Dietary phosphatidylcholine promotes breast cancer in the hyperlipidemic E0771 murine model

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Keywords: Breast cancer, Phosphatidylcholine, Casein, Autotaxin, lysophosphatidic acid, mass spectrometry

Posted Date: April 29th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-25261/v1>

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Dietary phosphatidylcholine promotes breast cancer in the hyperlipidemic E0771 murine model

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Running title: Phosphatidylcholine-rich dietary casein promotes breast cancer

Key words

Breast cancer; Phosphatidylcholine, Casein, Autotaxin, lysophosphatidic acid, mass spectrometry

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Conflict of interest statement: The authors declare no potential conflicts of interest.

24 **Non standard abbreviations**

25 PC: Phosphatidylcholine; ATX: Autotaxin; ENPP: ectonucleotide pyrophosphatase/phosphodiesterase; LDLR:

26 low-density lipoprotein receptor; LPA: Lysophosphatidic Acid; LPC: lysophosphatidylcholine; LysoPLD:

27 Lysophospholipase D; PAT-505: [3-((6-chloro-2-cyclopropyl-1-(1-ethyl-1H-pyrazol-4-yl)-7-fluoro-1H-indol-3-

28 yl) thio)-2-fluorobenzoic acid sodium salt]; PF8380: 4-[3-(2,3-dihydro-2-oxo-6-benzoxazolyl)-3-oxopropyl]-

29 (3,5-dichlorophenyl)methyl ester-1-piperazinecarboxylic acid; VFT: “Vitamin-Free” Test; WD: Western Diet;

30 HFD: High Fat Diet

31 **Abstract**

32 **Background**

33 Cancer cells are characterized by aberrant phosphatidylcholine (PC) metabolism. PC can be synthesized de
34 novo or absorbed from diet, after digestion, by the intestinal enterocytes. Here, we investigated the association
35 of dietary intake of PC and breast cancer development in mice.

36 **Methods**

37 We used tandem mass spectrometry methods to quantitate PC content of various fat sources used to
38 manufacture rodent diets. Rodent diets were then formulated with either casein or amino acids in place of
39 casein. To test the effects of dietary PC on tumor growth we fed low density lipoprotein receptor-null (LDLR–
40 /–) mice high fat diets formulated with casein (high PC) or amino acids in place of casein (low PC).
41 Endogenous PC biosynthesis and levels of total circulated plasma PC was monitored using stable isotope tracer
42 choline and mass spectrometry analysis. Tumors were induced in mice after 12 weeks of high fat diet feeding.
43 Since PC-derived molecules are important transducers of mitogenic signals, we tested the effects of inhibiting
44 production of lysophosphatidic acid (LPA) using a recently described autotaxin (ATX) inhibitor. Finally,
45 plasma inflammatory cytokine levels were analyzed to determine the effects of diets and ATX inhibition on
46 systemic cytokine milieu.

47 **Results**

48 We found that casein is the main source of PC when present in rodent diets. Replacing casein with amino acids
49 increased the relative proportion of endogenously biosynthesized PC in mouse plasma. Compared to diets
50 containing casein, amino acid-defined diets decreased primary tumor growth in the hyperlipidemic estrogen-
51 receptor positive E0771 breast cancer mouse model. Inhibition of autotaxin with the potent inhibitor PAT-505
52 did not attenuate breast cancer development in these hyperlipidemic mice. Further, replacing casein with amino
53 acids or treatment with PAT-505 significantly reduced systemic markers of inflammation.

54 **Conclusion**

55 Our results show that casein is a significant source of PC when present in rodent diets. Diets formulated with
56 amino acids in place of casein have higher proportion of circulating PC from the endogenous biosynthetic pool.
57 Casein-containing high fat diets promote primary breast tumor development in mice through mechanisms that
58 involve systemic inflammation but is independent of LPA production by autotaxin.

59

Introduction

Breast cancer is the most common cancer in women worldwide and the second highest cause of cancer mortality for women in the United States[1]. Abnormal phosphatidylcholine (PC) metabolism is emerging as one of the most consistent metabolic hallmarks that is associated with oncogenesis and tumor progression in multiple cancers including breast cancer[2]. Detection of total PC and PC metabolite levels have been proposed as putative diagnostic biomarkers for monitoring tumor progression and response to therapy. Although most of the PC can be synthesized de novo from free choline using cytidine diphospho-choline (Kennedy) pathway and by sequential methylation of phosphatidylethanolamine, humans require additional PC from dietary sources[3, 4]. As the predominant phospholipid of biological membranes PC is the most abundant phospholipid in a wide range of foods[5]. Adult human dietary intake of PC is approximately 6 to 10 g/day[6]. In addition, PC is also the major phospholipid of mammalian bile which can be secreted as much as 6 g/day during digestion to emulsify dietary triglycerides[7, 8]. Moreover, a PC enriched fraction produced through commercial purification, lecithin, is often added to food as an emulsifying agent or consumed as a nutritional supplement. Both dietary and bile derived PC can be absorbed as intact PC but the main mechanism of uptake involves hydrolysis by phospholipase A2 (PLA2) to generate LysoPC (LPC) and a free fatty acid both of which are actively assimilated by enterocytes[9].

PC is a significant source of the lipid mediator lysophosphatidic acid (LPA), a family of bioactive lysophospholipids that act as signaling molecules primarily through actions at cell-surface G-protein coupled receptors to promote cellular proliferation, migration, and survival[10]. There are two major pathways for LPA production from PC[11]. However, majority of the circulating LPA is produced by autotaxin (ATX), a secreted phospholipase D[12]. Encoded by the ecto-nucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2) gene, ATX hydrolyzes primarily LPC to LPA[13]. ATX was originally identified as an autocrine motility-promoting factor that causes mitosis and angiogenesis in cancer cells[14]. Many normal cells and several chronic inflammatory diseases and different types of cancer express ATX. Most biologic fluids and plasma also contain ATX. In mouse, embryonic development ATX expression is necessary, as ubiquitous genetic deletion of ATX is lethal. However, in adult mice, ubiquitous genetic deletion and pharmacological inhibition of ATX are well tolerated[15]. The importance of ATX has been demonstrated in many breast cancer studies[16-23]. Transgenic overexpression of ATX, and LPA receptors (LPA₁, LPA₂, or LPA₃) in mammary cells enhance spontaneous metastatic mammary tumors in mice[20]. ATX/LPA promote the migration of breast cancer cells in vitro and ATX is among the most upregulated genes in metastatic cancers. ATX has therefore emerged as a promising therapeutic target giving rise to the development of a large number of ATX inhibitors[16, 24, 25]. Inhibition of ATX was demonstrated for the first time to decrease breast tumor growth and metastasis in the syngeneic 4T1 orthotopic mouse model using the ATX inhibitor, ONO-8430506[26].

