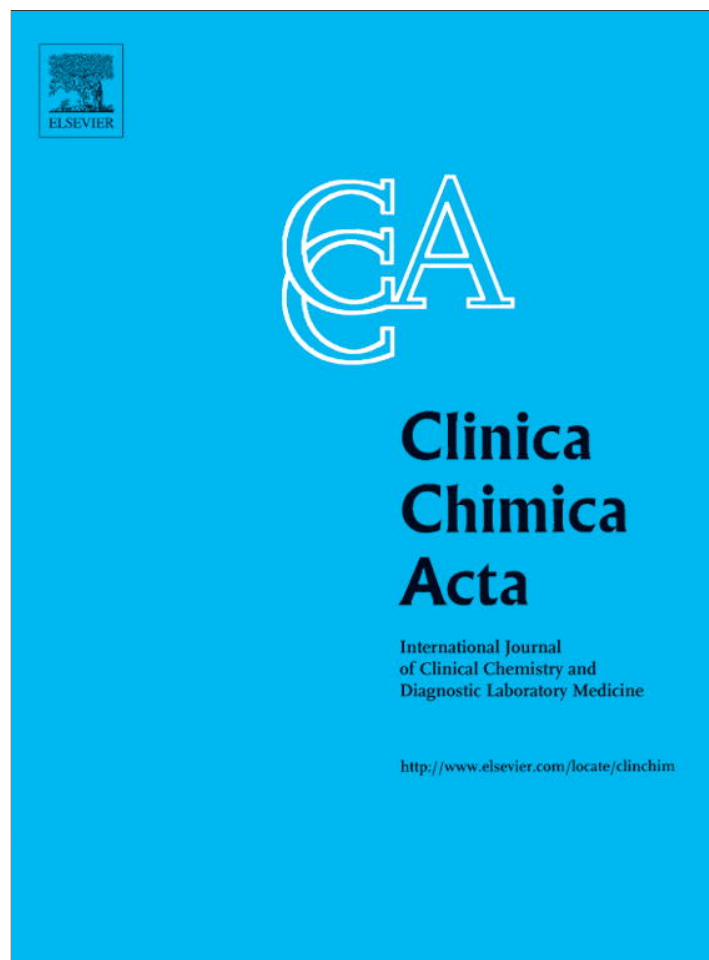


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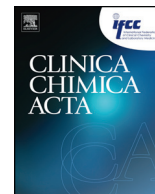
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Salivary markers of kidney function – Potentials and limitations

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ABSTRACT

Saliva can be collected non-invasively, repeatedly and without trained personnel. It is a promising diagnostic body fluid with clinical use in endocrinology and dentistry. For decades, it is known that saliva contains also urea, creatinine and other markers of renal function. Clinical studies have shown that the salivary concentrations of these markers could be useful for the assessment of kidney function without the need of blood collection. This article summarizes the clinical and experimental data on the use of saliva as a diagnostic fluid in nephrology and points out the advantages, pitfalls, technical requirements and future perspective for the use of saliva as a novel potential diagnostic biofluid.

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1. Introduction

Saliva is a body fluid with a broad diagnostic potential. It is used as a source of DNA, either for genotyping of human DNA or for the analysis of oral microbiome [1]. Salivary RNA is studied as a potential marker of oral

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malignancies [2]. Nucleic acids present in saliva are of local origin, although the cells from which they are derived might have originated from elsewhere such as blood or bone marrow [3]. Low molecular weight compounds present in saliva often originate from the systemic circulation [4]. The salivary concentration of such solutes partially correlates with their plasma concentrations. The most commonly clinically used salivary biomarkers are salivary steroids, such as cortisol, testosterone and estradiol. Numerous other molecules present in saliva are under investigation such as melatonin, oxytocin, interferon and interleukins [5].

Urea was found in saliva already in 1951 and a number of studies analyzed its changes as a potential marker of kidney diseases [6–9]. This review summarizes the available literature on the use of saliva for the measurement of renal function, outlines the obstacles that hindered the clinical use of saliva in nephrology and points out the main advantages of saliva that could spark future research and potential clinical applications.

2. Saliva secretion and composition

Daily salivary secretion is approximately 1000 ml, ranging from 800 ml to 1500 ml. Saliva is an aqueous solution with a pH of 6.0–7.0, which is the most suitable range for the digestive action of enzymes such as ptyalin [10]. Initial saliva has the same ion composition as plasma, i.e. it is isotonic. The final saliva is hypotonic with high potassium and bicarbonate and low sodium and chloride concentrations. Within the salivary glands, the acinar cells secrete initial saliva with proteins/peptides such as amylase, lipase, mucin glycoproteins, immunoglobulin A and kallikrein and the ductal cells modify the initial saliva to hypotonic final saliva via ion transporters, ion channels and a relative water impermeability [11].

The final saliva has a complex composition. In addition to solutes mentioned above, it includes magnesium, calcium, zinc, phosphates, urea, and ammonium [12]. Antibacterial substances like lysozymes, agglutinins, secretory immunoglobulin A, lactoferrin, peroxidase or cystatins and statherins are secreted into saliva. A fungal growth is inhibited by histidine-rich proteins, or histatins [13]. Saliva also contains mucin that coats food and thereby protects mucous epithelium against mechanical, thermal or chemical irritation [11] and proline-rich proteins [14]. To date, more than 2400 proteins were identified in saliva, and each of them might be potentially interesting as a biomarker [15].

