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Autophagy markers in umbilical cord blood-derived PBMCs and their association with preterm morbidities: a prospective multicenter cohort study

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ABSTRACT

Introduction Autophagy is a crucial cellular degradation pathway that maintains homeostasis during stress. Preterm infants are exposed to significant oxidative and metabolic stress during the perinatal transition. This study aimed to evaluate the levels of autophagy markers (Beclin-1, LC3B-I and LC3B-II) in the umbilical cord blood of preterm infants and investigate their association with neonatal morbidities and mortality.

Material and methods This prospective multicentre cohort study included 60 preterm infants. Umbilical cord blood samples were analysed for Beclin-1, LC3B-I and LC3B-II levels using Western blot. The LC3B-I/LC3B-II ratio was calculated as an indicator of autophagic flux. The relationship between these markers and major neonatal outcomes (respiratory distress syndrome (RDS), intraventricular haemorrhage (IVH), haemodynamically significant patent ductus arteriosus (hsPDA), early neonatal sepsis (ENS) and mortality) was evaluated using Mann-Whitney U tests and multivariable logistic regression adjusted for gestational age.

Results A significant positive correlation was observed between gestational age and Beclin-1 levels (r=0.406, p=0.005). In subgroup analyses, non-survivors exhibited significantly lower median Beclin-1 levels (0.45 vs 0.92, p=0.047) and a depressed LC3B-I/LC3B-II ratio (0.48 vs 1.90, p=0.018) compared with survivors. Similarly, infants with IVH had significantly lower LC3B-I levels (0.07 vs 0.65, p=0.011). However, in multivariable logistic regression analyses adjusted for gestational age, the independent statistical significance of these markers was attenuated, suggesting that the observed autophagic impairment is closely linked to gestational immaturity. No significant associations were found for RDS, hsPDA or ENS.

Discussion This study identifies lower cord blood Beclin-1 levels and an altered LC3B ratio as potential markers of vulnerability in preterm infants, particularly for mortality and IVH. The findings suggest that autophagic capacity at birth is developmentally regulated, with more immature infants displaying compromised autophagy initiation.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Effective clinical management of preterm infants requires reliable early biological indicators to predict risk and understand the cellular response to perinatal stress.

WHAT THIS STUDY ADDS

⇒ This study identifies that lower cord blood Beclin-1 levels and an altered LC3B ratio are significantly associated with a higher risk of mortality and intraventricular haemorrhage, reflecting a potential impairment in autophagic capacity at birth.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ These findings suggest that while autophagic maturity is closely linked to gestational age, these proteins could serve as valuable early-life biomarkers for identifying neonatal vulnerability and developing targeted therapeutic interventions.

INTRODUCTION

Autophagy is a highly conserved lysosomal degradation pathway that plays a fundamental role in cellular homeostasis by removing damaged organelles, misfolded proteins, and invading pathogens. This dynamic process is essential for cellular survival during stress conditions such as hypoxia, nutrient deprivation, and oxidative stress—common features in the perinatal transition and neonatal critical illness.[1–3] In the developing fetus and premature neonate, the capacity for autophagic regulation is particularly important, as rapid tissue remodeling, organ maturation, and metabolic shifts depend on the efficient clearance of cellular waste and recycling of biomolecules. Dysregulation of autophagy has been implicated in numerous perinatal

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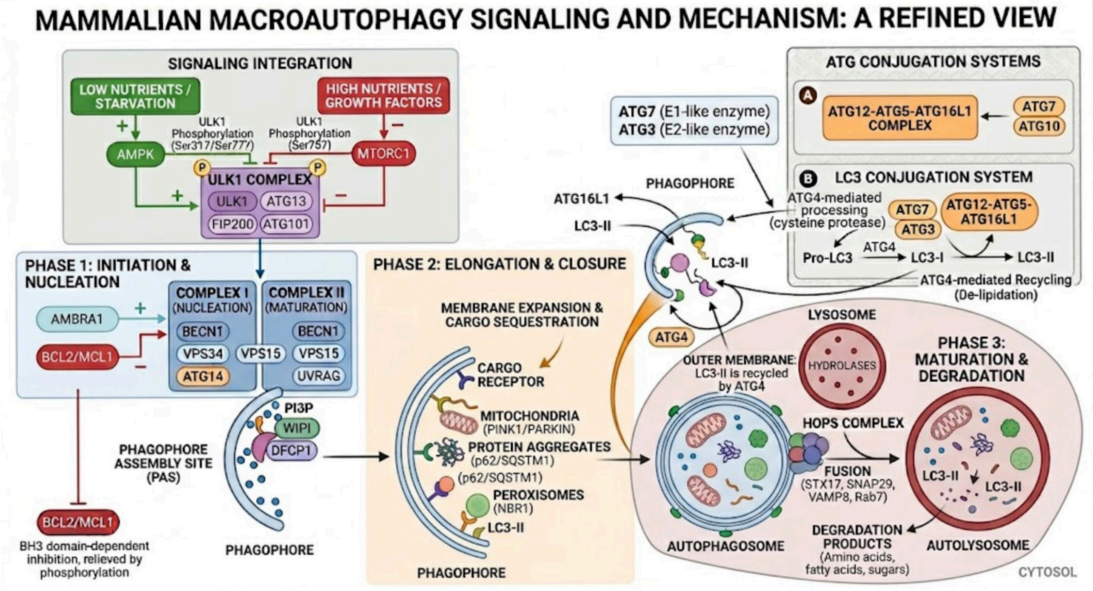

