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Saliva Can Be an Indicator for Aging. A Review

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ABSTRACT

Saliva plays an important role in the oral processing of food and consequently the sensory and textural experience. The ability to speak, swallw, masticate, taste food, and maintain a healthy oral cavity is related to the presence of saliva. Reduction in saliva results in many symptoms whose combined effect can drastically reduce quality of life. Several researches confirmed the presence of histological changes in the salivary glands induced by aging. Consequently, it is often assumed that the secretion and properties of saliva change with age, which can result in dry mouth conditions and taste aberrations. Such changes may result in reduced nutrient intake and malnutrition besides adversely affecting the quality of life. Some researchers have reported age-dependent changes on quantity (bulk salivary flow rate) as well as quality of saliva (e.g., composition, viscosity, lubrication) in healthy elderly individuals. However, recent research work has paid attention to the age-related salivary metabolites. This is due to lack of adequate understanding and characterization of endogenous factors, that is, the age-related changes in saliva, which may influence oral processing of food and subsequently nutrient intake. Few studies have comprehensively identified age-dependent changes in salivary metabolites. Hence, this review aims to survey the current state of knowledge concerning age- dependent changes in salivary glands structure, quantity and quality of saliva as well as salivary metabolites. The latter through analysis of saliva may act as an indicator for aging in population. Such insights will not only have clinical implications for maintaining optimal oral health in elderly population, but also serve to optimize food and to satisfy the needs of growing aging population.

Keywords: Aging, Saliva, dry mouth; Rheology; Tribology; Salivary metabolites.

1. Introduction

The population has undergone a fundamental change in its age structure globally, with a rapid increase in elderly population where the proportion of people aged over 60 years being estimated to double by 2050 (Lancet, 2014). Ageing, not only affects the physical and physiological condition, but also significantly impacts nutritional status. The latter could occur due to defective teeth or teeth loss, poor oral hygiene and lack of saliva resulting in impaired oral processing, taste and texture aberrations with subsequent reduction in nutrient intake and malnutrition (Coleman, 2002; Laguna *et al.,* 2015; Laguna *et al.,* 2016a; Laguna *et al.,* 2016b ; Mingioni *et al.,* 2016 and Laguna *et al.,* 2017c). Dysphagia and xerostomia are the most prevalent oral processing conditions encountered by aging. Xerostomia is a subjective sensation related to mucosal dehydration and reduced oral lubrication, which is not necessarily linked to salivary gland hypofunction (Pajukoski *et al.,* 2001; Nagler & Hershkovich, 2005; Liu *et al.,* 2012 and Villa *et al.,* 2015). Ageing could affect the ability to taste and smell due to diminished cognition, salivary hypofunction and diminished chewing ability due to the loss of dentures.

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All of these may lead to changes in the regulation of appetite provoking a lack of hunger, also known as "anorexia of ageing" (Malafarina *et al.,* 2013). It is often believed that xerostomia in elderly is ultimately associated with age-dependent changes in quantity and quality of salivary secretion (Vissink *et al.,* 1996).

Saliva is a complex biological fluid that is essential for eating to form a coherent, smooth and swallowable bolus (Prinz and Lucas, 1997). Saliva also plays an important role in sensory perception by diluting food components responsible for taste and aroma, allowing them to interact with the taste buds (Doyennette *et al.,* 2011and Neyraud, 2014). In addition to the eating-related functions, saliva secretion ensures continuous hydration of the mouth and demonstrates an antibacterial function (Dowd, 1999).

From a compositional viewpoint, saliva is a slightly acidic fluid mixture mainly composed of water (99.5%), proteins (0.3%), including mucins and enzymes and inorganic substances (0.2%) (Humphrey and Williamson, 2001). Saliva also adheres to oral surfaces and helps to maintain saliva pellicle thickness of 30-100 nm (Lendenmann *et al.,* 2000; Morzel *et al.,* 2014 and Hannig *et al.,* 2017) although the thickness may vary depending upon the pellicle's location in the mouth. Proteins, such as anionic glycosylated mucins, statherins render saliva with its rheological (viscosity, elasticity, stickiness), unique water-holding and lubrication properties (Douglas *et al.,* 1991; Sarkar & Singh, 2012; Sarkar *et al.,* 2016 and Laguna & Sarkar, 2017).

