Association between breath methane concentration and visceral fat area: a population-based cross-sectional study

Naoki Ozato1,2,7, Shinichiro Saito1, Tohru Yamaguchi3, Mitsuhito Katashima1,2, Itoyo Tokuda1, Kaori Sawada4, Yoshihisa Katsuragi2, Masanori Kakuta1, Seiya Imoto1, Kazushige Ihara4 and Shigeyuki Nakaji3

1 Department of Active Life Promotion Sciences, Graduate School of Medicine, Hirosaki University, Japan
2 Health Care Food Research Laboratories, Kao Corporation, Japan
3 Biological Science Research Laboratories, Kao Corporation, Japan
4 Department of Social Medicine, Graduate School of Medicine, Hirosaki University, Japan
5 Human Genome Center, Institute of Medical Science, University of Tokyo, Japan
6 Health Intelligence Center, Institute of Medical Science, University of Tokyo, Japan
7 Author to whom any correspondence should be addressed.
E-mail: ozato.naoki@kao.com

Keywords: intestinal microflora, visceral fat area, breath methane, methanogenic bacteria

Supplementary material for this article is available online

Abstract
High visceral fat area (VFA) is a stronger predictor of cardiovascular disease and overall mortality, compared with body mass index (BMI) and waist circumference (WC). Recent reports demonstrate that obesity is related to breath gas, which is produced by the intestinal microflora. However, these studies define obesity using BMI, not VFA. In this population-based cross-sectional study, we investigated the relationship between breath gases (methane and hydrogen) and both VFA and BMI. A total of 1033 participants (62% women; age [mean ± standard deviation] 54.4 ± 14.9 years) in the 2015 Iwaki Health Promotion Project in Japan were enrolled in the study. Breath samples were collected using a breath bag and analyzed by gas chromatography. VFA was measured using a visceral fat meter. The proportion of methanogenic bacteria to total intestinal microbiota was measured by polymerase chain reaction and 16S rRNA gene sequencing analysis. Our analysis revealed a significant association between high VFA and low breath methane, even after adjusting for confounding factors (B = −0.024 and P = 0.004). To identify the association between breath methane and VFA in participants with methane-producing bacteria in their intestinal microflora, participants were divided into two groups based on the presence or absence of methanogenic bacteria in their stool. The Methanogen + group was further divided into two subgroups with breath methane higher (Methane-UP) or lower (Methane-LO) than the median breath methane concentration. VFA was significantly lower in the Methane-UP group than in the Methane-LO group. In participants with methanogenic bacteria, breath methane concentration might be an independent biomarker of visceral fat accumulation.

Introduction
Visceral fat accumulation is a widely-recognized risk factor for mortality, likely independent of subcutaneous fat and waist circumference (WC) [1–3]. Metabolic risk factors, including hypertension, high blood glucose, and triglyceride (TG) concentrations, and low serum high-density lipoprotein (HDL)-cholesterol, are more strongly associated with visceral fat area (VFA) than higher subcutaneous fat, WC, or body mass index (BMI) [4–6]. Thus, reducing VFA decreases the risk of metabolic syndrome-induced fatal disease.

The intestinal microbiota is highly involved in host energy regulation and homeostasis, thereby affecting diabetes and/or obesity status [7]. Some studies report that the intestinal microbiota may cause obesity [8, 9]. However, the precise mechanisms underlying how the intestinal microbiota affects obesity are unclear. The
role of the intestinal microbiota in the development of obesity has been evaluated using a genome-based approach [8, 10]. Although VFA is a stronger predictor of cardiovascular disease and overall mortality than BMI is, it is difficult to obtain in a population-based cross-sectional study. The gold standard for measuring VFA is computed tomography (CT), which is expensive and associated with radiation exposure. Therefore, the association between the intestinal microbiota and obesity is usually studied using BMI instead of VFA.

Several studies evaluating the association between BMI and the intestinal microbiota revealed that Ruminococcus [11] and Akkermansia [12] are related to obesity. In mice, increased prevalence of Methanobrevibacter smithii, a methanogenic bacteria species, is associated with increased weight gain [13]. M smithii produces methane as a byproduct of hydrogen-requiring anaerobic metabolism. Methane produced in the intestine is expelled from the body by exhalation and flatus. Therefore, some studies have evaluated the association between BMI and breath methane concentration as a noninvasive measure of methanogenic bacteria activity. Breath methane-positive people with methanogenic bacteria account for approximately 8% to 50% of the population [14–17]. A human study found that participants positive for breath methane have a significantly higher BMI [18]. Similar results were reported in a larger study [19]. However, other studies demonstrate conflicting findings [16, 17]. In one study, the breath methane concentration was inversely associated with BMI [20]. In another study, no significant relationship was detected between BMI and breath methane concentration in participants with methanogenic bacteria [16]. Thus, studies on the relationship between BMI and breath methane concentration report conflicting results.

We measured VFA using an impedance method whose results highly correlate with using CT results (R > 0.8) [21] to investigate the association of breath gases (methane and hydrogen) with VFA and BMI in 1033 participants.

**Methods**

**Design, participants, and ethics**

The Iwaki Health Promotion Project was launched in 2005 as an annual health check-up for local residents, aiming to prolong a healthy lifespan. Participants were recruited from men and women at least 20 years of age living in the Iwaki region of Hirosaki City in the Aomori Prefecture, Japan [22–25]. VFA was first introduced as a health check-up parameter in 2015. We used data obtained from the 2015 health check-up as a population-based cross-sectional study. The Iwaki district has approximately 11 000 adults, and 1082 participated in this community health check-up in 2015. Of these, 49 participants did not complete the clinical assessment and were excluded from the analyses. Thus, 1033 participants (397 men and 636 women; mean age ± standard deviation, 54.4 ± 14.9 years) were enrolled into the analyses. In addition, 326 individuals who participated in the health check conducted in 2016, but not in 2015 (62% female, mean age 50.7 ± 17.5 years), were enrolled to confirm the findings obtained with the 2015 cohort.