Figure 1: Mammalian macroautophagy signaling and execution.

This schematic provides a simplified and integrative overview of mammalian macroautophagy signaling and execution pathways. Several molecular interactions and regulatory mechanisms have been intentionally simplified for visual clarity. In particular, the Beclin-1-associated phosphatidylinositol 3-kinase class III (PI3KC3) complexes are represented in a reduced format, and additional modulatory proteins involved in autophagosome maturation and trafficking (such as RUBICON and other endolysosomal regulators) are not fully illustrated. Likewise, selective autophagy adaptors beyond p62/SQSTM1 and NBR1 are omitted for simplicity.

Autophagy initiation is primarily regulated through AMPK–mTORC1–ULK1 signaling. Under nutrient deprivation or cellular stress, AMPK activates the ULK1 complex, whereas mTORC1 suppresses autophagy during nutrient-rich conditions. Activated ULK1 promotes formation of Beclin-1-containing PI3KC3 complexes, leading to phagophore nucleation through PI3P production. Membrane elongation and cargo sequestration are mediated by ATG conjugation systems and LC3 lipidation. LC3-II is incorporated into both inner and outer autophagosomal membranes; outer membrane LC3-II may subsequently undergo ATG4-mediated recycling, while inner membrane-associated LC3-II is degraded following lysosomal fusion. Finally, autophagosome–lysosome fusion enables degradation of engulfed cargo and recycling of metabolic substrates.

(Abbreviations: AMPK, AMP-activated protein kinase; mTORC1, mechanistic target of rapamycin complex 1; ULK1, unc-51-like kinase 1; PI3KC3, class III phosphatidylinositol 3-kinase complex; BECN1, Beclin-1; VPS34, vacuolar protein sorting 34; VPS15, vacuolar protein sorting 15; ATG, autophagy-related protein; LC3, microtubule-associated protein 1 light chain 3; PI3P, phosphatidylinositol-3-phosphate; WIPI, WD repeat domain phosphoinositide-interacting protein; DFCP1, double FYVE-containing protein 1; UVRAG, UV radiation resistance-associated gene protein; HOPS, homotypic fusion and protein sorting complex; SNAP29, synaptosomal-associated protein 29; STX17, syntaxin-17; VAMP8, vesicle-associated membrane protein 8; p62/SQSTM1, sequestosome-1; NBR1, neighbor of BRCA1 gene 1; MCL1, myeloid cell leukemia 1; PAS, phagophore assembly site.)

pathologies, suggesting that alterations in autophagic activity may contribute to neonatal morbidity and mortality.[4 5]

Autophagy proceeds through a sequential cascade beginning with ULK1 and mTOR-mediated initiation, followed by membrane nucleation orchestrated by the Beclin-1-containing PI3KC3 complex.[6–9] Beclin-1 serves as a pivotal scaffold protein essential for phagophore formation. Subsequent elongation and closure of the autophagosome are marked by the conjugation of LC3B-I to phosphatidylethanolamine, forming LC3B-II, which localises to the autophagosomal membrane and facilitates cargo recognition. In the final stages, LC3B-II-positive autophagosomes fuse with lysosomes, enabling the degradation and recycling of cytoplasmic components.[8] These steps and molecular regulators are schematically represented in figure 1. Disruption at any step—particularly reduced Beclin-1 expression or impaired LC3B lipidation—can compromise autophagic flux, leading to defective cellular stress responses. In the neonatal context, this may contribute to increased vulnerability to inflammation, hypoxia and ultimately, mortality.[3 10–12]

