pathway in macrophage. *Arterioscler Thromb Vasc Biol* 2008;28:871–7.
22. Mutoh M, Niho N, Komiya M, Takahashi M, Ohtsubo R, Nakatogawa K, Ueda K, Sugimura T, Wakabayashi K. Plasminogen activator inhibitor-1 (Pai-1) blockers suppress intestinal polyp fornmation in Min mice. *Carcinogenesis* 2008;29:824–9.
23. Bonnefoi MS, Kelley MF, Wells RE, Sanders JE, Jayyosi Z, Beys E, Kornbrust DJ, Langloss JM. Subchronic toxicity studies with the leukotriene D4 antagonist RG 12525. *Fundam Appl Toxicol* 1995;28: 129–38.
24. Leclercq IA, Field J, Enriquez A, Farrell GC, Robertson GR. Constitutive and inducible expression of hepatic CYP2E1 in leptin-deficient *ob/ob* mice. *Biochem Biophys Res Commun* 2000;268:337–44.
25. Kato H, Ohue M, Kato K, Nomura A, Toyosawa K, Furutani Y, Kimura S, Kadowaki T. Mechanism of amelioration of insulin resistance by β3-adrenoceptor agonist AJ-9677 in the KK-$A^y$/Ta diabetic obese mouse model. *Diabetes* 2001;50:113–22.
26. Hosogai N, Fukuhara A, Oshima K, Miyata Y, Tanaka S, Segawa K, Furukawa S, Tochino Y, Komuro R, Matsuda M, Shimomura I. Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation. *Diabetes* 2007; 56:901–11.
27. Hirose Y, Hata K, Kuno T, Yoshida K, Sakata K, Yamada Y, Tanaka T, Reddy BS, Mori H. Enhancement of development of azoxymethane-induced colonic premalignant lesions in C57BL/KsJ-*db/db* mice. *Carcinogenesis* 2004;25:821–5.
28. Kawamori T, Kitamura T, Watanabe K, Uchiya N, Maruyama T, Narumiya S, Sugimura T, Wakabayashi K. Prostaglandin E receptor subtype $EP_1$ deficiency inhibits colon cancer development. *Carcinogenesis* 2005;26:353–7.
29. Rosenberg DW, Giardina C, Tanaka T. Mouse models for the study of colon carcinogenesis. *Carcinogenesis* 2009;30: 183–96.
30. Bajou K, Lewalle JM, Martinez CR, Soria C, Lu H, Noël A. Foidart JM. Human breast adenocarcinoma cell lines promote angiogenesis by providing cells with uPA-PAI-1 and by enhancing their expression. *Int J Cancer* 2002;100:501–6.
31. Mignatti P, Rifkin DB. Plasminogen activators and matrix metalloproteinases in angiogenesis. *Enzyme Protein* 1996;49: 117–37.
32. Gustafson B, Hammarstedt A, Andersson CX, Smith U. Inflamed adipose tissue: a culprit underlying the metabolic syndrome and atherosclerosis. *Arterioscler. Thromb Vasc Biol* 2007;27: 2276–83.
33. Cowey S, Hardy RW. The metabolic syndrome: a high-risk state for cancer? *Am J Pathol* 2006;169:1505–22.
34. Popivanova BK, Kitamura K, Wu Y, Kondo T, Kagaya T, Kaneko S, Oshima M, Fujii C, Mukaida N. Blocking TNF-α in mice reduces colorectal carcinogenesis associated with chronic colitis. *J Clin Invest* 2008;118:560–70.
35. Fujisawa T, Endo H, Tomimoto A, Sugiyama M, Takahashi H, Saito S, Inamori M, Nakajima N, Watanabe M, Kubota N, Yamauchi T, Kadowaki T, et al. Adiponectin suppresses colorectal carcinogenesis under the high-fat diet condition. *Gut* 2008;57: 1531–8.

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# Increase of oxidant-related triglycerides and phosphatidylcholines in serum and small intestinal mucosa during development of intestinal polyp formation in Min mice

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**Recent epidemiological studies have shown a positive association of a high-fat diet with the risk of colon cancer. Indeed, increments in the serum levels of triglycerides (TG) and cholesterols are positively related with colon carcinogenesis. We previously reported that an age-dependent hyperlipidemic state is characteristic of Min mice, an animal model for human familial adenomatous polyposis (FAP). However, qualitative and quantitative changes of lipid metabolism are poorly understood in this state. Here, we provide detailed analysis of serum lipids in Min mice using reverse-phased liquid chromatography/electrospray ionization mass spectrometry (RPLC/ESI-MS). We also demonstrate local analysis of lipid droplets in the villi of the small intestine using laser capture microdissection and a sensitive chip-based nanoESI-MS system. As a result, oxidized phosphatidylcholines (PC) such as aldehyde and carboxylic acid types were increased, even at an early stage of intestinal polyp formation in serum. In addition, hydroperoxidizable TG precursors containing linoleic acid (18:2n-6) were deposited at the tip of the villi with aging, and these hydroperoxidized TG were also increased in serum. Meanwhile, increments of the oxidizable TG precursors in serum and small intestinal mucosa were suppressed by treatment with pitavastatin, a novel third generation lipophilic statin. These results suggest that quantitative and qualitative lipid changes such as hydroperoxidizable TG precursors are important in the course of intestinal polyp formation and oxidative stress might lead to the development of intestinal polyp formation in Min mice. (*Cancer Sci* 2011; 102: 79–87)**

The incidence and mortality of colon cancer, which is associated with obesity, a high-fat diet and hyperlipidemia according to several epidemiological studies, has increased in developed countries.[1–5] We previously reported an age-dependent hyperlipidemic state in *adenomatous polyposis coli* (*Apc*)-deficient Min and $Apc^{1309}$ mice, animal models of familial adenomatous polyposis (FAP).[6–8] Min mice develop large numbers of intestinal polyps due to truncation mutation in both alleles of the *Apc* gene, leading to activation of Wnt signaling to promote cell growth, which is increased by consumption of a high-fat diet.[9]

Although the direct link between *Apc*-deficiency and hyperlipidemia is yet to be clarified, it is notable that serum triglyceride (TG) levels in Min mice are almost 10 times higher than those observed in wild-type littermates (C57BL/6J) at 20 weeks of age, even though both mice were fed a non-high-fat diet, AIN-76A, including corn oil (5% of total components).[6] Both groups of mice ate almost the same amount of diet, but the mean bodyweight was 16% lower in Min mice compared with that of their wild-type littermates at 20 weeks of age, which might be due to the development of intestinal polyps. Consumption of a high-fat diet is associated with hyperlipidemia and leading obesity.[10] However, Min mice featured hyperlipidemia without a high fat-diet intake or obesity. The reasons for the pathology is correlated with decreased mRNA expression levels of lipoprotein lipase (LPL), which hydrolyzes TG into free fatty acids and monoglyceride, in the liver and small intestine.[11,12] We demonstrated that induction of LPL mRNA by peroxisome proliferator-activated receptor (PPAR)-$\alpha$ and -$\gamma$ agonists and selective LPL-inducing agent, NO-1886, which lacks potential for activating the PPAR pathways, suppressed the hyperlipidemic status, steatosis of the liver and intestinal polyp formation.[6–8] In addition, we indicated that a number of large lipid droplets were found in the surface epithelial cells of small intestinal polyps by Oil-red O staining and electron microscopy.[13] These results suggest that lipid metabolism changes might play important roles in intestinal polyp formation, but the quantitative and qualitative changes have not been well defined in the serum and small intestine.

In this study, we examined a detailed analysis of serum lipids in Min mice using electrospray ionization-mass spectrometry (ESI-MS) coupled with reverse-phased ultra-performance liquid chromatography (UPLC).[14,15] Moreover, lipid droplets in the villi of the small intestine were collected by laser microdissection (LMD) under direct microscopic visualization and analyzed using a sensitive chip-based nanoESI-MS system by neutral loss scanning of identical fatty acyl groups from individual TG molecular species and precursor ion scanning of phosphoryl choline from individual phosphatidylcholine (PC) molecular species.[16–22] The possible lipid markers for development of intestinal polyps in Min mice are discussed. Furthermore, the relationship between the markers and polyp formation in Min mice are demonstrated by administration of pitavastatin, a novel 3-hydroxy-3-methylglutaryl coenzyme-A (HMGCoA) reductase inhibitor, which possesses pleiotropic functions including anti-inflammation and anti-oxidant functions.[23–26]

## Materials and Methods

**Animals.** Male C57BL/6-$Apc^{Min/+}$ mice (Min mice) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA)

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at 6 weeks of age and genotyped as previously reported.[27] Heterozygotes of Min strain and wild-type (C57BL/6J) mice were acclimated to laboratory conditions for 1 week. Less than five mice were housed per plastic cage with sterilized softwood chips as bedding in a barrier-sustained animal room at 24°C ± 2°C and 55% humidity on a 12 h light/dark cycle.

To investigate lipid metabolism changes, male Min mice ($n$ = 9) and wild-type mice ($n$ = 6) at 5 weeks of age were given AIN-76A powdered basal diet (CLEA Japan, Tokyo, Japan). Min mice ($n$ = 3) and wild-type mice ($n$ = 2) were killed at 10, 15 and 20 weeks of age. To examine the effects of pitavastatin ([+]-monocalcium bis [3*R*,5*S*,6*E*]-7-[2-cyclopropyl-4-(4-fluorophenyl)-3-quinolyl]-3,5-dihydroxy-6-heptenoate), which was kindly provided by Kowa Pharmaceutical Co., Ltd (Aichi, Japan), male Min mice at 6 weeks of age were given pitavastatin, which was mixed well at concentrations of 40 p.p.m. for 14 weeks in AIN-76A. Min mice with pitavastatin treatment ($n$ = 3) and with pitavastatin untreatment ($n$ = 3) were killed at 20 weeks of age. Food and water were available *ad libitum*. The mice were observed daily for clinical signs and mortality. Bodyweights and food consumption were measured weekly. The experimental protocol was in accordance with the guidelines for Animal Experiments in the National Cancer Center and was approved by the Institutional Ethics Review Committee for Animal Experimentation.

**Materials.** All solvents for HPLC or MS grade were purchased from Wako Pure Chemicals (Osaka, Japan). Deionized water was obtained from a Milli-Q water system (Millipore, Milford, MA, USA).

**Extraction and isolation of serum and small intestinal mucosa lipids.** Total lipids from serum and small intestinal mucosa were extracted using Bligh and Dyer's method in the following procedure:[28] serum (75 μL) and small intestinal mucosa in the proximal segments (50 mg) removed by scraping were individually homogenized with 6 mL chloroform/methanol (1:2) for 10 strokes and left for 1 h at room temperature. In this process, 1 nmol of sphingomyelin (SM) (d18:1-12:0) was added as an internal standard. Each phase separation was achieved by adding 2 mL chloroform and 2 mL water. After vortexing, the mixture was centrifuged at 1000*g*. for 10 min. The bottom organic layer containing the total lipid extract was collected and dried under a gentle stream of nitrogen, and then dissolved by 200 μL chloroform-methanol (2:1) and normalized to the serum volume and intestinal mucosa weight.

