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Testosterone in human studies: Modest associations between plasma and salivary measurements

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Summary

Testosterone is involved in many processes like aggression and mood disorders. As it may easily diffuse from blood into saliva, salivary testosterone is thought to reflect plasma free testosterone level. If so, it would provide a welcome noninvasive and less stressful alternative to blood sampling. Past research did not reveal consensus regarding the strength of the association, but sample sizes were small. This study aimed to analyse the association in a large cohort. In total, 2,048 participants (age range 18–65 years; 696 males and 1,352 females) were included and saliva (using cotton Salivettes) and plasma were collected for testosterone measurements. Levels were determined by enzyme-linked immunosorbent assay and radioimmunoassay respectively. Free testosterone was calculated by the Vermeulen algorithm. Associations were determined using linear regression analyses. Plasma total and free testosterone showed a significant association with salivary testosterone in men (adjusted $\beta = .09$, $p = .01$; and $\beta = .15$, $p < .001$, respectively) and in women (adjusted $\beta = .08$, $p = .004$; and crude $\beta = .09$, $p = .002$ respectively). The modest associations indicate that there are many influencing factors of both technical and biological origin.

KEYWORDS
associations, large cohort, saliva, plasma, testosterone

1 | INTRODUCTION

Testosterone is secreted by the Leydig cells, under regulation of luteinising hormone (as part of the hypothalamo–pituitary–gonadal (HPG) axis). It is known for its key role in the sexual differentiation and sexual functioning, but plays an important role in social behaviour, such as in the maintenance of social status and aggression as well (Genazzani, Pluchino, Freschi, Ninni, & Luisi, 2007; Hofer, Lanzenberger, & Kasper, 2013). Moreover, as it affects the HPG axis, it seems to be involved in various psychiatric disorders including mood disorders. This is further supported by the gender differences found in the prevalence of the latter (Hofer et al., 2013). Testosterone is commonly measured in blood, but first reports on testosterone measurement in saliva appeared in the late sixties of the last century. The reason for collecting saliva is the less invasive, stressful and painful procedure compared with vena puncture, which is an important benefit especially in epidemiological studies. There are several arguments for the assumption that salivary testosterone is a good reflection of the plasma testosterone. First, testosterone is a lipophilic molecule that can easily pass the barrier between blood and salivary glands. Second, the salivary glands do not produce this hormone, implicating that all testosterone in saliva originates from the bloodstream largely through passive diffusion.

Testosterone in blood is bound to different proteins. In adult men, only 2%–3% of the circulating testosterone constitutes the free fraction. The remaining testosterone is strongly bound to sex hormone-binding protein (SHBG; 44%), and more loosely to albumin (50%) and cortisol binding globulin (3%–5%). Bioavailable testosterone is the free circulating hormone plus the (easily dissociated) albumin bound fraction (de Ronde et al., 2006). Several algorithms to calculate the free testosterone fraction have been proposed in the past, including

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the one used by Vermeulen et al. (Vermeulen, Verdonck, & Kaufman, 1999). However, their validity may largely depend on the accuracy of the measurements and the abundance of other hormones such as estradiol in the matrix (de Ronde et al., 2006). Other complicating factors are age, gender, body mass index (BMI), somatic disease processes and medication that modify testosterone levels.

Since the seventies of the last century, several studies have investigated the relation between blood and salivary testosterone, but results have varied. In males, marked associations between salivary testosterone and both free (FT) and total (TT) blood testosterone have been reported, with correlation coefficients ranging from $r = .74$ to almost 1.0 (Arregger, Contreras, Tumilasci, Aquilano, & Cardoso, 2007; Cardoso et al., 2011; Lane & Hackney, 2014; Shirtcliff, Granger, & Likos, 2002; Vittek, L'Hommedieu, Gordon, Rappaport, & Southren, 1985). Recently, more modest strengths of associations have also been reported (Cadore et al., 2008; Gonzalez-Sanchez et al., 2015). In females, much poorer associations were observed, with correlation coefficients ranging from $r = .23$ (Fiers et al., 2014; Flyckt et al., 2009; Shirtcliff et al., 2002; Szydlarska, Grzesiuk, Kondracka, Bartoszewicz, & Bar-Andziak, 2012) to $r = .79$ (Baxendale, Jacobs, & James, 1982; Granger, Schwartz, Booth, & Arentz, 1999; Vittek et al., 1985). This might relate to the generally much lower testosterone values and the accuracy of blood measurements within this range, but a more complex relation in females cannot be ruled out either. Limiting factors in most previously performed studies are the small to moderate sample sizes (ranging from $n = 12$ to 218) to analyse such relationships with precision (Lane & Hackney, 2014; Rilling, Worthman, Campbell, Stallings, & Mbizva, 1996) and not taking confounders into account.

