치의과학박사학위 학위논문

Relationships between Saliva and Blood Samples in the Analysis of Inflammatory and Oxidative Stress Biomarkers in Young Male Adults

성인 남성의 타액 내 염증 및 산화 스트레스 표지자 분석 시 타액과 혈액 검체의 관련성에 관한 연구

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ABSTRACT

Relationships between Saliva and Blood Samples in the Analysis of Inflammatory and Oxidative Stress Biomarkers in Young Male Adults

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Objectives: Diagnostic value of saliva depends on the reproducibility of data in repeatedly collected samples and predictable correlations between saliva and blood. The aim of study was to investigate the reliability, blood reflectance, and influence of blood contamination in the analysis of inflammatory and oxidative stress biomarkers in saliva samples.

Methods: In total, 37 healthy young male participants (26.7 ± 2.2 years) were included. Unstimulated whole saliva and blood samples were collected on the first visit, and saliva samples were collected again after 2-3 days. The concentrations of total protein and inflammatory [C-reactive protein (CRP), IL-1β, IL-6, and TNF-α] and oxidative stress [8-hydroxy-2'-deoxyguanosine (8-OHdG), malondialdehyde (MDA), and total antioxidant capacity (TAC)] biomarkers in saliva and blood, and as well as blood contamination biomarkers (transferrin and hemoglobin) in saliva were analyzed.

Results: The intra-class correlations of all examined biomarkers except TNF-α were fair to excellent. Significant positive correlations between CRP and IL-6 and between total protein and TAC were stable in the saliva samples collected on different days. Notably, IL-6 was the only biomarker that showed a significant correlation between saliva and blood. As the

concentration of salivary transferrin increased, the saliva/blood ratios of total protein and TAC also increased. The concentration of salivary hemoglobin did not affect the saliva/blood ratios of biomarkers.

Conclusions: The findings of this study are limited to healthy young males. For clinical applications, studies on salivary diagnostics should be performed for individual disease and health conditions, demographic characteristics, and biomarkers.

Keywords: Biomarker; Blood; Blood contamination; Inflammation; Oxidative stress; Saliva

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I. INTRODUCTION

There have been numerous efforts to utilize saliva for diagnostic purposes. The advantages of saliva sampling, such as non-invasiveness, cost-effectiveness, safety, and the ease of repetitive collection have facilitated its use as a diagnostic modality (Yoshizawa et al., 2013). Saliva-based diagnostics are advantageous for the elderly, physically handicapped individuals, and non-cooperative subjects such as children. Saliva contains components directly produced by the salivary glands and those transported from blood via passive diffusion, ultrafiltration, and active transport (Lee & Wong, 2009; Pfaffe, Cooper-White, Beyerlein, Kostner, & Punyadeera, 2011). These components can be used as biomarkers to diagnose oral and systemic diseases and for health surveillance (Prior & Cao, 1999; Sezer, Çiçek, & Çanakçi, 2012; Trivedi, Lal, Mahdi, Singh, & Pandey, 2015).

However, the use of salivary components as diagnostic biomarkers has many limitations. First, scientific consensus is lacking regarding the standardization of collection, storage, and analysis procedures for saliva samples (Palmieri & Sblendorio, 2007; Wang, Schipper, Velly, Mohit, & Gornitsky, 2015). Second, because the levels of salivary biomarkers are highly variable in patients and controls, it is difficult to determine the normal or pathologic range for each salivary biomarker (Kamodyová, Červenka, & Celec, 2015), e.g., according to previous studies, the normal level of salivary IL-6 ranges widely from 0.89 ± 1.72 (Riis et al., 2014) to 47.46 ± 18.74 pg/mL (Rhodus et al., 2005). Therefore, standardization of collection, storage, and analysis procedures for saliva samples and establishment of the concentrations of major salivary components with a diagnostic value in healthy individuals are crucial for the utilization of saliva as a reliable diagnostic medium.

The clinical value of salivary biomarkers highly depends on the reproducibility of results and predictable correlations between the same biomarkers in the blood and saliva, e.g., since the salivary levels of various stress and gonadal hormones are relatively good reflections of their blood concentrations, salivary analyses for these hormones have been widely employed for research purposes (Lee, Kim, Chang, & Kho, 2015). To extend the applicability of salivary diagnostics, “reproducibility” and “correlation” should be tested for each biomarker in different sexes and age groups, in healthy populations, and in various disease conditions. To this end, studies on the salivary biomarkers of healthy adults to rule out the influence of pathology-related factors and establish a reference are essential.

The development of methods to determine the blood contamination level in saliva is also necessary. Since the concentrations of some analytes in saliva are much lower than those in blood, leakage of blood into saliva due to gingival inflammation and deterioration of oral mucosal integrity may increase the level of salivary analytes (Behr et al., 2017; Kivlighan et al., 2004; Schwartz & Granger, 2004). Therefore, knowing whether changes in the concentrations of salivary biomarkers are due to the pathogenesis of targeted diseases or the effects of blood contamination is essential for accurate salivary diagnostics.

To investigate the reliability, blood reflection, and blood contamination issues in salivary diagnostics, we focused on inflammatory and oxidative stress biomarkers in saliva in healthy young adult males. Increased inflammation and the resultant increase in reactive oxygen species can induce or worsen the symptoms of many chronic diseases and accelerate the aging process. Therefore, numerous studies on diagnostics and health surveillance using saliva have been conducted to investigate inflammatory and oxidative stress biomarkers (Cullen, Thomas, Webb, & Hughes, 2015; Heitzer, Schlinzig, Krohn, Meinertz, & Münzel, 2001; Tulunoglu, Demirtas, & Tulunoglu, 2006). Only healthy young adult males were recruited as subjects in the present study to limit the uncontrollable variables such as the menstrual cycle and chronic diseases.

The purposes of this study were to investigate the reliability of saliva samples in repeated collections, the blood reflectance of saliva, and the effects of blood contamination on salivary biomarkers. This study can provide a basic but essential reference for monitoring the health status of patients with chronic diseases and elderly individuals experiencing difficulties with repetitive blood sampling.

II. REVIEW OF LITERATURE

1. Saliva as a diagnostic fluid

(1) Importance of saliva as a diagnostic fluid

Saliva is a biological fluid that has multiple functions including antimicrobial, lubrication, digestion, and gustatory functions. It is a hypotonic fluid composed of 99.5% of water and 0.5% of other various substances. Although salivary glands can produce their specific substances, most substances in blood also can be found in saliva due to various mechanisms such as passive diffusion, ultra-filtration, and active transport (Lee et al., 2009;

Pfaffe et al., 2011). The whole saliva also contains bacteria and food debris from the external environment. Therefore, saliva has been considered as “mirror of the body” and can be utilized as a diagnostic fluid, capable of monitoring both oral and systemic conditions.

Since the collection process of saliva is simple and non-invasive, non-professional individuals can perform it. Therefore, multiple, repetitive, and continuous monitoring using saliva samples is possible, and these advantages of salivary diagnostics are potent for children, elderly, and disabled patients who would experience difficulty in blood collection. Salivary diagnostics has been rapidly evolving with several technological advances in the fields of micro-methodology and molecular biology. In fact, various oral and general health condition-specific salivary biomarkers have been discovered and reported. Recently, saliva diagnosis has been used in many clinical fields, and various technical approaches have been attempted to broaden its application range (Hofman, 2001).

(2) Clinical application of salivary diagnostics

1) Infectious diseases

Salivary diagnostics have been used to screen infectious diseases by detecting salivary rRNAs or antigens of fungus, virus, and bacteria. For example, saliva has been traditionally used to diagnose oral candidiasis by swab and culture method. Also, saliva can be utilized to detect viral infections including HIV, hepatitis A, B, and C (Parizad et al., 2016), and cytomegalovirus (Cardoso et al., 2015), and bacterial infection (H. pylori) (Ferguson et al., 1993).

2) Hormones

Circulatory hormone levels can be monitored using saliva. Due to the simple and non-invasive nature of salivary diagnostics, hormones, which should be continuously monitored, are one of the most promising targets. Nowadays, aldosterone (Manolopoulou et al., 2009), parathyroid hormone (Agha-Hosseini et al., 2009), insulin (Hartman et al., 2016), progesterone (Mirzaii-Dizgah et al., 2011), estradiol, testosterone (CElEC et al., 2009), and cortisol (Elias et al., 2014) can be detected in saliva with a certain level of clinical reliability. Salivary progesterone is used to diagnose menstrual disorder. Estradiol and testosterone are used to diagnose and monitor hirsutism and menstrual cycle, and cortisol is used to diagnose Cushing’s syndrome and monitor its progression.

3) Drugs

There has been increasing interest in utilizing saliva for monitoring the level of medication and screening drug abuse. Measuring circulatory drug concentration using saliva sample may help to assess drug compliance, risks that can be resulted from the drug overdose, and ultimately to design precise and personalized therapeutic plans. Currently, there are many ongoing studies on comparing salivary and circulatory concentrations of carbamazepine, diazepam, digoxin, phenytoin, and valproic acid (Dwivedi et al., 2015; Yamada et al., 2015). Moreover, saliva can be used to monitor abuse of drugs including amphetamine, cocaine, methadone, and opiate (Christodoulides et al., 2015; Cone et al., 2007).

4) Other clinical situations

Diverse clinical situations may directly or indirectly cause physicochemical and biological alternations in saliva, and analyzing these alternations can be favorably used in salivary diagnostics. For example, saliva can be used to diagnose diabetes mellitus, one of the most typical metabolic diseases. Structural abnormality and alternation of salivary composition have been observed in diabetes patients (Kaczor-Urbanowicz et al., 2017). In addition, diabetic condition decreases periodontal integrity thus results in elevation of biomarkers in saliva such as myeloperoxidase, IgA, HbA1c, and the total amount of protein (Aitken-Saavedra et al., 2015; Malicka et al., 2015). Sjogren's syndrome, an autoimmune disease with compromised salivary and lacrimal glands, shows alternation of specific mRNA levels and significant reduction of salivary IL-6, IgA, IgG, IgM, Na+, lactoferrin, albumin, and beta2 microglobulin (Asashima et al., 2012; Riega-Torres et al., 2016). Utilization of salivary diagnostics on cancer diagnosis is also a promising field. It has been reported that salivary cyclin I, epidermal growth factor receptor, and fibroblast growth factor 19 are potential biomarkers to detect cancers of oral, pancreas, lung, and breast (Yong Zhang et al., 2016). In addition, the level of Aβ42 (Bermejo-Pareja et al., 2010) and α-synuclein and DJ-1 (Devic et al., 2011) were proposed as biomarker for Alzheimer's and Parkinson's disease, respectively. Further researches in this area should be continued. Further researches in this area should be continued.

