Immune profile of non-COVID-19 community-acquired pneumonia: A comparison study between breast and artificially fed children

Abstract

Objective: Pneumonia is the primary cause of illness and death in children under five years of age and is a significant public health concern**.** Although infant pneumonia-related deaths have decreased over the past ten years, mortality is still high in many places. This comparative study was planned to identify the immunological profile of children with NCCAP among BFB and AFB groups.

Materials and methods: This study was a multi-center, a cross-sectional investigation that enlisted 190 participants, separated into three groups based on the forms of feeding received: 80 were BFB, 97 received artificial feeding, and 13 received mixed feeding. The participants' ages ranged from one to thirty months. Blood samples were taken to assess WBCs, and immunological factors such as TLR2, TLR4, IL-5, CRP, IgA, and IgE levels, as well as to determine pneumonia's microbiological etiology.

Results: The majority of cases occurred within the first year of life, with an equal sex involvement and a mean/SD of 9.8/10.2 months. 42.1% of babies were breastfeeding. In the majority of the involved immune parameters and post-Hoc multiple comparisons, there was a significant difference among the three feeding groups of breast, artificial, and mixed ($p<0.05$). There were no substantial differences in the majority of the involved immune variables according to the etiology of NCCAP. The age revealed non-significant correlations with all studied immune variables. All immunological indicators had the inadequate, non-significant ability to distinguish between breast and artificial feeding, according to a ROC-curve investigation

Conclusion

There were favorable correlations of the immune profile between breastfeeding versus artificial feeding among NCCAP patients. Breastfeeding should be encouraged internationally as an adjuvant primary preventive, along with new immunoprophylaxis and maternal vaccination regimens, as both partial or exclusive breastfeeding benefit children who develop NCCAP.

Keywords: Community-acquired, bacterial pneumonia, immune, lower respiratory tract infection, viral pneumonia,

Introduction

Particularly in developing nations, pneumonia represents the primary reason for illness and mortality in children during their first five years of age and is a substantial public health concern [1-3]. Around 0.7 million children under five years of age died from pneumonia in 2019, with the Middle East and North Africa reporting the peak rates of pneumonia episodes among children annually [4]. Although infant pneumonia-related deaths have decreased over the past ten years,

mortality is still high in many places. With an average annual percentage change of 6.6%, pneumonia is one of the reemerging diseases in Iraq [5].

According to the patient's location at the time of infection, pneumonia is frequently divided into the categories of hospital-acquired, community-acquired, and ventilator-acquired pneumonia [6].

However, after the identification of a novel coronavirus as the cause of an outbreak that may have been fatal in December 2019 in Wuhan, China, and unique pneumonia, the illness was designated "coronavirus disease 2019 (COVID-19)" [7-9].

A frequent respiratory infectious disease is community-acquired pneumonia (CAP). The incidence in general varies from 1 to 25 incidents per 1000 people per year. Hospital admission is necessary for about 40% of CAP patients, and 5% of these patient populations are admitted to the unit of intensive care [10].

Toll-like receptors (TLRs), one type of “pattern-associated molecular patterns (PRRs)”, identify specific microbial constituents and immediately trigger immune cells. Transmembrane TLRs, which have been extensively identified, are PPRs that, when activated, cause the release of inflammatory cellular cytokines and draw inflammatory cell populations to the infection site [11]. While TLRs, in general, appear to be highly expressed in pediatric pneumonia as a first line of defense against invasive pathogens, other studies have found significant differences in WBCs and TLR2,4 as in [12, 13]; WBCs and IL-5 as individual parameters as in [14, 15]; and WBCs with CRP in others [14]; were identifiable biomarkers of pediatric pneumonia.

