

## Saliva: a reliable sample matrix in bioanalytics

Saliva is gaining increasing attention as a bioanalytical sample matrix. Mostly because of the easy and noninvasive collection, it is not only beneficial in endocrinological and behavioral science, but also in pediatrics. Saliva also has the advantage of being the only body fluid which can be collected even during physical exercise, for example, during sportive activities, and there are physiological characteristics that make it superior to serum/plasma or urine for specific scientific questions. This review provides an insight into the physiology of saliva formation, explaining how certain compounds enter this bodily fluid, and gives advice for collection, storage and analytical methods. Finally, it presents a number of reliable and proven applications for saliva analysis from scientific fields including endocrinology, sports medicine, forensics and immunology.

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Traditional biological matrices for the analysis of diagnostic substances, such as hormones, drugs or immunoglobulins, are serum, plasma and urine. Although serum and plasma provide information about the currently circulating concentration of the analyte, urine provides an overview of the accumulated excretory metabolites since the last act of urination. Over years, the analysis of saliva has attracted increased attention, in particular, among clinicians and researchers who consider saliva as a noninvasive and stress-free alternative to blood sampling.

Numerous publications have demonstrated the analysis of saliva to be a useful alternative for determining many endocrine parameters since saliva lacks the one main problem of blood collection, which is the invasiveness of the venipuncture resulting an increased response of stress hormones, namely glucocorticoids [1,2] and catecholamines [3]. To date, a definitive interpretation of salivary concentrations for daily routine purpose has not been

achieved for the majority of parameters. Therefore, it is still state of the art to assess saliva concentrations in combination with results obtained from matched plasma samples.

This review provides possibilities of saliva analysis in various fields of research and to show potentials for diagnostics and clinical trials. First, information is given on physiology of the salivary glands and saliva secretion. Subsequently, the methodological aspects for sampling and measurement of saliva are discussed. Finally, the diversity of saliva analysis is demonstrated with examples from the fields of endocrinology, forensic chemistry, therapeutic drug monitoring, sports medicine, metabolic diseases, immunology, and oral and gastrointestinal diseases.

### Anatomy & physiology of the salivary glands

The major part (65%) of saliva is produced by the submandibular glands. The parotid glands contribute between 20 and 50% to

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the total saliva volume, depending on stimulation [4,5]. Only a minor fraction of 5% is secreted by the sublingual glands. The remaining 10% derive from numerous small glands embedded in the oral cavity [5,6]. For routine applications, open-field studies or outpatient sampling, mixed saliva is the only suitable sample material, while gland-specific collection at the exit of the excretory ducts with cannulas or pipettes is extremely intricate and is only performed for specific otorhinolaryngologic investigations. This mixed saliva contains, to a small portion, exudate from the gingiva and peeled oral epithelial cells.

Saliva is produced in secretory end pieces of the glands, called acini. These acini drain into the salivary ducts, with small 'striated' ducts opening into wider intercalated and excretory ducts (Figure 1). The daily total volume ranges between 500 and 1500 ml, depending on water balance and stimulation [7,8].

