Title

Epidemiological and Mendelian randomisation studies of dihydrotestosterone and estradiol, and leucocyte telomere length in men.

Short title

Hormones, gene polymorphisms and telomere length

Authors

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Grants and funding

This study was funded by the Project Grant 1021326 from the National Health and Medical Research Council of Australia. The 1994/1995 Busselton Health Survey was supported and funded by the Health Promotion Foundation of Western Australia. BBY is recipient of a Clinical Investigator Award from the Sylvia and Charles Viertel Charitable Foundation, New South Wales, Australia. The funding sources had no involvement in the planning, analysis and writing of the manuscript.

Disclosures

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

Key words dihydrotestosterone; estradiol; aromatase; polymorphism; telomere length

Word count 3,489

Abstract

Context

Advancing age is accompanied by accumulation of ill-health and shortening of chromosomal telomeres signifying biological ageing. Testosterone (T) is metabolised to

dihydrotestosterone (DHT) by 5α-reductase (SRD5A2) and to estradiol (E2) by aromatase (CYP19A1). Telomerase preserves telomeres, and T and E2 regulate telomerase expression and activity *in vitro*.

Objectives

To establish whether circulating T or its metabolites DHT or E2, and single nucleotide polymorphisms (SNPS) in SRD5A2 or CYP19A1 associate with leucocyte telomere length (LTL) in men.

Participants and methods

Early morning serum T, DHT and E2 were assayed using mass spectrometry, and SRD5A2 and CYP19A1 snps and LTL analysed by PCR in 980 men from the Western Australian Busselton Health Survey. LTL was expressed as the T/S ratio.

Results

Men were aged (mean±SD) 53.7±15.6 years. LTL decreased linearly with age, from T/S ratio 1.89±0.41 at <30 years to 1.50±0.49 at 70 to <80 years (r=-0.225, p<0.0001). After adjustment for age, DHT and E2 were positively correlated with LTL (DHT r=0.069, p=0.030; E2 r=0.068, p=0.034). The SRD5A2 rs9282858 polymorphism was associated with serum DHT but not with LTL. Three dominant alleles of CYP19A1 were each associated with lower serum E2 and shorter LTL: rs2899470 T (E2 59.3 vs 68.6 pmol/L, p<0.0001; T/S ratio 1.54 vs 1.62, p=0.045), rs10046 C (60.5 vs 68.1 pmol/L, p=0.0005, 1.54 vs 1.62, p=0.035) and rs700518 A (59.9 vs 68.9 pmol/L, p<0.0001, 1.54 vs 1.63, p=0.020). A single copy haplotype C/T/I/A/T rs10046/rs2899470/rs11575899/rs700518/rs17703883 (52% prevalence) was associated with both lower E2 and shorter LTL.

Conclusions

In men, serum DHT and E2 correlate with LTL independently of age. Aromatase gene polymorphisms include 3 dominant alleles which are associated with both lower serum E2

and shorter LTL. E2 influences telomere length *in vivo* thus warranting further studies to examine whether hormonal interventions might slow biological ageing in men.

Introduction

Telomeres are essential DNA-protein complexes at the free ends of chromosomes comprising TTAGGG repeats, which protect the ends from fusion and degradation [1]. Conventional DNA replicative enzymes cannot fully replicate telomere ends, thus their length is progressively shortened with each mitotic cell cycle. Attrition of telomeres has been thought to result 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 [2,3]. Of note, telomere homeostasis is a dynamic process with telomere shortening being countered by the activity of telomerase, the reverse transcriptase enzyme responsible for elongating telomeres by addition of telomeric repeats to chromosomal ends [1]. Life stress has been associated with shorter telomeres [4]; conversely comprehensive lifestyle changes can influence telomerase activity and not only preserve, but increase telomere length over time [5]. Cross sectional and longitudinal studies have reported consistent reductions in telomere length with increasing age (for review, see [6]). However, it remains unclear whether chronological age alone drives the shortening of telomeres, as opposed to reflecting the cumulative influence of adverse environmental or physiological factors, and cardiovascular or other diseases [7]. Thus telomere length represents a cellular marker for biological ageing, and factors which predict increased telomere length offer potential avenues for interventions to preserve health.

