Title

Multiassay nutritional metabolomics profiling of low vitamin A status versus adequacy is characterized by reduced plasma lipid mediators among lactating women in the Philippines: A pilot study

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Metabolomics Reveals Altered Hepatic Bile Acids, Gut Microbiome Metabolites, and Cell Membrane Lipids Associated with Marginal Vitamin A Deficiency in a Mongolian Gerbil Model

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Scope: This study is designed to provide a broad evaluation of the impacts of vitamin A (VA) deficiency on hepatic metabolism in a gerbil model.
Methods and results: After 28 days of VA depletion, male Mongolian gerbils (*Meriones unguiculatus)* are randomly assigned to experimental diets for 28 days. Groups are fed a white-maize-based diet with ≈50 µL cottonseed oil vehicle either alone (VA−, $n = 10$) or containing 40 µg retinyl acetate (VA+, $n = 10$) for 28 days. Liver retinol is measured by high-performance liquid chromatography. Primary metabolomics, aminomics, lipidomics, bile acids, oxylipins, ceramides, and endocannabinoids are analyzed in post-mortem liver samples by liquid chromatography–mass spectrometry.
Results: Liver retinol is lower ($p < 0.001$) in the VA− versus VA+ group, with concentrations indicating marginal VA deficiency. A total of 300 metabolites are identified. Marginal VA deficiency is associated with lower bile acids, trimethylamine *N*-oxide, and a variety of acylcarnitines, phospholipids and sphingomyelins ($p < 0.05$). Components of DNA, including deoxyguanosine, cytidine, and *N*-carbomoyl-beta-alanine ($p < 0.05$), are differentially altered.
Conclusions: Hepatic metabolomics in a marginally VA-deficient gerbil model revealed alterations in markers of the gut microbiome, fatty acid and nucleotide metabolism, and cellular structure and signaling.

1. Introduction

Vitamin A (VA) is an essential micronutrient that plays a key role in many biological processes including growth, vision, reproduction, immunity, cellular differentiation and proliferation.[1] The deficiency of this micronutrient is a worldwide health concern, affecting over 190 million children and 19 million pregnant women.[2] Consequences of poor VA status include xerophthalmia, anemia, and weakened host resistance to infection, which can increase the severity of infectious diseases and risk of death.[3,4] In high income countries, VA deficiency is a common consequence of alcoholic liver disease, a major cause of liver damage worldwide, in addition to cystic fibrosis and pancreatic insufficiency.[5,6] The term VA or retinoids encompasses a variety of retinol analogs.[7] Retinoic acid is the primary active form of the vitamin, playing a critical role in the regulation of gene expression.[8] There has been substantial progress in the understanding of VA metabolism including advances in

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knowledge of the art of intestinal absorption and metabolism of retinoids within the enterocyte, uptake, processing, and storage within the liver.[9] The molecular implications of retinoids regulating transcriptional actions have received particular interest.[8] However, omics technologies have not been extensively used in the context of VA deficiency. The proteomic responses of retinol and carotenoid administration to an animal experimental model have been recently published,[10] but no metabolomic characterizations of VA deficiency have been reported. In the present study we used metabolomic analyses to further understand the influence of VA deficiency on overall metabolism in the liver. This study characterizes the hepatic metabolomic profile of marginal VA deficiency in Mongolian gerbils, an animal model commonly used in VA depletion studies and considered appropriate to extrapolate results to humans.[11,12]

2. Metabolomic Analysis

2.1. Primary Metabolomic, Aminomic, and Lipidomic Methods

Metabolomics assays for primary metabolomics, aminomics, and lipidomics were performed on liver samples using protein precipitation extraction with liquid chromatography tandem quadrupole mass spectrometry (LC-MS/MS) using modified published methods.[13,14] These three metabolomics assays are best described as semi-targeted since specific precursor and product ions were screened for hundreds of metabolites from several metabolite classes, including a total of 315 metabolites from 21 metabolite classes, and semi-quantitative data (peak area) was produced. More specifically, the primary metabolomics assay screens for primary metabolites such as carbohydrates, carboxylic acids, purines/pyrimidines/nucleotides/nucleosides, amino acid derivatives, sterols, and vitamins, in addition to other compounds. The aminomics assay screens for amine compounds including amino acids, amino acid derivatives, purines/pyrimidines/nucleotides/nucleosides, quaternary ammonium compounds, acylcarnitines, and imadazoles. The lipidomics assay detects complex lipids such as phospholipids, sphingomyelins, cholesteryl esters, diacylglycerols, and triacylglycerols.

2.1.1. Sample Preparation

Briefly, 40–60 mg liver was pulverized over dry ice prior to being added to Eppendorf tubes and spiked with 20 µL isotopically-labeled surrogates. Following the addition of 750 µL chilled methanol, samples were mixed by vortex 1 min prior to being centrifuged at 12 000 rpm for 10 min at 4 °C. The supernatant was transferred to 1.5 mL high-performance liquid chromatography (HPLC) amber glass vials, dried by centrifugal vacuum evaporation, and reconstituted in 3:1 methanol:acetonitrile containing 100 nM of 1-cyclohexylureido, 3-dodecanoic acid (CUDA; Sigma Aldrich). The reconstituted solution was mixed 1 min and filtered at 0.1 µm by centrifugation at 9500 rpm for 3 min through PVDF Durapore membranes (Millipore, Billerica, MA) at RT.

2.1.2. Sample Analyses

All UPLC-MS analyses were conducted on a Waters Acquity I-Class UPLC (Waters, Milford, MA) coupled with an API 4000 QTrap (Sciex, Framingham, MA) using multiple reaction monitoring (MRM) quantified with AB Sciex MultiQuant version 3.0. Specific MRMs have been previously published.[13] Primary metabolomics assay metabolites were separated using a 150 × 2.0 mm Luna NH2 column (Phenomenex, Torrance, CA) and detected by negative ion mode electrospray ionization.[13,15] Aminomics assay metabolites were separated using a 150 × 2.1 mm Atlantis HILIC column (Waters) and detected by positive ion mode electrospray ionization.[13,16] Lipidomics assay metabolites were separated using a 150 × 3.0 mm Prosphere HP C4 column (Grace, Columbia, MD) and detected by positive ion mode electrospray ionization.[13,17]

2.2. Bile Acids, Oxylipins, Ceramides, and Endocannabinoids Methods

Bile acids, oxylipins, ceramides, and endocannabinoids metabolomics assays were analyzed in liver tissues after enrichment with isotopically labeled surrogates, and elimination of phospholipids and proteins using a 96-well Ostro Pass-through Sample Preparation Plate (Waters; Milford, MA), and quantified by LC-MS/MS against authentic standards. These four metabolomics assays were targeted since they each analyzed single metabolite classes detected using specific precursor and product ions and generated quantitative data using calibrated standards for each metabolite.

