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METABOLIC IMPACT OF PARTIAL HEPATECTOMY IN THE NON-ALCOHOLIC STEATOHEPATITIS ANIMAL MODEL OF METHIONINE-CHOLINE DEFICIENT DIET

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Highlights

- The metabolic phenotype of an animal model of advanced stage after PH under MCD diet has been characterized
- Multiplatform metabolomics provided a large metabolite coverage of the metabolites associated to the model and the treatment
- Near 400 significant metabolites have been annotated
- Ratio phosphatidylcholine/phosphatidylethanolamine, triglycerides and amino acids show how liver regeneration under NASH proceeds

Abstract

In the liver, obesity is often manifested by the clinical disorder of the Non-Alcoholic Fatty Liver Disease (NAFLD). A proportion of NAFLD patients develop hepatic inflammation, known as Non-Alcoholic Steatohepatitis (NASH), which can end up in cirrhosis, or Hepatocellular Carcinoma (HCC). In this scenario, partial hepatectomy (PH) is an alternative to promote liver regeneration. However, as liver regeneration is impaired in NASH patients, more knowledge about its metabolic condition is needed to improve the regenerative response of the liver in this pathological condition. Although extensively employed, the panoply of molecular alterations involved in the regenerative response of the liver after partial hepatectomy PH is far from being fully characterized. Metabolic fingerprinting (metabolomics) is a powerful tool to help in the elucidation of complex metabolic networks, by means of a blind, naïve approach to study which metabolic nodes (metabolites) show the biggest variations between conditions. The objective of the present study was to gain deeper knowledge about the metabolic processes involved in the NASH animal model, and particularly in the effect of PH by using metabolomics. For achieving such information, twelve 8-week-old male C57BL/6J mice, fed commercial chow (control diet) or methionine and choline-Deficient

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diet (MCD) for three weeks were subjected to PH and sacrificed 2 weeks later. Livers were removed and submitted to metabolic profiling analysis through RP-LC/MS (qTOF), GC/MS (qTOF) and CE/MS(TOF). More than 3000 different features were detected and repeated measurements one-way ANOVA analysis was performed to unveil significant features. MCD diet induced changes ($p < 0.05$) in 46% of the detected features, whereas PH provoked significant changes in 85% of them. Most of the changes were detected through LC/MS and were associated to lipid metabolism. However, changes of metabolites virtually related to other metabolic routes (amino acids, carbohydrates, nucleotides) were found altered and detected by CE/MS and GC/MS. The changes associated to PH show a similar trend regardless of the diet, but in the context of the diet deficient in methionine and choline we have found results that point to a different ratio glycolysis/tricarboxylic acid cycle. Moreover, in the NASH model, the regeneration of the liver structures occurs at the expense of an increased phosphatidylethanolamines/phosphatidylcholines ratio.

1.1- Introduction

One of the clinical manifestations of obesity is the Non-Alcoholic Fatty Liver Disease (NAFLD). A proportion of NAFLD patients develop hepatic inflammation, known as Non-Alcoholic Steatohepatitis (NASH). NASH is characterized by lobular and portal inflammation, hepatocyte ballooning and variable degrees of fibrosis, which can end up in hepatic cirrhosis and finally to hepatocellular carcinoma (HCC) [1].

The different dietary animal models of NASH are relevant to mimic the pathogenesis of diet-induced obesity and associated metabolic disorders. To directly induce NASH, methionine and choline-deficient (MCD) has been extensively used, although it does not perfectly resemble the onset of human NASH linked to obesity due to weight loss and absence of insulin resistance [2]. As lifestyle interventions have limitations when newly diagnosed NASH is in advanced stage, it is still needed to evaluate new therapeutic strategies. Experimental (animal) models that can emulate human NASH are useful in this preclinical phase [2].

Once NAFLD progresses to advanced cirrhosis, liver transplant becomes in many cases necessary. Partial hepatectomy (PH) is an alternative to liver transplant since the liver has the unique capacity to regenerate with healthy hepatocytes. PH does not suffer from the problems associated to the lack of suitable donors, of enough quality, for long waiting periods [3].

In order to get insights on the metabolic regulation of liver regeneration upon resection of 70% of the liver tissue, several studies have evaluated the first hours/days after PH,

before the liver structure has been restored, mainly in mice or rats [4]. In such conditions, the balance between hepatic glucose and lipid metabolism is essential for energy supply during liver regeneration in mice [5], evolving from initial hypoglycaemia and hepatic triglyceride accumulation towards the restoration of normal liver metabolism. However, many of the metabolic processes involved in the full restoration of size and function are still under active research. In addition, metabolism of PH under NASH still needs more studies so that might become fully understood. It is known that the NASH condition worsens liver regeneration in patients and animal models [6], and elucidating the metabolic processes involved in the regeneration in naïve and NASH conditions will provide more alternatives to improve the outcome of patients submitted to such therapy. Metabolomics has already proven its powerful ability to highlight altered routes and help in biomarker discovery in plasma of human patients with NAFLD and/or NASH, even allowing to discriminate different NASH subtypes [7].

