Metabolomic analyses of plasma and liver of mice fed with immature *Citrus tumida* peel

Atsushi Toyoda[1,2,3]*, Mizuho Sato[1], Masaki Muto[1], Tatsuhiko Goto[1,2#], Yuji Miyaguchi[1,2,3], Eiichi Inoue[1,2,3]

Short title: Metabolomics of mice fed with Citrus peels

[1] College of Agriculture, Ibaraki University, Ami, Ibaraki 300-0393, Japan

[2] Ibaraki University Cooperation between Agriculture and Medical Science (IUCAM), Ami, Ibaraki 300-0393, Japan

[3] United Graduate School of Agricultural Science, Tokyo University of Agriculture and Technology, Fuchu-city, Tokyo 183-8509, Japan

[#] Present address: Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan.

*Corresponding Author:

E-mail: atsushi.toyoda.0516@vc.ibaraki.ac.jp (AT)

Abstract

Supplementing food with functional small molecules has been shown to prevent diseases and improve the quality of life, especially in elderly people. Citrus fruits and citrus fruit-products are popular food supplements across the world. In this study, we focused on a Japanese citrus fruit, *Citrus tumida* hort. ex Tanaka (*C. tumida*), and elucidated the effects of supplementation of the peels of immature *C. tumida* (PIC) on food intake, body and fat tissue weights, and metabolic profiles of plasma and liver in mice. Supplementation with 5% (w/w) PIC for 4 weeks significantly suppressed body weight gain and decreased adipose tissue weight, including that of the epididymal, perirenal, and subcutaneous fats. Metabolome analyses using capillary electrophoresis time-of-flight mass spectrometry showed that the level of 2-hydroxyvaleric acid was reduced in the blood plasma of mice fed with PIC. Supplementation with PIC significantly elevated the levels of dipeptides (Thr-Asp, Ser-Glu, and Ala-Ala), glucuronic acid (and/or galacturonic acid-2), and *S*-methylglutathione, and significantly reduced the levels of betaine aldehyde in the liver. In conclusion, PIC supplementation affects the metabolism of fatty acids, pectin, glutathione, and choline. Our study demonstrates the potential beneficial effects of PIC, especially in metabolic syndrome

and obesity. PIC may be developed as a functional food and used in the treatment of these diseases. Nutritional and metabolome studies are effective in studying the effects of specific dietary supplements and will contribute to the development of functional foods.

Introduction

Preventive and alternative medicines have shown to improve the quality of life, especially in the elderly population. Good health conditions may be attained by life habits, such as regular exercise, good sleep, and a proper diet. The effects of food on the quality of life have been extensively studied, and various functional foods and supplements are recommended to preserve or improve health. Fruits contain potent ingredients that affect our health. Citrus fruits are popular in several countries and have various health benefits [1]. The dried peels of *Citrus unshiu* and *C. reticulata*, which are used as natural medicines in Japan and China, show beneficial effects, including improved brain function [2]. The peel extract of *C. depressa* helps prevent obesity in mice fed with a high-fat diet [3]. Moreover, supplements of *C. unshiu* peel extract have been found to restore adenocarcinoma-induced weight loss in mice [4]. Citrus peels contain high amounts of flavonoids, which contribute to the health-beneficial effects [2]. Hesperidin and nobiletin flavonoids found in several citrus fruits show beneficial effects against some features of metabolic syndrome [1]. Interestingly, nobiletin shows a protective effect against metabolic syndrome by enhancing circadian rhythms [5], and oral administration of hesperidin reduces the levels of inflammatory markers in patients with metabolic syndrome [6]. In addition,

60 the peel of immature citrus fruits contains relatively high levels of flavonoids and
61 antioxidants than those in mature fruit peels [7,8].
62 *C. tumida* hort. ex Tanaka is a native citrus found around Mt. Tsukuba in Ibaraki
63 prefecture, Japan [9]. *C. tumida* contains high levels of hesperidin and nobiletin than
64 those in other species of citrus fruits [10]. However, the potential health benefits of *C.*
65 *tumida* have not been investigated. Therefore, we aimed to elucidate the health
66 benefits of *C. tumida*, especially for preventing obesity and depression, which are
67 linked to inflammation. We first carried out a comprehensive metabolite analysis of *C.*
68 *tumida*. Omics approaches are considered valuable tools to study the effects of food
69 and farm products on health [11]. Metabolomics is frequently employed to elucidate
70 food-induced alterations in global metabolism in humans and other animals. In this
71 study, we investigated the health benefits of the peel of immature *C. tumida* (PIC) by
72 evaluating the effects of diets supplemented with PIC on food intake (FI), body weight
73 gain (BWG), and the metabolome in plasma and liver of mice.
74

Materials and Methods

Animals and plant materials

This study was approved by the Animal Care and Use Committee of Ibaraki University and conforms to the guidelines of the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), Japan (Notification No. 71).

Male C57BL/6JJmsSlc (B6) mice (7-week-old) obtained from SLC Japan (Shizuoka, Japan) were housed at the animal facility of the College of Agriculture, Ibaraki University under a 12-h light-dark cycle (light on at 8:00 am). Prior to the experiments, the mice were individually housed in cages (143 × 293 × 148 mm, Charles River Laboratories Japan, Kanagawa, Japan) with wood chips. The mice were fed with a semi-purified diet (AIN-93G, Oriental Yeast, Tokyo, Japan). Food and water were available *ad libitum* and were weighed to monitor the daily consumption. Body weight was also determined daily to calculate BWG.

Immature *C. tumida* was harvested at an orchard in the eastern foothill of Mt. Tsukuba, Ibaraki prefecture, Japan, in early October 2015. Peels containing the outer orange layer and the inner white layer were manually collected and freeze-dried using a freeze-dryer (FDU-1110, TOKYO RIKAKIKAI, Tokyo, Japan). Dried peels were powdered using a centrifugal mill (ZM-1, Retsch technology GmbH, Haan, Germany).

Peel powder was stored at room temperature (23–26 °C) until use.