(3) Importance of inflammatory, oxidative stress biomarkers in saliva on the process of aging and diseases.

Reactive oxygen species (ROS) have multiple roles in proliferation, signal transduction, differentiation, gene expression regulation, and apoptosis of cell (Turrens, 2003). Under inflammatory conditions, increased number of pro-inflammatory cells such as phagocytic cells and neutrophils may elevate ROS stresses, and it consequently leads to the increased aging and prevalence of various diseases (Heitzer et al., 2001). There are increasing evidences that elevated the level of oxidative stress is related to atherosclerotic cardiovascular disease (Reuter et al., 2010), DM (Stephens et al., 2009), sterility (Aitken et al., 2006), chronic renal diseases, chronic inflammatory diseases (Oberg et al., 2004), and acceleration of aging process (Emerit et al., 2004). Although the molecular mechanisms on how ROS affects such diseases are yet to be fully discovered, it has been proved that DNA, protein, and cell membrane damages caused by ROS may be the principal mechanisms (Soule et al., 2007). Therefore, investigating the levels of inflammatory and oxidative stress biomarkers is not only highly advantageous for scrutinizing the process of disease development and aging, but also can be utilized in the field of health surveillance.

2. Inflammatory and oxidative stress biomarkers in saliva

(1) Inflammatory biomarkers in saliva

1) Interleukin-1β (IL-1β)

IL-1β is an 18 kDa-sized glycoprotein, a member of the IL-1R/TLR superfamily (Dinarello, 1998; O'Neill et al., 2000). IL-1β is a pro-inflammatory cytokine related to acute immune response, acute/chronic infection, and mild inflammatory state such as obesity (Darveau et al., 1997). In the oral cavity, IL-1β is produced and secreted by local periodontal connective tissue cells like fibroblast cells or infiltrating inflammatory cells (Tsai et al., 1995). The elevated level of IL-1β may trigger the secretion of other downstream cytokines and metalloproteinase (Dewhirst et al., 1985) and the activation of osteoclast to induce inflammatory tissue destruction (Gowen et al., 1986). Therefore, salivary IL-1β can be used as a biomarker for periodontal tissue destruction (Ng et al., 2007; Nishida et al., 2006). It has been reported that parotid acinar cells can produce and store IL-1β, which then released with saliva when they get α-and β-adrenergic stimulation (Tanda et al., 1998). However, the correlation between blood and salivary IL-1β is yet to be discovered.

2) Interleukin-6 (IL-6)

IL-6 is a versatilely glycosylated 22 to 27 kDa glycoprotein secreted by activated leukocyte, endothelial cells, and adipocytes. Interestingly IL-6 has two contradictory functions; it can trigger fever induction and production of CRP, one of acute inflammatory protein to initiate inflammatory immune responses (Chai et al., 1996; McCarty, 1999), but on the contrary, it can also suppress immune responses (Tilg et al., 1994). IL-6 is also involved in various disorders such as metabolic syndrome (Pedersen et al., 2012), clinical depression (Hiles et al., 2012), sleep dysfunction, and stress (EL‐SHEIKH et al., 2007; Groer et al., 2010; Sjögren et al., 2006). IL-6 in saliva may present by ultrafiltration from the blood or by synthesis by the locally infiltrated lymphoid cells or salivary glands. For instance, salivary gland epithelial cells are responsible for the elevated level of IL-6 in the patients with Sjogren's syndrome (Sun et al., 1998; Tishler et al., 1999). Many studies have tried to use salivary IL-6 as a biomarker that reflects blood IL-6. However results were controversial; some studies showed a weak correlation between salivary and blood IL-6 (Fernandez-Botran et al., 2011; Yuanyuan Zhang et al., 2008) but no such correlation was observed in other studies (Cox et al., 2008; Cullen et al., 2015; Sjögren et al., 2006). One study reported that both salivary IL-6 and blood IL-6 showed positive correlations with psychological risk factors rather than direct correlation (Sjögren et al., 2006).

3) Tumor necrosis factor-α (TNF-α)

Tumor necrosis factor-α is involved in the acute phase of systemic inflammation and also has critical roles for innate inflammatory response in vertebrates. TNF-α is mainly produced by activated macrophages but also can be produced by CD4+ lymphocytes, NK cells, neutrophils, mast cells, and eosinophils (Czermak et al., 1999; Heller et al., 1994). TNF-α is involved in various cellular processes including tissue remodeling and tumor necrosis (Goodsell, 2006). Dysregulation of TNF-α may induce a variety of diseases such as Alzheimer's disease (Swardfager et al., 2010), cancers (Locksley et al., 2001), major depression (Dowlati et al., 2010), and inflammatory bowel disease (IBD) (Brynskov et al., 2002).

Salivary TNF-α may originate from blood via ultrafiltration, or it can be produced by the salivary glands or focally infiltrated lymphoid cells. Also, it has been suggested that epithelial cells of the salivary glands take a role in the increase of TNF-α in the patients with Sjogren's syndrome (Wu et al., 1996).

Correlation between salivary and blood TNF-α has not been established yet. Although significant correlations between the level of the biomarkers in saliva and blood in patients with oral lichen planus were reported (Yuanyuan Zhang et al., 2008), no such correlation was observed in two other studies which subjected healthy individuals (Riis et al., 2014; Soto-Méndez et al., 2015).

4) C-reactive protein (CRP)

CRP is one of acute phase inflammatory protein synthesized by the liver, and it has been widely used as a biomarker for chronic inflammation (Hong et al., 2012; Ridker, 2003). The level of CRP is regulated by pro-inflammatory cytokines including IL-1, IL-6, and TNF-α (A. Kumar et al., 2011). Among them, IL-6 is considered the most critical activator (Volanakis, 2001). Several studies have indicated that an elevated level of CRP was associated with cardiovascular diseases, diabetes, autoimmune disorders, and malignancies (Kim et al., 2009). The presence of periodontitis in the oral cavity has been reported to increase the concentration of CRP in saliva (Giannobile et al., 2009; Higashi et al., 2008). Also, there has been increasing interest in utilizing CRP as a prognostic and pre-diagnostic biomarker for cancer (Kim et al., 2009). However, the role of CRP in the development and progression of oral cancer is still unclear.

Since the CRP is mainly produced in the liver, the concentration of CRP in saliva may reflect systemic inflammation. For instances, two studies reported a significant correlation between the level of CRP in blood and saliva (Byrne et al., 2013; Ouellet-Morin et al., 2011).

(2) Oxidative stress biomarkers in saliva

1) 8-Hydroxydeoxyguanosine (8-OHdG)

Reactive oxygen species (ROS) or reactive nitrogen species (RNS) attacks DNA and consequently produces chemical byproducts from purine and pyrimidine (Halliwell, 2000). The reaction of purine and ROS produces 8-hydroxydeoxyguanosine (8-OHdG). Although it is still controversial whether the 8-OHdG level can quantitatively reflect oxidative damage of DNA, the level of 8-OHdG in blood has been widely used to evaluate systemic oxidative DNA damage (Henderson et al., 2010).

Several studies tried to utilize 8-OHdG in saliva as a biomarker to evaluate DNA oxidative stress level in the oral cavity. One of them reported that there was a significant

correlation between periodontal health and concentration of 8-OHdG in saliva (Villa-Correa et al., 2015). Also, efforts have been made to explore whether the level of 8-OHdG in saliva reflect that in blood. According to the results using liquid chromatography/mass spectrometry study, a clear correlation between the level of 8-OHdG in saliva and blood was found in healthy individuals. Although these results were contradicting to the previous studies, the authors pointed that the technical errors such as inaccuracy of ELISA and interference by heavy weight molecules like carbohydrates and proteins might prevent previous researchers from discovering such correlation (Hu et al., 2010).

2) Malondialdehyde (MDA)

When ROS or RNS reacts with lipid, lipid hydroperoxides are formed, and the process of this reaction is called lipid peroxidation. Lipid peroxidation is a highly complicated reaction, and thus various byproducts are produced during the process (Dotan et al., 2004). Malondialdehyde (MDA), formed from fatty acid, is one of the most widely used lipid peroxidation biomarkers (Ayala et al., 2014). Although the most popular method to detect and quantify MDA is thiobarbituric acid reacting substances (TBARS) assay, developed by Yagi, aldehydes other than MDA also can be detected (Yagi, 1976). To overcome this limitation, new methods using LC or MS have been suggested, but still, conventional TBARS assay is considered as a reliable method (Akalın et al., 2007).

The level of MDA in saliva was increased in the patients with active caries or chronic periodontitis (Dinç et al., 2018; Guentsch et al., 2008; Saita et al., 2016). Only a few studies have been done for investigating the relationship between the concentration of MDA in saliva and other oral diseases. Also, the relationship between the level of MDA in saliva and blood has not yet been actively studied.

3) Total antioxidant capacity (TAC)

Since reactive species react with multifarious substances, it is not efficient to measure each individual anti-oxidative substance in a biological fluid. In addition, protective effect within a biological system is determined by the sum of individual antioxidation reaction. Therefore, measuring total antioxidant capacity rather than measuring individual ones has been considered as more rational and efficient way (Erel, 2004). However, since interpretation of results from total antioxidant capacity assay is not intuitive, cautions should be taken. For examples, when total antioxidant capacity is high, it can be interpreted as

healthy state, but this could be resulted by adaptive response due to long-term oxidative stress elevation. In opposite way, when total antioxidant capacity is decreased, it could be resulted from the lowered production of reactive species (Kamodyová et al., 2015).

