MEDICAL UNIVERSITY – PLEVEN
FACULTY OF MEDICINE
DEPARTMENT OF PEDIATRICS

GENA STOYKOVA PETKOVA

IMMUNOLOGICAL ASPECTS OF VITAMIN D DEFICIENCY IN CHILDREN WITH RESPIRATORY DISEASES

ABSTRACT

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Assoc. Prof. Boyko Shentov, MD, PhD

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CONTENTS

List of Abbreviations

ARI	Acute Respiratory Infections
WHO	World Health Organization
CAP	Community-Acquired Pneumonia
CERA	Committee on Ethics of Research Activity
VDR	vitamin D receptor
1,25(OH)2D3/1,25(OH)2D	1,25-dihydroxyvitamin D_3; calcitriol
UVB	ultraviolet B radiation
7-DHC	7-dehydrocholesterol
DBP	vitamin D-binding protein
SZA	solar zenith angle
CYP	cytochrome P450
25(OH)D3/25(OH)D	25-hydroxyvitamin D_3; calcidiol
CYP2R1	cytochrome P450 2R1; 25-hydroxylase
ALB	albumin
CYP27B1	1α-hydroxylase
CYP24A1	24-hydroxylase
PTH	parathyroid hormone
FGF23	fibroblast growth factor 23
RXR	retinoid X receptor
VDREs	vitamin D response elements
HPLC	high-performance liquid chromatography
LC-MS/MS	liquid chromatography–tandem mass spectrometry
DEQAS	Vitamin D External Quality Assurance Scheme
NIST	National Institute of Standards and Technology
VDSP	Vitamin D Standardization Program
NIH	National Institutes of Health
CDC	Centers for Disease Control and Prevention

IOM/NAM	Institute of Medicine / National Academy of Medicine
EFSA	European Food Safety Authority
SACN	Scientific Advisory Committee on Nutrition
ECTS	European Calcified Tissue Society
NNR	Nordic Nutrition Recommendations
PRRs	pattern recognition receptors
PAMPs	pathogen-associated molecular patterns
DAMPs	damage-associated molecular patterns
CAMP/LL-37	cathelicidin
DEFB2/DEFB4/HBD2	β-defensin 2
TLRs	Toll-like receptors
TNF-α	tumor necrosis factor alpha
IL-1β	interleukin-1β
IL-6	interleukin-6
CRP	C-reactive protein
M.tb.	Mycobacterium tuberculosis
RSV	respiratory syncytial virus
DC	dendritic cells
APCs	antigen-presenting cells
UNICEF	United Nations Children's Fund
ReSVinet	Respiratory Syncytial Virus Network
BTS	British Thoracic Society
BSS-ped	Bronchitis Severity Scale – pediatric version
RT-PCR	Reverse Transcription Polymerase Chain Reaction
ECLIA	Electrochemiluminescence Immunoassay
ELISA	Enzyme-Linked Immunosorbent Assay
Mean ± SD	mean ± standard deviation
Me	median
IQR	interquartile range
Spearman’s rho, rs	Spearman correlation coefficient

RVS	rhinoviruses
HBOV	human bocavirus
HMPV	human metapneumovirus
ADV	adenovirus
HPIV3	human parainfluenza virus type 3
S.aureus	Staphylococcus aureus
Influenza A	influenza A virus
Str. Pneumoniae	Streptococcus pneumoniae
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
AAP	American Academy of Pediatrics

I. Introduction

Respiratory diseases, and particularly acute respiratory infections (ARIs), are a leading cause of morbidity and mortality among children worldwide. In recent years, extensive debate has focused on the role of vitamin D in the immune response to these infections and the potential need for vitamin D supplementation. Most studies indicate that low vitamin D levels are highly prevalent among infants and children with respiratory infections. However, maintaining normal to elevated serum 25(OH)D concentrations appears to have a beneficial effect on the incidence and severity of some, but not all, respiratory infections.

Vitamin D is a fat-soluble vitamin primarily recognized for its essential role in the regulation of calcium and phosphorus metabolism, which is crucial for normal bone growth and development. In recent years, considerable attention has been directed toward its additional pleiotropic effects, following the discovery of vitamin D receptors (VDRs) in various peripheral (extra-renal) tissues. Examples include lymphoid and myeloid cells, antigen-presenting dendritic cells, and macrophages, which increase their expression of VDRs during infections. This enhances the synthesis of the active form of vitamin D and promotes the production of endogenous antibiotics (antimicrobial peptides), such as cathelicidin and defensins, which play an important role in pulmonary antiviral defense. Furthermore, vitamin D inhibits the production of pro-inflammatory cytokines by monocytes, including IL-1β, IL-6, and TNF-α.

Vitamin D deficiency and insufficiency represent a global public health challenge, not only because of their involvement in various diseases but also due to the limited data available on vitamin D status in the populations of many countries. Information regarding vitamin D status among Bulgarian children, particularly its association with the incidence of acute respiratory infections, remains scarce. Only a limited number of studies have investigated vitamin D status in Bulgarian children, most of which have focused on specific conditions such as idiopathic scoliosis, myopia, gastrointestinal diseases, nephrotic syndrome, and others. An additional challenge is the lack of universal consensus and limited guidance regarding the definition of vitamin D status in both children and adults. The Institute of Medicine and the European Calcified Tissue Society define vitamin D deficiency as serum 25(OH)D concentrations below 50 nmol/L, severe deficiency as concentrations below 30 nmol/L, and vitamin D insufficiency as concentrations between 52.5 and 72.5 nmol/L. In the present study, we adopted the criteria recommended by the Bulgarian Society of Endocrinology for adults, which define vitamin D deficiency as serum 25(OH)D

concentrations <25 nmol/L and vitamin D insufficiency as concentrations between 25 and 50 nmol/L.

The present study aimed to expand the available data regarding vitamin D status in relation to respiratory infections among the Bulgarian pediatric population, focusing on clinically significant respiratory diseases, including acute pneumonia, acute bronchiolitis, and acute bronchitis.

II. AIM AND OBJECTIVES

Aim: To analyze the role of vitamin D and selected inflammatory markers in the clinical course of acute lower respiratory tract infections in childhood.

The following objectives were established to achieve this aim:

1. To perform a clinical and epidemiological analysis of children with acute lower respiratory tract infections for the period from 01.07.2021 to 30.12.2023.
2. To determine vitamin D status in children with acute respiratory infections by measuring serum levels of 25(OH) vitamin D and parathyroid hormone (PTH).
3. To determine the serum concentrations of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α during the course of acute respiratory infections.
4. To investigate the relationship between vitamin D status, pro-inflammatory cytokine levels, and the clinical course of acute respiratory tract infections in children.

III. MATERIALS AND METHODS

1. MATERIALS

1.1 Study Design

The present study was a single-center, prospective clinical and epidemiological investigation. The study was designed as an analytical cross-sectional study.

The study was conducted at the Department of Pediatrics, University Hospital "Dr. Georgi Stranski" EAD, Pleven, during the period from July 1, 2021, to December 30, 2023.

The main stages of the clinical study are presented in Figure 1.

