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Title

Cross-sectional associations of sex hormones with leucocyte telomere length, a marker of biological age, in a community-based cohort of older men.

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Summary

Context

Telomeres protect chromosomes from damage, and shorter leucocyte telomere length (LTL) is a marker of advancing biological age. The association between testosterone (T) and its bioactive metabolites, dihydrotestosterone (DHT) and estradiol (E2) with telomere length, particularly in older men, is uncertain. The study aimed to clarify associations of sex hormones with LTL in older men.

Participants and methods

We used cross-sectional data from 2,913 men aged 76.7±3.2 years with morning blood samples assayed for T, DHT, E2 (mass spectrometry), and sex hormone-binding globulin (SHBG, immunoassay), to correlate sex hormones with LTL measured using PCR and expressed as T/S ratio in multivariable linear regression models adjusted for age, cardiometabolic risk factors and cardiovascular disease history.

Results

Average difference per decade of age was T -0.46 nmol/L, DHT -0.11 nmol/L, E2 -7.5 pmol/L, SHBG +10.2 nmol/L, and LTL (T/S ratio) -0.065. E2 correlated with T/S ratio (r=0.038, p=0.039) and SHBG was inversely correlated (r=-0.053, p=0.004). After multivariable adjustment, E2 was associated with T/S ratio (per 1SD increase E2: coefficient 0.011, p=0.043), T and DHT were not associated. When E2 and SHBG were simultaneously

included, E2 remained positively (coefficient 0.014, p=0.014) and SHBG inversely (coefficient -0.013, p=0.037) associated with T/S ratio.

Conclusions

In older men, neither T nor DHT are associated with LTL while E2 is independently associated with LTL and SHBG is inversely associated, thus relating sex hormone exposure to lower biological age. Further research is needed to determine causality and clarify the role of sex hormones in male ageing.

Key words: Androgen, Estrogen, Sex Hormone-Binding Globulin, Leucocyte Telomere Length,

Biological Age, Ageing Male

Introduction

Telomeres are essential DNA-protein complexes comprising TTAGGG repeats binding specific proteins, which protect the physical ends of chromosomes from fusion and degradation [1]. Conventional DNA replicative enzymes cannot fully replicate the DNA sequence at telomere ends, thus their length progressively shortens with each cell cycle [2]. Attrition of telomeres results in cellular senescence, characterised by alterations in gene expression, cell cycle arrest and ultimately loss of viability when telomere length declines to a critical value [1-3]. Measurement of telomere length can be performed in DNA extracted from leucocytes and the results correlate with telomere length measured in various tissues

[4,5]. Consistent with the relationship between ageing and shorter telomere length, age-related declines in leucocyte telomere length (LTL) are found in both men and women [6]. Furthermore, shorter LTL predicts higher risk of ill-health characteristic of advancing age, including incidence of myocardial infarction, stroke, type 2 diabetes, and, to some extent mortality [7,8]. Factors such as obesity and smoking are associated with telomere shortening, while tobacco abstinence and physical activity or fitness are associated with longer telomeres [9-11]. Telomere homeostasis is a dynamic process with telomere shortening countered by the activity of telomerase, the enzyme responsible for elongating telomeres by addition of telomeric repeats [1]. Thus telomere length is a biomarker for and putative mediator of biological ageing, and factors which predict increased telomere length offer potential avenues for interventions to preserve health.

Men tend to have shorter LTL than women of comparable age, raising the possibility of sex hormone exposure contributing to the regulation of telomere length [6]. In men circulating testosterone (T) declines and vulnerability to multiple comorbidities increases with advancing age [12]. In experimental studies using various cell lines, both testosterone (T) and its bioactive metabolite estradiol (E2) increased telomerase expression and/or activity [13-17]. Thus regulation of telomere length may be a mechanism by which reduced sex hormone exposure might accelerate biological ageing. However, there are few studies examining the association of sex hormones with LTL in men and those reported have contrasting results. In one study of 110 older men, neither T nor E2 measured using immunoassay were associated with LTL [18]. By contrast, an analysis of 980 predominantly middle-aged men from the Busselton Health Study found that circulating concentrations of both bioactive metabolites of T, dihydrotestosterone (DHT) and E2, assayed using mass spectrometry, correlated with LTL [19]. However a recent analysis of 499 adult men from the National Health and Nutrition

Examination Survey (NHANES) was contradictory, reporting an inverse association of circulating E2 measured using immunoassay with LTL [20]. A larger and more comprehensive analysis involving older men and using mass spectrometry for analysis of sex hormones would address this question.