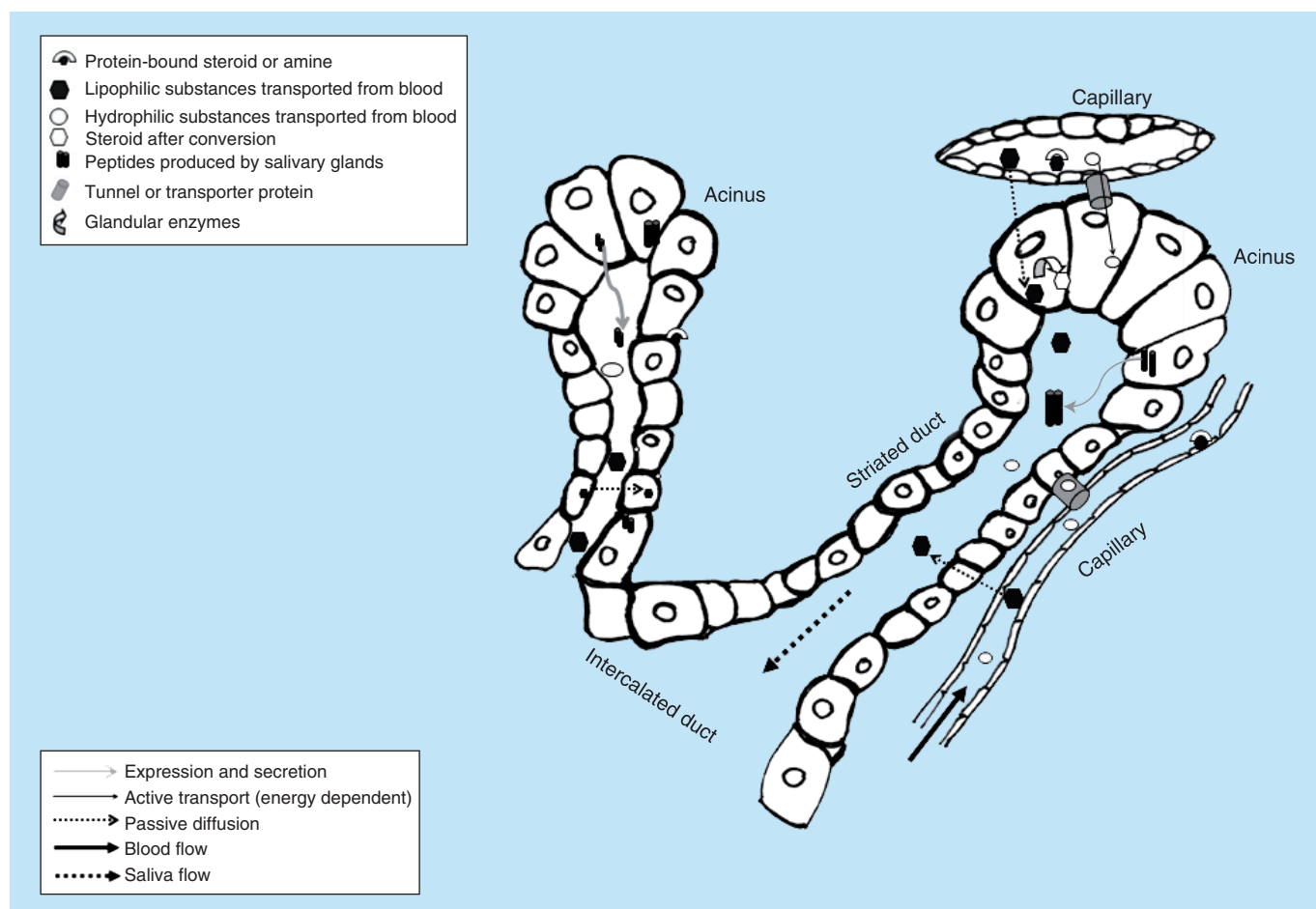
Various enzymes like amylase and lipase, preparing food for digestion, are supplied by saliva [9–11].

Furthermore, saliva contains factors of the immune response, such as secretory IgA [12], cathelicidin [13] or adrenomedullin [14], controlling the microbial growth on the epithelia.

The acini are surrounded by blood capillaries that enable the passage of substances from the circulation into the salivary glands (Figure 1) [15].

The concentration- or speed-limiting step of hormone transfer from blood into saliva is the passage through the capillary wall and the membrane of the glandular epithelial cells. Transfer of substances from the capillaries into the acini is driven by diverse mechanisms resulting in a complex fluid. These transport mechanisms are either passive diffusion, ultrafiltration through pores or active and energy dependent against concentration gradients [16].

In passive diffusion, lipid-soluble materials cross the cell membranes of capillaries and acini. The salivary concentrations of lipid-soluble, unconjugated steroids such as cortisol and testosterone approximate the unbound



**Figure 1. Transport mechanisms of substances into saliva.** Small lipophilic compounds, such as steroids, enter the glands by passive diffusion. Hydrophilic compounds (electrolytes and many proteins) are actively transported from the circulation to the glands. Enzymatic conversion has been reported (e.g., androstenedione to testosterone by 17-hydroxysteroid oxidoreductase). Peptides are either actively transported from blood to saliva (e.g., insulin) or are produced by the glands themselves (e.g., EGF).

plasma concentrations, while hydrophilic, conjugated steroids such as dehydroepiandrosterone sulfate only reach 1% of the unbound plasma concentrations [17].

Ultrafiltration drives the transport of small polar molecules into saliva through tight junctions at the apical pole of the acini. Mazariegos *et al.* [18] found these tight junctions in the resting rat parotid gland impermeable to tracers of molecular weights >2 kDa.

An active transport mechanism clearly applies for many electrolytes [19,20] and for some peptides such as insulin [21], but was also proven for some drugs [16]. Borzelleca and Cherrick [22] had already investigated the presence of antibiotics in saliva in 1965. The secretion of penicillin and tetracycline appeared to correlate with the concentration in blood. Since the secretion of penicillin by the salivary glands and by the kidney was prevented by probenecid, an inhibitor of renal activity, penicillin secretion in saliva is subjected to an active transport. Zuidema and Van Ginneken [23] confirmed this finding. Another inhibitor of renal activity, probenecid, showed no effect on the salivary secretion of diprophylline. These data suggested that the secretory mechanism in the kidney and in the salivary gland is not identical.

