

学位論文

**Age-related changes in human gingival blood flow parameters
measured using novel gingival blood flow measurement
technique based on laser speckle flowgraphy (LSFG)**

(レーザースペックルフローグラフィ(LSFG)を基にした
新規歯肉血流計測技術を用いて評価したヒト歯肉血流の加齢変化)

北里大学大学院医療系研究科分子薬理学
指導教授 馬嶋 正隆

申請者 大杉 侑子

著者の宣言

本学位論文は、著者の責任において実験を遂行し、得られた真実の結果に基づいて正確に作成したものに相違ないことをここに宣言する。

要旨

歯周病は糖尿病等の全身性慢性疾患の発症リスクを高めることが報告されており、口腔内の健康維持が全身の健康維持に有効であると考えられている。歯周病の症状である歯肉炎症時には、歯肉の血管構造、血流動態等が変化するため、歯肉の血流動態は口腔内の健康状態を把握するための重要な指標となり得ると考えられる。歯周病発症と歯肉の血流動態変化との因果関係は明らかではないが、歯周病患者数は加齢に伴い増加することから、歯肉の血流動態の加齢変化を明らかにすることは重要である。しかし、これまで安静時のヒト歯肉血流を個体間比較できる技術はなく、ヒト歯肉の血流動態の加齢に伴う変化は明らかでなかった。

本研究では、眼底血流測定に応用されるレーザースペックルフローグラフィ (LSFG) に着目した。LSFG は、二次元で広範囲の血流を非接触で連続的に測定でき、さらに、心臓の拍動に伴う血流の立ち上がりから立ち下りまでの変動を波形解析することで、測定部位に刺激を与えずに血流動態を測定することが可能である。その上、LSFG で得られる血流動態の指標は心拍で基準化されており、個体間の比較を行うことが可能である。

本研究ではまず、この LSFG の技術を基にしたヒト歯肉血流の測定技術を開発した。さらに、開発技術を用いて健常男性の安静時、および加温刺激時の歯肉血流を測定し、加齢に伴う歯肉の血流動態変化を明らかにした。

1. LSFG を基にした歯肉血流測定技術の開発

まず、唾液で濡れた歯肉表面の血流測定を可能にするために、LSFG に偏光フィルターを導入した。偏光フィルターを用いて唾液による鏡面反射光、および歯肉組織表面からの表面反射光を除去することで、歯肉表面の高精度な血流測定が可能になった。続いて、LSFG の測定値である mean blur rate (MBR) の、歯肉血流量の指標としての妥当性を評価した。試作装置で測定した歯肉の MBR の値は 400~1,800(a. u.) の範囲に分布するが、MBR はこの範囲においても速度較正器を用いて測定した速度と有意な直線関係を示し、MBR が歯肉血流量を示す指標として有効であることが示された。

2. 安静時歯肉血流の年齢変化

試作した LSFG を用いて、20-60 歳代の健常男性 124 名の上顎前歯部歯肉の安静時血流を測定した。血流波形解析を行い、歯肉血流量、血流の流れやすさの指標である血管コンダクタンス、および血流量の持続性を示す Blowout time (BOT) 等の血流指標を算出した。歯肉血流量には年齢による有意な差は認められなかったが、血管コンダクタンス、および BOT は被験者の年齢と有意な負の相関関係を示した。このことから、加齢に伴い安静時の歯肉血管コンダクタンスや歯肉組織への血流維持能力が低下する可能性が示された。これらの歯

肉血流指標と血圧、心拍数などの全身循環指標との関連性を重回帰分析により解析した結果、歯肉血管コンダクタンス、およびBOTは被験者の収縮期血圧、および脈圧と有意な負の相関関係を示し、歯肉血管コンダクタンスやBOTの低下には、加齢に伴う収縮期血圧や脈圧の上昇が関連している可能性が示唆された。

そこで、新たに安静時血圧が同等の健常男性を対象として、安静時の歯肉血流指標を比較した。安静時血圧が同等の若年男性、中高年男性25名の上顎前歯部歯肉の安静時血流を同様の手法で測定した結果、安静時の歯肉血管コンダクタンス、BOTには年齢による差が認められず、これらの指標には被験者の血圧、脈圧が影響を及ぼすことが確認された。

3. 加温刺激時の歯肉血流応答：若年層と中高年層での比較

続いて、歯肉局所刺激時の血流指標の変化を明らかにするために、安静時の歯肉血流維持能力に差が認められなかった上記健常男性25名の歯肉に、加温刺激を行った。歯肉を40℃で加温し、刺激に対する血流指標の変化を年齢で比較した。歯肉を加温すると血管が拡張し、若年、中高年ともの歯肉血流量、歯肉血管コンダクタンスが増加した。中高年群では加温による一時的な血管拡張時のBOTが有意に低下したのに対し、若年群ではこの変化が認められなかった。血圧が若年者と同等である中高年者の加温時の歯肉血流維持能力が、若年者と比較して有意な低下を示したことから、加齢に伴い歯肉血流の維持力が低下する可能性が示された。

本研究では、ヒト歯肉血流の個体間比較が可能なLSFGを開発し、安静時、および加温刺激時のヒト歯肉血流計測・血流動態解析を行うことにより、従来技術では困難であったヒト歯肉血流の年齢変化、即ち、ヒト歯肉では加齢に伴い歯肉組織への血流維持能力が低下する可能性があることを明らかにすることができた。本研究で開発したLSFG、および得られた知見は、今後、歯周病予防等のオーラルヘルスケア分野への応用が期待される。

Contents

	Page
1. Introduction	1
2. Development of novel gingival blood flow measurement technique based on laser speckle flowgraphy (LSFG)	
2-1. Introduction	3
2-2. Materials and methods	3
2-2-1. Laser speckle flowgraphy	3
2-2-2. Application of polarizing filter to LSFG	4
2-2-3. Evaluation of MBR using a speed calibrator	4
2-2-4. Evaluation of the stability of gingival MBR at different measurement distances	4
2-2-5. Statistical analysis	5
2-3. Results	5
2-3-1. Application of polarizing filter	5
2-3-2. Evaluation of MBR using the speed calibrator	5
2-3-3. Evaluation of the measurements from two distances	5
2-4. Discussion	5
2-5. Summary	6
3. Age-related changes in human gingival blood flow parameters	
3-1. Introduction	8
3-2. Materials and methods	8
3-2-1. Subjects	8
3-2-2. Laser speckle flowmetry	9
3-2-3. Gingival blood flow measurement by LSFG	9
3-2-4. Gingival blood flow analysis	9
3-2-5. Gingival blood flow parameters	10
3-2-6. Measurement of basal physiological values and the periodontal status	10
3-2-7. Statistical analysis	10
3-3. Results	11
Study 1	
3-3-1. Systemic basal physiological values and periodontal status	11
3-3-2. Gingival MBR and vascular conductance (MBR/MBP)	11
3-3-3. Pulse waveform analysis of gingival blood flow	11

3-3-4. Analysis of age-dependent changes in gingival blood flow parameters, including systemic hemodynamic parameters	12
3-3-5. Relationship between basal physiological values and gingival blood flow using age as a control variable	12
Study 2	
3-3-6. Systemic basal physiological values and periodontal status	12
3-3-7. Gingival blood flow under resting conditions	13
3-3-8. Changes in MBP, HR, gingival MBR, and vascular conductance (MBR/MBP) during thermal stimulation	13
3-3-9. Changes in pulse waveforms of gingival blood flow before and after thermal stimulation	13
3-4. Discussion	14
3-5. Summary	17
4. Conclusions	18
5. Future Perspectives	20
6. Acknowledgements	21
References	22
Figure legends	26
Figures	29
Tables	37

1. Introduction

Periodontal diseases are known to increase the risk of chronic systemic diseases such as diabetes and cardiovascular diseases¹⁻⁵. Oral health is therefore considered essential in maintaining the overall health of an individual⁶. Gingival inflammation is the primary symptom associated with periodontal disease, and is characterized by changes in the vascular structure and hemodynamics of the gingiva from the healthy state. For example, inflamed gingiva in dogs is characterized by an increase in the number and distribution of capillary loops⁷, as well as thickening of the vascular diameter⁸. Similarly in humans, it is characterized by reduced vascular oxygen saturation and increased hemoglobin concentration, which collectively cause the status of hyperemia⁹. Although the precise causal relationship remains unclear, the number of patients presenting with periodontal disease increases with age, suggesting a link between age-related hemodynamic changes in the human gingiva and periodontal disease¹⁰. However, there have been few studies investigating the effects of aging on human gingival blood flow under resting conditions¹¹. This may be because there have been relatively few tools available to measure human gingival blood flow *in vivo*.

Laser Doppler flowmetry (LDF) and laser speckle imaging (LSI) have both been widely used to measure gingival blood flow in humans¹²⁻¹⁶. LDF enables real-time measurement of relative changes in the gingival blood flow upon stress such as heating, cooling, brushing, or pressure. However, it is limited by its narrow measurement range and low reproducibility of data. The measurement is also time-consuming and burdensome for patients as it requires fixation of the measurement probe to the gingiva. On the other hand, LSI enables imaging of a wider region of interest without direct contact with the gingiva. However, neither measurement tool allows comparison of gingival blood flow between individuals under resting conditions, as they only measure the relative values to represent blood flow.

However, laser speckle flowgraphy (LSFG) may represent a new option to investigate gingival blood flow^{17, 18}. This technique has recently been used to measure blood flow levels and changes in hemodynamic responsiveness through waveform analysis of blood flow fluctuations. These fluctuations are generated through the normal function of the cardiac cycle^{19, 20}. LSFG allows comparison of blood flow without external stimuli, enabling investigation of human gingival hemodynamics under resting conditions across different age groups.

The aim of the present study was to examine age-related changes in gingival hemodynamics in healthy men. First, an LSFG-based system that enables measurements of gingival blood flow was developed in order to compare gingival blood flow under resting conditions among differently aged individuals. Using the system, gingival blood flow was measured in healthy male participants to characterize age-related changes in gingival

hemodynamics. Furthermore, hemodynamic responsiveness to thermal stimuli was measured and the differences in the response to thermal stimulus were examined by age groups.

2. Development of novel gingival blood flow measurement technique based on laser speckle flowgraphy (LSFG)

2-1. Introduction

This chapter describes the development of a technique based on laser speckle flowgraphy (LSFG) to enable inter-individual comparison of gingival blood flow in humans under resting conditions. Conventional LSFG systems, such as those used for the measurement of blood flow in the fundus or on the skin surface, cannot be used to measure the blood flow of the gingival surface because specular reflection caused by saliva interferes with measurement. Measurement must also be completed within a short period of time because individuals must have their mouths open during measurement to enable measurement without direct contact.

In order to remove specular reflection from saliva, a polarizing filter was incorporated in the prototype LSFG that was developed specifically for the measurement of gingival blood flow. Using the system, the mean blur rate (MBR), an index of tissue blood flow used in LSFG, was measured to assess whether it can also be used as an index of gingival blood flow. Lastly, gingival MBR measured at two distances using the prototype LSFG system were compared to evaluate the stability of gingival blood flow measurements.

2-2. Materials and methods

All experimental protocols were approved by the ethics committee of the Kao Corporation, Tokyo, Japan and conform to the principles of the Declaration of Helsinki (approval number: 14-17). Voluntary written consent was received from all subjects before participation.

2-2-1. Laser speckle flowgraphy²¹

LSFG comprises a probe unit that has a laser module with an 830-nm wavelength, color CMOS camera, and monochrome CCD camera; this probe emits class 1M laser light according to the IEC 60825-1 standard. The LSFG also comprises a control unit that controls the laser output and CCD camera of the probe, and a PC equipped with the measurement and analysis software (Softcare Co., Ltd, Fukuoka, Japan).

The principle of LSFG is shown in Figure 1. Using the 830-nm coherent laser light source, the speckle pattern of irradiated particles changes shape according to the motion of each particle. When erythrocytes in blood vessels are irradiated with laser light, they move in the blood vessels while changing positional relationships. Thus, the speckle pattern changes constantly and randomly. The measurement principle of LSFG is to calculate the blood flow through statistical analysis of the fluctuation of the speckle pattern.

