Description

Title of Invention: NOVEL MODIFYING REAGENTS FOR THE PRESENCE RATIO OF INTESTINAL MICROFLORA

Technical Field

[0001] The present invention relates to a pharmaceutical product for treating and/or preventing a diseases that are expected to have therapeutic benefit by altering the intestinal flora. More specifically, the present invention relates to a pharmaceutical product for treating and/or preventing obesity, diabetes, etc., in which the quinolone compound is the active ingredient.

Background Art

[0002] Recently, the relationship between enteric bacteria and various diseases has been reported, and these reports include the relationship between Akkermansia muciniphila and obesity or diabetes. Akkermansia is a novel genus which was proposed in 2004 (Non-Patent Literature 1). The bacterial strain belonging to this genus is about one genus, whose representative species is Akkermansia muciniphila. This bacterium is gram-negative obligate anaerobe which is nonmotile, non-sporulating, elliptic eubacterium. The main characteristics of this bacterium are that it is a mucin-metabolizing bacterium (hence the nomenclature), and it is thought to use mucin as a carbon source which is essential for its culture conditions.

[0003] It is known that the occupation ratio of Akkermansia muciniphila in intestinal flora is lowered in the diabetes or obesity patients. It has been reported that, when the level of Akkermansia muciniphila in the intestine flora of high-fat diet mouse was increased to average level of control group, then the body weight decreased, the body fat rate decreased, and the layer of intestinal mucus grew thick (Non-Patent Literatures 2 and 3). Further, according to other previous literatures, it is known that the increase of Akkermansia muciniphila level in the intestine flora is useful for the increase of mucin layer, the improvement of intestinal barrier function, and the treatment of inflammatory bowel disease (Non-Patent Literatures 4 and 5), fatty liver, hepatitis, appendicitis (Non-Patent Literature 6), and diabetes (Non-Patent Literature 7). In addition, the relationship between Akkermansia muciniphila and central nervous system disease such as epilepsy and amyotrophic lateral sclerosis (ALS) has been also studied (Non-Patent Literatures 8 and 9).

[0004] Patent Literature 1 discloses specific quinolone antimicrobials which exhibit antibacterial activity against Clostridioides difficile living in intestinal tract.

Citation List

Patent Literature

[0005] [PL 1] WO2013/029548

Non-Patent Literature

[0006] [NPL 1] M Derrien, M. International Journal of Systematic and Evolutionary Microbiology (2004). Akkermansia muciniphila gen. nov., sp. nov., a human intestinal mucin-degrading bacterium.

[NPL 2] Karlsson CL, et al. Obes (Silver Spring) (2012). The microbiota of the gut in preschool children with normal and excessive body weight

[NPL 3] Dao MC, et al. Gut (2016). Akkermansia muciniphila and improved metabolic health during a dietary intervention in obesity: relationship with gut microbiome richness and ecology.

[NPL 4] Png CW, et al. Am J Gastroenterol (2010). Mucolytic bacteria with increased prevalence in IBD mucosa augment in vitro utilization of mucin by other bacteria.

[NPL 5] Lyra A, et al. World J Gastroenterol (2012). Comparison of bacterial quantities in left and right colon biopsies and faeces.

[NPL 6] Swidsinski A, et al. Gut (2011). Acute appendicitis is characterised by local invasion with Fusobacterium nucleatum

[NPL 7] Zhang X, et al. PLoS One (2013). Human gut microbiota changes reveal the progression of glucose intolerance.

[NPL 8] Christine A. Olson,; Helen E. Vuong,; Jessica M. Yano,; Qingxing Y. Liang,; David J. Nusbaum,; Elaine Y. Hsiao, et al. The Gut Microbiota Mediates the Anti-Seizure Effects of the Ketogenic Diet, Cell, 2018, 173

[NPL 9] Eran Blacher,; Stavros Bashiardes,; Hagit Shapiro,; Daphna Rothschild,; Uria Mor,; Mally Dori-Bachash, et al. Potential roles of gut microbiome and metabolites in modulating ALS in mice. Nature, 2019, vol. 572

Summary of Invention

Technical Problem

[0007] The main purpose of the present invention is to provide a novel medicament which is expected to have therapeutic benefit on some diseases, such as obesity, diabetes, etc., by changing intestinal flora to increasing especially the layer of intestinal mucus.

Solution to Problem

[0008] The present inventors have extensively studied and then have found that a known quinolone antimicrobial, 1-cyclopropyl-6-fluoro-1,4-dihydro-8-methyl-7-(2-amino-3-cyano-5-pyridyl)-4-oxo-3-quinoline-carboxylic acid can improve intestinal flora, in particular, drastically increase the occupancy of Akkermansia which is known to thicken the mucin layer, and it is effectable for treating a disease which is expected to be treated through the

effect, such as obesity and diabetes. Based upon the new findings, the present invention has been completed.

[0009] The present invention includes the following embodiments.

(Item 1)

A pharmaceutical composition for intestinal flora improvement, comprising 1-cyclopropyl-6-fluoro-1,4-dihydro-8-methyl-7-(2-amino-3-cyano-5-pyridyl)-4-oxo-3-quinoline-carboxylic acid or a pharmaceutically acceptable salt thereof as an active ingredient.

[0010] (Item 2)

The pharmaceutical composition of Item 1, wherein intestinal flora is improved by increasing the intestinal occupancy of Akkermansia.

[0011] (Item 3)

The pharmaceutical composition of Item 1, wherein the layer of intestinal mucus is thickened by improving intestinal flora.

[0012] (Item 4)

A medicament for treating and/or preventing a disease which is expected to be improved by increasing the intestinal occupancy of Akkermansia, comprising 1-cyclopropyl-6-fluoro-1,4-dihydro-8-methyl-7-(2-amino-3-cyano-5-pyridyl)-4-oxo-3-quinoline-carboxylic acid or a pharmaceutically acceptable salt thereof as an active ingredient.

[0013] (Item 5)

A medicament for treating and/or preventing a disease which is expected to be improved by thickening the layer of intestinal mucus, comprising 1-cyclopropyl-6-fluoro-1,4-dihydro-8-methyl-7-(2-amino-3-cyano-5-pyridyl)-4-oxo-3-quinoline-carboxylic acid or a pharmaceutically acceptable salt thereof as an active ingredient.

[0014] (Item 6)

The medicament of Item 4 or 5, wherein the disease is obesity and/or diabetes.