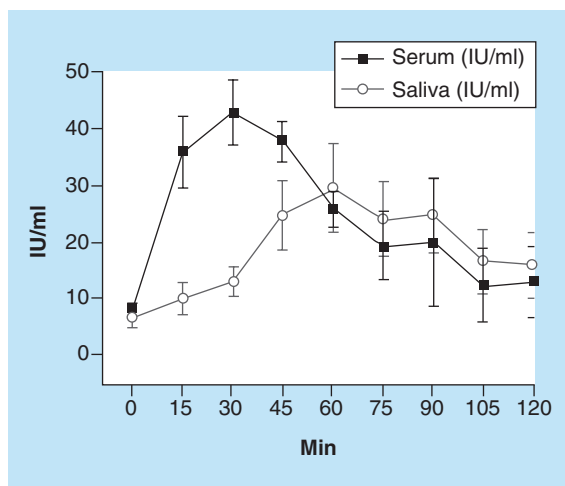
It is noteworthy that an active transport is not only energy-consuming, but also time-consuming, and can lead to a shift in the appearance of the compound, when matched plasma and saliva samples are plotted together, as indicated in Figure 2 for human insulin.

The movement of marker substances from blood to saliva and *vice versa* was investigated [24], describing permeability barriers in the glands that allow for some substances to pass readily into the saliva while other substances were held back. Some pharmaceuticals enhance saliva secretion by dilation of the tight junctions of the secretory end pieces [25], while some diseases such as Sjögren's syndrome cause a severe reduction of saliva production [26].

## Collection & analysis of saliva

### Collection precautions

Potential risk of infection for nurses collecting the sample or technicians performing the analysis of saliva is no greater than those associated with blood or urine samples. Trained personnel are required to draw blood samples, and particular care must be taken, for example, when donors are drug addicts with a significant risk of hepatitis B and HIV infections. Consequently, saliva is particularly attractive for a high-risk donor population, since the collection of blood appears more difficult due to thrombosed veins and since saliva effectively inhibits HIV infectivity [27]. The reason for this is not fully understood, but it is likely that secretory IgA is involved [28]. However, saliva does not display a



**Figure 2. Course of insulin in matched serum and saliva (n = 8 each) after oral glucose administration.** In saliva, the  $C_{Max}$  is delayed by approximately 30 min, reaching only lower concentrations compared with serum.

broad antiviral activity, since hepatitis B virus remains infectious in mixed saliva [29].

A prerequisite for conducting pharmacokinetic studies using saliva is to avoid residues of orally administered drugs in the oral cavity, as shown for amphetamines capsuled in gelatin [30]. Usually, the problem can be solved by rinsing the mouth with water before starting the collection, but the possibility of dilution effects needs to be considered [31]. The contribution of crevicular fluid from the gingiva to mixed saliva can be limited by avoiding tooth brushing prior to sampling and by rinsing the mouth with water prior to sampling [32,33]. To ensure robust and reproducible profiles, circadian rhythms as well in salivary gland activity [34] and production of compounds being transferred into saliva (e.g., steroid hormones [35,36] or melatonin [37]) need to be considered.

### Collection procedures

One main advantage of measuring substances in saliva relates to the noninvasive and easy sample collection. Several sampling methods have been described for mixed saliva, being outlined in the following. The oldest and easiest collection procedures use passive drooling and active spitting into containers. A lack of standardization in flow rate is compensated by the fact that no interference of the salivary substances with collection devices is expected. In order to achieve a constant glandular flow rate during (even multiple) collections, slight stimulation was introduced. Chewing paraffin wax or chewing gum [33] usually elicits flow rates between 1 and 3 ml/min. Citric acid or lemon drops effectively stimulate salivation, up to 10 ml/min [38]. In general, a standardized stimulation is advantageous for the interpretation of the

results compared with nonstandardized unstimulated saliva flow. Naturally, any stimulus on salivation must not absorb, modify or interfere with the substances of interest. In particular, Parafilm® absorbs lipophilic substances, causing false low results in analytics [39]. Moreover, acidic stimulation may cause problems when using immunoassays for analysis [40].

