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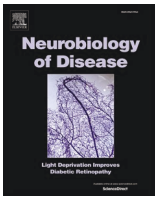

The diversity and abundance of gut microbiota are associated with the pain sensation threshold in the Japanese population

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ABSTRACT

Small fibre neuropathy (SFN) is an initial pathology of diabetic polyneuropathy (DPN). Serum lipopolysaccharide binding protein levels are positively correlated with the pain threshold in the foot, suggesting that the abundance of gut Gram-negative bacilli, which are a source of lipopolysaccharides, may be involved in the development of DPN. Furthermore, the abundance of the gut and oral microbiota is assumed to be involved in the pathogenesis of diabetes. Nevertheless, the association between SFN and the microbiota has not been clarified. A total of 1056 individuals were recruited in the 2018 Iwaki Health Promotion Project. Pain sensation was evaluated based on the pain threshold from intraepidermal electrical stimulation (PINT). Patients with PINT scores <0.15 mA were categorized into the low-PINT group ($n = 718$); otherwise, they were categorized into the high-PINT group ($n = 283$). Furthermore, each group was divided into the subjects with or without glucose tolerance based on HbA1c levels, fasting blood glucose levels and diabetic history. Principal coordinate analysis and α- and β-diversity of the microbiota were evaluated. The correlation between clinical and microbiota data was examined. Oral microbiota diversity showed no structural differences according to PINT scores, whereas principal coordinate analysis and α- and β-diversity revealed significant structural differences in gut microbiota ($p < 0.01$, $p < 0.05$ and $p < 0.05$, respectively), even after the participants with glucose intolerance were excluded ($p < 0.01$, $p < 0.05$ and $p < 0.05$, respectively). The relative abundance of the genus *Bacteroides* was significantly lower in high-PINT participants compared with low-PINT participants ($10 \pm 6.7\%$ vs. $11.3 \pm 7.0\%$, $p < 0.01$), even after the exclusion of subjects with diabetes and impaired fasting glucose ($10.0 \pm 6.5\%$ vs. $11.2 \pm 6.9\%$, $p < 0.05$). In univariate linear regression analyses, PINT was significantly correlated with metabolic syndrome parameters, eGFR, uric acid level and the abundance of *Bacteroides*. The correlation between *Bacteroides* and PINT scores remained significant after adjustment for multiple factors ($\beta = -0.07181$, $p < 0.05$). Changes of bacterial diversity and a low abundance of gut *Bacteroides* were correlated with elevated PINT scores in the Japanese population. This correlation may represent a new therapeutic option for SFN.

Abbreviations: DPN, diabetic polyneuropathy; SFN, small fibre neuropathy; IES, intraepidermal electrical stimulation; HbA1c, glycohemoglobin A1c; LBP, lipopolysaccharide binding protein; PINT, pain threshold from intraepidermal electrical stimulation; T2D, type 2 diabetes; IFG, impaired fasting glucose; BMI, body mass index; Fat, percent body fat; FBG, fasting blood glucose; sBP, systolic blood pressure; dBP, diastolic BP; Tc, total cholesterol; Tg, triglyceride; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; Hs-CRP, high sensitivity C-reactive protein; eGFR, estimated glomerular filtration rate; BDHQ, brief-type self-administered diet history questionnaire; ANOVA, analysis of variance; LPS, lipopolysaccharide; DPP4, dipeptydyl peptidase4; SGLT2, sodium-glucose co-transporter2; HFD, high fat diet.

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1. Introduction

Small nerve fibres consist of myelinated Aδ fibres and unmyelinated C fibres, and their function is to sense temperature changes and pain in the epidermis. Disorders of these nerve fibres manifest as small fibre neuropathy (SFN) in many pathologies, including diabetic polyneuropathy (DPN) (Strand et al., 2022). These nerve fibres are generally affected from the prediabetic state in DPN and result in spontaneous pain or loss of pain sensation as the symptoms of SFN (Hoitsma et al., 2004; Singleton et al., 2001). SFN can be evaluated by employing an electrode for intraepidermal electrical stimulation (IES) (Inui et al., 2002; Kukidome et al., 2016; Suzuki et al., 2016; Itabashi et al., 2019; Kudoh et al., 2020; Osonoi et al., 2020). We clarified that a normal high glycohemoglobin A1c (HbA1c) level, urine 8-hydroxy-2′-deoxy guanidine level indicative of oxidative stress and serum lipopolysaccharide binding protein (LBP) level indicative of inflammation and endotoxaemia correlated with an increased pain threshold from intraepidermal electrical stimulation (PINT) and represent biomarkers for SFN (Itabashi et al., 2019; Kudoh et al., 2020; Osonoi et al., 2020).

The human gut consists of trillions of bacteria residing in the gastrointestinal tract. The gut bacterial floor has various functions, including immune regulation, material absorption, and energy metabolism (Zhao, 2013; Sonnenburg and Bäckhed, 2016). Alterations in the composition of the healthy microbiota of the gut may lead to consequent systemic inflammation and local inflammation, which can change the functions of the central and peripheral nerves (Pane et al., 2022). An abundance of findings indicate that gut microbiota indeed plays a predominant role in the manifestation of acute and chronic pain, modulating the production of metabolites, neurotransmitters, cytokines and gut hormones (Lin et al., 2020). Interestingly, the transplantation of faecal microbiota from spared nerve injury rats to sham-operated germ-free mice can alter the severity of neuropathic pain, which means that regulation of the gut bacterial floor is a promising therapeutic target for neuropathic pain (Yang et al., 2019).

In addition, changes in the composition of the gut microbiota can be ascribed to the manifestation of hyperglycaemia (Gurung et al., 2020; Larsen et al., 2010). In subjects developing DPN, it is likely that the composition of gut microbiota exhibits a greater diversity and severe disruption of microbiota community richness compared with individuals with type 2 diabetes (T2D) alone (Wang et al., 2020).

On the other hand, the oral microbiota mirrors the dentate status, including its general development and periodontitis (Belstrøm, 2020). The oral microbiota also reflects the state of general diseases, including diabetes. A previous study reported that diabetes is associated with a decrease in the bacterial diversity of the oral microbiota (Ogawa et al., 2017; Sabharwal et al., 2019; Saeb et al., 2019). These changes in the composition of the oral microbiota may also provoke a low-grade general inflammatory reaction (Holmstrup et al., 2017).

These findings imply that the change in microbiota can explain the pathogenesis of SFN. Nevertheless, the correlation has not been evaluated. Herein, we evaluated PINT in a general Japanese population that included individuals with impaired fasting glucose (IFG) and overt diabetes. The correlation between PINT and the change in microbiota in the gut and oral cavity was determined in this study.

2. Material and methods

2.1. Ethics statement

This study was performed in accordance with the ethical standards of the Declaration of Helsinki and approved by the ethics committee at Hirosaki University Medical Ethics Committee (#2018–063). All patients provided written informed consent for this study.

2.2. Demographic characteristics of the study participants

We evaluated the medical data of volunteers from the Iwaki study, a health promotion study of Japanese citizens over 10 years of age. In this project, a health evaluation was conducted annually for participants living in the Iwaki area, a suburban area of Hirosaki in the Aomori Prefecture of northern Japan (Itabashi et al., 2019; Kudoh et al., 2020; Osonoi et al., 2020). Associations between clinical measurements and PINT scores were examined using the data from the 2018 Iwaki study.

2.3. Clinical profile

Fasting blood samples were collected in the morning from peripheral veins with the individual in the supine position. The following clinical measures were recorded: height; body weight; body mass index (BMI); percent body fat (fat); abdominal, waist and hip circumference; fasting blood glucose (FBG), HbA1c, C-peptide, creatinine, and blood urea nitrogen (BUN) levels; systolic blood pressure (sBP); diastolic BP (dBP); and serum levels of total cholesterol (Tc), triglyceride (Tg), high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c), uric acid, and high sensitivity C-reactive protein (Hs-CRP). Adipose tissue volume was measured with the bioelectricity impedance method using a Tanita MC-190 body composition analyser (Tanita Corp., Tokyo, Japan). Diabetes was diagnosed according to the 2010 Japan Diabetes Society criteria (IFG: fasting blood glucose levels 110–125 mg/dL, diabetes: fasting blood glucose levels ≥126 mg/dL or HbA1c levels ≥6.5%) (Committee of the Japan Diabetes Society et al., 2010). Those on medication for diabetes with a normal blood glucose level were also defined as having diabetes. HbA1c (%) was expressed as the National Glycohemoglobin Standardization Program value. None of the patients were diagnosed with type 1 diabetes or inherited diseases that affected HbA1c values. If the estimated glomerular filtration rate (eGFR) was <30 mL/min/1.73 m^2, which was calculated using the modified Japanese coefficient ($eGFR = 192 \times \text{serum creatinine}^{-1.094} \times \text{age}^{-0.287} \times 0.739$ [if female]), a diagnosis of severe renal failure was made (Matsuo et al., 2009; National Kidney Foundation, 2002). Hypertension was defined as blood pressure ≥ 140/90 mmHg or a history of treatment for hypertension. Hyperlipidaemia was defined as Tc ≥ 220 mg/dL, Tg ≥ 150 mg/dL, or history of treatment for hyperlipidaemia. Alcohol intake (current or nondrinker), smoking habits (current or nonsmoker), and subjective neuropathic symptoms were determined via questionnaire. Nutritional consumption data for the study participants were obtained using the brief-type self-administered diet history questionnaire (BDHQ) (Kobayashi et al., 2011). The BDHQ is a 4-page fixed-portion questionnaire that asks about the consumption frequency of selected foods, but not about portion size, to estimate the dietary intake of 58 food and beverage items during the preceding month, which are commonly consumed in Japan (Kobayashi et al., 2011; Kobayashi et al., 2012). Based on the standard tables of food composition in Japan revised in 2010, crude estimates for dietary intake of total energy and 42 selected nutrients were calculated (Watanabe, 2015).