The mean blur rate (MBR), an index of tissue blood flow used in LSFG, is calculated

from the spatial and temporal blur rate of a speckle pattern. MBR represents present blur in the photograph of a moving object. It is proportional to the erythrocyte moving speed. Modelling of the time-varying blur rate of the speckle pattern on an image sensor is shown in Figure 2. When erythrocytes move slowly, a relatively blur-free image is obtained, with a low MBR value. In contrast, when erythrocytes move rapidly, an image with higher blur is obtained, with a high MBR value.

2-2-2. Application of polarizing filter to LSFG

A prototype gingival blood flow measurement system (Softcare Co., Ltd, Fukuoka, Japan) using an LSFG-based laser speckle flowmetry system with an additional surface-reflective wire grid polarizing filter added to remove light reflected by saliva on the gingival surface or surface-reflected light from the gingival tissue was assessed²¹. For optimal measurement in the oral cavity, images were acquired with a narrow field of imaging and high resolution (measurement range: 650 x 520 mm at 160-mm distance, 360 x 300 mm at 80-mm distance; 700 x 480 pixel resolution).

2-2-3. Evaluation of MBR using a speed calibrator

MBR was evaluated using speed calibrator in the same manner as cutaneous MBR²². Speed was measured in a dark room shielded from outer light. As shown in Figure 3, the speed calibrator was connected to a high-speed motor and an opal glass plate (OGP). The speed calibrator and the LSFG probe unit were fixed on a table horizontally with a distance of 250 mm. For measurement by LSFG, the analysis area (68 x 64 pixels) was set with a radius 20 mm from the OGP center. Using the measurement software, the intensity of speckle light applied from the LSFG probe unit to the OGP was set at the vicinity of 100. The intensity of the light reflected from the OGP at varying speeds of the high-speed motor between 50 and 530 mm/s was detected by the light receiver, and the continuous speckle images (30 frames/s) were fed into the system for measurement of speckle light intensity and MBR.

2-2-4. Evaluation of the stability of gingival MBR at different measurement distances

The stability of gingival MBR was evaluated by measuring gingival blood flow in healthy male participants (N = 10, 31.4 ± 2.7 years) at different measurement distances. Each participant's head was immobilized to reduce artifacts by placing their chin on a rest. The mouth was then opened using an angle wider mouth opening instrument, and blood flow in the maxillary and mandibular anterior gingiva was measured at distances of 160 mm and 80 mm for 30 s at 30 frames per second (fps).

2-2-5. Statistical analysis

Statistical analyses were performed using SPSS Ver.23 (IBM, Armonk, NY, USA). The relationship between OGP speed and MBR, and the relationship between gingival MBR measured at distances of 160 mm and 80 mm were evaluated using Pearson's correlation analysis. A simple linear regression analysis was performed to examine whether the changes in OGP speed can explain the variance in MBR on the basis of the coefficient of determination. The significance level was set at 5% ($p = 0.05$).

2-3. Results

2-3-1. Application of polarizing filter

Representative images of gingival blood flow with and without the polarizing filter are shown in Figure 4. The use of the polarizing filter enabled removal of the light reflected from saliva, thus resulting in a wider field of imaging for gingival blood flow.

2-3-2. Evaluation of MBR using the speed calibrator

The correlation between MBR measured by LSFG and the velocity of the uniform circular motion of OGP measured using the speed calibrator is shown in Figure 5. MBR was used as the index of gingival blood flow. MBR and OGP speed had a strong correlation within the OGP speed range of 50- 530 mm/s (Figure 5, $r = 0.999$, $p < 0.001$). A simple linear regression analysis with OPG speed as the independent variable and MBR as the dependent variable revealed a direct relationship between OGP speed and MBR, represented by the following linear regression equation: $Y = 3.39X + 32.0$ ($r^2 = 0.998$)

2-3-3. Evaluation of the measurements from two distances

The x- and y-axes in Figure 6 represent scatter plots of MBR measured from 80 and 160 mm, respectively. There was a strong correlation between the measurements from the two distances ($r = 0.921$, $p < 0.001$).

2-4. Discussion

Surface-reflected light is a component of reflected light that reflects off of a surface of a substance and retains the polarization direction of incident light. On the other hand, the polarization direction of incident light is not retained in light re-emitted from scattering because it originates from lights scattered multiple times within a medium. Thus, the difference in the polarization properties can be used to distinguish the two types of reflected light.

In general, both surface-reflected and scattered lights are captured collectively on a camera once a laser light hits a body. As surface-reflected light reflects off of a surface of a

biological tissue, it does not contain any information on blood flow and is therefore treated as noise, specifically known as static scatter. Once a laser light enters the body, it scatters internally and some components of the scattered light are emitted back as scattered light. Thus, although scattered light does not retain the polarization properties of the incident light, surface-reflected light retains the direction of polarization (Figure 7). By placing the linear polarizer in front of the camera and perpendicular to the direction of polarization of the surface-reflected light, the amount of surface-reflected light that is captured by the light receiver can be limited or eliminated completely^{21, 23}. Thus, the accuracy of LSFG for the measurement of blood flow should improve by selectively capturing scattered light that carries the information about blood flow.

The present study demonstrated that the incorporation of the polarizing filter in the LSFG system eliminated specular reflection from saliva and enabled the measurement of blood flow within the oral mucosa. This also enabled highly accurate blood flow measurement within a short period of time by eliminating surface-reflected light from the surface of the gingiva.

In order to confirm whether MBR is an accurate index of blood flow, the OGP was set to undergo uniform circular motion in order to clarify the relationship between its velocity and MBR, which was calculated based on the intensity of light reflected from OGP. As a result, OGP speed and MBR had a linear relationship ($Y = 3.39X + 32.0$ ($r^2 = 0.998$)) within the OGP speed range of 50 and 530 mm/s, suggesting that MBR is an appropriate index of the velocity of blood flow within the range of 200 and 1,800. Gingival MBR likely falls within the range of 400 and 1,800 based on the measurement of gingival MBR from the two distances. Collectively, these findings suggest that MBR is an appropriate index of gingival blood flow within the range of 400 and 1,800.

The measurements of MBR from the two distances (80 and 160 mm) exhibited a linear relationship, suggesting that a constant value for MBR can be obtained when gingival blood flow is measured from either 80 or 160 mm. This stability of MBR measurement will enable comparisons of blood flow at different distances such as across different locations within the gingiva.

2-5. Summary

In this chapter, the polarizing filter was incorporated into the LSFG system, and the system was demonstrated to enable highly accurate measurement of blood flow from the surface of gingiva while eliminating artifacts from saliva. MBR, which is an index of tissue blood flow as determined by LSFG, had a linear relationship with the measurements made with a speed calibrator within the range of 50-530 mm/s. This suggests that MBR is an appropriate index of gingival blood flow within the range of 400 and 1,800. This study also demonstrated that a

constant value for MBR can be measured from two different distances, suggesting that it can compare gingival blood flow across different locations of the gingiva.

3. Age-related changes in human gingival blood flow parameters

3-1. Introduction

In this chapter, age-related changes in the maxillary anterior gingival blood flow were evaluated in healthy men using the prototype laser speckle flowgraphy (LSFG) system described in the previous chapter.

First, the maxillary anterior gingival blood flow was measured at rest in 124 healthy men aged between 22 and 69 years. Gingival MBR, vascular conductance, and pulse waveform parameters were analyzed in order to identify age-related changes (Study 1). Next, thermal stimuli were applied to the maxillary anterior gingiva of 25 healthy men in the age groups of 21-33 years and 55-65 years to examine whether there were age-related differences in the responses of gingival hemodynamics upon localized thermal stimuli to the gingiva. (Study 2).

3-2. Materials and methods

3-2-1. Subjects

All experimental protocols were approved by the ethics committee of the Kao Corporation, Tokyo, Japan and conform to the principles of the Declaration of Helsinki (approval numbers: 14-17, 15-51, S058-170721). Voluntary written consent was received from all subjects before participation.

Study 1: Gingival blood flow measurement at rest in 124 healthy men

The study involved 124 healthy Japanese men aged between 22 and 69 years. Exclusion criteria included attending medical institutions and dental clinics for periodontitis or cavity treatment, serious wounds in the oral cavity, acute gingival inflammation, spontaneous bleeding or pus discharge from periodontal pockets, and tooth mobility. Due to age-associated differences in basal physiological values, such as blood pressure, subjects were divided into three age groups: a young group (Y; 22-37 years, n = 45), a middle-aged group (M; 38-53 years, n = 43), and an elderly group (E; 54-69 years, n = 36).

All experiments were performed between 09:00 and 12:00 in a laboratory maintained at 25°C and 50% humidity. Participants were prohibited from ingesting alcohol, caffeine, or spicy food the day before measurements were taken. In addition, they were prohibited from ingesting food and drink (other than water) on the day of the measurement and were also asked not to perform any oral hygiene such as brushing. After acclimatizing for 15 min after arrival, blood pressure, pulse rate (PR), and gingival blood flow under resting conditions in a sitting position were measured for each individual. After the measurement of gingival blood flow, the periodontal status was recorded.

Study 2: Measurement of gingival blood flow at rest, and gingival blood flow response upon thermal stimulation in 25 healthy men with no differences in blood pressure

The study included 25 healthy Japanese men between 21 and 65 years. Prior to enrollment in the study, blood pressure measurements and oral check-ups were performed. As this study did not allow participants with age-related blood pressure increases, individuals with a systolic blood pressure (SBP) of 140 mmHg or higher or diastolic blood pressure (DBP) of 90 mmHg or higher were excluded. Based on an oral examination, those individuals currently following treatment at medical institutions or dental clinics, and those with serious wounds in the oral cavity, acute or severe gingival inflammation, spontaneous bleeding, pus discharge from periodontal pockets, or tooth mobility were also excluded from the study. The selected individuals were divided into two groups according to age (young group: 21–33 years old, $n = 12$; middle-age group: 55–65 years old, $n = 13$), and their periodontal status was recorded.

The experiment was performed in a laboratory with a room temperature of 25°C and humidity of 50%. Participants were prohibited from eating or drinking anything other than water and from performing any oral hygiene activity, such as brushing, 30 min before measurement. After entering the laboratory, participants were equipped with a continuous sphygmomanometer and an electrocardiograph to begin measuring their blood pressure and heart rate (HR). After 15 min of acclimation, gingival blood flow was measured in the sitting position. Then, a plastic tube with circulating warm water kept at a surface temperature of 40°C was placed into contact with the maxillary anterior gingiva for 1 min. After 1 min, the tube was removed, and gingival blood flow was measured again.

3-2-2. Laser speckle flowmetry

The prototype LSFG system described in the previous chapter was used.

3-2-3. Gingival blood flow measurement by LSFG

Each participant's head was immobilized to reduce artifacts by placing the chin on a rest. The mouth was then opened using an angle wider mouth-opening instrument and blood flow in the maxillary anterior gingiva was measured at a distance of 160 mm or 80 mm. For measurement at rest, gingival blood flow was measured for 30 s at 30 frames per second (fps)²⁴. Post-heating measurement was started 30 s after the thermal stimulus at 30 fps for 2 min²⁵.

3-2-4. Gingival blood flow analysis

The analytical region was set to the maxillary anterior gingiva (the area encompassed by a white line in Figure 8A; approx. 22 mm × 5 mm; 4000 pixels or greater). The mean blur rate (MBR),

an index of gingival tissue blood flow used for LSFG, and blood flow indices were calculated²¹. A higher MBR corresponds to an improved/faster blood flow. Heartbeats were extracted from heartbeat-induced blood flow fluctuations (Figure 8C) and the standardized average pulse waveforms per heartbeat (the pulse waveform) were assessed (Figure 8D).

3-2-5. Gingival blood flow parameters

Gingival vascular conductance was calculated by dividing the gingival MBR by the mean blood pressure (MBP). The four pulse waveform parameters, flow acceleration index (FAI), acceleration time index (ATI), falling rate, and blowout time (BOT), were calculated from the pulse waveforms obtained by LSFG (methods are shown in Figure 9). The indices shown in Figure 9 represent the maximal acceleration rate of the blood flow increase during the heartbeat, the peak positions of the pulse waveform, serial changes in the rate of decrease in blood flow, and the persistence of high blood flow, respectively^{22, 24, 26}.

