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Original Article

Relationship between placental autophagy and inflammasome activities with morbidity of extremely preterm infants

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ABSTRACT

Background. The placenta is the major regulatory element of the in-utero environment, and alterations in placental cellular functions in infection, inflammation, and hypoxemia lead to adverse preterm birth outcomes. The importance of regulation of autophagy and inflammasome activities has been shown in the pathogenesis of morbidities in immature animal models. This study aimed to determine the relationship between placental autophagy and inflammasome activities with morbidity in extremely preterm infants.

Methods. Premature infants born between 24th to 29th gestational weeks were evaluated prospectively. Placental LC3B and NLRP3 immunostainings were performed to assess autophagy and inflammasome activities. Preterm morbidities including respiratory distress syndrome (RDS), patent ductus ateriosus: (PDA), intraventricular hemorrhage (IVH), necrotizing enterocolitis (NEC), periventricular leukomalacia (PVL), sepsis, bronchopulmonary dysplasia (BPD), retinopathy of prematurity (ROP) and mortality were evaluated.

Results. Fifty-nine infants with a mean gestational age of 26.9 ± 1.5 weeks were included. Anti-LC3B staining scores were moderate or intense positive in 75% of the placentas. Anti-LC3B activity was not associated with the existance of evaluated neonatal morbidities or mortality. Autophagy and inflammasome coexistence were demonstrated in 35 placentas (59.3%). Anti-NLRP3 staining score was moderate or intensely positive in 75% of the placentas. Infants with BPD had a lower rate of positive anti -NLRP3 staining than infants without BPD (42.9 vs 57.1%, p=0.048). Infants who had hemodynamic significant patent ductus arteriosus (hsPDA) and surgical-NEC showed significantly intense anti-NLRP3 staining compared to infants who did not (18.8% vs 0%, p= 0.027 and 33% vs 7.5%, p=0.048 respectively).

Conclusions. The results showed that autophagy and inflammatory activities were present in varying amounts in the placenta of preterm infants. Association of decreased or increased rates of inflammasome activities with certain diseases such as BPD, hsPDA and surgical-NEC indicates the role of the intrauterin inflammatory process and the importance of critical balance in inflammation. Because of the complex pathophysiology of preterm morbidities, placental autophagy and inflammasome activities seem worthy of further investigation.

Key words: premature birth, morbidity, placenta, autophagy, inflammasome.

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Very low birth weight (VLBW) infants' survival rate has been increasing over the last few decades, but morbidities associated with prematurity have been seen in almost half of the survivors. In-utero exposures to conditions like inflammation and maternal medical diseases (hypertension, diabetes, preeclampsia) predispose an adverse outcome

for preterm infants during neonatal intensive care unit (NICU) stay and later in childhood. The placenta provides all vital supplements for fetal development during pregnancy, and plays a critical role in maintaining a healthy in-utero environment.[1]

During pregnancy, the development of the placenta is interrelated with the oxygen concentration and autophagy acts as a crucial process in the placenta which is frequently affected by oxidative stress.[2] Autophagy is an intracellular lysosomal degradation process that contributes to basal cellular and tissue homeostasis, as well as developmental regulation in higher organisms. Autophagy primarily acts as a protective mechanism that may prevent cell death.[3] However, an imbalance between the protective and destructive mechanisms of autophagy appears to be linked with pregnancy-related disorders such as preeclampsia and fetal growth restriction, fetal hypoxia and inflammation.[2,4,5]

Oxidative stress and inflammation play roles as leading mechanisms that cause preterm delivery and play a crucial role in the pathogenesis of preterm morbidities. Recently, the critical effects of autophagy on inflammation induction have started to be understood. Inflammasomes are intracellular multi-protein complexes that act as sensor molecules that initiate the inflammatory pathway. Recently, a critical crosstalk between autophagy and inflammasome induction has been indicated.[6] Placental inflammasome activity present both in microbial and sterile inflammation, is associated with spontaneous preterm delivery.[7] Increased placental inflammasome activity is also reported in pregnancies complicated with preeclampsia and diabetes.[8]

Placental histological abnormalities were previously described as a risk factor in preterm infants with adverse outcomes. In this study, we hypothesized that intrauterine autophagy and inflammasome activities, and their balance, play a function in the healthy development of infants. Therefore, examination of placental autophagy and inflammasome activities could offer early signals for the development of preterm morbidities. The main objectives of this study were to i. investigate the placental autophagy and inflammasome activities in preterm infants by characterizing the expression levels of LC3B and NLRP3, and ii. explore the relationship between autophagy and inflammasome activities with preterm morbidities.

Material and Methods

Study Population and Clinical Definitions

This prospective cohort study was conducted at Dokuz Eylül University Hospital between June 2017 to August 2018. All infants born between the 24th to 29th gestational weeks were evaluated for eligibility. Exclusion criteria were major congenital anomaly, parental refusal and transfer to another hospital.

The study protocol was approved by Dokuz Eylul University Faculty of Medicine, Local Ethical Committee on Human Research (No:2017/25-02). Written informed parental consent for experimentation with human subjects was obtained.