2.4. PINT measurement

For nociceptive stimulation, we used the same methodology that was employed in previous studies (Itabashi et al., 2019; Kudoh et al., 2020; Osonoi et al., 2020). The IES method was adopted using a disposable concentric bipolar needle electrode (NM-983 W; Nihon Kohden Corp., Tokyo, Japan) connected to a specific stimulator for cutaneous Aδ and C fibres as previously described (PNS-7000; Nihon Kohden) (Itabashi et al., 2019; Kudoh et al., 2020; Osonoi et al., 2020). The stimulator consisted of an outer ring anode (1.3 mm diameter), and the cathode included an inner needle that protruded 0.025 mm from the level of the outer ring. The IES electrode was placed onto the skin of the centre instep to deliver weak continuous electrical stimulations. The skin over

the extensor digitorum brevis was concurrently used for the evaluation in approximately half of the randomly selected participants. This stimulation can evoke a local pricking sensation. In instances where the keratinized layer of the skin was too thick and likely to interrupt the electronic stimulation, the electrode was moved to a location with less keratinization on the same foot. The participants were instructed to push the button as quickly as possible only when they felt a sensation. The electrical stimulation intensity started from 0.4 mA, which can be easily sensed by subjects who have normal pain sensation, and decreased stepwise by 0.05 mA until the participants reported a pricking sensation. The current intensity is directly proportional to the intensity of stimulation. PINT scores were defined as the minimum intensity at which the participants felt a pricking sensation in more than two trials. Therefore, PINT can evaluate the degree of hypoalgesia in response to electrical pain stimulation. A total of 20 well-trained staff members were involved in the measurement of PINT. For all subjects, the median PINT score was 0.10 mA, and the average PINT score for all subjects was 0.11 ± 0.09 mA with a 95% confidence interval from 0.11 to 0.12. The 95th percentile was 0.12 mA. Therefore, the subjects showing PINT scores of 0.15 mA or more were categorized as high-PINT subjects in this study.

2.5. Sample collection and DNA extraction

Faecal samples were collected from each subject in commercial containers 3 days before the health check (TechnoSuruga Laboratory Co., Ltd., Shizuoka, Japan) and suspended in guanidine thiocyanate solution [100 mM Tris–HCl (pH 8.0), 40 mM Tris–EDTA (pH 8.0), 4 M guanidine thiocyanate and 0.001% bromothymol blue] (Iino et al., 2019). Faecal samples were stored at 4 °C until the DNA was extracted as reported previously (Kawada et al., 2019). GTC buffer solutions containing faecal samples (800 μL of faeces) were added to tubes filled with zirconium beads. The tubes were then mixed at room temperature for 2 min at a speed of 5 m/s using a FastPrep 24 Instrument (MP Biomedicals, Santa Ana, CA, USA) (Iino et al., 2019). After cooling, the samples were centrifuged at 2350 ×*g* for 1 min. The DNA was then extracted from the bead-treated suspension using an automatic nucleic acid extractor (Precision System Science, Chiba, Japan). A MagDEA DNA 200 (GC) reagent kit (Precision System Science) was used for automatic nucleic acid extraction. The final concentration of each DNA sample was adjusted to 10 ng/μL. We completed the extraction of all sample DNA within 4 months (Iino et al., 2019). According to the methods described in previous reports, tongue plaque samples were also obtained by brushing the dorsal surface of the tongue 4–5 times with a swab on the morning of the survey before breakfast and tooth brushing after overnight fasting (Sato et al., 2020). Saliva is an aggregate of bacterial flora exfoliated from various parts of the oral cavity that can reflect the state of the bacterial flora in the whole oral cavity (Segata et al., 2012). Since the composition of the bacterial flora of saliva is known to be closer to that of the dorsum of the tongue coating, tongue plaque samples on the dorsal surface can represent the bacterial flora of the entire oral cavity (Yamanaka et al., 2012). Furthermore, tongue plaque samples on the dorsal surface have less fluctuation than other parts of the oral cavity (Zhou et al., 2013).

The swab head was then placed in a collection tube containing 4 M guanidium thiocyanate, 100 mM Tris-HCl (pH 8.0), 40 mM EDTA and 0.001% bromothymol blue. The samples were mixed with zirconia beads using a FastPrep 24 instrument (MP Biomedicals, Santa Ana, California, USA). DNA was extracted from the bead-treated suspensions using an automatic nucleic acid extractor and MagDEA DNA 200 (GC) (Precision System Science).

2.6. Next-generation sequence analysis and 16S rDNA-based taxonomic analysis

Next-generation sequence analysis was performed following the protocol of Ozato et al. (Ozato et al., 2019). Universal primer sets were used to amplify the V3–V4 region of the prokaryotic 16S rRNA gene, as described previously (Takahashi et al., 2014). The polymerase chain reaction (PCR) mixture and conditions were as described previously (Takahashi et al., 2014). To check the size of the amplified fragments, 2.0-μL aliquots of the PCR mixtures were electrophoresed on 1.0% agarose gels. The amplified fragments were purified using PCR Cleanup Filter Plates (Merck Millipore, Burlington, MA, USA). The purified PCR fragments were quantified by real-time quantitative PCR (q-PCR) using the methods described by Takahashi et al. (Takahashi et al., 2014). Illumina paired-end sequencing was performed using the 2 × 300 cycle paired-end method on the MiSeqTM system (Illumina, San Diego, CA, USA). The multiplexed paired-end reads from the Illumina MiSeq system were processed as follows. The adaptor sequences and low-quality bases (threshold = 20) were trimmed at the 3′-end of the reads by Cutadapt (version: 1.13). Reads containing N bases and shorter than 150 bases were discarded. The paired-end reads above the filter threshold were merged to form a single read called a "merged read". Merged reads shorter than 370 or longer than 470 were excluded by the fastq_mergepairs subcommand of VSEARCH (version: 2.4.3). Merged reads with more than one expected sequencing error were also excluded. After removing chimaera reads detected by the uchime_denovo subcommand of VSEARCH, the remaining merged reads were clustered at a sequence identity ≥97%. The taxa of the identified clusters were predicted by applying the database of microbiota named the Ribosomal Database Project (http://rdp.cme.msu.edu/) based on their representative reads. The results with a confidence value below 0.8 were treated as unclassified. In this study, *Ruminococcus* means a genus that belongs to *Ruminococcaceae,* and *Ruminococcus2* means a genus that belongs to *Lachnospiraceae*. The proportion of each genus of the gut microbiota is a composition ratio obtained by dividing the number of read counts of each genus by the total number of read counts. We compared the relative abundance of various intestinal bacteria in the gut microbiota and oral bacteria in the oral microbiota between SFN and non-SFN participants. The relative abundance is presented as the percentage composition of reads for each bacterium relative to the total number of reads. We investigated the whole gut and oral microbiota, and in the correlation analysis, microbiota with a relative abundance >1% were evaluated. These microbiota can influence the clinical pathogenesis of SFN.

2.7. Statistical analysis

Statistical analyses of the clinical data were performed using JMP ver. 12.1 (SAS Institute, Cary, NC) and R software (R Foundation for Statistical Computing, version R-3.4.3). The values of clinical measures are expressed as the means ± standard deviations. Normal distribution was evaluated by the Shapiro–Wilk normality test and Kolmogorov–Smirnov normality test. All kinds of bacterial abundance and PINT scores were not distributed normally. The statistical significance of the difference in values between two groups (parametric or nonparametric) and case–control associations among groups (nonparametric) was assessed by one-way analysis of variance (ANOVA) with post hoc tests and $\chi 2$ tests and Wilcoxon rank sum test, respectively. Correlations between PINT scores and clinical parameters were assessed by linear regression analyses, and the correlations were further assessed using multiple logistic regression analysis. Values were adjusted for factors associated with PINT scores using univariate regression analysis and accounting for potentially cofounding variables for SFN, as reported in a previous study (Itabashi et al., 2019; Kudoh et al., 2020; Osonoi et al., 2020). In addition, α-diversity was evaluated using the Shannon index and Chao1 index, and β-diversity was evaluated by principal coordinate analysis and statistically analysed using permutation multivariate analysis of variance. To match some clinical baseline characteristics of low-PINT and high-PINT cases, the propensity score was estimated by fitting a logistic regression model. One-to-one matching was performed using the nearest neighbour match on the propensity score with a calliper width set to 0.20 times the standard deviation of the propensity

score. A value of $p < 0.05$ was regarded as statistically significant.