Animal models are invaluable resources for cancer research and drug efficacy testing. However, many studies using animal models draw conclusions about dietary effects from comparisons of different diets without making considerations for PC composition in the diets. Typically, the PC content of laboratory animal diets is not reported in the literature or provided by the manufacturers. Therefore, we examined the PC composition of different fat sources in rodent diets and the effects of manipulating dietary PC levels on breast cancer development. This was extended to studies in which ATX inhibition was combined with dietary PC depletion. We used the hyperlipidemic C57BL/6 LDL receptor-null (LDLR^{-/-}) mice since these mice are reported to exhibit an accumulation of ATX-derived LPA when fed diets containing high fat contents[27, 28]. Our studies show that milk casein is the main source of PC when present in rodent diets. Stripping of casein and other sources of fat with denatured alcohol reduces PC levels contained in these constituents by up to 70%. Furthermore, replacing casein in diets with amino acids decreases PC contents by nearly 90%. The results of our study show that casein in rodent diets promotes greater tumor development compared to diets in which casein is replaced with amino acids. However, inhibition of ATX was without effect on primary tumor growth in PC-rich diet fed mice and reversed the beneficial effects of PC depletion on primary tumor growth. Feeding mice amino acid-defined diets or inhibiting ATX significantly reduced plasma inflammatory cytokine levels. Our findings suggest that the beneficial effects of replacing milk casein with amino acids is through a mechanism that is independent of the ATX-derived LPA signaling.

110 MATERIALS AND METHODS

111 Reagents

112 Unless otherwise indicated, reagents were from Millipore-Sigma. Recombinant mouse ATX was from R&D
113 Systems (Minneapolis, MN). Recombinant human ATX was from Echelon (Salt Lake City, UT). Lipids and
114 lipid standards were sourced from Avanti Polar Lipids (Alabaster, AL).

115 E0771 cell culture

116 E0771 murine breast cancer line originally isolated as a spontaneous tumor from C57BL/6 was purchased from
117 CH3 BioSystems (Buffalo, NY). The cells were maintained in RPMI 1640 medium supplemented with 10
118 mmol/L HEPES, 10% Fetal Bovine Serum, penicillin (100 units/ml) and streptomycin (100 µg/ml) cultured at
119 37°C in a humidified atmosphere of 5% CO₂ in air. Cells in cultures were washed with PBS, trypsinized and
120 resuspended in Hank's balanced salt solution (HBSS) at 5,000,000 cells/ml.

121 Rodent diet formulations

122 To test the hypothesis that casein-containing diets could promote tumor development in hyperlipidemic mice,
123 we formulated western and high fat diets in collaboration with Envigo diet manufacturers (Madison, WI).
124 Western diets (42% Kcal from fat) were modeled around the diet TD.88137[29] while high fat diets (60% kcal
125 from fat) were modeled on TD.06414[30]. Casein diets were formulated with casein or "Vitamin-free" Test
126 (VFT) Casein (Envigo, Madison, WI). The amino acid defined diets (isonitrogenous diets) were manufactured
127 using the amino acid profile of casein as formulated for diet TD.140088 with modifications equivalent to
128 casein-containing diets. Aspartic acid (Asp) and glutamine (Gln) are non-essential amino acids and not
129 included in the amino acid (AA) profile of casein. These amino acids were added to the diets at similar levels
130 found in other AA-defined diets (such as TD.99366). Asp was added at approximately half of the level of Asp
131 in other AA defined diets. Gln was added at a similar rate as Glu. Cysteine was added to the casein diet to
132 balance the amino acid profile - casein is limiting in sulfur AA. Since casein is a significant source of calcium
133 and phosphorus the levels of these minerals were also adjusted in the diets. VFT casein reduces fat and moisture
134 in the casein product and increases the concentration of protein. Therefore, we adjusted the inclusion rate of
135 VFT casein to better match the total protein levels. For all diets 50 g/kg maltodextrin was added to improve
136 pelleting of the amino acid and VFT-casein diets. Diets were color coded by adding food colors for ease of
137 identification. Detailed nutritional composition of the formulated diets can be found in Supplementary Tables 1-
138 2.

139

Rodent diets containing stable isotope labeled choline

PC in systemic circulation can be derived from diet or from de novo synthesis using choline as substrate. To interrogate the contribution of casein in rodent diets to plasma PC pool, high fat diets formulated with casein or amino acids were designed to contain deuterium labeled choline as the sole source of dietary free choline in the diets. The deuterium label in choline served as stable isotope tracer to track endogenous PC biosynthesis. Five-week-old female LDL receptor deficient (LDLR^{-/-}) C57BL/6 mice were purchased from Jackson laboratory (Bar Harbor, Maine) and maintained on 12-hour light/dark cycle at the University of Kentucky Animal Care Facility. The mice were fed water and standard rodent chow diet (2014 Teklad Harlan Rodent Diet) *ad libitum* for 1 week to acclimatize to the laboratory conditions before being assigned randomly into groups to receive the diets containing either labeled or unlabeled choline. After 8 weeks of *ad libitum* feeding, blood was drawn from mice, plasma prepared and frozen at -80°C for further lipid analysis as described below.

Animals and syngeneic orthotopic mouse model of breast cancer.

Animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Kentucky. Five-week-old female LDLR^{-/-} C57BL/6 mice were purchased from Jackson laboratory (Bar Harbor, Maine) and maintained on 12-hour light/dark cycle at the University of Kentucky Animal Care Facility. The mice were fed water and standard rodent chow diet (2014 Teklad Harlan Rodent Diet) *ad libitum* for 1 week to acclimatize to the laboratory conditions before being assigned randomly into groups to receive the assigned diets. Food consumption was monitored by weighing food pellets before and after replacement. Blood was drawn via submandibular bleeding into Ethylenediaminetetraacetic acid (EDTA) coated tubes (BD Microtainer) at the onset of high fat feeding and biweekly thereafter. Plasma was prepared immediately and stored frozen at -80°C until processing for measurements of lipids (PC, LPC and LPA), ATX inhibitors and other markers as needed. Twelve weeks after initiating feeding, mice were anesthetized, a small incision made and syngeneic estrogen receptor positive (ER+) E0771 tumor cells (500,000 cells suspended in 100 µl HBSS) were injected into the inguinal mammary fat pad using an insulin syringe. The incision was then closed with a wound clip. When tumors became palpable after 4-5 days, two orthogonal caliper measurements were taken and tumor volumes estimated using the formula $\text{width}^2 \times \text{length} / 2$. Primary tumors were allowed to grow until they reached the maximum allowable size of 1 cm³. The primary tumors were resected and animals kept for a further 2 weeks for spontaneous metastasis when the animals were euthanized and various organs harvested. To analyze lung metastatic burden, dissected lungs were stained with India ink to visualize metastatic nodules and the number of visible surface nodules counted.