Recently, Metabolomics has been introduced as a branch of chemical biology that profiles metabolites in cells and organisms (Dettmer *et al.,* 2007 and Patti *et al.,* 2012), using techniques such as liquid chromatography-mass spectrometry (LC–MS). It usually deals with molecules smaller than 1.5 kDa, and is an important tool for studying metabolic regulation. It has been reported that salivary metabolites appear to be linked to aging. Diverse age-linked changes occur in various tissues as blood (Chaleckis *et al.,* 2016; Darst *et al.,* 2019 and Srivastava, 2019) and urine (Teruya *et al.,* 2020), so salivary metabolites may also reflect changes due to aging, and are very likely distinct from age-linked metabolites of blood and urine (Teruya *et al.,* 2021). Human aging appears to be an outcome with many parameters. Increased, decreased, or missing metabolites likely affect the onset and progression of aging. Comprehensive analyses regarding aging of salivary metabolites have been scarce, although age-related changes in glutathione have been reported (Valdes *et al.,* 2013; Nassar *et al.,* 2014 and Srivastava, 2019).

The current basic concept of human aging is that it constitutes a composite of processes occurring in various tissues throughout the body, at molecular, cellular, and tissue levels (Rowe & Kahn, 1987; Aihie Sayer *et al.,* 1999 and Hofer *et al.,* 2003). Aging events often occur in an interdependent manner so that the overall outcome of human aging may be highly coordinated. Low molecular-weight metabolites participate in numerous metabolic pathways or networks, so that their declines and increases may constitute signatures of molecular events of human aging (Srivastava *et al.,* 2019). Identified metabolites may be implicated directly or indirectly in causes of human aging.

Because saliva can be collected non-invasively, it may enable a new way to comprehensively monitor human health and disease by measuring abundances of individual salivary metabolites. For example, patients of Covid-19 lose the ability to taste and smell (used as an indicator of infection). Salivary metabolites may reflect such changes, particularly for taste changes, if salivary metabolomics of patients are thoroughly examined. Previously, blood metabolomics has been developed to study human aging (Chaleckis *et al.,* 2016; Kameda *et al.,* 2020 and Kondoh *et al.,* 2021). Blood metabolites may be used if precautions are taken to avoid changes in labile chemical structures. In contrast, saliva contains metabolites such as sugars, amino acids, anti-oxidants, and high-energy compounds (Owen-Smith *et al.,* 1998; Kochańska *et al.,* 2000; Takeda *et al.,* 2009 and Dame *et al.,* 2015), some of which are important for tasting and digesting food. So far, no comprehensive method has been established to evaluate human aging based upon abundances of salivary metabolites. Since age-related metabolites in saliva may be distinct from those of blood, they may document different aspects of human aging, enabling them to be readily diagnosed (Mandel, 1990; Vissink *et al.,* 1996; Sreebny, 2000; Nagler & Hershkovich, 2005; Niccoli &Partridge, 2012 and Xu *et al.,* 2019). How they differ is of considerable interest.

To perform salivary studies, the collected saliva could be stimulated or unstimulated saliva (Smith *et al.,* 2013). Unstimulated saliva is mainly secreted from the sublingual and submandibular glands, while stimulated saliva is secreted mostly by the parotid gland. Stimulated saliva contains

lower quantities of protein (e.g. glycosylated mucin) and has a lower viscosity than that of the unstimulated counterpart. Many studies demonstrated differences in rheological and tribological properties between these two types of saliva (Prinz *et al.,* 2007and Silletti *et al.,* 2008). Unstimulated salivary flow rate is the most commonly employed measure for the quantity of saliva (Navazesh *et al.,* 1992). The rate of saliva secretion varies hugely among individuals, depending on an individual's health status and physiological conditions. The average saliva secretion rate ranges from 0.5 to 1.5 L/per day, showing its dependence on circadian rhythms (Pedersen *et al.,* 2002). For quality of saliva, researchers use different measures, such as material properties (e.g. viscosity, coefficient of friction) and/or chemical analysis (e.g. mucin, statherin concentration, degree of glycosylation) (Davies *et al.,* 2014 and Chaudhury *et al.,* 2015). Although there have been excellent reviews covering the physiological changes of saliva during ageing (Vissink *et al.,* 1996 and Nagler, 2004), it is important for food scientists to have a thorough understanding of the qualitative and quantitative changes in properties of this complex fluid upon aging. Such information is crucial to serve as a basis to optimize food design for the elderly population as the perception of the food may be driven significantly by the alteration of endogenous salivary properties and metabolites rather than the exogenous food properties.

Hence, the aim of this review is to understand age- dependent changes in salivary glands structure, quantity (flow rate), quality (composition) and material composition (rheology, lubrication) of saliva as well as salivary metabolites. The latter through analysis of saliva may act as an indicator for aging in population. In addition, this may highlight how such changes can impact the sensation of taste, smell and aroma to deepen the comprehension of salivary changes with ageing, so that generated insights can serve to design targeted food and/or new oral dryness therapies.