2.8. Data availability

Anonymized data not published within this article will be made available by request for any qualified investigator.

3. Results

3.1. Subject demographics

Out of 1056 volunteers from the Iwaki Study 2018, one thousand one subjects (419 men, 582 women), including overt diabetic subjects, were finally examined in this study (Fig. 1). The participants were further divided into 789 glucose tolerable subjects (327 men, 462 women) and 212 glucose intolerable subjects (92 men, 120 women). Each group was divided into the low-PINT group and the high-PINT group based on PINT levels (0.15 mA) as follows: (1) glucose tolerance, low PINT ($n = 580$): PINT <0.15 mA, (2) glucose tolerance, high PINT ($n = 209$): PINT ≥0.15 mA, (3) glucose intolerance, low PINT ($n = 138$): PINT <0.15 mA, and (4) glucose intolerance, high PINT ($n = 74$): PINT ≥0.15 mA. The clinical profiles of the men and women participants are shown in Table 1. The mean age was 52.2 ± 15.2 years for men and 53.2 ± 15.7 years for women. The frequency of subjective symptoms was comparable between men and women. PINT scores were significantly greater in men than women (0.12 ± 0.09 mA vs. 0.10 ± 0.09 mA, $p < 0.01$). However, the gap was 0.02 mA, and both scores were <0.15 mA, which was regarded as a clinically insignificant difference.

3.2. Subject demographics based on diabetic states

The clinical profiles of subjects based on diabetic status are shown in Table 2. The mean age was significantly higher in the diabetic group (DM) than in the nondiabetic group (nDM) (62.27 ± 12.98 years vs. 50.56 ± 2.68 years, $p < 0.01$). HbA1c levels were significantly higher in IFG subjects than in nDM subjects (6.12 ± 0.14% vs. 5.55 ± 0.23%, $p < 0.01$). HbA1c in the DM group was further increased compared with that in the IFG group (7.19 ± 1.14% vs. 6.12 ± 0.14%, $p < 0.01$). Intake of water, protein and carbohydrate was significantly increased in DM compared to nDM (1906.2 ± 626.2 g vs. 1683.1 ± 0.10 g, $p < 0.01$, 77.5 ± 27.9 g vs. 69.7 ± 25.0 g, $p < 0.05$, and 261.5 ± 95.9 g vs. 241.2 ± 82.2 g, $p < 0.05$, respectively). The main components of T2D treatment

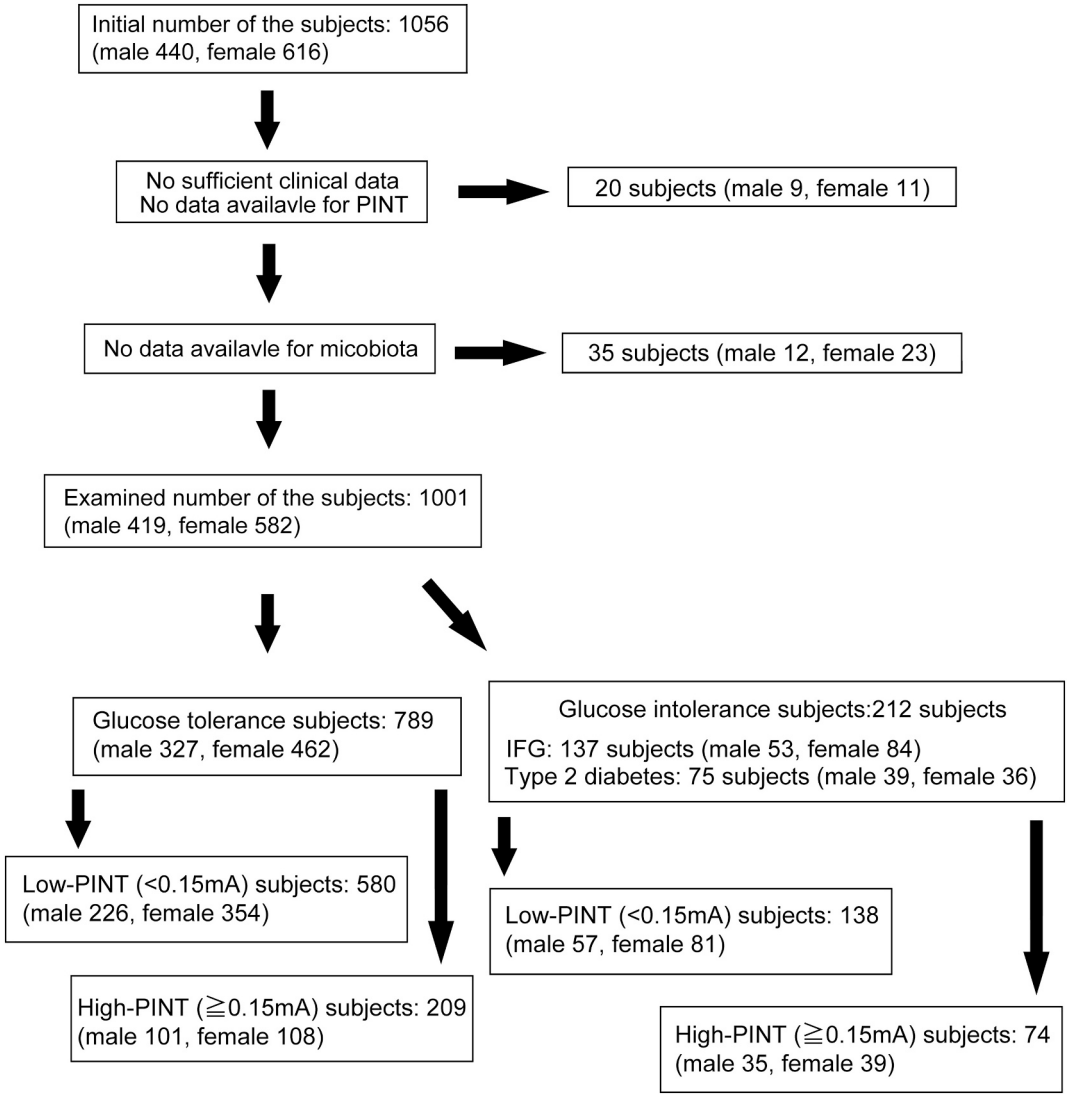

Fig. 1. Flow chart of subject selection.
Seven hundred eighty-nine normoglycemic participants (327 males, 462 females), 137 impaired fasting glucose participants (53 males, 84 females) and 75 type 2 diabetic participants (39 males, 36 females) were finally examined out of 1056 volunteers from the Iwaki Study 2018 in this study. PINT, pain threshold from intraepidermal electrical stimulation; IFG, impaired fasting glucose.

Table 1
Clinical profiles of the participants of IWAKI study.

	Men	Women	*P*
Numbers	419	582	–
Age (yrs)	52.20 ± 15.21	53.19 ± 15.65	*0.311*
Height (cm)	169.34 ± 6.57	156.57 ± 6.16	*<0.001*
Body weight (kg)	68.87 ± 11.07	54.55 ± 9.16	*<0.001*
BMI (kg/m^2)	23.98 ± 3.30	22.26 ± 3.56	*<0.001*
Fat (%)	23.73 ± 0.18	22.03 ± 0.15	*<0.001*
Abd. circumference (cm)	88.62 ± 9.35	81.75 ± 9.66	*<0.001*
Waist circumference (cm)	83.87 ± 9.24	73.61 ± 9.14	*<0.001*
Hip circumference (cm)	95.14 ± 6.00	92.46 ± 6.46	*<0.001*
FBG (mg/dL)	97.6 ± 17.1	92.7 ± 13.0	*<0.001*
HbA1c (%)	5.78 ± 0.70	5.74 ± 0.51	*0.352*
C-peptide (ng/mL)	1.6 ± 0.7	1.4 ± 0.5	*<0.001*
Cr (mg/dL)	0.81 ± 0.46	0.59 ± 0.28	*<0.001*
BUN (mg/dL)	14.73 ± 3.94	13.70 ± 4.20	*<0.001*
e-GFR	84.42 ± 17.24	87.44 ± 17.48	*0.006*
sBP (mmHg)	128.6 ± 17.8	122.5 ± 18.2	*<0.001*
dBP (mmHg)	81.9 ± 12.1	76.4 ± 11.3	*<0.001*
Tc (mg/dL)	201.36 ± 32.59	204.21 ± 35.46	*0.188*
Tg (mg/dL)	120.31 ± 88.72	80.83 ± 42.10	*<0.001*
HDL-c (mg/dL)	59.39 ± 16.37	70.10 ± 16.84	*<0.001*
LDL-c (mg/dL)	118.28 ± 28.46	116.59 ± 30.42	*0.367*
Uric acid (mg/dL)	6.04 ± 1.25	4.41 ± 0.99	*<0.0001*
Hs-CRP (mg/dL)	0.08 ± 0.11	0.06 ± 0.10	*0.030*
Water intake (g/day)	1978.9 ± 589.1	1524.0 ± 484.3	*<0.001*
Protein intake (g/day)	76.4 ± 26.6	61.7 ± 25.2	*<0.001*
Lipid intake (g/day)	56.7 ± 20.6	52.3 ± 18.0.5	*0.001*
Carbohydrate intake (g/day)	277.7 ± 88.0	219.6 ± 68.3	*<0.001*
Hypertension: n (%)	29.36 (123/419)	21.82 (127/582)	*0.330*
Dyslipidemia: n (%)	12.65 (53/419)	13.57 (79/582)	*>0.999*
Alcohol habit: n (%)	68.97 (289/419)	29.55 (172/582)	*<0.001*
Smoking habit: n (%)	27.21 (114/419)	8.25 (48/582)	*0.001*
Subjective symptoms: n (%)	1.19 (5/419)	1.03 (6/582)	*>0.999*
PINT scores (mA)	0.12 ± 0.09	0.10 ± 0.09	*0.001*