PAT-505 formulation and administration

PAT-505 [3-((6-chloro-2-cyclopropyl-1-(1-ethyl-1H-pyrazol-4-yl)-7-fluoro-1H-indol-3-yl) thio)-2-fluorobenzoic acid sodium salt was obtained from PharmAkea (San Diego, CA) under a material transfer agreement. For oral dosing of PAT-505 at 30-mg/kg body weight a 7.5 mg/mL solution was prepared by adding room temperature 0.5% methyl cellulose to PAT-505 in a scintillation vial and heating to ~60-70°C to dissolve. The solution was sonicated for 3-5 minutes until it became clear. The dosing solution was stored at room temperature for daily dosing for up to 1 week before a new solution could be prepared. One day prior to E0771 cell inoculation, treatment of animals with PAT-505 or 0.5% methyl cellulose (MC Vehicle) by oral gavage was initiated and continued twice daily until the animals were sacrificed.

Measurement of bioactive lipids and PAT-505 by mass spectrometry

PC and LPA measurements were performed using established methods previously described[31-38]. PC species and PAT-505 were measured using high performance liquid chromatography (HPLC) electrospray ionization (ESI) tandem mass spectrometry (MS) using AB Sciex 4000 Q-Trap linear ion trap triple quadrupole and AB Sciex 5600 Quadrupole time of flight mass spectrometers (Sciex, Framingham, MA). Deuterated lipids were initially analyzed using Fourier Transform Mass Spectrometry (FTMS) and validated using the Thermo Q-Exactive. The samples were injected by direct infusion into the UHRMS (THERMO Orbitrap Fusion™ Tribrid™, resolving power of 500,000 at m/z 200) via nanoESI (Advion TriVersa NanoMate®) in positive mode. Ten microscans were added to produce a single MS1 full scan (m/z 150–1600) with total acquisition time of 10 minutes. Lock mass (EASY-IC™) was used for improved reproducibility throughout the scans. Scans in each acquired spectrum were averaged using Xcalibur 4.1(THERMO Scientific™). LPA species were measured using the AB Sciex Triple Quad 6500 (Sciex, Framingham, MA). PC in the various rodent diets were extracted using modified Bligh and Dyer Method. Briefly, milligram amounts of diets were respectively weighed from randomly selected diet pellets and extracted in a solvent system consisting of chloroform/methanol/water 2:2:18 (v/v/v) after intermittent sonication in 8 ml glass tubes. To monitor analyte recovery, 17:0 LPC (Avanti Polar Lipids, Alabaster, AL) was included in each tube. Plasma PC and LPA were extracted from 50 µl of plasma using acidified organic solvents with the inclusion of 10 µl of Avanti Splash® Lipidomix® Mass spec Standard (for PC) and 17:0 LPA for LPA species (Avanti Polar Lipids, Alabaster, AL) as internal or recovery standards. Quantitation of lipids was done by selected ion monitoring mode HPLC MS/MS using calibration curves generated using independently quantitated standards. Data for rodent diet PC were quantified based on the recovery of 17:0 LPC and normalized to the weights of the diets respectively. Data for PC in diets or dietary constituents were normalized to their respective diet or constituent mass while data for plasma PC and LPA were normalized to the lipid standards contained in the deuterated Avanti Splash®

Lipidomix® standard mixture and 17:0 LPA. PAT-505 was extracted using a previously described method with the inclusion of PF8380 as recovery standard and analyzed using the AB Sciex 4000 Q-Trap linear ion trap triple quadrupole mass spectrometer[39]. Quantitation of PAT-505 was done using calibration curves from standards run independently.

Measurement of ATX activity

To validate the potency of PAT-505 *in vitro*, we analyzed the activities of recombinant mouse and human ATX against a panel of lysophosphatidylcholine (LPC) in the presence or absence of PAT-505. ATX catalyzes the hydrolysis of LPC to LPA and choline. The released choline is oxidized by choline oxidase to betaine and hydrogen peroxide which is detected in a color reaction commonly known as TOOS (N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline) assay[40]. We used LPC species with saturated fatty acyl chains 16:0 LPC and 18:0 LPC, unsaturated fatty acyl chain 18:1 LPC and the synthetic unnatural 17:0 LPC. The ATX/LysoPLD activity reaction containing ATX and LPC was performed in 100 µl buffer (50 mM Tris-HCl pH 8.0, 5 mM MgCl₂, 5 mM CaCl₂, 140 mM NaCl, 1 mg/ml Fatty acid free BSA, and 0.01% Brij-35 (w/v) for 2 hours at 37°C with intermittent shaking. To test the inhibitory effects of PAT-505, we pre-incubated PAT-505 with recombinant ATX for 30 minutes before adding the respective LPC substrates. The choline released was detected by adding 100 µl of detection/color reagent consisting of 50 mM Tris-HCL pH 8, 4.5 mM MgCl₂ 4.5 mM 4-aminoantipyrine, 2.7 mM TOOS, 20 units ml⁻¹ horseradish peroxidase and 3 units ml⁻¹ choline oxidase and incubating for 15 minutes before measuring the absorbance at 555 nm. Absorbance values were plotted against the log concentration of PAT-505.

Quantitation of plasma cytokines and chemokines by Multiplex assay

Plasma samples from tumor-induced PAT-505 and vehicle control-treated mice were analyzed for the levels of circulating cytokines and chemokines. Measurements were performed using the Milliplex Mouse Cytokine/Chemokine Premixed 32-Plex and TGF-beta 1, 2, 3 magnetic bead Kits (Millipore Corp, Billerica, MA) according to the manufacturer's protocol. Processed plates were measured on the Luminex Xmap MAGPIX system (Luminex Corp, Austin, TX), as per the manufacturer's instructions. The following cytokines/chemokines were measured: G-CSF, Eotaxin, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 p40, IL-12 p70, IL-13, IL-15, IL-17, LIF, LIX, IP-10, KC, MCP-1, MIP-1α, MIP-1β, M-CSF, MIP-2, MIG, RANTES, VEGF, TNF-α, TGF-β 1, TGF-β 2 and TGF-β 3. The values below standard curve were denoted as zero for statistical analyses while the data were reported as means ± standard errors of the means (SEM).