2. Ageing-Related Histological Changes in Salivary Glands

In human being, the three pairs of major salivary glands including parotid, submandibular and sublingual, are responsible for 92-95% of the secreted saliva, whereas minor salivary glands in the labial, buccal, palatal and lingual regions secrete the rest (Paula *et al.,* 2017). Salivary glands are predominantly composed of three types of cells involving acinar, ductal and myoepithelial, which contribute to salivary secretion into a series of duct system (Varga, 2015).

Saliva is primarily produced in the acinar cells and the type of secretion varies in the different glands, where the parotid gland produces serous secretions, the minor glands secrete mucous secretion and sublingual and submandibular gland produce mixed secretion (Mandel, 1987).

It has been reported that the glands of a young individual 23 ys showed a more even and compact lobar structure with uniform appearance of parenchymal elements when compared to an older individual 83 ys (Xu *et al.,* 2019). Also, with age, salivary gland histologically have shown an increase in the proportional volume of fat and fibrovascular tissue in the parotid and submandibular glands in elderly individuals (Scott *et al.,* 1987). In addition, the proportional volume of acinar cell secretion was reduced in elderly individuals, which can result in overall salivary gland hypofunction (Vissink *et al.,* 1996). This is considered as one of the major causes of dry mouth (Vissink *et al.,* 2010).

Sørensen *et al.,* (2014) studied the labial salivary gland of the lower lip in 190 elderly men (61 ys). They noticed 33% of the participants displayed moderate to severe acinar atrophy and fibrosis (31%). Although xerostomia was not significantly correlated with histology alterations of labial salivary glands, it was inversely related to the total nerve length in the glandular connective tissue. Salivary gland hypofunction in elderly population proved to be not only due to the histological glandular atrophy, but also due to other causes like the diminished intensity of the stimulation and reflex related to aging.With age, there is a reduction in the number of olfactory and taste receptors, diminished neuronal saliva stimulation (less transmitters acting on the receptors) and a decrease in the blood perfusion at the gland level. Furthermore, increase in ageing-related diseases and polypharmacy might also affect the gland function (Ekström *et al.,* 2017).

3. Ageing-related Changes in Quantity of Saliva

Changes in the quantity of saliva (salivary flow rate) has been reported with age (Xu *et al.,* 2019). However, there is a debate in several studies about the decrease in salivary flow rate with age.

This is may be due to the variations in the study design or saliva collection method. In a meta-analysis study including all the published work regarding saliva and age, 47 studies finally selected, and categorized the studies involving salivary flow rate in three groups, 1) submandibular and sublingual saliva; 2) parotid gland and 3) minor gland salivary flow rate (Affoo *et al.,* 2015). The authors reported that both, unstimulated and stimulated average saliva flow rates were significantly lower ($p < 0.001$) in older adults than younger, with the difference being 66% higher in stimulated than in unstimulated saliva. Such decrease in salivary flow rate was specifically attributed to saliva from submandibular and sublingual salivary glands. However, parotid and minor gland salivary flow rates did not appear to be significantly lower.

Polypharmacy, i.e. the use of multiple medications, such as antidepressants, diuretics, analgesics, antihypertensives, antianxiety drugs ect…, which are routinely administered in the elderly population could not fully explain the differences in salivary flow rates between younger and older adults. A recent study further confirmed this by comparing healthy elderly (70-92 ys.) and young subjects (22-55 ys.) (Vandenberghe Descamps *et al.,* 2016). The authors observed an average reduction of 38.5% in resting salivary flow rate and 38.0% of stimulated salivary flow rate in elderly subjects as compared to young subjects. They attributed the salivary flow decline by age to loss of acinar cells, loss of secretory tissue and adiposity increase as well as neurophysiological deterioration in salivary glands. Such decrease in salivary flow rate has an indirect influence of the quality of saliva.

4. Ageing-related Changes in Quality of Saliva

Qualitative changes have been reported to include composition, rheology and lubrication properties of saliva (Xu *et al.,* 2019).