Subjects from this study stem from a large prospective cohort study with a sample size of more than 2,000 participants. This study therefore has enough power to address the following questions: (i) Is salivary testosterone a valid marker for plasma testosterone in large scale cohort studies? and (ii) Do sociodemographic, sampling and lifestyle factors confound the values of salivary or plasma testosterone and therefore confound the association?

2 | PATIENTS AND METHODS

Data were collected from the Netherlands Study of Depression and Anxiety (NESDA; www.nesda.nl). NESDA is a multicentre study which investigates the aetiology and development of depressive and anxiety disorders. Participants, who were not fluent in Dutch or suffered from one of the following diseases, were excluded: psychotic disorder, obsessive compulsive disorder, bipolar disorder or severe addiction disorder (Penninx et al., 2008). The study design was approved by the Ethical Review Board of the VU University Medical Centre and thereafter by the local ethical committees of the participating centres. All participants gave verbal and written informed consent.

In the present analysis, a subset of the NESDA sample was used. We excluded 923 participants who did not collect saliva samples ($n = 826$ (27.7%) and/or had missing values on plasma hormone data ($n = 96$ (3.3%)) and those with suspected marked abnormalities in plasma protein composition, as in chronic kidney failure or liver diseases/cirrhosis, or during pregnancy to calculate reliable free testosterone values (Vermeulen et al., 1999). In total, 2,048 participants (age range 18–65 years; 696 males and 1,352 females) were included.

2.1 | Assessment of testosterone in plasma and saliva

Vena puncture took place in the morning after an overnight fast. The mean time of waking up was 06:57 a.m. [2.5 and 97.5th percentile 05:30–08:00], and the mean time of sampling was 08:36 a.m. [2.5 and 97.5th percentile 08:00–09:35]. The blood samples were processed and stored at −80°C. TT was measured in duplicate after performing a diethylether extraction with an in-house radioimmunoassay (RIA) using a polyclonal antitestosterone–antibody. Inter-assay coefficients of variation (CV) were estimated <10% for SHBG and between 14% and 19% for total testosterone (TT), and the analytical sensitivity was 0.5nmol. Free testosterone (FT) was calculated from TT and SHBG using the algorithm by Vermeulen et al. (1999). Because albumin concentration was not measured in the NESDA sample, a constant albumin concentration of 43 g/L (0.62 mmol/L) was assumed. According to Vermeulen et al. (1999), variations in albumin concentration smaller than 25% will not significantly influence estimated FT values. To verify whether the albumin concentration in our sample was within 25%, albumin concentrations of 30 randomly selected participants were measured in serum (7). Variation in this sample was 19% (mean 45.17, *SD* 8.6), well within the recommended borders.