**Laser capture microdissection of the small intestine.** The intestinal tracts of male Min mice at 10 and 20 weeks old ($n$ = 3) were removed and separated into the small intestine. Each small intestine was divided into the proximal segment (4 cm in length), and then opened longitudinally and fixed flat between sheets of filter paper in 10% buffered formalin for further Oil-red O

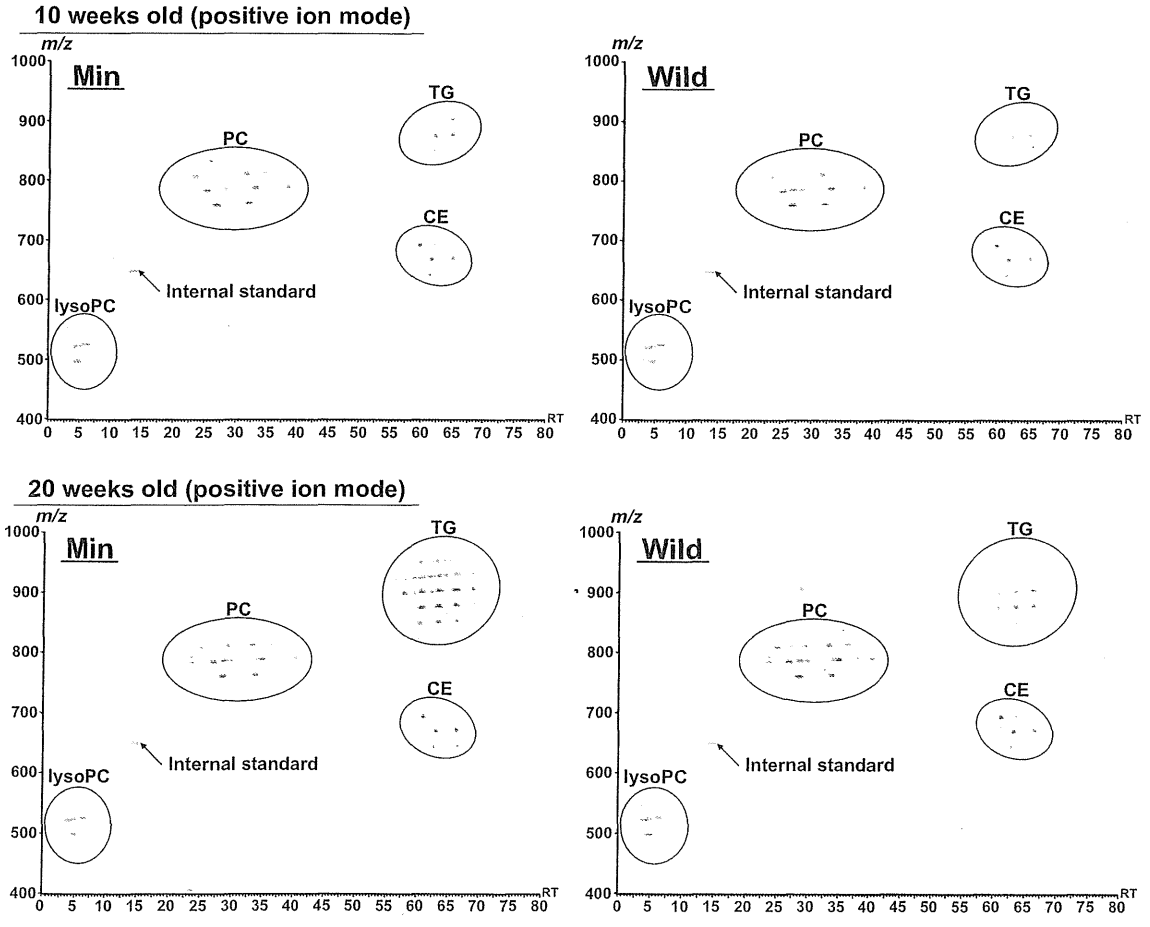


**Fig. 1.** 2-D profiling analysis of serum lipids from Min mice by reverse-phased liquid chromatography/electrospray ionization mass spectrometry (RPLC/ESI-MS) in positive ion mode. Each of the lipid classes was detected in the following order: lysophosphatidylcholine (lysoPC) > phosphatidylcholine (PC) > triglyceride (TG), cholesteryl ester (CE). The TG of Min mice were increased at 10 weeks of age and highly elevated at 20 weeks of age. 2-D lipid maps were constructed with X (retention time) and Y (*m/z* value) axes, and the intensity of these peaks was adjusted by color density spots.



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staining and LMD.[6] Lipid droplets of villi in the small intestine were observed using modified Oil-red O staining method for confirmation of the locus.[29] In brief, each frozen section as Swiss rolls was treated with 60% isopropanol for 1 min, and stained with Oil-red solution for 15 min at room temperature. The dye was then removed, and the section was washed with PBS and 60% isopropanol each for 2 min. For local analysis of the villi, each frozen section was mounted on DIRECTOR LMD slide (AMR Inc., Tokyo, Japan), and lipid droplets from the tip and basis of the villi were collected by Leica LMD system, LMD 6000 (Leica Microsystems, Wetzlar, Germany) with a pulsed 355-nm diode laser and individually extracted by methanol.[22] The correction intensity in each graph was calculated by the ratio of TG to the endogenous standard (16:0-18:2 PC).

**Reverse-phased liquid chromatography/ESI-MS (RPLC/ESI-MS) conditions.** The RPLC/ESI-MS analysis was performed using a quadrupole/time-of-flight hybrid mass spectrometer, Q-TOF micro (Waters Corporation, Milford, MA, USA) with an ACQUITY UPLC system (Waters Corporation).[14] The scan range of the instrument was set at $m/z$ 200–1100, the scan duration of MS and MS/MS was at 0.5 s and the collision gas used for the MS/MS experiments was at $7.5 \times 10^5$ mbar (argon). The capillary voltage in positive ion mode was set at 3.5 kV, cone voltage at 30 V and collision energy of MS/MS at 30 V, whereas capillary voltage was at −2.5 kV, cone voltage at −30 V and collision energy of MS/MS at −30 V in negative ion mode.

The RPLC separation was achieved using an ACQUITY UPLC BEH C18 column (150 × 1.0 mm inner diameter [i.d.], Waters Corporation) at 45°C. Two microlitre of total lipids normalized to serum volume was individually injected. The mobile phase was acetonitrile/methanol/water : 19/19/2 (0.1% formic acid + 0.028% ammonia) (A) and isopropanol (0.1% formic acid + 0.028% ammonia) (B), and the composition was produced by mixing these solvents. The gradient consisted of holding solvent (A/B : 90/10) for 7.5 min, then linearly converting to solvent (A/B : 70/30) for 32.5 min and finally linearly converting solvent (A/B : 40/60) for 50 min. The mobile phase was pumped at a flow rate of 40–50 μL/min. The MS data processing was applied by Mass++ software (http://masspp.jp/) to detect each chromatogram peak with quantitative accuracy. The correction intensity in each graph was calculated by the ratio of TG or phosphatidylcholine (PC) to the internal standard.

**Multiple reaction monitoring (MRM) conditions.** The MRM analysis was performed using a quadrupole-linear ion trap hybrid mass spectrometer, 4000Q TRAP (AB SCIEX, Foster City, CA, USA) with the same ACQUITY UPLC system as previously reported.[15] Ten microlitre of total lipids normalized to serum volume was individually injected. The mobile phase was acetonitrile/methanol/water : 2/2/1 (0.1% formic acid + 0.028% ammonia) (A) and isopropanol (0.1% formic acid + 0.028% ammonia) (B), and the composition was produced by mixing these solvents. The gradient consisted of holding solvent

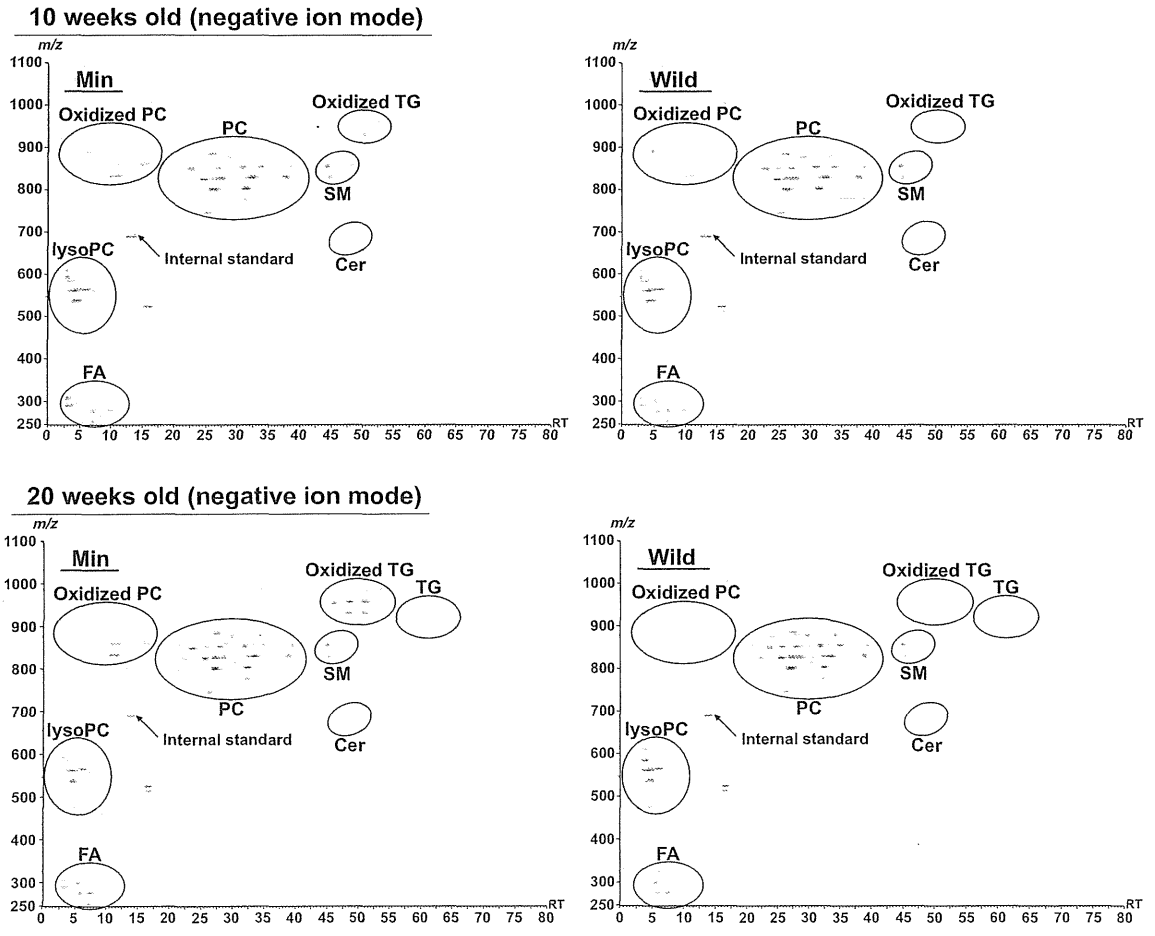


**Fig. 2.** 2-D profiling analysis of serum lipids from Min mice by reverse-phased liquid chromatography/electrospray ionization mass spectrometry (RPLC/ESI-MS) in negative ion mode. Each of the lipid classes was detected in the following order: oxidized phosphatidylcholine (PC), lysophosphatidylcholine (lysoPC), fatty acid (FA) > PC > sphingomyelin (SM), ceramide (Cer), oxidized triglyceride (TG) > TG. Oxidized PC and TG of Min mice were highly increased even at 10 weeks of age and highly elevated at 20 weeks of age.