With regard to PH, metabolomics with ^1H NMR has been applied to the study of the metabolic impact of PH at 24/48 h post-surgery [8], showing the rapid accumulation and disappearance of triglycerides in the liver. Nevertheless, the whole metabolic status of the hepatectomized liver once the size has been restored (beyond 8 days post-surgery), has not been characterized yet.

We have previously applied a metabolomics approach with GC-MS and CE-MS to respective studies addressed to evaluate potential improvements of the regenerative response of the liver of mice under MCD diet, receiving treatments with either a new glucagon receptor agonist [9] or a glucagon-like peptide 1/glucagon receptor dual agonist [10]. In the context of a complete histological, molecular and physiological characterization of the response, by means of the metabolomics approach we showed the effect of the treatments in some nucleotides and hydrocarbons, as well as metabolites from the central carbon metabolism and amino acid metabolism, but the detailed changes in lipid composition were not studied.

The objective of the present work was to deepen the knowledge about the metabolites differentially expressed in mice taking MCD diet, before and after (15 days) having been submitted to 70% PH, by means of a multiplatform (GC-MS, CE-MS, RPLC-MS) metabolomics approach

1.2- Material and methods

1.2.1-Animals

Twelve 8-week-old male C57BL/6 mice, purchased to Charles River Laboratories (Charles River, Barcelona, Spain), were maintained in light/dark (12 h light/12 h dark),

temperature (22 °C) and humidity-controlled rooms, fed ad libitum with free access to drinking water.

Mice were fed chow diet (Chow; SAFE A04-10 Panlab, Barcelona) or methionine choline-deficient diet (MCD; TD-90262 Harland-Tecklad, Indianapolis, IN) for three weeks (Supplementary Fig. S2). After that, mice were subjected to a standard 70% PH under isoflurane anaesthesia. The animals were maintained for two more weeks with their corresponding diet. At the end of the experiment, animals were sacrificed, and livers were removed and weighed [9, 10].

All animal experimentation was controlled following the recommendations of the Federation of European Laboratory Animal Science Associations (FELASA) on health monitoring, whereas use of animals in experimental procedures was approved by the Ethical Committee at Consejo Superior de Investigaciones Científicas (CSIC) Animal Care and Use Committee and was conducted accordingly to accepted guidelines for animal care of Comunidad de Madrid (Spain).

1.2.2- Sample treatment and instrumental analysis.

A general overview of sample treatment and instrumental analysis process is depicted in Fig. 1. The multiplatform approach is based on a single homogenization step, followed by specific steps to make the sample compatible with the analytical conditions. Sample collection and preparation protocol was performed as previously described [11] with slight modifications.

1.2.3- Homogenization.

Livers were frozen in liquid nitrogen immediately after removal and stored at -80°C. Liver samples were thawed the same day of the analysis and mashed in to small pieces using mortar and pestle after dipping them in liquid nitrogen. Water: methanol, 50:50 (WM) was used as solvent for homogenization. 45-50 mg from each sample were collected and added with 1:10 ratio (1mg liver tissue:10µl solvent) in WM solvent. Homogenization (Qiagen TissueLyser LT, Germany) was performed adding 3 mm beads per sample at 50-Hz speed for 2 min and samples were rested on ice for 1-2 min (x4 series).

1.2.4- Preparation for analysis

For LC/MS (QTOF) 100 µL of liver homogenate was vortexed with 100 µL of methanol for 2 min. 100 µL of methyl-tert-butyl ether (MTBE) was added and vortexed for 1 h at room temperature. After that, samples were centrifuged at 4000 × g for 20 min at 20 °C. Then, 100 µL of supernatant was transferred to a chromatography vial for LC-MS analysis.

For CE/MS (TOF) 100 µL of liver homogenate was centrifuged at 14000 × g for 15 min at 15 °C. 80 µL of homogenate was transferred to an Eppendorf tube and dehydrated for

2 h in a Savant™ SPD131DDA SpeedVac™ (ThermoFisher Scientific, Waltham, MA USA). Samples were re-suspended in a 100 µL solution of 0.2 mM methionine sulfone (internal standard) and 0.1 mM formic acid. Then, re-suspended samples were vortexed for 5 min and transferred to an ultrasound bath for 2 min. After that, samples were centrifuged at 16000x g for 20 min at 20 °C. 100 µL of supernatant was collected and transferred to a Chromacol® vial for CE-MS analysis.

