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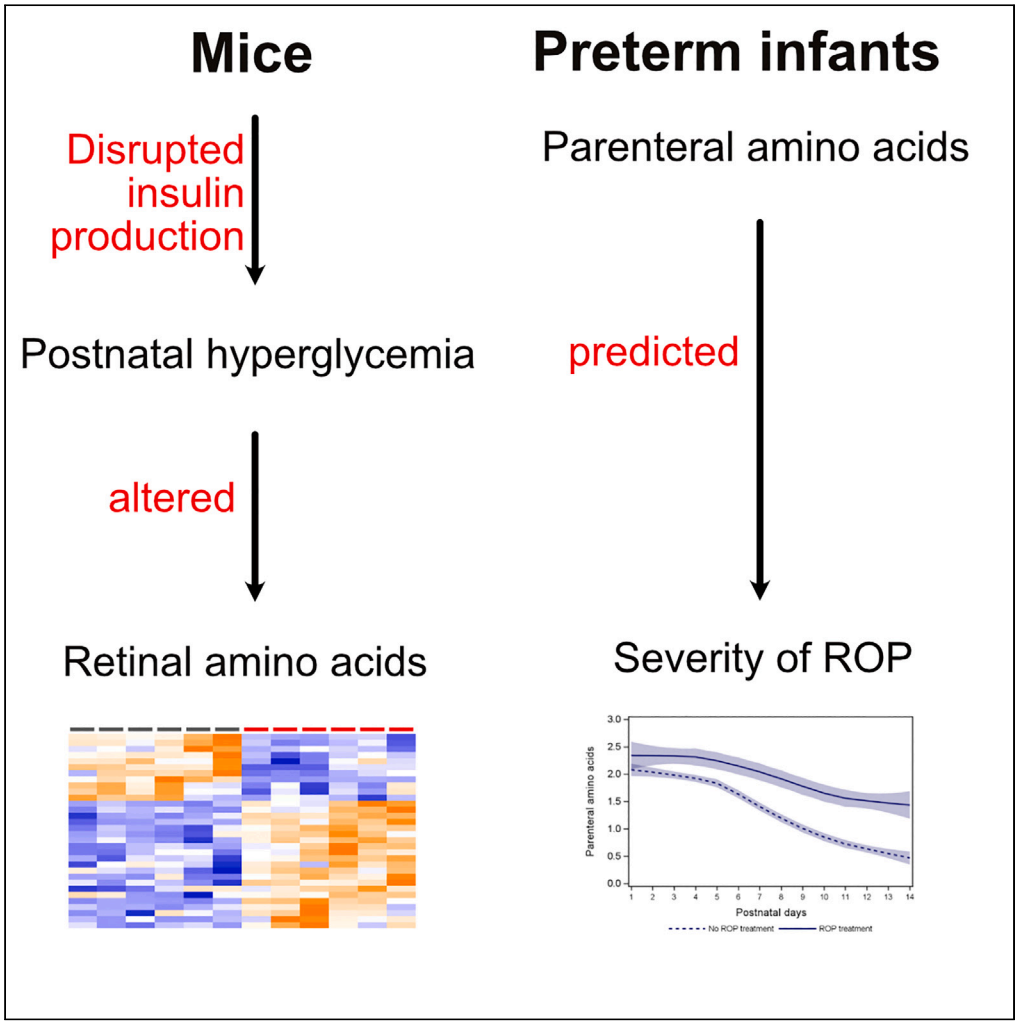

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Highlights

Hyperglycemia altered amino acid levels in neonatal mouse retinas

Parenteral L-isoleucine suppressed retinal vessel growth in hyperglycemic mouse retinas

Parenteral amino acid in preterm infants correlated with ROP requiring treatment

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Postnatal hyperglycemia alters amino acid profile in retinas (model of Phase I ROP)

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SUMMARY

Nutritional deprivation occurring in most preterm infants postnatally can induce hyperglycemia, a significant and independent risk factor for suppressing physiological retinal vascularization (Phase I retinopathy of prematurity (ROP)), leading to compensatory but pathological neovascularization. Amino acid supplementation reduces retinal neovascularization in mice. Little is known about amino acid contribution to Phase I ROP. In mice modeling hyperglycemia-associated Phase I ROP, we found significant changes in retinal amino acids (including most decreased L-leucine, L-isoleucine, and L-valine). Parenteral L-isoleucine suppressed physiological retinal vascularization. In premature infants, severe ROP was associated with a higher mean intake of parenteral versus enteral amino acids in the first two weeks of life after adjustment for treatment group, gestational age at birth, birth weight, and sex. The number of days with parenteral amino acids support independently predicted severe ROP. Further understanding and modulating amino acids may help improve nutritional intervention and prevent Phase I ROP.

INTRODUCTION

Retinopathy of prematurity (ROP) is a leading cause of blindness in children worldwide, affecting about 15 million infants annually.[1] ROP is a two-phase disease (Figure 1A). Phase I ROP begins after preterm birth with suppression of the growth of the immature retinal vasculature. The growth suppression is secondary to loss of necessary growth factors and nutrients typically provided *in utero*,[2] as well as oxygen supplementation, damaging to retinal vascular growth but necessary for the survival of the preterm infant. Efforts are made to optimize oxygen supplementation in preterm infants, but the best balance between decreasing mortality with high oxygen and preventing ROP with lower oxygen is still unknown.[3–6] In the avascular regions of the underdeveloped neural retina, oxygen and nutrient requirements are unmet, triggering the growth of compensatory but often pathological retinal vessels (neovascularization, Phase II ROP).[7,8] The well-established oxygen-induced retinopathy model[7,9–11] has been used for mechanistic investigations and evaluation of intervention for neovascular Phase II ROP. Vascular endothelial growth factor (VEGF) was found to be a significant contributor to retinal neovascularization with the use of the oxygen-induced retinopathy model[7,12–15] and anti-VEGF therapy can significantly suppress neovascular ROP in Phase II ROP. However, there are also adverse effects as VEGF is an important growth factor for normal tissue development and neuronal survival.[16–20] Therefore, better understanding of how to prevent physiological vascular growth suppression (Phase I ROP) will lead to safer therapeutic interventions that will prevent the development of neovascularization.

In premature infants, hyperglycemia in the early postnatal weeks, independent of oxygen, is strongly correlated with later development of retinal neurovascular Phase II ROP.[21–24] There is a higher frequency of hyperglycemia in infants with ROP vs. no ROP.[22] Hyperglycemia in the first week of life, independent of gestational age (GA), oxygen therapy, respiratory support and poor weight gain is a significant risk factor for ROP.[23] Our recent studies have demonstrated that postnatal hyperglycemia delays physiological retinal vascularization and is associated with neovascular ROP.[2,25,26] If we can improve physiological retinal vascularization at Phase I, we can prevent pathological neovascularization at Phase II (Figure 1A).

Metabolic dysfunction resulting from early hyperglycemia, hormone deprivation and malnutrition, leads to abnormal retinal development. As the neural retina continues to develop, there are increasing metabolic demands, triggering Phase II ROP, which generally coincides with the rapid development of rod photoreceptors (very rich in mitochondria) during postmenstrual weeks 30–32.[27,28] In the rat model of oxygen-induced retinopathy, photoreceptor dysfunction predicts subsequent retinal neovascularization.[29] Therefore, satisfying retinal metabolic

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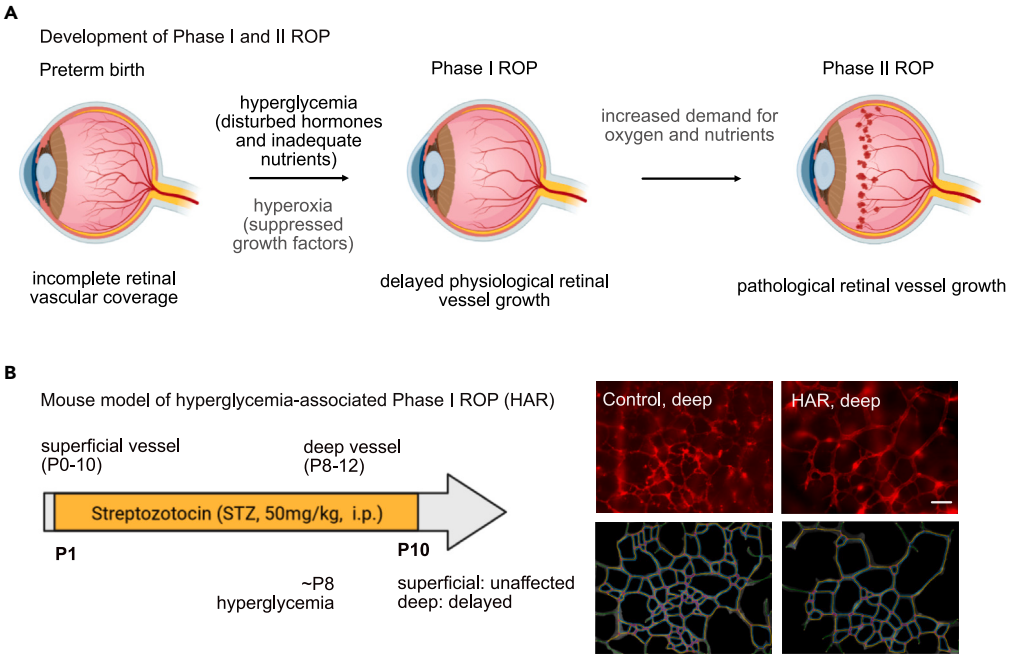

Figure 1. Schematics of ROP development and mouse model of hyperglycemia-associated retinal vessel growth delay in Phase I ROP (HAR)

(A) Schematics of human ROP development. Retinal vasculature is incomplete due to the preterm birth. Hyperglycemia and hyperoxia suppress physiological retinal vascularization and lead to Phase I ROP. Increased demand for oxygen and nutrients triggers compensatory but pathological retinal neovascularization (Phase II ROP). Graph was generated using BioRender.com.