Salivary collections took place in the morning at home. Participants were instructed verbally as well as through detailed written information accompanying the Salivettes. Saliva was collected immediately after awakening ("the moment you can open your eyes") and 30, 45 and 60 min later. The mean time of sampling for the first timepoint was 07.26 a.m. [2.5 and 97.5th percentile 06.20–10.10]. The mean time of sampling for the fourth timepoint was 08.27 a.m. [2.5 and 97.5th percentile 06.30–11.15]. All subjects were instructed not to eat, smoke, drink and brush teeth within 15 min of sampling. The Salivettes were stored at −20°C and returned by regular mail. After receipt, Salivettes were thawed and centrifuged at 2,000 *g* for 10 min to remove the mucus and obtain clear liquids which could be pipetted. The saliva was aliquoted and stored at −80°C. To smooth the episodic secretion, 75 μl of each of the four samples was mixed to yield one sample. If one of the samples was missing, a corresponding volume of the other sample(s) was taken. Biochemical analysis of testosterone in saliva was measured in duplo by the testosterone in saliva assay from Diagnostic Biochem Canada (EiAsy Testosterone Saliva, DBC: CAN-TE-300) using 2 × 100 μl material. The sensitivity of the kit is 1.0 pg/ml, while displaying low cross-reactivities with other steroids. In every assay, a standard control was used, with a mean of 26.9 pg/ml (*SD* 2.1) that was reproducible with a coefficient of variation (CV) of 7.8%. The intra-assay precision was 7.1%, 3.4% and 6.7% at the concentrations of resp. 14, 38 and 123 pg/ml ($n = 10$). The mean CV difference over all testosterone measurements was 10.2%.

In most cases during the data collection, there was a median time interval of 8 days (inter-quartile range 4–22) between the blood and the saliva collection.

2.2 | Covariates

Sociodemographic, sampling and lifestyle variables were analysed to determine whether they significantly impacted testosterone levels. Research performed in the same cohort revealed that age was strongly and inversely associated with morning salivary testosterone levels in both men and women (Giltay et al., 2012). To analyse which variables significantly covaried plasma testosterone levels, we included the following baseline factors; age, North-European ancestry, level of education, body mass index (BMI), time of awakening, time of blood sampling, fasting, smoking status, alcohol use, chronic diseases and the physical activity.

Level of education was specified in years of education. Time of awaking was demarcated as the moment you can open your eyes. Fasting was defined as an overnight fast. BMI was calculated as the weight (kg) divided by the squared height (m). Smoking status was dichotomised into non- or current smoker. Alcohol use was defined as daily alcohol intake (units). Chronic disease was prevalent when suffering from diabetes mellitus, stroke or other cardiovascular diseases or cancer. Physical activity was assessed using the International Physical Activity Questionnaire (Craig et al., 2003) and expressed per 1,000 Metabolic Equivalent of Task (MET)/min a week. A MET/min is defined as the metabolic equivalent of the number of calories consumed by a person (of 60 kg) per minute in an activity relative to basal metabolic rate.

In addition, menstrual status, defined as pre- or post-menopausal with or without the use of oral contraceptives (OCP), was taken into account only in women.

2.3 | Statistical analysis

Baseline sociodemographic and lifestyle characteristics and hormone levels were described using number of cases (percentages), mean ± standard deviation (*SD*) or median + inter-quartile range (IQR), when appropriate in men and women. Values of salivary testosterone were log-transformed to provide normal distribution.

Univariate analyses were carried out using univariate linear regression in subjects free of psychopathology to evaluate the effects of twelve sociodemographic, sampling and lifestyle factors on plasma testosterone levels for men and women separately. We performed multiple linear regression analyses to assess independent determinants of plasma testosterone. A variable was defined as a confounder when a two-sided *p*-value was <.10 both in the uni- and multivariate analyses for both plasma and salivary testosterone. Bonferroni correction ($\alpha = 0.10/12$ (0.008)) was performed to adjust the results of multiple comparisons.

To determine the association between salivary and plasma testosterone, we calculated standardised beta-coefficients, using linear regression, separately for strata of men and women. Multivariate analyses were performed adjusting for the determined confounders. To estimate the impact of different confounders, we calculated the per cent change in beta-coefficients (i.e., $[\Delta^b\ 100\%]/\Delta^a$ of more than 10%, i.e., $\%\Delta > 10$).

Finally, we determined whether the association between salivary and plasma testosterone differed between participants with a depression or anxiety disorder and healthy controls and between participants with a different time sampling frame. Regression analyses were repeated in the subgroup of participants free of psychopathology and in the subgroup of participants with a time interval of maximum 2 weeks for plasma and saliva sampling. In a last sensitivity analysis, we excluded 7 outliers in men (plasma testosterone levels <5.0 nmol/L) and 8 outliers in women (plasma testosterone levels >7.0 nmol/L). Multivariate analyses were performed adjusted for determined confounders.