For GC/MS (QTOF) 40 µL of liver homogenate was transferred to an Eppendorf tube and 120 µL of cold acetonitrile was added. Samples were vortexed for 2 min and let stand on ice for 5 min. Following, liver samples were centrifuged at 15400x g for 10 min at 4°C. 100 µL of the supernatant was transferred to a GC vial and dehydrated for 2 h. 10 µL of O-methoxy amine hydrochloride in pyridine solution (15 mg/ml) was added to the dried samples and vortexed for 1 min. Vials were ultra-sonicated for 10 s and vortexed for 2 min (x3 series). Samples were then transferred to a dark place and incubated for 1 h. 10 µL of N, O-Bis (trimethyl silyl) trifluoroacetamide (BSTFA) with 1% of trimethylchlorosilane (TMCS) was added to the vials and mixed vigorously for 5 min. After that, samples were incubated for 60 min at 70 °C. During incubation, internal standard (10 ppm solution of C18:0 methyl ester) was injected to confirm the retention time and the peak are in the GC-MS. After that, samples were cooled for 1 h in a dark place. 100µL of internal standard (10 ppm) was added to each vial and mixed vigorously for 2 min. Vials were centrifuged at 2500x g for 15 min at 18 °C.

1.2.5- Instrumental conditions

The final instrumental conditions for liver samples were optimized from methods for LC/MS; GC/MS and CE/MS previously established in our laboratory [11].

1.2.6- Quality control samples:

QCs samples for CE were prepared by pooling equal volumes of homogenates from each sample from all groups. QCs were processed as all the samples. In the case of LC, it was decided to run separate experiments for samples before and after PH, and therefore 2 different sets of QCs were prepared. In the case of GC, a larger set of samples was used to prepare the pool, which are not included in the present study. They were analyzed throughout the whole worklist to provide a measurement not only of the system's stability and performance but also of the reproducibility of the sample treatment procedure.

1.2.7- Data treatment

1.2.7.1- LC/MS (QTOF)

Chromatograms of resulting data files were analysed with Mass Hunter Qualitative Analysis software (B.04.00, Agilent). Background data and unrelated ions were cleared

using the Molecular Feature Extraction (MFE) tool included in this software. Data files were processed with Mass Hunter DA Reprocessor (Agilent). Data alignment and filtering were performed with Mass Profiler Professional software (B.06.00, Agilent). Features were filtered selecting the data presented in at least 80% of the QCs and in 80% of the samples in one of the four groups.

1.2.7.2- CE/MS (TOF)

A library of standards and a comprehensive list of metabolites previously obtained by other authors in CE/MS[12], were used to find features in the data files through the Find by Formula and Extract Ion tools included in Mass Hunter Qualitative Analysis software (B.04.00, Agilent). To check the assurance of the process we used methionine sulfone (MetS) as internal standard (IS), and the signal and migration time (MT) of all compounds are corrected by the abundance and MT of this IS.

1.2.7.3- GC/MS (QTOF)

The identification is assured by the reproducibility of EI mass spectra and RTL method (according to the Internal Standard—C18:0 methyl ester). Background data and unrelated ions were cleared using the Molecular Feature Extraction (MFE) tool included in Mass Hunter Qualitative Analysis software (B.04.00, Agilent). Deconvolution and compound identification were performed using Mass Hunter Unknown Analysis (B.04.00, Agilent Technologies). Fiehn and NIST libraries were used in the identification. Mass Profiler Professional software (B.06.00, Agilent Technologies) were used to perform the data alignment and filtering (described in LC-MS section). Assignment selective ion and integration were done with Mass Hunter Qualitative Analysis (B.04.00, Agilent Technologies).

1.2.8- Statistical analysis.

Data are reported by all the comparisons of the p-values of estimated means according to the linear regression model that underlies ANOVA of repeated measures with correction of Greenhouse-Geisser arising with respect to time before and after PH (within variable) and diet (between variable). The p-values are given for all variables, within each group (diet), within each time (PH) and within each group-time (diet-PH). They correspond to the significant values selected according to the p-value of the Mauchly sphericity contrast with Greenhouse-Geisser correction. All statistical analyses were performed using the MATLAB® R2015a software.

In addition, multivariate analysis (Principal Component Analysis) from all sets of data were performed to check for possible bias or trends of the data, as well as to evaluate the clustering of QCs (Supplementary Fig. S3).

1.2.9- Annotation

Compounds detected in GC-MS were identified as aforementioned (section 1.2.7- Data treatment). The statistically significant compounds from CE-MS or LC-MS were putatively annotated by searching their accurate mass against public databases i.e., METLIN (<https://metlin.scripps.edu/>), KEGG (<https://www.genome.jp/kegg/>), LIPIDMAPS (<https://www.lipidmaps.org/>) and HMDB (<https://www.hmdb.ca/>), all simultaneously accessed through an in-house developed search engine, CEU Mass Mediator (<https://www.ceumass.eps.uspceu.es/>) [13, 14].

