Journal of Developmental Origins of Health and Disease

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Original Article

Cite this article: Hegde PS, Agni MB, Rai P, Upadhyay SS, Aravind A, Keshava Prasad TS, and Gowda KMD. (2024) Supplementation of diet with Astaxanthin and DHA prevents gestational and lactational undernourishment-induced metabolic derangements in dams: a metabolomic approach. *Journal of Developmental Origins of Health and Disease* **15**: e30, 1–11. doi: 10.1017/S2040174424000345

Received: 7 June 2024
Revised: 11 September 2024
Accepted: 9 October 2024

Keywords:
Astaxanthin; docosahexaenoic acid; gestational period; lactational period; lipid metabolism metabolic disorders; untargeted metabolomics

Corresponding author:
K.M. Damodara Gowda;
Email: dr_damodar@nitte.edu.in

Supplementation of diet with Astaxanthin and DHA prevents gestational and lactational undernourishment-induced metabolic derangements in dams: a metabolomic approach

Pramukh Subrahmanya Hegde[1], Megha Bhat Agni[1], Praveen Rai[2], Shubham Sukerndeo Upadhyay[3], Anjana Aravind[3], Thottethodi Subrahmanya Keshava Prasad[3] and K.M. Damodara Gowda[1]

[1]Department of Physiology, KS Hegde Medical Academy, Nitte (Deemed to be University), Karnataka, Mangalore, India; [2]Division of Infectious Diseases & Microbial Genomics, Nitte University Centre for Science Education and Research (NUCSER), Nitte (Deemed to be University), Mangalore, Karnataka, India and [3]Center for Systems Biology and Molecular Medicine, Yenepoya Research Centre, Yenepoya (Deemed to be University), Mangalore, Karnataka, India

Abstract

Nutrition is the critical nongenetic factor that has a major influence on the health status of an organism. The nutritional status of the mother during gestation and lactation plays a vital role in defining the offspring's health. Undernutrition during these critical periods may induce chronic metabolic disorders like obesity and cardiovascular diseases in mothers as well as in offspring. The present study aims to evaluate the impact of undernutrition during gestational and lactational periods on the plasma metabolic profile of dams. Additionally, we investigated the potential synergistic mitigating effects of astaxanthin and docosahexaenoic acid (DHA) on dysregulated plasma metabolic profiles. Evaluation of plasma lipid profile revealed that undernourishment resulted in elevated levels of total cholesterol, triglycerides, low density and very low-density lipoproteins in dams. Liquid chromatography-tandem mass spectrometry (LC–MS/MS) based untargeted metabolomics illustrated that pathways related to lipid metabolism, such as cholesterol metabolism, steroid biosynthesis and metabolism of amine-derived hormones, were dysregulated by undernourishment. Additionally, pathway enrichment analysis predicted that there is a high incidence of development of desmosterolosis, hypercholesterolaemia, lysosomal acid lipase deficiency and Smith–Lemli–Opitz syndrome in the offspring, reflecting predisposition in mothers. However, synergistic supplementation of astaxanthin and DHA ameliorated these adverse effects by regulating a separate set of metabolic pathways associated with lipid metabolism. They included branched chain amino acid degradation such as valine, leucine and isoleucine, metabolism of alpha-linolenic acid, lipoic acid, lysine degradation, biosynthesis, elongation and degradation of fatty acids.

Introduction

Women have unique dietary needs at different stages of their lives, particularly during pregnancy and lactation, during which they are most susceptible to nutritional deficiencies. It is essential for mothers to have access to nutritious foods and suitable care is crucial for promoting their well-being and that of their children.[1] During pregnancy and lactation, there is a heightened need for energy and nutrients, which are important for the well-being of both the mother and the progeny.[2] Large number of pregnant women are vulnerable to malnutrition due to lack of access to essential nutrition services that are crucial for their own well-being and the well-being of their infants. Hence, mothers find difficulty to satisfy their increased nutritional demands and restore their nutrient reserves while they are lactating. Maternal undernutrition is a significant risk factor for intrauterine growth restriction (IUGR), a common and severe pregnancy condition. Infants who have undergone IUGR are more susceptible to perinatal complications, as well as long-term physical and cognitive disabilities. Additionally, it can lead to stillbirths, low birth weight and other developmental delays in offspring.[3]

However, environmental insults such as undernutrition during gestation and lactation period may increase the likelihood of mothers developing metabolic syndrome like gestational diabetes, preterm delivery, frequent miscarriages, obesity, adverse cardio metabolic outcomes, anaemia, pre-eclampsia, haemorrhage and maternal mortality.[4] This also suggests that therapeutic dietary approaches may be useful to combat against the adversities caused by the

undernutrition. Enhancing the dietary intake of women, ensuring them access to nutrition services and care practices before and throughout pregnancy, as well as during lactation is essential for avoiding malnutrition and/or undernutrition-induced adverse effects among the most susceptible mothers and infants.

In this context, present study aimed to investigate the synergistic effect of FDA-approved nutraceuticals, astaxanthin (AsX) and docosahexaenoic Acid (DHA), on dams subjected to undernourishment during pregnancy and lactation using Albino Wistar rats as experimental model. We examined alterations in plasma metabolomic profile of dams in response to undernourishment and supplementation with AsX and DHA. AsX is ketocarotenoid, which has greater antioxidant efficacy in comparison to other dietary antioxidants.[5] AsX is naturally found in algae, yeast, salmon, trout, krill, shrimp and crayfish.[6] Use of AsX as a nutraceutical encompasses several health benefits such as prevention of cardiovascular disease, anti-inflammatory properties, antioxidant effects, protection for the skin, eye health promotion, antidiabetic and potential anticancer properties.[5,7,8]

DHA is an omega-3, long chain Polyunsaturated Fatty cid mostly present in deep water fish such as salmon, mackerel, sardine and tuna.[9] The potential beneficial effects encompass neuroprotection, cardiovascular disease prevention, anti-inflammatory, anti-cancer, anti-asthmatic and immune-boosting properties.[10] Recent studies in animals and clinical investigations have demonstrated that the inclusion of DHA in the diet reduces body fat mass, accumulation of fatty acids in the liver by reducing fatty acid synthase and plasma triglycerides,[11–13]and also DHA treatment during pregnancy may impact neonatal birth weight, duration of pregnancy and the likelihood of postpartum depression.