A two-sided *p*-value <.05 was considered statistically significant. Data were analysed using IBM SPSS Statistics 22 (SPSS Inc., Chicago, IL, USA).

3 | RESULTS

In Table 1, the socio-demographic and lifestyle characteristics of 2,048 participants are depicted according to gender. The mean age was 44.9 ± 12.6 years in men and 42.7 ± 13.1 years in women. In total, 76.3% of the participants suffered from a current anxiety and/or depressive disorder.

The associations between sociodemographic, sampling and lifestyle variables on plasma total and free testosterone levels were analysed in subjects free of psychopathology as they may be considered as confounders in the subsequent association analyses with salivary testosterone. Previous research performed in the same cohort revealed that age was a potential confounder, as it was inversely correlated with salivary testosterone (Giltay et al., 2012). Also current smoking was associated with higher and the use of oral contraceptives with lower salivary testosterone levels. With regard to plasma levels, age was inversely associated with plasma TT in men and women, which was statistically significant (after Bonferroni correction) in both univariate analyses ($p < .001$ for men and $p = .001$ for women), but not in multivariate analyses ($p = .01$ for men and $p = .07$ for women). Age was inversely associated with plasma FT in men as well, both in the univariate and in the multivariate analyses (p < .001) See Tables 2 and 3.

Table 4 shows the associations of modest strength between plasmaand salivary testosterone levels in 2,048 participants according to gender. Age was included in the adjusted regression, as only this variable was associated with both plasma and salivary testosterone in men (TT and FT) and women (TT) and therefore might confound the association between plasma and salivary testosterone. Plasma TT showed a significant association with salivary testosterone in men (adjusted $\beta = .09$, $p = .01$) and in women (adjusted $\beta = .08$, $p = .004$). Plasma FT showed a significant association with salivary testosterone both in men (adjusted $\beta = .15$, $p < .001$) and in women (crude $\beta = .09$, $p = .002$). Figure 1 depicts the association between plasma FT and salivary testosterone (with testosterone levels categorised for illustrative purposes only). The size of the confounding effect of age on the association between salivary and plasma TT and FT was $\%\Delta\beta = -25.0\%$

TABLE 1 Sociodemographic and lifestyle characteristics and testosterone levels of 2,048 participants according to gender

	Men (*N* = 696)	Women (*N* = 1,352)
Sociodemographics		
Age (years)	44.9 ± 12.6	42.7 ± 13.1
North-European ancestry	669 (96.1%)	1,286 (95.1%)
Education (years)	12.3 ± 3.3	12.4 ± 3.3
Sampling variables plasma		
Time of awakening	06:56 ± 00:53	06:58 ± 00:40
Time of blood sampling	08:36 ± 01:18	08:35 ± 01:19
Fasting	660 (95.0%)	1,307 (97.0%)
Sampling variables saliva		
Time of awakening	07:23 ± 01:14	07:29 ± 01:15
Time of blood sampling point 1	07:23 ± 01:13	07:28 ± 01:14
Time of blood sampling point 4	08:27 ± 01:14	08:29 ± 01:10
Lifestyle indicators		
Comorbid anxiety or depressive disorder	509 (73.1%)	1,053 (77.9%)
Body mass index (kg/m^2)	26.2 ± 4.5	25.2 ± 5.3
Currently smoking (*n*)	261 (37.5%)	420 (31.1%)
Daily alcohol intake (units)	1.5 ± 1.8	0.8 ± 1.1
Chronic diseases (*n*)	145 (20.8%)	187 (13.8%)
Physical activity (1,000 MET-min/week)	3.7 ± 3.3	3.7 ± 2.9
Menstrual status		
Post-menopausal	–	509 (8.0%)
Pre-menopausal with OCP	–	338 (25.2%)
Pre-menopausal without OCP	–	492 (36.7%)
Salivary testosterone (pg/ml)	16.14 [10.27–26.68]	16.11 [10.30–26.77]
Serum total testosterone (nmol/L)	19.10 [15.5–23.6]	2.80 [2.20–3.40]
Serum free testosterone (nmol/L)	0.40 [0.32–0.48]	0.03 [0.02–0.04]

Data are number of cases (percentages), mean ± standard deviation (*SD*) or median [inter-quartile range [IQR)], when appropriate.

and %Δ β = −20.0% in men and %Δβ = −27.3% for FT in women respectively.