4.1 Salivary Composition

Although most of the researches on salivary changes are conducted with patients with burning mouth syndrome (Nagler, 2004), Sjögren's Syndrome (Chaudhury *et al.,* 2016), or salivary analysis after radiotherapy (Eliasson *et al.,* 2005), yet, Studies on the changes in salivary composition in healthy elderly individuals are relatively limited. The available studies compared changes in salivary composition in elderly and young populations (Nagler & Hershkovich, 2005). The authors found a significant increase in concentration of inorganic components in elderly (n=25, 70-86 ys.)as compared to that of young population (n=26, 20-29 ys.). They attributed this increase in ionic concentration of saliva to reduced salivary volume. As the water secretion pathway was affected with less salivary flow rate, there was a subsequent concentration effects on the ions. However, more recently, Nassar *et al.,* (2014) found a decrease in calcium ($Ca2+$) when comparing the two age groups of population (old: n=20, 60-80 ys., young: n=20, 30-60 ys.) in the case of unstimulated saliva, which was not in agreement with the data reported by Nagler & Hershkovich (2005). Although the mechanism for salivary calcium decrease is unclear, such reduction of calcium has been previously observed in serum of healthy elderly subjects (Barbagallo *et al.,* 1999). Furthermore, it should be noted that there is no standardized age category in the two mentioned studies, resulting in a potential source of variability in defining the elderly population.

Concerning the organic components, Nagler & Hershkovich (2005) found differences when reported as concentration versus total amount secreted (output). For instance, although the concentration of salivary proteins increased with age, this was not significant. However, when presented as output of secretion, salivary proteins decreased significantly ($p < 0.05$).

For amylase, this was opposite i.e. the concentration was significantly higher but the output increase was not significant. This suggests that in some cases there is an age-dependent influence on secretion of one specific component, whereas, in others, the concentration effect is mainly driven by the decreased salivary output.

There was a consensus particularly with respect to mucin concentration decrease with ageing. The salivary mucins (a group of different glycoproteins in saliva that covers the oral mucosa), form an immobile pellicle retained on epithelial cells (membrane-associated mucins: MUC1, MUC3, MUC4, MUC12) and a mobile salivary film (secreted soluble mucins: MUC2, MUC5A, MUC5B, MUC6, MUC7) (Cárdenas *et al.,* 2007; Macakova *et al.,* 2010; Morzel *et al.,* 2014 and Laguna & Sarkar, 2017). The MUC1 was reported to decrease in the elderly subjects; increasing the development of oral mucosal diseases in the aged population (Chang *et al.,* 2011).In addition, Denny *et al.,* (1991) and

Navazesh *et al.,* (1992) found that both levels of MUC1 and MUC2 in unstimulated saliva were significantly lower in the healthy aged group (65-83 years old) as compared to young adults (18-35 years old).

From the clinical point of view, salivary components are important in maintaining oral health. Salivary proteins, such as mucin, lactoferrin, lysozyme, peroxidase are required for non immunological bacteria-defence system (Wu *et al.,* 1994 and Proctor, 2016).Various researchers observed reduced lactoferrin and peroxidase activity in the healthy elderly subjects, thus the modification in the balance between salivary antimicrobial agents contribute to the impairment of oral tissues (Salvolini *et al.,* 2000; Nagler, 2004 and Dodds *et al.,* 2005).

These findings are in agreement with age-related histological and physiological changes in the salivary glands as previously mentioned. Not only the quantity, but also the properties of mucins may influence dryness perception with the latter being associated with the changes in residual salivary composition and ions (Chaudhury *et al.,* 2015). Patients with dry mouth exhibit altered saliva rheological properties and reduced mucosal hydration, indicating functionally impaired saliva, largely linked to reduction in MUC5B and MUC7, as indicated in the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Reduced amount of mucin results in a thinner adsorbed layer on the anterior hard palate i.e. thickness in the normosalivator is in the range of 7.6-57.2 μ m, whereas in the case of hyposalivation, it is in the range of 3.4-25.7 μm. This is known as "enamel pellicle", which induces weak oral mucosa protection and enamel demineralization (Lee *et al.,* 2002; Lindh *et al.,* 2014 and Proctor, 2016).

With reference to enamel demineralization, salivary calcium is required in reformation of enamel pellicle with salivary proteins. As discussed earlier, evidence of declined levels of calcium in healthy elderly along with the reduced salivary protein secretion thus may significantly affect oral health in elderly population (Al-Hashimi & Levine, 1989; Lendenmann *et al.,* 2000 and Nagler & Hershkovich, 2005). Therefore, the lack of mucins may not only lead to oral disease due to lack of defence but also dryness of the oral mucosa.