(B) Schematics of mouse model of hyperglycemia-associated Phase I ROP (HAR). STZ was i.p. injected from P1 to P9. Hyperglycemia was induced around P8. At P10, delayed retinal vascularization at the deep plexus was found. Representative images of deep retinal vascular network at P10 were shown (top right, red, isolectin-stained vessels) and the images were quantified using ImageJ (bottom right). Scale bar, 50μm.

demand prevents abnormal retinal vessel growth. Targeted metabolomics analysis shows massive alterations in retinal metabolic profile and highly induced amino acid levels in mouse oxygen-induced retinopathy.[30] But the impact of metabolic dysregulation on retinal vascular pathology is not fully understood. In diabetic retinopathy there are significant alterations in amino acid metabolic pathways.[31] Vitreous metabolomics demonstrates that amino acid pathways are significantly altered in patients with proliferative diabetic retinopathy, including decreased creatine.[30] Oral creatine suppresses retinal neovascularization in mouse oxygen-induced retinopathy, partially modeling the neovascular aspect of diabetic retinopathy.[30] Therefore, we examined the impact of hyperglycemia on the amino acid profile in neonatal retinas in a model of Phase I ROP.

The reason for postnatal hyperglycemia in preterm infants is multifactorial including deficient insulin secretion, insulin resistance and limited peripheral glucose uptake.[32–35] To expand our knowledge of the retinal-specific metabolic responses to hyperglycemia during early ROP, we need to minimize the influences of the multiple contributors such as nutritional variations observed in clinics. Animal models allow us to study isolated ROP risk factors. The specific mechanisms underlying hyperglycemia's role in suppressing retinal growth are understudied, partially because of a lack of models of Phase I ROP. The oxygen-induced retinopathy model represents the oxygen suppression not the hyperglycemic suppression of physiological vascular growth in Phase I ROP. We have recently established a mouse model of hyperglycemia-associated retinopathy (HAR, Phase I retinopathy) with delayed retinal neural development and suppressed physiological retinal vascularization[2] (Figure 1B). mimicking hyperglycemia in newborns. In this model, streptozotocin (STZ), a compound preferentially toxic to insulin-producing β cells, is used to decrease insulin production and trigger postnatal hyperglycemia around postnatal day (P) 8 (~200 mg/dL blood glucose in HAR pups vs. 140 mg/dL in normal control pups). Significantly delayed retinal vascularization (decreased number of meshes and total vessel length per field) at the deep retinal vascular plexus (formation starts at P8) is observed at P10. Insulin treatment from P7 to P9 can restore the retinal vascular developmental delay. No significant changes in retinal vessel growth are found when STZ is directly delivered into the vitreous.[2] These findings suggest the delayed retinal vascularization is secondary to hyperglycemia or insulin deficiency, not STZ toxicity. In this model, supplementation of essential lipids and metabolic regulators lacking in premature infants improves retinal metabolism and neurovascular development.[2,25,36] Here, in the same Phase I ROP mouse model, we utilized targeted metabolomics to investigate the retinal metabolic responses to postnatal hyperglycemia, which can help optimize nutrient supply to preterm infants.

Further exploration of the nutrients and metabolites associated with ROP could help optimize maternal and infant diets to prevent ROP, since emerging evidence suggests nutritional interventions may prevent developmental pathology of the retina.[25,37–39] However, there are limited studies focused on correlating blood metabolites with ROP in premature infants. Recently, serum metabolomics has been used to study the preterm infant's metabolome and its relation to the development of severe ROP but no significant association between ROP

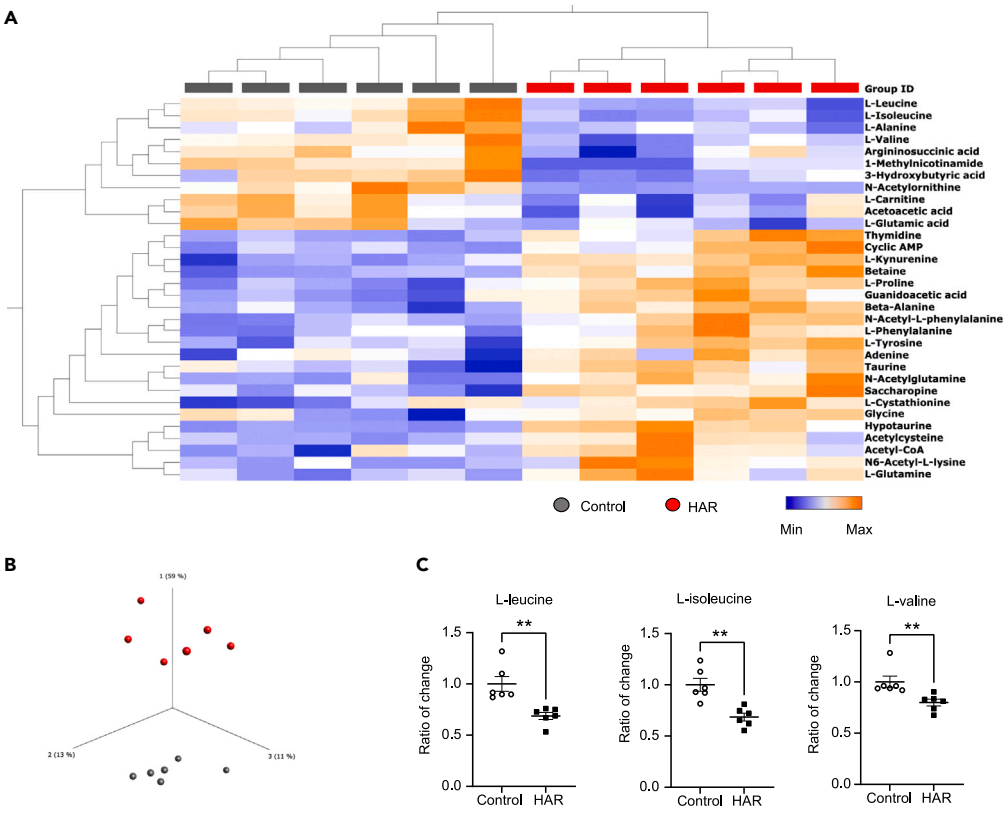

Figure 2. Altered metabolic profile in mouse retinas with hyperglycemia-associated retinal vessel growth delay (HAR, Phase I ROP)

(A) MS-based metabolomics demonstrated unique differences in metabolic signatures of the HAR mouse retina. Heatmap highlighting metabolite abundances with significant differences ($p < 0.05$) in HAR (red nodes, right) versus control (gray nodes, left) mice was shown. High levels of metabolites are indicated by orange, while low levels are indicated by blue. Each row represented a unique metabolite while each column represented a biological sample. Six retinas were pooled as n = 1, n = 6 for each group.

(B) Principal Component Analysis (PCA) of metabolomics data showed significant differences between metabolite profiles of control mice (gray nodes) and HAR mice (red nodes). Each respective node was representative of a single biological replicate. High-variance filtering in Qlucore Omics explorer ($p < 0.05$) was applied.

(C) Branched-chain amino acids (L-leucine, L-isoleucine, and L-valine) changed in HAR vs. normal control retinas. Data were represented as mean ± SEM. Normality (quantile-quantile plot) and F-test were first conducted. Unpaired t-test or Mann-Whitney test was then used for comparison. *$p < 0.05$, **$p < 0.01$.

and metabolite levels in the first month of life were found.[40] Meanwhile, the nutritional management in the neonatal period has profoundly influenced the serum metabolome.[40] The duration of parenteral nutrition is a strong predictor of ROP.[41] The mechanisms linking parenteral nutrition duration to ROP outcome are unknown. Here, we further explored the impact of early parenteral nutrition (particularly amino acids) on severe ROP requiring treatment in preterm infants.

