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Chapter 6

Female Salivary Testosterone: Measurement, Challenges and Applications

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http://dx.doi.org/10.5772/53648

1. Introduction

Testosterone (T) is one of the most important naturally circulating steroid hormones. Exerting both androgenic and anabolic activities it is secreted into the blood and in men is produced primarily by the testes. In women, by contrast, production occurs in the ovaries and particularly from peripheral conversion of the T precursors androstenedione, DHEA and DHEA-S (Burger, 2002). T is no longer regarded as a male only hormone, and similarly estradiol is no longer a female only hormone (Fausto-Sterling, 2000). While it is true that men generally have higher levels of T and lower concentrations of oestrogen and progesterone than women, all these sex steroid hormones play essential roles in both sexes (Ullis et al., 1999).

Testosterone has now been well established as having an essential function in wide-ranging areas of female health. For example, it is an important determinant of female sexuality, critical for development and maintenance of bone mineralisation, contributes to menstrual cycle regulation and to behavioural changes in premenstrual syndrome (Bachmann & Leiblum, 1991; Slemend et al., 1996). Therefore, T and other androgen replacement therapies in women with or without oestrogen have become widely recommended for a variety of women experiencing androgen deficiency syndrome (Somboonporn et al., 2006; Hickok et al., 1993).

It has been shown that sex hormones levels can be influenced by diet, exercise, age, BMI, ethnicity and others (Allen & Key, 2000; Kraemer et al., 1998). Various studies have suggested that increasing dietary fibre intake could influence total and/or bio-available T and oestradiol levels (Rock et al., 2004) or T and SHBG levels (Longcope et al., 2000). Recently, Wang et al. (2005) reported that a low-fat high-fibre diet decreased serum and urine androgens in men. The majority of these studies on T were conducted in men and the data published were conflicting. These equivocal findings resulted, at least in part, from different study designs and protocols (fibre content and type, subject compliance, lack of control af-

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fecting other dietary components) and the complex mechanisms involved in steroid metabolism. Subsequently, we have performed a pilot study to investigate the effect of increasing dietary fibre and found a moderate increase in urinary T excretion of healthy women taking a mixed high fibre diet for two weeks (unpublished observations), suggesting a potential correlation between dietary fibre intake and androgen status. This could be due to an effect on the enterohepatic cycle as hypothesised by some investigators in delaying the excretion of steroids and hence a modest increase in plasma and urinary levels (Adlercreutz et al., 1987; Groh et al., 1993). Other researchers have suggested that low-fat high-fibre diet might indirectly increase bio-available serum androgen levels by preventing the development of insulin resistance which is associated with reduced SHBG levels (Haffner et al., 1994; Pasquali et al., 1995). Conversely, some workers proposed that low-fat high-fibre diet reduced circulating steroid hormone concentrations due to higher faecal excretion of conjugated steroids (Pusateri et al., 1990) or an increase in the synthesis of SHBG (Berrino et al., 2001).

Quantitative determination of circulating T is possible from an assortment of biological material; i.e. plasma, serum, hair, saliva, and urine. However, early attempts at determining concentrations of circulating T, primarily for clinical purposes, traditionally utilised plasma or serum. As blood samples require time-consuming and often stressful venipuncture, obtaining invasive multiple samples over a period of hours can be painful and this procedure can be unattractive to participants (Dabbs, 1990). Moreover, measurement tends to be of the total rather than free, biologically active, fraction of T. Faced with these challenges there has been a growing awareness of the potential value of utilising saliva for measuring hormone concentrations (Mandel, 1993; Collins, 2000). Consequently, the use of saliva as a diagnostic tool in clinical and bio-behavioural research has grown significantly during the last two decades (Quissell, 1993). However, salivary T measurements can be markedly influenced during the process of sample collection with interference effects caused by mucupolysaccharides and leakage of blood into saliva, storage conditions and random daily fluctuations (Granger et al., 2004).

There are limited data establishing normal androgen values for women at different ages, thus hampering the ability to define those with androgen deficiency (Guay, 2002), and there is definitely a clear need for additional information on normal reference ranges of female T levels at different age groups. Moreover, little is known about detailed daily patterns in female T throughout the menstrual cycle (Davis, 1999). One of the aims of this review article is therefore to discuss the importance of estimating salivary T in women and the challenges posed by its measurement in female saliva, including a summary of our in-house ELISA technique and the optimisation needed for the estimation of salivary T. We also discuss female salivary T circadian dynamics showing our own data in this regard. Finally, we would like to highlight the importance of sampling protocol; multiple female salivary T sampling versus single saliva sampling.

2. Role of testosterone in women's health and well-being

Whilst androgens are known to play a significant role in wide-ranging aspects of male health (Nieschlag, 1998; Isidori et al., 2008), there is an increasing realisation that they are

also critical for mental and sexual health as well as physical well-being in females (Christiansen, 2004; Davis & Tran, 2001). The following examples provide a non-exhaustive illustration of testosterone significance for females.

2.1. Epidemiology and clinical importance of abnormal testosterone levels in females

Female sexual dysfunction is thought to affect over 40% of women in the United States, according to a study by Laumann and colleagues (1999). As experts evaluate women with potential sexual interest disorders, there is a growing body of literature to guide them in how to understand, diagnose and treat these problems. Androgens are known to be involved in women's arousability, response, intensity and ease of orgasm, as well as in initial spontaneous desire, the active neurovascular smooth muscle response of swelling, increased lubrication and genital sexual sensitivity. T is thought to be the most important hormone for maintaining sex drive or libido in women and a deficiency can cause impaired sexual function (Snyder, 2001). In this regard, T may decrease vaginal atrophy as well as inflammation, itching and pain of the vulva (Leiblum et al., 1983). However, excessive amounts may increase the risk of endometrial cancer due to hyperinsulinemia (Ciampelli & Lanzone, 1998).

In a recent Cochrane systematic review (Somboonporn et al., 2010), it was concluded that adding T to hormone therapy has a beneficial effect on sexual function in postmenopausal women. However, the combined therapy is associated with a higher incidence of hair growth, acne and a reduction in high-density lipoprotein (HDL) cholesterol. These adverse events may vary with differing doses, routes of T administration and individual differences. Reflecting on the theoretical and conceptual challenges of relating specific hormone levels to behaviour, there is no doubt that a relationship has not been established between levels of T and symptoms of sexual dysfunction in women. In the brain, T has a role in maintaining mood and memory. Indeed, high levels of T exert a significant negative effect on mood, personal sense of well being, interpersonal relationships, self-confidence and self-worth, and depression is a major symptom associated with low levels in women (Sands & Studd, 1995). In the heart, T has relaxing (vasodilating) effect on coronary arteries (Sarrel, 1998; White et al., 1998), and thus, it can reduce symptoms of angina. Unstable hormonal fluctuations can be observed after menopause (Overlie et al., 1999). These fluctuations are usually associated with increased incidence of migraine headache, obesity, mood changes and bleeding disturbances in perimenopausal and postmenopausal women (Fettes, 1999; Vliet & Davis, 1991). It has been suggested that postmenopausal women who are not receiving some T therapy may have greater risk of developing coronary heart disease (Rako, 1998). The effects associated with administering exogenous T are not especially straightforward however. For example, when T is administered alone, it can increase the risk of atherosclerosis and decrease HDL levels (Crook & Seed, 1990). Conversely, when T is administered with oestrogens, the increased risk of heart disease diminishes (Sarrel, 1998; Davis, 2011).

In the Michigan Bone Health Study (1992–1995), the authors examined the correlates of T in pre- and perimenopausal women (i.e., age, menopausal status, body composition, and lifestyle behaviours) in aged 25–50 years (n=601). Body composition measures were found to be significantly and positively associated with total T concentrations in a dose-response man-

ner (Sowers et al, 2001). Hysterectomy with oophorectomy was associated with significantly lower T concentrations. For bones and osteoporosis, T (and the metabolite DHT) directly stimulates receptors on the osteoblasts (bone building) cells to promote bone growth, bone mineralisation and repair of damaged bone (Gasperino, 1995; Hui et al., 2002). In light of this, T replacement may markedly decrease osteoporosis in postmenopausal women, and together with oestrogens the steroids can preserve and rebuild the cartilages between bones (Tremollieres et al., 1992). In the skin, T can improve the overall skin appearance by preserving collagen and protecting against thinning of the skin as well as sebaceous glands activity that lubricates the skin (Brincat et al., 1987). As we begin to more fully understand the above actions, the therapeutic use of exogenous T in women is becoming increasingly widespread, although not always without often unwanted side effects. Thousands of women have been treated with T, the majority experience symptom improvement, improved sexual well-being (Davis and Davison, 2012). Other possible beneficial effects of T therapy (reduced fracture risk, improved cognitive and cardiovascular function), necessitate further investigation. Reduced T levels have been found in bilateral oophorectomy, adrenal insufficiency, hypopituitarism, use of combination oral contraceptive pills or systemic glucocorticosteroids, and premature ovarian failure (Fogle et al, 2007; Labrie et al, 2011).