Salivary total antioxidant capacity seems to reflect the local oxidative stress status of the oral cavity, rather than the systemic oxidative stress of the circulatory system (Lettrichová et al., 2016). According to the previous studies TAC was increased in the patients with dental caries (Hegde et al., 2009; D. Kumar et al., 2011; Preethi et al., 2010), but TAC is increased (Wei et al., 2010; Yoshizawa et al., 2013) or decreased (Baltacıoğlu et al., 2014; Sculley et al., 2003) in the patients with chronic periodontitis. Therefore relationship between pathologic conditions and salivary TAC seems very complicated.

3. Challenges of salivary diagnostics that should be overcome to have real clinical significance

(1) Standardization of saliva collection and analyze process

Since saliva is advantageous over other biological fluids, numerous attempts have been made to utilize saliva as a diagnostic fluid clinically (Pfaffe et al., 2011). However, clinical application of salivary diagnostics is limited by several obstacles. Above all, lack of standardized saliva collection and analysis process is one of the most critical limitations in salivary diagnostics.

Saliva can be categorized into unstimulated and stimulated saliva based on the conditions during the collection process. Also, saliva is categorized into whole saliva and glandular saliva based on the origin of collected saliva; the whole saliva represents the mixture of all glandular saliva, crevicular fluid, exfoliated epithelial cells, and microorganisms in the oral cavity, but glandular saliva only represents pure saliva produced by specific salivary glands, collected via cannulation of glandular ducts or collection devices (Navazesh, 1993). There are various methods to collect whole saliva; drooling, spitting, swabbing, and suction methods (Pfaffe et al., 2011). In this way, there may be differences in the results of saliva analysis depending on the types and methods of the various saliva collecting methods. Also, levels of salivary biomarkers such as IL-6 and CRPmay fluctuate depending on circadian rhythm (Boyer et al., 1976; Riis et al., 2014) and show intra and inter-individual variability (Lettrichová et al., 2016). Therefore, to study salivary biomarkers, strict conditioning of experimental subjects is required. First, participants' age, gender, and health

status should be controlled, and secondly, saliva should be collected at a specific time and condition. Finally, it is critical to select saliva collection methods suitable for experimental or clinical purposes. Also, cautions should be taken when using ELISA kit designed for blood diagnosis because the concentration of substances in saliva is relatively lower than that in blood. Many proteins, inhibitors, and enzymes mixed in saliva may also cause serious errors or misinterpretation.

However, up to present days, those cautions that should be taken during the collection and analysis of saliva are not always considered due to the several practical issues, and thus the levels of salivary biomarkers in normal conditions are inconsistent in many studies (Byrne et al., 2013; Cullen et al., 2015; Riis et al., 2014). If saliva collection and analysis processes are standardized, the clinical value and applicability will become more robust.

(2) Processing potential confounders

To apply salivary biomarkers in clinical diagnoses, it is critical to eliminate or exclude intraoral potential confounders. Local intraoral lesions may greatly undermine the accuracy and value of salivary diagnostics applied to diagnose systemic diseases. For example, salivary biomarkers such as CRP, MMP-8, MMP-9, and IL-1β are used to diagnose acute myocardial infarction, but their concentrations may be increased by periodontitis (Christodoulides et al., 2005; Górska et al., 2006; Herr et al., 2007; Miller et al., 2006). On the contrary, biomarkers used to detect intraoral diseases may be interfered by systemic diseases. For instance, salivary CRP and α2-macroglobulin levels are elevated in patients with post-traumatic stress disorder (Delaissé et al., 2000).

Blood contamination is another factor that may interfere with the accuracy of the levels of salivary biomarkers. Since the concentrations of biomarkers in blood is relatively higher than those in saliva, the levels of salivary biomarkers could be largely altered when the blood smear into saliva due to periodontitis or loss of oral mucosal integrity. Previous studies have pointed out that the alternation of blood contamination markers induces a significant change in salivary hormones, oxidative stress, and inflammatory biomarkers (Behr et al., 2017; Suh et al., 2009). Transferrin is one of the most commonly used indicators for blood contamination in saliva samples, and hemoglobin has been also suggested.

Based on these issues, it should be pointed out that it is difficult to evaluate oral or systemic diseases solely based on the increment or decrement of salivary biomarker levels. To apply salivary diagnostics on clinical fields, it is necessary to accurately evaluate many possible confounders.

4. Perspective

Field of salivary diagnostics is rapidly growing, enabling us to perform population-based screening, confirmatory diagnosis, monitoring disease prognosis, and evaluating treatment results. Like other diagnostic modalities, accuracy, cost-effectiveness, and ease of use will be the most important issue in salivary diagnostics. Particularly, when discovering biomarkers linked with disease onset and progression, validation of biomarkers should be prioritized to minimize false negative and false positive. However, most of the studies on saliva diagnosis are limited to simply discover individual biomarkers by cross-sectional design, so research for investigating the combination of biomarkers and the verification through long-term longitudinal studies are needed. Furthermore, targeted studies unveiling correlations among a distinct biological phase of the disease and a set of key biomarkers should be conducted. Way to normalize circadian or day to day variation and confounders also should be studied. Discovery of new salivary components such as transcriptome, microbiome, proteome, and exosome is another expanding area in salivary diagnostics.

As the importance of salivary diagnostics grows, the role of the dentist as an interpreter of diagnostic results will be more emphasized. Approximately 20% of the total American population regularly visit dentists, and it is higher than the number of patients who attends internal medicine doctor (Greenberg et al., 2010), considered as primary health care professionals. If salivary diagnostics gets successfully established as a reliable clinical tool, salivary POCT (Point of Care Testing) will rapidly grow, and dentists will be able to diagnose life-threatening diseases in advance. As mentioned previously, the pre-evaluation of confounders is critical and thus the clinical value of dentistry will be more emphasized in the overall scheme of primary health care.

III. MATERIALS AND METHODS

1. Participants

Thirty-seven healthy young men (26.7 ± 2.2 years) participated in this study; all of them were nonsmokers, had no specific oral diseases, and had no history of serious illness. To control for the influence of the menstrual cycle, female participants were excluded. In addition, participants with the following conditions were excluded: those taking medications that can affect salivary secretion in the past three months (e.g., psychiatric and neurological medications, antihistamines, anticholinergics, and antihypertensives), and those wearing dentures or orthodontic appliances.

2. Study design

All participants visited twice. On the first visit, unstimulated whole saliva (UWS) and venous blood samples were collected from the participants. Saliva samples were collected again from the same participants after two or three days. The research protocol was reviewed in compliance with the Helsinki Declaration and approved by the Institutional Review Board of the Seoul National University Dental Hospital (#CRI16015) on 1 Dec., 2016. Informed consent was obtained from all participants.

3. Collection of whole saliva and blood samples

All saliva samples were collected from 8:30 am to 9:30 am to minimize circadian variation in salivary composition. The participants were not allowed to eat or drink anything except water and to perform oral hygiene activities including tooth brushing on the day of collection. UWS was collected using the spitting method for 10 minutes. Salivary flow rates were recorded in mL/min. Cellular debris in saliva samples were removed by centrifuging at 4,000 x g for 20 minutes at 4℃ and supernatants were aliquoted and stored at -70℃ until further analyses. Overnight fasting blood samples were taken from 9:00 am to 10:00 am, i.e., immediately after the collection of saliva samples.

4. Determination of total protein concentration in saliva and blood samples

Total protein concentration in the saliva and blood samples was determined with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA) using bovine serum albumin as a standard.

5. Analyses of inflammatory and oxidative stress biomarkers in saliva and blood samples

C-reactive protein (CRP), IL-1β, IL-6, and TNF-α were included as inflammatory biomarkers and 8-hydroxy-2'-deoxyguanosine (8-OHdG), malondialdehyde (MDA), and total antioxidant capacity (TAC) were used as oxidative stress biomarkers. Immunoassay kits were used to quantify the inflammatory biomarkers in saliva (CRP, IL-1β, and IL-6, Salimetrics, State College, PA, USA; TNF-α, R&D Systems, Minneapolis, MN, USA) and blood (CRP, IL-1β, IL-6, and TNF-α, R&D Systems). Immunoassay kits (8-OHdG, Abcam, Cambridge, MA, USA; MDA, Cell Biolabs, San Diego, CA, USA) and an assay kit for measuring copper ion reduction activity (TAC, Abcam) were used to measure the concentrations of oxidative stress biomarkers in saliva and blood. The salivary secretion rate and protein proportional ratio of each biomarker were calculated by multiplying the corresponding salivary flow rates and dividing by the corresponding total protein concentrations, respectively.

6. Determination of blood contamination in saliva samples

Immunoassay kits were used to measure transferrin (Salimetrics) and hemoglobin (Avivasysbio, San Diego, CA, USA) concentrations in saliva samples for determination of the blood contamination level.

7. Statistics

Normal distribution of data was tested using the Kolmogorov-Smirnov normality test, and parametric and non-parametric tests were used according to the results. To analyze the reliability of the repeated saliva sampling, intra-class correlation (ICC) coefficients were analyzed. To analyze the differences between two different samples from the same subject, paired T-test and Wilcoxon signed rank test were used. The relationships between the components were analyzed with Pearson's and Spearman's correlation tests, then adjusted for multiple comparisons with the Bonferroni correction method. To determine the degree of association between saliva and blood, Spearman's correlation coefficients between the

biomarkers of saliva and blood were calculated. The Bland-Altman method (Bland and Altman, 1986) was also applied, plotting the differences between the normalized levels in saliva and blood (y-axis) versus the average of the normalized levels in saliva and blood (x-axis). In addition, to investigate the influence of blood contamination on the salivary biomarkers for inflammation and oxidative stress, the change in the saliva/blood ratio for each biomarker was evaluated according to the increase of transferrin and hemoglobin concentrations using the linear regression method. Statistical analyses were performed using SPSS software 20.0 (SPSS Inc., Chicago, IL, USA). *P*-values less than 0.05 were considered statistically significant.