4.2 Material Properties of Saliva

Saliva has been studied as a material by measuring its viscosity, elasticity, adherence and spinnbarkeit (Gohara *et al.,* 2004 and Stokes &Davies, 2007). The first step in such studies is the collection of saliva and its preservation. This is because saliva properties differ as a function of the collection method (Stokes &Davies, 2007and Proctor, 2016). Many studies demonstrated distinct results (e.g. rheology, tribology) when using stimulated versus unstimulated saliva (Prinz *et al.,* 2007and Silletti *et al.,* 2008). In addition, the difference of salivary stimulation is magnified in the elderly subjects. For example, mild stimulation, such as the use of lemon-drop resulted in 39% age-dependent decrease in salivary flow rate (Pedersen *et al.,* 1985). While, using strong stimuli, such as pilocarpine hydrochloride, the capability of salivary glands in drug-free elderly was also reduced (Nagler & Nagler, 1999 and Nagler & Hershkovich, 2005). Once the saliva was collected, changes in the composition naturally occur due to the proteolysis and bacterial metabolism (Gohara *et al.,* 2004; Schipper *et al.,* 2007; Takehara *et al.,* 2013 and Wagner & McKinley, 2017).

In order to minimize the contamination, pre-treatments (e.g. centrifugation, dehydration) and storage conditions (e.g. freezing) are required in saliva preservation. However, these actions also lead to changes in some salivary parameters. For example, with the storage time and after being frozen, the salivary viscosity was found to decrease significantly (Stokes & Davies, 2007).

The macromolecules i.e. mucins affect the rheological properties of saliva. Shear viscosity (i.e. energy dissipated during flow) using simple capillary viscometer (Waterman *et al.,* 1988) and elasticity (i.e. energy stored) using uni-axial elongational flow (Zussman *et al.,* 2007) have been measured in saliva from elderly individuals. An age-related reduction in salivary flow rate, accompanied by an increase in salivary viscoelasticity and protein content were reported. This age-dependent increase in salivary viscosity was also further supported using elongational thread viscometer by Kazakov *et al.,* (2009).

Currently oral tribology is emerging as a promising tool to quantify friction and lubrication of food-saliva mixtures in the oral mucosa using in vitro polymeric set up (Laguna *et al.,* 2017a; Laguna & Sarkar, 2017 and Laguna *et al.,* 2017b). Studies involving Xerostomia patients have reported reduced sulfation of salivary mucins (both MUC1 and MUC2), which may affect the lubricating

property of saliva (Chaudhury *et al.,* 2016). However, there is no tribological work published to date, regarding the change of saliva with age. Considering that mucin, calcium content and mono-valent ionic concentration of saliva differs significantly in elderly individuals as compared to young population, lubrication and adsorption properties of saliva are likely to be affected in elderly individual. This might affect the textural perception of food significantly, however, such hypothesis needs to be validated with well-coordinated instrumental and sensory studies with healthy elderly subjects.

The loss of negatively-charged glycan residues transforms mucins from extended polymers into more tightly packed globular aggregates, which causes a reduction of water retention capacity of mucin in the residual saliva and oral dryness (Coles *et al.,* 2010). This may also provide an explanation for patients suffering from dry mouth conditions even with high mucin concentrations (Saari *et al.,* 1997). Furthermore, the degradation of sulfation of mucins is proposed as the causative factors in changes of rheological properties of saliva (Yamada, 1980). On the other hand, the spinnability (fibrosity) of saliva measures the adhesive and elastic properties of saliva to enable its adsorption to the oral surfaces and form a pellicle (Vijay *et al.,* 2015). Studies have indicated that unstimulated saliva in dry mouth patients has significantly lower spinnability. This is largely attributed to the reduction as well as degradation of moisture-retaining mucin proteins (Carpenter, 2013 and Chaudhury *et al.,* 2015). On the other hand, the highly concentrated and viscous saliva lacks the ability to flow freely in the oral cavity (Zussman *et al.,* 2007). This results in the localized areas of dryness and followed by the perception of dry mouth (Närhi, 1994). This suggests that the treatments for dry mouth patients and rational design for artificial saliva should include not only the quantity, but also the ability of saliva to be retained on surfaces.