As women age, the dramatic drop in T level is thought to result from a decline in the adrenal production of T precursors, DHEA and DHEAS (Zumoff, et al., 1995; Davison et al., 2005). This marked fall in peripheral androgens is associated with a number of conditions including metabolic syndrome and osteoporosis (Davy & Melby, 2003). In addition, the Women's Health Initiative (2002) published their results from a prospective randomised prevention trial of more than 16000 healthy postmenopausal women which was intended to study the long term effects of HRT given as a combination of conjugated equine oestrogens and medroxyprogesterone acetate. Although decreased risks of colorectal cancers and hip fractures were reported, many women came off their hormone supplements and explored alternatives because of the reported increased risk of stroke and invasive breast cancers. Likewise, a large-scale UK study (Beral, 2003) found that current use of HRT, but not past use, was associated with an increased risk of breast cancer. Similar to many other therapeutic agents, HRT has its benefits and risks. The public is constantly in search of alternative forms of food or herbal supplements to alleviate menopausal symptoms or to prevent long-term complications of ovarian failure.

2.2. Clinical contribution of monitoring salivary T in females for pathological and physiological conditions

To date, a definite relationship has not been established between a specific level of T and symptoms of T excess or deficiency such as sexual dysfunction in women and premenstrual symptoms. There is no established level of free T below which a woman can be said to be deficient, nor any level to which a woman should be restored that determines she is replete. Thus the diagnosis of these disorders due to low testosterone remains a clinical diagnosis of exclusion. In the absence of a reliable free /total testosterone assay the limitations of availa-

http://dx.doi.org/10.5772/53648

ble assays should be understood, and the measurement of testosterone used to exclude the use of testosterone in women in whom therapy might result in testosterone excess.

There is a paucity of research which investigates salivary female T in pathological and physiological conditions. For this reason we would like to highlight the following examples of clinical monitoring of female salivary T. Salivary T has been used to monitor treatment of children with congenital adrenal hyperplasia (CAH), Salivary T was found to be a useful additional biochemical marker with 17OHP to indicate the levels of free T (Perry et al., 2005). In a study comparing salivary and total plasma testosterone levels in healthy controls and patients with Klinefelter's syndrome (Wellen et al.,1983) provided indirect evidence that in Klinefelter patients levels of salivary T and androstenedione correlated well with the reported free plasma levels. This suggests that measurement of salivary steroids may be useful in evaluating endocrine function in both healthy and disease states. Monitoring the menstrual cycle status of female athletes by salivary steroid determination following a 21km run (De Crée et al., 1990) reported an increase of salivary T of 15.2%. These findings corroborate earlier studies, which found higher post-exercise plasma sex steroid levels.

Several studies have published data on the applicability and clinical value of salivary T measurements for the diagnosis and follow-up of therapy of idiopathic hirsutism, late-onset hypogonadism and androgen deficiency in end-stage renal disease (e.g. Shibayama et al., 2009; Luisi et al., 1982; Cardoso et al., 2011). In addition, Teoh et al. (2005) and Gayriloya and Lindau (2009) carried out physiological studies to assess population levels of salivary T in children, adult females and males. They reported high co-operation rates with the in-house salivary specimen collection.

3. Testosterone production and mechanisms of action in women

Circulating levels of plasma T in pre-menopausal women originate from multiple sites; the ovaries (20-25%), adrenal cortex (20-25%) and the remainder (50-60%) from the peripheral conversion of T precursors (androstenedione, DHEA and DHEA-Sulphate) (Burger, 2002; Longcope, 1986; Simpson, 2002). T circulates in women with around 66-74% bound strongly to sex hormone binding globulin (SHBG) and 24-30% bound weakly to albumin with only about 1-3% being free in the blood (Pardridge & Demers, 1991; Vermeulen, 1998).

Under certain conditions the bio-available T dissociates from its carrier protein (mostly albumin) and becomes free. Manni and colleagues (1985) indicated that albumin-bound T was available to tissues such as the brain in conditions where the free T level was negligible and in the absence of albumin. This hypothesis was contested by Ekins (1990) and Mendel (1989) based on the assumption that steroids can act only through their genomic mechanism. Besch et al. (1982) stated that only the non-protein-bound or 'free' fraction of a hormone, such as a steroid enters the cell of the target tissue and interacts with its specific receptor protein. As an adjunct to this latter point it should be noted that around 50% of pre-menopausal (and almost 100% of post-menopausal) T is synthesised by peripheral conversion, distinct from any endocrine function (Labrie et al., 2000). In addition, the fact that many steroids are now

shown to exert some effects through the non-genomic pathway (see below) leads us to suppose that some actions, particularly in the female, are mediated by the albumin-bound fraction of T. Moreover, in a recent review Lepage (2006) demonstrated clearly the importance of measuring the bio-available T as compared to total and free T. Thus, free and non-SHBG-bound (sometimes called bio-available) T measures are likely to be the most reliable indicators of tissue exposure to T (Collins, 2000). As an adjunct, recent finding suggest that female androgens made locally in large amounts in peripheral tissues from DHEA/S act in the same cells where synthesis takes place (Labrie et al., 2003). It was therefore concluded that the measurement of androgen glucuronides instead of T perhaps better reflects the androgenic activity in women (Labrie et al., 2006).

Steroid hormones in the periphery are believed to equilibrate rapidly between tissues and blood (Goncharov et al., 2006; Miler et al., 2004; Schurmeyer & Nieschlag, 1982). Total concentrations of T in peripheral tissue and body fluids are mainly dependent upon the levels of binding proteins such as sex hormone binding globulin (SHBG) and albumin. These binding proteins can act as a reservoir for the steroid and protect it against extensive metabolism of active (free) steroids during passage of the blood through the liver (Mendel, 1989). It is now widely accepted that the free steroid form represents the biologically active fraction. However, it is believed that the albumin bound T (bio-available) can bind to the receptor (possibly the membrane bound) and exert its activity because the affinity of the steroid towards its receptor is far greater than its affinity to albumin (Author's unpublished data; Heinlein & Chang, 2002; Fix et al., 2004). In fact, T can also be regarded as a circulating prohormone that can be converted either to DHT for androgenic activity or to oestradiol which is the principal endogenous ligand for oestrogen receptors (Hiipakka & Liao,1998; McPhaul & Young, 2001).

T, like other steroids, may exert its action in living cells by either the well-known genomic pathway, involving hormones binding to a cytosolic receptor and subsequent modulation of gene expression followed by protein synthesis (genomic actions). Or through pathways that do not act on the genome (non-genomic actions) (Lösel et al., 2003). T has recently been reported to exert effects through interactions with receptors in the cell membrane (Heinlein & Chang, 2002). Rapid effects of androgens have been shown on calcium fluxes (Guo et al., 2002), intracellular phosphorylation cascades (Castoria et al., 2003), secretion of GnRH by pituitary cells (Shakil et al., 2002) and others. It seems that in these cells, androgens can exert their effect at very low concentrations that are not sufficient to stimulate gene transcription.

Androgen dynamics in women are usually controlled by 4 temporal phenomena: ovarian function, hypothalamic-pituitary-adrenal axis, age decline of adrenal androgens and depletion of ovarian follicles after menopause (Burger & Casson, 2004). T and its precursor, DHEA/S are now widely accepted as vital for normal female development (lower certain body fat, maintain muscle mass, healthy-skin, boost energy levels and mood), sexual health (substrate for oestrogen production, enhance sex drive and relieve menopausal symptoms) (Laumann, et al., 1999; Davis & Tran, 2001) and might have a role to play in alleviating the aging process of men and women (Kirkwood, 2005; Ullis et al., 1999).

http://dx.doi.org/10.5772/53648

4. Measurement of female salivary testosterone: Challenges and problems

Quantitative determination of circulating T can be achieved utilising a variety of different procedures such as; radioimmunoassay, luminescence immunoassay, fluorescence immunoassay, enzyme-linked immunosorbant assay, gel filtration, equilibrium dialysis and centrifugal ultrafiltration for serum free T, protein precipitation, gas-chromatography mass-spectrometry (including isotope dilution) and more recently LC/MS-Tandem spectrophotometry (Edwards, 1985; Kemeny, 1991; Sinha-Hikim et al., 1998), each with their own inherent strengths and weaknesses.

Once a medium in which to analyse T has been selected (saliva in this case) the question then becomes what type of assay should be employed? Although the bewildering array of techniques can make selecting the most appropriate test perplexing, as Edwards (1985) stated 'A systematic and objective approach will...indicate the most appropriate technique for the particular application in mind' (p.2). In both saliva and blood, RIA was until comparatively recently the method of choice for determining concentrations of circulating T. Despite a number of limitations, traditional analogue RIA is still widely used (Rosner, 2001). Several authors, however, have ardently expressed concern that this method is insensitive at the lower end of the T range; precisely where female salivary T falls (Sinha-Hikim et al., 1998). This situation arises because most T RIA's were designed for the measurement of serum levels in men and thus lack the sensitivity required for the precise measurement of the low levels prevalent in women.