We aimed to assess whether circulating concentrations of T, DHT and E2, and their principal carrier protein sex hormone-binding globulin (SHBG), were associated with LTL in community-dwelling older men in relatively good health, independently of age and cardiometabolic risk factors.

Participants and methods

Study population

The Health In Men Study (HIMS) is a population-based cohort study of community-dwelling older men from Perth, Western Australia [21]. 12,203 men completed a questionnaire and attended for physical examination in Wave 1 (W1, 1996-1999). During Wave 2 (W2, 2001-2004), 4,246 of these men then aged 70-89 years completed a second questionnaire, and attended for physical examination and venesection. After exclusions, the final study sample comprised 2,913 men. Men were predominantly of Caucasian ethnic origin. The University of Western Australia Human Research Ethics Committee approved the study, and all men gave written informed consent.

Definition of medical comorbidities and physical activity levels

Blood pressure, height and weight recorded at W2 were analysed. Body mass index (BMI) was defined as weight (kg) divided by height (m) squared. A history of cardiovascular disease (CVD) was defined as any self-reported history of heart attack or stroke, heart bypass surgery or balloon angioplasty, aortic aneurysm, or surgery to the aorta, carotid or lower limb arteries at W2. Use of hypertensive medications was recorded from questionnaire responses at W2. Men diagnosed with diabetes, reporting use of glucose-lowering medication, or with fasting or non-fasting glucose at W2 of ≥7 mmol/L or ≥11.1 mmol/L respectively, were considered to have diabetes. Physical activity was assessed using questionnaire in W1. Physical activity was the sum of the number of hours in a usual week of non-vigorous and 2x the number of hours of vigorous physical activities, reflecting the higher exercise intensities associated with vigorous activities.

Laboratory assays for circulating sex hormones

Blood samples were collected between 08:00 and 10:30 h at W2. Plasma and serum aliquots were prepared immediately after phlebotomy and stored at -80°C until assayed. Plasma total T, DHT, and E2 concentrations were quantified using liquid chromatography-tandem mass spectrometry (LC-MS/MS) as reported previously [22]. Details of the methodology, validation, accuracy, stability and quality control have been published previously [23,24]. Precision profiles displayed coefficients of variation <6% for T concentrations (>0.4 nmol/L), <13% for DHT concentrations (>0.7 nmol/L), and <8% for E2 concentrations (>25pmol/L). Serum SHBG had been determined previously by chemiluminescent immunoassay on an Immulite 2000 analyzer (DPC-Biomediq) with coefficient of variation <7%. Calculated free testosterone (cFT) was obtained using an empirical formula FTZ derived from a panel of

3,975 consecutive blood samples from a routine diagnostic laboratory service for which direct measurement of free T by centrifugal ultrafiltration was available together with contemporaneous results of total T and SHBG [25]. The formula was validated in a separate set of 2,159 samples from another laboratory [26]. The optimal formula whose results best fitted directly measured free T was: cFT = 24.00314 x T/log10SHBG – 0.04599 x T2 [25].

Measurement of leucocyte telomere length (LTL)

From blood samples collected at W2 aliquots of leucocyte DNA were prepared and stored at -80^{o}C until required. An optimised PCR-based methodology for accurate measurement of LTL utilising the protocol described by Cawthon et al [27] was employed. Briefly, telomere lengths of the leucocyte DNA samples were measured by a multiplex quantitative PCR method [19]. Each sample was amplified for telomeric DNA and for beta-globin, a single-copy control gene, which was used as an internal control to normalize the starting amount of DNA. The K562 cell line was used as a standard [28]. Periodic reproducibility experiments were performed to confirm adequate normalization. All samples, standards, and controls were run in triplicate, and the median value used for analyses. A standard curve derived from K562 cell line was used to transform the cycle threshold into nanograms of DNA. The amount of telomeric DNA (T) was divided by the amount of single-copy control gene DNA (S), producing a relative measurement of the telomere length (T/S ratio). The coefficient of variation for the quantitative PCR across all batches was <10%. As some men did not provide a leucocyte DNA sample at W2 (choosing to provide blood samples only for plasma and serum) DNA for analysis of LTL were available for 3,894 men.