2.2.1. Sample Preparation

Liver (≈20–25 mg) was pulverized over dry ice prior to being weighed into 2 mL polypropylene tubes. The aliquot was

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enriched with 5 µL of 0.2 mg mL^{-1} butylated hydroxytoluene and ethylenediaminetetraacetic acid in 1:1 methanol (MeOH)/water, and 10 µL of 1000 nM deuterated surrogates in MeOH. A total of 50 µL of MeOH was added (including the surrogate) and the tube was homogenized at −80 °C with three 1/8" size stainless steel balls for 30 s at 1350 rpm on a Geno/Grinder 2000 (SPEX Sample Prep, Metuchen, NJ). Isopropanol with 10 mM ammonium formate and 1% formic acid (550 µL) and water (100 µL) were then added, and the sample was homogenized for an additional 30 s. Tissue debris was removed by 5 min centrifugation at 10 000 g at room temperature and supernatants were transferred into the Ostro Plate wells.

The sample was eluted into 1.0 mL glass inserts containing 10 µL 20% glycerol by applying a vacuum at 15 mm Hg for 10 min. The eluent was dried by centrifugal vacuum evaporation on a Genovac EZ-2 (SP Scientific; Stone Ridge, NY) for 35 min at the medium boiling point setting, followed by an aqueous setting for an additional 35 min. The residue was reconstituted with methanol containing the internal standards CUDA and 1-phenyluredio, 3-hexanoic acid at 100 nM (1:1 methanol/acetonitrile), vortexed 1 min, and filtered by centrifugation through a 0.1 µm PVDF membrane (Millipore; Billerica, MA) for 3 min at 6 °C at <4500 g (rcf), and stored at −20 °C until analysis by LC-MS/MS.

2.2.2. Sample Analyses

Analytes in a 100 µL extract aliquot were separated by reverse phase chromatography using modifications of a previously published protocols for oxylipins,[18] endocannabinoids,[18] ceramides,[19] and bile acids.[20,21] Samples were held at 10 °C. Separated residues were detected by electrospray ionization in negative mode for oxylipins and bile acids and positive mode for endocannabinoids and ceramides using multiple reaction monitoring on an API 4000 QTrap (AB Sciex, Framingham, MA, USA). Analytes were separated using a 2.1 × 150 mm, 1.7 µm BEH C18 column for oxylipins and endocannabinoids, 2.1 × 100 mm, 1.7 µm BEH C18 column for bile acids, and 2.1 × 100 mm, 1.7 µm BEH C8 column for ceramides (Waters). Analytes were quantified using internal standard methods with 5 to 8 point calibration curves. Calibrants and internal standards were either synthesized [10,11-DHN, 10,11-DHHep, 10(11)-EpHep and CUDA] or purchased from Loradan Fine Lipids (Malmo, Sweden), Cayman Chemical (Ann Arbor, MI), Avanti Polar Lipids Inc. (Alabaster, AL), Steraloids (Newport, RI), Medical Isotopes (Pelham, NH), or Sigma-Aldrich (St. Louis, MO), unless otherwise indicated. Data were processed with AB Sciex MultiQuant version 3.0.

2.3. Statistical Analysis, Data Visualization, and Network Analyses

The data were log-transformed and the Distributions of the variables were checked with the Shapiro-Wilk test. A two-sided *t*-test or Mann–Whitney U test with adjustment for multiple hypothesis testing according to Benjamini and Hochberg at a false discovery rate (FDR) of 5%[22] were used to compare the measures of central tendency of the VA− versus VA+ groups. Differences in hepatic and serum retinol concentrations were tested using one-sided *t*-tests. Heatmap correlation matrixes were built using Spearman correlations to identify the metabolites that were connected to VA status and hepatic variables as well as to identify connections within metabolite assays. Enrichment analyses were used to identify the main metabolic pathways involved using the Holm adjusted *p*-values and the FDR procedure. *p*-values <0.05 were considered statistically significant. Metabolic pathways interconnections were plotted for network visualizations. Partial least squares discriminant analysis (PLS-DA) was performed on Johnson transformed data and included analytes with variable importance in projection scores greater than 1.25. Univariate and multivariate analyses were performed with the software JMP 13.1.0 (SAS Institute Inc., Cary, NC, USA). Heatmap correlations, enrichment analyses and network visualizations were performed with the R software version 3.6.1,[23] Metaboanalyst 4.0 (http://www.metaboanalyst.ca/, Canada),[24,25] and ChemRICH (http://chemrich.fiehnlab.ucdavis.edu/, USA).[26]

3. Results

3.1. Physical Characteristics and Vitamin A Status

Post-mortem analyses indicated that the two groups did not differ in body weight, but gerbils treated with VA had 15.5% lighter livers ($p < 0.01$) and a 14.2% lower liver to body weight ratio ($p < 0.05$) than the non-treated gerbils (**Figure** 1A). Liver retinol concentrations were higher ($p < 0.001$) in the VA+ versus the VA− group as expected. All gerbils in the VA+ group presented hepatic retinol concentrations ≥0.1 µmol g^{-1} (mean±SD; 0.45 ± 0.11 µmol g^{-1}). In contrast, in the VA− group all gerbils with the exception of one that presented liver retinol concentrations = 0.18 µmol g^{-1} had very low liver retinol concentrations (<0.1 µmol g^{-1}, 0.064 ± 0.05 µmol g^{-1}) (Figure 1B). In spite of the confirmed presence of low retinol concentrations in the liver, serum retinol concentrations of 1.14 ± 0.2 µmol L^{-1} in the VA− group and 1.24 ± 0.15 µmol L^{-1} in the VA+ group were not different ($p = 0.095$). Since the VA deficiency cutoff point of 0.1 µmol g^{-1} liver is not internationally accepted and serum retinol levels between VA− and VA+ groups were not different, we have defined the gerbils included in this study as marginally VA deficient.[27] Liver retinol concentrations negatively correlated with liver weight ($r = -0.71$, $p < 0.01$) (Figure 1C).[28] To follow-up on the alteration in liver weight, indicators of changes in adiposity were investigated. However, no differences between groups were detected for lipid mass, as well as total triglycerides and total cholesterol concentrations (results not shown). Hydroxyproline concentration, an indicator of fibrosis, was also not different between groups.