For compounds included in our in-house libraries, retention/migration times were also compared, and this adds more confidence to the annotation. In addition, an LC-MS/MS experiment was setup, and the fragmentation pattern was analysed for deeper annotation of some statistically significant features. Therefore, for each annotated metabolite there is a different level of confidence in the annotation. We have followed the proposal from Schrimpe-Rutledge *et al.* [15], and therefore the 5 levels of confidence that we have used are:

- Level 1: highest confidence identification. Validated identification extracted from reliable databases confirmed with a pure reference standard.
- Level 2: putative identification. Validated identification extracted from reliable databases plus predictive or externally acquired structure evidence from MS/MS analysis (providing information about reliable neutral losses or known fragmentation patterns).
- Level 3: Tentative identifications. Validated identification extracted from reliable databases, compatible with all the variables (biological, chemical, instrumental) of the study.
- Level 4: Molecular structure candidates: After processing the signal from the instrument, only molecular empirical formula can be provided.
- Level 5: Unknown features: only deconvoluted experimental m/z, abundancies and retention time could be known from a deconvoluted feature.

According to these levels, we can consider that we have annotated a metabolite if we have been able to achieve a tentative identification (level 3). Furthermore, a feature can be considered a compound only if we are able to propose a molecular formula (level 4).

1.3- Results and Discussion

With our multiplatform approach, we have been able to obtain information for more than 3000 features, ideally compounds (Table 1). Each platform was suitable to find differences in a different set of compounds from different metabolic families, expanding therefore the metabolite coverage of the study. From the total 3297, 1530 resulted

significant because of the MCD diet (*i.e.*, 46% of the features) and 2814 were significant due to PH (85% from the total) (Table 2). LC/MS was the platform that provided more significant features, 2819 were significant ($p < 0.05$) due to MCD diet and PH (1353 from MCD diet, 2509 from PH). 119 from CE/MS were significant ($p < 0.05$) due to MCD diet and PH (62 from MCD diet, 82 from PH), whereas GC/MS provided 269 compounds that resulted to be significant ($p < 0.05$) (115 from MCD diet, 223 from PH) (Table 2).

The corresponding annotation strategies were applied to all significant ($p < 0.05$) features detected by CE/MS (49) and GC/MS (183), whereas for LC results, the 174 most significant features ($p < 0.0001$) were selected for further annotation. Furthermore, 186 LC-MS/MS spectra were processed, from which 21 showed enough quality as to provide additional information to raise the level of confidence in the annotation from that provided by the coincidence in databases (Supplementary Table S1).

In the case of CE/MS, 43 out of 49 significant compounds were identified making use of the CMM tool and the putative annotation was compared with an in-house database. From the 43 identified, 20 could be confirmed from both sources (Supplementary Table S2).

Supplementary Table S3 summarizes all the information from all significant putatively annotated compounds (392), grouped by the metabolic route(s) where they can be primarily involved. The average abundance, the p -value associated to each factor under study, as well as the $\log_2$ of the Fold Change (FC) is also included. One code was assigned to each feature in each technique, which is used across the manuscript and tables to facilitate the quest for information. Part of the changes associated to MCD diet discussed herein can be seen in Figure 2. Supplementary Materials contains more detailed information about the annotation parameters for all features (Supplementary Table S4), as well as a heat map where the abundances and the fold changes are graphically depicted (Supplementary Fig. S4).

The impact of PH to the biochemical classes is summarised in table 3, which shows the average (within the biochemical class) of $\log_2(\text{FC})$ of PH in both groups (CT or MCD). We could find common trends and differences mainly in the lipids (Fatty acids, glycerolipids - GL, sterols - ST) and amino acids (AA). Regarding these results, it is noticeable that PH induces the same type of changes (increase/decrease), no matter which diet were the mice taking. The differences between both groups when submitted to PH are in the extent of the changes. The signal of glycerophospholipids (GP) and AAs was in general higher after PH, but a mild decrease of triglycerides (TG) could be seen. Most of the GPs could not be discriminated between glycerophosphatidylcholines

(PC) and glycerophosphatidylethanolamines (PE) because of poor intensity of characteristic adducts and/or fragments in MS, but part of them could be unequivocally identified: All PCs were decreased after PH, whereas all PEs were increased, regardless of the diet, as previously described by Maldonado *et al.* [16]. Although the trends can be similar, the relative abundance of the lipids is very different. There are less GPs in the MCD group, of any type (except glycerophosphoinositols, PI), in any moment (before/after PH). See also Fig. 3 for a graphical representation of the changes associated to PH in both diets. These results point to a defective GPs metabolism in mice receiving MCD diet that likely present compromised PC and PE synthesis due to choline/methionine deficiency that impact in the hepatocytes both in quiescent (before PH) and proliferating (after PH) stages. Importantly, this could explain the defective hepatic regeneration of MCD diet-fed mice that requires a boost in PC/PE synthesis in agreement with our previous data [8, 10].