Besides substances that are produced and/or secreted by the salivary glands, saliva contains also compounds originating from other body compartments. Desquamated or death epithelial cells of the oral cavity and buccal-pharyngeal mucosa are found in the saliva, including released organelles or microvesicles such as exosomes [16]. Blood or serum components can get into saliva by either passive diffusion, active transport or by ultrafiltration of extracellular fluids induced by hydrostatic pressure through tight junctions between acinar cells. The gingival crevicular fluid, i.e. the exudate from the gingival margin termed crevice, along with the bronchial or nasal fluids are further components of whole saliva [17]. Depending on the status of the gingiva and microbial colonization, the crevicular fluid can be either a serum transudate with low protein content or exudate with higher protein content due to local inflammation, both containing serum constituents, e.g. microRNAs [18], cytokines or steroid hormones such as cortisol or sex hormones [19].

3. Factors influencing saliva secretion

Saliva secretion, especially its flow, is mostly increased because of chewing or taste [20]. Apart from these, another major factor influencing the speed of saliva secretion is autonomic nervous system, where sympathetic stimulation generally down-regulates and parasympathetic stimulation on the contrary up-regulates the salivary flow [11]. Indeed, the autonomic nervous system with brainstem solitary tract nuclei is linked with other – higher brain centers as is amygdala, or

orbitofrontal cortex. Such salivary flow is a constant process and when not further increased by chewing, it is referred to resting salivary flow. On contrary, the chewing stimulation of salivary secretion, it is important for food to stimulate the receptors in periodontal ligament. Additionally, the taste salivary secretion is dependent on diet composition [21]. In studies, acids, i.e. citric acid are the most potent salivary flow stimulants. However, since such stimulants do not commonly occur in the diet in its acidic form, the chewing and taste can be equally efficient. Smell is another factor that through orbitofrontal cortex can trigger salivation, although to smaller extent than aforementioned factors [11]. Although the Pavlovian type response can influence the salivary flow, this is true in animals, e.g. dogs. In humans, it seems that mouthwatering during the sight or thought of food is induced by smell sense [22]. Additionally, the mouthwatering when smell stimulus is missing, is not considered as increased salivary flow, rather it is due to the contraction of skeletal facial muscles that squeeze the salivary ducts resulting in increased saliva in mouth. From other physiologic properties of human body, the circadian cycle also contributes to the speed of resting salivary flow [23]. While salivary flow is highest in the afternoon hours, it diminishes to very low speed during sleep thus copying the activation of autonomous nervous system during daytime. Gender also influences the salivary flow, with females having smaller salivary flow when compared to males, however, this is simply explained by smaller salivary glands in females [21]. The effect of age on salivary flow currently remains obscure. Several studies found that elderly people have decreased salivary flow when compared to young controls. Nevertheless, this contributed to higher and continuous medication such as hypertensive, antipsychotic, and anxiolytic use in the elderly rather than the direct effect of age [24]. Indeed, overall status and systemic diseases may as well contribute to the variability in salivary flow. Of these, dehydration is one of the most important factors, where decrease in body fluids by approximately 8% leads to dramatic reduction in salivary flow [21].

The relatively high number of components renders saliva a promising diagnostic biological fluid. The various factors influencing salivary composition might be, however a source of biased results. As exemplified for urea, its salivary concentration can be decreased by bacteria of oral cavity possessing urease activity [25]. Some substances, considered as uremic toxins such as malondialdehyde or advanced oxidation protein products [26], can also be directly or indirectly influenced by the oral microbiome. Although the long-term dietary habits seem not to influence the oral microbiome [27], short-term changes in diet, in particular of those containing microbiota, may influence the oral microbiome resulting in change of malondialdehyde and advanced oxidation protein products [28]. Thus, the major salivary source of markers of renal function is derived from circulation, however, other factors have to be taken into account when interpreting these parameters. Under normal circumstances, metabolic degradation products such as urea, creatinine, and nitrites excreted by the kidneys into the urine. In diseased kidneys, these compounds accumulate in the systemic circulation and get into saliva, either directly or are excreted by salivary glands. Exogenous sources or sources of potential bias are food contamination or salivary microbiome composition.

For further details, there are several comprehensive high quality review articles regarding saliva physiology, composition and functions [11,17,21,29–31].

4. Kidney disease classification

Currently, the use of serum creatinine as a marker of kidney function is inadequate. Since the serum creatinine is influenced by creatinine secretion or extrarenal secretion, the real glomerular filtration rate must decline by half to detect elevated creatinine serum concentrations [32]. Therefore, the glomerular filtration rate (GFR) is the measure of choice to determine overall kidney status. In clinical practice, Kidney Disease Outcomes Quality Initiative (K/DOQI) guidelines recommended the use of serum creatinine based estimates of GFR, which is referred to

as eGFR [33]. The most commonly used formula is the one by Cockcroft and Gault [34]. It classifies the CKD into 5 stages according to glomerular filtration rate, where GFR more than 90 ml/min/1.73 m² is considered as stage 1 and kidney disease with normal or increased GFR. Stage 2 belongs to GFR 60–89 ml/min/1.73 m² and represents mild kidney damage, stage 3 with GFR 30–59 ml/min/1.73 m² and moderate kidney damage. Stage 4 is classified as severe kidney damage with GFR 15–29 ml/min/1.79 m² and kidney failure (stage 5) belongs to GFR less than 15 ml/min/1.73 m² or on dialysis [35].

5. Biomarkers of renal functions in saliva

Clinical studies analyzing the potential usefulness of salivary markers of renal functions are summarized in Table 1.

