Title: Association analysis between type 2 diabetes and the gut microbiota: a community-based cross-sectional study in Japan

Authors: Koji NAGASHIMA[1], Takayoshi HISADA[1], Jun MOCHIZUKI[1], Tatsuya MIKAMI[2], Yoshinori TAMADA[2] and Shigeyuki NAKAJI[2]

[1]TechnoSuruga Laboratory Co., Ltd., 388-1 Nagasaki, Shimizu-ku, Shizuoka 424-0065, Japan

[2]Innovation Center for Health Promotion, Graduate School of Medicine, Hirosaki University, 5 Zaifu-cho, Hirosaki City, Aomori 036-8562, Japan

Running head: Association between type 2 diabetes and the gut microbiota

Corresponding Author: Koji NAGASHIMA

E-mail: koji.nagashima@tecsrg.co.jp

Keywords: gut microbiota, type 2 diabetes, *Lactobacillales*, community-based cross-sectional study

Abstract: There are a few studies on the association between type 2 diabetes (T2D) and the gut microbiota but no community-based cross-sectional study. In the Iwaki Health Promotion Project (Hirosaki, Japan), we surveyed medical information and lifestyle and analyzed the gut microbiota using the terminal restriction fragment length polymorphism technique or next-generation sequencing. Based on these data, multivariable logistic regression analysis was performed with or without T2D as the dependent variable. Consequently, it was suggested that the *Lactobacillales* population in the gut microbiota was positively associated with T2D, consistent with the results of case-control studies.

Type 2 diabetes (T2D) is caused by insulin resistance or impaired insulin secretion. T2D is related to genetic factors and lifestyle, such as overeating or lack of exercise. In Japan, the sum of patients with T2D and potential diabetics was 20 million in 2016 (The National Health and Nutrition Survey in Japan, 2018). The prevalence is higher in males and with increasing years.

Research on the association between various diseases and the gut microbiota has advanced [1] and become increasingly important. Also, case-control studies on the association between T2D and the gut microbiota have been reported [2-10]. These studies commonly indicated an increased *Lactobacillales* population in the fecal microbiota in T2D, so *Lactobacillales* have the potential to be a novel target for prevention and therapy of T2D.

Community-based health examination surveys (in Hirosaki, Japan) have been performed for many years, called “the Iwaki Health Promotion Project.” In this project, about ten hundreds of people participate every year, and data from more than 2000 items

related to vital and medical information, dietary habits, and lifestyle are collected. These also include data on the gut microbiota obtained using the terminal restriction fragment length polymorphism (T-RFLP) technique [11, 12] or next-generation sequencing (NGS) [13].

There has been no community-based cross-sectional study on the association between T2D and the gut microbiota. Therefore, in this study, multivariable logistic regression analysis was performed with or without T2D as the dependent variable using Iwaki Health Promotion Project data.

This survey was intended for the Iwaki Health Promotion Project (in Hirosaki, Japan) participants in 2012, 2014, and 2016. Subjects were inquired about sex, age, medical history, drug intake, smoking, alcohol drinking, exercise, etc. Weight and height were measured to calculate the body mass index (BMI). The number of subjects by sex and age is indicated in Table 1. This study was approved by the Ethics Committee of Hirosaki University Graduate School of Medicine. Written informed consent was obtained from all participants.

Fecal DNA was prepared as described previously [14]. Feces were collected into a 4 M guanidine thiocyanate solution, followed by bead-beating and extraction using magnetic beads. The fecal microbiota was analyzed by T-RFLP [11, 12] or NGS [13], targeting the 16S rRNA gene.