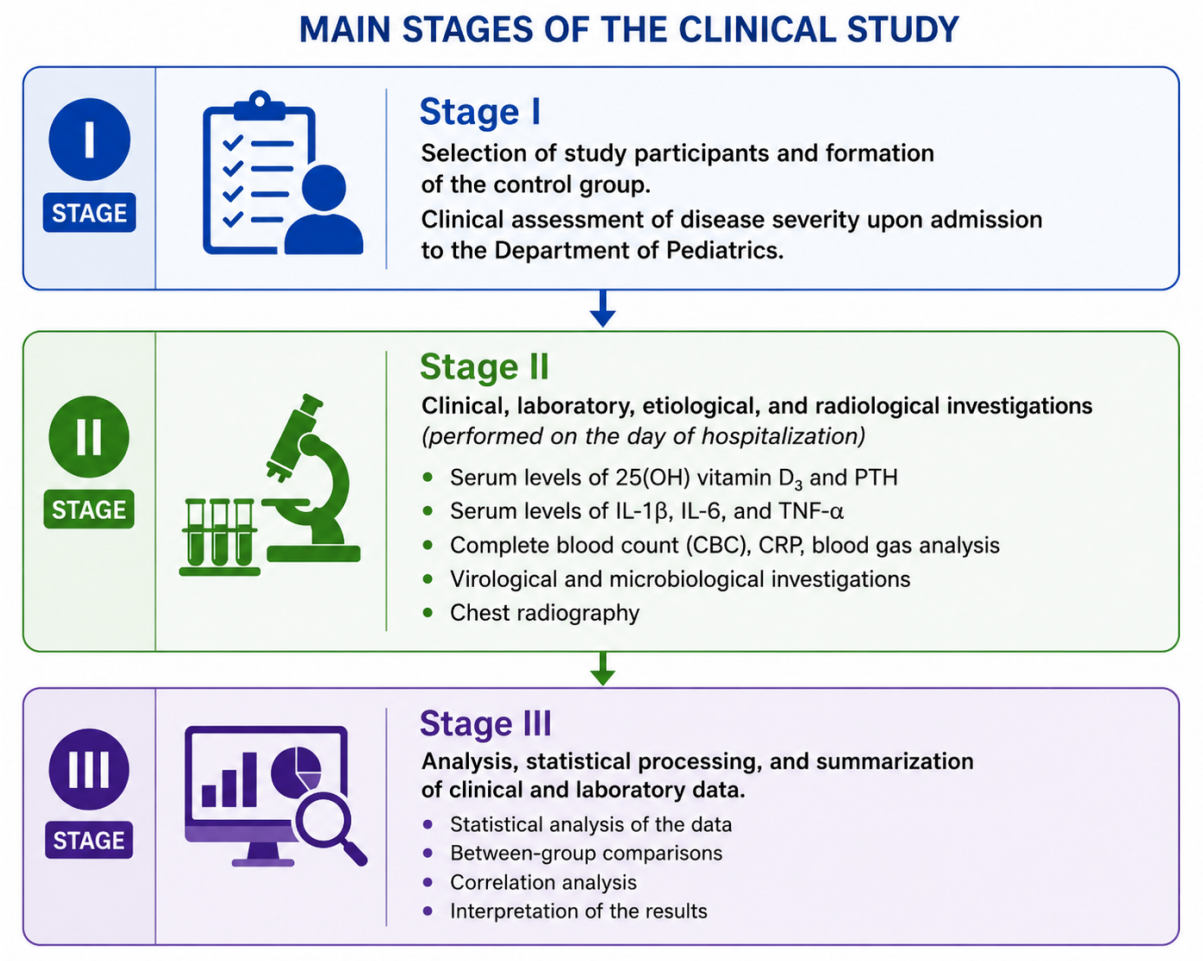


Figure 1. Main stages of the clinical study.

1.2. Patients

A total of 129 patients aged 0–17 years, hospitalized at the Department of Pediatrics for acute lower respiratory tract infections, were included in the study. The participants were allocated into four groups (Figure 2):

- Acute bronchopneumonia (n = 42)

- Acute bronchiolitis (n = 32)
- Acute bronchitis (n = 25)
- Control group (n = 30)

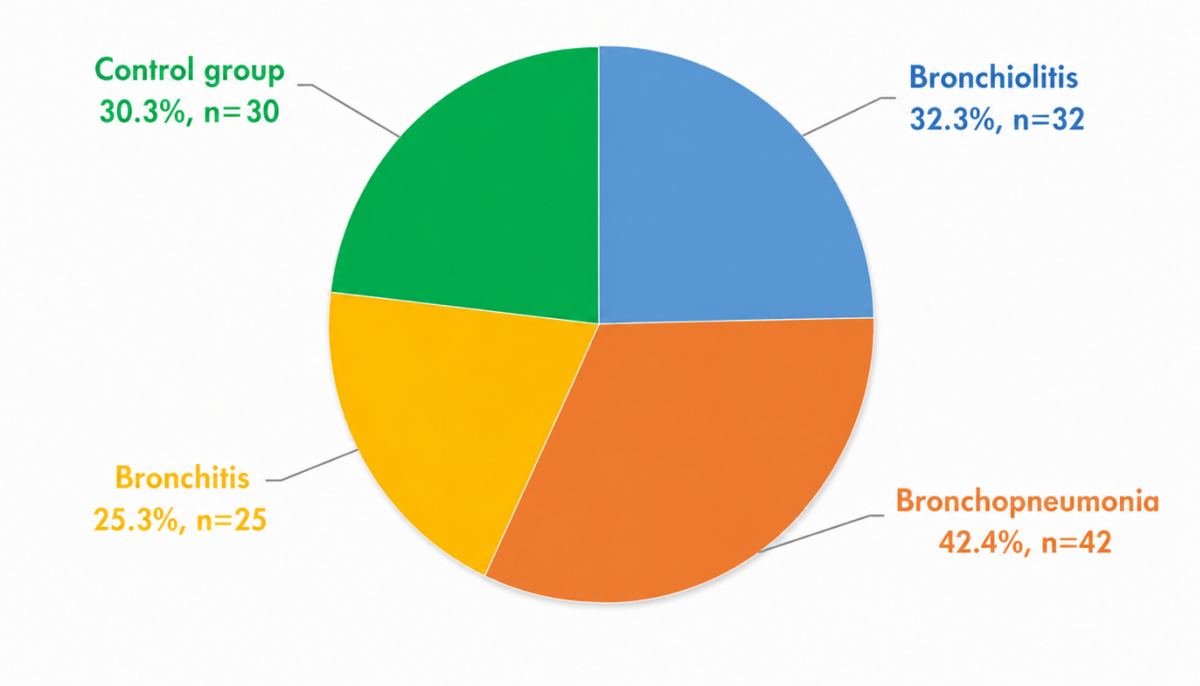


Figure 2. Distribution of the study participants by group.

The following inclusion and exclusion criteria were established for patient selection:

1. Inclusion Criteria - children aged 1 month to 17 years who were hospitalized with acute lower respiratory tract infections were included in the study. The diagnosis of the individual disease entities was established on the basis of generally accepted clinical and radiological criteria.

2. Exclusion Criteria

- Patients with acute non-infectious respiratory diseases (foreign body aspiration, congenital anomalies);
- Patients with chronic respiratory and cardiovascular diseases (bronchial asthma, cystic fibrosis, primary ciliary dyskinesia, primary immunodeficiency disorders, congenital heart defects);
- Patients with renal and endocrine diseases that could adversely affect vitamin D metabolism and alter its serum levels.

The control group consisted of healthy children with an age distribution comparable to that of the study participants, who underwent routine preventive examinations at the Department of Pediatrics.

2. METHODS

2.1. Clinical and radiological methods used for the classification of the study participants according to their diagnosis

2.1.1. Clinical Data

- **Medical history** – data were collected regarding the course of the acute illness, physical development, family history, history of previous and concomitant diseases, as well as current vitamin D supplementation.
- **Season of hospitalization** – for the purposes of the analysis, patients were categorized according to the season of admission to the Department of Pediatrics as follows: winter (December–February), spring (March–May), summer (June–August), and autumn (September–November). Seasonality was used as an additional factor in the assessment of serum 25(OH) vitamin D levels.
- **Physical examination** – a standard clinical examination was performed, including assessment of the patient's general condition, the presence of physical signs of an acute inflammatory process involving the lower respiratory tract, and evaluation of disease severity. Height and body weight were measured to assess physical development.