In addition to the changes in the lipidic metabolites, we have seen changes that affect to a similar extent to most of the proteinogenic amino acids: they are mostly decreased due to MCD diet (as compared to CT), and after PH the levels of amino acids are higher than before, in both groups. But methionine (Met, G063) and threonine (Thr, C11/G041), did not follow this trend: they were higher in MCD groups than in CT, and were lower after PH. Met is one of the 9 essential amino acids and is necessary for the synthesis of S-adenosylmethionine (SAm), the main methyl donor abundant in the liver. The transfer of the methyl group (for instance, to DNA for epigenetic regulation) is driven by S-adenosyl methyl transferases and produces S-adenosylhomocysteine (SAH). To regenerate SAm, a cycle of hydrolysis (SAH hydrolase), methylation (Betaine-homocysteine methyl transferase, or 5-methyltetrahydrofolate-homocysteine methyl transferase) and transfer (SAm synthetase) is carried out. In this cycle, choline is the precursor of betaine and sarcosine, and although it can be obtained from phospholipids, the nutritional needs for this choline are very high, and it is considered essential.

The importance of SAm in NAFLD and NASH has been revealed in different studies such as those performed in mice lacking SAm synthetase and Glycine N-methyl transferase (GNMT) that develop NASH, showing that advanced NASH is characterized by the inhibition of SAH hydrolase gene, and increased levels of SAH [17]. In our study we did not find statistically significant differences in SAm, although we previously found that the treatment of partially hepatectomized mice under MCD diet with a dual GLP1 receptor/glucagon receptor induced a dramatic increase of SAm [10].

In our study, we found that the group of mice that received MCD diet showed increased levels of SAH (C41), versus the control group mice fed a chow diet. Taking together with the differences in homocysteine (Hcy, C02) and Met (G063), such increase in MCD mice versus the control is pointing to an impaired regulation of the cycle. In the context of a diet deficient in Met, it may become paradoxical the presence and variation of Met and Hcy in the liver (Supplementary Table S3). In absence of supply from diet, such essential amino acids must come either from the proteolysis of proteins already synthesized before the animals started with the MCD diet and/or the gut microflora activity (see below).

Regarding proteolysis, mice that develop NASH under MCD diet develop a hyper metabolic state [18] because they lose weight even though the diet has an adequate caloric supply. Therefore, catabolism of proteins is exacerbated in liver to provide both energy supply and carbon skeleton for other biomolecules such as glucose (gluconeogenesis). All amino acids can be metabolized to amphibolic intermediates, Acetyl CoA and intermediates of the tricarboxylic acid cycle. Serine (Ser, C07) and Thr (C11) will produce pyruvate (G005), which can be reduced by NADH to lactate (G007) or decarboxylated in the mitochondria to Acetyl CoA. In contrast, amino acids which are catabolized in the mitochondria to Acetyl CoA or to one of the intermediates of the TCA cycle, *i.e.*, Valine (Val, C04/G026), Histidine (His, C12), Leucine (Leu, C05), Isoleucine (Ile, G040), Phenylalanine (Phe, C06/G082), Tyrosine (Tyr, G126), proline (Pro, C08), glutamic acid (Glu, C09) and aspartic acid (Asp, G101) were found decreased in MCD group as compared to CT group. The higher levels of citrate (G096), together with the decrease of malic (G062) and fumaric (G047) acids, indicate excessive activity of the TCA cycle, in common with humans with NAFLD [19]. This impaired metabolism of amino acids is related to the elevated ALT and AST [20]. With our methodology, we cannot know whether elevated transaminases are a cause or a consequence of the altered levels of amino acids, because there is a feedback in the activity of these enzymes and the levels of amino acids and their metabolites.

Despite elevated protein catabolism in the liver of mice fed the MCD diet, it is not enough to supply the needs for an essential amino acid as methionine and gut microflora can provide an extra supply of such amino acid. Of note, the possibility to obtain Hcy from other sulphur sources such as cysteine (through cystathionine) is only present in mammals. Nevertheless, several common bacterial species express cystathionine gamma synthase [21], which synthesizes cystathionine from cysteine. In such context, it is worth mentioning that the gut microflora is critical for mice fed an

MCD diet because one antibiotic-treatment can produce higher NASH and excessive fibrosis [22].