In silico T-RFLP using bacterial 16S rRNA gene sequences obtained by random cloning analysis of the fecal microbiota provided correspondence between operational taxonomic units (OTUs) in T-RFLP and phylogenetic bacterial groups [11, 12]. This study classified these OTUs into eight bacterial functional groups (B-1–B-8; see Table 2) based on the organic acid-producing activity [15]. Conversion equations are given below.

$$B1 = OTU366 + OTU469 \times 0.97 + OTU853$$

B2 = OTU317

B3 = OTU106 + OTU168 + OTU338 + OTU369 × 0.33 + OTU494 × 0.84 + OTU650 + OTU657 × 0.08 + OTU754 × 0.52 + OTU920 × 0.8 + OTU940 × 0.28 + OTU955 × 0.92

B4 = OTU124

B5 = OTU332 + OTU520 + OTU657 × 0.92

B6 = OTU369 × 0.33 + OTU494 × 0.14 + OTU505 × 0.5 + OTU517 + OTU640 + OTU749 + OTU754 × 0.1 + OTU940 × 0.24 + OTU955 × 0.08

B7 = OTU110 × 0.96 + OTU369 × 0.17 + OTU469 × 0.03

B8 = OTU369 × 0.17 + OTU494 × 0.02 + OTU505 × 0.5 + OTU754 × 0.38 + OTU920 × 0.2 + OTU940 × 0.48 + OTU990

Multivariable logistic regression analysis (forward selection method) was performed with or without T2D as the dependent variable. The number of subjects with or without T2D and with healed T2D is shown in Table 3. Subjects with healed T2D were excluded from the analysis due to the difficulty of attributing to either of the binary variables. The functional bacterial groups in Table 2 in T-RFLP or seven orders, including *Bacteroidales*, *Bifidobacteriales*, *Clostridiales*, *Coriobacteriales*, *Enterobacteriales*, *Lactobacillales*, and *Selenomonadales*, in NGS were the independent variables. The sequences derived from these seven orders accounted for >90% of the total. Age, BMI, smoking, alcohol drinking, and exercise were used as moderators in the regression analysis. In 2016, when subjects were inquired about drug intake, α-glucosidase inhibitor (α-GI), a diabetes drug, was also used as a moderator. JUSE-StatWorks/V5E (The Institute of JUSE, Tokyo, Japan) was used for statistical analysis. The significance level was set at $p < 0.05$.

In Table 3, the T2D ratio in subjects was 3.5% to 4.2% in females and 7.2% to 7.5% in

94 males, which is almost the same level as the ratio of outpatients with T2D to the
95 population (>20 years old in 2016) in Aomori Prefecture: 3.1% in females and 5.4% in
96 males

97 This study used multivariable logistic regression to assess the association between T2D
98 and the gut microbiota (OTUs) in the Iwaki Health Promotion Project participant
99 population for 3 years. A summary of the results is presented in Table 4. The functional
100 bacterial group B-5 (*Lactobacillales*, refer to Table 2) was commonly extracted as a
101 significant variable in males and females in case of 2012 and 2014, in which T-RFLP
102 analysis was performed, with odds ratios [OR; 95% confidence intervals (95% CIs)] was
103 1.062 (1.025–1.100), 1.068 (1.019–1.20), 1.085 (1.032–1.142), and 1.069 (1.020–1.121),
104 respectively. In case of 2016, in which NGS analysis was performed, *Lactobacillales* was
105 a significant variable in males with ORs (95% CIs) of 1.174 (1.019–1.351), but it was an
106 insignificant variable in females, with p-value of a slightly over 10% (p = 0.109). The
107 functional bacterial group B-4 (*Bifidobacterium* spp.) was also a significant variable in
108 some cases, that is, males and females in 2012 and males in 2014 and 2016, with ORs
109 (95% CIs) of 1.150 (1.064–1.244), 1.075 (1.007–1.146), 1.053 (1.013–1.095), and 1.074
110 (1.024–1.125), respectively. As for the rest, the functional bacterial group B-3
111 (*Clostridium* clusters IV, XI, XIVa, and XVIII) in females in 2014, *Bifidobacterium* and
112 *Coriobacteriales* in males in 2016, and *Bacteroidales* in females in 2016 were significant
113 variables.

114

115 Although previous studies on the association between T2D and the gut microbiota were
116 performed in various countries (Austria, Iran, Denmark, Japan, and Sweden) with
117 various techniques (NGS, quantitative polymerase chain reaction, and T-RFLP), a
118 positive association with *Lactobacillales* was indicated in any studies, suggesting a

considerably high certainty of the results. In this study, a multivariable logistic regression analysis was performed to assess the association between T2D and the gut microbiota in a cross-sectional study intended for people in Hirosaki, Japan. Similar results to previous studies were obtained.

