche Diagnostics Australia) with CVs of 1.0-2.0%. Biochemical and hematological profiles used routine auto-analyzer methods. Non-protein bound ("free") T (FT) was calculated using the empirical FTZ formula [18] which more accurately estimates FT measured by either centrifugal ultrafiltration [19] or equilibrium dialysis [20] than equations which assume the valid applicability of equilibrium

binding theory. As FTZ is a calculated index, rather than a measured hormonal variable, it has no measure of reproducibility. Biochemical analytes (hemoglobin, lipids, renal and liver function tests, iron studies) were measured by standard autoanalyser methodologies.

Data analysis

The primary data analysis was by mixed model analysis of variance taking age and BMI as within-individual, fasting and smoking (never, ex, current) status as between-individual factors and participants as a random effect using NCSS software (Kaysville, UT). The models were fitted by restricted maximum likelihood with an autoregressive covariance structure allowing for differences between timepoints. Serial data were also analyzed by repeated measures analysis of variance and factorial analysis of variance with appropriate post-hoc testing as required in which the serum steroids were the repeated time-dependent variables whereas all covariates were measured only at baseline.

RESULTS

Participant characteristics

All participants (n=325) reported excellent or very good health at screening to Q1 of the SF-36 questionnaire to be eligible for entry (table 1). Subsequent written responses to the same question confirmed excellent (39%), very good (46%) or good (15%) health. Participants provided >99% (2900/2925) of scheduled blood samples. Obesity was present in 36 (11.1%) and hypertension in 62 (19.1%) of participants. No adverse events were reported in the study.

Hormones

The between-individual and within-individual variability of serum T, DHT, E_2 and E_1 in the 325 men (Table 2) demonstrated no difference within day whether fasting or not, and between week, month or at 3 month intervals for non-fasting (repeated measures ANOVA, all $p>0.05$). The T/DHT ratio (11.9 ± 0.08, median 11.3, interquartile range 9.6 – 13.3) was also stable over time ($p=0.75$).

Using mixed model ANOVA for the within-individual data (2900 samples) and accounting for 9 samples per man (n=325), serum T levels showed no trend (slope 0.17% per year, p=0.24) with age (figure 1 & 2). The study power (two sided α=0.05) to exclude a decrease in serum T was over 80% for an annual decrease of 0.5% or greater. When separated into decades of age, there were no changes in mean serum T and small but significant increases in serum DHT, E_2 and E_1 with age (figure 2). In models including covariables, serum T levels were significantly higher in fasting state, lower with increasing BMI and marginally reduced among ex-smokers but age had no significant effect (table 3). Compared with the non-fasting state, fasting significantly (all $p<0.05$) increased serum T (1.5 nmol/L, 9%), DHT (0.14 nmol/L, 9%), E2 (14 pmol/L 16%) and E1 (16 pmol/L, 13%). The fasting effect was significantly reduced at higher BMI (BMI x fasting interaction, p=0.043).

Serum DHT levels were not significantly different over 3 months (table 2) but within-individual were significantly increased with age and fasting but decreased with BMI and only marginally reduced in ex-smokers (table 3).

Serum E_2 and E_1 were not significantly different between individuals over 3 months (table 2). Within-individual serum E_2 levels were significantly increased by age, fasting and BMI but smoking had no effect (table 3). Within-individual serum E_1 levels were significantly increased by age, fasting, BMI and in current (but not ex) smokers (table 3).

Non-steroids were measured at baseline only and, when analyzed by age decade, serum LH, FSH and SHBG were not significantly different through the 7^{th} decade with FSH and SHBG significantly increased in the 8^{th}, and LH increased in the 9^{th} decade of age (figure 3). The calculated FT was not significantly different until it decreased in the 9^{th} decade.

Obesity has virtually identical pattern of effects in each model whether using BMI or fat mass (by bioimpedance) in absolute (kg) or relative (% of body weight) terms as measures of adiposity (data not shown).

Systolic or diastolic blood pressure was not associated with changes in any sex steroid concentrations except for diastolic blood pressure which was associated with a small decrease in blood DHT (0.01 nmol/L for every mm mercury, p=0.024).

Smoking effects examined by categorising smoking status into two continuous variables (pack-years, cessation time) showed marginally significant negative effects on serum T of cessation time (p=0.06) and on serum DHT of pack-years (p=0.04) and cessation time (p=0.07) whereas smoking effects were non-significant for serum E_2 and marginally positive for pack-years (p=0.07) on serum E_1.

