**Title:** Distribution of Salivary Testosterone in Men and Women in a British General Population-Based Sample: the Third National Survey of Sexual Attitudes and Lifestyles (Natsal-3)

**Short title**: Salivary testosterone men and women

**Key words:** Saliva, testosterone, LC-MS, population

**Word Count:** 3921 words

**Author list:** BG Keevil*[1], S Clifton[2], C Tanton[2], W Macdowall[3], AJ Copas[2], D Lee[4], N Field[2], KR Mitchell[3,7], P Sonnenberg[2], J Bancroft[5], AM Wallace†[6], CH Mercer[2], AM Johnson[2], K Wellings[3] and FCW Wu[8].

1 Department of Clinical Biochemistry, University Hospital South Manchester, Manchester Academic Health Science Centre, The University of Manchester, Manchester, UK

2 Research Department of Infection and Population Health, University College London, London UK

3 Department of Social and Environmental Health Research, London School of Hygiene and Tropical Medicine, London UK

4 Cathie Marsh Institute for Social Research, School of Social Sciences, The University of Manchester, Manchester, UK

5 Kinsey Institute, Indiana University, Bloomington USA.

6 Department of Clinical Biochemistry, Royal Infirmary, Glasgow G4 0SF, Scotland, U.K.

7 MRC/CSO Social and Public Health Sciences Unit, University of Glasgow, Glasgow, UK

8 Andrology Research Unit, Manchester Centre of Endocrinology and Diabetes, Manchester Academic Health Science Centre, The University of Manchester, Manchester, UK

***Corresponding author contact details:** Brian Keevil, Department of Clinical Biochemistry, University Hospital South Manchester, Southmoor Rd, Manchester M23 9LT.

Tel: 0161 291 2135 Email: brian.keevil@uhsm.nhs.uk

† Deceased

**Conflicts of interest**

S.C. is supported by the National Institute for Health Research (NIHR Research Methods Programme, Fellowships and Internships, NIHR-RMFI-2014-05-28). The views expressed in this publication are those of the author(s) and not necessarily those of the NHS, the National Institute for Health Research or the Department of Health. A.M.J. has been a Governor of the Wellcome Trust since 2011. FCWW has acted as a consultant for Bayer-Schering, Eli Lilly and Besins Healthcare and also participated in advisory board meetings and lectured on their behalf. FCWW has received lecture fees from Bayer-Schering and Besins Healthcare. FCWW has received grant support (2010-2014) from Bayer Schering AG and Besins Healthcare.

**Acknowledgements**

We thank the study participants; the team of interviewers from NatCen Social Research, operations and computing staff from NatCen Social Research; and the study funders. Natsal-3 is a collaboration between University College London, the London School of Hygiene & Tropical Medicine, NatCen Social Research, Public Health England (formerly the Health Protection Agency), and the University of Manchester. The study was supported by grants from the Medical Research Council [G0701757]; and the Wellcome Trust [084840]; with contributions from the Economic and Social Research Council and Department of Health. We gratefully acknowledge the important technical contributions of Halina McIntyre and Anne Kelly, Department of Clinical Biochemistry, Royal Infirmary, Glasgow G4 0SF, Scotland, U.K. Philip Macdonald, Department of Clinical Biochemistry, University Hospital South Manchester, Manchester UK. SC is supported by the National Institute for Health Research (NIHR Research Methods Programme, Fellowships and Internships, NIHR-RMFI-2014-05-28). NF is supported by an academic clinical lectureship. The views expressed in this publication are those of the author(s) and not necessarily those of the NHS, the National Institute for Health Research or the Department of Health. Since September 2015, KM has been core funded by the UK Medical Research Council (MRC); MRC/CSO Social & Public Health Sciences Unit, University of Glasgow (MC_UU_12017-11).

**Abstract** [252 words]

*Introduction*

Measurement of salivary testosterone (Sal-T) to assess androgen status offers significant potential advantages in epidemiological research. Utility of the method depends upon interpretation of results against robustly determined population distributions, which are currently lacking.

*Aim*

To determine age-specific Sal-T population distributions for men and women.

*Methods*

Morning saliva samples were obtained from participants in the third National Survey of Sexual Attitudes and Lifestyles (Natsal-3): a probability-sample survey of the British general population. Sal-T was measured using LC-MS/MS; linear and quantile regressions were used to determine age-specific 2.5$^{th}$ and 97.5$^{th}$ percentiles for the general population = (1,675 men, 2,453 women) and the population with health exclusions (1,145 men, 1,276 women).