IV. RESULTS

Salivary flow rate, mean concentrations, and the distributions of all examined biomarkers in the saliva and blood samples are shown in Table 1 and Fig. 1. The secretion rates and the ratios of biomarkers out of the total protein in saliva are shown in Table 2 and Table 3, respectively. There were no significant differences (P = 0.110 - 0.891) in the concentrations of all examined biomarkers between the saliva samples collected on two different visits. When comparing the concentrations in blood and saliva, total protein (d = 2.210, $P < 0.001$), CRP (d = 0.637, $P < 0.001$), and TAC (d = 10.187, $P < 0.001$) were more abundant in the blood while IL-1β (d = 1.025, $P < 0.001$), IL-6 (d = 0.645, $P < 0.001$), and MDA (d = 0.779, $P < 0.001$) were more abundant in saliva (Table 1). In contrast, the protein proportion of CRP (d = 0.322, $P < 0.001$) was higher in saliva (Table 3).

The ICC coefficients between the concentrations, secretion rates, and protein proportions of analytes in the saliva samples on both visits are given in Table 4. The ICC values for the concentrations of CRP and IL-6 were excellent (≥ 0.90). The ICC values for the salivary flow rate (0.880) and concentrations of MDA (0.872), total protein (0.797), IL-1β (0.706), transferrin (0.693), and TAC (0.664) were good. Those for hemoglobin (0.569) and 8-OHdG (0.517) were fair, whereas that of TNF-α was poor (0.098). Similar patterns were observed in the ICC values for the secretion rate and protein proportion.

Table 5 shows the correlations between the salivary flow rate, total protein, and biomarkers of inflammation, oxidative stress, and blood contamination. Among 55 total pairs, CRP/IL-6 and total protein/TAC showed statistically significant correlations in the saliva samples collected on both days. The correlations in the secretion rate were more prominent

(Table 6). Among the 45 pairs, five pairs showed statistically significant correlations in both saliva samples. The secretion rates of total protein, TNF-α, and TAC showed statistically significant correlations with each other in both samples. In addition, CRP/IL-6 and IL-1β/hemoglobin showed statistically significant correlations in both samples (Table 6).

In the context of protein proportion, a statistically significant correlation was found only between CRP and IL-6 (Table 7). It was noteworthy that a statistically significant relationship was found between CRP and IL-6 in terms of concentration, secretion rate, and protein ratio.

Table 8 shows the correlations between salivary and blood biomarkers on the first visit. IL-6 was the only biomarker that showed a statistically significant correlation between saliva and blood in the aspects of concentration ($r = 0.356$, $P = 0.031$) and protein proportion ($r = 0.416$, $P = 0.010$). This correlation was stronger and more significant for the protein ratio. The Bland-Altman graphs showed that when the average of the normalized levels of IL-6 or CRP in saliva and blood samples was close to zero, the difference between the normalized levels in saliva and blood samples was nearly zero. On the other hand, when the average of the normalized levels becomes larger than 0.2, the difference has a tendency to diverge (see Fig. 2).

It could be assumed that the saliva/blood ratio of total protein, CRP, and TAC would increase as the degree of blood contamination in saliva increased because these three components are highly concentrated in blood relative to saliva. As the salivary concentration of transferrin increased, the saliva/blood ratios of total protein ($P = 0.018$) and TAC ($P = 0.080$) were also elevated, however, that of CRP did not increase ($P = 0.869$). The increase in the concentration of salivary hemoglobin did not significantly affect the saliva/blood ratios of total protein ($P = 0.876$), CRP ($P = 0.300$), and TAC ($P = 0.433$) (Fig 3).

V. DISCUSSION

In this study, The reliability of saliva samples, correlations between the biomarkers in saliva and blood, and the influence of blood contamination on the saliva/blood ratios of biomarkers were evaluated.

The ICC coefficients of every biomarker except TNF-α in the saliva samples ranged from “fair” to “excellent” (Yen & Lo, 2002). Inflammatory biomarkers showed higher ICC

coefficients in saliva samples than oxidative stress biomarkers. These findings were consistent with previous studies. Although saliva showed lower reliability than blood (Riis et al., 2014), considerable inter-/intra- individual variability (Lettrichová et al., 2016), and higher sensitivity to emotional tasks (Riis, Granger, DiPietro, Bandeen‐Roche, & Johnson, 2015), the overall reliability of saliva samples was moderately high (Fernandez-Botran, Miller, Burns, & Newton, 2011; Izawa, Miki, Liu, & Ogawa, 2013; Lettrichová et al., 2016; Riis et al., 2014; Riis et al., 2015). One study, in which saliva samples were collected for 30 consecutive days and salivary oxidative stress biomarkers were analyzed, reported high variability in their concentrations (Lettrichová et al., 2016). Several studies have shown that the salivary levels of oxidative stress biomarkers can fluctuate due to local conditions rather than systemic states (Iannitti, Rottigni, & Palmieri, 2012; Zukowski, Maciejczyk, & Waszkiel, 2018). Immune mechanisms related to periodontal inflammation are one of the main sources of reactive oxygen species in the oral cavity. Alcohol and food consumption, smoking, and dental treatment and materials are also sources of oxidative stress in the mouth (Zukowski, Maciejczyk, & Waszkiel, 2018). Thus, the reliability of saliva samples as biomarkers of oxidative stress may be low. One possible explanation as to why the present study had higher reliability for oxidative stress biomarkers in the saliva than previous studies (Behuliak et al., 2009; Lettrichová et al., 2016) is that the collections of saliva samples were performed in a short interval of two to three days. The collection of saliva samples from healthy young adults, whose TNF-α concentrations may be too low to be detected by immunoassay kits, and the lack of specific immunoassay kits developed for TNF-α in saliva samples may have caused the low reliability of TNF-α. Additionally, alkaline phosphatase in saliva samples may have impaired the accuracy of the immunoassay.

The inter-correlational stability between different salivary biomarkers within each saliva sample collected on two different days was steadily maintained in terms of concentration, secretion rate, and protein proportion. Although biomarkers in the saliva samples collected from the same individual exhibited a certain level of reproducibility, some degree of variability was observed, e.g., the concentrations of IL-β and hemoglobin showed a statistically significant positive correlation only in the saliva collected on the first visit. Therefore, repeated measurements may be necessary to ensure the reliability of the saliva sample. In addition, although a statistically significance level was not reached, a relatively high correlation coefficient was observed between TNF-α and MDA in both samples collected on two different days. It could be explained by the influence of common local

factors such as periodontal inflammation (Gümüş, Nizam, Lappin, & Buduneli, 2014; Miricescu et al., 2014). Because young healthy male subjects were included in the present study, the correlation between the two biomarkers could not be strong.

IL-6 was the only biomarker that showed a significant, albeit, weak correlation between saliva and blood. Some studies have suggested that the IL-6 level in saliva reflects that in the blood (Aleksandra Nielsen, Nederby Nielsen, Schmedes, Brandslund, & Hey, 2005; Fernandez-Botran et al., 2011; Williamson, Munro, Pickler, Grap, & Elswick, 2012; Zhang et al., 2008). One study reported that IL-6 in saliva and blood showed a weak positive correlation in healthy individuals (Williamson et al., 2012). However, the sample collection time was not controlled in the study and a lower correlation coefficient ($r = 0.31$, $P < 0.05$) than the result of the present study was reported. A significant positive correlation has also been reported between the levels of IL-6 in saliva and blood in patients with ulcerative colitis (Aleksandra Nielsen et al., 2005) and oral lichen planus (Zhang et al., 2008). In contrast, some studies have reported that there were no significant correlations between the levels of IL-6 in saliva and blood in healthy children (Soto-Méndez et al., 2015), adolescent girls (Riis et al., 2014), and adults under a broad range of psychological states (Sjögren, Leanderson, Kristenson, & Ernerudh, 2006), as well as before and after strenuous exercise (Cullen et al., 2015). However, the studies that failed to report significant correlations between salivary and blood IL-6 levels did not control saliva collection timing to exclude the effects of circadian rhythm or did not use immunoassay kits developed for saliva samples. In two of the studies that described the sample collection time, saliva samples were collected around noon in one (Riis et al., 2014), and the subjects were asked to control sample collection time and to handle the collected samples by themselves in the other (Sjögren et al., 2006), which might impair the credibility of the data. The salivary concentrations of IL-6 and CRP in healthy adults show a diurnal pattern, which is highest early in the morning (7:00 to 10:00 am) and low during the daytime (Izawa et al., 2013). Therefore, lack of control over saliva collection time in these studies might hinder the correlation between salivary and plasmatic levels of IL-6. In addition, the concentrations of biomarkers in saliva are usually low, and saliva contains various substances that might interfere with reactions in immunoassay kits developed for blood. Indeed, two studies (Riis et al., 2014; Soto-Méndez et al., 2015) used the multiplex immunoassay method, which has several advantages over singleplex immunoassay. However, the method has some disadvantages due to the difficulty of quality control and the possibility

of cross-reactivity (Tighe, Ryder, Todd, & Fairclough, 2015). Furthermore, the imprecision was even greater in saliva compared to blood (Browne et al., 2013).

Several studies involving healthy or depressed participants have shown that CRP in saliva could have a strong correlation with that in blood under certain conditions, e.g., individuals with high serum CRP [more than 7338.66 (Byrne et al., 2013) and 2994.68 ng/mL (Ouellet-Morin, Danese, Williams, & Arseneault, 2011)] showed a strong correlation between salivary and serum CRP levels while those with low serum CRP showed weak or no such correlation (Byrne et al., 2013; Ouellet-Morin et al., 2011). Based on these findings, it could be explained that the lack of significant correlation between the levels of salivary and blood CRP in the population of healthy young adults was related with low average serum CRP concentration (805.2 ± 1423.1 ng/mL). In addition, these findings suggest to us that research on salivary diagnostics should be performed for individual disease and health conditions, demographic characteristics, and biomarkers.