In a sensitivity analysis performed in a subgroup of participants who had a ≤2-week interval between both samplings and on the same day, the strengths of the associations were slightly attenuated (see Table 4). A further sensitivity analysis performed in the subgroup of subjects free of psychopathology (men *n* = 187 and women *n* = 299) showed that the strengths of the associations between salivary testosterone and plasma TT remained the same. The adjusted β was .12 (*p* = .11) in men and .06 (*p* = .28) in women for the association between salivary testosterone and plasma TT and .15 (*p* = .09) in men for the association between salivary testosterone and plasma FT. The exclusion of 7 outliers in men (TT adjusted β = .10, p = .001 and FT adjusted β = .16, p < .001, n = 689) or after exclusion of 8 outliers in women (TT adjusted β = .12, p < .001, n = 1344) did neither remarkably change the results.

4 | DISCUSSION

The aim of the present study was to investigate the relation between salivary testosterone and plasma total and free testosterone (TT/FT) in a large sample. A second objective was to investigate whether sociodemographic, sampling and lifestyle factors covaried the values of salivary or plasma testosterone and therefore could be stated as confounders. Although the associations were modest, they were consistent across analyses in subjects with or without psychopathology and participants who had a ≤2-week interval between both samplings. Only age significantly confounded the association between plasma and salivary testosterone, however, not between plasma FT and salivary testosterone in women.

To find an explanation for these modest associations relative to the generally much stronger associations that have been reported previously (Arregger et al., 2007; Cardoso et al., 2011; Lane & Hackney, 2014; Shirtcliff et al., 2002), both methodological and technical issues should be discussed.

Strengths of this study are that several precautions were taken in the NESDA study including not brushing teeth, smoking or eating within 15 min of saliva collection to prevent contamination of the saliva samples by blood. Salivary samples are extremely sensitive for contamination by blood (Granger, Shirtcliff, Booth, Kivlighan, & Schwartz, 2004), as testosterone levels in blood are higher than in saliva. Importantly, not all previous studies (Lane & Hackney, 2014; Rilling et al., 1996; Shirtcliff et al., 2002; Vittek et al., 1985) have taken such precautions and this might have influenced the correlations. In addition, we included a large sample (*n* = 2,048) of participants, which was much larger than prior studies. Consequently, this study was sufficiently powered to examine confounding effects of sociodemographics, sampling and lifestyle factors.

However, there are shortcomings of our study which might have influenced the correlation. First, the use of cotton Salivettes instead of a passive drool approach has been linked with measurement errors and inflated values and therefore deemed to be less reliable (Fiers et al., 2014; Groschl & Rauh, 2006). However, control studies have shown that cotton Salivettes increase testosterone values in a stable and predictable manner. As this absolute increase in measured salivary testosterone is similar, this may explain the larger relative increases that we saw in women compared to men, as well as a probably larger measurement error in women than in men. Nevertheless, their rank order stability was partly preserved, which makes Salivettes still applicable for testosterone measurements within large cohort studies (Giltay

TABLE 2 Associations with plasma (free) testosterone levels in 187 men free of lifetime psychopathology