*Results*

In the general population, mean Sal-T in men decreased from 322.6 pmol/L in 18 year olds to 153.9 pmol/L in 69 year olds. In women, the decrease in the geometric mean Sal-T was from 39.8 pmol/L in 18 year olds to 19.5 pmol/L in 74 year olds. The annual decrease varied with age with an average of 1.0-1.4% in men and 1.3-1.5% in women. For women, the 2.5$^{th}$ percentile fell below detection limits (<6.5 pmol/L) from age 52 years. Mean Sal-T was approximately six times higher in men than women, and this remained constant over the age range. Sal-T was lowest for men, and highest for women, in summer. Results were similar for the general population with exclusions.

*Conclusions*

This is the first study to describe sex and age-specific distributions for Sal-T in a large representative population using a specific and sensitive LC-MS/MS technique. The present data can inform future population research by facilitating the interpretation of Sal-T results as a marker of androgen status.

## Introduction

Use of saliva in the investigation of testosterone (T) status is attractive because sample collection is convenient, requires minimal training and can be easily undertaken at home. Measurement of salivary testosterone (Sal-T), therefore, offers great potential in facilitating epidemiological and biomedical research at a population level.

Most T circulating in the blood is bound to sex hormone binding globulin (SHBG) and albumin, rendering only a small free (unbound) fraction (~1-2%)[1] which can diffuse across capillaries into target tissues where it exerts biological activity. Direct measurement of serum free-T (the 'gold standard') is technically challenging and expensive hence serum free-T is usually derived from mathematical formulae based on association constants of T with its binding proteins.[2] However, the relationship of calculated serum free T to directly-measured free T, as well as their clinical significance, has not been universally accepted.[3] T circulating in the body readily diffuses across capillaries and salivary ducts resulting in a salivary fraction containing free unbound T.[4] Measurement of Sal-T may thus provide an alternative to measuring serum total-T, free-T or bioavailable-T in the assessment of androgen status. Concerns have been raised regarding the reliability of Sal-T measurement using immunoassay methods.[5] However, recent methodological advances have allowed Sal-T to be reliably and accurately measured by more specific and sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS).[6-8] In both men and women, Sal-T correlates well with calculated serum free-T[8] and does not correlate with SHBG.[9] High correlation between Sal-T and serum free-T measured by equilibrium dialysis in both men and women has also been confirmed, but there was a significant systematic positive bias in women, which may reflect the influence of salivary protein binding to the lower female concentrations of Sal-T.[8] Whether Sal-T can be a surrogate for circulating free-T, or a valid measure of tissue bioavailable-T, can now be further investigated.

Application of Sal-T measurements for the assessment of androgen status in men and women is critically dependent upon the interpretation of results against rigorously determined age-specific

population distributions. Using relatively small numbers of samples from hospital personnel or clinic attenders for this purpose is convenient but problematic due to inherent selection bias and inadequate statistical power. Population ranges based on probability samples are more representative of the general population; they have only become available recently for serum-T measurements in both men[10] and women[11] and as yet, have not been widely utilised. The present study aimed to determine age-specific population distributions for Sal-T in a large sample of adult men and women from the general population in Britain using a highly sensitive and specific LC-MS/MS method. We provide population distributions for the general population with exclusions (excluding those with self-reported medical conditions or using medications that can alter T levels) as well as the general population across the full age range to maximise usefulness for a broad range of research studies.

## Methods

*Study Population*

The third National Survey of Sexual Attitudes and Lifestyles (Natsal-3) is a stratified probability sample survey of 15,162 men and women aged 16-74 years resident in Britain, which used the Postcode Address File as its sampling frame. Participants were interviewed between September 2010 and August 2012 using computer-assisted personal interviewing (CAPI), including a computer-assisted self-interview (CASI) for the more sensitive questions. The response rate was 57.7%. Full details of the methods are described elsewhere.[12, 13]

After the interview a subsample of men and women aged 18-74 years, who did not regularly work night shifts, were invited to provide a saliva sample to test for T, without return of results. Consenting participants were given a self-collection pack and asked to provide their sample before 10am, to minimise diurnal variation in T.[7] They were asked not to brush their teeth, eat or chew before giving the sample, and to spit directly into a plain polystyrene tube. Saliva samples were posted to the laboratory where they were prepared and frozen at -80° C until analysis.[7] On receipt of the sample, participants were sent a £5 voucher as a token of appreciation.

Of 13,431 participants aged 18-74 years who did not regularly work at night, 9,170 people were invited to provide a saliva sample. In total 4,591 samples were received and matched to the survey data (50.1% of those invited to provide a sample). Of those, 463 samples (10.1%) were excluded (insufficient volume (n=154); sample discoloured/bloody (n=91); sample recorded as taken after 10.30am (n=34); period between sample being taken and received by the laboratory more than 5 days or unknown due to missing date of collection (n=172); not tested due to error (n=12)), leaving 4,128 (45.0%) with a T result.

