Saliva diagnostics:

Coming closer to the clinic.

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Saliva diagnostics: coming closer to the clinic.

Steroid analysis using a saliva sample first appeared in the scientific literature more than 40 years ago.[1] Saliva sampling and analysis presents an attractive alternative to blood testing because it is non-invasive, easily repeatable, allows for the collection of multiple samples, and it can be performed in settings that may not be conducive to blood sampling. It is also less stressful and more convenient for patients.[2]

Saliva-based diagnostics are revolutionizing the field of personalized medicine because they can now be used to evaluate a huge variety of different physiological conditions, such as sex hormone imbalances, stress, and depression. This is because saliva contains various hormones and other chemicals that can serve as direct indicators or indirect biomarkers of human health, biochemical imbalances, as well as certain disease states.[3]

In recent years, the uptake of saliva testing has expanded well beyond basic sex hormone testing into much broader fields that include occupational medicine, sports medicine, and sleep disorders.[4,5] In this paper we will cover some of the significant tests which are already available, as well as look at new trends in saliva testing.

HOW COME SALIVA TESTING IS SUCH A GREAT IDEA?

Saliva-based diagnostic assays are already available to detect and measure medically relevant compounds such as steroids, including the adrenal hormones cortisol and dehydroepiandrosterone (DHEA), the sex hormones estradiol, progesterone, and testosterone, as well as melatonin, alpha amylase, and IgA.

All these tests benefit from the key advantages of saliva testing: it is non-invasive, easy, painless, and can be done virtually any time or anywhere, including home collection by a lay person, which is very convenient when measures are required to be taken outside of normal working hours. Many tests also benefit from the fact that saliva measurements reflect biologically active steroid levels.[6] In effect, most steroid hormones in the bloodstream are bound to carrier proteins and are metabolically inactive. Only a small amount of any hormone in the blood is unbound and able to enter cells and affect their function.[7] Therefore, standard blood tests do not measure bioavailable hormone levels.

In contrast, only free, unbound hormones leave the bloodstream via the membranes and make their way into saliva. Thus, saliva testing provides a measure of biologically active hormones. Furthermore, established methods of saliva testing have proven to be highly accurate.[6] As saliva contains much lower concentrations of hormones than are in blood— a mere 1-5% of total hormone levels—saliva-based assays need to be very sensitive to measure these low levels. This is summarized in Fig 1.

Blood	Saliva
• Total steroid fraction	• **Free steroid fraction**
• Modified by binding proteins	• Independent from binding proteins
• 95-98% of steroids biologically inactive	• **Biologically active hormone fractions**
• Analytical problems at low concentrations	• Sensitive measurement by Luminescence immunoassay and ELISA.

Figure 1: Steroids in blood and saliva: the case for saliva testing

MARKET ACCEPTANCE: WHY ISN'T EVERYONE DOING SALIVA TESTING?

The overall market acceptance of saliva testing in the clinic has been hampered by the lack of accepted SOPs and knowledge in the clinical community. Hence, the established SOPs used in the clinical setting still tend to be blood-based.

This contrasts with the "Wellness" clinical setting, where saliva-based testing is well-established. In effect, the number of labs using saliva for hormonal testing is relatively small in the established medical arena, compared with that in "Wellness" space. We see a mismatch, where wellness practitioners are happy to perform saliva testing: it is easy for them to do it, but they cannot prescribe, and on the other hand, physicians, who can prescribe, will tend to use blood.

It is easy to see how we have arrived at this dichotomy. One reason is that as "Wellness" has become our 21st century goal, thousands of online tests have appeared, with DNA and hormone tests leading the charge.[8] However, some of these tests come with an extraordinary lack of scientific foundation, let alone standardization.[9]

Let us now consider several key application areas where saliva testing has become routine, such as for male and female sex hormones, and others where it is relatively new, such as in studies on stress, and sleep disorders.

FEMALE HORMONE TESTING: FROM MANAGING INFERTILITY TO MENOPAUSE

Female hormone levels have been measured for decades, using blood tests.[10] However, blood sampling is costly, invasive, and often logistically difficult, so there has been a shift towards the adoption of tests based on more convenient and cost-efficient sample types, such as saliva. Saliva-based tests are a reliable and proven method for measuring female hormone levels as well as being highly accurate and painless for the patient. Figure 2 shows a simplified version of how hormone levels change with age. Measurement of these, and other related hormones will give a medical professional an indication of any hormone imbalances, and lead to a recommended course of treatment.