The study was approved by the Ethics Committee of Hirosaki University School of Medicine and conducted in accordance with the principles of the Declaration of Helsinki (2014–377). Written informed consent was obtained from all participants prior to the study. This study was registered in the University Hospital Medical Information Network (UMIN-CTR, https://www.umin.ac.jp) prior to the analyses (UMIN ID: UMIN000030351).

**Measurements obtained other than the methanogenic bacteria ratio**

All participants attended a health check-up early in the morning after fasting for at least 9 h. VFA was measured using a EW-FA90 visceral fat meter (Panasonic Corporation, Osaka, Japan). This instrument is an authorized medical device in Japan (No. 22500BZX00522000) based on high correlation with the CT method [21], which is the gold standard for VFA measurement. Breath samples were collected using a breath bag (Otsuka Pharmaceutical Corporation, Tokyo, Japan). All participants underwent training to provide end-alveolar breath before sample collection. The breath was immediately transferred to a gas-tight glass syringe and 1 ml breath was injected into a gas chromatograph with a semiconductor detector (TRilyzer mBA-3000, Taiyo Ltd, Osaka, Japan) to measure breath hydrogen and methane concentrations. The following clinical characteristics were measured as metabolic risk factors: BMI (calculated from height and weight), WC, VFA, fasting serum glucose, glycated hemoglobin (HbA1c), systolic blood pressure (SBP), diastolic blood pressure (DBP), serum TG, HDL-cholesterol, and low-density lipoprotein (LDL)-cholesterol. Blood samples were collected from the peripheral veins of the participants in the morning after at least 9 h fasting. All laboratory tests were outsourced to LSI Medience Co. (Tokyo, Japan). Smoking (cigarettes/d), sleep time (hours/d), exercise amount (Mets/h/w), and habitual medicine use (e.g. medicine for hypertension, hyperlipidemia, diabetes, rheumatism, dementia, or allergy) were determined from questionnaires or daily journals. Daily carbohydrate, protein, fat, alcohol, and total dietary fiber intake were estimated from the Brief Diet History Questionnaire [26, 27].

**Measurements of the methanogenic bacteria ratio**

**Sample collection and DNA extraction**

Fecal samples from each participant were collected within 3 d prior to the study using a commercial tube
kit (TechnoSuruaga Laboratory Co., Ltd., Shizuoka, Japan) and cotton swabs. Sample kits were filled with 3 ml GTC solution (100 mM Tris-HCl [pH 8.0], 40 mM Tris-EDTA [pH 8.0], 4 M guanidine thiocyanate, and 0.001% bromothymol blue). Cotton swabs were saturated with 1 ml GTC solution. Fecal samples were stored at 4°C until DNA extraction.

GTC buffer solutions (800 µl feces) were added to tubes filled with zirconium beads. The tubes were then mixed at room temperature for 2 min at 5 m s⁻¹ using a FastPrep 24 Instrument (MP Biomedicals, Santa Ana, CA, USA). After cooling, samples were centrifuged at 5000 rpm for 1 min. DNA was then extracted from the bead-treated suspension using an automatic nucleic acid extractor (Precision System Science, Chiba, Japan). MagDEA DNA 200 (GC) (Precision System Science) was used for automatic nucleic acid extraction. The final DNA concentration in each sample was adjusted to 10 ng µl⁻¹.

**Polymerase chain reaction amplification and sequencing**

To amplify the V3-V4 region of the prokaryotic 16S rRNA gene, universal primer sets were used as described previously [28]. Polymerase chain reaction (PCR) assays were performed as described previously [28]. To check amplicon size, 2.0 µl aliquots of the PCR reaction 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 as described by Takahashi et al [28]. MiSeq system (Illumina, San Diego, CA, USA) was used for 2 × 300 cycle paired-end sequencing.

**Taxonomic classification and calculation of the methanogenic bacteria ratio**

The multiplexed paired-end reads from the Illumina MiSeq system were processed as follows. The adapter sequences and low-quality bases (threshold = 20) were trimmed from the 3’-ends using Cutadapt (version: 1.13). Reads containing N bases and shorter than 150 bases were discarded. The filtered reads were merged to form a single read, called a ‘merged read’. Merged reads shorter than 370 bp or longer than 470 bp were excluded using the VSEARCH fastq_mergepairs subcommand (version: 2.4.3). Merged reads with more than one expected sequencing error were also excluded. After removing chimeric reads detected using the VSEARCH uchime_denovo subcommand, the remaining merged reads were clustered at a sequence identity ≥ 97%. The taxa of the identified clusters were predicted using the RDP Classifier (commit hash: 701e229de7cbe53-d426130e23459fd916159994). Identified taxa with a confidence value < 0.8 were treated as unclassified. Methanogenic bacteria amount was calculated by combining the total Methanomassiliicoccus, Methanobrevibacter, and Methanosphaera levels, which are commonly known human methanogenic bacteria [29–32]. The proportion of methanogenic bacteria was calculated as the total methanogenic bacteria divided by the total intestinal microbiota.

**Statistical analysis**

Participant characteristics are reported as means ± standard deviation (SD). Wilcoxon rank-sum tests were performed to compare the two groups. Because VFA is an area dimension (square measure), we transformed the VFA values by square root transformation to improve normality. The square root-transformed VFA value normality was confirmed by the Kolmogorov–Smirnov test. VFA values were normally distributed after square root transformation (P = 0.143). The square root-transformed VFA values were used for statistical analyses. The associations between VFA and breath gases (hydrogen and methane) were assessed by multiple regression analysis with a stepwise variable selection method using VFA as an objective variable, and breath gases (hydrogen and methane) and covariates as explanatory variables.