This and previous studies suggested the causal relationship between T2D and *Lactobacillales*, but the cause or result is unknown. First, whether *Lactobacillales* are innate bacteria or bacteria originating from foods, such as yogurt, was discussed. Sato et al. [6] and Adachi et al. [9] considered that these bacteria are innate because the number of subjects who consumed yogurt was not significantly different between the control and T2D groups and significantly fewer in the latter group. Furthermore, the number of probiotic bacteria taken from supplements or foods was ~10^{10} cells/day. These bacteria cannot colonize and proliferate in the gut to remain a minor population. Therefore, the idea that these bacteria are innate is deemed to be appropriate.

Morita et al. [16] indicated that lactate and pyruvate, produced in the small intestinal in a bacteria-dependent manner, enhanced immune responses by inducing dendrite protrusion of intestinal $CX3CR1^{+}$ mononuclear cells. These findings speculated a possible causal relationship as follows: an excess of bacteria, including lactic acid bacteria in the small intestine, induces the overstimulation of immune responses, followed by chronic inflammation, resulting in the T2D onset (17, 18).

If that is the case, why do bacteria such as lactic acid bacteria proliferate abnormally in the small intestine? Usually, gastric acid strongly prevents bacterial entry to the small intestine from the oral cavity so that almost no bacterial proliferation occurs. However, gastric acid secretion declines with aging or is decreased by stress, which is considered to cause the proliferation of these bacteria in the intestines [19-22].

On the one hand, some studies suggested that α-GI increases *Bifidobacterium* spp. and

144 *Lactobacillales* population in the fecal microbiota [6, 9, 10]. In this project in 2016, the
145 number of subjects with T2D who did or did not take α-GI was 12 (10 males and 2
146 females) and 45 (23 males and 22 females), respectively. *Bifidobacterium* spp. population
147 in the fecal microbiota in males was higher in subjects with T2D taking α-GI than those
148 not taking α-GI [mean ± standard deviation (SD), 24.3 ± 7.9 vs. 9.3 ± 11.0; $p = 0.003$,
149 Wilcoxon rank-sum test]. Similarly, in *Lactobacillales* in males, the population was
150 higher in subjects with T2D taking α-GI (mean ± SD, 29.4 ± 14.6 vs. 8.5 ± 14.9; $p < 0.001$).
151 No significant difference was observed in females. From these facts, here we accessed
152 using α-GI as an additional moderator for the regression analysis in 2016, in which
153 subjects were inquired about drug intake.

154 On the other hand, α-GI is not involved in the other studies that indicate the positive
155 association between T2D and *Lactobacillales* [5, 7, 8]. Furthermore, the *Lactobacillales*
156 population in the fecal microbiota also increased in a mouse model of insulin resistance
157 [23]. Considering these findings, α-GI is deemed to have only limited effectiveness in this
158 and previous studies on the association between T2D and the gut microbiota.

159

160 Taken together, multivariable logistic regression analysis was performed in the
161 community-based cross-sectional study to assess the association between T2D and the
162 gut microbiota. It was suggested that the *Lactobacillales* population was positively
163 related to T2D, as indicated in previous studies. It is expected that future analyses will
164 be performed with more subjects, and studies on the elucidation of the causal
165 relationship are making progress. As such, novel techniques for prevention and therapy
166 targeted to the bacteria are being developed.

167

ACKNOWLEDGMENTS

This study was supported by JST COI (grant no. JPMJCE1302). We thanks Mr. Ken-ichi Kudo (Hirosaki University) for advice on statistical analysis.

AUTHER CONTRIBUTIONS

K.N. played a role in the investigation, conceptualization and writing original draft. T.H. played a role in the data curation, formal analysis and investigation. J.M., T.M. and Y.T. played a role in the project administration and reviewing. S.N. played a role in the project administration and supervision.

CONFLICT OF INTEREST

The authors declare no competing interests.

REFERENCES

(1) Wang X, Zhang A, Miao J, Sun H, Yan G, Fang-fang Wu F, Wang X. 2018. Gut microbiota as important modulator of metabolism in health and disease. RSC Adv 8: 42380-42389.