Nevertheless, higher levels of immunoglobulin E (IgE) are linked to lower respiratory tract infections in general and to a higher rate of readmission in children with bronchopneumonia in particular [16]. Meanwhile, the immunoglobulins taken during nursing offer immediate defense against germs found in the gastrointestinal system because they are directly bound by the IgA in mother milk. Contrarily, to protect against infections that are localized in these locations, the immunoglobulins swallowed should be absorbed via the intestinal epithelium and transported to body organs through the blood (such as the lungs). But still, data indicates that the intestinal IgA absorption pathway is inefficient [17].

Recent studies in addition to several preceding pieces of research have demonstrated the association of breastfeeding with CAP incidence or severity [18-22] or with a lower incidence of bronchiolitis [23]. Meanwhile, other studies disagreed with such outcomes and reported that the cumulative incidence of lower or upper respiratory tract infections did not significantly or clinically relevantly reduce according to newborn feeding methods [24]. Similarly, being breast milk fed when mothers have respiratory infections may raise the risk of spread, acting as a proxy for close associates [17].

Nevertheless, discrepancy among the revisions was observed and no analysis has been conducted to describe an immunological comparison between BFB babies (BFB) and artificially fed babies (AFB) in cases with non-COVID-19 community-acquired pneumonia (NCCAP). Therefore, it was intended for this comparative study to determine the immunological profile of kids with NCCAP in the BFB and AFB groups.

Materials and methods

Study plan and patients' characteristics

The study was a cross-sectional, multi-center investigation that was part of a long line of investigations into the non-COVID-19 CAP. The study enlisted 190 participants, separated into three groups based on the forms of feeding that the children engaged received: 80 were BFB, 97 received artificial feeding, and 13 received mixed feeding. All participants were carefully selected with a history of full-term and normal weight at birth. The participants' ages ranged from one to thirty months. From November 2020 to April 2021, cases were chosen from pediatric hospitals in Babylon, in the center of Iraq. The pediatrician's identification of CAPn was made depending on the patient's history, clinical examination, lab blood tests, and plain chest X-rays. Additional information, such as their age, gender, history of feedings, and the length of the disease was obtained from the applicants. As well, blood samples were taken to assess WBCs, immunological factors such as TLR2, TLR4, IL-5, CRP, IgA, and IgE levels, as well as to determine pneumonia's microbiological etiology. Those with pneumonia caused by COVID-19 or due to other causes like aspiration pneumonia, tracheoesophageal fistula, foreign body inhalation, tuberculous pneumonia, pneumonia with septicemia, and immune-compromised children were excluded from this study. Children with inadequately defined feeding histories or whose caregivers were not close relatives were also excluded.

Ethical respects

All participants provided verbal informed consent, and the institutional health directorate and the hospital scientific committee in the public hospitals both accepted the study plan. The College of Pharmacy/University of Babylon's ethical scientific committee also gave its approval to the entire study methodology.

Hematological assays

Polymerase chain reaction tests were used to exclude patients with COVID-19 infection. An automated Coulter counter from "Beckman Coulter Analyzer® Life science-USA", was applied to assess the WBCs counts. Levels of serum CRP were calculated using the "Automatic Fluorescence Immunoassay technique by the immuno-analyzer AFIAS-6®" (Korea). All other parameters including IgE, IgA, TLR2, TLR4, and IL-5 were analyzed based on corresponding ELISA kits from "Bioassay Technology Laboratory Shanghai-China". To identify the microbial causation of pneumonia, the "indirect immunofluorescent assay, (Vircell®, Spain)" was used.

Statistical analysis

Microsoft Excel 2021 and SPSS IBM software for Windows, Version 21.0, USA, were used to conduct the statistical analysis. Student's t-test and Mann-Whitney U-tests were used to compare quantitative indicators based on the distribution. A chi-square test was applied to match qualitative traits. Multiple Post Hoc comparisons were used to assess the variations among the variables using an ANOVA technique. The ROC curve analyses were performed to estimate the efficacy of the constructed model as well as to determine the differentiating abilities of quantitative immunological parameters between BFB and AFB. Statistically, the 0.05 p-value was regarded as significant.