A sexual dimorphism exists above fifty years of age when men have shorter telomeres (and life expectancy) compared with women [8]. Hormonal regulation of telomerase activity and

hence telomere length could be considered as a possible explanation. Testosterone (T) is the principal male sex hormone whose production is regulated by pituitary luteinising hormone (LH), and which circulates bound to sex hormone-binding globulin (SHBG). T is metabolised by 5α-reductase (SRD5A2) to the more potent androgen dihydrotestosterone (DHT), and by aromatase (CYP19A1) to the most potent estrogen, estradiol (E2) [9]. T increased telomerase expression and activity in ovarian cancer cells [10], while both the synthetic androgen methyltrienolone and E2 increased telomerase activity in cultured peripheral blood lymphocytes [11]. In breast, prostate and liver cells, E2 increased telomerase expression and activity [12-14].

Although the experimental data are suggestive, human data exploring the association of hormones with telomere length are limited. Peripheral blood is a convenient source of DNA in which to assess leucocyte telomere length (LTL) which correlates with telomere length in skin and other tissues [15-17]. In post-menopausal women use of hormone replacement therapy has been associated with preservation of LTL [18,19]. In a study of 110 men aged 71-86 years, telomere length was inversely correlated with age, but not serum T or E2 measured with immunoassay, and shorter LTL was associated with bone loss [20]. However, immunoassays for sex steroid hormones such as T may exhibit non-specificity and method-dependent bias thus a larger sample size and accurate measurement of sex hormones using mass spectrometry would be preferable.

While both T and E2 have been identified as hormones increasing telomerase activity in cells, it remains uncertain whether either influences LTL *in vivo* in men. Furthermore, an association of DHT with LTL has not been explored. The question arises as to whether in men, lower levels of T or its biologically active metabolites, DHT and E2, might be related to

shorter telomere length; and if so whether shorter LTL could mediate associations of low T with ill-health. Functional polymorphisms of the CYP19A1 gene vary the activity of the enzyme in converting T to E2 [21]. This equates to a genetically determined exposure since birth, allowing an analysis of outcomes at a specific time-point to encompass a lifetime of exposure to risk. There are fewer recognised polymorphisms of the SRD5A2 gene which influence catalysis of T to DHT [22]. We tested the hypothesis that higher concentrations of sex hormones measured using mass spectrometry would be independently associated with longer LTL in men, then extended these findings by performing Mendelian randomisation studies to explore causality using CYP19A1 and SRD5A2 polymorphisms affecting circulating E2 and DHT respectively.

Methods

Study population

The Busselton Health Study (BHS) is based in the coastal region of Busselton in Western Australia with a predominantly Anglo-Celtic population [23]. A series of cross-sectional surveys were conducted over 1966-1987 in this population. Surviving participants of these surveys were invited to participate in a follow up survey in 1994/95. On this occasion, 2,143 men aged 17 to 97 years participated and provided blood and leucocyte DNA samples for analysis. The 1994/95 survey was approved by the Human Research Ethics Committee of the University of Western Australia (Ethics 05/05/004/B74) and all participating men provided written consent.

Assessment of medical comorbidities

Methods used in the Busselton Health Survey have previously been described [23]. A comprehensive health and lifestyle questionnaire and physical assessment were completed.

The questionnaire identified smoking history, alcohol consumption, minutes of modest- and vigorous- intensity leisure time physical activity per usual week, diabetes and medications. Alcohol consumption was labelled 'light' if consumption was ≤140g/week and 'heavy' if consumption was >140g/week. Blood pressure, height and weight were recorded. Body mass index (BMI) was defined as weight (kg) divided by height (m) squared. Further assessment of medical comorbidities was performed using the Western Australian Hospital Morbidity Data System, which records all hospital admissions to public and private hospitals in Western Australia [24]. Hypertension was defined based on self-reported use of antihypertensive medications at the survey or a history of hospital admissions with hypertension (ICD-9 codes 401-405). Diabetes was based on self-reported doctor-diagnosed diabetes or use of glucose-lowering treatment at the survey, or a history of hospital admissions with a diagnosis of diabetes (ICD-9 code 250). History of CVD was defined as having any hospital admission for CVD (ICD-9 codes 390-459) during the 15 years before the survey (i.e. 1980-1994).