Blood contamination in saliva can significantly hinder the sensitivity and specificity of salivary diagnostics and is one of the most important but underestimated issues in this field. Transferrin has been widely used as a biomarker to identify the presence of blood contamination in saliva samples (Behr et al., 2017; Kang, Kim, Chang, & Kho, 2017; Kivlighan et al., 2004; Schwartz & Granger, 2004; Suh, Kim, & Kho, 2009). As the amount of transferrin in the saliva increases, so do the saliva/blood ratios of total protein and TAC concentrations. These results confirm that the level of salivary transferrin can be a good indicator for blood contamination in saliva. In contrast, the expected trend for CRP was not observed, possibly because the concentrations of CRP in saliva and blood collected from healthy adults were lower than those reported in other studies (Byrne et al., 2013; Ouellet-Morin et al., 2011). It may also be due to the incompleteness of transferrin as a blood contamination biomarker. In fact, in addition to the amount of blood contamination, other factors that affect the concentration of transferrin in whole saliva have been reported. One animal study showed that acinar cells in the parotid glands produce transferrin and that the transferrin produced by hepatocytes can also be actively transported to parotid gland acinar cells (Nashida, Yoshie, Imai, & Shimomura, 2009). Additionally, salivary flow rate, gonadal hormones, age, chewing performance, and oral microorganisms can affect the concentration of transferrin in saliva (Kang, Lee, & Kho, 2018), which could add complexity to the interpretation of results. Hemoglobin has been proposed as another biomarker capable of displaying blood contamination in saliva samples (Choi, Kim, & Donnelly, 2018; Nam et al.,

2015). The result of the present study, however, showed that hemoglobin may not be as useful as transferrin as a biomarker of blood contamination in saliva samples. However, It could not be fully explained why hemoglobin did not effectively reflect blood contamination in saliva. The immunoassay for hemoglobin detects hemoglobin molecules released from hemolyzed RBC in hypotonic saliva. Therefore, the molecular stability of hemoglobin in saliva might affect the assay results. Further studies are required on this issue.

There are limitations in the present study. First, the results of this study are limited to men, not women because gender differences have been reported in salivary levels of oxidative stress biomarkers (Lettrichová et al., 2016). Second, the severity of gingival inflammation was not evaluated in this study. Instead, the salivary levels of transferrin and hemoglobin were analyzed. A previous study has reported the relationship between the level of transferrin in saliva and the severity of gingival inflammation (Kang, Lee, & Kho, 2018). Not only that, the present study included only healthy young males at the age of 20's who usually have low levels of gingival inflammation. Finally, although IL-6 shows a significant correlation between saliva and blood, the possibility of local production, e.g. from the salivary glands, should always be considered.

VI. CONCLUSIONS

The analyses of salivary levels of inflammatory and oxidative stress as well as blood contamination biomarkers were reproducible. However, the investigated salivary inflammatory and oxidative stress biomarkers, with the exception of IL-6, did not reflect the biomarkers present in blood. The level of IL-6 showed a significant, but relatively low, correlation between saliva and blood. The saliva/blood ratios of the biomarkers more concentrated in blood than saliva tended to increase as the concentration of transferrin increased in saliva samples.

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Table 1. Salivary flow rate and concentrations of total protein and inflammatory, oxidative stress, and blood contamination biomarkers in saliva and concentrations of total protein and inflammatory and oxidative stress biomarkers in blood

N = 37	Saliva at visit 1	Saliva at visit 2	*P* value [a]	Saliva average	Blood at visit 1	*P* value [b]
Salivary flow rate (mL/min) - unstimulated whole saliva	0.47 ± 0.23	0.52 ± 0.24	0.021	0.50 ± 0.24		
Total protein (mg/mL)	0.99 ± 0.40	0.98 ± 0.35	0.695	0.99 ± 0.37.	116.1 ± 14.4	<0.001
Inflammatory marker						
CRP (ng/mL)	15.9 ± 23.4	14.7 ± 21.6	0.434	15.3 ± 22.4	805.2 ± 1423.1	<0.001
IL-1β (pg/mL)	267.5 ± 260.8	220.4 ± 187.0	0.377	243.9 ± 226.6	0.12 ± 0.01	<0.001
IL-6 (pg/mL)	35.2 ± 53.3	28.7 ± 43.1	0.158	31.9 ± 48.2	1.00 ± 0.82	<0.001
TNF-α (pg/mL)	1.40 ± 2.40	1.77 ± 4.39	0.546	1.59 ± 3.51	1.82 ± 2.14	0.280
Oxidative stress marker						
8-OHdG (ng/mL)	51.3 ± 50.1	70.1 ± 101.9	0.856	60.7 ± 80.3	54.7 ± 7.0	0.040
MDA (pmol/mL)	392.8 ± 401.1	438.7 ± 452.4	0.777	415.7 ± 425.2	78.7 ± 38.7	<0.001
TAC (mmol/L)	2.09 ± 0.85	2.11 ± 0.66	0.913	2.10 ± 0.76	163.2 ± 15.8	<0.001
Blood contamination marker						
Transferrin (mg/dL)	1.89 ± 1.46	2.31 ± 1.82	0.509	2.10 ± 1.65		
Hemoglobin (μg/dL)	113.3 ± 149.5	73.8 ± 115	0.145	93.6 ± 134		

CRP, C-reactive protein; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; MDA, malondialdehyde; TAC, total antioxidant capacity

[a] The Wilcoxon signed rank test was used to analyze differences between the variables of Saliva at visit 1 and Saliva at visit 2.

[b] The Wilcoxon signed rank test was used to analyze differences between the variables of Saliva at visit 1 and Blood at visit 1.

Table 2. Secretion rates of total protein and inflammatory, oxidative stress, and blood contamination biomarkers in saliva

N = 37	Visit 1	Visit 2	*P* value	Average
Total protein (mg/min)	0.43 ± 0.23	0.47 ± 0.20	0.093	0.45 ± 0.22
Inflammatory marker				
CRP (ng/min)	6.60 ± 9.45	6.83 ± 9.48	0.099	6.71 ± 9.40
IL-1β (pg/min)	127.8 ± 149.3	109.8 ± 118.3	0.934	118.8 ± 134.1
IL-6 (pg/min)	16.1 ± 26.7	14.3 ± 20.5	0.952	15.2 ± 23.7
TNF-α (pg/min)	0.72 ± 1.29	1.01 ± 2.90	0.261	0.87 ± 2.23
Oxidative stress marker				
8-OHdG (ng/min)	21.8 ± 17.6	29.8 ± 33.6	0.267	25.8 ± 26.9
MDA (pmol/min)	216.7 ± 292.7	226.7 ± 279.5	0.958	221.7 ± 284.2
TAC (nmol/min)	941.4 ± 545.8	1026.1 ± 440.3	0.099	983.8 ± 494.3
Blood contamination marker				
Transferrin (μg/min)	7.55 ± 5.84	10.70 ± 9.10	0.099	9.13 ± 7.76
Hemoglobin (ng/min)	6.23 ± 9.40	3.94 ± 7.19	0.315	5.09 ± 8.39

CRP, C-reactive protein; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; MDA, malondialdehyde; TAC, total antioxidant capacity

The Wilcoxon signed rank test was used to analyze differences between the variables of Saliva at visit 1 and Saliva at visit 2.

Table 3. Protein proportions of inflammatory and blood contamination biomarkers in saliva and blood

N = 37	Saliva at visit 1	Saliva at visit 2	*P* value [a]	Saliva average	Blood	*P* value [b]
Inflammatory marker						
CRP (x 10^{-5} in saliva) (x 10^{-5} in blood)	1.68 ± 2.44	1.84 ± 3.11	0.988	1.76 ± 2.77	0.72 ± 1.32	0.013
IL-1β (x 10^{-4} in saliva) (x 10^{-6} in blood)	2.86 ± 2.90	2.44 ± 2.35	0.327	2.65 ± 2.63	1.07 ± 0.13	<0.001
IL-6 (x 10^{-5} in saliva) (x 10^{-6} in blood)	4.05 ± 6.79	3.72 ± 6.36	0.198	3.89 ± 6.54	8.96 ± 7.92	0.001
TNF-α (x 10^{-6} in saliva) (x 10^{-5} in blood)	1.54 ± 2.55	1.89 ± 4.59	0.898	1.72 ± 3.69	1.64 ± 1.96	<0.001
Blood contamination marker						
Transferrin (x 10^{-2})	2.00± 1.39	2.40 ± 1.88	0.255	2.20 ± 1.65		
Hemoglobin (x 10^{-5})	1.24 ± 1.79	0.81 ± 1.25	0.105	1.02 ± 1.55		

CRP, C-reactive protein

[a] The Wilcoxon signed rank test was used to analyze differences between the variables of Saliva at visit 1 and Saliva at visit 2.

[b] The Wilcoxon signed rank test was used to analyze differences between the variables of Saliva at visit 1 and Blood at visit 1.

Table 4. ICC coefficients between total protein and biomarkers of inflammation, oxidative stress, and blood contamination in saliva samples on the first and second visits in terms of concentration, secretion rate, and protein proportion.