Visceral fat is affected by a variety of environmental factors, such as age [33], sex [33], BMI [21], WC [21], smoking [34], alcohol consumption [35], exercise [36], and dietary habits [37], including total dietary fiber intake [38, 39]. Furthermore, the human gut microbial composition is affected by a variety of environmental factors, including age [40], dietary habits [41, 42], and habitual medicine use [43]. Thus, correlations among the explanatory variates, including breath gases (hydrogen and methane), age, sex, carbohydrate intake, fat intake, protein intake, and total dietary fiber intake, smoking, alcohol consumption, exercise, and habitual medicine use, were assessed by Spearman’s correlation coefficient. The correlation coefficients were all less than 0.8, confirming that multicollinearity was not a problem. P < 0.05 (two-sided) was considered statistically significant. All analyses were performed using R software (version 3.3.4).

**Results**

**Participant characteristics**

Study participant characteristics (N = 1033, 62% female) are summarized in table 1. The proportion of overweight individuals (BMI ≥ 25) was 30.2% for men and 18.8% for women. The obesity rate (BMI ≥ 30) was 4.0% for men and 3.0% for women. The rates are comparable to the 2010 Japanese national survey (overweight and obesity rate in subjects 30–69 years of age: 33.5% for men and 20.5% for women) [44]. Mean VFA was 83.0 ± 42.5 cm³, lower than the value defined as visceral obesity (≥100 cm³) by the Japan Society for the Study of Obesity [45]. The frequency of a person taking one or more daily medications (e.g. medicine for hypertension, hyperlipidemia, diabetes, rheumatism, dementia, or allergy)
was 31%. The participants were divided into two groups based on the presence (Methanogen+, n = 141, 14% of all participants) or absence (Methanogen−, n = 892, 86% of all participants) of methanogenic bacteria in their stool, as determined by 16S rRNA gene sequencing analysis (Table 1). A simple comparison analysis with no confounder adjustment revealed that the Methanogen+ group exhaled significantly more methane gas, but metabolic risk factors BMI, WC, VFA, SBP, DBP, serum glucose, TG, HDL-cholesterol, and LDL-cholesterol were not significantly different. The HbA1c level was slightly, but significantly higher in the Methanogen+ group (P = 0.030). In the Methanogen+ group, the proportion of methanogenic bacteria relative to all microbiota was significantly inversely associated with VFA (Spearman rank test, r = −0.21, P = 0.012).

### Table 1. Participant characteristics.

| Characteristics              | All (1033/397/636) | Methanogen+ (141/55/86) | Methanogen− (892/342/550) | P value* |
|-----------------------------|--------------------|-------------------------|---------------------------|----------|
| Age (y)                     | 54.4 ± 14.9        | 58.0 ± 14.4             | 53.8 ± 14.9               | 0.002**  |
| Height (cm)                 | 160.5 ± 9.3        | 159.7 ± 9.2             | 160.6 ± 9.3               | 0.322    |
| Body weight (kg)            | 59.1 ± 11.6        | 59.7 ± 12.6             | 59.0 ± 11.5               | 0.742    |
| Metabolic risk factors:     |                    |                         |                           |          |
| BMI (kg m⁻²)                | 22.8 ± 3.5         | 23.3 ± 3.9              | 22.8 ± 3.4                | 0.310    |
| Waist circumference (cm)    | 77.5 ± 10.1        | 78.3 ± 10.5             | 77.4 ± 10.0               | 0.393    |
| VFA (cm)                    | 8.81 ± 2.32        | 8.81 ± 2.32             | 8.87 ± 2.39               | 0.705    |
| Glucose (mmol L⁻¹)          | 4.56 ± 0.84        | 4.63 ± 0.83             | 4.56 ± 0.84               | 0.100    |
| HbA1c (%)                   | 5.75 ± 0.56        | 5.80 ± 0.52             | 5.74 ± 0.56               | 0.030*   |
| SBP (mmHg)                  | 122.2 ± 17.5       | 124.3 ± 18.9            | 121.8 ± 17.3              | 0.218    |
| DBP (mmHg)                  | 74.9 ± 11.6        | 75.6 ± 12.0             | 74.8 ± 11.6               | 0.433    |
| TG (mmol L⁻¹)               | 1.10 ± 0.85        | 1.16 ± 1.04             | 1.09 ± 0.81               | 0.501    |
| HDL-cholesterol (mmol L⁻¹)  | 1.73 ± 0.45        | 1.68 ± 0.42             | 1.74 ± 0.45               | 0.332    |
| LDL-cholesterol (mmol L⁻¹)  | 3.04 ± 0.74        | 3.05 ± 0.70             | 3.04 ± 3.15               | 0.977    |
| Lifestyle habit:            |                    |                         |                           |          |
| Smoking habit (stick/d)     | 5.8 ± 10.4         | 5.9 ± 14.4              | 5.8 ± 9.6                 | 0.791    |
| Sleep time (h/d)            | 7.0 ± 7.0          | 6.9 ± 1.2               | 7.0 ± 1.1                 | 0.410    |
| Amount of exercise (Mets h/w)| 5.2 ± 12.8         | 5.6 ± 14.5              | 5.1 ± 12.5                | 0.861    |
| Total energy intake (kJ/d)  | 7740 ± 2382        | 8142 ± 2679             | 7678 ± 3235               | 0.070    |
| Alcohol consumption (g/d)   | 11 ± 19            | 10 ± 18                 | 11 ± 19                   | 0.257    |
| Carbohydrate intake (g/d)   | 253 ± 82           | 268 ± 92                | 251 ± 80                  | 0.046*   |
| Fat intake (g/d)            | 51 ± 19            | 53 ± 21                 | 51 ± 19                   | 0.251    |
| Protein intake (g/d)        | 68 ± 26            | 73 ± 30                 | 68 ± 25                   | 0.062    |
| Total dietary fiber intake (g/d) | 11 ± 5          | 12 ± 5                  | 11 ± 4                    | 0.048*   |
| Breath Gas:                 |                    |                         |                           |          |
| Hydrogen (ppm)              | 9.57 ± 10.7        | 8.6 ± 8.9               | 9.71 ± 11.0               | 0.677    |
| Methane (ppm)               | 3.84 ± 7.65        | 14.5 ± 17.3             | 2.16 ± 0.509              | <0.001***|
| Methanogen genera ratio:    |                    |                         |                           |          |
| Total (%)                   | 0.097 ± 0.508      | 0.711 ± 1.210           | ND                        | <0.001***|
| Methanomassiliicoccus (%)   | 0.003 ± 0.036      | 0.023 ± 0.094           | ND                        | <0.001***|
| Methanobrevibacter (%)      | 0.086 ± 0.475      | 0.626 ± 1.150           | ND                        | <0.001***|
| Methanosphaera (%)          | 0.008 ± 0.071      | 0.062 ± 0.185           | ND                        | <0.001***|