BMI, body mass index; Abd. Circumference, abdominal circumference; FBG, fasting plasma glucose; Cr, creatinine; e-GFR, estimated glomerular filtration rates; sBP, systolic blood pressure; dBP, diastolic blood pressure; Tc, total cholesterol; Tg, triglyceride; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; Hs-CRP, high sensitivity C-reactive protein; PINT, pain threshold from intraepidermal electrical stimulation.

were dietary therapy (53.3%), oral antidiabetic agents (36.7%), including metformin (16.0%), dipeptydyl peptidase4 (DPP4) inhibitor (30.7%), sodium-glucose co-transporter2 (SGLT2) inhibitor (8.0%) and insulin (10.7%). A total of 25.3% of subjects were treated with multiple types of agents, including insulin. PINT scores of IFG were significantly higher than those of nDM (0.12 ± 0.11 mA vs. 0.11 ± 0.08 mA, $p < 0.05$). The PINT scores of DM were greater than those of IFG (0.15 ± 0.13 mA vs. 0.12 ± 0.11 mA, $p < 0.05$).

3.3. Subject demographics based on PINT scores

Age; metabolic syndrome-related parameters, such as BMI, waist and hip circumference; sBP; Tg; diabetes-related parameters, such as FBG, HbA1c, and c-peptide; and renal failure-related parameters, such as BUN and Cr, were significantly higher in high-PINT subjects than in low-PINT subjects (Table 3). PINT scores were significantly increased in high-PINT subjects compared with low-PINT subjects (0.22 ± 0.10 mA vs. 0.07 ± 0.02 mA, $p < 0.001$).

3.4. Oral bacterial floor and peripheral pain sensation

In the oral bacterial floor, no microbial structural differences were observed in the principal coordinate analysis between low-PINT and high-PINT participants (Fig. 2A). After the exclusion of subjects with diabetes and IFG, no significant differences in microbial structure were noted between low-PINT and high-PINT participants (Fig. 2A). No significant differences in the Shannon index ($p = 0.22$) or the Chao1 index ($p = 0.91$) were observed between low-PINT and high-PINT subjects

Table 2
Clinical profiles of the subjects divided by diabetic states.

	nDM	IFG	DM
Numbers (male/female)	789 (328/461)	137 (53/84)	75 (39/36)
Age (yrs)	50.56 ± 2.68	60.39 ± 12.13*	62.27 ± 12.98*
Height (cm)	162.27 ± 8.92	160.15 ± 8.12†	161.01 ± 9.82
Body weight (kg)	59.44 ± 11.96	63.86 ± 12.32*	65.66 ± 12.75*
BMI (kg/m^2)	22.45 ± 3.29	24.79 ± 3.67*	25.26 ± 4.09*
Abd. circumference (cm)	83.09 ± 9.55	89.46 ± 9.75*	91.48 ± 10.77*
Waist circumference (cm)	76.32 ± 10.01	82.78 ± 9.96*	85.29 ± 10.28*
Hip circumference (cm)	92.96 ± 6.21	95.77 ± 6.18*	95.95 ± 7.30*
FBG (mg/dL)	90.49 ± 8.50	100.94 ± 8.90*	127.85 ± 28.86*‡
HbA1c (%)	5.55 ± 0.23	6.12 ± 0.14*	7.19 ± 1.14*‡
C-peptide (ng/mL)	1.34 ± 0.52	1.77 ± 0.77*	1.89 ± 0.97*
Cr (mg/dL)	0.67 ± 0.15	0.67 ± 0.19	0.89 ± 1.25*‡
BUN (mg/dL)	13.64 ± 3.76	15.68 ± 4.31*	16.51 ± 5.78*
e-GFR	87.46 ± 16.83	82.49 ± 16.83	79.88 ± 22.28†§
sBP (mmHg)	122.70 ± 17.42	132.04 ± 18.47*	136.46 ± 18.24*
dBP (mmHg)	77.93 ± 11.94	81.57 ± 11.88§	81.11 ± 11.16$^{\#}$
Tc (mg/dL)	201.09 ± 35.34	209.34 ± 30.05$^{\#}$	202.21 ± 28.78
Tg (mg/dL)	90.52 ± 62.06	119.86 ± 86.66*	125.05 ± 76.89*
HDL-c (mg/dL)	66.91 ± 17.25	62.66 ± 18.15†	57.78 ± 15.71* **
LDL-c (mg/dL)	116.06 ± 30.09	123.10 ± 27.21†	119.85 ± 27.70
Uric acid (mg/dL)	5.01 ± 1.31	5.39 ± 1.56$^{\#}$	5.31 ± 1.44
Hs-CRP (mg/dL)	0.06 ± 0.10	0.08 ± 0.10$^{\#}$	0.12 ± 0.14*§
Water intake (g/day)	1683.1 ± 0.10	1774.3 ± 549.6	1906.2 ± 626.2†
Protein intake (g/day)	69.72 ± 25.02	77.13 ± 29.76†	77.18 ± 27.93$^{\#}$
Lipid intake (g/day)	55.49 ± 19.14	56.7 ± 20.40	55.87 ± 21.91
Carbohydrate intake (g/day)	241.22 ± 82.23	248.26 ± 72.39	261.54 ± 95.88$^{\#}$
Diabetic therapy			
Dietary therapy			53.33% (40/75)
Metformin			16.00% (12/75)
DPP4 inhibitor			30.67% (23/75)
SGLT2 inhibitor			8.00% (6/75)
Other oral agents			14.67% (11/75)
Insulin			10.67% (8/75)
2 or more agents			25.33% (19/75)
Hypertension: n (%)	18.76 (148/789)	48.18 (66/137)*	48.00 (36/75)*
Dyslipidemia: n (%)	9.00 (71/789)	23.36 (32/137)†	38.67 (29/75)* **
Alcohol habit: n (%)	47.40 (374/789)	41.61 (57/137)	40.00 (30/75)
Smoking habit: n (%)	15.72 (124/789)	18.18 (23/137)	20.00 (15/75)
Subjective symptoms: n (%)	1.01 (8/789)	1.40 (2/143)	1.33 (1/75)
PINT scores (mA)	0.11 ± 0.08	0.12 ± 0.11$^{\#}$	0.15 ± 0.13* **

nDM, non-diabetic subjects; IFG, impaired fasting glucose subjects; DM, type 2 diabetic subjects; BMI, body mass index; Abd. Circumference, abdominal circumference; FBG, fasting plasma glucose; Cr, creatinine; e-GFR, estimated glomerular filtration rates; sBP, systolic blood pressure; dBP, diastolic blood pressure; Tc, total cholesterol; Tg, triglyceride; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; Hs-CRP, high sensitivity C-reactive protein; DPP4, dipeptidyl peptidase4; SGLT2, sodium-glucose co-transporter2; PINT, pain threshold from intraepidermal electrical stimulation. *$p < 0.001$ vs nDM, $^{\dagger}p < 0.01$ vs nDM, $^{\ddagger}p < 0.001$ vs IFG, $^{\S}p<0.01$ vs IFG, #$p < 0.05$ vs nDM, **$p < 0.05$ vs IFG.