Preterm neonates experience a profound shift in oxygenation, metabolic demands and immune exposure immediately after birth, yet their cellular defence systems remain functionally immature. Among these, autophagy represents a critical homeostatic process that mitigates oxidative stress, facilitates organ maturation and sustains energy balance during nutrient scarcity.[13] Insufficient or dysregulated autophagy in this population may impair mitochondrial quality control, disrupt intracellular signalling and exacerbate vulnerability to hypoxic or inflammatory injury.[14] Given the central roles of Beclin-1

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and LC3B in mediating autophagic flux, altered expression or activity of these markers in the neonatal period may reflect maladaptive responses that could influence short-term survival or long-term outcomes. Understanding the dynamics of autophagy in this context may yield insight into the cellular mechanisms underlying neonatal morbidity and mortality.

In clinical studies involving vulnerable populations such as preterm neonates, direct assessment of autophagy in target tissues (eg, lung or brain) is invasive and ethically restricted.[15] Therefore, we prioritised peripheral blood mononuclear cells (PBMCs) isolated from umbilical cord blood as a non-invasive surrogate to evaluate systemic autophagic status.[13] The rationale for this selection is twofold. First, preterm infants are exposed to high levels of oxidative stress and reactive oxygen species during the transition to extrauterine life, which are potent inducers of autophagy as a cytoprotective mechanism.[15] Second, PBMCs are central effectors of the systemic immune response. Autophagy in these cells plays a critical role in regulating inflammation, specifically by modulating the NLRP3 inflammasome and preventing excessive cytokine release.[12] Consequently, alterations in PBMC autophagy markers serve as a biological mirror reflecting the infant's systemic capacity to manage perinatal stress and inflammation.

MATERIAL AND METHODS

Study population

This prospective, multicenter observational cohort study was conducted across three Neonatal Intensive Care Units (NICUs) in Turkey between November 2021 and November 2022. To capture center-based variability in morbidity and mortality and increase the generalizability of the results, data were collected from tertiary-level perinatal units that shared similar delivery room protocols and antenatal care standards.

Preterm infants born at ≤32 weeks of gestation and admitted to the NICU within the first hours of life were eligible for inclusion. Exclusion criteria were: major congenital anomalies, lack of antenatal follow-up, referral to an external hospital or missing parental consent. Patients or the public were not involved in the design, or conduct, or reporting, or dissemination plans of our research.

Sample size estimation

Based on nationwide data from the Turkish Neonatal Society reporting a 22.8% mortality rate in infants <1500 g,[16] a priori power analysis was performed using G*Power V.3.1. Assuming a medium effect size (Cohen's d=0.5), α=0.05, and 80% power for a two-tailed comparison—consistent with conventional standards and recent cord-blood autophagy studies—the required sample size was 63 infants per group (126 total). Given the constraints of prospective recruitment in a limited preterm population, the final cohort (n=60) should be considered exploratory and not adequately powered for confirmatory inference.

Data collection and clinical manifestations

Maternal and neonatal demographic and clinical data of the infants included in the study were collected from electronic and written files. For the diagnosis and follow-up of respiratory distress syndrome (RDS), intraventricular haemorrhage (IVH), early and late neonatal sepsis, necrotising enterocolitis, haemodynamically significant patent ductus arteriosus (hsPDA), early neonatal sepsis (ENS), periventricular leukomalacia, bronchopulmonary dysplasia, retinopathy of prematurity, which were evaluated as significant morbidities, the recommendations in the Diagnosis and Treatment Guidelines of the Turkish Neonatology Society, which are also in accordance with international definitions, were used.[16–20]

All participating tertiary-level NICUs followed standardised clinical protocols based on the Turkish Neonatal Society Guidelines to ensure uniformity of care. Delivery room management, resuscitation and respiratory support for RDS were performed according to these shared national standards. Similarly, a uniform diagnostic approach for IVH was maintained through serial cranial ultrasound examinations performed by experienced specialists on postnatal days 3, 7 and prior to discharge. These consistent clinical and diagnostic standards across all study sites minimised centre-based variability in both management and outcomes.

