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REVIEW ARTICLE

A review of the role of extracellular vesicles in neonatal physiology and pathology

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Extracellular vesicles (EVs) are cell-derived membrane-bound particles, extensively investigated across many fields to improve the understanding of pathophysiological processes, as biomarkers of disease and as therapeutic targets for pharmacological intervention. We aim to describe the current knowledge of EVs detected in the body fluids of human neonates, both term and preterm, from birth to 4 weeks of age. To date, EVs have been described in several neonatal body fluids, including cerebrospinal fluid, umbilical cord blood, neonatal blood, tracheal aspirates and urine. These studies demonstrate some important roles of EVs in the neonatal population, particularly in haemostasis. Moreover, some studies have demonstrated the pathophysiological mechanisms and the identification of potential biomarkers of neonatal disease. We must continue to build on this knowledge, evaluating the role of EVs in neonatal pathology, particularly in prematurity and during the perinatal adaption period. Future studies should use larger numbers, robust EV characterisation techniques and always correlate the findings to clinical outcomes.

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IMPACT:

- This article summarises the current knowledge of the effect of EVs in neonates.
- It describes the potential compensatory role of EVs in neonatal haemostasis.
- It also describes the role of EVs as mediators of pathology and as potential biomarkers of perinatal and neonatal disease.

INTRODUCTION

Extracellular vesicles (EVs) are now recognised as mediators of physiological and pathological processes, biomarkers of disease and therapeutic targets.[1–3] EVs are nanoparticles surrounded by a lipid bilayer, which are released from cells but cannot replicate.[4] EVs contain proteins, lipids and microRNA (miRNA).[5] The biological roles of EVs vary depending on their cell or membrane of origin.[6] Platelet-derived EVs (PDEVs), thought to account for >70% of plasma EVs,[7] play an important role in haemostasis by increasing both the phospholipid surface available for secondary haemostasis and the amount of tissue factor (TF) present in the environment.[8] EVs also have a role in pathological processes, including inflammation and tumour metastasis.[9,10]

EV profiles have been used as biomarkers of disease. For example, in tumours such as glioblastoma, a diagnostic EV profile is detectable in the cerebrospinal fluid.[11] Moreover, there have been advances in the use of EVs as therapeutic targets, particularly in pathologies of prematurity such as bronchopulmonary dysplasia (BPD).[12] Numerous studies have demonstrated the potential efficacy of mesenchymal stem cell EVs in the treatment of necrotising enterocolitis (NEC) and BPD in pre-clinical models of disease.[13–15] In addition, EVs from microglia (rich in miR-24-3p) have been shown to attenuate retinopathy of prematurity (ROP) in a mouse model.[16]

For the purposes of clarity, the terms small EVs (SEVs) and large EVs (LEVs) are used in this review, although frequently referred to as exosomes and microparticles, respectively, in the literature. SEVs are isolated by ultracentrifugation at 100,000 × *g* and are generally <100–150 nm in size, while LEVs are isolated at 20,000 × *g* and measure up to 1000 nm.[4,17] SEVs are predominantly derived from inward blebbing of multivesicular bodies and participate in intercellular communication.[18] LEVs typically derive from the plasma membrane and play a role in inflammation and coagulation.[19]

The International Society of Extracellular Vesicles (ISEV) has published position statements on the minimal information for studies of extracellular vesicles (MISEVs), to improve the reliability and reproducibility of the results of EV studies.[4,20,21] These guidelines recommend detailed reporting of the collection, separation and storage methods used.[4,20] Moreover, MISEV recommends the characterisation of EVs using global EV markers and at least two single EV characterisation techniques.[21]

Several techniques are available to characterise EVs. Nanoparticle tracking analysis (NTA) uses Brownian motion to calculate the size and concentration of SEVs (<300 nm, but it can visualise particles up to 1000 nm).[22] The fluorescent mode of NTA utilises fluorophores (quantum dots) attached to antibodies, to fluorescently label SEVs, allowing determination of the EV phenotype.[22]

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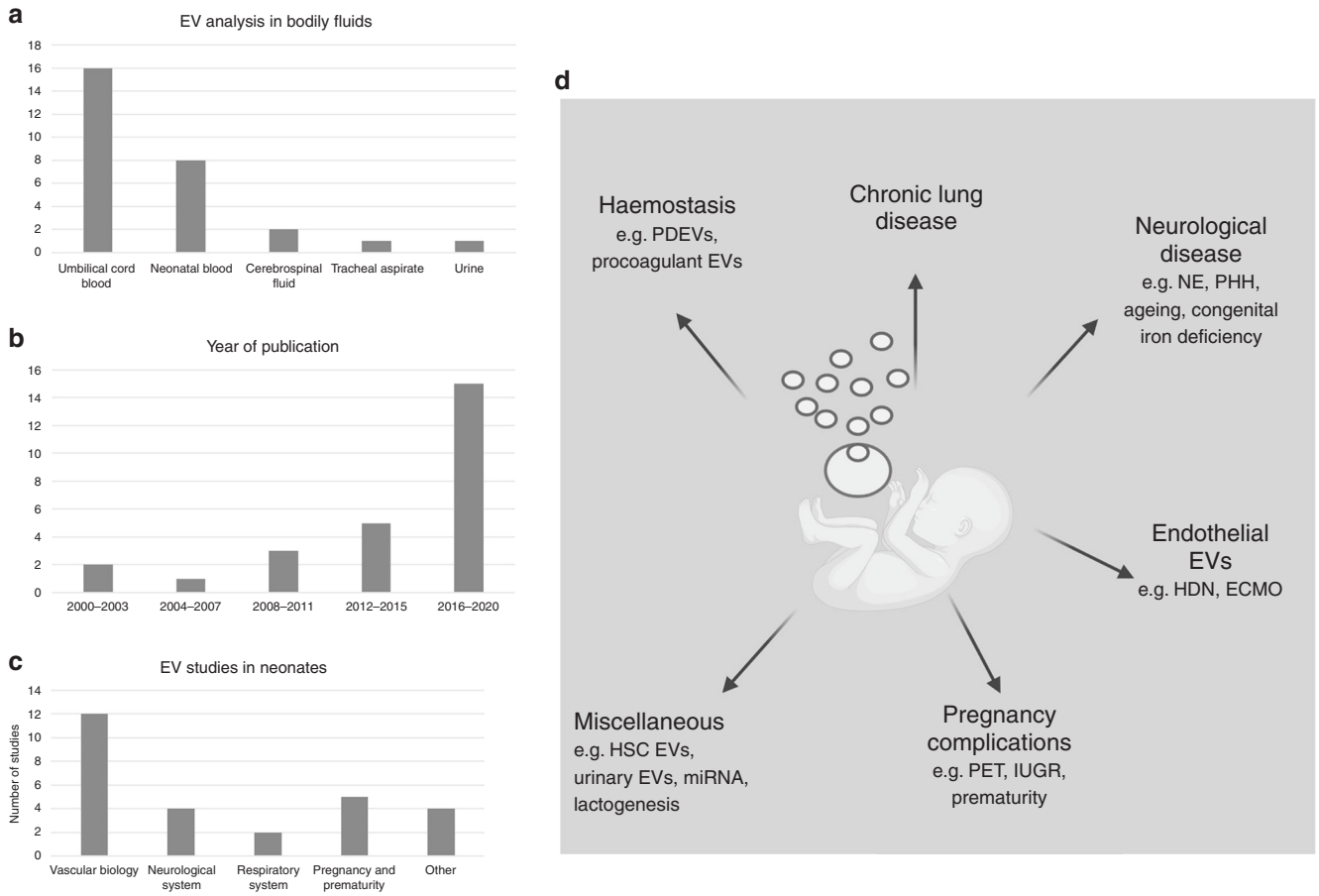