RESULTS

Metabolism is altered in hyperglycemia-associated neonatal mouse retinas with suppressed physiological retinal vessel growth (Phase I ROP)

To determine the extent to which postnatal hyperglycemia induces retinal metabolic disturbance in neonatal mice, we performed mass spectrometry-based targeted metabolomics in HAR vs. normal control mouse retinas at P10, when delayed physiological retinal vascularization is observed (Figure 1B).[2] MS-analysis identified a total of 147 metabolites from retinal homogenates, however, 14 metabolites identified were considered low confidence because they were only detected in low abundance in a single sample and were therefore excluded from the analysis. When considering metabolites achieving statistical significance ($p < 0.05$), there were 32 metabolites identified. A heatmap of metabolites ($p < 0.05$) revealed differential abundances between the HAR and control retinas (Figure 2A). A corresponding principal component analysis of HAR and control mice (Figure 2B) further highlighted the distinct metabolic phenotype induced by postnatal hyperglycemia, evidenced by the node clustering in each respective group.

Interestingly, we found that the three essential branched-chain amino acids (BCAAs: L-leucine, L-isoleucine, and L-valine) were remarkably decreased in HAR vs. control retinas (Figures 2A and 2C). L-leucine is ultimately converted to acetyl-CoA (fuel of the tricarboxylic acid [TCA] cycle) and acetoacetate. L-isoleucine forms acetyl-CoA and succinyl-CoA (an intermediate of the TCA cycle). Partial degradation of L-valine

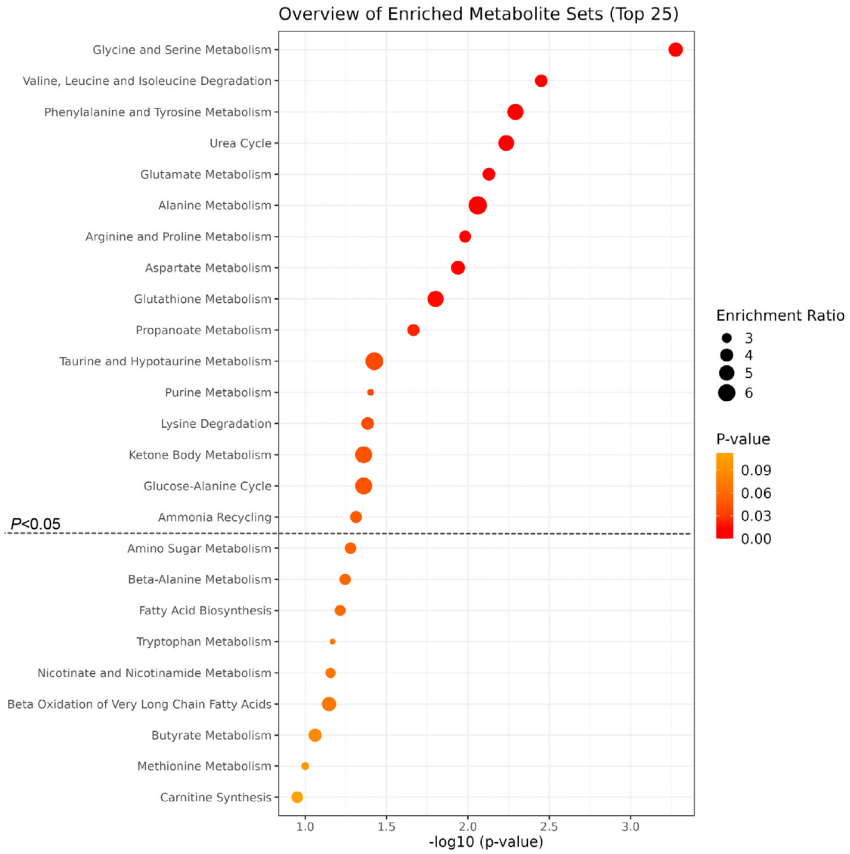

Figure 3. Amino acid metabolic pathways altered in mouse retinas with hyperglycemia-associated retinal vessel growth delay (HAR, Phase I ROP)

All the metabolites found to be significantly ($p < 0.05$) altered in HAR retina were loaded into MetaboAnalyst using the SMPDB reference database to perform enrichment analysis to map the connections between differentially expressed metabolites and the top 25 functional metabolic pathways ($p < 0.05$ for pathways above the dash line). The dot plot graphed enrichment ratios, which reflected the number of significant metabolites and the total number of metabolites in a given pathway, with -Log10 (p-value). The higher the enrichment ratio, the more significant a given pathway was enriched.

generates 3-hydroxybutyric acid. Acetoacetate and 3-hydroxybutyric acid can be subsequently converted to acetyl-CoA to fuel the TCA cycle. Correspondingly, we observed higher acetyl-CoA levels in HAR vs. control retinas (Figure 2A). We also found that in HAR retinas there were lower levels of the branched non-essential amino acid L-carnitine, which is mainly taken from the diet or synthesized from lysine and methionine, transporting long-chain fatty acids (LCFAs) into mitochondria for energy production. Moreover, there were other amino acids (L-glutamine, L-proline, L-phenylalanine, and L-tyrosine) that were increased in HAR retinas. L-glutamine and L-proline can be converted to α-ketoglutarate (an intermediate of the TCA cycle), while L-phenylalanine and L-tyrosine form acetoacetate and fumarate (an intermediate of the TCA cycle). Together, these observations suggested that retinal amino acid metabolism was significantly changed under hyperglycemic conditions in neonatal mice with suppressed physiological retinal vessel growth.

Enrichment analysis revealed key pathway involvement of hyperglycemia-associated altered metabolites

To further identify the major pathways affected by hyperglycemia in neonatal mouse retinas, the differentially abundant metabolites were loaded into MetaboAnalyst for pathway analysis. The pathways identified (based on the SMPDB repository) were based on enrichment ratios (Figure 3), which reflected the total number of metabolites in a given pathway and the number of metabolites identified/differentially abundant within the dataset. The dot plot format graphed enrichment values (overlap) against the -log10(p value). The top five pathways enriched in HAR retinas included: *1. Glycine and serine metabolism* ($p < 0.001$), 2. *Valine, leucine and isoleucine degradation* ($p < 0.01$), 3. *Phenylalanine and tyrosine metabolism* ($p < 0.01$), 4. *Urea cycle* ($p < 0.01$), and 5. *Glutamate metabolism* ($p < 0.01$).

Intraperitoneal supply of L-isoleucine delayed physiological retinal vessel growth in hyperglycemia-associated retinas (Phase I ROP)

To further examine the impact of amino acids on physiological retinal vessel growth under hyperglycemic condition, we intraperitoneally supplied BCAAs (L-leucine, L-isoleucine, and L-valine), which were significantly decreased in HAR retinas, to neonatal mouse pups from P7