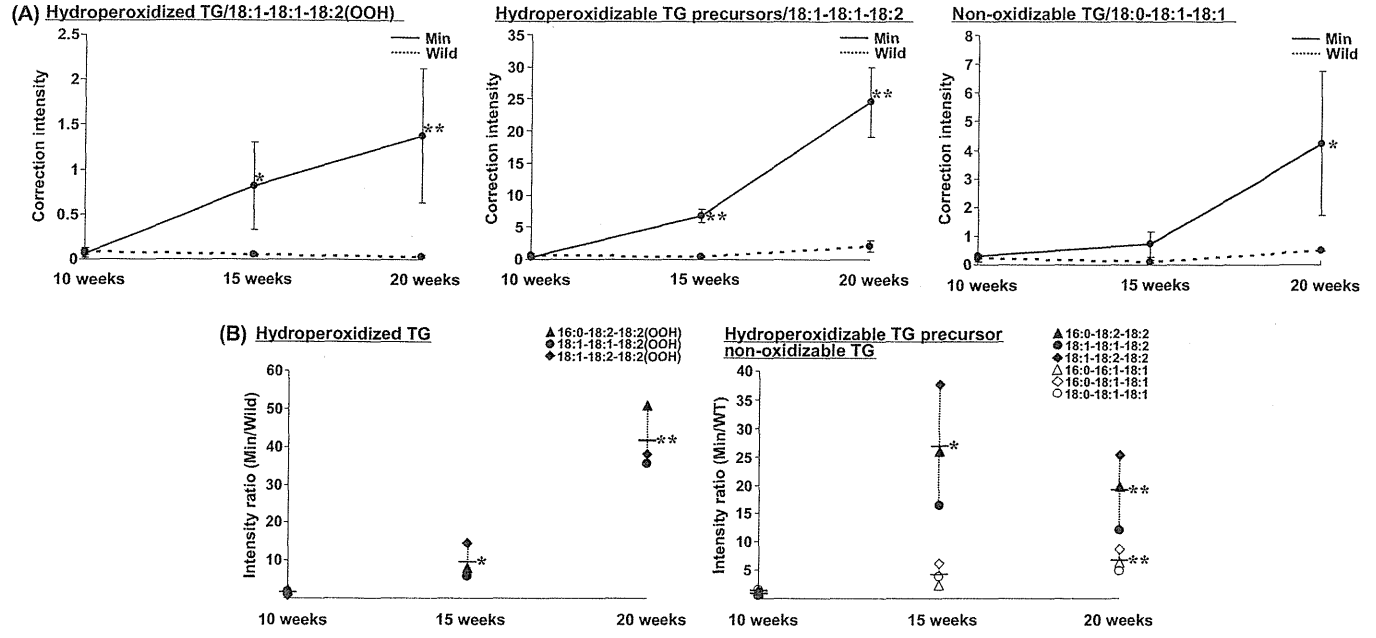


**Fig. 3.** Quantitative and qualitative profiles of serum triglyceride (TG) molecular species of Min mice in the course of intestinal polyp formation. (A) Compared with wild-type mice, hydroperoxidized TG and hydroperoxidizable TG precursors of Min mice were highly increased from 15 weeks of age, whereas non-oxidizable TG of Min mice were somewhat elevated at 20 weeks of age. Values are mean ± SD ($n$ = 3; $*P < 0.05$, $**P < 0.01$ versus correction intensity of Min mice at 10 weeks of age). (B) Regarding ratios of Min mice to wild-type mice in serum TG molecular species, hydroperoxidized TG ratios were elevated in a time-dependent manner, and hydroperoxidized TG of Min mice at 20 weeks of age were 30–50 times larger than those of wild-type mice. Hydroperoxidizable TG precursor ratios were highly increased at 15 weeks of age and then somewhat decreased at 20 weeks of age, whereas non-oxidizable TG ratios were gradually increased in a time-dependent manner. Values are mean ± SD ($n$ = 3; $*P < 0.05$, $**P < 0.01$ versus intensity ratio at 10 weeks of age) and the individual mean values of these TG are indicated by crossbars.

(A/B : 100/0) for 5 min, then linearly converting to solvent (A/B : 50/50) for 20 min and finally holding solvent (A/B : 50/50) for 34 min at a flow rate of 70 μL/min and column temperature of 30°C.

**NanoESI-MS conditions.** Chip-based nanoESI-MS analysis was performed using a 4000Q TRAP with chip-based ionization source, TriVersa NanoMate (Advion BioSystems, Ithaca, NY, USA). The ion spray voltage was set at 1.25 kV, gas pressure at 0.3 pound per square inch (psi) and flow rates at 200 nL/min. The scan range was set at $m/z$ 400–1100, declustering potential at 100 V, collision energies at 50–70 V and resolutions at Q1 and Q3, "unit". The mobile phase composition was chloroform/methanol: 1/2 (0.1% ammonium formate). Total lipids were directly subjected by flow injection and selectivity analyzed by neutral loss scanning of identical fatty acyl groups from individual TG molecular species and precursor ion scanning of phosphorylcholine from individual PC molecular species.[16–21]

**Statistical analysis.** The student's $t$-test was used for statistical analysis. Values with $P < 0.05$ were considered to be statistically significant.

## Results

**Global analysis of serum lipids from Min mice by 2-D profiling.** Intestinal polyp counts in Min mice were obviously increased with age compared with wild-type mice due to down-regulation of LPL expression. Indeed, the average number of intestinal polyps developed in Min mice was 82.4 ± 8.4 (mean ± SE). However, none of the wild-type mice developed intestinal polyps.

To further examine the effects on lipid metabolism at the molecular species level, we globally analyzed serum lipids at 10, 15 and 20 weeks of age by RPLC/ESI-MS and then created 2-D lipid maps of the individual precursor ion peaks for searching quantitative and qualitative changes. The 2-D lipid maps were constructed with X (retention time) and Y ($m/z$ value) axes, and the intensity of these peaks was adjusted by color density spots.

As a result, TG increments in Min mice were detected on the map of positive ion mode (55–70 min) even at 10 weeks of age and were markedly found at 20 weeks of age (Fig. 1). In addition, several spots were abundantly observed on the map of negative ion mode (5–15 and 45–55 min) in Min mice compared with wild-type mice (Fig. 2). These distinctive spots were analyzed by MS/MS and respectively identified as oxidized PC and TG, which we first detected from mouse TG in white adipose tissue as previously reported.[14] At 20 weeks of age, these oxidized PC and TG were highly increased in Min mice, suggesting that enhancement of oxidative stress might be caused in the course of intestinal polyp formation.

**Fig. 4.** Quantitative and qualitative profiles of serum oxidized phosphatidylcholine (PC) molecular species of Min mice in the course of intestinal polyp formation. (A) Oxidized PC derived from peroxidation of polyunsaturated fatty acids (PUFA) such as linoleic acid (18:2n-6), arachidonic acid (20:4n-6) and docosahexaenoic acid (22:6n-3) at the $sn$-2 position were analyzed by multiple reaction monitoring. Aldehyde (CHO) and carboxylic acid (COOH) types of Min mice ($n$ = 3) were increased even at 10 weeks of age compared with wild-type mice ($n$ = 2). Values are mean ± SD. (B) In addition to these types, hydroperoxide (OOH) types of Min mice ($n$ = 3) were elevated at 20 weeks compared with wild-type mice ($n$ = 2). Values are mean ± SD. (C) The PC ratios of Min mice to wild-type mice were highly increased at 20 weeks of age compared with 10 weeks of age in the following order: carboxylic acid types > aldehyde types > hydroperoxide types. Individual mean values of these types are indicated by crossbars.


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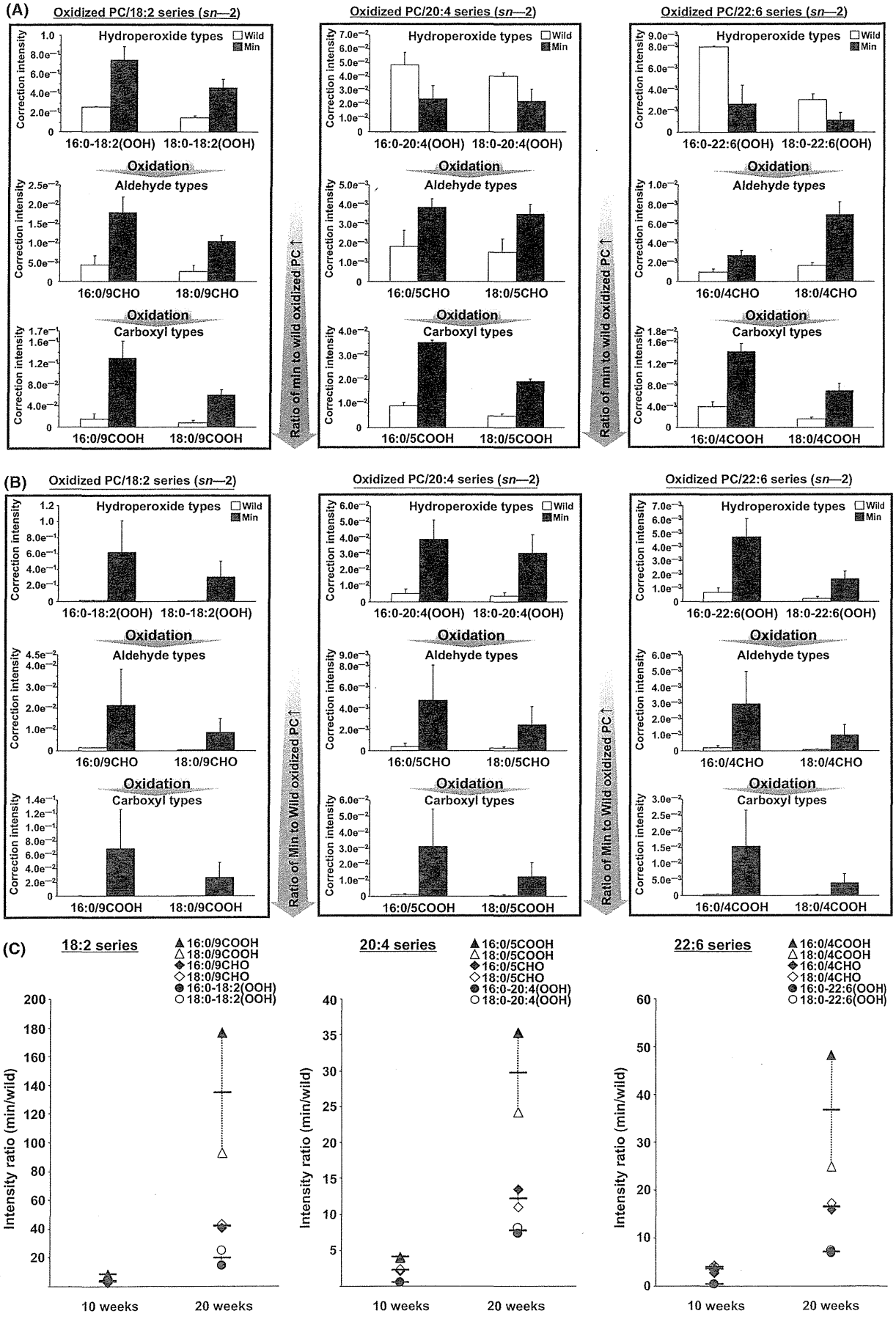