5. Effect of Ageing-related Salivary Changes on Food Flavor Perception

Food substances need to be dissolved in the saliva in order to reach the taste receptors (Neyraud, 2014). Moreover, saliva plays a role in mouth sensations caused by food components, such as astringency that occurs by the complexation of food polyphenols and salivary proteins (Brossard *et al.,* 2016; Rutuja *et al.,* 2016 and Laguna & Sarkar, 2017). On the other hand, the effect of concentration changes of inorganic ions might elevate the taste thresholds and decrease supra-threshold intensities that give an explanation of elderly people suffering from taste aberrations. It has been also shown that this age-degenerated taste sensitivity is not only related to the decrease of number of taste buds (Shin *et al.,* 2012), but also of the salivary cell production resulting in a time lag in the turnover of taste receptor cells. It is well-recognized that the response to sucrose and sweetness perception is pH dependent. Hence, changes in salivary inorganic composition resulting in the changes of salivary pH can explain its influence on the receptor's stimulation indirectly affecting sweetness perception in elderly population (Tierney & Atema, 1988 and Matsuo &Yamamoto, 1992). Although it has not been linked with old age, changes in the salivary flow has shown a significant positive correlation between saliva flow and time to reach maximum intensity of sweetness and cherry flavour in chewing gums (Guinard *et al.,* 1997). Therefore, age-dependent changes in salivary composition can have a direct influence on taste perception and might consequently affect food intake (Boesveldt *et al.,* 2018). In a systematic review (Muñoz-González *et al.,* 2018), it was inferred that salivary hypofunction was associated with a decrease of the objective chewing and swallowing abilities and taste perception. Interestingly, rare attention has been paid in literature to investigate the relationship between salivary flow and texture or aroma on one side and salivary composition, taste/texture perception and food intake on the other side. This suggests that more research is needed in this field to understand the role of hyposalivation on potential decline of aroma/ texture perception and its consequences on food intake.

6. Ageing-Related Changes in Salivary Metabolites

Recently human age-related saliva metabolic markers have been studied. In this study, comprehensive quantification of human salivary metabolites was investigated using LC–MS. Among 99 metabolites, 21 proved to be age-related. All of these age-linked metabolites declined with age except ATP which increased in the elderly (Teruya *et al.,* 2021).

6.1 Saliva Sample collection and characteristics of subjects

Teruya *et al.,* (2021) collected saliva samples from 27 healthy volunteers in Onna Village, Okinawa. The average age of 14 elderly persons was 75.8 ± 3.9 year, whereas the other 13 persons were young (30.6 ± 3.2 year). Their urinary metabolites had been analyzed previously (Teruya *et al.,* 2020).The gender ratio (male/female) was 10/17 and the average BMI of subjects was 22.5 for young subjects and 24.5 for elderly. Blood glucose levels were within normal ranges (plasma glucose, 69–117mg/dL; HbA1c, 5.1–5.8%). Two saliva samples were collected from each of the subjects at the same time. Saliva samples were quenched at – 40 °C and their extracts were stored at − 80 °C until non-targeted, comprehensive LC-MS analysis of metabolites using procedures described for blood and urinary metabolites (Chaleckis *et al.,* 2014; Chaleckis et al 2016 ; Teruya *et al.,* 2019 and Teruya *et al.,* 2020). Procedures of LC-MS data analysis, compound identification, and statistical treatment with MZmine 2 were done as previously reported (Pluskal *et al.,* 2010; Pluskal *et al.,* 2012 and Pluskal & Yanagida, 2016). Replicates of all samples showed essentially identical quantification data for salivary metabolites (Teruya *et al.,* 2021).

6.2 Groups of salivary metabolites

Interestingly, it has been reported that ninety-nine salivary metabolites, comprising 14 subgroups, were identified recently (Teruya *et al.,* 2021). These included 4 nucleotides, 3 nucleotide-sugar derivatives, 12 nucleosides, nucleobases, and derivatives, 4 sugar derivatives, 10 sugar phosphates, 4 vitamins and coenzymes, 5 choline and ethanolamine derivatives, 6 carnitines, 5 organic acids, 2 antioxidants, 17 standard amino acids, 10 methylated amino acids, 6 acetylated amino acids, and 11 other amino acids. Abundances of metabolites were quantified in terms of peak areas (10^6–10^9 arbitrary units, AU) and were also represented semi-quantitatively (low L, medium M, high H) following definitions by Chaleckis *et al.,* (2016). Six compounds (urate, phosphocholine, arginine, phenylalanine, proline, tyrosine) were relatively abundant, ranging from H to M (H–M). Five compounds (dimethyl-xanthine, histidine, betaine, citrulline, taurine) were M (medium), while 33 additional compounds were in the range of M to L (medium to low). The remaining 55 compounds, or about half, were of low (L) abundance.