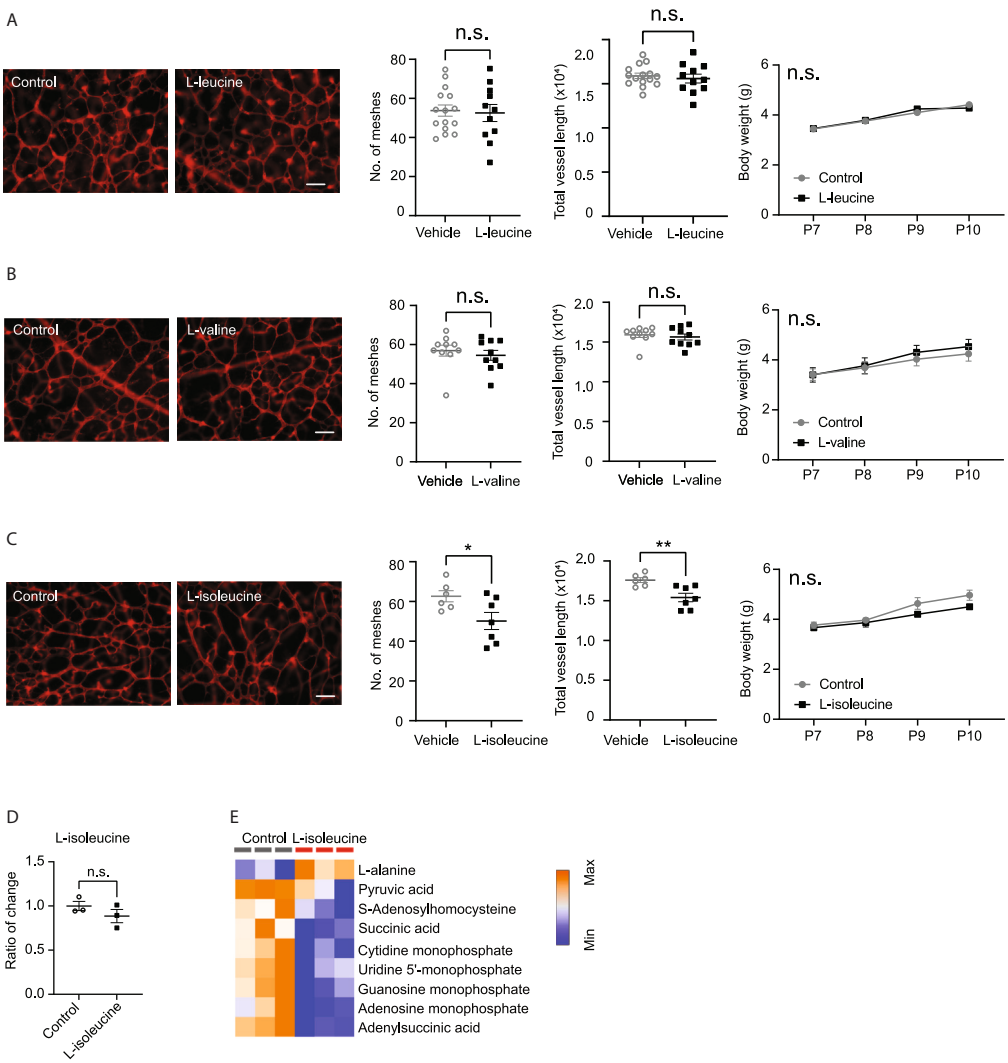

Figure 4. Parenteral (i.p.) supply of decreased BCAAs in mouse pups with hyperglycemia-associated retinal vessel growth delay (HAR, Phase I ROP)

(A–C) In mouse HAR, BCAAs ((A) L-leucine vs. vehicle; (B) L-valine vs. vehicle; (C) L-isoleucine vs. vehicle) were administered i.p. into mouse pups from P7 to P9. At P10, no. of meshes and total vessel length per field at the deep retinal vascular plexus were quantified using ImageJ. Data were represented as mean ± SEM. Normality (quantile-quantile plot) and F-test were first conducted, unpaired t-test or Mann-Whitney test was used to compare the groups. *$p < 0.05$, **$p < 0.01$. n.s., not significant. n = 11–15 retinas per group (A), n = 10 retinas per group (B), n = 6 retinas per group (C). Scale bar, 50μm.

(D and E) Littermate HAR mice were treated with L-isoleucine or vehicle control (PBS) i.p. daily from P7. At P10, retinas were collected for metabolomic analysis. Eight retinas were pooled as n = 1. n = 3 per group.

(D) Retinal L-isoleucine levels were unchanged. Data were represented as mean ± SEM. Unpaired t test. n.s., not significant.

(E) Significantly changed retinal metabolites ($p < 0.05$) between L-isoleucine- and vehicle control (PBS)-treated HAR mice were displayed in the heatmap.

to P9, respectively. At P10, there was no statistically significant impact of parenteral L-leucine and L-valine treatment on retinal vessel growth (Figures 4A and 4B). Parenteral L-isoleucine supplementation decreased number of meshes and total vessel length per field in HAR mice (Figure 4C), suggesting of worsened physiological retinal vascularization. No statistically significant differences in postnatal body weight gain from P7 to P10 (Figure 4) and blood glucose levels at P10 were found between the treatment and their littermate control groups: L-leucine (blood glucose, 297 mg/dL) vs. control (blood glucose, 212 mg/dL); L-valine (blood glucose, 250 mg/dL) vs. control (blood glucose, 294 mg/dL); L-isoleucine (blood glucose, 201 mg/dL) vs. control (blood glucose, 174 mg/dL). To further examine if parenteral delivery of L-isoleucine altered retinal metabolome in HAR mice, L-isoleucine- and vehicle control-treated littermate mice were collected at P10 for metabolomics analysis. No significant change was found in retinal levels of L-isoleucine possibly due to its fast depletion in tissues[42] (Figure 4D). There were significantly decreased levels of pyruvic acid and succinic acid (TCA cycle metabolites), as well as nucleotides (cytidine monophosphate (CMP), uridine 5′-monophosphate (UMP), guanosine monophosphate (GMP), adenosine monophosphate (AMP), adenylosuccinic acid ((an intermediate in the interconversion of IMP and AMP), S-adenosylhomocysteine (the precursor

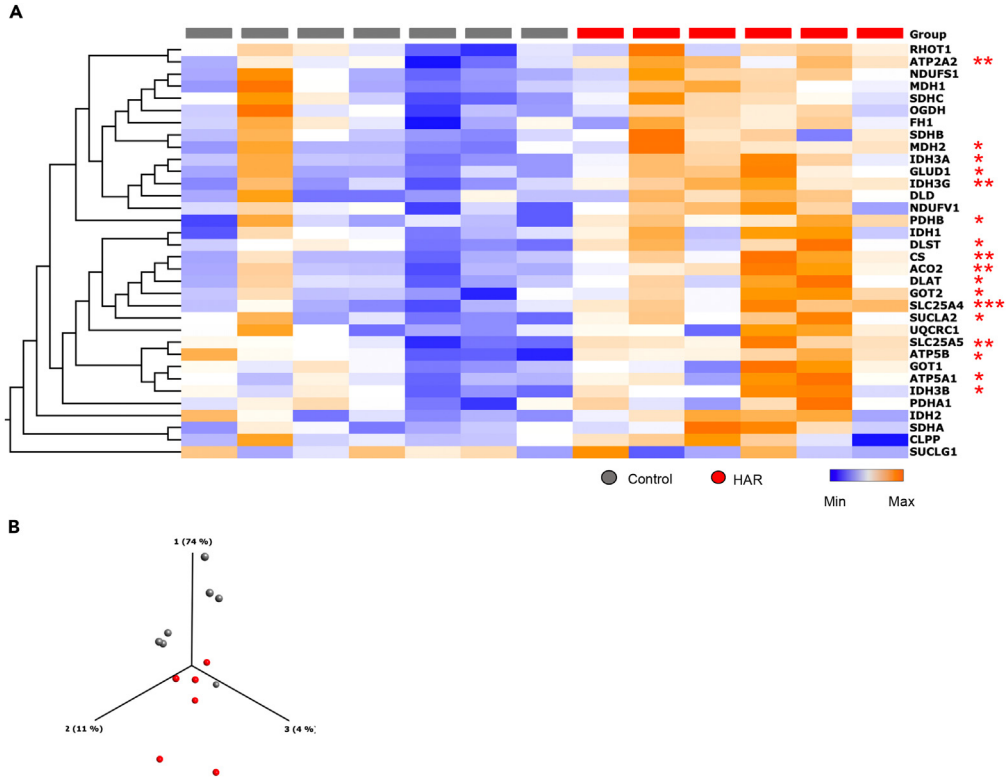

Figure 5. Retinal mitochondria-related proteins increased in mouse retinas with hyperglycemia-associated retinal vessel growth delay (HAR, Phase I ROP)

(A) Heatmap highlighting high abundance of proteins (shown in orange) and low abundance of proteins (shown in blue) in control mice (gray, left) and HAR mice (red, right) was shown. Two retinas from the same mouse were pooled as n = 1. Control n = 7, HAR n = 6. Unpaired t test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (B) Principal Component Analysis (PCA) of proteomics data showed distribution of protein profiles of control mice (gray nodes) and HAR mice (red nodes). Each respective node is representative of a single biological replicate. See also Figures S1 and S2.

of adenosine)) (Figure 4E). We also found nicotinic acid in vehicle control-treated group, but the level was below the detection limit in L-isoleucine-treated group (data not shown).

Mitochondria-related proteins accumulated in hyperglycemia-associated neonatal mouse retinas with suppressed physiological retinal vessel growth (Phase I ROP)

Since significantly altered amino acid profile was suggested in HAR retinas, we next examined if there were any compensatory responses in retinal metabolic proteins. We have previously shown that the levels of metabolic proteins were decreased in mice with worsened physiological retinal vascularization in HAR mice.[2] Targeted proteomic analysis was performed to measure the abundance of proteins involved in mitochondrial function, glucose, and lipid metabolism. 21 proteins were found to be statistically different between groups ($p < 0.05$). Seventeen proteins were involved in the mitochondrial function (Figure 5). Only 4 were involved in glucose and lipid metabolism (See also Figures S1 and S2). There were increased abundances of mitochondria-related proteins in HAR vs. control retinas (Figure 5A). The corresponding PCA showed apparent differences between protein profiles of the two groups (Figure 5B). Taken together, the increase in metabolic proteins suggested an effort to increase mitochondrial energy production in HAR retinas. We also speculated that predominantly changed amino acids (Figure 2A) might alter the energy production in HAR retinas. In HAR retinas, glutamic-oxaloacetic transaminase 2 (GOT2), which contributes to amino acid metabolism and the urea and TCA, was increased ($p < 0.05$). In addition, glutamate dehydrogenase 1 (GLUD1), which catalyzes the oxidative deamination of glutamate to α-ketoglutarate and ammonia, was also higher in HAR retinas ($p < 0.05$), in line with decreased L-glutamic acid found in metabolomics analysis (Figure 2A).