[0015] (Item 7)

A medicament for treating and/or preventing obesity and/or diabetes, comprising 1-cyclopropyl-6-fluoro-1,4-dihydro-8-methyl-7-(2-amino-3-cyano-5-pyridyl)-4-oxo-3-quinoline-carboxylic acid or a pharmaceutically acceptable salt thereof as an active ingredient.

[0016] (Item 8)

The medicament of any one of Items 4 to 7, which is for oral administration.

[0017] (Item 9)

The medicament of any one of Items 4 to 8, wherein the daily dose of the active ingredient is 0.1 mg - 30000 mg.

[0018] (Item 10)

A method for treating and/or preventing obesity and/or diabetes, comprising administering a therapeutically effective amount of 1-cyclopropyl-6-fluoro-1,4-dihydro-8-methyl-7-(2-amino-3-cyano-5-pyridyl)-4-oxo-3-quinoline-carboxylic acid or a pharmaceutically acceptable salt thereof to a patient in need thereof.

[0019] (Item 11)

Use of 1-cyclopropyl-6-fluoro-1,4-dihydro-8-methyl-7-(2-amino-3-cyano-5-pyridyl)-4-oxo-3-quinoline-carboxylic acid or a pharmaceutically acceptable salt thereof in the manufacture of a medicament for treating and/or preventing obesity and/or diabetes.

[0020] (Item 12)

1-Cyclopropyl-6-fluoro-1,4-dihydro-8-methyl-7-(2-amino-3-cyano-5-pyridyl)-4-oxo-3-quinoline-carboxylic acid or a pharmaceutically acceptable salt thereof for use in treating and/or preventing obesity and/or diabetes.

Effect of Invention

[0021] The oral administration of the present compound can drastically increase the occupancy of Akkermansia quickly in intestinal flora, and reorganize the intestinal flora wherein Akkermansia muciniphila dominates. And, that reconstitution of the intestinal flora can increase mucin production. Thus, the present invention is expected as a medicament for treating and/or preventing obesity or diabetes through the improvement of the above intestinal flora. Furthermore, the present compound is a poorly absorbable drug, and thereby it is distributed in a high concentration in the intestinal tract when it is orally administered, but it has low blood transferability. Thus, the present compound also has a merit, i.e., a low risk of generalized side effect, which is a problem in existing quinolone antibacterial agents.

Brief Description of Drawings

[0022] [Fig. 1] Fig. 1 shows the result of the variation in the amount of mucin in Example 3.

[Fig. 2] Fig. 2 shows the result of the variation in body-weight in Example 5 (** $p<0.01$, * $p<0.05$).

[Fig. 3] Fig. 3 shows the result of the variation in blood glucose level in Example 5 (** $p<0.01$).

[Fig. 4] Fig. 4 shows the result of the variation in HbA1c in Example 5 (** $p<0.01$, * $p<0.05$).

[Fig. 5] Fig. 5 shows the result of the variation in casual blood glucose level in Example 6 (** $p<0.01$).

[Fig. 6] Fig. 6 shows the result of the variation in fasting blood glucose level in

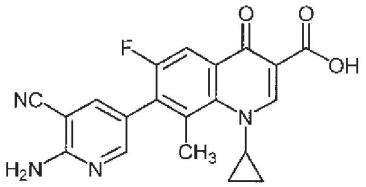
Example 6 (** p<0.01).

[Fig. 7] Fig. 7 shows the result of the variation in HbA1c in Example 6 (** p<0.01).

Description of Embodiments

[0023] The present compound 1-cyclopropyl-6-fluoro-1,4-dihydro-8-methyl-7-(2-amino-3-cyano-5-pyridyl)-4-oxo-3-quinoline-carboxylic acid has the structure of formula (1), which is disclosed as Compound number 2-18 in Patent Literature 1 that also discloses its process and its antibacterial activity for Clostridium difficile.

[Chem.1]



(1)

[0024] The compound of the present invention may be in the form of hydrate and/or solvate, and hence the present compound also encompasses a hydrate and/or solvate thereof.

In addition, the compound of the present invention in which any one or more ^{1}H atoms are replaced by $^{2}H(D)$ atoms is also within the scope of the present invention.

There may exist a polymorphism in a crystal of the compound of the present invention or a pharmaceutically acceptable salt thereof, and hence such crystal polymorphism is also within the scope of the present invention.

[0025] The "pharmaceutically acceptable salt" includes, as an acid addition salt, a salt with inorganic acid such as hydrochloride, hydrobromide, hydroiodide, sulfate, perchlorate, and phosphate; a salt with organic acid such as oxalate, malonate, maleate, fumarate, lactate, malate, citrate, tartrate, benzoate, trifluoroacetate, acetate, methanesulfonate, p-toluenesulfonate, and trifluoromethanesulfonate; and a salt with amino acid such as glutamate and aspartate; and as a salt with a base, an alkali metal salt such as sodium salt and potassium salt; alkaline-earth metal salt such as calcium salt; and an ammonium salt.

[0026] The "intestinal flora" is a generic term for the population of bacteria that live in the intestine of humans or animals. The "intestinal flora" herein means a complex gut microbial ecosystem constituting by about 100 trillion enteric bacteria consisting about 1,000 bacterial species which live in intestine of humans or animals, mainly in large intestine, and maintain a close relationship and balance with each other. In the present invention, the "improvement of intestinal flora" means increasing the intestinal occupancy of Akkermansia as the main improvement effect, in which the representative species of Akkermansia is Akkermansia muciniphila. Akkermansia is mucin-

metabolizing bacterium, and the increase of the intestinal occupancy of Akkermansia is expected to accelate the increase in mucin layer and the improvement of intestinal barrier function, further bring in the therapeutic effect of obesity, diabetes, inflammatory bowel disease, fatty liver, hepatitis, appendicitis, diabetes, epilepsy, amyotrophic lateral sclerosis (ALS), autism, atopic dermatitis, etc. and the anticancer activity (potentiation of immune checkpoint inhibitor such as PD1 antibody).

[0027] The administration route of the present compound may be selected from oral administration or directly rectal administration such as enema preparation and suppository, preferably oral administration. The daily dose depends on the compound, administration route, condition of patient, age of patient, etc. In case of oral administration, for example, it may be generally administered in a dose of about 0.02 mg - about 500 mg, preferably about 0.01 mg - about 200 mg, more preferably about 1 mg - about 10 mg, even more preferably about 2 mg - about 20 mg, per kg of human or mammal's body weight, in one to several portions. For example, the daily dose of human includes about 0.1 mg - about 30000 mg, preferably about 5 mg - about 12000 mg, more preferably about 50 mg - about 6000 mg, even more preferably about 100 mg - about 1200 mg.