Umbilical cord blood processing and autophagy protein assessment

Umbilical cord blood (2–4 mL) was collected immediately after delivery under sterile conditions, stored at 4°C, and processed within 24 hours. PBMCs were isolated using Ficoll-based density-gradient centrifugation, washed with PBS, subjected to red blood cell lysis and lysed in RIPA buffer supplemented with protease inhibitors. Lysates were stored at –80°C until further analysis.

Protein concentrations were measured using the BCA assay. Equal protein amounts were separated on 8%–15% SDS-PAGE gels, transferred to polyvinylidene fluoride membranes, and blocked with 5% BSA or non-fat dry milk. Membranes were incubated overnight at 4°C with primary antibodies against Beclin-1 and LC3B, followed by horseradish peroxidase-conjugated secondary antibodies. Bands were visualized using enhanced chemiluminescence and imaged with a Chemidoc system. Densitometric quantification was performed using ImageJ. To ensure accurate quantification and correct for potential variations in protein loading, all target protein signals were normalized to the corresponding beta-actin signal, which served as the internal loading control. The final data are presented as the ratio of the target protein's optical density to that of beta-actin for each sample (Figure 2).

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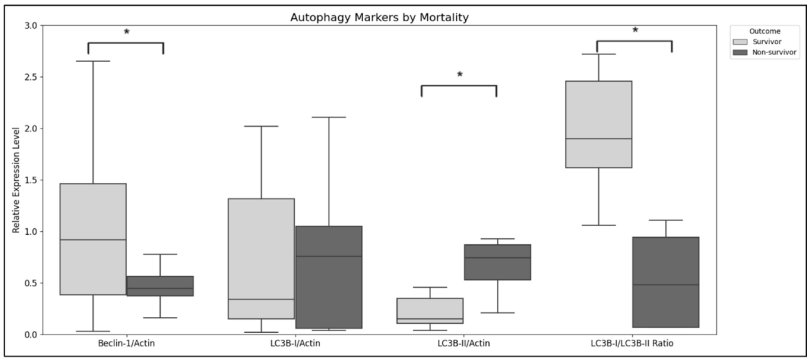

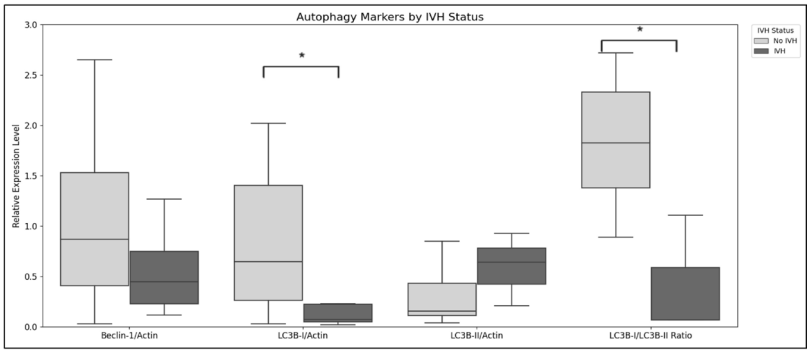

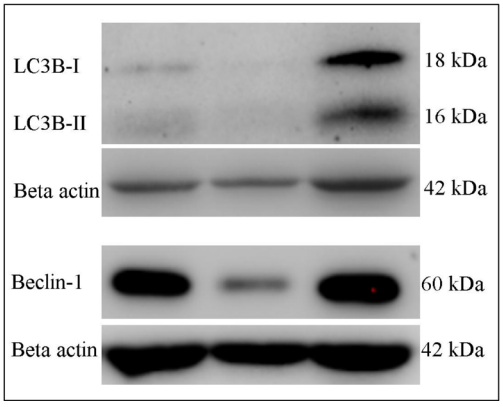

Figure 2: Autophagy marker expression levels in cord blood-derived peripheral blood mononuclear cells associated with mortality and intraventricular hemorrhage (IVH).