N = 37	ICC coefficient (95% CI)					
	Concentration	*P* value	Secretion rate	*P* value	Protein proportion	*P* value
Total protein	0.797 (0.606-0.895)	<0.001	0.672 (0.362-0.831)	0.001		
CRP	0.971 (0.943-0.985)	<0.001	0.966 (0.934-0.983)	<0.001	0.963 (0.927-0.981)	<0.001
IL-1β	0.706 (0.428-0.848)	<0.001	0.759 (0.532-0.876)	<0.001	0.837 (0.683-0.916)	<0.001
IL-6	0.929 (0.862-0.963)	<0.001	0.890 (0.786-0.943)	<0.001	0.922 (0.849-0.960)	<0.001
TNF-α	0.098 (-0.751-0.536)	0.379	0.096 (-0.756-0.535)	0.382		
8-OHdG	0.517 (0.062-0.751)	0.016	0.553 (0.132-0.770)	0.009		
MDA	0.872 (0.752-0.934)	<0.001	0.874 (0.755-0.935)	<0.001		
TAC	0.664 (0.348-0.827)	0.001	0.701 (0.420-0.846)	<0.001		
Transferrin	0.693 (0.403-0.842)	<0.001	0.430 (-0.107-0.706)	0.048	0.590 (0.203-0.789)	0.005
Hemoglobin	0.569 (0.163-0.778)	0.007	0.588 (0.200-0.788)	0.005	0.547 (0.120-0.767)	0.010

CRP, C-reactive protein; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; MDA, malondialdehyde; TAC, total antioxidant capacity
ICC, Intra-class correlation; CI, Confidence interval

Table 5. Correlations between the salivary flow and concentration of total protein and inflammatory, oxidative stress, and blood contamination biomarkers in saliva

(A) Saliva at visit 1

N = 37	Total protein	CRP	IL-1β	IL-6	TNF-α	8-OHdG	MDA	TAC	Transferrin	Hemoglobin
Flow rate	-.364	-.104	-.006	.073	.350	-.117	.356	-.215	-.463	.249
Total protein		.002	.308	-.016	.111	.471	-.018	.616*	.248	.273
CRP			.166	.878*	-.067	.083	-.092	-.037	-.044	.298
IL-1β				.176	.060	.106	-.073	.085	.103	.558*
IL-6					-.006	-.015	-.050	-.084	-.080	.435
TNF-α						-.130	.353	.243	.245	.116
8-OHdG							.253	.368	-.046	-.056
MDA								.105	-.019	-.248
TAC									.315	.045
Transferrin										-.031

(B) Saliva at visit 2

N = 37	Total protein	CRP	IL-1β	IL-6	TNF-α	8-OHdG	MDA	TAC	Transferrin	Hemoglobin
Flow rate	-.518	-.158	-.208	.010	.144	-.195	-.111	-.419	-.310	-.070
Total protein		-.183	.237	-.287	.278	.282	.189	.764*	.375	.163
CRP			.061	.867*	-.153	.051	-.165	-.221	.148	.256
IL-1β				.014	-.037	.118	-.097	.038	.022	.477
IL-6					-.159	-.082	-.221	-.304	-.071	.328
TNF-α						-.150	.327	.257	-.099	-.168
8-OHdG							-.117	.021	.283	.094
MDA								.321	-.020	-.278
TAC									.366	-.060
Transferrin										-.160

Statistical analysis was performed using Spearman's correlation test and the Bonferroni correction of α was applied to control the multiplicity problem. Consequently, $^{*}P < 0.000909$ was considered statistically significant.

CRP, C-reactive protein; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; MDA, malondialdehyde; TAC, total antioxidant capacity

Table 6. Correlations between secretion rates of total protein and inflammatory, oxidative stress, and blood contamination biomarkers in saliva

(A) Saliva at visit 1

N = 37	CRP	IL-1β	IL-6	TNF-α	8-OHdG	MDA	TAC	Transferrin	Hemoglobin
Total protein	.107	.485	.188	.603*	.596*	.469	.836*	.115	.522*
CRP		.299	.903*	.175	.174	.043	.271	-.078	.376
IL-1β			.326	.354	.375	.204	.420	.089	.616*
IL-6				.235	.141	.094	.303	-.007	.475
TNF-α					.239	.521*	.739*	.384	.343
8-OHdG						.491	.575*	.000	.296
MDA							.589*	.198	-.024
TAC								.148	.383
Transferrin									.113

(B) Saliva at visit 2

N = 37	CRP	IL-1β	IL-6	TNF-α	8-OHdG	MDA	TAC	Transferrin	Hemoglobin
Total protein	.029	.350	.025	.701*	.308	.237	.808*	.372	.355
CRP		.071	.885*	.124	.062	-.055	.018	.106	.270
IL-1β			.104	.205	.186	-.029	.197	.029	.569*
IL-6				.115	-.046	-.083	.029	-.065	.371
TNF-α					.164	.449	.719*	.203	.143
8-OHdG						.086	.149	.351	.263
MDA							.461	-.023	-.164
TAC								.268	.200
Transferrin									-.136

Statistical analysis was performed using Spearman's correlation test and the Bonferroni correction of α was applied to control the multiplicity problem. Consequently, $^{*}P < 0.001111$ was considered statistically significant.

CRP, C-reactive protein; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; MDA, malondialdehyde; TAC, total antioxidant capacity

Table 7. Correlations between protein proportions of inflammatory and blood contamination biomarkers in saliva

(A) Saliva at visit 1

N = 37	IL-1β	IL-6	TNF-α	Transferrin	Hemoglobin
CRP	.117	.847*	.031	-.075	.272
IL-1β		.166	-.005	.002	.435
IL-6			.073	-.086	.393
TNF-α				.319	-.046
Transferrin					-.149

(B) Saliva at visit 2

N = 37	IL-1β	IL-6	TNF-α	Transferrin	Hemoglobin
CRP	.090	.916*	.090	.177	.316
IL-1β		.069	-.086	-.080	.460
IL-6			.106	-.013	.392
TNF-α				-.127	-.159
Transferrin					-.279

Statistical analysis was performed using Spearman's correlation test and the Bonferroni correction of α was applied to control the multiplicity problem. Consequently, $^{*}P < 0.003333$ was considered statistically significant.

CRP, C-reactive protein

Table 8. Correlations between concentrations of total protein and inflammatory and oxidative stress biomarkers in saliva and blood and correlations between protein proportions of inflammatory biomarkers in saliva and blood

N = 37	Concentration		N = 37	Protein proportion	
	Pearson's r	Significance		Spearman's r	Significance
Total protein	0.043	0.802			
CRP	-0.171	0.313	CRP	-0.169	0.318
IL-1β	0.093	0.583	IL-1β	-0.046	0.787
IL-6	0.356	0.031*	IL-6	0.416	0.010*
TNF-α	-0.148	0.382	TNF-α	-0.152	0.368
8-OHdG	-0.285	0.088			
MDA	-0.010	0.954			
TAC	-0.017	0.921			

* $P < 0.05$

CRP, C-reactive protein; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; MDA, malondialdehyde; TAC, total antioxidant capacity

Figure legends

Fig. 1. Distribution of the levels of total protein, CRP, IL-1β, IL-6, TNF-α, TAC, 8-OHdG, MDA, transferrin, and hemoglobin in the saliva and blood samples.

CRP, C-reactive protein; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; MDA, malondialdehyde; TAC, total antioxidant capacity

Fig. 2. The Bland-Altman graphs for the levels of biomarkers in saliva and blood samples. All values of both samples were normalized. The x-axis shows the average level of saliva and blood, whereas the y-axis represents differences between levels in saliva and blood. The limits of agreement (mean difference ± 2 standard deviations) are indicated by dotted horizontal lines.

CRP, C-reactive protein; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; MDA, malondialdehyde; TAC, total antioxidant capacity

Fig. 3. Changes in the saliva/blood ratios of total protein, CRP, and TAC with increases in the concentrations of transferrin and hemoglobin.

The solid line indicates the trend line of linear regression, and the dotted lines indicate the 95% confidence band.

CRP, C-reactive protein; TAC, total antioxidant capacity

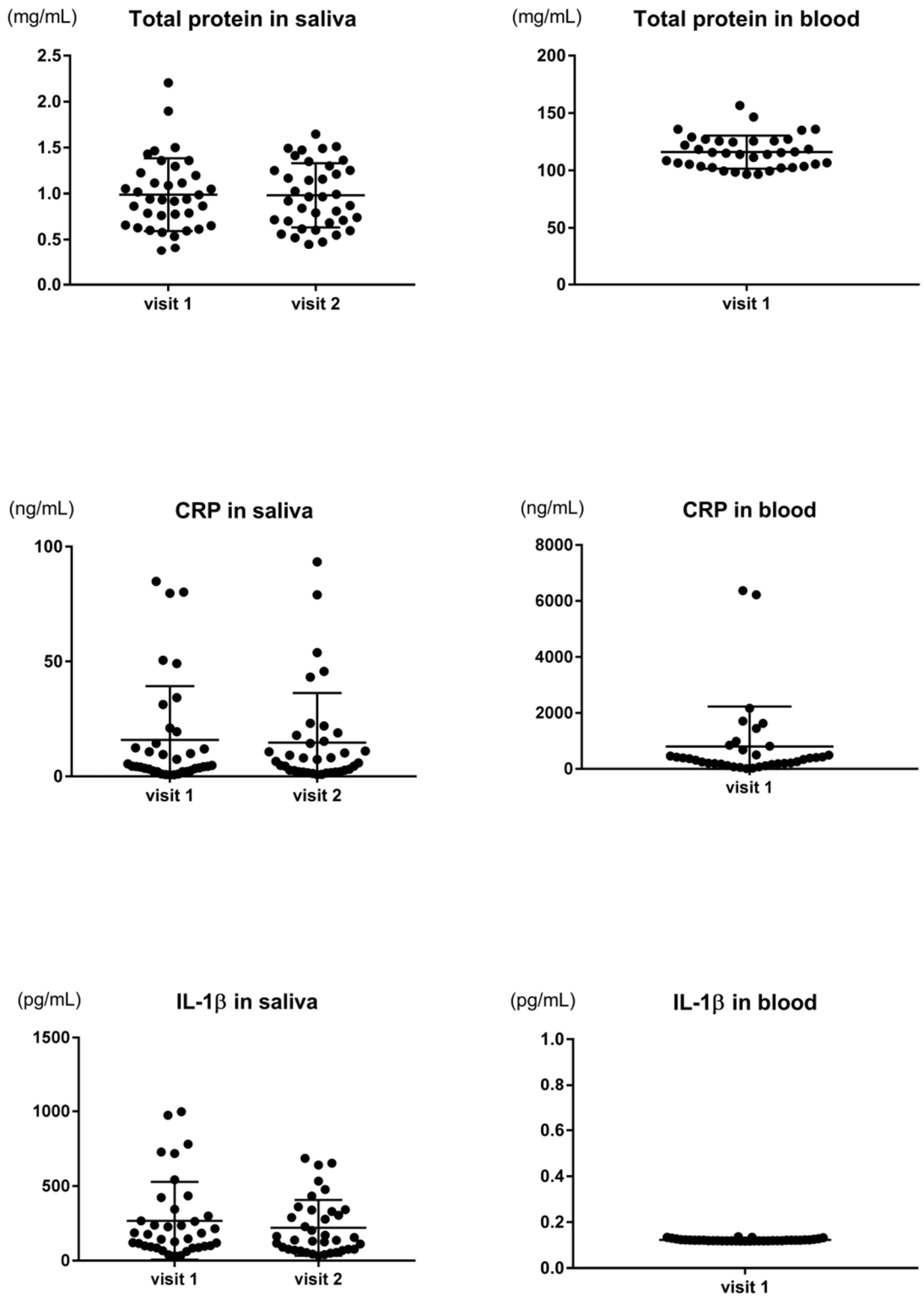
(mg/mL)
Total protein in saliva
2.5
2.0
1.5
1.0
0.5
0.0
visit 1
visit 2
(mg/mL)
Total protein in blood
200
150
100
50
0
visit 1
(ng/mL)
CRP in saliva
100
50
0
visit 1
visit 2
(ng/mL)
CRP in blood
8000
6000
4000
2000
0
visit 1
(pg/mL)
IL-1β in saliva
1500
1000
500
0
visit 1
visit 2
(pg/mL)
IL-1β in blood
1.0
0.8
0.6
0.4
0.2
0.0
visit 1

Fig. 1.