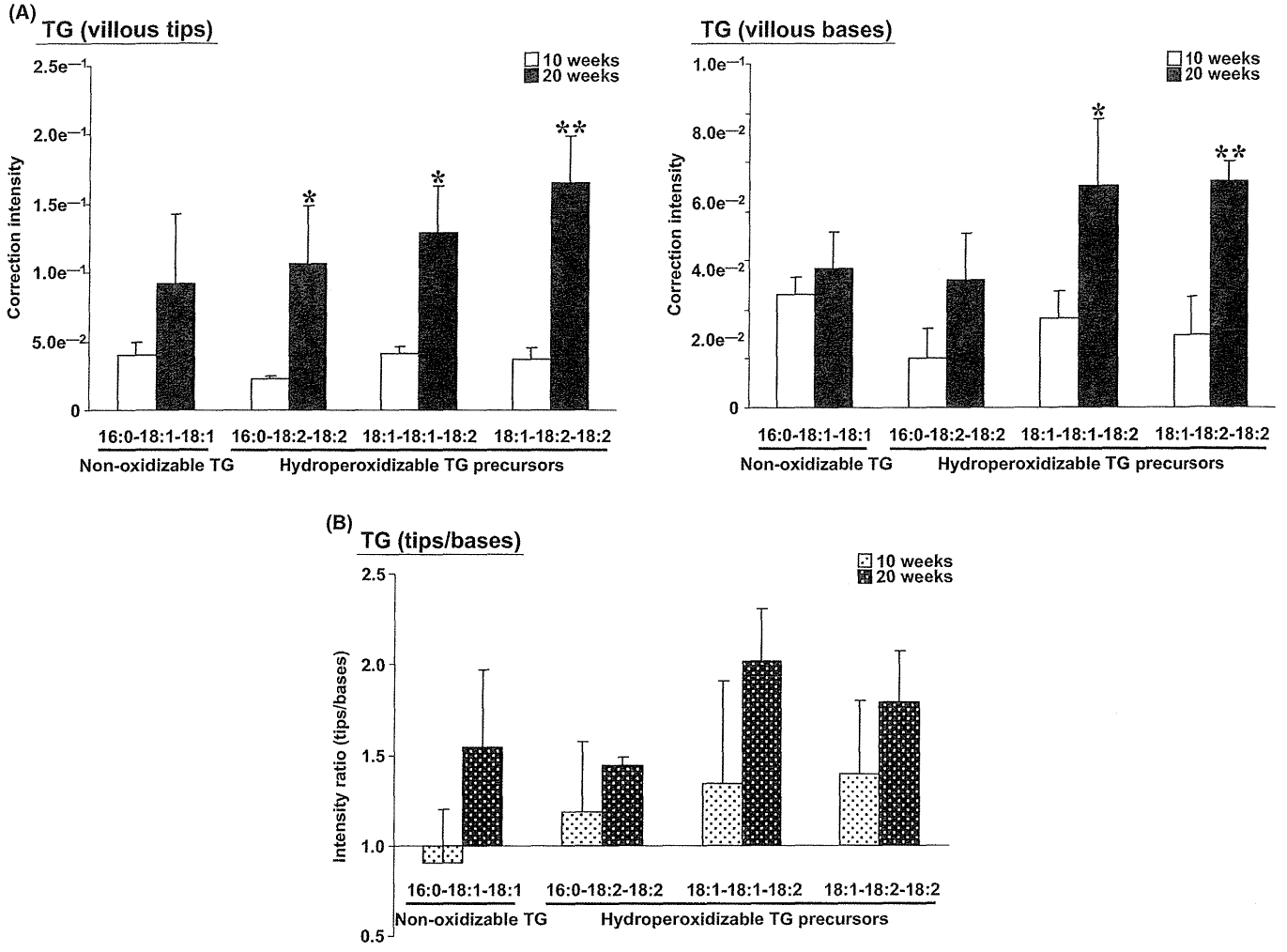


**Fig. 5.** Local analysis of lipid droplets in Min mice by laser microdissection and the nano-electrospray ionization-mass spectrometry (nanoESI-MS) system. (A) Hydroperoxidizable triglyceride (TG) precursors at villous tips and bases of Min mice were more increased at 20 weeks of age than 10 weeks of age. Values are mean ± SD ($n = 3$; $^*P < 0.05$, $^{**}P < 0.01$ versus correction intensity at 10 weeks of age). (B) From hydroperoxidizable TG precursor ratios of villous tips to villous bases, hydroperoxidizable TG precursors of villous tips were 1.5–2 times larger than those of villous bases at 20 weeks of age. Values are mean ± SD ($n = 3$).

**Quantitative and qualitative changes of serum TG molecular species of Min mice in the course of intestinal polyp formation.** These spots were more precisely quantified by Mass++, MS data processing software to detect individual chromatogram peaks with quantitative accuracy. In Min mice, serum TG molecular species such as hydroperoxidized (OOH) TG, which were derived from peroxidation of intramolecular linoleic acid (18:2n-6), and hydroperoxidizable TG precursors containing 18:2, which were oxidizable to the hydroperoxidized TG, were increased in a time-dependent manner (Fig. 3A). In particular, hydroperoxidizable TG precursors were highly elevated from 15 weeks of age. Meanwhile, non-oxidizable TG containing saturated fatty acid (SFA) and monounsaturated fatty acid (MUFA) were quantitatively unchanged until 15 weeks of age compared with wild-type mice and somewhat increased at 20 weeks of age. These results suggest that individual TG molecular species were not uniformly elevated, but oxidant-related TG were preferentially increased. As for the TG ratios of Min mice to wild-type mice, hydroperoxidized TG ratios were elevated in a time-dependent manner, and hydroperoxidized TG of Min mice at 20 weeks of age were 30–50 times larger than those of wild-type mice (Fig. 3B). Hydroperoxidizable TG precursor ratios were highly increased at 15 weeks of age and then somewhat decreased at 20 weeks of age, whereas non-oxidizable TG ratios were gradually elevated in a time-dependent manner. There were significant differences between hydroperoxidized TG ratios and non-oxidizable TG ratios at 20 weeks of age, and between hydroperoxidizable TG precursor ratios and non-oxidizable TG ratios at 15 weeks of age. It seems that these ratios might be effective indications for the pathology of intestinal polyp formation.

**Quantitative and qualitative changes of serum PC molecular species of Min mice in the course of intestinal polyp formation.** Regarding serum PC molecular species, hydroperoxidizable PC precursors containing 18:2 and non-oxidizable PC containing SFA were quantitatively unchanged between Min and wild-type mice at 10 and 20 weeks of age (Fig. S1). Meanwhile, oxidized PC of Min mice were abundantly observed on the 2-D map, and then a large variety of these oxidized types such as OOH, aldehydes (CHO) and carboxylic acids (COOH) were analyzed in detail by MRM with theoretically expanded data sets as previously reported.[15] These oxidized types were derived from enzymatic and non-enzymatic reactions of peroxidation of polyunsaturated fatty acids (PUFA) such as 18:2,


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arachidonic acid (20:4n-6) and docosahexaenoic acid (22:6n-3) at the *sn*-2 position and important in inflammatory biomarkers for the physiological and pathological phenomena.[30,31] Interestingly, aldehyde and carboxylic acid types derived from peroxidation of 18:2, 20:4 and 22:6 were increased even at 10 weeks of age in Min mice (Fig. 4A), indicating that oxidative stress might occur at the early stage of polyp formation. Besides these aldehyde and carboxylic acid types, hydroperoxide types were highly elevated at 20 weeks in Min mice (Fig. 4B). In addition, PC ratios of Min mice to wild-type mice in these oxidized types were increased at 20 weeks of age compared with 10 weeks of age in the following order: carboxylic acid types > aldehyde types > hydroperoxide types (Fig. 4C). These data support the ideas that Min mice at 20 weeks of age were at a high oxidative stress level and these oxidants might also be effective indications of the exacerbation stage and inflammatory state.

**Local analysis of lipid droplets in the villi of Min mice.** Because serum lipid contents might be affected by dietary fat absorption in the small intestine, detailed analysis of the villi were investigated at 10 and 20 weeks of age in Min mice by LMD, which permits procurement of the locus from frozen sections of Swiss-rolled middle and distal parts of the small intestines. Lipid accumulation was observed at the tip of the villi in the small intestine with Oil-red O staining and the locus was collected. These lipid mixtures were individually extracted by methanol from the collected tissues and analyzed using a chip-based nanoESI-MS system. As a result, PC molecular species were quantitatively and qualitatively unchanged at 10 and 20 weeks of age in Min mice (Fig. S2). Meanwhile, hydroperoxidizable TG precursors were more abundant at 20 weeks of age than those at 10 weeks of age in Min mice (Fig. 5A). Moreover, the TG at the tip of the villi were 1.5–2.5 times larger than those at the basis at 20 weeks of age (Fig. 5B). These results suggest that hydroperoxidizable TG precursors were comparatively deposited at the tip of the villi with age in Min mice and might be subjected to oxidative stress and inflammation in the small intestine. As discussed previously, hydroperoxidized TG and hydroperoxidizable TG precursors in the serum of Min mice were increased at 20 weeks of age, which might be caused by the increments of hydroperoxidizable TG precursors at the tip of the villi.