Analysis for the general population included all 4,128 participants (1,675 men and 2,453 women) with useable T results. To generate the distribution for a general population with exclusions (those who did not report health conditions or taking medication which may influence T levels), 530 men and 1,177 women were excluded from analysis as follows (individuals may be excluded for more than one reason): prostate cancer (13 men); prostate enlargement (90 men); prostate surgery (20 men); polycystic ovaries (35 women); treatment for any of the following in the past year: cancer (25 men; 49 women), thyroid conditions (27 men; 183 women), testicular or pituitary conditions (16 men), ovarian or pituitary conditions (23 women); prescription medication for epilepsy (15 men; 15 women); hysterectomy and HRT (to indicate oophorectomy, 181 women) and unprompted reporting use of T (1 man). We did not ask participants directly about their use of T. Additionally excluded were 363 men with a body mass index (BMI) <18.5 or >30 kg/m$^2$ and 118 women with a BMI <18.5 or >40 kg/m$^2$. Women reporting current use of either HRT (62 women) or hormonal contraception (pill, mirena, injections, implants, patch: 535 women) and those who were currently pregnant (42 women) were also excluded. Finally, individuals who did not answer any of the above questions were excluded (42 men; 134 women), leaving 1,145 men and 1,276 women included in analysis of the general population with exclusions.

*Measurements*

The LC-MS/MS Sal-T assay was developed using strict validation criteria.[7, 14] Sample preparation using liquid–liquid extraction entailed adding sample (200 μL), $D_5$-testosterone internal standard (10 μL, 340 pmol/L) and methyl-tert-butyl ether (1 mL). After vortexing for 5 minutes the organic layer was transferred and evaporated and the residue reconstituted with a 500 mL/L methanol mobile phase (80 μL) and transferred to a 96-well microtiter plate.

Liquid chromatography was performed with an ACQUITY™ Ultra Performance Liquid Chromatography system coupled to a Xevo TQ-S™ mass spectrometer (Waters Corporation, Manchester, UK) operated in positive ionization mode. The lower limit of quantification was 6.5 pmol/L and the assay was linear to at least 52,000 pmol/L. Inter-assay CV (SD) bias was 12.9% (1.7) 1.2%; 9.8% (2.5) 0.4%; and 4.5% (12.0) 1.9% at concentrations of 12.9, 26.0 and 260 pmol/L respectively. Intra assay CV(SD) bias was 9.5% (1.3) 0.8%; 5.5% (1.6) 12.6%; and 2.1% (6.2) 11.1% at concentrations of 12.9, 26.0 and 260 pmol/L respectively. Recovery was 104% (98.3 – 108.9 range).[7]

*Statistical analyses*

Statistical analyses were carried out using STATA (version 13.1) accounting for the complex survey design (stratification, clustering, and weighting of the sample) [StataCorp. 2013. *Stata Statistical Software: Release 13*. College Station, TX: StataCorp LP]. We applied two weights when analysing the data: the survey weight corrects for unequal probability of selection and differential response (by age, sex, and region) to the survey itself; the saliva weight corrects for unequal probability of selection and differential response to the saliva sample. A number of factors were found to be associated with providing a sample, including age at interview, ethnicity, general health, and sexual function measured using the Natsal-SF.[15] Full details of these weights and their calculation have been published elsewhere.[12]

We used two statistical approaches to estimate the 2.5$^{th}$ to 97.5$^{th}$ percentiles for the population distributions for Sal-T in men and women: linear regression and quantile regression, as previously reported for calculating serum-T reference ranges.[10, 11] Both analyses were carried out to produce the distributions limits for the general population and the general population with exclusions.

Linear regression, as a parametric technique, may be unduly affected by extreme values. Therefore, very high Sal-T values were censored so that for each 10-year age group stratified by sex, values above the 99$^{th}$ percentile were replaced by the 99$^{th}$ percentile (17 men; 26 women). The 99$^{th}$ percentile values ranged from 587.4 pmol/L in the youngest men to 352.6 pmol/L in the oldest men, and 233.2 pmol/L to 104.6 pmol/L in the youngest and oldest women respectively. The Sal-T data for men were approximately normally distributed, however the distribution for women was skewed and so values were transformed on the natural log scale for analysis, then back-transformed to generate the final population distribution limits. As quantile regression is a non-parametric approach, there was no need to censor the extreme high values of Sal-T, or to transform the data for women.

Three men (all aged >60 years, all included in the general population with exclusions) and 76 women (distributed across the 18-74 years age range, 33 of whom were included in the general population with exclusions) had Sal-T levels below the limit of detection (<6.5pmol/L). Interval regression was used assigning these cases to the range 0 to 6.5pmol/L for the linear regression for men. The lower bound among women was set as 0.5 so that values could be log transformed. For the quantile regression these cases were assigned a value of 3.25pmol/L (half the limit of detection).