Statistical analysis

Results were expressed as mean $\pm$ standard error of the means (SEM). Statistical analyses were performed using the Prism 7 package (GraphPad, La Jolla, CA) and SigmaStat (San Jose, CA, USA). Unpaired two-tailed Student's *t*-test or analysis of variance (one way or multiple comparisons) was performed for comparisons between the groups. Comparisons in tumor growth kinetics between groups were performed using 2-way ANOVA with post hoc Bonferroni-corrected *t* test. Values of $p < 0.05$ were considered statistically significant.

Results

Casein is the major source of Phosphatidylcholine when added as a constituent of rodent diets

We obtained the ingredients commonly used as fat sources to formulate rodent diets from Envigo (Madison, WI) and quantitated PC in the separate components. As shown in Table 1, PC was detected in all of the preparations tested. Lard and anhydrous milk, which are common fat sources in high fat diets, are rich in PC. Other components used in rodent diets to substitute for lard and anhydrous milk also contain detectable PC amounts. The most striking observation however is that PC is highly abundant in casein. Casein is usually added to diets as a protein source. In our analysis, the quantities of PC in casein were approximately 100-fold higher compared to traditional fat sources. We also observed that PC levels can be significantly reduced in the respective sources by stripping with organic solvents. Levels of PC were reduced in “Vitamin-Free” Test (VFT) Casein by about 70%. VFT-casein is produced by extracting casein obtained from lactic acid precipitation of skim milk with hot denatured ethanol. Compared to regular casein, VFT-casein is reported by the manufacturer to contain less fat, less moisture and more protein. As with hot denatured ethanol, we also observed that tocopherol stripping of lard and corn oil reduced PC levels in these constituents substantially by up to 50-75% (Table. 1). Analysis of the individual PC species present in these dietary components reveal the presence of PCs of different carbon lengths and fatty acyl chain saturations, with PC (34:1) being the most abundant (Table 2).

Having established that casein is the major source of PC we formulated different types of rodent diets based on the composition of the widely used western diet TD.88137 (42% Kcal from fat) and high fat diet TD.06414 (60% Kcal from fat), respectively. The casein content in the western diet and high fat diet were kept at 195 g/kg and 295 g/kg, respectively. Western diets were formulated with 210 g/kg anhydrous milkfat while high fat diets contained 310 g/kg lard. Three different types of western diet were designed based on the amino acid composition of casein. One of the diets contained casein (Casein Diet). The second diet was made with VTF-Casein while the third diet was constituted by replacing casein with amino acids (AA diets) based on the amino acid profile of casein as provided Envigo. Amino acid composition was adjusted to be equivalent to the levels found in casein. Care was taken to also match the levels of calcium, phosphorus and sucrose in both diets. High fat diets on the other hand were formulated with either casein or amino acids. To determine whether the expected alteration of PC levels occurred in the different formulated diets, lipids were extracted from these diets and their PC compositions analyzed by LC/MS/MS. The results of our analysis show that both the western and high fat diets containing casein had the highest PC contents, while diets formulated with VFT Casein or amino acids had significantly reduced levels of PC (Fig 1). Whereas casein is used primarily as a source of protein in standard laboratory rodent diets from multiple manufacturers these observations identify casein as also the major contributor of dietary PC in these diets.

Dietary casein contributes to circulating plasma PC.

To monitor the contribution of dietary casein to systemic PC pool, we formulated high diets with casein or amino acids based on the amino acid profile of casein and assessed incorporation of dietary choline into plasma PC using Fourier Transform Mass Spectrometry. (FTMS). Fig. 2A depicts a representative section of the spectrum of the MS Scan and labeling of PC(34:2), one of the most abundant PC in the plasma of a mouse fed amino acid defined diet containing deuterium labeled choline. From their calculated exact masses, the highlighted peaks in the spectrum are the signals for D0-PC(34:2) at m/z 758.56927 formed from unlabeled choline, and D9-PC(34:2) at m/z 767.62579 which would have been generated from the deuterated choline supplied in the diets. We further analyzed these samples by LC/MS/MS using the Thermo Q-Exactive All-ions fragmentation (AIF) in positive mode to generate the product ions. As shown in Fig. 2 B and C, we observed a pattern corresponding to the D0- and D9-choline present in the PC(34:2), represented by the m/z 184 and m/z 193 masses of the choline ion moieties respectively. These observations validated biosynthesis of PC from exogenously provided choline and confirm that tracer choline is utilized at high levels and at various intensities. To establish the relative quantities of individual PC species in plasma, we used the high resolution AB Sciex 5600 Quadrupole time of flight (TOF) mass spectrometer. TOF MS data were acquired in positive mode, ions corresponding to the m/z ratios of individual PCs extracted and analyzed using the AB Sciex OS Analytics program. A comparison of the ratios of deuterated PC to the unlabeled PC of the most abundant PCs showed that there was a higher degree of labeled PC in plasma from mice that were fed with the amino acid-defined diets Fig. 2D-I.

Casein promotes breast cancer in E0771 hyperlipidemic mice fed high fat diet.

To understand the effects of dietary casein and inhibition of autotaxin on breast cancer development in hyperlipidemic mice, we fed age-matched LDLR^{-/-} mice with high fat diets (60% kcal from fat) formulated with casein or defined amino acids in place of casein (AA diets). After 12 weeks of feeding, breast cancer was induced in these mice by injection of the syngeneic E0771 mammary tumor cells into the inguinal mammary fat pads while treating with either 30 mg/kg ATX inhibitor PAT-505 or methyl cellulose vehicle control twice daily. In the liver fibrosis mouse models, PAT-505 was efficacious and shown to inhibit ATX activity in plasma and liver tissues after oral administration[39]. We observed reduced primary tumor growth in mice that were fed amino acid defined diets compared to casein-containing diet (Fig. 3A). There was no significant difference in primary tumor volumes and excised tumor masses between PAT-505-and vehicle-treated mice when the animals were fed with casein-containing diet. However, treatment with PAT-505 abolished the tumor growth reducing effects of amino acid-defined diet since tumor volume (Fig. 3A) and excised tumor masses

(Fig. 3B) were higher in PAT-505 treated mice compared to the vehicle-treated mice. Previous studies using a potent ATX inhibitor ONO-8430506 and the highly metastatic 4T1 breast cancer models reported that long term treatment with the ATX inhibitor did not result in significant difference in primary tumor size[26]. The authors observed an initial reduction of tumor growth in ONO-8430506-treated mice which eventually caught up to the vehicle group resulting in comparable primary tumor development. In our studies, treatment with PAT-505 increased the number of metastatic nodules in the lungs of mice that received casein-containing diet but did not have significant metastatic effects in mice that were fed amino acid-defined diet (Fig. 3C).