2.1.2. Radiological Data

Chest radiography was performed at the Department of Diagnostic Imaging, University Hospital “Dr. Georgi Stranski” EAD, Pleven.

2.2. Clinical and Laboratory Assessment of Disease Severity

2.2.1. Acute Bronchiolitis

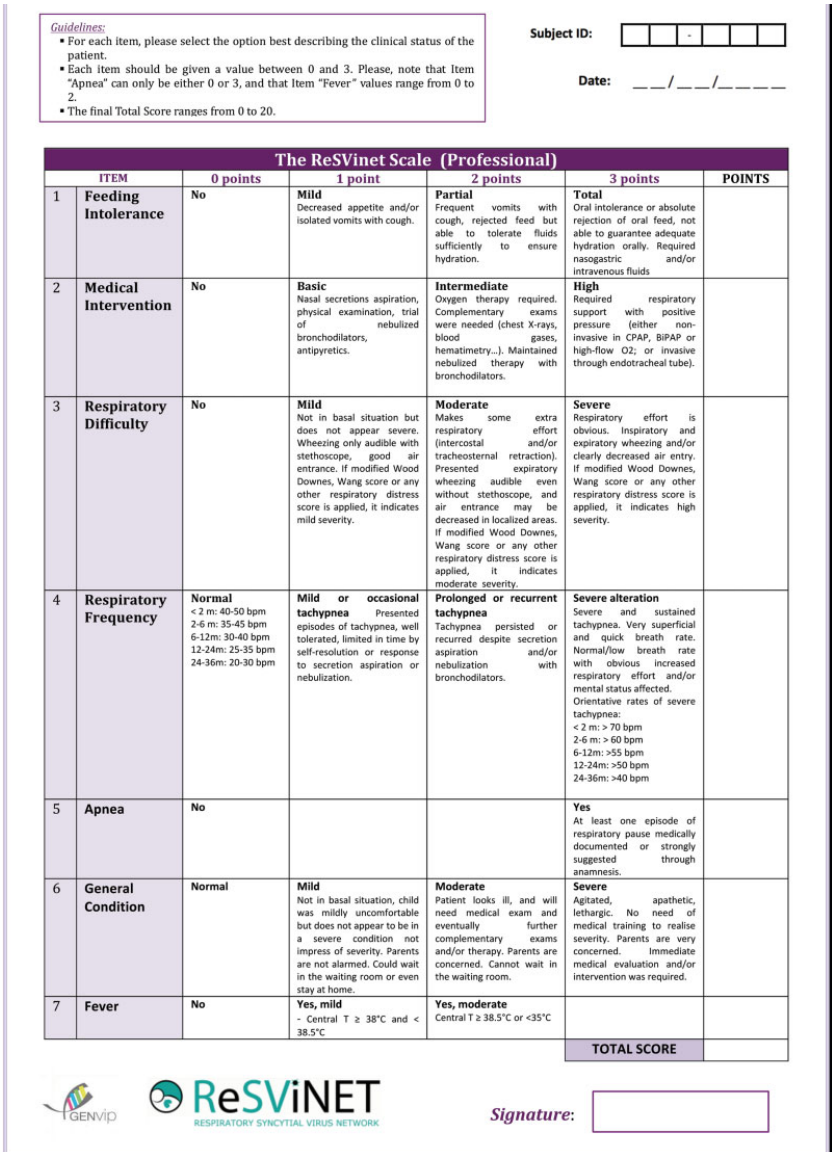
Disease severity was assessed using the Respiratory Syncytial Virus Network (ReSVinet) scale.The scale includes seven parameters: feeding difficulties, need for medical intervention, respiratory distress, respiratory rate, apnea, general condition, and body temperature (Table 1).

Each parameter is scored from 0 to 3 points, with higher scores indicating greater disease severity, except for apnea (scored as 0 or 3 points) and body temperature (scored as 0,

1, or 2 points). The total score, calculated as the sum of all components, ranges from 0 to 20 points.

The cut-off values for severity assessment were as follows:

- 0–6 points – mild disease;
- 7–13 points – moderate respiratory distress;
- 14–20 points – severe respiratory involvement.



Guidelines:
- For each item, please select the option best describing the clinical status of the patient.
- Each item should be given a value between 0 and 3. Please, note that Item "Apnea" can only be either 0 or 3, and that Item "Fever" values range from 0 to 2.
- The final Total Score ranges from 0 to 20.

Subject ID: ☐☐☐-☐☐☐

Date: __/__/____

The ReSVinet Scale (Professional)

	ITEM	0 points	1 point	2 points	3 points	POINTS
1	**Feeding Intolerance**	**No**	**Mild** Decreased appetite and/or isolated vomits with cough.	**Partial** Frequent vomits with cough, rejected feed but able to tolerate fluids sufficiently to ensure hydration.	**Total** Oral intolerance or absolute rejection of oral feed, not able to guarantee adequate hydration orally. Required nasogastric and/or intravenous fluids	
2	**Medical Intervention**	**No**	**Basic** Nasal secretions aspiration, physical examination, trial of nebulized bronchodilators, antipyretics.	**Intermediate** Oxygen therapy required. Complementary exams were needed (chest X-rays, blood gases, hematimetry...). Maintained nebulized therapy with bronchodilators.	**High** Required respiratory support with positive pressure (either non-invasive in CPAP, BiPAP or high-flow O2; or invasive through endotracheal tube).	
3	**Respiratory Difficulty**	**No**	**Mild** Not in basal situation but does not appear severe. Wheezing only audible with stethoscope, good air entrance. If modified Wood Downes, Wang score or any other respiratory distress score is applied, it indicates mild severity.	**Moderate** Makes some extra respiratory effort (intercostal and/or tracheosternal retraction). Presented expiratory wheezing audible even without stethoscope, and air entrance may be decreased in localized areas. If modified Wood Downes, Wang score or any other respiratory distress score is applied, it indicates moderate severity.	**Severe** Respiratory effort is obvious. Inspiratory and expiratory wheezing and/or clearly decreased air entry. If modified Wood Downes, Wang score or any other respiratory distress score is applied, it indicates high severity.	
4	**Respiratory Frequency**	**Normal** < 2 m: 40-50 bpm 2-6 m: 35-45 bpm 6-12m: 30-40 bpm 12-24m: 25-35 bpm 24-36m: 20-30 bpm	**Mild or occasional tachypnea** Presented episodes of tachypnea, well tolerated, limited in time by self-resolution or response to secretion aspiration or nebulization.	**Prolonged or recurrent tachypnea** Tachypnea persisted or recurred despite secretion aspiration and/or nebulization with bronchodilators.	**Severe alteration** Severe and sustained tachypnea. Very superficial and quick breath rate. Normal/low breath rate with obvious increased respiratory effort and/or mental status affected. Orientative rates of severe tachypnea: < 2 m: > 70 bpm 2-6 m: > 60 bpm 6-12m: >55 bpm 12-24m: >50 bpm 24-36m: >40 bpm	
5	**Apnea**	**No**			**Yes** At least one episode of respiratory pause medically documented or strongly suggested through anamnesis.	
6	**General Condition**	**Normal**	**Mild** Not in basal situation, child was mildly uncomfortable but does not appear to be in a severe condition not impress of severity. Parents are not alarmed. Could wait in the waiting room or even stay at home.	**Moderate** Patient looks ill, and will need medical exam and eventually further complementary exams and/or therapy. Parents are concerned. Cannot wait in the waiting room.	**Severe** Agitated, apathetic, lethargic. No need of medical training to realise severity. Parents are very concerned. Immediate medical evaluation and/or intervention was required.	
7	**Fever**	**No**	**Yes, mild** - Central T ≥ 38°C and < 38.5°C	**Yes, moderate** Central T ≥ 38.5°C or <35°C		
					TOTAL SCORE	