3.2. Change in Metabolite Concentrations in Response to Marginal Vitamin A Deficiency

A total of 300 metabolites were identified from the seven metabolomics assays used. Group significant differences were found for 25 metabolites (**Table 1**). The semi-targeted assays for primary metabolomics, aminomics, and lipidomics revealed various metabolites changed with marginal VA deficiency. The marginal VA deficiency metabolomic profile of primary metabo-

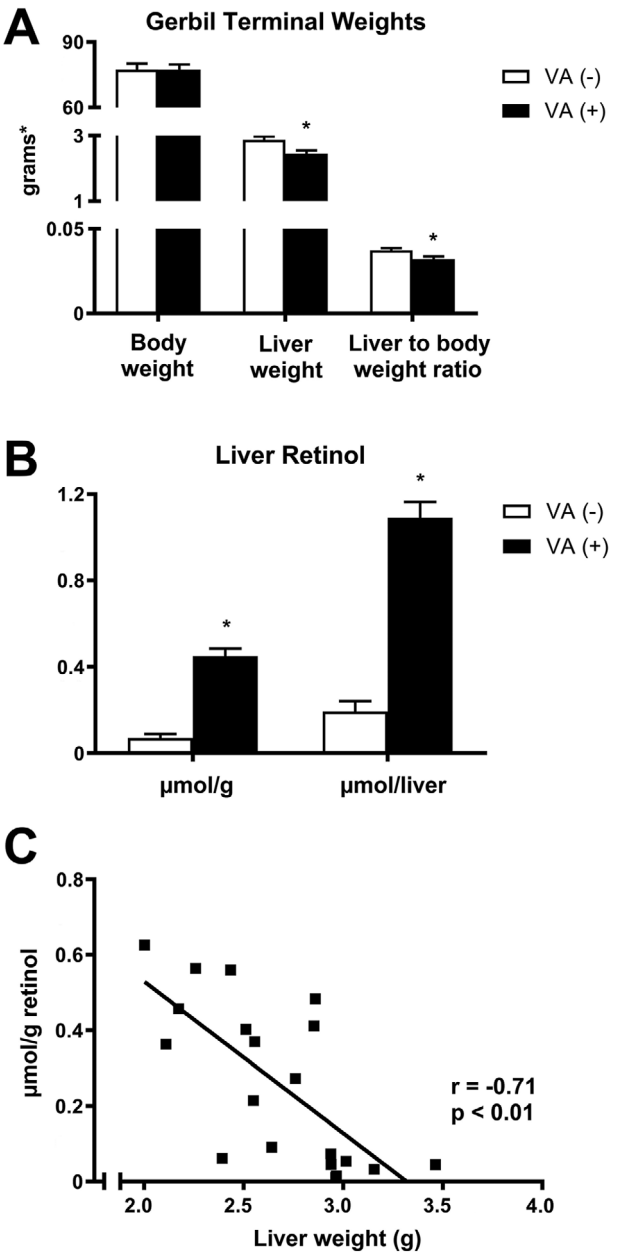

Figure 1. A). Gerbil terminal weights and B) liver retinol levels.

lites was characterized by lower levels ($p < 0.05$) of glycocholic acid, SUM glycochenodeoxycholate/glycodeoxycholate, cytidine, dihydroxyacetone phosphate, SUM maltose/lactose/sucrose in the VA− versus VA+ group. In the case of aminomics, thiamine monophosphate, deoxyguanosine, trimethylamine-N-oxide (TMAO), and stearoylcarnitine were lower ($p < 0.05$) and citrulline, taurine, N-carbomoyl-beta-alanine, betaine, and guanosine monophosphate higher ($p < 0.05$) in the VA− versus the VA+ group. For lipidomics analyses, C18:0 sphingomyelin, C21:0 sphingomyelin, C22:6 lysophosphatidylethanolamine, C32:2 phosphatidylcholine, C34:4 phosphatidylcholine, and C34:1 phosphatidylcholine were lower ($p < 0.05$) in the VA− group.

Data from these semi-targeted analyses suggested that lipids were most dramatically altered in response to marginal VA deficiency, including cell membrane lipids such as phospholipids, lysophospholipids and sphingomyelins, and 3 glycine conjugated bile acids. The targeted metabolomics performed focused on lipid classes that include cell membrane lipids such as ceramides, phospholipid- and fatty acid-derived oxylipins and endocannabinoids, and bile acids. For bile acids analysis, cholic acid, chenodeoxycholic acid, and tauroursodeoxycholic acid were lower ($p < 0.05$) in the VA− group. Ursodeoxycholic acid showed a trend decrease in the VA− group ($p = 0.053$). Ceramide C:18:0 was also lower in the VA-group ($p < 0.05$). There were no significant differences in any of the oxylipin or endocannabinoid assay metabolites. Malonylcarnitine passed after FDR adjustment ($p < 0.0001$). Table S2, Supporting information shows the complete list of metabolites found not to be significantly different between the two groups. Although PLS-DA analysis separated groups along latent variable 1 (Figure S3, Supporting Information), the model had low predictive ability (data not shown).

3.3. Heatmap Visualization between Physical Characteristics, Vitamin A Status, and Metabolomics

Heat map correlation analyses for significant and non-significant Spearman correlations allowed to visualized patterns associating metabolites changes with liver retinol concentration and liver and body weight (**Figure 2**). Correlation analyses for the metabolites that presented at least one significant correlation are presented in Figure S4; Tables S3and S4, Supporting information. In VA depletion (VA− group) the metabolites directly ($p < 0.05$) correlated with liver retinol were reduced glutathione ($r = 0.86$), isobutyrylcarnitine ($r = 0.79$), and butyrylcarnitine ($r = 0.75$), while adipate ($r = -0.86$), guanine ($r = -0.89$), uridine ($r = -0.86$), inosine ($r = -0.86$), hypoxanthine ($r = -0.86$), kynurenic acid ($r = -0.86$), thiamine monophosphate ($r = -0.93$), SUM fructose/glucose/galactose ($r = -0.79$), creatinine ($r = -0.79$), pantothenate ($r = -0.71$), deoxyuridine ($r = -0.75$), and xanthine ($r = -0.64$) were inversely ($p < 0.05$) correlated with liver retinol. In VA-sufficient gerbils (VA+ group) reduced glutathione ($r = 0.58$) was directly ($p < 0.05$) correlated with liver retinol, while CMP ($r = -0.63$), cytidine ($r = -0.78$), ornithine ($r = -0.78$), 3-hydroxydodecenoylcarnitine ($r = -0.70$), and C:36:2 DAG ($r = -0.76$) were inversely correlated. In regards to liver to body weight ratio in the VA− group, largely energetic and pentose phosphate pathway-related metabolites and serotonin plus its derivatives were positively correlated while a variety of amines were inversely correlated ($p < 0.05$). In the VA+ group, predominantly lipids were inversely correlated ($p < 0.05$), including a total of 15 phosphatidylcholines, six sphingomyelins, two cholesteryl esters, three diacylglycerols, seven triacylglycerols, and five oxylipins.

3.4. Heat Map Visualization between Metabolites within Metabolomics Assays

Heat map correlation analyses for significant and non-significant correlations within assays showed both VA− and VA+ groups

Table 1. Comparison of metabolites between vitamin A negative (VA−) and vitamin A positive (VA+) groups.