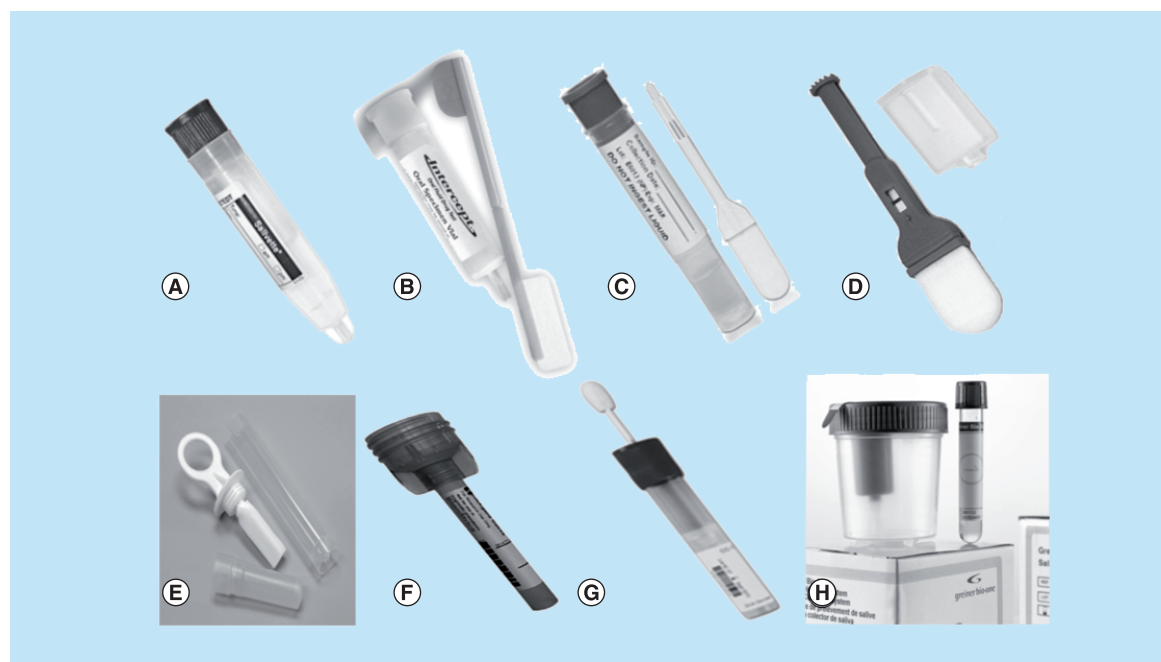
Since a substantial social barrier exists in donors to the described spitting/drooling, life science companies came up with a number of dedicated saliva collection devices, being exemplarily illustrated in Figure 3 & Table 1, which will be described in the following.

Cotton rolls, as used by dentists, were the first devices for collecting, leading to the well-known Salivette® (Figure 3A). The donor slightly chews on the roll for about half a minute to soak it with saliva. The roll gets applied to the perforated insert of a polypropylene container and centrifuged to obtain the matrix in the bottom of the container. The original cotton swab has meanwhile been replaced due to the significant interference or low recovery of several substances, for example, 17-hydroxyprogesterone [50], testosterone [51] and insulin [52]. Problems were also reported

for the Salivette pretreated with citric acid, when measuring dehydroepiandrosterone [53]. However, the new Sarstedt (Nümbrecht, Germany) product consists of a sponge of fine polystyrene fibers, which now convinces with a good recovery of a broad panel of salivary analytes, while absorbing up to 2 ml of saliva [52].

The Intercept® (Figure 3B) absorbs approximately 1.0 ml and may collect a mixture of gingival fluid and saliva rather than pure saliva, due to its positioning between cheek and gums. The retrieval of the matrix again appears via centrifugation. The intended use is for salivary drug testing, where it has proven good results [54].

Very similar is the Quantisal® (Figure 3C), albeit supplied with a defined volume of storage buffer and, very beneficial, a small window displaying a blue marker line, when sufficient saliva has been collected. Good recovery could be demonstrated for a panel of hormones (steroids and peptides) and therapeutic drugs [52,54]. The same can be said about the Versi SAL® (Figure 3D), which the biggest advantage, compared with the Quantisal, is the better durability of the adsorptive pad. The Sani-Sal® (Figure 3E) surprises with a pacifier-like appearance;



**Figure 3. Compilation of currently available commercial collection devices (A–H) for saliva.** For names and vendors, see the corresponding details in Table 1.

- (A) Reproduced with permission from [41] © Sarstedt.
  - (B) Reproduced with permission from [42] © Ora SureTechnologies, Inc.
  - (C) Reproduced with permission from [43] © Immunalysis.
  - (D) Reproduced with permission from [44] © Oasis Diagnostics.
  - (E) Reproduced with permission from [45] © Health-ChemDiagnostics, LLC.
  - (F) Reproduced with permission from [46] © Biomatrix.
  - (G) Reproduced with permission from [47] © Dnagenotek.
  - (H) Reproduced with permission from [48] © Greiner Bio-One.
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Table 1. Vendors for commercial saliva collection devices and their intended application (based on vendor information).

Nr	Brand	Application	Ref.
1	Salivette®	Hormones, drugs of abuse and therapeutics	[41]
2	Intercept®	Drugs of abuse	[42]
3	Quantisal®	Drugs of abuse and therapeutics	[43]
4	Versi SAL®	Proteomics	[44]
5	Sani-Sal®	Drugs of abuse and HIV test	[45]
6	DNAgard®	DNA (includes stabilizer)	[46]
7	ORACollect•DNA	DNA (includes stabilizer)	[47]
8	Saliva Collection System®	Liquid based system for drugs of abuse and therapeutics	[48]
9	SalivaBio Oral Swab	Identical to Salivette	[49]
10	SimpliOFy™	DNA (includes stabilizer)	[44]
11	Micro•SAL™	Specific for collection in laboratory animals	[44]
12	Pedia•SAL™	Specific for collection in infants	[44]

Numbers 1–8 correspond to Figure 3A–H, respectively.  
Nr: Number.

however, the vendor claims an intended use for drugs of abuse and HIV tests [55]. Both DNAgard® (Figure 3F) and ORACollect•DNA (Figure 3G) have been designed for DNA analysis from salivary epithelial cells. While the ORACollect•DNA uses a swab for a standard smear test, the DNAgard is designed to drain the saliva through a funnel into the collection vessel. Here the 'old fashioned' passive drooling is revitalized. In contrast to all above-mentioned devices, the Saliva Collection System® (Figure 3H) uses a liquid-based approach. It appears very elaborate and requires far more training than any other described device; however, the analytical data achieved with this system were reliable for most steroids and peptide hormones [52].

