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Metabolomic Characterization of Human Model of Liver Rejection Identifies Aberrancies Linked to Cyclooxygenase (COX) and Nitric Oxide Synthase (NOS)

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Statistical Analysis C
Data Interpretation D
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Background: Acute liver rejection (ALR), a significant complication of liver transplantation, burdens patients, healthcare payers, and the healthcare providers due to an increase in morbidity, cost, and resources. Despite clinical resolution, ALR is associated with an increased risk of graft loss. A unique protocol of delayed immunosuppression used in our institute provided a model to characterize metabolomic profiles in human ALR.

Material/Methods: Twenty liver allograft biopsies obtained 48 hours after liver transplantation in the absence of immunosuppression were studied. Hepatic metabolites were quantitated in these biopsies by liquid chromatography and mass spectroscopy (LC/MS). Metabolite profiles were compared among: 1) biopsies with reperfusion injury but no histological evidence of rejection (n=7), 2) biopsies with histological evidence of moderate or severe rejection (n=5), and 3) biopsies with histological evidence of mild rejection (n=8).

Results: There were 133 metabolites consistently detected by LC/MS and these were prioritized using variable importance to projection (VIP) analysis, comparing moderate or severe rejection *vs.* no rejection or mild rejection using partial least squares discriminant statistical analysis (PLS-DA). Twenty metabolites were identified as progressively different. Further PLS-DA using these metabolites identified 3 metabolites (linoleic acid, γ-linolenic acid, and citrulline) which are associated with either cyclooxygenase or nitric oxide synthase functionality.

Conclusions: Hepatic metabolic aberrancies associated with cyclooxygenase and nitric oxide synthase function occur contemporaneous with ALR. Additional studies are required to better characterize the role of these metabolic pathways to enhance utility of the metabolomics approach in diagnosis and outcomes of ALR.

MeSH Keywords: **Graft Rejection • Liver Transplantation • Metabolomics • Nitric Oxide Synthase • Prostaglandin-Endoperoxide Synthases**

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Background

Acute liver rejection (ALR) following liver transplantation occurs in approximately 4–20% of patients [1,2]. According to the United Network for Organ Sharing (UNOS), in 2017 the number of liver transplants performed in the USA was 8082, representing an increase of 24.5% since 2016. Currently, the number of patients waiting for liver transplant is approximately 13 914 and the average waiting time for liver transplant is 511 days. Therefore, although the incidence of acute liver rejection is low, the high volume of transplants coupled with increased frequency and limited donors encourages the maximization of graft survival by addressing ALR, a major cause of graft damage.

This study aimed to characterize metabolomic aberrancies in a human model of liver rejection to guide future studies aimed at addressing graft damage congruent with ALR. Between 2008 and 2012, the standard immunosuppression protocol for patients receiving liver transplants at Indiana University Hospital was begun 2 days following transplant and prior to collection of liver biopsy at the time of fascial closure. The hypothesis was that a delay in immunosuppression would induce tolerance [3]. This immunosuppression delay protocol coupled with the collection of a fascial closure biopsy provides an opportunity to characterize ALR consequences in a human model of early liver rejection, specifically to utilize a targeted liquid chromatography/mass spectrometry (LC/MS) platform for metabolomics to profile and quantify hepatic metabolites in order to identify metabolic signatures associated with ALR.