Fig. 1 A summary of the neonatal EV studies to date. a describes the bodily fluids in which EVs have been described in neonates. Image created with GIMP. **b** demonstrates the increasing number of publications on the topic of neonatal EVs in recent years. Image created with GIMP. **c** describes the biological systems studied in the neonatal population. Image created with GIMP. **d** graphically represents the clinical areas of neonatal EV research to date. Image created with Biorender.com. EV extracellular vesicle, PDEVs platelet-derived extracellular vesicle, NE neonatal encephalopathy, PHH post-haemorrhagic hydrocephalus, HDN haemolytic disease of the newborn, ECMO extracorporeal membrane oxygenation, PET pre-eclamptic toxaemia, IUGR intrauterine growth restriction, HSC EVs haematopoietic stem cell extracellular vesicles, miRNA microRNA.

Flow cytometry is used to measure LEVs in the range 300–1000 nm.[23] Individual EVs pass a laser beam that produces light scatter, used to calculate the size, and fluorescent parameters, to detect the cellular origin.[24] This is performed using fluorescently conjugated antibodies, for example, CD144 (endothelial-derived EVs[25]) and CD41/CD42b/CD61 (PDEVs).[26] Dynamic light scattering is a technique that measures the size of particles (1 nm to 6 µm) in a solution using Brownian motion, but does not give information regarding the concentration or the origin of the EVs.[27] Transmission electron microscopy (TEM) and immunoblotting are also methods used to confirm the presence of EVs within a sample.[21]

Several reviews have discussed the possible therapeutic applications of EVs in neonatology.[12,28–30] However, there is a paucity of information regarding circulating EVs in the neonatal period. In this review, we will describe the current knowledge of EVs released into the bodily fluids of neonates in the first month of life and the clinical implications of what is known to date (Fig. 1).

THE ROLE OF EVS IN NEONATAL VASCULAR BIOLOGY

PDEVs and procoagulant EVs

Nine studies evaluated the role of PDEVs or procoagulant activity of neonatal EVs. The first used flow cytometry to evaluate large PDEVs in umbilical cord blood (UCB) from preterm compared with term infants and adults.[31] After activation there was a significant increase in PDEVs (CD42b), in both preterm and term infants.

The procoagulant activity of the EVs was measured using a novel method of flow cytometry, which detected the binding of fluorescein isothiocyanate-labelled factor V/Va to the surface of PDEVs (Gp1b positive). At baseline, there was no significant difference in the procoagulant effect; however, after stimulation, there was a significant reduction in the procoagulant effect of EVs in the preterm group. The reduction in procoagulant effect was corrected by the addition of adult plasma or factor V.[31] This suggested a plasma/factor deficiency as the cause for the reduced procoagulant effect.

Schmugge et al.[32] determined the percentage of PDEVs in both UCB and neonatal samples in healthy term infants. Using flow cytometry (CD41), a higher proportion of PDEVs was reported in both UCB and neonatal samples compared with adults. It was found that neonatal platelets displayed greater platelet activation than adult platelets at baseline and it was hypothesised that this may have in part been due to difficulties in sampling neonatal blood.

In 2008, Wasiluk et al.[33] described the number of PDEVs in preterm and term UCB samples using flow cytometry (CD61). A significant increase in the number of PDEVs in the preterm infants was demonstrated. Moreover, the number of PDEVs was not dependant on the number of platelets present.

Most recently, O'Reilly et al.[34] described the EVs released in preterm infants on day of life (DOL) one and three. While there was an increase in the total number of both SEVs and LEVs

between DOL 1 and 3, demonstrated using both NTA and flow cytometry, there was a reduction in the number of PDEVs (CD41) over the same time. This is suggestive of an early platelet activation event following preterm delivery. Unfortunately, these findings were not investigated in term controls. It is therefore unclear if the changes seen are due to normal adaption to extrauterine life.

Over the past decade, five studies have evaluated the procoagulant activity of neonatal EVs. Schweintzger et al.[35] characterised EV procoagulant activity in term UCB samples compared with adults. Using flow cytometry, no significant difference in the total EV number (Annexin V), between the groups, was found. Moreover, this study used two techniques to evaluate the functional EV procoagulant activity. The enzyme-linked immunosorbent assay (ELISA) (XYMUPHEN-MP activity kit) measured the procoagulant EV phospholipid content in the plasma.[35,36] Computer-automated thrombography (CAT) is a global assay of coagulation that uses a fluorogenic substrate to measure thrombin generation in the plasma.[37] Both ELISA and CAT demonstrated significantly increased procoagulant activity of the neonatal EVs.