[0028] The dosage form in the present invention includes tablet, capsule, granule, powder, liquid, syrup, suspension, enema, and suppository. These dosage forms can be prepared in a conventional manner. If the dosage form is a liquid one, it may be a formulation to prepare a solution or suspension in use by mixing it with water, appropriate water-solution, or other appropriate solvent. The tablet and the granule may be coated in a well-known manner. The dosage form may be prepared in known manner with pharmaceutically acceptable additives.

The additives used herein include, according to the intended use, excipients, disintegrating agents, binders, fluidizer, lubricants, coating agents, colorants, solubilizers, solubilizing agents, thickeners, dispersants, stabilizing agents, sweeteners, and flavors. For example, they include lactose, mannitol, calcium hydrogen phosphate, microcrystalline cellulose, low-substituted hydroxypropylcellulose, cornstarch, partly pregelatinized starch, carmellose calcium, croscarmellose sodium, crospovidone, sodium starch glycolate, hydroxypropylcellulose, hydroxypropyl methylcellulose, polyvinyl alcohol, light anhydrous silicic acid, magnesium stearate, calcium stearate, sodium stearyl fumarate, polyethylene glycol, propylene glycol, titanium oxide, talc, iron sesquioxide, and yellow ferric oxide.

[0029] In case that the present compound is formulated into a single dosage form, the dosage form may include the present compound in 0.1 - 85 % (w/w) per the whole composition, but the present invention is not limited thereto. Preferably, it is 10 - 70 % (w/w) per the whole composition.

[0030] In addition, the present compound may be used in concomitant with another drug or as a combination with another drug for the purpose of enhancing the effect and/or reducing the side effects.

Examples

[0031] The present invention is explained in the following in detail by referring to Examples, however, the present invention should not be limited thereto. The present compound used herein (hereinafter, referred to as "test substance") and the reference drug were obtained as shown below.

Test substance

[1-cyclopropyl-6-fluoro-1,4-dihydro-8-methyl-7-(2-amino-3-cyano-5-pyridyl)-4-oxo-3-quinoline-carboxylic acid]: gained from Otsuka Pharmaceutical Co., Ltd.

[0032] Example 1. Effect of test substance to intestinal flora in normal mouse

The test substance was administered to a normal Balb/c mouse over 21 days, and feces excreted during the period were analyzed to evaluate the effect of the test substance on the intestinal flora.

(Test method)

To a Balb/c mouse was orally administered 5 % Gum Arabic which was a vehicle for administration or the test substance (10 mg/kg) once a day, and feces were collected on the 7th day and the 21st day. The collected feces were milled/homogenized with a buffer in EZ-beads (Promega), and the supernatant was treated with Maxwell™ RSC automatic nucleic acid purification system (Promega) following its instruction manual to extract bacterial genome DNA.

The concentration of the obtained bacterial genomic DNA sample was measured with DropSense96 (SCRUM). The concentration of the sample was then arranged to 5 ng/μL, and used as PCR template to prepare amplicons by PCR enrichment of the V4 region of the ribosomal RNA gene. The prepared amplicon (5 μL), Nextera Index 1 adapters (N7xx) (5 μL), Nextera Index 2 adapters (S5xx) (5 μL), 2 × KAPA HiFi HS ReadyMix (25 μL), and Nuclease free water (10 μL) were mixed to prepare a reaction solution, and the reaction solution was reacted with a thermal cycler in the steps of 95°C × 3 min, 8 cycles of (95°C × 30 sec, 55°C × 30 sec, 72°C × 30 sec), and 72°C × 5 min to prepare a library.

The obtained library was purified with Agencourt AMPure XP, measured about its concentration with DropSense 96, and then evaluated about its quality with 2100 Bio-analyzer electrophoresis system (Agilent).

The concentration of each library was arranged to 1 nM, and the same amount of each was mixed to prepare a sequencing library for analysis (NGS library). The NGS library was denatured with 0.1 N NaOH to obtain single-stranded product, the product

was neutralized, and the concentration of the neutralized product was re-adjusted to about 1.5 pM, which was analyzed with a next generation sequencer MiniSeq (Illumina) following its instruction manual. The sequencing runs were carried out by using a next-generation sequencer (MiniSeq), and nucleotide sequence files (.fastq) were automatically generated in the sequencer. Using the above sequence files (.fastq) of V4 region on 16S rRNA gene, the typing of bacteria species in intestinal flora was carried out by using the application software for metagenomics, 16S Metagenomics (Illumina, Inc.).

[0033] (Result)

The analytical results of intestinal flora on the 7th day and 21st day are shown in Tables 1 and 2 below. As for the vehicle-administered mice, there was no change over time in intestinal flora. On the other hand, the intestinal flora of the test substance-administered mice was obviously changed on the 7th day, compared with the vehicle control group. The occupancy of Akkermansia in the intestinal flora of the test substance administration group's feces was increased to more than 50 %. On the 21st day, the occupancy rate decreased, but the rate was still higher than that of the vehicle control group.

Table 1. Result on 7th day (%)

genus	vehicle control 1	vehicle control 2	vehicle control 3	test substance 1	test substance 2	test substance 3
Akkermansia	2.0	2.5	1.5	70.5	54.4	69.4
Lactobacillus	33.0	63.7	48.8	16.8	28.5	12.9
Blautia	16.7	5.7	11.4	0.3	4.8	2.8
Coprobacillus	0.2	0.1	0.5	0.0	0.0	0.0
Prosthecobacter	0.3	0.4	0.2	5.4	4.5	5.3
Bacteroides	7.5	6.5	4.4	0.5	0.5	1.5
Clostridium	2.0	0.8	3.2	0.3	0.5	0.2
Parabacteroides	4.5	3.9	2.8	0.3	0.3	0.9
Acetobacterium	0.2	0.2	0.1	0.0	0.0	0.0
Luteolibacter	0.0	0.0	0.0	1.1	0.9	1.1
Erysipelothrix	0.4	0.1	0.8	0.1	0.1	0.0
Alkaliphilus	4.4	1.2	4.0	0.1	0.0	0.1
Ruminococcus	3.8	1.4	3.0	0.0	0.0	0.1
Rubritalea	0.0	0.0	0.0	0.9	0.8	0.9
Mucispirillum	0.0	0.0	0.0	0.0	0.0	0.0
Pediococcus	0.8	1.5	0.9	0.1	0.1	0.1
Macrococcus	0.1	0.0	0.1	0.0	0.0	0.0
Oscillospira	1.8	1.0	1.2	0.0	0.1	0.1
Others	13.4	5.8	10.0	1.0	1.6	1.8
Unclassified	8.8	5.4	7.1	2.6	2.8	2.8