3-2-6. Measurement of basal physiological values and the periodontal status

Upper arm SBP, DBP, MBP, pulse pressure (PP), and PR of the 124 participants in Study 1 were measured using a non-invasive sphygmomanometer (Oscillometric methods, Dinamap Pro100, GE Medical Systems Information Technologies Inc., Milwaukee, WI, USA). The SBP, DBP, MBP, and PP of the 25 participants in Study 2 were measured using a non-invasive continuous sphygmomanometer (Finometer MIDI; Finapres Medical Systems BV, Enschede, The Netherlands). Values were converted into brachial blood pressure. In addition, HR and electrocardiogram measurements were performed using a memory HR monitor (LRR-03; GMS Co., Ltd., Tokyo, Japan). In both Studies 1 and 2, the participants' body mass index (BMI) values were calculated from their heights and body weights. The probing pocket depth (PPD) and gingival index (GI) were used as indices of gingival inflammation²⁷. These were measured for all teeth with a periodontal probe and a protocol outlined in Oral Health Surveys Basic Methods²⁸.

3-2-7. Statistical analysis

Statistical analyses were performed using SPSS Ver.23 (IBM, Armonk, NY, USA). The basal physiological values and gingival blood flow parameters for each subject are presented as the means \pm S. E. In Study 1, to compare the three age groups, a one-way factorial ANOVA was performed, and Bonferroni's method was used for multiple comparisons. ANCOVA was performed to compare the three age groups accurately using the basal physiological values as covariates. The relationships among MBR, gingival vascular conductance (MBR/MBP), and blood flow waveform parameters with age were evaluated using Pearson's correlation analysis.

Stepwise multiple regression analyses were performed to determine independent factors affecting the gingival blood flow parameters. For the stepwise multiple regression analyses, age, BMI, systemic hemodynamic parameters (SBP, DBP, MBP, PP, and PR), and gingival parameters (PPD and GI) were used as independent factors. To evaluate the relationships among basal physiological values, MBR, gingival vascular conductance (MBR/MBP), and blood flow waveform parameters, a partial correlation analysis using age as a control variable was performed. In Study 2, for comparisons of basal physiological values, periodontal status, and gingival blood flow parameters between the two age groups, two-sample *t*-tests were performed. For comparisons of each parameter before and after thermal stimulation, paired *t*-tests were performed. The significance level was set at 5% ($p = 0.05$).

3-3. Results

Study 1

3-3-1. Systemic basal physiological values and periodontal status

A summary of the characteristics and gingival blood flow parameters of the three age groups is shown in Table 1. BMI, DBP, and MBP were significantly higher in groups M and E than in group Y ($p < 0.05$). SBP and PPD were significantly higher in group E than in groups Y and M ($p < 0.05$), whereas PP was significantly higher in group E than in group M ($p < 0.05$). No significant differences were observed in PR or GI among the three groups. Gingival inflammation was judged as absent or mild in all subjects and the mean GI did not exceed 2.0 for any participant.

3-3-2. Gingival MBR and vascular conductance (MBR/MBP)

There were no significant differences observed in gingival MBR in the three age groups (Table 1). However, gingival vascular conductance (MBR/MBP) was significantly lower in groups M and E relative to that in group Y ($p < 0.05$, Table 1). Further analysis of the relationships between age and gingival vascular conductance (MBR/MBP) revealed a negative correlation with age ($r = -0.25$, $p < 0.01$, Table 2). This suggests that there is an increase in vascular resistance with age.

3-3-3. Pulse waveform analysis of gingival blood flow

The mean pulse waveforms of the three age groups are shown in Figure 10. The acceleration rates during increased blood flow were slower in groups M and E than in group Y. In addition, the time to reach peak blood flow was longer in older groups, whereas the blood flow reduction after reaching a peak was faster. Although no significant differences were observed in gingival MBR among the three groups, differences were noted in the shapes of the pulse waveforms.

Based on these data, several blood flow waveform parameters were examined, including ATI, falling rate, and BOT. ATI and falling rate were significantly higher in group E than in group Y, although the average BOT was significantly lower ($p < 0.001$, Table 1). There was a significant correlation between age and all parameters (ATI: $r = 0.37$, $p < 0.001$, falling rate: $r = 0.39$, $p < 0.001$, BOT: $r = -0.39$, $p < 0.001$, Table 2).

3-3-4. Analysis of age-dependent changes in gingival blood flow parameters, including systemic hemodynamic parameters

The results of stepwise multiple regression analysis for factors, including age and systemic hemodynamic parameters independently contributing to gingival blood flow parameters are shown in Table 3. MBR was positively correlated with PR and gingival vascular conductance (MBR/MBP) was negatively correlated with SBP, but neither was correlated with age. However, the falling rate exhibited a positive correlation with PP and age, and BOT had a negative correlation with SBP and age. ATI was positively correlated with age, whereas no relationships were observed between ATI and systemic hemodynamic parameters.

3-3-5. Relationship between basal physiological values and gingival blood flow using age as a control variable

The partial correlation coefficients of MBR, gingival vascular conductance (MBR/MBP), pulse waveform parameters, and varying basal physiological values, with age as a control variable, are shown in Table 4. No correlation was observed among BMI, PPD, GI, and the pulse waveform parameters. MBR/MBP was negatively correlated with SBP, DBP, MBP, and PP (SBP: $r = -0.27$, $p < 0.01$, DBP: $r = -0.26$, $p < 0.01$, MBP: $r = -0.22$, $p < 0.05$, PP: $r = -0.21$, $p < 0.05$). The falling rate was positively correlated with PP ($r = 0.19$, $p < 0.05$), whereas the BOT was negatively correlated with SBP and PP (BOT vs. SBP: $r = -0.21$, $p < 0.05$, BOT vs. PP: $r = -0.21$, $p < 0.05$). MBR and MBR/MBP were negatively correlated with PR (MBR vs. PR: $r = 0.22$, $p < 0.05$, MBR/MBP vs. PR: $r = 0.19$, $p < 0.05$).

Study 2

3-3-6. Systemic basal physiological values and periodontal status

The characteristics and gingival blood flow parameters in the two age groups are shown in Table 5. The BMI in the middle-aged group was significantly higher than that in the young group ($p < 0.05$). There were no significant differences in SBP, DBP, MBP, PP, HR, PPD, or GI between the two groups. All participants had no or mild gingival inflammation, and the mean GI did not exceed 2.0 for any participant.

3-3-7. Gingival blood flow under resting conditions

There were no significant differences in gingival MBR between the two age groups. No significant differences in gingival vascular conductance (MBR/MBP), FAI, BOT, or falling rate were observed between the two groups. In contrast, ATI was significantly higher in the middle-aged group than in the young group ($p < 0.01$, Table 5).

3-3-8. Changes in MBP, HR, gingival MBR, and vascular conductance (MBR/MBP) during thermal stimulation

The MBR, HR, and gingival blood flow parameters for the two age groups at rest, and at 30 s after discontinuation of localized heating are shown in Table 6. Average changes over time in MBP, HR, gingival MBR, and gingival vascular conductance (MBR/MBP) in participants in the young and middle-aged groups before and after localized thermal stimulus to the gingiva are shown in Figure 11. The MBP did not change after localized stimulation was applied to the gingiva, whereas HR temporarily decreased immediately after placing the heated plastic tube on the gingiva ($p < 0.05$). In both the young and middle-aged groups, gingival MBR and vascular conductance (MBR/MBP) significantly increased upon thermal stimulation of the maxillary anterior gingiva when compared with their pre-stimulus values (Rest; $p < 0.01$; Table 6 and Figure 11). Gingival MBR was significantly higher than its value at rest for approximately 1 min after localized heating of the gingiva was discontinued in both groups, and neither group exhibited differences. There were also no differences between the two groups regarding how much their gingival MBR and vascular conductance (MBR/MBP) values increased in response to stimulation.

3-3-9. Changes in pulse waveforms of gingival blood flow before and after thermal stimulation

To compare the pulse waveforms of gingival blood flow at rest and at 30 s after discontinuation of the thermal stimulus in detail, standardized pulse waveforms of gingival blood flow in each group were evaluated (Figure 12). In the young group, there were no significant changes in the increase or decrease of gingival blood flow induced by the heartbeat before or after thermal stimulation.

Although the increase in blood flow induced by the heartbeat became rapid after thermal stimulation in the middle-aged group, the outflow of blood also became rapid after reaching its peak, and the time at which MBR was 50% or more was reduced (Figure 12). Although no significant differences were noted between the groups in terms of changes in gingival MBR or vascular conductance (MBR/MBP) before or after thermal stimulation, some differences were observed in the shapes of the pulse waveforms after thermal stimulation. Based

on these results, gingival blood flow parameters related to blood inflow, outflow, and persistence were compared before and after thermal stimulation (Table 6). In both groups, FAI temporarily increased in response to heating of the gingiva ($p < 0.01$; Table 6). Although there were no changes in ATI, falling rate, or BOT before or after the thermal stimulus in the young group, the BOT decreased in the middle-aged group compared with its pre-stimulus value (Rest; $p < 0.05$). In addition, although there were no differences in falling rates at rest between the two groups, the falling rate after heating was significantly higher in the middle-aged group than in the young group ($p < 0.05$).

3-4. Discussion

Laser Doppler flowmetry (LDF) and laser speckle imaging (LSI) have both been widely used to measure blood flow in the skin and orofacial areas of animals and humans such as the gingiva and lips^{12,13,15,16,29}. However, LDF does have some limitations, including a narrow measurement range and low reproducibility^{30,31}. Although this suggests that LSI may be better suited to analyzing gingival blood flow, especially as the technique enables non-contact measurements of blood flow within a wide range, it cannot be used to analyze the waveforms of blood flow fluctuations. As this is essential for understanding differences in hemodynamics between younger and older individuals at rest, LSFG was used in the present study. It revealed no significant differences in gingival MBR across the three age groups examined (Tables 1 and 2), supporting the previous study by Matheny et al¹¹. However, the gingival vascular conductance (MBR/MBP) was significantly lower in the middle-aged (M) and elderly (E) groups than in younger individuals (Y). It was also negatively correlated with age (Tables 1 and 2). This suggests that peripheral vascular conductance in the gingiva decreases with age, causing blood circulation in the gingiva to degenerate.

Furthermore, the ATI, falling rate, and BOT (each calculated using pulse waveform analysis) changed in response to aging. In particular, increases in gingival blood flow induced by the heartbeat occurred more slowly in older participants. The time to reach peak MBR (an increase in ATI) was also delayed, whereas blood flow reduction from the peak was faster (reflected in an increase in falling rate). Lastly, the duration of high blood flow was shortened in older individuals (shown by a decrease in BOT). Together, these results suggest that an LSFG-based method can be used to measure age-related changes in hemodynamics in the gingiva, as well as the reduction in gingival vascular conductance. These results support our previous data demonstrating age-related decreases in facial skin blood flow in response to cooling load³². Therefore, any degeneration in hemodynamics due to age may also occur in the gingiva, as well as the facial skin.

To investigate potential mechanisms responsible for the changes in gingival blood

flow parameters due to aging, stepwise multiple regression analyses were performed for factors independently affecting gingival blood flow parameters and a partial correlation analysis using age as a control variable. The multiple regression analysis revealed that gingival vascular conductance (MBR/MBP) depended on SBP more than on age (Table 3). The partial correlation analysis also demonstrated that MBR/MBP was negatively correlated with blood pressure (SBP, DBP, and MBP) and PP (Table 4). Thus, the age-related decrease in MBR/MBP observed in this study was due to the age-related increase in SBP. The BOT and falling rate were also correlated with blood pressure and PP. Negative correlations were observed between BOT and SBP, whereas a positive correlation was observed between the falling rate and PP (Tables 3 and 4). Large arterial stiffness progresses with age and is accompanied by increased SBP and widening of PP to maintain blood supply³³. The results suggest the involvement of aortic stiffness in the observed decreases in MBR/MBP and BOT, and in the increases in the falling rate of gingival blood flow. In addition, increases in SBP and PP due to increased age-related arterial stiffness may widen the range of heartbeat-induced blood flow fluctuations, effectively sharpening the peak of the pulse waveform and accelerating blood flow reduction from the peak³⁴. These changes in the aortic pulse waveform may therefore influence the local pulse waveform in the gingival microcirculation, which explains the differences observed in the study. However, while MBR/MBP shows strong dependence solely on SBP (Table 3), the BOT and falling rate depend on age, in addition to SBP or PP (Table 3). Furthermore, we noted no correlation among ATI, blood pressure, and PP (Tables 3 and 4), suggesting that other factors are involved in the age-associated decrease in BOT and increases in ATI and falling rate.