Earlier we reported the effects of astaxanthin alone on the lipid profile, brain lipid concentrations,[14] and DHA supplementation during pregnancy has been recommended by several health organisations due to its role in neural, visual and cognitive development.[15] Since DHA is highly unsaturated and very susceptible to free radical-induced peroxidation, we assume that the use of antioxidant AsX, with DHA could better balance the beneficial effect of improving lipid profiles and reducing adipocyte dysfunction through their synergistic antioxidant, anti-inflammatory and lipid-modulating effects. Later we reported that astaxanthin and DHA synergistically neutralised the effects of Reactive Oxygen Species (ROS) in adipocytes effectively by reducing oxidative damage and preventing the dysfunction of these cells.[16]

In the current study, we evaluated the synergistic effect of dietary supplementation of AsX and DHA. The synergistic effect would be better than the individual effect of DHA, because antioxidant capabilities of AsX might prevent the oxidation of DHA to exert its maximum effect in combination. Combining DHA with AsX, a potent antioxidant, can enhance its defense against oxidative stress, preserving its anti-inflammatory properties and reducing inflammation.[16] Further, since DHA is a highly unsaturated fatty acid and is highly susceptible to peroxidation caused by free radicals,[17] we hypothesise that the combination of antioxidant AsX and DHA could more effectively balance the positive effects of lipid profile improvement and adipocyte dysfunction reduction through their synergistic antioxidant, anti-inflammatory and lipid-modulating actions. Barros et al., 2012 reported synergistic use of AsX and fish oil resulted in a reduction in lipid and cholesterol levels in the blood plasma. Additionally, it led to an increase in the phagocytic activity of activated neutrophils, which was not seen when AsX or fish oil were used individually.[18] Also, DHA supplementation during pregnancy has been recommended by several health organisations due to its role in neural, visual and cognitive development. Low levels of DHA and AA in maternal plasma and cord blood has been related to lower head circumference, lower birth weight, lower placental weight,[19] and less cognitive and visual maturation during childhood.[20,21] Other studies found associations between omega-3 FA intake during pregnancy and lower risks of IUGR, preterm birth, allergies and asthma in children.[19–21]

High throughput techniques such as Liquid Chromatography-Tandem Mass Spectrometry (LC–MS/MS) based metabolomics offer comprehensive insights into biochemical alterations, potential biomarkers for both acute and chronic illnesses. Examining fluctuations in a mother's metabolome profiles throughout pregnancy and lactation could assist to find novel biomarkers for potential treatment targets and important times for prophylactic measures. Furthermore, this study could reveal the probable pathways involved in the inheritance of metabolic abnormalities from one generation to another.

Materials and methods

Ethical statement

All animal experiments adhere to the ARRIVE guidelines and are conducted in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals. The Institutional Animal Ethics Committee of KS Hegde Medical Academy, Nitte (Deemed to be University), Mangalore, approved the study protocol (Ref No: KSHEMA/IAEC/06/2020).

Experimental animals

A total of 18 females and 6 adult male Albino Wistar rats of 2–3 months old, weighing 200–250 g were procured from our institutional animal care facility for breeding. Female and male rats were housed separately (3 rats per cage). After 10 days of acclimatisation, rats were kept for breeding in the ratio of 3:1 (Female: Male) for 4–5 days. Pregnancy was confirmed in the dams by vaginal smear study to mark the gestational day 1 (GD1). On the same day, male rats were separated and dams were divided into three different groups with 6 dams per group. Animals were maintained on a 12 h of dark and light schedule and controlled temperature (22 ± 2°C). Animals were permitted free access to food and water. Pregnant rats were then housed in individual cages and subjected to undernourishment and/or supplementation from gestational day 1 (GD1) to postnatal day 21 (PD21) respectively (Fig. 1a).

The perinatal undernourishment was ensured by reducing 30% of the actual food consumption and AsX (24mg/Kg BW/Day & DHA (500mg/Kg BW/Day) administered orally during the perinatal period of 42 days (21 prenatal + 21 postnatal days). Olive oil was used as vehicle. The experimental animals (dams, $n = 6$) were segregated into three different groups as Group I- Control rats (C): Normal dams fed with rat feed and water *ad libitum;* Group II-UN: Dams were undernourished during their gestation and lactation; Group III- UN + AD: Dams under nourished during their gestation and lactation supplemented with AsX & DHA (24mg/Kg BW/day and 500mg/Kg BW/ day respectively). Throughout the experimental period all the animals had free access to water *ad libitum*.

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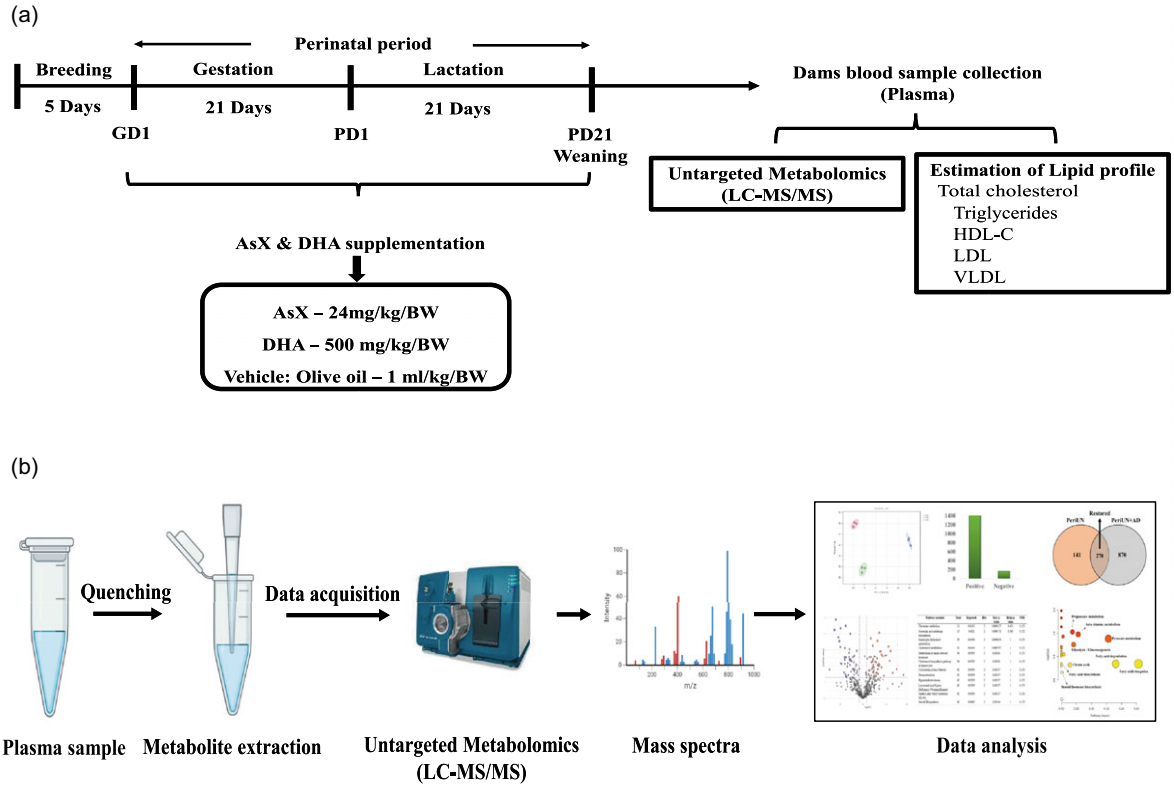