Results

Table 1 lists the general demographic and clinical appearances of children with NCCAP. The majority of cases occurred within the first year of life, with an equal sex involvement and a mean/SD of 9.8/10.2 months. 42.1% of babies were breastfeeding. Most of the patients' WBC counts were not significantly raised, and their immunological profiles were also shown in the table.

Table 1: General clinical and demographic features of pediatric patients with NCCAP

Characteristics	Mean	Std. Deviation	Range	Minimum	Maximum

Age/Months	9.8	10.2	59.0	1.0	30.0
Age classes No %	Less than 6 M	80	42.1%		
	6 - 12	68	35.8%		
	12 - 24	18	9.5%		
	24 - 36	13	6.8%		
	36 - 48	8	4.2%		
	> 48	3	1.6%		
Sex No %	Males	92	48.4%		
	Females	98	51.6%		
Types of feeding No %	Breast	80	42.1%		
	Artificial	97	51.1%		
	Mixed	13	6.8%		
Residency No %	Rural	97	51.1%		
	Urban	93	48.9		
Etiology of NCCAP No %	Negative	37	46.3%		
	Bacterial	26	32.5%		
	Viral	17	21.2%		
Period/days	7.7	8.0	58.0	2.0	60.0
WBC	7.3	2.8	14.9	3.2	18.1
TLR4	1.3	0.7	2.0	1.0	3.0
TLR2	1.5	0.7	2.0	1.0	3.0
Interleukin-5	2.3	0.7	2.0	1.0	3.0
IgA	108.1	146.6	659.2	5.2	664.4
IgE	2185.2	1568.5	4564.0	306.0	4870.0
CRP	9.9	21.2	159.0	0.5	159.5

The distribution of the immune parameters among the studied NCCAP children according to types of feeding was shown in Table 2. In the majority of the involved immune parameters and Post Hoc multiple comparisons, there was a substantial difference among the types of feeding ($p<0.05$).

Table 2: Distribution of the immune parameters among the studied NCCAP children according to types of feeding

Characteristics Mean (SE)	**Types of feeding**			**P-value**	**Post Hoc Tests**
	Breast (N-80)	Artificial (N-26)	Mixed (N-17)		
Age/Months	9.0 (1.0)	10.7 (1.1)	7.2 (3.5)	0.33	-
WBC	6.9 (0.3) *	7.4 (0.3)	8.8 (1.0) *	0.06	* 0.02
TLR4	5.5 (0.7) *	4.1 (0.4)	9 (2.8) *	0.011	* 0.046, 0.005
TLR2	11.2 (1.5) *	21.9 (1.8) *	17.4 (5.8)	0.001	* 0.001
Interleukin-5	120 (15.1)	105 (12.3) *	192.6 (39.6) *	0.07	* 0.023
IgA	109 (17.9) *	91.5 (13) *	226.6 (40.8) *	0.007	* 0.007, 0.002
IgE	2366.4 (162.9)	2116.4 (168.6)	1583.1 (397.6)	0.20	-
CRP	10.6 (2.6)	8.8 (1.9)	15.3 (5.7)	0.55	-

The distribution of the immune parameters among the studied NCCAP children according to the etiology of NCCAP was shown in Table 3. In the majority of the involved immune parameters and Post Hoc multiple comparisons, there were no substantial differences, except for the IL-5, which revealed significantly higher levels among bacterial NCCAP.