Biochemical assessments

Blood samples were collected in the early morning after an overnight fast and serum was subsequently stored at -70°C until time of analysis. Serum T, DHT and E2 were quantified within a single LC-MS run without derivatization using atmospheric pressure photo-ionization for positive mode for androgens and negative mode for estrogens, from 200 μL samples as previously described [25]. Between-run imprecision was T 8.6% at 5.3 nmol/L and 7.9% at 26.9 nmol/L, DHT 11.3% at 1.3 nmol/L and 9.1% at 5.3 nmol/L, E2 14.5% at 73 pmol/L and 9.9% at 279 pmol/L. Sex hormone binding globulin (SHBG) was assayed using a solid-phase, two-site enzyme immunometric assay with chemiluminescent substrate (Immulite 2000xPi; Siemens Healthcare, Bayswater, Victoria, Australia) with between-run imprecision of 3.4% at 39.4nmol/L. Luteinising hormone (LH) was assayed using a two-step

noncompetitive chemiluminometric immunoassay (Abbott Architect, Abbott Diagnostics, North Ryde, NSW, Australia) with between-run imprecision of 5.6% at 4.8 IU/L. Fasting serum cholesterol, high-density lipoprotein (HDL) and triglycerides (TG) were determined by standard enzymatic methods on a Hitachi 747 analyser (Roche Diagnostics, Castle Hill, NSW, Australia).

Analysis of polymorphisms in the 5α-reductase (SRD5A2) and aromatase (CYP19A1) genes

SRD5A2 and CYP19A1 SNPS were analysed using Taqman® SNP genotyping assays, designed and supplied by Applied Biosystems (ABI proprietary sequences). Taqman genotyping was performed in 384-well plates according to the manufacturer's protocol. Following PCR amplification, an allelic discrimination plate read was performed using an Applied Biosystems 7900HT Fast System. Genotyping was successful >98% of samples. Haploview [26] was used to determine the linkage disequilibrium between the CYP19A1 SNPS. Analysis was restricted to common haplotypes observed at a frequency >5%.

Measurement of leucocyte telomere length (LTL)

We optimised a PCR-based methodology for accurate measurement of LTL utilising the protocol described by Cawthon et al [27]. Briefly, telomere lengths of the leucocyte DNA samples were measured by a multiplex quantitative PCR method. 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 the analyses. A standard curve derived from K562 cell line was used to transform the cycle threshold into ng 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%. We measured LTL in a random sample of 1,146 men of the 2,143 men in the 1994/95 survey.

Statistical analysis

SAS version 9.4 was used to analyse the data. Results were expressed as mean and standard deviation (SD) for continuous data, and percentages for categorical data. Correlation coefficients were calculated for associations of age and hormones with T/S ratio, and then hormone associations adjusted for age. There was no evidence of non-linearity. For the Mendelian randomisation and haplotype analyses linear regression models with T, DHT and E2 as the outcome, and also with T/S ratio as the outcome, were fitted and included the categorical SRD5A2 and CYP19A1 SNP variables. Models were adjusted for age, smoking, vigorous exercise, alcohol, BMI, SBP, diabetes, hypertension, use of lipid-lowering medication and cardiovascular disease, as factors influencing health status in older men. A p-value of <0.05 was considered significant.

Results

Characteristics of the study population

We measured LTL in a random sample of 1,146 of the 2,143 men who participated in the survey. After excluding men who were taking androgens and anti-androgens (n=7), men who had a history of orchidectomy or prostate cancer (n=22) and men missing key variables (n=137), there were 980 men aged (mean±SD) 53.7±15.6 years who had hormones, SRD5A2 and CYP19A1 snps, and LTL assayed. Baseline demographic, physical and biochemical data

are shown (Table 1). Mean BMI was in the overweight range, and the prevalences of diabetes and CVD were 7.7% and 20.0%, respectively.