Statistical analysis

SAS version 9.4 was used (SAS Institute, Cary, NC, USA). Characteristics of the study population are presented as mean±standard deviation (SD) for continuous data, and n (%) for categorical data. The distribution of LTL was close to normal. The chi-square test was used to compare categorical variables and t-test for quantitative variables. In analyses of age vs hormones and LTL data are expressed as mean and the standard error of the mean (SE). Correlation coefficients were calculated for associations of hormones with T/S ratio. Linear regressions of T/S ratio on hormones were performed with adjustment for age and cardiometabolic risk factors. The adjustment variables were age, BMI, CVD history, alcohol, smoking, physical activity, cholesterol, high density lipoprotein cholesterol (HDL), lipid medication, diabetes, systolic BP, and hypertension medication. Additional multivariate adjusted linear regression analyses including both T and SHBG, and E2 and SHBG, were performed. Results are presented as the estimated coefficient (p-value) where the coefficient represents the change in T/S ratio for a 1 SD change in the hormone variable. A p-value of <0.05 was considered significant.

Results

Characteristics of the study population

After excluding men taking androgen-related medications or with history of orchidectomy or prostate cancer there were 3,532 men. However, some men had missing data on hormones, LTL or key covariates and after excluding these there were 3,018 community-dwelling men for inclusion in the initial analysis. Characteristics of these 3,018 men and the 514 men excluded due to missing key data are shown (Table 1). In total 34% had a history of CVD and

14.0% diabetes. Excluded men had a similar history and cardiovascular profile to the study population. Of the study population, 2,913 were aged 70-84 years and 105 were aged 85-89 years.

Associations of age with sex hormones and LTL

The relationships of age to sex hormones, SHBG and LTL are shown (Table 2). T, cFT, DHT and E2 showed consistent declines with age, while SHBG increased with age. T/S ratio showed a decline with age from 70-74, 75-79 and 80-84 years. Of note, there were only 105 men in the age stratum 85-89 years, and these men showed stable sex hormone and SHBG concentrations and higher T/S ratios compared to men aged 80-84 years (Table 2). This phenomenon, consistent with a healthy survivor effect, has been observed in previous epidemiological studies [6]. Therefore, as these men aged 85-89 years may be healthy survivors and less representative of the population of older men in general, subsequent analyses were conducted on the 2,913 men aged 70-84 years (mean±SD 76.7±3.2 years). In these men the estimated average difference per decade of age was T -0.46 nmol/L, cFT -17.8 pmol/L, DHT -0.11 nmol/L, E2 -7.5 pmol/L, SHBG +10.2 nmol/L and LTL (T/S ratio) -0.065.

Correlations of sex hormones with LTL

In the 2,913 men aged 70-84 years, E2 showed a positive correlation with T/S ratio (r=0.038, p=0.039). After excluding highest and lowest 1% of values, the correlation between E2 and T/S ratio was largely unchanged (r=0.039, p=0.037). SHBG was inversely correlated with T/S ratio (r=-0.053, p=0.004). After excluding highest and lowest 1% of values, the

correlation between SHBG and T/S ratio was largely unchanged (r=-0.055, p=0.004). T, cFT and DHT were not correlated with LTL (r=-0.019, p=0.313; -0.004, p=0.817, and -0.008, p=0.658 respectively).

Multivariate analyses of hormones and LTL

Linear regressions of T/S ratio on hormones after adjustment for age, cardiometabolic risk factors and CVD history are shown (Table 3). E2 showed a significant multi-variable adjusted association with LTL (Table 3). The estimated difference in T/S ratio was 0.011 per 1 SD increase in E2. T, cFT, DHT and SHBG were not associated with LTL. Results for cFT mirrored those for T and did not provide any additional information [29].