Metabolomics is the study of a large number of small molecule metabolites in biofluids and tissue to identify biomarkers associated with altered metabolic pathways. As metabolites are modulated by protein and enzymatic function, they reflect many of the alterations caused by disease or other biological stresses. Metabolites are exquisitely sensitive to different biological states and therefore represent a promising approach to identify biopathology contemporaneous with rejection [4–6]. Several analytical techniques such as nuclear magnetic resonance (NMR), LC-MS, and gas chromatography-mass spectrometry (GC-MS) have been used to detect metabolic changes [7]. Several studies have used a variety of analytical techniques to elucidate aberrant pathways associated with cancer [8]. While a few of these studies have used animal models [9–11], the majority have focused on humans [4,10,12–22] using NMR [4,16], LC-MS [12,13,15,17,21,22], and GC-MS [13–15,18,19], or HPLC methods [20]. These studies have reported alterations in numerous metabolic pathways, including glycolysis, amino acid, fatty acid, and bile acid metabolism. While there are few reports that have focused exclusively on altered metabolic pathways associated with liver rejection, there have been studies relating to rejection-associated events. Previous reports have documented metabolic pathways and individual metabolites that modulate immune cell function and immune responses [23]. For example, modulation of T cells has been well documented in conjunction with rejection, as has the role of metabolism and nutrient availability upon T cell activation and function [5,24]. Moreover, activation of T cells requires metabolic reprogramming in order to increase glycolytic flux, lactate, lipids, proteins, nucleic acid, and carbohydrates [25]. These changes in metabolic profiles also direct signaling. For example, increased intracellular leucine metabolism controls mammalian target of rapamycin (mTOR) signaling required to induce Th1, Th2, and Th17 CD4(+) T effector cell differentiation [26]. Therefore, there is a need to better understand changes in hepatic biochemical pathways associated with rejection, and metabolomics is an established analytical modality available to identify key compounds. Focusing on hepatic tissue prior to immunosuppression in patients provides a unique opportunity to improve our understanding of graft damage and loss.

Material and Methods

All patients were reviewed and approved by the Indiana University Institutional Review Board protocol # 1011004278 "Acquisition and storage of liver tissue and blood for research". Sixty patients were recruited at the time of listing for liver transplant and received a liver transplant. Underlying liver etiology and clinical correlates were collected from medical records. Liver biopsies were collected 2 days following liver transplant at the time of fascial closure, frozen in liquid nitrogen, and stored at –86°C. Patients did not receive immunosuppression prior to fascial closure. Biopsies were evaluated for histological evidence of rejection or other pathological aberrances as per clinical standard of care. Indiana University Department of Pathology notes were collated for evidence of rejection and reperfusion injury. Matched biopsies were chosen from 21 patients, based on the standard of care routine pathology report of 2-day biopsy. Biopsies were reviewed again by a pathologist (RS) per international working party on the terminology of hepatic allograft rejection [27]. The pathologist was not able to classify reperfusion injury or rejection for 1 biopsy and this biopsy was excluded from LC/MS metabolomic analysis. Remaining biopsies were stratified into 3 groups based on pathologist (RS) rating. Group 1: no histological evidence of rejection (N=7), Group 2: evidence of mild rejection (N=8), and Group 3: patients with evidence of moderate or severe rejection (N=5).

Patients and procedures

In all liver transplants, the muscle layer was left open and skin was closed immediately after transplantation to prevent compartment syndrome. All patients were taken back to the operating room for delayed fascial closure on the 2nd post-transplant

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Table 1. Patient demographics.

	Control reperfusion injury	Mild rejection	Moderate or severe rejection
Number	7	8	5
Age (years)	64±6.4	62±2.9	63±7.9
Gender (M/F)	5/2	6/2	4/1
MELD score	23±4.4	21±8.9	20±4.5
AST	23±2.1	25±3.8	19±4.0
Warm ischemia time (min)	17±2.8	18±3.16	20±3.3
Cold ischemia time (min)	320±58.6	312±77.2	383±61.9

Data represents mean ±SD.

day [28]. During fascial closure, a second allograft biopsy was performed. A part of the second liver allograft biopsy was frozen for future use. Patients did not receive any immunosuppression prior to fascial closure [3]. For the purpose of this study, the day 2 biopsies were divided in to 3 groups (n=5–8 per group): Group 1 (control), patients with no evidence of rejection; Group 2, patients with histological evidence of mild rejection; and Group 3, patients with evidence of moderate or severe rejection. To evaluate the metabolic changes associated with liver rejection, we performed LC-MS analysis, targeting 216 metabolites in liver biopsies taken 2 days after liver transplantation.