TABLE 1

Inverse association of leucocyte telomere length with age

There was a progressive decline in LTL with increasing age, from T/S ratio 1.89±0.41 at <30 years to 1.50±0.49 at 70 to <80 years (Table 2). The estimated linear regression was: T/S ratio = 2.13 – 0.0081 age ($p<0.0001$). Thus for an increase of a decade in age, T/S ratio was lower by approximately 0.08.

TABLE 2

Associations of hormones with leucocyte telomere length

Serum T and DHT were positively correlated with LTL (T r=0.098, p=0.002; DHT r=0.075, p=0.018) (Table 3). Of note, serum SHBG and LH were inversely correlated with age (SHBG r=-0.064, p=0.043; LH r=-0.079, p=0.013). After adjustment for age, serum DHT and E2 remained positively correlated with LTL (DHT r=0.069, p=0.030; E2 r=0.068, p=0.034), but serum T, SHBG and LH did not.

TABLE 3

Associations of SRD5A2 and CYP19A1 polymorphisms with circulating hormones

In regression models adjusting for age, smoking, exercise, alcohol, BMI, blood pressure, hypertension, diabetes and CVD, one SRD5A2 and six CYP19A1 polymorphisms were

identified which were associated with lower serum DHT or E2, respectively (Supplemental Table 1). In the case of the SRD5A2 rs9282858 polymorphism, two men with the AA allele were excluded from the analysis. The GA allele was associated with lower serum DHT compared with GG. In each of the CYP19A1 polymorphisms, the results fit a dominant model, with lower serum E2 in men with both the minor allele homozygote and the heterozygote genotypes, compared with the unexposed major allele homozygote genotype. For rs2470152 men with CT or TT had lower E2 concentrations compared with CC. Comparable results were seen for the other five polymorphisms: rs17703883 TC, CC vs TT, rs2899470 GT, TT vs GG, rs10046 CT, CC vs TT, rs700518 GA, AA vs GG and rs11575899 ID, DD vs II. The dominant allele model was applied subsequently to the analysis of genotype associations with LTL.

SUPPLEMENTAL TABLE 1

Mendelian randomisation analyses of telomere length

In regression models adjusting for age and other covariates, the SRD5A2 rs9282858 polymorphism was not associated with any difference in LTL (Table 4). In the adjusted analysis three dominant alleles of CYP19A1 were associated with both lower serum E2 and shorter LTL: rs2899470 GT+TT vs GG (E2 59.3 vs 68.6 pmol/L, $p<0.0001$; LTL 1.54 vs 1.62, $p=0.045$), rs10046 CT+CC vs TT (60.5 vs 68.1 pmol/L, $p=0.0005$, 1.54 vs 1.62, $p=0.035$) and rs700518 GA+AA vs GG (59.9 vs 68.9 pmol/L, $p<0.0001$, 1.54 vs 1.63, $p=0.020$).

TABLE 4

Haplotype analyses of telomere length

Deviations from Hardy-Weinberg equilibrium (HWE) at p=0.05 level were observed for the CYP19A1 SNPs (Supplemental Table 2). A linkage disequilibrium map shows that these SNPs are in high linkage equilibrium (Supplemental Figure 1). The four most common haplotypes with a frequency cut-off >5% were analysed in relation to circulating E2 and LTL (Table 5). There were two 2 copy haplotypes which were associated with differences in E2 but not LTL. One 1 copy haplotype was associated with shorter LTL but no difference in E2 (T/G/I/G/T rs10046/rs2899470/rs11575899/rs700518/rs17703883: T/S ratio 1.51 vs 1.62, p=0.013). The remaining three 1 copy haplotypes were associated with lower circulating E2. Of these, one that was present in 52% of the study population was associated with both lower E2 and shorter LTL (C/T/I/A/T rs10046/rs2899470/rs11575899/rs700518/rs17703883: T/S ratio 1.53 vs 1.61, p=0.024).