The association of T with LTL remained non-significant when included in a model that also included SHBG. The model with both E2 and SHBG showed significant associations for E2 and SHBG (inversely) with LTL (Table 3). The full fitted model including both E2 and SHBG is shown (Table 4). Age, BMI, HDL and SHBG were inversely associated with LTL, while total cholesterol and E2 were positively associated in the full fitted model. The effect size expressed as difference in T/S ratio was comparable for E2 with these other covariates (Table 4). The increase in T/S ratio associated with a 1 SD higher plasma E2 concentration was comparable in magnitude to the decrease associated with having a BMI 3.6 kg/m^2 higher, and was two thirds the decrease associated with being 3.6 years older.

Discussion

In this analysis of 2,913 community-dwelling men aged 70-84 years, T, DHT and E2 concentrations declined with age, with a parallel decline in LTL expressed as the T/S ratio. Plasma E2 concentrations were independently associated with longer LTL, while SHBG concentrations were inversely associated. T and DHT were not associated with LTL in older men. The correlations were modest with correlation coefficients of 0.038 for E2 and -0.053 for SHBG with T/S ratio respectively. These were largely unchanged in the trimmed analyses excluding the highest and lowest 1% of values, thus were not influenced by extreme outliers. In the multivariable model, E2 remained positively and SHBG inversely associated with LTL indicating that the associations are independent of age, BMI and cardiometabolic risk factors. Of note, the magnitude of increase in T/S ratio associated with a 1 SD higher plasma E2 concentration was comparable with having a BMI 3.6 kg/m^2 lower, and two thirds that associated with being 3.6 years younger. Therefore, these findings in an epidemiological study illuminate an association of sex hormones with biological age in older men (see Figure). However, the predictive value of E2, SHBG and LTL in any individual older man is unproven and likely to be limited in view of the moderate associations and large inter-individual variations observed in these parameters.

These findings are in contrast to previous reports (see Table 5) which reported neutral or inverse associations of E2 with LTL in men [18,20]. In the study by Bekaert [18] of 110 men with median age 75.0 years, circulating T and E2 were measured using immunoassay, bioavailable (non-SHBG bound) T and E2 were calculated, and LTL was assayed using Southern blots. Neither calculated bioavailable T nor E2 correlated with LTL [18]. Coburn [20] studied 499 men from NHANES 1992-2002 surveys with median age 42.0 years, with

circulating T and E2 concentrations assayed using immunoassays and LTL measured using PCR. In that study circulating T was not associated with LTL, whereas circulating E2 was inversely associated with LTL in analyses adjusting for age, ethnicity, education, BMI and smoking status [20]. Limitations of those analyses include smaller sample sizes and use of immunoassay for T and E2. Immunoassays for sex steroids such as T and E2 can suffer from non-specificity and method-dependent bias, and mass spectrometry is considered more accurate especially at low sex steroid concentrations [30-32]. Conversely, in 980 men from the Busselton Health Study with mean age 53.7 years where circulating T, DHT and E2 were assayed using mass spectrometry and LTL measured using PCR [19], both DHT and E2 correlated with LTL after adjusting for age. Therefore, the current analysis involving a different cohort of 2,913 men with mean age of 76.7 years from the Health In Men Study establishes the positive association of plasma E2 concentration with LTL independent of age and other covariates, specifically in a community-derived population of older men.

Of note, in the current analysis we did not observe any association of either T or DHT with LTL. As expected, circulating DHT in our study population of older men was lower than in the predominantly middle-aged men from the Busselton analysis (mean DHT 1.42 nmol/L vs 1.77 nmol/L) and telomere length was shorter (mean T/S ratio 1.18 vs 1.69) [19]. It is possible that the association of DHT with LTL is less apparent in older men who have shorter telomeres. However, the association of E2 with longer telomeres remains consistent in older men.

The inverse association of SHBG with telomere length is of interest, and is independent of E2. A similar scenario is seen with E2, SHBG and bone strength such that lower circulating E2 and higher SHBG concentrations are independently associated with fracture risk in men [33]. Therefore it seems feasible that E2 and SHBG should be reciprocally associated with LTL, with both modulating the effect of hormonal exposure on a specific outcome in a distinct fashion.