Immunosuppression protocol

The induction immunosuppression consisted of 3 doses of 2 mg/kg rabbit anti-thymocyte globulin (rATG) every 48 hours starting on post-transplant day 2 along with a single dose of 1.5 mg/m^2 BSA of rituximab on post-transplant day 3. Premedication for rATG was given immediately before its administration in the form of solumedrol [500 (first dose), 250 (second dose), and 120 mg (third dose)], acetaminophen (650 mg), and diphenhydramine (25 mg). Maintenance immunosuppression was also initiated on post-transplant day 2 in the form of tacrolimus monotherapy, although some recipients received additional mycophenolate mofetil. The goal trough levels for tacrolimus were 7 to 10 ng/mL in the first 3 months and 6 to 8 ng/mL thereafter [3].

LC/MS

Day 2 liver allograft biopsies were frozen in liquid nitrogen and stored at –86°C. Tissue was transported on dry ice to the Northwest Metabolomics Research Center (NW-MRC) at the University of Washington for analysis. Briefly, targeted LC/MS/MS was performed according to methods developed at the University of Washington Metabolomic Research Center as per Zhu et al. [29] Targeted aqueous metabolite profiling analysis was performed using an Agilent 1260/AB-Sciex 5500 Qtrap Liquid Chromatography-Mass spectroscopy/mass spectroscopy (LC-MS/MS) instrument and standard operating procedures we developed previously [29]. The LC-MS/MS analysis is based on hydrophilic interaction chromatography (HILIC), and targets 216 metabolites located in more than 35 different metabolic pathways. This system provides detailed information on metabolites involved in glycolysis, tricarboxylic acid cycle (TCA), and pentose phosphate shunt, as well as amino acid, fatty acid, and nucleic acid metabolism, and other pathways. Twenty-six isotope-labeled internal standards were included to monitor sample preparation steps and system performance, as well as to provide absolute quantitation of a number of amino and organic acids.

Data analysis for metabolomics

The intensity of tissue peaks in each data set were normalized to tissue weight. Statistical analysis was performed using XLSTAT software. Each data set was mean-centered before the analysis. Univariate analysis of the individual metabolites was performed using the *t* test to identify metabolites for multivariate analysis. The statistical differences are expressed as p-values. Multivariate partial least squares - discriminant analysis (PLS-DA) was performed using XLSTAT software using metabolites identified by variable importance to projection (VIP) analysis.

Results

There were 3 deaths within the 3 cohort groups (Table 1). None of the deaths were related to rejection and occurred 9–51months after transplant. One patient died 4 years 3 months after transplantation, with no evidence of rejection in the entire post-transplant course. One patient had an HCV recurrence and died 3 years 1 month after transplantation, with no evidence of rejection in the entire post-transplant course. The third patient died from a viral infection 9 months after transplantation, and was not linked to an isolated episode of moderate/severe rejection diagnosed 2 days post-transplant.

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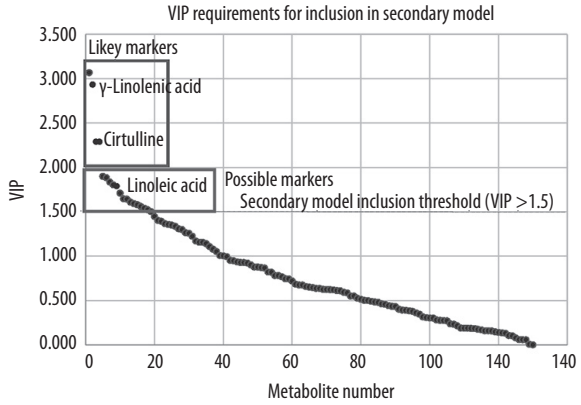

Figure 1. Initial modelling to exclude irrelevant variables. Plot of all metabolite VIP valuables in the initial model. Metabolites identified as biomarkers in the final model are labeled. This step was used to remove variables unlikely to contribute to the identification of rejection. The remaining variables could then be investigated for significance with reduced interference.