Study 1 suggested that age-related increases in SBP and PP caused the changes in gingival blood flow parameters, and that the resting blood pressure influenced gingival blood flow at rest. Therefore, in Study 2, gingival blood flow was compared between younger and middle-aged participants, with no differences in normal blood pressure among the groups (young group: 21–33 years, $n = 12$; middle-aged group: 55–65 years, $n = 13$).

There were no significant differences in gingival MBR, gingival vascular conductance (MBR/MBP), BOT, or falling rate between the two age groups with no differences in normal blood pressure. These results were consistent with the hypothesis that the blood pressure strongly influences these three parameters. The ATI was significantly higher in the middle-aged group than in the young group, as previously noted in Study 1, suggesting that the inflow of blood to the gingival tissue is slower in middle-aged individuals. As there were no differences in systemic hemodynamic parameters between the two groups, differences in ATI were caused by other age-related changes in the peripheral gingival tissue.

Many studies have reported that blood flow increases with the application of thermal stimulation, electrical stimulation, capsaicin, or nitric oxide (NO) in human and animal gingiva^{12, 35-37}. In these studies, however, age-related changes in responsiveness during stimulation were not evaluated. In forehead skin, which is dominated by parasympathetic vasodilator fibers in addition to sympathetic fibers, similar to the gingiva³⁶, the reactivity of the skin blood vessels to cooling stimuli is reported to decrease with age³². Therefore, in addition to measuring blood flow at rest, changes in responsiveness upon simple stimulation to the gingival blood vessels were compared. A method was selected in which the gingival area was locally heated for a short time with a plastic tube kept at a constant temperature with warm water, which was able to be performed without difficulty and posed low physical burden on the participant. By heating the maxillary anterior gingiva for 1 min at 40°C, a temporary increase in blood flow was confirmed in both groups without changes in blood pressure, demonstrating that this increase in blood flow was caused by temporary peripheral vasodilation in the gingiva. The HR decrease during thermal stimulation suggested that this vasodilation occurred via activation of parasympathetic nerves. Moreover, as warming at 40°C did not cause pain and the temperature was lower than the threshold for the activation of transient receptor potential cation channel subfamily V member 1 (< 43°C)³⁸, nociceptive c-fibers were not likely to be involved in this vasodilation in the gingiva.

Contrary to the report on the response in forehead skin, in this study, no significant differences were observed between the two age groups in terms of changes in gingival MBR and vascular conductance (MBR/MBP) before or after thermal stimulation. Systemic factors, such as hypertension, have been reported to reduce cutaneous vasodilation by sympathetic vasodilator fibres³⁹. If a similar effect were to occur in the parasympathetic vasodilator fibers involved in gingival vasodilation, the two groups in the current study having no differences in blood pressure may explain the lack of differences in responsiveness to the thermal stimulus. Moreover, the thermal stimulus in the current study being mild (40°C for 1 min) can explain why there were no age-related differences in response to its application.

Significant increases in FAI after localized heating of the gingiva were observed in both groups, suggesting that blood inflow to the gingival tissue became more rapid after the stimulus due to decreases in vascular resistivity induced by peripheral vasodilation in the gingiva. Although no differences were observed in outflow parameters in the young group, the BOT temporarily decreased immediately after thermal stimulation in the middle-aged group and the falling rate after stimulus was significantly higher in the middle-aged group than in the young group. This demonstrated that during temporary vasodilation in the gingiva due to thermal stimulation, blood outflow from the gingival blood vessels in the middle-aged group became more rapid compared with that in the young group and at rest, suggesting that the

ability to maintain blood flow in the gingival vessels was reduced.

This decrease in the ability to maintain blood flow in the gingiva during localized thermal stimulation may be a result of the decreased extensibility of gingival arterioles and/or decreased gingival vascular regulatory function. The length of the elastic fiber in gingival tissue has been reported to decrease with age, leading to a decline in the elasticity of the gingiva⁴⁰. In addition, calcification in the arterioles of human dental pulp has been reported to start around the age of 40, together with a thickening of the intima and its elastic fibres⁴¹. These reports suggest that age-related hardening of arterioles and decrease in blood vessel extensibility occur in the gingiva as well. The reduction in the ability to maintain blood flow to the gingiva after temporarily applying a thermal stimulus observed in the middle-aged group may be explained by the decrease in the extensibility of gingival blood vessels caused by the temporary vasodilation. According to Nagashima et al.,³² the age-related decrease in the responsiveness of forehead cutaneous blood vessels to cooling stimuli is caused by the aging of vasoconstrictor and vasodilator nerves, such as decreases in responses of skin sympathetic nervous activity to the stimuli, decreases in noradrenaline release from nerve terminals of vasoconstrictive fibers, and decreases in the sensitivity of α receptors of cutaneous smooth muscles to noradrenaline, in advance of age-related histoanatomical changes in the cutaneous blood vessels. A reduction in NO-producing activity has also been reported in studies on *in vitro* aging in cultured human umbilical vein endothelial cells⁴². Thus, the production of NO in the gingiva, a vasorelaxant, and the reactivity of blood vessels to neurotransmitters are likely to decrease with age, and these neural factors may play a role in the decrease in blood flow persistence during temporary vasodilation by thermal stimuli.

3-5. Summary

In this chapter, LSFG was demonstrated to successfully measure the blood flow in human maxillary anterior gingiva in healthy participants. This study confirmed that there are age-related changes in gingival blood flow parameters at rest and in the response upon application of localized thermal stimulation to the gingiva, suggesting that the ability to maintain gingival blood flow decreases with age.

4. Conclusions

The overall aim of this study was to clarify age-related changes in gingival hemodynamics in healthy men.

First, an LSFG-based system enabling comparison of human gingival blood flow at rest was developed. The polarizing filter was incorporated into the system to perform blood flow measurements of the gingival surface while eliminating artifacts from saliva. This enabled accurate measurement of blood flow on the surface of the gingiva. Then, the use of MBR measured by LSFG as an index of tissue blood flow was examined using the speed calibrator, and MBR was demonstrated to be an appropriate index of gingival blood flow.

Using the prototype LSFG system, the maxillary anterior gingival blood flow at rest in 124 healthy male participants aged between 22 and 69 years was measured to examine age-related changes in the gingival hemodynamics. Elderly participants had lower gingival blood flow parameters, including gingival vascular conductance and BOT, suggesting that the ability to maintain gingival blood flow at rest decreases with age. The changes in these parameters were positively correlated with SBP and PP. Therefore, using the same technique, the maxillary anterior gingival blood flow at rest was evaluated in 25 healthy participants divided into young and middle-aged groups who had an equivalent blood pressure and PP at rest. Our study found no difference between the two age groups in terms of gingival vascular conductance or BOT when measured at rest. This suggests that the resting blood pressure and PP affect parameters that represent gingival vascular conductance and the persistence of high blood flow in the gingiva.

In order to further assess hemodynamic responsiveness to localized thermal stimulation, thermal stimuli were applied to the gingiva of the 25 male participants in young and middle-aged groups who had an equivalent BOT at rest. Specifically, the gingiva was heated to 40°C to assess age-related changes in the parameters of blood flow upon thermal stimuli. Heating of the gingiva caused vascular dilation, resulting in increased gingival blood flow and vascular conductance in both young and middle-aged groups. Although the BOT significantly decreased in the middle-aged groups during transient vascular dilation, this change was absent in the young group. Therefore, the ability of the gingiva to maintain blood flow during heating was significantly reduced in the middle-aged participants even though their blood pressure was equivalent to that of the young participants. This suggests that aging reduces the ability of the gingiva to maintain blood flow.

As described above, we developed an LSFG-based method that enables inter-individual comparison of gingival blood flow at rest, and measured gingival blood flow in healthy male participants aged between 21 and 69 years. We confirmed that aging may reduce

the ability of the gingival tissue to maintain blood flow, which was difficult to achieve using previously available techniques.

5. Future Perspectives

In the present study, we developed an LSFG system that enables measurement of gingival blood flow, and demonstrated that, unlike conventional techniques, this system can be used to measure the resting gingival blood flow and age-related changes in the vascular response to thermal stimuli. Future studies may include examination of age-related blood flow changes in women and individuals with differing oral health statuses to clarify how gingival blood flow and gingival status are associated with systemic health.

Due to improvements in healthcare and infrastructure, the elderly population is predicted to increase worldwide. This will be accompanied by an increase in the number of patients with systemic chronic diseases, including periodontal disease. Importantly, it has previously been reported that periodontal disease increases the risk of other chronic diseases such as diabetes and cardiovascular diseases¹⁻⁵. This suggests that maintaining oral health in the elderly population will help prevent other systemic chronic diseases⁶. Although the main cause of periodontal disease is plaque-forming pathogens, certain environmental factors are also regarded as risk factors, including lifestyle and stress^{43, 44}. Understanding the relationships among these factors, gingival microcirculation, and gingival conditions may therefore lead to new oral health care regimens that may assist in preventing periodontal disease. The current study suggests that LSFG is an important tool that can be used for such investigations, enabling inter-individual comparisons of blood flow under resting conditions to evaluate human gingival microcirculation.

6. Acknowledgements

I would like to express my gratitude to those who were involved in my thesis.

First, I would like to thank Professor Masataka Majima, Department of Molecular Pharmacology, Kitasato University Graduate School of Medical sciences, for providing guidance in writing this thesis. I would also like to thank Professor Masayoshi Shichiri, Department of Endocrinology, Diabetes and Metabolism, Kitasato University Graduate School of Medical Sciences, the chief examiner, as well as the secondary examiners Professor Naoki Hiki, Department of Surgery, Kitasato University School of Medicine, Professor Akira Takeda, Department of Plastic Surgery, Kitasato University School of Medicine, and Professor Noriko Takahashi, Department of Physiology, Kitasato University School of Medicine for their guidance.

This study was performed within Kao Corporation. I would like to acknowledge the past and present members of the Personal Health Care Products Research Laboratories, Kao Corporation, specifically Mr. Akira Fuji, the current Director, Mr. Koji Maeda, the former Director, and Mr. Yoshifumi Niki, the project leader who provided me with the opportunity to conduct the study. I thank Dr. Yoshinao Nagashima from the laboratory for providing guidance in numerous aspects of research, including experimental protocols, interpretation of the results, and manuscript preparation. I also thank other members of the laboratory, including Dr. Yoshitaka Yano, Mr. Hidetake Fujinaka, Dr. Susumu Nakatsu, Dr. Kayo Sato, Ms. Atsuko Chiba, Mr. Tsutomu Oyama, and Ms. Makiko Hiraishi for their cooperation and advice.

I would like to extend my gratitude to Dr. Sachiko Oh-ishi, Emeritus Professor of Kitasato Institute and Dr. Hiroshi Izumi, Emeritus Professor of Health Sciences University of Hokkaido for guiding me consistently and kindly in my research activities and in preparing my thesis. Lastly, I would like to thank my family for their support throughout my journey.