GENVIP ReSViNET RESPIRATORY SYNCYTIAL VIRUS NETWORK

Signature:

Table 1. Assessment of Acute Bronchiolitis Severity Using the ReSVinet Scale

Parameter	**0 points**	**1 point**	**2 points**	**3 points**
Feeding / food intake	Normal	Slightly reduced intake	Markedly reduced intake	Inability to feed
Medical intervention	Not required	Observation only	Oxygen therapy required	Intensive care / mechanical ventilation required

Respiratory distress	Absent	Mild	Moderate	Severe
Respiratory rate	Normal for age	Slightly increased	Moderately increased	Markedly increased / apnea
Apnea	Absent	—	—	Present
General condition	Good	Mildly impaired	Moderately impaired	Severely impaired / lethargy
Body temperature	< 37.5°C	37.5–38.0°C	38.1–38.5°C	> 38.5°C

2.2.2. Acute Pneumonia

The severity of the clinical course was assessed on the basis of generally accepted clinical criteria, in accordance with the recommendations of the World Health Organization (WHO) and the British Thoracic Society (BTS). The assessment included evaluation of the general condition, respiratory rate, use of accessory respiratory muscles, presence of expiratory grunting, cyanosis, altered level of consciousness, as well as blood gas analysis parameters and oxygen saturation (Table 2).

Table 2. Summary of Clinical Criteria for the Assessment of Pneumonia Severity in Children According to the Recommendations of the World Health Organization and the British Thoracic Society

Clinical Parameter	**Mild Disease**	**Moderate Disease**	**Severe Disease**
General condition	Preserved	Moderately impaired	Severely impaired, lethargy/altered consciousness
Respiratory rate	Normal for age: < 2 months: < 60/min 2–12 months: < 50/min 1–5 years: < 40/min	Increased for age: ≥ 60/min (< 2 months) ≥ 50/min (2–12 months) ≥ 40/min (1–5 years)	Markedly increased above age-specific norms or apnea
Respiratory distress	Absent or mild	Presence of chest retractions	Marked chest retractions, use of accessory respiratory muscles
Expiratory grunting	Absent	May be present	Frequently pronounced
Nasal flaring	Absent	May be present	Pronounced
Cyanosis	Absent	Perioral (possible)	Central cyanosis
Feeding/fluid intake	Preserved	Reduced	Inability to feed
Oxygen saturation (SpO_2)	≥ 94%	90–93%	< 90%
Blood gas analysis (pO_2)*	≥ 80 mmHg	60–79 mmHg	< 60 mmHg

Capillary refill time	< 2 sec	Delayed	≥ 2 sec
Other signs	Absent	Mild dehydration	Seizures, severe dehydration

* The pO_2 values are indicative and should be interpreted with caution when derived from capillary blood samples.

2.2.3. Acute Bronchitis

The severity of acute bronchitis was assessed using the BSS-ped (Bronchitis Severity Scale – pediatric version), based on the evaluation of three principal clinical symptoms—cough, wheezing on auscultation, and dyspnea—each scored on a five-point scale ranging from 0 to 4 points. The total score ranges from 0 to 12 points and reflects the severity of the disease (Table 3).

Table 3. Assessment of Acute Bronchitis Severity in Childhood (BSS-ped)

Clinical Parameter	Mild Disease	Moderate Disease	Severe Disease
Cough	Mild, occasional	Frequent, pronounced	Severe, paroxysmal
Wheezing on auscultation	Sparse or absent	Present, bilateral	Pronounced, diffuse
Dyspnea	Absent or mild	Moderate	Severe
Total score (BSS-ped)	2–4 points	5–7 points	≥ 8 points

2.3.Identification of the Etiological Agents of Infection

The etiological agents of infection were identified using the following methods:

- Examination of nasopharyngeal secretions for the detection of viral pathogens at the National Reference Laboratory "Influenza and Acute Respiratory Diseases", National Center of Infectious and Parasitic Diseases (NCIPD), Sofia, using Real-Time RT-PCR (Reverse Transcription Polymerase Chain Reaction);
- Microbiological examination of throat swab specimens at the Microbiology Laboratory of University Hospital "Dr. Georgi Stranski", Pleven;
- Examination of nasopharyngeal secretions for the detection of viral pathogens using antigen tests for SARS-CoV-2, Influenza A, and Influenza B at the Microbiology Laboratory of University Hospital "Dr. Georgi Stranski", Pleven.

2.4. Laboratory Methods

2.4.1 Quantitative Determination of Serum 25(OH) Vitamin D_3 and Parathyroid Hormone (PTH) Levels

The assessment of serum 25(OH) vitamin D_3 and parathyroid hormone (PTH) levels was carried out within the framework of Research Project No. 8/2021, entitled "Assessment of Vitamin D Status in Children with Acute Respiratory Infections", conducted by the Department of Pediatrics at the Medical University – Pleven and the Clinical Laboratory of Exacta Medica Medical Center JSC, Pleven. The project was funded by the Medical University – Pleven.

The study was conducted at the Department of Pediatrics, University Hospital "Dr. Georgi Stranski", Pleven, and the Clinical Laboratory of Exacta Medica Medical Center JSC, Pleven. The study protocol was approved by the Research Ethics Committee of the Medical University – Pleven (see Appendix 1). Participants were enrolled voluntarily after obtaining informed consent from their parents or legal guardians (see Appendix 2). A study-specific patient clinical record form was developed and completed for each participant (see Appendix 3).

Venous blood samples were collected from all patients on the day of hospitalization. Following centrifugation, serum samples were used for the determination of 25(OH) vitamin D and PTH levels.

2.4.1.1. Serum 25(OH)D Level

The determination of serum 25(OH) vitamin D_3 was performed using an in vitro competitive immunoassay on a Roche Cobas e411 immunoassay analyzer. The method is based on an electrochemiluminescence immunoassay (ECLIA), in which the concentration of the analyte is automatically calculated using a calibration curve generated through two-point calibration and reference information encoded in the reagent barcode.

Analytical characteristics of the method:

- Measuring range: 4–100 ng/mL (10–250 nmol/L);
- Lower limit of detection: 4 ng/mL (10 nmol/L);
- Analytical accuracy: up to 4 ng/mL.

Conversion factors:

- ng/mL × 2.5 = nmol/L;
- nmol/L × 0.4 = ng/mL.

Vitamin D status was assessed according to the recommendations of the Bulgarian Society of Endocrinology (2019), as follows:

- >120 nmol/L – hypervitaminosis;
- 50–120 nmol/L – sufficient level;
- 25–49 nmol/L – insufficiency;

- <25 nmol/L – deficiency.

2.4.1.2. Serum Parathyroid Hormone (PTH) Level

The serum PTH level was determined using an electrochemiluminescence immunoassay (ECLIA) performed on a Roche Cobas e411 analyzer. The method is based on a sandwich immunoassay for the quantitative determination of intact PTH. Hormone concentrations were calculated using a calibration curve generated by two-point calibration and reference information encoded in the reagent barcode. Serum obtained from venous blood samples was used for the analysis. Blood samples were centrifuged immediately after collection due to the short half-life of PTH.

Analytical characteristics of the method:

- Measuring range: 1.20–5000 pg/mL;
- Lower limit of detection: 1.20 pg/mL.

The results were interpreted according to the reference values of the assay:

- 15–65 pg/mL – normal range;
- >65 pg/mL – elevated level;
- >100 pg/mL – markedly elevated level.