Even now, simple and routine methods for determining free steroid concentrations in plasma have not been fully developed and widely validated. Consequently, most current procedures involve technically demanding and time consuming centrifugal ultrafilitration or equilibrium dialysis (Miller et al., 2004; Hammond et al., 1980; Riad-Fahmy et al., 1982); processes which in themselves yield results with varying degrees of accuracy. In an effort to circumvent the problem of not easily being able to measure free-T some authors use mathematical equations, such as the free androgen index, mass action formulation, or the Sodegaard equation (Ho et al., 2006; Vermeulen et al., 1999). All are mathematical formulae of varying complexity for working out the free component of T based on the measurement of total T and, as a minimum, SHBG. However, because these equations rely on an affinity constant (SHBG in the case of T) and because estimations of the affinity constant varies widely, there are very real problems with these approximations (Besch et al., 1982; Rinaldi et al., 2002). As Vermuelen et al. (1999) point out, total serum T concentration is subject to variations in the concentration of the binding proteins such as SHBG and CBG; it is not, therefore, a reliable index of bio-available T. Faced with these challenges, there has been a growing awareness of the potential value of utilising saliva for measuring hormone concentrations (Mandel, 1993). Consequently, the use of saliva as a diagnostic tool in bio-behavioural and clinical research has grown significantly during the previous two decades (Quissell, 1993).

In a variety of research arenas, accurate measurement of female free-T is widely regarded as problematic (Matsumoto & Bremner, 2004). In a clinical paper on screening for androgen insufficiency, Guay (2002) argued that a major problem in assessing female T is the inaccuracy of the measurements by current assays. Recent articles published by Taieb et al. (2002) and

Herold and Fitzgerald (2003) illustrate that when measured by automated processes, T concentrations may be inaccurate to the order of magnitude of 200-500%. This issue of measurement therefore acts as one potentially serious limiting factor in the confidence that we can place in the results of hormone-behaviour studies, clinical studies and for female's screening before T therapy. Although, as technology advances and measurement continues to become somewhat more straightforward there is still a need for highly sensitive, reliable, and efficient immunoassays with accessible reagents and materials for the determination of T in females (Granger et al., 1999; Guay, 2002).

Given the difficulties associated with accurately measuring female free and bio-available T, the enzyme-linked immunosorbant assay (ELISA) protocol has a number of features to recommend it over RIA. For example, reagents are cheap in comparison with RIA, the laboratory in which our research is undertaken employs staff with substantial expertise in ELISA development, validation and trouble-shooting. Moreover, there is no hazard of radiation, and because of the low concentrations of antigens in saliva HIV and hepatitis infections are much less of a danger from saliva than from blood (Major et al., 1991). Indeed, unless visibly contaminated with blood, human saliva is not considered a class 2 biohazard, affording researchers and institutions administrative and safety benefits. As a consequence of these factors the ELISA method stands out as the most appropriate routine method of choice for use in the majority of research institutes conducting bio-behavioural research and clinical work on T.

There are a number of features that are required of an effective assay. As Kemeny and Chantler (1988) note, the type of assay should be closely tailored to the particular task for which it is required; and the requirements of diagnostic laboratories are often very different than those of bio-behavioural research laboratories. For example, in bio-behavioural studies that require assessing chronobiological changes in the very low levels of free salivary T in females, issues such as ease of use and speed are less important characteristics than sensitivity and accuracy. Subsequently, one of the additional aims of this chapter is to describe the development, optimisation, and validation of an extremely sensitive in-house ELISA, designed specifically for determining salivary T in women. In particular, the ELISA is evaluated for its accuracy, specificity, and precision.

Salivary sampling regimens have several obvious and distinct advantages over blood sampling. They accommodate frequent and easy collection by non-invasive, relatively stress free-techniques, thereby facilitating short term dynamic tests, pharmacokinetic analyses, and studies of chronobiological changes (Riad-Fahmy et al., 1982). In addition, it has been reported that the majority of subjects find little difficulty in salivating directly into collection containers, providing adequate volumes (between 3mL and 5mL) for determining a steroid hormone profile in less than 5 minutes (Dabbs, 1991). Nonetheless, a number of challenges and problems associated with saliva methodology do exist (Granger et al., 2004). For example, compliance with salivary sampling protocols has been identified as a potential challenge. Compliance to collection protocols is essential for accurate determination of T levels in saliva and concerns amount collected, condition of sample and precautions taken prior to collection (i.e. rinsing of the mouth, not eating etc.). In relation to collection, compliance has been investigated by the use of an electronic monitoring device, and it was reported that only 74% of subjects were found to comply with the sampling instructions and 26% failed at

http://dx.doi.org/10.5772/53648

least once to obtain the correct saliva sample (Kudielka et al., 2003; Broderick et al., 2003). Our own experience is that engagement with participants and the provision of detailed oral and written instructions alongside the opportunity to practice with an investigator present to answer questions and provide guidance ameliorates significantly issues of compliance.

Although diurnal and monthly patterns of salivary T generally parallel serum values, absolute ranges show variability across several studies. Few studies of normal individuals, controlling for known variables, such as pH, time of day, month and medications, have been performed using the recently developed high sensitivity enzyme immunoassays, such as those sold by Diagnostic Systems Laboratories (Webster, TX), Salimetrics (State College, PA) and American Laboratory Products Co. (ALPCO) (Windham, NH). Below are several factors which need to be taken into consideration either when using commercial kits or developing in-house assay methodology.

4.1. Blood-saliva correlation

As Albumin is a small protein, with a molecular weight of approximately 69,000, it can pass through the salivary membrane. Conversely, SHBG (to which the majority of circulating T is bound) is a large carrier protein with a molecular weight of 150-200,000. As this steroid-binding protein cannot pass easily through the salivary membrane, one of the advantages in using saliva as the biological fluid in study centres would be around the claim that it contains concentrations of analyte similar to, or even identical with, the non-protein bound (free) concentrations in blood (Baxendale, Reed, & James, 1980; Vittek et al., 1985; Longcope et al., 1987). These claims generally relate to males, however. Although there are fewer studies examining this association in females, it has been suggested that correlations between T levels in serum and saliva may be significantly lower than in males (Granger et al., 1999; Miller et al., 2004). Shirtcliff and co-workers (2002) published a study which appears to cast doubt on the veracity of this correlational relationship in females, stating that regardless of assay method, salivary T levels are modestly correlated with serum levels for males but not necessarily females. The absolute concentration, whilst reflecting accurately the unbound fraction in the plasma (and also the fraction which is not bound to SHBG) is approximately twice the concentration of free-T in plasma, in contrast to the findings in male subjects (Baxendale, Jacobs & James, 1982). One major implication of these findings is that substitution of saliva for serum T levels in at least biobehavioural studies may estimate the T-behaviour relationship differently for females than males because substitution of saliva assay results for serum values markedly underestimates known T-behaviour associations. In addition, there might be some ethnic variation in total and free T concentration in that Heald et al. (2003) found that both total and calculated free T were lower in Pakistani men than in Europeans.

4.2. Contamination of salivary samples

Clinical researchers have established that collection techniques can affect the integrity of salivary samples, which subsequently interferes with ability to accurately determine hormone levels. For example, given the difference in T concentration between blood and salivary samples, leaking of blood or serum into the mouth (i.e. due to gum disease, injury,

consuming very chewy meals, caffeine intake, or vigorous cleaning) can affect the integrity of quantitative estimates of salivary concentrations (Granger et al., 2004; Lac et al., 1993). In response to a question about the incidence of haemoglobin contamination in salivary samples, at a round table discussion on assay development and collection procedures (Tenovus workshop, 1982), Schürmeyer suggested that researchers might reasonably expect a contamination incidence of around 5-10%. The majority of bio-behavioural studies investigating the role of T in women propose that single time-point or very limited sampling is sufficient. Whilst this issue is explored in more detail later in this chapter, it is worth noting here that potential contamination of salivary samples by blood further complicates the use of single samples. In addition, it is noted that where samples have been found with aberrant levels, researchers have on occasion been treated by diluting the samples (i.e. Gladue et al., 1989). Whilst this step may be appropriate for male salivary samples, dilutions of female salivary samples which may already contain levels of T at the utmost sensitivity of the assay are rendered un-determinable.

Whilst the contamination of salivary samples by blood is widely recognized as a potential problem in the scientific fields driving assay development there appears to be something of a malaise regarding the issue in a wide range of bio-behavioural studies. Indeed, with only a handful of notable exceptions a large number of authors have neglected to report how they have dealt with this serious confound. In contrast, Mazur et al. (1980, 1987) and Gladue et al. (1989) paid careful attention to the issue of contamination, adopting what, at the time, must have appeared a sound approach. In utilising measurement strips called Hemastix® they attempted to ascertain which, if any, of their salivary samples may have become contaminated with blood. Unfortunately, Kivlighan et al. (2004) cast doubt on the suitability of this approach and reported that the confounding effects of blood leakage cannot be adequately screened or controlled by visual inspection of sample discoloration or using the Hemastix® approach. Other methods have recently been developed to assess blood contamination in salivary samples. For example, Salimetrics now offer an assay to determine blood contamination in salivary samples (Schwartz & Granger, 2004), although this approach adds substantially to the cost of assaying samples. Perhaps the best approach to reducing blood contamination is avoiding its occurrence in the first place. In our own lab, and following suggestions by Adlercreutz (1990), our subjects were asked to adhere to a number of steps including refraining from brushing their teeth prior to sample collection, rinsing their mouths thoroughly several times prior to collection, not consuming large meals, and not smoking or drinking caffeine. These steps effectively reduced the amount of samples seen in the assays with abnormal levels; indeed following these steps parallel to detailed instructions enhanced collection to the extent that blood contamination in well over 10,000 samples was effectively reduced to an incidence of below 1%.