References

- 1) Preshaw PM, Alba AL, Herrera D, Jepsen S, Konstantinidis A, Makrilakis K, et al: Periodontitis and diabetes: a two-way relationship. *Diabetologia* 2012; 55: 21–31.
- 2) Saremi A, Nelson RG, Tulloch-Reid M, Hanson RL, Sievers ML, Taylor GW, et al: Periodontal disease and mortality in type 2 diabetes. *Diabetes Care* 2005; 28: 27–32.
- 3) Demmer RT, Desvarieux M, Holtfreter B, Jacobs DR, Wallaschofski H, Nauck M, et al: Periodontal status and A1C change: longitudinal results from the study of health in Pomerania (SHIP). *Diabetes Care* 2010; 33: 1037–43.
- 4) Liljestrand JM, Havulinna AS, Paju S, Männistö S, Salomaa V, Pussinen PJ: Missing teeth predict incident cardiovascular events, diabetes, and death. *Journal of Dental Research* 2015; 94: 1055–62.
- 5) Asai K, Yamori M, Yamazaki T, Yamaguchi A, Takahashi K, Sekine A, et al: Tooth loss and atherosclerosis: the Nagahama study. *Journal of Dental Research* 2015; 94: 52S–8S.
- 6) Petersen PE, Yamamoto T, Improving the oral health of older people: the approach of the WHO Global Oral Health Programme. *Community Dentistry and Oral Epidemiology* 2005; 33: 81–92.
- 7) Egelberg J, The blood vessels of the dento-gingival junction. *Journal of Periodontal Research* 1966; 1: 163–79.
- 8) Hansson BO, Lindhe J, Brånemark PI, Microvascular topography and function in clinically healthy and chronically inflamed dentogingival tissues, A vital microscopic study in dogs. *Periodontics* 1968; 6: 264–71.
- 9) Hanioka T, Shizukuishi S, Tsunemitsu A, Hemoglobin concentrations and oxygen saturation of clinically healthy and inflamed gingiva in human subjects. *Journal of Periodontal Research* 1990; 25: 93–8.
- 10) Grossi SG, Zambon JJ, Ho AW, Koch G, Dunford RG, Machtei EE, et al: Assessment of risk for periodontal disease. I. Risk indicators for attachment loss. *Journal of Periodontology* 1994; 65: 260–7.
- 11) Matheny JL, Johnson DT, Roth GI, Aging and microcirculatory dynamics in human gingiva. *Journal of Clinical Periodontology* 1993; 20: 471–5.
- 12) Baab DA, Oberg PA, Holloway GA, Gingival blood flow measured with a laser Doppler flowmeter. *Journal of Periodontal Research* 1986; 21: 73–85.
- 13) Baab DA, Oberg A, Lundström A, Gingival blood flow and temperature changes in young humans with a history of periodontitis. *Archives of Oral Biology* 1990; 35: 95–101.
- 14) Perry DA, McDowell J, Goodis HE: Gingival microcirculation response to tooth brushing measured by laser Doppler flowmetry. *Journal of Periodontology* 1997; 68: 990–5.

- 15) Patiño-Marín N, Martínez F, Loyola-Rodríguez JP, Tenorio-Govea E, Brito-Orta MD, Rodríguez-Martínez M, A novel procedure for evaluating gingival perfusion status using laser-Doppler flowmetry. *Journal of Clinical Periodontology* 2005; 32: 231–7.
- 16) Molnár E, Fazekas R, Lohinai Z, Tóth Z, Vág J: Assessment of the test-retest reliability of human gingival blood flow measurements by Laser Speckle Contrast Imaging in a healthy cohort. *Microcirculation* 2018; 25: e12520.
- 17) Fujii H, Visualisation of retinal blood flow by laser speckle flowgraphy. *Medical & Biological Engineering & Computing* 1994; 32: 302–4.
- 18) Konishi N, Tokimoto Y, Kohra K, Fujii H: New laser speckle flowgraphy system using CCD camera. *Optical Review* 2002; 9: 163–9.
- 19) Shiba T, Takahashi M, Hori Y, Maeno T: Pulse-wave analysis of optic nerve head circulation is significantly correlated with brachial–ankle pulse-wave velocity, carotid intima–media thickness, and age. *Graefe's Archive for Clinical and Experimental Ophthalmology* 2012; 250: 1275–81.
- 20) Tsuda S, Kunikata H, Shimura M, Aizawa N, Omodaka K, Shiga Y, et al: Pulse-waveform analysis of normal population using laser speckle flowgraphy. *Current Eye Research* 2014; 39: 1207–15.
- 21) Nagashima Y, Ohsugi Y, Hiraishi M, Niki Y, Fuji A, Majima M, et al: Development of laser speckle blood flowmeter for evaluating the physiological function of skin. *Biomedical Physics & Engineering Express* 2019; 5: 055012.
- 22) Nagashima Y, Ohsugi Y, Niki Y, Maeda K, Okamoto T: Assessment of laser speckle flowgraphy: Development of novel cutaneous blood flow measurement technique. *Biophotonics Japan 2015 Proceedings* 2015; 9792: 979218-1–11.
- 23) Lee MC, Konishi N, Fujii H: Blood flow analysis of skin tissue under the sacrum using laser speckle flowgraphy. *Optical Review* 2003; 10: 562–6.
- 24) Ohsugi Y, Nagashima Y, Nakatsu S, Sato K, Chiba A, Fujinaka H, et al: Age-related changes in gingival blood flow parameters measured using laser speckle flowmetry. *Microvascular Research* 2019; 122: 6–12.
- 25) Ohsugi Y, Nagashima Y, Chiba A, Oyama T, Hiraishi M, Yano Y, et al: Gingival blood flow response upon thermal stimulation: comparison between young and middle-aged healthy men. *Kitasato Medical Journal* in press.
- 26) Sugiyama T: Basic technology and clinical applications of the updated model of laser speckle flowgraphy to ocular diseases. *Photonics* 2014; 1: 220–34.
- 27) Loe H, Silness J: Periodontal disease in pregnancy I. Prevalence and severity. *Acta Odontologica Scandinavica* 1963; 21: 533–51.
- 28) World Health Organization: Oral Health Surveys: Basic Methods, 5th edition. Geneva: WHO

Press; 2013; 48–50.

- 29) Izumi H, Karita K: Somatosensory stimulation causes autonomic vasodilatation in cat lip. *Journal of Physiology* 1992; 450: 191–202.
- 30) Forrester K, Doschak M, Bray R: In vivo comparison of scanning technique and wavelength in laser Doppler perfusion imaging: measurement in knee ligaments of adult rabbits. *Medical & Biological Engineering & Computing* 1997; 35: 581–6.
- 31) Roustit M, Blaise S, Millet C, Cracowski JL: Reproducibility and methodological issues of skin post-occlusive and thermal heperemia assessed by single-point laser Doppler flowmetry. *Microvascular Research* 2010; 79: 102–8.
- 32) Nagashima Y, Yada Y, Suzuki T, Sakai A: Evaluation of the use of an integration-type laser-Doppler flowmeter with a temperature-loading instrument for measuring skin blood flow in elderly subjects during cooling load: comparison with younger subjects. *International Journal of Biometeorology* 2003; 47: 139–47.
- 33) Safar ME: Pulse pressure in essential hypertension: clinical and therapeutical implications. *Journal of Hypertension* 1989; 7: 769–76.
- 34) Izzo Jr JL, Shykoff BE: Arterial stiffness: clinical relevance, measurement, and treatment. *Reviews in cardiovascular medicine* 2001; 2: 29–40.
- 35) Izumi H, Karita K: The effects of capsaicin applied topically to inferior alveolar nerve on antidromic vasodilatation in cat gingiva. *Neuroscience Letters* 1990; 112: 65–9.
- 36) Izumi H: Reflex parasympathetic vasodilatation in facial skin. *General Pharmacology* 1995; 26: 237–44.
- 37) Gánti B, Molnár E, Fazekas R, Mikecs B, Lohinai Z, Mikó S, et al: Evidence of spreading vasodilation in the human gingiva evoked by nitric oxide. *Journal of Periodontal Research* 2019; 54: 499–505.
- 38) Tominaga M, Caterina MJ: Thermosensation and pain. *Journal of Neurobiology* 2004; 61: 3–12.
- 39) Holowatz LA, Kenney WL. Local ascorbate administration augments NO- and non-NO-dependent reflex cutaneous vasodilation in hypertensive humans. *The American Journal of Physiology-Heart and Circulatory Physiology* 2007; 293: H1090–6.
- 40) Gogly B, Godeau G, Gilbert S, Legrand JM, Kut C, Pellat B, et al: Morphometric analysis of collagen and elastic fibers in normal skin and gingiva in relation to age. *Clinical Oral Investigations* 1997; 1: 147–52.
- 41) Bernick S. Age changes in the blood supply to human teeth. *Journal of Dental Research* 1967; 46: 544–50.
- 42) Sato I, Morita I, Kaji K, Ikeda M, Nagao M, Murota S: Reduction of nitric oxide producing activity associated with in vitro aging in cultured human umbilical vein endothelial cell.

Biochemical and Biophysical Research Communications 1993; 195: 1070–6.

- 43) Bergström J: Cigarette smoking as risk factor in chronic periodontal disease. Community Dentistry and Oral Epidemiology 1989; 17: 245–7.
- 44) Genco RJ, Ho AW, Grossi SG, Dunford RG, Tedesco LA: Relationship of stress, distress, and inadequate coping behaviors to periodontal disease. Journal of Periodontology 1999; 70: 711–23.

Figure legends

Figure 1. The principle and outline of LSFG. (A) shows the principle of LSFG. A laser irradiates the erythrocytes in the gingiva through the LSFG blood flow measurement device (C), and the random speckle pattern of reflected scattered light created by erythrocytes in the blood vessels is detected by the image sensor. (B) shows mapping images (blood flow image is superimposed onto the gingival image). (C) shows the probe unit of the prototype LSFG-based gingival blood flow measurement system (width 55 mm x depth 150 mm x height 90 mm).

Figure 2. Calculation of MBR, an index of tissue blood flow used in LSFG. MBR represents present blur in the photograph of a moving object, and it is proportional to the erythrocyte moving speed. (A) When erythrocytes move slowly, a relatively blur-free image is obtained, with a low MBR value. (B) In contrast, when erythrocytes move rapidly, an image with higher blur is obtained, with a high MBR value.

Figure 3. Composition of the speed calibrator. It comprises a high-speed motor and an opal glass plate (OGP). The intensity of the light reflected from the OGP at varying speeds of the high-speed motor was detected by the light receiver.

Figure 4. Gingival blood flow image measured using LSFG, (A) without the application of polarizing filter and (B) with the application of polarizing filter. The area outlined in blue in (A) indicates the area in which gingival blood flow was unable to be measured due to specular reflection. The use of the polarizing filter enabled the measurement of gingival blood flow in the same area as outlined in white in (B).

Figure 5. Relationship between the velocity measured using the speed calibrator and MBR measured by LSFG. The x-axis indicates the speed at which OGP underwent uniform circular motion, as measured by the speed calibrator (mm/s). The y-axis indicates MBR, the index of gingival blood flow, that was measured by LSFG. r : Pearson's correlation coefficient.

Figure 6. Relationship between MBR measured at two different distances. The x- and y-axes indicate gingival MBR measured at 80 and 160 mm, respectively. r : Pearson's correlation coefficient.

Figure 7. Polarization of surface-reflected and scattered light. Incident light from the laser source is scattered within the tissue and is partially re-emitted out from the tissue. Thus, the

direction of polarization is maintained in surface-reflected light but not in scattered light.

Figure 8. Typical analysis of gingival blood flow in a 32-year-old²⁴. (A) Gingival blood flow was measured in the region of the maxillary anterior gingiva enclosed by the white line. (B) A heartbeat map superimposed on a gingival image. The red area indicates high MBR, whereas the blue area indicates low MBR (excluding teeth). (C) Pulses were detected from the fluctuations in blood flow. (D) A representative standardized pulse waveform. MBR: mean blur rate.

Figure 9. Characteristics of pulse waveform parameters^{24, 25}. (A) The flow acceleration index (FAI) indicates the maximal blood flow increase per frame (1/30 s) within a single heartbeat. (B) The acceleration time index (ATI) indicates the peak position of blood flow and is derived from the ratio of the time before reaching the peak to a single heartbeat. (B) The falling rate is derived from the falling area of the waveform and indicates serial changes in the rate of decrease in blood flow. It was defined as the ratio of the area above the curve (S_F) relative to the entire area (S_{all}) after the peak. (C) The blowout time (BOT) is an index of the persistence of high blood flow, and represents the time at which the wave maintains more than half of the mean of the maximum and minimum MBR during a beat.

Figure 10. Mean pulse waveforms for a single heartbeat in the young (Y), middle-aged (M), and elderly groups (E)²⁴. Time was plotted on the horizontal axis with the duration of one heartbeat set as 100. Blood flow (MBR [%]) was plotted on the vertical axis using 0 and 100 as the minimum and maximum values of the mean pulse waveform, respectively (means \pm S. E.). The acceleration rates of increases in blood flow were faster in group Y. In addition, reductions in flow after reaching the peak were slower relative to those in other groups. In contrast, blood flow slowly increased in group E and the time to reach the peak was delayed. However, blood flow reduction after the peak was rapid. MBR: mean blur rate.