2.4.2. Quantitative Determination of Interleukin-1 Beta (IL-1β), Interleukin-6 (IL-6), and Tumor Necrosis Factor Alpha (TNF-α)

The assessment of IL-1β, IL-6, and TNF-α was carried out within the framework of Research Project No. 21/2022, entitled “Immunological Aspects of Vitamin D Deficiency in Children with Acute Respiratory Diseases”, conducted by the Department of Pediatrics at the Medical University – Pleven and the Immunology Laboratory of University Hospital “Dr. Georgi Stranski” EAD, Pleven. The project was funded by the Medical University – Pleven.

The project was conducted at the Department of Pediatrics and the Immunology Laboratory of University Hospital “Dr. Georgi Stranski” EAD, Pleven.

The study protocol was approved by the Research Ethics Committee of the Medical University – Pleven (see Appendix 4). Participants were enrolled voluntarily after obtaining informed consent from their parents or legal guardians (see Appendix 5). A study-specific patient clinical record form was developed and completed for each participant (see Appendix 6).

Venous blood samples were collected from all patients on the day of hospitalization. Following centrifugation, serum samples were stored at −20°C until analysis.

Laboratory method:

Quantitative determination of serum IL-1β, IL-6, and TNF-α levels was performed using enzyme-linked immunosorbent assay (ELISA) with commercially available assay kits from Thermo Fisher Scientific (Invitrogen). The method is based on a sandwich immunoassay, in which the target cytokine in the sample binds to specific antibodies immobilized on a microplate, followed by the addition of a biotinylated antibody and a streptavidin–HRP conjugate. After the addition of the substrate (TMB), a colorimetric reaction develops that is proportional to the concentration of the analyzed cytokine. Optical density was measured at 450 nm (reference wavelength 620–630 nm) using a Stat Fax 2100 ELISA reader, and cytokine concentrations were determined from a calibration curve.

Measuring range:

- IL-1β: 3.9 – 250 pg/mL
- IL-6: 1.56 – 100 pg/mL
- TNF-α: 7.8 – 500 pg/mL

Limit of detection:

- IL-1β: 0.3 pg/mL
- IL-6: 0.92 pg/mL
- TNF-α: 2.3 pg/mL

Due to the absence of standardized reference ranges for serum IL-1β, IL-6, and TNF-α levels, the results were analyzed as continuous quantitative variables and compared among the study groups.

2.4.3. Other Laboratory Investigations

Standard laboratory investigations were performed at the Clinical Laboratory of University Hospital "Dr. Georgi Stranski", Pleven, including blood gas analysis, complete blood count, and C-reactive protein (CRP), using standard laboratory methods:

- C-reactive protein (CRP) measurement by an immunoturbidimetric method;
- Complete blood count (CBC) performed using an automated hematology analyzer (3-diff and 5-diff);
- Blood gas analysis performed by a potentiometric method using a Medica Easy Blood Gas analyzer.

2.5. Statistical Methods for Data Analysis

The data were entered and analyzed using **IBM SPSS Statistics for Windows, version 26.0** and **Microsoft Excel 2019**. A p-value of <0.05 was considered statistically significant, and the null hypothesis was rejected at this level.

The following statistical methods were applied:

• **Descriptive analysis** – qualitative variables were summarized as absolute and relative frequencies (number and percentage), whereas quantitative variables were presented as mean ± standard deviation (Mean ± SD) for normally distributed data and as median (Me) with interquartile range (IQR) for non-normally distributed data.

• **Tests of normality** – the Kolmogorov–Smirnov test was used to assess the distribution of quantitative variables.

• **Parametric methods** – Student's t-test and analysis of variance (ANOVA) were applied for normally distributed data.

• **Non-parametric methods** – the Mann–Whitney U test and the Kruskal–Wallis test were used for non-normally distributed data.

• **Analysis of categorical variables** – the χ^2 (Chi-square) test was applied to evaluate associations between categorical variables.

• **Correlation analysis** – correlations were assessed using Spearman's rank correlation coefficient (Spearman's rho, rs), while Pearson's correlation coefficient was used for normally distributed variables.

IV. RESULTS AND DISCUSSION

1. Results and Discussion for Objective 1:

To perform a clinical and epidemiological analysis of children with acute lower respiratory tract infections for the period from July 1, 2021, to December 30, 2023.

The study included 129 children with acute respiratory infections who were treated at the Department of Pediatrics, University Hospital "Dr. Georgi Stranski" EAD, Pleven, during the period from July 1, 2021, to December 30, 2023.

1.1 Age Distribution of the Study Children

The mean age of the study population (0–17 years) was 2.58 ± 3.90 years. Children with acute respiratory infections (patients) had a mean age of 2.63 ± 3.65 years, whereas the mean age of the healthy control group was 2.38 ± 4.69 years. Age is presented in months in Table 4.

Patients with acute bronchiolitis were between 2 months and 2 years of age, corresponding to the typical age distribution of this disease. The inclusion of these patients contributed to the lower mean age of the study population.

No statistically significant differences were found between patients and healthy controls with regard to age, height, or body weight (Table 4). This indicates good comparability between the study groups in terms of the main demographic and anthropometric characteristics.

The absence of statistically significant differences suggests that these variables did not act as major confounding factors in the investigated associations, thereby allowing a more reliable interpretation of the study findings.

Table 4. Descriptive Statistics of the Demographic Characteristics of the Study Participants

Characteristics	Patients (n=99)	Healthy Controls (n=30)	Statistical Significance (p-value)
Demographic characteristics			
Sex, n (%)			>0.05
• Female	40 (40.4%)	12 (40.0%)	
• Male	59 (59.6%)	18 (60.0%)	
Age (months), Mean ± SD; Me (IQR)	31.6 ± 43.815; 12 (29)	28.6 ± 56.284; 5 (13)	>0.05
Height (cm), Mean ± SD; Me (IQR)	85.1 ± 28.585; 75 (33)	74.9 ± 40.955; 60 (21)	>0.05
Body weight (kg), Mean ± SD; Me (IQR)	14.8 ± 13.623; 11 (7.8)	16.3 ± 23.555; 6.2 (5.2)	>0.05

1.2 Sex Distribution of the Study Population

The sex distribution of the study population showed a predominance of males, with 77 children (59.7%), compared with 52 females (40.3%). A similar pattern was observed in the group of children with acute respiratory infections, where males accounted for 59.6% of cases, compared with 40.4% for females (Table 4).

These findings are consistent with data from the international literature, which report a higher incidence of acute respiratory infections among male children. The observed differences have been attributed to sex-related variations in the immune response, with males being more susceptible to infections caused by viruses, bacteria, and other pathogens. Prospective studies have identified male sex as one of the factors most frequently associated with hospitalization due to respiratory syncytial virus (RSV) infection. Recent studies of children with bronchiolitis have likewise demonstrated higher hospitalization rates among boys. In addition, long-term epidemiological analyses have reported a higher incidence of acute respiratory infections in males, particularly during early childhood.

1.3. Etiological Characteristics of the Infections

To identify the etiological agents of infection, all patients underwent microbiological examination of throat swab specimens for bacterial pathogens and antigen testing for viral pathogens. In addition, all children with acute bronchiolitis underwent PCR testing for respiratory viruses to enable a more precise determination of the viral etiology of the disease.

- Viral pathogens were identified in all patients with acute bronchiolitis (n=32) by PCR testing. In two of these patients, Staphylococcus aureus was also isolated from throat swab specimens. Among the viral pathogens, RSV was the most frequently detected, followed by rhinovirus (RV), whereas the remaining viruses were identified less frequently.
- In the bronchopneumonia group (n=42), viral pathogens were detected in four children (Influenza A virus and SARS-CoV-2), while Streptococcus pneumoniae and Staphylococcus aureus were isolated from throat swab specimens in two additional children.
- In the acute bronchitis group (n=25), an etiological pathogen was identified in three children. Two children were diagnosed with SARS-CoV-2 infection, and Streptococcus pneumoniae was isolated from the throat swab of one child (Figure 3).