Supplemental Table 2

Supplemental Figure 1

Table 5

Discussion

In community-dwelling men serum DHT and E2 correlate with LTL independently of chronological age, while some polymorphisms in the aromatase gene which reduce circulating E2 are associated with shorter LTL. These findings implicate exposure to DHT, and more particularly E2 as potential determinants of biological ageing in men.

Our results contrast with the previous study of 110 men aged 71-86 years by Bekaert et al which measured serum T and E2 using immunoassay, and LTL using telomere restriction

fragment length analysis [20]. In that study, while age was inversely correlated, neither serum T nor E2 were associated with LTL. In our study age was inversely correlated with LTL, an apparent correlation of serum T with LTL was not robust after adjustment for age, while higher serum DHT and E2 remained associated with longer LTL independent of age. Our cohort was larger, and we measured T, DHT and E2 using mass spectrometry thus minimising the risk that immunoassay-related non-specificity or bias might have obscured an underlying association. The inverse associations of SHBG and LH with LTL were also nullified by adjustment for age, indicating the importance of the respective hormones, DHT and E2.

In older men, the circulating androgens T and DHT can exhibit parallel associations with specific health outcomes, for example both low T and low DHT are independent predictors of incident stroke [29]. However, their predictive utility for poorer health outcomes can also diverge, with higher DHT but not T being independently associated with reduced mortality from ischaemic heart disease in older men [30]. Our results demonstrate an association of circulating DHT, rather than T, with LTL. The Mendelian randomisation analysis did not show any effect of the SRD5A2 rs9282858 AG vs GG on LTL, despite its association with lower serum DHT. However, the proportion of men carrying the AG allele was relatively small (7.2% of the cohort).

The age-independent association of E2 with LTL in our cohort of men also is novel and the Mendelian randomisation analyses involving CYP19A1 polymorphisms offer some support for the concept of causality: that genetically determined differences in exposure to higher E2 may result in better preservation of LTL. These findings *in vivo* are consistent with cellular studies demonstrating actions of E2 on telomerase expression and activity [12-14]. In other

cell models, androgens increase telomerase expression [10,11], in part via aromatisation to estrogen [11]. Three of the CYP19A1 polymorphisms we examined rs2470152, rs17703883 and rs11575899 were associated with serum E2, but not with LTL. Of the three CYP19A1 polymorphisms associated with both serum E2 and LTL, rs2899470 correlated with rs2470152 which has been associated with E2 in younger and older men [31], rs10046 has been associated with blood pressure in women [32] and rs700518 with E2 and bone density in men [33,34]. Our findings extend the recognised role of aromatase and E2 to regulate bone density in men [34], prompting consideration of a potential role for E2 in a broader context of biological ageing involving multiple tissues where telomere length mirrors LTL such as skin and synovium [15], vasculature [16] and muscle [17]. For the three CYP19A1 polymorphisms influencing LTL, the dominant alleles were associated lower serum E2 approximating 10 pmol/L and a shorter T/S ratio at around 0.08. Thus a modest reduction in circulating E2 was associated with a difference in LTL corresponding to an increase of a decade of chronological age.

These findings need to be interpreted with care, as the relevant aromatase snps rs10046 and rs700518, and rs2899470 and rs10046, were in linkage disequilibrium with each other. The haplotype analysis identified one commonly expressed haplotype which was associated with both lower E2 and shorter LTL. However the overall results were not entirely consistent. Not all haplotypes associated with lower E2 were associated with shorter LTL, and one haplotype associated with shorter LTL was not associated with lower circulating E2. One possible explanation would be that circulating E2 and LTL are affected by common variables including age and BMI [6,25], and other unmeasured factors including life stress and lifestyle behaviours in the case of LTL [4,5]. Replication of these results in other large prospective cohorts would be important. We cannot fully discount the possibility that the results are

chance or coincidental findings, nevertheless the conjunction of age-adjusted associations between circulating hormones with LTL, and suggestive findings from some of the Mendelian randomisation studies, would allow us to postulate an underlying relationship between the two.