Table 3: Distribution of the immune parameters among the studied NCCAP children according to the etiology of NCCAP

Characteristics Mean (SE)	Etiology of NCCAP			P-value	Post Hoc tests
	Negative (N-37)	Bacterial (N-37)	Viral (N-37)		
Age/Months	11.5 (2.1)	10.7 (2.7)	7.8 (2.8)	0.61	-
WBC	8.9 (0.6)	8.5 (0.8)	8.2 (0.7)	0.81	-
TLR4	9.1 (1.5)	5.6 (1.3)	8.6 (2.1)	0.23	-
TLR2	17.5 (2.9)	17.3 (3.9)	17.4 (4.8)	1.0	-
Interleukin-5	216.6 (25.8)	245 (29.9) *	134.8 (24.5) *	0.052	* 0.017
IgA	208.1 (28.3)	246.3 (40.7)	154.9 (37.6)	0.27	-
IgE	2113.9 (287.5)	2131.8 (302.7)	2003.8 (423.8)	0.96	-
CRP	22.4 (5.9)	12.1 (4.1)	13.4 (8.1)	0.38	-

Among the studied immune parameters, TLR4, IL-5, and IgE was significantly varied between males and females of the studied NCCAP children, Table 4.

Table 4: Distribution of the immune parameters among the studied NCCAP children according to the gender of NCCAP

Characteristics Mean (SE)	Males (N-92)	Females (N-98)	P-value
Age/Months	8.9 (1.0)	10.6 (1.1)	0.24
WBC	7.2 (0.3)	7.4 (0.3)	0.59
TLR4	5.9 (0.6)	4.2 (0.4)	0.05*
TLR2	14.8 (1.7)	19.2 (1.8)	0.07
Interleukin-5	90.4 (12.4)	143.3 (13.6)	0.005*
IgA	106.6 (15.1)	110.4 (15.1)	0.82
IgE	1873.4 (160.7)	2477.9 (156.1)	0.008*
CRP	9.0 (1.9)	10.9 (2.4)	0.53

When the cases of CAP were classified into the suboptimal BFB (N-93) and purely AFB (N-97), the immune profile revealed non-significant variations (p-0.05) other than the levels of TLR2 (p-0.023) and TLR4 (p-0.001), Table 5.

Table 5: Compared distribution of the immune parameters among the studied NCCAP children between those on suboptimal breastfeeding and those on pure artificial feeding

Characteristics Mean (SE)	Suboptimal Breast (N-93)	Artificial (N-97)	P-value
Age/Months	8.7 (1.0)	10.7 (1.1)	0.17
WBC	7.2 (0.3)	7.4 (0.3)	0.62
TLR4	6.0 (0.8)	4.1 (0.4)	0.025*
TLR2	12.0 (1.5)	21.9 (1.8)	0.001*
Interleukin-5	130.2 (14.3)	105.6 (12.3)	0.19
IgA	125.4 (16.9)	91.5 (13)	0.11
IgE	2256.9 (152.6)	2116.4 (168.6)	0.53
CRP	11.2 (2.4)	8.8 (1.9)	0.42

When the cases of CAP were classified into those on pure breastfeeding (N-93) and those on mixed feeding (N-97), again the immune profile revealed non-significant variations (p-0.05) other than the levels of TLR2 (p-0.001), Table 6.

Table 6: Compared distribution of the immune parameters among the studied NCCAP children between those on pure breastfeeding and those on mixed feeding

Characteristics Mean (SE)	Pure Breast (N-80)	Mixed feeding (N-110)	P-value
Age/Months	9.0 (1.0)	10.3 (1.1)	0.36
WBC	6.9 (0.3)	7.5 (0.3)	0.11
TLR4	55.5 (0.7)	4.7 (0.5)	0.32
TLR2	11.2 (1.5)	21.4 (1.8)	0.001*
Interleukin-5	120 (15.1)	115.9 (12.0)	0.82
IgA	109 (16.9)	107.4 (13)	0.94
IgE	2366.49 (162.9)	2053.4 (156.3)	0.17
CRP	10.6 (2.7)	9.6 (1.8)	0.74

Worth mentioning, in all compared comparisons aforementioned in Tables 3, 4, and 5, the differences were not corrected by the ages of the NCCAP children. As

well, age revealed non-significant correlations with all studied immune variables, Table 7.

Table 7: The correlation of the ages of NCCAP children with the study's immune parameters.