Detection of flavonoids by high-performance liquid chromatography

To determine the flavonoid levels in PIC, concentrations of nobiletin, narirutin, geosmin, hesperidin, and tangeretin were simultaneously analyzed by high-performance liquid chromatography (HPLC) (Hitachi Chromaster System, Hitachi, Tokyo, Japan). Flavonoid standards were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Dried PIC powder (100 mg) was mixed and extracted with 4 mL methanol:dimethyl sulfoxide (1:1) with agitation for 12 h at room temperature using a shaker (NR-10, Taitec, Tokyo, Japan). After the eluted solution was centrifuged at 1000 *g* for 5 min, the supernatant was collected and filtered through a membrane filter (Millex-GS 0.22 µm, Merck Millipore, Darmstadt, Germany). Samples were stored at −80 °C until analysis. A 10-µL sample aliquot was injected into the HPLC apparatus and analyzed with a photodiode array detector. ZORBAX SB-C8 (150 × 3.0 mm i.d.) (Agilent Technologies, Tokyo, Japan) was used as a separation column, and Agilent Hardware Kit High Press was used as a guard column (Agilent Technologies). The temperature of the column oven was set at 40 °C and spectra from

111 200 to 450 nm were obtained. The linear gradient elution program consisted of an
112 initial 20 min (mobile phase, from 80% and 20% to 0% and 100% of formic acid and
113 methanol, respectively) followed by 5 min of 100% methanol at a flow rate of 1.0
114 mL/min. Concentrations of the compounds were calculated from integrated peak areas
115 of the sample and the corresponding authentic standards.
116

117 Experimental design

118 After acclimatization to the environment of the animal facility in Ibaraki University for
119 one week, the B6 mice were divided into two groups: control (*n* = 6) and PIC-fed (*n* =
120 7). The PIC-fed group was fed 5% (w/w) PIC powder in the AIN93G powder diet,
121 whereas the control group was fed only AIN93G powder for four weeks.
122

123 Tissue sampling

124 After fasting from 9:00 am to 12:00 am, the mice were sacrificed by decapitation and
125 trunk blood was collected in a tube on ice. Final concentration was set at 0.13%
126 EDTA-2K. The sample was centrifuged at 1,200 *g* at 23 °C for 10 min. The supernatant
127 blood plasma was collected and stored at −80 °C until use. Approximately 50 mg from
128 the left lobe of the liver was removed and immediately frozen in liquid nitrogen and

129 stored at −80 °C until use. Epididymal, perirenal, and subcutaneous fats were collected
130 and weighed. Tissue weight was normalized to body weight (BW) on the day of
131 sampling.
132

133 Metabolomic analysis of plasma and liver

134 Nine representative mice were selected for the metabolome analysis. The plasma and
135 liver samples (*n* = 5 in the control group, *n* = 4 in PIC-fed group) were subjected to
136 metabolomic analysis as previously reported [12]. Sample preparation and
137 metabolome analysis were carried out by HMT (Human Metabolome Technology Inc.
138 Tsuruoka, Japan). Capillary electrophoresis time-of-flight mass spectrometry
139 (CE-TOFMS) analysis of the metabolome was performed using an Agilent CE Capillary
140 Electrophoresis System equipped with an Agilent 6210 Time of Flight mass
141 spectrometer (Agilent Technologies, Waldbronn, Germany) at HMT following
142 previously described protocols [12–15]. Briefly, approximately 50 mg frozen liver
143 sample was immersed in 1800 µL 50% acetonitrile in Milli-Q water (Millipore-Japan,
144 Tokyo, Japan) containing internal standards (H3304-1002, HMT). The tissue was
145 homogenized using the BMS-M10N21 homogenizer (BMS, Tokyo, Japan) and then
146 centrifuged at 2300 *g* for 5 min at 4 °C. Next, 800 µL of the upper layer was filtered by

centrifugation using an HMT 5-kDa cut-off filter (UFC3LCCNB-HMT, HMT) at 9100 *g* for 120 min at 4 °C. The filtrate was resuspended in 50 µL Milli-Q water for CE-MS analysis. For plasma, 50 µL sample was added to 450 µL methanol containing internal standards (H3304-1002, HMT). The solution was mixed with 500 µL chloroform and 200 µL Milli-Q water and centrifuged at 2300 *g* for 5 min at 4 °C. Next, 400 µL of the upper layer was filtered through an HMT 5-kDa cut-off filter as described above. The filtrate was then resuspended in 25 µL Milli-Q water for CE-MS analysis.

The identified metabolites from the metabolome library were assigned to the Kyoto Encyclopedia of Genes and Genomes (KEGG), facilitating the search for the corresponding metabolic pathways [16].

Statistical analysis

BWG, FI, water intake (WI), and tissue weights were analyzed by an unpaired two-tailed Student's *t*-test. Data were analyzed using Excel (Microsoft, WA) and are shown as mean ± SEM. For metabolomic analyses, Welch's *t*-tests were used to compare the “supplementation” factor. To control for *P*-values of multiple comparisons, the false discovery rate was determined based on previously published studies [17,18]. The significance threshold was set to $Q < 0.1$.

Results

Concentrations of major flavonoids in PIC

We analyzed the major flavonoids, including nobiletin, narirutin, geosmin, hesperidin, and tangeretin, in PIC using HPLC (Table 1). Unfortunately, nobiletin and narirutin could not be separated under the HPLC conditions described above. Previous data showed that the concentration of nobiletin is approximately 3 times that of narirutin in *C. tumida* [41]; hence, nobiletin is relatively a major flavonoid in PIC.

Table 1. Flavonoid content in peels of immature *Citrus tumida* (100 mg).

Flavonoids			**(mg)**
Nobiletin + Narirutin	0.674	±	0.015
Geosmin	0.078	±	0.003
Hesperidin	0.206	±	0.010
Tangeretin	0.249	±	0.009

BWG, FI, WI, and feed efficiency

We measured cumulative BWG, FI, and WI to evaluate the effect of PIC supplementation. Mice from the PIC-fed group showed lower BWG (control: 4.45 ± 0.47 g vs. PIC: 3.20 ± 0.22 g, $P = 0.0267$, Fig 1A) and a tendency for lower FI (control: 126.18 ± 3.69 g vs. PIC: 117.76 ± 1.70 g, $P = 0.0517$, Fig 1B) when compared to control mice. No significant difference was observed in WI (control: 121.40 ± 4.52 g vs. PIC: 125.10 ± 1.90 g, $P = 0.4470$, Fig 1C). Mice from the PIC-fed group also showed a tendency to have a lower feed efficiency than that in control mice (control: 0.035 ± 0.0036 vs. PIC: 0.027 ± 0.0020, $P = 0.0706$, Fig 1D).