Parenteral nutrition at early life predicted ROP treatment in preterm infants

Next, we used clinical data from extremely preterm infants to further explore the connection between amino acid metabolism and ROP. A total of 178 infants born <28 weeks' gestation was eligible for analysis, and out of these, 36 (20.2%) were diagnosed with Type I ROP and treated with laser photocoagulation and/or anti-VEGF therapy (ROP treatment) (See also Figure S3; Table 1). Infants not treated for ROP

Table 1. Demographic data of preterm infants

	Measure	Total N = 178	No ROP treatment N = 142	ROP treatment N = 36	*p*-value[a]
Gestational age (weeks)	mean (SD)	25.6 ± 1.4	25.9 ± 1.4	24.5 ± 1.1	<0.0001
	median (range)	25.7 (22.6–27.9)	26.1 (22.6–27.9)	24.4 (22.9–26.6)	
Sex					0.040
Boys	n (%)	101 (56.7%)	75 (52.8%)	26 (72.2%)	
Girls	n (%)	77 (43.3%)	67 (47.2%)	10 (27.8%)	
Birth weight (g)	mean (SD)	805.8 ± 199.6	839.4 ± 200.1	673.2 ± 132.2	<0.0001
	median (range)	786 (425–1345)	823 (455–1345)	670 (425–975)	

SD = standard deviation; ROP = retinopathy of prematurity.
[a]Statistical test performed: Fisher's non-parametric permutation test for continuous variables and Fisher's exact test for dichotomous variables.

had a mean (SD) GA at birth of 25.9 (1.4) weeks and BW of 839 (200) g, which were significantly higher than infants who required ROP treatment (GA at birth 24.5 (1.1) weeks and 673 (132) g).

Since our HAR mouse experiments identified altered amino acid homeostasis related to retinal pathogenesis, and parenteral nutrition intake impacts infant blood amino acid levels,[40,43,44] we explored the potential correlation between ROP outcome and intake of parenteral amino acids. Infants who developed ROP requiring treatment had significantly higher intake of parenteral amino acids in the neonatal period compared to infants without need for ROP treatment (during postnatal days 2–7, 2–14 and 2–28, Figure 6A; Table 2). These differences remained significant for day 2–14 and day 2–28 in analyses adjusted for treatment group, GA at birth, BW and sex (Table 2). Also, the total number of days with parenteral amino acid support differed significantly between groups, where ROP treated infants on average received parenteral amino acids for an additional 15 days (18.6 ± 17.2 vs. 33.6 ± 22.2 days, unadjusted $p < 0.0001$; adjusted analysis OR 1.19 (95% CI 1.03–1.38), $p = 0.018$).

Using days with parenteral amino acids support to predict ROP treatment in a receiver operating characteristic (ROC) analysis performed similarly to using GA at birth alone, with c-statistics of 0.79 in both analyses (Figure 6B). Combining days with parenteral amino acids with treatment group, GA at birth, birth weight, and sex yielded a c-statistics of 0.86 (Figure 6B; Table 2).

DISCUSSION

To better understand the retinal nutritional needs and metabolism during neonatal hyperglycemia at the early stage of ROP, we analyzed retinal metabolic changes in the mouse HAR model with suppressed physiological retinal vascularization (Phase I ROP). Hyperglycemia changed retinal amino acid levels in mouse neonates. Parenteral supply of the decreased amino acid L-isoleucine worsened physiological retinal vessel growth. Our clinical data showed that preterm infants with severe ROP (requiring treatment) had a higher intake of parenteral versus enteral amino acids in the neonatal period, and that the number of days on parenteral amino acid support, independent of treatment group, sex, GA, and BW, strongly predicted severe ROP (requiring treatment). In fact, using days on parenteral amino acids to predict ROP treatment was as strong as GA alone. This observation raises the concern that current parenteral nutrition may be suboptimal in promoting retinal health.

The fetus uses amino acids for both energy and growth. After preterm birth, human milk alone cannot supply enough protein/amino acids to meet the high requirements of the infant, therefore nutritional support with parenteral amino acid solutions is needed in the first days or weeks of life. Premature infants without exogenous amino acids become catabolic and lose about 1% of protein stores daily.[45] Parenteral supplementation of amino acids within the first 52 h of life at 3 g/kg/day in very low BW infants restores plasma amino acid concentrations to levels seen during the second and third trimesters of pregnancy.[46] Preterm infants receiving parenteral amino acid supplementation within 24 h of life have greater weight gain than infants receiving the supplementation after 24 h of life.[47] But knowledge of the optimal amino acid composition for preterm growth is still limited.[48] As poor postnatal weight gain predicts severe ROP and improved weight gain reduces ROP risk,[49,50] early amino acid supplementation may help prevent ROP. Experimental studies have shown that the dipeptide arginine-glutamine decreases the retinal avascular area and inhibits retinal neovascularization in mouse oxygen-induced retinopathy.[38,51] Suppression of glutamine use by inhibiting glutaminase-1 (converting glutamine to glutamate and ammonia) causes sprouting defects in retinal angiogenesis.[52] Loss of endogenous serine production in endothelial cells leads to impaired retinal vessel growth, increase cell apoptosis and defects in mitochondrial respiration.[53] Together, these observations suggest modulation of amino acid availability may control physiological retinal vessel growth.

BCAAs (leucine, isoleucine, valine) are essential amino acids that cannot be synthesized in mammals and must be obtained from the diet. BCAAs provide building blocks for protein synthesis and serve as fuel sources for energy production. The products of BCAA metabolism fuel the TCA cycle. BCAA shortage may result in impaired growth and neurological development.[54] We here found decreased BCAAs in HAR vs. control neonatal retinas, possibly resulting from accelerated use and/or decreased uptake of BCAAs from circulation. Preterm infants with GA<25 weeks and an increased risk for ROP have higher levels of serum BCAAs than those with GA>25 weeks,[40] suggesting that 1) there

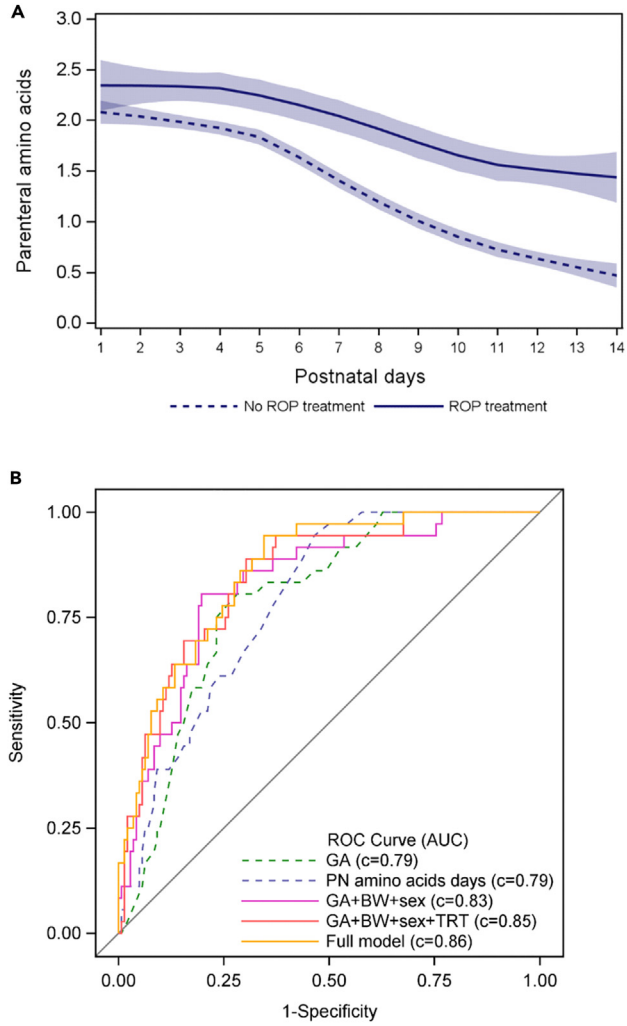

Figure 6. Parenteral amino acid intake in preterm infants in relation to ROP outcome

(A) Administration of parenteral amino acids. No ROP treated vs. ROP treated infants.