*P < 0.1, P < 0.05, <0.01 and <0.001 are indicated by †, ‡, ‡‡ and ‡‡‡, respectively. Data represent mean ± SD.

*P value was evaluated between Methanogen+ and Methanogen−.

** Adjusted assessment between breath gases (hydrogen or methane) and VFA

Associations between breath gases (hydrogen or methane) and VFA adjusted for confounding factors; age, sex, carbohydrate intake, fat intake, protein intake, and total dietary fiber intake, smoking, alcohol consumption, exercise, and habitual medicine use were assessed by multiple regression analysis with a stepwise variable selection method using all 1033 participants (Table 2). In Model 1 (breath methane versus VFA), the objective variable was VFA, while breath methane and covariates such as age, sex, dietary fiber intake, and habitual medicine use were explanatory variables. Five variables (breath methane, age, sex, dietary fiber intake, and habitual medicine use) were significantly associated with VFA. Breath methane, female sex, and dietary fiber intake were significantly inversely related to VFA (P = 0.004, <0.001, and 0.018, respectively). Age and habitual medicine use were significantly positively associated with VFA (P ≤ 0.001 for both). In Model 2 (breath hydrogen versus VFA), the objective variable was VFA, while breath hydrogen and covariates such as age, sex, dietary fiber intake, and habitual medicine use for yes were significantly related to VFA. Female sex and...
dietary fiber intake were significantly inversely related to VFA (P ≤ 0.001 and 0.006, respectively). Age was significantly positively associated with VFA (P < 0.001). Among these factors, breath methane was significantly inversely associated with VFA, but breath hydrogen was not (P = 0.004 for breath methane and 0.050 for breath hydrogen).

### Table 2. Multiple regression analysis of the association between VFA and breath gas (Hydrogen and Methane) concentrations, including related factors.

| Model 1: Methane | Model 2: Hydrogen |
|------------------|-------------------|
| β                | 95% CI            | P value<sup>a</sup> |
| Methane          |                   |                   |
| Age              | −0.024            | −0.040 ∼ −0.007   | 0.004** |
| Sex (female)     | 0.021             | 0.012 ∼ 0.031     | <0.001** |
| Total dietary fiber intake | −2.110 | −2.393 ∼ −1.827 | <0.001** |
| Smoking habit    | −0.034            | −0.063 ∼ −0.006   | 0.018*  |
| Habitual medicine use (yes) | 0.010 | −0.003 ∼ 0.023 | 0.139  |
|                   | 0.918             | 0.618 ∼ 1.218     | <0.001** |
| Hydrogen          |                   |                   |
| Age              | 0.011             | 0.000 ∼ 0.025     | 0.050. |
| Sex (female)     | 0.020             | 0.010 ∼ 0.030     | <0.001** |
| Total dietary fiber intake | −2.217 | −2.470 ∼ −1.965 | <0.001** |
| Smoking habit    | −0.040            | −0.068 ∼ −0.011   | 0.006** |
| Habitual medicine use (yes) | 0.934 | −0.634 ∼ 1.235 | <0.001** |

P < 0.1, P < 0.05 and < 0.01 are indicated by ‘*, **’ and ‘***’, respectively.

Data represent mean ± SD.

β = regression coefficient.

Square root-transformed VFA was used.

<sup>a</sup> Stepwise method based on AIC criterion was used.

**Adjusted assessment between breath methane with VFA in participants with methanogenic bacteria**

Associations between methane and VFA in participants harboring methanogenic bacteria were assessed by multiple regression analysis with a stepwise variable selection method (table 4). Model 1 was used to determine the association between breath methane and VFA. Three variables (breath methane, sex, and habitual medicine use) were significantly related to VFA. Breath methane and female sex were significantly inversely related to VFA (B = −0.033 and P = 0.002 for breath methane, B = −1.979 and P < 0.001 for sex). Habitual medicine use was significantly positively associated with VFA (B = 1.100 and P = 0.011). Among these factors, VFA was significantly inversely associated with breath methane after adjusting for confounding factors; age, sex, carbohydrate intake, fat intake, protein intake, and total dietary fiber intake, smoking, alcohol consumption, exercise, and habitual medicine use.

**Confirmation group analysis**

The confirmation group characteristics were similar to the main study group (S1 table is available online at stacks.iop.org/JBR/14/026008/mmedia). Breath gases (methane and hydrogen), VFA, BMI, and WC of the confirmation group were similar to the main study sample. After adjusting for confounding factors as shown in Model 3, breath methane concentration was significantly inversely associated with VFA (P = 0.015). However, the breath hydrogen concentration was not significantly associated with VFA. Similar to the main study sample, 43 (13%) participants had methanogenic bacteria, and their breath methane concentrations were significantly inversely associated with VFA concentration after adjusting for the confounding factors in Model 1 (P < 0.001).

