



Defect in Ser312 phosphorylation of *Tp53* dysregulates lipid metabolism for fatty accumulation and fatty liver susceptibility: Revealed by lipidomics

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ABSTRACT

The *Tp53* gene is a well-known tumour suppressor, mutation of which (e.g. prevention of Ser312 phosphorylation) induces deletion or expression of an inactive *p53* protein to increase the susceptibility of tumour occurrence. However, the role of *Tp53* gene in maintaining metabolic homeostasis for regulating physiopathological activities is still not well-understood. This study aimed to use the lipidomics study as a systematic approach to understand the relationship between the phenotypic effects of *Tp53* mutation on lipid-related endogenous metabolites. Plasma and liver samples from mice carrying a *Tp53* Ser312 to Ala mutation and wild type mice were collected, lipids were extracted by liquid-liquid extraction method and analyzed by the RPLC-LTQ-FTMS for the lipidomics study. Our results indicated that defect in Ser312 phosphorylation of *Tp53* leads the lipid disturbance (e.g. triacylglycerols) for fatty accumulation and fatty liver susceptibility, which is with preference of females. Histological observation by staining with haematoxylin and eosin further validated our lipidomics findings. To our conclusion, fatty liver occurrence may have different phenotypes, one of which is strongly linked with the *Tp53* mutation and is susceptible in females. Lipidomics as a technique to detect a great number of endogenous compounds provides precise metabolic information that may further help improve personalized diagnosis of Chronic hepatic diseases.

1. Introduction

The tumour suppressor protein *p53* gene (*Tp53* or *p53*) is the most frequently mutated gene in human cancer. As a transcription factor, *Tp53* is able to transactivate and transrepress hundreds of genes that are involved in a diverse array of biological outcomes including cell cycle arrest, DNA repair, apoptosis, aberrant oncogene activation, senescence, and metabolic regulation [1]. In cells, *Tp53* protein acts as a master sensor of stress and it responds to stress signals such as DNA damage,

replication stress, hypoxia and metabolic stress. One of the most known functions of *Tp53* is its ability to maintain genomic stability, by preventing transmission of damage to daughter cells. When the *Tp53* gene is mutated so that the *p53* protein is inactive or absent, this damage persists and can result in deregulated cell growth and tumour development. A reflection of the importance of *Tp53* is the high mutation rate of the *Tp53* gene in cancers and germline mutation leads to cancer susceptibility in humans. Experimentally mice lacking *Tp53* are highly tumour prone, resemble that seen in humans.

Abbreviations: DG, diacylglycerols; LLE, Liquid-liquid extraction; LPC, lysophosphatidylcholines; LPE, lysophosphoethanolamines; OPLS-DA, Orthogonal Partial Least Square-Discriminant Analysis; PC, Phosphatidylcholine; PCA, Principal component analysis; PE, phosphoethanolamines; SM, sphingomyelins; TG, triacylglycerols; *Tp53*, Tumour protein 53.

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In vivo, there is a strong tissue context dependent activity of *Tp53*. In response to the same stimuli, gamma radiation for example, *Tp53* expression is induced in some tissues but not the others. Additionally, even in the tissues with induced *Tp53*, elevated *Tp53* can only induce apoptosis in some tissues such as intestine and thymus but not in the other tissues such as lung. All these illustrate the complexity of *Tp53* regulation [2,3]. Consistent with this, *Tp53* mutation frequency also varies from one tumour type to another. For example, the incidence of *Tp53* mutation is over 80 % in ovarian adenocarcinomas, around 30 % in breast and liver cancers, and <10 % in neuroblastomas and myeloid cancers [4].

It has been well established that *Tp53* activity is highly regulated at its protein level and it is susceptible to multiple post-translational modifications, including multiple phosphorylation sites clustered at the N- and C-termini. Phosphorylation at these sites regulates the stability of *Tp53* and they are primarily phosphorylated by stress kinases such as ATM in response to DNA damage [2]. To date, the precise function of phosphorylation of *Tp53* on these sites remains largely unknown due to the mild phenotypes observed in *Tp53* phosphorylation sites mutation carrying mice [5]. One of the exceptions, however, is the mouse carrying a knock in Ser312 to Ala mutation in their *Tp53* gene (*Tp53Ser312A* mice, equivalent to human codon 315), preventing phosphorylation on Ser312 in mouse. Following exposure to ionising radiation or DNA alkylating agent, *Tp53Ser312A* mice principally develop T cell lymphomas, which are typical of the tumours that develop in mice with compromised *Tp53* function.

Metabolomics has been used as a part of systems biology, especially to study small molecule mediators for the identification of diseases, exploring potential biological pathways and treatment mechanisms [6]. A wide range of endogenous molecules, so-called metabolites (such as amino acids [7], vitamins and hormones [8], oxylipins [9], fatty acids [10], etc.), have been reported to be involved in the metabolic process in various diseases. Among the dysregulated metabolites, some lipids are considered as potential biomarkers and therapeutic targets of inflammatory diseases including cancer [11]. There are many different classes of lipids that related to diseases, such as cholesterol, triacylglycerols, phospholipids, glycerol (phosphor) lipids, sterol lipids, sphingolipids, lipoprotein associated fatty acids, etc. [12]. Recently, a robust metabolomics protocol has been established to detect endogenous membrane lipidomics in mice plasma, where various classes of lipids including phosphatidylcholines (PCs), phosphoethanolamines (PEs), lysophosphatidylcholines (LPCs), lysophosphoethanolamines (LPEs), diacylglycerols (DGs), triacylglycerols (TGs), sphingomyelins (SMs), and cholesteryl esters (ChoEs) were detected [13]. The potential ability of wild type *Tp53* to act as a regulator of endogenous metabolism especially lipidomics is emerging, which is partly achieved through its ability to regulate the expression and/or activities of a number key players of lipidomics such as SREBP1, C6PD and Sirt1. Although the reason why *Tp53Ser312A* mice are susceptible to liver cancer development in response to gamma irradiation is still unknown, however one hypothesis is that the mutation of *Tp53* has ability to dysregulate the lipidome in *Tp53Ser312A* mice, which process is most prominent in liver. If this is indeed the case, one would detect an altered lipid profile and a liver phenotype in *Tp53Ser312A* mice compared with *Tp53wt* mice even in the absence of gamma radiation. Thus, we took an advantage of this recently developed technology to investigate whether mutation of *Tp53* by preventing of phosphorylation at Ser312 is able to influence its ability to regulate lipidomics *in vivo* by comparing 1) the lipidomics profiles and 2) the liver histology between WT *Tp53wt* and *Tp53Ser312A* mice. We found many different species of lipids that relate to fatty liver were significantly dysregulated by the *Tp53* mutation, which are sex-associated and hepatic diseases-associated. Our lipidomics findings were further validated by the histological staining with haematoxylin and eosin. This may raise the importance of the *p53* mutation in the hepatic diseases such as fatty liver. Importantly, phenotyping and personalized medicine for the hepatic diseases should be drawn more to

people's attention.

2. Materials and methods

2.1. Mice

A total of 21 wild-type (WT) mice (including 10 female and 11 male) and 21 homozygous *Tp53*^{312A/A} mice (including 10 female and 11 male) were kindly provided by Ludwig Institute for Cancer Research (United Kingdom). The detailed *Tp53*^{312A/A} knock in mice were as described previously [5]. The mice were on a mixed C57BL/6; 129/Sv; FVB strain background. Animals were fed with standard diet. All animal procedures were given ethical approved by the University of Oxford and licensed by the U.K. Home Office.