(B) Receiver operating characteristic (ROC) curve for various models predicting ROP treatment: 1) gestational age (GA) at birth alone, 2) parenteral nutrition (PN) amino acids number of days alone, 3) GA, birth weight (BW) and sex, 4) GA, BW, sex and randomized treatment AA/DHA or control (TRT), and 5) PN amino acid number of days, GA, BW, sex and TRT. AUC = area under the curve. See also Figure S3.

is a decreased peripheral uptake of BCAAs or 2) there is a higher nutritional contribution of BCAAs. In the same study, it was reported that serum isoleucine levels positively correlate with enteral nutrition intake.[40] High dose of parenteral amino acid supplementation also increased blood BCAAs.[43,44] The impact of accumulated circulating BCAAs on retinal maturation is unknown. In male type 2 diabetic adults, high serum leucine levels may suggest a lower risk for diabetic retinopathy.[55] Under conditions of starvation, type 1 and 2 diabetes, there are increased blood BCAAs levels. It is still unclear if BCAAs are biomarkers of diabetes or contribute to disease pathogenesis.[56] Current literature covers both detrimental and beneficial effects of BCAAs on metabolic health. Oral intake of isoleucine not leucine decreases plasma glucose levels and increases glucose uptake in the skeletal muscle in food-deprived three-week-old rats, while leucine not isoleucine increases the glucose incorporation into glycogen in the rat skeletal muscles *in vivo*.[57] Another report has also shown that reduced dietary isoleucine or valine, but not leucine, increases hepatic insulin sensitivity and ketogenesis, as well as energy expenditure in adult mice.[58] Leucine improves while valine supplementation reduces insulin sensitivity in adult mice under high-fat conditions.[59] Therefore, it is necessary to determine the impact of specific BCAAs on unique health conditions. Our current data have shown that parenteral supplementation of L-isoleucine suppressed while L-leucine and L-valine did not affect physiological retinal vascularization in neonatal HAR mice, further confirming the specific roles of each BCAAs in neonatal retinal growth. In addition, the amount of specific BCAAs also needs to be optimized based on postnatal age. In term infants, an enterally nutritional supplementation of isoleucine:leucine:valine at the ratio of 1:1.3:1 is recommended in the first month.[60] Better understanding of the role of specific BCAA and their combination in retinal vascular development is needed to optimize the BCAA composition in parenteral nutrients.

Table 2. Relation between parenteral nutrition, glucose, and need for ROP treatment, unadjusted and adjusted (FAS population with final ROP examination)

				Unadj.[a]	Adjusted[b]		
	Measure	No ROP treatment N = 142	ROP treatment N = 36	p value	OR (95% CI)	p value	AUC ROC
Mean parenteral amino acids (g/kg) 2–7 days	mean (SD)	1.85 ± 0.79	2.27 ± 0.66	0.0042	1.34 (0.73–2.48)	0.35	0.85
	median (range)	1.8 (0.2–3.5)	2.2 (1.0–4.0)				
Mean parenteral amino acids (g/kg) 2–14 days	mean (SD)	1.26 ± 0.79	1.91 ± 0.80	<0.0001	1.74 (0.99–3.05)	0.05	0.86
	median (range)	1.0 (0.1–3.5)	1.7 (0.7–3.9)				
Mean parenteral amino acids (g/kg) 2–28 days	mean (SD)	0.82 ± 0.68	1.45 ± 0.95	<0.0001	1.75 (1.01–3.02)	0.046	0.86
	median (range)	0.5 (0.0–3.0)	1.0 (0.5–3.4)				
Total days with parenteral amino acids	mean (SD)	18.6 ± 17.2	33.6 ± 22.2	<0.0001	1.19 (1.03–1.38)	0.018	0.86
	median (range)	14 (3–122)	26 (12–95)				

Unadj = Unadjusted; ROP = retinopathy of prematurity; AUCROC = Area under the Receiver Operating Characteristic Curve.
[a]Statistical test performed: Fisher's non-parametric permutation test.
[b]Statistical test performed: Logistic regression adjusting for treatment group, gestational age at birth, birth weight and sex.

In addition to BCAAs, we also observed increased L-glutamine, L-proline, L-phenylalanine, and L-tyrosine in HAR retinas. Disturbed glutamine metabolism causes defects in retinal vessel growth.[52] Enteral and intraperitoneal supplementation of glutamine together with arginine inhibits uncontrolled retinal vessel growth.[38,51] Both glutamine and arginine can be converted to α-ketoglutarate to fuel the TCA cycle. We speculated that glutamine might be an alternative fuel source of mitochondrial respiration in HAR retinas. In addition to glutamine, retinal L-proline was also higher in HAR vs. control mice. Genetic defects in proline synthesis lead to retinal pigment epithelium (RPE) dysfunction and retinal degeneration in humans.[61] Proline is a key and favorable nutrient to support the TCA cycle in RPE.[62] Dietary proline improves visual function and decreases photoreceptor death after sodium-iodate-induced RPE damage in mice.[62] We, therefore, speculated that there might be potential positive impact of accumulated proline on HAR retinas. Moreover, L-phenylalanine, an essential amino acid obtained from the diet, was increased in HAR. Phenylalanine is hydroxylated to tyrosine, which then can be converted to fumarate and acetoacetate to fuel the TCA cycle. Tyrosine is also a precursor of various neurotransmitters. Disturbed conversion of phenylalanine to tyrosine leads to various eye symptoms including retinal thinning.[63] Supplementation of phenylalanine in newborn rats caused damage to the retinal neurons.[64] Therefore, the impact of accumulated L-phenylalanine on neonatal HAR retinas can be both positive and negative.

In agreement with a previous study,[41] we found that prolonged parenteral amino acid supplementation is a strong independent predictor of future ROP treatment. Fetal amino acid homeostasis is regulated by active fetal-placental amino acid exchange,[65] a function that is untimely lost in the case of premature birth. There is yet no consensus regarding optimal amino acid composition of parenteral solutions given to preterm infants, nor the maximal dosing. The beneficial effects of increasing parenteral amino acid intake above 3.5 g/kg/d are unclear and may even show negative outcomes.[66] Moreover, the requirements for amino acids from total parenteral nutrition are lower than those from enteral feeding as the gut is bypassed.[67–70] However, current amino acid composition of parenteral nutrition lacks scientific support, and suboptimal supplementation may cause adverse impacts on body protein synthesis during growth. Hence, optimizing the specific amino acid supplementation with postnatal age may help prevent retinal abnormalities.

In addition to alterations in fuel substrates observed by metabolomics, we also found remarkable increase of mitochondria-related protein abundances (including GOT2 and GLUD1 for amino acid metabolism) in HAR retinas with delayed physiological retinal vessel growth, further suggesting efforts to increase retinal energy production in HAR. Interestingly, there were mild changes in proteins involved in glucose and lipid metabolism. Taken together, our data suggested that amino acids might serve as major alternative energy sources for mitochondrial respiration in HAR. Further tests are needed to examine the functional outcome and whether this response is long-term or not. In our recent investigation of HAR vs. control retinas using single-cell transcriptomics, we found overall decreased expression of metabolic genes in all types of retinal neurons at the same time point as investigated in the current study.[25] There is a well-known discrepancy between mRNA and protein levels.[71–73] Posttranslational modification can occur as an instant response to cellular stress and result in changed protein turnover and protein levels. Therefore, we speculated that accumulated proteins might function as instant responses to hyperglycemia while decreased gene expression of metabolic proteins might predict long-term responses. Future longitudinal functional studies are needed to reveal the metabolic outcomes in HAR.

Metabolic disturbances in ROP development and progression reflected in the blood circulation have been documented with the use of metabolomics, proteomics and lipidomics.[8] Targeted metabolomic analysis of the plasma from preterm infants has revealed that amino acids and their derivatives may correlate with ROP.[74–77] However, these results need to be carefully interpreted as parenteral and enteral nutrition intake may affect blood metabolome[40,43,44] and not all studies have reported this information. Our current study showed that parenteral nutrition, especially prolonged provision of parenteral amino acids, correlated with the higher need for ROP treatment. Correspondingly, there were remarkable alterations in retinal amino acid levels in mouse neonates after postnatal hyperglycemic induction leading to retinopathy.

Parenteral amino acid supplementation may exert distinct effects on retinal health as shown in our BCAAs treatment in HAR model. Therefore, optimization of specific amino acids in parenteral nutrition would be needed to better prevent ROP.