6.3 Twenty-one Age-Linked Salivary Metabolites

Twenty-one of 99 metabolites have been statistically reported as age-related ($p < 0.05$, fold change < 0.66 or > 1.5). They comprise 3 nucleotides (ATP, AMP, UMP), 3 nucleosides (dimethyl-xanthine, adenosine, *N*-methyl-adenosine), 5 sugar phosphates, 2 vitamins /coenzymes (NAD^+, nicotinamide), 1 carnitine, 2 standard amino acids (glutamate, threonine), 1 methylated amino acid (*N*-methyl-histidine), 1 anti-oxidant (glutathione disulfide), and 3 other amino acids (citrulline, acetyl-carnosine, creatinine (Teruya *et al.,* 2021). The authors found that the 21 metabolites manifesting age differences varied between significantly higher (red), lower (blue), and unchanged metabolites (gray) in the elderly group. In blood, 42 of these 99 compounds were enriched in RBCs (Chaleckis *et al.,* 2014 and Chaleckis *et al.,* 2016), so many RBC-enriched compounds are also found in saliva. These are all sugar phosphates, nucleotide-sugars, and anti-oxidants. Metabolites of saliva thus somewhat resemble those of whole blood.

6.4 Gender Differences in Age-related Salivary Metabolites

Gender differences have been reported in 2 salivary metabolites, acetyl-carnosine and creatinine, which decrease significantly in females. Both are also linked to age. Since these compounds are mainly localized in muscle, the appearance of both age and gender differences may be due to differences in oral organ or systemic muscle mass (Teruya *et al.,* 2021).

6.5 Coefficients of Variation of Salivary Metabolites among subjects

Coefficients of variation (CV) represent measures of individual variability of metabolites abundances (Chaleckis *et al.,* 2016). The abundance of each saliva metabolite was obtained from LC–MS data and CVs were subsequently calculated. Salivary compounds were classified into 6 sub-groups, according to the magnitude of their CVs. Of 21 age-related salivary metabolites, only two, creatinine and *N*-methyl-histidine, belonged to the least variable group, which included 12 metabolites (CV, 0.3–0.5).Two other compounds, citrulline and UMP, were in the next least variable group, which

comprised 14 compounds (0.5–0.6).Sixteen metabolites were relatively more variable, with CVs ranging from 0.60 to 1.0. Only one, acetyl-carnosine, was in the most variable (CV > 1.0) subgroup, which comprised 19 compounds. Thus, CVs of the great majority of salivary compounds (17/21) were relatively variable (CV 0.6–1.0) (Teruya *et al.,* 2021).

6.6 Correlations of age-related salivary metabolites

About half of age-linked saliva metabolites were highly correlated forming a correlation network. Five sugar phosphates suggest that the pentose phosphate and glycolysis/gluconeogenesis pathways are active in saliva and that their activities decline in elderly people. Other age-linked metabolites such as NAD^+, nicotinamide, adenosine and AMP also declined in elderly subjects, supporting the notion that antioxidation and energy are important for salivary metabolism and diminish in elderly subjects (Teruya *et al.,* 2021). These metabolic pathways in saliva have not been recognized previously.

Amino acids (glutamate, threonine, citrulline, carnitine) are also age-related. Glutamate is the major excitatory neurotransmitter in the brain, involved in functions such as motor behavior, cognition, and emotion. It may be affected in the course of normal aging. Glutamate concentration decreases with age predominantly in the gray matter motor cortex region (Kaiser *et al.,* 2005). Threonine produces glycine in the process of catabolism to pyruvate and is used to synthesize collagen, glutathione, creatine, and so on. Citrulline is a by-product of nitric oxide (NO) synthesis in the urea cycle, and a citrulline deficiency can cause a reduction in the bioavailability of NO. Carnitine is related to mitochondrial function in muscle and brain (Bremer, 1983; Virmani & Binienda 2004 ; Stephens *et al.,* 2007 and Ferreira & McKenna, 2017). Hence oral functions related to these metabolites may decline in the elderly. Comprehensive quantitative analyses of salivary metabolites are scarce so many of age-linked metabolites have not been reported in the literature (Nassar *et al.,* 2014).