2.2. Preparation of liver tissue and plasma samples

After sacrifice of the mice, a total of 42 plasma samples and 42 liver samples from wild-type (WT) and homozygous *Tp53*^{312A/A} mice were kindly collected and provided by Ludwig Institute for Cancer Research (United Kingdom), specifically 10 female WT mice vs 10 female *Tp53*^{312A/A} mice, and 11 male WT mice vs 11 male *Tp53*^{312A/A} mice. Plasma samples were immediately combined with heparin (LEO Pharma, The Netherlands) after the collection to prevent coagulation and all plasma and liver tissue samples were stored at −80 °C as soon as possible until required.

2.3. Chemicals and lipid internal standards

HPLC-grade dichloromethane and UPLC-MS grade methanol, acetonitrile and isopropanol were obtained from Biosolve (Valkenswaard, The Netherlands). Internal standards containing cholesteryl heptadecanoate ChoE (17:0), 1,2,3-Tripentadecanoylglycerol TG (15:0/15:0/15:0) and 1,2,3-triheptadecanoylglycerol TG (17:0/17:0/17:0) were purchased from Sigma-Aldrich Chemie B.V. (Zwijndrecht, the Netherlands). The other lipids including 1-heptadecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine PC (17:0/0:0), 1,2-diheptadecanoyl-*sn*-glycero-3-phosphocholine PC (17:0/17:0), 1-nonadecanoyl-2-hydroxy-*sn*-glycero-3-phosphocholine PC (19:0/0:0), 1,2-dinonadecanoyl-*sn*-glycero-3-phosphocholine PC (19:0/19:0), 1,2-dipentadecanoyl-*sn*-glycero-3-phosphoethanolamine PE (15:0/15:0), 1,2-diheptadecanoyl-*sn*-glycero-3-phosphoethanolamine PE (17:0/17:0), 1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) PG (14:0/14:0), 1,2-diheptadecanoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) PG (17:0/17:0) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA).

2.4. Lipid extractions

The liquid–liquid extraction (LLE) method for acquiring plasma lipidome was mainly performed according to the previous study by Hu et al. [13]. Generally speaking, 30 µL heparin plasma was taken into a 2 ml-micro tube (Eppendorf, Hamburg, Germany) and was combined with 30 µL of mixture of internal standards (concentrations were as described by Hu et al. [13]), followed by the addition of methanol (190 µL) and dichloromethane (380 µL). The solutions were vortexed and 120 µL of water was added to obtain a two-phase system. The lower organic phase (300 µL) that contained virtually all the lipids was then centrifuged at 6000 g for 10 min at 10 °C for further analysis. Twenty-five µL of the lipid extracts was diluted with 475 µL acetonitrile-isopropanol-water (65:30:5, v/v/v) and stored at −20 °C until analysis and 10 µL was injected for the UPLC-MS analysis.

Extraction of lipids from the frozen liver samples was achieved by liquid–liquid-extraction (LLE) with methanol-dichloromethane (1:2, v/v) based on Wei et al. [14]. In general, 10 mg of the lyophilized frozen liver was crushed into a powder and put into a 1.5 ml-micro tube. A

mixture of methanol (180 μ L)-dichloromethane (360 μ L) was added, followed by 60 μ L of internal standards mixture and vortexed. Concentrations of the internal standard mixture were the same as mentioned by Wei et al. [14]. The suspension was placed in an ultrasonic bath at 4 °C for 5 min and shaken continuously in a shaker at 4 °C for 45 min. After centrifuging (6000 g) at 10 °C for 10 min, 500 μ L of the supernatant was transferred into a new microtube and 100 μ L of 0.9 % NaCl solution (w/v) was added to obtain a two-phase system. The lower organic phase (300 μ L) containing virtually all the lipids were then centrifuged at 2000 g at 10 °C for 10 min before further analysis. Finally, a volume of 10 μ L of the lipid extracts was injected into the LC-MS system after diluting 40 times from the final extracts.

2.5. LC-MS lipid profiling

Each lipid extract sample was analyzed by reversed-phase liquid chromatography on an Ascentis Express column (C8 2.1 \times 150 mm, particle size of 2.7 μ m, Sigma-Aldrich Chemie B.V., Zwijndrecht, the Netherlands), coupled with linear ion trap-Fourier transform ion cyclotron resonance-mass spectrometry (RPLC-LTQ-FTMS) (Thermo Fisher, San Jose, CA, USA). An auto-sampler equipped in the LC system was used for sample injections into the LC-MS system. A binary gradient with Eluent A (water-acetonitrile (40:60, v/v) containing 10 mM ammonium formate) and Eluent B (acetonitrile-isopropanol (10:90, v/v) containing 10 mM ammonium formate) was used as a mobile phase. A Mass spectrometer equipped with an ESI source was used for the full profiling of lipids in positive ion mode. The detailed LC separation condition and the identifications of the detected lipid peaks were as described in previous report [13]. This method covered eight different lipid classes including Cholesteryl esters (ChoEs), lysophosphatidylcholines (LPCs), lysophosphoethanolamines (LPEs), phosphatidylcholines (PCs), phosphoethanolamines (PEs), Diacylglycerols (DGs), Triacylglycerols (TGs), and Sphingomyelins (SMs).

2.6. Data processing and statistics

Peaks were determined using the software LCQuan v2.5 (Thermo Fisher), and peak areas were exported and normalized using the relevant internal standards. Statistical data visualization and analysis were based on metaboanalyst (V4.0) (<https://www.metaboanalyst.ca>). The log transformation (G-log) with Pareto-scaling (mean-centred and divided by the square root of the standard deviation of each variable) was used in order to get a normal distribution of the original data. Univariate analysis (two-tailed unpaired Student's *t*-test) was employed to evaluate significant differences between two groups for each individual lipid (determined by $p < 0.05$). Box-plot chart analysis was performed using GraphPad Prism 7 software (Graph Pad Software, San Diego, CA, USA). Principal component analysis (PCA) and Orthogonal Partial Least Square-Discriminant Analysis (OPLS-DA) were performed in order to further investigate the discrimination of the lipidomics data between groups [15,16]. A permutation test with 100 iterations was performed to estimate the null distribution, by randomly permuting the class labels of the observations. *p*-values of each pair of comparison in the permutation test were calculated to evaluate the null hypotheses. The most contributed lipids to the group separation were selected by the correlation score ($p[\text{corr}]$) of the loading plot for the variable influence based on the OPLS-DA analysis.

2.7. Staining and microscopic observation of livers from mice

Livers from four *Tp53* wild type mice (2 male, 2 female), thirteen heterozygous *Tp53*^{312A/S} (5 male, 8 female) and six homozygous *Tp53*^{312A/A} (3 male, 3 female) mice were collected for the histological staining experiments. The collected livers were firstly fixed in neutral buffered formalin, then were dehydrated through increasing ethanol concentrations, followed by xylene and embedded in paraffin. Sections

were cut and stained with haematoxylin and eosin [17]. Hepatic steatosis (fatty liver) was assessed by microscopic inspection. Significance was calculated using Fisher's exact test.

3. Results

Mutations of *Tp53* gene have been detected in precancerous stages of several cancers. Experimentally mice lacking *Tp53* are highly tumour prone especially in liver, which is well known. But if and how the *Tp53* mutation dysregulate the lipidomic metabolism is still unclear. We hypothesised that a perturbation of lipidomics profile may differ between *Tp53* WT versus *Tp53*Ser312A mice, which is with tumour susceptibility. Additionally, Plasma is a complex bio-fluid composed of a variety of metabolites, including lipids. It is now emerging that perturbation of lipidomics in circulating plasma is associated with fatty liver condition such as non-alcohol associated fatty liver disease (NAFLD) (Feldstein et al., 2010). Therefore, lipidomics profiling can be used as a read out of liver homeostasis. We hypothesised that a perturbation of lipidomics profile in circular plasma may differ between *Tp53* WT versus *Tp53*Ser312A mice. To test our hypothesis, we first generated lipidomics profiles from the liver and plasma of 42 mice (10 female WT mice vs 10 female homozygous *Tp53*^{312A/A} mice, and 11 male WT mice vs 11 male *Tp53*^{312A/A} mice) respectively, and detected a total of 147 hepatic lipids and a total of 155 plasma lipids.