Maternal and neonatal demographic and clinical data were collected from written and electronic folders. Data included i) neonatal demographics: gestational age determined as date of last menstrual period and/or first trimester ultrasonography, birthweight, gender, Apgar score at 5 minutes, mode of delivery, full course of antenatal corticosteroid administration[9], intrauterin growth status[10], multiple pregnancy, in-vitro fertilization (IVF) pregnancy; ii) maternal demographics: maternal age, preterm prelabour rupture of membrane (PPROM) and duration, chorioamnionitis, gestational hypertensive disorder, gestational diabetes, chronic conditions needed medical supervision; iii) neonatal morbidities: respiratory distress syndrome (RDS)[11], clinical and culture proven early onset neonatal sepsis

(EONS), hemodynamic significant patent ductus arteriosus (hsPDA) requiring treatment[12], intraventricular hemorrhage (IVH) > Grade II according to Papile staging[13], periventricular leukomalacia (PVL)[14], culture proven late-onset neonatal sepsis (LONS), retinopathy of prematurity (ROP) needed treatment[15], necrotizing enterocolitis (NEC) stage 2-3 according to Bell criteria[16], moderate and severe BPD at 36 weeks' postmenstrual age according to NICHD definition[17] and iv) neonatal mortality during hospital stay. Compound outcome was defined as any morbidity related to oxidative stress (IVH > grade 2, hsPDA, surgical NEC, cystic PVL, moderete-severe BPD and treatment required ROP), and/or mortality.

Placental Examination and Immunohistochemically Assessment

Placental histopathological evaluations were performed by two pathologists who were blinded to the neonatal clinical data. Placental tissue samples were fixed in 10% buffered formalin for 24 hours and embedded in paraffin. Tissue sections (4 μm) were cut from the maternal side, fetal side and umbilical cord, and then mounted on poly-L-lysine coated slides. Hematoxylin and eosin (HE) stained slides were examined under a light microscope (Olympus BX51; Olympus Corp. Tokyo Japan) at 100× magnification for histopathological evaluation. Placental histopathological findings were classified according to Amsterdam Placental Workshop Group Consensus Statement.[18] Villous maldevelopment such as infarcts, villous dysmaturity, accelerated villous maturation and vascular intramural fibrin deposition were classified as maternal/fetal malperfusion. Grading and staging of inflammation were defined as acute/chronic chorioamnionitis (or chorionitis) with/without a fetal inflammatory response.[18]

Immunohistochemical staining was performed for autophagy activity using LC3B antibody (rabbit monoclonal, Cell Cignaling, Lausen, Switzerland) at a dilution of 1:50 for 1 hour[19], and for inflammasome activity marker with NLRP3 antibody (rabbit polyclonal, Novus Biologicals, Littleton, CA, USA) at a dilution 1:200 for 1 hour.[20]

Placental tissues from four different sites for each patient were obtained for protein expression evaluation. Two pathologists scored LC3B and NLRP3 staining for all placental sites, and median values were used for the final score. Cytoplasmic NLRP3 staining in trophoblastic cells was considered positive and graded as negative, weak positive and strongly positive, due to the intensity of staining semi-quantitatively. Nuclear LC3B staining in trophoblastic cells was considered positive and scored due to the extensity of staining as 0: negative or positive in less than 10% of the cells, 1: positive in 10-50% of the cells and 2: positive in more than 50% of the cells.[21]

Statistics

SPSS version 22.0 (IBM SPSS Statistics, Chicago, IL, USA) was used in the study. Continuous data were expressed as mean ± standard deviation (SD) or median (interquartile range) according to the normal distribution pattern of data using the Shapiro Wilk test and analyzed with Student t or Mann Whitney U tests according to the distribution of data. Categorical unrelated data were analyzed using the chi-square or Fisher's exact tests as appropriate, and related data were analyzed using McNemar test. All reported p values were two-sided with a significance level of 0.05.

Results

Clinical characteristics

Among 62 eligible infants during the study period, 59 infants were included in the study. Two infants were transferred to other clinics and one infant was excluded due to major congenital abnormalities. The mean gestational age and birth weight of the infants were 26.9 ±1.5 weeks and 958±270 grams. Twenty-one of the infants (35.6%) were between 24-26 weeks of gestational age and the remaining were between

27-29 weeks of gestational age. Demographic and clinical characteristics of neonatal and maternal data are listed in Table I.

Placental Histopathological Findings

Fifty-nine placentas were examined, of which 32 (54%) had vascular malperfusion, 8 (13%) had inflammation (chorioamnionitis), 17 (29%) had vascular malperfusion plus inflammation, 1 (2%) had placenta previa and 1 (2%) was normal (Fig. 1. A, B).

Table I. Neonatal and maternal characteristics.

Characteristics	n=59
Gestational age (weeks) *	26.9 ± 1.5
Birth weight (grams) *	958 ± 270
Apgar score at 5min **	8 (7-8)
C/S delivery, n (%)	50 (87.7%)
Male gender, n (%)	25 (42.4%)
SGA, n (%)	6 (10.2%)
Multiple births, n (%)	16 (27.1%)
IVF pregnancy, n (%)	14 (23.7%)
Antenatal steroid, n (%)	32 (54.2%)
RDS, n (%)	50 (87.7%)
Surfactant dose **	1 (1-2)
hsPDA, n (%)	32 (54.2%)
IVH (> Grade 2), n (%)	2 (3.4%)
PVL, n (%)	6 (10.8%)
Early-onset sepsis, n (%)	15 (25.4%)
Late-onset sepsis, n (%)	19 (32.2%)
NEC (≥ Stage 2), n (%)	5 (8.4%)
BPD at 36w, n (%)	7 (11.8%)
Postnatal steroid treatment, n (%)	18 (30.5%)
ROP requiring treatment, n (%)	6 (10.2%)
The duration of NICU stay, days *	65 ± 24
Gestational age at discharge, weeks *	36 ± 2
Mortality, n (%)	3 (5.0 %)
Maternal age, (years) **	29 ± 6
PPROM, n (%)	22 (37.3%)
PPROM duration, (h) **	39 (26-120)
Histologic chorioamnionitis, n (%)	25 (42.3%)
Gestational hypertensive disorders, n (%)	19 (32.2%)
Gestational diabetes, n (%)	3 (5.0%)
Maternal obesity (BMI>30 kg/m^2), n(%)	7 (11.8%)
Maternal chronic disease, n (%)	4 (6.7%)

*Values are presented as mean±standard deviation
**Values are presented as median, an IQR (inter quartile range) 25-75 are given in parenthesis

Anti-LC3B and Anti-NLRP3 Immunohistochemical Staining

Overall, anti-LC3B and/or anti-NLRP3 immunohistochemical staining were positive in 53 patients (Fig. 2A). The intersection between the inflammasome and autophagy activities was shown through a Venn diagram in Figure 2B.