Hcy must be methylated, and the methyl group may be donated by 5-methyltetrahydrofolate (5-MTHF) or betaine, as previously mentioned. As the MCD diet was deficient in choline, levels of liver betaine (C01) and sarcosine (G014) were found lower in MCD-fed mice as compared to controls (Supplementary Table S3). In the absence of betaine, glycine is the main source of methyl groups for the synthesis of methionine, because it can be metabolized into 5,10-methylenetetrahydrofolate, which in turn can be converted into 5-MTHF by Methylenetetrahydrofolate reductase. We found a profound decrease in the levels of betaine, and sarcosine, as well as glycine (G134) in the MCD group before PH. Furthermore, the conversion of dUMP (G172) into dTMP was found reduced in MCD group. 5,10-MTHF is the source also for such conversion, but in a situation of Met deficiency this conversion seems under regulated. We also found differences in the arginine metabolism, and differences between MCD and control groups in putrescine (G052), spermidine (C24) and spermine (C26). As one derivative of SAME is needed for the conversion of spermidine into spermine, these results indicate that SAME pool is not enough to attend to all the reactions where the methyl transfer is needed. In such conditions, putrescine is catabolized through an alternative pathway into 4-aminobutanoic acid (C48).

Recently, Dong *et al.* [23], analysing urine and blood of non-diabetic NAFLD with normal liver function as well as NASH and HC subjects with impairment of liver function, identified biomarkers whose presence was different according to the NAFLD stage. They showed by ROC analysis that 3-indoleacetic acid, L-carnitine, pyro glutamic acid, and indole lactic acid might discriminate NASH from NAFLD. Lake *et al.* [24] also studied the progression of NAFLD towards NASH by metabolomics and transcriptomic analysis and reported increased levels of leucine, isoleucine, and valine from NAFLD to NASH. Similarly, carnitine metabolites were significantly elevated in NASH compared to NAFLD.

Lipotoxicity, oxidative stress and activation of the immune system are believed to play key roles in the activation and production of pro-inflammatory cytokines as well as in the metabolic adaptations triggered to stop or revert fat accumulation and production of reactive oxygen species (ROS) from mitochondrial fatty acid oxidation which are main contributors of hepatocyte injury leading to cell death by apoptosis [25].

Oxidative stress promoted by lipid accumulation is a key driver of progression from simple steatosis to NASH [26]. In this regard, inflammation markers such as 11-HETE,

oxidative stress related compounds (glutathione metabolites), bile acids and carbohydrate-related metabolites were found different in plasma of both types of patients. Different studies have also shown increased primary bile acids in circulation in NASH [27]

In our present study we could find changes in glutathione (G142) and some metabolites related to either its synthesis (2-hydroxybutyric, G013, pyro glutamic acid, G064) or its further modification (hydroxymethylglutathione, C37) (ophthalmic acid C36) or catabolism [20]. Under MCD diet, although there is higher availability of glutathione, its synthesis is lower than in controls, and its depletion is higher. PH involved an increase in the availability of glutathione, but under MCD diet this increase was lower, and the depletion was higher. A combination of factors: substrate (Cys, Gly, Glu) availability, ROS production (from mitochondria), NADPH synthesis (from pentose phosphate) simultaneously concur to modulate glutathione synthesis and reduction. Therefore, under MCD diet the amount of GSH available to counteract oxidative stress is lowered, and to be able to increase such availability could provide therapeutic opportunities in NASH, as well as in conditions strongly related to NASH such as HCC [20].

In the liver under physiological conditions, glycogenolysis and gluconeogenesis are activated to maintain body glucose homeostasis when blood glucose levels decrease upon fasting. Coordinated and well-working glucose metabolism is essential for normal liver regeneration. It is long known that after PH the decrease in liver tissue is accompanied by complex hormonal changes producing an increase in glycogenolysis and gluconeogenesis in the remnant liver [28], as well as a shift of glucose utilization towards the pentose phosphate pathway in order to provide NADPH for lipid and DNA synthesis. Nevertheless, these studies have been carried out mainly in the early stages after PH, before the liver reaches the Hepatostat [29], whereas we show that in our study, even though the liver had reached 100% of the liver weight/body weight [10] differences in the carbohydrate-related metabolism are still present: glucose (G140) and glucose-containing oligosaccharides (sucrose - G162, maltose - G162, lactose - G164, maltotriose - G182) were lower after PH, whereas phosphorylated sugars (erythrose phosphate G143, glucose phosphate G151), and compounds related to pentose phosphate pathway were increased (gluconolactone G114, galactonolactone G122, galacturonic acid G130, gluconic acid G133). On the other hand, ribose-phosphate G139 was decreased after PH. All together, these results point to an intense activity of nucleic acid synthesis associated to cell replication. In all cases, MCD diet

does not change the trend, or direction of the change (increase/decrease) of each carbohydrate metabolite, associated to PH. The differences between both diets are in the relative abundances.