3.1. Hepatic and plasma lipidomics associate with sex differences

Sex effects on metabolomics have been mentioned in some studies including lipids (Yetukuri et al., 2007). We evaluated firstly how the sex factor on the hepatic and plasma lipidomics in our *Tp53*^{312A/A} and/or WT mice respectively. In order to generally visualize the group discrimination by the hepatic and plasma lipidomics profile, PCA score plot analysis was performed on the 147 hepatic lipids and 155 detected plasma lipids respectively. As shown in Fig. 1, the unsupervised PCA score plot displayed a sex-associated clustering, although in the WT mice 2 female samples were misclassified into the male group (Fig. 1A). This analysis revealed that sex is the most profound contributing factor that causes the PCA clustering regardless of the genotype.

Next, to ascertain which hepatic or plasma lipid molecules made the biggest contributions to the sex difference, univariate analysis with students' *t*-test was performed to analyze the significance of the change in level for each individual lipid. A supervised multiple variate analysis (OPLS-DA) was also performed to filter the important variables that contribute to the group classification in a different way (Supplementary Fig. 1). The Q², R²Y and *p*-values in the 100 times permutation indicated a good performance, without any over-fittings (Supplementary Fig. 1). The hepatic lipids that were significantly either up or down regulated are listed in Table 1, and changes to each individual lipid are displayed in plots in Fig. 2A and 2B. The plasma lipids whose levels were significantly up or down regulated are listed in Table 2, and the relative levels for each lipid are shown in boxplots in Fig. 2C and 2D.

As shown in Table 1 and comparing the Fig. 2A with 2B, the change in hepatic lipid levels with respect to sex was more pronounced in *Tp53*^{312A/A} mice, as these mice had a greater number of lipids with significantly changed levels between males and females (Fig. 2B). Levels of eight of these lipids were significantly changed in both WT and *Tp53*^{312A/A} mice, including elevated levels of TGs ($n = 7$) and attenuated ChoE (18:1) in the males. This may indicate that these lipids contribute to sex differences but not to phenotypic differences. Hepatic lipids revealed a considerable difference between genders, with a diversity of lipids that specifically appeared. For instance, only 2 PCs and 1 SM changed significantly in the WT group with a relatively lower level in the male than that in the female, while the *Tp53*^{312A/A} mutant mice appeared to have a large number of PCs ($n = 18$) and SMs ($n = 8$) which were all elevated in male mice. An additional attenuated PE (36:3) in male was shown only in mutant mice but not in the WT group. These

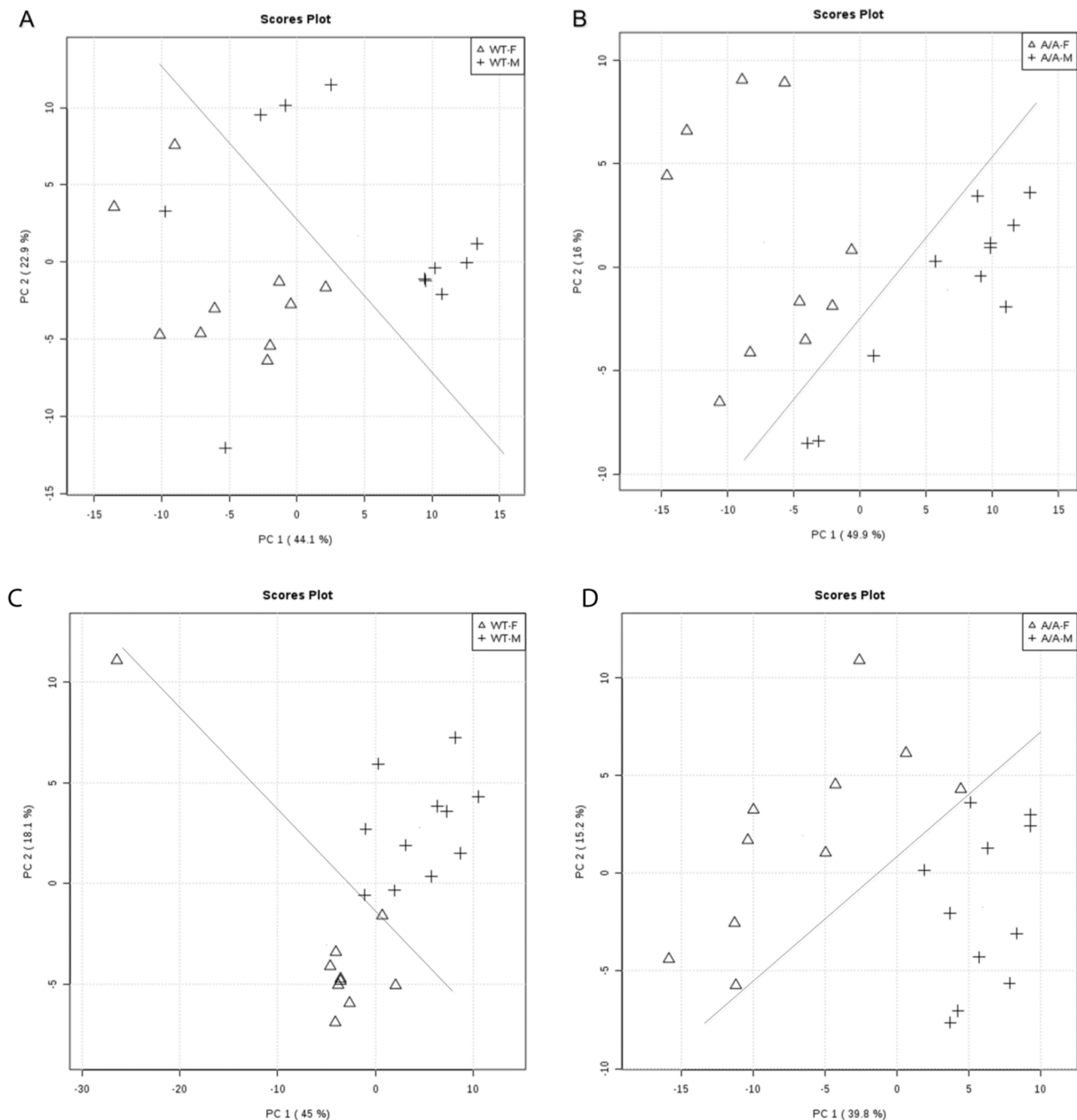


Fig. 1. PCA score plot of lipidomics data for sex classifications. (A) Hepatic lipidomics data of WT female mice vs WT male mice; (B) Hepatic lipidomics data of *Tp53*^{312A/A} female mice vs *Tp53*^{312A/A} male mice; (C) plasma lipidomics data of WT female mice vs WT male mice; (D) plasma lipidomics data of *Tp53*^{312A/A} female mice vs *Tp53*^{312A/A} male mice. Peak areas were exported and normalized using the relevant internal standards.

lipids may not only contribute solely to the sex difference but to the sex-associated (tumour) disease considering their presence only in the *Tp53*^{312A/A} mice group.