Fig 1. Effects of supplementation of the peels of immature *Citrus tumida* (PIC) on (A) cumulative body weight gain, (B) food intake, (C) water intake, and (D) feed efficiency (*n* = 6–7 in each group). Control: mice fed with AIN93G diet; PIC: mice fed with AIN93G supplemented with 5% PIC (w/w) diet. Data are expressed as mean ± SEM. $^{\dagger}P < 0.10$, $^{*}P < 0.05$, $^{**}P < 0.01$ versus control.

Liver and fat weights

As shown in Fig 2A, liver weight (as % of BW) of PIC-fed mice was higher than that of control mice (control, 4.01 ± 0.13% vs. PIC, 4.57 ± 0.057 %, $P = 0.0015$). The weight of epididymal, perirenal, and subcutaneous fats (as % BW) in PIC-fed mice was significantly lower than that in control mice (epididymal fats – control: 1.85 ± 0.15 % vs. PIC: 1.40 ± 0.047 %, $P = 0.0104$; perirenal fat – control: 0.60 ± 0.067 % vs. PIC: 0.35 ± 0.010 %, $P = 0.0021$; subcutaneous fat – control, 0.32 ± 0.026 % vs. PIC, 0.22 ± 0.029 %, $P = 0.0404$) (Fig 2B, 2C, and 2D).

Fig 2. Effects of supplementation of the peels of immature *Citrus tumida* (PIC) on (A) cumulative liver weight, (B) epididymal fat weight, (C) perirenal fat weight, and (D) subcutaneous fat weight (*n* = 6–7 in each group). Control: mice with fed AIN93G diet; PIC: mice fed with AIN93G containing 5% PIC (w/w) diet. Data are expressed as mean ± SEM. *$P < 0.05$, **$P < 0.01$ versus control.

Metabolomics

CE-TOFMS revealed 191 metabolites in the plasma. A single metabolite, 2-hydroxyvaleric acid, showed significantly ($P < 0.05$ and $Q < 0.1$) lower levels in PIC-fed mice than in control mice (Table 2).

Table 2. Metabolites affected by ingestion of peels of immature *Citrus tumida* (*Q*<0.1).

metabolite	sample	ratio (supl/cont)	*p*	*Q*
2-Hydroxyvaleric acid	plasma	0.8	6.0326E-05	0.01152
Thr-Asp (Ser-Glu)	liver	2.4	1.1E-04	0.02762
Daminozide (Ala-Ala)	liver	1.4	4.5E-04	0.05596
Glucuronic acid (Galacturonic acid)	liver	1.6	4.6E-04	0.03793
Betaine aldehyde	liver	0.5	8.3E-04	0.05161
S-Methylglutathione	liver	1.6	9.1E-04	0.04538

210

211 A total of 250 metabolites were detected by CE-TOFMS in the liver. As shown in Table

212 1, 5 metabolites showed significant differences in levels between PIC-fed and control

213 mice ($P < 0.05$ and $Q < 0.1$). The relative amounts of Thr-Asp (and/or Ser-Glu),

214 daminozide (and/or Ala-Ala), glucuronic acid (and/or galacturonic acid-2), and

215 S-methylglutathione were significantly higher in PIC-fed mice than in control mice. In

216 contrast, the relative amount of betaine aldehyde was significantly lower in PIC-fed

217 mice than in control mice.

Discussion

In this study, we investigated the health benefits of a local citrus fruit, *C. tumida*, and focused on the dietary effects of PIC, which contains flavonoids, such as nobiletin and hesperidin. As shown in Table 1, PIC contained high amounts of nobiletin and narirutin compared to other flavonoids, although the peaks of nobiletin and narirutin could not be separated under the conditions described in the Methods. Supplementation of PIC significantly suppressed BWG compared to that observed with the control diet ($p < 0.05$, Fig 1A); although FI and feed efficiency were reduced slightly, the difference was not statistically significant ($p < 0.1$, Fig 1B and 1D). In addition, PIC intake significantly decreased the weight of adipose tissues ($p < 0.05$, Fig 2B, 2C, and 2D). These effects may result from the presence of flavonoids in PIC, which can affect lipid metabolism. In particular, *C. tumida* contains high levels of hesperidin, nobiletin, and tangeretin [10]. Hesperidin has several biological and pharmacological properties, such as anti-inflammatory, anti-carcinogenic, anti-oxidative, vascular protective, and lipid-lowering activities [19–21]. Moreover, nobiletin and hesperidin repress the expression of genes related to lipid synthesis, such as stearoyl-CoA desaturase [22]. In general, several citrus fruit-flavonoids have anti-inflammatory, insulin-sensitizing, and lipid-lowering activities [1]. Therefore, the flavonoids in PIC may reduce body fat

237 deposition and suppress BWG in mice.

238 Metabolomics is widely used in food science and nutrition research [23], and the use
239 of CE-TOFMS reveals the global effects of food on metabolism [11]. In this study, the
240 plasma and liver metabolites of PIC-fed and control mice were analyzed and compared
241 comprehensively using CE-TOFMS, and significant differences were detected,
242 suggesting that this approach may provide important metabolic information about the
243 effects of oral PIC supplementation. In humans, the metabolomic analysis of the
244 effects of diets, including citrus fruits, was similarly successful [24].