As this is a cross-sectional analysis from an observational study, causality cannot be determined. Nevertheless several lines of evidence support a role for E2 to regulate telomerase activity and thereby telomere length. An estrogen-response element is present in the promoter of the catalytic subunit of the telomerase enzyme, thus E2 acting transcriptionally could stimulate telomerase activity [34]. Indeed, E2 increased telomerase activity and/or expression in several cell lines *in vitro* [13,15-17]. In an animal model, aromatase knockout mice who are E2 deficient have reduced ovarian telomerase expression and telomere length, which are reversed by E2 therapy [35]. Also, whilst telomerase activity is repressed in many somatic tissues during extra-uterine life, it is present in highly proliferative tissues including the haematopoietic system, gastrointestinal tract and skin [36], providing a pathway by which telomerase induction by exposure to sex hormones could result in greater circulating LTL and tissue telomere length. Thus there are recognised mechanisms by which E2 could regulate telomere length. Our findings are consistent with a role for circulating E2 to modulate telomere length in older men, raising the prospect that this bioactive metabolite of T may influence biological ageing *in vivo*.

Exogenous T undergoes aromatisation to E2 in a manner similar to the endogenous hormone [37]. However, it is unclear whether T treatment either in itself, or via provision of substrate for aromatisation to E2, would influence telomere length. In a Phase I/II study which enrolled 27 patients with a rare aplastic anaemia associated with short LTL, treatment with the androgenic anabolic steroid danazol slowed attrition of LTL over a period of 24 months in the first 12 patients, leading to the study being halted early [38]. However, the implications of this finding in patients with a defined haematological disease to the broader population of men in the community are unclear. In a study of 40 men commencing androgen deprivation therapy (ADT) for prostate cancer, there was no change in LTL over 24 months when compared to 25 radiotherapy-matched controls [39]. However, the sample sizes were small, and dropouts occurred such that at 12 months there were 28 ADT-treated men and 22 radiotherapy-matched controls, and at 24 months there were 7 and 12 respectively [39]. Additional studies and ultimately randomised controlled trials are needed to test whether T treatment in middle-aged or older men would affect telomere length directly or via conversion to E2, and whether or not this would preserve health during ageing.

We acknowledge several limitations of this study. Blood samples were taken at a single time point, and we did not have serial measurements. This is a cross-sectional analysis and causation cannot be inferred. Nevertheless, there are mechanistic data in the literature supporting a role for E2 in regulation of telomere length [13,15-17]. We measured LTL and did not have tissue samples with which to measure telomere length: however, LTL correlates with telomere length in several tissues, including skin, synovium, buccal cells and fibroblasts, thus is an informative marker for epidemiological studies [4,5]. Although 8.3% of men did not provide DNA samples, there is no reason to suspect this could have biased the findings. We observed that the 105 men aged 85-89 years had longer mean LTL than men aged 80-84

years, consistent with a healthy survivor effect. For this reason we excluded them from the analysis to reduce potential confounding of the results. Therefore our final study population of 2,913 men aged 70-84 years represents a demographic group of older men, but we cannot extend these results to men aged 85 years or more. Finally HIMS comprises men of predominantly Caucasian ethnicity, thus we cannot extrapolate our results to men of other ethnic origins, nor to women.

Strengths of this study are the large study cohort of older community-dwelling men, use of LC-MS/MS for assay of sex hormones, and the comprehensive adjustment for multiple covariates in the analysis. As we adjusted for age and for factors including BMI, physical activity and history of diabetes and CVD it is less likely that our results were confounded by age, underlying health or physical factors. The PCR methodology for measurement of LTL is appropriate for epidemiological studies and results from this technique correlate strongly with LTL measured using Southern blot [27].

Conclusions

E2 is independently associated with LTL while SHBG is inversely associated, suggesting that sex hormone exposure is associated with lower biological age in older men. However, causality cannot be inferred from an observational, cross-sectional study, thus further research is needed to explore whether interventions that modulate sex hormone exposure might preserve telomere length in men.