**Suppression of hydroperoxidizable TG precursor production in the serum and small intestinal mucosa of Min mice by pitavastatin treatment.** To examine the relationship between oxidative stress and polyp formation in Min mice, detailed analysis of total lipid extract from the serum and small intestinal mucosa of Min mice treated with pitavastatin at doses of 40 p.p.m. for 14 weeks were investigated by a chip-based nanoESI-MS system. Intestinal polyp formation in the pitavastatin-treated groups was significantly suppressed (the data will be presented in a separate report). The PC molecular species were quantitatively and qualitatively unchanged between the pitavastatin-treated and untreated groups (data not shown). Meanwhile, the intensity ratios of hydroperoxidizable TG precursors to non-oxidizable TG (16:0-18:1-18:1) were significantly decreased in the serum and intestinal mucosa of the pitavastatin-treated groups compared with the untreated groups (Fig. 6). These results suggest that hydroperoxidizable TG precursors are important in developing intestinal polyp formation and hydroperoxidized TG derived from the oxidizable TG precursors might induce oxidative stress and inflammation.

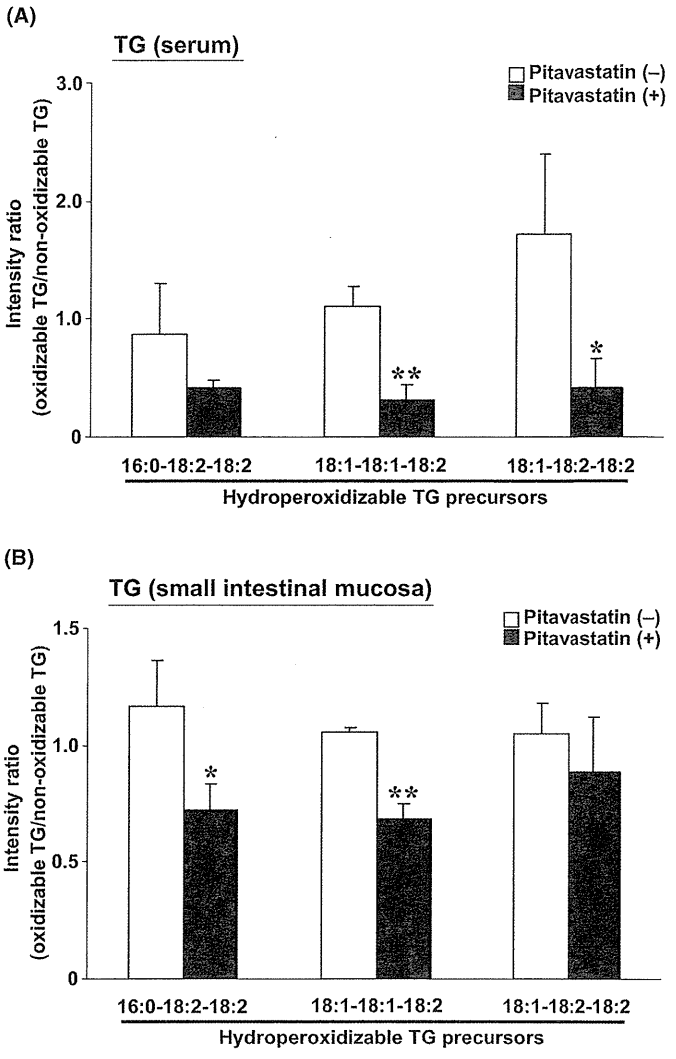


**Fig. 6.** Suppression of hydroperoxidizable triglyceride (TG) precursor production in serum and the small intestinal mucosa of Min mice by pitavastatin treatment. (A) Intensity ratios of hydroperoxidizable TG precursors to non-oxidizable TG (16:0-18:1-18:1) were significantly decreased in the serum of the pitavastatin-treated groups at doses of 40 p.p.m. for 14 weeks along with suppression of intestinal polyp formation compared with the untreated groups. (B) Similarly, the intensity ratios in the intestinal mucosa of the pitavastatin-treated groups were significantly reduced. Values are mean ± SD ($n = 3$; $^*P < 0.05$, $^{**}P < 0.01$ versus intensity ratios of the pitavastatin-untreated groups).

## Discussion

In this study, oxidant-related TG and oxidized PC such as aldehyde and carboxylic acid types were increased even at the early stage of intestinal polyp formation in the serum of Min mice. The oxidized PC of Min mice were highly elevated even at 10 weeks of age. In particular, hydroperoxidized TG and hydroperoxidizable TG precursors were significantly increased from 15 weeks of age in a time-dependent manner. We previously reported that Min mice had higher levels of 18:2 in plasma compared with wild-type mice by gas–liquid chromatography method.[32] These results suggest that oxidant-related TG in the blood increased in the course of intestinal polyp formation, and these TG might be useful as biological markers for the formation and development of intestinal polyps.

The present study also showed that hydroperoxidizable TG precursors were comparatively deposited at the tip of villi with age in Min mice from our local analysis using LMD and sensitive chip-based nanoESI-MS system. Dietary fat mainly constituted of TG is absorbed at the tip of the villi and accumulates in the cytoplasm of intestinal epithelial cells, but not in stromal cells. The origin of the accumulated lipids seems to be derived

from the contents in the digestive tract, not from blood vessels. Therefore, our detailed analysis of the villi in Min mice is thought to be effective for directly detecting qualitative and quantitative changes. To our knowledge, local lipid analysis of the villi has not yet been reported and accumulation of hydroperoxidizable TG precursors with age was first detected in Min mice. In addition, detailed analysis of serum and the intestinal mucosa in Min mice treated with pitavastatin revealed that hydroperoxidizable TG precursors were significantly decreased along with suppression of intestinal polyp formation. It is speculated that accumulation of hydroperoxidizable TG precursors in the small intestine mucosa leads to an increased serum level of hydroperoxidized TG, and oxidative stress and inflammation might be systemically induced by these oxidized TG. For one reason, the fact that malabsorption of the hydroperoxidizable TG precursors occurs at the tip of the villi, which might evoke a source for increments of hydroperoxidized TG in serum. For another reason, hydroperoxidizable TG precursor increments at the tip of the villi lead to an elevated influx into the general circulation, and hydroperoxidized TG might be abundantly generated by peroxidation of these precursors. Moreover, hydroperoxidized TG derived from these precursors could induce DNA damage, which might link to truncation mutation in both alleles of the *Apc* gene. However, further experiments are needed to sensitively detect oxidized PC and TG from the small regions of the villi by our local analysis, and to clarify the role of hydroperoxidized TG in intestinal polyp formation.

In conclusion, hydroperoxidizable TG precursors of Min mice in serum and the small intestine mucosa were increased in the course of intestinal polyp formation, and increase of these oxidizable TG precursors was suppressed by pitavastatin. These oxidant-related lipids might be useful as biological indications for the formation and development of intestinal polyps. Furthermore, hydroperoxidized TG, hydroperoxidizable TG precursors and oxidized PC such as aldehyde and carboxylic acid types were highly increased even at 10 weeks of age, and these lipids might be possible markers for early intestinal polyp formation. These results also suggest that qualitative changes of TG and PC are important in the course of intestinal polyp formation and might lead to their development in Min mice.

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## Disclosure Statement

The authors have no conflict of interest.

## References

1 Le Marchan L, Wilkens LR, Kolonel LN, Hankin JH, Lyu LC. Associations of sedentary lifestyle, obesity, smoking, alcohol use, and diabetes with the risk of colorectal cancer. *Cancer Res* 1997; **57**: 4787–94.
2 Abu-Abid S, Szold A, Klausner J. Obesity and cancer. *J Med* 2002; **33**: 73–86.
3 Yamada K, Araki S, Tamura M *et al.* Relation of serum total cholesterol, serum triglycerides and fasting plasma glucose to colorectal carcinoma *in situ*. *Int J Epidemiol* 1998; **27**: 794–8.
4 Kaye JA, Meier CR, Walker AM, Jick H. Statin use, hyperlipidaemia, and the risk of breast cancer. *Br J Cancer* 2002; **86**: 1436–9.
5 Otani T, Iwasaki M, Ikeda S *et al.* Serum triglycerides and colorectal adenoma in a case–control study among cancer screening examinees (Japan). *Cancer Causes Control* 2006; **17**: 1245–52.
6 Niho N, Takahashi M, Kitamura T *et al.* Concomitant suppression of hyperlipidemia and intestinal polyp formation in *Apc*-deficient mice by peroxisome proliferator-activated receptor ligands. *Cancer Res* 2003; **63**: 6090–5.
7 Niho N, Takahashi M, Shoji Y *et al.* Dose-dependent suppression of hyperlipidemia and intestinal polyp formation in Min mice by pioglitazone, a PPAR gamma ligand. *Cancer Sci* 2003; **94**: 960–4.
8 Niho N, Mutoh M, Takahashi M, Tsutsumi K, Sugimura T, Wakabayashi K. Concurrent suppression of hyperlipidemia and intestinal polyp formation by NO-1886, increasing lipoprotein lipase activity in Min mice. *Proc Natl Acad Sci USA* 2005; **102**: 2970–4.
9 Mutanen M, Pajari AM, Oikarinen SI. Beef induces and rye bran prevents the formation of intestinal polyps in Apc(Min) mice: relation to beta-catenin and PKC isozymes. *Carcinogenesis* 2000; **21**: 1167–73.
10 Keys A, Menotti A, Karvonen MJ *et al.* The diet and 15-year death rate in the seven countries study. *Am J Epidemiol* 1986; **124**: 903–15.
11 Gehrisch S. Common mutations of the lipoprotein lipase gene and their clinical significance. *Curr Atheroscler Rep* 1999; **1**: 70–8.
12 Mead JR, Irvine SA, Ramji DP. Lipoprotein lipase: structure, function, regulation, and role in disease. *J Mol Med* 2002; **80**: 753–69.
13 Mutoh M, Komiya M, Teraoka N *et al.* Overexpression of low-density lipoprotein receptor and lipid accumulation in intestinal polyps in Min mice. *Int J Cancer* 2009; **125**: 2505–10.
14 Ikeda K, Oike Y, Shimizu T, Taguchi R. Global analysis of triacylglycerols including oxidized molecular species by reverse-phase high resolution LC/ESI-QTOF MS/MS. *J Chromatogr B Analyt Technol Biomed Life Sci* 2009; **877**: 2639–47.
15 Nakanishi H, Iida Y, Shimizu T, Taguchi R. Analysis of oxidized phosphatidylcholines as markers for oxidative stress, using multiple reaction monitoring with theoretically expanded data sets with reversed-phase liquid chromatography/tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 2009; **877**: 1366–74.
16 Houjou T, Yamatani K, Nakanishi H, Imagawa M, Shimizu T, Taguchi R. Rapid and selective identification of molecular species in phosphatidylcholine and sphingomyelin by conditional neutral loss scanning and MS3. *Rapid Commun Mass Spectrom* 2004; **18**: 3123–30.
17 Houjou T, Yamatani K, Imagawa M, Shimizu T, Taguchi R. A shotgun tandem mass spectrometric analysis of phospholipids with normal-phase and/or reverse-phase liquid chromatography/electrospray ionization mass spectrometry. *Rapid Commun Mass Spectrom* 2005; **19**: 654–66.
18 Taguchi R, Nishijima M, Shimizu T. Basic analytical systems for lipidomics by mass spectrometry in Japan. *Methods Enzymol* 2007; **432**: 185–211.
19 Han X, Gross RW. Global analyses of cellular lipidomes directly from crude extracts of biological samples by ESI mass spectrometry: a bridge to lipidomics. *J Lipid Res* 2003; **44**: 1071–9.
20 Murphy RC, James PF, McAnoy AM, Krank J, Duchoslav E, Barkley RM. Detection of the abundance of diacylglycerol and triacylglycerol molecular species in cells using neutral loss mass spectrometry. *Anal Biochem* 2007; **366**: 59–70.
21 Ejsing CS, Sampaio JL, Surendranath V *et al.* Global analysis of the yeast lipidome by quantitative shotgun mass spectrometry. *Proc Natl Acad Sci USA* 2009; **17**: 2136–41.
22 Espina V, Wulfkuhle JD, Calvert VS *et al.* Laser-capture microdissection. *Nat Protoc* 2006; **1**: 586–603.
23 Flores NA. Pitavastatin Nissan/Kowa Yakuhin/Novartis/Sankyo. *Curr Opin Investig Drugs* 2002; **3**: 1334–41.
24 Yasui Y, Suzuki R, Miyamoto S *et al.* A lipophilic statin, pitavastatin, suppresses inflammation-associated mouse colon carcinogenesis. *Int J Cancer* 2007; **121**: 2331–9.
25 Iglesias P, Diez JJ. New drugs for the treatment of hypercholesterolaemia. *Expert Opin Investig Drugs* 2003; **12**: 1777–89.
26 Demierre MF, Higgins PDR, Gruber SB, Hawk E, Lippman SM. Statin and cancer prevention. *Nat Rev Cancer* 2005; **5**: 930–42.
27 Moser AR, Pitot HC, Dove WF. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science* 1990; **247**: 322–4.
28 Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 1959; **37**: 911–7.
29 Green H, Kehinde O. An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion. *Cell* 1975; **5**: 19–27.
30 Smith WL, Murphy RC. Oxidized lipids formed non-enzymatically by reactive oxygen species. *J Biol Chem* 2008; **283**: 15513–4.
31 Schneider C, Porter NA, Brash AR. Routes to 4-hydroxynonenal: fundamental issues in the mechanisms of lipid peroxidation. *J Biol Chem* 2008; **283**: 15539–43.
32 Kuriki K, Mutoh M, Tajima K, Wakabayashi K, Tatematsu M. Relationships between intestinal polyp formation and fatty acid levels in plasma, erythrocytes, and intestinal polyps in Min mice. *Cancer Sci* 2008; **99**: 2410–6.