It is clear from Table 2 and Fig. 2C and 2D that a large number of plasma lipids contributed also to the sex difference, irrespective of whether the sample was from the *Tp53*^{312A/A} or WT group. Generally speaking, levels of LPCs (lysophosphatidylcholines), PCs (phosphatidylcholines), LPEs (lysophosphoethanolamines), ChoEs (cholesteryl esters) and TGs (triacylglycerols) were higher in male samples than in female. An increased DG (diacylglycerol) (O-42:7) in males was present only in the WT group, while an increased PE (phosphoethanolamine) (38:4) in males was present only in the *Tp53*^{312A/A} group but not in WT mice. The same sphingomyelins (SM) showed the same up/down

regulations in WT and in *Tp53*^{312A/A} group. While these SMs were neither always increased together nor decreased together, but with diversity in trends: some were relative higher in the male, others were lower in male. For instance, male mice have relatively lower levels of SMs (d18:1/14:0, d18:1/15:0, d18:1/16:0, d18:1/18:1, d18:1/18:0, d18:0/24:1 and d18:0/25:0) but higher levels of d18:1/20:0, d18:1/22:1 and d18:1/22:0 in both WT and in *Tp53*^{312A/A} mice. These may indicate the important contributions of SMs to the sex effect. However, their biological significance needs to be further investigated.

Another interesting observation related to sex was that we found more special plasma lipids contributed to the sex difference in *Tp53*^{312A/A} group (ChoE (18:2), ChoE (20:4), LPC (22:6/0:0), PC (38:4), PE (38:4), SM (d18:1/17:0), SM (d18:0/24:2), TG (56:7), TG (58:6)).

Table 1Hepatic lipids significantly affected by sex in wild type and in $Tp53^{312A/A}$ mice.

| Hepatic lipids | Gender difference in $p53^{312A/A}$ mice (Male vs Female) | Gender difference in WT mice (Male vs Female) |
|-----------------|---|---|
| ChoE_(18:1) | ↓** | + ↓*** |
| TG_(56:1) | ↑** | + ↑*** |
| TG_(58:4) | ↑*** | + ↑*** |
| TG_(58:3) | ↑*** | + ↑*** |
| TG_(58:2) | ↑*** | + ↑*** |
| TG972.89 | ↑*** | + ↑*** |
| TG974.91 | ↑*** | + ↑*** |
| TG_(60:3) | ↑*** | + ↑*** |
| PC_(38:4) | — | + ↓*** |
| PC_(40:6) | — | + ↓*** |
| PC_(32:2) | ↑*** | — |
| PC_(32:1) | ↑*** | — |
| PC_(O-34:3) | ↑*** | — |
| PC746.61 | ↑*** | — |
| PC_(34:4) | ↑*** | — |
| PC_(O-36:3) | ↑*** | — |
| PC_(O-36:2) | ↑*** | — |
| PC_(36:6) | ↑*** | — |
| PC_(36:5) | ↑*** | — |
| PC_(O-38:7) | ↑*** | — |
| PC_(O-38:6) | ↑** | — |
| PC_(38:7) | ↑*** | — |
| PC_(38:2) | ↑*** | — |
| PC_(O-40:6) | ↑*** | — |
| PC_(40:8) | ↑*** | — |
| PC_(40:5) | ↑** | — |
| PC_(40:4) | ↑*** | — |
| PC_(O-42:5) | ↑*** | — |
| PE_(36:3) | ↓** | — |
| SM_(d18:1/16:1) | ↑*** | — |
| SM_(d18:1/17:0) | ↑** | — |
| SM_(d18:1/18:2) | ↑*** | — |
| SM_(d18:1/20:1) | ↑*** | — |
| SM_(d18:1/21:0) | ↑*** | — |
| SM_(d18:1/23:1) | ↑*** | — |
| SM_(d18:1/23:0) | ↑*** | — |
| SM_(d18:0/25:0) | ↑*** | — |
| SM811.67 | — | + ↓*** |
| TG738.66 | — | + ↓*** |

The hepatic lipids showed sex differences existing in WT (left hand column) and $Tp53^{312A/A}$ mice (right). The changes of lipids were marked with arrow (↑ or ↓), in order to indicate their regulations in male versus female. The important contributors were filtered by both OPLSDA $p[\text{corr}] > 0.7$ and t -test ($p < 0.05$). The hepatic lipids were displayed in bold font if they are altered in both WT or $Tp53^{312A/A}$ mice. ***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$; +: Important contributors were filtered only by OPLSDA ($p[\text{corr}] > 0.7$).

However, considering that these lipids are present only in the $Tp53^{312A/A}$ mice group, these plasma lipids may not only contribute purely to the sex differences but may contribute to the sex-associated diseases (e.g., a tumour: such as breast cancer or ovarian cancer in females).

3.2. Defect in Ser312 phosphorylation of $Tp53$ dysregulates hepatic lipids

Given the sex effect upon hepatic lipids, we explored phenotypic differences in males and females separately to avoid the impact that sex may have on any lipid phenotype in the $Tp53^{312A/A}$ mice. We examined the effect of the $Tp53^{312A/A}$ mutation on hepatic lipidomics using the univariate student's t -test ($p < 0.05$) as well as the supervised OPLS-DA (Supplementary Figure 2A and 2B) to give a list of contributory lipids. Table 3 lists the hepatic lipids that were significantly up or down regulated with the $Tp53^{312A/A}$ mutation in males and females

respectively. Changes of each individual are also displayed in boxplots in Figure 3A and 3B. As shown in Table 3, Figure 3A and 3B, a total number of 11 lipids in male and 12 lipids in female mice were significantly altered in the $Tp53^{312A/A}$ mice compared with the WT mice. All the eleven hepatic lipids, consisting 6 PCs (54.5 %), 4 TGs (36.4 %) and 1 SM (d18:1/18:2), were elevated in the $Tp53^{312A/A}$ male mice (Table 3 and Fig. 3A). In contrast, the significantly changed hepatic lipids in females consisted of 10 TGs (83.3 %), 1 PC (8.3 %) and 1 DG (8.3 %), all of which showed an increase in the $Tp53^{312A/A}$ mutant mice but with an exception of the decreased PC (40:6). This may indicate the importance of the hepatic TG levels that associate strongly with the $Tp53^{312A/A}$ mutation in both males and females, while hepatic PCs were more important only in males carrying the $Tp53^{312A/A}$ mutation. Four TGs (TG (56:0), TG 948.89, TG 978.94 and TG (60:1)) got our attention, which were elevated in both $Tp53^{312A/A}$ male and $Tp53^{312A/A}$ female mice compared with WT (Table 3, marked with bold font and listed on top of the table). This may further indicate the potential importance of TGs dysregulated by the $Tp53$ mutation.

3.3. Defect in Ser312 phosphorylation of $Tp53$ alters plasma lipids

We also explored plasma phenotypic differences in male and female mice separately due to the sex variance observed in plasma lipid levels. With consistence of the hepatic analysis, we evaluated the effect of the $Tp53^{312A/A}$ mutation on the plasma lipid species according to the univariate student's t -test ($p < 0.05$) as well as the supervised OPLS-DA (Supplementary Fig. 2C and 2D). Table 4 lists the plasma lipids that were significantly up or down regulated in the $Tp53^{312A/A}$ mice compared with WT in males and in females respectively. Change for each individual lipid is displayed graphically in Fig. 3C and 3D. Generally, the $Tp53^{312A/A}$ female mice displayed more numbers of the plasma lipids that were significantly changed than the males with the same mutation (Table 4 and Fig. 3C and 3D). In details, a total of 21 lipids in $Tp53^{312A/A}$ females and 6 lipids in $Tp53^{312A/A}$ male mice were considered as the main plasma lipids that associate with the $Tp53^{312A/A}$ mutation. Among the 21 lipids filtered in the $Tp53^{312A/A}$ female mice, most of the important contributors to the $Tp53$ mutation phenotype were TGs ($n = 9$) and LPCs ($n = 8$). This indicates again the important role of TGs, as well as LPCs that in relation to the $Tp53$ mutation.