**Discussion**

Methane is produced from hydrogen by methanogenic bacteria such as *M smithii* [16]. Therefore, we investigated if VFA and breath gas (hydrogen and methane) are associated. After analysis of all participant data, the association between VFA and breath hydrogen concentration was not significant, but participants with higher VFA had significantly lower breath methane concentrations (table 2). Breath methane is a product of methanogenic bacteria, which are not present in all individuals [16]. Therefore, we divided the study participants into two groups according to the presence or absence of methanogenic bacteria in their stool. Methanogenic bacteria were present in 14% of all participants, a lower percentage than reported in previous studies (25%–50%) [14–16]. Asians have a
Square root-transformed VFA was used. A Stepwise method based on AIC criterion was used.

**Table 3.** Characteristics of the subgroups with higher or lower breath methane concentration.

| Characteristics               | Methane-UP | Methane-LO | P value<sup>a</sup> |
|-------------------------------|------------|------------|--------------------|
| Number (male/female)          | 70(23/47)  | 71(32/39)  |                    |
| Age (y)                       | 60.7 ± 13.9| 55.3 ± 14.5| 0.030<sup>*</sup>  |
| Height (cm)                   | 159.1 ± 9  | 160.3 ± 9.5| 0.517              |
| Body weight (kg)              | 57.9 ± 12.3| 61.5 ± 12.7| 0.049<sup>*</sup>  |
| Metabolic risk factors:       |            |            |                    |
| BMI (kg m<sup>−2</sup>)       | 22.7 ± 3.6 | 23.9 ± 4.2 | 0.106              |
| WC (cm)                       | 76.8 ± 9.7 | 80 ± 11.1  | 0.134              |
| VFA (cm)                      | 8.28 ± 2.33| 9.45 ± 2.31| 0.006**            |
| Glucose (mmol l<sup>−1</sup>) | 4.6 ± 0.65 | 4.65 ± 0.97| 0.874              |
| HbA1c (%)                     | 5.79 ± 0.36| 5.81 ± 0.64| 0.585              |
| SBP (mmHg)                    | 124.5 ± 20.8| 124.2 ± 16.9| 0.792              |
| DBP (mmHg)                    | 74.6 ± 12.4| 76.5 ± 11.6| 0.163              |
| TG (mmol l<sup>−1</sup>)      | 0.98 ± 0.57| 1.34 ± 1.34| 0.008**            |
| HDL-cholesterol (mmol l<sup>−1</sup>) | 1.7 ± 0.46 | 1.66 ± 0.38 | 0.952              |
| LDL-cholesterol (mmol l<sup>−1</sup>) | 2.92 ± 0.55 | 3.17 ± 0.8  | 0.15               |
| Lifestyle habit:              |            |            |                    |
| Smoking habit (stick/d)       | 6.5 ± 18.7 | 5.4 ± 8.3  | 0.996              |
| Sleep time (h/d)              | 6.8 ± 1.2  | 7 ± 1.2    | 0.376              |
| Amount of exercise (Mets h/w) | 5.9 ± 16   | 5.4 ± 12.9 | 0.293              |
| Total energy intake (kJ/d)    | 8343 ± 2354| 7944 ± 2968| 0.266              |
| Alcohol consumption (g/d)     | 6 ± 13     | 13 ± 21    | 0.061              |
| Carbohydrate intake (g/d)     | 280 ± 87   | 257 ± 95   | 0.132              |
| Fat intake (g/d)              | 54 ± 20    | 52 ± 21    | 0.571              |
| Protein intake (g/d)          | 77 ± 29    | 69 ± 31    | 0.085              |
| Total dietary fiber intake (g/d) | 12 ± 5   | 12 ± 6     | 0.274              |
| Breath Gas:                  |            |            |                    |
| Hydrogen (ppm)               | 7.7 ± 7.9  | 9.6 ± 9.9  | 0.237              |
| Methane (ppm)                | 25.9 ± 18.4| 3.2 ± 1.3  | <0.001**           |
| Methanogen genera ratio:      |            |            |                    |
| Total                         | 1.29 ± 1.289| 0.142 ± 0.391| <0.001**         |
| Methanomassiliicoccus (%)     | 0.044 ± 0.129| 0.003 ± 0.023| <0.001**       |
| Methanobrevibacter (%)        | 1.157 ± 1.424| 0.103 ± 0.291| <0.001**       |
| Methanospirillum (%)          | 0.088 ± 0.195| 0.036 ± 0.172| <0.001**       |

<sup>a</sup> P value was evaluated between Methane-UP and Methane-LO.

| Model 1: VFA          | β     | 95% CI       | P value<sup>a</sup> |
|-----------------------|-------|--------------|---------------------|
| Methane               | −0.033| −0.054 − 0.012| 0.002**             |
| Age                   | 0.016 | −0.013 − 0.045| 0.277               |
| Sex (female)          | −1.979| −2.730 − 1.229| <0.001**            |
| Total dietary fiber intake | −0.058| −0.127 − 0.012| 0.164              |
| Habitual medicine use (yes) | 1.100| 0.254 − 1.945| 0.011*             |

<sup>a</sup> Stepwise method based on AIC criterion was used.

P < 0.1, P < 0.05 and < 0.01 are indicated by *, ** and *** respectively. Data represent mean ± SD. β = regression coefficient. Square root-transformed VFA was used.