In vivo Bioavailability of PAT-505 in breast cancer mouse model of cancer and *in vitro* drug potency.

Because of the results in Fig. 3 showing a lack of breast tumor growth inhibition by PAT-505, we decided to develop methods to determine the bioavailability of PAT-505 in harvested plasma and tissues and also validate the drug's potency *in vitro*. The concentration of PAT-505 was assessed in plasma obtained from mice (n=7-9/group) implanted with tumor and receiving twice daily oral administration of the drug. Using tandem MS methods we confirmed the bioavailability of PAT-505 in plasma and tissues harvested from our mice (Fig. 4A). We observed very high levels of circulating PAT-505 in plasma, reaching concentration levels of 60 μ M after one week of drug administration (Fig. 4B). These levels are consistent with the reported pharmacokinetic (PK) of PAT-505 as assessed in mice and rats[39]. We also determined PAT-505 levels in tissues that were harvested after the animals were sacrificed. PAT-505 was detected in the all the organs analyzed with the highest levels detected in the intestines and liver, while the lowest levels were present in the brain and spleen (Fig. 4C). PAT-505 is reported to have nanomolar potency (in vitro IC₉₀ of ~630nM) therefore the very high concentrations of PAT-505 we determined to be present in mouse blood should have been sufficient to efficiently inhibit circulating ATX activity. Previous studies with PAT-505 administered at doses of 30 mg/kg once daily resulted in robust antifibrotic activity[39]. These studies evaluated plasma samples taken 24 hours after the last dose (trough) for ATX lysoPLD activity and trough concentrations in plasma and liver tissues, confirming good oral bioavailability and potency. The potency of PAT-505 against ATX lysoPLD activity was evaluated *in vitro* using recombinant mouse ATX and 16:0 LPC, 17:0 LPC, 18:0 LPC and 18:1 LPC as substrates. PAT-505 inhibited the ATX lysoPLD activity for all the LPC in a concentration dependent manner (Fig. 4D and 4E). Similar to published reports, PAT-505 potency was at nanomolar concentrations with the average IC₅₀ values of 2.0 – 3.0 nM. Despite high dosing for a prolonged period, we observed very modest reduction in the levels of LPA in plasma. Even though the total level of LPA was reduced, only the levels of the species 18:2 LPA and 22:5 LPA were significantly reduced (Fig. 5). This is consistent with the findings of Katsifa et. al., who reported that out of the LPA species they measured following treatment of mice with PF8380, only LPA 18:2 was significantly reduced[15, 41].

Casein increases circulating levels of pro-tumorigenic cytokines and chemokines.

Cytokines and chemokines are increasingly implicated in cancer pathogenesis and present a target for innovative diagnostic and chemotherapeutic agents. Cancer cells can respond to circulating cytokines secreted by the host to promote growth, attenuate apoptosis and facilitate invasion and metastasis. Conversely certain cytokines that are released in response to infection, inflammation and immunity can inhibit cancer development and progress. Therefore, we examined the plasma concentration of a panel of cytokines in plasma from mice fed the casein or amino acid-defined high fat diets in combination with PAT-505 after tumor induction. Using the Magpix multiplex technology (Luminex Corp, Austin, TX) we determined that TGF- β 1 and TGF- β 2 levels were significantly reduced in mice that received amino acid-defined diets compared to casein diets (Figure 6). Treatment with PAT-505 reduced the levels of both TGF- β 1 and TGF- β 2 in casein mice to levels observed in AA-mice but did not have further reduction in AA-mice. In addition, feeding mice with amino acid-defined diets decreased the levels of pro-inflammatory cytokines IL-1 α , IL-12 p40 and M-CSF, and chemokines MCP-1 (CCL2) and KC (CXCL1) compared with casein diets (Figure 6). Treatment with PAT-505 also decreased the levels of TGF- β 1, TGF- β 2, IL-1 α , IL-12 p40, MCP-1 and KC compared with vehicle control (Figure 6).

Discussion

We quantitated PC levels in various fat sources used to manufacture rodent diets and the current findings demonstrate that casein is the major source of PC when present in rodent diets. We further show that replacing casein with amino acids based on casein profile in rodent high fat diets decreases the development of breast cancer in the estrogen-receptor positive syngeneic mouse model.

Although PC is the most abundant phospholipid in eukaryotic membranes and is critical for cell growth and organelle function there are no reports in the literature on the exact composition of PC in rodent diets. Most dietary formulations indicate the contents of choline but not phosphatidylcholine. Casein is usually added to diets as a standard source of protein with no consideration of the levels of PC it adds to diets. The quantities of casein included in diet formulas vary depending on the desired protein composition in these diets. For example, diets used to induce obesity such as western diets (45% kcal from fat) contain approximately 195 g/kg, while high fat diets (60% kcal from fat) are formulated with about 265 g/kg diet weights. In some cases, casein is replaced with defined amino acids. These adjustments in casein quantities and composition would also alter PC composition of the rodent diets. The observation that casein is a significant source of PC may also be important to consider when explaining findings in the literature showing that choline-deficient diets containing higher levels of casein are resistant to induction of fatty liver in the nonalcoholic steatohepatitis (NASH) models[42-45]. This is because casein, loaded with PC, would effectively be a rich precursor that replenishes the omitted choline in the choline-deficient diets.