(2) Larsen N, Vogensen FK, van den Berg FWJ, Nielsen DS. Andreasen AS, Pedersen BK, Al-Soud WA, Sørensen SJ, Hansen LH, Jakobsen M. 2010. Gut microbiota in human adults with type 2 diabetes differs from non-diabetic adults. PLoS ONE 5: e9085.

(3) Sasaki M, Ogasawara N, Funaki Y, Mizuno M, Iida A, Goto C, Koikeda S, Kasugai K, Joh T. 2013. Transglucosidase improves the gut microbiota profile of type 2 diabetes mellitus patients: a randomized double-blind, placebo-controlled study. BMC Gastroenterol 13: 81.

(4) Karlsson F, Tremaroli V, Nookaew I. Bergström G, Behre CJ, Fagerberg B, Nielsen J, Bäckhed F. 2013. Gut metagenome in European women with normal, impaired and diabetic glucose control. Nature 498: 99-103.

(5) Remely M, Dworzak S, Hippe B, Zwielehner J, Aumüller E, Brath H, Haslberger A. 2013. Abundance and diversity of microbiota in type 2 diabetes and obesity. J Diabetes Metab 4: 253.

(6) Sato J, Kanazawa A, Ikeda F, Yoshihara T, Goto H, Abe H, Komiya K, Kawaguchi M, Shimizu T, Ogihara T, Tamura Y, Sakurai Y, Yamamoto R, Mita T, Fujitani Y, Fukuda H, Nomoto K, Takahashi T, Asahara T, Hirose T, Nagata S, Yamashiro Y, Watada H. 2014. Gut dysbiosis and detection of "live gut bacteria" in blood of Japanese patients with type 2 diabetes. Diabetes Care 37: 2343-2350.

(7) Hartstra AV, Bouter KEC, Bäckhed F, Nieuwdorp M. Insights into the role of the microbiome in obesity and type 2 diabetes. Diabetes Care. 2015; 38: 159-165.

(8) Sedighi M, Razavi S, Navab-Moghadam F, Khamseh ME, Alaei-Shahmirid F, Mehrtashe A, Amirmozafari N. 2017. Comparison of gut microbiota in adult patients with type 2 diabetes. Microbial Pathogenesis 111: 362-369.

(9) Adachi K, Sugiyama T, Yamaguchi Y, Tamura Y, Izawa S, Hijikata Y, Ebi M, Funaki Y, Ogasawara N, Goto C, Sasaki M, Kasugai K. 2019. Gut microbiota disorders cause type 2 diabetes mellitus and homeostatic disturbances in gut-related metabolism in Japanese subjects. J Clin Biochem Nutr 64: 231-238.

213 (10) Hashimoto Y, Hamaguchi M, Kaji A, Sakai R, Osaka T, Inoue R, Kashiwagi S,
214 Mizushima K, Uchiyama K, Takagi T, Naito Y, Fukui M. 2020. Intake of sucrose
215 affects gut dysbiosis in patients with type 2 diabetes. J Diabetes Investig 11:1623-
216 1634.

217 (11) Nagashima K, Hisada T, Sato M, Mochizuki J. 2003. Application of new primer-
218 enzyme combinations to terminal restriction fragment length polymorphism
219 profiling of bacterial populations in human feces. Appl Environ Microbiol 69: 1251-
220 62.

221 (12) Nagashima K, Mochizuki J, Hisada T, Suzuki S, Shimomura K. 2006. Phylogenetic
222 analysis of 16S ribosomal RNA gene sequences from human fecal microbiota and
223 improved utility of terminal restriction fragment length polymorphism profiling.
224 Biosci Microflora 25: 99-107.

225 (13) Takahashi S, Tomita J, Nishioka K, Hisada T, Nishijima M. 2014. Development of a
226 prokaryotic universal primer for simultaneous analysis of bacteria and archaea
227 using next-generation sequencing. PLoS ONE 9: e105592.

228 (14) Hisada T, Endoh K, Kuriki K. Inter- and intra-individual variations in seasonal and
229 daily stabilities of the human gut microbiota in Japanese. 2015. Arch Microbiol 197:
230 919-934.