When mice under MCD diet were subjected to PH, the aforementioned changes in amino acids due to active protein catabolism, together with the alterations in carbohydrate metabolism lead to differences also in the levels of lactic(G007): pyruvic (G005) ratio (lower in MCD than in CT, largely increased after PH in CT, only mild increase after PH in MCD). Under MCD diet, PH involves more activity of the mitochondrial TCA cycle: whereas citric, malic, and fumaric acids were decreased after PH in CT group, their levels were increased after PH in the MCD group. In the early stages after PH, there is an increase of the gluconeogenic and decrease of the glycolytic capacity [28], but our results show that after that early adaptation to a large growth activity, new adaptations occur, and glycolysis and lactate production are increased, and the modulation of the metabolic flux towards the mitochondria should be tampered to enhance the efficiency of the PH [13].

Concerning the lipidic content, again we should emphasize that most of the compounds that were found significantly different after PH were lipids (more than 90%) from which we annotated only the 174 most significant. In a recent study Alonso et al.[10], studied metabolomic data obtained from experimental animals as well as human samples, and stated that there are two subtypes of NASH according to their circulating pattern of triglycerides, diglycerides, fatty acids, ceramides, and oxidized fatty acids.

With the analytical approach employed, most of the lipids were detected by means of LC/MS (both positive and negative), and most of the signals detected with our LC/MS method could be attributed to lipids (Fig. 3). The intracellular triglyceride accumulation characteristic of NAFLD is usually expressed as corrected by the amount of proteins, and in these mice the ratio of hepatic triglyceride to proteins was shown to be increased [9]. For the sake of the global metabolic interpretation of the results, we decided to not correct any of the abundances of the metabolites, therefore reflecting the accumulation in the whole organ. With our metabolomics approach we found only moderate increase in 3 monoglycerides, and a decrease in 5 triglycerides (highly significant, $p < 0.0001$), which reflect the behaviour of all those lipids with $p < 0.05$. Our results are not as paradoxical as might seem, because mice with MCD diet-induced NASH also develop hepatic fibrosis [30]. Therefore, ratio lipids/protein in fibrosis might be increased whereas the total amount of lipids per mass unit of liver (as reflected by the total abundance) could show a different trend.

Our results show a significant increase in lipid metabolism, particularly glycerophospholipids as the main lipids that constitutes cell and organelles membranes; amino acids metabolism and glucose metabolites metabolism, which suggest that liver regeneration is being exacerbated by these pathways in order to quickly replace the removed damaged tissue, as a fast production of new healthy hepatocytes is needed.

1.4- Conclusion

In a mouse model of NASH, decreased hepatocyte damage and enhanced liver mass recovery after PH are likely related to multiple homeostatic mechanisms and adaptive metabolic responses including glucose metabolism. Such adaptation allows the regenerating liver to mainly focus on synthesis of lipids and DNA.

In our previous studies, we showed that part of these changes could be counteracted by a dual GLP1 receptor/glucagon receptor agonist [10], and to a lesser extent by a GLP1 receptor agonist [9]. In those studies, we focused mainly in the compounds that were different, associated to the treatments. In the present study we have deepened in the metabolic alterations under MCD diet and the impact of PH.

Both MCD diet and PH have a profound impact on the liver metabolism, although we found greater metabolic changes due to PH than to MCD diet. The multiplatform metabolomics approach has expanded the metabolite coverage, and CE/MS has allowed to detect changes in amino acid (proteinogenic and non-proteinogenic) and methylation pathways, whereas lipid metabolism (sphingolipid, phospholipid, neutral lipid, acyl carnitines, etc.) has been evaluated by means of LC/MS, and GC/MS has provided data about several carbohydrates and related small non-aminated compounds.

The present study has provided solid evidences of the metabolomics profile of the regenerating liver in the context of MCD-induced NASH. We have expanded the number of annotated compounds to a number close to 400, among those that significantly changed associated to NASH development and advanced liver regeneration after PH. This work will facilitate the study of the animal model of PH under MCD diet, in the quest for better therapeutic approaches to liver regeneration. This study has permitted to enlarge the knowledge about the metabolic routes that are involved in the advanced stages of liver regeneration after PH, especially in an animal model of NASH.