Our results reveal that formulating rodent diets with amino acids in place of casein reduce breast cancer development. The results are consistent with previous findings that suggest an association between higher dietary casein intake and increased risk of cancer[46-52]. Our study provides further evidence that the tumor promoting effects of casein is due to nutrients contained in casein since supplementing diets with all amino acids that are present in casein as replacement for casein decreases breast tumor development in mice. Casein is a significant source of calcium, phosphorus and vitamins. Therefore, we adjusted the levels of these nutrients by adding vitamin premix with choline and the two minerals to minimize variability among the diets we formulated. Dietary PC has been associated with increased cardiovascular disease risk[53]. We were interested in establishing whether casein also can promote tumor development by being a rich precursor source of PC metabolites. Orally administered PC is hydrolyzed in the intestinal lumen by phospholipase A₂ to form LPC which is absorbed by enterocytes[54]. Some of the absorbed LPC is reacylated to form PC while the rest is hydrolyzed to LPA majorly by the enzyme ATX. Because of the potential of ATX to contribute to many diseases, a number of inhibitors have been described[24, 40, 55-58]. Many ATX inhibitors have been developed some with better in vivo bioavailability. We used the ATX inhibitor PAT-505 which has demonstrated 24-hour

maximal inhibition of ATX activity with once daily dosing[39]. We reasoned that targeting LPA production by depleting PC in diet and inhibiting ATX activity with a potent drug would decrease tumor growth. We used LDLR knockout mice as they have higher circulating levels of LPA[27, 28]. PAT-505 treatment of mice only had modest effects as manifested by only the decreases of plasma concentrations of the saturated species of LPA (18:2-LPA and 22:5-LPA). The PAT-505 effects on other LPA species including unsaturated species was modest but statistically insignificant. However, *in vitro* PAT-505 inhibition assays showed nanomolar potency for both saturated and unsaturated LPC substrates. Our results reveal the existence of a discrepancy in potency of ATX inhibition *in vitro* and *in vivo*. Even though the ATX-dependent pathway is thought to be responsible for most of the LPA present in circulation, a possible explanation for the modest *in vivo* ATX inhibitory effect might be due to the mouse model we used. Given the large excess of substrate availability in hyperlipidemic mice fed high fat diet, it is very likely that the intended ATX inhibition was not achieved since the substrate was not a limiting factor for ATX-mediated LPA production. Furthermore, the presence of an ATX-independent pathway for LPA formation may lead to some resistance to ATX inhibition[59]. In this pathway, PC and other phospholipids are hydrolyzed by phospholipase D (PLD₁ or PLD₂) to generate phosphatidic acid (PA). LPA is then produced from PA by PLA₁ (acting typically on the sn-1 position of PA) or PLA₂ (acting on the sn-2 position). This hypothesis is also supported by the findings of Navab et al., who reported that the conversion of LysoPC 18:0 to LPA 18:0 was not altered by the ATX inhibitor PF8380 suggesting that the conversion is ATX independent *in vivo*[60]. In conditional ATX knockout mice, levels of ATX protein in all tissues tested were substantially reduced yet only LPA 18:2 levels were lowered to levels which were statistically significant[15]. Furthermore, in clinical trials that showed efficacy of the ATX inhibitor GLPG1690 in idiopathic pulmonary fibrosis, drug potency was evaluated by analyzing levels of only LPA 18:2 species[61]. Our observation of a lack of significant inhibition of primary tumor growth by PAT-505 may also be due to the fact that in the E0771 mouse tumor model we were unable to capture the delayed primary tumor growth observed with 4T1 mouse model. Benesch et al., showed in a study using the ATX inhibitor ONO-8430506 in the more aggressive 4T1 mouse breast cancer model that primary tumor growth was suppressed by the ATX inhibitor in the initial phase[26]. After 12 days of tumor growth, even with the continued treatment with ONO-8430506, the primary tumor growth caught up with the vehicle-treated growth with no significant differences thereafter. Furthermore, in our studies we also did not obtain conclusive data on metastasis to the lungs following treatment with PAT-505 suggesting a lack of efficacy of ATX inhibition in the hyperlipidemic E0771 mouse model. However, ATX inhibition with ONO-8430506 in mice fed standard diet, decreases the number of 4T1 metastatic nodules to the lungs. Our results support the theory that factors other than ATX may be more important in driving the long term primary tumor growth when breast tumors invade the abdominal musculature. In addition, the threshold of ATX inhibition required to effect blockade of tumor growth *in vivo* is still unknown.

Inflammation is a known risk factor associated with cancer initiation and progression[62-69]. Our results demonstrate that dietary casein contributes to increased levels of inflammatory mediators in plasma. Several cytokines and chemokines such as IL-1 α , IL-1 β , TGF- β 1, TGF- β 2, IL-10, IL-12p40, LIX, IP-10, KC, MCP-1, MIP-1 β , M-CSF, MIG, RANTES and VEGF were significantly altered between casein and amino acid-defined diets suggesting that casein contributes to modifying levels of these factors in circulation. Some of these cytokines such as IL-1 and VEGF are known to promote angiogenesis, tumor growth and metastasis in experimental models. ATX/LPA nexus upregulates inflammatory signaling[70]. TGF- β has dual role as a tumor suppressor and pro-oncogenic factor and is considered as a predictive biomarkers for progressive tumorigenesis[71]. In the early phase of epithelial tumorigenesis, it inhibits primary tumor development and growth. However, in the late stages of tumor progression, the role of TGF- β becomes one of tumor promotion. Inhibition by PAT-505 also significantly reduced levels of the many of the cytokines and chemokines, although we did not observe a clear potentiation effects of combining ATX inhibition and replacing casein on the levels of circulating cytokines/chemokines.

Conclusions

Our study demonstrates for the first time that casein is the major source of PC when included as a protein ingredient in experimental diets. Levels of PC can be significantly reduced by either solvent stripping of fats from casein or replacing casein with amino acids. These findings provide the basis for adjusting levels of PC when formulating rodent diets especially in studies involving utilization of choline deficient diets. The rate of biosynthesis of PC by the endogenous Kennedy pathway is increased in mice receiving diets in which casein has been replaced by amino acids. We also showed that casein promotes primary tumor growth in the hyperlipidemic murine breast cancer models through a mechanism that involves pro-inflammatory signaling. The cancer-promoting effects of casein does not depend on production of LPA by ATX as inhibition of ATX did not attenuate primary tumor growth either singly or in combination with casein-replacement in these mice. However, the current study does have some limitations. For example, casein contains complex nutrient composition. Although the PC levels in casein is quite profound, it is also possible that the tumor enhancing effects of casein might be due to other factors/nutrients present in casein. A future study using amino acid defined control diets with added PC would be useful in validating the PC effects on tumor growth. Further studies to investigate the link between casein and breast cancer will be able to establish the metabolic processes associated with diets rich in casein and thus provide the basis for dietary approaches to mitigate cancer development and identifying biomarkers for therapeutic interventions.