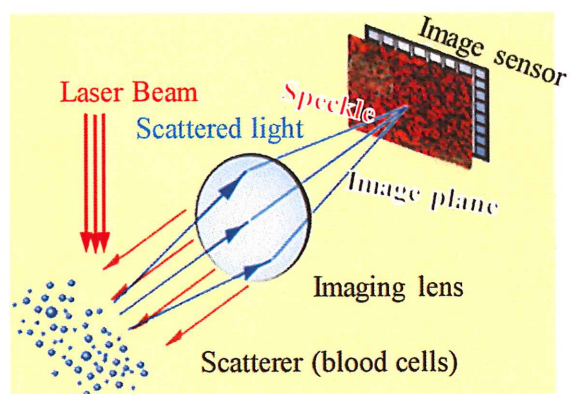
Figure 11. Time-course changes in MBP, HR, gingival MBR, and vascular conductance (MBR/MBP) in the young group (A) and middle-aged group (B) before and after localized thermal stimulation to the gingiva (i.e. heating)²⁵. MBP (A1 and B1), HR (A2 and B2), gingival MBR (A3 and B3), and vascular conductance (MBR/MBP) (A4 and B4). * $p < 0.05$ versus rest (paired t-test). HR, heart rate; MBR, mean blur rate; MBP, mean blood pressure. Young group (n = 12), middle-aged group (MBP: n = 12, HR: n = 13, MBR: n = 12, MBR/MBP: n = 11). MBP data for the middle-aged group are the average for 12 participants because the blood pressure sensor for one participant deviated during measurement. MBR data for the middle-aged

group are the average for 12 participants because another participant in this group moved his face upon measuring gingival blood flow after thermal application, making it impossible to measure gingival blood flow for more than 1 min after the stimulation. Thus, MBR/MBP data for the middle-aged group are the average for 11 participants.

Figure 12. Mean pulse waveforms for a single heartbeat at rest and at 30 s after heating in the young group (A) and middle-aged group (B)²⁵. Time was plotted on the horizontal axis with the duration of one heartbeat set as 100. Blood flow (MBR [%]) was plotted on the vertical axis using 0 and 100 as the minimum and maximum values of the mean pulse waveforms, respectively (means \pm standard errors). In the middle-aged group (B'), reductions in the blood flow after reaching the peak became faster at 30 s after heating than that at rest. In addition, the duration of high blood flow was shorter than that at rest. In contrast, in the young group (A'), reductions in the blood flow after reaching the peak did not change after the thermal stimulus. MBR: mean blur rate.

Figures

Figure 1



(C)



(B)