Figure 1. Study design. a. Undernourishment and/or AsX & DHA supplementation plan and b, Study design of metabolomics analysis.

The dose selection was based on previous studies. Brendler T. 2019 determined that supplementing with 24 mg/kg/day of astaxanthin is not harmful in human subjects.[24] Neto J. et al., 2021 conducted a study examining the impact of DHA/EPA supplementation at a dosage of 500 mg/kg body weight (BW) on the metabolic profile of adult obese rats.[25] Also in our previous study, we demonstrated that the administration of astaxanthin at a dose of 24 mg/kg BW combined with DHA at 500 mg/kg BW significantly improved the proteomic profile associated with adipose tissue dysfunction induced by perinatal undernutrition in adult male rats.[26]

Estimation of biochemical parameters

After the 21 ± 2 days of lactation period, pups were separated and dams ($n = 6$) were subjected to euthanasia, and blood sample was collected in plain and EDTA coated vacutainers by cardiac puncture. The blood samples were centrifuged at 3000 r.p.m. for 10 min to obtain the serum and plasma. And the samples were stored at − 80°C until analysis. The serum lipid profile [total cholesterol (TC), triglycerides (TG's) and high-density lipoprotein (HDL-C)] was performed using commercially available kits as per the manufacturer's guidelines (Agappe Diagnostics, India). Whereas, very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) levels were calculated by the formula, VLDL = TG/5 and LDL = TC-(HDL + VLDL) respectively.

Sample preparation for metabolomic analysis

Metabolite extraction was carried out using the triple solvent method[27] with certain modifications. Briefly, the LCMS grade solvents acetonitrile, methanol and water were used in the ratio of 2:2:1. From each group 3 animals were considered for the experiment. 15 µl of plasma sample was taken from each animal and pooled it group wise. Therefore, the total volume of plasma sample taken from each group is 60 µl. Then 1 ml of triple solvent was added to 60 µl of the plasma from each group. Following steps were common for both gut and plasma sample preparation. Samples were vortexed for 15 min, followed by water bath sonication for 15 min. The mixture was then incubated at −20°C overnight for protein precipitation. Next day, the mixture was then centrifuged at 12,000 r.p.m. for 20 min at 4°C. The supernatant was then transferred to a new 1.5 ml vial and was vacuum dried using a SpeedVac concentrator. The samples were resuspended in 250 µl of 0.1% formic acid and were further diluted 3 times before acquisition. Fig. 1b represents study design of metabolomics analysis.

Data acquisition

Analysis of the metabolites extracted from the plasma samples was performed using liquid chromatography followed by MS/MS analysis using QTRAP 6500 mass spectrometer (ABSciex) coupled with Agilent 1290 infinity II liquid chromatography system with a C18 RRHD Zorbax column (20 × 150 mm, 1.8 µm particle size). Analyst software version 1.6.3 was used for data acquisition and the Analyst Device Driver for setting the parameters for the analysis. The separation of the metabolites was carried out using a 25-min LC (liquid chromatography) method. The solvent A was 0.1% formic acid in LCMS grade water and solvent B was 0.1 % formic acid in 90 % LCMS grade acetonitrile, the flow rate was set to 0.25 mL/ min. The injection volume was set to 10 µl. The LC method was set was set to 25 min with the following gradient: 2% B for 1–10 min, 30% B at 10–14 min, 60% B at 14–18 min, 95% B for 18–21

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min and 2% B for 21–25 min. The mass spectrometry data acquisition was carried out with IDA method (Information dependent acquisition) in low mass mode. The IDA method was built using the EMS (enhanced mass spectra) to EPI (enhanced product ion) modes. The top five spectra from the EMS mode were used for analysis in the EPI (MS/MS) mode, using high energy CID i.e. collision-induced dissociation. The metabolite data was acquired in both positive and negative polarities at 4500 V and −4500 V respectively, with a probe temperature of 450°C. The compound parameters were set at a declustering potential (DP) of 100 V, collision energy (CE) of 10 V. The injection volume was set to 10 µl. Epicatechin was used as an internal standard and was spiked in the samples at a concentration of 250 ng/ml. Quality Control (QC) samples were prepared by pooling equal quantities of plasma metabolite extracts. Data acquisition was initiated by queuing two QC samples post blank injection and then single QC after each batch. This acquisition scheme was employed for both positive and negative polarities.

Data analysis

Data were represented as Mean ± SEM. The comparison of all the data between the groups was done by One-way ANOVA and to compare between the different time points Two-way multiple ANOVA was performed followed by Tukey's multiple comparison test. The p value < 0.05 was considered as the level of significance and all the statistical calculations were performed with GraphPad Prism software for Windows (Version 8.0).