For both men and women, the standard deviation (SD) of Sal-T was not constant with age. Therefore, after fitting the linear regression for the mean values we calculated the SD of Sal-T for each year of age and used these values as the outcome in a second linear regression to predict the SD as a function of age. The predicted 2.5$^{th}$ and 97.5$^{th}$ percentiles for each year of age were calculated as the predicted mean Sal-T minus the predicted SD for that age multiplied by $b_l$ and the predicted mean plus the predicted SD multiplied by $b_u$ respectively, where $b_l$ and $b_u$ were selected so that across all ages 2.5%

of the population had T values below the lower bounds and 2.5% of the population had T values above the upper bounds. We tried different values of each multiplier, $b_l$ and $b_u$, starting with 1.96 which corresponds to the normal distribution and iteratively increasing or decreasing the values until we achieved the desired coverage. For men in the general population the values were: $b_l = 2.00$ and $b_u = 2.30$, and for women in the general population they were $b_l = 2.11$ and $b_u = 1.96$. The values for the men in the general population with exclusions were: $b_l = 2.09$ and $b_u = 2.25$, and for women were: $b_l = 2.10$ and $b_u = 1.96$.

For men, the SD of Sal-T decreased with age up to a point (from around age 70), then increased again in the oldest age group. We were unable to adequately model this increase in SD to accurately calculate the $2.5^{th}$ and $97.5^{th}$ percentiles (which are based on SD), and consequently truncated the population distribution analysis for men at age 69. There was no equivalent increase in the SD among older women; therefore data are presented for the full age range, 18-74. Truncation was not necessary for analysis of mean testosterone levels, including associations with season.

To allow for a possible non-linear relationship between Sal-T and age, we explored two different functions of age (in addition to a linear function) in both the linear and quantile regressions: a quadratic function and a restricted cubic spline function. For the latter, three knots were specified at the $10^{th}$, $50^{th}$, and $90^{th}$ percentiles of age (the default placement for three knots). The population distribution produced by the models using the quadratic and cubic spline functions were similar; therefore, we opted to use the simpler quadratic function in the final models. For women, analyses were performed on log-transformed data then back-transformed, therefore geometric means are presented.

To assess seasonal variation in T, mean (geometric mean for women) testosterone and 95% confidence intervals were plotted by season for the general population and linear regression was used to test for differences. Season was defined as: Winter (December, January, February); Spring (March, April, May); Summer (June, July, August); Autumn (September, October, November). In order to

explore potential geographical differences, participants were grouped into three broad regions of residence: Scotland and North of England, Midlands and Wales, and East and South of England (including London).

*Ethics statement*

Natsal-3 was approved by the Oxfordshire Research Ethics Committee A (reference: 09/H0604/27). Written informed consent was obtained for anonymised testing of saliva samples, without return of results.

**Results**

Distributions of mean (SD) and median (IQR) Sal-T in the general population by 10-year age groups is shown in Table 1, and for the general population with exclusions in appendix Table 1. Sal-T for both men and women showed a distinct age-related decline with clear demarcation in mean levels between men and women. The mean Sal-T concentration was approximately six times higher in men compared to women, this relationship remained constant over the six decades studied in both the general population and the general population with exclusions (Table 1 and appendix Table 1).

Sal-T distributions based on linear and quantile regression analyses for men and women in the general population are shown in Figure 1. For both men and women, the linear and quantile regressions produced similar population distributions. Appendix Table 2 shows age-specific values for the 2.5$^{th}$ and 97.5$^{th}$ percentiles of the distribution for the general population produced by the linear regression (those produced by the quantile regression are not shown). Sal-T distributions for men and women in the general population with exclusions are shown in appendix Figure 1 and values for the 2.5$^{th}$ and 97.5$^{th}$ percentiles of the distribution are shown in appendix Table 2. For women, the 2.5$^{th}$ percentile fell below the limit of detection (<6.5pmol/l) from age 52 onwards in the general population and age 54 onwards in the general population, with exclusions and so values are not given.

There were a wide range of Sal-T values above the 97.5$^{th}$ percentile among women under the age of 55 years, but for those over that age most of the high values were clustered just above the 97.5$^{th}$ centile line (Figure 1). Although detailed information on menstrual phase was not collected, our questionnaire enabled identification of women who provided saliva samples within seven days of starting their last menstrual period (presumed early follicular phase). Very few of the high values in the pre-menopausal women were among those in the early follicular phase (data not shown) suggesting that they may reflect mid-cycle T peaks.[16]

Over the full age range examined, mean Sal-T decreased by around 50-60% in both the general population with exclusions and the general population of men and women. As our models of the association between Sal-T and age included a non-linear function of age, the predicted year-on-year decline in testosterone varied by age. For men in the general population, the predicted decrease in average Sal-T for each year of age was 1.3-1.5%; the predicted decline between age 18 and 19 was 1.4% (322.6 to 318.0 pmol/L), between age 45 and 46 was 1.5% (216.9 to 212.7 pmol/L) and between age 68 and 69 was 1.3% (156.0 to 153.9 pmol/L). For women in the general population, the predicted decrease in average Sal-T for each year of age was 1.0-1.4%; the decline between age 18 and 19 was 1.0% (39.8 to 39.4pmol/l), between age 45 and 46 was 1.4% (28.9 to 28.5pmol/l) and between age 73 and 74 was 1.0% (19.7 -19.5pmol/l).