Ambitious companies meanwhile offer various devices for the specific use in pediatric or even non-human saliva donors, as compiled in Table 1. A very comprehensive review on saliva collection devices has recently been published by Slowey [56]. It will turn out whether these devices keep what they promise. In general, own experience shows that researchers need to test up front, which of the available devices shows the best performance, with regard to easy utility, highest substance recovery and lowest interference in the assay. This task definitely pays off with reliable and reproducible results.

### Storage

Once saliva has been collected, proper storage until analyses is mandatory. Long-term storage at room temperature has been examined by Chen *et al.* [57] for salivary cortisol. After 16 weeks, the concentra-

tion declined by >90% in samples without preservatives. However, salivary cortisol remains stable as long as 3 weeks at room temperature when stabilizers were added [32], allowing for out-patient sampling and subsequent shipping by mail. It is not as simple to guarantee sample stability for salivary peptides or amines, such as insulin [58] or melatonin [59]. Peptides are more likely to adsorb to the surface of collection tubes than small molecules, causing significant losses. Moreover, saliva contains proteolytic enzymes [60–63] responsible for a very rapid degradation of these peptides. To overcome these problems, it is recommended to use an appropriately tested collection device, to use low-protein binding cryotubes for storage and to add preservatives such as aprotinin to keep enzymatic degradation at a minimum.

Regularly, saliva is stored frozen to allow for long-term storage [64,65] and some data exist covering freeze–thaw cycles (for steroids [32]) with either centrifugation before freezing, or after thawing and prior to analysis, to break down the obstructive mucins and to separate out relicts of epithelial cells.

### Immunological methods

Ligand-binding assays (LBAs) are frequently used for monitoring hormones or immunoglobulins in saliva, because they are easy to use, require small sample volume and are sufficiently sensitive for the low salivary concentrations. However, LBAs do not always yield the required specificity for distinguishing the desired substance in the presence of cross-reactants or metabolites, as commonly seen in neonates, during pregnancy or in certain diseases.

The first RIAs for saliva applications were in-house developments for the analysis of steroid hormones, based either on in-house antibodies and tracers [66,67] or adaptations of commercial plasma RIAs for the saliva matrix [68–70]. RIAs meanwhile have been replaced by ELISA and other nonradioactive LBAs. Several kit manufacturers [49,71,72] offer US FDA approved ELISAs for salivary steroids, amylase, secretory IgA and some endogenous peptides. Salivary assays for peptides and proteins will be especially beneficial for clinicians investigating biomarkers in oral or head and neck cancers. However, LBAs for therapeutic drugs are rarely commercially available and, moreover, are very unlikely designed for saliva. Here, in-house developments are required to obtain sensitive and selective LBAs for saliva applications.

### Chromatographic methods

While LBAs are more frequently used for endogenous compounds, such as steroids, peptides and proteins, therapeutic drugs and drugs of abuse are clearly the domain of chromatographic methods.

Historically, the introduction of GC/MS allowed for first time to reach the required sensitivity and selectivity required for the detection of salivary parameters.

The GC/MS analysis of nitrazepam in saliva after conversion to *N*-butylnitrazepam can be done to a limit of detection of 0.5 ng/ml [73]. Also other drugs of abuse, namely clobazam [74] and cocaine [75], could be determined reliably in saliva using this technique.

In parallel, HPLC was applied to measure salivary drug levels. Thompson and Cone [76] utilized HPLC with electrochemical detection for the determination of D9-tetrahydrocannabinol. HPLC was also employed to separate caffeine and its metabolites, paraxanthine and theophylline, in saliva [77].

Today, investigations of the saliva proteome use either MALDI-TOF [78] or SELDI-TOF MS [79]. While these techniques have more focus on qualitative determinations, LC-MS/MS is superior for quantification of salivary compounds, such as steroids [80–83], peptides or amines with a molecular weight below 5 kDa, such as ghrelin [84] or melatonin [85], therapeutic drugs [86] and drug metabolites [87]. Apart from the fact that some problems described for LBAs do not appear when using LC-MS/MS, the possibility of running multianalyte measurements (different substances or substance and metabolites) is a huge advantage to support saliva analysis.