Box plots displaying the relative densitometric levels of Beclin-1, LC3B-I, LC3B-II, and the LC3B-I/LC3B-II ratio, stratified by mortality (survivor vs. non-survivor) and intraventricular hemorrhage status (IVH vs. no IVH). These groups are presented specifically due to the statistically significant differences observed in their marker profiles. The central line represents the median, box boundaries indicate the interquartile range (IQR), and whiskers extend to the minimum and maximum values. Significance: Asterisks (*) denote a statistically significant difference ($p < 0.05$) between the two groups, as determined by the Mann-Whitney U test. Representative Western blot images requested for validation, showing the protein bands for Beclin-1, LC3B-I, and LC3B-II in the corresponding groups. Beta-actin was used as the loading control for normalization. The visual band intensities corroborate the quantitative findings shown in the box plots. Hemorrhage status (IVH vs. no IVH). These groups are presented specifically due to the statistically significant differences observed in their marker profiles. The central line represents the median, box boundaries indicate the interquartile range (IQR), and whiskers extend to the minimum and maximum values. Significance: Asterisks (*) denote a statistically significant difference ($p < 0.05$) between the two groups, as determined by the Mann-Whitney U test. Representative Western blot images requested for validation, showing the protein bands for Beclin-1, LC3B-I, and LC3B-II in the corresponding groups. Beta-actin was used as the loading control for normalization. The visual band intensities corroborate the quantitative findings shown in the box plots.

Statistical analysis

All statistical analyses were conducted using IBM SPSS Statistics V.24 (IBM, Armonk, New York, USA). The distribution of continuous variables was evaluated using the Shapiro–Wilk test and visual inspection of histograms and Q–Q plots. Continuous variables are presented as mean±SD or median (IQR), depending on their distribution. Categorical variables are summarised as frequencies and percentages.

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Comparisons between infants with and without mortality or morbidity were performed using the Mann-Whitney U test for non-normally distributed variables. Categorical variables were compared using the χ^2 test or Fisher's exact test, as appropriate. Morbidities with very low incidence were excluded from statistical analysis.

Spearman's rank correlation coefficient was used to evaluate associations between autophagy-related protein levels and clinical parameters. A two-tailed p value <0.05 was considered statistically significant for all analyses.

Univariate and multivariate logistic regression analyses were conducted to determine independent predictors of clinical outcomes. Adjusted ORs with 95% CIs were reported, and p<0.05 was considered statistically significant.

RESULTS

Clinical characteristics

Cord blood samples were collected from 65 preterm infants born at ≤32 weeks of gestation. However, due to technical limitations, biomarker analysis could only be successfully performed in 60 cases. Therefore, all subsequent analyses in the study were conducted based on these 60 infants. The median gestational age was 30.0 weeks (24–32), and the median birth weight was 1460 g (505–2180). The mean maternal age was 30.4±6.4 years. Female infants accounted for 51.7% of the population. Antenatal steroid administration was documented in 80% of cases, and 11.7% were born following preterm premature rupture of membranes. Maternal comorbidities included gestational hypertensive disorders (21.7%), gestational diabetes (21.7%) and hypothyroidism (3.3%). Detailed clinical characteristics of the study population are presented in table 1.

When infants were stratified based on survival status, the median gestational age was 27.0 weeks in the mortality group and 30.0 weeks in the survival group (p=0.002). Median birth weight was 875 g among non-survivors and 1550 g among survivors (p<0.001). The proportion of female infants did not differ significantly between groups (p=0.747).

Table 1: Baseline demographic, perinatal and clinical characteristics of the study population

	Variable	Value
Perinatal and natal characteristics	Maternal age	30.4±6.4
	Maternal disease	
	► Gestational hypertensive disorders, n (%)	13 (21.7)
	► Gestational diabetes, n (%)	13 (21.7)
	► · Hypothyroidism	2 (3.3)
	Gestation week	30.0 (24–32)
	Birth weight (g)	1460 (505–2180)
	Gender (Female), n (%)	24 (51.7)
	Antenatal steroid, n (%)	48 (80)
	PPROM, n (%)	7 (11.7)
	Apgar score - first minute	6 (1–9)
	Apgar score - fifth minute	8 (1–10)
Neonatal outcomes	RDS, n (%)	34 (56.7)
	hsPDA, n (%)	14 (23.3)
	IVH (any grade), n (%)	12 (20)
	Early neonatal sepsis, n (%)	14 (23.3)
	Late neonatal sepsis, n (%)	9 (15)
	NEC (≥ Stage 2), n (%)	4 (6.7)
	BPD (mild and severe), n (%)	5 (9.7)
	ROP (requiring treatment), n (%)	2 (3.3)
	Mortality, n (%)	12 (20)

Continuous variables were assessed for normality using the Shapiro–Wilk test. Variables found to follow a normal distribution are presented as mean±SD, while those not normally distributed are expressed as median (minimum–maximum). Categorical variables are summarised as numbers and percentages (n, %). BPD, bronchopulmonary dysplasia; hsPDA, hemodynamically significant patent ductus arteriosus; IVH, intraventricular hemorrhage;NEC, necrotizing enterocolitis; PROM, premature rupture of membranes; RDS, respiratory distress syndrome; ROP, retinopathy of prematurity.