4.3. Collection methods

With the increased use of salivary measures in clinical practice, bio-behavioural and clinical research, several research teams have sought to advance our understanding of the circumstances and conditions that may influence the validity of salivary assessments (e.g. Lipson & Ellison, 1989; Shirtcliff et al., 2001). The use of a range of stimuli has been reported to stimulate salivary flow where necessary; i.e. paraffin wax, rubber bands, sugar-free gum, cotton

http://dx.doi.org/10.5772/53648

swabs, and citric acid (Malamud & Tabak, 1993; Navazesh, 1993). Granger and co-workers (2004) found that materials commonly used in the literature to absorb saliva (cotton and polyester swabs) or stimulate saliva (powdered drink-mix crystals, citric acid and chewing gum) have the potential to change salivary T results. Typically, saliva is collected by having a participant deposit between 3-5mL into a collection container (less may be required in some circumstances); this step is usually reported as taking between 3-8 minutes. The saliva is then stored frozen prior to assay. While clinical subjects may be willing and able to provide un-stimulated samples that can be immediately frozen, this protocol is often unpractical for field collection (Lipson & Ellison, 1989). As such, collection of saliva samples under these conditions may necessitate certain changes from common clinical practices. Although collection of saliva is often referred to as un-stimulated, this is somewhat misleading. If the salivary gland is un-stimulated it does not produce saliva; what is meant essentially is saliva produced by minimal stimulation (Read, 1989). Small amounts of saliva can be collected without the need to externally stimulate production, but the amount usually collected often requires subjects to stimulate saliva production in some way. This issue is particularly pertinent when investigating changes in concentration of salivary hormones over a certain period of time or prior to competition, where time constraints exist and feelings of stress or anxiety may make saliva production more troublesome. We have optimised our own salivary samples collection method (see Table 1) that ensures the quality of saliva, reproducibility, minimises blood contamination and stress during collection.

A Precautions
DO NOT exercise 24 hours prior to or on the day of salivary sample collections. (Exercise is defined as anything more than low-moderate physical activity e.g. swimming, cycling, yoga, sex etc.)
DO NOT consume alcohol 24 hours prior to or on the day of salivary sample collections or drink coffee, milk, yoghurt or meals within 45mins of collection
Each time you collect a saliva sample the method should be identical.
Procedure:
B Step by step guide to successful saliva sample colection
Step 1 - Do not eat or brush your teeth 60 minutes prior to saliva sample collection.
Step 2 - Rinse and swill mouth out thoroughly three times with tap or bottle water.
Step 3 - Chew a quarter of a stick of sugar free gum provided, as this will aid the production of saliva.
Step 4 - Retain the sugar free gum in your mouth, and spit away the first mouthful of saliva into the waste container provided. This will remove unwanted cellular elements in the mouth and from the chewing gum.
Step 5 - Continue chewing the gum and spit into the collection container provided, until you have deposited 3-5 millilitres (ml) of saliva. Ensure the cap is replaced tightly.
Step 6 - Please mark the date and time of sample on the label of the collection container.
Step 7 - Please store saliva samples in the fridge 4°C. Samples will then be collected by the researcher and frozen until analysed.

Table 1. Optimised in-house method of salivary samples collection

4.4. Storage of saliva samples

It is now known that bacterial growth can occur if saliva samples are stored above 4˚C (i.e. at room temperature) for extended periods of time. In a recent report, it was found that bacterial growth in saliva increased by incubation at room temperature over 10 days, and this caused a significant decrease in salivary levels of both cortisol and T (Whembolua et al., 2006). Some workers have shown that salivary steroid levels (cortisol, progesterone and 17-OH-P) decreased significantly in the course of 3 weeks under different storage conditions (native or centrifuged saliva, saliva with triflouroacetate and saliva combined with 0.05% NaN_3), and that the decrease was clinically significant from the second week onwards (Groschl et al., 2001). After repeated freezing and re-thawing, only cortisol decreased significantly ($p<0.001$) presumably due to enzymatic conversion to metabolites. On the other hand, a study by Granger et al. (2004) investigating the week-to-week change in salivary T levels of samples stored at 4˚C for 4 weeks, -20˚C and -40˚C up to 24 months found that there was a linear increase in T levels across the 4 weeks for samples stored at 4˚C ($R^2=0.88$, $p<0.05$). By the end of 4 weeks, there was an increase of 330% in T concentration using a Salimetric assay. However, they reported a dramatic decrease in measured T levels over the 6 months and 24 months in pooled samples stored at -20 and -40˚C ($R^2=0.85$, $p<0.05$). Clearly, in order for researchers to have confidence in their assay results a good deal of attention has to be paid to issues of collection, storage and compliance.

5. Direct vs. In-direct technique

Solid phase and coated-technology assays can adopt either a direct or an in-direct approach to determining levels of T. Whilst several assays, including commercially available kits, employ a direct method (that is, salivary samples are not treated in any way prior to assay) there are a number of limitations with this approach, especially when examining female androgens. Whilst the benefits of utilising saliva over serum have previously been described, saliva is a far from inert substance; it contains a variety of contaminants, such as bacteria, leukocytes, mucins, and very importantly for enzyme assays, endogenous enzymes. All of which can interfere with assays based on the ELISA technique. As a consequence, salivary samples are rendered extremely susceptible to interfering agents such as pH imbalance which yields results that are, unpredictably, either too high or low (alkaline samples, for example, tend to yield low results). Schwartz et al. (1998) reported that when pH falls below 4 or rises above 9, then assay performance is likely to be compromised. At a round table discussion of sex hormones and corticosteroid assays, Adlercreutz (1990) citing a range of studies, noted that T assays do not work well in non-extracted plasma. Moreover, Jones et al. (2004) reported that some samples from female subjects gave falsely high results when measured with direct immunoassay.

Taib et al. (2003) found that 7 out of 10 immunoassay kits tested had overestimated T concentrations by up to 46%, and that the target values were missed by 200-500%. This has prompted some authors to doubt the validity of female T assays and suggest perhaps guessing levels

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in women to be cheaper and more accurate! (Herold & Fitzgerald, 2003). However, Dabbs and colleagues (1995) reported on the reliability of salivary T assays evaluated by nine laboratories and found acceptable overall agreement (r=0.87 for men and r=0.78 for women). We suggest that one of the reasons commercial assays invariably produce T results that appear high compared with in-house protocols is because of the cross-reactivity with DHEA/DHEA-S. The importance of the cross-reactivity findings depends not only on the % cross-reactivity, but on the relative concentration of the compound compared against T. For example, DHEA-S occurs in plasma at approximately 500 times the amount of T. Hence, a cross-reactivity of 0.72% is potentially of more importance than the 2.3 % cross-reactivity found in DHT, which occurs at levels below T in plasma. Using the extraction procedure removes the ability of DHEA-S to interfere with the 'in-house' assay optimised in our laboratory.

6. Development of ELISA based assays for the measurement of testosterone in saliva

A simple, reliable, easy to perform, sensitive and highly specific ELISA type assay for the measurement of female salivary T has been developed in our lab and utilized in several wide-ranging research projects investigating T levels in the menstrual cycle, circadian rhythm and bio-behavioural studies. This has enabled us to screen large number of samples within a short time at relatively low cost and with high sensitivity and assured specificity. The principle of the salivary T assay optimised in our laboratory is based on the in-direct, competitive binding technique (Al-Dujaili, 2006; Al-Dujaili et al., 1988; O'Sullivan et al., 1979). Essentially, the T present in salivary samples competes with a fixed and limited amount of T coated on the micro-titre plate, for binding sites on an antibody. Because the concentration of the T coated to the wells is held constant, while the concentration of T in the salivary samples vary, the amount of enzyme labelled second antibody bound to the first antibody is inversely proportional to the concentration of the unlabelled analyte present in the sample.

Extensive experimentation was conducted in our laboratory to optimise this assay. Because of the often extremely low levels of T in female saliva, one of the particular requirements for quantitative determination is that the assay be especially sensitive. However, the ELISA process sits within a complex web of inter-locked parameters, and achieving this sensitivity requires a constant balancing act between reagents and conditions to arrive at and maintain the final protocol. For example, one of the ways to minimise the impact of interfering factors is to extract the samples prior to assay. In line with guidelines taken from Al-Dujaili et al. (1988) all assay reagents and conditions have been optimised to produce the required sensitivity, precision, accuracy and reliability including the amount of testosterone-bovine serum albumin (BSA) conjugate needed to coat the plate, volume of sample, amount of antibody, incubation temperature and incubation times. Below are our optimized protocols for sample preparation, plate preparation and ELISA procedure:

6.1. Sample preparation

1. Frozen samples are thawed, centrifuged at 3500rpm for 10mins, and aliquoted.
2. Aliquots centrifuged at 6000rpm for 2mins.
3. 0.5mL of sample combined with 4mL of diethylether.
4. Vortex mix for 10mins and freeze at -80˚C.
5. Decant unfrozen ether and place in 45˚C water bath until evaporated under nitrogen gas.
6. Reconstitute with 0.5mL assay buffer.
7. Stand at room temp for 30mins and finally vortex mix to equilibrate.