			VA−		VA+			
Metabolite	Assay	Units	Mean	SD	Mean	SD	Fold-change[a]	*p*-value[b]
Malonylcarnitine	Aminomics	PA	2 065 667.3	820 914.7	4 075 175.8	377 224.1	0.51	0.0001[c]
Citrulline	Aminomics	PA	15 535 332.9	4 179 249.3	7 595 308.3	4 048 369.5	2.05	0.001
Taurine	Aminomics	PA	243 077 508.4	28 940 571.7	190 245 658.7	39 495 413.7	1.28	0.007
N-carbamoyl-beta-alanine	Aminomics	PA	324 518 353.0	58 313 891.4	247 889 579.9	50 786 662.1	1.31	0.011
Thiamine monophosphate	Aminomics	PA	1 433 354.6	290 566.5	1 781 799.4	232 082.6	0.80	0.015
Deoxyguanosine	Aminomics	PA	4 017 220.9	1 397 001.2	8 278 568.5	4 248 483.5	0.49	0.016
Glycocholic acid	Primary	PA	5 699 381.1	7 732 340.5	35 201 216.7	37 482 988.1	0.16	0.019
Cytidine	Primary	PA	195 907.5	139 035.5	537 974.1	523 903.0	0.36	0.025
Cholic acid	Bile Acids	pmol g^{-1}	3936.5	4111.2	11 330.0	8324.0	0.35	0.025
SUM glycodeoxycholate/glycochenodeoxycholate	Primary	PA	919 765.3	749 119.8	5 521 518.0	6 009 046.6	0.17	0.026
Trimethylamine N-oxide	Aminomics	PA	147 218.9	129 689.1	414 573.5	281 306.8	0.36	0.027
Chenodeoxycholic acid	Bile Acids	pmol g^{-1}	70.0	59.5	207.5	156.2	0.34	0.029
C18:0 sphingomyelin	Lipidomics	PA	266 900 000.0	435 892 971.8	557 875 000.0	387 637 694.4	0.48	0.033
C22:6 lysophosphatidyethanolamine	Lipidomics	PA	72 405 000.0	96 627 959.1	158 238 750.0	126 239 567.1	0.46	0.034
Dihydroxyacetone phosphate	Primary	PA	32 819.7	15 241.6	59 187.1	28 864.8	0.55	0.034
C32:2 phosphatidycholine	Lipidomics	PA	134 075 000.0	214 872 425.6	268 605 625.0	180 728 743.0	0.50	0.038
C34:4 phosphatidycholine	Lipidomics	PA	116 812 500.0	153 660 167.1	222 407 500.0	122 850 838.2	0.53	0.040
Betaine	Aminomics	PA	1 138 694 298.3	199 063 810.7	962 200 012.4	122 247 636.4	1.18	0.041
C34:1 phosphatidycholine	Lipidomics	PA	332 762 500.0	401 274 284.3	669 743 750.0	405 508 054.7	0.50	0.042
Guanosine monophosphate	Aminomics	PA	850 436.1	859 980.9	281 486.0	132 414.4	3.02	0.043
SUM maltose/lactose/sucrose	Primary	PA	9 806 141.03	10 040 665.2	17 001 903.5	7 804 706.4	0.58	0.048
Tauroursodeoxycholic acid	Bile Acids	pmol g^{-1}	534.0	493.4	1042.6	722.6	0.51	0.049
C21:0 sphingomyelin	Lipidomics	PA	71 610 000.0	113 076 311.5	168 887 500.0	145 247 635.2	0.42	0.049
Stearoylcarnitine	Aminomics	PA	76 006 270.4	14 083 941.8	95 821 341.0	22 481 555.3	0.79	0.049
C18:0 ceramide	Ceramides	pmol g^{-1}	2532.2	606.5	3232.0	790.9	0.78	0.049

[a] Fold-change VA−/VA+; [b] two-sided *t*-test or Mann–Whitney U test with adjustment for multiple hypothesis testing according to Benjamini and Hochberg at a FDR of 5%. Ranked by *p*-value; [c] passed FDR.

had strong assay positive correlations for phosphatidylcholines, sphingomyelins, oxylipins, and primary metabolites (**Figure 3**). In the VA− group, metabolomics assay positive correlations occurred between endocannabinoid assay monocylglycerols and lipidomics assay complex lipids. Negative correlations existed between lipidomics assay complex lipids and endocannabinoid assay acylethanolamides (Figure 3A). Unique to VA+, metabolomics assay negative correlations occurred between lipidomics assay complex lipids and oxylipin assay metabolites (Figure 3B).

3.5. Network and Metabolic Pathways Visualization Analysis

Pathway enrichment analyses were performed contrasting the VA− and VA+ groups. MetaboAnalyst did not reveal any significantly changed pathways (data not shown). Because complex lipids cannot be included in the MetaboAnalyst pathway enrichment analysis, we used ChemRICH to perform a chemical similarity enrichment analysis on the lipidomics data set. The only significantly impacted lipidomics metabolite cluster was phosphatidylcholines ($p < 0.0001$) (Table S4, Supporting Information).

4. Discussion

In this study, multiple metabolomics assays were used to characterize the hepatic metabolome of marginal VA deficiency in gerbils. To our knowledge, this is the first metabolomics study to extensively investigate the metabolic alterations that occur in response to depleted levels of this micronutrient in its primary storage site. Conducted in an appropriate well-known animal model for VA deficiency, a total of 300 identified metabolites in combination with relevant postmortem outcomes were compared between two groups with varying VA status. Through the use of univariate, heat map correlation and metabolic enrichment analyses, this experiment provides novel insights into the metabolic pathways underlying VA deficiency. Our study is useful to confirm already known metabolic implications, and also serves as a hypothesis generating study.