Seasonal differences in mean Sal-T were observed ($p<0.0001$ for both men and women; Figure 2), however these differed by gender, with lowest levels in the summer for men, and the highest levels in the summer for women. We found no associations between mean Sal-T and broad geographical region ($p=0.2432$ Figure 2).

**Discussion**

This is the first study to establish age-specific population distributions for LC-MS-analysed Sal-T in men and women from a large general population sample. Our findings show only minor overlap

between the age-specific male and female population distributions, mirroring those seen with serum T, and lending support to the validity of our Sal-T measurements. The six-fold higher Sal-T found in men compared to women is also similar to that observed for serum-T,[17] reflecting the markedly higher daily T blood production rate in men.[18] A distinct age trend in Sal-T was observed in both sexes. The rate of cross-sectional decline in Sal-T with age was similar to the decline in Sal-T with age in other smaller studies in men[19-21] and women,[20] but greater than the reported decline in serum-T in men[22-24] and women.[11, 25, 26]

The age-associated decline in serum T has been implicated in a variety of physiological changes in ageing men,[27, 28] but this has been disputed by some[29, 30] who suggest that the apparent decline is largely due to co-morbidity with healthy elderly men showing little change in their circulating T levels. Although we found that men over the age of 45, who did not report any of the exclusion health conditions , had slightly higher levels of Sal-T compared to the whole sample, Sal-T in these men nevertheless halved between ages 18 and 69 years. This suggests that the widely reported serum total and free T fall over the life course of 17% and 35% respectively,[22-24] may under-estimate the ageing-associated decline in testicular function or testosterone bioavailability at the tissue level. An important corollary of this compelling age-trend in Sal-T is to reinforce the view that a Z-score approach, using age-specific population ranges, may be more appropriate and physiologically meaningful than the hitherto-preferred T score approach based on comparison with ranges derived from young (<40yr) healthy men.[10]

In pre-menopausal women, we observed some extreme high values of Sal-T, extending far above the 97.5$^{th}$ percentile, which possibly reflect mid-menstrual cycle peaks in T.[16, 26, 31] We did not collect detailed information on menstrual phase and so were unable to control for variation in T across the menstrual cycle in our analysis. In broad agreement with serum T from other large population-based studies,[11, 25, 26] we found that the decline in Sal-T in women is steepest in the early reproductive years, subsequently flattening out in midlife. In agreement also with serum-T from other studies,[11, 26, 32] we did not observe a significant effect of the menopausal transition on Sal-T. The percentage change in

serum-T previously found in healthy women aged between 20-60 years was 30%,[11] whereas the percentage change in Sal-T in our study was ~60% for a similar age range. Thus, as in men, the age-related fall in Sal-T in women who did not report any of the exclusion health conditions is appreciably greater than that observed for serum-T. The principal sources of androgens in post menopausal women are the adrenal gland and the ovary.[33] An increase in free T may also arise from a relative fall in SHBG compared to T, a finding that is consistent with the trend of decreasing SHBG across the menopausal transition.[34]

The seasonal variation in Sal-T observed in men showed the opposite trend to that seen in women, with an increase in the summer and a fall in the winter. Previous studies examining seasonal variation in serum-T levels in men and women have yielded inconsistent results; with either no seasonal variation found,[35] or with peak levels in the winter[36] or summer.[37] In the only Sal-T study, peak levels were found in October and December for women and men respectively.[38] Though statistically significant, the magnitude of the observed seasonal differences in Sal-T is relatively small (~20 pmol/L in men and ~8 pmol/L in women) and the variation may not be biologically or clinically significant. Given these inconsistencies, we do not feel it would be appropriate to provide separate Sal T population distributions by season.

Natsal-3 is broadly representative of the British population including in terms of ethnicity[13] but was not specifically designed to examine ethnic variations in testosterone. We found no association with broad geographical region, which is perhaps unsurprising given that Britain is a small country in terms of area, and previous research into geographical variation has been on a global scale.[39]

Strengths of this study are the large general population sample size, the state-of-the-art LC-MS/MS measurement of Sal-T and rigorous statistical analysis techniques. To enable the fullest application in future investigations, we established population distributions not only in the entire general population but also after exclusions of conditions and medications that can affect Sal-T. This ensures

applicability of this novel information to a wide range of epidemiological and biomedical studies in future.