An alternative ELISA (ACTICHROME Microparticle activity kit) was used to evaluate the procoagulant activity of EVs in UCB of healthy term infants compared with maternal blood and healthy non-pregnant females.[36] Again, the procoagulant effect of EVs was higher in UCB. However, this study only performed a functional analysis of EVs and did not characterise EVs using any other technique, as recommended by MISEV.[21]

Karlaftis et al.[38] investigated the procoagulant activity of EVs in healthy term infants on DOL 1 and 3, comparing them with older children and adults. This study used the "STA-Procoag phospholipid kit". In this assay, patient plasma is added to phospholipid-depleted human plasma and the phospholipid content of the EVs impacts thrombin generation. Reduced procoagulant activity was demonstrated in neonatal samples on DOL 1 compared with older children and adults. Similar to the last study,[36] only one functional technique was used to evaluate EVs. Unlike the ELISAs previously described, this assay does not pre-select the EVs by Annexin V binding.

In 2015, Campello et al.[39] described the EVs released in UCB in infants born to mothers with and without preeclampsia (PET). The procoagulant activity of UCB EVs was assessed using the STA-ProCoag phospholipid kit. There was a higher procoagulant activity in the PET group, compared with healthy controls. Moreover, this study also used flow cytometry to characterise the EVs and found that the proportion of large PDEVs (CD61) was significantly higher in the PET group.

Finally, Korbal et al.[40] described the number of TF bearing EVs, between preterm and term UCB samples. An ELISA (XYMUPHEN-MP TF kit) was used to demonstrate a marginally increased TF-EV content in preterm infants. Again, only one functional technique was used. Moreover, none of the three studies[36,38,40] measured the total EV concentration, thus it is not clear whether the number or procoagulant activity of the circulating EVs was altered (Table 1).

Endothelial EVs

Haemolytic disease of the newborn (HDN) is a serious condition whereby an infant's red cells are haemolysed by maternal antibodies, resulting in anaemia, hyperbilirubinaemia and kernicterus if left untreated. Awad et al.[25] used flow cytometry to detect large endothelial EVs (eEVs) ($CD144^+$) in neonates with ABO HDN compared with infants with Rhesus HDN and controls without HDN. It was hypothesised that ABO-mediated haemolysis would result in endothelial dysfunction due to the presence of the A and B antigens on endothelial cells. A significant increase in eEVs in HDN compared with term controls was found and infants with ABO HDN had significantly higher levels of eEVs compared to Rhesus HDN. Although this study highlights a potential pathophysiological mechanism of ABO HDN-mediated endothelial injury, only one EV characterisation method was used and there was no clinical evaluation of endothelial dysfunction. However, these findings were replicated by Zhu et al.[41] in Chinese neonates with ABO-mediated HDN, which strengthens the results.

Vítková et al.[42] described the release of eEVs as a marker of endothelial injury in infants undergoing extracorporeal membrane oxygenation (ECMO). Flow cytometry was used to detect large eEVs in patients receiving ECMO compared with healthy term infants. A significant increase in the total number of LEVs was found in ECMO patients. There was a trend towards increased eEV markers (CD105, CD31, CD309) and a significant increase in mucosal vascular addressin cell adhesion molecule 1-positive EVs in ECMO patients compared with controls. The heterogeneity of clinical indications for ECMO in this study may have confounded the results. Moreover, in patients receiving ECMO, the identification of EVs released in response to interaction with the ECMO circuit vs. disease progression can be a challenge and was discussed as a limitation of this study. An increased production of EVs in response to ECMO circuits has been shown in animal models.[43]

THE ROLE OF EVS IN NEONATAL RESPIRATORY DISEASE

Chronic lung disease (CLD) (or BPD) is defined as the ongoing requirement for respiratory support at 36 weeks corrected gestational age (CGA). It is a serious complication of prematurity, causing respiratory and neurodevelopmental morbidity, and occurs in 26 - 28 % of infants <1500 g.[44]

In 2018, Lal et al.[45] described the role of EVs in neonates with BPD. First, NTA was used to demonstrate that tracheal aspirates (TAs) from infants with severe BPD (ventilated at 36 weeks CGA) had a reduced modal EV size (65 v 105 nm) and higher particle concentration of SEVs than controls. Using EV-depletion techniques, it was inferred that 63% of the SEVs were derived from epithelial cells (mucin 4). Subsequently, in a prospective cohort of extremely preterm infants, TAs were taken within 6 h of delivery and the infants were divided into BPD-susceptible and -resistant groups based on the outcome of BPD at 36 weeks. Forty differentially expressed miRNA were identified, and in the validation cohort, low miR 876-3p was identified as the most sensitive predictor of severe BPD in early TA. Following the identification of miR 876-3p, a significant reduction of EV miR 876-3p in the 36-week TA of infants with severe BPD compared with controls was confirmed. In addition, epithelial cell culture experiments and a mouse model of BPD were used to demonstrate that both hyperoxia and lipopolysaccharide exposure reduces EV miR 876-3p. Finally, the gain of miR 876-3p (through the intranasal administration of mimic miR 876-3p) in the mouse models, exposed to both hyperoxia and hyperoxia/LPS, resulted in reduced alveolar hypoplasia, compared with mice without the miR gain. Through the robust methodology, this study successfully identified a possible underlying pathological mechanism of BPD, a biomarker of BPD and a therapeutic target.

Go et al.[46] compared serum from preterm infants who developed CLD with preterm infants who did not. Samples were collected from UCB and neonatal samples at DOL 28 and 36 weeks CGA. At DOL 28, a significant increase in miR-21, compared with levels at birth, was found in infants with CLD. Using a mouse model, increased miR-21 was also found in the lung tissue after exposure to hyperoxia. Thus, a serum EV biomarker of CLD was described. There was a low rate of prenatal steroid administration (59–74%) in this study, which may limit the generalisation of the findings.