Table 2. Result on 21st day (%)

genus	vehicle control 1	vehicle control 2	vehicle control 3	test substance 1	test substance 2	test substance 3
Akkermansia	1.3	1.4	1.7	23.4	17.5	18.6
Lactobacillus	50.0	60.3	34.9	13.6	20.7	18.4
Blautia	10.9	6.0	18.2	38.6	31.3	35.0
Coprobacillus	0.1	0.0	0.3	6.6	12.4	10.5
Prosthecobacter	0.1	0.3	0.3	2.0	1.4	1.6
Bacteroides	4.6	6.4	6.5	0.6	0.6	0.7
Clostridium	0.9	1.7	1.5	2.1	1.3	1.1
Parabacteroides	2.6	4.3	3.5	0.2	0.2	0.2
Acetobacterium	0.1	0.1	0.2	1.3	0.9	1.3
Luteolibacter	0.0	0.0	0.0	0.4	0.3	0.3
Erysipelothrix	0.9	0.7	1.1	0.6	1.1	1.0
Alkaliphilus	1.6	1.1	1.8	0.1	0.1	0.1
Ruminococcus	1.9	0.9	3.2	0.0	0.1	0.1
Rubritalea	0.0	0.1	0.0	0.3	0.3	0.3
Mucispirillum	0.0	0.0	0.0	1.0	0.5	0.5
Pediococcus	0.9	1.5	1.1	0.2	0.3	0.3
Macrococcus	0.0	0.0	0.1	0.5	0.9	0.8
Oscillospira	1.4	0.6	2.0	0.1	0.1	0.1
Others	16.4	7.4	14.6	4.1	4.6	4.0
Unclassified	6.1	7.3	8.8	4.1	5.6	5.2

[0034] Example 2. Effect of test substance to intestinal flora in colitis-model mouse

(Test method)

From the spleen of Balb/c mouse, naive T cell (CD4 + CD62L + CD44−cell) was taken out with Naive CD4+ T Cell isolation kit, mouse (Miltenyi Biotec), which was transplanted into the abdominal cavity of an immunocompromised mouse (SCID mouse) in 500 μL/body (5 x 105 cells/body) to prepare a colitis-model mouse. 14 days after the cell-transplantation, the mice were separated into groups based on their body weights. To each grouped mouse was orally administered 5 % Gum Arabic which was a vehicle for administration or the test substance (10 mg/kg) once a day for continuous 21 days. The untreated mouse means a SCID mouse which received no transplantation. The collected feces were analyzed about intestinal flora with a next generation sequencer. The detailed analysis and analytical method were the same as Example 1.

[0035] (Result)

The analytical results of intestinal flora for 21 days are shown in Tables 3 to 5 below. As for the vehicle control group and the untreated group, there was no significant change over time in intestinal flora. On the other hand, the intestinal flora of the test substance-administered mice was obviously changed. On the 7th day or later, the occupancy of Akkermansia in the intestinal flora of feces was markedly increased in the test substance administration group.

Table 3. Vehicle control group (%)						
genus	0 day	1 day	3 days	7 days	14 days	21 days
Akkermansia	0.0	0.0	0.0	0.0	0.0	0.0
Bacteroides	26.6	28.5	31.5	24.2	19.7	11.7
Lactobacillus	7.6	18.8	18.5	32.0	23.8	48.5
Blautia	29.4	19.9	14.0	14.9	11.8	4.7
Parabacteroides	7.3	7.0	10.5	7.8	17.3	9.0
Ruminococcus	4.6	2.9	3.2	2.8	2.6	1.4
Adlercreutzia	2.9	3.5	3.4	2.8	1.5	1.1
Oscillospira	2.8	1.8	2.2	1.6	1.8	0.6
Alkaliphilus	0.5	1.9	1.7	1.5	2.0	0.4
Johnsonella	1.0	0.6	0.8	0.3	0.4	0.2
Rhodothermus	1.1	0.6	0.5	0.4	0.3	0.0
Clostridium	0.8	0.6	0.8	0.5	0.7	0.7
Pseudobutyrivibrio	1.7	1.0	1.1	0.4	0.3	0.0
Sphingobacterium	0.7	0.8	0.9	0.7	0.7	0.4
Slackia	0.8	0.7	0.6	0.5	0.3	0.2
Pediococcus	0.4	0.6	0.5	0.6	0.5	0.7
Escherichia	0.1	0.2	0.2	0.1	0.8	3.9
Emticicia	0.6	0.3	0.2	0.3	0.6	0.3
Others	5.0	4.8	4.0	3.9	5.6	7.7
Unclassified	6.2	5.6	5.6	4.7	9.3	8.3

Table 4. Test substance administration group (10 mg/kg) (%)						
genus	0 day	1 day	3 days	7 days	14 days	21 days
Akkermansia	0.0	0.0	0.0	13.7	26.2	21.1
Bacteroides	15.1	71.9	80.8	70.0	49.2	37.7
Lactobacillus	5.0	1.3	0.1	0.1	4.4	4.2
Blautia	39.4	6.0	3.4	0.9	0.5	0.9
Parabacteroides	3.2	8.1	7.1	6.6	9.6	25.3
Ruminococcus	6.9	1.3	0.9	0.1	0.0	0.0
Adlercreutzia	3.2	0.0	0.0	0.0	0.0	0.0
Oscillospira	3.7	1.8	0.2	0.1	0.1	0.4
Alkaliphilus	0.8	0.1	0.0	0.0	0.0	0.0
Johnsonella	1.6	0.4	0.1	0.1	0.1	0.1
Rhodothermus	1.4	0.2	0.1	0.0	0.0	0.0
Clostridium	0.8	1.2	0.5	0.4	0.6	0.9
Pseudobutyrivibrio	1.9	0.1	0.0	0.0	0.0	0.0
Sphingobacterium	0.3	0.7	0.6	0.7	0.6	0.4
Slackia	1.0	0.1	0.0	0.0	0.0	0.0
Pediococcus	0.4	0.1	0.0	0.0	0.2	0.1
Escherichia	0.1	0.9	0.0	0.0	0.0	0.0
Emticicia	0.0	0.0	0.0	0.0	0.0	0.0
Others	8.0	2.1	2.6	3.7	4.5	3.9
Unclassified	7.2	3.7	3.5	3.7	4.0	4.9