(Fig. 2B), even after the exclusion of subjects with diabetes and IFG (Shannon index, $p = 0.10$; Chao1 index, $p = 0.83$) (Fig. 2C). A total of 283 genera were identified in the oral microbiota. In total, 14 genera had a relative abundance of >1%. There was no significant difference in the relative abundance of those genera between low-PINT and high-PINT participants regardless of the presence of diabetes and IFG

Table 3
Clinical profiles of low-PINT participants and high-PINT participants.

	low-PINT (< 0.15 mA)	high-PINT (≧0.15 mA)	*P*
Numbers (male/female)	718 (284/434)	283 (136/147)	–
Age (yrs)	51.61 ± 15.47	56.05 ± 14.83	*<0.001*
Height (cm)	161.73 ± 8.63	162.00 ± 9.63	*<0.001*
Body weight (kg)	59.61 ± 12.25	62.77 ± 11.75	*0.002*
BMI (kg/m^2)	22.68 ± 3.58	23.76 ± 3.32	*<0.001*
Abd. circumference (cm)	83.628 ± 10.05	87.16 ± 9.65	*<0.001*
Waist circumference (cm)	76.85 ± 10.41	80.56 ± 10.07	*<0.001*
Hip circumference (cm)	93.07 ± 6.41	94.87 ± 6.10	*<0.001*
FBG (mg/dL)	93.79 ± 13.85	97.2 ± 17.31	*<0.001*
HbA1c (%)	5.73 ± 0.52	5.84 ± 0.73	*0.006*
C-peptide (ng/mL)	1.42 ± 0.60	1.51 ± 0.70	*0.037*
Cr (mg/dL)	0.66 ± 0.16	0.73 ± 0.68	*0.014*
BUN (mg/dL)	13.88 ± 3.95	14.83 ± 4.50	*0.002*
e-GFR	86.77 ± 17.32	84.47 ± 17.74	*0.082*
sBP (mmHg)	123.86 ± 17.63	127.92 ± 19.22	*0.001*
dBP (mmHg)	78.26 ± 11.72	79.86 ± 12.44	*0.053*
Tc (mg/dL)	202.53 ± 34.15	204.49 ± 34.33	*0.459*
Tg (mg/dL)	94.52 ± 61.09	104.41 ± 84.98	*0.041*
HDL-c (mg/dL)	66.39 ± 17.48	63.79 ± 17.26	*0.029*
LDL-c (mg/dL)	116.72 ± 29.52	118.86 ± 29.90	*0.326*
Uric acid (mg/dL)	5.06 ± 1.37	5.17 ± 1.35	*0.325*
Hs-CRP (mg/dL)	0.06 ± 0.10	0.07 ± 0.11	*0.256*
Water intake (g/day)	1699.41 ± 578.03	1747.51 ± 568.53	*0.231*
Protein intake (g/day)	71.28 ± 26.72	71.38 ± 24.59	*0.942*
Lipid intake (g/day)	54.62 ± 20.10	53.04 ± 17.88	*0.257*
Carbohydrate intake (g/day)	242.32 ± 82.81	247.27 ± 80.72	*0.381*
Hypertension: n (%)	20.89 (150/718)	35.34 (100/283)	*0.063*
Dyslipidemia: n (%)	11.83 (85/718)	16.60 (47/283)	*0.314*
Alcohol habit: n (%)	46.66 (335/718)	44.53 (126/283)	*0.777*
Smoking habit: n (%)	16.85 (121/718)	14.49 (41/283)	*0.558*
Subjective symptoms: n (%)	0.98 (9/718)	1.06 (3/283)	*>0.999*
PINT scores (mA)	0.07 ± 0.02	0.22 ± 0.10	*<0.001*

PINT, pain threshold from intraepidermal electrical stimulation; BMI, body mass index; Abd. Circumference, abdominal circumference; FBG, fasting plasma glucose; Cr, creatinine; e-GFR, estimated glomerular filtration rates; sBP, systolic blood pressure; dBP, diastolic blood pressure; Tc, total cholesterol; Tg, triglyceride; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; Hs-CRP, high sensitivity C-reactive protein.

(Table 4). The genera that were 1% or less abundant are shown in Supplemental Tables 1 and 2. The abundance of the 17 genera was different between the low- and high-PINT groups, while that of the 18 genera was different in the subjects after exclusion of subjects with diabetes and IFG. However, there were only nine genera that displayed similar changes, which suggests that abnormal glucose metabolism could influence the composition of oral microbiota.

3.5. Gut bacterial floor and peripheral pain sensation

Principal coordinate analysis revealed significant microbial composition differences between low-PINT and high-PINT participants, including subjects with diabetes and IFG ($p < 0.01$) (Fig. 3A). If subjects with diabetes and IFG were excluded, the significant difference was still preserved ($p < 0.01$) (Fig. 3A). Significant differences in the Shannon index ($p < 0.05$) and the Chao1 index ($p < 0.05$) were noted between low-PINT and high-PINT participants, including subjects with diabetes and IFG (Fig. 3B). These significant differences were maintained if the participants with diabetes and IFG were excluded ($p < 0.05$ for the Shannon index and $p < 0.05$ for the Chao1 index) (Fig. 3C). Furthermore, 317 genera were identified in the gut microbiota based on next-generation sequencing analysis. In total, 17 genera were identified with a relative abundance >1% (Table 5). Among these genera with >1% abundance, significant differences were noted in the relative abundance of three genera between low-PINT and high-PINT participants. The relative abundance of the genus *Bacteroides* was significantly higher in low-PINT participants than in high-PINT participants (11.3 ± 7.0% vs. 10.0 ± 6.7%, $p < 0.01$). This difference was still preserved after the exclusion of subjects with diabetes and IFG (11.2 ± 6.9% vs. 10.0 ± 6.5%, $p < 0.05$). The relative abundance of the genus *Prevotella* was significantly higher in high-PINT participants than in low-PINT participants (5.8 ± 1.1% vs. 4.8 ± 1.0%, $p < 0.05$). This difference was also preserved after the exclusion of subjects with diabetes and IFG (5.4 ± 10.0% vs. 4.6 ± 9.9%, $p < 0.05$). The relative abundance of the genus *Bifidobacterium* was comparable between low-PINT and high-PINT participants (8.1 ± 7.6% vs. 7.7 ± 8.3%, $p = 0.08$), while the relative abundance was significantly higher in low-PINT participants than in high-PINT participants after the exclusion of subjects with diabetes and IFG (8.4 ± 7.9% vs. 7.6 ± 8.1%, $p < 0.05$). Because this suggests that the abundance of *Bifidobacterium* would be changed by abnormal glucose metabolism, the abundance of *Bifidobacterium* was excluded from further correlation analysis. The genera that were 1% or less abundant are shown in Supplemental Tables 3 and 4. The abundance of 22 genera was different between the low- and high-PINT groups, while that of 23 genera was different in the subjects after exclusion of subjects with diabetes and IFG. However, there were only 17 genera that displayed similar changes, which also suggests that abnormal glucose metabolism could influence the composition of gut microbiota.

3.6. Correlation of PINT score with clinical parameters, including the relative abundance of Bacteroides

Because the abundance of *Bacteroides* also significantly increased in women compared with men (10.1% vs. 10.9%, $p < 0.05$), it is necessary to adjust the correlation between the PINT score and the abundance of *Bacteroides* with other clinical factors. Univariate regression analysis revealed a significant correlation between the PINT score and clinical measures, such as sex, age, BMI, abdominal circumference, HbA1c, C-peptide, e-GFR, sBP, uric acid, and relative abundance of *Bacteroides* (Table 6). The correlation between the PINT score and the relative abundance of *Bacteroides* ($\beta = -0.07181$, $p < 0.05$) or age ($\beta = 0.128088$, $p < 0.01$) also remained significant after adjustment for multiple factors correlated with PINT in univariate analysis (sex, BMI, abdominal circumference, HbA1c, c-peptide, e-GFR, sBP, uric acid, and dyslipidaemia).

3.7. Correlation of the relative abundance of Bacteroides with clinical measurements

Univariate regression analysis was performed to explore the factors that correlated with the relative abundance of *Bacteroides* in the gut microbiota. Univariate regression analysis revealed a significant correlation between the relative abundance of *Bacteroides* and clinical measures, such as age, BMI, abdominal circumference, waist circumference, FBG, HbA1c, BUN, e-GFR, sBP, Hs-CRP, water intake and protein intake (Table 7). The correlation between the relative abundance of *Bacteroides* and age ($\beta = -0.08922$, $p < 0.05$) remained significant after adjustment for multiple factors (BMI, abdominal circumference, FBG, HbA1c, BUN, e-GFR, sBP, Hs-CRP, water intake and protein intake).

3.8. Propensity score matching based on diabetic factors or age-matched subject demographics divided by PINT scores

The patients were matched according to the characteristics of diabetes (HbA1c and FBG) or age (Supplemental Tables 5 and 6). In cases matched for the either parameter, the relative abundance of *Bacteroides* was significantly lower in high-PINT participants than in low-PINT participants ($p < 0.01$, respectively).