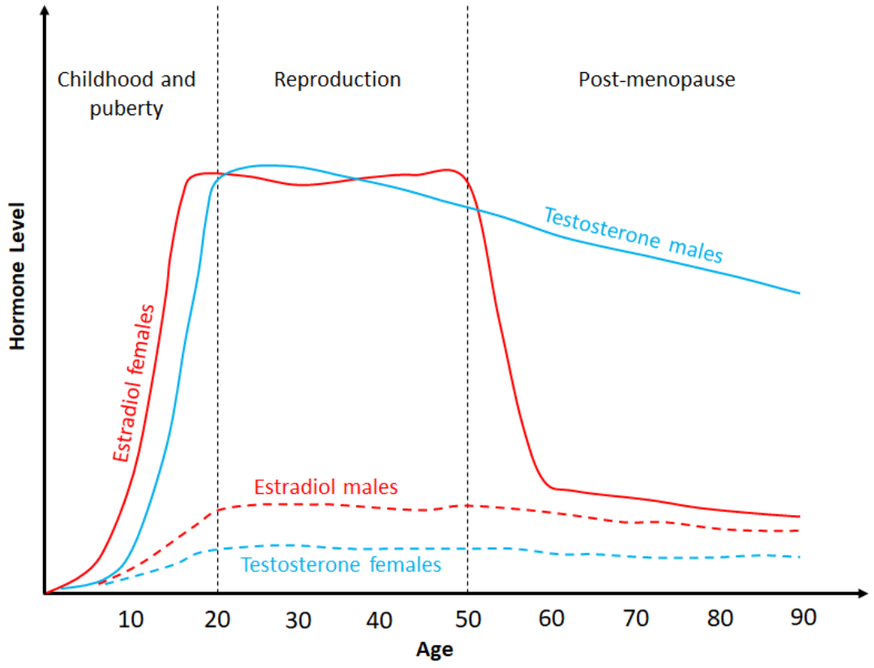

Figure 2: Variation in sex hormone levels with age

Once the symptoms of a potential hormone imbalance have been identified, such as infertility, excessive menstrual bleeding, or hot flashes in menopause, the medical practitioner will first want to measure the level of specific hormones.

Typically, blood samples will be taken, and serum-based tests will be performed. However, in the search for less invasive and more cost-effective ways of measuring female hormones, saliva-based hormone testing was born. A saliva test consists of three parts: sample collection, hormone measurement, and downstream analysis.

Sample collection has been a source of huge variation in the past, which has hindered its uptake. However, SOPs are now in place regarding sample collection methods and the devices used for collection, as well as recommended operating procedures for the saliva testing labs themselves.

The recommended platforms for measuring and analyzing female—and indeed other—hormone levels in saliva samples can be either enzyme immunoassay (EIA, e.g., ELISA) or mass spectrometry-based e.g., LC-MS/MS. Most start-up labs can be organized to do ELISA tests very effectively, either manually or with automated workstations, or very high-volume centralized service labs may offer more expensive LC-MS/MS.

Once the samples have been analyzed and hormone levels are compared with normal ranges, the results can be shared with the clinician, who will take further actions and prescribe treatment if necessary, such as hormone replacement therapy (HRT).

MALE HORMONE TESTING: FROM MANAGING INFERTILITY TO ANDROPAUSE

Testosterone is a steroid hormone produced in the testes of males and in smaller quantities in the ovaries of women. In both men and women, a substantial amount is produced by the conversion of circulating DHEA-S, DHEA and androstenedione. Testosterone shows a diurnal rhythm, with highest levels in the morning and a nadir around midnight.

Testosterone plays an important role in the development of male reproductive tissues, supports increased muscle mass, bone mass, and hair growth and is essential for health, stamina, sexual function, cardiovascular health, and a healthy immune system.

The measurement of testosterone is used in the diagnosis and the treatment of disorders such as primary and secondary hypogonadism, disorders originating from testicular problems or defects of the hypothalamus or pituitary glands.

Andropause, the so-called male menopause, signifies the retreat of the key male hormone testosterone. As a man ages, his body naturally produces less testosterone (see Fig. 1). In fact, by the time a man is in his mid-forties, testosterone levels can be down by 40%. Lifestyle factors such as excessive stress, weight gain and lack of exercise can lower levels even further—impacting stamina, drive and virility.