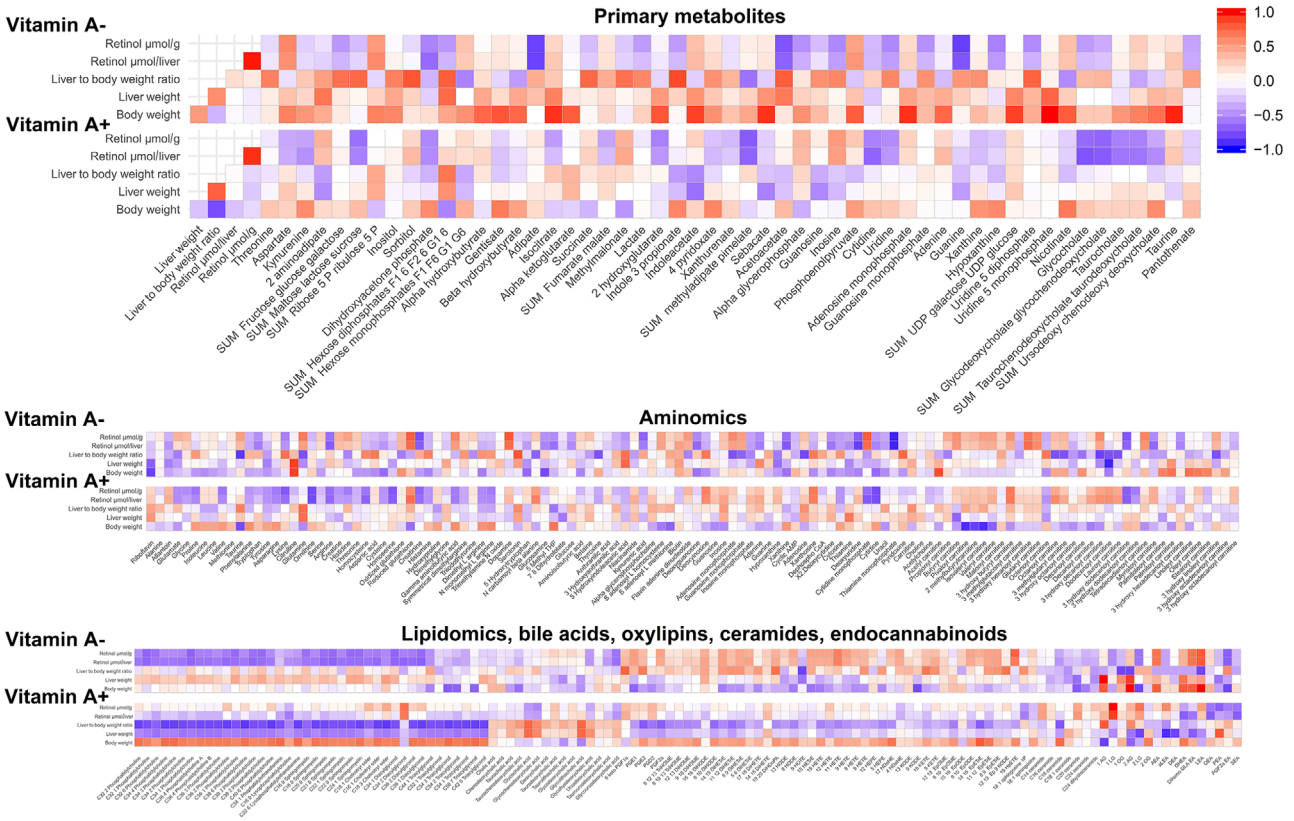

Figure 2. Heatmap correlation matrix metabolites versus vitamin A status and hepatic variables. Metabolites are placed in order by metabolite classes within each assay.

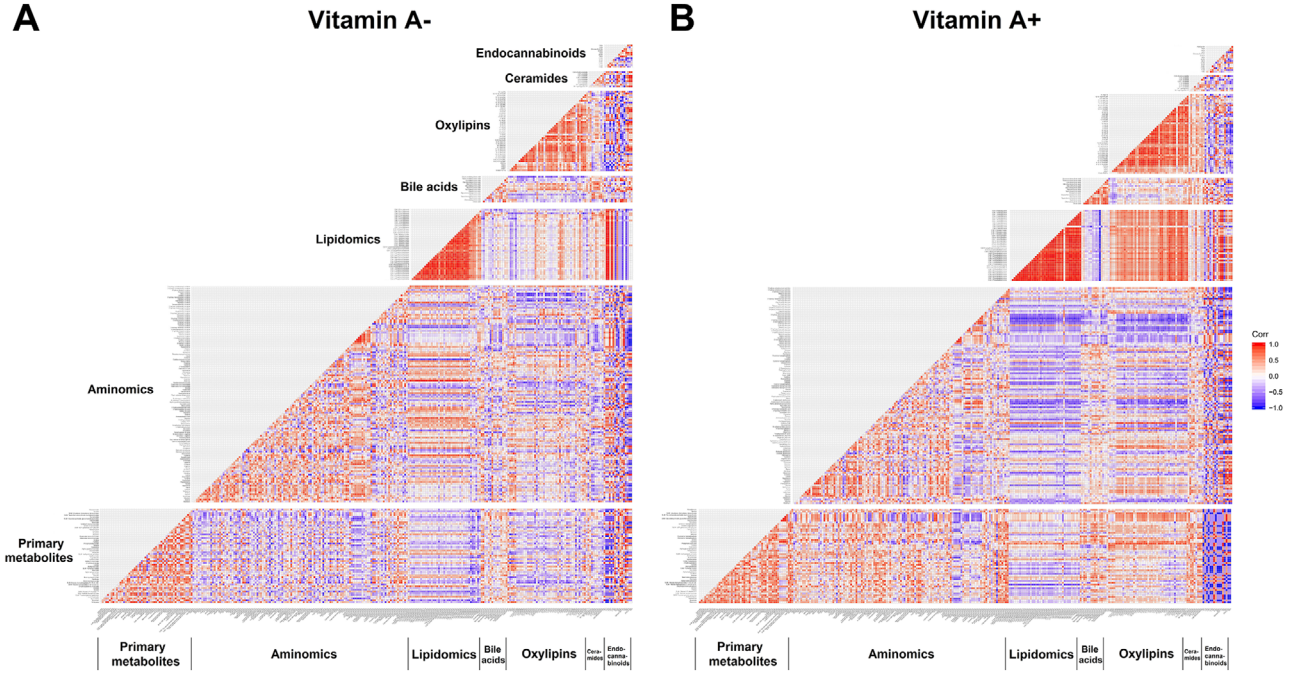

Figure 3. Heatmap correlation matrix metabolites versus metabolites in VA− and VA+ groups. Metabolites are placed in order by metabolite classes within each assay.