Anti-LC3B staining was scored as 0 in 15 placentas (25%), 1 in 25 placentas (43%) and 2 in 19 placentas (32%). Anti-NLRP3 staining was negative in 15 placentas (25%), weakly positive in 38 placentas (64%) and strongly positive in 6 placentas (11%) (Fig. 2. A, B, C).

Relationship Between Placental LC3B And NLRP3 Expressions With Preterm Morbidities And Mortality

Overall, anti-LC3B staining score was 1 or 2 positive in 75% of the placentas. Maternal illness, antenatal steroid administration, type of delivery, intrauterine growth status, and presence of histological chorioamnionitis did not significantly affect the staining pattern. When the relationship between morbidities and anti-LC3B activity was evaluated for each morbidity or mortality separately, anti-LC3B positivity rate was not associated with the existence of neonatal morbidities including RDS, hsPDA, IVH, PVL, sepsis, NEC, BPD, ROP or mortality. Anti- LC3B staining rate was 72.2% in the group of preterm infants who had compound outcomes and 75.6% for the infants who did not (p=0.783). Considering the intensity of autophagy, no significant relationship was found between the intensity of autophagy and evaluated morbidities.

Overall, anti-NLRP3 staining score was moderate or intensely positive in 75% of the placentas. Maternal illness, antenatal steroid administration, type of delivery, intrauterine growth status, and presence of histological chorioamnionitis did not significantly affect the results. Infants with BPD had a lower rate of positive anti-NLRP3 staining, than infants

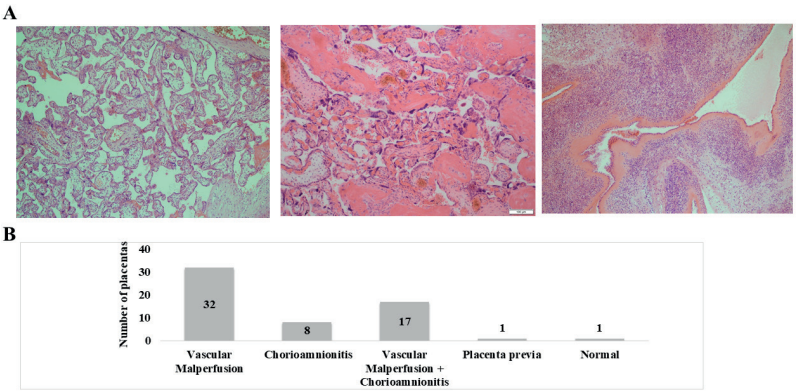

Fig. 1. A. Placental microscopic images (1x10 HE) representing a. normal placenta, b. vascular malperfusion and c. chorioamnionitis; B. Distribution of placental histological findings.

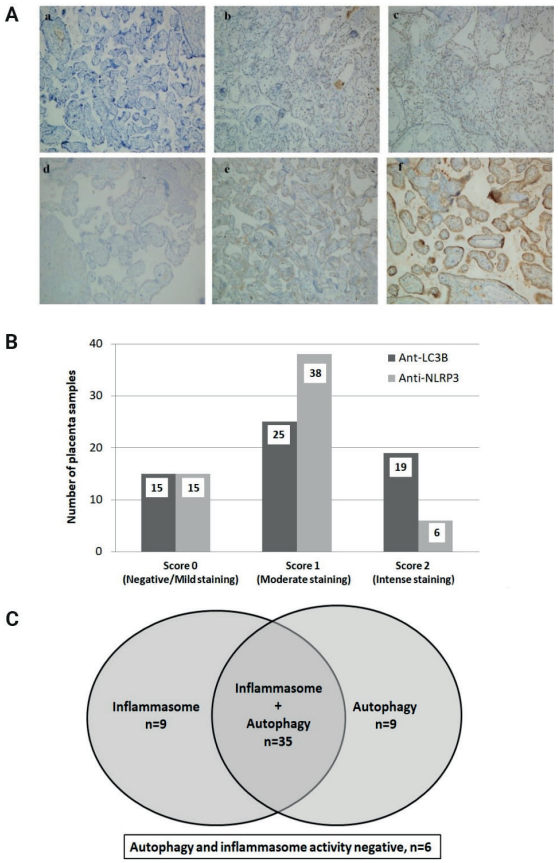

Fig. 2. Evaluation of Placental Anti-LC3B and Anti-NLRP3 activities: A. Evaluation of staining of placental anti-LC3B and anti-NLRP3 immunostainings (1x20): a. Anti-LC3B score 0 b. Anti-LC3B score 1 c. Anti-Anti-LC3B score 2 d. Anti-NLRP3 negative e. Anti-NLRP3 weak positive f. Anti- NLRP3 strong positive, B. Distribution of Anti-LC3B, Anti-NLRP3 scores across placentas C. Venn diagram demonstrating the overlapping between inflammasome and autophagy activities.

without BPD (42.9 vs 57.1%, p=0.048) (Table II). Infants who had compound outcome, positive anti-NLRP3 staining rate were 66.7% and the rate was 76.9 % for infants who did not (p=0.355). When an intense NLRP3 activity was evaluated, cases with early sepsis, hemodynamic significant PDA and surgical NEC had shown significantly increased NLRP3 activity. For infants who had hsPDA showed significantly intense anti-NLRP3 staining compared to infants who did not (18.8% vs 0%, p=0.027). Similarly, infants who developed NEC requiring surgery had a higher rate of intense inflammasome activity (33% vs 7.5%, p=0.048). All infants with mortality (n=3) had shown intense placental NLRP3 activity (p=0.279) (Table III).