Histological evidence of rejection

Of the 60 patients recruited and transplanted, routine pathology reports described histological evidence of rejection in 14 patients (23%). Biopsies were stratified based on these reports into 3 groups: 1) no histological evidence of rejection, 2) evidence of mild rejection, and 3) patients with evidence of moderate or severe rejection. Seven samples from each group were selected for pathology review and metabolomic analysis. A pathologist (RS) reviewed all 21 fascial closure liver biopsies taken 2 days post-transplant. Her analysis found 5 biopsies had unequivocal evidence of moderate or severe rejection (endothelialitis, cell infiltration, or bile duct injury). Seven biopsies had no evidence of rejection but had reperfusion damage. One sample was indeterminable, did not reach criteria for rejection, and had no reperfusion injury. The remaining 8 biopsies had histological evidence of mild rejection. The 1 biopsy that was indeterminable was not included in either rejection or control groups and data were not included in the metabolomic analysis.

The biopsies in the rejection group were characterized by the presence of a mixed inflammatory infiltrate in portal tracts that comprised variable combinations of lymphocytes, eosinophils, and neutrophils. Endothelialitis and bile duct damage were present in varying degrees of severity. Control biopsies showed features of reperfusion damage that included variable combinations and severity of portal edema, peribiliary neutrophils, perivenular hepatocellular necrosis, and presence of lobular neutrophils. One biopsy showed mild macrovesicular steatosis with necrosis and neutrophils. The biopsies were characterized into 3 groups for the purpose of metabolomic analysis: Group 1 was reperfusion injury only (N=7), Group 2 was categorical histological evidence of moderate or severe rejection (N=5), and Group 3 was histological evidence of mild rejection (N=8).

LC/MS-based metabolomics

The LC-MS/MS method was optimized to target a total of 216 metabolites in the liver biopsy samples. However, after deleting metabolites that were not detected, metabolites below the signal to noise cutoff, and metabolites inconsistently detected in the samples, 133 metabolites were quantified. We assessed and compared differences in metabolites between 1) biopsies with mild rejection or with moderate or severe rejection when compared to tissues with reperfusion injury and 2) biopsies with mild rejection when compared to biopsies with moderate or severe rejection. Each metabolite was ranked by its variable importance in the projection (VIP) score via partial least squares-discriminative analysis (PLS-DA) using XLSTAT Biomed software (Figure 1). Twenty-one metabolites with VIP scores above 1.5 were included in a secondary PLS-DA analysis comparing no rejection (reperfusion injury) to both moderate to severe rejection (Figure 2A) and mild rejection (Figure 2B). Of these metabolites, linoleic acid, γ-linolenic acid, and citrulline emerged as providing the strongest predictive model of rejection (Figure 2). The differences between these metabolites in rejection (mild, moderate, and severe) and control biopsies were examined individually (Figure 3). They were then used to construct a final sample model by PLS-DA. Cross-validation of this model was then used to estimate how closely the 3 metabolites, taken as a group, correlate with the biopsy histology report from the pathologist (RS) (Figure 4). The resulting aggregate indicates that, taken together, the 3 metabolites can accurately identify the rejection status of each patient in our sample group, and that this is likely to be the case for independent samples.

Discussion

This study represents a unique model of human liver rejection due to the unique immunosuppression and surgical protocol that was followed. There are no previously published data on human liver rejection in this setting. In the absence of immunosuppression, changes occurring in the liver biopsies in the setting of cellular rejection are novel and intriguing. Using 2-day protocol liver biopsies, targeted LC/MS-based metabolomics analysis, and PLS-DA, we identified 3 aberrant metabolites (linolenic acid, γ-linolenic acid, and citrulline) contemporaneous with liver rejection.