A smaller proportion of methanogenic bacteria than other races [16, 17], and the rate observed in the Japanese participants in our study was similar to previous reports. There were no significant differences in BMI or VFA between groups with and without methanogenic bacteria. Similar results were previously reported for BMI [16], but VFA was not previously evaluated. After adjusting for confounding factors, we detected a significant association between breath methane concentration and VFA. However, no association was present with HbA1c, suggesting that the results differ depending on whether or not statistical adjustments are performed. The Methanogen + group was further divided into two subgroups according to the median breath methane concentration: upper half (Methane-UP) or lower half (Methane-LO) of the breath methane concentration. The mean age of the Methane-UP group was significantly higher than that of the Methane-LO group. BMI did not differ significantly between the two breath methane concentration groups. However, VFA significantly differed between the Methane-UP and Methane-LO groups, suggesting that VFA is more closely associated with breath methane concentration than BMI on the basis of a non-adjusted simple
comparison. Even after adjusting for confounding factors, VFA remained significantly associated with breath methane, independent of the confounding factors age and sex [16, 18]. Breath methane concentration may be an independent biomarker of visceral fat accumulation and a possible indicator of metabolic risks. Further investigations, including analyses of follow-up data, are required to determine the relationship between baseline breath methane concentration and high VFA over time. Breath methane is a product of methanogenic bacteria, which have a syntrophic relationship with hydrogen-producing bacteria, thus converting hydrogen to methane [46]. We hypothesized that breath methane concentration is inversely associated with breath hydrogen concentration. The data may support this hypothesis, but further investigation is needed. Hydrogen is a source of methane production, short chain fatty acids (SCFA), and hydrogen sulfide [47], which might indicate a non-linear correlation between breath methane and hydrogen. Methanogenic bacteria change SCFA composition, acetate, and propionate, by coexisting with SCFA-producing microflora [48, 49]. Furthermore, SCFA suppresses obesity-related factors by regulating G-protein coupled receptors 41 and 43 [50, 51]. In addition, our results showed that participants with high breath methane harbored a higher ratio of methanogenic bacteria (table 3). Participants with low breath methane harbored low methanogenic bacteria and increased VFA by altering SCFA composition. Furthermore, there is a report that methane can directly stimulate the secretion of glucagon-like peptide-1, which is related to obesity [52]. Although it is completely a contrasting idea, there is a research that methanogenic bacteria promote the increase in SCFA production, which leads to fat accumulation as an additional energy source, suggesting that further research is necessary. The reproducibility of our observations in this study was assessed using an independent confirmation group enrolled from the 2016 health check. The confirmation group analyses were similar to the main study group.

There seems to be a commonly held belief that methane is linked to obesity and some reports support this hypothesis [18, 19]. However, other studies demonstrate conflicting findings [16, 17]. Therefore, the relationship between breath methane and BMI is inconclusive in previous reports [16, 18–20]. Therefore, we investigated the relationship between breath methane and VFA, which correlates with BMI but varies with race [53]. Thus, VFA may be a confounding factor for the relationship between breath methane and BMI and could explain the previous inconsistencies [16, 18–20], although further investigation is required in populations including different races. Our study revealed no significant association between BMI and breath methane concentration, whereas VFA was significantly inversely associated with breath methane concentration.

In some intestinal microflora studies, diversity is related to BMI as an obesity index [54, 55]. Therefore, we evaluated the association between methanogenic bacteria and VFA. The proportion of methanogenic bacteria was significantly inversely associated with VFA in the Methanogen + group, suggesting that methanogenic bacteria is related to this diversity. If methanogenic bacteria maintain or improve VFA status, these bacteria might be a new target/index for obesity and diabetes.

Previously, total dietary fiber intake significantly increased breath methane concentrations [16]. However, we did not observe this relationship in the present study (table 4). After adjusting for confounding factors, total dietary fiber intake tended toward a positive relationship with breath methane. Thus, increasing dietary fiber intake may lead to a higher breath methane concentration and thereby suppress visceral fat accumulation.

Our study has several key strengths and some limitations. The large number of participants and the use of an independent confirmation group are advantageous to our study. It has been reported that exposure to CT may be a risk factor for cancer [56, 57]. Therefore, in a study for relatively healthy people, using our method is superior from the ethical point of view. Therefore, another strength was VFA measurement using an impedance method, which highly correlates with gold standard methods such as CT [21]. One limitation is that we used a cross-sectional study and not a longitudinal study, nor could we assess whether subjects with low methane currently having low VFA will go on to develop higher VFA in the future. A longitudinal study is necessary to determine causality and the associated mechanisms. Because this study was performed in a single country and a single race, reproducibility should be confirmed in other demographic settings. Another limitation is that, although the correlation between breath methane levels and VFA can be determined in only the methane producer population, which is 8–50% of the population depending on country, this was not done. Furthermore, a recent study reported that methane can be produced directly by cellular processes in the absence of microbes and that this could be further linked to products of oxidative stress [58]; however, we did not consider this viewpoint.

We are the first to report that the human gut microbial composition is associated with VFA and is more strongly associated with metabolic risk factors than higher subcutaneous fat, WC, or BMI in a relatively large population. The association between breath methane and VFA was evaluated, including the effect of methanogenic bacteria. Reproducibility was confirmed in an independent population that participated in the health check conducted in a different year.
Conclusions

Our large study suggests that breath methane is significantly inversely associated with VFA in participants harboring methanogenic bacteria. Further, breath methane is more closely associated with VFA than BMI in these participants. However, a longitudinal study is necessary to determine causality and the associated mechanisms.

Acknowledgments

Authors N O, S S, T Y, M K, and Y K were employed by Kao Corporation (Tokyo, Japan). All other authors declare no competing interests. There are no patents, products in development, or marketed products to declare. This does not alter our adherence to all journal policies on sharing data and materials.

This work was supported by JST, Center of Innovation Program (JPMJCE1302), and Kao Co. (Tokyo, Japan). DNA extraction to sequence analysis was conducted at TechnoSuruga Laboratory Co., Ltd., Shizuoka, Japan.