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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Quantitative and qualitative profiles of serum phosphatidylcholine (PC) molecular species of Min mice in the course of intestinal polyp formation. The PC molecular species in the serum of Min mice ($n$ = 3) were quantitatively and qualitatively similar to wild-type mice ($n$ = 2). Values are mean ± SD. The PC containing docosahexaenoic acid (22:6n-3) was not included in the AIN-76A diet and the correction intensities were very small.

**Fig. S2.** Local analysis of phosphatidylcholine (PC) molecular species at the villous tips and bases of Min mice by laser microdissection and the nano-electrospray ionization-mass spectrometry (nanoESI-MS) system. The PC molecular species at the villous tips and bases of Min mice were quantitatively and qualitatively unchanged at 10 and 20 weeks of age. There was no significant difference between the villous and base values. Values are mean ± SD ($n$ = 3). The PC containing 22:6 was not included in the AIN-76A diet and the intensities were very small.

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*Review*

# Experimental Animal Models of Pancreatic Carcinogenesis for Prevention Studies and Their Relevance to Human Disease

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**Abstract:** Pancreatic cancer is difficult to cure, so its prevention is very important. For this purpose, animal model studies are necessary to develop effective methods. Injection of *N*-nitrosobis(2-oxopropyl)amine (BOP) into Syrian golden hamsters is known to induce pancreatic ductal adenocarcinomas, the histology of which is similar to human tumors. Moreover, K-*ras* activation by point mutations and *p16* inactivation by aberrant methylation of 5' CpG islands or by homozygous deletions have been frequently observed in common in both the hamster and humans. Thus, this chemical carcinogenesis model has an advantage of histopathological and genetic similarity to human pancreatic cancer, and it is useful to study promotive and suppressive factors. Syrian golden hamsters are in a hyperlipidemic state even under normal dietary conditions, and a ligand of peroxizome proliferator-activated receptor gamma was found to improve the hyperlipidemia and suppress pancreatic carcinogenesis. Chronic inflammation is a known important risk factor, and selective inhibitors of inducible nitric oxide synthase and cyclooxygenase-2 also have protective effects against pancreatic cancer development. Anti-inflammatory and anti-hyperlipidemic agents can thus be considered candidate chemopreventive agents deserving more attention.

**Keywords:** pancreatic cancer; hyperlipidemia; iNOS; hamster; BOP

## 1. Introduction

In recent years, pancreatic cancer has increased to become the fifth leading cause of cancer mortality in Japan [1]. Since the five-year-survival rate is very low, elucidation of the mechanisms of pancreatic carcinogenesis and development of prevention methods are important high priority tasks. Factors affecting pancreatic cancer development have been studied using several *in vivo* animal models [2]. Use of *N*-nitrosobis(2-oxopropyl)amine (BOP) in the Syrian golden hamster is known to be unique for development of pancreatic ductal adenocarcinomas, the histology of which is similar to that in human cases [3-6]. In the hamster model, early lesions such as focal hypertrophy, hyperplasia, goblet cell metaplasia, atypical hyperplasia and *in situ* carcinoma sequentially develop in the common duct, pancreatic duct and ductules, but not in acinar cells [7]. Transplacental induction of pancreatic ductal cancer by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and ethanol in Syrian golden hamster is also an interesting model to investigate a synergistic effect of cigarette smoking and alcohol drinking on fetuses [8]. In rats, the azaserine-induced pancreatic cancer model is well-known, but the lesions are acinar cell carcinomas [9]. A nitrosourea amino acid carcinogen, *N*-delta-(*N*-methyl-*N*-nitrosocarbamoyl)-L-ornithine (MNCO), has further been shown to cause pancreatic acinar cell carcinomas in rats [10] and ductal carcinomas in hamsters [11]. Different from in hamsters, BOP mainly induces thyroid gland tumors in rats [12,13] and lung and liver tumors in mice [14]. There is thus a species specificity in the types of pancreatic neoplasm induced in rodents [15-17]. The 7,12-dimethylbenzanthracene (DMBA)-induced pancreatic cancer model can also be employed as a chemical carcinogenesis model in rats [18,19] and mice [20]. In this case, direct implantation of the carcinogen into the head of the pancreas causes tubular complexes in acini and induces pancreatic neoplasms of ductal phenotype in which cytokeratin 19 is expressed [21] and K-*ras* gene mutations are present [22]. Recently, genetically engineered mouse (GEM) models of pancreatic exocrine cancer have been developed and used to elucidate mechanisms of pancreatic carcinogenesis, although the pathology is somewhat different from human cases [23]. Mouse models with pancreas-specific expression of mutant K-*ras* from the embryonic stage frequently develop acinar-to-ductal metaplasia and pancreatic intraductal neoplasms (PanINs), but few pancreatic cancers under normal conditions [24-26]. Additional alterations in tumor-suppressor genes, such as *p16* [27], *p53* [28], *dpc4* [29], and TGF-β receptor II [30], or pancreatitis [31] in the GEM models have been shown to cause quite high incidences of pancreatic cancers. On the other hand, conditional expression of mutant K-*ras* in the adult phase hardly induces PanINs and cancer if without pancreatitis [31]. Transgenic rats that express a mutated Ha- or K-*ras* oncogene regulated by the *Cre/lox* system have also been demonstrated to develop pancreatic ductal carcinomas upon injection of a *Cre*-carrying adenovirus into the pancreatic ducts and acini via the common bile duct [32,33]. In these rat models, mutant *Ras* is conditionally expressed in the pancreas of young adult rats and neoplastic lesions arise in pancreatic duct epithelium, intercalated ducts and centroacinar cells, but not acinar cells [32].

Here, we focus on the BOP-induced pancreatic cancer model in hamsters and discuss its utility for cancer prevention studies.

## 2. Genetic Alterations in Pancreatic Ductal Carcinomas of Humans and BOP-treated Hamsters

Pancreatic carcinogenesis is known to be a multi-step process involving multiple genetic alterations in humans [34-37] and similar genetic alterations have been found in hamsters [38,39]. Findings for genetic alterations in pancreatic ductal cancers in the two species are summarized in Table 1.

**Table 1.** Gene alterations in pancreatic cancers in humans and hamsters [34-60].

| Gene | Alterations | Frequency in (%) | |
|---|---|---|---|
| | | Human | Hamster (BOP-treated) |
| *K-ras* | Mutation | 75–100 | 70–95 |
| *p16$^{INK4A}$/CDKN2A* | CpG methylation/Deletion/Mutation | 80–95 | 93 |
| *DPC4/SMAD4* | Deletion / Mutation + LOH | 50 | 8 |
| *DCC* | Deletion | 50 | 53 |
| *P53* | Mutation + LOH | 40–75 | 0 |
| *FHIT* | Aberrant transcripts | 62 | 73 |

LOH: loss of heterozygosity

K-*ras* is quite frequently mutated in pancreatic ductal carcinomas in hamsters (70–95%) [40-42] as well as humans (75–100%) [43-45], resulting in activation of downstream signaling proteins such as elements in the Raf/MEK/MAPK and PI3K/Akt pathways. K-*ras* mutations are also observed in early lesions, such as atypical ductal hyperplasia in hamsters and humans [41,46]. The major K-*ras* mutation in BOP-induced pancreatic carcinomas in hamsters is predominantly a G to A transition in the second position of codon 12, while both G to A transitions and G to T transversions at the second position of codon 12 are frequently observed in human pancreatic cancers [43,44].

The *p16$^{INK4A}$/CDKN2A* is known to be a tumor suppressor gene located at chromosome 9p that is inactivated in most pancreatic ductal carcinomas in humans (80–95%) by intragenic mutations (40%), homozygous deletions (40%) or hypermethylation of its promoter region (15%) [45,47,48]. The protein encoded by *p16* is an inhibitor of cyclin-dependent kinase and regulates the cell cycle by activation of RB proteins. Frequent alteration of *p16* (~93%) has also been reported in BOP-induced pancreatic tumors in hamsters and the majority of changes involve aberrant methylation (47%) or homozygous deletion (37%) [49].

*DPC4/SMAD4* is a tumor suppressor gene located at chromosome 18q21.1 which encodes a protein associated with the TGF-β signaling pathway. *DPC4/SMAD4* is inactivated in 50% of pancreatic adenocarcinomas in humans by homozygous deletions (30%) or intragenic mutations in one allele coupled with loss of heterozygosity (LOH) (20%) [50]. On the other hand, *Dpc4/Smad4* alterations are rare in BOP-induced pancreatic tumors in hamsters (8%) [51].