Declaration of interests

The authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figure legend

Shorter telomeres are associated with advanced biological age. In a cross-sectional analysis of 2,913 men aged 70-84 years, higher plasma estradiol concentrations were associated with longer leucocyte telomere length independently of age, cardiometabolic risk factors and cardiovascular disease history, while higher sex hormone-binding globulin concentrations were associated with shorter leucocyte telomere length.

Table 1

Characteristics of full study population (n=3,018), the final analysis cohort of men aged 70-84 years (n=2,913), and those excluded due to missing key data (n=514). Table shows mean±SD or n (percent).

Characteristic		Full study sample (n=3,018)	Final study sample (n=2,913)	Excluded subjects (n=514)	p-value*
Age (years)		77.0 ± 3.6	76.7 ± 3.2	77.4 ± 3.6	0.018
BMI (kg/m^2)		26.5 ± 3.6	26.5 ± 3.7	26.5 ± 3.5	0.746
Cardiovascular disease history		1,016 (33.7)	972 (33.4)	168 (32.7)	0.664
Alcohol (g/day)		11.8 ± 15.4	11.9 ± 15.5	10.7 ± 14.8	0.157
Smoking	Never	1,004 (33.3)	966 (33.2)	157 (30.5)	0.171
	Former	1,839 (60.9)	1773 (60.9)	334 (65.0)	
	Current	175 (5.8)	174 (6.0)	23 (4.5)	
Physical activity (h/week)		7.03 ± 7.14	7.06 ± 7.16	6.67 ± 7.64	0.306
Cholesterol (mmol/L)		4.89 ± 0.95	4.89 ± 0.94	4.91 ± 0.99	0.716
HDL (mmol/L)		1.39 ± 0.36	1.39 ± 0.36	1.40 ± 0.35	0.580
Lipids medication		1,168 (38.7)	1141 (39.2)	181 (35.2)	0.132
Diabetes		422 (14.0)	410 (14.1)	81 (15.8)	0.271
Systolic blood pressure (mmHg)		147 ± 20	147 (20)	148 ± 20	0.601
Hypertension medication		1,665 (55.2)	1,602 (55.0)	274 (53.3)	0.433
Testosterone (nmol/L)		13.1 ± 4.9	13.1 ± 4.9	12.7 ± 5.1	0.119
Calculated free testosterone (pmol/L)		185 ± 55	186 ± 55	177 ± 59	0.007
Dihydrotestosterone (nmol/L)		1.42 ± 0.73	1.42 ± 0.73	1.48 ± 0.73	0.087
Estradiol (pmol/L)		73.2 ± 28.7	73.4 ± 28.9	73.1 ± 32.0	0.977
SHBG (nmol/L)		42.6 ± 17.1	42.4 ± 17.0	42.4 ± 16.8	0.838
LTL (T/S ratio)		1.18 ± 0.30	1.18 ± 0.30	1.19 ± 0.31	0.711

* Comparison of full study sample with excluded group for those with data on this variable.

P-values are from chi-square tests for categorical variables and from t-tests for quantitative variables.

Table 2

Relationship with age for hormones and leucocyte telomere length (expressed as the T/S ratio) in 3,018 community-dwelling men aged 70-89 years. Table shows mean (SE).

Age (years)	n	T (nmol/L)	cFT (pmol/L)	DHT (nmol/L)	E2 (pmol/L)	SHBG (nmol/L)	LTL (T/S ratio)
70 – 74	1,138	13.3 (0.14)	191 (1.61)	1.45 (0.022)	75.5 (0.85)	39.7 (0.44)	1.20 (0.009)
75 – 79	1,283	13.1 (0.14)	185 (1.50)	1.42 (0.021)	73.3 (0.81)	42.5 (0.46)	1.18 (0.008)
80 – 84	492	12.8 (0.23)	175 (2.55)	1.33 (0.032)	68.6 (1.27)	48.2 (0.95)	1.15 (0.012)
85 – 89	105	12.3 (0.43)	169 (5.01)	1.39 (0.073)	68.8 (2.20)	48.1 (1.74)	1.20 (0.030)
All	3,018	13.1 (0.09)	185 (0.99)	1.42 (0.013)	73.2 (0.52)	42.6 (0.31)	1.18 (0.005)
Average change per decade of age*	3,018	-0.55 (0.25)	-17.8 (2.74)	-0.088 (0.037)	-6.87 (1.44)	9.21 (0.84)	-0.045 (0.015)
Average change per decade of age#	2,913	-0.46 (0.28)	-17.8 (3.14)	-0.107 (0.042)	-7.46 (1.66)	10.16 (0.96)	-0.065 (0.017)

* Estimated from linear regression on age.