The hidden imbalances contributing to these factors generally include low testosterone levels, which can then lead to decreased stamina and libido, fatigue and erectile dysfunction. It is not only testosterone which is affected. There is also an interplay of different hormone pathways. Hence there may be a higher estrogen level, which in men can result in weight gain, increased chest and belly fat, hot flashes, night sweats &

excessive need to urinate. High cortisol levels can result in insomnia, anxiety, sugar cravings, and increased belly fat. Or conversely, low cortisol causes chronic fatigue, low energy, food and sugar cravings, poor exercise tolerance or recovery and lowered immunity to diseases.

Only 1-2% of the total testosterone is unbound from plasma proteins and therefore biologically active.[11] It is released via the salivary glands and mainly transformed to 5a-dihydrotestosterone. The concentration of testosterone in saliva, reflects however the level of free testosterone in plasma.[12]

Due to the pulsatile dynamics of testosterone secretion, repeated saliva sampling is recommended. It is advisable that 3 to 5 saliva samples be collected within 2 hours, and then, in the laboratory, equal volumes of the individual saliva samples can be pooled. This mixed sample will result in a mean free testosterone value, which represents the active hormone concentration in a reproducible way.

FROM COGNITIVE DIFFICULTIES TO FEELING STRESSED OUT: GO WITH THE FLOW!

The hormone levels in your saliva say a lot about you, your health, and how, for example, you manage stress. Cortisol, which can be measured in saliva, is often called the "stress hormone." When the body is under stress, whether due to physical, mental, or emotional reasons, cortisol levels rise.[13] Combined with the actions of other stress-related hormones, such as DHEA (the anti-stress hormone), they affect various bodily functions including regulation of the immune system, sleep, mood, fat and protein metabolism.

Chronic stress can lead to an imbalance in cortisol and DHEA levels and a risk of adrenal dysfunction. For healthy people, cortisol levels are at their highest in the morning and decline throughout the day, rising again after midnight. Stress testing, using salivary cortisol measurements, is an increasingly common method for evaluating worker burnout in occupational medicine.

Levels of cortisol, DHEA, testosterone, and α-amylase have been found to not only correlate with chronic stress, but also with post-traumatic stress disorder (PTSD), behavior, and cognitive function (Figure 3).[14-17]

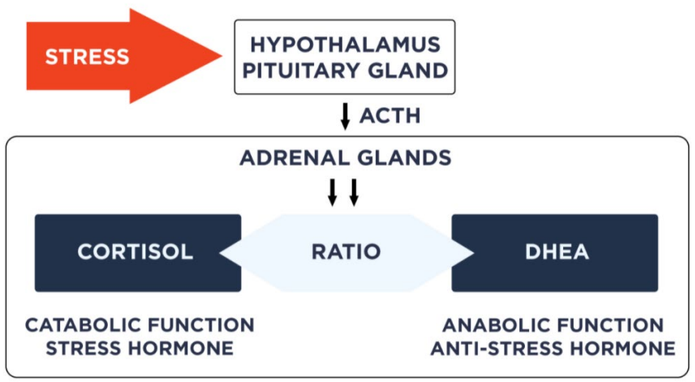

Figure 3: Relationship between stress, cortisol and DHEA.

Measuring the salivary concentrations of these hormones and recognizing their circadian patterns is therefore key.

One simple example is shown in Figure 4, where cortisol concentrations are found to be higher when an individual is subjected to a stressful situation and then remain elevated for several hours after the source of stress is removed.[13]

Individual reactions to stress can be vastly different, so a standardized test, the Trier Social Stress Test (TSST) is often used. This test can be applied to all participants.[18] There are also suggestions that other factors can influence the level of cortisol in response to stress in humans. Gender, as well as the menstrual cycle, seem to have an impact on the cortisol profile. The application of drugs such as oral contraceptives seem to diminish the cortisol response to stress.[19]

COULD SALIVA TESTS BE USED TO DETECT AND MONITOR DEPRESSION?

Research reports suggest that depression is the second most common disease in the world after heart failure and may even take the lead in 2030.[20-22] Whilst there are no specific laboratory tests to date that would be helpful in the diagnosis of anxiety and depression, in a recent review, Chojnowska et al. (2021) did find salivary biomarkers that could be helpful.[5] The authors chose saliva since it was seen as an ideal biological fluid for diagnostic purposes: it does not coagulate; it is stable for 24 h at room temperature and for a week at 4°C.