245 The blood plasma levels of 2-hydroxyvaleric acid (2-hydroxypentanoic acid) decreased
246 in PIC-fed mice ($p < 0.05$, $Q < 0.1$, Table 2). 2-Hydroxyvaleric acid is present in human
247 fluids and is related to the pathophysiological metabolites in acidosis [25]. The
248 biological function of 2-hydroxyvaleric acid in animal tissues is unclear. Non-obese
249 diabetic (NOD) mice are widely used in type 1 diabetes studies, and they are divided
250 into progressor and non-progressor groups depending on whether or not the animal
251 shows disease progression [26]. The authors showed that plasma levels of
252 2-hydroxyvaleric acid are lower in non-progressor than in progressor NOD mice, and
253 therefore, 2-hydroxyvaleric acid may be a good predictor of type 1 diabetes. In
254 addition, plasma levels of 2-hydroxyvaleric acid in humans are decreased by

255 simvastatin, a statin that reduces LDL-cholesterol levels and the risk of cardiovascular
256 disease [27]. It is possible that simvastatin, or a metabolite of the drug, inhibits an
257 enzyme that produces 2-hydroxyvaleric acid. Although the precise mechanism
258 underlying these effects is unknown, PIC may modify the fatty acid metabolism and
259 downregulate the 2-hydroxyvaleric acid synthesis pathway, resulting in lower levels in
260 the plasma.
261 Thr-Asp and/or Ser-Glu levels increased in the liver of PIC-fed mice compared to those
262 in the liver of control mice ($p < 0.05$, $Q < 0.1$, Table 2). PIC may increase proteolysis in
263 the liver, leading to increased dipeptide levels. Daminozide and/or Ala-Ala levels also
264 increased in the liver of PIC-fed mice ($p < 0.05$, $Q < 0.1$, Table 2). Daminozide is used as
265 a pesticide and is unlikely to be normally present in animal tissues. Ala-Ala is a
266 dipeptide derived from proteolysis, and increased levels may be the result of increased
267 protein catabolism. D-Ala-D-Ala is an important component of peptidoglycan in the
268 bacterial cell wall [28,29]. A previous study reported that vancomycin-sensitive
269 gram-positive gut bacteria may promote hepatocellular carcinoma through the
270 enterohepatic circulation of gut bacterial metabolites or toxins [30]. D-Ala-D-Ala in the
271 gut may, therefore, be carried to the liver via the enterohepatic circulation. Dietary
272 plant fibers, including pectin, a major component in citrus peels, strongly affect the

intestinal bacterial ecosystem [31] and thus increase the Ala-Ala levels in the liver of PIC-fed mice.

The levels of glucuronic and UDP-glucuronic acids increased in the liver after PIC supplementation ($p < 0.05$, Table 2, Table S2). Since both of them play a pivotal role in the elimination of toxic substances [32], increased levels of glucuronic and UDP-glucuronic acids by PIC may contribute to enhanced detoxification in the liver.

PIC also increased the levels of galacturonic acid in the liver ($p < 0.05$, $Q < 0.1$, Table 2). Galacturonic acid is the main component of pectin [33], which is digested by several fibrolytic enzymes produced by intestinal microorganisms [34]. Pectin is a major component of citrus peels. Therefore, the digestion of PIC produces galacturonic acid in the mouse intestine, and this metabolite is possibly transported to and accumulated in the liver. It has been reported that orally fed pectin and galacturonic acid inhibit hepatic lipogenesis in rats [35]. Lipid metabolism should be studied in PIC-fed mice to clarify these and other issues. The functions of other dietary fibers in PIC should also be investigated.

Reduced glutathione (GSH), a major protectant against oxidative stress, is methylated in the SH group of its cysteine moiety and converted to S-methylglutathione. Levels of S-methylglutathione increased in PIC-fed mice compared to those in control mice ($p <$

291 0.05, $Q < 0.1$, Table 2). In addition, oxidized glutathione (GSSG) levels in the liver of
292 PIC-fed mice were slightly higher than those in control mice ($p < 0.1$, Table S4). PIC may
293 thus be able to increase anti-oxidative stress activity via glutathione metabolism in the
294 liver. In fact, chronic administration of *C. unshiu* extracts reduces oxidative stress in the
295 liver of diabetic rats by increasing glutathione levels [36]. The function of
296 S-methylglutathione in the liver is unclear. However, in the central nervous system,
297 S-methylglutathione is released upon hypoxia and depolarization [37] and central
298 administration of S-methylglutathione mitigates acute stress in neonatal chicks [38].
299 Therefore, S-methylglutathione may play an important role in the central nervous
300 system. Future studies are necessary to assess the function of S-methylglutathione in
301 the liver and the effects of increased S-methylglutathione levels induced by PIC on the
302 liver function.
303 Supplementation of PIC downregulated choline metabolism in the liver (Table 2, Table
304 S2, Fig 3). GPCho is a product of the breakdown of phosphatidylcholine and is
305 converted to choline by GPCho phosphodiesterase. Choline dehydrogenase produces
306 betaine aldehyde from choline [39], and glycine betaine is converted from betaine
307 aldehyde by aldehyde dehydrogenase [40]. The mechanism underlying the
308 downregulation of choline metabolism in the liver by PIC supplementation remains

309 unclear. Future studies will focus on evaluating the expression of genes related to
310 choline metabolism in the liver of PIC-fed mice, and critical ingredients in PIC
311 downregulating choline metabolism should be discovered.
312
313 **Fig 3. Choline-related metabolites in the liver.** The pathway of choline-related
314 metabolites in the liver is shown. Welch's *t*-tests were used to compare "supplement,"
315 using both peels of immature *Citrus tumida* (PIC)-fed mice (*n* = 4) and control mice (*n* =
316 5). Control: mice fed with AIN93G diet; PIC: mice fed with AIN93G containing 5% PIC
317 (w/w) diet. Data are expressed as mean ± SEM.
318
319 In summary, we evaluated the effects of PIC supplementation on BWG, FI, WI, adipose
320 tissue weight, and metabolomics of blood plasma and liver in mice and concluded that
321 PIC influences body and adipose tissue weights and promotes metabolic alterations.
322 Especially, plasma 2-hydroxyvaleric acid and liver choline metabolism including betaine
323 aldehyde were significantly decreased by dietary PIC. In the future, we will focus on
324 elucidating the mechanisms by which PIC supplementation reduces body fat and
325 changes the levels of some metabolites in mice. This study used a small cohort and
326 might have missed some critical metabolites that are significantly altered by dietary

327 PIC. Therefore, in future studies we will try to carry out metabolomic analyses using
328 larger cohorts of mice and humans to elucidate PIC functions, especially in fat
329 metabolism. These studies will contribute to establish the effects of PIC on human and
330 animal health and provide a better understanding of the health benefits of citrus fruits.

Acknowledgments

We thank H. Shimonishi and T. Fujii (Ibaraki University) for valuable technical assistance.