Declaration of interests

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.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Abbreviations

AA	Amino Acids
ANOVA	Analysis Of Variance
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
CDP	Cytidine diphosphate
CE/MS	Capillary Electrophoresis Mass Spectrometry
CMM	CEU Mass Mediator
DMT2	Diabetes Mellitus type 2
FFA	Free Fatty Acids
GC/MS	Gas Chromatography Mass Spectrometry
GP	Glycerophospholipids
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C virus
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LPA	Lysoglycerophosphatidic acid
LPC	Lysoglycerophosphocholine
LPE	Lysoglycerophosphoethanolamine
LPS	Lysoglycerophosphoserine
MCD	Methionine Choline Deficient
MFE	Molecular Feature Extraction
NAFLD	Non-Alcoholic Fatty Liver Disease
NASH	Non-Alcoholic Steato Hepatitis
NEG	Negative ion mode
PC	Glycerophosphocholine
PE	Glycerophosphoethanolamine
PG	Glycerophosphoglycerol
PH	Partial Hepatectomy
PI	Glycerophosphoinositol
PIP	Glycerophosphoinositol phosphate
POS	Positive ion mode
PS	Glycerophosphatidylserine
QC	Quality Control
qTOF	Quadrupole Time of Flight
RTL	Retention Time Locking
ROS	Reactive Oxygen Species
RP-LC/MS	Reverse Phase- Liquid Chromatography Mass Spectrometry
TG	Triacylglycerides
TOF	Time of Flight

Captions

Table 1: Total found features, per instrumental technique

Table 1: Total found features, per instrumental technique

PLATFORM	Detected features	
	total (n°)	Total (%)
CE/MS	207	6.3%
LC/MS.neg	1300	39.4%
LC/MS. Pos	1519	46.1%
GC/MS	271	8.2%
TOTALS	3297	100%

Table 2: Total significant ($p < 0.05$) features, per instrumental technique and factor

Table 2: Total significant ($p < 0.05$) features, per instrumental technique and factor

PLATFORM	Significant features		
	MCD Diet	PH	Interaction
CE/MS	62	82	40
LC/MS.neg	621	1224	551
LC/MS. Pos	732	1285	572
GC/MS	115	223	56
TOTALS	1530	2814	1219

Table 3: Summary of most affected groups of metabolites due to partial hepatectomy (PH). Values are the average of the individual $\log_2$ (Fold Change); Fold change = quotient between the average of the signals after PH and before PH, for each diet. CT = Control; MCD = Methionine-Choline Deficient.

Table 3: Summary of most affected groups of metabolites due to PH. Values are the average of the individual $\log_2$ (Fold Change); Fold change = quotient between the average of the signals after PH and before PH, for each diet

Metabolic family	$\log_2(\text{FC}[\text{PHCT vs. CT}])$ PH in CT	$\log_2(\text{FC}[\text{PHMCD vs MCD}])$ PH in MCD
Fatty acid	7.0	4.6
GL (DG)	6.6	5.0
GL (MG)	2.3	1.6
GL (TG)	-2.8	-1.7
GP (PC)	-4.0	-5.9
GP (PC//PE)	5.9	6.7
GP (PE)	3.5	3.4

GP (PI)	12.1	9.5
GP (PS)	8.9	8.9
ST (bile acids and der.)	26.7	26.7
ST (Chol. and der.)	2.7	5.4
AA	1.0	0.7

Fig.1: Sample preparation and data acquisition scheme for multiplatform analysis.

Fig.2: Summary of main metabolic differences between MCD diet vs. Control diet. Ala: Alanine; Pyr: Pyruvate; Lac: Lactate; Glc: Glucose; AcCoA: Acetyl-CoA; GOL: Glycerol; MG: Monoglycerides; DG: Diglycerides; TG: Triglycerides; PL: Phospholipids; FA: Fatty Acids; Fum: Fumarate; Mal: Malate; Cit: Citrate; DMG: DiMethyl Glycine; Gly: Glycine; Ser: Serine; SAME: S-Adenosyl Methionine; SAH: S-Adenosyl Homocysteine; Hcy: Homocysteine; Cys: Cysteine; GSH: Glutathione reduced; Met: Methionine; 5-MTHF: 5- Methyl Tetrahydrofolate; 5,10-MTHF: 5,10- Methylene tetrahydrofolate; dUMP: Uridine monophosphate; dTMP: Thymidine monophosphate; DHF: Dihydrofolate; SAMethionamine: S-Adenosyl Methionamine; PUT: Putrescine; 4-AB: 4-Amino butyric.

Fig.3. Graphical representation of the three most relevant groups of metabolites involved in different metabolic changes before and after PH. Panel A: changes

comparing groups fed with control diet. Panel B: changes comparing groups fed with MCD diet. GP, Glycerophospholipids; AA, Amino Acids; TG, Triglycerides. Circles size increase proportionally to the logarithm of the relative abundance of metabolites before and after PH. Circle colors represent the metabolic changes within each diet due to PH: blue means decrease while red means increase.