Metabolomics data analysis

The raw files were converted to. mzml format using MSConvert (https://bio.tools/msconvert PMID:23051804). These converted files were imported in MZmine 2.53 (https://mzmine.github.io PMID: 20650010). The feature detection was carried out with an m/z tolerance of 0.5 Da. It was followed by chromatogram deconvolution using the Noise amplitude algorithm with amplitude of noise as 1.5E2. Isotopes were detected using Isotopic peak grouper with an m/z tolerance of 0.25 Da and retention time tolerance of 0.2 min. Alignment of peaks was performed Join Aligner with an m/z tolerance of 0.05 Da and a retention time tolerance of 0.5 min. After processing, the data was searched against the Human Metabolome Database (PMID:34986597) using the MS2 compound software (PMID:34115523). The metabolite identification and annotation were carried out with a precursor mass tolerance of 0.05 Da and fragment ion tolerance of 0.5 Da. [M+H] and [M−H] adducts were used as adducts for positive and negative modes respectively. Turboputative (PMID:36158581) was used to filter out exogenously present metabolites such as drugs, nutrients, plant metabolites, contaminants such as halogenated compounds and peptides. This helps in focusing on metabolites which are present in the system endogenously. Statistical, enrichment, and pathway analysis was carried out using Metaboanalyst 5.0 (PMID:34019663).

Results

Effect of maternal undernourishment and AsX and DHA supplementation during gestation and lactation period on lipid profile

Maternal undernourishment during gestation and lactation period impacted their lipid profile. One-way ANOVA with Tukey's multiple comparison test revealed that UN dams showed significantly increased levels of TC (133.9 ± 10.92, $p < 0.0001$), TG (72.77 ± 9.66, $p = 0.0034$) VLDL (10.61 ± 1.10, $p = 0.0041$), LDL (6.66 ± 0.91, $p = 0.006$), and decreased level of HDL-C (31.60 ± 7.85, $p = 0.0086$) in relation to control dams (Fig. 2). However, AsX and DHA supplementation reversed UN effect on these parameters by bringing back to normal level: TC (70 ± 10.92, $p = 0.006$), TG's (23.86 ± 9.66, $p = 0.0018$) VLDL (4.77 ± 1.10, $p = 0.0013$), LDL (3.72 ± 0.91, $p = 0.0253$) and HDL-C (49.50 ± 7.85, $p = 0.1105$).

Effect of maternal undernourishment and AsX and DHA supplementation during gestation and lactation period on plasma metabolomic profile

Global metabolomics facilitated the identification and alignment of features that had MS/MS information using Metaboanalyst 5.0. The analysed mass spectrometry data revealed a total of 1,408 aligned m/z features in the positive mode and 173 aligned m/z features in the negative mode. Untargeted metabolomic analysis was used to determine the metabolic cues associated with undernourishment-induced metabolic stress and the protective effects of AsX and DHA against it. Principal component analysis (PCA) of the data obtained from positive and negative modes revealed distinct separation and clustering between the experimental groups. The clustering of the UN condition was noticeably away from the control, as well as the AsX and DHA supplementation conditions (Fig. 3a and 3b).

Differential regulation of metabolites by undernourishment and asX and DHA supplementation

We conducted an in-depth analysis of differentially regulated metabolites in response to undernourishment and supplementation with AsX and DHA. Metabolites were considered significantly upregulated ($p \leq 0.05$) and a fold change (FC), $FC \geq 1.25$, and significantly downregulated ($p \leq 0$ 0.05 and a $FC \leq 0.8$. We found, totally 1404 metabolites were differentially regulated in +ve ion mode, whereas only 165 metabolites in −ve ion mode (Fig. 3c). Similarly, undernourishment led to differential regulation of 419 metabolites, whereas supplementation with AsX and DHA resulted in differential regulation of 1148 metabolites (Fig. 3d). These differentially regulated metabolites are depicted in volcano plots (Fig. 3e–h). We observed differential regulation among various classes of lipid molecules across the experimental groups, primarily belongs to the family of diacylglycerols, triacylglycerols, glycerophospholipids. Undernutrition resulted in upregulation of these lipid species, whereas AsX and DHA treatment down-regulated some of these molecules bringing them to normal level. However, we did not quantify individual molecules to see their actual differential regulation pattern. Supplementary tables S1 and S2 provides a partial list of differentially regulated plasma metabolites by undernourishment and AsX and DHA supplementation respectively.

Metabolite pathway enrichment analysis

Pathway enrichment analysis was done to determine the importance of the metabolites that were restored and regulated differently by AsX and DHA in response to undernourishment. The pathways that were enriched upon undernourishment and AsX & DHA supplementation are represented in Tables 1 and 2. Several key metabolic pathways are found to be differentially regulated by undernourishment. These pathways include

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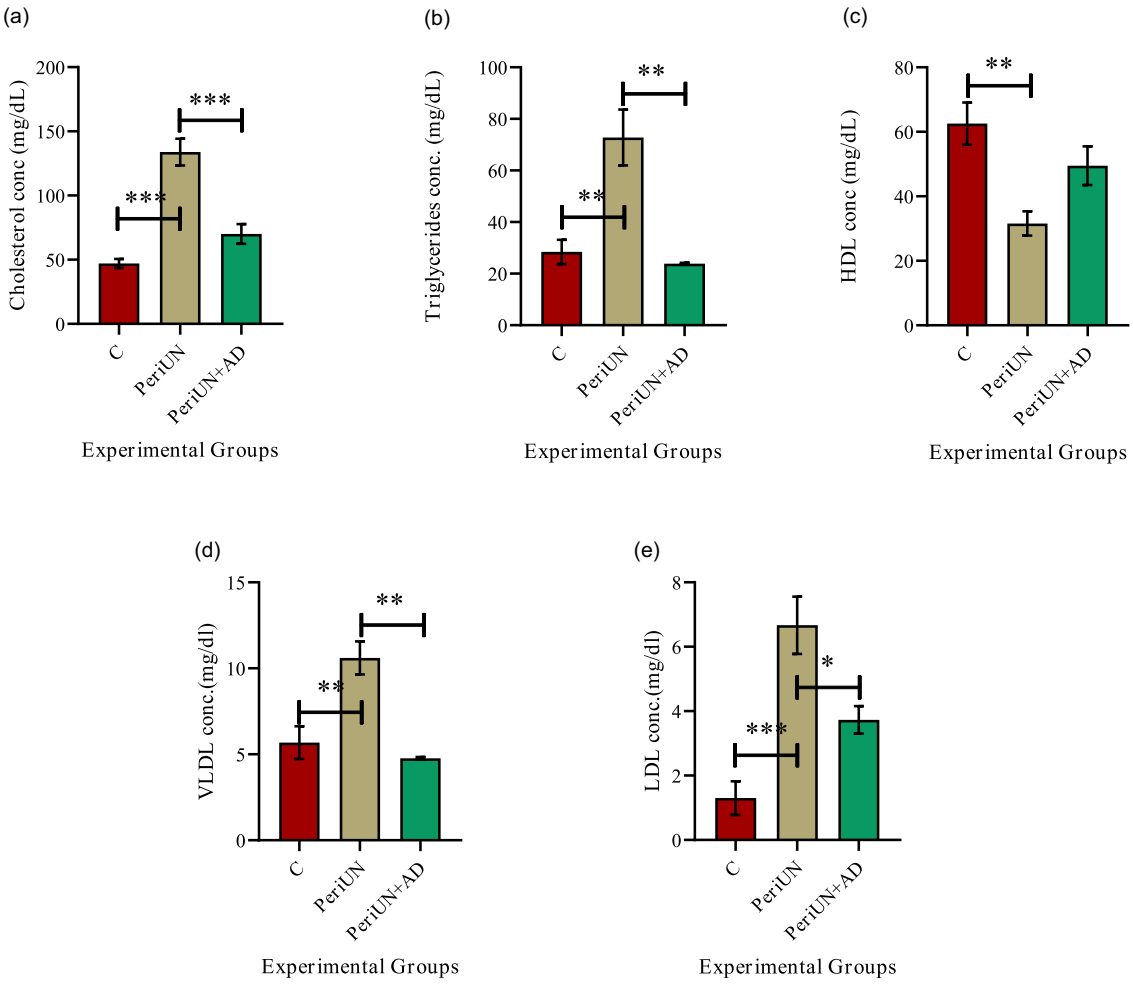