### Specific areas of application

#### Endocrinology

The assessment of hormones in saliva has the longest tradition of all saliva analytics and has previously been

extensively reviewed [17,88]. Back to the 1980s, behavioral research has focused on salivary steroid hormones, since the noninvasive character of sampling was considered not to impact the stress hormone response of the participants to the actual stressor. Moreover, steroids in blood plasma are predominantly bound to specific binding globulins, which make them unable to pass from the blood through cell membranes. With steroid receptors located in the cytosol or inside the nucleus, only the unbound plasma fraction of steroids can perform its physiological activity. Since the same unbound fraction passes from plasma into saliva by the described passive diffusion, salivary steroids became attractive to researchers interested in measuring the physiological active fraction.

Adrenal-derived cortisol is by far the most frequently reported hormone in saliva. Its main function is to increase gluconeogenesis during energy-demanding situations, colloquially called 'stress'. Any influence of sample collection must be avoided when studying hormonal responses to stress or depression. Consequently, the noninvasive, stress-free sampling of saliva has become prevalent especially in psychoendocrinology [89,90]. There is consensus in numerous studies on stress and endocrinology that salivary cortisol significantly increases during chronic stress, as observed in people facing life-threatening conditions [91,92], in disadvantaged or isolated subjects [93,94] or in patients suffering from depression [95].

Gonadal sex steroids, such as estrogens, androgens and gestagens, have also successfully been investigated in saliva. For example, during the ovarian cycle, a clear differentiation between the follicular and luteal phases could be demonstrated for both estradiol [96] and progesterone [97], introducing reliable biomarkers for ovarian functionality. Salivary testosterone, on the other hand, is a proven biomarker for male hypogonadism. It has been successfully used to establish cut-off values to differentiate between eugonadic and hypogonadic men [98]. Although these results are very convincing, it must be noted that a definite interpretation of salivary androgen levels remains difficult, as defined age- and gender-dependent reference intervals are rare [99]. Further difficulties appear due to steroid conversion by enzymes in the salivary gland (Figure 1). The salivary glands contain, among others, 11  $\beta$ -hydroxysteroid dehydrogenase, converting cortisol to cortisone as the hormone passes through the gland [100]. Swinkels *et al.* explained higher levels of salivary testosterone by significant glandular conversion of androstenedione into testosterone by 17-hydroxysteroid oxidoreductase [101,102].

Since the iodinated hormones produced in the thyroid have slight chemical similarities with steroids,

it is not surprising that thyroxine (T4) and triiodothyronine (T3) follow the same diffusion transport. Consequently, it has been postulated that the low salivary T4 and T3 concentrations also reflect the non-protein-bound fraction in plasma [103]. The presence of thyroid-derived hormones in saliva may be related to the fact that these hormones have a direct effect on the functioning of the salivary glands [104,105]. Finally, there was evidence that salivary thyroid-derived hormones can reliably be used to assess thyroid dysfunction [106,107].

In addition to steroids and thyroxines, an increasing number of publications describe the analysis of peptide hormones from saliva. The pancreatic insulin, which regulates the blood glucose level, is one key analyte for evaluating diabetes. In 1967, Sweeney and Antoniadis successfully detected insulin in human saliva [108]. There was some controversy, whether this insulin was solely pancreas or partially salivary gland derived, since the salivary glands have anatomic similarities to the pancreas [21,109], but there is firm conviction that the salivary insulin was transferred from the blood to saliva. A hint for an energy-dependending and time-consuming transport is shown in **Figure 2**. The kinetics of insulin after the oral glucose tolerance test is shown for matched samples in serum and saliva with saliva reaching the  $C_{Max}$  later and at a lower extent than serum [M GRÖSCHL, UNPUBLISHED DATA]. The reliability of salivary insulin measurements, mostly performed with adapted commercial serum immunoassays, was repeatedly confirmed by numerous groups [110–112]. Despite these convincing data, salivary insulin analysis has not been transferred into routine applications or into clinical trials – that is, to investigate pharmacokinetics of synthetic insulin substitutes, such as insulin aspart or insulin detemir.

Other peptide hormones, deriving from various glands and tissues, have also been reliably analyzed from saliva. Two important regulators of energy expenditure and body weight, the cytokine leptin, predominantly derived from the white adipose tissue, and its 'counterpart' ghrelin, mostly expressed in the gastric mucosa, are in the focus of research on metabolic syndrome and obesity. For both peptides, the salivary glands were identified as independent sources [113–115], and surprisingly both peptides showed distinct physiological activity with regard to oral epithelial proliferation [113,116] and wound healing [117], similar to the cytokines VEGF [118] and EGF [119]. Finally, there is evidence that the salivary gland expressed and secreted leptin is a promising biomarker for specific oral tumors, namely parotid tumors, pleomorphic adenomas and adenolymphomas, with significantly increased leptin levels in mixed saliva compared with

healthy controls [120,121]. Similar findings were made for IL-6 [122], which has a close biochemical familiarity to leptin. But other salivary cytokines are also proven biomarkers, such as EGF, in the diagnosis and therapy of inflammatory bowel diseases [123]. For further information on the physiological functioning of salivary hormones in the oral cavity and upper digestive tract, please refer to [124].