Authors' contribution

A. Toyoda, M. Sato, and T. Goto designed the studies. A. Toyoda, M. Sato, M. Muto, Y. Miyaguchi, and E. Inoue performed the experiments. A. Toyoda and T. Goto analyzed the data. A. Toyoda wrote the manuscript.

References

1. Mulvihill EE, Burke AC, Huff MW. Citrus Flavonoids as Regulators of Lipoprotein Metabolism and Atherosclerosis. Annu Rev Nutr. 2016;36: 275–299. doi:10.1146/annurev-nutr-071715-050718

2. Ito A, Shin N, Tsuchida T, Okubo T, Norimoto H. Antianxiety-Like Effects of Chimpi (Dried Citrus Peels) in the Elevated Open-Platform Test. Molecules. 2013;18: 10014–10023. doi:10.3390/molecules180810014

3. Lee Y-S, Cha B-Y, Saito K, Choi S-S, Wang XX, Choi B-K, et al. Effects of a Citrus depressa Hayata (shiikuwasa) extract on obesity in high-fat diet-induced obese mice. Phytomedicine. 2011;18: 648–54. doi:10.1016/j.phymed.2010.11.005

4. Kim A, Im M, Gu MJ, Ma JY. Citrus unshiu peel extract alleviates cancer-induced weight loss in mice bearing CT-26 adenocarcinoma. Sci Rep. 2016;6: 24214. doi:10.1038/srep24214

5. He B, Nohara K, Park N, Park Y-S, Guillory B, Zhao Z, et al. The Small Molecule Nobiletin Targets the Molecular Oscillator to Enhance Circadian Rhythms and Protect against Metabolic Syndrome. Cell Metab. 2016;23: 610–21. doi:10.1016/j.cmet.2016.03.007

6. Rizza S, Muniyappa R, Iantorno M, Kim J, Chen H, Pullikotil P, et al. Citrus

polyphenol hesperidin stimulates production of nitric oxide in endothelial cells while improving endothelial function and reducing inflammatory markers in patients with metabolic syndrome. J Clin Endocrinol Metab. 2011;96: E782-92. doi:10.1210/jc.2010-2879

7. Choi S-Y, Ko H-C, Ko S-Y, Hwang J-H, Park J-G, Kang S-H, et al. Correlation between flavonoid content and the NO production inhibitory activity of peel extracts from various citrus fruits. Biol Pharm Bull. 2007;30: 772–8.

8. Barreca D, Bellocco E, Caristi C, Leuzzi U, Gattuso G. Flavonoid composition and antioxidant activity of juices from Chinotto (Citrus x myrtifolia Raf.) fruits at different ripening stages. J Agric Food Chem. 2010;58: 3031–6. doi:10.1021/jf9044809

9. Tanaka C. Species problem in citrus a critical study of wild and cultivated units of citrus, based upon field studies in their native homes. Japanese Society for the Promotion of Science, 9, 139. Tokyo: Japanese Society for the Promotion of Science; 1954.

10. Kawaii S, Tomono Y, Katase E, Ogawa K, Yano M. Quantitation of flavonoid constituents in citrus fruits. J Agric Food Chem. 1999;47: 3565–71.

11. Takahashi S, Saito K, Jia H, Kato H. An Integrated Multi-Omics Study Revealed

377 Metabolic Alterations Underlying the Effects of Coffee Consumption. Chowen JA,
378 editor. PLoS One. 2014;9: e91134. doi:10.1371/journal.pone.0091134
379 12. Goto T, Kubota Y, Toyoda A. Plasma and liver metabolic profiles in mice
380 subjected to subchronic and mild social defeat stress. J Proteome Res. 2015;14:
381 1025–32. doi:10.1021/pr501044k
382 13. Soga T, Heiger DN. Amino acid analysis by capillary electrophoresis electrospray
383 ionization mass spectrometry. Anal Chem. 2000;72: 1236–41.
384 14. Soga T, Ohashi Y, Ueno Y, Naraoka H, Tomita M, Nishioka T. Quantitative
385 metabolome analysis using capillary electrophoresis mass spectrometry. J
386 Proteome Res. 2003;2: 488–94.
387 15. Soga T, Ueno Y, Naraoka H, Ohashi Y, Tomita M, Nishioka T. Simultaneous
388 determination of anionic intermediates for Bacillus subtilis metabolic pathways
389 by capillary electrophoresis electrospray ionization mass spectrometry. Anal
390 Chem. 2002;74: 2233–9.
391 16. Kanehisa M, Goto S, Sato Y, Kawashima M, Furumichi M, Tanabe M. Data,
392 information, knowledge and principle: back to metabolism in KEGG. Nucleic
393 Acids Res. 2014;42: D199–D205. doi:10.1093/nar/gkt1076
394 17. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and

395 Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society.
396 Series B (Methodological). WileyRoyal Statistical Society; 1995. pp. 289–300.
397 doi:10.2307/2346101
398 18. Storey JD, Tibshirani R. Statistical significance for genomewide studies. Proc Natl
399 Acad Sci U S A. 2003;100: 9440–5. doi:10.1073/pnas.1530509100
400 19. Miyake Y, Yamamoto K, Tsujihara N, Osawa T. Protective effects of lemon
401 flavonoids on oxidative stress in diabetic rats. Lipids. 1998;33: 689–95.
402 20. Chiba H, Uehara M, Wu J, Wang X, Masuyama R, Suzuki K, et al. Hesperidin, a
403 citrus flavonoid, inhibits bone loss and decreases serum and hepatic lipids in
404 ovariectomized mice. J Nutr. 2003;133: 1892–7. doi:10.1093/jn/133.6.1892
405 21. Morand C, Dubray C, Milenkovic D, Lioger D, Martin JF, Scalbert A, et al.
406 Hesperidin contributes to the vascular protective effects of orange juice: a
407 randomized crossover study in healthy volunteers. Am J Clin Nutr. 2011;93:
408 73–80. doi:10.3945/ajcn.110.004945
409 22. Nichols LA, Jackson DE, Manthey JA, Shukla SD, Holland LJ. Citrus flavonoids
410 repress the mRNA for stearoyl-CoA desaturase, a key enzyme in lipid synthesis
411 and obesity control, in rat primary hepatocytes. Lipids Health Dis. 2011;10: 36.
412 doi:10.1186/1476-511X-10-36