Limitations of the study

There are several limitations in the current study. First, the mouse model does not fully represent human ROP development. Premature birth leads to incomplete retinal vascularization and the cause of postnatal hyperglycemia is multifactorial (insulin deficiency, insulin resistance, limited peripheral glucose uptake, hormonal disturbance, and malnutrition).[2,26,32–35] In mouse hyperglycemia-associated retinal vascularization delay (Phase I retinopathy), hyperglycemia was triggered by insulin deficiency in full-term mice. The mouse retina was fully vascularized at the superficial layer when hyperglycemia was induced around P8, and the retinal vessel growth delay was found at the deep vascular plexus, which did not fully represent clinical phenotypes of Phase I ROP. In addition, mouse pups received milk from well-fed mothers while preterm infants received nutrition from both parenteral and enteral intake routes at early life. In most cases, the nutrients are insufficient in preterm infants due to the suboptimal composition of total parenteral nutrition and immature guts. Second, our clinical data demonstrated that the prolonged parenteral (versus enteral) amino acid intake was associated with severe ROP. In mouse hyperglycemia-associated retinal vascularization delay (Phase I retinopathy), we only examined the impact of parenteral supply of BCAAs (i.p.) on retinal vasculature development. Direct enteral supplementation of nutrients to small mouse pups (P7 to P9) is technically challenging. However, comparison of parenteral versus enteral amino acids in neonatal pups would be needed to explain why parenteral amino acid supplementation may exert a negative impact on physiological retinal vascularization. Future investigation of adjusting maternal diets with BCAA (and other amino acids) levels may help better elucidate the role of BCAAs (and other amino acids) in retinal vessel growth. Moreover, the current dose of parenteral BCAAs supplementation in mouse pups was based on circulating levels in mouse blood from prior publications. Measurement of plasma and retinal BCAAs (and other amino acids) after supplementation may help optimize the dose of amino acids supplied. However, the feasibility of analysis would also be technically challenging depending on if there is fast depletion of certain amino acids in the circulation. Future studies regarding if hyperglycemic retinas uptake BCAAs (and other amino acids) directly to influence retinal development, or if there is indirect circulating impact of BCAAs (and other amino acids) supplementation on retinal vascularization are also needed to better understand the underlying mechanisms.

Mass spectrometry provides valuable insight into quantitative changes across a wide range of molecules captured in a single moment in time. It is important to note, especially in the case of proteins, that the changes measured are reflective of abundance differences and not necessarily function. Further functional validation would be needed to confirm the ultimate metabolic outcomes. In the follow-up studies, we will investigate longitudinal metabolic responses in the presence of specific amino acids in HAR retinas using BaroFuse analysis,[78–81] a microfluidic system with consistent nutrient and oxygen supply to preserve retinal viability. Other nutrients like LCFAs would also be intriguing targets as L-carnitine, which transports LCFAs to mitochondria for fatty acid beta-oxidation and acetyl CoA production, was decreased in HAR retinas. In addition, metabolic proteins (ECHS1, ETFA, and ECH1) involved in mitochondrial fatty acid oxidation were higher in HAR retinas, suggesting that lipid metabolism might also be induced as an immediate compensatory response to increase retinal energy production. Therefore, L-carnitine or specific LCFA supplementation may help improve retinal development in early life. Further validation is needed before drawing the conclusion.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

Conceptualization: A.H. and Z.F.; Data curation: J.H., A.P., A.K.N., M.B., H.Y., J.Y., M.K., K.N., and M.K.; Formal analysis: J.H., A.P., and M.K.; Writing – original draft: Z.F., J.H., A.P., and A.K.N.; Writing – review and editing: M.K., A.H., and Z.F.; Funding acquisition: Z.F. All authors reviewed and approved the submission.

DECLARATION OF INTERESTS

All authors have no conflicts of interest.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
isolectin GS-IB4	Invitrogen	I21413(RRID:AB_2313921)
Chemicals, peptides, and recombinant proteins		
Leucine	Sigma	L6914
Isoleucine	Sigma	I5281
Valine	Sigma	V4638
Vaminolac	Fresenius Kabi	082077 (old: 574396)
Primene	Baxter Medical AB	B05BA01
Streptozotocin	Sigma	S0130
Deposited data		
Proteomics	This paper	Zenodo.org. DOI was https://doi.org/10.5281/zenodo.7662483
Metabolomics of HAR vs. control retinas	This paper	Metabolomics Workbench, datatrack_id:3745, study ST002497. https://doi.org/10.21228/M8BB1T
Metabolomics of L-isoleucine- vs. control-treated retinas	This paper	Metabolomics Workbench, datatrack_id:4215 study_id:ST002830, https://doi.org/10.21228/M8NT5Z
Experimental models: Organisms/strains		
C57BL/6J	Jackson Laboratory	RRID: IMSR_JAX: 000664
Software and algorithms		
Prism v9.0	GraphPad Software, Inc.	https://www.graphpad.com/updates/prism-900-release-notes
SAS software v9.4	SAS Institute Inc.	https://www.sas.com/en_us/software/viya.html?utm_source=other&utm_medium=cpm&utm_campaign=non-cbo-us&dclid=&gclid=Cj0KCQjwl8anBhCFARIsAKbbpyRZWJ0vrRGEkrkPAnky-7Kfr00q7MIHcDlvPTNT-oB2efnnpvDZ6iwaAlPzEALw_wcB
Qlucore omics explorer (v3.8)	Qlucore	https://qlucore.com/qlucore-omics-explorer3.8
MetaboAnalyst (v5.0)	Jianguo Xia's lab	https://www.metaboanalyst.ca/
ImageJ 1.47	National Institutes of Health	imagej.nih.gov/ij/
Nutrium software	Nutrium AB	https://nutrium.com/en

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Zhongjie Fu (zhongjie.fu@childrens.harvard.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Datasets are publicly available. Proteomics data: https://doi.org/10.5281/zenodo.7662483. Raw data of metabolomics was deposited at Metabolomics Workbench[82] (Metabolomics data of HAR vs. normal control retinas: https://doi.org/10.21228/M8BB1T; Metabolomics data of L-isoleucine- vs. vehicle control-treated HAR retinas: https://doi.org/10.21228/M8NT5Z).
- This paper does not report the original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animals

C57BL/6J (RRID: IMSR_JAX: 000664, Jackson Laboratory, Bar Harbor, ME) mice, of each sex, aged 10-12 weeks, were purchased, housed and bred in the institutional vivarium and maintained on a 12hour/12hour light/dark cycle with mouse chow provided *ad libitum*. All procedures were approved by our Institutional Animal Care and Use Committee and adhered to ARRIVE guidelines and the NIH Guide for the Care and Use of Laboratory Animals. Neonatal littermates were randomly assigned to experimental groups. The cages were located at close spots to minimize the potential housing influences. Mice with weight range 4 to 5 grams at P10 and both sexes were used for analysis. With conditions tested with β=0.8 and α=0.05, at least n=6 per group will be needed for the analysis.

Study participant

Preterm infant data were collected from the Mega Donna Mega randomized trial[37] (ClinicalTrials.gov Identifier: NCT03201588). The study was performed following the ethical principles of the Helsinki Declaration[83] with permission from the Regional Ethics Review Board at the University of Gothenburg (Dnr 303-11, T570-15). Signed informed consent to participate was collected from parents/guardians of all included infants. The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of work are appropriately investigated and resolved. All participants are born in Sweden, and other demographic information including age and gender are provided in Table 1. Patient population is provided in method details and Figure S3.

METHOD DETAILS

Mouse HAR (Phase I ROP model)

To study the metabolic alterations occurring in hyperglycemia-associated Phase I ROP, we applied quantitative metabolomics and proteomics on mouse retinas from HAR and normal control mice. Induction of hyperglycemia was accomplished as previously described.[2] Neonatal mice were intraperitoneally injected with 50mg/kg/day streptozotocin (STZ, Sigma, S0130) consecutively from P1 to P9 using a 34-G needle (Hamilton syringe) (Figure 1B). Vehicle control animals received equal volumes of vehicle phosphate-buffered saline (PBS, Gibco, Waltham, MA). Hyperglycemia is induced around P8 and delayed retinal vascularization is found at P10 [2]. Control was re-named as group 1 and HAR was re-named as group 2 for analysis.