		WBC	IgA	IgE	CRP	Interleukin-5	TLR2L	TLR4L
Age/Months	Pearson Correlation	0.03	0.03	0.08	0.13	0.13	0.05	0.11
	Significance	0.73	0.65	0.29	0.07	0.07	0.50	0.18

Table 8 displays the Pearson correlations among the study variables with each other among patients with CAP. All the results were not corrected by the effect of the age of the children at 0.01 and 0.05 significance levels.

Table 8: Pearson correlations among the study variables with each other among patients with NCCAP

Variables	Correlation	WBC	IgA	IgE	CRP	TLR2	TLR4	Interleukin-5
WBC	Pearson Correlation	-						
	Significance							
IgA	Pearson Correlation	0.029	-					
	Significance	0.801						
IgE	Pearson Correlation	0.193	0.200	-				
	Significance	0.087	0.075					
CRP	Pearson Correlation	0.003	0.238*	0.025	-			
	Significance	0.980	0.034	0.822				
TLR2	Pearson Correlation	-0.073	0.444**	.323**	0.164	-		
	Significance	0.522	0.000	0.003	0.146			
TLR4	Pearson Correlation	0.002	0.414**	0.323**	0.188	0.604**	-	
	Significance	0.989	0.000	0.004	0.095	0.000		
Interleukin-5	Pearson Correlation	0.087	0.432**	0.222*	0.192	0.420**	0.296**	-
	Significance	0.444	0.000	0.047	0.088	0.000	0.008	

*. Significant (2-tailed) correlation at the 0.05 level
**. Significant (2-tailed) correlation at the 0.01 level

The ROC curves (Figure 1) were made to further assess the potential differentiating value of all the studied immune parameters in predicting children breastfeeding from children on a bottle or mixed feeding in patients with NCCAP. The accuracy, sensitivity, specificity, significance, and 95%CI were displayed for each parameter.