This study also has some limitations. Health conditions were self-reported and single morning saliva samples do not take into account the intra-individual variations due to circhoral, diurnal and circannual rhythms. The lack of accurate information on the timing of samples in relation to the menstrual cycle and of clinical information on possible PCOS among women may have introduced added noise in the distributions. Although our sample was similar to the census with respect to ethnicity, health and marital status after weighting,[12, 13] as with any general population survey our data are susceptible to some participation biases - individuals in residential or nursing care were not included in the sampling frame and poor health could have affected willingness to participate i.e. our population distributions for the general population might refer to a slightly healthier sample than the true British general population. The final response rate to the saliva study was 45%, the saliva data were therefore weighted during analysis to minimise potential non-response bias.[12]

Age and sex specific population distributions are important as a baseline against which other analyses and other research studies can be compared. The array of background information, particularly with respect to age and BMI, will be important when considering important research questions such as the variations in Sal-T at the population level with respect to the frequency of sexual activity at the extremes of the age spectrum, sexual satisfaction, and number of sexual partners; some of these are currently being addressed in our ongoing analyses.

The present data describe the distribution of Sal T in the general population as part of a large study to investigate determinants of variations in sexual lifestyle and practices in men and women. The information is not intended to be applied to the clinical setting (without further stringent clinical evaluation) particularly with respect to hormone replacement in older individuals. Indeed the very clear decline in Sal T with age lend support to the view that lower levels of T is a physiological change and argue against the use of hormone replacement in older individuals.

We have determined age-specific population distributions for Sal-T in a large, representative population of men and women using a highly specific and sensitive LC-MS/MS technique. The relative simplicity of saliva collection has important implications for large population-based studies where serum collection has been impractical or too expensive. These population data, which can be harmonised with those from other laboratories using validated LC-MS/MS methods, provide a benchmark for ensuring the appropriate interpretation and comparisons of Sal-T results in future research. An essential first step has now been taken to pave the way for the application of Sal-T in investigating the potential importance of androgen exposure in many aspects of sexual behaviour and general health in large scale population surveys of men and women.

**Acknowledgements**

This study was funded by the Medical Research Council and the Welcome Trust

We gratefully acknowledge the important technical contributions of Halina McIntyre and Anne Kelly, Department of Clinical Biochemistry, Royal Infirmary, Glasgow G4 0SF, Scotland, U.K.

**References**


1. Hammond GL, Wu TS, Simard M. Evolving utility of sex hormone-binding globulin measurements in clinical medicine. *Current opinion in endocrinology, diabetes, and obesity*. 2012 Jun;19(3):183-9.

2. Vermeulen A, Verdonck L, Kaufman JM. A critical evaluation of simple methods for the estimation of free testosterone in serum. *The Journal of clinical endocrinology and metabolism*. 1999 Oct;84(10):3666-72.

3. Hackbarth JS, Hoyne JB, Grebe SK, Singh RJ. Accuracy of calculated free testosterone differs between equations and depends on gender and SHBG concentration. *Steroids*. 2011 Jan;76(1-2):48-55.

4. Vining RF, McGinley RA, Symons RG. Hormones in saliva: mode of entry and consequent implications for clinical interpretation. *Clinical chemistry*. 1983 Oct;29(10):1752-6.

5. Davison S. Salivary testing opens a Pandora's box of issues surrounding accurate measurement of testosterone in women. *Menopause* (New York, NY). 2009 Jul-Aug;16(4):630-1.

6. Macdonald PR, Owen LJ, Wu FC, Macdowall W, Keevil BG. A liquid chromatography-tandem mass spectrometry method for salivary testosterone with adult male reference interval determination. *Clinical chemistry*. 2011 May;57(5):774-5.

7. Keevil BG, MacDonald P, Macdowall W, Lee DM, Wu FC, Team N. Salivary testosterone measurement by liquid chromatography tandem mass spectrometry in adult males and females. *Annals of clinical biochemistry*. 2014 May;51(Pt 3):368-78.

8. Fiers T, Delanghe J, T'Sjoen G, Van Caenegem E, Wierckx K, Kaufman JM. A critical evaluation of salivary testosterone as a method for the assessment of serum testosterone. *Steroids*. 2014 Aug;86:5-9.

9. Keevil BG, Fiers T, Kaufman JM, Macdowall W, Clifton S, Lee D, et al. Sex hormone-binding globulin has no effect on salivary testosterone. *Annals of clinical biochemistry*. 2016 Apr 26.

10. Bhasin S, Pencina M, Jasuja GK, Travison TG, Coviello A, Orwoll E, et al. Reference ranges for testosterone in men generated using liquid chromatography tandem mass spectrometry in a community-based sample of healthy nonobese young men in the Framingham Heart Study and

applied to three geographically distinct cohorts. *The Journal of clinical endocrinology and metabolism.* 2011 Aug;96(8):2430-9.