451 **Acknowledgements**

452 We thank Dr. Andrew J. Morris for providing mass spectrometry facilities, sharing expertise regarding LPA
453 metabolism and critical review of the manuscript. We thank also Dr. Sony Soman and Courtney Hammill for
454 support with mass spectrometry analysis. Dr. Colleen McMullen assisted in preparation and editing of this
455 article.

456 **Funding**

457 This work was supported by grants from NCI K01 CA197073 (FOO); and NCI CRCHD P30CA177558 pilot
458 grant to FOO. The content is solely the responsibility of the authors and does not necessarily represent the
459 official views of the NIH.

460 **Availability of data and materials**

461 The datasets used and/or analyzed during this study are included in this published article or available from the
462 corresponding author on reasonable request.

463 **Contributions**

464 FOO originated the study and was responsible for data collection, analysis, interpretation, and drafting the
465 manuscript. FOO, EB, BY, and EA performed the experiments and participated in writing the manuscript.
466 FOO and LC analyzed the data, edited the manuscript and discussed the data. All authors read and approved
467 the manuscript.

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470 **Ethical declarations**

471 **Ethical approval and consent to participate**

472 Not applicable

473 **Competing interests**

474 The authors declare that they have no competing interests

475

476

477

Figure Legends

Fig. 1. Levels of PC in Western and High Fat Diets formulated with casein or amino acids based on casein profile. The concentration of PC was normalized to the weights of diet pellets. Data shown are means $\pm$ SEM of triplicate determinations. Statistical significance evaluated by Student's *t*-test (* $P < 0.001$)

Fig. 2. Dietary deuterated choline contributes to plasma PC by the two distinct biosynthetic pathways. A. Full scan spectrum indicating some of the most abundant lipids. Peaks for D9-PC(34:2) and D0-PC(34:2) are highlighted. **B and C.** product ion spectra showing the non-deuterated and D9-choline, respectively. **D to I.** Relative proportions of some of the most abundant PCs in mouse plasma from mice fed a diet containing deuterated choline. Comparison between groups was performed by Student *t*-test or repeated measures ANOVA [panels D-I], * $P \leq 0.05$, ** $p \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

Fig. 3. Effects of manipulating dietary casein and ATX inhibition on the growth of breast cancers and lung metastasis. A. Primary tumor volumes of high fat diet-fed mice injected with E0771 tumor cells into the mammary fat pad and gavaged twice daily with either vehicle or PAT-505 30 mg/kg. Tumor volumes were calculated from caliper measurements using $\text{width}^2 \times \text{length} / 2$. Results are expressed as means $\pm$ SEM for 7-8 mice/group. **B.** Masses of primary tumors resected on d 19 after implantation. Tumors resected from mice fed amino acid-defined diet and treated with PAT-505 were heavier than vehicle treated mice. $P = 0.016$. **C.** Tumor burden in the lungs measured by the number of visible lung metastatic nodules in mice one week after primary tumors were resected at d 19, showing higher metastasis in mice fed casein-containing high fat diet. $P < 0.01$.

Fig. 4. PAT-505 plasma and tissue concentrations and in vitro ATX lysoPLD activity. PAT-505 in plasma and tissues was analyzed after continuous twice daily oral administration of PAT-505 to mice at a dosage of 30 mg/kg. **A.** Representative mass spectrometric chromatogram showing peaks for PAT-505 and PF8380 used for quantitation of PAT-505. **B.** Plasma PAT-505 concentration after one week of PAT-505 oral administration to mice ($n = 7-9$ /group). The drug concentration has been normalized using PAT-505 offline calibration and inclusion of PF8380 during sample extraction to monitor analyte recovery. **C.** PAT-505 concentration in tissues harvested after 21 days of treatment with PAT-505 or vehicle control. Values have been normalized to tissue weight. **D and E.** Effects of PAT-505 on in vitro lysoPLD activity was determined using recombinant mouse and human ATX, respectively, in the TOOS assay. Data represent the mean $\pm$ SEM of triplicate determinations from at least three independent experiments.

Fig. 5. Effects of treating mice with 30 mg/kg PAT-505 on plasma molecular species of LPA. Mice were treated twice daily with 30 mg/kg PAT-505 and blood was collected after 7 days of continuous treatment. Control mice were gavaged with 0.5 % Methylcellulose. Plasma LPA levels was determined by UPLC-MS/MS

and concentrations expressed relative to C17:0-LPA internal standard. Results are means $\pm$ SEM for 9-10 mice/group. *P<0.05 vs. corresponding vehicle-treated mice.

Fig. 6. Effects of manipulating casein in high fat diets and PAT-505 treatment on inflammatory mediators in tumor bearing mice. Blood was collected from mice (n=7-9/group) 7 days after twice daily treatment with 30mg/kg PAT-505. Circulating cytokines and chemokines were analyzed and compared between the two diet groups receiving vehicle or PAT-505 treatment. Values are expressed as means $\pm$ SEM of duplicate determinations. Statistical significance was determined by Student's t-test. *P<0.05 vs. corresponding vehicle-treated mice.

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Component	Concentration (pmol/mg)
Lard	14.47
TS - Lard	0.50
Beef tallow	1.63
Cocoa butter	0.18
Coconut oil	2.15
Palm oil	0.12
Corn oil	1.19
TS - corn oil	0.35
Casein	605.65
VFT Casein	166.19

Table 1. Concentration of PC in various fat sources used to manufacture rodent diets

PC Species	Concentration (pmol/mg)			
	Casein	VFT Casein	Lard	Anhydrous Milk
34:1 PC	157.97	62.98	4.09	2.83
32:0 PC	63.84	13.19	0.62	0.66
32:1 PC	38.70	8.91	0.35	0.34
34:0 PC	33.62	7.26	0.25	0.23
34:2 PC	20.58	3.54	0.78	0.33
36:1 PC	68.41	17.90	1.97	0.32
36:5 PC	46.38	11.05	0.07	0.16
36:2 PC	52.54	11.27	3.80	0.36

Table 2. Comparison of the most abundant PC species in various dietary components