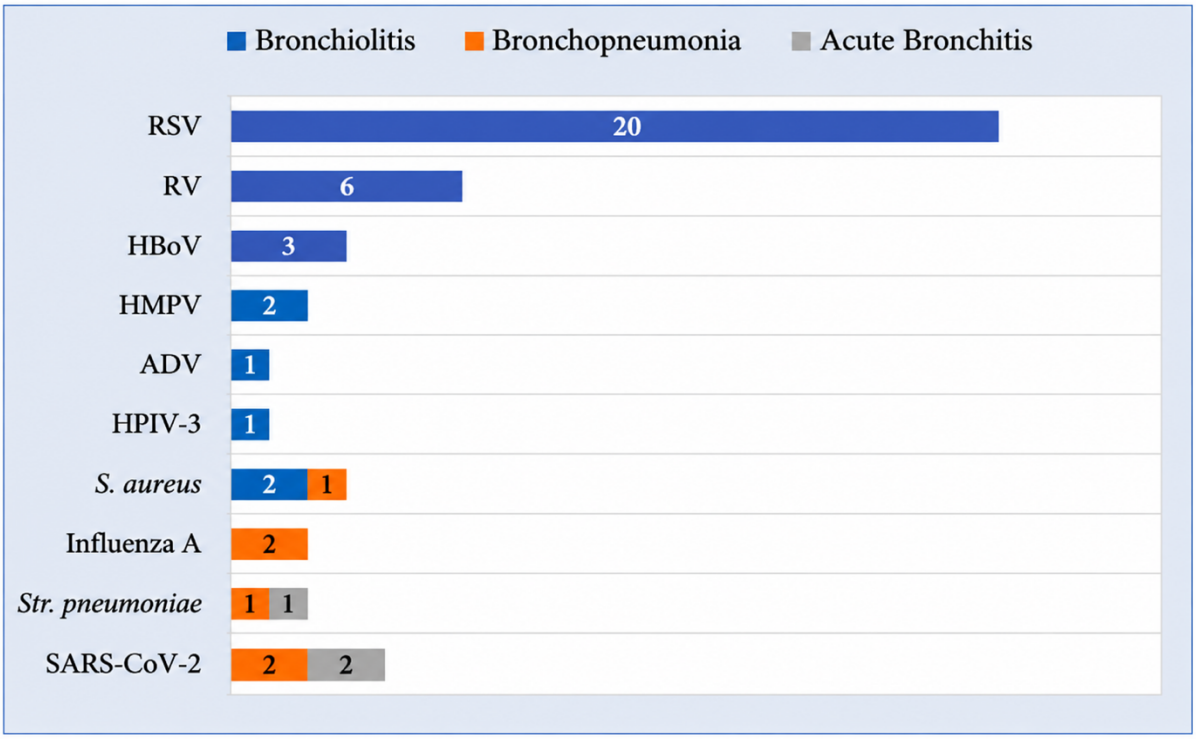


Figure 3. Distribution of the Identified Etiological Pathogens Among the Three Groups of Children with Acute Respiratory Infections

RSV – Respiratory syncytial virus; RV – Rhinovirus; HBoV – Human bocavirus; HMPV – Human metapneumovirus; ADV – Adenovirus; HPIV-3 – Human parainfluenza virus type 3; S. aureus – Staphylococcus aureus; Influenza A – Influenza A virus; Str. pneumoniae – Streptococcus pneumoniae; SARS-CoV-2 – Severe acute respiratory syndrome coronavirus 2 (the causative agent of COVID-19).

The results demonstrated a predominance of viral etiology among children with acute bronchiolitis, with respiratory syncytial virus (RSV) being the most frequently identified pathogen. This finding is consistent with the literature, which recognizes RSV as the leading cause of bronchiolitis during infancy and early childhood.

In the bronchopneumonia group, an etiological pathogen was identified in a relatively small number of patients. This is likely attributable to the limitations of the diagnostic methods employed and to the fact that, in a considerable proportion of pediatric pneumonia cases, the causative pathogen remains unidentified despite microbiological and virological investigations. The low rate of microbiological confirmation observed in children with bronchopneumonia is consistent with previous reports indicating that etiological agents frequently cannot be identified because of difficulties in obtaining appropriate lower

respiratory tract specimens, prior antibiotic treatment, or the limited sensitivity of available diagnostic methods.

Similarly, an etiological pathogen was identified in only a limited number of patients with acute bronchitis. This is likely related to the limitations of the diagnostic methods used, differences in the stage of infection at the time of specimen collection, and the fact that not all viral pathogens can be detected by the diagnostic tests employed in the present study.

1.4. Assessment of Disease Severity

1.4.1. Acute Bronchiolitis

Among the patients with acute bronchiolitis (n=32), the following distribution according to disease severity was observed (Figure 4):

- Mild disease – 7 children (21.9%);
- Moderate disease – 21 children (65.6%);
- Severe disease – 4 children (12.5%).

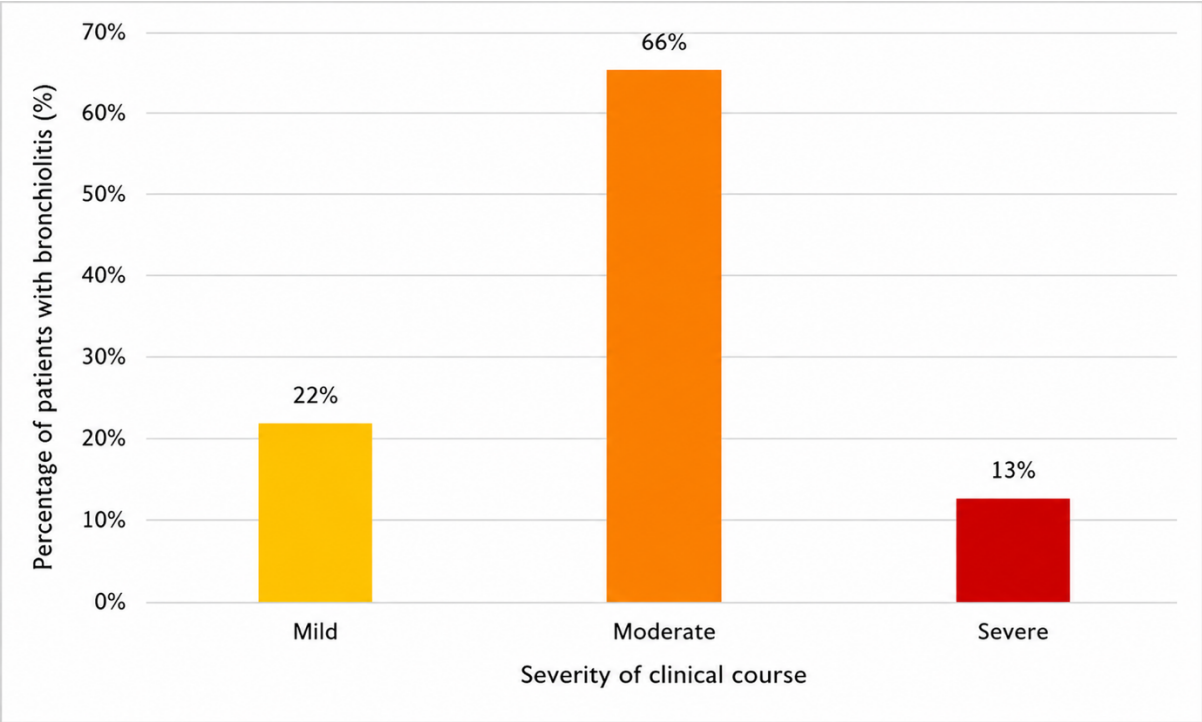


Figure 4. Distribution of Patients with Acute Bronchiolitis According to Disease Severity (n=32).