Table 5. Untreated group (%)						
genus	0 day	1 day	3 days	7 days	14 days	21 days
Akkermansia	0.0	0.0	0.0	0.0	0.1	0.0
Bacteroides	21.7	21.2	27.0	10.1	12.8	6.9
Lactobacillus	38.1	41.4	9.2	33.5	45.4	22.4
Blautia	14.5	14.8	29.3	27.4	17.7	23.4
Parabacteroides	3.5	2.8	4.6	1.6	2.2	13.5
Ruminococcus	3.3	3.3	6.0	4.7	3.0	5.3
Adlercreutzia	3.3	2.0	2.0	2.0	2.7	1.5
Oscillospira	1.5	1.2	3.3	2.7	1.4	2.5
Alkaliphilus	0.3	0.3	0.6	0.5	0.8	4.8
Johnsonella	0.9	0.7	1.7	1.9	1.1	0.6
Rhodothermus	0.9	0.8	1.3	1.4	1.0	1.4
Clostridium	0.5	0.4	1.1	0.8	0.4	0.7
Pseudobutyrivibrio	0.2	0.3	0.6	0.6	0.2	0.1
Sphingobacterium	0.4	0.3	0.4	0.2	0.2	0.3
Slackia	0.7	0.6	0.7	0.8	0.7	0.7
Pediococcus	0.8	0.7	0.3	0.6	0.8	0.4
Escherichia	0.0	0.0	0.0	0.0	0.0	0.0
Emticicia	0.0	0.0	0.0	0.0	0.4	0.6
Others	4.0	4.4	5.6	5.4	4.3	6.4
Unclassified	5.3	4.8	6.3	5.9	5.1	8.4

[0036] Example 3. Analysis of intestinal flora and measurement of mucin amount in feces of rat to which test substance (100 mg/kg) is administered twice a day

To a normal SD rat was administered a vehicle for administration (5 % Gum Arabic) or the test substance (100 mg/kg) twice a day, and feces were collected every day, which were analyzed about intestinal flora in feces and measured about mucin amount in feces.

(Test method)

To a SD rat was orally administered 5 % Gum Arabic which was a vehicle for administration or the test substance (100 mg/kg) twice a day, and feces were collected every day. The collected feces were milled/homogenized with a buffer in EZ-beads (Promega), and the obtained supernatant was treated with Maxwell™ RSC automatic nucleic acid purification machine (Promega) following its instruction manual to extract bacterial genome DNA.

The obtained bacterial genome DNA sample was analyzed about its concentration with DropSense96 (SCRUM), the concentration of the sample was arranged to 5 ng/μL, and then the V4 region of the ribosomal RNA gene was concentrated with PCR to prepare an amplicon. The prepared amplicon (5 μL), Nextera Index 1 adapters (N7xx) (5 μL), Nextera Index 2 adapters (S5xx) (5 μL), 2 × KAPA HiFi HS ReadyMix (25 μL), and Nuclease free water (10 μL) were mixed to prepare a reaction solution, and the reaction solution was reacted with a thermal cycler in the steps of 95°C × 3 min, 8

cycles of (95°C × 30 sec, 55°C × 30 sec, 72°C × 30 sec), and 72°C × 5 min to prepare a library.

The obtained library was purified with Agencourt AMPure XP, analyzed about its concentration with DropSense 96, and then evaluated about its quality with 2100 Bioanalyzer electrophoresis system (Agilent).

The concentration of each sample library was arranged to 1 nM, and the same amount of each was mixed to prepare a NGS library. The NGS library was denatured with 0.1 N NaOH to obtain single-stranded product, the product was neutralized, and the concentration of the neutralized product was re-arranged to about 1.5 pM, which was analyzed with a next generation sequencer MiniSeq (Illumina) following its instruction manual. Using the nucleotide sequence files of V4 region, the typing of bacterium in the intestinal flora was carried out by using an application software for metagenomics, 16S Metagenomics (Illumina, Inc.).

For analysis of the mucin amount in feces, feces were collected every day, which were lyophilized, and the lyophilized samples were measured with FecalMucin Assay kit (Cosmo Bio Co., Ltd.).

[0037] (Result)

The results of the vehicle control group and the test substance administration group are shown in Tables 6 and 7 below.

The result showed that the feces of the vehicle control group have a variety of intestinal flora, but there was no significant variation from the 0 day to the 7th day. The result also showed that the occupancy of Akkermansia was very low.

On the other hand, the test substance administration group (100 mg/kg) showed transient decrease in diversity/bacterial species of intestinal flora. On the 1st day, the occupancy of Akkermansia markedly increased, Lactobacillus increased, and Parabacteroides a little increased. These three species occupied 90 %, and the diversity of intestinal flora decreased. On the 3rd day, the occupancy of Akkermansia further increased to about 40 %, Lactobacillus markedly decreased, and Parabacteroides a little increased. Then, on the 7th day, the occupancy of Akkermansia a little decreased, Parabacteroides a little increased, and the occupancy of the other species also increased. The diversity had a tendency toward recovery.