Sample preparation for MS-based metabolomics

Retinas were collected at P10 following a single incision across the sclera and immediately snap frozen in liquid nitrogen and stored at -80°C until sample processing.[84] Samples were processed and analyzed by LC-MS/MS by the NYU Metabolomics Core Resource Laboratory, New York, NY, USA, as described previously.[30,85,86] Briefly, samples were homogenized using a bead blaster for 10 cycles with 30 seconds on and 30 seconds off. Metabolites were extracted using 80% methanol and dried down using a speedvac. Next, samples were reconstituted in 50 µL MS-grade water and sonicated for two minutes. Samples were then spun down in a centrifuge at 21KG for 3 min and finally transferred to MS vials for analysis. MS analyses were carried out by coupling the LC system to a Thermo Q Exactive HF™ mass spectrometer operating in heated electrospray ionization mode (HESI). Method duration was 30 min with a polarity switching data-dependent Top 5 method for both positive and negative modes. Spray voltage for both positive and negative modes was 3.5kV and capillary temperature was set to 320°C with a sheath gas rate of 35, aux gas of 10, and max spray current of 100 µA. The full MS scan for both polarities utilized 120,000 resolution with an AGC target of 3e6 and a maximum IT of 100 ms, and the scan range was from 67-1000 *m/z*. Tandem MS spectra for both positive and negative mode used a resolution of 15,000, AGC target of 1e5, maximum IT of 50 ms, isolation window of 0.4 m/z, isolation offset of 0.1 m/z, fixed first mass of 50 m/z, and 3-way multiplexed normalized collision energies (nCE) of 10, 35, 80. The minimum AGC target was 1e4 with an intensity threshold of 2e5. All data were acquired in profile mode. Internal standards were used for correction of retention time and identification of metabolites, followed by a quantitative assessment of reproducibility given by coefficient of variation (CV%). Tissues were extracted according to a fixed ratio of tissue mass (mg) to extraction solution (mL), but we *a priori* specified a compositional normalization (percent sum) due to the low tissue masses for HAR and normal control retinas. The sum of the metabolite intensities of a sample were calculated so that each sample had its own sum value, then used to normalize each metabolite intensity per sample. For L-isoleucine- and vehicle control-treated retinas, the corrected metabolite intensity was used for further analysis. Six to eight retinas were pooled as one replicate to reduce biological variability for metabolomics analysis in each group, n=6 per group.

Sample preparation for MS-based proteomics

Both retinas from each mouse were collected, pooled, and prepared for targeted MS-based proteomics as described previously.[2] Retinas were homogenized in Holt's lysis buffer and 75 µg protein lysate was used for analysis. LC-tandem MS was performed using selected reaction monitoring (SRM)[87] on a Thermo Scientific TSQ Vantage mass spectrometer equipped with an Eksigent splitless nanoflow HPLC system. 7 µL aliquots of each sample were injected onto a 10 cm x 75 µm i.d. capillary column packed with Phenomenex Jupiter C18 reversed phase beads. The column was eluted at 150 nL/min with a 60 min linear gradient of acetonitrile in 0.1% formic acid. The SRM assays were developed and validated to monitor two peptides per protein. Each peptide was monitored in a 6-min window centered on the known elution time of the peptide.[88–90] Approximately 30 protein assays are grouped into a panel of proteins that are measured in a single LC-tandem MS run.

Data processing of metabolomics data

Initial data analysis (metabolite identification & quantification) was performed by the NYU metabolomics facility.[30,91–93] Subsequent downstream bioinformatic analysis was performed using MetaboAnalyst R-based statistical and pathway analysis (v5.0).[94] Differences in metabolite levels between groups were assessed using unpaired *t* test and considered statistically significant if $P<0.05$. For pathway analysis SMPDB database was reviewed, applying Fisher's Exact Test for mapping. Only metabolites meeting *P*-value criteria were loaded for the analysis.

Data processing of proteomics data

Peptide quantification was performed using the program Skyline to determine the respective chromatographic peak areas.[95] The response for each protein was taken as the geometric mean of the two peptides monitored. Changes in the relative abundance of the proteins were determined by normalization to the bovine serum albumin internal standard. All confidently identified proteins were loaded into Qlucore omics explorer for visualization. One data in HAR group failed a 3 sigma statistical test and be considered an outlier. Two retinas from a single mouse were pooled as n=1, n=6-7 mice per experimental group (HAR vs. control).

Visualization of metabolomic and proteomic dataset

All data visualization including heatmaps, principal component analysis, and pathway analysis were performed using the online R-based platform of MetaboAnalyst (v5.0) and/or Qlucore omics explorer (v3.8).

Amino acid supplementation in mouse HAR

To examine the impact of amino acids on retinal vascular development, the most significantly decreased amino acids L-leucine, L-isoleucine and L-valine in HAR retinas were intraperitoneally administered into neonatal HAR mice. Intraperitoneal delivery in animals provides a relatively simple and efficient route to study parenteral nutrition in humans.[96] In HAR, mouse pups received L-leucine (1.5 μg/g, Sigma, L6914), or L-isoleucine (0.6 μg/g, Sigma, I5281), or L-valine (2.4 μg/g, Sigma, V4638) from P7 to P9. The dose was estimated based on circulating levels of these amino acids in mice: L-leucine (~200μM),[97] L-isoleucine (80-100μM),[97,98] and L-valine (200-400μM).[97,99,100] At P10, retinas were stained with isolectin GS-IB_4 (vessel marker, Invitrogen, I21413). Littermate mice were injected with vehicle control PBS. Both female and male pups were used. Body weight and blood glucose levels were recorded. Retinal vasculature (parameters: number of meshes, total vessel length per field) was evaluated using "*Angiogenesis Analyzer*" plugin in Image J as previously described.[2,25]

Patient population

The Mega Donna Mega randomized trial[37] included 207 infants born before 28 weeks of gestation at three study centers in Sweden. Recruitment was between December 2016 and August 2019. Infants were randomized to receive either arachidonic acid and docosahexaenoic acid (AA:DHA) enteral supplementation starting within three days after birth and lasting up to 40 weeks of postmenstrual age or conventional nutrition. Inclusion and exclusion criteria have been described.[37] The current study included all infants that had final ROP examination performed, n=178.

Nutrition strategy and glucose monitoring

The strategy to deliver enteral and parenteral nutrition has been described in detail.[37,101,102] Briefly, parenteral nutrition was commenced to all infants as soon as possible after birth, and gradually increased over 3-4 days in compliance with Swedish national guidelines to reach target intakes of lipids (3-4 g/kg/d), protein (3.5-4 g/kg/d), and carbohydrates (11-16 g/kg/d). The parenteral amino acid solutions used were Vaminolac (2g/100ml Fresenius Kabi, Uppsala, Sweden) or Primene (3.1g/100ml, Baxter Medical AB, Kista, Sweden), two solutions with overall similar amino acid compositions.[66] Minimal enteral feeds with human milk were introduced, when possible, the first day of life and gradually increased according to the infant's enteral feeding tolerance, replacing the parenteral nutrition, with a target volume of 160-180 ml/kg/d. Mother's own milk was the firsthand choice when available, otherwise pasteurized donor human milk was provided. All nutritional data were prospectively registered in the Nutrium Software (Nutrium AB, Umeå, Sweden). Mean intake of parenteral amino acids (g/kg) days 2-7, days 2-14, days 2-28 and total days on parenteral amino acid intake were studied.

ROP screening and classification

Weekly or biweekly retinal examinations for signs of ROP started at 5-6 weeks postnatal age (earliest at 31 weeks postmenstrual age) according to the national guidelines and lasted until the retina was fully vascularized. Classification of ROP according to disease stages followed the international consensus classification.[103] Type I ROP as defined by ETROP criteria constituted the study endpoint, ROP treatment.[104]

QUANTIFICATION AND STATISTICAL ANALYSIS

For animal studies, data was presented as Mean ± SEM. Normality (quantile-quantile (QQ) plot) and F-test (for variance) was first conducted, and parametric unpaired t-test (or Welch's t-test) and non-parametric Mann-Whitney test was used to compare the groups (Prism v9.0; GraphPad Software, Inc., San Diego, CA). $P<0.05$ was considered as statistically significant. For clinical studies, statistical analyses were performed using SAS software version 9.4 (SAS Institute Inc., Cary, NC, USA). For test between 2 groups with respect to dichotomous variables

Fisher's exact test was used, and for continuous variables Fisher's non-parametric permutation test. Multivariable logistic regression was applied for investigation of association between parenteral amino acid and ROP treatment. Adjustment was made for randomized treatment group, GA, BW and sex. Results were described by odds ratios (OR), 95% CI, p-values and c-statistics. A model with c-statistics 0.70-0.80 is considered as a good model, 0.80-0.90 excellent, and >0.90 an outstanding model. Receiver operating characteristic (ROC) curve analysis was performed for GA alone, parenteral amino acid number of days alone, GA + BW + sex, GA + BW + sex + treatment, and full model including GA + BW + sex + treatment + parenteral amino acid number of days. $P<0.05$ was considered as statistically significant. All tests were two-tailed.

ADDITIONAL RESOURCES

The Mega Donna Mega randomized trial[37] (ClinicalTrials.gov Identifier: NCT03201588). URL https://clinicaltrials.gov/study/NCT03201588?cond=NCT03201588&rank=1.