### Sports medicine & doping control

Monitoring hormonal changes during sporting activity provides valuable information on training success to the sport medicine specialists supporting athletes and trainers. Drawing blood or urine before and after training or competition is therefore common practice. To assess changes during training, however, these sample matrices are inadequate, as an interruption of the activity would be required. Saliva is the only sample matrix that enables direct access to the trainee in action, as proven in several publications where saliva samples were collected during diverse sporting activities such as marathon running [125], soccer [126,127], rugby [128], volleyball and tennis [129,130], paragliding [131] or mountaineering [132], and analyzed for androgens and glucocorticoids.

Regrettably, physiological alterations are not the only subject for hormone analysis in sports medicine. In doping control, the most commonly used biological matrix is urine, having the advantages of easy and noninvasive collection. Nevertheless, collection of a specimen for doping control demands a control person monitoring the athlete to prevent substitution or falsification of the sample with – for example, unnumbered urine from a third person [133]. In any case, the presence of a controller during urination might be considered as a violation of privacy of the athlete. Other disadvantages of urine are the dependence on the renal clearance of drugs, and the fact that not all drugs are excreted via the kidneys. For example, lipid-soluble  $\beta$ -blockers are rapidly eliminated by the liver metabolism [134]. Therefore, saliva may offer a fast, undisturbed access to the athlete. Around 1910, Alfons Bukowski established a method to measure alkaloids in equine saliva, which was immediately used to introduce a doping test for racing horses [135].

It is prohibited to deploy androgens, either to gain more muscle mass or to reduce pain during extreme physical exertion. Long-term administration of testosterone clearly affects the androgen profile, being observed in the quotient between urinary testosterone and epitestosterone. Under physiological conditions, this quotient is approximately '1' [136]. If this quotient shifts toward testosterone ( $>>1$ ), then this androgen was most likely administered to the athlete's body.

Since both androgens can reliably be measured from saliva, it might be advantageous to use this matrix during training or sportive competition instead of urine, collected behind a curtain.

Synthetic androgens, such as nandrolone or trenbolone, interfuse the epithelia-like endogenous androgens. Again saliva offers easy access for screening purposes, without compromising the athlete's privacy. However, there is currently no information available on the official pages of the World Anti-Doping Agency [137], whether official programs exist to use saliva in systematic doping control.

Exogenous peptides have been used to either enhance muscle growth (hGH), or to increase the number of erythrocytes (EPO). Few publications demonstrate the presence of these peptides in saliva [138,139] in traces (% of the plasma concentrations). Since these peptides are not expressed and produced by the salivary glands, a transport from the blood vessels through the epithelia into the salivary system must exist. If it is possible for endogenous peptides to pass through the epithelia, then recombinant hGH or EPO may also be transported equivalently.

Although sports medicine and doping control appear to be most promising fields for salivary hormone analysis [140], there is obviously a long way to go before this approach receives similar acceptance as in other medical disciplines.

### Forensic applications

The most common forensic application for saliva analytics is definitely the detection of DNA at a crime scene. This topic alone can fill textbooks. Since the analytics does not appear from the saliva matrix but from detached epithelial cells, it will not be in the scope of this paper. There is a definite need for quick, sensitive and robust qualitative than truly quantitative methods for finding drug-influenced drivers guilty by the police. Due to the described transport modes of different substances from blood into the glands, saliva should be very appropriate for detection of drugs of abuse. Published experiences of German police using the saliva rapid test gave convincing results for very sensitive detection of tetrahydrocannabinol, opiates and amphetamines with different commercial quick tests [141]. Morphine and codeine [142] along with methamphetamine, buprenorphine and methadone [143] are detectable in saliva at relatively high concentration using LC-MS/MS. Over all, for the determination of drugs of abuse, saliva is a proven and trusted sample matrix [129], again due to the rapid and easy sample collection, combined with concentration similar or equal to serum concentration, not requiring specifically sensitive analytical methods.

### Drug monitoring

There are two aspects of drug monitoring, for which saliva might be an interesting sample matrix. On the one hand, clinical studies prior to official authority approval may utilize saliva for pharmacokinetic and pharmacodynamic questions for a new drug, such as dose finding and creation of metabolites. On the other hand, therapy control may use saliva as a diagnostic tool to noninvasively assess the reaching of a therapeutic target value [144].

In short, clinical studies do not utilize saliva very commonly. This might be related to the fact that clinics performing studies by order of pharmaceutical clients rarely have experience with collection and proper handling of this sample matrix. Moreover, the pharmaceutical customer may simply not be aware of this alternative opportunity. However, at least in epidemiologic studies, recruiting study participants in rural areas of developing countries would benefit from the easy collection procedures for saliva compared with blood or urine.

It is crucial to assess the correct therapeutic concentrations when cytotoxic drugs, such as busulfan, are administered to leukemic patients undergoing stem cell transplantation. Blood samples are drawn in short time intervals during administration in order to monitor for the correct concentration to be achieved. This procedure is a strain on the already weakened patient. In 2006, we demonstrated that the kinetic profile for busulfan can be assessed same, as good in saliva as in plasma, yielding a strong linear correlation between the matched samples from both matrices [86]. In order to reduce the stress for the patient, replacing blood collection by saliva would be a benefit at least from an ethical point of view.