Figure 2. Effect of undernourishment and AsX & DHA supplementation on biochemical parameters: (a) Total Cholesterol, (b) Triglyceride, (c) HDL-C, (d) VLDL, and (e) LDL. Results were represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

threonine catabolism, serotonin and melatonin biosynthesis, enterocyte cholesterol metabolism, cholesterol metabolism, steroid biosynthesis, lovastatin action pathway and the metabolism of amine-derived hormones. Additionally, conditions such as hypercholesterolaemia, lysosomal acid lipase deficiency (Wolman disease), Smith–Lemli–Opitz Syndrome (SLOS), desmosterolosis and hypercholesterolaemia were also enriched by undernourishment (Table 1). AsX and DHA supplementation upon undernourishment resulted in differential regulation of valine, lysine, leucine and isoleucine degradation, metabolism of alpha-linolenic acid, butanoate, lipoic acid, tryptophan, citrate cycle (TCA cycle), fatty acid biosynthesis, elongation and degradation etc. (Table 2).

In order to enhance comprehension, we conducted integrated metabolite joint pathway impact analysis. This analysis involves using enrichment analysis approaches to discover the metabolic pathways that have the most significant influence. The impact is determined using pathway impact and adjusted p-values. The Figures were created via MetaboAnalyst 5.0. Pathway impact refers to the combined results of centrality and pathway enrichment. Higher impact values indicate the relative importance of the pathway.

The size of the circle reflects the impact of the pathway, while the colour represents its significance (with more intense red indicating a lower p-value). The joint pathway analysis revealed that 87 metabolites associated with steroid hormone biosynthesis. Additionally, 47 metabolites were linked fatty acid biosynthesis, 39 metabolites belong to fatty acid degradation and fatty acid elongation pathways. The citrate cycle (TCA cycle) encompassed 20 metabolites and pyruvate metabolism was represented by 23 metabolites. Furthermore, 26 metabolites were related to glycolysis or gluconeogenesis, 22 metabolites were involved in propanoate metabolism and 23 metabolites were associated with beta-alanine metabolism, which was differentially regulated by the undernourishment and AsX-DHA supplementation (Fig. 4a, 4b). Detailed results of impact analysis have been mentioned in Supplementary Table S3 and S4.

Discussion

The nutritional quality and accessibility throughout the perinatal period, which includes gestation and lactation, significantly influence the health outcomes of both mothers and newborns. Environmental variables, including undernutrition during this critical period, may trigger the onset of chronic illnesses in both mothers and their infants. It also compromises the maternal immune system, increasing susceptibility to infections and other complications during pregnancy and childbirth. Consequently, this may result still birth, preterm birth and increased offspring's

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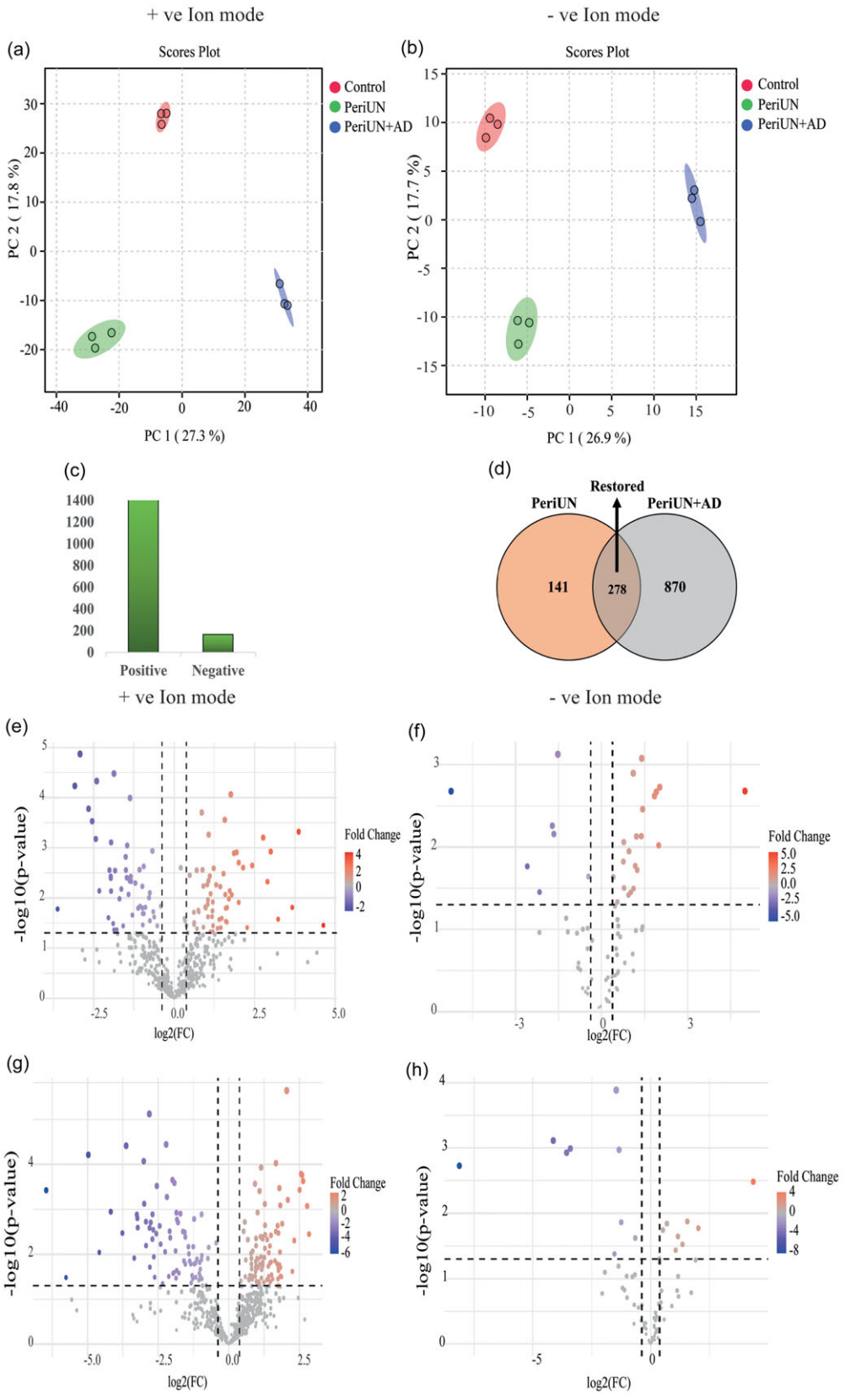