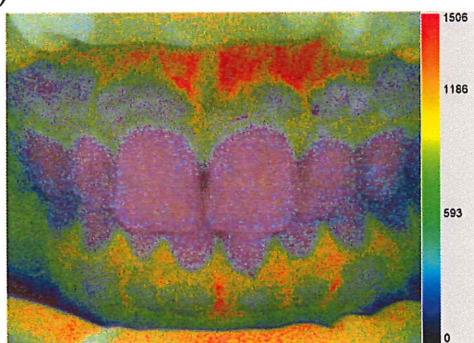


Figure 2

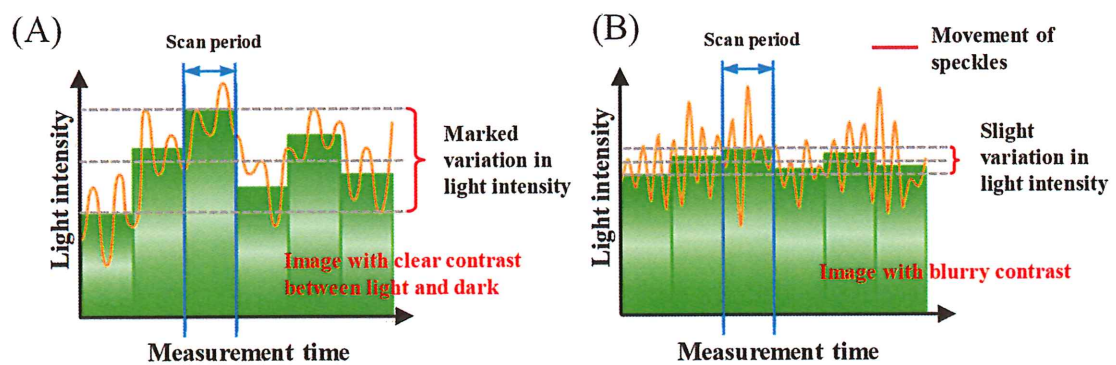


Figure 3

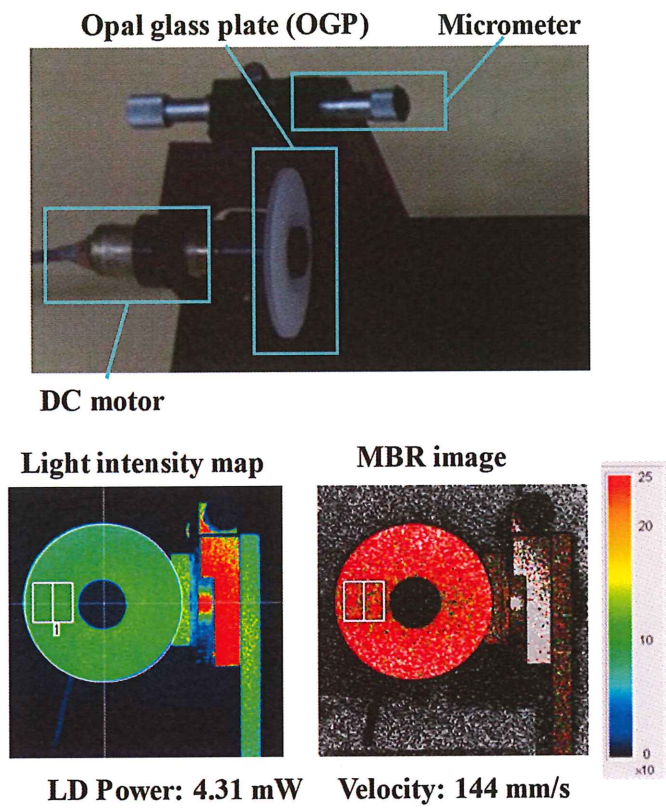


Figure 4

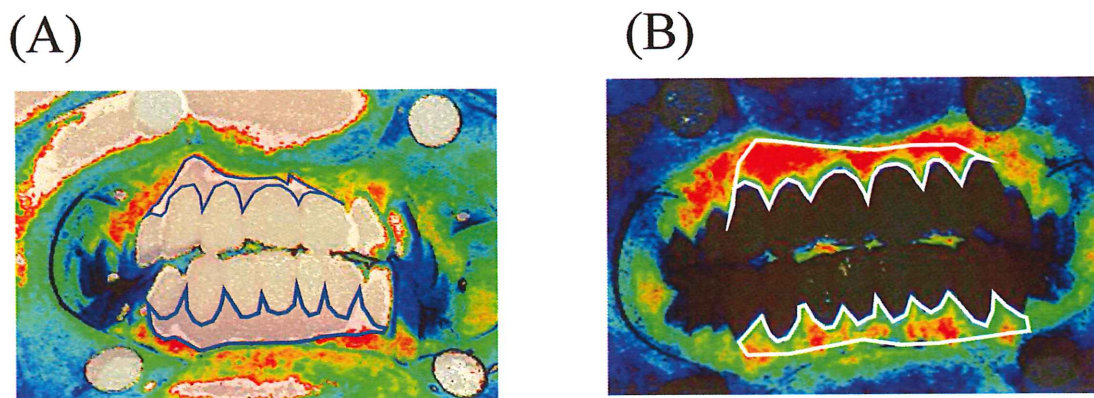


Figure 5

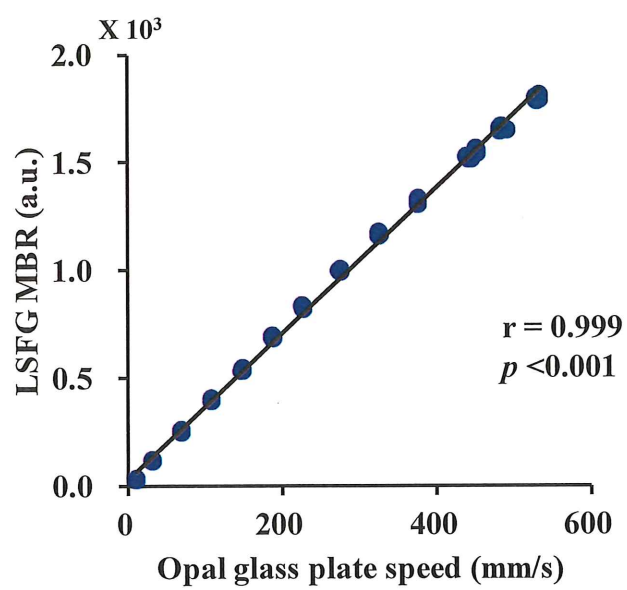


Figure 6

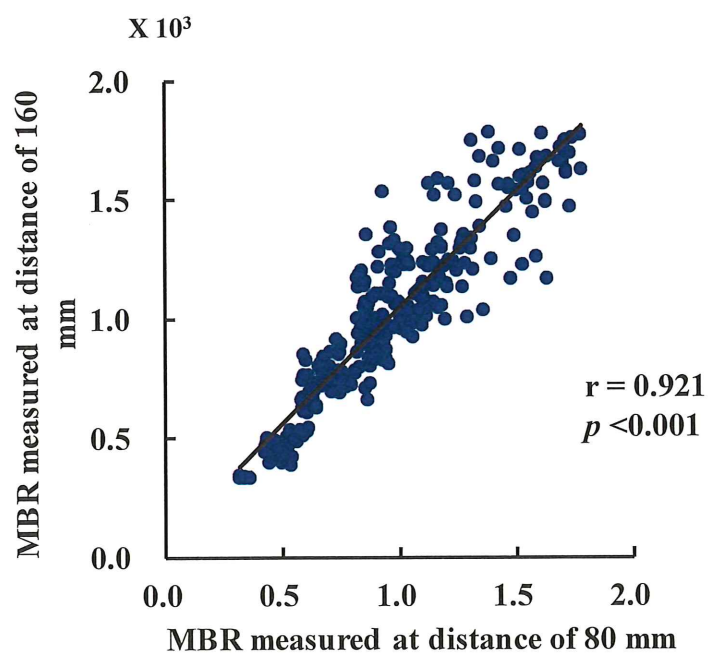


Figure 7

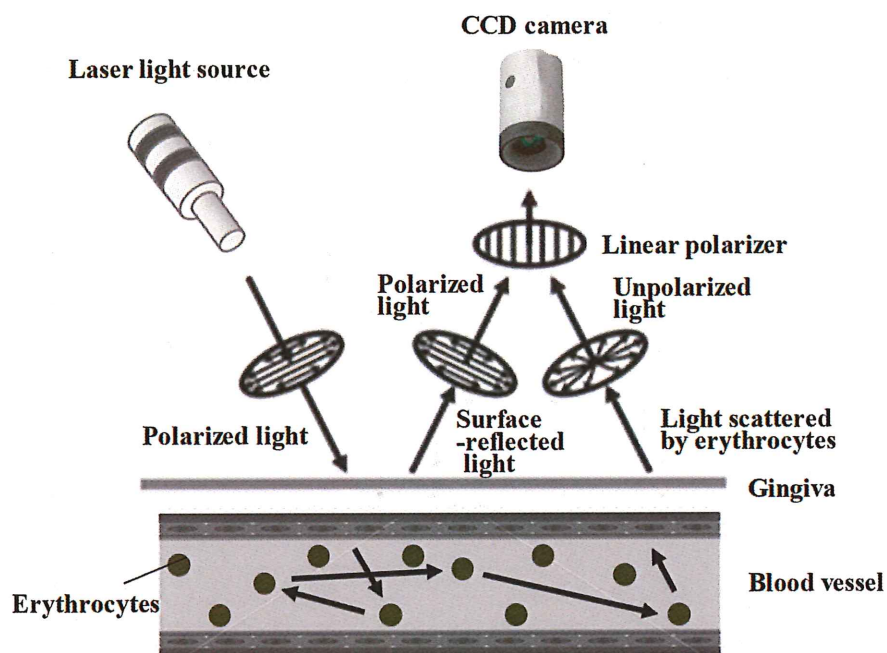


Figure 8

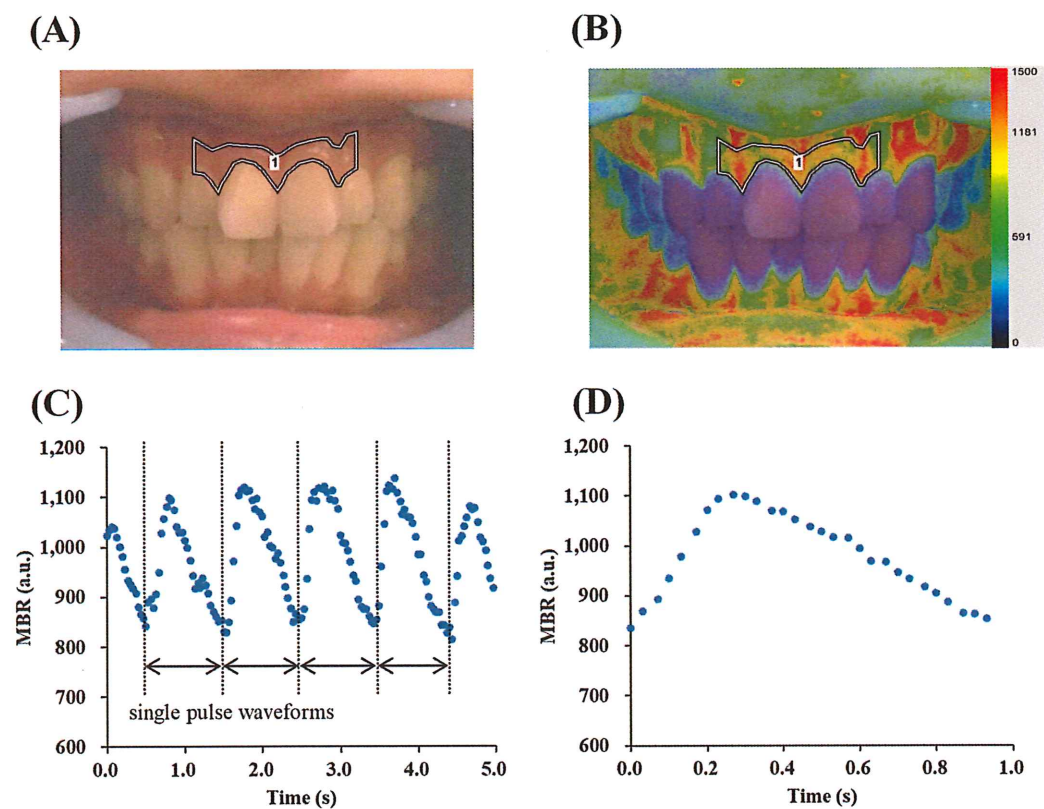
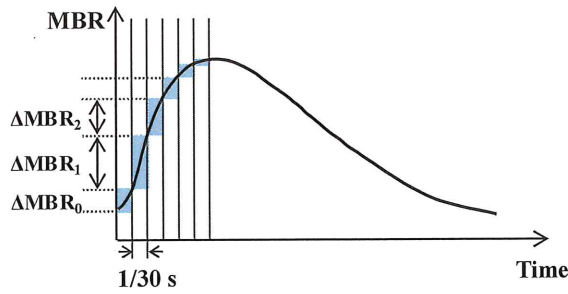


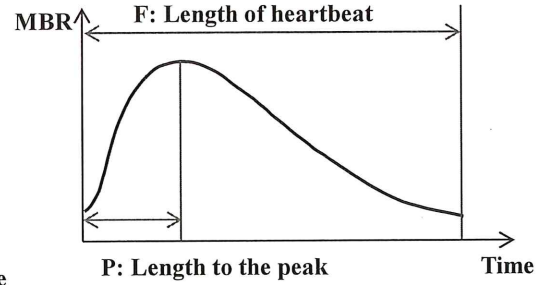
Figure 9

(A) Flow acceleration index (FAI)



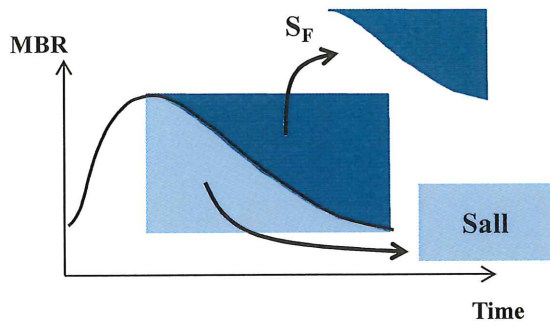
$$FAI = \text{Max} (\Delta MBR_i)$$

(B) Acceleration time index (ATI)



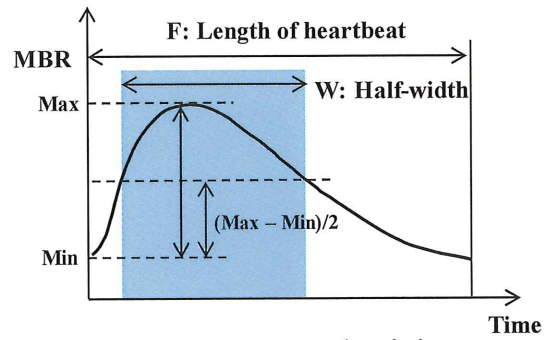
$$ATI = C \times (P/F)$$

(C) Falling rate



$$\text{Falling rate} = C \times (S_F/S_{all})$$

(D) Blowout time (BOT)