413 23. Wishart DS. Metabolomics: applications to food science and nutrition research.
414 Trends Food Sci Technol. Elsevier; 2008;19: 482–493.
415 doi:10.1016/J.TIFS.2008.03.003
416 24. May DH, Navarro SL, Ruczinski I, Hogan J, Ogata Y, Schwarz Y, et al. Metabolomic
417 profiling of urine: response to a randomised, controlled feeding study of select
418 fruits and vegetables, and application to an observational study. Br J Nutr.
419 2013;110: 1760–70. doi:10.1017/S000711451300127X
420 25. Asano K, Miyamoto I, Matsushita T, Murakami Y, Minoura S, Wagatsuma T, et al.
421 Succinic acidemia: a new syndrome of organic acidemia associated with
422 congenital lactic acidosis and decreased NADH-cytochrome c reductase activity.
423 Clin Chim Acta. 1988;173: 305–12.
424 26. Grapov D, Fahrmann J, Hwang J, Poudel A, Jo J, Periwal V, et al. Diabetes
425 Associated Metabolomic Perturbations in NOD Mice. Metabolomics. 2015;11:
426 425–437. doi:10.1007/s11306-014-0706-2
427 27. Trupp M, Zhu H, Wikoff WR, Baillie RA, Zeng Z-B, Karp PD, et al. Metabolomics
428 reveals amino acids contribute to variation in response to simvastatin treatment.
429 Oresic M, editor. PLoS One. 2012;7: e38386. doi:10.1371/journal.pone.0038386
430 28. Wright GD, Walsh CT. D-Alanyl-D-alanine ligases and the molecular mechanism

of vancomycin resistance. Acc Chem Res. American Chemical Society; 1992;25: 468–473. doi:10.1021/ar00022a006

29. Kumar I, Josephine HR, Pratt RF. Reactions of peptidoglycan-mimetic beta-lactams with penicillin-binding proteins in vivo and in membranes. ACS Chem Biol. 2007;2: 620–4. doi:10.1021/cb7001347

30. Yoshimoto S, Loo TM, Atarashi K, Kanda H, Sato S, Oyadomari S, et al. Obesity-induced gut microbial metabolite promotes liver cancer through senescence secretome. Nature. 2013;499: 97–101. doi:10.1038/nature12347

31. Koropatkin NM, Cameron EA, Martens EC. How glycan metabolism shapes the human gut microbiota. Nat Rev Microbiol. 2012;10: 323–35. doi:10.1038/nrmicro2746

32. Rowland A, Miners JO, Mackenzie PI. The UDP-glucuronosyltransferases: their role in drug metabolism and detoxification. Int J Biochem Cell Biol. 2013;45: 1121–32. doi:10.1016/j.biocel.2013.02.019

33. Mohnen D. Pectin structure and biosynthesis. Curr Opin Plant Biol. 2008;11: 266–77. doi:10.1016/j.pbi.2008.03.006

34. Matsuura Y. Pectic acid degrading enzymes from human feces. Agric Biol Chem. Japan Society for Bioscience, Biotechnology, and Agrochemistry; 1991;55:

885–886. doi:10.1271/bbb1961.55.885

35. Suzuki M, Kajuu T. Suppression of hepatic lipogenesis by pectin and galacturonic acid orally-fed at the separate timing from digestion-absorption of nutrients in rat. J Nutr Sci Vitaminol (Tokyo). 1983;29: 553–62.

36. Sugiura M, Ohshima M, Ogawa K, Yano M. Chronic administration of Satsuma mandarin fruit (Citrus unshiu Marc.) improves oxidative stress in streptozotocin-induced diabetic rat liver. Biol Pharm Bull. 2006;29: 588–91.

37. Kanazawa A, Kakimoto Y, Nakajima T, Sano I. Identification of gamma-glutamylserine, gamma-glutamylalanine, gamma-glutamylvaline and S-methylglutathione of bovine brain. Biochim Biophys Acta. 1965;111: 90–5.

38. Yamane H, Tomonaga S, Suenaga R, Denbow DM, Furuse M. Intracerebroventricular injection of glutathione and its derivative induces sedative and hypnotic effects under an acute stress in neonatal chicks. Neurosci Lett. 2007;418: 87–91. doi:10.1016/j.neulet.2007.03.003

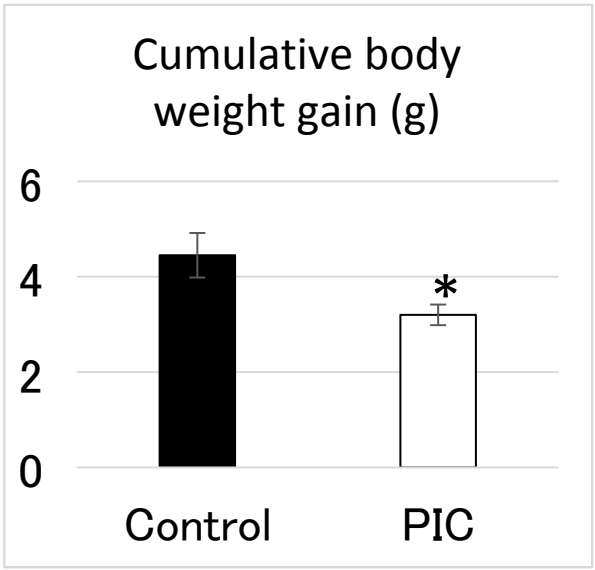
39. Yilmaz JL, Bülow L. Enhanced stress tolerance in Escherichia coli and Nicotiana tabacum expressing a betaine aldehyde dehydrogenase/choline dehydrogenase fusion protein. Biotechnol Prog. 2002;18: 1176–82. doi:10.1021/bp020057k