Figure 3. PCA score plot representing clustering of metabolite sets among experimental groups: (a) +ve Ion mode and (b) −ve Ion mode; (c) Bar plot represents total number of differentially regulated metabolites in both +ve and −ve ion mode; (d) Venn diagram represents number of differentially regulated metabolites identified in UN group and AsX and DHA supplemented group along with restored metabolites; (e) and (f) Volcano plot represents differentially regulated metabolites by undernutrition in both +ve and −ve ion modes respectively; (g) and (h) Volcano plot represents differentially regulated metabolites by AsX and DHA supplementation upon undernutrition in both +ve and −ve ion modes respectively.

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Table 1. Pathways enriched by undernourishment during gestation and lactation

Pathway enriched	Total	Expected	Hits	Raw *p* value	Holm *p* value	FDR
Threonine catabolism	13	0.0182	2	0.000127	0.421	0.152
Serotonin and melatonin biosynthesis	15	0.021	2	0.000171	0.565	0.152
Enterocyte cholesterol metabolism	29	0.0406	2	0.000654	1	0.152
Cholesterol metabolism	32	0.0448	2	0.000797	1	0.152
Metabolism of amine-derived hormones	36	0.0505	2	0.00101	1	0.152
Cholesterol biosynthesis pathway in hepatocytes	36	0.0505	2	0.00101	1	0.152
Cerivastatin Action Pathway	42	0.0589	2	0.00137	1	0.152
Desmosterolosis	42	0.0589	2	0.00137	1	0.152
Hypercholesterolemia	42	0.0589	2	0.00137	1	0.152
Lysosomal Acid Lipase Deficiency (Wolman Disease)	42	0.0589	2	0.00137	1	0.152
Smith-Lemli-Opitz Syndrome (SLOS)	42	0.0589	2	0.00137	1	0.152
Steroid Biosynthesis	43	0.0603	2	0.00144	1	0.152

Total: total number of compounds in the pathway; Hits: the actually matched number from uploaded data; Raw *p*: original *p* value calculated from enrichment analysis; Holm *p*: *p* value adjusted by Holm–Bonferroni method.

Table 2. Pathways enriched by AsX and DHA supplementation upon undernourishment during gestation and lactation

Pathway enriched	Total	Expected	Hits	Raw *p*	Holm *p*	FDR
Valine, leucine and isoleucine degradation	39	0.177	2	0.0121	0.958	0.485
Alpha-linolenic acid metabolism	13	0.0591	1	0.0578	1	0.626
Butanoate metabolism	15	0.0682	1	0.0664	1	0.626
Citrate cycle (TCA cycle)	20	0.091	1	0.0877	1	0.626
Beta-alanine metabolism	21	0.0955	1	0.0919	1	0.626
Propanoate metabolism	21	0.0955	1	0.0919	1	0.626
Pyruvate metabolism	23	0.105	1	0.1	1	0.626
Glycolysis / Gluconeogenesis	26	0.118	1	0.113	1	0.626
Glutathione metabolism	28	0.127	1	0.121	1	0.626
Lipoic acid metabolism	28	0.127	1	0.121	1	0.626
Lysine degradation	30	0.136	1	0.129	1	0.626
Fatty acid elongation	38	0.173	1	0.161	1	0.69
Fatty acid degradation	39	0.177	1	0.165	1	0.69
Tryptophan metabolism	41	0.186	1	0.173	1	0.69
Fatty acid biosynthesis	47	0.214	1	0.196	1	0.745

Total: total number of compounds in the pathway; Hits: the actually matched number from uploaded data; Raw *p*: original *p* value calculated from enrichment analysis; Holm *p*: *p* value adjusted by Holm–Bonferroni method.

mortality.[28,29] Majority of research has concentrated on the predisposition of wide range of chronic diseases across different age group of offspring's. However, very few studies have focused on the maternal aspects of undernourishment. Here we have made an attempt to evaluate the effect of undernutrition during gestation and lactation on maternal plasma metabolic profile. Additionally, we have evaluated the potential synergistic effect of AsX and DHA upon undernourishment.