4. Discussion

We investigated the association between gut or oral microbiota

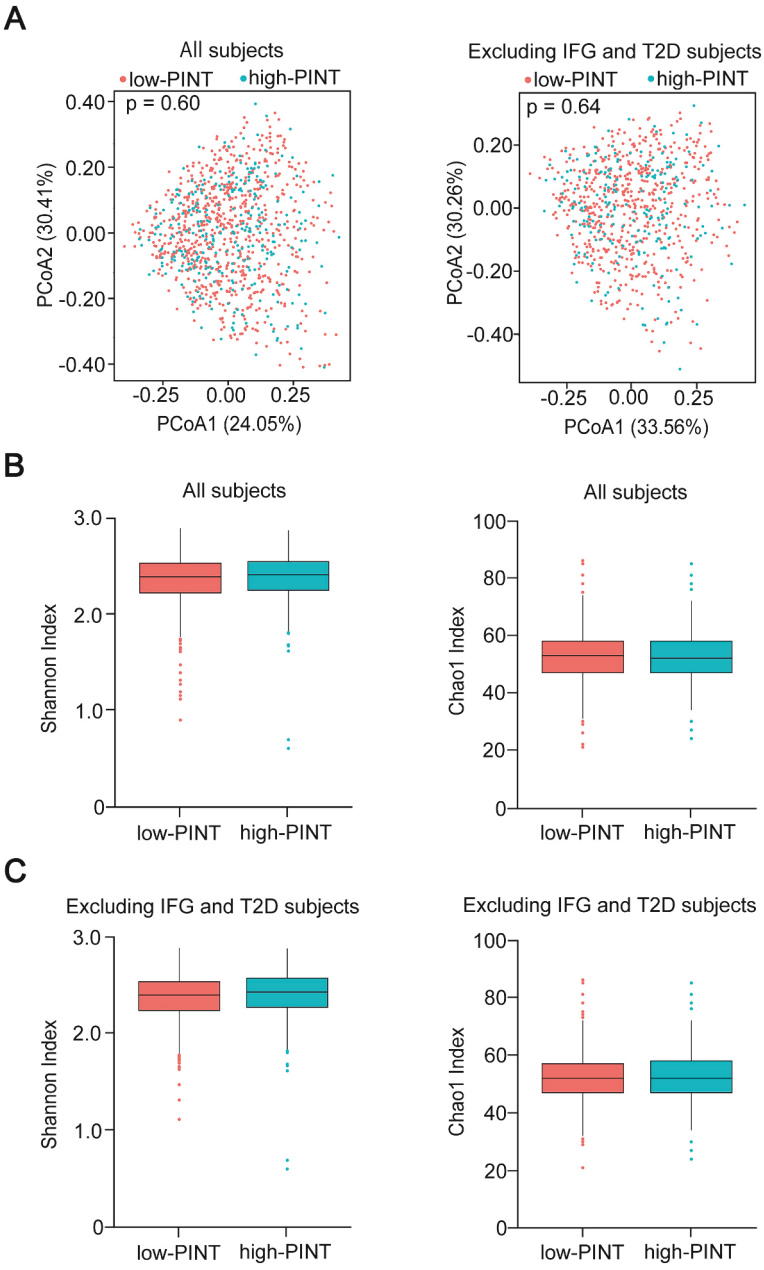

Fig. 2. Variety of salivary microbiota abundance.
Principal coordinate analysis between low-PINT (< 0.15 mA) and high-PINT (≥ 0.15 mA) participants is shown (A). The Shannon index and Chao1 index of oral microbiota are shown when all subjects (B) and the subjects excluding abnormal glucose subjects (C) were divided into the low-PINT and high-PINT participants. IFG, impaired fasting glucose; T2D, type 2 diabetes; PINT, pain threshold from intraepidermal electrical stimulation. The bar shows the maximum value and the minimum value.

diversity and the pain threshold of the foot in this study. Regarding oral microbiota, microbiota diversity and composition were not associated with the pain threshold. However, we first revealed significant differences in gut microbial variety and composition between low-PINT and high-PINT participants. Among the genera of gut microbiota identified by next-generation sequencing, the percent composition of the genus *Bacteroides* was significantly lower in high-PINT participants than in low-PINT participants. Although the abundance of *Prevotella* was increased in high-PINT participants compared to low-PINT participants, the difference was not significant in correlation analysis. The abundance of *Bifidobacterium* was also varied, but the difference was assumed to be an abnormal glucose metabolism. Interestingly, these changes were independent of the presence of abnormal glucose metabolism and age.

Many reports show that changes in the microbiome population in the gut are associated with the pathophysiology of T2D (Gurung et al., 2020). Although the inconsistency of the results regarding the composition of gut microbiota in T2D is reportedly due to ethnicity, the negative association between the *Bacteroides* genus and T2D seems to be the commonly and consistently reported in previous studies (Candela et al., 2016; Zhang et al., 2013; Lippert et al., 2017). Moreover, previous reports show that abnormal glucose states, including normal-high HbA1c, are associated with worse PINT scores (Kukidome et al., 2016; Suzuki et al., 2016; Itabashi et al., 2019; Kudoh et al., 2020; Osonoi et al., 2020). Therefore, it was unexpected that the diversity of the microbiota and the relative abundance of *Bacteroides* in the gut were changed in high-PINT participants independent of abnormal glucose metabolism. The underlying aetiologies of SFN are diverse and include immunological, toxic, infectious, paraneoplastic, and hereditary causes in addition to abnormal glucose metabolism (Lippert et al., 2017). This finding suggests that the unidentified factor(s) other than abnormal glucose metabolism can modify the diversity of the bacteria in the gut of the high-PINT subjects.

Wang et al. reported that the development of DPN, which was assessed by NCVs and subjective symptoms, can result in greater differences in diversity and severe disruption of microbiota community richness compared with T2D alone (Wang et al., 2020). In DPN subjects,

Table 4
Mean relative abundance of oral microbiota with 1% occurrence in the whole population (percentage of the total bacterial reads) between Low-PINT and High-PINT subjects.

	All subjects			Excluding glucose intolerable subjects		
Genus (>1%)	low-PINT (*n* = 746)	high-PINT (*n* = 291)	Wilcoxon rank sum test	low-PINT (*n* = 602)	high-PINT (n = 291)	Wilcoxon rank sum test
Unclassified	2.8 ± 1.9	2.7 ± 2.1	0.242	2.8 ± 1.9	2.7 ± 2.1	0.339
Actinomyces	7.0 ± 3.7	7.2 ± 3.9	0.842	7.1 ± 3.6	7.2 ± 4.0	0.943
Atopobium	2.2 ± 2.1	2.2 ± 1.9	0.326	2.1 ± 2.1	2.1 ± 1.9	0.570
Fusobacterium	2.2 ± 1.7	2.4 ± 1.8	0.255	2.3 ± 1.7	2.5 ± 1.8	0.075
Gemella	1.1 ± 1.1	1.2 ± 0.9	0.103	1.1 ± 0.9	1.2 ± 0.9	0.076
Granulicatella	1.5 ± 1.0	1.5 ± 1.0	0.798	1.5 ± 1.0	1.5 ± 1.1	0.974
Haemophilus	5.1 ± 4.6	5.1 ± 4.7	0.534	5.5 ± 4.8	5. 1 ± 4.6	0.172
Neisseria	8.1 ± 7.7	8.0 ± 7.6	0.834	8.3 ± 7.7	8.1 ± 7.7	0.653
Prevotella	15.3 ± 7.9	15.2 ± 8.4	0.727	15.1 ± 7.8	15.1 ± 8.6	0.810
Porphyromonas	2.4 ± 2.7	2.4 ± 2.6	0.908	2.5 ± 2.7	2.6 ± 2.7	0.755
Rothia	5.7 ± 6.0	6.2 ± 6.2	0.280	5.5 ± 5.6	5.8 ± 6.0	0.621
Saccharibacteria_genera_incertae_sedis	7.6 ± 6.4	6.9 ± 5.9	0.261	7.5 ± 6.2	7.0 ± 5.8	0.551
SR1_genera_incertae_sedis	1.1 ± 3.1	0.8 ± 2.0	0.540	1.2 ± 3.3	1.0 ± 2.3	0.299
Streptococcus	21.3 ± 9.0	21.7 ± 9.9	0.681	21.1 ± 8.6	21.5 ± 10.4	0.836
Veillonella	9.0 ± 4.0	9.0 ± 3.8	0.977	9.0 ± 4.0	8.7 ± 3.9	0.467

PINT, pain threshold from intraepidermal electrical stimulation.

the abundance of *Bacteroides* and *Faecalibacterium* was significantly decreased, whereas that of *Escherichia-Shigella*, *Lachnoclostridium*, *Blautia*, *Megasphaera* and *Ruminococcus torques* was increased. Although the influences of diabetes should be taken into account, our results may reveal the initial changes in gut microbiota in SFN, including DPN, because SFN is assumed to be an initial manifestation of DPN. These results suggest that the composition of the gut microbiota can drastically change as SFN progresses to large fibre neuropathy. Regarding treatment, the composition of the gut microbiota may be more easily fixed in the early stage of neuropathies.

Our previous report showed that serum LBP levels are increased in prediabetes and diabetes patients and are positively correlated with the pain threshold evaluated by PINT (Kudoh et al., 2020). LBP can reflect the level of lipopolysaccharide (LPS), which is a cell-associated glycolipid that comprises the outer leaflet of the outer membrane of Gram-negative bacteria. We expected that the population of Gram-negative bacilli might be increased in participants with high PINT scores, and we elucidated that the population of *Bacteroides*, a Gram-negative bacilli, in the gut was significantly decreased in high-PINT participants compared with low-PINT participants in this study. On the other hand, because a proportional amount of bacteria was used instead of the absolute number in this study, the absolute effects of low *Bacteroides* abundance on PINT are still unclear. Considering the slight difference in *Bacteroides* abundance and bacterial composition, the clinical significance of gut microbiota changes may be limited to PINT abnormalities.