Autophagy –inflammasome interplay

There was a significant overlap between autophagy and inflammasome activity (Mc Nemar test, p 0.05). Considering the relationship between autophagy and inflammasome, we formed four groups: i. Autophagy (without inflammasome): n=9 (15.3%) ii. Inflammasome (without autophagy): n=9 (15.3%), iii. Autophagy with inflammasome: n=35 (59.3%) iv. Negative or mild staining for autophagy inflammasome: n=6 (10.2%) (Fig. 2B). The above four groups did not show significant differences in terms of the diseases assessed.

Autophagy and/or inflammasome activities did not show a significant overlap with other placental histopathological findings (chorioamnionitis or vascular malperfusion) (Mc Nemar Test, p< 0.05).

Table II. The distribution of Anti-LC3B and Anti-NLRP3 staining, across neonatal outcome categories.

Neonatal Outcome	Anti-LC3B Score 1 or 2 Positive n (%)*	p	Anti-NLRP3 Weak or Strong Positive n (%)*	p
RDS				
No (n= 10)	6 (60.0)**	0.245	8 (80.0)	0.667
Yes (n=49)	38 (77.6)		36 (73.5)	
hsPDA requiring treatment				
No (n= 27)	20 (74.1)	0.935	20 (74.1)	0.935
Yes (n=32)	24 (75.0)		24 (75.0)	
IVH (> Grade 2)				
No (n= 57)	42 (73.7)	0.999	42 (73.7)	0.999
Yes (n=2)	2 (100.0)		2 (100.0)	
PVL				
No (n=51)	38 (74.5)	0.999	38 (74.5)	0.648
Yes (n=6)	5 (83.3)		4 (66.7	
Early-onset sepsis				
No (n=45)	34 (75.6)	0.757	31 (68.9)	0.072
Yes (n=14)	10 (71.4)		13 (92.9)	
Late-onset sepsis				
No (n=19)	12 (63.2)	0.285	15 (84.2)	0.478
Yes (n=40)	32 (80.0)		27 (67.5)	
NEC (≥ Stage 2)				
No (n=53)	40 (75.5)	0.638	40 (75.5)	0.478
Yes (n= 6)	4 (66.7)		4 (66.7)	
BPD at 36w				
No(n=50)	38 (76.0)	0.792	39 (78.0)	**0.048**
Yes (n=7)	5 (71.4)		3 (42.9)	
ROP requiring treatment				
No (n=51)	38 (74.5)	0.999	37 (72.5)	0.999
Yes (n=6)	5 (83.3)		5 (83.3)	
Mortality				
No (n=56)	42 (75.0)	0.999	41 (73.2)	0.564
Yes (n=3)	2 (66.7)		3(100.0)	
Compound outcome				
No (n=39)	29 (74.4)	0.957	30 (76.9)	0.563
Yes (n=20)	15(75.0)		14 (70.0)	

* % within outcomes (RDS, hs PDA, IVH, PVL, NEC, early sepsis, late sepsis, BPD, ROP, all morbidities, mortality, morbidies)

** existence of one of these outcomes or mortality: IVH > grade 2, hsPDA, surgical NEC, cystic PVL, moderete-severe BPD, ROP requiring treatment

RDS: respiratory distress syndrome, hsPDA: hemodynamically significant patent ductus arteriosus, IVH: intraventricular hemorrhage, PVL: periventricular leukomalacia, NEC: necrotizing enterocolitis, BPD: bronchopulmonary dysplasia, ROP: retinopathy of prematurity

Table III. The distribution of intense Anti LC3B and AntiNLRP3 staining acros selected neonatal outcome categories.

Neonatal Outcome	Anti-LC3B Score 2 Positive, n (%)*	p	Anti-NLRP3 Strong-Positive, n (%)*	p
hsPDA requiring treatment				
No (n= 27)	10 (37.0)	0.465	0 (0)	0.027
Yes (n=26)	9 (28.1)		6 (18.8)	
Early-onset sepsis				
No (n=45)	17 (37.8)	0.188	2 (4.4)	0.024
Yes (n=14)	2 (14.3)		4 (28.6)	
NEC (≥ Stage 2)				
No (n=53)	16 (30.2)	0.376	4 (7.5)	0.048
Yes (n= 6)	3 (50.0)		2 (33)	

* % within outcomes
# hsPDA: hemodynamically significant patent ductus arteriosus, NEC: necrotizing enterocolitis

Discussion

The placenta is the key regulatory element of the in-utero environment. Cellular homeostasis mechanisms such as the oxidative defense system, inflammatory pathway and autophagy are highly linked to neonatal morbidities.[22] This is the first study investigating the relationship between preterm morbidities and placental autophagy and/or inflammasome activities. The results demonstrated that placental autophagy and inflammasome activities were intersected significantly, and existed in the majority of the extremely premature infant population in varying degrees. Overall, no significant relationship was found between preterm morbidities and placental autophagy. Placental inflammasome activity seemed reduced in infants who developed BPD whereas intense inflammasome activity rate was significantly higher in infants who developed early sepsis, hsPDA and surgical NEC.