	Plasma total testosterone				Plasma free testosterone			
	Univariate models		Multivariate models		Univariate models		Multivariate models	
	β	*p*-value	β	*p*-value	β	*p*-value	β	*p*-value
Sociodemographics								
Age (years)	−.31	<.001	−.21	.01	−.56	<.001	−.52	<.001
North-European ancestry	−.17	.02	−.19	.01	−.01	.89	−.05	.44
Education (years)	.08	.28	.06	.44	.00	.95	.00	1.00
Sample variables								
Time of awakening	.08	.26	.04	.55	.08	.29	.03	.62
Time of blood sampling	.12	.11	.11	.10	.12	.11	.12	.05
Fasting	−.01	.94	−.00	.99	−.09	.23	−.07	.27
Lifestyle indicators								
Body mass index (kg/m^2)	−.37	<.001	−.26	.001	−.28	<.001	−.04	.62
Currently smoking (*n*)	.10	.16	.10	.15	.04	.57	.05	.46
Daily alcohol intake (units)	.01	.94	.06	.38	−.04	.63	−.02	.80
Chronic diseases (*n*)	−.19	.01	−.03	.71	−.28	<.001	−.06	.42
Physical activity (MET-minutes)	.03	.64	.09	.19	−.04	.61	−.01	.93

Data are standardised beta-coefficients using univariate and multivariate linear regression.

TABLE 3 Associations with plasma (free) testosterone levels in 299 women free of lifetime psychopathology

	Plasma total testosterone				Plasma free testosterone			
	Univariate models		Multivariate models		Univariate models		Multivariate models	
	β	*p*-value	β	*p*-value	β	*p*-value	β	*p*-value
Sociodemographics								
Age (years)	−.20	.001	−.15	.07	−.02	.79	−.02	.81
North-European ancestry	.07	.20	.06	.30	.02	.71	.02	.74
Education (years)	.06	.31	.05	.42	.03	.56	.04	.52
Sample variables								
Time of awakening	.03	.59	−.03	.65	−.04	.45	−.06	.34
Time of blood sampling	−.04	.46	−.06	.35	−.01	.93	−.04	.50
Fasting	.00	.98	−.00	.95	.07	.22	.04	.54
Lifestyle indicators								
Body mass index (kg/m^2)	−.13	.03	−.08	.22	.06	.29	.07	.29
Currently smoking (*n*)	.06	.30	.04	.47	.03	.58	.04	.55
Daily alcohol intake (units)	−.03	.67	−.01	.87	.01	.84	.04	.58
Chronic diseases (*n*)	−.04	.47	−.00	.97	.02	.72	.02	.75
Physical activity (MET-minutes)	.04	.45	.05	.41	.02	.68	.03	.69
Menstrual status	−.13	.02	−.01	.94	−.06	.30	−.08	.31

Data are standardised beta-coefficients using univariate and multivariate linear regression.

et al., 2012; Smeets-Janssen et al., 2015). Second, differences in the sampling times of plasma and saliva could have been an important factor. The time span between these collections often exceeded 2 weeks, leading to assessment within different phases of the menstrual cycle. However, subgroup analyses in sampling within 2 weeks or even on the same day did not reveal better correlations, pointing towards other factors that played a more important role. Besides the average time interval of half an hour with the morning, saliva and blood collections could have diluted associations in the present study (Dabbs, 1990; Diver, Imtiaz, Ahmad, Vora, & Fraser, 2003) as testosterone has a diurnal rhythm with concentration drops to 50% from morning to evening. However, most other studies did not collect both media at the exact

TABLE 4 Associations between serum and salivary (free) testosterone levels in men and women

	Men			Women		
	β	*p*-value	%Δ[a]	β	*p*-value	%Δ[a]
All subjects	***N* = 696**			***N* = 1,352**		
Total testosterone						
Crude	.12	.002		.11	<.001	
Adjusted for age	.09	.01	−25.0	.08	.004	−27.3
Free testosterone						
Crude	.18	<.001		.09	.002	
Adjusted for age	.15	<.001	−20.0	–	–	–
Sampling within 2 weeks	***N* = 396**			***N* = 623**		
Total testosterone						
Crude	.10	.048		.07	.05	
Adjusted for age	.07	.14	−30.0	.06	.12	−14.3
Free testosterone						
Crude	.16	.002		.06	.08	
Adjusted for age	.12	.04	−25.0	–	–	–
Sampling on same day	***N* = 23**			***N* = 41**		
Total testosterone						
Crude	−.05	.81		.08	.62	
Adjusted for age	−.07	.77	−40.0	.14	.42	+75.0
Free testosterone						
Crude	−.06	.86		.04	.80	
Adjusted for age	−.04	.86	+33.3	–	–	–
Free of psychopathology	***N* = 187**			***N* = 299**		
Total testosterone						
Crude	.14	.050		.09	.19	
Adjusted for age	.12	.11	−14.2	.06	.28	−33.3
Free testosterone						
Crude	.17	.02		.13	.02	
Adjusted for age	.15	.09	−11.7	–	–	–