A couple of study found that *Bacteroide* abundance increased on a carbohydrate-restricted low-calorie diet for one year and a diet containing a minimum of 35% fat for 2 years (Ley et al., 2006; Haro et al., 2017). In our study, the amounts of water and protein intake, but not carbohydrate and fat, were correlated with *Bacteroides* abundance in univariate analysis, while none of them was correlated in multivariate analysis. This may be ascribed to the difference in ethnicity and the methods used to evaluate nutritional intake. On the other hand, age was also disproportionally correlated with the abundance of *Bacteroides* even in multivariate analysis in this study. The abundance of *Bacteroides* in the context of ageing is inconsistent in the literature. Early investigations regarding the gut microbiome and ageing reported an increased dominance of *Bacteroides* in older persons relative to healthy younger controls (Claesson et al., 2011; Zwielehner et al., 2009), whereas Wilmanski T et al. reported that the abundance of *Bacteroides* was significantly depleted in healthy ageing subjects (Wilmanski et al., 2021). Intriguingly, retaining a high proportion of *Bacteroides* even in older age implies decreased survival in a 4-year follow-up. These findings may suggest that the presence of both healthy and less-healthy elderly individuals may influence the bacterial abundance in early investigations. Our results also can reflect the healthy conditions of our participants. Because the participants in our study are volunteers in a health promotion study and not participants in an ordinary health check-up, the study participants could be healthier than the general population.

Various studies have shown that antidiabetic agents, including metformin, α-glucosidase inhibitors, glucagon-like peptide-1 receptor agonists, DPP4 inhibitors and SGLT2 inhibitors, can affect the composition and function of gut microbiota (Forslund et al., 2015; Liu et al., 2022). This finding suggests that antidiabetic therapy may influence the PINT score by modulating the composition of the gut microbiota. Our data showed that DPP4 inhibitors were the most often used (30.7%), but the effects of DPP4 inhibitors on gut bacterial composition, particularly on *Bacteroides*, have not been consistent. Liao et al. demonstrated that DDP4 inhibitors improved glucose metabolism by increasing the abundance of gut *Bacteroidetes* and substantially reversing the changes in the gut microbiota induced by a high-fat diet (HFD) (Liao et al., 2019). In another report, sitagliptin treatment decreased the phylum *Bacteroidetes*, while it increased *Firmicutes* and *Tenericutes*, resulting in a partial correction of the dysbiosis of microbiota in HFD-fed rats with T2D. (Yan et al., 2016). It is difficult to evaluate the effects of diabetic therapy on bacterial composition and PINT in this study, because there was not a sufficient number of diabetic precipitants due to the nature of the IWAKI study. Future studies including a sufficient number of diabetic subjects are required to explore the association.

Regarding the correlation between diabetes and oral microbiota, previous studies report diabetic state-induced alterations in the oral microbiome, such as increased *Capnocytophaga (*Mashimo et al., 1983*)*, *P. gingivalis* and *Tannerella forsythia (*Campus et al., 2005; da Cruz et al., 2008*)* and increased *Capnocytophaga, Pseudomonas, Bergeyella, Sphingomonas, Corynebacterium, Propionibacterium*, and *Neisseria* in hyperglycaemic individuals (Ganesan et al., 2017). In contrast, diabetes reduces *Porphyromonas, Filifactor, Eubacterium, Synergistetes, Tannerella*, and *Treponema* genera (Casarin et al., 2013). A recent comprehensive analysis suggested that oral bacterial diversity was reduced or unchanged in the diabetic state compared to the normal state (Ganesan et al., 2017; de Groot et al., 2017). Thus, previous human studies have not revealed consistent changes in microbial composition in diabetes. Despite the various reasons for these inconsistent results, such as the difference in methods and subject populations, these inconsistencies may indicate that the change in oral microbiota minimally contributes to the pathogenesis of diabetes. Furthermore, reports regarding the association between the alteration of oral microbiota and onset of DPN or

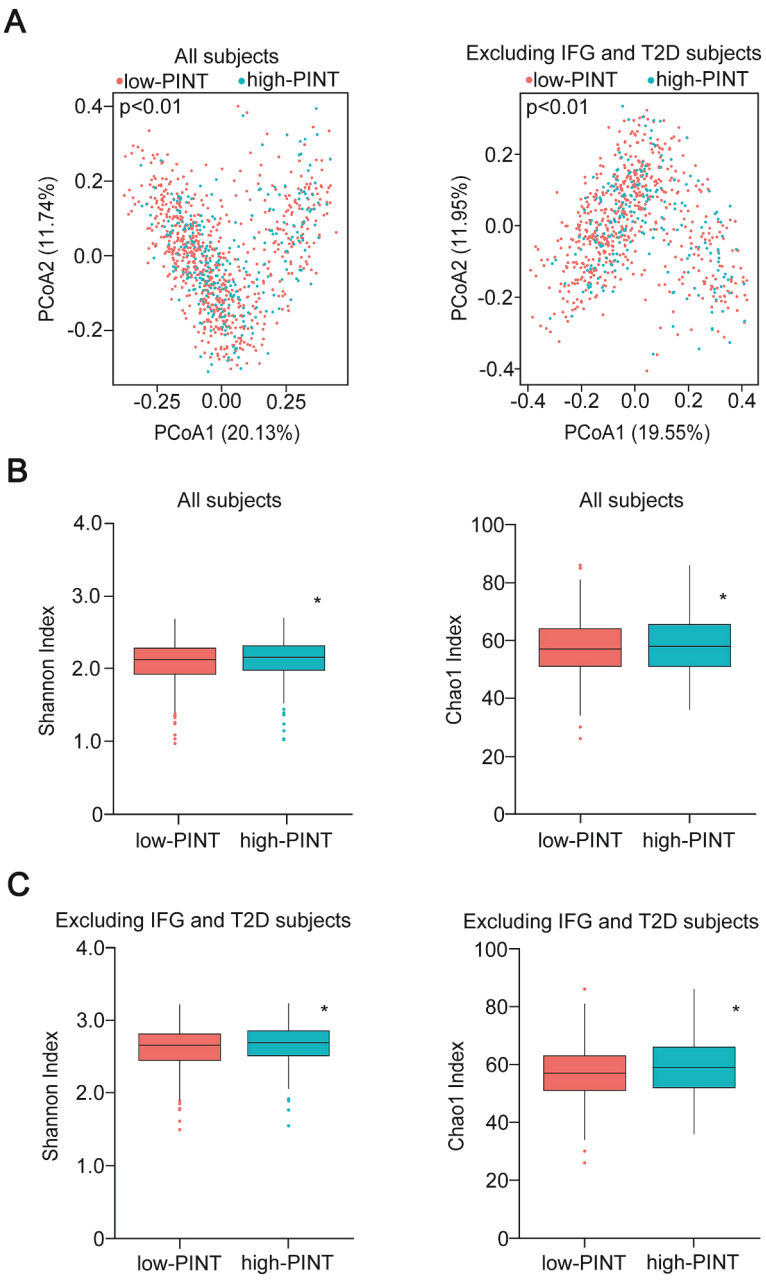

Fig. 3. Variety of gut microbiota abundance.
Principal coordinate analysis between low-PINT (< 0.15 mA) and high-PINT (≥ 0.15 mA) participants is shown (A). The Shannon index and Chao1 index of gut microbiota are shown when all subjects (B) and the subjects excluding abnormal glucose subjects (C) were divided into the low-PINT and high-PINT participants. *$p < 0.05$ vs. low-PINT participants. IFG, impaired fasting glucose; T2D, type 2 diabetes PINT, pain threshold from intraepidermal electrical stimulation. The bar shows the maximum value and the minimum value.

nondiabetic peripheral neuropathy are lacking to the best of our knowledge. Consistent with these results, no significant correlation was noted between the change in composition of the oral bacterial floor and the pain threshold in this study. Considering these findings, alterations in the pain thresholds of healthy and diabetic subjects may not be strongly associated with the alteration of oral microbiota composition.

Our study has several limitations. First, this study is a population-based cross-sectional observation. Participants in this study were primarily categorized as heathy volunteers. Therefore, sufficient numbers of subjects with overt diabetes or prediabetes were not included. Furthermore, it is unclear whether the development of SFN is associated with changes in the gut microbiota. Thus, longitudinal observations are necessary in the future. Second, invasive evaluations, such as nerve conduction velocities and molecular and pathological changes in the skin or the sural nerve, were not performed in this study. In addition to the evaluation of small fibre function, it would be useful to evaluate structural and molecular changes in the skin to understand the precise pathophysiology of SFN induced by changes in gut microbiota. Third, this study clarified the correlation between the change in the composition of gut microbiota and attenuation of the pain threshold; however, it is unclear whether these changes in microbiota can reflect the changes in the absolute number of microbiota and if they are the pathological cause for the deterioration of pain threshold. In vivo or in vitro studies should be performed to confirm the contribution of gut microbiota changes to the pathogenesis of small nerve dysfunction in the future.

In conclusion, our current study first clarified that changes in the gut microbiota, but not the oral microbiota, were significantly associated with elevated PINT scores in a general Japanese population, and this association may be independent of diabetic conditions and ageing. Nevertheless, it is still unclear whether these changes can be directly linked to the clinical manifestation of SFN. Thus, further experimental confirmation is required for future therapeutic applications.

Funding

This study is principally funded by grants from JSPS Kakenhi grant

Table 5
Mean relative abundance of gut microbiota with 1% occurrence in the whole population (percentage of the total bacteria) between Low-PINT and High-PINT subjects.