5.1. Salivary urea

Already in 1951, it has been shown that urea can be detected in saliva, although the sensitivity was rather low and large volumes of saliva had to be collected [6]. Salivary urea is significantly affected by age and body mass index but mostly reflects serum urea [36,37]. The correlation between serum and salivary concentrations of urea is significant in both, patients and control subjects, with a correlation coefficients of $r = 0.74$ in healthy volunteers and a nearly complete correlation ($r = 0.99$) in patients with renal diseases [38]. Urea can be metabolized in saliva by bacterial ureases [39], but the magnitude of this effect and the diagnostic implications are not yet clear. In subjects or patients with normal serum urea, potential other sources might significantly influence salivary concentrations, whereas in patients with high serum urea this effect is less important.

The largest clinical study analyzed urea in as few as 50 μ l of saliva in more than 150 saliva samples from adult controls and patients with CKD using a standard colorimetric method [40]. The analytical parameters were impressive with coefficients of variation well below 5% for both inter-assay variability and intra-assay variability with a detection limit of 0.6 mmol/l. The cut-off value for salivary urea in this study was 7.5 mmol/l and all patients were above with a mean concentration 3-fold higher compared to controls. In another study in adults, salivary urea correlated well also with creatinine clearance ($r = -0.7$) with a cut-off value discriminating patients with CKD from healthy controls of approximately 14 mmol/l [41]. Compared to salivary creatinine, salivary urea was more sensitive marker of CKD, particularly in earlier stages [42]. Similarly to adults, compared to healthy children, children with CKD had 5-fold higher salivary urea concentrations [43]. Hemodialysis decreased salivary urea by 40% in patients, although even after hemodialysis the concentrations remained twice as high as in controls [44]. Compared to patients on hemodialysis, renal transplantation significantly reduced salivary urea. However, compared to healthy controls, the salivary urea remained significantly increased even 1 year after transplantation [9].

Animal experiments in dogs showed that the different salivary glands vary in the rate of urea excretion [45]. Submandibular and sublingual saliva contained significantly less urea compared to saliva from the parotid gland. Exogenous manipulation of plasma urea resulted in rapid changes in the salivary urea concentrations and stimulation of salivation increasing the salivary flow rate resulted in changes of salivary urea concentrations. In human volunteers, it was found that if the salivary concentration of urea is high enough, the excretion can be reversed and urea can even be reabsorbed by the oral mucosa [46]. Taken together, salivary urea is the best known and currently the most promising salivary parameter of renal function with potential use in both screening and monitoring of renal function.

5.2. Salivary creatinine

Using the automated Jaffé method salivary creatinine was measured in healthy subjects and 25 patients with renal disease with increased

serum creatinine [47]. In this study, the salivary creatinine concentrations represented 10–15% of the serum concentrations and the correlations between serum and salivary concentrations were only significant in patients ($r = 0.78$) but not in healthy controls. Healthy subjects had salivary creatinine between 6 and 18 μ mol/l, whereas patients had much higher concentrations between 18 and 591 μ mol/l. Other studies found similar findings, correlation between salivary creatinine and serum creatinine was found only in patients ($r = 0.73$), the salivary creatinine concentrations were considerably lower than in serum and patients had on average 5-fold higher concentrations compared to controls [39,48]. The ratios of serum vs. salivary creatinine concentrations were reported between 4 and 30 indicating a high inter-individual variability [49]. Since creatinine is a large molecule with low lipid solubility, it is not possible for creatinine to easily pass the membrane or tight intercellular junctions of the cells and get into saliva. However, in CKD patients, several mechanisms can lead to increased diffusion [48]. Firstly, the increased concentration gradient facilitates diffusion to saliva and secondly, permeability of the salivary gland cells is altered [50]. Also, in the healthy population, low plasma concentrations of creatinine might be impossible to correctly measure in saliva. Therefore, from a clinical perspective, salivary creatinine could be used particularly in screening programs as a qualitative outcome indicating healthy vs. diseased patients, rather than a true quantitative marker.

5.3. Small molecules, uremic toxins and drugs

Several non-protein organic molecules could be found in saliva. Uric acid is a highly abundant low molecular weight molecule with radical-scavenging activity [51]. Uric acid can be reliably detected in saliva and is an important contributor to its antioxidant status, since it accounts for 80% of overall salivary antioxidant capacity [52]. Salivary uric acid was much lower compared to serum and showed significant correlations between serum and saliva only in azotemic patients [39]. In patients with CKD, salivary total antioxidant status and uric acid were reduced by hemodialysis or peritoneal dialysis [53,54]. The authors suggested salivary uric acid as a potential measure of antioxidant status in patients with CKD. Further clinical studies are required to evaluate to potential diagnostic value of salivary uric acid.

No correlation was found between salivary and serum concentrations of sodium, potassium and chloride, likely due to active transport of these ions during saliva production. Compared to controls, patients with CKD have significantly higher concentrations of these ions, whereas salivary calcium was lower [42]. Salivary phosphate correlated well with serum creatinine and was 3-times higher in CKD patients compared to control subjects [55,56]. Patients with CKD had higher amylase activity in saliva in comparison to healthy controls [42], however, no changes during hemodialysis were found [57] and it is unlikely that this parameter might be sensitive and specific marker of renal function.

Interestingly, only very few studies analyzed the presence of uremic toxins in saliva. Purine nucleotide N-methyl-2-pyridone-5-carboxamide was found to be increased in uremic patients vs controls in both serum and saliva [58]. The analysis of salivary uremic toxins might be a promising field of research.