$$BOT = C \times (W/F)$$

C: the constant of proportionality

Figure 10

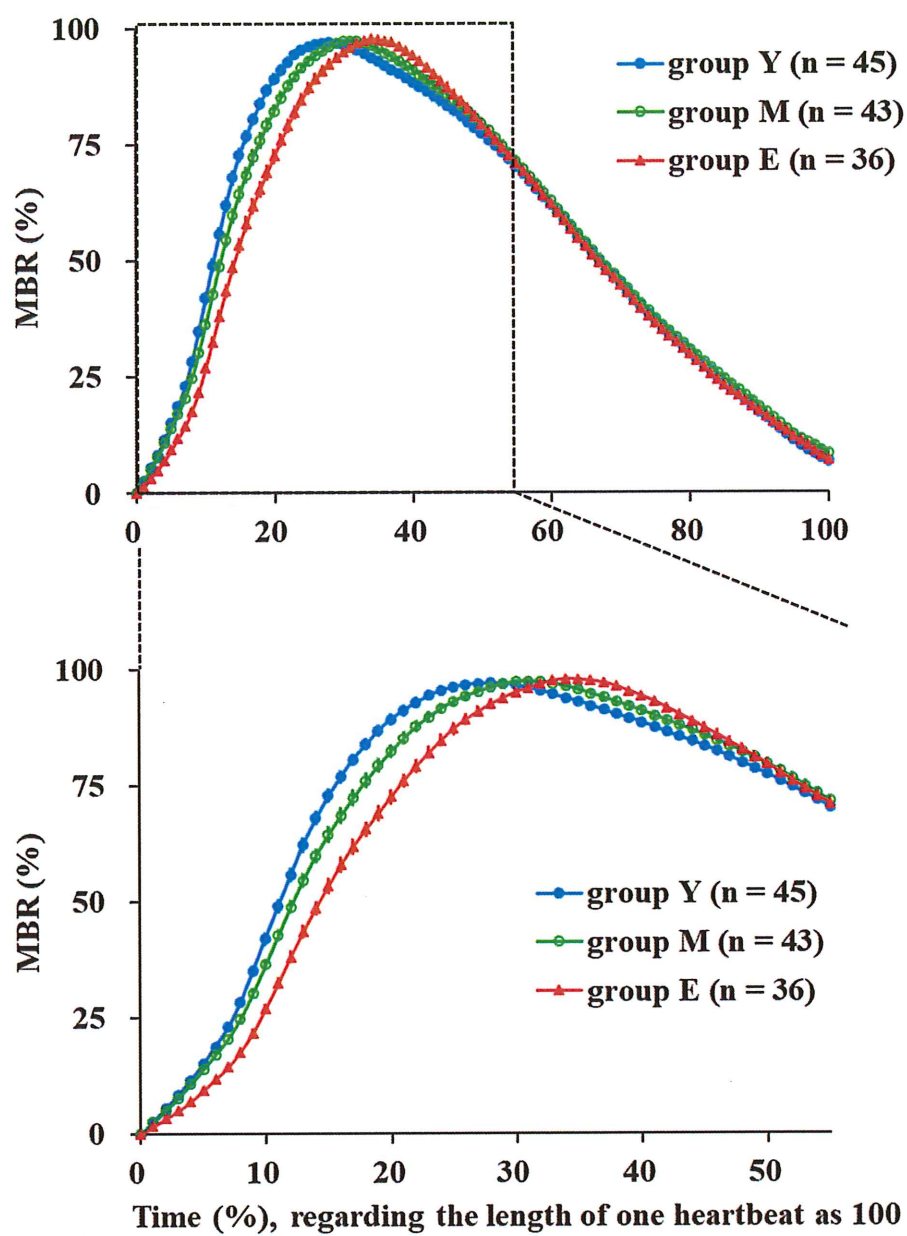


Figure 11

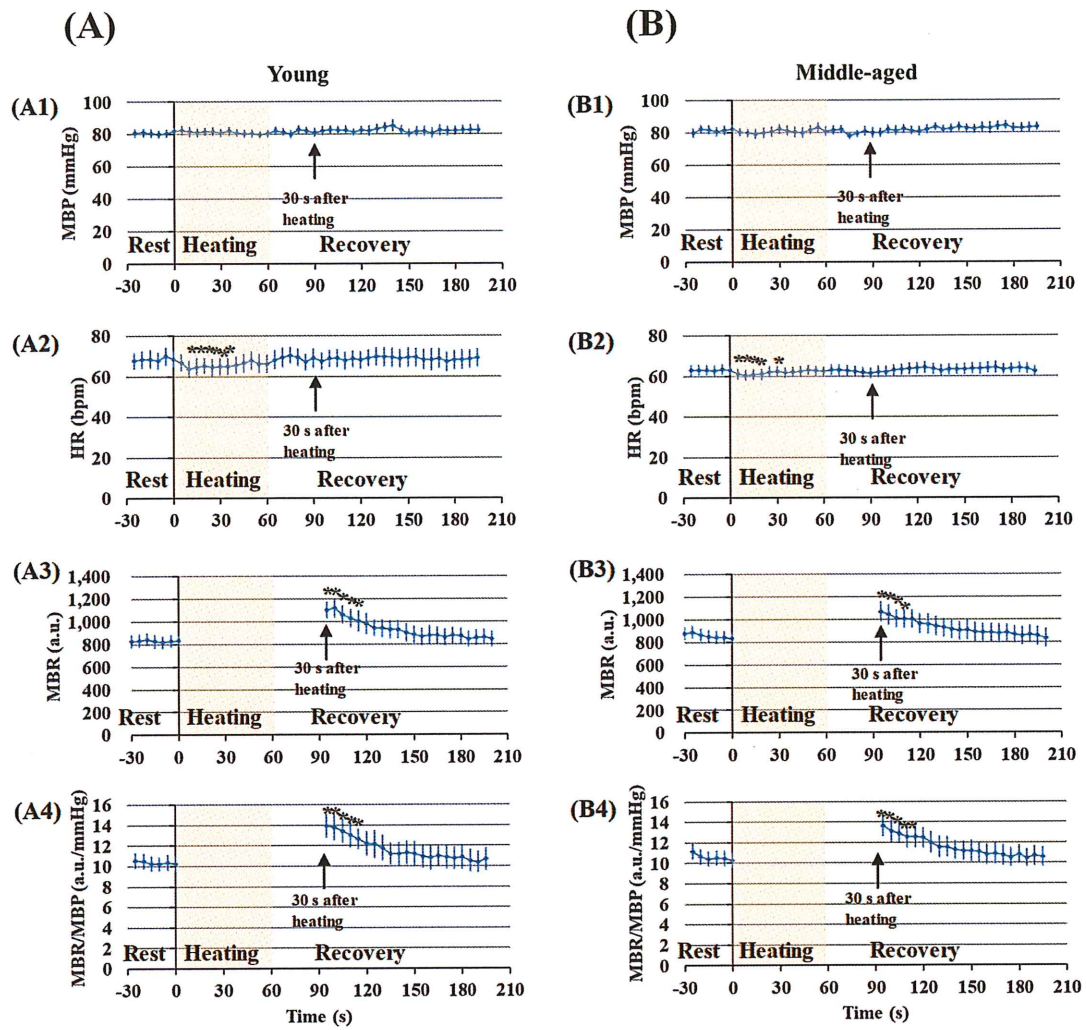
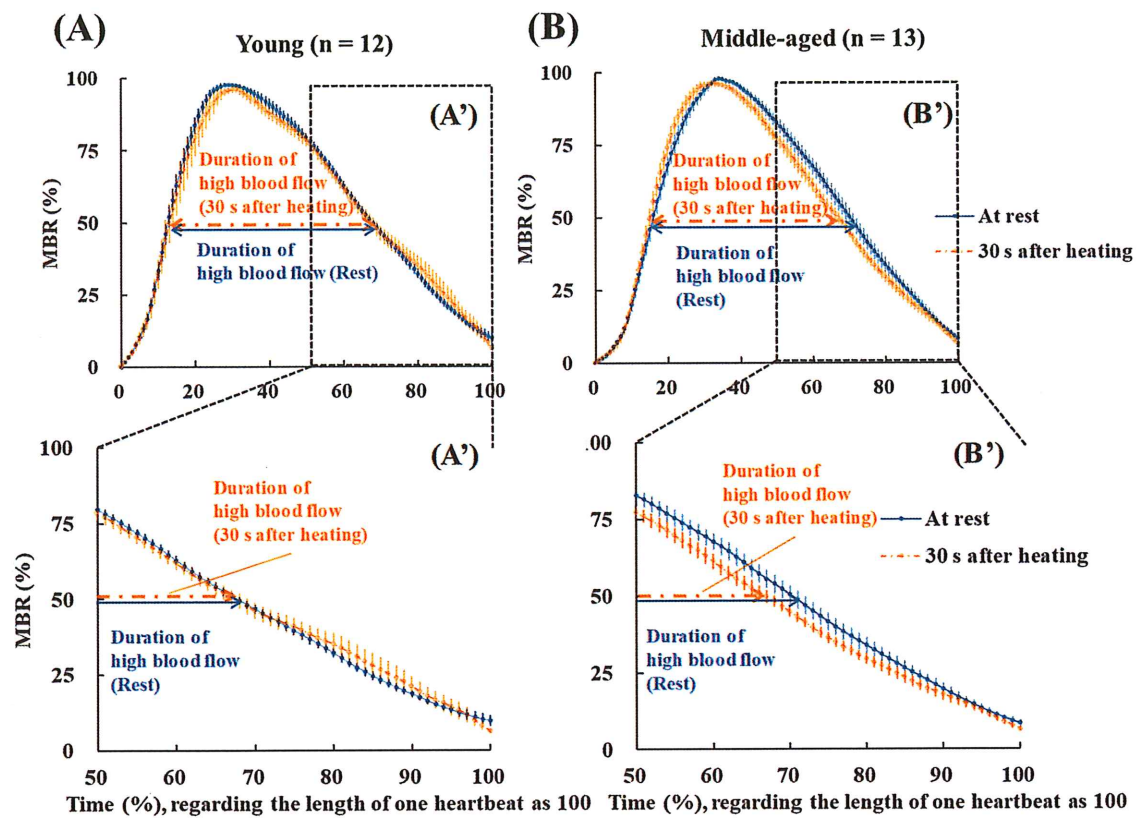


Figure 12



Tables

Table 1. A summary of the characteristics and gingival blood flow parameters in the young (Y), middle-aged (M), and elderly groups (E)²⁴.

	Group Y (n = 45)	Group M (n = 43)	Group E (n = 36)	One-way ANOVA	Multiple comparisons		
					Y vs. M	Y vs. E	M vs. E
Age (years)	22 - 37	38 - 53	54 - 69	p	p		
BMI (kg/m ²)	21.2 ± 0.3	23.2 ± 0.4	23.1 ± 0.4	< 0.001	< 0.001	< 0.01	1.000
SBP (mmHg)	116.3 ± 1.5	122.0 ± 2.0	130.3 ± 3.2	< 0.001	0.174	< 0.001	< 0.05
DBP (mmHg)	73.5 ± 1.0	80.7 ± 1.2	83.1 ± 1.7	< 0.001	< 0.01	< 0.001	0.191
MBP (mmHg)	87.8 ± 1.1	94.6 ± 1.4	98.7 ± 2.1	< 0.001	< 0.001	< 0.001	0.678
PP (mmHg)	42.8 ± 1.0	41.3 ± 1.1	47.3 ± 2.1	< 0.05	1.000	0.085	< 0.05
PPD (mm)	1.98 ± 0.03	2.01 ± 0.04	2.20 ± 0.07	< 0.01	1.000	< 0.01	< 0.05
MBR (a.u.)	983.6 ± 39.4	895.5 ± 37.9	917.1 ± 38.4	0.231	-	-	-
MBR/MBP (a.u./mmHg)	11.3 ± 0.5	9.6 ± 0.4	9.4 ± 0.4	< 0.01	< 0.05	< 0.05	1.000
ATI	30.6 ± 0.8	33.1 ± 0.7	35.4 ± 0.7	< 0.001	0.056	< 0.001	0.108
Falling rate	11.9 ± 0.1	12.2 ± 0.1	12.7 ± 0.1	< 0.001	0.399	< 0.001	< 0.05
BOT	55.8 ± 0.5	54.6 ± 0.6	51.9 ± 0.7	< 0.001	0.479	< 0.001	< 0.01

Means ± S. E. (one-way ANOVA was used for comparing groups, and Bonferroni's method was used for multiple comparisons). BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, MBP: mean blood pressure, PP: pulse pressure, PPD: probing pocket depth, MBR: mean blur rate, MBR/MBP: gingival vascular conductance, ATI: acceleration time index, BOT: blowout time.

Table 2. Results of Pearson's correlation analysis among MBR, gingival vascular conductance (MBR/MBP), and blood flow waveform parameters with age²⁴.

	R	p
MBR (a.u.)	0.09	-
MBR/MBP (a.u./mmHg)	-0.25	< 0.01
ATI	0.37	< 0.001
Falling rate	0.39	< 0.001
BOT	-0.39	< 0.001

R: Pearson's correlation coefficient. ATI: acceleration time index, BOT: blowout time, MBR: mean blur rate, MBP: mean blood pressure.

Table 3. Results of stepwise multiple regression analysis for factors independently affecting each blood flow parameter²⁴

Variable				
Dependent	Independent	β	p	R ²
MBR/MBP	SBP	-0.34	< 0.001	0.114
ATI	age	0.37	< 0.001	0.139
Falling rate	age	0.34	< 0.001	0.175
	PP	0.18	< 0.05	
BOT	age	-0.30	< 0.01	0.182
	SBP	-0.21	< 0.05	

β : standard partial regression coefficient, R²: multiple coefficient of determination, MBR: mean blur rate, MBR/MBP: gingival vascular conductance, ATI: acceleration time index, BOT: blowout time, PR: pulse rate, SBP: systolic blood pressure, PP, pulse pressure.

Table 4. Partial correlation coefficients of blood flow parameters, blood pressure, and pulse pressure using age as a control variable²⁴

	MBR (a.u.)	MBR/MBP (a.u./mmHg)	ATI	Falling rate	BOT
BMI (kg/m ²)	0.14	0.01	0.00	-0.06	-0.08
SBP (mmHg)	0.04	-0.27**	0.07	0.16	-0.21*
DBP (mmHg)	0.09	-0.26**	0.01	0.07	-0.12
MBP (mmHg)	0.06	-0.22*	0.04	0.12	-0.16
PP (mmHg)	-0.03	-0.21*	0.09	0.19*	-0.21*
PR (bpm)	0.22*	0.19*	-0.15	-0.09	0.13

*p < 0.05, **p < 0.01. BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, MBP: mean blood pressure, PP: pulse pressure, PR: pulse rate, MBR: mean blur rate, MBR/MBP: gingival vascular conductance, ATI: acceleration time index, BOT: blowout time.

Table 5. Summary of participant characteristics and gingival blood flow parameters in the young and middle-aged groups²⁵

	Young group (n = 12)	Middle-aged group (n = 13)
Age (years)	21–33	55–65
BMI (kg/m ²)	20.9 ± 0.6	22.7 ± 0.5*
SBP (mmHg)	106.6 ± 2.9	110.5 ± 3.7 ^a
DBP (mmHg)	63.2 ± 2.0	61.5 ± 2.0 ^a
MBP (mmHg)	80.8 ± 2.3	80.9 ± 2.6 ^a
PP (mmHg)	43.5 ± 1.9	49.0 ± 2.1 ^a
HR (bpm)	67.7 ± 4.1	62.9 ± 2.6
PPD (mm)	1.82 ± 0.06	1.87 ± 0.04
MBR (a.u.)	862.7 ± 59.1	887.4 ± 55.2
MBR/MBP (a.u. /mmHg)	10.8 ± 0.8	11.0 ± 0.7 ^a
FAI (a.u.)	64.1 ± 8.9	53.8 ± 7.0
ATI	29.1 ± 1.3	34.6 ± 1.3**
Falling rate	12.3 ± 0.2	12.3 ± 0.3
BOT	55.3 ± 0.9	53.9 ± 1.3

Means ± standard error, * $p < 0.05$, ** $p < 0.01$ versus the young group (t -tests). au: arbitrary unit. ^aThe blood pressure data for the middle-aged group are the average for 12 participants because the blood pressure sensor for one participant deviated during measurement. BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, MBP: mean blood pressure, PP: pulse pressure, HR: heart rate, PPD: probing pocket depth, MBR: mean blur rate, MBR/MBP: gingival vascular conductance, FAI: flow acceleration index, ATI: acceleration time index, BOT: blowout time.

Table 6. Changes in gingival blood flow before and after thermal stimulus in the young and middle-aged groups²⁵

	Young group (n = 12)		Middle-aged group (n = 13)	
	Rest	30 s after heating	Rest	30 s after heating
MBP (mmHg)	80.8 ± 2.3	81.0 ± 2.6	80.9 ± 2.6 ^a	80.0 ± 3.1 ^a
HR (bpm)	67.7 ± 4.1	68.7 ± 4.7	62.9 ± 2.6	62.1 ± 2.8
MBR (a.u.)	862.7 ± 59.1	1097.6 ± 72.9 ^{**}	887.4 ± 55.2	1095.4 ± 86.2 ^{**}
MBR/MBP (a.u./mmHg)	10.8 ± 0.8	13.8 ± 1.1 ^{**}	11.0 ± 0.7 ^a	14.0 ± 1.1 ^{a, **}
FAI (a.u.)	64.1 ± 8.9	101.5 ± 14.1 ^{**}	53.8 ± 7.0	80.0 ± 9.3 ^{**}
ATI	29.1 ± 1.3	27.9 ± 1.1	34.6 ± 1.3 [‡]	30.9 ± 1.4
Falling rate	12.3 ± 0.2	11.8 ± 0.3	12.3 ± 0.3	12.7 ± 0.3 [‡]
BOT	55.3 ± 0.9	55.9 ± 2.4	53.9 ± 1.3	50.6 ± 1.0 [*]

Means ± standard error, ^{*}*p* < 0.05, ^{**}*p* < 0.01 versus Rest (paired *t*-test). [†]*p* < 0.05, [‡]*p* < 0.01 versus the young group (*t*-test). MBP: mean blood pressure, HR: heartbeat, MBR: mean blur rate, FAI: flow acceleration index, ATI: acceleration time index, BOT: blowout time, au: arbitrary unit. ^aMBP data for the middle-aged group are the average for 12 participants because the blood pressure sensor for one participant deviated during measurement.