LC/MS/MS-based metabolomics provides broad-based coverage of the important small molecule metabolites in biofluids

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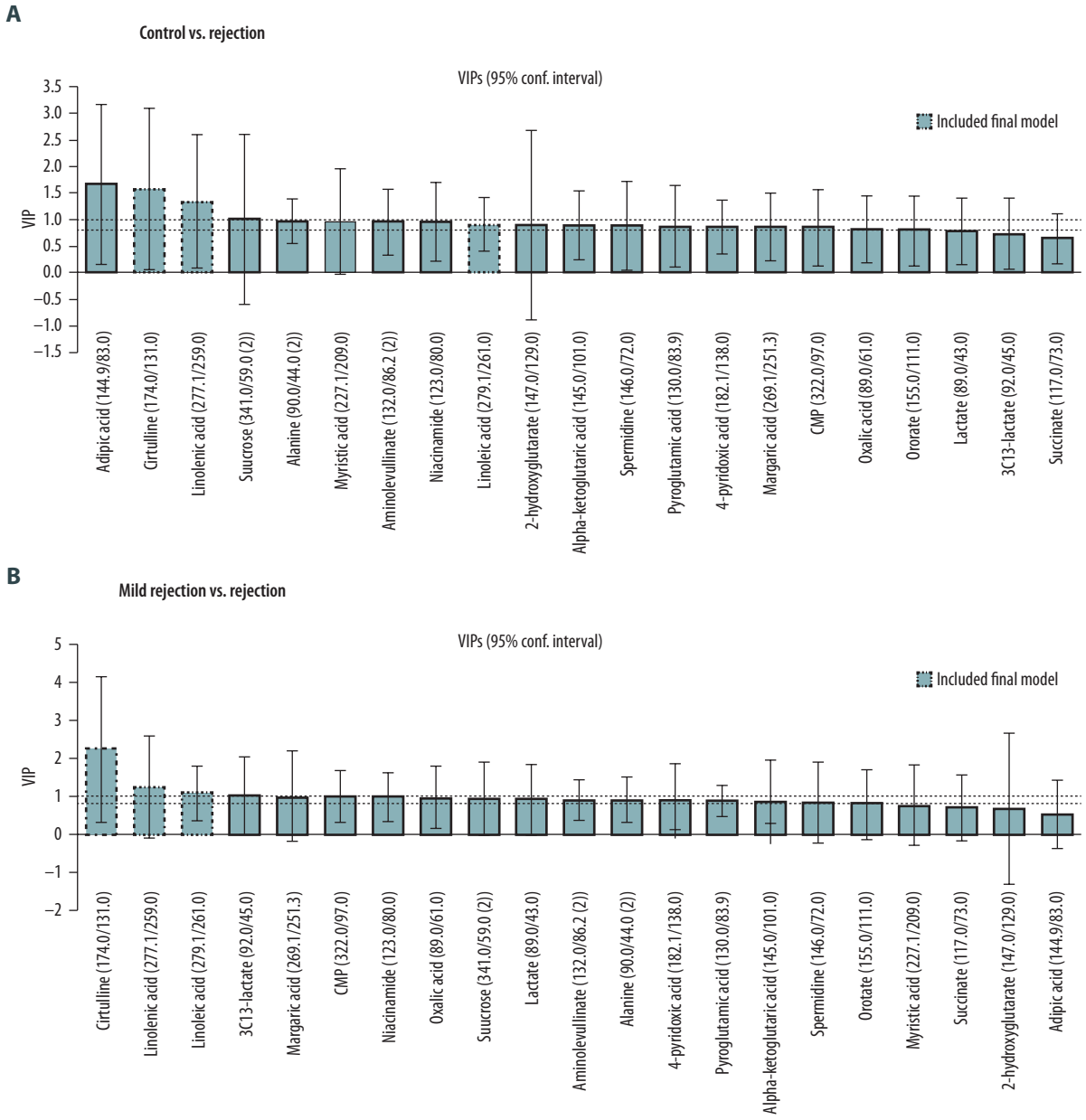