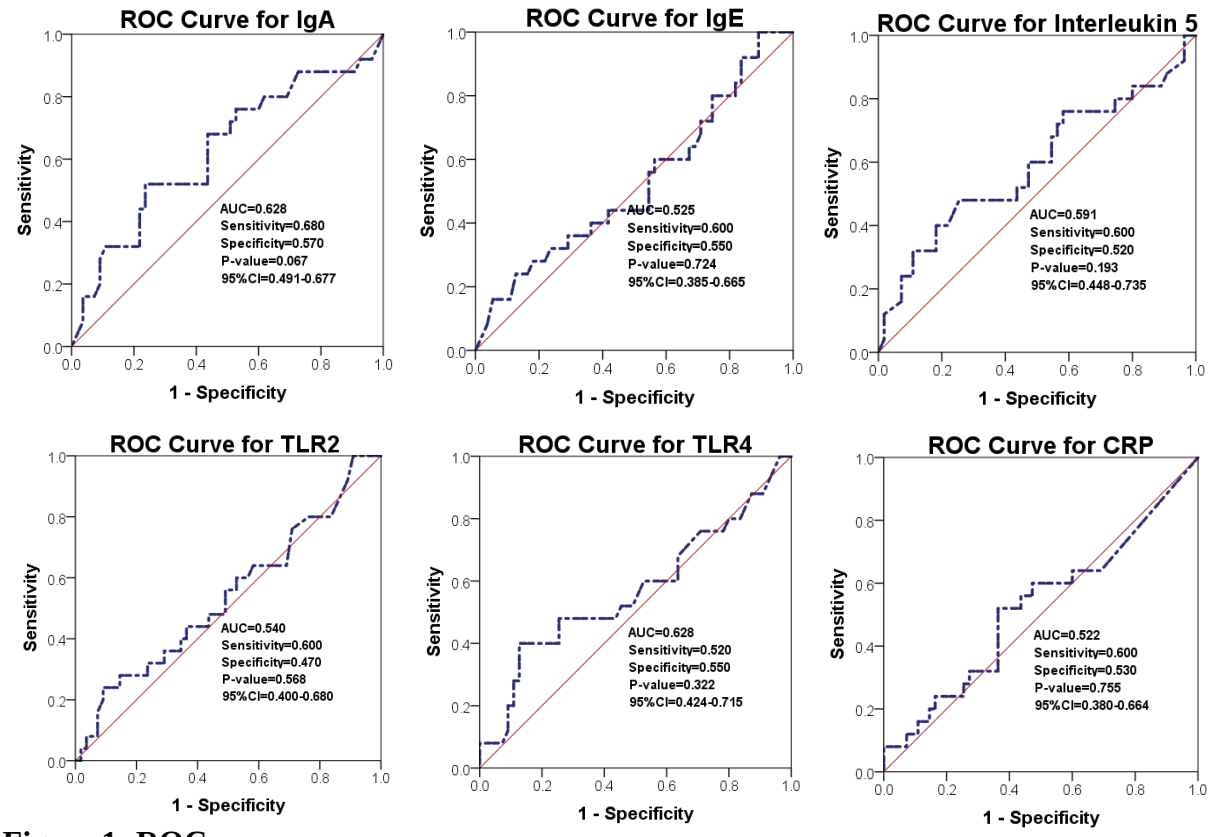

Figure 1: ROC curve analysis of several immunological study factors to distinguish between infants breastfed and infants fed artificially

Discussion

According to our knowledge, this study is the first to examine the host immunological profile of NCCAP in children in Iraq who were BFB with those who were fed artificially. The extensive immunological biomarkers and very specific inclusion and exclusion criteria were key strengths of our investigation.

In pediatric groups, our studies identified the circulatory immune indicators that were most commonly related to CAP severity. The stimulation of immunological responses causes enhanced immunopathology and imbalanced systemic inflammation in pneumonia [24].

Acute respiratory failure, mortality, and/or lung tissue injury are all consequences of these dysregulated immune responses. The plasma of critically ill pneumonic infants contains significant quantities of WBCs in addition to various cytokines

that were identified as relevant in our analysis, such as CRP, TLR2, TLR4, and IL-5, and these cytokines often signify hyperactivation of innate immune responses. Yet, given the abundant historical research that shows that breast milk provides significant immuno-protection, our results are intriguing.

Acute-phase protein (CRP) has significant multisystemic clinical utility, CRP is activated in response to nonspecific inflammation and/or tissue injury. Although earlier research revealed that CRP levels in adults' bronchial asthma, cardiovascular risk, psychological wellness, and oral health might be correlated [25-29], The relationship between CRP and early feeding is not well understood [30]. TNF and interleukins trigger the majority of CRP production in the liver [31, 32]. Several investigations on pediatric CAP have demonstrated that increased CRP readings are linked to poor outcomes and CAP severity. BFB and bottle-fed infants in our study had higher plasma concentrations of CRP that were comparable, and there were no discernible differences between any two study groups. Our findings corroborate earlier research by Roszkowska R. et al., who found no appreciable difference in hs-CRP levels between the groups of women who had never nursed and those who had done so [30]. In agreement with this, Martin RM. et al. identified no link between nursing and inflammation [33]. Similar findings were reported, and Lee et al. investigation confirmed that this biomarker does not appear to have a role in predicting secondary outcomes [34].

Contrarily, Rudnicka, et al. showed that breastfeeding for at least a month was linked to decreased CRP [35]. But so far, since both of the earlier trials were conducted on adult women, we can infer that nursing benefits cannot be discovered until later in infancy and childhood.

The lack of an association between breastfeeding and inflammatory indicators could have several causes. Breastfeeding may only have an impact on inflammatory markers in demographic subgroups with more prominent low-grade inflammatory status [35]. Another theory is that infants are too young to notice a significant reduction in NCCAP risk from nursing.