231 (15) Kettle H, Louis P, Holtrop G, Duncan SH, Flint HJ. 2015. Modelling the emergent
232 dynamics and major metabolites of the human colonic microbiota. Environ
233 Microbiol 17: 1615-1630.

234 (16) Morita N, Umemoto E, Fujita S, Hayashi A, Kikuta J, Kimura I, Haneda T, Imai T,
235 Inoue A, Mimuro H, Maeda Y, Kayama H, Okumura R, Aoki J, Okada N, Kida T,
236 Ishii M, Nabeshima R, Takeda K. 2019. GPR31-dependent dendrite protrusion of
237 intestinal CX3CR1+ cells by bacterial metabolites. Nature 566: 110-114.

238 (17) Kim M, Galan C, Hill AA, Wu W, Fehlner-Peach H, Song HW, Schady D, Bettini ML,
239 Simpson KW, Longman RS, Littman DR, Diehl GE. 2018. Critical Role for the
240 Microbiota in CX3CR1+Intestinal Mononuclear Phagocyte Regulation of Intestinal
241 T Cell Responses. Immunity 49: 151-163.

242 (18) Donath MY, Shoelson SE. 2011. Type 2 diabetes as an inflammatory disease. Nature
243 Rev Immunol. 11: 98-107.

244 (19) Freedberg DE, Toussaint NC, Chen SP, Ratner AJ, Whittier S, Wang TC, Wang HH,
245 Abrams JA. 2015. Proton pump inhibitors alters specific taxa in the human
246 gastrointestinal microbiome: A crossover trial. Gastroenterology 149: 883-885.

247 (20) Jackson MA, Goodrich JK, Maxan M, Freedberg DE, Abrams JA, Poole AC, Sutter
248 JL, Welter D, Ley RE, Bell JT, Spector TD, Steves CJ. 2016. Proton pump inhibitors
249 alter the composition of the gut microbiota. Gut 65: 749-756.

250 (21) Otsuka T, Sugimoto M, Inoue R, Ohno M. 2017. Influence of potassium-competitive
251 acid blocker on the gut microbiome of *Helicobacter pylori*-negative healthy
252 individuals. Gut 66: 1723-1725.

253 (22) Shin AS, Gao X, Bohm M, Lin H, Gupta A, Nelson DE, Toh E, Teagarden S, Siwiec
254 R, Dong Q, Wo JM. 2019. Characterization of proximal small intestinal microbiota
255 in patients with suspected small intestinal bacterial overgrowth: A cross-sectional
256 study. Clin Transl Gastroenterol 10:e00073.

257 (23) Maeda T, Miki S, Morihara N, Kagawa Y. 2019. Aged garlic extract ameliorate fatty
258 liver and insulin resistance and improves the gut microbiota profile in a mouse
259 model of insulin resistance. Exp Ther Med 18: 857-866.

260

Table 1. The number of subjects by sex and age each year

Years		2012		2014		2016	
No. of subjects		940		1,111		1,120	
Sex		Male	Female	Male	Female	Male	Female
Age	～20	16	19	27	43	26	37
	30	51	68	73	84	86	100
	40	56	86	78	102	76	113
	50	73	132	78	130	83	128
	60	96	167	105	198	106	179
	70	45	104	49	107	47	97
	80～	12	15	14	23	18	24
Sum		349	591	424	687	442	678

Table 2. Classification of OTUs in T-RFLP into the bacterial functional groups

Bacterial functional groups	Phylogenetic bacterial groups	Organic acid producing activity	OTUs in T-RFLP *
B-1	*Bacteroides*	Acetate, Propionate, Succinate	366, 469, 853
B-2	*Prevotella*	Acetate, Propionate, Succinate	317
B-3	*Clostridium* cluster IV, XI, XIVa, XVIII	Acetate, Succinate	106, 168, 369(25.0), 490(87.0), 754(52.3), 940(28.0), 955(91.9)
B-4	*Bifidobacterium*	Acetate, Lactate	124
B-5	*Lactobacillales* (*Enterococcus*, *Lactobacillus*, *Streptococcus*)	Lactate	332, 520, 657
B-6	*Clostridium* cluster IV, XI, XIVa, XVIII	Butyrate, Lactate, Formate	369(50.0), 490(11.1), 517, 749, 754(9.5), 955(8.1), 940(24.0), 990
B-7	*Clostridium* cluster XIVa	Acetate, Butyrate, Formate Lactate, Acetate → Butyrate	369(25.0), 494(1.8), 754(38.1), 940(48.0)
B-8	*Clostridium* cluster IX	Acetate, Propionate	110