Figure 2. (**A, B**) Secondary models of potentially significant metabolic markers. Variable Importance in the Projection (VIP): Are estimates of the relative predictive power of each variable in a partial least squares model. Citrulline, linolenic acid, and linoleic acid (highlighted in violet) were selected for use in a final regression model based on their high VIP in the initial model, and relatively high VIP in discriminating between both control data and rejection, and full rejection *vs.* mild rejection. Data from comparison between mild rejection and control are omitted, as the 2 could not be easily distinguished.

and tissue to allow the identification of altered metabolic pathways. As metabolites are modulated by protein function, they reflect many of the alterations caused by disease or other biological stresses [4–6]. Analysis using PLS-DA is appropriate when large numbers of potentially correlated variables must be analyzed. It is especially well suited to cases where the number of variables exceeds the number of samples, which would otherwise produce overfitting using conventional regression models. We used VIP scores, which represent the effect of a particular variable on the PLS-DA model, to eliminate non-predictive variables from our dataset, and to identify the variables with the highest degree of predictive power at the level of individual patients. This analysis revealed 3 metabolites: linoleic acid, γ linolenic acid, and citrulline. Linoleic acid

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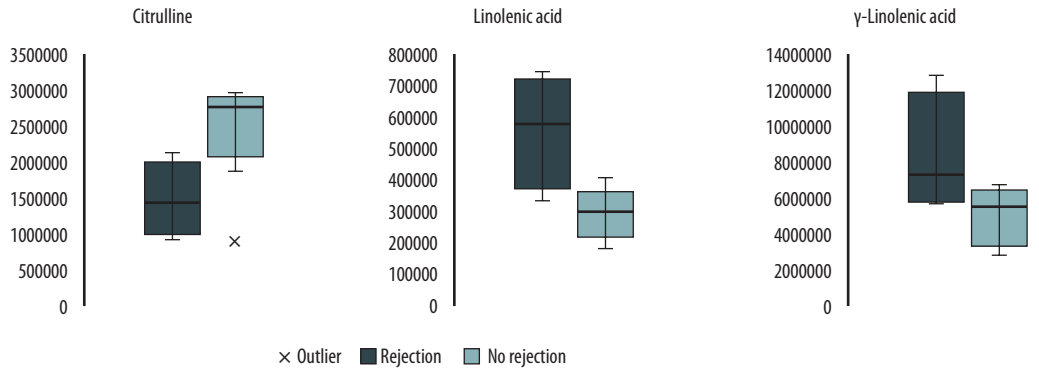

Figure 3. BOX and whisker plots for 3 major metabolites associated with rejection. Box-and-whisker plots showing the distribution of the selected metabolites in both rejection and non-rejection samples. The boxes display the 25th through 75th percentiles, with the whiskers showing the 5th through 95th percentile.

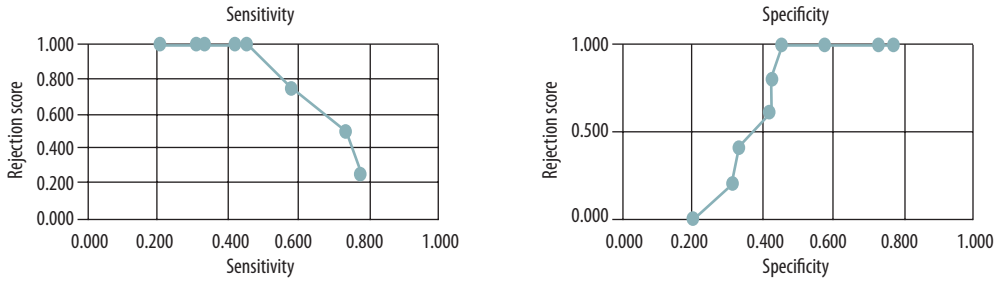

Figure 4. ROC curve for PLS-DA analysis of Linolenic acid, Linoleic acid, and Citrulline. Sensitivity and specificity of the model for different cutoff values of the aggregate rejection score. Optimizing the threshold for rejection results in zero false positives and zero false negatives in jackknife cross-validation of the final PLS-DA analysis (AUROC=1).