Evidence from previous studies has shown that the maternal inflammatory response, specifically chorioamnionitis, correlated with BPD, IVH, PVL and ROP.[23,24] Placental vascular malperfusion was also found to be a risk factor for BPD and IVH.[25,26] In this study, placental histological findings indicating chorioamnionitis and vascular malperfusion were not significantly associated with preterm morbidities. Most likely, the complicated intrauterine processes of the infants included in the study and the presence of at least one placental pathology in almost all of them may have suppressed the distinguishing feature of placental histology.

Autophagy plays a critical function in health and disease since it can be either a protector or detrimental.[27] Autophagy is one of the main mechanisms for maintaining cellular homeostasis, and is defined as the degradation of damaged intracellular proteins, organelles and microbial organisms. Autophagosome formation (double membrane vesicle) is essential for the process, and several proteins such as LC3B, Beclin-1, and p53 involve in molecular signaling. These proteins are widely used to assess autophagy activity. Microtubule-associated protein light chain 3B (LC3B) is the most commonly used marker for autophagosome formation in studies.[28] Autophagy is essential for normal placentation throughout pregnancy, and it contributes to operating appropriately in stressful conditions.[29] Placental autophagy is triggered by many pregnancy-related complications like preeclampsia, fetal growth restriction (FGR) and gestational diabetes.[30] Recent studies have demonstrated a close relationship between abnormal autophagy and prematurity, and autophagy has been found to be protective against preterm labor

by promoting the synthesis of progesterone.[31,32] In this study, we detected a high rate of placental autophagy activity in a premature infant population regardless of the existence of morbidity or mortality. Although varying in severity, placental autophagy activity was found in 75% of these babies. These data are inconsistent with previous ones indicating that the decrease in autophagy activity increases the risk of prematurity. Since our entire population was preterm, we could not demonstrate the effect of prematurity on autophagy.

In addition to the relationship between autophagy dysregulation and preterm birth, disorders in autophagy regulation are shown to be associated with certain preterm diseases. Previous preclinical studies showed impaired autophagy in oxygen-induced retinopathy model and BPD model, enhanced autophagy in the white matter injury model and NEC model.[33-36] Autophagy gene (ATG16L1) and NLRP3 gene were evaluated in preterm infants diagnosed with NEC, and a functional variant in ATG16L1 was associated with NEC.[37] In this cohort study, no relationship was found between autophagy activity and morbidities related to prematurity. The coexictance of autophagy and inflammasome did not make a significant difference. When we categorize the patients according to the severity of autophagy, the results did not change. Our evaluation in a partially homogeneous group consisting of entirely risky pregnancies and a small sample size may have prevented us from detecting meaningful results.

Gestational diabetes mellitus (GDM) and maternal obesity are risk factors for both mother and neonatal outcomes. Lipotoxicity associated oxidative stress results in altered autophagy in obese patients. Few studies have investigated autophagic activity from placental samples from obese pregnant women and women diagnosed with GDM.[38] In a previous study, decreased apoptosis and autophagy in the placentas from GDM women with LGA infants compared to those from normal pregnant women. However, it was reported that the biological significance of concomitant autophagy and apoptosis decreases in GDM placentas remains unclear. Several other studies showed inconsistent results in the placentas of women with gestational diabetes mellitus (GDM).[39] Anti-LC3B stainings in placental tissues from 10 women with GDM and obesity in our cohort resulted in 70% positivity. Overlapped conditions such as placental inflammation and vascular malperfusion rates were relatively high in our study, so it could affect our findings. Larger sample size studies are needed to pursue more detailed information about the association between preterm morbidities and placental apoptosis and autophagy in women with GDM and obesity.

Inflammasomes are cytosolic proteins that are part of the innate immune system regulating inflammation. NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3) is a member of the inflammasome complex that activates proinflammatory pathways mainly via Caspase-1 activation. The NLRP3 is the most frequent marker to assess inflammasome formation.[40] The inflammasome activity is involved in implantation and as well as pregnancy.[41] Altered placental NLRP3 activation is also involved in placental disorders such as preeclampsia and preterm delivery with chorioamnionitis.[8,42] Rare data exist regarding preterm morbidities and inflammasome activation. The inflammasome activity was involved in preclinical studies including hyperoxic lung injury, endotoxin-induced lung injury and neonatal hypoxic-ischemic brain injury models.[43-45] In our study, we assessed the placental NLRP3 activity and its association with preterm morbidities. Our results showed a significantly increased high-intense inflammasome activity in patients who had surgical NEC and hsPDA. Surprisingly, a majority of the infants with BPD had lower inflammasome activity (Table II). Although decreased placental inflammasome activity in infants developing BPD may seem unexpected due to the role of antenatal inflammation in the pathogenesis of BPD development, it may be considered that exposure to fetal inflammation

may have both harmful and beneficial effects on the immature lung.[46] Repeated exposure to LPS or chronic chorioamnionitis in experimental animals reduces the development of BPD by leading to immune tolerance and a reduced inflammatory response. Exposure to antenatal inflammation can also induce a maturation effect through proinflammatory cytokines such as IL-1, IL-6, IL-8, and TNF-alpha, which enhance surfactant protein and lipid synthesis. Antenatal inflammation can correspondingly lead to structural modifications in the fetal lung and affect the expression of growth factors that are required for branching such as transforming growth factor-beta, connective tissue growth factor, fibroblast growth factor-10.[47]

Poor in-utero conditions cause increased placental oxidative stress with increased production of reactive oxygen species (ROS).[48] ROS is a major determinant of cellular damage by triggering autophagy and inflammation. Autophagy regulates inflammation by clearance of damaged mitochondria, ROS and inflammasome components. In this study, we demonstrated a significant overlap between autophagy and inflammasome activities. We also assessed the inflammasome activity with autophagy activity since the interaction between autophagy and inflammasome is complicated and two-sided. However, accompanying autophagy to inflammasome did not significantly change the results.