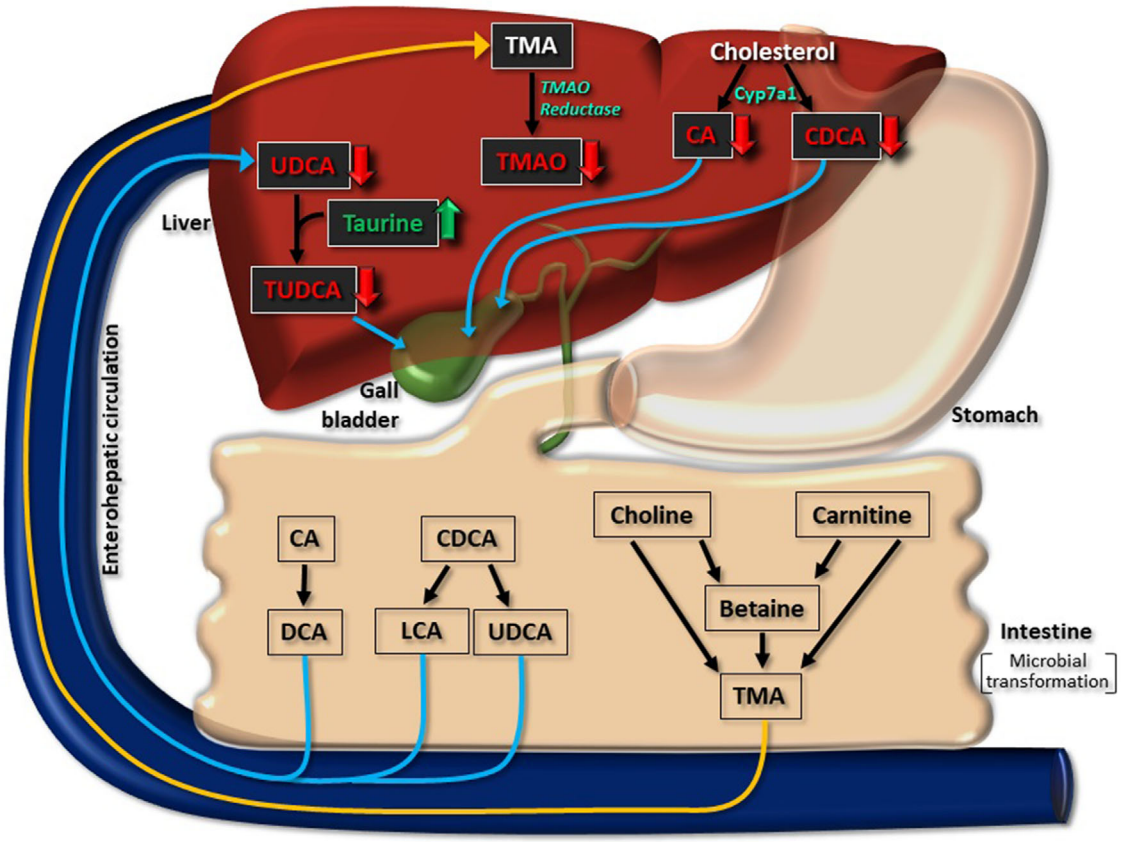

Figure 4. Effect of marginal vitamin A deficiency on liver metabolites, including pathways for metabolites known to be produced by the gut microbiota. Metabolites were not measured in the intestinal tissue. Arrows indicate directional change of statistically significant metabolites ($p < 0.05$). Note: ursodeoxycholic acid showed a trend decrease in the VA− group ($p = 0.053$).

4.1. Hepatic Primary Bile Acids are Decreased with Marginal Vitamin A Deficiency

Interestingly, the decreased concentration of the primary bile acids' cholic acid, chenodeoxycholic acid, and glycocholic acid with VA marginal deficiency in the VA− group suggests decreased activity of cholesterol 7 alpha-hydroxylase (CYP7A1), the rate-limiting enzyme in bile acid synthesis (**Figure 4**).[29] Most literature testing the influence of VA treatment on CYP7A1 has been conducted in direct retinoic acid administration in cell culture or short-term retinyl palmitate or all-trans retinoic acid consumption that has resulted in a down-regulation of CYP7A1[30–32] through means such as the activation of FXR by cholic acid and chenodeoxycholic or fibroblast growth factor 15/19 (FGF15/19) and small heterodimer partner.[33] In addition, RXRα-deficient mice had elevated bile acid synthesis.[34] However, long-term intake resulting in sufficient VA status has not replicated this change in the liver.[35] As a result, it is possible the long-term intake of VA in the form of retinyl acetate may actually result in its accumulation, as we observed, either by mechanisms other than CYP7A1 such as decreased action of bile acid-CoA:amino acid N-acyltransferase and bile salt export protein (BSEP) that are responsible for its export from the liver. In fact, administration of 9-cis-retinoic acid to C57BL/6J mice decreased expression of BSEP[36] and thus may explain why VA+ group had higher bile acid levels than the VA− group in our study. Since tauroursodeoxycholic acid and ursodeoxycholic acid are FXR antagonists,[37] they would be acting in opposition of the primary bile acids and thus may have influenced CYP7A1 expression. Further complicating interpretation is that CYP7A1 is mediated not only through 9-cis-retinoic acid, but also FXR/RXR-dependent and independent mechanisms, as well as a multitude of nuclear factors and receptors such as hepatocyte nuclear factor 4-alpha (HNF4α).[31] Last, the mean liver retinol concentration of 0.064 μmol g^{-1} in the VA− group was at a level where bile acid excretion from the liver is low since research indicates biliary excretion of VA up until the range of liver VA concentrations of 0.035–0.11 μmol g^{-1} is limited.[38,39]

4.2. Effect of Marginal Vitamin A Deficiency on Gut Microbiome-Altered Metabolites

Since this study did not evaluate the gut microbiome, we are unable to determine if its diversity or composition were altered, nor can we exclude the possibility of a leaky gut being present due to the deficiency.[40] Research indicates VA may influence

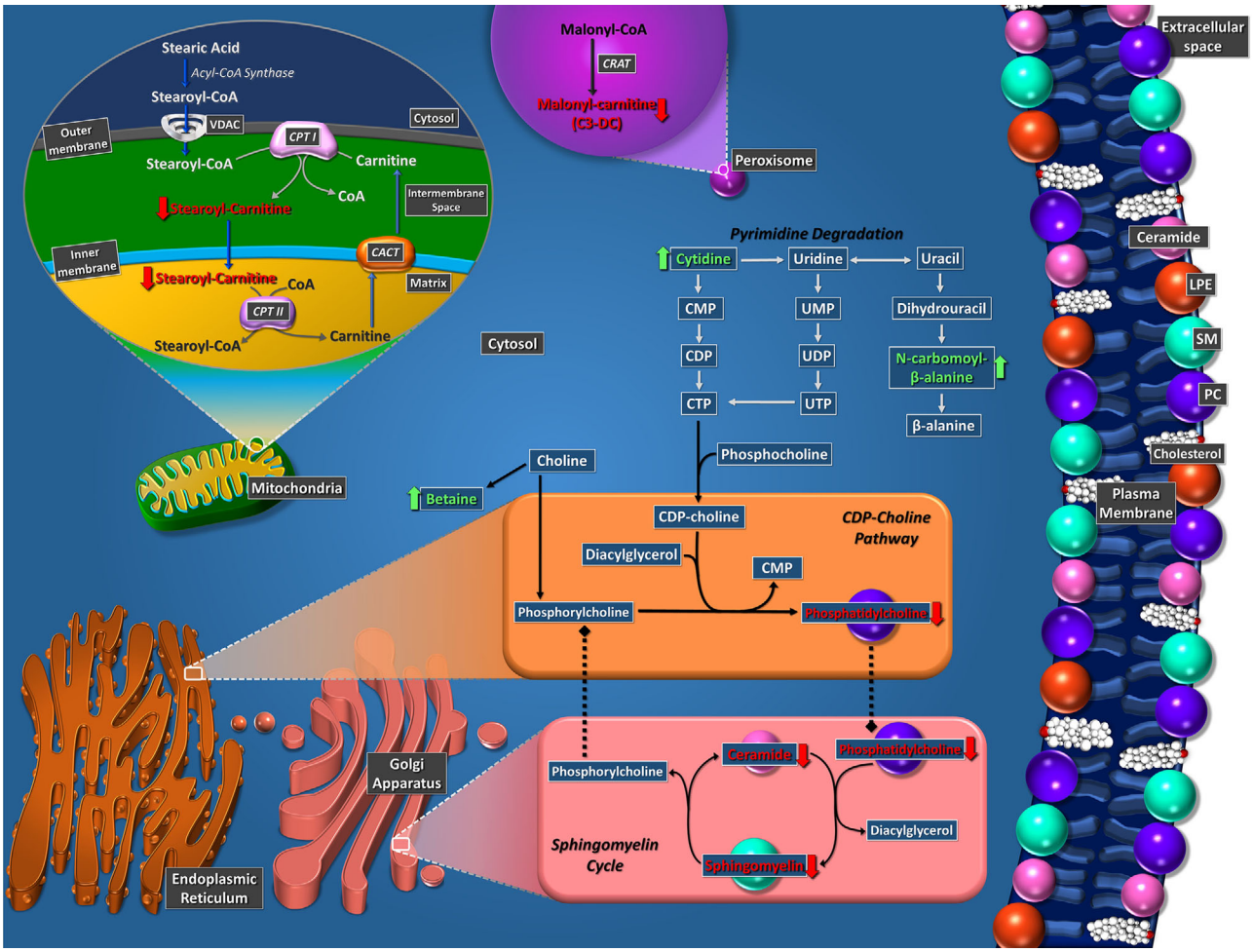

Figure 5. Proposed intersecting pathways altered in liver with marginal vitamin A deficiency, including the sphingomyelin cycle, CDP-choline pathway, and pyrimidine catabolism. Arrows indicate directional change of statistically significant metabolites ($p < 0.05$).