Saliva could be potentially used to track the concentration of various drugs, e.g. antibiotics or immunosuppressive drugs [59]. To date only one study in pediatric renal transplant patients found, that the measurement of salivary tacrolimus did not relate to blood concentrations and was not recommended to be used in clinic [60]. The usefulness of saliva for drug monitoring is dependent on the particular drug and its particular physical-chemical properties and metabolism and deserves further studies.

5.4. Peptides, proteins and hormones

Amyloidosis is a major problem in patients with CKD, in particular those treated with hemodialysis. One of the main proteins causing

Table 1
Clinical studies of salivary markers in renal diseases.

Salivary marker	Groups	Patient number	Result	Interpretation	Year/reference
Urea (mmol/l)	Controls	78	5.36	Salivary urea reliable factor for testing azotemic states in CKD patients	2009/[40]
	GFR < 50 ml/min	154	17.86		
Urea (mmol/l)	Controls (GFR > 90 ml/min)	186	4.42	Salivary urea is stable marker with application in CKD patients	2013/[36]
	GFR < 90 ml/min	96	16.33		
Urea	Controls	56	r = 0.735	Significant correlation of urea and salivary urea in CKD patients	1987/[38]
	Diabetes/hypertension/CKD	50	r = 0.99		
	Hemodialysis	50	r = 0.99		
Urea (mmol/l)	Controls	N/A	39.27	(Children with CKD)	1987/[43]
	Before hemodialysis	10	183.14		
	After hemodialysis	10	86.04		
Urea (mmol/l)	GFR < 10 ml/min/1.73 m ²	11	Cut-off for salivary urea 14.28 mmol/l and GFR < 80 ml/min/1.73 m ²	Salivary urea is useful for discrimination of CKD patients from healthy population	2012/[41]
	GFR 11–20 ml/min/1.73 m ²	10			
	GFR 21–50 ml/min/1.73 m ²	12			
	GFR 51–80 ml/min/1.73 m ²	26			
	GFR 81–170 ml/min/1.73 m ²	42			
Urea (mmol/l)	Controls	64	0.008	Higher salivary concentrations of urea in CKD patients than controls, conditioned by severity of CKD	2008/[42]
	GFR 15–60 ml/min	22	0.017		
	GFR < 15 ml/min	28	0.026		
Urea (mmol/l)	Before hemodialysis	94	25	Significant effects of dialysis on salivary urea concentrations	2007/[44]
	After hemodialysis	94	15.3		
Urea (mmol/l)	Controls	10	12.21	Salivary urea is useful non-invasive marker	2014/[9]
	Hemodialysis	20	272.13		
	Kidney transplantation	15	25.54		
Creatinine (μmol/l)	Controls	23	8.5	Salivary creatinine as useful marker to distinguish CKD patients, cut-off value 16.8 μmol/l	1996/[47]
	CKD	25	84		
Creatinine (μmol/l)	Creatinine vs salivary creatinine in CKD		r = 0.784	Higher concentrations of salivary creatinine in CKD patients, correlation between salivary and plasma creatinine	2014/[48]
	Controls	37	10.61		
	GFR < 30 ml/min/1.73 m ²	105	58.34		
Creatinine (μmol/l)	Creatinine vs salivary creatinine		r = 0.731	Higher concentrations of salivary creatinine in CKD patients compared to healthy people	2008/[42]
	Controls	64	31.19		
	GFR 15–60 ml/min	22	30.07		
Sodium (mmol/l)	GFR < 15 ml/min	28	99.01	Higher concentrations of sodium in saliva of CKD patient than in healthy population	2008/[42]
	Controls	64	8.03		
	GFR 15–60 ml/min	22	11.82		
Potassium (mmol/l)	GFR < 15 ml/min	28	15.48	Higher concentrations of potassium in saliva of CKD patient than in healthy population, influenced by the severity of CKD	2008/[42]
	Controls	64	22.48		
	GFR 15–60 ml/min	22	27.03		
Chloride (mmol/l)	GFR < 15 ml/min	28	34.14	Higher concentrations of chloride in saliva of CKD patient than in healthy people	2008/[42]
	Controls	64	1.78		
	GFR 15–60 ml/min	22	0.9		
Calcium (mmol/l)	GFR < 15 ml/min	28	0.66	Lower concentrations of calcium in saliva of CKD patient compared to healthy controls	2008/[42]
	Controls	30	Cut-off for control subjects 4.76		
	Various stages of CKD	110			
Phosphate (mmol/l)	Chronic hemodialysis	68		Salivary phosphate is useful for monitoring hyperphosphatemia in CKD patients	2008/[55]
	N/A	N/A	Increased		
Phosphorus	Controls	64	129,031	Increased salivary phosphorus in CKD patients	2009/[56]
	GFR 15–60 ml/min	22	145,681		
	GFR < 15 ml/min	28	222,750		
Amylase activity (units/l)	Controls	N/A	Increased	Composition of saliva is influenced by the CKD	2008/[42]
	CKD	N/A	N/A		
N-methyl-2-pyridone-5-carboxamide	Controls	64	129,031	Higher salivary N-methyl-2-pyridone-5-carboxamide in CKD patients compared with controls, positive correlation with serum creatinine	2010/[58]
	GFR 15–60 ml/min	22	145,681		
	GFR < 15 ml/min	28	222,750		
Beta(2)-microglobulin (mg/l)	Controls	7	0.38	Salivary beta(2)-microglobulin test is useful for CKD-related amyloidosis prediction	2007/[62]
	Diabetes mellitus	7	0.68		
	CKD	20	0.83		
	Hemodialysis	19	1.62		
Testosterone (nmol/l)	Controls eugonadic	112	0.413	Morning salivary testosterone as reliable marker of androgen deficiency in CKD patients	2011/[80]
	Controls hypogonadic	40	0.134		
	Terminal CKD with androgen deficiency	60	0.338		
Hepatocyte growth factor (ng/ml/protein)	Controls	20	0.68	Salivary HGF as index of periodontitis without CKD and in patients on peritoneal dialysis	2012/[81]
	Chronic hemodialysis	26	1.06		
	Continuous peritoneal dialysis	15	0.85		
	Predialysis CKD	28	0.83		
	Advanced periodontitis	26	1.87		
Epidermal growth factor (ng/mg/protein)	Controls	30	5	No significant difference in EGF concentration, however a salivary bioactivity of EGF significantly depressed in patients on hemodialysis	2001/[82]
	Chronic hemodialysis	47	4.2		
	Controls	EGF activity	1.55		
	Chronic hemodialysis	EGF activity	0.59		