When looking into the up-down regulations, $Tp53^{312A/A}$ mutant female mice showed mostly increased lipids (mainly TGs, LPC and LPEs) compared with the female WT mice. While in $Tp53^{312A/A}$ mutant male mice, however, LPCs showed no significant changes and only 2 TG (46:0 and MS974.91) were elevated, the rest (4 out of 6) lipids decreased significantly (DG_O-42:7, PE36:3, SM17:0, SM18:1). Importantly, two plasma lipids, TG (46:0) and DG (O-42:7), were significantly altered in both males and females of the $Tp53^{312A/A}$ mutant mice. In details, TG (46:0) levels were elevated in $Tp53^{312A/A}$ mutant mice versus WT mice, irrespective of sex factor. DG_ (O-42:7), however, was down regulated in male mutant mice but conversely was increased in the female mutant mice. Therefore, TG (46:0) has a potential as an important indicator for the $Tp53^{312A/A}$ mutation. Our lipidomics study reveals that defect in Ser312 phosphorylation of $Tp53$ causes dysregulation of lipids profile.

3.4. Phosphorylation of $Tp53$ at Ser312 prevents fatty liver incidence in female mice

Among its many functions, the liver is responsible for producing bile, which aids in the breakdown and detoxification of lipids. The liver is also one of the main sites where tumours are observed in $Tp53^{312A/A}$ mice following irradiation [5]. Our lipidomics results in this study displayed many elevated plasma and hepatic lipids (such as TGs) in the $Tp53^{312A/A}$ mice, which is with preference in females. The lipids accumulation in liver may suggest the morphology changes of liver tissues. For validating this hypothesis, an additional histological experiment was achieved for evaluating the histological appearances of the fatty

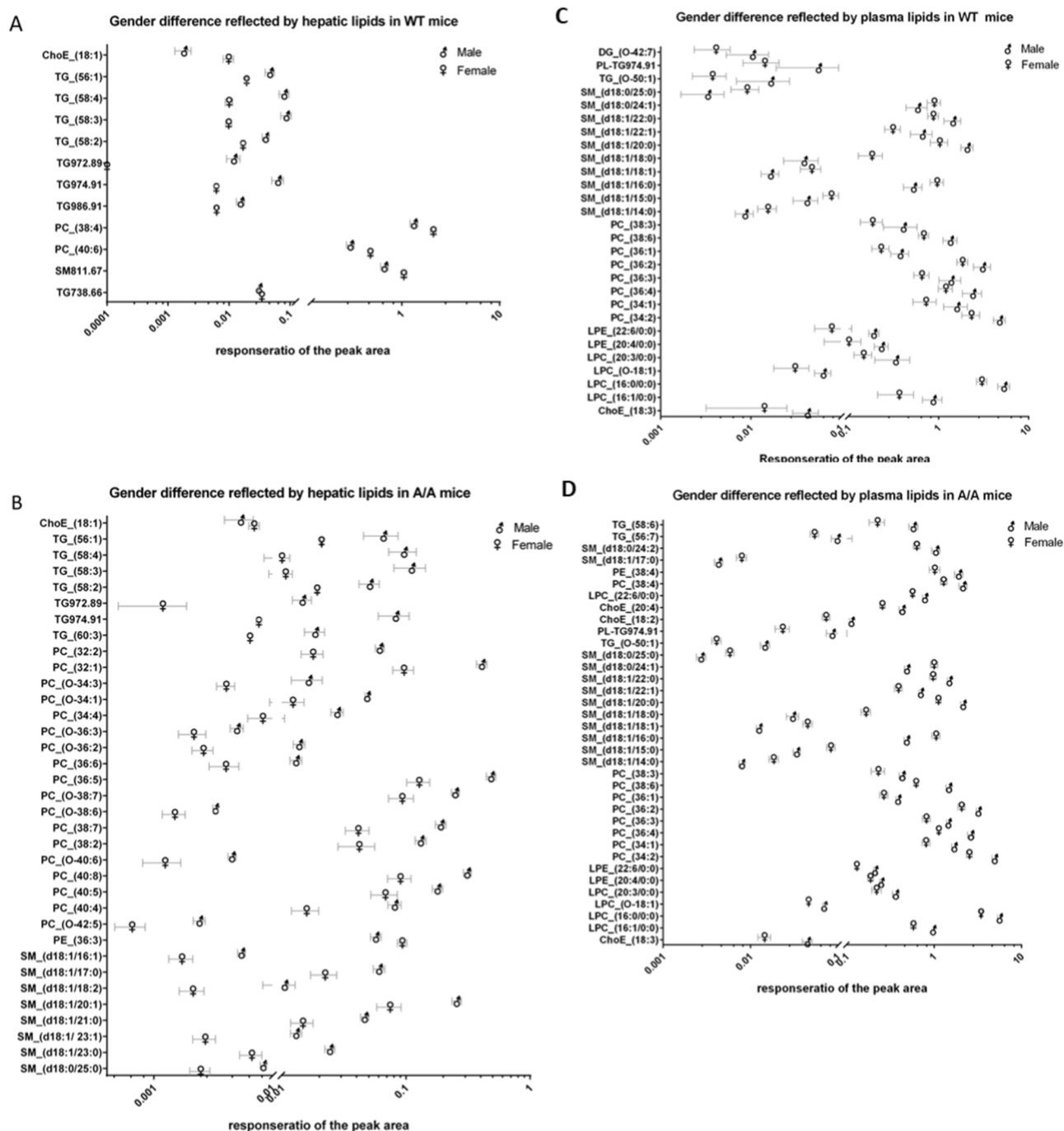


Fig. 2. Sex difference reflected by hepatic and plasma lipids profiles. (A) hepatic data in WT mice; (B) hepatic data in *Tp53*^{312A/A} mice; (C) plasma data in WT mice; (D) plasma data in *Tp53*^{312A/A} mice. Peak areas were exported and normalized using the relevant internal standards. Hepatic and plasma lipids in the corresponding sex were significantly different, evaluated by both student's *t*-test ($p < 0.05$) as well as $p[\text{corr}] > 0.7$ based on the loading plot from OPLS-DA. The data were expressed as mean value \pm SEM.

accumulation and fatty liver and their relation to sex preference. Livers derived from transgenic mice which carry one or two copies of Ser312 mutated *Tp53*; *Tp53*^{312A/S} and *Tp53*^{312A/A} mice respectively were compared to that of wild type *Tp53* mice. Livers from four *Tp53* wild type mice (2 male, 2 female), thirteen heterozygous *Tp53*^{312A/S} (5 male, 8 female) and six homozygous *Tp53*^{312A/A} (3 male, 3 female) mice, were embedded in paraffin and stained with haematoxylin and eosin. Fatty liver (hepatic steatosis), as recognised by the presence of large circular vesicles (see Fig. 4, Table 5), was observed in 5/7 of the *Tp53*^{312A/A} mice, compared to 2/5 wild type. 5/13 heterozygous showed some signs of fatty liver (see Fig. 1, Table 1). Although the highest proportion of

animals with fatty liver came from the *Tp53*^{312A/A} cohort, this was not significant. When the sex of the animals was compared, however, the majority of animals showing fatty liver were female ($p < 0.05$). The only male to show fatty liver was a *Tp53*^{312A/A} homozygote. These results suggest that *Tp53* Ser312 phosphorylation plays an important role in maintaining lipids homeostasis in liver, and defect in this will result in a significant enrichment of fatty liver incidence with sex preference as that seen in female *Tp53*^{312A/S} and *Tp53*^{312A/A} mice.