Autophagy markers

Umbilical cord blood samples obtained from 60 preterm infants were analysed for the relative expression levels of Beclin-1, LC3B-I, LC3B-II and the LC3B-I/LC3B-II ratio, normalised to beta-actin. The relationship between these markers and five major neonatal morbidities, including mortality, RDS, IVH, hsPDA and ENS, was evaluated (table 2).

Demographic analysis revealed that non-survivors and infants who developed IVH had significantly lower gestational ages and birth weights compared with the control groups (p<0.01). However, no statistically significant differences were observed regarding gender distribution or antenatal steroid administration rates between the groups.

In the subgroup analysis based on mortality, significant differences in autophagy markers were identified, as illustrated in figure 2. Specifically, Beclin-1/beta-actin levels were significantly lower in the non-survivor group (median 0.45) compared with survivors (median 0.92; p = 0.048). Conversely, LC3B-II/beta-actin levels were

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Table 2 Comparison of umbilical cord blood autophagy marker levels according to neonatal morbidities and mortality in preterm infants

Condition	Marker	With condition	Without condition	P value
Mortality	Beclin-1/beta-actin	0.45 (0.16–0.78)	0.92 (0.03–5.72)	**0.048***
	LC3B-I/beta-actin	0.76 (0.04–2.11)	0.34 (0.02–5.08)	0.886
	LC3B-II/beta-actin	0.74 (0.21–0.93)	0.15 (0.04–0.84)	**0.034***
	LC3B-I/LC3B-II Ratio	0.48 (0.07–1.11)	1.90 (1.06–16.23)	**0.012***
IVH	Beclin-1/beta-actin	0.45 (0.12–1.27)	0.87 (0.03–5.72)	0.055
	LC3B-I/beta-actin	0.07 (0.02–2.11)	0.65 (0.03–5.08)	**0.009***
	LC3B-II/beta-actin	0.64 (0.21–0.93)	0.15 (0.04–0.85)	0.161
	LC3B-I/LC3B-II Ratio	0.07 (0.07–1.11)	1.82 (0.89–16.23)	**0.048***
RDS	Beclin-1/beta-actin	0.58 (0.03–5.72)	0.98 (0.25–2.47)	0.192
	LC3B-I/beta-actin	0.30 (0.02–2.11)	0.84 (0.03–5.08)	0.214
	LC3B-II/beta-actin	0.16 (0.05–0.93)	0.41 (0.04–0.85)	0.731
	LC3B-I/LC3B-II Ratio	1.90 (0.07–16.23)	1.27 (0.89–1.75)	0.527
hsPDA	Beclin-1/beta-actin	0.45 (0.04–3.74)	0.92 (0.03–5.72)	0.078
	LC3B-I/beta-actin	0.18 (0.02–1.38)	0.38 (0.03–5.08)	0.068
	LC3B-II/beta-actin	0.40 (0.05–0.93)	0.26 (0.04–0.85)	0.808
	LC3B-I/LC3B-II Ratio	0.98 (0.07–16.23)	1.62 (0.89–2.72)	0.762
ENS	Beclin-1/beta-actin	0.66 (0.12–5.72)	0.90 (0.03–3.74)	0.267
	LC3B-I/beta-actin	1.23 (0.04–4.52)	0.30 (0.02–5.08)	0.058
	LC3B-II/beta-actin	0.50 (0.15–0.85)	0.26 (0.04–0.93)	0.606
	LC3B-I/LC3B-II Ratio	1.40 (0.89–1.90)	1.62 (0.07–16.23)	0.889

Data are presented as medians (min-max). P values were calculated using the Mann-Whitney U test. An asterisk indicates a statistically significant difference ($p < 0.05$) between groups.
ENS, early neonatal sepsis; hsPDA, hemodynamically significant patent ductus arteriosus; IVH, intraventricular hemorrhage;RDS, respiratory distress syndrome.

significantly higher in non-survivors (0.74) compared with the survivor group (0.15; p=0.034). Consequently, the LC3B-I/LC3B-II ratio was significantly depressed in non-survivors (0.48) compared with survivors (1.90; p=0.012). No significant difference was observed in LC3B-I/beta-actin levels (p=0.886).