THE ROLE OF EVS IN NEONATAL NEUROLOGY

In 2014, Tietje et al.[47] described the variation of CSF EVs with increasing age. Using NTA, no difference in the size of SEVs

Table 1. Summary of included studies.

Publication	Source	Number of samples	Number of patients	CGA of patients included	Contents/surface markers examined	Proposed effect/findings
Publications examining EVs in neonatal vascular biology						
Michelson et al.[31]	Umbilical cord blood (neonates) Peripheral blood (adults)	15 neonatal samples	$N = 8$ preterm neonates $N = 7$ term neonates $N = 13$ adults	Term: 38–41 weeks Preterm: 24–30 weeks	GPIb +ve Functional assay using factor V/Va	Preterm platelet-derived EVs are more abundant but have less of a procoagulant effect
Schmugge et al.[32]	Umbilical cord blood and peripheral blood (neonates) Peripheral blood (adults)	38 neonatal samples DOL 1 19 neonatal samples d2–d3 (15 matched, 4 unmatched) 15 children samples 22 adult samples	$N = 42$ term neonates $N = 15$ children $N = 22$ adults	39.3 weeks (36–42 weeks)	CD41	Increased PDEVs in neonates
Wasiluk et al.[33]	Umbilical cord blood	51 preterm samples 55 term samples	$N = 51$ preterm $N = 55$ term	Preterm: 25–36 weeks Term: 38–41 weeks	CD61	Preterms produced more activated platelet-derived EVs
O'Reilly et al. 2018[34]	Peripheral blood	29 preterm samples	$n = 8$ matched DOL 1 and DOL 3 $n = 7$ unmatched DOL1 $n = 6$ unmatched DOL 3	Less than 27 weeks (not described in abstract)	Annexin V^+/ $CD41^+$	Change in number, size and composition of EVs between DOL 1 and DOL 3
Schweintzger et al.[35]	Umbilical cord blood (neonates) Peripheral blood (adults)	31 neonatal samples 28 adult samples	$N = 28$ adults $N = 31$ newborns	38–40 weeks gestational age	Annexin V^+	Term EVs are procoagulant
Uszyński et al.[36]	Umbilical cord blood (neonates) Peripheral blood (mothers and healthy controls)	28 neonatal samples 38 adult samples	$N = 28$ mother–infant dyads $N = 10$ non-pregnant females	39.3 (38–41.6) weeks gestational age	Procoagulant activity	Procoagulant EVs are present in higher concentrations in infants than their mothers or healthy controls
Karlaftis et al.[38]	Peripheral blood	120 samples total (20 neonatal samples)	$N = 10$ D1 neonates $N = 10$ D3 neonates $N = 20 < 1$ year $N = 20$ 1–5 years $N = 20$ 6–10 years $N = 20$ 11–16 years $N = 20$ Adults	>37 week	Procoagulant activity (non-selective)	Neonatal samples were hypocoaguable (prolonged clotting times vs. other age groups)
Campello et al.[39]	Umbilical cord blood (neonates) Peripheral blood (mothers)	32 neonatal samples 32 adult samples)	$N = 32$ mother–infant dyads (16 with preeclampsia, 16 without preeclampsia)	38 weeks ± 6 days (pre-eclamptic group) 37 ± 1 day (normotensive)	$CD61^+$ (platelet derived) P-$Selectin^+$ (activated platelet derived) E-$Selectin^+$ (endothelial) $CD45^+$ (leucocyte) $CD142^+$ (tissue factor bearing) Annexin V^+	Increased total EV content and procoagulant activity in preeclampsia affected infants
Korbal et al.[40]	Umbilical cord blood	23 preterm samples 25 term samples 48 samples	$N = 23$ preterm $N = 25$ term	Term: 40 weeks (39–41 weeks) Preterm: 34 (32–36)	Tissue factor-bearing microparticles	Increased levels of tissue factor-bearing EVs in preterm infants
Awad et al.[25]	Peripheral samples	85 samples			$CD144^+$ (VE-cadherin)	