Table 6. Vehicle control group (%)					
genus	0 day	1 day	3 days	5 days	7 days
Parabacteroides	19.7	22.8	20.6	27.2	24.2
Lactobacillus	16.7	15.8	14.5	11.8	11.4
Blautia	12.9	12.0	15.1	11.3	12.4
Akkermansia	0.8	0.9	0.6	0.8	0.4
Clostridium	6.6	6.1	5.4	3.8	5.0
Ruminococcus	3.7	3.8	4.8	4.8	5.2
Bacteroides	2.8	2.9	2.4	3.6	3.0
Prevotella	2.9	4.1	3.0	4.4	3.7
Turicibacter	2.9	3.2	4.0	2.9	5.1
Dysgonomonas	2.0	2.1	1.9	2.5	1.7
Oscillospira	2.0	2.8	2.9	3.2	3.3
Lachnospira	2.1	2.0	2.2	1.6	1.6
Sarcina	3.3	2.2	1.6	0.7	1.0
Alkaliphilus	1.7	1.8	1.9	2.1	2.3
Prosthecobacter	0.1	0.1	0.2	0.1	0.1
Phascolarctobacterium	0.0	0.0	0.1	0.1	0.0
Providencia	0.0	0.0	0.0	0.0	0.0
Johnsonella	1.5	1.5	1.8	1.5	1.7

Table 7. Test substance (100 mg/kg) administration group (%)					
genus	0 day	1 day	3 days	5 days	7 days
Parabacteroides	20.8	31.4	35.3	41.0	44.2
Lactobacillus	14.5	28.6	5.2	3.6	3.1
Blautia	15.2	0.8	0.1	0.9	0.9
Akkermansia	1.3	23.3	41.2	20.0	16.0
Clostridium	5.0	1.3	0.0	0.2	0.1
Ruminococcus	4.2	0.3	0.0	0.0	0.0
Bacteroides	3.1	0.6	2.2	6.1	5.6
Prevotella	3.3	0.1	0.0	0.1	0.1
Turicibacter	1.5	0.4	0.0	0.0	0.0
Dysgonomonas	2.2	1.9	2.0	4.2	2.5
Oscillospira	2.6	0.4	0.0	0.1	0.5
Lachnospira	2.7	0.1	0.0	0.0	0.0
Sarcina	2.0	0.6	0.0	0.0	0.0
Alkaliphilus	1.3	0.1	0.0	0.0	0.0
Prosthecobacter	0.3	3.0	5.6	4.3	2.1
Phascolarctobacterium	0.0	0.0	0.3	5.0	9.8
Providencia	0.0	0.0	1.7	5.5	6.6
Johnsonella	1.8	0.1	0.0	0.0	0.0

[0038] The variation of each mucin amount of rats in the vehicle control group and the test substance administration group is shown in Figure 1. The mucin amount in feces of the vehicle control group had no significant variation from the 0 day to the 7th day. On the other hand, the mucin amount in feces of the test substance administration group (100

mg/kg) increased from the 1st day, the amount reached about maximum on the 3rd day, and reached the peak on the 7th day.

The result indicated that there is a significant and potent relationship between the increase in the occupancy of Akkermansia and the mucin amount in intestine ($r=0.52$, $P<0.05$) in the test substance administration group.

[0039] Example 4. Analysis of intestinal flora in feces of DSS-induced colitis-model rat to which test substance (1, 3, 10 mg/kg), SASP (300 mg/kg), CPFX (500 mg/kg) twice a day, or DEX (1 mg/kg) is administered once a day

To a colitis-model rat prepared by having Dextran sulfate sodium (DSS) orally taken was administered a vehicle for administration (5 % Gum Arabic) or the test substance (1, 3, 10 mg/kg) twice a day, and feces of each administration group were collected every day, which were analyzed about intestinal flora in feces. In addition, as comparative agents of the present test substance, SASP (sulfasalazine), CPFX (ciprofloxacin), and DEX (dexamethasone) were also evaluated.

(Test method)

The rats were adapted to an environment with access to feed-water bottles for three days, and then divided into groups. After the grouping, symptoms of colitis were induced by allowing rats to freely drink 3 % DSS solution put into the feed-water bottle (the start day of drinking was set as the 0 day). To the rats in the untreated group, water for injection in the feed-water bottle was freely drunk during the test period. From the next day of the grouping to the last day of the test, the test substance (1, 3, 10 mg/kg), SASP (300 mg/kg) or CPFX (500 mg/kg) as a comparative agent, or the vehicle (5 % Gum Arabic) was orally administered to the rat with a feeding needle twice a day. A control agent, DEX (1 mg/kg) was orally administered to the rat with a stomach tube once a day. Feces of each rat were collected every day, and intestinal flora were analyzed in the collected feces with a next generation sequencer. The detailed analysis and analytical method were the same as Example 3.

[0040] (Result)

The results of each group are shown in Tables 8 to 15 below. The results showed that the variation of intestinal flora in feces is different among each group. The intestinal flora in feces of the untreated group showed a variety of intestinal flora, but there was no significant variation from the 0 day to the 7th day. In the vehicle control group, the occupancy of Bacteroides increased over time. On the other hand, in the test substance administration group (1, 3, 10 mg/kg), the occupancy of Akkermansia markedly increased from the 1st day. A dose-dependent in the occupancy of Akkermansia was observed. In the comparative agent administration groups (the SASP group and the DEX group), there was a time-course variation in the intestinal flora, but the occupancy of Akkermansia had no significant variation. In the CPFX group, the

occupancy of Akkermansia had no significant variation until the 7th day though the dose was very high, i.e., 500 mg/kg.

Table 8. Untreated group (%)				
genus	0 day	1 day	3 days	7 days
Lactobacillus	16.9	17.8	12.3	9.1
Parabacteroides	17.1	18.8	12.0	14.3
Blautia	9.3	6.3	8.1	6.0
Akkermansia	3.6	3.5	0.9	1.7
Bacteroides	3.0	3.5	6.9	10.3
Clostridium	4.9	4.6	4.6	3.5
Allobaculum	2.5	2.7	1.2	0.7
Prevotella	2.2	3.3	2.6	5.5
Dysgonomonas	2.8	3.2	4.8	5.7
Slackia	1.7	1.4	1.8	1.0
Ruminococcus	2.3	2.0	0.5	1.0
Turicibacter	2.1	3.4	2.8	2.3
Pediococcus	1.3	1.5	2.4	1.8
Oscillospira	1.2	1.0	1.0	1.9
Bifidobacterium	0.6	0.7	1.5	1.1

Table 9. Solvent control group (%)				
genus	0 day	1 day	3 days	7 days
Lactobacillus	14.4	14.7	16.5	12.6
Parabacteroides	19.9	19.8	13.7	4.6
Blautia	11.6	10.4	9.5	11.7
Akkermansia	1.3	1.3	1.8	0.3
Bacteroides	3.1	2.6	11.2	20.2
Clostridium	5.1	5.6	4.2	3.5
Allobaculum	1.7	4.4	5.0	5.1
Prevotella	2.8	2.7	3.7	0.1
Dysgonomonas	2.8	2.5	1.5	1.5
Slackia	1.5	0.6	0.5	1.7
Ruminococcus	2.1	2.9	2.2	0.3
Turicibacter	1.2	1.9	2.5	2.1
Pediococcus	1.4	1.5	1.5	2.0
Oscillospira	2.2	3.8	3.3	2.1
Bifidobacterium	0.6	0.7	0.6	1.6