11. Haring R, Hannemann A, John U, Radke D, Nauck M, Wallaschofski H, et al. Age-specific reference ranges for serum testosterone and androstenedione concentrations in women measured by liquid chromatography-tandem mass spectrometry. *The Journal of clinical endocrinology and metabolism*. 2012 Feb;97(2):408-15.

12. Erens B, Phelps A, Clifton S, Hussey D, Mercer CH, Tanton C, et al. The third National Survey of Sexual Attitudes and Lifestyles (Natsal-3): Technical Report 2013 [avilable at http://www.natsal.ac.uk/natsal-3/methodology.aspx]

13. Erens B, Phelps A, Clifton S, Mercer CH, Tanton C, Hussey D, et al. Methodology of the third British National Survey of Sexual Attitudes and Lifestyles (Natsal-3). *Sexually transmitted infections.* 2014 Mar;90(2):84-9.

14. Food and Drug Administration. Guidance for Industry: Bioanalytical Method Validation. 2001.

15. Mitchell KR, Ploubidis GB, Datta J, Wellings K. The Natsal-SF: a validated measure of sexual function for use in community surveys. *European journal of epidemiology*. 2012 Jun;27(6):409-18.

16. Liening SH, Stanton SJ, Saini EK, Schultheiss OC. Salivary testosterone, cortisol, and progesterone: two-week stability, interhormone correlations, and effects of time of day, menstrual cycle, and oral contraceptive use on steroid hormone levels. *Physiology & behavior*. 2010 Jan 12;99(1):8-16.

17. Vierhapper H, Nowotny P, Waldhausl W. Determination of testosterone production rates in men and women using stable isotope/dilution and mass spectrometry. *The Journal of clinical endocrinology and metabolism*. 1997 May;82(5):1492-6.

18. Southren AL, Gordon GG, Tochimoto S, Pinzon G, Lane DR, Stypulkowski W. Mean plasma concentration, metabolic clearance and basal plasma production rates of testosterone in normal young men and women using a constant infusion procedure: effect of time of day and plasma concentration

on the metabolic clearance rate of testosterone. *The Journal of clinical endocrinology and metabolism.* 1967 May;27(5):686-94.

19. Matsui F, Koh E, Yamamoto K, Sugimoto K, Sin HS, Maeda Y, et al. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay for simultaneous measurement of salivary testosterone and cortisol in healthy men for utilization in the diagnosis of late-onset hypogonadism in males. *Endocrine journal.* 2009;56(9):1083-93.

20. Lee S, Kwon S, Shin HJ, Park J, Lim HS, Lee KR, et al. Quantitative measurement of salivary testosterone in Korean adults by stable isotope-dilution liquid chromatographyelectrospray-tandem mass spectrometry. *BMB reports.* 2010 Nov;43(11):761-5.

21. Shibayama Y, Higashi T, Shimada K, Odani A, Mizokami A, Konaka H, et al. Simultaneous determination of salivary testosterone and dehydroepiandrosterone using LC-MS/MS: Method development and evaluation of applicability for diagnosis and medication for late-onset hypogonadism. *Journal of chromatography B, Analytical technologies in the biomedical and life sciences.* 2009 Sep 1;877(25):2615-23.

22. Harman SM, Metter EJ, Tobin JD, Pearson J, Blackman MR, Baltimore Longitudinal Study of A. Longitudinal effects of aging on serum total and free testosterone levels in healthy men. Baltimore Longitudinal Study of Aging. *The Journal of clinical endocrinology and metabolism*. 2001 Feb;86(2):724-31.

23. Travison TG, Araujo AB, O'Donnell AB, Kupelian V, McKinlay JB. A population-level decline in serum testosterone levels in American men. *The Journal of clinical endocrinology and metabolism.* 2007 Jan;92(1):196-202.

24. Wu FC, Tajar A, Pye SR, Silman AJ, Finn JD, O'Neill TW, et al. Hypothalamic-pituitary-testicular axis disruptions in older men are differentially linked to age and modifiable risk factors: the European Male Aging Study. *The Journal of clinical endocrinology and metabolism.* 2008 Jul;93(7):2737-45.

25. Kushnir MM, Blamires T, Rockwood AL, Roberts WL, Yue B, Erdogan E, et al. Liquid chromatography-tandem mass spectrometry assay for androstenedione, dehydroepiandrosterone, and testosterone with pediatric and adult reference intervals. *Clinical chemistry*. 2010 Jul;56(7):1138-47.

26. Rothman MS, Carlson NE, Xu M, Wang C, Swerdloff R, Lee P, et al. Reexamination of testosterone, dihydrotestosterone, estradiol and estrone levels across the menstrual cycle and in postmenopausal women measured by liquid chromatography-tandem mass spectrometry. *Steroids*. 2011 Jan;76(1-2):177-82.