Discussion

In this study 325 men aged 40 years and over who reported very good or excellent health had morning blood sample taken 9 times over 3 months with the observation that older male age was associated with no change in serum T and mild increases in serum DHT, E_2 and E_1 until the 8th decade of life. The study was large and sensitive enough to detect consistent but small effects on serum steroid levels of (a) fasting which increased all 4 steroid hormones, (b) obesity which decreased androgens (T, DHT) and increased E_2, (c) smoking with subtle effects mainly due to ex-smokers and (d) small but consistent within and between day variability in serum estrogens (E_2, E_1). Hence the failure to detect any decrease in sex steroid levels with older male age is unlikely to be due to lack of study power. While reporting very good or excellent health, participants were not necessarily in objectively excellent health as shown by the prevalence of hypertension and obesity although at rates much lower than among the age-matched Australian male population [21, 22]. If inclusion of men with asymptomatic underlying disease reduced serum sex steroids, this further strengthens our conclusion. Instead the present findings predict that no decrease in blood sex steroids should be expected among men who believe they remain in very good or excellent health as they age. The present findings suggests that the well known decline in serum T with male ageing observed in many representative populations [1] may be due to the co-morbidities that accumulate with progressive male ageing rather than age per se. Consequently, the present evidence fails to support the Andropause hypothesis but is more consistent with the alternative interpretation that the decline in serum T with male ageing is a non-specific effect of the common co-morbidities that accumulate during ageing. Nevertheless, as observational findings, the present study cannot refute the Andropause hypothesis nor even discount the possibility that, when age-related co-morbidities accumulate, any decline in serum T may still contribute to physical symptoms or signs or even that there are subsets of men who do demonstrate age-related falls in serum sex steroids despite our failure to observe any evidence for that possibility. The present findings may contribute to shifting the focus regarding the potential utility, efficacy and safety of T treatment for older men from ageing per se to an adjunct

treatment for the co-morbidities of ageing such as obesity, metabolic syndrome, type 2 diabetes, cardiovascular disease or heart failure.

The concept that age per se may have minimal influence on circulating T is consistent with previous smaller studies [23] and some cross-sectional cohorts [24]; however, other previous studies report a persistent age effect even after adjustment for known co-morbidities and predefined symptoms [5, 11]. The small increases we observed in the other sex steroids (DHT, E_2) associated with male ageing reinforce the view that age per se may not necessarily decrease male reproductive steroids or their metabolites. The distribution of serum T is consistent with that observed previously among young (18-35 yr) Australian men in excellent general and reproductive health [25] (8.2-28.2 nmol/L vs. 10-30 nmol/L, 95% confidence interval), both using MS-based steroid measurement. The slightly lower levels in the present older population may reflect the more rigorous requirement for normal reproductive function and/or exclusion of obesity in the study of younger but not older men.

Beyond this study's primary focus on the intensive within-person measurement of serum sex steroids in 9 samples per man, limited observations of other reproductive hormones based on only a single baseline blood sample per man showed no significant changes in serum level of LH, FSH and SHBG through the 7th decade with increases confined to the 8th decade (FSH, SHBG) or later (LH, calculated FT) consistent with most previous studies of male ageing [1] including a large Australian study which disregarded symptoms and used suboptimal assay methods for T and FT [26]. However, none focused on men reporting to be in very good or excellent health. These late life changes confined to the 8th or later decade of life is congruent with the findings in unselected men dying suddenly where reduced testicular volume attributable to age per se was also confined to the 8th or later decade [27]. The increased serum gonadotrophins without any decrease in blood androgens or estrogens is most consistent with subtle defects in testicular function such as compensated Leydig cell failure, whereby increased gonadotrophin secretion overcomes mild latent decreases in Leydig cell T secretion without overt clinical AD . The clinical significance of any change in calculated FT, considered as if it were a hormonal variable, was confined to a modest reduction in asymptomatic men over the age of 80 years and remains speculative [20]. Nevertheless, the present data suggests that age alone is not associated with, nor likely to explain, quantitative decreases in circulating T, DHT or E_2 levels.

A distinctive feature of the present study consistent with our wider focus on characterising the significance of non-specific symptoms associated with AD [7], is its elite recruitment strategy to focus on the impact of positive assertion of very good or excellent health by the participants. While some previous population representative cohort studies have inferred the participants apparent good health by the absence of predefined symptoms or known diseases, such retrospective categorisation of good health does not ensure consistency with the participants own views of their health. The gap between such retrospective inference and the positive assertion of very good health may explain, through residual confounding, the difference between our present findings of no apparent decline in blood T with male ageing and other cohort studies reporting age effects per se after adjustment for known co-morbidities and certain predefined symptoms [5, 6, 11].