Regarding IVH, the distribution of markers is also presented in figure 2. Significant alterations were observed in LC3B-I levels and the conversion ratio. LC3B-I/beta-actin levels were significantly reduced in infants with IVH (median 0.07) compared with those without (median 0.65; p=0.009). Similarly, the LC3B-I/LC3B-II ratio was significantly lower in the IVH group (0.07) compared with the non-IVH group (1.82; p=0.048). Beclin-1/beta-actin levels showed borderline significance (p=0.055), while LC3B-II/beta-actin levels did not differ significantly (p=0.161).

For the other investigated morbidities, no statistically significant differences were detected in any of the autophagy markers (Table 2).

Multivariable analysis with minimal adjustment

Logistic regression analyses were performed to evaluate the associations between autophagy markers and neonatal outcomes. To minimise the influence of potential confounders, three distinct multivariable adjustment models were implemented: Model 1 (adjusted for gestational age), Model 2 (adjusted for gestational age, sex and antenatal steroid use) and Model 3 (adjusted for birth weight). Detailed stratification analyses for all five morbidity groups (RDS, IVH, hsPDA, ENS and mortality), including comprehensive demographic characteristics, biomarker distributions and adjusted p values, are provided in the online supplemental tables S1–S5).

In the unadjusted analysis, Beclin-1, LC3B-II, and the LC3B-I/LC3B-II ratio were significantly associated with mortality. However, after adjusting for gestational age, the independent statistical significance of these markers was attenuated (Beclin-1: aOR=0.22, p=0.171; LC3B-II: aOR=120.7, p=0.33). Similarly, for IVH, the significant associations observed with LC3B-I and the LC3B-I/LC3B-II ratio in univariate analysis did not retain statistical significance after adjustment for gestational age (LC3B-I: aOR=0.19, p=0.103; Ratio: aOR=0.03, p=0.251) (Table 3).

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Table 3 Multivariable logistic regression analysis of selected autophagy markers associated with mortality and intraventricular haemorrhage

Outcome	Biomarker	Unadjusted OR (95% CI)	Unadjusted P-value	Adjusted OR (95% CI)*	Adjusted P-value
Mortality	Beclin-1/beta-actin	0.15 (0.02 to 1.20)	0.075	0.22 (0.03 to 1.92)	0.171
	LC3B-II/beta-actin	92.49 (0.78 to 10,987.4)	0.063	120.76 (0.01 to 1000)	0.330
	LC3B-I/LC3B-II Ratio	<0.01 (0.00 to 1000)	0.378	<0.01 (0.00 to 1000)	0.999
IVH	LC3B-I/beta-actin	0.31 (0.06 to 1.68)	0.173	0.19 (0.03 to 1.39)	0.103
	LC3B-I/LC3B-II Ratio	0.01 (0.00 to 6.88)	0.174	0.03 (0.00 to 12.22)	0.251

*The adjusted model includes the biomarker and gestational age as covariates. Statistical significance was set at $p < 0.05$. Wide CIs observed in some markers are attributed to the limited sample size and low event frequency.
IVH, intraventricular hemorrhage.

Correlation analysis

To investigate whether autophagy activity in umbilical cord blood is influenced by gestational maturity, Spearman's rank correlation analyses were performed between gestational age and the relative expression levels of key autophagy markers. The analysis revealed a significant positive correlation between gestational age and Beclin-1 levels (r=0.406, p=0.005), indicating that the capacity to initiate autophagy tends to increase with gestational maturity.

In contrast, no statistically significant correlations were observed for LC3B-I (r=−0.004, p=0.981) or LC3B-II (r=−0.425, p=0.148). Although LC3B-II levels exhibited a trend towards a negative correlation, this did not reach statistical significance in the current cohort. Similarly, the LC3B-I/LC3B-II ratio did not show a significant correlation with gestational age (r=0.543, p=0.084).

DISCUSSION

The present study demonstrates that autophagy markers in PBMCs derived from umbilical cord blood are significantly altered in preterm infants who experience mortality or develop IVH. Our findings highlight that lower levels of Beclin-1 and a depressed LC3B-I/LC3B-II ratio at birth are associated with increased vulnerability to severe neonatal outcomes. Interestingly, while these markers showed strong associations in univariate analyses, their significance was attenuated after adjustment for gestational age. This suggests that the autophagic profile at birth is closely linked to fetal maturity, where the degree of prematurity serves as a primary driver of both impaired autophagy and adverse clinical outcomes. To our knowledge, this is the first study to systematically evaluate these specific markers in cord blood in relation to a broad spectrum of neonatal morbidities.