Table 1. continued

Publication	Source	Number of samples	Number of patients	CGA of patients included	Contents/surface markers examined	Proposed effect/findings
			$N = 45$ ABO Haemolytic disease $N = 20$ Rhesus haemolytic disease $N = 20$ Healthy newborns	Term babies (37.3–38.2)		Release of endothelial EVs may be a result of IgG endothelial injury parallel to erythrocyte destruction
Zhu et al. [41]	Peripheral samples	72 samples	$N = 29$ ABO haemolytic disease $N = 22$ rhesus haemolytic disease $N = 21$ healthy newborns	Term: 38–40 weeks mean gestational age	$CD144^+$ (VE-cadherin)	Same hypothesis as above, Chinese population
Vítkova et al.[42]	Peripheral samples	26 samples	$n = 13$ infants on ECMO $n = 13$ term healthy newborns	Term: ~39 weeks gestation	Annexin V^+ $PECAM^+/CD31^+$ $Mad\text{-}CAM^+$ $VEGFR2^+$	Higher concentrations of mucosal-associated ($Mad\text{-}CAM^+$) endothelial EVs may suggest activation or damage of endothelial cells in critically unwell infants receiving ECMO
Publications examining EVs in neonatal respiratory disease						
Lal et al.[45]	Tracheal aspirates	50 samples for characterisation of respiratory EVs 80 samples for miRNA in prediction of bronchopulmonary dysplasia study	EV characterisation $N = 25$ severe bronchopulmonary dysplasia $N = 25$ term newborns intubated for non-respiratory surgical indications miRNA study $N = 18$ Discovery $N = 12$ Validation	Term: Ex 37 weeks ± 2 days with corrected age 38 weeks ± 2 days BPD: Ex 25 week ± 2 days corrected 37 weeks ± 2 days miRNA study: mean 24–25 weeks	Surface markers (depletion/cell of origin study): MUC4 (epithelial origin), CD66 (neutrophil origin) RNA: 802 exosomal miRNAs examined	Lower levels of miRNA 876-3p at birth predicts the development of BPD in at-risk infants
Go et al.[46]	Umbilical cord blood and peripheral blood (DOL 28 sample)	146 samples	Chronic Lung disease =3 9 Non-CLD = 34	CLD group: 25 ± 0.3 weeks Non-CLD group 28.2 ± 0.3	Surface marker: $CD9^+$, $CD63^+$ RNA: 62 miRNA transcripts identified as universally expressed in human EVs, of which only one miR-21 upregulated on DOL 28 in CLD vs. non-CLD	miR-21 expression on DOL 28 may predict the development of CLD
Publications examining EVs in neurological disease						
Tietje et al.[47]	Cerebrospinal fluid	23 samples	$N = 11$ (11 days old–2 years $N = 3$ (10 years old–15 years old) $n = 9$ (70–100 years old)	Nil described (11 days old–2 years)	EV markers: $ALIX^+$, $CD9^+$, $CD133^-$ RNA: 42 miRNAs uniquely expressed in under 2 year olds, 32 unique to>70-year-old group	EV concentration and miRNA concentrations fluctuate with age. hnRNPA2/B1 containing EVs are produced by the choroid plexus and decline with age
Goetzl et al.[49]	Central venous samples	42 samples	$n = 14$ undergoing therapeutic hypothermia sampled at 8, 10 and 14 h following commencement of hypothermia	36–41.3 weeks gestational age	Neuronal biomarkers: Synaptopodin, Synaptophysin, Neuronal specific Enolase, Cytochrome C Oxidase (COX IV)	Falling Synaptopodin levels in EVs may act as the biomarker for neuronal injury secondary to Hypoxic Ischaemic Encephalopathy
Spaull et al.[50]	Cerebrospinal fluid	7 samples (5 samples from 1 patient, 1 sample from 2 other patients)	$N = 3$	23–24 weeks	EV markers: $CD63^+/CD81^+$ RNA: 5 miRNA transcripts examined; miR-17, miR-26a, miR-124, miR-9, miR-1911	EVs and EV miRNA present in CSF in PHH. EV concentration reduces and miR1991 increases over time following PHH

Table 1. continued

Publication	Source	Number of samples	Number of patients	CGA of patients included	Contents/surface markers examined	Proposed effect/findings
Marell et al.[52]	Umbilical cord blood	79 samples	$N = 79$	36.3–41.6 weeks	EV marker: $CD81^+$ Neuronal marker: $CNTN\text{-}2^+$, $BDNF^+$	CNTN-2 and BDNF EVs in cord blood may be biomarkers for brain iron stores
Neonatal EVs and preeclampsia						
Jia et al.[57]	Umbilical cord blood	20 samples	$N = 10$ cases $N = 10$ controls All born by caesarean section	Preeclampsia cases 34.3 ± 4.6 weeks Controls 38.6 ± 5.5 weeks	Proteomics: 221 transcripts investigated, 29 differentially expressed proteins associated with enzyme regulation/binding. Further analysis identified 9 proteins associated with complement and coagulation cascades (C4BP, C4BPB, F13B, FGA, FGB, FGG, MBL2, PROS1, VWF)	Proteins associated with complement and coagulation cascades implicated in preeclampsia
Xueya et al.[58]	Umbilical cord blood/ paired maternal peripheral blood	38 neonatal samples 38 maternal samples	$N = 20$ controls $N = 18$ preeclampsia	Control 39 ± 0.22 Pre-eclampsia 33.3 ± 1.07	EV markers: $CD9^+$, $TSG101^+$, $Alix^+$ RNA: 1043 transcripts examined, 25 differentially expressed, 11 novel, 11 upregulated compared to control, 14 downregulated. VEGF signalling pathway identified among 10 by KEGG and GO analysis and examined due to the previous bio-plausibility	miR125a inhibits VEGFA expression leading to the impaired placental formation and the development of preeclampsia
Neonatal EVs examining the role of maternal dietary interventions						
Bruschi et al.[61]	Umbilical arterial blood	32 samples	$N = 12$ term infants $N = 10$ preterm infants with threatened preterm labour $N = 10$ preterm infants with threatened preterm labour treated with polyunsaturated fatty acids (n-3 PUFA) for 2 weeks	Term 39 weeks + 3 days (38–40 + 3) Preterm 31 + 3 weeks (30–32 + 5) Preterm PUFA 32 + 3 (31–34 + 2)	EV markers: $CD81^-/CD45^+$ Proteomics: 2312 transcripts identified, 1341 present in all samples, 165 unique to term infants, 206 unique to untreated preterm infants, 121 unique to treated preterm infants	PUFA treatment leads to lower oxidative damage and increases glutathione synthase activity
Neonatal EVs in intrauterine growth restriction						
Miranda et al.[64]	Umbilical cord blood Maternal peripheral blood	30 neonatal samples 30 maternal samples	$N = 10$ uncomplicated $N = 10$ foetal growth restriction $N = 10$ small for gestational age	Term deliveries 38.7–40.1 weeks median gestation	EV markers: $CD63^+$, $Flotillin\text{-}1^+$, $TSG101^+$, $Grp94^-$ Placental EV marker: $PLAP^+$	Ratio of placental exosomes to total exosomes positively correlates with neonatal and placental weight
EVs associated with umbilical cord blood stem cells						
Xagorari et al.[67]	Umbilical cord blood	37 samples	N = 37	NR	EV markers: Annexin V^+, $CD34^+$ (umbilical cord stem cell origin) RNA: miR106b family transcripts, miR 221, miR	$CD34^+$ EVs exist in cord blood and carry miRNAs