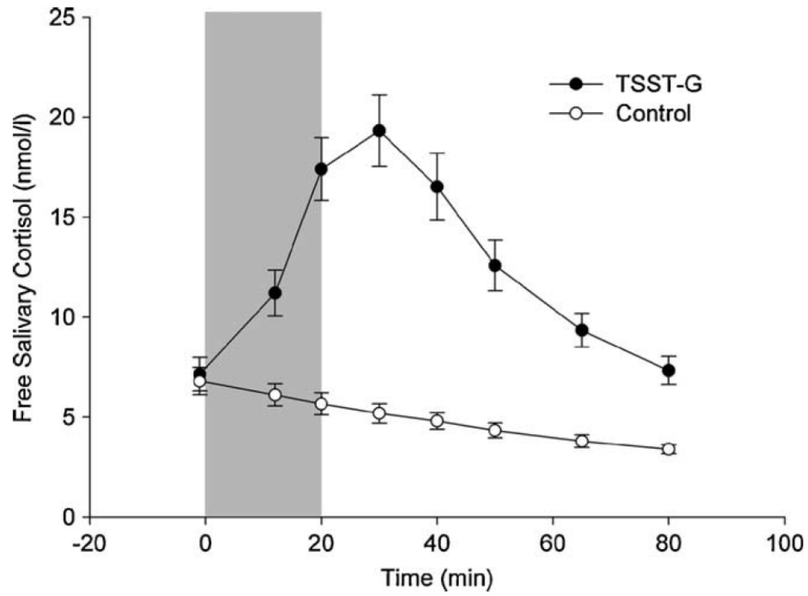

Figure 4: Cortisol levels stay high after source of stress has been removed (TSST-G), compared with non-stressed control.[13]

The conclusion from this initial study is that there are indeed potential salivary markers of stress, which include cortisol, lysozyme, sAA and CgA, although the authors advise additional analyses to allow the development of different assay profiles, separate for specific mental disorders, which would define concentration/activity ranges of individual markers in such a way that they could be used to distinguish between acute and chronic stress, anxiety and depression. As part of that development, the optimal times of day for sampling would need to be determined, as some of the markers show a circadian rhythm.[5]

SALIVA SAMPLING IN SPORTS MEDICINE

Saliva sampling is an easy, non-invasive choice for evaluating physical conditions during exercise. Salivary cortisol, DHEA and testosterone determinations are useful in sports medicine research. The use of these biological markers assumes that these salivary steroids may reasonably be taken to reflect the hypothalamic pituitary-adrenal (HPA) axis function.

Indeed, in the diagnostic setting, salivary cortisol levels correlate with those in plasma following ACTH and CRH stimulation and exercise-induced stress.[6] During physical exercise, free salivary cortisol, DHEA and testosterone concentrations increase with the intensity of exercise. It is particularly important in sports medicine to note that hormone levels and exercise-induced hormone changes vary among subjects with different types of training.[23]

Figure 4 (page 07) shows the hormone profiles from periodic saliva sampling an endurance-trained versus an untrained subject during physical activity. Endurance-trained subjects display a less pronounced hormone response to exercise.

Observing hormone levels using saliva testing during sport sessions is also a useful tool for optimizing athletes' training status and identifying any excessive exercise resulting in overtraining syndrome. In one

study, hormonal levels of athletes exhibiting overtraining syndrome were compared with a control group, before and during similar training regimes. The study revealed that no significant differences existed prior to beginning the intensive training; nor did the hormonal levels of the control subjects alter significantly ($P>0.05$) due to the training. The overtrained subjects, however, did have a significant ($P<0.01$) decline in testosterone (6.8±1.0 to 4.4±1.0 ng/ml; MEAN ± SE). Furthermore, prolactin was found to significantly ($P<0.05$) elevate in the overtrained subjects (8.1±2.0 to 13.2±1.1. ng/ml), while the testosterone/cortisol ratio showed a significant ($P<0.005$) decline (0.83±0.26 to 0.36±0.08).24 Besides looking at these hormone levels during exercise, measurement of the IgA levels in saliva samples will also provide useful information concerning the athlete's immune status.

MEASURING INSOMNIA: SALIVA TESTING TO SURVEY YOUR SLEEP

Occupational diseases include sleep disorders. They occur mostly in shift workers and leading to psychological and physical stress. In this kind of disorder, it is helpful to evaluate individual circadian rhythms, combining measurements of melatonin and cortisol concentration. Both hormones are produced according to a circadian rhythm, with contrary circadian patterns.