6.2. Plate preparation

1. Coat plates with T conjugate and leave to incubate overnight at 4˚C
2. Wash three times with wash buffer
3. Block for 1hr at 37˚C
4. Discard blocking buffer

6.3. Optimized ELISA Procedure

1. 100μL of standard and previously extracted and reconstituted sample into wells in duplicate. Standards run at 0.00, 1, 5, 10, 50, 250, 1000pg/mL
2. Add 100μL of antibody in assay buffer
3. Shake and incubate at 37˚C for 1hr
4. Discard and wash 4 times
5. Add 100μL of enzyme (horseradish peroxidase- linked second antibody)
6. Shake and incubate at 37˚C for 1hr
7. Discard and wash 4 times
8. Add 100μL of substrate (tetramethylbenzidine)
9. Incubate at room temperature for 15mins
10. Add 50μL of stop solution (H_2SO_4)
11. Read at 450nm on MRX Dynex plate reader

The validity of the salivary assay for T was confirmed by Al-Dujaili (2006) using the same antibody and technique in urine and additionally by the correlation between the results obtained with the in-house ELISA and those assayed by Salimetrics ELISA kit (Salim $_{ELISA}$ =

1.12x In-house $_{ELISA}$ – 0.042, $R^2 = 0.95$, n= 58). Cross-reactivity data with major interfering steroids were minimal (see Table 2) except for testosterone-3-glucuronide (58.8%), dihydrotestosterone (2.3%) and androstenedione (4.6%). The average recovery of T in this assay was 104.0% (range 97.5% to 110%). The average intra-assay coefficient of variation was 5.78%. Inter-assay imprecision of 8.7% was determined from the mean of averaged duplicates for 50 separate runs for male and female aliquots. Both inter and intra assay coefficients of variation are at levels comparable with the best commercially available assay kits. Assay sensitivity was determined and an un-related *t*-test revealed the differences between the zero standard and 0.5 pg/mL concentration were significantly different: t (1, 11) = 9.098, p<0.001. By this method the assay sensitivity is 0.5 pg/mL(1.74 pmole). The working sensitivity of the assay, corresponding to the mass of testosterone required to give a decrease in the %B of tracer of 2.5SD of the zero point signal was 1.24 pg/mL(4.33pmole). See typical standard curve in figure 1. The applications of our salivary T ELISA can be seen in a wide-range of clinical and bio-behavioural studies (e.g. Moore at al., 2011; Conway et al., 2007; Deady et al., 2006; Sharp & Al-Dujaili, 2010). In addition, applications of the assay are discussed in sections 8 and 9 of this chapter.

Steroid hormone	**% Cross- reactivity**
Testosterone	100
Testosterone-3-glucuronide	58.8
Nandrolone	12
Dihydrotestosterone	2.3
Androstenedione*	4.6
DHEA	1.05
DHEA-sulphate*	0.72
Cortisol	0.001
Cortisone	0.03
Pregnenolone	0.05
Progesterone	0.02
Corticosterone	0.24
Prednisolone	0.3
Estradiol-17B	0.52
11-Deoxy-Cortisol	0.2
17-OH-Progesterone	0.02
11-deoxy-Corticosterone	0.06
Cholesterol	0.04

Table 2. Cross-reactivity between Testosterone and related steroid hormones

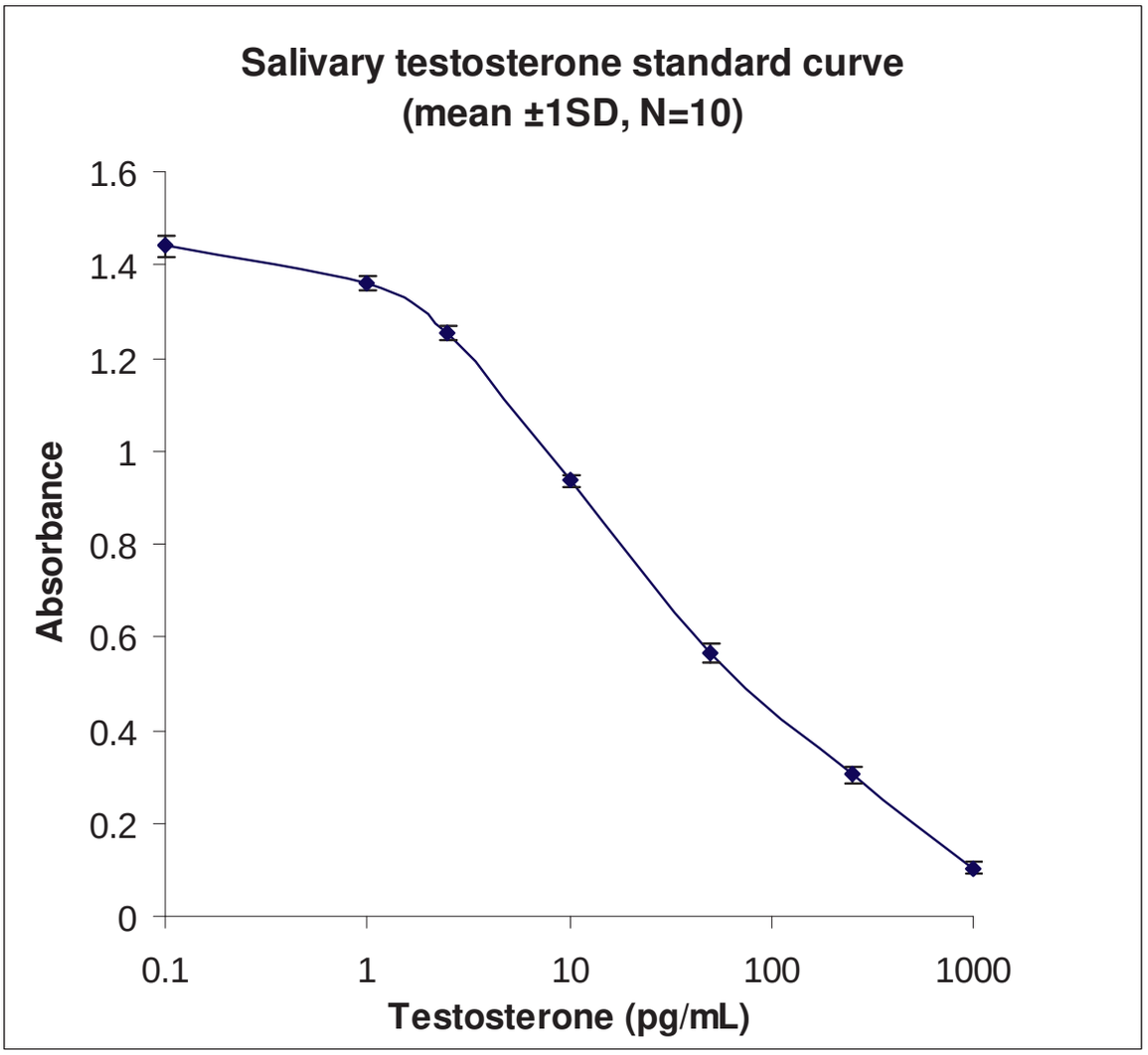


Figure 1. Typical testosterone ELISA standard curve (mean with SD, N=10)

7. Female salivary testosterone: Single vs. multiple sampling

Establishing a baseline for salivary T is not entirely straightforward. Whilst T production is partially under genetic control (Meikle et al., 1988) it is also responsive to a range of biological, environmental, and psychosocial stimuli; the relative influences of which are not yet fully understood, in either males or females. Amongst those factors identified as having a role in modifying T levels are: fasting, diet, sexual activity, alcohol, competition, behaviour intended to increase status, aggression, physical exercise, cognition, stress, immune function, and mood. In line with this, T concentrations have been shown to vary with time of day (Ahokoski et al., 1998; Walker et al., 1980), season of the year (Dabbs, 1991), sexual activity (Morris et al., 1987), and they fluctuate, in males at least, in a pulsatile fashion over minutes and hours (Veldhuis et al., 1987). Moreover, among women, T concentrations are

thought to increase around the middle of the menstrual cycle (Massafra et al., 1998; Vermeulen & Verdonck, 1976; section 9 of this chapter).

Dabbs (1990) encapsulates some of the difficulties in designing suitable salivary sampling regimens when he states, '...variability introduces error into behavioral studies, where stable measures are needed to characterise individual differences and changes over time. Without more information on these changes one cannot know how many participants to run, how many measurements to take, and when to take measurements' (p.83). For a T baseline to be meaningful additionally depends, at least in part, on what purpose it is required to serve. In certain clinical practices, for example, researchers have advocated collecting single samples each day over the course of a week and pooling the samples to provide a weekly average. In order to ascertain if an individual has levels of T that might indicate a risk of a particular clinical condition this approach is appropriate. However, when attempting to examine chronobiological changes in relation to behavioural indices this sampling protocol would be unsuitable.

Comparatively little accurate research is available concerning detailed daily patterns of T in females; particularly the biologically active free and bio-available components, as measured in saliva. In the absence of reliable information, studies investigating the relationship between androgens and female behaviour have formulated methods that tend to employ either single time-point or very limited sampling protocols. However, given that females may also experience temporal fluctuation in T levels, similar to males, this salivary sampling protocol may be inappropriate. In order to address questions of salivary sampling schedules for one of our research programmes in female hormone/dominance research, we sought to provide a comprehensive picture of potential circadian activity and episodic fluctuation in female salivary T over one and then two non-consecutive days.