Legend: CKD – chronic kidney disease, GFR – glomerular filtration rate, N/A – not available, r – correlation coefficient, EGF – epidermal growth factor.

amyloidosis in these patients is beta(2)-microglobulin [61]. In patients on hemodialysis the salivary concentrations of beta(2)-microglobulin were more than 3-fold higher compared to healthy controls [62]. Although the correlation between serum and salivary concentrations was not significant [63], it was suggested that salivary beta(2)-microglobulin might help identify patients with high risk amyloidosis. Salivary beta(2)-microglobulin was only weakly related to serum urea and creatinine and cannot be used as an indicator of CKD [64].

Cystatins, members of cysteine protease inhibitor family, are also present in saliva [65]. Cystatin C is proposed as a better non-invasive marker for estimation of GFR, since its concentrations in body fluids are more stable [66]. Production of cystatin C for example is not affected by diet, infections or body fat content and therefore more tightly correlates with GFR than serum creatinine [67,68]. Similarly, neutrophil gelatinase and associated lipocalin (NGAL) is a small protein that can be found also in renal tubules and it was found to increase its plasma and urinary concentration during acute kidney injury as well as in chronic kidney disease [69]. NGAL is detectable in saliva. However, both markers related to the kidney functions have been studied in saliva in association with the periodontal status and not with renal functions [70,71]. Analysis of these markers in saliva with regard to kidney diseases should be conducted soon.

In saliva, kallikrein secretion is mostly maintained by the cells with intense electrolyte transport in major salivary glands [72]. In humans, elevated kallikrein secretion in saliva was associated with essential hypertension and hypertension with decreased kidney function. Experimental renovascular or genetic hypertension in rats also led to increased kallikrein secretion into saliva [73,74]. Human studies in patients with Sjorgen syndrome [75] or rheumatoid arthritis [76] observed higher concentrations of salivary kallikrein compared to controls.

Cortisol, the major stress steroid hormone, can be reliably detected in saliva. In patients with CKD, salivary cortisol can be used as a measure of adrenal insufficiency occurring in some patients [77]. Patients with CKD often suffer from hypogonadism that can be diagnosed by measuring testosterone. The measurement of free bio-available testosterone in serum or plasma is technically challenging and mostly calculated from total testosterone, albumin and sex hormone binding globulin. An alternative is to measure salivary testosterone, as only the free fraction of testosterone can pass from blood to saliva. If blood contamination is prevented/excluded, the salivary testosterone concentration correlates with the free plasma fraction [78,79]. This has been confirmed in a population of patients with CKD showing that saliva testosterone can be reliably used to discriminate patients with androgen deficiency [80]. Assessment of salivary steroid hormones, already used in endocrinology, is one of the most promising fields with direct clinical implications.

Growth factors present in saliva were associated with oral or gastrointestinal complications of renal diseases. Hepatocyte growth factor (HGF) is higher in patients with CKD, especially those on dialysis [81]. Salivary HGF concentrations are related to clinical indices of periodontal health such as gingival index and papillary bleeding index. Although the mechanism and the causality are not yet clear, it might be that salivary HGF is involved in the pathogenesis of periodontitis in patients with CKD (see below). Patients on hemodialysis have lower bioactivity of epidermal growth factor in saliva, suggested to be associated with higher incidence of peptic ulcers in these patients [82]. Whether this association is causative is also unknown.

6. Periodontitis and xerostomia

CKD was associated with a higher risk of periodontitis and tooth plaque formation [83,84]. Despite lower salivary flow rate, patients on hemodialysis had similar clinical indices of dental health and caries risk compared to controls [85]. Similarly, compared to hemodialysed patients, even two years after renal transplantation no differences were found in the oral health between the two groups [86]. Salivary pH measurement was able to discriminate patients with CKD and

controls comparably to salivary urea [42]. The observed higher salivary pH and higher buffering capacity of saliva in patients with CKD might explain the lack of differences in dental health. It also might point to a different mechanism of periodontitis compared to population with normal kidney function. Certainly, one can argue why the salivary pH is increased when patients with CKD suffer metabolic acidosis. This can be explained by at least 2 mechanisms. Firstly, the increased urea in saliva is subjected to bacterial ureases [39] where carbon dioxide and ammonium ion are produced, both having an alkalinizing effect [87]. Secondly, hydrogen ion production falls up to tenfold in patients with CKD due to the increased salivary urea, which in turn alters the metabolism of carbohydrates to acid catabolites [88].