Melatonin is the key molecule promoting night-time sleep; it is extremely low during the day and will often rise rapidly around 11-12 p.m. (up to ten- or twenty-times daytime levels), although there are individual variations.25 Normal cortisol concentration in human saliva during the day is highly dynamic. Cortisol typically peaks in the early morning. The timing of this cortisol peak does not depend on clock time, nor is it influenced by daylight. It is set by the individual's time of waking. Both hormones are regarded as stress markers. Recent studies have shown that melatonin can influence cortisol levels. These results are useful in the diagnosis and treatment of sleep disorders. Fig. 5 shows a typical profile measuring melatonin and cortisol using Tecan ELISA kits.

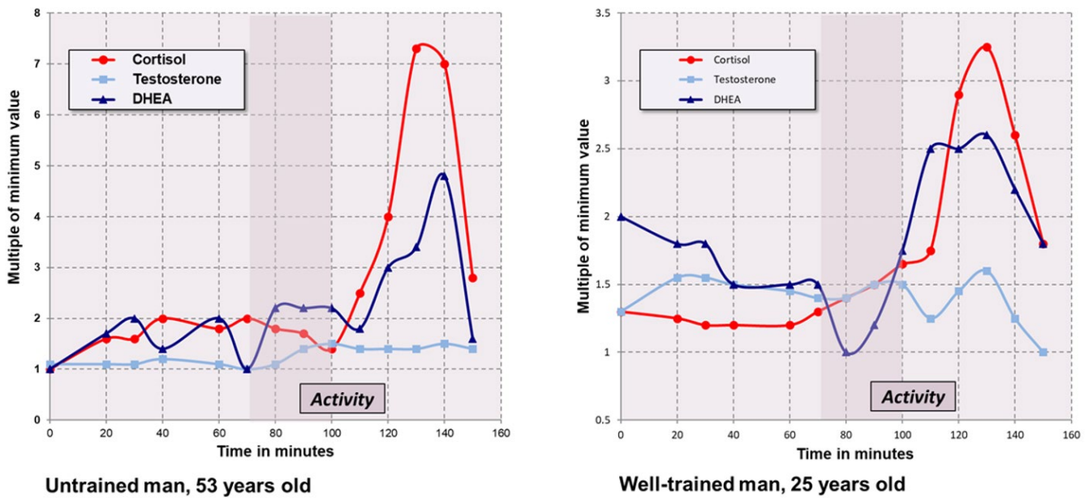

Figure 5:. Physical activity on a bicycle ergometer: untrained vs trained

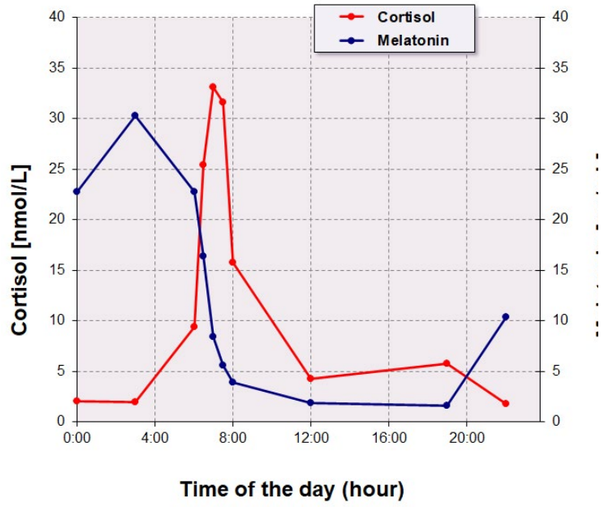

Figure 6: Variation in cortisol and melatonin during a 24-hour period, measured using Tecan Melatonin direct Saliva ELISA and Cortisol Saliva ELISA kits

FROM BLOOD TO SALIVA: FLICK THE SWITCH

Saliva-based testing is enjoying a growth in market acceptance in many application areas, such as the monitoring of sex hormones, of cortisol in stress and sleep disorders, and even for the detection of pathogens such as SARS-CoV-2 in the recent COVID-19 pandemic.26 Saliva diagnostics still have a long way to go before being considered the “go-to” technology for some classic serum-based tests, but their versatility, ease of use and an increasing body of scientific literature are all set to drive this acceptance.

Interested in setting up saliva-based diagnostics in your lab? Check out Tecan's **broad range of saliva-based tests**. See our range of assays specific to the **endocrine system**. If you need higher throughput assays, check out our automation **solutions**.

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