Table 2Plasma lipids significantly affected by sex in wild type and in *Tp53*^{312A/A} mice.

| Plasma lipids | Gender difference in <i>p53</i> ^{312A/A} mice (Male vs Female) | Gender difference in WT mice (Male vs Female) |
|------------------------|---|---|
| ChoE_(18:3) | ↑ *** | ↑ *** |
| LPC_(16:1/0:0) | ↑ *** | ↑ *** |
| LPC_(16:0/0:0) | ↑ *** | ↑ *** |
| LPC_(O-18:1) | ↑ *** | ↑ *** |
| LPC_(20:3/0:0) | ↑ ** | ↑ *** |
| LPE_(20:4/0:0) | ↑ *** | ↑ *** |
| LPE_(22:6/0:0) | ↑ *** | ↑ *** |
| PC_(34:2) | ↑ *** | ↑ *** |
| PC_(34:1) | ↑ *** | ↑ *** |
| PC_(36:4) | ↑ *** | ↑ *** |
| PC_(36:3) | ↑ *** | ↑ *** |
| PC_(36:2) | ↑ *** | ↑ *** |
| PC_(36:1) | ↑ *** | ↑ *** |
| PC_(38:6) | ↑ *** | ↑ *** |
| PC_(38:3) | ↑ *** | ↑ *** |
| SM_(d18:1/14:0) | ↓ *** | ↓ *** |
| SM_(d18:1/15:0) | ↓ *** | ↓ *** |
| SM_(d18:1/16:0) | ↓ *** | ↓ *** |
| SM_(d18:1/18:1) | ↓ *** | ↓ *** |
| SM_(d18:1/18:0) | ↓ *** | ↓ *** |
| SM_(d18:1/20:0) | ↑ *** | ↑ *** |
| SM_(d18:1/22:1) | ↑ *** | ↑ *** |
| SM_(d18:1/22:0) | ↑ *** | ↑ *** |
| SM_(d18:0/24:1) | ↓ *** | ↓ *** |
| SM_(d18:0/25:0) | ↓ *** | ↓ *** |
| TG_(O-50:1) | ↑ *** | ↑ *** |
| TG974.91 | ↑ *** | ↑ ** |
| DG_(O-42:7) | — | + ↑ * |
| ChoE_(18:2) | ↑ *** | — |
| ChoE_(20:4) | ↑ *** | — |
| LPC_(22:6/0:0) | ↑ *** | — |
| PC_(38:4) | ↑ ** | — |
| PE_(38:4) | ↑ ** | — |
| SM_(d18:1/17:0) | ↓ ** | — |
| SM_(d18:0/24:2) | ↑ ** | — |
| TG_(56:7) | ↑ ** | — |
| TG_(58:6) | ↑ *** | — |

Plasma lipids showed sex differences existing in WT (left hand column) and *Tp53*^{312A/A} mice (right). The changes of lipids were marked with arrow (↑ or ↓), in order to indicate their regulations in male versus female. The important contributors were filtered by both OPLSDA $p[\text{corr}] > 0.7$ and t -test ($p < 0.05$). Plasma lipids contributing to the sex difference in both WT and *Tp53*^{312A/A} mice were displayed in bold font. Significant variables were selected by p -value from t -test, degree of significance indicated with an asterisk. ***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$; +: Additional important contributors were filtered by correlation scores ($p[\text{corr}] > 0.7$) based on the OPLS-DA.

4. Discussion

The relationship between cancer susceptibility and lack of a fully functioning *Tp53* gene is well known. In addition to the inhibition of tumour pathways, the *Tp53* has been reported to participate in the pathways that associate with glucose metabolism and reactive oxygen

Table 3List of hepatic lipids significantly affected by *Tp53*^{312A/A} mutation.

| Hepatic lipids | Regulations in male (<i>p53</i> ^{312A/A} vs WT) | Regulations in female (<i>p53</i> ^{312A/A} vs WT) |
|------------------|---|---|
| TG_(56:0) | ↑ * | ↑ * |
| TG 948.89 | ↑ * | ↑ ** |
| TG 978.94 | ↑ * | ↑ ** |
| TG_(60:1) | + ↑ | ↑ ** |
| PC_(O-34:3) | ↑ * | — |
| PC_(O-34:1) | ↑ * | — |
| PC_(O-38:5) | ↑ ** | — |
| PC_(O-38:4) | ↑ * | — |
| PC_(O-40:6) | ↑ * | — |
| PC_(40:4) | ↑ * | — |
| SM_(d18:1/18:2) | ↑ ** | — |
| DG_(O-42:7) | — | ↑ *** |
| PC_(40:6) | — | ↓ * |
| TG_(62:3) | — | + ↑ * |
| TG_(48:4) | — | ↑ * |
| TG_(48:3) | — | ↑ * |
| TG_(50:5) | — | ↑ * |
| TG_(58:1) | — | ↑ ** |
| TG972.89 | — | ↑ ** |

Important hepatic contributors were selected by both p -value from t -test and co-efficiency from OPLS-DA ($p[\text{corr}] > 0.7$). The changes of lipids were marked with arrow (↑ or ↓), in order to indicate their regulations in *Tp53*^{312A/A} mice versus WT mice. ***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$; +: Important contributor from OPLSDA ($p[\text{corr}] > 0.7$).

species [18]. Metabolomics especially lipidomics is an emerging approach which provides useful information that associates with diseases by integrating information, thus its application is raising in diagnosing and looking for specific biomarkers for diseases. However, little is known about the regulation of individual plasma and hepatic lipid species by the defect in Ser312 phosphorylation of *Tp53*.

The liver plays important roles in the lipids production, transportation and clearance. While plasma as an “integrative centre of storage” may provide different information to supplement or support the information provided by the liver. In this study, we analysed the lipids profiles from both plasma and liver tissue to study how the mutant *p53* could dysregulate the lipid perturbation. However, sex effect needs to be taken into account as it may affect both plasma and hepatic lipid profiles. Through the lipidomics study, firstly, we demonstrated that both plasma and hepatic lipids displayed a sex difference, involving LPCs, PCs, SMs and TGs. Our study reported the relative higher levels of TGs in male mice, which could be supported by Gonzalez-Covarrubias’s study with healthy subjects, although the detailed species (i.g., TG (52:1), TG (54:6) and TG (57:2)) were with a certain extent of differences [19]. While Gonzales-Covarrubias found a relevant higher level of PE, PC and SM in female subjects, but in our mice study, almost all these lipids were higher in male rather in the female. Additionally, we found some TGs were with relative higher levels in liver but lower levels in plasma in male compared with the female. This may indicate a different capability of lipid productions and circulations in relation to sex. The sex-association of SMs and other species of lipids (PCs, PEs, ChoEs, DGs and TGs) has been found also in the plasma of human subjects [20], which partially support our results of sex effects on plasma lipid profiles. Additionally, we found female mice have a relative lower plasma level of ChoE (18:3), but a higher hepatic level of ChoE (18:1) than male. The liver ChoE can be circulated/transferred from liver to blood, thus our results may indicate that females have a weaker ability to circulate (esterified) cholesterol or an easier hepatic storage of ChoE than males. In fact, an accumulation of ChoE may lead to a ChoE storage disease [21], and to develop further the transitions to fibrosis and, finally, into cancer [22]. Thus, our founding of the relative higher hepatic ChoE level in the female may indicate a higher risk of cholesteryl ester storage disease of females (e.g., Fatty liver), via which way to raise even the susceptibility of cancer. A cohort study has supported our opinion via a