40. Chern MK, Pietruszko R. Human aldehyde dehydrogenase E3 isozyme is a

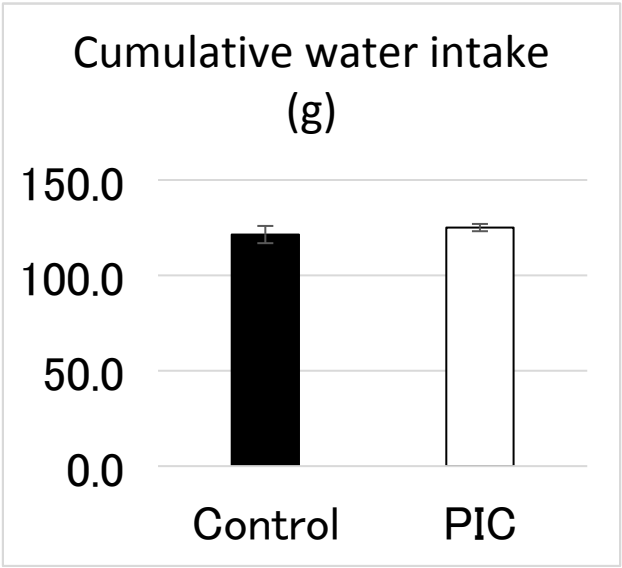
betaine aldehyde dehydrogenase. Biochem Biophys Res Commun. 1995;213: 561–8. doi:10.1006/bbrc.1995.2168

41. Sakai S, Nakagawa R. Studies on the functional ingradients in the farm products in Ibaraki prefecture. Bulletins of industrial technology innovation center of Ibaraki prefecture. 2011;40. Available from: http://www.kougise.pref.ibaraki.jp/periodical/reseach/40/vol40-04.pdf (in Japanese)

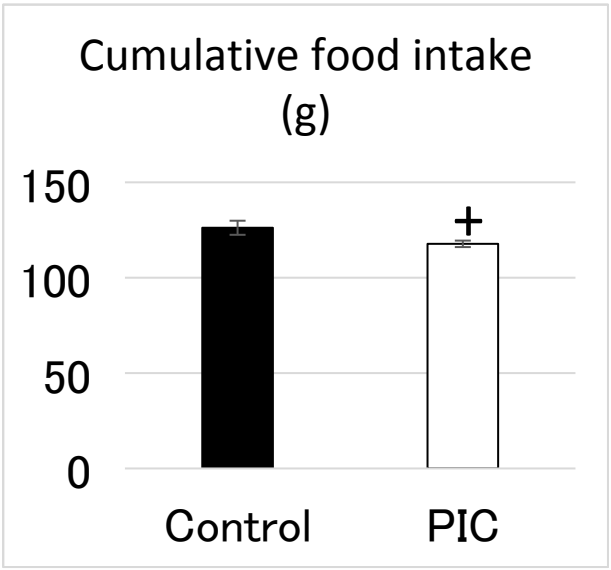
A



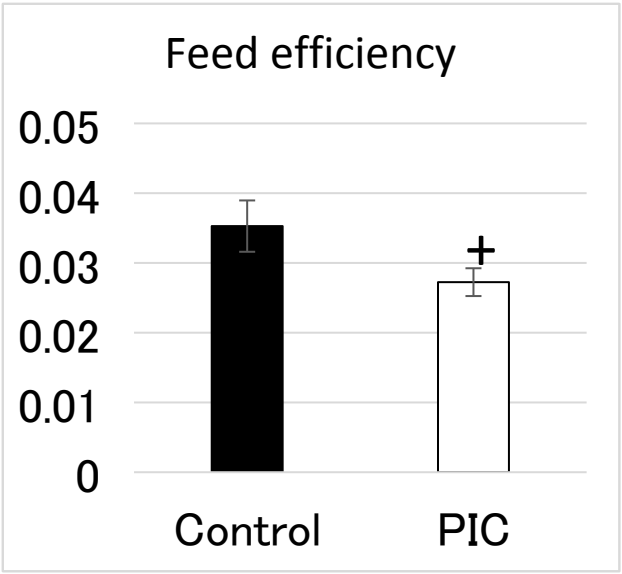
C



B

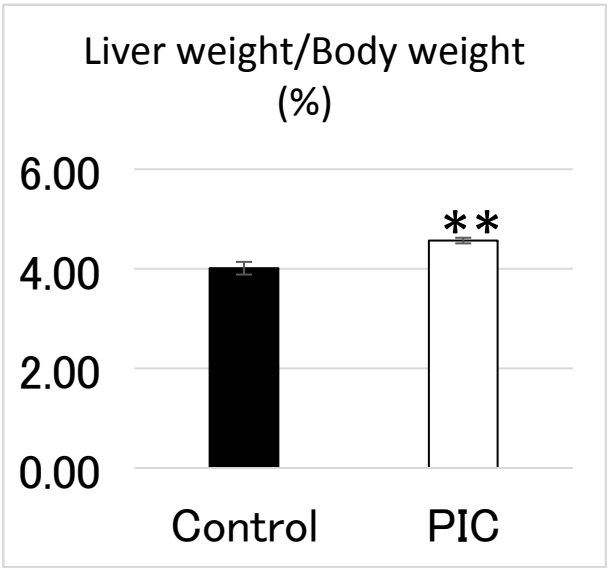


D

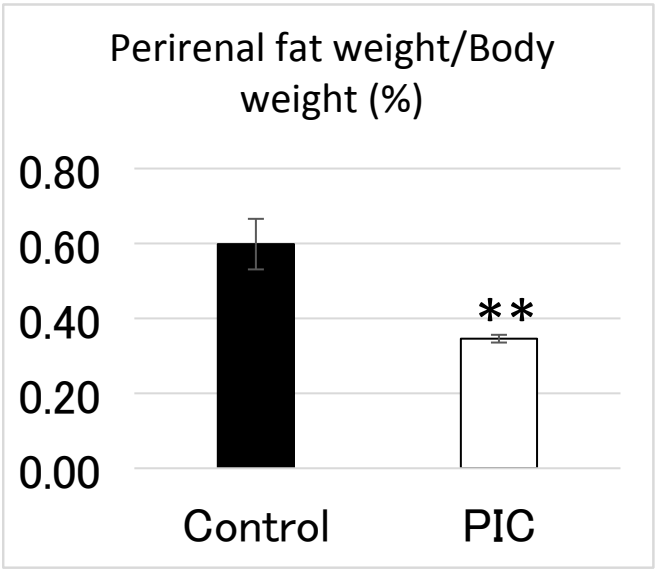


Toyoda et al. Fig. 1.