TLRs constitute the majority of significant receptors that are expressed either on the cell membrane (TLR1, 2, 4, 5, and 6) or within the cytoplasmic vesicles (TLR3, 7, 8, and 9) [36]. TLRs constitute the majority of significant receptors that are expressed either on the cell membrane (TLR1, 2, 4, 5, and 6) or within the cytoplasmic vesicles (TLR3, 7, 8, and 9) [36]. Transmembrane TLRs are PPRs that, when turned on, release inflammatory cytokines from cells and attract immune cell populations to the location of the infection. Several TLRs

communicate with the adaptor protein MyD88 to influence the production of cytokines. The critical role of NF-B in numerous physiological and pathological processes, including the control of inflammatory molecules, apoptosis, stress responses, and tumor growth inhibition, is found downstream of the TLR signal [11]. The MyD88-dependent circuit is a crucial pathway by which TLRs activate NF-kB. Research has demonstrated that the TLR/NF-kB pathway is directly associated with the incidence of lung disorders such as acute lung damage, chronic obstructive pulmonary disease, asthma, and lung cancer [37]. Activated NF-kB governs the expression of numerous inflammatory markers. By the release of TNF- and IL-1, which have been linked to asthma, and the translocation of NF-kB [11] and the release of TNF-α and IL-1β, which have a verified role in bronchial asthma [31, 32, 38, 39].

In a prior study, the correlation matrix between gestational age and the proportion of TLR2 and TLR4 positive cells failed to demonstrate any predictive value. Also, the study showed that pediatric illnesses, particularly bacterial pneumonia, had greater TLR levels [40]. TLR2 and TLR4 have dual functions in the CAP infection, in the induction of adequate immune responses to eradicate Mycoplasma pneumoniae, and in the induction of complications from Mycoplasma pneumoniae (hyperresponsiveness) [41]. However, no correlation between TLRs levels and age was found in our investigation. Moreover, Mycoplasma pneumoniae and the up-regulation of TLR2 and TLR4 were positively correlated, according to Ming C. et al. [11]. However, no correlation between TLRs levels and age was found in our investigation. Additionally, Mycoplasma pneumoniae and the up-regulation of TLR2 and TLR4 were positively correlated, according to Ming C. et al. [11]. TLR-2 and TLR-4 salivary analyses are useful in the identification of severe pneumonia, particularly in children. Recent research by Ozlem N. et al. has shown that measuring TLR-2 and TLR-4 levels in saliva can help diagnose severe pneumonia, particularly in children [42].

Although there were significant differences in the levels of IL- 5between participants receiving breast milk and those receiving artificial feeding in the current study, these differences were insufficient to distinguish between the two feeding methods using ROC curve analysis. Similar alterations in immunomodulatory components of human milk in response to an active illness in the breastfeeding newborn were described by Riskin A. et al. [43]. A newborn infant fed cow's milk had a selectively high level of blood IL-5, according to Yasunori K. et al. However, only neonatal infants with a history of allergy to cow milk were included in his study [44]. In contrast, infants who were BFB had lower

levels of IL-5 in their stool samples than infants who were fed formula according to research published in the American Journal of Clinical Nutrition [45]. Contrarily, a different study indicated that BFB children had higher amounts of IL-5 in their cord blood compared to formula-fed newborns, according to research published in the Journal of Allergy and Clinical Immunology [46].

Breastfeeding protects young children from pneumonia, especially in the first few months of life, according to various epidemiological studies that looked at the relationship between age and pediatric CAP [47-49]. Our study was unable to confirm these findings. This could be a result of the early establishment of intensive antibiotic therapy during NCCAP in our circumstances or as a result of the development of a new conjugate vaccine for children under the age of two, which could support the idea that the etiology of CAP is unrelated to the child's age [50].

Reviews of the literature and current clinical guidelines have identified areas of uncertainty in the management and investigation of children with CAP. Further research is required to answer the unresolved clinical questions so that clinicians can move further toward practicing evidence-based medicine [51].