7.1. Methodology

Subjects were seventy-three healthy females (age range=18-29 with a mean age of 23.5). None were hirsute, had serious acne or were overweight. Particular attention was paid to factors that can affect circulating SHBG levels (i.e. history of kidney or liver disease, restriction of calorific intake) and none of the participants has administered any form of hormonal medication during the previous 9 months. Eight 4mL salivary samples were collected throughout the course of the day; one every two hours from 9am until 11pm. Following the same protocol, fifty-three healthy female participants (age range=18-28; mean=24.7) with a history of regular menstrual cycles lasting between 26-34 days also collected saliva on a second non-consecutive day. T concentrations were determined utilising our 'in-house' ELISA, described earlier in the chapter.

7.2. Findings

T concentration showed a circadian rhythm similar to that found in males. Perhaps more importantly, throughout the course of the day T concentrations were highly variable with

episodic fluctuation of individual data points exceeding 83% of 9am levels. Mean T concentrations over day 1 and 2 were 140.5 and 148.2 picomoles/L respectively.

Consistent with findings from previous studies, and resulting at least in part from the spread of samples collected across menstrual cycle phase, there was considerable inter-individual variation in levels of T. Even so, table 3 illustrates that within the group mean, female participants demonstrate a clear circadian profile, with levels higher in the early morning and lower at 9pm, before starting to rise at 11pm. In the current data set percentage change from mean 9am levels reached a level of 34.4% at 9pm with individual percentage change from mean 9am levels reaching over 100%.

	9am	11am	1pm	3pm	5pm	7pm	9pm	11pm
T (pmole/L)	205.8	0.188	176.2	176.1	152.4	150.6	136.2	141.4
Mean (SEM)	(11.0)	(10.7)	(10.8)	(11.5)	(10.8)	(10.5)	(9.5)	(10.9)

Table 3. Female Circadian Salivary Testosterone (Mean ± SEM, n=71)

	9am	11am	1pm	3pm	5pm	7pm	9pm	11pm
Day1 T (pmole/L)	160.9	144.6	149.9	148.3	136.5	140.6	126.8	118.9
Mean (SEM)	(13.8)	(12.1)	(12.7)	(13.0)	(10.8)	(12.0)	(12.1)	(13.4)
Day2 T (pmole/L)	171.9	171.8	166.5	166.1	130.1	125.4	128.9	125.6
Mean (SEM)	(14.9)	(12.6)	(12.9)	(13.2)	(12.9)	(12.1)	(12.6)	(11.1)

Table 4. Testosterone Over Two Non-Consecutive Days (Mean ± SEM, n=53)

Contrary to the oft-cited claim that T is less labile in the afternoons our own results indicated that individual variability is at its most pronounced between 1pm and 7pm, which is the time it has been suggested studies take place in order to account for circadian variation and during which time 1 sample is presumed to represent basal levels. For study 2, Table 4 illustrates that across both days T followed a circadian profile appears only marginally similar.

7.3. Implications

In utilising single salivary measurements as somehow representative of basal female T levels we argue that studies attempting to correlate biological markers with behavioral indices have introduced a potentially serious confound into their methodological design. This confound is occasioned not only by normal circadian activity which, when considered in isolation we propose may be the wrong component of analysis on which to base the design of these studies, but results from the temporal fluctuation evident in individual salivary T. By adopting a more comprehensive sampling regimen than has previously been available the two studies reported here provide a more detailed representation of circadian activity in the free component of female T. Study 1 revealed evidence of a pronounced, though far from uniform, circadian profile in female T, the magnitude of which from 9am to 9pm was ap-

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proximately 34%. In a study on circadian and menstrual variation Dabbs and de La Rue (1991) reported that morning T levels were 80% higher than evening samples. The magnitude of this circadian profile appears surprisingly large, however. It is, for example, much greater than the male equivalent (Bremner et al., 1983) and more than double the magnitude of our circadian findings. It is worth considering why.

Findings from early bio-behavioral studies attempting to assess the free (and/or bio-available) component T tended to suffer from limitations in the assay technology available to them. In the case of the Dabbs and de La Rue study (*ibid*.) the assay performance appeared to be a major limiting factor in placing any confidence in the findings. Not only did the extraction step recover only 85% of the original analyte, but within and between-assay coefficients of variation (CV) of 13.4% and 14.8% respectively appear excessively high. Moreover, with only two samples collected per day, the collection schedule was extremely limited. Indeed, rather than collect samples at predetermined times the participants followed their own schedules. As such, collection of the evening samples had an 85-minute standard deviation. These limitations provide a basis for recommending caution when interpreting the magnitude of their circadian findings.

The year before, Dabbs (1990) had written another paper on the circadian activity of T in both men and women and found a much smaller circadian profile. With specific reference to the female participants there were serious methodological anomalies. Data were presented from three independent studies: one group of participants collected salivary samples at 7am and 10am; a second group collected samples at 7am, 10am, and 10.30am. A final group collected samples at 10am, 4pm, and 10pm. Dabbs reported that female T levels were high in the early morning followed by a drop in the afternoon and early evening: 'Mean testosterone concentration dropped about 50% from morning to evening for both sexes, with largest drops early in the day' (p.83). However, in creating a circadian profile, the data from these separate studies were combined. In adopting this approach Dabbs fashioned a situation whereby a 7am sample for subject A must have been compared against a 10pm sample from subject B. Given the widespread acknowledgment of, often extreme, inter-individual variation in T levels, this procedure seems curious, at best. Moreover, despite earlier in the paper providing several references which pointed to the fluctuation of hormone levels with time of year, the data Dabbs collected, and subsequently combined, were collected at differing times of the year (autumn and spring). Indeed, as Dabbs notes, the results emanated from separate studies in which 'Data were collected over a 2½ year period' (p.83). Concerning the validity of findings from these studies there are additional questions concerning the assay performance and procedures. Firstly, in choosing to analyse aliquots of differing amounts of saliva (from 0.05mL to 0.4mL) Dabbs effectively altered the concentration of T being determined. He proceeded to suggest that the assay was run under analogue conditions (that is, one standard curve is provided and against which all results are determined). Secondly, the assay performance itself had an inter-assay variation in excess of 20% after recovering only 80-85% of the analyte following extraction. If these events were not problematic enough, Dabbs states 'There were changes in assay materials and in lab technicians and procedures

over the course of the studies' (p.84). We argue, that these limitations essentially render the results impossible to interpret.

Interestingly, and we would argue critically, Dabbs makes the following point, 'This kind of variability should give us pause in working with single measurements from each subject, where it is not possible to recognise a score as deviant from a subject's mean' (1990, p.85). In attempting to determine the validity of utilising single salivary samples for determining baselines there are two issues that arise from this comment. Dabbs appears to be suggesting here that single time-point sampling can be problematic, if for no other reason than assay techniques can and do throw up erroneous results that can be extremely difficult to detect unless they are considered in relation to other scores from the same subject. The second issue is still whether circadian variation or episodic fluctuation confounds study design. It is not possible to make a judgement based on these two studies; firstly because of the considerable limitations in the study (design, measurement, methodology, interpretation) but also due to the lack of a comprehensive sampling regimen. It is further worth noting that Dabbs (1991) appear to contradict his earlier position, stating '…single measurements are reliable enough for use in behavioral research' (p.815).

In order to control for circadian activity several authors have indeed attempted to collect samples during the course of the afternoon, when T levels have been assumed to be less labile than in the mornings. In this regard Booth et al. (1989) state that '…it is helpful that matches are played in the afternoon' (p.558). Echoing this theme, Mazur, Booth, and Dabbs (1992) made explicit reference to their attempts to '…ensure that reported effects are not artefacts of…diurnal variation' (p.72). Recently, a study on the relationship between T and personality characteristics in which single T measures were collected, Sellers et al. (2007) stated 'To minimize the effects of diurnal fluctuations in T levels, all participants were assessed between the hours of Noon and 4pm' (p.5). Zitmann and Nieschlag (2001) put forward an alternative proposal arguing, in their review paper of T and behavioral characteristics, that T samples should be collected during the morning in order to minimise the effects of diurnal variation. Of the three published hormone-competition studies involving female participants, Mazur et al. (1997) claimed that by collecting samples between 1pm and 10pm they had effectively controlled for diurnal variation in T, which they clearly had not.

As a result of the more comprehensive circadian profile from our own data we are able to demonstrate that, far from being relatively stable, women experience considerable moment-to-moment variability in T levels. Even so, within the group mean of study 1 the significant differences generally existed between time points at the beginning of the day and late afternoon (i.e. 5pm) onwards. One interpretation of this finding would be that single measurements, especially if collected during early to mid-afternoon, would suffice in bio-behavioral research; collection at one moment in time being likely to yield much the same T level as any other. However, in the present study the lack of a statistically significant difference between T levels in the afternoon (between 1pm and 5pm) owes as much to the high standard deviations produced by the often extreme inter-individual variation as it does to a lack a difference in levels of T between time points. It is our contention, therefore, that when considered in isolation, mean circadian data may mislead researchers into believing that single sam-

http://dx.doi.org/10.5772/53648

pling is appropriate in the design of bio-behavioral studies. We maintain that the episodic fluctuation (or, perhaps more correctly, random variability as the mechanism for this fluctuation is currently unknown in females) occurs at an order of magnitude that reduces confidence in the stability of a single salivary sample. It is for this reason we propose researchers utilize multiple samples in the determination of baseline T. Indeed, as Riad-Fahmy (1982) note, 'The wide episodic fluctuations in circulating steroid levels make analysis of single samples useful only in screening procedures' (p.367).