intestinal mucosa colonization with commensal bacteria by bolstering intestinal immunity and epithelial integrity.[41] Therefore, we can only state that metabolites known to be altered by gut microbiota were altered as a result of VA status. More specifically, the secondary bile acids tauroursodeoxycholic acid and ursodeoxycholic acid as well as TMAO were changed (Figure 4). Ursodeoxycholic acid is produced when primary bile acids are converted by gut bacteria to secondary bile acids while the microbial conversion of betaine, choline, or carnitine to TMA precedes the production of TMAO in the liver.[42,43] Provided the role of VA in the gut described previously, the impact of low VA status on the enterohepatic circulation of secondary bile acids and TMA may potentially alter their liver concentrations.[44] The fact that the production of ursodeoxycholic acid was altered while other secondary bile acids such as lithocholic acid and deoxycholic acid did not change suggests gut microbial composition may have also been impacted.[45] Ursodeoxycholic acid has many beneficial roles in the liver;[46,47] thus a decrease with VA deficiency may further compromise liver health. Although elevated TMAO is associated with negative health effects, it is suspected that the decrease in its levels in this study with marginal VA deficiency are more a reflection of decreased gut integrity rather than an effect considered beneficial for health.[42,48]

4.3. Cell Membrane Lipids are Decreased with marginal Vitamin A Deficiency

Various cell membrane lipids associated with the Kennedy pathway (CDP-choline pathway), including phosphatidylcholines (C32:2, C34:1, and C34:4), and a lysophosphatidylethanolamine (C22:6), were decreased with VA depletion (**Figure 5**). In agreement with our results, past research has observed a decrease in liver phospholipid content with VA deficiency and an increase with VA intake in rat and guinea pig models, respectively.[49–51] In fact, research indicates the lower phospholipid content may stem from the downregulation of acetyl-CoA carboxylase due to VA deficiency.[52] A lack of fatty acid production may consequently impact the ability to produce phospholipids. At the mitochondrial level, the total phospholipid concentration of liver mitochondria may also be decreased with VA deficiency.[52] It has been suggested that VA deficiency may cause mitochondrial dysfunction

through decreased production of the phospholipid cardiolipin, an important component of oxidative phosphorylation.[51]

Novel findings of this study included alterations in sphingomyelin cycle-associated sphingomyelins (C18:0 and C21:0) and a ceramide (C18:0). These metabolites have known functions in G-protein coupled receptor function, receptor-mediated ligand uptake, and protein sorting.[53] Ceramides, also a component of the lipid bilayer, have numerous cell signaling capabilities that may impact oxidative stress, inflammation, and apoptosis.[54] Potentially related to observed lower liver weight in the VA+ group, C18:0 ceramide is a particularly effective inducer of apoptosis.[55] Interestingly, the metabolism of phosphatidylcholine and these two sphingoid bases intersect in the sphingomyelin cycle (Figure 5). Therefore, the lack of sphingomyelin may be a consequence of decreased phosphatidylcholine and ceramide availability. Moreover, similar to phosphatidylcholine, it may be related to lack of fatty acid availability.[52]

4.4. Other Metabolites Altered by Marginal Vitamin A Deficiency

Compounds associated with utilization of fat for energy were decreased with VA depletion, including the short-chain malonylcarnitine and long-chain stearoyl-carnitine) (Figure 5). VA deficiency has been shown to downregulate the genes involved in β-oxidation.[56] Mice fed a VA deficient diet for 9 weeks showed that genes encoding for enzymes involved in beta-oxidation within the mitochondria and the peroxisome had decreased transcript levels in the VA deficient liver, including carnitine octanoyl-transferase.[56] In another study, mice supplemented with all-trans-retinoic acid had increased gene expression of carnitine palmitoyl-transferase 1a.[57] Malonylcarnitine, an acylcarnitine observed in our study, was associated with serum retinol levels.[58] In contrast, research has shown VA deficiency decreases acetyl-CoA carboxylase, the enzyme in the rate-limiting step of fatty acid synthesis, expression while increasing carnitine palmitoyltransferase-I expression.[52]

Interestingly, numerous nucleotides and nucleosides were differentially altered, including deoxyguanosine (Figure 4). Studies have shown a connection between VA status and oxidative stress.[59] Supportive of this potential association were strong positive correlations between the antioxidant reduced glutathione and liver retinol levels in the study described herein. This may suggest defense against reactive oxygen species may be compromised with VA deficiency in the gerbils. Related to our study, in the presence of oxidative stress, deoxyguanosine may be oxidized to its product 7,8-dihydro-2-deoxyguanosine (8-oxodG).[60] Therefore, one hypothesis for the decreased deoxyguanosine in the study may be that it was being oxidized to 8-oxo-dG as a result of oxidative stress in the VA deficient gerbils. In fact, increased presence of 8-oxodG has been observed in the livers and leukocytes of VA deficient rats[61,62] and studies have observed an inverse relationship between blood levels of VA and total DNA adducts, including 8-oxodG, in non-smokers, as well as smokers.[63]

Furthermore, urea-cycle associated citrulline and the urea-derivative of beta alanine (N-carbomoyl-beta-alanine) were both elevated in the marginal VA deficient group. In a similarly designed study that performed a proteomic analysis of VA deficiency, Bohn et al.[10] observed increased OTC and ASL enzymes in response to VA administration. This supports a potential decrease in urea cycle function with VA deficiency.