Metabolites such as ATP, creatinine, acetyl-carnosine, glutathione disulfide, and methyl-histidine do not participate in a clear correlation network (r < 0.7), but depict salivary aging demonstrated by their decline or increase (ATP) (Teruya *et al.,* 2021). This confirmed a previous report on the decline of glutathione disulfide (Nassar *et al.,* 2014). Not only glutathione, but other anti-oxidative compounds are quite abundant in saliva, such as anserine, which contains *N*-methyl-histidine, acetyl-carnosine. Acetyl-carnosine, creatinine, and *N*-methyl-histidine are also authentic muscle-related metabolites, perhaps required to support tasting and other lingual and oral activities. The reduction of these three muscle specific metabolites in saliva is symbolic among the aging markers identified. *N*-Methyl-histidine is a component of the muscle dipeptide, anserine. *N*-Methyl-histidine concentration in saliva increases after exercise (Ra *et al.,* 2014), as well as in blood and urine (Dohm *et al.,* 1985). It has been recently reported that the urinary *N*-methyl-histidine level in elderly people was significantly lower than in young people (Teruya *et al.,* 2020). Thus, a decrease in salivary *N*-methyl-histidine in the elderly may reflect a decrease in systemic basal muscle mass and physical activity with age. Interestingly, acetyl-carnosine and creatinine also showed a gender difference. Acetyl-carnosine with antioxidant activity is mainly localized in muscles and has a role in suppressing inflammation or fatigue (O'Dowd *et al.,* 1988). Non-invasive measurement of free carnosine in muscle by proton magnetic resonance spectroscopy (1H-MRS) showed that carnosine content increased dramatically in puberty (8–20 year) boys, but not in girls. In addition, a decrease was observed from young adults (21–30 year) to adults (31–50 year) for both men and women, but there was no significant change in adults and elderly (60–83 year) (Baguet *et al.,* 2012). Creatinine is a metabolite of creatine phosphate, which is a source of muscle energy, and its serum and urinary concentrations are highly correlated with skeletal muscle mass (Baxmann *et al.,* 2008 and Patel *et al.,* 2013). Hence, the age and gender differences of these two muscle metabolites in saliva may be closely related to differences in oral organ or systemic muscle mass. In addition, amino acids or peptides, such as anserine and glutamate are involved in taste, so their decline suggests that elderly people lose their ability to taste. Some of these age-linked compounds (14/21) formed a clear high correlation network, but others did not. When PCA was tested in more than 20 combinations from the 21 compounds, it was found that even as few as 4 compounds, ATP, citrulline, creatinine, and glutathione disulfide, roughly discriminate age. Judging from the diversity of age-linked salivary

metabolites, numerous physiological mechanisms are reflected in salivary aging and appear coordinated. Anti-oxidative, redox, and energy production diminish in elderly subjects.

As elderly saliva contained reduced levels of metabolites related to gluconeogenesis, glycolysis, the pentose phosphate pathway, and nitrogen metabolism, it is apparent that these metabolic pathways are less active in the mouths of elderly people. ATP was the sole exception, as it increased in abundance in saliva of elderly subjects. In addition, certain nucleosides/nucleotides, such as adenosine, nicotinamide, AMP, and UMP, are correlated, and they also diminished with age so that nucleoside/nucleotide metabolism seems to be less active.

Age-dependent salivary metabolites thus reflect nutrition (carbohydrate, amino acid, nucleoside, lipid), phosphate metabolism, energy demands, redox reactions, and anti-oxidation. These are consistent with the presence of ATP, NAD^+, glutathione disulfide, carnitine, and creatinine. Notably, these age-related salivary metabolites lessen with age, consistent with the notion that aging in saliva might be caused by the loss of some essential metabolic (digestion, energy, muscular) features of oral activities. The one exception is the increase of ATP. The reason for this increase is unclear, but possibly it is due to reduced catabolism of ATP to ADP and AMP (Teruya *et al.,* 2021).

6.7 Heatmap of metabolites reveals the mode of oral aging

To estimate the degree of salivary aging among14 elderly and 13 young individuals, a heatmap approach was taken (Teruya *et al.,* 2021). Abundances of 21 age-linked salivary metabolites were colored, reflecting the degree of deviation from average value (50), white, with blue representing abundances below average, and red, higher than average. Young subjects clearly display higher levels of age-related metabolites (subjects 15–27, red) and senior subjects (1–14) show decreased metabolites (blue). Two elderly female subjects (13 and 14, 80 and 75 years, respectively) revealed surprisingly young patterns, particularly no. 13. Scrutinizing the heatmap of no.13, rather high levels of energy-related metabolites (*N*-methyl-adenosine, adenosine, AMP, UMP, sedoheptulose-7-phosphates, glucose-6-phosphate, and fructose-6-phosphates), and redox and muscle metabolites (nicotinamide, carnitine, and creatinine) are evident. This level of nucleotide-related metabolism may be exceptional.

7. Conclusions

In conclusion, age related changes in quantity and quality of saliva could affect sensorial/ textural perception and consequently food intake, which is critical to design optimized food and oral therapies for maintaining optimal oral health and nutritional status in elderly population. In addition, the age-linked salivary metabolites together illuminate a metabolic network that reflects a decline of oral functions during human aging. Saliva may greatly help to assess the degree of human metabolic aging. Salivary metabolites may also prove useful to better understand pediatric matters. Human characteristics monitored via salivary metabolites undoubtedly have broad significance, since these salivary metabolites can be used as indicator for aging.

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