Patients on hemodialysis are a potential target group for salivary analyses. However, these patients suffer from reduced salivary secretion [83]. The reduced function of salivary glands was confirmed by quantitative scintigraphy [89,90]. Both, the spontaneous salivary production and stimulated salivary production are diminished in hemodialysis patients compared to controls [91]. Interestingly, smokers among the patients had a significantly higher stimulated salivary flow rate than non-smokers. Not surprisingly, the only therapeutic option that helped to decrease thirst and xerostomia in these patients was renal transplantation [86].

The mechanisms leading to xerostomia in patients on hemodialysis are unknown, although medications and fibrotic changes in the salivary glands may lead to reduced salivary flow. A cross-sectional study identified several factors that were independently associated with reduced salivary flow rate. These include age, the use of sevelamer, serum urea and a number of parameters related to bone metabolism [92]. In contrast, a small case-control study showed that when patients are correctly matched with controls, there was no significant difference in salivary flow rate between patients with CKD and controls [93]. This negative finding was later confirmed in a larger study [42]. It is not yet clear whether there is a difference in salivary flow between patients on hemodialysis and control subjects. The negative findings might be a consequence of high variability of the methods that are used for its assessment or of the prevalence of xerostomia in this patient population. The subjective feeling of dry mouth is associated with low salivary bicarbonate and higher salivary calcium concentrations. In addition, higher sodium concentrations in saliva were associated with nausea [94]. This small study indicated that the composition of saliva might be important and should be further studied in CKD patients.

Xerostomia is an important, yet, not well understood problem in hemodialysis patients with local (oral) and systemic consequences and without an effective long-term treatment apart from renal transplantation [95]. Hemodialysis itself can at least partially improve some biochemical alternations of saliva [96], and it rapidly increases the salivary flow rate by 30% but only for a very short time [44]. Oral diseases, such as periodontitis, might affect salivary concentrations and should be interpreted with caution.

7. Limitations of saliva as diagnostic fluid

It is not possible to use saliva for the analysis of large molecular weight or charged molecules which do not get from plasma to saliva. The salivary concentration of many of the smaller molecules is much lower than in plasma, albeit this might be overcome using more sensitive analytical techniques or a higher sample volume. Bias might come from eating, drinking, gum-chewing or even from tooth brushing (Fig. 1) [97,98]. Problem of bias also comes from blood contamination of saliva, either after tooth-brushing or other oral hygiene technique, or because of oral cavity inflammation such as periodontitis or gingivitis with subsequent bleeding. However, as a rule of thumb, it is recommended to exclude samples with visible blood contamination. The spectrum of external and internal factors influencing selected marker concentration in saliva is more comprehensive. Remaining substances from food might not only interfere with the measured markers in saliva,

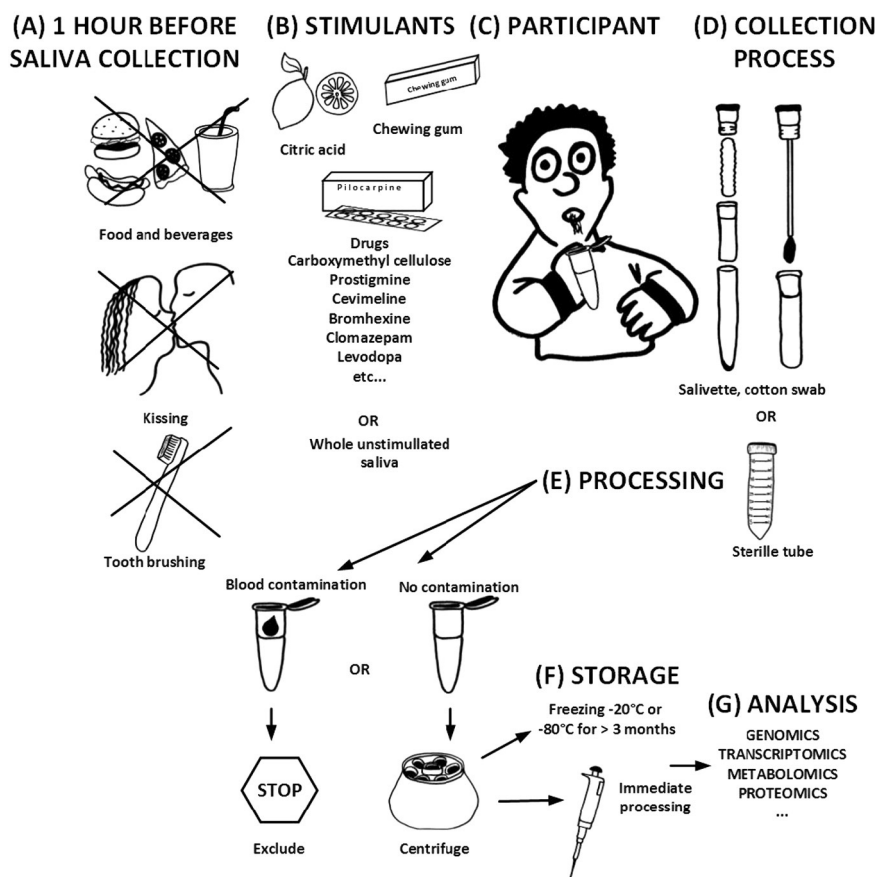


Fig. 1. Saliva collection and processing. One hour before saliva collection, factors that could influence the saliva composition or flow, i.e. food, tooth brushing, kissing, should be avoided (A). Whole saliva, or stimulated saliva can be collected into the sterile tube (B). Saliva collection systems are available, however, some of them can artificially influence the salivary composition (C). If blood contamination occurs, the samples should be excluded from further analysis (E). Not contaminated samples are to be further processed by centrifugation and then either frozen (-20°C if storing <3 months or -80°C >3 months) or immediately processed (F) for genome, transcriptome, proteome or metabolome analysis (G).