The present study provides the most detailed view so far of within-individual variability of serum androgens and estrogens in men and the impact of fasting, obesity and smoking. The use of mass spectrometry, the reference method for steroid hormones [28], provides assurance that the effect estimates are free from the non-specificity and limited method intercomparability of serum T [25] and E_2 [29] immunoassays. Although our findings suggest that non-fasting serum androgen levels are stable over 3 months among healthy older men, among others with significant co-morbidities or during intercurrent illness, such stability may no longer apply.

The present study provides the first detailed MS-based estimates of between and within individual variability of serum sex steroids whereas previous systematic studies have been based on immunoassays including the least reliable direct (non-extraction) assays [30, 31]. As all samples for any individual were measured in this study within a single run, the estimates indicating greater variability over longer periods of time are likely to reflect genuine biological variability, rather than artefactual measurement "noise". The within-individual variability increased for time periods up to a month and then plateaued so that variability was no greater at 3 than at 1 month. While we observed a systematic effect of fasting to increase morning serum sex steroids, the within-day variability of circulating sex steroids was significantly less in the fasted than non-fasting state. These estimates may provide a useful basis for sample size estimates for clinical trials of T treatment.

A consistent effect of an overnight fast on serum sex steroids in healthy older men has not, to our knowledge, been reported previously and explains the observation that glucose ingestion

acutely lowers fasting serum testosterone [32]. However, our finding based on triplicate blood samples in fasting and non-fasting states on consecutive mornings in over 300 healthy older men provides strong evidence that it both increased and reduced variability of serum sex steroid levels. Previous studies have reported decreased blood T after prolonged (48+ hr) fasting in men [33] with obese [34] men more resistant to such decreases consistent with our findings that obesity attenuated the effect of fasting. In other small studies (n=7-10) of healthy young athletes, mild fluid volume depletion had minimal effect on serum T [35]. These differences most probably reflect differences in study power, age of participants and type of T assays. As the sequence (fasting prior to non-fasting) was fixed rather than in random order and sampling was restricted to mornings, we cannot exclude the unlikely possibility of a sequence or time of day effects. Our present findings underline the need for caution in extrapolating results regarding blood T or other sex steroids from the fasted to the non-fasting state. The mechanism underlying the uniform but modest increase in all 4 sex steroids is most consistent with an effect of fluid volume depletion although changes in steroid metabolizing enzymes (eg CYP 3A4) cannot be excluded. Interestingly, the fasting state markedly reduced within-individual variability of serum hormone levels with implications for future study power and sample size.

Obesity has a well known association with reduced serum T and other sex steroids from population-based and more detailed but smaller analytical studies [36]. Increased expression of aromatase in adipose or other (muscle, brain, skin, bone) tissue may explain the increase in circulating serum estrogens without change in their aromatisable androgen precursors.

We observed consistent but small effects of ex-smoking status on serum T and DHT; however, when smoking status was categorized into two continuous variables (pack-years and time since cessation of smoking), no consistent, significant effects were identified suggestive of either a small effect size and/or nullification by misclassification of smoking status. As smoking status was self-defined and not verified by objective measurement, misclassification between current and ex-smoker status may explain the inconclusive findings. In previous studies the effects of smoking on serum T and other sex steroids remain unsettled. In previous studies increases in serum T have been reported most consistently among smokers with a dose relationship with pack-years [37]; however no effects has been consistently defined in ex-smokers.

The covariable effects observed in this study associated with changes in circulating androgens in healthy men have been less studied with regard to their effect on estrogens. In this respect a major consideration is the unreliability of E_2immunoassays, notably direct, non-extraction immunoassays, when applied to low circulating E_2levels such as in men as well as menopausal women [29]. Our finding measuring E_2 and E_1 using mass spectrometry in serum of older men are consistent with previous reports using GC-MS measurement in the Labrie laboratory [38, 39]; however, those studies were restricted to single blood samples.

The strengths of this study include the use of the reference method of MS for steroid measurement, the intensive repeated measures design and the distinctive focus on men in very good or better health. While MS remains more laborious and costly than direct immunoassays, the improved sensitivity now matching that of steroid immunoassay and our improved method that allows for both androgen and estrogens to be measured in a single run without derivatization, ensures improved efficiency and robustness of the hormonal data free from the method dependence of steroid immunoassays.