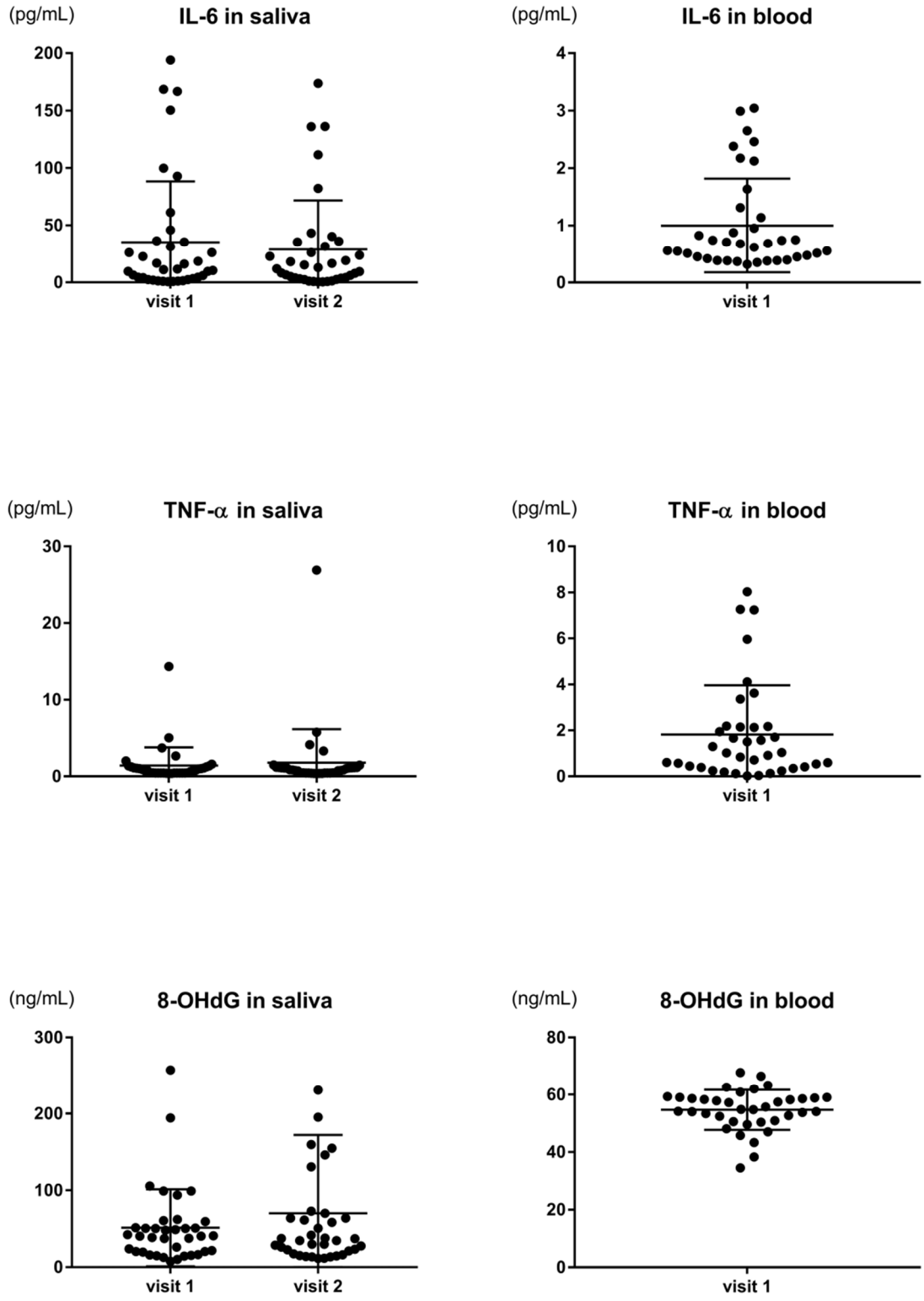

Fig. 1. (Continued)

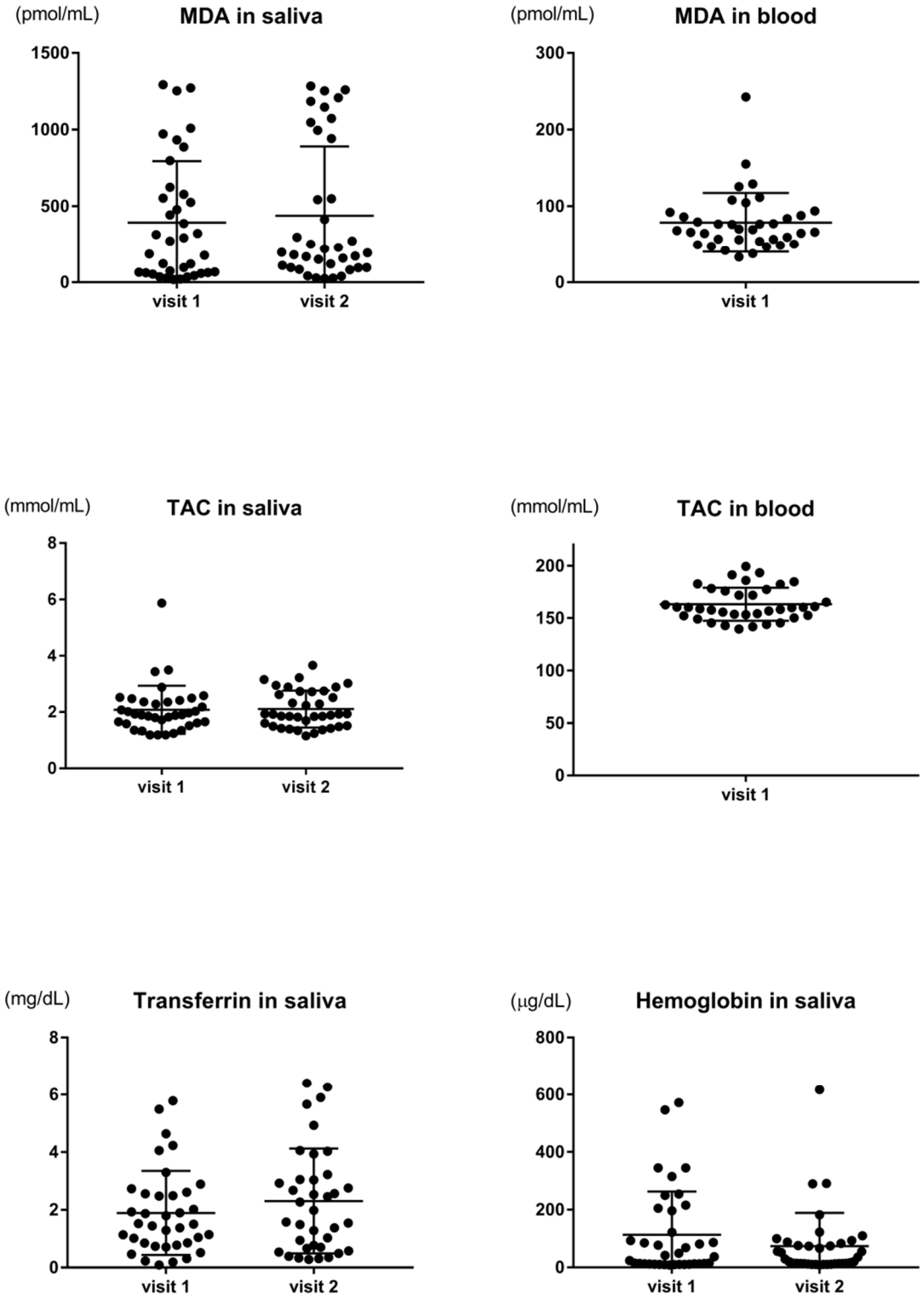

Fig. 1. (Continued)