7.4. Reliability across days

Dabbs (1990) reported correlations for female T across two consecutive days as ranging from r=.55 though r=.73. Study 2 sought to examine the stability of T levels on two non-consecutive days demonstrated the relative lack of stability in levels of free T providing evidence that, contrary to earlier reports, the day-to-day stability of female T may not be especially high. In particular, the reliability between those subject who collected data on day 4 and then 14 of their cycle the reliability is, at some time points, low. There is some question as to whether levels of free T change across the menstrual cycle. Several studies have reported that T varies in a predictable manner throughout the course of a menstrual cycle (Alexander et al., 1990; Bloch et al., 1998), although the magnitude and subsequent relationship to behaviour is, at present, unclear and we address this in the following section. In a study examining plasma T changes across the menstrual cycle, Vermeulen and Verdonk (1976) stated, 'It is evident from this study that T…plasma levels do show statistically significant cyclical variations with maximal variations around ovulation' (p.493). Just over ten years later this finding was echoed in a study by Morris, et al. (1987), in which they indicated a rise in T levels around the mid- point of the cycle. Dabbs and de La Rue (1991), whilst finding a mid-cycle rise in salivary T, suggested that, as this variation was smaller than circadian variation, 'Menstrual cycle effects can be ignored in most research relating psychological and behavioural variables to individual differences in testosterone' (p.182). Our data appear to cast doubt on these recommendations.

In providing a more comprehensive profile of the temporal activity of female salivary free T the two studies reported here deal with an issue not satisfactorily addressed in the extant literature. Study 1 demonstrated that not only is there a distinct circadian rhythm but individual temporal variability can be pronounced. We contend it is this temporal variability that researchers need to account for in the design of salivary sampling protocols, and not only circadian activity. Study 2 demonstrated that this temporal profile, whilst similar over non-consecutive days 48hrs apart, reveals low reliability when samples are collected on more disparate occasions. Hence, a sample collected at 10am on day 1 may well not correspond particularly closely to a sample collected at 10am on a second day, especially if those days occur during different phases of a menstrual cycle. And yet, this is precisely the type of sampling regimen evident in the hormone-competition literature (e.g. Bateup et al., 2002). Combined, these data support the position that our understanding of the relationship between T and behavior in women is seriously hindered by the use of single-time point sampling methodology. Hence, over and above reporting evidence of circadian activity, the

highly erratic nature of female salivary T levels throughout the day is an important consideration in sampling design. Indeed, these results suggest that females also exhibit episodic or random fluctuation at levels which call into question the use of single T measurement in female bio-behavioural studies.

8. Establishing female salivary testosterone circadian rhythm profiles in the menstrual cycle: Evidence of decline during ageing

In an attempt to further our understanding of circadian and menstrual dynamics the aim of the study was to establish circadian profiles and normal levels of salivary T in healthy women from the age of 19 through 69 at 3 points during the menstrual cycle, by means of a highly sensitive ELISA (described in section 7). Also, we wanted to investigate whether salivary T levels decline with age.

Five main groups of females: ages, 19-29, 30-39, 40-49 (pre and postmenopausal), 50-59 and 60-69 years were investigated. All subjects provided 8 saliva samples per day on the 4th, 14th and 21st day of their cycle according to their first day of menses, and post-menopausal women collected saliva samples on the 4th, 14th and 21st day of the calendar month. The women were not on any medication including the contraceptive pill and HRT, and did not suffer from any major illness. T levels were determined by our in-house ELISA method. The data indicated that female salivary T concentration showed a circadian rhythm similar to that found in males, although at lower levels (Fig. 2). Perhaps, more importantly, throughout the course of the day T levels were highly variable with episodic fluctuations of individual data points exceeding the 09.00 hours levels on some days (see Fig. 3). There was marked variation in T concentration between day 4, 14 and 21 of the cycle, though not statistically significant except for the age group of 30-39 year ($p<0.02$,) (see Fig. 4). When a repeated measure ANOVA with time and day as within subject factors and age group as between subject factors was applied, it was found that there was a significant difference in salivary T levels for age groups 19-29 and 40-49 years ($p = 0.01$), and between 30-39 and 40-49 years ($p = 0.02$). A Bonferroni correction was used for multiple comparisons.

Normal ranges for salivary T in females from the age of 19 through 69 year old at 3 different distinct days of the cycle (4, 14 and 21) for menstruating women, and 3 calendar days for post-menopausal women have been established. The results indicate that female salivary T concentration show a circadian rhythm similar to that found in males, although perhaps more importantly, throughout the course of the day T levels were highly variable with episodic fluctuations of individual data points exceeding the 09.00 hours levels. These findings lead us to suggest that normal ranges of T should only be determined from multiple samples as single measurements may be potentially misleading as they are subject to too much error variance. This view was reported by the authors in another study (Sharp & Al-Dujaili, 2004) and supported by Hoffman (2001) who suggested that because of diurnal and monthly variations, several steroid hormones need multiple samples to give meaningful results.

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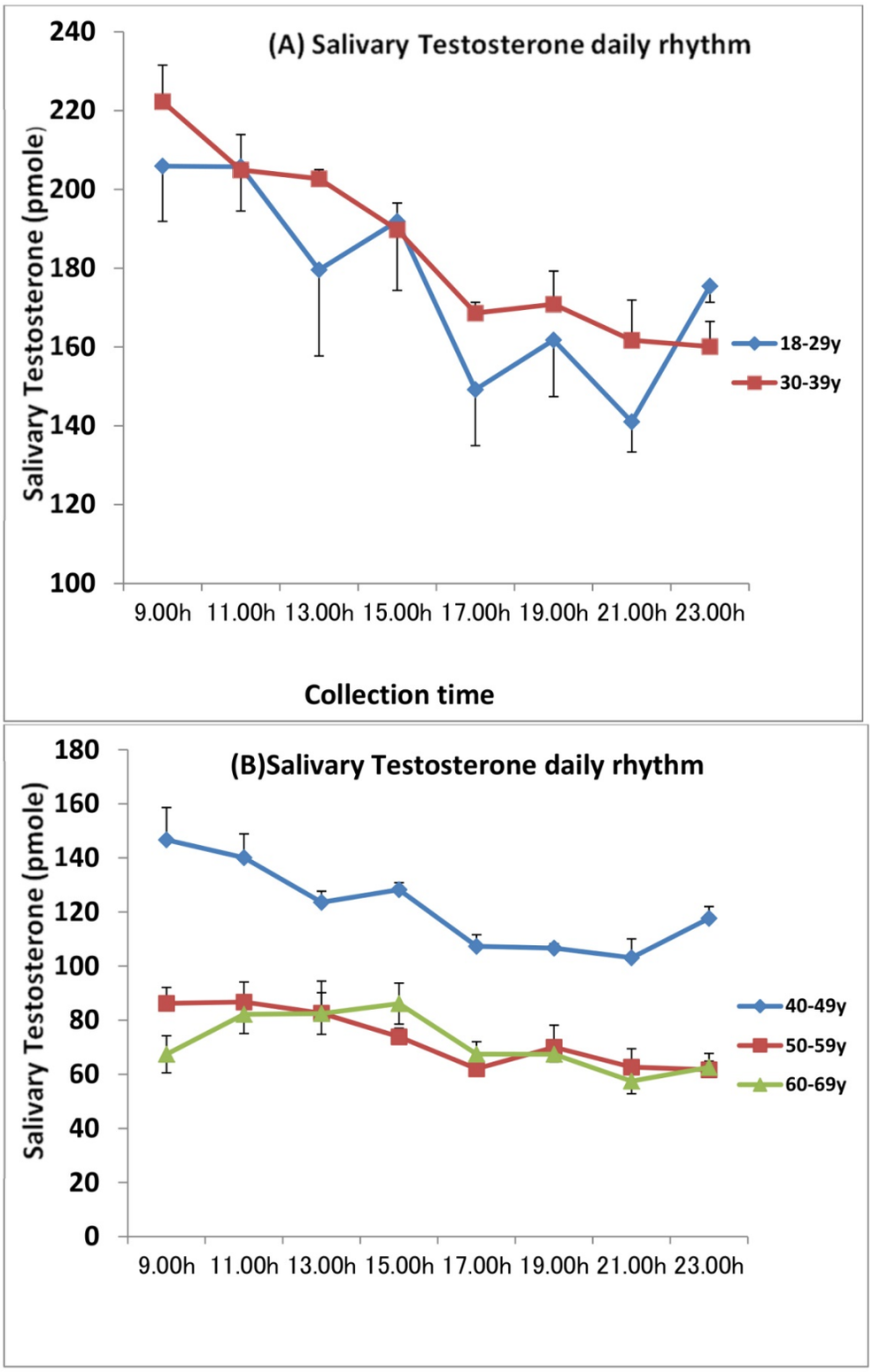


Figure 2. Female Salivary daily Testosterone rhythm throughout the menstrual cycle for A) pre-menopausal women (age group 19-29, 30-39 years) and B) postmenopausal women (age groups 44-49, 50-59 and 60-69). Some pre-menopausal women 40-44 years data were included in the graph of 40-49 years. All data represent mean ±sem of day 4, 14 and 21 of the cycle.