	Casein WD 21% AMF Diet (0.2% Chol) (TD.09821)	VFT-Casein WD 21% AMF Diet (VFT, 0.2% Chol, Green) (TD.160726)	AA – WD 21% AMF Diet (AA, 0.2% Chol, Red) (TD.160725)
Macronutrient Composition	(% by weight)	(% by weight)	(% by weight)
Protein	17.3%	17.3	17.2
Carbohydrate	48.7%	49.3	47.4
Fat	21.2.0%	21.0	21.0
	(% kcal from)	(% kcal from)	(% kcal from)
Protein	15.2	15.2	15.4
Carbohydrate	42.9	43.3	42.4
Fat	41.9	41.5	42.3
Kcal/g	4.5	4.6	4.5
Nutrients	g/Kg Diet	g/Kg Diet	g/Kg Diet
Casein	195		
Casein, “Vitamin-Free” Test		188.56	
DL-Methionine	3	3.0	
L-Alanine			4.69
L-Arginine			5.48
L-Asparagine			5.29
L-Aspartic Acid			10.61
L-Cystine			0.55
L-Glutamic Acid			33.85
Glycine			2.82
L-Histidine HCl, monohydrate			5.91
L-Isoleucine			8.02
L-Leucine			14.12
L-Lysine HCl			16.28
L-Methionine			7.5
L-Phenylalanine			7.97
L-Proline			17.29
L-Serine			8.78
L-Threonine			6.43
L-Tryptophan			1.84
L-Tyrosine			8.74
L-Valine			9.7
L-Glutamine			28.68
Sucrose	341.46	341.36	345.49
Corn Starch	100.0	106.44	84.875
Maltodextrin	50.0	50.0	50.0
Anhydrous Milkfat	210.0	210.0	210.0
Cholesterol	1.5	1.5	1.5
Cellulose	50.0	50.0	50.0
Mineral Mix, Ca-P Deficient (79055)			13.37

Mineral Mix, AIN-76 (170915)	35	35.0	
Calcium Phosphate, monobasic, monohydrate			21.75
Calcium Carbonate	4.0	4.0	8.325
Vitamin Mix, Teklad (40060)	10.0	10.0	10.0
Ethoxyquin, antioxidant	0.04	0.04	0.04
Green Food Color		0.1	
Red Food Color			0.1

Supplementary Table 1. Nutritional composition of the casein western diet (WD) (TD.09821), VFT-casein WD (TD.160726) and Amino Acid (AA)-WD (TD.160725)

	60% High Fat Diet (TD.170339)	60% High Fat Diet (AA defined) (TD.170341)
Macronutrient Composition	(% by weight)	(% by weight)
Protein	23.5	23.1
Carbohydrate	27.5	26.4
Fat	34.3	34.0
	(% kcal from)	(% kcal from)
Protein	18.3	18.4
Carbohydrate	21.5	21.0
Fat	60.2	60.7
Kcal/g	5.1	5.0
Nutrients	g/Kg Diet	g/Kg Diet
Casein	265.0	
L-Alanine		6.37
L-Arginine HCl		7.45
L-Asparagine		7.19
L-Aspartic Acid		14.42
L-Cystine	4.0	4.15
L-Glutamic Acid		46.0
Glycine		3.83
L-Histidine HCl, monohydrate		8.03
L-Isoleucine		10.9
L-Leucine		19.19
L-Lysine HCl,		22.13
L-Methionine		6.11
L-Phenylalanine		10.83
L-Proline		23.5
L-Serine		11.93
L-Threonine		8.74
L-Tryptophan		2.5
L-Tyrosine		11.88
L-Valine		13.18
L-Glutamine		38.97
Sucrose	90.0	100.6
Maltodextrin	161.9	150.745
Lard	310.0	310.0
Soybean Oil	30.0	30.0
Cellulose	65.5	65.5
Mineral Mix, AIN-93G-MX (94046)	48.0	
Mineral Mix, w/o Ca & P (98057)		18.36
Calcium Phosphate, dibasic	3.4	20.92
Vitamin Mix, AIN-93-VX (94047)	21.0	21.0
Choline Chloride	1.1	1.1
Food Color	0.1	0.1

Calcium Carbonate		4.375
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Supplementary Table 2. Nutritional composition of the casein High Fat Diet (TD.170339) and Amino Acid (AA) defined-High Fat Diet (TD.170341)

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figures

Figure 1

The results of our analysis show that both the western and high fat diets containing casein had the highest PC contents, while diets formulated with VFT Casein or amino acids had significantly reduced levels of PC

Figure 2

Fig. 2A depicts a representative section of the spectrum of the MS Scan and labeling of PC(34:2), one of the most abundant PC in the plasma of a mouse fed amino acid defined diet containing deuterium labeled choline. From their calculated exact masses, the highlighted peaks in the spectrum are the signals for D0-PC(34:2) at m/z 758.56927 formed from unlabeled choline, and D9-PC(34:2) at m/z 767.62579 which would have been generated from the deuterated choline supplied in the diets. We further analyzed these samples by LC/MS/MS using the Thermo Q-Exactive All-ions fragmentation (AIF) in positive mode to generate the product ions. As shown in Fig. 2 B and C, we observed a pattern corresponding to the D0- and D9-choline present in the PC(34:2), represented by the m/z 184 and m/z 193 masses of the choline ion moieties respectively. TOF MS data were acquired in positive mode, ions corresponding to the e m/z ratios of individual PCs extracted and analyzed using the AB Sciex OS Analytics program. A comparison of the ratios of deuterated PC to the unlabeled PC of the most abundant PCs showed that there was a higher degree of labeled PC in plasma from mice that were fed with the amino acid-defined diets Fig. 2D-I.

Figure 3

Figure 4

Using tandem MS methods we confirmed the bioavailability of PAT-505 in plasma and tissues harvested from our mice (Fig. 4A). We observed very high levels of circulating PAT-505 in plasma, reaching concentration levels of 60 μM after one week of drug administration (Fig. 4B). These levels are consistent with the reported pharmacokinetic (PK) of PAT-505 as assessed in mice and rats[39]. We also determined PAT-505 levels in tissues that were harvested after the animals were sacrificed. PAT-505 was detected in

the all the organs analyzed with the highest levels detected in the intestines and liver, while the lowest levels were present in the brain and spleen (Fig. 4C).

Figure 5

imilar to published reports, PAT-505 potency was at nanomolar concentrations with the average IC₅₀ values of 35 2.0 – 3.0 nM. Despite high dosing for a prolonged period, we observed very modest reduction in the levels of LPA in plasma. Even though the total level of LPA was reduced, only the levels of the species 18:2 LPA and 22:5 LPA were significantly reduced (Fig. 5).

Figure 6

Using the 347 Magpix multiplex technology (Luminex Corp, Austin, TX) we determined that TGF- β 1 and TGF- β 2 levels were significantly reduced in mice that received amino acid-defined diets compared to casein diets (Figure 6).