Our preliminary investigation of maternal lipid profile revealed that, perinatal undernourishment impairs plasma TC, TG's, HDL-C, LDL and VLDL levels. Increased lipoprotein, cholesterol and triglycerides and reduced HDL-C levels are characteristic biomarker of obesity.[30] Undernourishment can result in metabolic and physiological programming of the mother as well as the offspring's, with short term or lifelong effects on the risk of development of chronic diseases such as obesity, cardiovascular diseases, high blood pressure and other metabolic diseases. Abnormal lipid profiles may serve as a possible underlying mechanism that explains these relations. However, in the present study, AsX and DHA supplementation demonstrated the potential to restore the impaired plasma lipid levels. This observation is aligning with our previous findings, where we demonstrated that, perinatal

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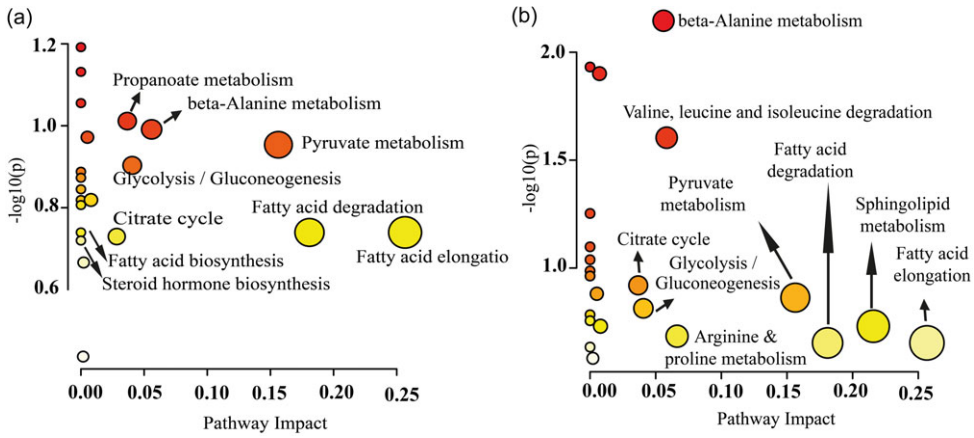

Figure 4. Bubble plot represents metabolite pathway impact analysis in (a) Undernourishment and (b) AsX and DHA supplementation groups.

undernourishment led to dyslipidemia and adipose tissue dysfunction, mediated by differentially regulating the genes or proteins involved in the lipid metabolism among adult male of F1 generation.[26]

The untargeted metabolomics analysis highlighted the differential regulation of metabolite abundances with respect to undernourishment and AsX-DHA supplementation. Undernourishment lead to upregulation of certain classes of lipid molecules, including diacylglycerols (DAGs), triacylglycerols (TAGs) and glycerophospholipids. Whereas, class of compounds which belongs to phosphatidylinositols were downregulated. DAGs, which act as intermediates in the synthesis of TAGs and glycerophospholipids, are upregulated, suggesting enhanced lipid synthesis. This is likely due to undernutrition, where energy storage is limited. TAGs, the main fat storage form, are also upregulated, suggesting a shift towards fat storage. Dysregulation of DAGs and glycerophospholipids can affect processes like inflammation, cell proliferation and apoptosis.[17,31] Phosphatidylinositols are precursors to phosphatidylinositol 3,4,5-trisphosphate (PIP3), a key lipid involved in the activation of the PI3K/Akt signaling pathway leading to increased apoptosis of cardiomyocytes and endothelial cells, contributing to heart failure and other cardiovascular complications.[32] Along with downregulating aforementioned metabolites, AsX and DHA supplementation resulted in the upregulation of entities belongs to branched fatty acid esters of hydroxy fatty acids (e. g. FAHFA(18:1(9Z)/9-O-18:0). FAHFAs, have been recently discovered lipids that have anti-inflammatory and moderating effects on diabetes. Recent research has shown that FAHFAs have the ability to promote autophagy and produce antioxidant effects in neuronal cells and cardiomyocytes.[33]

The altered dynamics of metabolites involved in sphingolipid and beta-alanine metabolism, fatty acid biosynthesis, glycolysis or gluconeogenesis, phenylalanine metabolism, citrate cycle (TCA cycle), cholesterol metabolism has been found in previous findings of gestational obesity and preeclampsia.[34,35] Consistent with these findings, our pathway enrichment analysis revealed that, the pathways involved in the lipid metabolism and glucose metabolism has been affected by undernourishment as well as AsX-DHA supplementation. In particular, pathways involved in the metabolism and biosynthesis of cholesterol, melatonin and serotonin, metabolism of amine-derived hormones as well as the biosynthesis of steroid were enriched in response to undernourishment.

Disrupted cholesterol metabolism as evidenced by the abnormal levels of lipoproteins (LDL, VLDL, HDL) and triglycerides validated in this study, can precipitate cardiovascular diseases like atherosclerosis, high blood pressure and heart failure. Adipose tissue plays a crucial role in the synthesis and metabolism of steroid hormones. Estrogens and other sex steroids play a vital part as they regulate the metabolism of fats (lipolysis) and the storage of fat in adipose tissue throughout the body.[36] Glucocorticoids play a crucial role in regulating lipid homeostasis. However, an excessive amount of glucocorticoids may lead to elevated levels of free fatty acids in the bloodstream and cause the buildup of lipids in skeletal muscle and liver. These effects are linked to the development of insulin resistance and obesity.[37] Furthermore, the investigation forecasted an elevated likelihood of abnormalities such as lysosomal acid lipase deficiency (Wolman Disease), Smith–Lemli–Opitz Syndrome (SLOS) and desmosterolosis. All of these disorders are stem from altered lipid metabolism and are commonly seen in the offspring's who have experienced IUGR during their early developmental stages.[38–40] It indicates that, maternal undernourishment during critical period may predispose aforementioned disorders in the offspring.

On the other hand, AsX and DHA supplementation resulted in the enrichment of separate set of pathways which are associated with fatty acid metabolism. Which includes, degradation of branched chain amino acids (BCAA's) such as valine, leucine and isoleucine. Previous studies have shown a positive correlation between elevated levels of branched chain amino acids in the bloodstream and the development of insulin resistance in individuals who are obese or have diabetes.[41,42] Hence, degradation of BCAA's may reduce the chance of development of metabolic disorders. Alpha-linolenic acid (ALA) metabolism may enhance lipid metabolism by modulating fatty acid oxidation and adipogenesis pathways. Research has shown that consumption of ALA may enhance the process of mitochondrial fatty acid oxidation and boost energy expenditure,[43] consequently reduces the accumulation of fat inside the body. Furthermore, previous findings suggest that depletion of essential amino acids may help in reducing the BW and visceral fat accumulation.[44–47] In accordance with these findings, in the present study we observed AsX-DHA supplementation resulted in enrichment of lysine degradation pathway, which lowers the incidence of obesity by suppressing fat accumulation and transcription of adipogenic genes in adipocytes.[48] Lipoic acid (particularly alpha-lipoic acid) is a necessary cofactor for mitochondrial respiratory enzymes, which enhances mitochondrial activity. Several studies have reported that alpha lipoic acid supplementation has promising effect on mitigating the obese phenotype.[49,50] In line with these findings, present study illustrated that AsX-DHA supplementation resulted in enrichment of lipoic acid metabolism. Other than above discussed pathways,

https://doi.org/10.1017/S2040174424000345 Published online by Cambridge University Press

AsX and DHA supplementation enriched certain pathways which are directly involved fatty acid metabolism, such as: fatty acid biosynthesis, elongation and degradation. Pathways enriched among glucose metabolism include glycolysis or gluconeogenesis, TCA cycle and pyruvate metabolism.