Table 10. SASP group (%)				
genus	0 day	1 day	3 days	7 days
Lactobacillus	20.9	30.6	18.6	16.0
Parabacteroides	18.3	15.5	1.1	0.1
Blautia	9.3	3.5	11.3	13.9
Akkermansia	2.0	0.6	0.0	0.0
Bacteroides	3.3	2.6	1.3	1.7
Clostridium	5.1	2.7	2.6	1.5
Allobaculum	1.7	7.9	8.1	8.2
Prevotella	2.8	0.3	0.0	0.1
Dysgonomonas	3.0	3.1	0.8	0.1
Slackia	1.6	2.6	1.6	0.3
Ruminococcus	2.2	0.8	0.2	0.2
Turicibacter	1.1	0.8	1.5	4.2
Pediococcus	1.4	3.1	3.0	2.4
Oscillospira	1.0	1.0	0.5	0.5
Bifidobacterium	0.5	3.5	16.7	13.3

Table 11. DEX group (%)				
genus	0 day	1 day	3 days	7 days
Lactobacillus	20.3	21.8	16.9	11.2
Parabacteroides	20.4	13.0	5.5	7.3
Blautia	10.6	6.5	6.4	4.8
Akkermansia	1.5	3.0	1.6	1.4
Bacteroides	2.7	2.7	11.2	19.6
Clostridium	4.1	4.7	4.3	4.8
Allobaculum	2.0	5.0	3.3	5.7
Prevotella	2.3	2.3	2.6	0.3
Dysgonomonas	2.7	3.6	1.8	2.1
Slackia	1.5	1.0	0.7	1.0
Ruminococcus	2.0	0.9	0.2	0.3
Turicibacter	1.9	2.1	2.6	2.8
Pediococcus	1.6	3.5	3.6	2.5
Oscillospira	1.3	1.7	2.1	1.1
Bifidobacterium	0.6	1.4	2.4	2.5

Table 12. CPFX group (%)				
genus	0 day	1 day	3 days	7 days
Lactobacillus	15.5	53.3	66.6	51.7
Parabacteroides	19.7	3.1	0.0	0.2
Blautia	10.1	6.0	1.1	2.0
Akkermansia	1.2	0.8	0.0	1.0
Bacteroides	3.3	0.4	0.9	8.6
Clostridium	4.0	3.7	1.8	0.7
Allobaculum	3.8	1.9	0.4	0.1
Prevotella	2.5	0.3	0.1	0.0
Dysgonomonas	2.6	0.5	0.1	0.1
Slackia	1.5	0.6	0.1	0.1
Ruminococcus	2.2	2.4	0.0	0.0
Turicibacter	3.8	2.3	0.4	0.1
Pediococcus	1.0	3.8	8.2	6.9
Oscillospira	1.1	2.2	0.8	0.3
Bifidobacterium	1.4	0.7	1.3	0.4

Table 13. Test substance (1 mg/kg) group (%)				
genus	0 day	1 day	3 days	7 days
Lactobacillus	18.2	24.1	15.2	6.0
Parabacteroides	18.7	23.1	16.8	11.1
Blautia	9.5	4.3	0.5	3.0
Akkermansia	2.0	4.9	12.4	7.1
Bacteroides	3.0	4.7	11.2	8.5
Clostridium	5.2	3.3	1.2	6.2
Allobaculum	2.4	0.8	0.1	0.0
Prevotella	2.7	5.8	1.3	0.1
Dysgonomonas	2.9	3.4	1.5	3.2
Slackia	1.6	0.7	7.3	1.0
Ruminococcus	1.9	1.2	0.5	1.1
Turicibacter	1.1	0.5	0.3	0.1
Pediococcus	1.5	1.6	0.8	1.0
Oscillospira	1.3	1.6	0.6	0.5
Bifidobacterium	0.6	0.4	5.6	5.4

Table 14. Test substance (3 mg/kg) group (%)				
genus	0 day	1 day	3 days	7 days
Lactobacillus	17.3	27.3	14.4	5.5
Parabacteroides	16.6	27.7	20.9	12.5
Blautia	9.7	1.4	0.6	1.8
Akkermansia	1.5	6.1	16.3	5.2
Bacteroides	3.4	3.1	5.3	11.4
Clostridium	5.1	1.4	0.5	5.9
Allobaculum	2.9	0.4	0.1	0.0
Prevotella	2.2	6.2	0.3	0.2
Dysgonomonas	2.5	3.8	1.4	3.1
Slackia	1.6	1.7	11.1	0.6
Ruminococcus	1.5	1.1	0.6	1.1
Turicibacter	1.7	0.3	0.0	0.0
Pediococcus	1.6	1.6	0.7	0.7
Oscillospira	1.8	0.8	0.1	0.3
Bifidobacterium	0.9	0.2	5.5	5.2

Table 15. Test substance (10 mg/kg) group (%)				
genus	0 day	1 day	3 days	7 days
Lactobacillus	18.9	19.1	12.6	5.7
Parabacteroides	16.7	28.1	20.9	14.2
Blautia	7.8	0.6	0.5	1.3
Akkermansia	3.7	20.2	20.0	6.4
Bacteroides	3.3	2.4	0.8	7.5
Clostridium	4.8	0.7	0.2	4.1
Allobaculum	2.0	0.1	0.0	0.0
Prevotella	1.8	1.8	0.0	0.2
Dysgonomonas	2.8	2.9	1.4	3.9
Slackia	1.6	1.5	14.6	1.3
Ruminococcus	2.0	0.3	0.0	0.2
Turicibacter	3.3	0.4	0.0	0.0
Pediococcus	1.7	1.1	0.6	0.6
Oscillospira	0.9	0.4	0.0	0.2
Bifidobacterium	0.5	0.1	4.9	5.5

[0041] Example 5. Effect of test substance on obesity-model mouse induced by eating high-fat diet

In order to evaluate the efficacy of the test substance for obesity or diabetes, the test substance was orally administered to an obesity-model mouse which was induced by

eating with high-fat diet, and the blood glucose level and the hemoglobin A1c (HbA1c) were analyzed.