Estimated from linear regression on age, excluding men aged 85-89 years.

Table 3

Multi-variable adjusted associations between hormones and T/S ratio in 2,913 men aged 70-84 years. Table shows estimated coefficient (p-value) where coefficient represents the change in T/S ratio for a 1 SD change in the hormone variable. The adjustment variables were age, BMI, CVD history, alcohol, smoking, physical activity, cholesterol, HDL, lipid medication, diabetes, systolic blood pressure, and hypertension medication. Additional multi-variable models including both T and SHBG, and E2 and SHBG, are shown.

Hormone	Coefficient (p-value)
T (SD=4.9 nmol/L)	-0.004 (0.553)
cFT (SD=55 pmol/L)	-0.001 (0.806)
DHT (SD=0.73 nmol/L)	-0.001 (0.890)
E2 (SD=28.7 pmol/L)	**0.011 (0.043)**
SHBG (SD=17.1 nmol/L)	-0.009 (0.125)
Model including T and SHBG	
T	0.003 (0.723)
SHBG	-0.011 (0.145)
Model including E2 and SHBG*	
E2	**0.014 (0.014)**
SHBG	**-0.013 (0.037)**

* The full fitted model for study sample (n=2,913) is given in Table 4.

Table 4

Fitted multi-variable linear model for T/S ratio on E2, SHBG in 2,913 men aged 70-84 years, with adjustment variables as tabulated.

Variable		Effect size*	p-value
Age (SD=3.6 years)		**-0.021**	**0.001**
BMI (SD=3.6 kg/m^2)		**-0.014**	**0.019**
Cardiovascular disease history (Yes vs No)		-0.001	0.913
Alcohol (SD=15.4 gms/day)		0.002	0.757
Smoking	(Current vs Never)	0.019	0.445
	(Former vs Never)	0.010	0.423
Physical activity (SD=7.14 hrs/week)		0.001	0.909
Cholesterol (SD=0.95 mmol/L)		**0.015**	**0.018**
HDL (SD=0.36 mmol/L)		**-0.021**	**0.001**
Lipids medication (Yes vs No)		0.019	0.147
Diabetes (Yes vs No)		0.002	0.880
Systolic blood pressure (SD=20 mmHg)		0.006	0.258
Hypertension medication (Yes vs No)		0.022	0.055
Estradiol (SD=28.7 pmol/L)		**0.014**	**0.014**
SHBG (SD=17.1 nmol/L)		**-0.013**	**0.037**

* Effect size is calculated as (adjusted) difference in T/S ratio from reference category for categorical variable and for a 1 SD increase (i.e. coefficient*SD) for continuous variable.

Table 5

Selected studies examining associations of circulating sex hormone concentrations with leucocyte telomere length in men. T=testosterone, E2=estradiol, SHBG=sex hormone-binding globulin, LTL=leucocyte telomere length.

Author, year and reference	Age range (years)	Age mean or median (years)	N	Hormone assay	Results
Bekaert S, et al. 2005 [18]	71-86	75.0 (median)	110	Immunoassay	No association of LTL with T or E2
Yeap BB, et al. 2016 [19]	17-97	53.7 (mean)	980	Mass spectrometry	Higher E2 associated with longer LTL, aromatase polymorphisms associated with lower E2 also associated with shorter LTL
Coburn SB, et al. 2018 [20]	≥20	42.0 (median)	499	Immunoassay	No association of T with LTL, higher E2 associated with shorter LTL
Yeap BB, et al (current analysis*)	70-84	76.7 (mean)	2,913	Mass spectrometry	Higher E2 associated with longer LTL, higher SHBG associated with shorter LTL

* current analysis is from the Health In Men Study, a different cohort from the Busselton Health Study reported in [19]

Figure 1