Further, pathway impact analysis illustrated that both undernourishment and AsX-DHA supplementation influenced similar set of pathways. Key pathways such as TCA cycle, pyruvate metabolism, glycolysis, fatty acid biosynthesis, degradation and elongation, degradation of BCAA's valine, leucine and isoleucine, beta-alanine metabolism and steroid hormone biosynthesis has been influenced by undernourishment as well as supplementation. Additionally, AsX-DHA supplementation influenced metabolism of sphingolipids, arginine and proline. These findings clearly states that undernourishment impairs metabolism of certain amino acid along with glucose and mainly it affects lipid metabolism. Dysregulation of lipid metabolism results in abnormal fat deposition inside the body subsequently leads to metabolic disorders like obesity and CVD's. However, AsX and DHA supplementation during gestation and lactation showed potential to mitigate this adverse effect of undernourishment by modulating regulatory pathways of lipid metabolism.

The mechanisms of action for AsX and DHA remain speculative. AsX, a powerful antioxidant, is expected to reduce oxidative stress, therefore protecting the integrity of cells and stabilising lipids crucial for preserving cellular homeostasis. DHA, a kind of omega-3 fatty acid, is acknowledged for its ability to reduce inflammation by decreasing the production of pro-inflammatory cytokines, which aids in regulating and harmonising the body's inflammatory response. Both AsX and DHA are postulated to stimulate peroxisome proliferator-activated receptors (PPARs),[51,52] which are crucial regulators of lipid metabolism. During periods of undernourishment, lipid metabolism is often compromised. However, AsX and DHA potentially improve lipid utilisation and energy balance. In addition, research has shown that DHA regulates Sterol Regulatory Element-Binding Proteins (SREBPs),[53] resulting in a decrease in the production of fats and the buildup of triglycerides. This modulation also entails the activation of AMP-Activated Protein Kinase (AMPK), which stimulates catabolic processes such as the breakdown of fatty acids and suppresses anabolic activities such as the production of lipids.

The combined actions of AsX and DHA on PPARs and AMPK signalling are anticipated to enhanced fatty acid oxidation, which is crucial for supplying energy under metabolic stress. In addition, DHA's control on adipogenesis helps to maintain homeostasis of lipid storage, limiting excessive accumulation of fat as a programming for undernutrition. Thus, the cumulative effect of AsX and DHA safeguards homeostasis of lipids and energy in the body, in conditions of undernutrition or metabolic stress.

Conclusion

The present study addressed the disordering of plasma metabolites driven by gestational and lactational undernourishment during pregnancy and lactation and subsequent restoration by supplementing the dams with AsX and DHA. Supplementation with AsX and DHA influenced important pathways related to the metabolism of fatty acids/lipids, exhibiting potential in reducing the risk of metabolic disorders including obesity and CVD's. The results indicate that AsX and DHA supplementation to the dams who suffered undernourishment during pregnancy and lactation could reduce the chances of development of metabolic disorders in their offspring.

Limitations of the study

Like any scientific study, there may be certain inherent constraints, such as the use of animal models, which may not accurately reflect human physiology. Inducing undernourishment in human beings is not ethically feasible. Furthermore, accurately assessing the degree to which an individual has experienced undernutrition in relation to specific dietary needs is quite challenging. The kind and degree of undernutrition will not be identical across human beings. Additionally, the genetic background of a person significantly influences the degree of undernutrition and their reaction to supplements. Further, the specific dosage of AsX and DHA administered, as well as the length of time for which they are supplemented, might potentially impact the observed outcomes. Therefore, further research is needed in various settings to completely confirm the validity of these results. Notwithstanding these overarching factors, the research seems to have mitigated possible biases, establishing a robust basis for its results. Moreover, utilising targeted metabolomics strategy may reveal crucial metabolites and molecular pathways governed by undernutrition, as well as those affected by AsX and DHA. Conducting longitudinal studies at various time points during gestation and lactation might give more comprehensive insights of dyslipidaemia and development of metabolic disorders need further exploration and studies focussed on long-term outcomes, exploring additional dietary interventions to provide deeper mechanistic insights to fully understand the mitigating effects of maternal undernutrition.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S2040174424000345.

Data Availability. The datasets generated during and/or analysed during the current study are included in the manuscript and supplementary file.

Acknowledgements. The authors acknowledge the support of the institutional animal care facility, Nitte (Deemed to be University) and laboratory facilities at Nitte University Centre for Science Education and Research (NUCSER) and Center for Systems Biology and Molecular Medicine, Yenepoya Research Centre, Yenepoya (Deemed to be University) for providing the laboratory and instrumental facilities for LC-MS experiments.

Author contribution. All authors contributed to the study's conception and design P.S.H. and M.B.A. carried out animal handling and treatment experiments, performed statistical analysis and preparation of figures and tables and contributed to the drafting and editing of the manuscript. S.U and A. A helped to carry out metabolomics experiments and the data analysis. D.G. K M, P.R. and, T.S.K.P provided scientific advice and contributed to designing the research work, conceptualising the ideas, reviewing the analysed data, drafting and editing the manuscript. T.S.K.P supervised metabolomics experiments. D.G.K. M. led the entire research. All the authors read and approved the final manuscript.

Financial support. This study was supported by grants from Nitte (Deemed to be University), N/RG/NUFR2/KSHEMA/2021/03 dated 23.09.2021. Author Damodara Gowda K M has received research support from Nitte (Deemed to be University).

Competing interests. The authors declare no competing interests.

Ethical standard. Animal experiments were approved by the Institutional Animal Ethics Committee, KS Hegde Medical Academy, Nitte (Deemed to be University), Mangalore (approval number Ref. KSHEMA/IAEC/06/2020).

Consent for publication. Not applicable as there was no content obtained from other sources. All the authors consented to the publication.

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