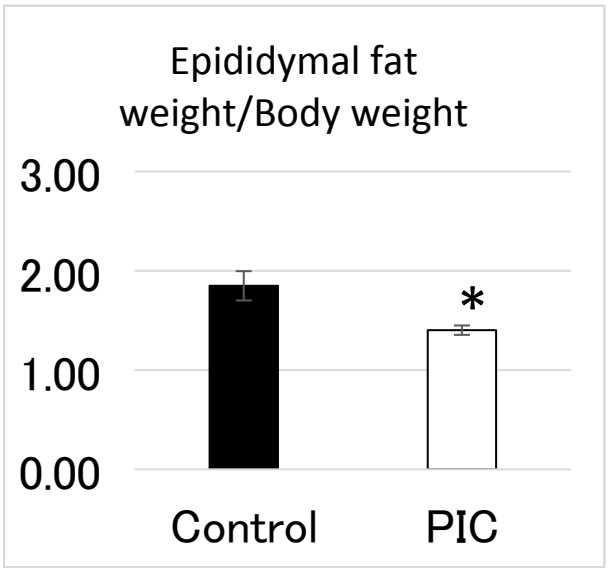
A



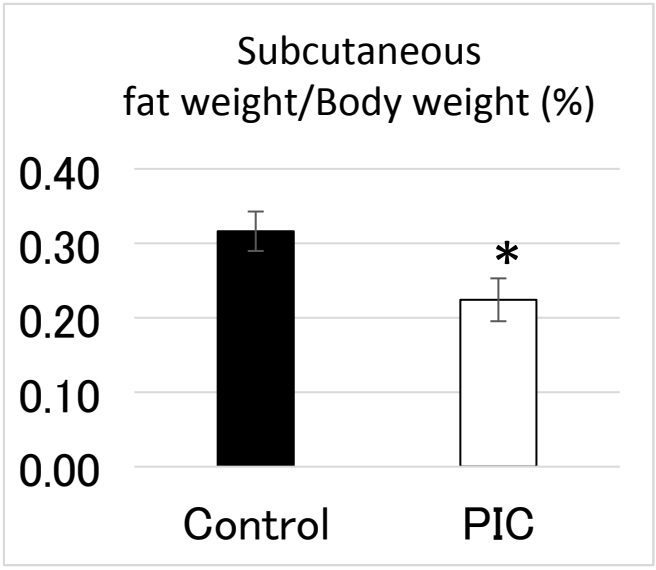
C



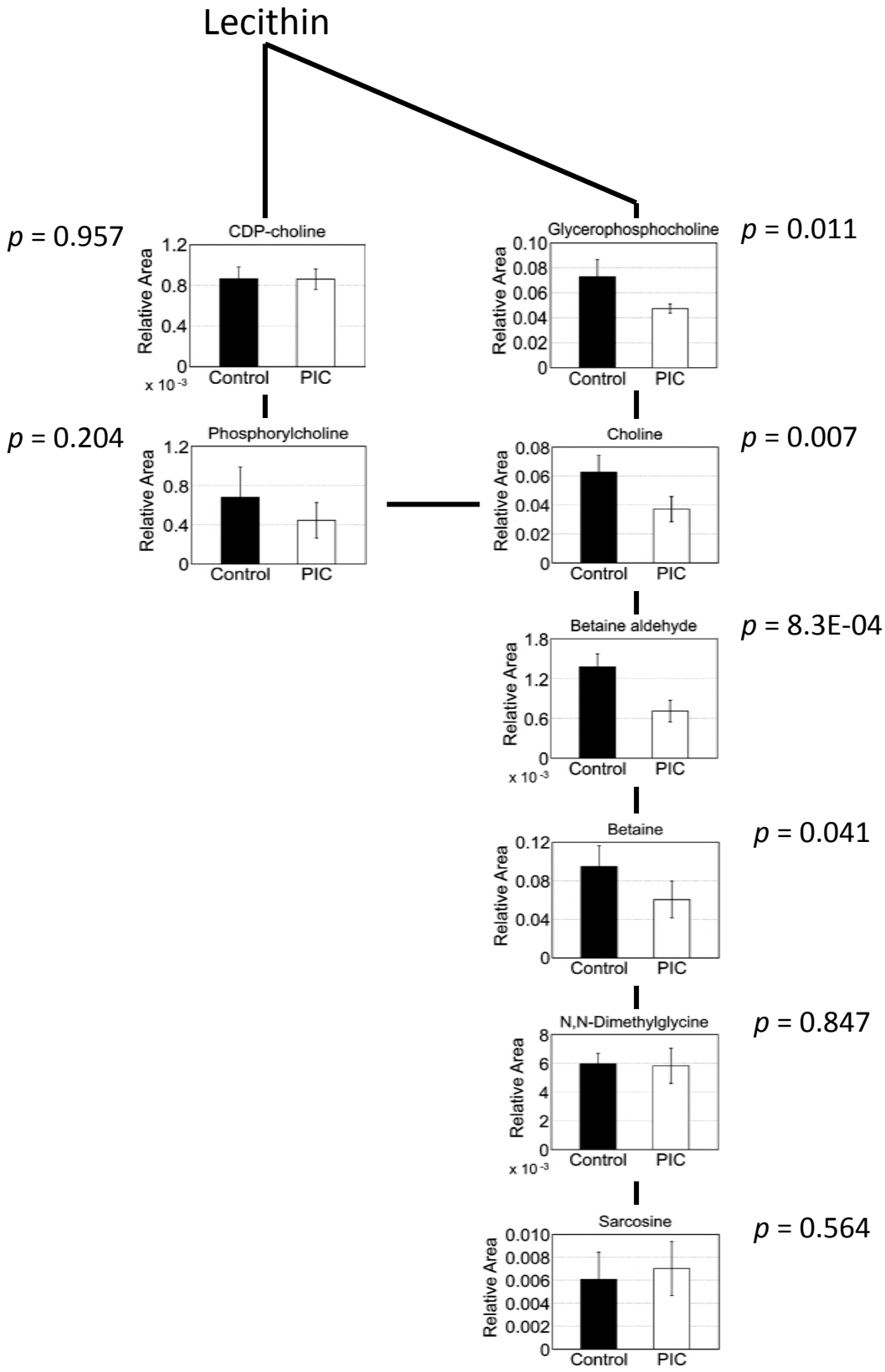
B



D



Toyoda et al. Fig. 2.



Toyoda et al. Fig 3