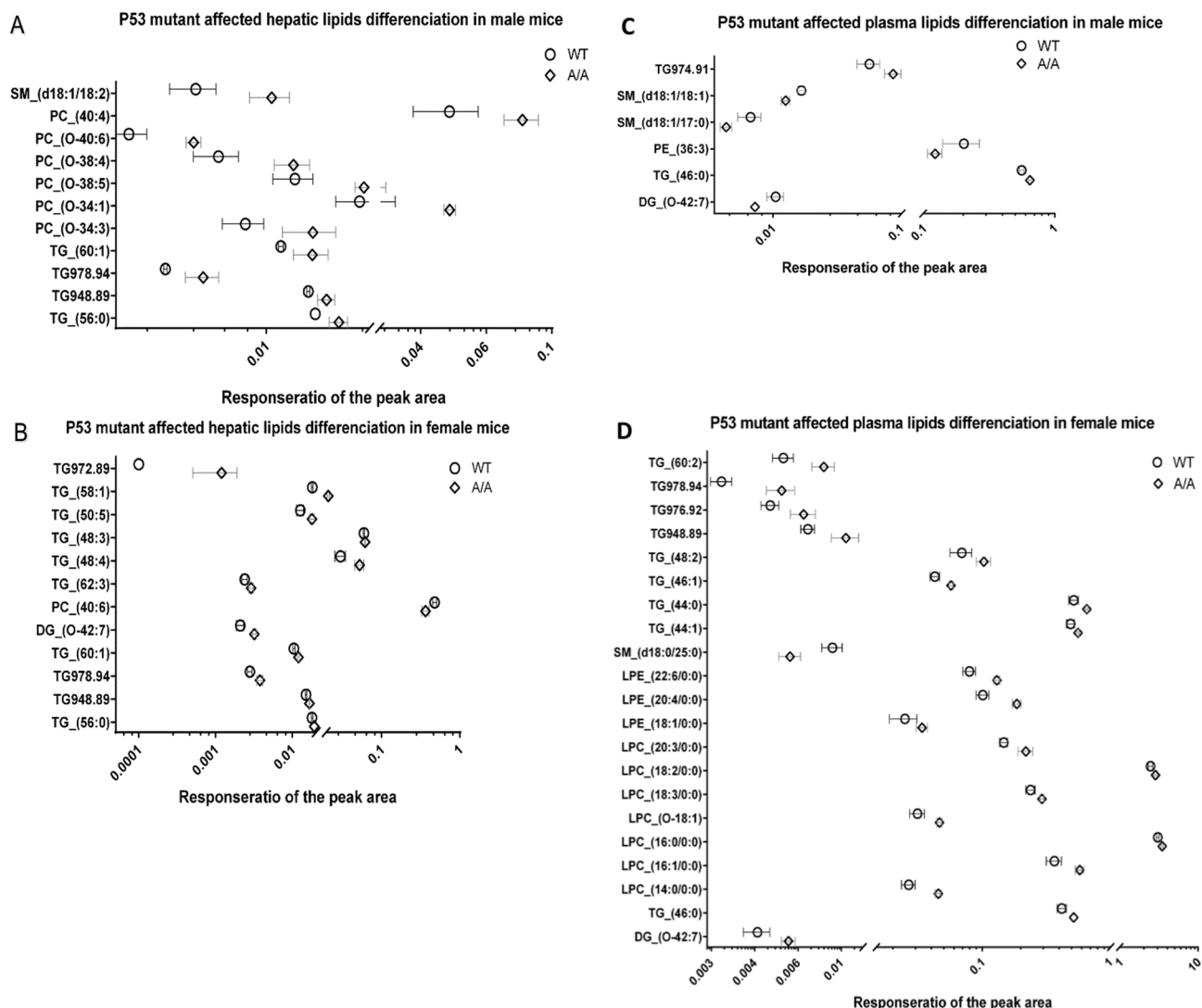


Fig. 3. Dysregulated lipids associate with *Tp53* mutation in (A) liver of male mice; (B) liver of female mice; (C) plasma of male mice; (D) plasma of female mice. Hepatic and plasma lipids in the corresponding to mutation were significantly different defined by $p < 0.05$ from student's *t*-test and $p[\text{corr}] > 0.7$ based on loading plot from OPLS-DA. Peak areas were exported and normalized using the relevant internal standards. The data were expressed as mean value \pm SEM.

different way that female *Tp53* mutation carrier has indeed a higher cancer risk [23]. However, the susceptibility of fatty liver by the *TP53* mutation, especially relating to sex, has been ignored for a long time. In summary, the lipids with sex effect need to be taken into account for novel biomarkers exploring when doing disease-associated studies. While sex effect may not only indicate metabolic differences in sex, but a different risk of diseases occurrence that is sex-associated.

Interestingly in this study, when looking into data in the male and the female respectively, we found a great number of lipids that belong to a variety of species are with significantly perturbations, in plasma and liver respectively. Especially, *Tp53*^{312A/A} mice are with more significant elevated levels of hepatic and plasma TGs. Additionally, we observed a great number of elevated plasma LPCs existed only in female mutant mice and numbers of hepatic PCs existed only in male mutant mice, which is significantly dysregulated by the *p53* mutation. TGs are important esters of fatty acids with glycerol and can be transported by chylomicrons and VLDL to the cellular membrane of adipose cells and to muscle cells. They are also present in the blood, aiming to help with the transference of adipose fat and blood glucose between the liver and blood. The dysregulation of lipids has been associated with various diseases with different up/down regulations in different lipid species. For instance, the elevated levels of plasma/ hepatic TGs and LPCs have

been considered to highly associated with liver disease such as hepatic steatosis (with syndrome of diabetes mellitus) [24], hepatoma and liver hyperplasia [25,26]; but were also considered as important biomarkers in other disease such as obesity/diabetes [25,27], atherosclerosis/hypertension [28], stroke [29] and cardiovascular diseases [30,31]. The overexpression of LPCs have been considered as potential biomarkers for sex-related cancer such as ovarian cancer, cervical cancer in females [32,33], and prostate cancer in males [34]. The plasma LPCs have been reported to also stimulate the G-protein coupled receptors to induce migration of breast cancer [35]. Hence, our results may indicate the *Tp53* may not only susceptible to a tumour but also a high risk of dysregulation-associated disease such as fatty liver, cardiovascular and diabetes. What is more, a sex-related disease may occur in *Tp53* mutant mice. However, literature not always showed consistent results of the role of lipids in all cancers, for instance, some studies report the LPCs were lower in colorectal cancer [36], lung cancer [37], etc. This may due to they are different cancer type, but may also due to the sex effects were not given into account and this may influence significantly on the results.