*DCC* is a tumor suppressor gene located at chromosome 18q21.3, which encodes a protein with homology to cell adhesion receptors. Expression has been found to be lost in 50% of human pancreatic

adenocarcinomas [52] and also in 50% of BOP-induced pancreatic tumors in hamsters [53]. In addition, DCC expression is reduced or lost in poorly differentiated or undifferentiated pancreatic cancer cell lines, whereas it is conserved in the more differentiated ones [52,37].

*p53* is the most frequently altered tumor suppressor gene in various cancers, its protein being a transcription factor which regulates cell cycle and apoptosis. *p53* is located at chromosome 17p and frequently inactivated by LOH and mutations in 40 to 75% of pancreatic adenocarcinomas in humans [34,45,54-56]. Overexpression of p53 protein can be detected in the nuclei of p53-mutated cells [54,55]. On the other hand, there is no evidence of p53 mutations in primary tumors in BOP-treated hamsters [57].

*FHIT* gene is a putative tumor suppressor gene located at chromosome 3p14, which is expressed in normal pancreatic ductular cells and is altered in pancreatic cancers [58]. Exogenous expression of *FHIT* in human pancreatic cancer cells causes cell cycle arrest and apoptosis [59] and loss of full length transcripts is frequent in primary pancreatic cancers of humans (62%) [58] and BOP-treated hamsters (73%) [60].

In addition to these gene alterations, increased protein expression, such as telomerase [61,62], midkine [63,64], cyclooxygenase-2 (COX-2) [65], metalloproteinase (MMP)-2, MMP-9 and membrane type 1-MMP [66,67] are shown in hamsters as in humans.

These findings indicate that multiple gene alterations and changes in protein expression observed in human pancreatic cancers are similarly involved in the BOP-induced hamster pancreatic ductal carcinogenesis model, underlining its utility for studying methods for pancreatic cancer prevention.

## 3. Modifying Factors in the Experimental Pancreatic Carcinogenesis Models

In addition to cigarette smoking, a well-known cause of pancreatic cancer, epidemiological studies have shown that chronic pancreatitis, obesity and diabetes mellitus are risk factors [68]. Using experimental animal models including mainly the BOP-induced pancreatic carcinogenesis model in hamsters, these and other possible promotive and suppressive factors in pancreatic carcinogenesis have been studied.

### *3.1. Obesity and Diabetes*

Dietary fat has modifying effects on pancreatic carcinogenesis. It has been shown that a high-corn oil diet increased pancreatic ductal adenocarcinoma development in BOP-treated hamsters as compared with a low-corn oil diet [69]. Furthermore, a diet containing beef tallow has been shown to increase pancreatic cancer development compared with a diet containing corn oil [70]. Type and composition of fat are considered to be important. Fish oil rich in n-3 polyunsaturated fatty acids has been demonstrated to reduce pancreatic tumor incidences and hepatic metastasis in the BOP-treated hamster model [71]. Enhancing effects of high fat diet and suppressive influence of n-3 polyunsaturated fatty acid-rich fish oil on development of precancerous lesions, PanINs, in K-*ras* mutated GEM models have also been reported [72,73]. Obesity-mediated enhancement of PanIN lesion development is associated with increased inflammation, and abrogation of TNFR1 signaling blocks tumor promotion [72]. On the other hand, n-3 polyunsaturated fatty acids ameliorate

inflammation through inactivation of the NF-κB signaling pathway and inhibit cell proliferation through induction of cell cycle arrest and apoptosis [73,74].

Streptozotocin is known to induce diabetes through damage to islet cells and its modifying effects on pancreatic carcinogenesis have been studied in the BOP-treated hamster model, though the results are somewhat controversial. It has been reported that administration of streptozotocin alone caused islet cell tumors (44%), pseudoductules (40%), and ductular adenomas (12%), while simultaneous treatment with streptozotocin (single i.v. injection, 30 mg/kg body weight) and BOP (single s.c. injection, 10 mg/kg body weight) resulted in a significantly higher incidence of ductular carcinomas than induced by BOP alone [75]. On the other hand, pretreatment with streptozotocin at a diabetogenic dose (50 mg/kg body weight, three-times i.p. injection) prevented pancreatic cancer development when BOP was subsequently administered [76]. These inhibitory effects of pretreatment were dependent on the severity of the diabetes and could be blocked with nicotinamide [77]. These findings indicate that streptozotocin has a tumorigenic activity at relatively low dose, but when administered before BOP treatment, streptozotocin-induced diabetes/loss of insulin production could prevent BOP-induced pancreatic cancer development through killing islet cells. However, enhancing effects of diabetes and insulin-resistance observed in obesity on growth of transplantable pancreatic cancer cells are nevertheless convincing [78-80].

### *3.2. Pancreatitis*

Cerulein is an analogue peptide of cholecystokinin, and its chronic intraperitorial injection causes pancreatic hypertrophy, characterized by increased pancreatic weight, increased amylase content and acinar cell hyperplasia. Moreover, cerulein augments the carcinogenicity of *N*-nitrobis(2-hydroxypropyl)amine (BHP) in the hamster pancreas [81]. It is also reported that chronic pancreatitis caused by cerulein induces development of pancreatic ductal adenocarcinomas in GEM mice expressing K-*ras*$^{G12V}$ in acinar/centroacinar cells [31]. On the other hand, pancreatitis caused by common duct ligation before BOP injection decreased carcinoma development, while repeated induction of pancreatitis by common duct ligation after BOP administration resulted in enhanced development of carcinomas, with reference to both number and size [82].

Heavy alcohol drinking and cigarette smoking are major causes of pancreatitis in humans [83]. Epidemiological studies have shown that smoking and chronic pancreatitis are risk factors, whereas alcohol consumption itself has no direct relation [83,84]. However, in a transplacental induction model of pancreatic ductal cancer featuring NNK and EtOH treatment in the Syrian golden hamster, EtOH alone caused pancreatitis and hyperplasia, while NNK alone did not induce either [8], indicating a strong enhancing effect of pancreatitis on pancreatic carcinogenesis. It has also been reported that EtOH and nicotine promote pancreatic carcinogenesis in the DMBA-implanted mouse model [85,86].

In addition, repeated induction of pancreatitis with choline-deficient diet combined with DL-ethionine and L-methionine after initiation with BOP has been demonstrated to cause rapid production of pancreatic carcinomas in hamsters [87].

### *3.3. Others*

There is limited evidence suggesting that red meat is a cause of pancreatic cancer [88,89]. In addition to total intake, the method of meat preparation is also important. Grilled red meat is a risk factor [90]. Effects of mutagenic heterocyclic amines (HCA) formed during cooking of meat on pancreatic carcinogenesis were studied in the BOP-treated hamster model. Among HCAs, 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1) and 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx) caused increase in pancreatic carcinoma development in BOP-treated hamsters [91]. Dietary intake of DiMeIQx has also been shown to be associated with pancreatic cancer risk in man [92].

High intake of fruits, vitamin C and vitamin E are suggested to protect against pancreatic cancer [88,93,94] and both vitamins have been found to exert protective effects on BOP-induced pancreatic cancer development in hamsters [95].

## 4. Cancer Prevention Targets for Humans and Evaluation in Experimental Pancreatic Carcinogenesis Models

From the etiology of pancreatic cancer, possible methods for prevention are: (1) avoiding carcinogenic *N*-nitrosoamine exposure such as cigarette smoke; (2) body weight control by diets and physical activity; (3) use of anti-hyperlipidemic and/or anti-diabetic agents; (4) use of anti-inflammatory agents.

Epidemiological studies have suggested that several agents having anti-hyperlipidemic, anti-diabetic or anti-inflammatory activities may have chemopreventive potential against pancreatic cancer [96]. Statins are cholesterol-lowering agents and also inhibit membrane-binding of the Ras protein, and are reported to reduce pancreatic cancer cell invasion and metastasis [97]. A case-control study of half a million veterans demonstrated a significant reduction of pancreatic cancer risk in statin users (adjusted OR = 0.37) [98], while meta-analysis of 12 studies showed no evidence of association between statin use and pancreatic cancer risk (RR = 0.88) [99]. Aspirin is a most frequently used nonsteroidal anti-inflammatory drug (NSAID) and has been reported to reduce cancer risk in several organs such as in the colon [100]. In the pancreas, epidemiological data on aspirin use are controversial [101,102]. A cohort study of post-menopausal women has shown that current use of aspirin is associated with a reduced risk of pancreatic cancer (adjusted RR = 0.57) [103], whereas another cohort study of nurses demonstrated that more than 20 years of regular aspirin use is associated with increased risk (RR = 1.58) [104]. Metformin, an anti-diabetic drug, activates AMP-activated protein kinase (AMPK) and inhibits pancreatic cancer growth [105,106]. A hospital-based case-control study has shown that metformin use is associated with reduced risk (OR = 0.38), while insulin or insulin secretagogue use is associated with increased risk (OR = 1.78) of pancreatic cancer in diabetic patients [107]. However, there is still no report of cohort study or randomized-control trial on metformin use. Since incidence of pancreatic cancer is relatively low compared with colon, breast and prostate cancers, prospective studies need quite a large population. In addition, randomized control studies are difficult, because the diseases such as hyperlipidemia and diabetes should be properly cared for. Therefore, evidences provided by preclinical studies including *in vivo*

carcinogenesis studies using animal models are considered to be very important to evaluate the chemopreventive efficacy and mechanisms of these agents. Factors related to insulin resistance and inflammation are candidate targets for pancreatic cancer prevention. Table 2 shows chemopreventive agents evaluated in BOP-induced pancreatic carcinogenesis.