The limitations of this study include the observational and cross-sectional design for the non-steroidal measures (LH, FSH, SHBG, FTZ) measured only once and the choice of a self-selected, elite healthy population which disallows direct extrapolation to unselected men of similar age. Although this study population was not formed by having objectively excellent health (as judged by medical testing), they represent a previously unstudied segment of the older male population who do not suffer the nonspecific, putative AD symptoms and believe they are in very good or excellent health. However, the study's finding characterizing the relationship between male ageing and trends in serum T when the older men are free from the concomitant non-specific symptoms that accumulate with ageing, remains provide a distinctive and novel insight.

We conclude that, among men in self-reported very good or excellent health, male ageing per se is not associated with any reduction in serum sex steroids. This raises the suggestion that observed changes with ageing in representative population cohorts may rather be attributable to the co-morbidities that accumulate during ageing. We also provide strong evidence that overnight fasting in men increases blood sex steroids whereas obesity and (ex)smoking reduce serum androgens.

Table 1 – Baseline Characteristics of Participants

Variable	Median (Q1, Q3)#	Mean ± SEM	Range
n = 325			
Age (yr)	60 (51, 67)	60 ± 1	40 - 97
Height (cm)	176 (171, 181)	176 ± 0.4	156 - 203
Weight (kg)	81.2 (74.5, 88.3)	82.0 ± 0.6	57.8 - 121.8
BMI (kg/m^2)	26.1 (24.2, 28.2)	26.3 ± 0.2	19.5 - 40.6
BSA (m^2)	2.00 (1.91, 2.10)	2.01 ± 0.01	1.66 - 2.51
Fat-free mass (kg)	62.2 (57.3, 67.7)	62.7 ± 0.4	41.3 - 92.8
Fat mass (kg)	18.7 (14.4, 23.2)	19.2 ± 0.4	4.5 - 47.6
Waist circumference (cm)	95 (88, 100)	95 ± 1	71 - 131
Waist-hip ratio	0.93 (0.89, 0.97)	0.93 ± 0.03	0.71 - 1.11
Systolic blood pressure (mm)	125 (120, 140)	129 ±1	90 - 188
Diastolic blood pressure (mm)	80 (75, 85)	81 ± 1	60 - 110
LH (IU/L)	4.6 (3.5, 6.3)	5.2 ± 0.2	1.2 - 26.8
FSH (IU/L)	5.4 (3.9, 8.0)	6.7 ± 0.3	1.0 - 52.9
SHBG (nmol/L)	40.3 (30.5, 52.0)	42.1 ± 0.9	2.9 - 133
Hb (g/L)	148 (141, 155)	147 ± 1	112 - 173
PSA (ng/mL)	1.1 (0.65, 2.25)	1.8 ± 0.1	0.05 - 16.2
Total cholesterol (mmol/L)	5.3 (4.6, 5.9)	5.28 ± 0.05	2.9 - 9.6
HDL cholesterol (mmol/L)	1.3 (1.1, 1.5)	1.33 ±0.01	0.7 - 2.6
LDL cholesterol (mmol/L)	3.4 (2.8, 3.9)	3.4 ± 0.05	1.4 - 7.2
Triglycerides (mmol/L)	1 (0.8, 1.5)	1.23 ± 0.04	0.3 - 5
Urea (mmol/L)	6.5 (5.7, 7.6)	6.8 ± 0.1	3.6 - 14.9

Creatinine (μmol/L)	85 (78, 94)	87 ± 1	59 - 151
Bilirubin (μmol/L)	11 (8, 15)	12 ± 0.3	4 - 38
Albumin (g/L)	46 (44, 48)	46.0 ±0.1	37 - 53
Protein (g/L)	73 (70, 76)	72.8 ± 0.2	62 - 84
ALP (IU/L)	62 (53, 74)	65 ± 1	25 - 165
Gamma GT (IU/L)	21 (16, 30)	26 ± 1	5 - 131)
ALT (IU/L)	21 (15, 28)	22 ± 1	5 - 66
AST (IU/L)	22 (19, 26)	23 ± 0.4	10 - 51
Iron (mmol/L)	18 (15, 22)	19 ±0.3	5 - 55
Transferrin (g/L)	2.6 (2.4, 2.8)	2.61 ± 0.02	1.6 - 3.9
Ferritin (mg/L)	157 (83, 253)	186 ± 8	11 - 679