The strength of our study was its prospective design and relatively homogenous study population of preterm infants between 24-29 gestational age, who are especially at higher risk of adverse outcomes. However, several limitations exist. The first limitation was the small size of the study population. Autophagy and inflammasome activities are dynamic responses to certain triggers and they change activity over time. Studying the placenta at a single time point may have prevented us from demonstrating the dynamic processes taking place over time. Another limitation was the assessment of autophagy activation only via immunohistochemical LC3B staining. Autophagy is a dynamic process in human tissues, therefore using a single marker and method is challenging. Therefore, authophagy guideline recommends the use of multiple assays, whenever possible.[49]

In this study antenatal inflammatory process was assessed by only placental histopathological examination and inflammasome activity. Several studies in the literature have investigated umbilical cord inflammatory markers such as Il-6, procalcitonin and CRP, and the results were mostly controversial in terms of correlation between histological chorioamnionitis and neonatal outcomes.[50,51] Umbilical cord IL-6 is the most studied biomarker for predicting histological chorioamnionitis, and its sensitivity and specificity vary between 64-84%.[52] Therefore it is recommended that the histological examination of the placenta is more accurate and essential for predicting neonatal outcome. In regard to this limitation, the relationship between placental autophagy and the umblical cord inflammatory biomarkers are needed to be evaluated in future studies.

An important limitation was the lack of evaluation of postnatal factors in preterm morbidities in our cohort. Preterm morbidities are widely multifactorial affected by both antenatal and postnatal conditions. The aim of our study was evaluating placental autophagy and inflammasome as early biomarkers for predicting preterm morbidities. Hence various antenatal risk factors could affect placental findings, we evaluated antenatal risk factors in our cohort and found no relationship regarding placental findings. Further studies are needed to be carried out including postnatal risk factors for preterm morbidities based on our preliminary results on placental autophagy and inflammasomes.

In our study population, almost all placentas had an abnormality, demonstrating either inflammation or placental malperfusion findings. It is well known that these two conditions are involved in neonatal adverse outcomes. Autophagy and inflammasome

activities from pathological placentas could affect our results in terms of predicting preterm morbidities. Because of the natural causes of prematurity, a control group consisting of age-matched healthy placentas could not be set up. Because of the characteristics of our sample (high sectio rate, lower rate of antenatal steroid, higher complication rate) the data from this study population may be difficult to translate more widely.

In conclusion, the results signify the physiological role of these mechanisms in the intrauterine period. Association of decreased or increased rates of inflammasome activities with certain diseases such as BPD, hsPDa and surgical NEC indicates the role of the intrauterine inflamattory process and the importance of critical balance around the inflammation. Alongside conventional placental histopathological findings, the investigation of the placenta in terms of autophagy and inflammasome activity could bring new insights to clinical practice in neonatology. Understanding the interplay between autophagy and inflammasome in the pathogenesis of preterm morbidities may benefit a better understanding of molecular pathways, contribute to the development of new biomarkers, and provide new therapeutic options.

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Ethical approval

This study was approved by the Ethics Committee of Dokuz Eylul University Medical Faculty. (No:2017/25-02).

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: FT, HO, ND, EO; data collection: BD, AA; analysis interpretation of results: BD, FT, HO, ND, draft manuscript preperation: BD, FT. All authors reviewed the results and approved the final version of the manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

REFERENCES

1. Koc E, Demirel N, Bas AY, et al. Early neonatal outcomes of very-low-birth-weight infants in Turkey: a prospective multicenter study of the Turkish Neonatal Society. PLoS One 2019; 14: e0226679. https://doi.org/10.1371/journal.pone.0226679
2. Wu F, Tian FJ, Lin Y. Oxidative stress in placenta: health and diseases. Biomed Res Int 2015; 2015: 293271. https://doi.org/10.1155/2015/293271
3. Choi AMK, Ryter SW, Levine B. Autophagy in human health and disease. N Engl J Med 2013; 368: 651-662. https://doi.org/10.1056/NEJMra1205406
4. Gong JS, Kim GJ. The role of autophagy in the placenta as a regulator of cell death. Clin Exp Reprod Med 2014; 41: 97-107. https://doi.org/10.5653/cerm.2014.41.3.97
5. Oh SY, Roh CR. Autophagy in the placenta. Obstet Gynecol Sci 2017; 60: 241-259. https://doi.org/10.5468/ogs.2017.60.3.241
6. Netea-Maier RT, Plantinga TS, van de Veerdonk FL, Smit JW, Netea MG. Modulation of inflammation by autophagy: consequences for human disease. Autophagy 2016; 12: 245-260. https://doi.org/10.1080/15548627.2015.1071759
7. Gomez-Lopez N, Romero R, Panaitescu B, et al. Inflammasome activation during spontaneous preterm labor with intra-amniotic infection or sterile intra-amniotic inflammation. Am J Reprod Immunol 2018; 80: e13049. https://doi.org/10.1111/aji.13049
8. Shirasuna K, Karasawa T, Takahashi M. Role of the NLRP3 inflammasome in preeclampsia. Front Endocrinol (Lausanne) 2020; 11: 80. https://doi.org/10.3389/fendo.2020.00080

9. Committee on Obstetric Practice. Committee Opinion No. 713: antenatal corticosteroid therapy for fetal maturation. Obstet Gynecol 2017; 130: e102-e109. https://doi.org/10.1097/AOG.0000000000002237