* In silico T-RFLP using the 16S rRNA gene sequences obtained by the random cloning analysis of feacal microbiota provides a correspondence between the OTUs in T-RFLP and the phylogenetic bacterial groups (11, 12). In this research, these OTUs were further classified into the 8 bacterial functional groups (B-1 to B-8) on the basis of the organic acid producing activity. Even if the clones from the bacterial 16S rRNA gene belong to the same OTU, they are allocated to the different functional group in some cases because of the different organic acid producing activity. Numbers in parenthese indicate the allocation ratios (%), which were calculated from the number of clones belonging to each functional group.

Table 3. The number of subjects with or without T2D and with healed T2D by sex and years

T2D		2012		2014		2016	
		No.	Ratio (%)	No.	Ratio (%)	No.	Ratio (%)
With	Male	25	7.2	31	7.3	33	7.5
	Female	21	3.6	29	4.2	24	3.5
Without	Male	323	92.6	388	91.5	405	91.6
	Female	567	95.9	655	95.3	649	95.7
Healed	Male	1	0.3	5	1.2	4	0.9
	Female	3	0.9	3	0.7	5	1.1
Sum	Male	349	100	424	100	442	100
	Female	591	100	687	100	678	100

Table 4. Multivariable logistic regression analysis for the presence of T2D

Year	Sex	Variables	B	SE	*P*		OR	95% CI	
								Lower	Upper
2012	Male	B-4 (*Bifidobacterium*)	0.140	0.040	0.00045	*	1.150	1.064	1.244
		B-5 (*Lactobacillales*)	0.060	0.018	0.00075	*	1.062	1.025	1.100
		Constant	-15.195	2.810	0.00000				
	Female	B-5 (*Lactobacillales*)	0.066	0.024	0.00603	*	1.068	1.019	1.120
		B-4 (*Bifidobacterium*)	0.072	0.033	0.03159	*	1.075	1.007	1.146
		B-6 (*Clostridium*)	0.057	0.032	0.07192		1.059	0.994	1.127
		Constant	-16.395	2.974	0.00000				
2014	Male	B-5 (*Lactobacillales*)	0.082	0.026	0.00163	*	1.085	1.032	1.142
		B-4 (*Bifidobacterium*)	0.052	0.020	0.00788	*	1.053	1.013	1.095
		Constant	-11.749	2.397	0.00000				
	Female	B-3 (*Clostridium*)	-0.094	0.029	0.00128	*	0.910	0.860	0.964
		B-5 (*Lactobacillales*)	0.067	0.024	0.00498	*	1.069	1.020	1.121
		B-8 (*Clostridium*)	-0.053	0.033	0.10939		0.948	0.889	1.012
		B-4 (*Bifidobacterium*)	0.024	0.018	0.19678		1.024	0.989	1.061
		Constant	-11.465	2.269	0.00000				

continued

2016	Male	*Bifidobacteriales*	0.071	0.024	0.00382	*	1.074	1.024	1.125
		Coriobacteriales	0.160	0.072	0.02529	*	1.174	1.019	1.351
		Lactobacillales	0.036	0.018	0.04820	*	1.037	1.001	1.074
		Constant	-8.968	1.352	0.00000				
	Female	*Bacteroidales*	-0.063	0.025	0.01043	*	0.939	0.894	0.986
		Lactobacillales	0.033	0.021	0.10876		1.034	0.992	1.077
		Constant	-4.960	1.609	0.00206				

Refer to Table 2 with respect to the variables of B-3, B-4, B-5, B-6 and B-8.

Age, BMI, the habits of smoking, alcohol drinking and exercise, and the medication of α-GI (only in the case of 2016) were used as the moderator variable.

The variables with $p < 0.2$ were presented. * indicate $p < 0.05$.

Abbreviations: SE, standard error; OR, odds ratio; CI, confidence interval.