	All subjects			Excluding glucose intolerable subjects		
Genus (>1%)	low-PINT (n = 746)	high-PINT (n = 291)	Wilcoxon rank sum test	low-PINT (n = 602)	high-PINT (n = 291)	Wilcoxon rank sum test
Unclassified	8.2 ± 5.2	8.4 ± 5.5	0.592	8.3 ± 5.5	8.3 ± 5.1	0.693
Alistipes	2.0 ± 2.8	2.0 ± 2.9	0.390	2.1 ± 3.0	2.1 ± 3.0	0.246
Anaerostipes	5.3 ± 5.5	5.0 ± 5.4	0.389	5.4 ± 5.5	5.1 ± 5.5	0.599
Bacteroides	11.3 ± 7	10 ± 6.7	**0.001**	11.2 ± 6.9	10.0 ± 6.5	**0.001**
Bifidobacterium	8.1 ± 7.6	7.7 ± 8.3	0.077	8.4 ± 7.9	7.6 ± 8.1	**0.034**
Blautia	8.3 ± 4.3	8.0 ± 4.4	0.878	8.2 ± 4.1	8.1 ± 4.6	0.694
Clostridium.IV	2.3 ± 3.7	2.4 ± 3.3	0.514	2.5 ± 3.9	2.4 ± 3.5	0.868
Collinsella	5.1 ± 4.9	5.1 ± 5.0	0.209	5.2 ± 4.9	5.2 ± 5.1	0.138
Faecalibacterium	5.9 ± 5.0	6.8 ± 4.8	0.959	6.7 ± 5.0	6.8 ± 4.9	0.875
Fusicatenibacter	2.4 ± 2.8	2.3 ± 2.3	0.237	2.3 ± 2.6	2.4 ± 2.3	0.227
Gemmiger	2.5 ± 2.6	2.7 ± 3.0	0.072	2.5 ± 2.5	2.7 ± 3.0	0.093
Lachnospiracea_incertae_sedis	1.8 ± 1.1	1.8 ± 1.2	0.872	1.7 ± 1.1	1.8 ± 1.2	0.963
Megamonas	1.2 ± 4.1	1.1 ± 4.0	0.756	1.0 ± 3.6	1.0 ± 2.8	0.735
Prevotella	4.8 ± 1.0	5.8 ± 1.1	**0.029**	4.6 ± 9.9	5.4 ± 10.0	**0.017**
Roseburia	3.9 ± 4.1	4.3 ± 4.1	0.396	3.9 ± 4.2	4.4 ± 4.1	0.334
Ruminococcus	3.8 ± 5.2	4.0 ± 5.3	0.151	3.7 ± 4.9	4.1 ± 5.4	0.095
Ruminococcus2	5.8 ± 5.1	5.2 ± 5.5	0.352	5.8 ± 2.9	5.1 ± 5.3	0.200
Streptococcus	1.9 ± 3.0	2.1 ± 3.8	0.898	1.8 ± 2.9	2.2 ± 1.8	0.714

PINT, pain threshold from intraepidermal electrical stimulation.

Table 6
Clinical factors correlated with PINT.

	Univariate		Multivariate	
Characteristics	β	*p*	β	*p*
Sex	0.087383	0.005	0.034969	0.377
Age (yrs)	0.147265	<0.001	0.128088	0.003
Height (cm)	0.01777	0.570	–	–
Body weight (kg)	0.140553	<0.001	–	–
BMI (kg/m^2)	0.172617	<0.001	−0.00434	0.954
Abd. circumference (cm)	0.197602	<0.001	0.116269	0.148
Waist circumference (cm)	0.198351	<0.001	–	–
Hip circumference (cm)	0.142397	<0.001	–	–
FBG (mg/dL)	0.12257	<0.001	–	–
HbA1c (%)	0.105048	0.001	−0.0008	0.982
C-peptide (ng/mL)	0.128062	<0.001	0.038789	0.339
Cr (mg/dL)	0.036701	0.238	–	–
BUN (mg/dL)	0.077756	0.012	–	–
e-GFR	−0.07136	0.022	0.02652	0.494
sBP (mmHg)	0.128605	<0.001	-0.00602	0.871
dBP (mmHg)	0.087919	0.005	–	–
Tc (mg/dL)	0.017313	0.578	–	–
Tg (mg/dL)	0.091762	0.003	–	–
HDL-c (mg/dL)	−0.095	0.002	–	–
LDL-c (mg/dL)	0.028162	0.365	–	–
Uric acid (mg/dL)	0.080618	0.009	−0.00847	0.842
Hs-CRP (mg/dL)	0.056078	0.071	–	–
Water intake (g/day)	0.038576	0.217	–	–
Protein intake (g/day)	0.015809	0.613	–	–
Lipid intake (g/day)	−0.03637	0.245	–	–
Carbohydrate intake (g/day)	0.038356	0.220	–	–
Hypertension (+/−)	0.173348	<0.001	–	–
Dyslipidemia (+/−)	0.086921	0.005	0.029994	0.366
Alcohol habit (+/−)	−0.01498	0.630	–	–
Smoking habit (+/−)	−0.00662	0.832	–	–
Subjective symptoms (+/−)	−0.03389	0.276	–	–
Bacteroides (%)	−0.10898	0.006	–0.07181	0.023
Prevotella (%)	0.036705	0.238	–	–

PINT, pain threshold from intraepidermal electrical stimulation; BMI, body mass index; Abd. Circumference, abdominal circumference; FBG, fasting plasma glucose; Cr, creatinine; e-GFR, estimated glomerular filtration rates; sBP, systolic blood pressure; dBP, diastolic blood pressure; Tc, total cholesterol; Tg, triglyceride; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; Hs-CRP, high sensitivity C-reactive protein.

Table 7
Clinical factors correlated with *Bacteroides*.

	Univariate		Multivariate	
Characteristics	β	*p*	β	*p*
Gender	0.054802	0.081	–	–
Age (yrs)	−0.15711	<0.001	−0.0913	0.031
Height (cm)	0.014189	0.652	–	–
Body weight (kg)	−0.04681	0.138	–	–
BMI (kg/m^2)	0.07997	0.017	−0.0062	0.935
Abd. circumference (cm)	−0.08916	0.005	−0.0332	0.663
Waist circumference (cm)	−0.08255	0.009	–	–
Hip circumference (cm)	−0.04991	0.112	–	–
FBG (mg/dL)	−0.07316	0.020	0.0198	0.718
HbA1c (%)	−0.07751	0.014	-0.0199	0.705
C-peptide (ng/mL)	−0.02766	0.378	–	–
Cr (mg/dL)	−0.05724	0.068	–	–
BUN (mg/dL)	−0.10536	0.001	–0.0137	0.714
e-GFR	0.131444	<0.001	0.0611	0.113
sBP (mmHg)	−0.09246	0.003	–0.0200	0.595
dBP (mmHg)	−0.05004	0.111	–	–
Tc (mg/dL)	0.040738	0.194	–	–
Tg (mg/dL)	0.030056	0.338	–	–
HDL-c (mg/dL)	0.039213	0.212	–	–
LDL-c (mg/dL)	0.012889	0.682	–	–
Uric acid (mg/dL)	−0.00856	0.785	–	–
Hs-CRP (mg/dL)	−0.06995	0.026	–0.0464	0.162
Water intake (g/day)	−0.0634	0.046	−0.0151	0.721
Protein intake (g/day)	−0.07105	<0.001	−0.0218	0.606
Lipid intake (g/day)	−0.03505	0.271	–	–
Carbohydrate intake (g/day)	−0.04793	0.132	–	–
Hypertension (+/−)	−0.11501	0.001	–	–
Dyslipidemia (+/−)	−0.03985	0.204	–	–
Alcohol habit (+/−)	−0.01844	0.557	–	–
Smoking habit (+/−)	−0.00117	0.970	–	–
Subjective symptoms (+/−)	−0.02928	0.351	–	–

BMI, body mass index; Abd. Circumference, abdominal circumference; FBG, fasting plasma glucose; SUIT, secretory units of islets in transplantation; Cr, creatinine; e-GFR, estimated glomerular filtration rates; sBP, systolic blood pressure; dBP, diastolic blood pressure; Tc, total cholesterol; Tg, triglyceride; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; Hs-CRP, high sensitivity C-reactive protein.

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Disclosure

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CRediT authorship contribution statement

Yuki Takeuchi: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Visualization. **Hiroki Mizukami:** Conceptualization, Methodology, Validation, Formal analysis, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Kazuhiro Kudoh:** Investigation, Methodology, Visualization, Funding acquisition. **Sho Osonoi:** Investigation, Visualization. **Takanori Sasaki:** Investigation. **Hanae Kushibiki:** Investigation. **Saori Ogasawara:** Investigation. **Yutaro Hara:** Investigation. **Akiko Igawa:** Investigation. **Xuekai Pan:** Investigation. **Takahiro Yamada:** Investigation. **Keisuke Yamazaki:** Investigation. **Tatsuya Mikami:** Supervision, Writing – review & editing. **Makoto Daimon:** Supervision, Writing – review & editing. **Soroku Yagihashi:** Supervision, Writing – review & editing. **Kenichi Hakamada:** Supervision, Writing – review & editing. **Shigeyuki Nakaji:** Conceptualization, Formal analysis, Supervision, Funding acquisition.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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