10. Villar J, Cheikh Ismail L, Victora CG, et al. International standards for newborn weight, length, and head circumference by gestational age and sex: the newborn cross-sectional study of the INTERGROWTH-21st Project. Lancet 2014; 384: 857-868. https://doi.org/10.1016/S0140-6736(14)60932-6

11. Sweet DG, Carnielli V, Greisen G, et al. European Consensus guidelines on the management of respiratory distress syndrome - 2016 update. Neonatology 2017; 111: 107-125. https://doi.org/10.1159/000448985

12. Köksal N, Aygün C, Uras N. Turkish Neonatal Society guideline on the management of patent ductus arteriosus in preterm infants. Turk Pediatri Ars 2018; 53: S76-S87. https://doi.org/10.5152/TurkPediatriArs.2018.01808

13. Papile LA, Burstein J, Burstein R, Koffler H. Incidence and evolution of subependymal and intraventricular hemorrhage: a study of infants with birth weights less than 1,500 gm. J Pediatr 1978; 92: 529-534. https://doi.org/10.1016/s0022-3476(78)80282-0

14. de Vries LS, Eken P, Dubowitz LM. The spectrum of leukomalacia using cranial ultrasound. Behav Brain Res 1992; 49: 1-6. https://doi.org/10.1016/s0166-4328(05)80189-5

15. Koç E, Baş AY, Özdek Ş, Ovalı F, Başmak H. Turkish Neonatal and Turkish Ophthalmology Societies consensus guideline on the retinopathy of prematurity. Turk Pediatri Ars 2018; 53: S151-S160. https://doi.org/10.5152/TurkPediatriArs.2018.01815

16. Bell MJ, Ternberg JL, Feigin RD, et al. Neonatal necrotizing enterocolitis. Therapeutic decisions based upon clinical staging. Ann Surg. 1978; 187: 1-7. https://doi.org/10.1097/00000658-197801000-00001

17. Jobe AH, Bancalari E. Bronchopulmonary dysplasia. Am J Respir Crit Care Med 2001; 163: 1723-1729. https://doi.org/10.1164/ajrccm.163.7.2011060

18. Khong TY, Mooney EE, Ariel I, et al. Sampling and Definitions of Placental Lesions: Amsterdam Placental Workshop Group Consensus Statement. Arch Pathol Lab Med 2016; 140: 698-713. https://doi.org/10.5858/arpa.2015-0225-CC

19. Schläfli AM, Berezowska S, Adams O, Langer R, Tschan MP. Reliable LC3 and p62 autophagy marker detection in formalin fixed paraffin embedded human tissue by immunohistochemistry. Eur J Histochem 2015; 59: 2481. https://doi.org/10.4081/ejh.2015.2481

20. Weel IC, Romão-Veiga M, Matias ML, et al. Increased expression of NLRP3 inflammasome in placentas from pregnant women with severe preeclampsia. J Reprod Immunol 2017; 123: 40-47. https://doi.org/10.1016/j.jri.2017.09.002

21. Avagliano L, Danti L, Doi P, et al. Autophagy in placentas from acidotic newborns: an immunohistochemical study of LC3 expression. Placenta 2013; 34: 1091-1094. https://doi.org/10.1016/j.placenta.2013.09.004

22. D'Angelo G, Chimenz R, Reiter RJ, Gitto E. Use of melatonin in oxidative stress related neonatal diseases. Antioxidants (Basel) 2020; 9: 477. https://doi.org/10.3390/antiox9060477

23. Ogunyemi D, Murillo M, Jackson U, Hunter N, Alperson B. The relationship between placental histopathology findings and perinatal outcome in preterm infants. J Matern Fetal Neonatal Med 2003; 13: 102-109. https://doi.org/10.1080/jmf.13.2.102.109

24. Ko HS, Cheon JY, Choi SK, et al. Placental histologic patterns and neonatal seizure, in preterm premature rupture of membrane. J Matern Fetal Neonatal Med 2017; 30: 793-800. https://doi.org/10.1080/14767058.2016.1186634

25. Catov JM, Scifres CM, Caritis SN, Bertolet M, Larkin J, Parks WT. Neonatal outcomes following preterm birth classified according to placental features. Am J Obstet Gynecol 2017; 216: 411.e1-411.e14. https://doi.org/10.1016/j.ajog.2016.12.022

26. Chisholm KM, Heerema-McKenney A, Tian L, et al. Correlation of preterm infant illness severity with placental histology. Placenta 2016; 39: 61-69. https://doi.org/10.1016/j.placenta.2016.01.012

27. Wirawan E, Vanden Berghe T, Lippens S, Agostinis P, Vandenabeele P. Autophagy: for better or for worse. Cell Res 2012; 22: 43-61. https://doi.org/10.1038/cr.2011.152

28. Berezowska S, Galván JA. Immunohistochemical detection of the autophagy markers LC3 and p62/SQSTM1 in Formalin-Fixed and Paraffin-Embedded tissue. Methods Mol Biol 2017; 1560: 189-194. https://doi.org/10.1007/978-1-4939-6788-9_13

29. Hung TH, Hsieh TT, Chen SF, Li MJ, Yeh YL. Autophagy in the human placenta throughout gestation. PLoS One 2013; 8: e83475. https://doi.org/10.1371/journal.pone.0083475

30. Nakashima A, Tsuda S, Kusabiraki T, et al. Current understanding of autophagy in pregnancy. Int J Mol Sci 2019; 20: 2342. https://doi.org/10.3390/ijms20092342

31. Gawriluk TR, Rucker EB. BECN1, corpus luteum function, and preterm labor. Autophagy 2015; 11: 183-184. https://doi.org/10.4161/15548627.2014.984269