and γ-linolenic acid are associated with cyclooxygenase (COX) pathways, while citrulline is associated with nitric oxide synthase (NOS) pathways.

Linoleic acid is an octadecadeinoic fatty acid and a precursor for arachidonic acid, which is a substrate for COX enzymes and subsequent biosynthesis of vasoactive molecules. Changes in arachidonic acid are linked to numerous pathologies of the liver, including portal hypertension and liver cirrhosis [30,31]. Linoleic acid regulates the COX-2/VEGF/MAP kinase pathway [32] and endothelial vasodilatory function [33]. Studies have shown that COX-2 was significantly increased in a rodent model of liver rejection [34]. However, whether increased COX is beneficial or not is controversial. Some studies have shown that increased COX-2 is protective [35], while others have found that inhibition of COX-2 increases graft survival in animal models [34,36]. Moreover, linoleic acid is also associated with pathologies independent of COX, as it is synthesized from phosphatidylcholine via phospholipase A2 or phospholipase A1. Aberrancies of phospholipase A2 are associated with Parkinson disease, peroxisomal beta-oxidation enzyme deficiency, neurodegeneration with brain iron accumulation, and peroxisomal acyl-CoA oxidase deficiency [37,39].

The second metabolite identified by PLS-DA was γ-linolenic acid, which is an all-cis-6,9,12-octadecatrienoic acid designated as 18: 3 and is synthesized from linoleic acid by introduction of a (third) double bond at the delta 6 position under the catalytic influence of delta-6-desaturase enzyme. This step is believed to be the rate-limiting stage in the metabolic pathway. Aging, obesity, diabetes, high alcohol intake, stress-related hormones, and viral infections are known to reduce conversion of linoleic acid to γ-linolenic acid [40–43]. γ-linolenic acid is known to inhibit angiogenesis, partly via the decrease in the expression of VE-cadherin and beta-catenin [44], potentially due to the elimination of the precursor, -linoleic acid. Hepatocyte expression of insulin growth factor-I, insulin growth factor-II, growth hormone receptor, insulin receptor, Insulin growth factor binding protein-3, and Insulin growth factor binding protein-4 mRNAs are all upregulated by linoleic acid [45]. Conversion of linoleic acid to γ-linolenic acid is known to be beneficial for human health [46]. Linolenic acid attenuates endothelial apoptosis *in*

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vitro via the PI3K/Akt/eNOS pathway [47]. In terms of targeting linoleic acid/γ-linolenic acid, there are a number of compounds aimed at modulating the activity of PLA2, including varespladib [48], giripladib [49], and efipladib [50], which would limit the biosynthesis of both linoleic acid and γ-linolenic acid.