Strengths of our study include the study of a large cohort of community-dwelling men, availability of early morning serum T, DHT and E2 measured by mass spectrometry, and SRD5A2 and CYP19A1 polymorphism data in addition to LTL results. Genetic assays were performed rigorously including the use of triplicates for LTL assay samples. We were able to undertake correlative analyses of hormone concentrations with LTL, and Mendelian randomisation analyses using SRD5A2 and CYP19A1 polymorphisms and LTL. Limitations of our study include the use of a single blood sample, albeit taken early in the morning to minimise effects of circadian variation on hormone concentrations, and the lack of additional informative SRD5A2 polymorphisms with only the rs9282858 polymorphism demonstrating differences in serum DHT. Several aromatase polymorphisms were in linkage disequilibrium, and not all the results of the genetic analyses were informative. We did not have serial blood samples to determine longitudinal changes in either hormone concentrations or LTL. Our study population is predominantly Caucasian and therefore our findings may not apply to other populations comprising other ethnicities or to women.

Cellular senescence has been postulated as a consequence of telomere shortening below a critical threshold [2,3]. Even before telomere shortening reaches this stage, inactivation of telomerase results in accelerated ageing [35]. Consistent with a biomarker or a possible contributing factor for biological ageing, shorter LTL predicts age-related poorer health outcomes such as dementia and to an extent, with mortality [36,37]. Telomere length is

heritable, and loci affecting LTL are also associated with increased risk of coronary artery disease [38]. In that genome-wide meta-analysis, no CYP19A1 polymorphisms were identified as being associated with LTL [38]. Our results raise the question of whether interventions which increase circulating E2 would favour longer LTL, and thereby slow the process of biological ageing in men. Notably in this context, as an estrogen-response element is present in the promoter of the catalytic subunit of the telomerase enzyme, estrogen acting transcriptionally could stimulate telomerase activity [39]. In addition, whilst telomerase activity is repressed in many somatic tissues during extra-uterine life, it is present in highly proliferative tissues such as the haematopoietic system, testis and skin [40], thus potentially linking telomerase induction by estrogen with greater circulating LTL.

Observational studies of men who were castrated have suggested an association with extended lifespan [41,42]. However, the studies were limited by potential selection biases, behavioural confounders and use of grouped controls [41,42]. Of note, individual case-control studies of European castrati singers have shown no difference in life expectancy [43,44]. By contrast men with Klinefelter Syndrome exhibit increased mortality risk and reduced survival [45]. Our results warrant confirmatory studies in other populations, and provide a rationale for randomised placebo-controlled clinical trials to determine whether interventions which raise concentrations of T and its metabolites DHT or E2 could slow biological ageing and improve health outcomes in men.

Conclusions

In men, serum DHT and E2 correlate with LTL independently of age. Aromatase gene polymorphisms include 3 dominant alleles which are associated with both lower serum E2 and shorter LTL. Haplotype analysis demonstrated one common haplotype which was

associated with lower serum E2 and LTL. While replication in other cohorts and further investigation of the effects of DHT are required, these results suggest a putative role for circulating E2 in the regulation of telomere length *in vivo*. Further studies are warranted to examine whether interventions involving T supplementation via its metabolism to DHT and E2 might slow biological ageing and thereby preserve health in men.

Acknowledgements

We thank Surya Sutanto, University of Sydney, NSW, and the staff of PathWest Laboratory Medicine, Sir Charles Gairdner Hospital, WA, and the ANZAC Research Institute, NSW, for their excellent technical assistance. We thank the staff of the Health Department of Western Australian Data Linkage Branch, the Hospital Inpatient Data Collection system and Registrar General for their assistance in providing linked data. The authors especially thank the Busselton men who took part in the survey and the Busselton Population Medical Research Institute for access to the survey data and specimens.

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Legend for Supplemental Figure 1

Linkage disequilibrium map of aromatase (CYP19A1) polymorphisms analysed in this study.