32. Romero R, Dey SK, Fisher SJ. Preterm labor: one syndrome, many causes. Science 2014; 345: 760-765. https://doi.org/10.1126/science.1251816

33. Wang S, Ji LY, Li L, Li JM. Oxidative stress, autophagy and pyroptosis in the neovascularization of oxygen-induced retinopathy in mice. Mol Med Rep 2019; 19: 927-934. https://doi.org/10.3892/mmr.2018.9759

34. Zhang L, Soni S, Hekimoglu E, Berkelhamer S, Çataltepe S. Impaired autophagic activity contributes to the pathogenesis of bronchopulmonary dysplasia: evidence from Murine and Baboon models. Am J Respir Cell Mol Biol 2020; 63: 338-348. https://doi.org/10.1165/rcmb.2019-0445OC

35. Descloux C, Ginet V, Rummel C, Truttmann AC, Puyal J. Enhanced autophagy contributes to excitotoxic lesions in a rat model of preterm brain injury. Cell Death Dis 2018; 9: 853. https://doi.org/10.1038/s41419-018-0916-z

36. Yuan Y, Ding D, Zhang N, et al. TNF-α induces autophagy through ERK1/2 pathway to regulate apoptosis in neonatal necrotizing enterocolitis model cells IEC-6. Cell Cycle 2018; 17: 1390-1402. https://doi.org/10.1080/15384101.2018.1482150

37. Sampath V, Bhandari V, Berger J, et al. A functional ATG16L1 (T300A) variant is associated with necrotizing enterocolitis in premature infants. Pediatr Res 2017; 81: 582-588. https://doi.org/10.1038/pr.2016.260

38. Diceglie C, Anelli GM, Martelli C, et al. Placental antioxidant defenses and autophagy-related genes in maternal obesity and gestational diabetes mellitus. Nutrients 2021; 13: 1303. https://doi.org/10.3390/nu13041303

39. Hung TH, Huang SY, Chen SF, Wu CP, Hsieh TT. Decreased placental apoptosis and autophagy in pregnancies complicated by gestational diabetes with large-for-gestational age fetuses. Placenta 2020; 90: 27-36. https://doi.org/10.1016/j.placenta.2019.12.003

40. Sutterwala FS, Haasken S, Cassel SL. Mechanism of NLRP3 inflammasome activation. Ann N Y Acad Sci 2014; 1319: 82-95. https://doi.org/10.1111/nyas.12458

41. Gomez-Lopez N, Motomura K, Miller D, Garcia-Flores V, Galaz J, Romero R. Inflammasomes: their role in normal and complicated pregnancies. J Immunol 2019; 203: 2757-2769. https://doi.org/10.4049/jimmunol.1900901

42. Gomez-Lopez N, Romero R, Xu Y, et al. A role for the ınflammasome in spontaneous preterm labor with acute histologic chorioamnionitis. Reprod Sci 2017; 24: 1382-1401. https://doi.org/10.1177/1933719116687656

43. Liao J, Kapadia VS, Brown LS, et al. The NLRP3 inflammasome is critically involved in the development of bronchopulmonary dysplasia. Nat Commun 2015; 6: 8977. https://doi.org/10.1038/ncomms9977

44. Lin Y, Yang Y. MiR-24 inhibits inflammatory responses in LPS-induced acute lung injury of neonatal rats through targeting NLRP3. Pathol Res Pract 2019; 215: 683-688. https://doi.org/10.1016/j.prp.2018.12.028

45. Chen D, Dixon BJ, Doycheva DM, et al. IRE1α inhibition decreased TXNIP/NLRP3 inflammasome activation through miR-17-5p after neonatal hypoxic-ischemic brain injury in rats. J Neuroinflammation 2018; 15: 32. https://doi.org/10.1186/s12974-018-1077-9

46. Kramer BW, Kallapur S, Newnham J, Jobe AH. Prenatal inflammation and lung development. Semin Fetal Neonatal Med 2009; 14: 2-7. https://doi.org/10.1016/j.siny.2008.08.011

47. Kunzmann S, Collins JJP, Kuypers E, Kramer BW. Thrown off balance: the effect of antenatal inflammation on the developing lung and immune system. Am J Obstet Gynecol 2013; 208: 429-437. https://doi.org/10.1016/j.ajog.2013.01.008

48. Schoots MH, Gordijn SJ, Scherjon SA, van Goor H, Hillebrands J-L. Oxidative stress in placental pathology. Placenta 2018; 69: 153-161. https://doi.org/10.1016/j.placenta.2018.03.003

49. Klionsky DJ, Abdel-Aziz AK, Abdelfatah S, et al. Guidelines for the use and interpretation of assays for monitoring autophagy (4th edition). Autophagy 2021; 17: 1-382. https://doi.org/10.1080/15548627.2020.1797280

50. Howman RA, Charles AK, Jacques A, et al. Inflammatory and haematological markers in the maternal, umbilical cord and infant circulation in histological chorioamnionitis. PLoS One 2012; 7: e51836. https://doi.org/10.1371/journal.pone.0051836

51. Oh JW, Park CW, Moon KC, Park JS, Jun JK. The relationship among the progression of inflammation in umbilical cord, fetal inflammatory response, early-onset neonatal sepsis, and chorioamnionitis. PLoS One 2019; 14: e0225328. https://doi.org/10.1371/journal.pone.0225328

52. Tasci Y, Dilbaz B, Uzmez Onal B, et al. The value of cord blood interleukin-6 levels for predicting chorioamnionitis, funisitis and neonatal infection in term premature rupture of membranes. Eur J Obstet Gynecol Reprod Biol 2006; 128: 34-39. https://doi.org/10.1016/j.ejogrb.2005.11.049