Table 1. continued

Publication	Source	Number of samples	Number of patients	CGA of patients included	Contents/surface markers examined	Proposed effect/findings
Huang et al.[68]	Umbilical cord blood Peripheral adult blood	5 neonatal samples 5 adult samples	$N = 5$ neonates $N = 5$ adults	39.74 ± 0.7	517c,miR 519d, miR 520 h highly expressed EV markers: $CD63^+$, $Alix^+$ RNA: 65 RNA transcripts differentially expressed, 6 umbilical specific miR-219a-2-3p, miR-3157-5p, miR 369-3p, miR 4746-5p, miR 485-5p, miR-668-3p	KEGG and GO analysis suggested the differentially expressed UCB miRNA were involved in pregnancy, cell mobility and nervous system development
Umbilical EVs associated with lactation						
Wang et al.[70]	Umbilical cord blood	70 samples	$N = 70$	38.51 ± 1.67	EV markers: $CD63^+$ $CytC^+$ RNA: 337 miRNAs identified, 85 "lactation related" based on alternative animal work and KEGG and GO analysis	Umbilical EVs may represent an important regulatory pathway for lactogenesis
EVs in neonatal urine/amniotic fluid						
Keller et al.[71]	Amniotic fluid Newborn/adult urine	4 amniotic fluid samples 5 neonatal samples NR adult samples	$N = 4$ foetus $N = 5$ neonates NR adults	NR (week 16 amniotic sampling)	EV markers: $CD9^+$, $HSP70^+$, Annexin I^+ Aquaporin 2^+ $CD24^+$	$CD24^+$ EVs are secreted by the kidney in amniotic fluid and urine

NR not recorded, *EV* extracellular vesicles, *GPIb* glycoprotein 1b, *CD* cluster of differentiation, *PDEV* platelet-derived extracellular vesicle, *DOL* day of life, VE-cadherin vascular endothelial cadherin, *PECAM* platelet endothelial cell adhesion molecule, *Mad-CAM* mucosal vascular adressin cell adhesion molecule, *VEGFR* vascular endothelial growth factor receptor, *MUC4* mucin 4, *miR* microRNA, *Alix* Alg-2-interacting protein X, *hnRNPA2/B1* heterogenous nuclear riboproteins A2/B1, *CSF* cerebrospinal fluid, *PHH* post-haemorrhagic hydrocephalus, *CNTN-2* contactin 2, *BDNF* brain-derived neurotrophic factor, *C4BP* C4-binding protein, *C4BPB* C4-binding protein beta, *F13B* factor XIIIb, *FGA* fibrinogen alpha chain, *FGB* fibrinogen beta chain, *FGG* fibrinogen gamma chain, *MBL2* mannose-binding lectin 2, *PROS1* protein S, *VWF* von Willebrand factor, *TSG101* tumour susceptibility gene 101, *KEGG and GO* Kyoto Encyclopaedia of Genes and Genomes-Gene Ontology, *PUFA* polyunsaturated fatty acids, *Grp94* glucose-regulated protein 94, *PLAP* placental alkaline phosphatase, *CytC* cytochrome *C*, *HSP70* heat-shock protein 70.

between age groups was identified. However, the number of SEVs in the CSF of children <2 years was significantly increased compared with teenagers and adults. Several differentially expressed miRNAs between the youngest and oldest groups were also identified. However, the indication for lumbar puncture in the youngest group was not clear. Without this information, confounding factors, such as febrile illness, may be missed.

Therapeutic hypothermia (TH) is a treatment that reduces the risk of death or disability, in infants with moderate to severe neonatal encephalopathy.[48] Goetzl et al.[49] described the release of neural SEVs using ELISA in peripheral blood of term infants undergoing TH at 8, 10, and 14 h after the initiation of treatment. It was shown that a decreasing slope of synaptopodin, a cytoskeletal protein and mediator of synaptic plasticity, over time was significantly associated with a longer LOS, higher need for anti-epileptics and a worse diffusion-weighted imaging summary score. Both NTA and ELISA were used to characterise EVs. Although this study did not describe the long-term outcomes, it identifies a potential biomarker of short-term outcomes in infants undergoing TH.

In 2019, Spaull et al.[50] analysed the CSF EVs of preterm neonates with post-haemorrhagic hydrocephalus (PHH). PHH is a progressive dilatation of the ventricles, which can occur after an intraventricular haemorrhage and is associated with a high risk of neuro-disability.[51] A heterogenous size and concentration of SEVs between the patients were shown using NTA. While two patients displayed a similar modal size of EV, all patients displayed similar concentrations of EVs within the 30–100 nm size. One infant had serial CSF analysis and there was a decrease in the particle concentration over time, with a corresponding increase in particle size. Although multiple techniques were used to characterise EVs, only three patients were included and only one had serial samples. Moreover, there was no correlation to the clinical outcomes.

The final study used UCB EVs to evaluate brain health in infants at risk of congenital iron deficiency.[52] Contactin 2 (CNTN-2) and brain-derived neurotrophic factor (BDNF) were used as markers of brain health. It was hypothesised that risk factors for congenital iron deficiency would result in lower levels of CNTN-2 and BDNF. ELISA was used to measure EV CNTN-2 and BDNF levels in UCB EVs and the results were compared to cord ferritin levels, the marker of congenital iron deficiency used. It was shown that low levels of EV CNTN-2 and high levels of EV BDNF were associated with low ferritin levels and thus markers of congenital brain iron deficiency. While this is a novel method of assessing possible brain iron deficiency, it was not possible to definitively measure the brain iron stores in this study. Similar to the last two studies,[49,50] the inclusion of the developmental outcomes would have strengthened the case for these two EV markers.

THE ROLE OF EVS IN PRENATAL AND PERINATAL DISEASE

EVs in preeclampsia

PET occurs in 3% of pregnancies and can be life-threatening.[53] It is responsible for 20% of preterm deliveries <1500 g and infants born to mothers with PET have a higher risk of intrauterine growth restriction (IUGR) and perinatal mortality.[54–56]

As previously discussed, Campello et al.[39] described the EVs released in UCB in infants born to mothers with and without PET. Using flow cytometry, a significant increase in the total LEV count (Annexin V), PDEVs (CD61), activated platelet-derived (CD62P), leucocyte-derived (CD45) and TF bearing EVs (CD142) was shown in the PET group, compared with healthy controls. Surprisingly, there was no difference in the number of eEVs (CD62E) in UCB between PET and healthy pregnancy.