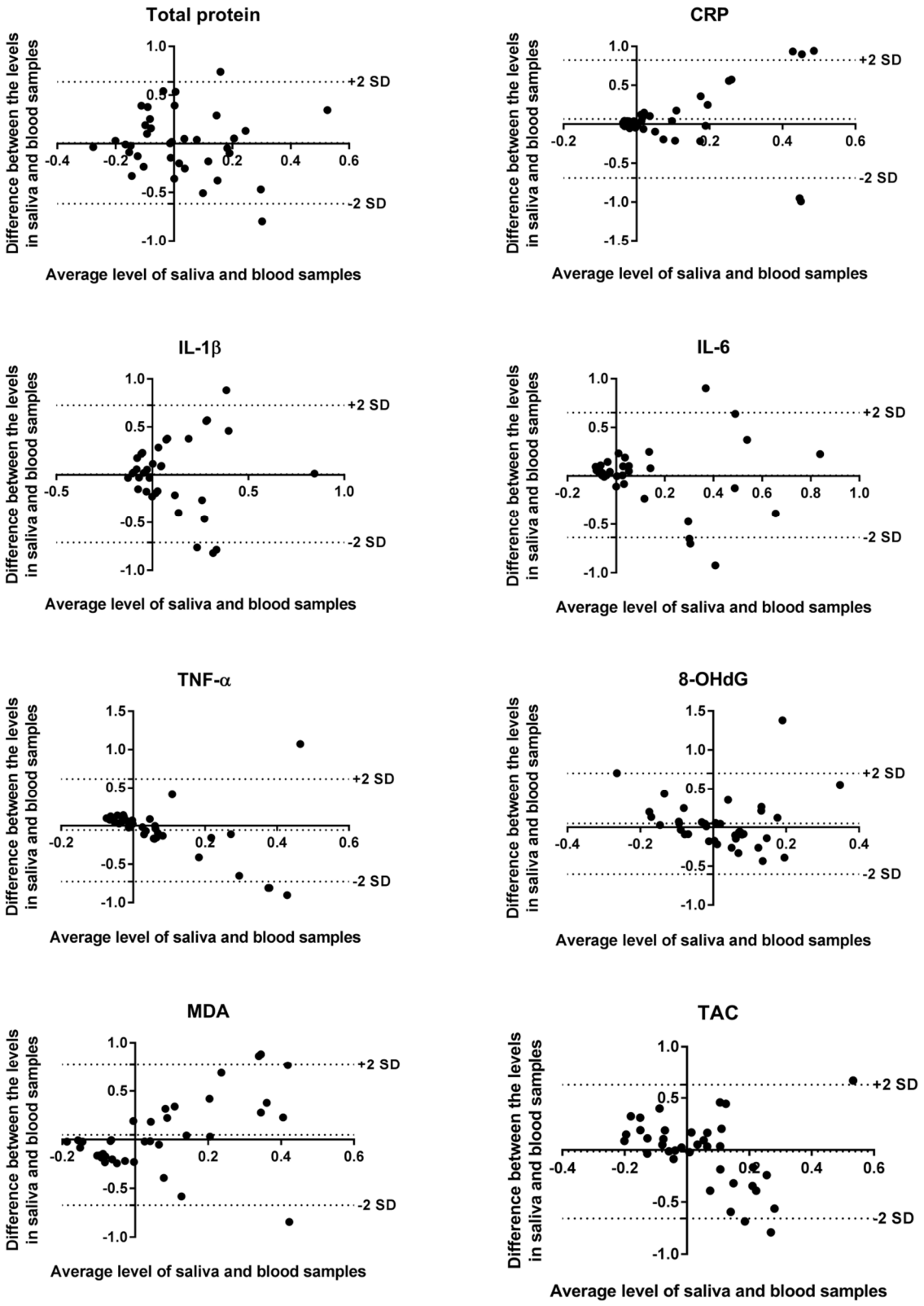
Total protein
Difference between the levels in saliva and blood samples
Average level of saliva and blood samples
+2 SD
-2 SD
CRP
IL-1β
IL-6
TNF-α
8-OHdG
MDA
TAC

Fig. 2.

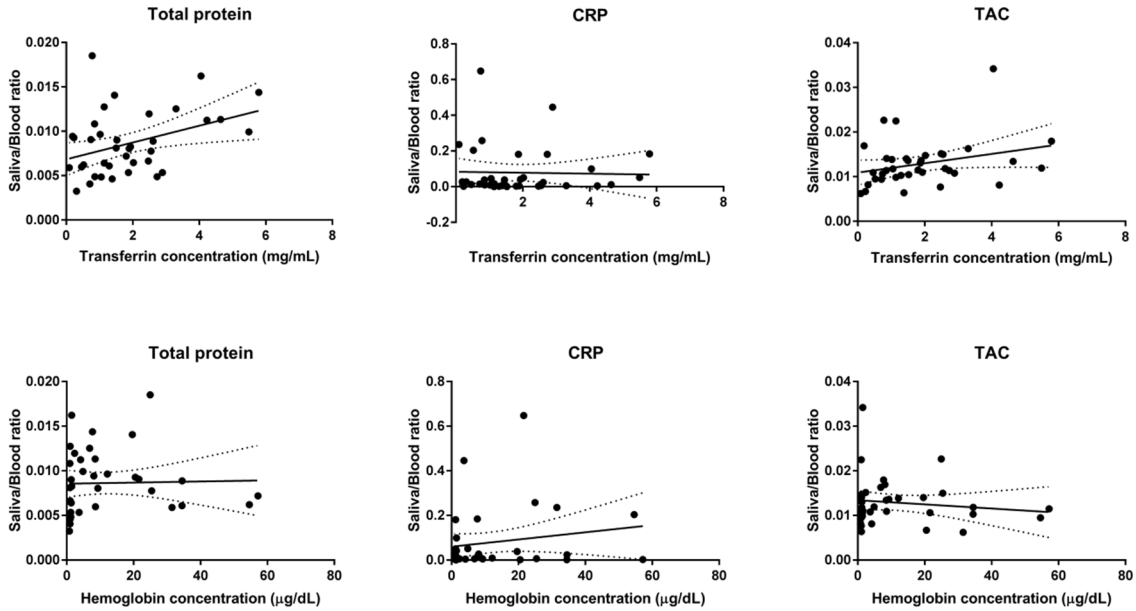
Total protein
CRP
TAC
Saliva/Blood ratio
Transferrin concentration (mg/mL)
Hemoglobin concentration (μg/dL)

Fig. 3.

국문초록

성인 남성의 타액 내 염증 및 산화 스트레스 표지자 분석 시 타액과 혈액 검체의 관련성에 관한 연구

남 윤

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타액 검체의 수집은 비침습적이고, 비용 효율적이며, 안전하고, 반복 채취가 용이하므로 타액을 질병 및 건강 상태 평가를 위한 진단 검체로 활용하려는 노력이 지속되어 왔다. 특히 여러 질병 및 노화 과정에 관련된 염증 및 산화 스트레스 생체 표지자의 타액 내 농도는 여러 질병 및 노화 과정에 관련되어 있음이 기존의 연구를 통해 밝혀져 있으므로 질병 및 건강상태 모니터링 등에 활용 가능성이 크다.

그러나 타액의 수집, 저장 및 보관 과정의 표준화가 아직 확립되지 않았고, 건강 상태에 따른 타액 내 생체 표지자들의 표준 농도가 아직 확립되지 않았다는 한계를 가지고 있어 실제 임상에의 적용은 제한적이다. 타액 내 생체 표지자의 분석이 진정한 임상적 가치를 가지기 위해서는 반복적으로 수집된 타액 검체에서 재현가능한 결과가 얻어져야 하며, 타액 내 생체 표지자의 농도와 혈액 내 생체 표지자의 농도 간의 상관관계가 예측 가능해야 한다. 또한, 일반적으로

타액 내의 생체 표지자 농도는 혈액 내 보다 매우 낮은 경향이 있으므로, 치은의 염증 혹은 구강 점막의 연속성이 훼손되어 혈액이 구강 내로 노출 되는 경우, 타액 내 생체 표지자의 농도가 크게 증가될 수 있다. 따라서 타액 내의 혈액 오염 정도와 그에 따른 타액 내 생체 표지자의 영향을 평가하는 방법을 개발해야 한다.

본 연구에서는 건강한 성인에서 염증 및 산화 스트레스 생체 표지자를 대상으로 타액 검체의 신뢰도, 타액 검체와 혈액 검체의 관련성 및 타액 검체의 혈액 오염이 타액 검체 내 생체 표지자 분석에 미치는 영향을 조사하는 것을 목적으로 하였다.

본 연구는 건강한 37 명의 남성(평균 나이 26.7 ± 2.2 세)을 대상으로 하였으며 첫 방문 시에는 비자극 전타액과 정맥혈을, 2-3 일 후 두 번째 방문 시에는 비자극 전타액을 채취하였다. 이를 토대로 타액과 혈액 내의 총 단백질, 염증성 물질 [C-reactive protein (CRP), IL-1β, IL-6 및 TNF-α], 산화 스트레스 물질 [8-hydroxy-2'-deoxyguanosine (8-OHdG), malondialdehyde (MDA) 및 총 항산화 용량 (total Antioxidant Capacity, TAC)]과 혈액 오염 표지자 물질 (transferrin 및 hemoglobin) 농도를 분석하였다.

타액 검체의 신뢰도와 관련하여, TNF-α를 제외한 모든 생체 표지자의 급내상관 (intra-class correlation) 수준이 신뢰성 있게 나타났으며 두 번의 방문 시 채취된 타액 검체 모두에서 CRP 와 IL-6 및 총 단백질과 총 항산화 용량 간의 통계적으로 유의미한 양의 상관관계가 나타났다. 또한, 타액 검체의 IL-6 농도는 혈액 검체의 IL-6 농도와 유의미한 상관관계를 보였으며, 혈액 오염

표지자인 타액 내 transferrin 농도가 증가할수록, 총 단백질과 총 항산화 용량의 타액/혈액 비율이 함께 증가되었다. 반면, 타액 내 hemoglobin 의 농도는 본 연구에서 분석된 생체 표지자의 타액/혈액 비율에 영향을 주지 못하였다.

결론적으로 본 연구를 통해 타액 분석 결과가 재현가능하며 신뢰성이 있음을 확인하였다. IL-6 는 본 연구에서 포함된 여러 생체 표지자 중 타액과 혈액 농도 간에 유의미한 상관관계를 보인 유일한 생체 표지자였으며, 건강한 젊은 성인 남성에서 transferrin 이 hemoglobin 에 비해 더 신뢰할 수 있는 혈액 오염의 생체 표지자인 것으로 확인되었다. 그러나, 본 연구는 건강한 젊은 남성만을 대상으로 시행되었으므로 연구결과 해석에 제한이 있다. 따라서 향후 본 분야의 임상 적용을 확립하기 위해서는 다양한 연령 및 성별에서 여러 건강 및 질병 상태를 대상으로 각 생체 표지자 들에 대한 후속 연구가 수행되어야 한다.

주요어: 생체 표지자, 타액, 혈액, 염증, 산화 스트레스, 혈액 오염

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