Data are standardised beta-coefficients by linear regression analysis.

same time either, with time spans of 1–2 hr (Arregger et al., 2007; Cadore et al., 2008; Cardoso et al., 2011; Gonzalez-Sanchez et al., 2015; Granger et al., 1999; Szydlarska et al., 2012). Importantly, the diffusion rate from testosterone between the blood and saliva barrier is unknown and therefore the optimal time sampling frame between blood and saliva collection neither. Third, the majority of participants have psychopathology which is thought to influence hormone levels (Giltay et al., 2012; Gordon et al., 2015; Joshi et al., 2010). However, in a subsample of participants free of mental health problems, correlations stayed the same. Fourth, immunoassays for testosterone are considered to be less reliable than liquid or gas chromatography with tandem mass spectrometry (GC-MS or LC-MS/MS) analyses in particular at the lower concentrations generally measured in women (<5 nmol/L) (Rautenberg & Lentjes, 2007; Thijssen, 2000). Problems noted with this method are inadequate specificity, interferences with hetero and auto-antibodies and most importantly cross-reactivity with other steroid hormones. Measurement of salivary testosterone with LC-MS/MS could hopefully broaden its implication for diagnostics in endocrine abnormalities as well. Determination of the andrological status also requires measurement of other steroid hormones, for example estradiol and DHT, which assessment may be insufficiently reliable when using immunoassays, in part due to their lower values (Hsing et al., 2007). Consequently, immunoassays are more and more replaced by LC-MS/MS, which is a more specific and sensitive analysis technique (Ketha, Kaur, Grebe, & Singh, 2014) but more expensive and time-consuming as well (Kushnir, Rockwood, & Bergquist, 2010). Hsing et al. compared the measurement of testosterone in serum between RIA and GC-MS. Although the intraclass correlation