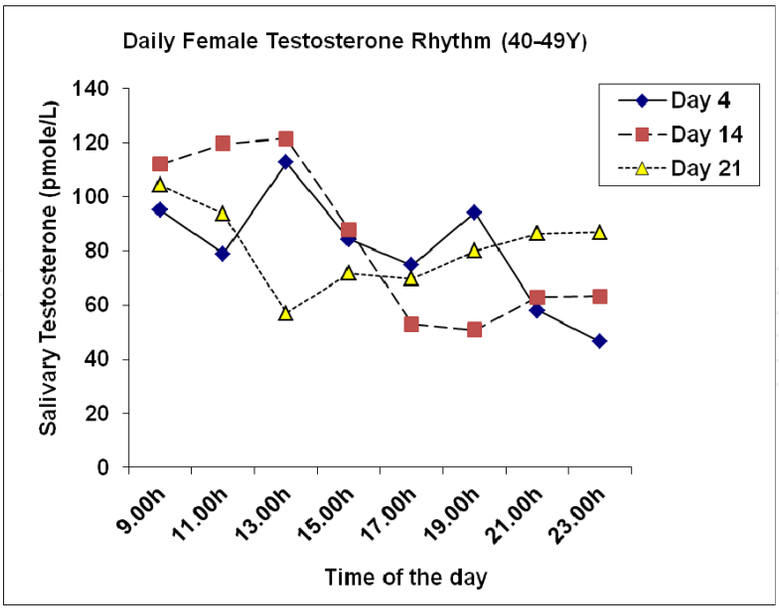


Figure 3. Female salivary testosterone levels in women at 40-49 years showing episodic fluctuations of individual data points exceeding the 09.00 hours levels on some days (Data are mean of 12 females).

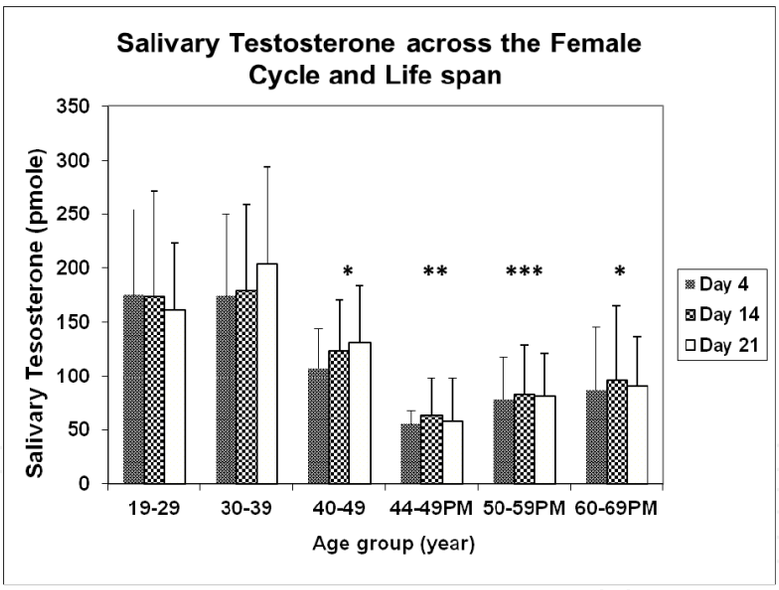


Figure 4. Summary of Salivary Testosterone results in females: Average testosterone concentration per day in pmole. (* = P< 0.05, ** = p< 0.01, *** = p< 0.001). Student paired t-tests were done between these groups and group 19-29years)

In terms of absolute salivary T levels obtained by our ELISA method and bearing in mind the high specificity of the T antibody employed, the values seem to be somewhat higher than those reported elsewhere, particularly for younger women. We believe that this could be due to the fact that our ELISA might be measuring some of the bio-available T concentration (i.e. the free fraction plus the albumin-bound T: Pardridge & Demers, 1991). A view

http://dx.doi.org/10.5772/53648

supported by earlier findings by Ruutiainen et al. (1987) who found that concentrations of salivary T (without chromatographic prepurification) in hirsute women exceeded free T in plasma by 10-fold. However, Swinkels and co-workers (1991) found that salivary T levels were 3-fold higher than plasma free T due to the metabolism of androgen precursors (e.g., androstendione) during passage through the salivary gland. We believe that both of the above mechanisms may be involved.

Our results demonstrated an effect of age on salivary T similar to that found in serum in which there is a significant decline in salivary T level between younger groups of females and those after menopause and older groups. These results were also found by other researchers (e.g. Labrie et al., 1997). However, some workers (Burger, 2002) stated that serum T does not change significantly in relation to the menopausal transition but it falls slowly with age. Such difference could be explained by the fact that they were measuring serum total T rather than the biologically active hormone. Another study by Campbell and Ellison (1992) found that salivary T was higher in anovulatory cycles compared with ovulatory cycles, and that T levels tend to be higher at the midcycle phase.

The results of this study have clearly demonstrated that there was a significant decline in female salivary T across age groups and particularly after the age of 39 years. These findings are supported in a study by Labrie and colleagues (1997) in which they compared serum T levels in women of 20-30 years old with 70-80 years old, and found that there was a marked decline in C19 steroids in the older group with smaller changes after the age of 60 years. Other studies (e.g. Zumoff et al., 1995; Longcope, 1998) have also shown a decline in serum T after the age of 30 years and just before the menopause varying from 15-50%. We have also shown that there was a significant drop in female salivary T concentration in post-menopausal women compared to menstruating women within the age group of 40-49 years ($p< 0.02$ to $p<0.05$). However, Burger et al. (2000) showed no change in serum total T levels 5 years before and 7 years after the menopause. Our results, on the other hand, showed a slight increase in salivary T at the age group of 60-69 years compared to other post-menopausal women. This was mainly due to 2 subjects (ages 61 and 64) who consistently exhibited higher T values that could be due to undeclared use of HRT or non-compliance with instructions. Several studies have shown possible vital roles for androgens - and in particular T- in women (Synder, 2001; Braunstein, 2002; Burger, 2002). Specifically, to increase libido, increasing bone mineralization, effect on muscle mass and strength and sense of general well being. We also think that it is absolutely necessary to assess female T before any hormone replacement therapy, and our results demonstrate without doubt that some women had seriously low T levels that might precipitate deleterious effects on the health and life quality of these women.

In conclusion, our results suggest that females do exhibit episodic fluctuations in daily salivary T throughout the menstrual cycle, and there is marked variation in T concentration between day 4, 14 and 21 of the cycle. Moreover, single T measurements appear to be of little value representing only the level at the time they were collected. There is however, a significant decline in female salivary T after the age of 39 years. The data obtained will hopefully allow clinicians to have much greater confidence in their ability to determine the necessity

of androgen replacement therapy (HRT) for post-menopausal women and those with suspected insufficiency. It is extremely important for women embarking on taking any form of HRT to consult their physician who can now assess by simple screening method the endogenous level of T in order to administer the correct dose of HRT. This might minimise any side effects.

9. Summary

We commenced our chapter with an up-to-date revision on the role of testosterone (T) in women's health, illustrating both the complexity and breadth of physiological and psychological actions. Although not exhaustive in scope it is clearly evident from these examples that T can no longer be considered as male only hormone and a greater emphasis on the role of T in female behaviours is warranted. Second, we described T production in females and discussed recent advances in our understanding of mechanisms of action such as non-genomic pathways. Here we also considered the importance of incorporating both free and bio-available fractions in the measurement of salivary T. Third we discussed the use of saliva as a diagnostic fluid and highlighted that despite its utility there are a number of problems and challenges related to the collection, storage and measurement of androgens in saliva, which researchers must be aware of if we are to have confidence in studies across several disciplines and particularly in those bio-behavioural studies which seek to draw often controversial inferences about the role of women in society based upon levels of circulating T. Fourth we dealt with the assay technology generally and more specifically the development and optimization of our own enzyme-linked immunosorbant assay (ELISA), specifically designed for use in the quantitative determination of female salivary T. Finally, we discussed the circadian dynamics of female salivary T. Very little research is available about detailed daily patterns of T in females; particularly the biologically active free component, as measured in saliva. In the absence of reliable information, hormone replacement therapy programmes and bio-behavioural studies involving female participants have tended to formulate salivary sampling strategies taken directly from research involving males. We were able to draw upon our own innovative work on the circadian dynamics of female T to illustrate the limitations of single or limited time-point sampling by demonstrating the large intra and inter subject variability of diurnal rhythms and further show that merely sampling in the afternoon as a means of reducing error is fraught with complications. Our findings allowed us to highlight the need for comprehensive multiple sampling design for effective protocols. Following this examination of circadian dynamics we also described our work on menstrual fluctuations in salivary and circadian dynamics across the lifespan, which demonstrated the significant difference in T levels across the menstrual cycle and decline across age. We hope that the ELISA protocol described here, the challenges in measurement and collection discussed and our findings on circadian dynamics in women are of some assistance to researchers in helping them reflect upon and design meaningful studies involving women and T.

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