but it also might be a source of technical problem for further processing. Due to irritating effect of tobacco, smoking should also be considered when assessing measured markers from saliva. Tobacco and nicotine can influence the saliva composition either directly by interacting with the measured substances, or indirectly through increased salivary flow [99]. As was found recently, kissing that leads to exchange of biological fluid can be a source of bias and variability [100]. Furthermore, newly developed salivary collection systems are being introduced. However, as found out, the cotton swabs either with or without stimulants that are used in such systems can artificially bind some of the salivary components with subsequent artificially decreased concentrations [1,78,101]. Type of collection device should be wisely chosen based on measured marker. Nevertheless, before any wide clinical use of salivary markers, for each marker individually the influence of these particular factors has to be analyzed.

Microbial urease could affect the measurement of urea by local intraoral degradation of urea [39]. This suggests that individual microbiome patterns, but also oral hygiene, could affect the measured concentration of urea, and potentially other metabolites, in saliva. However, clinical studies suggest that this effect might be rather low, at least for urea. Salivary urea concentrations are also affected by the time from collection to measurement. The observed effect, even after a very short time, could limit the possibilities of saliva as a diagnostic fluid for kidney diseases [102]. The reason might be the loss of carbon dioxide dissolved in the sample leading to an increase in pH and decrease in buffering capacity, but an experimental proof for that is lacking. Solutions stabilizing saliva for DNA or RNA isolation already exist [103]. The use of test strips and analyses of fresh saliva can solve this issue.

Some patients have problems with collecting saliva and spitting, some require a long time until enough sample volume is reached and some inadvertently swallow during saliva collection, which might bias the measured concentrations [46]. However, most of the patients preferred saliva collection compared to blood taking [104]. Xerostomia might be another limitation, in particular if repeated sampling in hemodialysis patients would be required.

The continuous production of saliva enables real time monitoring. On the other hand, this might be also a limitation in cases where rapid changes in concentrations might bias a measurement where an "average" concentration is needed for the diagnosis. This can be overcome by multiple sample collections, as shown for salivary steroid hormones, where 5 samples at different time points have to be collected and the concentration is measured only in the pool from aliquots [105]. Other molecules, e.g. urea, do not require such repeated samples.

This straight forwardness is affected by the fact that different salivary glands variously contribute to the final composition of oral (saliva) fluid [106]. In the case of unstimulated saliva, up to 70% of salivary flow is influenced by the submandibular glands, 20% by parotid gland, up to 8% by sublingual glands and less than 10% by the minor salivary glands. On the other hand, when using some stimulants, these contributions of salivary glands to salivary flow are altered and more than 50% of salivary secretion is due to the parotids [21,107].

8. Advantages of saliva as diagnostic fluid

In comparison to blood, saliva has several practical advantages. The collection of samples does not require any specific materials, needles

or anticoagulants. A clean collection tube that can be sealed is sufficient. The patients or volunteers can collect the sample at home and no trained personnel or medical supervision is required [108]. Although blood taking is not dangerous, it is still associated with a small risk of complications and some patients do not tolerate the blood-taking procedure well. Saliva collection is truly non-invasive and is especially suitable for children and elderly patients [109]. It is useful when repeated collections are needed, e.g. during dynamic studies. When collected, the saliva samples are easy to handle, store and transport, depending on the analytical molecule. Some molecules are very robust and stable against external conditions, e.g. steroid hormones or DNA, some more sensitive, e.g. RNA or markers of oxidative stress. Compared to blood taking the collection of saliva is cheap [110]. Urine is an essential fluid for diagnostics of renal diseases. While both, saliva and urine are easy to obtain, in some instances urine collection is less convenient. For analyses of some parameters saliva might be better suited, e.g. urine contains degradation products of molecules of interest, such as nucleic acids for genetic and gene expression analyses [111].

The pros and cons of the use of saliva as a fluid for biomarker analyses are summarized in Table 2.

9. Collection of saliva and technical details

Numerous studies in the past have shown that the pre-analytical phase is crucial for the outcome of biochemical analyses, especially in saliva [1,78,98]. Although the conditions differ for various biomarkers, in general, the patient has to abstain from eating, drinking and kissing as a source of potential contamination at least 30 min (optimum 1 h) before sampling. Previous studies suggested that the cotton or polypropylene swabs affect the measured concentrations of analytical targets, e.g. sex hormones or salivary markers of oxidative stress [78,101] albeit this has not been clearly shown for markers of kidney function. However, the best method for saliva sampling is the collection of whole unstimulated saliva by spitting into a sterile tube. In experimental conditions, salivary flow rate manipulation in animals changed salivary urea concentrations [45]. In clinical studies, however it was shown convincingly that stimulation of salivary flow does not affect the salivary urea concentration [40]. This suggests that most of the salivary urea is not of local oral origin, but comes from circulation. This is of clinical importance, since some patients with low salivary production require induction of salivation using e.g. gum, citric acid or pilocarpine [112,113].