The significant reduction of Beclin-1 levels in infants who did not survive suggests an impairment in the initiation phase of autophagy. Beclin-1 is a critical scaffold protein required for the formation of the PI3K complex, which triggers the autophagic process.[7] In the context of neonatal mortality, a deficiency in Beclin-1 may reflect an inadequate cellular response to the transition from the intrauterine to the extrauterine environment, particularly in the most immature infants. Furthermore, the observed increase in LC3B-II levels alongside lower Beclin-1 in the mortality group underscores a complex dysregulation. While the initiation might be downregulated, the accumulation of LC3B-II—the membrane-bound form of LC3B—often indicates either an upregulation of autophagosome maturation or a blockade in autophagic flux.[8 9]

The "opposite behaviour" of LC3B-I and LC3B-II observed in our study, particularly the significant decrease in the LC3B-I/LC3B-II ratio in both mortality and IVH cases, provides important mechanistic insights. LC3B-I is converted to LC3B-II through lipidation to form the autophagosomal membrane.[1] A lower ratio typically signifies an accelerated conversion of LC3B-I to LC3B-II, suggesting that the machinery for autophagosome maturation remains active or is even compensatory under high stress, even if early initiation (Beclin-1) is compromised. This shift may represent a cellular adaptation to stress where autophagic flux is pushed to its limits to maintain cellular homeostasis. However, in cases of severe IVH or mortality, this compensatory shift appears insufficient to prevent cellular injury or death, reflecting a state of "exhausted" or "impaired" autophagy. Such impairment in endothelial or glial cells has been previously shown to enhance capillary fragility and oxidative injury, which are key mechanisms in the pathogenesis of IVH.[14]

Regarding other neonatal morbidities, such as RDS, hsPDA and ENS, no statistically significant differences were found in cord blood autophagy levels. This was unexpected, given that autophagy plays a vital role in surfactant homeostasis[21–23] and the regulation of inflammation.[12 24] The lack of significance may be attributed to the timing of the sample collection; cord blood reflects the intrauterine environment and the immediate perinatal transition, whereas conditions like ENS or hsPDA often manifest or intensify in the days following birth.

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Therefore, while cord blood autophagy levels may serve as early indicators for immediate risks like mortality and early-onset IVH, they may be less predictive of morbidities that evolve later in the neonatal period.

A notable finding in our updated analysis was the positive correlation between Beclin-1 levels and gestational age. This indicates that as a fetus matures, the capacity to initiate autophagy increases, potentially providing better protection against perinatal stress. This finding contrasts with a recent study by Künstle *et al*, which reported no gestational age-dependent differences in autophagy markers between preterm and term infants.[25] However, our data suggest that within the preterm population itself, "autophagic maturity" is a fundamental component of physiological resilience. When we adjusted our analysis for gestational age, the independent predictive power of the markers decreased, confirming that the protective effect of autophagy is intrinsically tied to developmental maturity.

CONCLUSION

In conclusion, our study identifies cord blood Beclin-1 and the LC3B-I/LC3B-II ratio as potential markers of neonatal vulnerability in preterm infants. The significant association of these markers with mortality and IVH suggests that a diminished autophagic capacity at birth—linked to gestational immaturity—is a key factor in the pathogenesis of severe preterm complications. The observed shift in LC3B maturation indicates an active but potentially failing compensatory response to stress. Future research involving larger populations and longitudinal sampling is warranted to determine if these markers can be used to identify high-risk infants and to explore therapeutic strategies aimed at modulating autophagy to improve preterm outcomes.

Strengths and limitations of the study

The primary strength of this study is its prospective, multicentre design and its focus on cord blood, which offers a non-invasive "snapshot" of the neonate's autophagic status at the moment of birth. However, several limitations must be acknowledged. The study is exploratory in nature, and the relatively small sample size of 60 subjects limits the statistical power of multivariable adjustments. As a result, the wide CIs observed in some logistic regression models suggest that these findings should be interpreted with caution and require validation in larger cohorts. Additionally, cord blood analysis provides information only at a single time point; serial measurements of autophagy markers during the first week of life would provide a more dynamic view of how these infants adapt to postnatal stressors. Finally, due to the limited volume of preterm cord blood samples, we were unable to measure p62 (SQSTM1) levels, which would have allowed for a more definitive distinction between increased autophagic induction and blocked flux.

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