There is growing evidence, while it is not definite, that infants who are BFB have lower IgE levels than newborns who are formula-fed, which may minimize their chance of developing allergy disorders. In comparison to formula feeding, breastfeeding was linked to decreased levels of total IgE and specific IgE to food allergens, according to a systematic review and meta-analysis published in the journal Nutrients in 2021 [52]. Even after correcting for mother allergy and other circumstances, a study published in the Journal of Human Lactation in 2020 revealed that BFB children had lower levels of specific IgE to cow's milk protein than infants who were formula-fed [53]. Even after correcting for mother allergy and other circumstances, a study published in the Journal of Human Lactation in 2020 revealed that BFB children had lower levels of specific IgE to cow's milk protein than infants who were formula-fed [53]. The authors failed to provide evidence to support these conclusions and found no differences between children who were bottle-fed and those who were BFB. To properly comprehend the variations in IgE levels between BFB and formula-fed infants, more research is required.

Besides this, as shock and/or sepsis, which manifest later, may share inflammation-related characteristics with severe CAP, these markers may be employed as early indicators of these outcomes. Further research is necessary to verify this theory.

Therefore, more research is still required to confirm the efficacy of such biomarkers as diagnostics for breast milk or prognostic indicators of illness severity.

Conclusion

There were favorable correlations of the immune profile between breastfeeding versus artificial feeding among NCCAP patients. Breastfeeding should be promoted internationally as adjuvant primary prevention, along with new immunoprophylaxis and maternal vaccination regimens, as both exclusive and partial breastfeeding benefit infants who develop NCCAP.

Acknowledgements

This study was performed as a part of the CAG (Clinical Academic Group) Center for Endotheliomics under the Greater Copenhagen Health Science Partners (GCHSP). Sources of Funding The study was supported by the Novo Nordisk Foundation (grants NNF14CC0001 and NNF17OC0027594) and the Innovation Fund Denmark (grant 5153-00002B). The funders played no role in the conduct of the study. Funding Novo Nordisk Foundation and the Innovation Fund Denmark

Additional information

Competing interests

Pär Ingemar Johansson: has received grants from the AP Møller Foundation, Innovation Fund Denmark and Novo Nordisk Foundation. The author has been issued the following patents: Publication no: 20110201553, 20110268732, 20130040898, 20130261177, 20150057325, 20160113891, 9381166, 9381243, 20160250164, 9433589, 20160303040 and US20090053193A1. PI Johansson reports ownership of stocks in Trial-Lab AB, Endothel Pharma ApS, TissueLink ApS, and MoxieLab ApS. PI Johansson declares that the financial interests listed have no impact on the submitted work. The author has no other competing interests to declare. The author declares that the financial interests listed have no impact on the submitted work. Søren Brunak: participates on the Danish National Genome Center advisory board and is the Chairman for the data infrastructure board. The author has stock in Intomics A/S, Hoba Therapeutics Aps, Novo Nordisk A/S, Lundbeck A/S and ALK Abello. The author participates on the board of directors for both Proscion A/S and Intomics A/S. The author has no other competing interests to declare. SB declares that the financial interests listed have no impact on the submitted work. The other authors declare that no competing interests exist.

Funding Funder Grant reference number Author Novo Nordisk Fonden NNF14CC0001 Søren Brunak Novo Nordisk Fonden NNF17OC0027594 Søren Brunak Innovation Fund Denmark 5153-00002B Søren Brunak The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Funding

Funder	Grant reference number	Author
Novo Nordisk Fonden	NNF14CC0001	Søren Brunak
Novo Nordisk Fonden	NNF17OC0027594	Søren Brunak
Innovation Fund Denmark	5153-00002B	Søren Brunak

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

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Ethics

Human subjects: This is a register-based study and informed consent for such studies is waived by the Danish Data Protection Agency. Data access was approved by the Danish Patient Safety Authority (3-3013-1731), the Danish Data Protection Agency (DT SUND 2016-50 and 2017-57) and the Danish Health Data Authority (FSEID 00003092 and FSEID 00003724).

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