Q1 and Q3 are the 1st and 3rd quartiles of distribution

Table 2

Between and Within Individual Variability of Serum Steroid Levels

CV (%)	T (nmol/L)	DHT (nmol/L)	E_2 (pmol/L)	E_1 (pmol/L)
Between individual*				
Mean ± SEM	16.7 ± 0.3	1.5 ± 0.04	90 ± 2	124 ± 2
Range	2.9 – 33.9	0.23 – 7.3	29 – 197	43 – 464
Quartiles	13.2, 16.4, 19.6	1.1, 1.43, 1.83	70, 86, 106	98, 118, 143
95% CI	8.2 – 28.2	0.6 – 3.0	39 – 153	62 – 211
Within individual#				
All samples	11.8	15.9	16.5	14.2
All non-fasting	10.8	15.2	14.4	12.2
Within-day (fasting)	4.9	9.7	8.7	7.1
Within-day (non-fasting)	5.4	10.4	8.8	6.9
Between day	11.8	14.1	14.5	12.8
Between week	8.8	11.5	11.0	9.3
Between month	10.3	12.2	12.1	10.6
Between 3 months	9.7	12.3	12.7	10.4

* Descriptive statistics based on grand mean of 9 samples per man for 325 men.

Within-individual coefficient of variability (CV, as %) averaged across all 325 men. Definition of estimates: "All samples" includes 9 samples over 3 months. "All non-fasting" includes 6 non-fasting samples over 3 months. "Within-day (fasting)" includes 3 samples within the first day in fasting state. "Within-day (non-fasting)" includes 3 samples within the second day in non-fasting state. "Between day" indicates variability over 6 samples including 3 fasting on day 1 and 3 non-fasting on day 2 (disregarding fasting state). "Between week" includes the mean of 3 non-fasting samples on 2nd day and one non-fasting sample a week later. "Between month" includes the mean of 3 non-fasting samples on 2nd day and one non-fasting sample a month later. "Between 3 months" includes the mean of 3 non-fasting samples on 2nd day and one non-fasting sample 3 months later.

Table 3

Effects of Covariables on Serum Steroid Levels

Serum Hormone	Age	Fasting	BMI	Smoking	
				Ex-Smoker	Current
T	-0.1 (-0.6, 0.3)	**1.5 (1.3, 1.7)**	**-0.5 (-0.6, -0.3)**	**-1.5 (-2.7, -0.4)**	0.0 (-2.5, 2.4)
DHT	**0.10 (0.03, 0.16)**	**0.14 (0.11, 0.16)**	**-0.05 (-0.07, -0.02)**	**-0.2 (-0.3, -0.1)**	0.0 (-0.4, 0.3)
E_2	**4 (1, 7)**	**14 (13, 16)**	**2 (1,3)**	1 (-5, 8)	3 (-11, 18)
E_1	**7 (3, 11)**	**16 (14, 18)**	**2 (1,3)**	3 (-7, 12)	**25 (4, 45)**

Data is tabulated as effects size (nmol/L for T and DHT, pmol/L for E_2 and E_1) with 95% confidence intervals in parentheses below. Age effect is per 10 years, fasting effect is vs non-fasting in morning samples, BMI effect is per BMI unit (kg/m^2) and smoking effect is current or ex-smoker vs never smoker. Statistically significant effects (ie exclude null effect) are highlighted in bold font.

Figure Legends

Figure 1 – Plot of serum T versus age. Each filled circle represents the average of 9 serum T measurements over 3 months for each of the 325 healthy men. Data from one man over 90 years of age is omitted from the figure for clarity but is included in all calculations. For further details see text.

Figure 2 –Box plot of serum T (upper left), DHT (upper right), E_2 (lower left), estrone (lower right) versus age in decades. Box outlines indicate quartiles, whiskers indicate 10^{th} and 90^{th} centiles, filled circles indicate 95% confidence limits.All steroid data represents the average steroid level (based on 9 samples over 3 months per man) separated into decades of age asterisk indicates significant difference ($p<0.05$, Newman-Keuls posthoc test). The number of men in each decade age group is indicated in parentheses at the bottom of each histogram bar. For further details see text.

Figure 3 – Box plots of serum LH (upper left), FSH (upper right), SHBG (lower left) and calculated FTZ (lower right) versus age in decades. Box outlines indicate quartiles, whiskers indicate 10^{th} and 90^{th} centiles, filled circles indicate 95% confidence limits and asterisk indicates significant difference ($p<0.05$, Newman-Keuls posthoc test). The LH, FSH and SHBG data are measured by immunoassay and the FT calculated from the FTZ formula using single baseline samples per man. For further details see text.

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