After centrifugation of saliva, the supernatant can be frozen and stored. Post-collection processing by centrifugation is yet another important factor. While it is difficult to obtain precisely predefined amount of mucous saliva for further analysis, the centrifugation step helps to clear saliva from "large" debris. Additionally, multiple freeze-thawing of examined salivary samples could affect the results of measured biomarkers, however it may depend upon specific substance analyzed. For example, the cortisol or alpha-amylase concentrations in saliva are not influenced by repeated freeze-thaw cycles [114,115]. In general, it is suggested to use fresh saliva as soon as possible since even a short time delay can result in changes of some analytes, e.g. urea due to bacterial degradation, RNA due to RNase degradation or changes in enzyme activities [102]. If analysis of fresh samples is not possible, immediate freezing at $-80\text{ }^{\circ}\text{C}$ is the best option, possibly avoiding further freeze-thaw cycles, while freezing to $-20\text{ }^{\circ}\text{C}$ or $-80\text{ }^{\circ}\text{C}$ seems quite efficient in conserving the saliva [116], from practical point of view, this is possible only at laboratory practice and research, not in standard clinical settings or outpatient units. In clinical practice, therefore, several preservatives can be used. For example, adding the sodium azide prevents further bacterial growth [31]. Since saliva possess proteinase activity, the addition of protein inhibitors such as aprotinin, leupeptin, antipain, and EDTA is required [17]. Sodium benzoate with citric acids or ethyl and propyl paraben are another saliva preservative options [117]. Still, these preservatives are not kidney marker specific, rather they are universal and tried with other than kidney marker analytes in

Table 2

Advantages and disadvantages of saliva as a diagnostic fluid.

	Advantages	Disadvantages
General	Non-invasive and convenient sampling Repeated sampling possible	Some patients require a longer time for sample collection Contamination from food, beverages, kissing etc. Low concentration of some analytes
	Easy collection without the need for trained personnel Number of established salivary markers	Only small and uncharged molecules get from plasma to saliva Microbial urease can bias measurements
Specific for kidney diseases	Urea, creatinine and uric are present and measurable in saliva Cheap and easy to use test strips for immediate analysis are available Stimulation of salivary flow does not affect salivary urea Changes in serum during hemodialysis rapidly mirrored in saliva	Short delays in analysis might affect the results Xerostomia in some patients on hemodialysis Have to store at $-80\text{ }^{\circ}\text{C}$ as soon as possible after collection

saliva. Hence, once again, before wide clinical use, stability and influence of preservation molecules should be examined individually. On the other hand, to overcome the problem of preservation, the saliva tests more focused on point-of-care testing should be developed.

The whole process is shown in Fig. 1. A long transport at room temperature may result in the growth of microflora that can affect the concentrations of some markers, such as salivary urea. Already within 30 min changes in pH of the saliva samples occur and affect the measurement of markers such as urea [102]. The main goal of using saliva as diagnostic fluid is the development of simple screening tests using strips that can be evaluated either by the patient or by the physician without the need for a specific equipment or biochemical laboratory.

10. Test strips

The use of salivary markers of kidney function depends on its easy practical application such as test strips using dry chemistry with a fast development of easily visible color reaction change. If combined with a spectrophotometric detection of the color reaction the analytical parameters of the urea measurement in saliva were very promising. The coefficients of variations were below 10% for both, intra- and inter-assay variability and the results were comparable to the use of serum [7]. The same study has shown that the measurement is affected by the ambient temperature, which can be solved by measurements performed at room temperature, i.e. at $25\text{ }^{\circ}\text{C}$.

For salivary urea a semiquantitative test strip has already been developed with 6 colors as a potential outcome. In a clinical study the so-called salivary urea nitrogen (SUN) correlated well with blood urea nitrogen ($r = 0.63$) and was useful for identifying patients at various stages of chronic renal kidney disease. The result was visible within 1 min by comparing the test pad with 6 standardized blocks, similarly to the widely used urinary strips. Inter-observer variation was low and the prize for the strip was below 1 USD [8].

A similar test strip was prepared for the monitoring of salivary uric acid and nitrites [118]. In a case-control study using these strips, the salivary uric acid was not different in CKD patients in comparison to control subjects. However, there was a significant decrease in both, salivary uric acid and nitrites after hemodialysis, suggesting a potential use for the monitoring of hemodialysis effectivity in CKD patients.

11. Conclusion and future outlook

Renal diseases represent an enormous medical, economic and social problem world-wide [119]. Screening and early diagnosis are very important global challenges. When advantages and limitations of saliva are taken into account, research points that salivary biomarkers of

renal functions still can be distinguished in whole unstimulated saliva thus to have a potential of being a potential fluid for monitoring of patients diagnosed with CKD or on hemodialysis or for diagnosis of specific consequences, e.g. hypogonadism under home conditions. Certainly, it is not clear whether home-based monitoring is an effective tool in monitoring of renal diseases, which definitely needs further studies. However, from studies regarding diabetes mellitus, it is known that diabetes prevention interventions using mHealth approach may have beneficial impact on overweight in such patients [120] and such mHealth approach is more effective than the traditional approach [121]. Furthermore, saliva-based detection systems are already being developed. Recently, a compact smartphone accessory for salivary cortisol was created [122] and we think that it is only a matter of time, until similar devices will be developed for renal parameters.

Therefore, in our opinion, further research should focus on the technical side of salivary measurement and development of easy to use mobile applications and accessories. Additionally, larger clinical studies focusing on the potential applications, the influence of limiting factors and testing the utility of the available platforms such as test strips are required. Also, the decreasing costs and better availability of high throughput sequencing, mass spectrometry and metabolome might result in identification of novel potential salivary biomarkers of kidney diseases. In the near future, saliva might become another diagnostic biofluid in nephrological practice.

Declarations

The authors confirm that there are no conflicts of interest. This paper has not been published previously in whole or in part.

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