(Test method)

C57BL/6J mice were divided into groups based on their body weights, blood glucose levels, and HbA1c levels. Soon after the grouping, high-fat diet was started, and the oral administration of the vehicle (control) or the test substance (10 mg/kg) once a day was started. Twice a week, the body weight and the food consumption were weighed. The blood glucose level was analyzed by measuring blood taken from tail vein on the initial day of the administration and then every two weeks. The HbA1c level was analyzed by measuring blood taken every 4 weeks from tail vein with DCA Vantage (Siemens Healthcare Diagnostics).

[0042] (Result)

The weight increase in the untreated mice (which took usual diet) was a little until the 63rd day, while the weight increase in the vehicle control group taking high-fat diet was significantly higher, compared with that of the untreated group (on the 63rd day, $p<0.01$). The weight increase in the test substance administration group taking high-fat diet was significantly suppressed, compared with that of the vehicle control group (on the 63rd day, $p<0.05$).

As for the blood glucose level, the vehicle control group taking high-fat diet showed a significant increase of the blood glucose level, compared with that of the untreated group (on the 63rd day, $p<0.01$). In the test substance administration group taking high-fat diet, the increase of the blood glucose level was significantly suppressed, compared with that of the vehicle control group (on the 63rd day, $p<0.01$), which was the same level as the untreated group.

Also as for the HbA1c level, the vehicle control group taking high-fat diet showed a significant increase of the HbA1c level, compared with that of the untreated group (on the 63rd day, $p<0.01$). In the test substance group taking high-fat diet, the increase of the HbA1c level was significantly suppressed, compared with that of the vehicle control group (on the 63rd day, $p<0.05$). Since the administration of the test substance brought in the significant suppression of the weight increase, the significant suppression of the increase in the blood glucose level, and the significant suppression of the increase in the HbA1c level, compared with that of the solvent control group taking high-fat diet, it was suggested that the test substance was useful for treating obesity and diabetes.

[0043] Example 6. Effect of test substance on diabetes-model mouse (db/db mouse)

In order to evaluate the efficacy of the test substance for diabetes, the test substance was orally administered to a diabetes-model mouse (db/db mouse), and the blood glucose level and the hemoglobin A1c (HbA1c) which are indicators of diabetes were

analyzed.

(Test method)

db/db Mice were divided into groups based on their body weights, blood glucose levels, and HbA1c levels. Soon after the grouping, the vehicle (control) or the test substance (10 mg/kg) were orally administered once a day. After the administration was started, the blood glucose level was measured every two weeks and the HbA1c level was measured every four weeks. On the 8th and 12th weeks, however, the test mice were fasted from the previous day, and the blood glucose levels were measured as fasting blood glucose level.

[0044] (Result)

The results are shown in Figures 5 to 7. The increases of the casual blood glucose level and the fasting blood glucose level in the test substance group were significantly suppressed, compared with the vehicle control group (on the 70th day in the casual blood glucose level, $p<0.01$; and on the 84th day in the fasting blood glucose level, $p<0.01$）.

Regarding also the HbA1c, the increase of the level was suppressed significantly in the test substance administration group, compared with that of the vehicle control group (on the 84th day, $p<0.01$). Since the administration of the test substance significantly suppressed the increase in the blood glucose level and the increase in the HbA1c level, compared with the vehicle control group, it was suggested that the test substance was useful for treating diabetes.

Claims

[Claim 1] A pharmaceutical composition for intestinal flora improvement, comprising 1-cyclopropyl-6-fluoro-1,4-dihydro-8-methyl-7-(2-amino-3-cyano-5-pyridyl)-4-oxo-3-quinoline-carboxylic acid or a pharmaceutically acceptable salt thereof as an active ingredient.

[Claim 2] The pharmaceutical composition of claim 1, wherein intestinal flora is improved by increasing the intestinal occupancy of Akkermansia.

[Claim 3] The pharmaceutical composition of claim 1, wherein the layer of intestinal mucus is thickened by improving intestinal flora.

[Claim 4] A medicament for treating and/or preventing a disease which is expected to be improved by increasing the intestinal occupancy of Akkermansia, comprising 1-cyclopropyl-6-fluoro-1,4-dihydro-8-methyl-7-(2-amino-3-cyano-5-pyridyl)-4-oxo-3-quinoline-carboxylic acid or a pharmaceutically acceptable salt thereof as an active ingredient.

[Claim 5] A medicament for treating and/or preventing a disease which is expected to be improved by thickening the layer of intestinal mucus, comprising 1-cyclopropyl-6-fluoro-1,4-dihydro-8-methyl-7-(2-amino-3-cyano-5-pyridyl)-4-oxo-3-quinoline-carboxylic acid or a pharmaceutically acceptable salt thereof as an active ingredient.

[Claim 6] The medicament of claim 4 or 5, wherein the disease is obesity and/or diabetes.

[Claim 7] A medicament for treating and/or preventing obesity and/or diabetes, comprising 1-cyclopropyl-6-fluoro-1,4-dihydro-8-methyl-7-(2-amino-3-cyano-5-pyridyl)-4-oxo-3-quinoline-carboxylic acid or a pharmaceutically acceptable salt thereof as an active ingredient.

[Claim 8] The medicament of any one of claims 4 to 7, which is for oral administration.

[Claim 9] The medicament of any one of claims 4 to 8, wherein the daily dose of the active ingredient is 0.1 mg - 30000 mg.

[Claim 10] A method for treating and/or preventing obesity and/or diabetes, comprising administering a therapeutically effective amount of 1-cyclopropyl-6-fluoro-1,4-dihydro-8-methyl-7-(2-amino-3-cyano-5-pyridyl)-4-oxo-3-quinoline-carboxylic acid or a pharmaceutically ac-

ceptable salt thereof to a patient in need thereof.

[Claim 11] Use of 1-cyclopropyl-6-fluoro-1,4-dihydro-8-methyl-7-(2-amino-3-cyano-5-pyridyl)-4-oxo-3-quinoline-carboxylic acid or a pharmaceutically acceptable salt thereof in the manufacture of a medicament for treating and/or preventing obesity and/or diabetes.

[Claim 12] 1-Cyclopropyl-6-fluoro-1,4-dihydro-8-methyl-7-(2-amino-3-cyano-5-pyridyl)-4-oxo-3-quinoline-carboxylic acid or a pharmaceutically acceptable salt thereof for use in treating and/or preventing obesity and/or diabetes.