Jia et al.[57] described the proteomic content of UCB EVs in pregnancies complicated by PET vs. healthy controls. NTA showed a higher concentration of SEVs in the PET group (statistical significance not described). This supports the findings in the previous study,[39] although flow cytometry and NTA measure particles of different sizes. The proteomic analysis identified a differential expression in 29 proteins and the pathways most associated with the PET group were the complement and coagulation pathways.

In 2020, Xueya et al.[58] described the differential EV miRNA expression in UCB between infants born to mothers with PET and without. Following EV miRNA analysis, 25 differentially expressed miRNA were identified, including miR125a-5p, which was increased in the PET group. The relatively increased expression of miR125a-5p was also demonstrated in maternal peripheral blood and placental tissue in the PET group. Using cell culture techniques, the authors demonstrated that miR125a-5p may inhibit angiogenesis by regulating VEGFA (vascular endothelial growth factor) and may be involved in the progression of PET.

It is important to note that these studies were designed to assess the pathophysiological mechanisms and maternal outcomes of preeclampsia and not the neonatal clinical outcomes. In each case, the controls were not gestational age matched.[39,57,58] The infants with PET were born at an earlier gestation, thus prematurity may confound the findings.

EVs in prematurity

Extremely preterm infants (born <28 weeks gestation)[59] are at high risk of neonatal death and long-term physical disability and neurodevelopmental impairment.[60] Bruschi et al.[61] described the effect of polyunsaturated fatty acid (PUFA) supplementation to mothers with threatened preterm labour. The EVs in UCB of the treated group ($n = 10$) were compared with term infants ($n = 12$) and untreated preterm infants ($n = 10$). Using mass spectrometry analysis, glutathione synthetase (GSS) was identified as the most discriminating marker between the groups. An ELISA of GSS showed that the levels were highest in the untreated preterm group, followed by treated preterm infants and then term infants. Moreover, higher levels of protein oxidation were demonstrated in the untreated preterm group. Antenatal treatment with PUFA may ameliorate some of the biochemical oxidative changes in preterm blood EVs and reduce inflammation. The clinical outcomes of the infants or safety data for the treatment were not described in this study.

EVs in IUGR

IUGR is defined as "a foetus with an estimated foetal weight <10th percentile that, because of a pathologic process, has not attained its biologically determined growth potential".[62] IUGR is associated with an increased risk of intrauterine death, intrapartum asphyxia, and poorer long-term neuro-development.[63] Miranda et al.[64] investigated the role of UCB EV markers in infants with IUGR compared with healthy controls (gestational age matched). Using fluorescence NTA with quantum dots bound to CD63 (EV marker) and placental-type alkaline phosphatase (PLAP), no differences in the total number of placental SEVs in UCB were found. However, the percentage of placental SEVs in UCB was significantly lower in IUGR babies and correlated with the severity of the growth restriction. Similar findings were shown in the maternal blood samples at the time of delivery, and thus identified the proportion of placental EVs in maternal blood as a potential diagnostic marker of IUGR. However, the findings would need to be replicated in maternal samples earlier in pregnancy and in larger numbers to be a useful clinical marker.

OTHER ROLES OF EVS IN NEONATOLOGY

Haematopoietic stem cells (HSCs) are multipotent cells, which generate all of the cellular blood components.[65,66] In 2019, Xagorari et al.[67] described the presence of HSC-LEVs (CD34) using flow cytometry in UCB of healthy term infants. Moreover, haematopoiesis-specific miRNA were identified in both $CD34^+$ cells and $CD34^+$ LEVs.

One study compared EV miRNA between UCB from healthy term infants and adults.[68] Using NTA, no difference in the size or concentration of SEVs was found. miRNA sequencing identified that the 30 most abundant miRNAs were similar between groups. Sixty-five differentially expressed miRNA were identified, and following functional analysis, using KEGG and GO (Kyoto Encyclopaedia of Genes and Genomes-Gene Ontology) pathways, the differentially expressed miRNAs were involved in pregnancy and reproduction, cell mobility, biogenesis of exosomes and nervous system pathways. Moreover, the authors showed that miRNA in UCB exosomes were very similar to the miRNA identified in UCB plasma identified in another study, reinforcing the miRNA enrichment of SEVs.[69]

Wang et al.[70] described EV miRNA related to lactogenesis, the process by which breast milk is produced, in UCB in healthy term infants. Sixty-nine lactation-related miRNA were identified in UCB LEVs. Moreover, the application of these EVs to epithelial mammary cells increased the production of b-casein, an important component of human breast milk. However, the lactation-related miRNA were not investigated in the maternal circulation during the peripartum period. This would have provided further insight into the regulation of human lactogenesis as the placenta is expulsed very shortly after delivery.

Finally, Keller et al.[71] demonstrated the presence of urinary SEVs during foetal life (amniotic fluid at 16 weeks gestation) and in neonatal and adult urine samples and the preservation of CD24 urinary EVs across species, detected here in a mouse model.

FUTURE DIRECTIONS OF NEONATAL EV RESEARCH AND CLINICAL APPLICATIONS

To date, coagulation and haemostasis are the most studied role of EVs in neonatology. Premature neonates are at high risk of haemorrhage, particularly IVH.[72] Although preterm infants have reduced levels of coagulation factors,[73,74] several studies have shown that thrombin generation is similar between preterm and term infants.[73,75,76] The procoagulant role of LEVs has been well described,[8] and it has been suggested that EVs may play a compensatory role in the preterm neonatal haemostatic system.[33,35,40]

The use of multiple platelet markers (CD42b,[31] CD41[32,34] and CD61[33,39]) and assays of procoagulant function (flow cytometry,[31] CAT,[35] ELISA[35,36,40] and procoagulant phospholipid assay[38,39]) may account for some of the variation in findings. Interestingly, none of the studies, which examined procoagulant EV function, described the incidence of clinically significant haemorrhage or thrombosis, although three included preterm infants,[31,39,40] at high risk of both haemorrhage[72] and thrombosis.[77,78]

Most studies demonstrated an increase in PDEVs in neonates, particularly preterm neonates.[31–33,39] Similarly, four demonstrated increased procoagulant activity of neonatal EVs.[35,36,39,40] Although two studies described reduced procoagulant activity in preterm[31] and term[38] infants compared with adults, the first suggests a plasma factor deficit rather than an EV deficit as the cause, and the second used a non-selective EV assay as the only evaluation technique.