Our results displayed increase of sex-associated TG (TG 974.91) and DG (O-42:7), as well as the mutation-associated TG (TG 978.94), which were detected in both the plasma and the liver of the mutant mice. Thus,

Table 4Plasma lipids significantly changed in *Tp53*^{312A/A} mice versus WT mice.

| Plasma lipids | Regulations in male (p53 ^{312A/A} vs WT) | Regulations in female (p53 ^{312A/A} vs WT) |
|--------------------|--|---|
| DG (O-42:7) | ↓* | ↑* |
| TG (46:0) | ↑** | ↑** |
| LPC (14:0/0:0) | — | ↑** |
| LPC (16:1/0:0) | — | ↑** |
| LPC (16:0/0:0) | — | ↑* |
| LPC (O-18:1) | — | ↑** |
| LPC (18:3/0:0) | — | ↑* |
| LPC (18:2/0:0) | — | ↑* |
| LPC (20:3/0:0) | — | ↑* |
| LPE (18:1/0:0) | — | △↑* |
| LPE (20:4/0:0) | — | ↑*** |
| LPE (22:6/0:0) | — | ↑*** |
| PE (36:3) | + ↓* | — |
| SM (d18:1/17:0) | + ↓* | — |
| SM (d18:1/18:1) | ↓** | — |
| SM (d18:0/25:0) | — | △↓* |
| TG (44:1) | — | + ↑* |
| TG (44:0) | — | ↑* |
| TG (46:1) | — | ↑** |
| TG (48:2) | — | △↑* |
| TG948.89 | — | △↑* |
| TG974.91 | ↑* | — |
| TG976.92 | — | + ↑* |
| TG978.94 | — | ↑** |
| TG (60:2) | — | ↑* |

Important plasma contributors were selected by both *p*-value from *t*-test and coefficient from OPLS-DA ($p[\text{corr}] > 0.7$). Lipids were displayed in bold font if they altered in both male and female mice. The changes of lipids were marked with arrow (↑ or ↓), in order to indicate their regulations in *Tp53*^{312A/A} mice versus WT mice. ***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$; △: Important contributor from only *t*-test +: Important contributor from only OPLS-DA.

detecting lipid profiles in different organ/body fluid helps to double confirm the results. Although in our study, both plasma and liver provide lipid information to discriminate the *Tp53* Ser312 mutation from the WT mice, there are still large differences on the detailed lipid species in blood versus that in liver. For instance, the significant changes of LPC and LPE in our study were unique existed in the plasma but was not shown in hepatic lipid profile. Therefore, our study indicates that the hepatic lipids may contribute differently to that in the plasma/serum, thus it is necessary to integrate lipids profiles from different organism for a better understanding of mechanism of disease that will lead to develop a precise diagnosis tool in future research.

Additional to the lipidomics results, we evaluated the histological appearances of the fatty accumulation and fatty liver susceptibility, thus to conclude that the Phosphorylation of *Tp53* at Ser312 prevents fatty liver incidence in female mice. To support, we also checked some literatures to link *Tp53* mutations and liver diseases (especially sex-related). To date, The *Tp53* mutant mice have been considered more susceptible to a later stage of liver hyperplasia, as well as a higher hepatic tumour occurrence especially in the female mutant mice (about 4/

Table 5

Sex and genotypes of mice with fatty liver.

| Sex | Genotype | Fatty liver? | | Grand Total |
|--------------|----------|--------------|----|-------------|
| | | N | Y | |
| Female | A/A | | 3 | 3 |
| | A/S | 3 | 5 | 8 |
| | WT | 1 | 2 | 2 |
| Female Total | | 4 | 10 | 14 |
| Male | A/A | 2 | 2 | 4 |
| | A/S | 5 | | 5 |
| | WT | 2 | | 2 |
| Male Total | | 9 | 2 | 11 |
| Grand Total | | 13 | 12 | 25 |

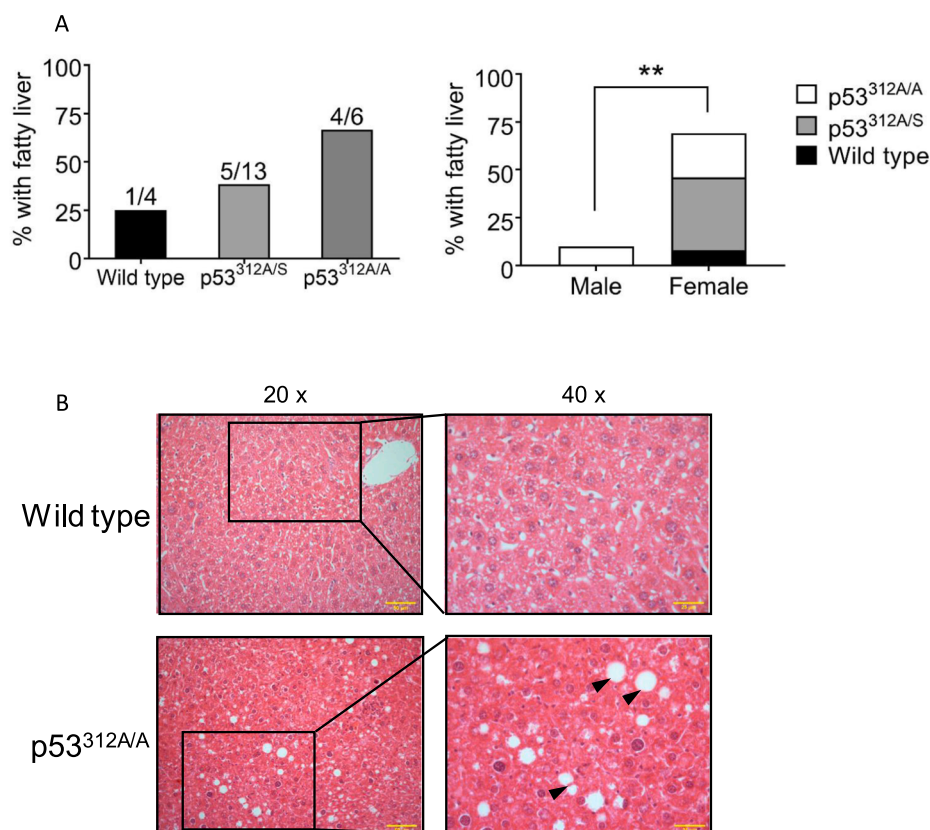


Fig. 4. Incidence of fatty liver (steatic hepatosis) in the livers of aged *Tp53*^{312A/A} mice (A) The livers of an aged cohort of mice were examined by H and E staining. (Left) Fatty liver was identified in 5/7 (71 %) homozygous *Tp53*^{312A/A} mice, compared with 2/5 (40 %) wild type and 5/13 (38 %) heterozygous *Tp53*^{312A/S} mice. Although a higher proportion of homozygous mice developed fatty liver, this was not significant. (Right) when sex was compared, it was found that female mice were significantly more likely to develop fatty liver than males ($p < 0.05$). In addition, the only male to develop fatty liver was a homozygous mutant. (B) An example of the steatosis that was observed in *Tp53*^{312A/A} female mice is shown in comparison with wild type, with 20x and 40x magnification respectively, Scale bar indicates 25 μm and 50 μm in the 40x and 20x figures respectively.

5 were female who suffered from a hepatic tumour) [5]. The higher occurrence of a tumour in female mice than that in male mice was observed on p53 mutant mice model when giving irritation stimuli [38]. What is more, the important gene of *E2f1* has been found, which contributed importantly to the tumour occurrence in female [39]. These may all partially support our findings in this manuscript.

In summary, using lipidomics, the relationship between *Tp53* gene mutation and lipid perturbations are linked together. The defect in Ser312 phosphorylation of *Tp53* perturbed a great species of lipids, which indicates not only a risk of tumour occurrence by the *Tp53* mutation, but also high occurrence of other sex-associated diseases such as fatty liver by the same mutation, which could be supported by our histological staining results. Therefore, our results demonstrated a single gene defect is able to cause biological network disturbance in whole system, and cancer therapies should consider a systems approach. Importantly, although some prevalent studies has also shown some symptoms of advanced cancer that related to sex [40–42], few clinical applications have really taken into account this as an important issue. Lipidomics/metabolomics as an integrative method may provide some important evidence for early and personalized diagnose and therapies for clinical application, and the sex factor needs to be taken into account.

CRedit authorship contribution statement

Min He: Formal analysis, Funding acquisition, Visualization, Writing – original draft. **Elizabeth A. Slee:** Validation. **Mengmeng Sun:** Analysis and editing. **Chunxiu Hu:** Methodology. **Wen-Te Chang:** Review. **Guowang Xu:** Review. **Xin Lu:** Supervision. **Mei Wang:** Conceptualization, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jchromb.2022.123491>.

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