1.4.1.1. Assessment of Disease Severity in the Subgroup of Children with Bronchiolitis Caused by Respiratory Syncytial Virus (RSV)

In the subgroup of children with acute bronchiolitis (n = 32), respiratory syncytial virus (RSV) was identified in 20 patients. To provide a more detailed analysis, disease severity was also assessed within this subgroup.

According to the ReSVinet score:

- 2 children (10%) had severe disease;
- 6 children (30%) had mild disease;
- 12 children (60%) had moderate disease.

Cases with moderate disease severity predominated, whereas severe cases accounted for a relatively small proportion of the subgroup.

The results are presented in Figure 5.

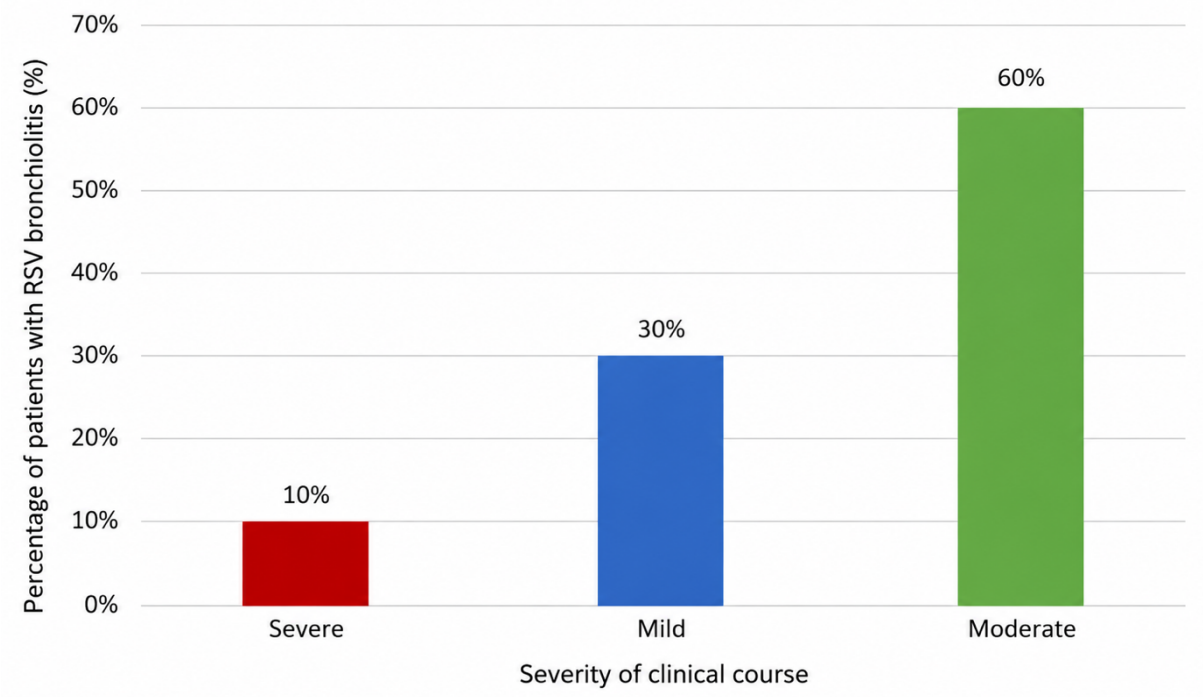


Figure 5. Distribution of Patients with RSV Bronchiolitis According to Disease Severity (n = 20).

1.4.2. Acute pneumonia

According to the summarized clinical criteria based on the recommendations of the World Health Organization (WHO) and the British Thoracic Society (BTS), all patients with acute bronchopneumonia were classified as having moderate disease severity.

1.4.3. Acute Bronchitis

According to the BSS-ped score, all patients with acute bronchitis were classified as having mild disease.

The predominance of moderately severe disease among patients with bronchiolitis is consistent with published data indicating that severe cases among hospitalized children are relatively uncommon. Most cases of bronchiolitis are mild and resolve spontaneously within a few days, with only a small proportion of patients requiring hospitalization. Among hospitalized children, progression to severe disease with respiratory failure requiring intensive care occurs in only a limited percentage of cases (approximately 3–10%).

Similarly, the predominance of moderate disease severity among patients with bronchopneumonia is consistent with findings reported in the literature. For example, a multinational prospective cohort study involving 73 emergency departments across 14 countries demonstrated that mild and moderate forms of community-acquired pneumonia predominate in children, whereas severe cases are relatively uncommon (approximately 5%). Other studies have reported that the need for intensive care among children with pneumonia varies considerably, with 10.3% to 37.2% of cases requiring admission to a pediatric intensive care unit. Another prospective study found that only 6.0% of hospitalized children met the criteria for severe pneumonia.

The mild clinical course observed in patients with acute bronchitis is consistent with the clinical characteristics of the disease as a self-limiting infection that typically resolves within 2–3 weeks.

2. Results and Discussion for Objective 2

To determine the vitamin D status of children with acute respiratory infections by measuring serum 25(OH) vitamin D and parathyroid hormone (PTH) levels.

2.1. Comparison of Serum 25(OH)D and PTH Levels Between Children with Acute Respiratory Infections (ARIs) and Healthy Controls

The comparison of serum 25(OH) vitamin D levels between children with acute respiratory infections (ARIs) and healthy controls showed the following distribution (Figure 6):

- 28% of children with ARIs had insufficient serum 25(OH) vitamin D levels, compared with 10% of healthy controls.
- 4% of children with ARIs had vitamin D deficiency, compared with 3% of children in the control group.
- A higher proportion of children with elevated serum 25(OH) vitamin D levels (hypervitaminosis) was observed among healthy controls (27%) compared with children with ARIs (19%).
- Sufficient serum 25(OH) vitamin D levels were observed in 60% of healthy children, compared with 48% of children with ARIs.