ORCID iDs

Naoki Ozato https://orcid.org/0000-0002-2038-4705

References

[1] Kuk J L, Katzmarzyk P T, Nichaman M Z, Church T S, Blair S N and Ross R 2006 Visceral fat is an independent predictor of all-cause mortality in men Obesity 14 336
[2] Koster A, Murphy R A, Eiriksdottir G, Aspelund T, Friis S, Sigurdsson S, Lang T F, Guadnason V, Launer L J and Harris T B 2015 Fat distribution and mortality: the AGES-Reykjavik study Obesity 23 893
[3] McNeely M J, Shofer J B, Leonetti D L, Fujimoto W Y and Boyko E J 2012 Associations among visceral fat, all-cause mortality, and obesity-related mortality in Japanese Americans Diabetes Care 35 296
[4] Matsushita Y, Nakagawa T, Yamamoto S, Takahashi Y, Yokoyama T, Noda M and Mizoue T 2010 Associations of visceral and subcutaneous fat areas with the prevalence of metabolic risk factor clustering in 6292 Japanese individuals: the Hitachi health study Diabetes Care 33 2117
[5] Shah B V et al 2014 Visceral adiposity and the risk of metabolic syndrome across body mass index: the MESA study JACC Cardiovasc Imaging 7 1221
[6] Saito S, Morii A, Osaki N and Katsuragi Y 2017 Diacylglycerol enhances the effects of alpha-linolenic acid against visceral fat: a double-blind randomized controlled trial Obesity 25 1667
[7] Remely M, Aumueller E, Aderlov C, Dworzak S, Hippe B, Zanner J, Pointner A, Brath H and Haslberger A G 2014 Effects of short chain fatty acid producing bacteria on epigenetic regulation of FFAR3 in type 2 diabetes and obesity Gene 537 85
[8] Turnbaugh P J, Ley R E, Mahowald M A, Magrini V, Mardis E R and Gordon J I 2006 An obesity-associated gut microbiome with increased capacity for energy harvest Nature 444 1027
[9] Hildebrandt M A et al 2009 High-fat diet determines the composition of the murine gut microbiome independently of obesity Gastroenterology 137 1716
[10] Ley R E, Turnbaugh P J, Klein S and Gordon J I 2006 Microbial ecology: human gut microbes associated with obesity Nature 444 1022
[11] Schwietz A, Taras D, Schafer K, Beijer S, Bos N A, Donus C and Hardt P D 2010 Microbiota and SCFA in lean and overweight healthy subjects Obesity 18 190
[12] Plovier H et al 2017 A purified membrane protein from Akkermansia muciniphila or the pasteurized bacteria improves metabolism in obese and diabetic mice Nat. Med. 23 107
[13] Samuel B S and Gordon J I 2006 A humanized gnotobiotic mouse model of host-archaeal-bacterial mutualism Proc. Natl Acad. Sci. USA 103 1001
[14] Stewart J A, Chadwick V S and Murray A 2006 Carriage, quantification, and predominance of methanogens and sulfate-reducing bacteria in faecal samples Lett. Appl. Microbiol. 43 58
[15] Scanlan P D, Shanahan F and Marchesi J R 2008 Human methanogen diversity and incidence in healthy and diseased colonic groups using mcrA gene analysis BMC Microbiol. 8 79
[16] Fernandez J, Wang A, Su W, Rozenbloom S R, Taibi A, Comelli E M and Wolfe T M 2013 Age, dietary fiber, breath methane, and fecal short chain fatty acids are interrelated in Archaea-positive humans J. Nutr. 143 1259
[17] Nishijima S, Suda W, Oshima K, Kim S W, Hirose Y, Morita H and Hattori M 2016 The gut microbiome of healthy Japanese and its microbial and functional uniqueness DNA Res 23 125
[18] Basseri R J et al 2012 Intestinal methane production in obese individuals is associated with a higher body mass index Gastroenterol. Hepatol. 8 22
[19] Mathur R, Amichai M, Chua K S, Miroja J, Barlow G M and Pimentel M 2013 Methane and hydrogen positivity on breath test is associated with greater body mass index and body fat J. Clin. Endocrinol. Metab. 98 e698
[20] Wilder-Smith C H, Olesen S S, Materna A and Drewes A M 2018 Breast methane concentrations and markers of obesity in patients with functional gastrointestinal disorders United Eur. Gastroenterol. J. 6 593
[21] Ryo M et al 2005 A new simple method for the measurement of visceral fat accumulation by bioelectrical impedance Diabetes Care 28 451
[22] Urushidate S et al 2010 Association between concentration of trace elements in serum and bronchial asthma among Japanese general population J. Trace Elem. Med. Biol. 24 236
[23] Daimon M et al 2016 Association between pituitary-adenal axis dominance over the renin-angiotensin-aldosterone system and hypertension J. Clin. Endocrinol. Metab. 101 889
[24] Daimon M et al 2017 Dominance of the hypothalamus-pituitary-adenal axis over the renin-angiotensin-aldosterone system is a risk factor for decreased insulin secretion Sci. Rep. 7 11360
[25] Sugawara N, Maruo K, Sugai T, Suzuki Y, Ozeki Y, Shimoda K, Someya T and Yasui-Furukori N 2018 Prevalence of underweight in patients with schizophrenia: A meta-analysis Schizophr. Res. 195 67
[26] Sasaki S, Yanagibori R and Amanno K 1998 Self-administered diet history questionnaire developed for health education: a relative validation of the test-version by comparison with 3-day diet record in women J. Epidemiol. 8 203
[27] Kobayashi S, Murakami K, Sasaki S, Okubo H, Hirota N, Notsu A, Fukui M and Date C 2011 Comparison of relative validity of food group intakes estimated by comprehensive and brief-type self-administered diet history questionnaires against 16 dietary records in Japanese adults Public Health Nutr. 14 1200
[28] Takahashi S, Tomita I, Nishioka K, Hisada T and Nishijima M 2014 Development of a prokaryotic universal primer for simultaneous analysis of Bacteria and Archaea using next-generation sequencing PloS One 9 e105592
[29] Miller T L and Wolin M J 1983 Stability of Methanobrevibacter smithii populations in the microbial flora excreted from the human large bowel Appl. Environ. Microbiol. 45 317
[30] Miller T L and Wolin M J 1985 Methanospirillum stadtmanae gen. nov., sp. nov.: a species that forms methane by reducing methanol with hydrogen. *Arch. Microbiol.* 141 116