The third metabolite identified by PLS-DA was citrulline, a key intermediate in the urea cycle produced via the metabolism of ornithine and carbamoyl phosphate. Moreover, citrulline is a by-product of the enzymatic production of nitric oxide from the amino acid arginine. As citrulline is a part of the urea cycle and urea is a marker of liver failure, it is not unexpected that rejection is associated with increased citrulline. However, urea levels were not significantly higher in patients with rejection. Because citrulline is involved in many biological pathways, it is impossible to accurately hypothesize the pathobiology, physiological, and biochemical milieu associated with changes to hepatic linoleic acid, γ-linoleic acid and citrulline based on biopsies. However, the fact that they are connected to important hepatic perfusion regulators suggests that changes impart/reflect a response to tissue stress, damage, and/or acute graft rejection. Arginine is the predominate substrate for the production of nitric oxide (NO), a well-documented vasodilator associated with liver perfusion and portal hypertension [51,52]. The role of NO in liver perfusion is well documented and focuses on sinusoidal stellate cell control of sinusoidal dilation and thus an increase in resistance to portal venous blood flow. A reduction in citrulline might be indicative of a modulation of NO biosynthesis. Reduced citrulline could be reflective of a reduction of NOS activity, as citrulline is the biproduct of the conversion of arginine to NO. In contrast, as citrulline is also the substrate, a reduction could be indicative of an increase in NOS activity. What we do know is that a change in NO within the liver will modulate perfusion and affect ischemia and hypoxia and impart an additional stress to the liver. Moreover, endothelial NOS (eNOS) is also known to "uncouple" when co-factors are absent, leading to the formation of oxygen free radicals [53]. The conversion of arginine to NO and citrulline is a 2-step process involving N-hydroxy-l-arginine as an intermediate; therefore, uncoupling of endothelial NOS could result in a reduction in citrulline.

The data do not suggest that either linolenic acid or citrulline should replace current markers of acute liver rejection. LC-MS/MS is unlikely to be quicker or cheaper than histology and liver functional tests. Nevertheless, there is utility in investigating linolenic acid and citrulline, as both have been shown to be markers of interest in other pathologies. For example, the ratio of linolenic acid to deoxycholic acid species is a potential biomarker for metabolic abnormalities in obesity [54] and hepatic steatosis [55]. Moreover, the circulating citrulline concentration is a biomarker of intestinal functionality [56,57]. What the data may reveal is hepatic response to acute liver rejection by the modulation of vasodilators to maintain liver perfusion. However, we are cognizant that differences in metabolomics signatures between control livers and livers with rejection could be independent of rejection. It is possible that these differences are linked to other aspects of liver disease. For example, sarcopenia, which is associated with modulated metabolism, poorer outcomes, and changes in the levels of citrulline and linoleic acid, occurs in patients with liver disease [58–61]. A preliminary analysis of sarcopenia in the patients within this study, based on measurement of the psoas muscle at the C3, as previously described [62,63], was performed and identified 3 patients with sarcopenia. Two patients within the early rejection group had sarcopenia. One patient with no evidence of rejection was identified with sarcopenia. Because the frequency of overlapping sarcopenia within the 3 cohorts is sporadic, it is difficult to determine if sarcopenia is an independent variable in hepatic metabolites associated with hepatic response to rejection.

Additional research is required to further elucidate our findings and to better understand any connection among metabolic changes, acute liver rejection, and graft survival. Further research is likely to focus on metabolomic quantification post-transplant in rodent models of liver rejection [64]. This is because rejection rates observed in clinical programs are very low; therefore, to expand this project using patient samples only would be prohibitive. Moreover, the delayed immunosuppression protocol is controversial and delayed immunosuppression and 2-day protocol biopsies are not the standard of care at our institute at present.

Finally, the immunosuppression protocol deserves further explanation. The premise behind delayed introduction of immunosuppression was to allow immune activation of recipient lymphocytes in the allograft. It was thought that the potent rATG would then lead to apoptosis and death of recipient lymphocytes within the graft, allowing operational tolerance in the long term. Although this approach permits a degree of rejection in the allograft, this is a desired effect and has no adverse effects in the long term, which was demonstrated in our larger study involving 1000 patients [3]. Based on this large-sample experience, we do not believe that deaths that occurred in this study cohort were due to the delayed immunosuppression.

Conclusions

Contemporaneous with acute liver rejection, increases in linoleic acid and γ-linolenic acid are observed alongside a decrease in citrulline. These metabolites are connected to pathways that regulate liver perfusion.

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