**Table 2.** Chemopreventive agents of *N*-nitrosobis(2-oxopropyl)amine (BOP)-induced pancreatic carcinogenesis in hamsters.

| Compounds | Mechanistic categories | Ref. |
|---|---|---|
| Anti-hyperlipidemic/diabetic agents | | |
| Pioglitazone | PPARγ ligand | [113] |
| Metformin | AMPK activator | [114] |
| Anti-inflammatory agents | | |
| Indomethacin | NSAID | [119] |
| Phenylbutazone | NSAID | [119] |
| NO-ASA | NO-NSAID | [121] |
| Nimesulide | COX-2 inhibitor | [118] |
| Celecoxib/Zileuton | COX-2/5-LOX inhibitors | [127] |
| ONO-1714 | iNOS inhibitor | [131] |
| Others | | |
| OPB-3206 | MMP-2 inhibitor | [66] |
| Protochatechuic acid | Antioxidant | [135] |
| GTE | Antioxidant | [136] |
| BHA | Antioxidant | [137] |
| Sarcophytol A | Anti-tumor promoter | [138] |
| Methionine | Essential amino acid | [139] |
| PEITC | Cytochrome P450 suppressor | [140] |
| PPITC | Cytochrome P450 suppressor | [143] |
| PBITC | Cytochrome P450 suppressor | [144] |
| BITC | Cytochrome P450 suppressor | [145] |
| Sulforaphane | Anti-oxidative enzyme inducer | [145] |
| Aloe arborescens | Detoxyfiying enzyme inducer | [146] |
| Oltipraz | Nrf2 activator | [147] |

### *4.1. Anti-Hyperlipidemic/Diabetic Agents*

It has been reported that high cholesterol intake is associated with an increased risk of pancreatic cancer [108]. Smoking is associated with metabolic syndrome, and nicotine elevates serum triglyceride levels [109,110]. Obesity and diabetes are also closely associated with hyperlipidemia and hyperinsulinemia [111,112]. Interestingly, Syrian golden hamsters are in a hyperlipidemic state even under normal diet conditions, and pioglitazone, a ligand of peroxizome proliferator-activated receptor (PPAR) γ, has demonstrated to improve hyperlipidemia and suppress development of ductal adenocarcinomas in BOP-treated hamsters; the ductal adenocarcinoma incidences in the BOP + 800 ppm pioglitazone group and the BOP alone group were 38% *vs.* 80% ($P < 0.01$) and the multiplicities were 0.55 ± 0.15 *vs.* 1.37 ± 0.22 ($P < 0.01$), respectively [113]. In addition, the incidences of bile duct tumors in BOP-treated hamsters were clearly suppressed by pioglitazone [113]. Metformin, an

activator of AMPK, has also been shown to decrease serum insulin levels and suppress development of hyperplastic, dysplastic and malignant ductal lesions in the pancreas of BOP-treated hamsters on a high fat diet condition [114]. Pioglitazone and metformin are both anti-diabetic drugs which improve insulin resistance [115].

### *4.2. Anti-inflammatory Agents*

Expression of COX-2 is up-regulated in PanIN and adenocarcinomas in humans and BOP-treated hamsters [64,116-118] and inhibition of prostanoid synthesis by NSAIDs, such as indomethacin and phenylbutazone, has been shown to reduce the development of precancerous lesions (atypical hyperplasia) and adenocarcinoma in the hamster model [119,120]. Whereas suppressive effects of aspirin were not significant, nitric oxide (NO)-donating aspirin, NO-ASA, has potent activity to prevent pancreatic cancer, especially arresting the transition from PanIN2 to PanIN3 and carcinoma, in BOP-treated hamsters [121]. It has also been reported that another COX-inhibitor, ibuprofen, reduces pancreatic cancer development in the hamster transplacental model with NNK + EtOH [122]. In GEM models, aspirin treatment has been shown to delay progression of PanINs in *LsL-Kras*$^{G12D}$*; Pdx1-Cre* mice and to partially inhibit development of invasive cancers in *LsL-Kras*$^{G12D}$*; LsL-Trp53*$^{R172H}$*; Pdx1-Cre* mice [123]. Furthermore, a selective COX-2 inhibitor, nimesulide, has been demonstrated to suppress development of precancerous lesions (atypical hyperplasia) and adenocarcinoma in BOP-treated hamsters [118]. In addition, inhibition of COX-2 by nimesulide delayed the appearance of PanIN-2 and PanIN-3 lesions in a conditional K*ras*$^{G12D}$ mouse model, indicating the importance of prostaglandin synthesis by COX-2 in the early stage of pancreatic carcinogenesis [124]. In addition to COX-2, 5-LOX is also up-regulated in the ductal cells of PanIN and adenocarcinomas in humans, BOP-treated hamsters and Elastase-K*ras* mice [125,126]. Receptors of the downstream 5-LOX metabolite, leukotriene $B_4$, have been reported to be expressed in human pancreatic cancer tissues [125] and combination of COX-2-inhibition by Celebrex and 5-LOX-inhibition by Zyflo has shown to significantly decrease liver metastasis by pancreatic cancers in BOP-treated hamsters [127]. MK886, an inhibitor of 5-LOX activating protein FLAP, also reduced pancreatic cancer development in the hamster transplacental model with NNK + EtOH [122].

Increased expression of iNOS is also observed in pancreatic adenocarcinomas in humans and hamsters [128-131], perhaps involving K-*ras* activation [132]. Inhibition of iNOS by a selective iNOS inhibitor ONO-1714 suppressed development of precancerous lesions (atypical hyperplasia) and invasive adenocarcinomas in BOP-treated hamsters [131].

### *4.3. Others*

Expression of MMP-2 is increased in precancerous lesions and adenocarcinomas, and proMMP-2 is highly activated in pancreatic carcinomas in humans and hamsters [133,66]. Inhibition of proMMP-2 activation by the MMP inhibitor OPB-3206 has been demonstrated to suppress pancreatic cancer development in BOP-treated hamsters under a rapid production protocol [66]. Another MMP inhibitor, RO 28-2653, has been reported to inhibit liver metastasis in the BOP-induced pancreatic carcinogenesis model, directly indicating roles for MMP-2 in cancer progression [134].

Protochatechuic acid, green tea extracts (GTE) and butylated hydroxyanisole (BHA) are anti-oxidative agents which have demonstrated inhibitory effects on pancreatic cancer development during the post-initiation stage of the BOP-initiated hamster model [135-137]. Sarcophytol A, which is known to be an anti-tumor promoter, and methionine, which is an essential amino acid and associated with anti-oxidation, have also been shown to suppress pancreatic carcinogenesis in the BOP-treated hamster model [138,139].

Phenethyl isothiocyanate (PEITC), a natural constituent of cruciferous vegetables, has been demonstrated to be a potent chemopreventive agent in the initiation phase of pancreatic carcinogenesis in hamsters initiated with BOP [140,141], while not affecting the post-initiation phase [142]. Synthetic analogues of PEITC, such as 3-phenylpropyl isothiocyanate (PPITC), 4-phenylbutyl isothicyanate (PBITC) and benzyl isothicianate (BITC), and sulforaphane, Aloe arborescens and oltipraz have also been shown to suppress the initiation phase of BOP-induced pancreatic carcinogenesis through inhibition of activating (phase I) enzymes or activation of detoxifying (phase II) enzymes related to metabolism of BOP [143-147].

Nicotine-derived nitrosamine NNK stimulates release of noradrenaline/adrenaline by binding to alpha7 nicotinic acetylcholine receptors and activates beta-adrenergic receptors, resulting in proliferation of human pancreatic epithelial cells through cAMP-dependent signaling [148,149]. A beta-blocker propranolol has been shown to suppress the development of pancreatic cancer induced in the hamster transplacental model with NNK + EtOH [150].

Angiotensin-I-converting enzyme (ACE) and angiotensin II type 1 receptor are upregulated in human pancreatic cancer tissues and co-localized with vascular endothelial growth factor (VEGF) in malignant ducts and in stromal cells [151]. The ACE inhibitor enalapril has been demonstrated to delay progression of PanINs in *LsL- Kras$^{G12D}$; Pdx1-Cre* mice and to partially inhibit development of invasive cancer in *LsL- Kras$^{G12D}$; LsL-Trp53$^{R172H}$; Pdx1-Cre* mice [123].

An epidermal growth factor receptor inhibitor, gefitinib, has been demonstrated to suppress development of PanINs and cancer in *LsL- Kras$^{G12D}$; p48-Cre* mice [152]. Furthermore, a src kinase inhibitor, dasatinib, has been shown to suppress metastasis in *LsL- Kras$^{G12D}$; LsL-Trp53$^{R172H}$; Pdx1-Cre; Z/EGFP* mice, although there are no effects on proliferation and no survival advantage [153]. In addition, synthetic oleanane triterpenoids CDDO-methyl ester or CDDO-ethyl amide, the rexinoid LG100268, or the combination have been shown to increase survival in *LsL- Kras$^{G12D}$; LsL-Trp53$^{R172H}$; Pdx1-Cre* mice [154].

## 5. Conclusions

As shown above, the BOP-induced pancreatic carcinogenesis model in Syrian golden hamsters has genotypic and phenotypic similarities to the human case, and is a useful animal model for investigation of cancer prevention, even though the mechanistic analyses are a little difficult due to its limited genetic information. In this model, both precancerous lesions and advanced ductal carcimomas are assessable, and most of the BOP-treated hamsters develop pancreatic ductal carcinomas within six months. On the other hand, DMBA-induced pancreatic carcinogenesis models in rats and mice are considered to be not suitable for prevention studies, from the viewpoints of pathological origin of cancers and technical difficulty with neoplastic lesions developing only where carcinogen is implanted.

GEM models are powerful for verifying the oncogenic mechanisms, but the process of carcinogenesis is pathologically different from the vast majority of human cases. Recently, several chemoprevention studies using GEM models have been reported [73,123,124,126, 152-154], mainly of two types. One focuses on suppression of PanIN development in *LsL- Kras$^{G12D}$; Pdx1-Cre* mice or *LsL- Kras$^{G12D}$; p48-Cre* mice. In this system, incidences of pancreatic cancer are low (~20% at one year) [155], and therefore, it is difficult to obtain statistically significant results for cancer development. The PanIN lesions in GEM mice have similar phenotypes to humans, such as COX-2 [124] and LOX-5 [126] expression, but the pathological process of development of early lesions is quite different from human cases. Thus, the usefulness of this model may be limited regarding early detection and prevention of human pancreatic cancer. In suppression studies on cancer development or prolonged survival with *LsL- Kras$^{G12D}$; LsL-Trp53$^{R172H}$; Pdx1-Cre* mice, the GEM animals mimic the genetics of human pancreatic cancer and quickly develop pancreatic ductal carcinomas. This model may be more suitable for therapeutic studies than for prevention.

In humans, a number of epidemiological studies have suggested reduced pancreatic cancer risk with use of anti-hyperlipidemic/diabetic or anti-inflammatory agents. However, this is difficult to prove in randomized-control studies, because of the relatively low incidence of pancreatic cancer in humans and the absence of early biomarkers to predict pancreatic cancer. Thus, *in vivo* carcinogenesis studies using animal models are important to support the epidemiological findings and provide direct evidence. Some anti-hyperlipidemic and anti-inflammatory agents have indeed been shown to exert suppressive effects on pancreatic carcinogenesis in animal models including that with BOP-initiation in the hamster, indicating that factors related to hyperlipidemia, insulin resistance and inflammation are candidate targets for pancreatic cancer prevention.

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## References

1. Matsuno, S.; Egawa, S.; Shibuya, K.; Shimamura, H.; Sunamura, M.; Takeda, K.; Katoh, H.; Okada, S.; Suda, K.; Nakao, A.; Isaji, S.; Hiraoka, T.; Hosotani, R.; Imaizumi, T. Pancreatic cancer: Current status of treatment and survival of 16071 patients diagnosed from 1981–1996, using the Japanese National Pancreatic Cancer Database. *Int. J. Clin. Oncol.* **2000**, *5*, 153-157.
2. Standop, J.; Schneider, M.B.; Ulrich, A.; Pour, P.M. Experimental animal models in pancreatic carcinogenesis: Lessons for human pancreatic cancer. *Dig. Dis.* **2001**, *19*, 24-31.