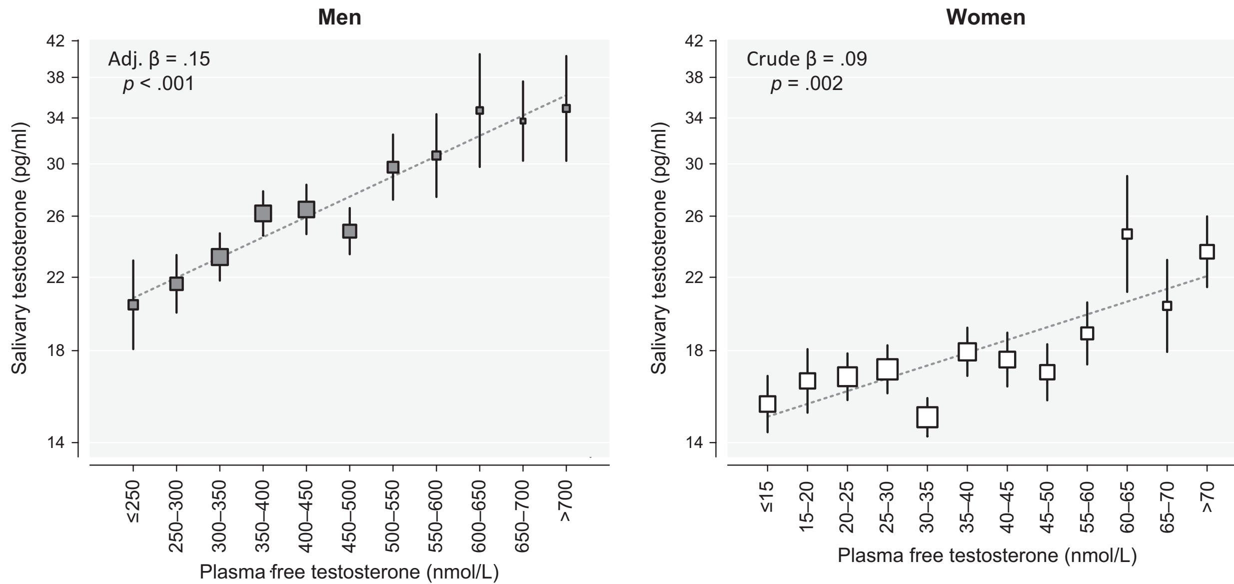

FIGURE 1 Relationship between serum free testosterone and salivary testosterone levels in 696 men and 1,352 women. Serum free testosterone levels were categorised for visualisation purposes only, as all analyses were carried out with continuous data. Salivary testosterone levels were $\log_e$-transformed before analysis, and back-transformed geometric mean levels are presented on logarithmic scales. Error bars represent standard errors (*SE*). The sizes of the boxes are proportional to the number of participants

coefficients (ICC) were generally higher within the batch measured by GC-MS, the correlation coefficients (CV) were similar. Moreover, the Pearson's and Spearman's correlation coefficients between both methods were high (>.9) and all statistically significant (Hsing et al., 2007). These high correlation coefficients were confirmed in a later studies (Huhtaniemi et al., 2012; Khosla et al., 2008). To the best of our knowledge, less is known about the direct comparability between salivary testosterone levels measured by ELISA versus mass spectrometry. Probably, same methodological issues when using RIA on saliva samples were of importance, but were not elaborated further in the article, making it difficult to interpreted their results (Fiers et al., 2014). Besides, most previous studies have used immunoassays for the detection of testosterone (Arregger et al., 2007; Baxendale et al., 1982; Cadore et al., 2008; Cardoso et al., 2011; Flyckt et al., 2009; Gonzalez-Sanchez et al., 2015; Granger et al., 2004; Lane & Hackney, 2014; Rilling et al., 1996; Shirtcliff et al., 2002; Szydlarska et al., 2012; Vittek et al., 1985). Fifth, different immunoassays for the saliva and plasma testosterone measurements in the NESDA study have been used. Arguably, the relation between saliva and blood measurements will suffer when cross-reactivity profiles of the immune assays are markedly different.

We would advise that future research should use passive drool samples or synthetic rather than cotton Salivettes. Arguably, measurement of testosterone in hair using LC-MS/MS (Noppe, de Rijke, Dorst, van den Akker, & van Rossum, 2015) might be an alternative and unique chance to estimate testosterone production over longer time periods. Future research should reveal the association between testosterone measured in hair, saliva and blood. Clearly, such studies must be performed with LC-MS/MS to reduce analytical errors in the lower concentration range. Moreover, saliva and blood collection should ideally be performed at the same day, ideally within hours. Conversely, this would also challenge the worldwide clinical practice of measuring testosterone level at a single time point and advocate the measurement of testosterone in hair. Finally, age should be taken into account as a confounder for the association between salivary and plasma testosterone. Unknown yet but perhaps important, the matrix might be more complex for plasma than saliva, indicating that specificity issues might play a more prominent role with the plasma measurements.

In summary, although statistically significant associations were found between plasma TT/FT and salivary testosterone in the present study, the associations were much more modest as compared to previous studies (Arregger et al., 2007; Cardoso et al., 2011; Lane & Hackney, 2014; Shirtcliff et al., 2002). Only age significantly confounded the association between plasma and salivary testosterone, however, not between plasma FT and salivary testosterone in women. These results indicate that the association is not a simple one and likely influenced by many factors of both technical (e.g., the use of Salivettes instead of passive drools of saliva and other issues of sample collection protocols) and biological origin. Until we know the impact of these factors, the determination of testosterone in blood continues to be the method of choice, especially in clinical settings.

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