4.5. Strengths and Limitations of this Research

The design of our study corresponds to a well-known animal model used in the context of VA studies.[11] The animal liver retinol concentration was confirmed to be at a marginally deficient status, reflective of very low reserves. In ideal conditions it would be best to have a similar design in humans, however, this is impossible due to ethical reasons. Although our experimental design was appropriate, it would be ideal to have metabolomics measurements before (baseline) and after intervention (follow-up) in order to compare dependent groups. Our study was limited to the comparison of two independent groups. Our sample size of 10 gerbils per group may have resulted in low power for some metabolites, reducing our capacity to detect differences associated with VA deficiency. A strength of the metabolomics analysis is that we spanned the metabolome from polar to nonpolar metabolites and high to low concentration metabolites using seven assays. That said, we acknowledge that maybe there are more metabolic pathways induced by VA deficiency. Another limitation for identifying metabolomic pathways was the absence of untargeted metabolomics. Having untargeted metabolic would have likely increased the number of metabolites to match with the bioinformatics libraries increasing the number of identified pathways. It would be ideal to profile metabolomics in other tissues and biofluids such as plasma, adipose tissue, or gut. Evaluating the gut microbiome composition and diversity is also needed. The conduction of a study in humans or a large animal model with physiology more similar to humans such as primates or pigs should be performed. Our study provides important hepatic metabolic information. We included liver and serum retinol measurements and some physical characteristics but we did not include clinical functional outcomes of deficiency or excess, thus our findings are limited to metabolic effects.

4.6. Potential Health Effects and Future Perspectives

The purpose of this metabolomics study was predominantly to investigate pathways altered by marginal VA deficiency in order to generate new hypotheses concerning the effects of this condition in the liver. Therefore, the physiological implications of these impacts on metabolism cannot be determined at this time. Future studies need to address the specific mechanisms altered by VA deficiency that caused the reduction in the bile acids, phospholipids and acylcarnitines, in addition to the effect on liver histology and function. The changes in these metabolites may contribute to the development of chronic liver diseases associated with VA deficiency.[64] These diseases are characterized by loss of hepatic stellate cell vitamin levels due to a switch to myofibroblast production. In fact, it is possible that the observed higher liver weights in the VA− group in our study may be related to the development of liver fibrosis. Alterations in numerous gut-liver axis metabolites in this study require a follow-up study that

utilizes a combination of microbiome and metabolomics analysis in order to validate findings and better understand implications for gut health. Assessment of metabolite changes with severe VA deficiency is also warranted. From a clinical and public health perspective, knowledge of the widespread metabolic effects of VA deficiency can help support rapid and optimal recovery from deficiency by addressing not only micronutrient status but also concomitant physical consequences of associated disorders. Furthermore, this knowledge may also help define healthy nutrient status to support overall health. In general, more efforts are needed to apply metabolomics profiling into micronutrient research since few metabolomics analyses have been used to assess micronutrient deficiencies.[65,66] In addition, various omics platforms are currently available to be integrated to study systems biology. More evidence is needed to translate this knowledge at the population level in a context of translational and personalized medicine.

5. Conclusions

To the best of our knowledge, this study is the first metabolomic characterization of VA depletion. The study described herein represents the most comprehensive metabolomics assessment of marginal VA deficiency to date. A variety of novel changes, as well as alterations supportive of previous research were observed. In particular, changes indicated disruption of metabolites associated with gut-liver axis, cell structure, and energy metabolism. The observed alterations provide a more complete view of the metabolic consequences of this micronutrient deficiency. Future research is needed to follow-up on these potential mechanisms of VA deficiency and explore potential biomarkers.

6. Experimental Section

Experimental Animal Model and Study Design: An experimental in vivo study randomly assigned treatment to VA-depleted male Mongolian gerbils (*Meriones unguiculatus*) (29–35 d) purchased from Charles River Laboratories (Kingston, NY).[67] Gerbils were group housed during both VA depletion (2–3 per cage) and repletion (2 per cage) in hanging wire-bottom cages to limit coprophagy. Feed intake was measured daily. Body weights were measured daily day for the first 2.5 weeks and three times per week for the remainder of the study. A 12 h light/dark cycle was utilized and room temperature and humidity remained constant. After the 28 d depletion, animals were weight-matched and allocated into treatment groups. Treatment groups included a VA positive control (VA+, $n = 10$) and marginally deficient negative control (VA−, $n = 10$) whose diets were both white maize-based (VA+, 40 µg provided as retinyl acetate in ≈50 µL cottonseed oil; VA−, given the same volume of cottonseed oil alone). After 28 d repletion (study d 56), a final sacrifice ($n = 20$) was performed by exsanguination under isoflurane (Figure S1, Supporting Information). Experiments were conducted in accordance with the institutional guidelines for the care and use of laboratory animals and were approved by the College of Agriculture and Life Sciences Animal Care and Use Committee at the University of Wisconsin (UW)-Madison.

Gerbil feed was specially formulated to contain 50% white maize by weight with no additional VA by Harlan–Teklad, (Madison, WI). Specific components of the feed are listed in Table S1, Supporting Information.

The white maize was a common variety available in Zambia that was processed using whole grain (known as roller) milling methods by a small mill that serves a rural community. Maize was subsequently stored in a freezer (−20 °C) until hand-carried to the UW-Madison. The total concentration of fatty acids in the feed was 24.3 µg mg^{-1}. The fatty acid composition of the diet consisted primarily of palmitate and oleate, along with lower amounts of stearate and linoleate (Figure S2, Supporting information), with the remaining detected 7% of fatty acids consisting of 16:1n7, 18:1n7, 20:0, 22:0, and 24:0.

Dosage Information: Daily doses of VA were administered orally with a positive displacement pipette. Specifically, 40 µg of VA (0.52 µg g^{-1} BW at end of study) in the form of retinyl acetate was provided in ≈50 µL cottonseed oil. The estimate human equivalent dose is 162.1 µg per day (540 IU per day) for a 60 kg human.

Biochemical Vitamin A Analysis of Liver and Serum: All sample analysis for retinoids was conducted under gold fluorescent lighting. Liver total retinol was analyzed by extracting and saponifying an aliquot using a modified published procedure.[68] Briefly, a transverse section of liver (≈0.25 g liver analyzed in singlet per animal) was ground with ≈3 g anhydrous sodium sulfate and C23 β-apo-carotenol as an internal standard with a mortar and pestle. Samples were extracted repeatedly with dichloromethane and filtered to achieve 25 mL. A 5 mL aliquot was dried under nitrogen, resuspended in 100 µL methanol:dichloromethane (50:50, v:v), and 20 µL were injected on the HPLC system. The procedure for serum retinol was as published.[28] Briefly, 250 µL serum and C23 β-apo-carotenol as an internal standard were extracted twice with 300 µL hexanes, dried under nitrogen, resuspended in 50 µL methanol:dichloromethane (75:25, v:v), and 35 µL were injected on the HPLC system. The HPLC system for retinol analysis used a Gracesmart C18 (5-µm, 4.6 × 250-mm) column with 92:8, v:v, acetonitrile:water at 1 mL min^{-1} combined with a photodiode array detector set at 325 nm.[67] Very low hepatic retinol concentrations were defined as <0.1 µmol g^{-1}.[27]

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

B.G. and S.T. designed and conducted the feeding trial and performed retinol analysis. M.R.L., C.J., B.W., T.L.P., R.K.F., and J.W.N. processed and analyzed liver samples and metabolomics data. M.R.L., A.B., C.J., and J.W.N. performed statistical analyses, interpreted information, and wrote the manuscript. M.R.L. conceived the main study and has final responsibility for all parts of this research.

Keywords

liver, metabolomics, nutritional metabolomics, omics, vitamin A

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