[31] Eckburg P B, Bik E M, Bernstein C N, Purdom E, Dethlefsen L, Sargent M, Gill S R, Nelson K E and Relman D A 2005 Diversity of the human intestinal microbial flora. *Science* 308 1655

[32] Dridi B, Henry M, Richet H, Raout D and Drancourt M 2012 Age-related prevalence of Methanomasulicoccus luminyensis in the human gut microbiome. *APMIS* 120 773

[33] Yamada M, Moriguchi Y, Mitani T, Aoyama T and Arii H 2014 Age-dependent changes in skeletal muscle mass and visceral fat area in Japanese adults from 40 to 79 years-of-age. *Geriatr. Gerontol. Int.* 14 8

[34] Nakamichi K, Nishida M, Ohata T, Moriyama T and Yamauchi-Takihara K 2014 Smoking associates with visceral fat accumulation especially in women. *Circ. J.* 78 1259

[35] Sumi M et al 2019 Association of alcohol consumption with fat deposition in a community-based sample of Japanese men: the shiga epidemiological study of subclinical atherosclerosis (SESSA). *J. Epidemiol.* 26 6

[36] Xiao T and Fu Y F 2015 Resistance training vs. aerobic training and role of other factors on the exercise effects on visceral fat. *Eur. Rev. Med. Pharmacol. Sci.* 19 1779

[37] Veen V L et al 2017 Visceral adiposity and metabolic syndrome after very high-fat and low-fat isocaloric diets: a randomized controlled trial. *Am. J. Clin. Nutr.* 105 85

[38] Galisteo M, Moron R, Rivera L, Romero R, Anguera A and Zarzuelo A 2010 *Plantago ovata* ovata husks-supplemented diet ameliorates metabolic alterations in obese Zucker rats through activation of AMP-activated protein kinase. Comparative study with other dietary fibers. *Clin. Nutr.* 29 261

[39] Hairston K G, Vitolins M Z, Norris J M, Anderson A M, Hanley A J and Wagenknecht L E 2012 Lifestyle factors and 5-year abdominal fat accumulation in a minority cohort: the IRAS family study. *Obesity* 20 421

[40] Odamaki T, Kato K, Sugahara H, Hashikura N, Takahashi S, Xiao J Z, Fumiaki A and Osawa R 2016 Age-related changes in gut microbiota composition from newborn to centenarian: a cross-sectional study. *BMC Microbiol.* 16 90

[41] Wu G D et al 2011 Linking long-term dietary patterns with gut microbial enterotypes. *Science* 334 105

[42] David L A et al 2014 Diet rapidly and reproducibly alters the human gut microbiome. *Nature* 505 599

[43] Dethlefsen L and Relman D A 2011 Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. *Proc. Natl Acad. Sci. USA* 108 4554

[44] Ministry of Health, Labour and Welfare 2011 Japan National Health and Nutrition Survey 2010. (Japan: MHLW)

[45] Examination Committee of Criteria for ‘Obesity Disease’ in Japan; Japan Society for the Study of Obesity 2002 New criteria for ‘obesity disease’ in Japan. *Circ. J.* 66 987

[46] Deppenmeier U 2002 The unique biochemistry of methanogenesis. *Prog. Nucleic Acid Res. Mol. Biol.* 71 223

[47] Carbonero F, Benefiel A C and Gaskins H R 2012 Contributions of the microbial hydrogen economy to colonic homeostasis. *Nat. Rev. Gastroenterol. Hepatol.* 9 504

[48] Chen M and Wolin M J 1977 Influence of CH4 production by Methanobacterium ruminantium on the fermentation of glucose and lactate by Selenomonas ruminantium. *Appl. Environ. Microbiol.* 34 756

[49] Wolever T M, Robb P A, Ter Wal P and Spadafora P G 1993 Interaction between methane-producing status and diet on serum acetate concentration in humans. *J. Nutr.* 123 681

[50] Kimura I, Inoue D, Maeda T, Hara T, Ichimura A, Miyachi S, Kobayashi M, Hirasawa A and Tsujimoto G 2011 Short-chain fatty acids and ketones directly regulate sympathetic nervous system via G protein-coupled receptor 41 (GPR41). *Proc. Natl Acad. Sci. USA* 108 8030

[51] Kimura I et al 2013 The gut microbiota suppresses insulin-mediated fat accumulation via the short-chain fatty acid receptor GPR3. *Nat. Commun.* 4 1829

[52] Lavender R, Mezouari A, Carson M A, Basiliko N and Gagnon J 2017 A role for methanogens and methane in the regulation of GLP-1. *Endocrinol. Diabetes Metab. 1* e00056

[53] Tanaka S, Horimi C and Katsukawa F 2003 Ethnic differences in abdominal visceral fat accumulation between Japanese, African-Americans, and Caucasians: a meta-analysis. *Acta Diabetol.* 40 5302

[54] Turnbaugh P J et al 2009 A core gut microbiome in obese and lean twins. *Nature* 457 480

[55] Le Chatelier E et al 2013 Richness of human gut microbiome correlates with metabolic markers. *Nature* 500 541

[56] Pearce M S et al 2012 Radiation exposure from CT scans in childhood and subsequent risk of leukaemia and brain tumours: a retrospective cohort study. *Lancet* 380 499

[57] Miglioretti D L et al 2013 The use of computed tomography in pediatrics and the associated radiation exposure and estimated cancer risk. *JAMA Pediatr.* 167 700

[58] Boros M and Keppler F 2019 Methane production and bioactivity-a link to oxidative-reductive stress. *Front. Physiol.* 10 1244