These findings support the compensatory role of EVs in the neonatal haemostatic system. Future studies should use multiple methods to evaluate the procoagulant nature of EVs, in conjunction with appropriate EV identification and characterisation techniques (see discussion on MISEV below) and compare the results to clinical outcomes of haemorrhage and thrombosis.

Several studies described potential biomarkers of neonatal disease, including two which identified biomarkers of CLD.[45,46] The advantage of miR 876-3p in TA is its measurement in the first 6 h of life, thus allowing the early targeted treatment of at-risk infants.[45] Although the identification of a serum biomarker of CLD would be advantageous, allowing the diagnosis of CLD in infants who do not require early ventilation, there are some limitations of miR-21.[46] The usefulness of a biomarker of CLD, which is tested at DOL 28, is questionable, as the current treatments are instituted at an earlier time point,[79] and it may be apparent by DOL 28 which infants are likely to develop severe CLD. Two studies identified potential EV biomarkers of PET and IUGR; however, neither measured EVs in early pregnancy to confirm the findings in UCB.[58,64] Moving forward, the investigation of EV biomarkers should focus on clinically relevant time points and evaluate them in larger populations to determine whether they allow tailoring of treatment and improve clinical outcomes.

The biofluid of choice has not been established in neonates. Both UCB and neonatal blood were used in these studies. UCB may not be the optimal fluid for the evaluation of neonatal EVs, except in conditions such as PET, where placental EVs are relevant. Although the use of UCB as a surrogate for neonatal blood would be beneficial, due to the blood-volume limitations in infants,[80] obtaining adequate UCB samples from extremely preterm infants in the delayed cord clamping era may be a challenge.[81] It is not yet clear whether UCB EVs are representative of neonatal EVs in the first DOL and further work is required to evaluate this.

With regards to the MISEV guidelines,[4,20,21] there were variations in the pre-analytical variables in the studies described. Blood samples were collected in a variety of anti-coagulants, including sodium citrate,[25,31,34–36,38–42] CTAD (citrate, theophylline, adenosine and dipyridamole),[32,33] citrate phosphate dextrose,[67,68] EDTA (ethylenediaminetetraacetic acid),[46,49,52,58,64] and in three cases the anticoagulant used was not described.[57,61,70] The ideal anticoagulant for EV studies is not yet agreed upon, except advising against the use of heparin.[20,82–84] In addition, two studies described a prolonged period between sample collection and processing (up to 8 days and 48 h, respectively).[52,67] It has been shown that a delay between sample collection and processing alters the EV content.[85] Moreover, EVs were analysed in whole blood,[25,31–33,41] plasma[34–36,38–40,42] and serum,[46,49] and several studies isolated EVs.[46,49,52,57,58,61,64,67,68,70] The ISEV consider plasma as the optimal fluid to analyse blood EVs.[20] For those studies that isolated EVs, differential centrifugation,[57,61,67,68,70] density gradient centrifugation[64] and precipitation solutions[46,49,52] were used. In addition, the recommendation for general EV characterisation and the use of at least two techniques to characterise single EVs[21] were followed in some studies,[50,58,64,67,68,70] but not universally. While neonates are a unique group with significant challenges including blood-volume limitations, difficult phlebotomy and timing of samples with clinical samples, every effort should be made to comply with the recommendations,[4,20,21] to improve the quality and reliability of the results produced.

MISEV recommends the use of an appropriate control group.[20] This can be a challenge in neonatal studies, as intercurrent morbidities and gestational age may contribute to differences in results. Lal et al.[45] used term infants ventilated for non-respiratory pathology, and while these are not truly "healthy" controls, the rationale was well explained. Unfortunately, three studies did not include a control group, thus limiting the interpretation of the findings.[34,49,50] While some studies clearly described the timing of postnatal samples in cases and controls,[42] others did not state the age at which neonatal samples were collected.[25,41] Evidence suggests that postnatal age is relevant, as the concentration and composition of EVs changed between DOL 1 and DOL 3.[34] Similarly, the clinical indication for blood sampling in the controls was not always described. The studies of HDN highlight the difficulties in using infants with jaundice as controls, as even mild ABO HDN with a negative Direct Coombs test and not requiring phototherapy had significantly elevated number of eEVs.[25,41]

The clinical implications of EVs studied should always be described. The study by Lal et al.[45] demonstrates the clinical potential of EVs to describe the pathogenesis of neonatal disease

and identify useful biomarkers. However, little clinical information was provided in other studies.[47,50] Similarly, the long-term outcome of infants studied, particularly in neurological diseases, were not available and would have been useful to identify biomarkers of long-term prognosis.[49,50,52] Finally, the sample size chosen in many studies was small, with sizes ranging from 3 infants to 79 infants.

CONCLUSION

This review highlights the important function of EVs in the neonatal period. Many studies indicate a potential compensatory role of EVs in the neonatal haemostatic system, due to increased numbers of PDEVs,[31–33] TF-EVs[40] and procoagulant EV activity.[35,36] In addition, several biomarkers of neonatal pathologies have been described—miR 876-3p in TA and miR-21 in serum as markers of BPD and synaptopodin as a biomarker of short-term outcome of infants undergoing TH.[45,46,49] We must continue to build on this knowledge, evaluating the role of EVs in neonatal pathology, particularly in prematurity and during the perinatal adaption period. Moving forward, the use of larger numbers, robust EV characterisation techniques (according to MISEV guidelines[4,20,21]) and appropriate controls will improve the validity of results. The correlation of the EV profile with important clinical outcomes will broaden our understanding of the impact of EVs in this vulnerable population.

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AUTHOR CONTRIBUTIONS

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ADDITIONAL INFORMATION

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