27. Institute of Medicine Committee on Assessing the Need for Clinical Trials of Testosterone Replacement T. In: Liverman CT, Blazer DG, editors. Testosterone and Aging: Clinical Research Directions. Washington (DC): National Academies Press (US) Copyright 2004 by the National Academy of Sciences. All rights reserved.; 2004.

28. Kaufman JM, Vermeulen A. The decline of androgen levels in elderly men and its clinical and therapeutic implications. *Endocrine reviews.* 2005 Oct;26(6):833-76.

29. Sartorius G, Spasevska S, Idan A, Turner L, Forbes E, Zamojska A, et al. Serum testosterone, dihydrotestosterone and estradiol concentrations in older men self-reporting very good health: the healthy man study. *Clinical endocrinology*. 2012 Nov;77(5):755-63.

30. Yeap BB, Alfonso H, Chubb SA, Handelsman DJ, Hankey GJ, Norman PE, et al. Reference ranges and determinants of testosterone, dihydrotestosterone, and estradiol levels measured using liquid chromatography-tandem mass spectrometry in a population-based cohort of older men. The *Journal of clinical endocrinology and metabolism*. 2012 Nov;97(11):4030-9.

31. Abraham GE. Ovarian and adrenal contribution to peripheral androgens during the menstrual cycle. *The Journal of clinical endocrinology and metabolism.* 1974 Aug;39(2):340-6.

32. Longcope C. Androgen metabolism and the menopause. *Seminars in reproductive endocrinology*. 1998;16(2):111-5.

33. Davison SL, Bell R, Donath S, Montalto JG, Davis SR. Androgen levels in adult females: changes with age, menopause, and oophorectomy. *The Journal of clinical endocrinology and metabolism.* 2005 Jul;90(7):3847-53.

34. Lasley BL, Crawford S, McConnell DS. Adrenal androgens and the menopausal transition. *Obstetrics and gynecology clinics of North America.* 2011 Sep;38(3):467-75.

35. Lee DM, Tajar A, Pye SR, Boonen S, Vanderschueren D, Bouillon R, et al. Association of hypogonadism with vitamin D status: the European Male Ageing Study. *European journal of endocrinology / European Federation of Endocrine Societies.* 2012 Jan;166(1):77-85.

36. Svartberg J, Jorde R, Sundsfjord J, Bonaa KH, Barrett-Connor E. Seasonal variation of testosterone and waist to hip ratio in men: the Tromso study. *The Journal of clinical endocrinology and metabolism.* 2003 Jul;88(7):3099-104.

37. Garde AH, Hansen AM, Skovgaard LT, Christensen JM. Seasonal and biological variation of blood concentrations of total cholesterol, dehydroepiandrosterone sulfate, hemoglobin A(1c), IgA, prolactin, and free testosterone in healthy women. *Clinical chemistry.* 2000 Apr;46(4):551-9.

38. Stanton SJ, Mullette-Gillman OA, Huettel SA. Seasonal variation of salivary testosterone in men, normally cycling women, and women using hormonal contraceptives. *Physiology & behavior.* 2011 Oct 24;104(5):804-8.

39. Orwoll ES, Nielson CM, Labrie F, Barrett-Connor E, Cauley JA, Cummings SR, et al. Evidence for geographical and racial variation in serum sex steroid levels in older men. *The Journal of clinical endocrinology and metabolism.* 2010 Oct;95(10):E151-60.

**Figure 1: Distribution of salivary testosterone in men (A) and women (B) in the general population**

Curves created using linear regression (solid line) for the fitted mean (men) or geometric mean (women), 2.5th and 97.5th percentiles, and quantile regression (dashed line) for the median, 2.5th percentile and 97.5th percentile.

Observed values (x) of 1526 men and 2543 women are displayed

**Figure 2: Mean (men) or geometric mean (women) salivary testosterone (pmol/l) by season (A for men and B for women) and by region (C for men and D for women) in the general population**

**Appendix figure 1: Distribution of salivary testosterone in men (A) and women (B) in the general population with exclusions***

Curves created using linear regression (solid line) for the fitted mean (men) or geometric mean (women), 2.5th and 97.5th percentiles, and quantile regression (dashed line) for the median, 2.5th percentile and 97.5th percentile.

Observed values (x) of 1074 men and 1276 women are displayed

* Men with a body mass index (BMI) <18.5 or >30 $kg/m^2$ and women with a BMI <18.5 or >40 $kg/m^2$; prostate cancer, prostate enlargement, prostate surgery; polycystic ovaries; treatment for cancer, thyroid, testicular/ovarian or pituitary conditions in the past year; medication for epilepsy; women reporting both hysterectomy and HRT use; HRT; and hormonal contraception. All self-reported. See 'study population' section of methods for further details of exclusion criteria.