The protease inhibitor, indinavir, is supplied during highly active antiretroviral therapy in patients with HIV. Similar to busulfan, a strong linear correlation between serum and saliva was found [145,146], allowing us to monitor indinavir concentrations easily. In addition to the ethical aspect, it needs to be reminded that saliva was proven to inhibit HIV infectivity, reducing the risk for both nurse and laboratory staff compared with common serum analysis.

Apart from the described substances, there are plenty of other drugs, which can reliably be monitored from saliva with tacrolimus [147], carbamazepine [148] abacavir, tenofovir and darunavir [149] being only a few examples.

### Immunology

The most commonly measured immunoglobulin in human saliva is the dimeric secretory IgA. It displays antibacterial [150], antiviral [28] and antifun-



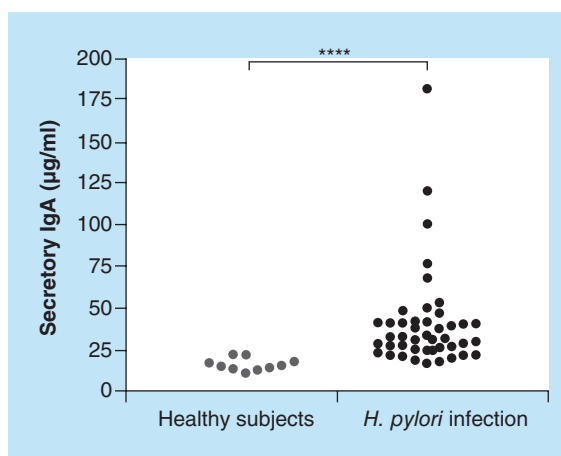
gal [151] activities in the gastrointestinal and respiratory tracts. Consequently, it can be used to investigate the responsiveness of the patient toward infections, for example, oral Streptococci [152,153] or *Neisseria meningitidis* [154]. Again, a big benefit of using saliva is also the ability to access study cohorts which might not be accessible for ordinary blood donation. For example, Brandt *et al.* [155] investigated the IgA response against specific Streptococci in communities of Australian aboriginals. Similar experience was made in a study conducted in Amerindians in the Venezuelan rainforest [156]. Unpublished data from this project show a significant increase in secretory IgA in *Helicobacter pylori* infected subjects compared with healthy controls (Figure 4). Goto *et al.* [157] published this correlation between *H. pylori* specific IgA and the protection against this microbe.

In addition to the immune response against 'air-born' intruders, intended vaccination against bacterial or viral germs can be monitored successfully in saliva. Serotype-specific antibodies against *Streptococcus pneumoniae* have been assessed in saliva in parallel to serum after vaccination [158]. Salivary and serum correlated positively, leading to the assumption that salivary IgG derived from serum, while secretory IgA was locally produced by leucocytes, present in the gingival crevicular fluid. The same group and others [159,160] showed the same reliability of *N. meningitidis* serotype-specific immunoglobulin analysis from saliva, making again this sample matrix a valuable alternative in large epidemiological studies.

## Conclusion

The aim of this article was to present an overview on the diversity of saliva analysis, presenting applications from endocrinology, sports and legal medicine, pharmaceutical research and drug monitoring.

For specific questions, especially in psychiatry, stress research or in pharmacokinetics, saliva can provide equal or better results than blood. There are a couple of strong arguments for using saliva. First of all, the noninvasive and easy sampling was performed by nonmedical study personnel, which allows for accessing donors afraid of venipuncture, especially children, or study participants in remote areas. The same is true for access to samples under conditions where blood collection is impossible – that is, during sporting activity. Second, the suitability for rapid short-interval sampling includes direct access to substances not bound to blood proteins. Multiple samplings per day to observe circadian rhythms or for kinetic profiles are feasible and, in trained participants, can be easily performed at home.



**Figure 4. Secretory IgA in Venezuelan Amerindians, comparing subjects with and without *Helicobacter pylori* infection.**

\*\*\*\* $p < 0.0001$ .

*H. pylori*: *Helicobacter pylori* infection.

## Future perspective

Modern collection devices will facilitate access to saliva for nurses and physicians, increasing the acceptance for this sample matrix from a technical point of view. Nevertheless, a strict definition of the collection procedure, including training of the study participants, is mandatory. Fortunately, not all substances measurable in saliva require extremely sensitive analytical methods. Most pharmaceuticals can easily be detected in saliva at concentrations equal to serum.

For endogenous compounds, such as hormones, the establishment of reference values in relation to age, gender and daytime, and the implementation of round-robin trials, is required.

In summary, with adequate standardization of both collection and analysis, a very reliable and robust approach is available to achieve full acceptance of saliva as a reliable and valuable sample matrix in bioanalytical research.

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