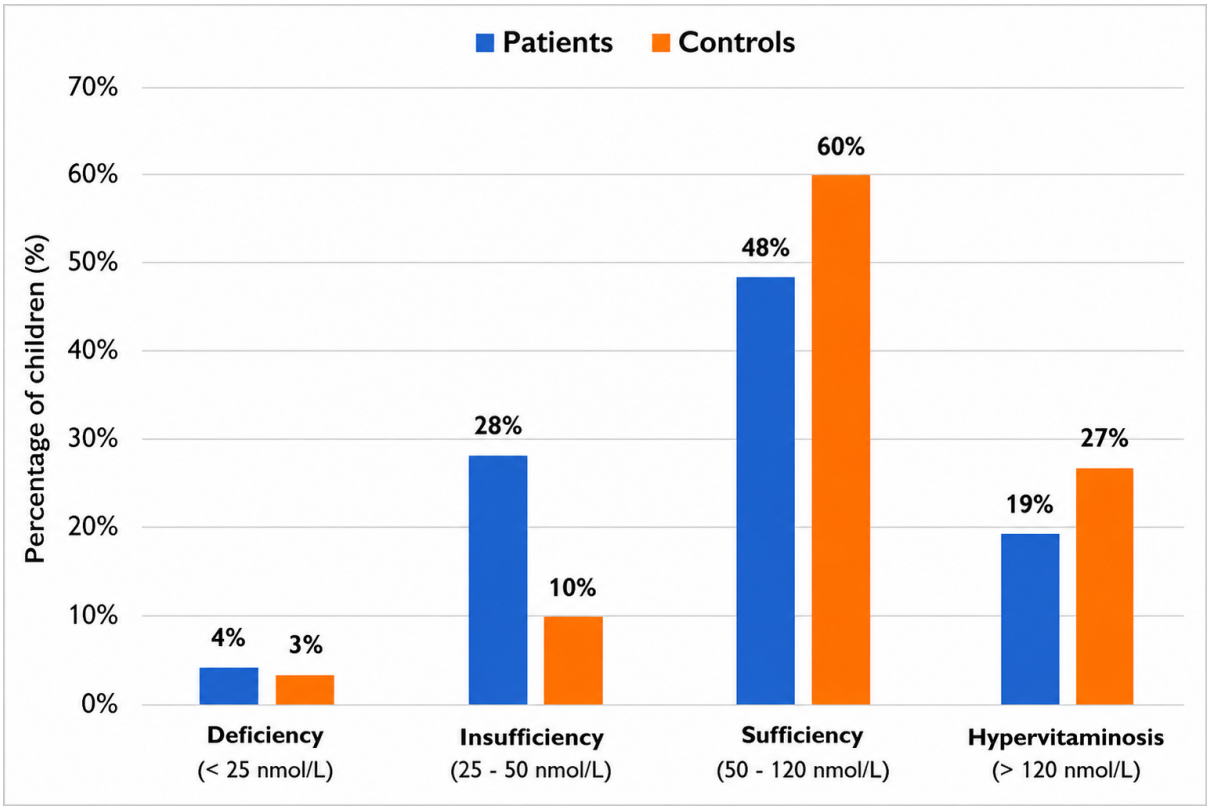


Figure 6. Vitamin D Status in the Overall Population of Children with Acute Respiratory Infections (ARIs) (n=99) Compared with the Healthy Control Group (n=30).

No statistically significant differences were found in the mean serum levels of 25(OH) vitamin D and PTH between children with acute respiratory infections (ARIs) and healthy controls (Table 5).

Table 5. Comparative Analysis of Serum 25(OH) Vitamin D and Parathyroid Hormone (PTH) Levels in Children with Acute Respiratory Infections and Healthy Controls

Parameters	Patients (n=99)	Healthy Controls (n=30)	Statistical Significance (p-value)
25(OH)D **(Mean ± SD; Me, IQR)**	81.1 ± 47.018 69.2, 67.8	93.7 ± 55.974 81.5, 60.1	>0.05
PTH **(Mean ± SD; Me, IQR)**	18.9 ± 15.914 16.8, 15.1	25.3 ± 19.337 21.1, 13.5	>0.05

However, analysis of the distribution of vitamin D status categories revealed statistically significant differences between the two groups (Table 6):

- A significantly higher proportion of children with insufficient serum 25(OH) vitamin D levels was observed among patients with ARIs (28.3%) compared with healthy controls (10.0%) ($\chi^2 = 20.161$, $p < 0.001$).
- Sufficient serum 25(OH) vitamin D levels were more frequently observed in healthy children (60.0%) than in patients with ARIs (48.5%) ($\chi^2 = 13.636$, $p < 0.001$).
- A higher proportion of children with elevated serum 25(OH) vitamin D levels (hypervitaminosis) was found among healthy controls (26.7%) compared with patients with ARIs (19.2%) ($\chi^2 = 5.538$, $p = 0.019$).
- No statistically significant difference was found between the two groups in the proportion of children with vitamin D deficiency ($p > 0.05$).

Table 6. Distribution of Vitamin D Status Among Children with Acute Respiratory Infections and Healthy Controls (Number and Percentage)

Vitamin D Status [25(OH)D]	**Healthy Controls, n (%)**	**Patients, n (%)**	**Statistical Significance (p-value)**
Deficiency (<25 nmol/L)	2 (3.3)	4 (4.0)	>0.05
Insufficiency (25–50 nmol/L)	3 (10.0)	28 (28.3)	<0.001
Sufficiency (50–120 nmol/L)	18 (60.0)	48 (48.5)	<0.001
Hypervitaminosis (>120 nmol/L)	7 (26.7)	19 (19.2)	<0.05

Total	30 (100.0)	99 (100.0)	—

2.2. Analysis of Serum 25(OH)D Levels According to the Month of Assessment

To evaluate the effect of seasonality on serum 25(OH) vitamin D levels, an analysis was performed according to the month in which the samples were collected. The results demonstrated a clear seasonal variation. The lowest serum 25(OH) vitamin D levels were observed during the winter months, followed by a gradual increase throughout spring and early summer.

Higher 25(OH) vitamin D levels were observed during the summer and autumn months, accompanied by considerable interindividual variability. The widest distribution of serum concentrations was recorded in June, August, October, and December.

These findings confirm the significant influence of seasonality on serum 25(OH) vitamin D levels in the study population (Figure 7).

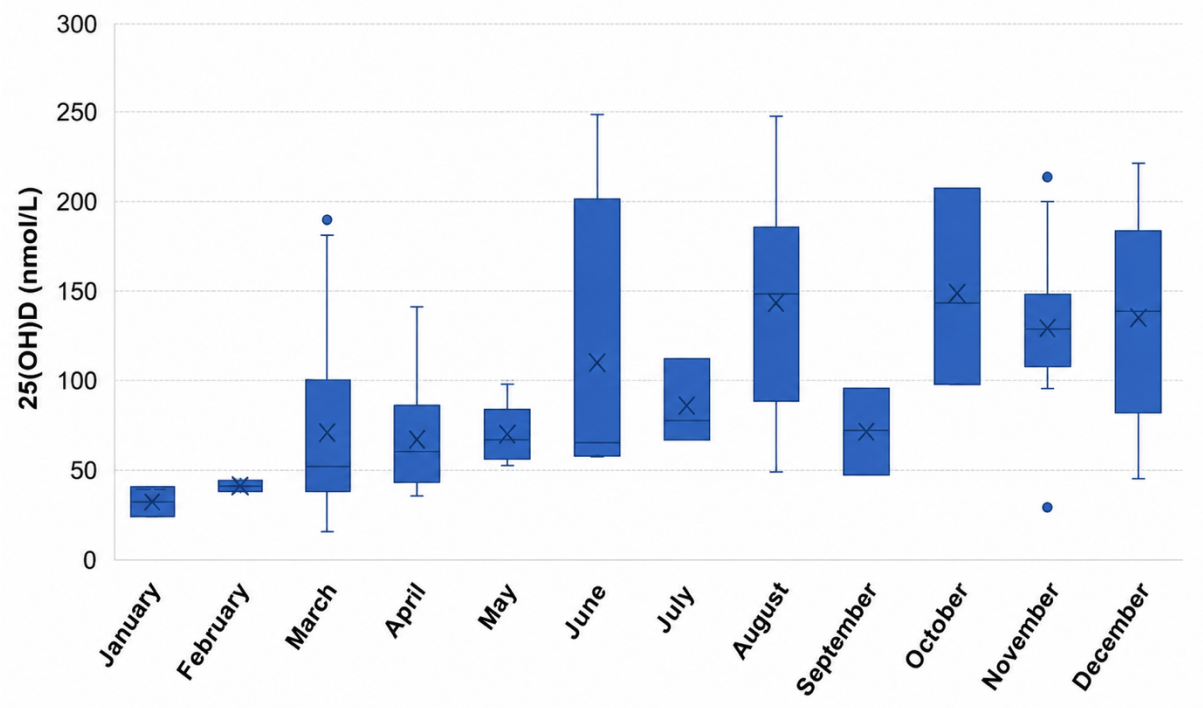


Figure 7. Distribution of Serum 25(OH) Vitamin D Levels in the Study Population According to the Month of Assessment (nmol/L).

2.3. Comparison of Serum 25(OH)D Levels Between the Individual Diagnostic Groups and the Healthy Control Group

A further analysis was performed to compare serum 25(OH) vitamin D levels among the individual diagnostic subgroups (Figure 8):

- In the acute bronchitis group, 9 children (36%) had insufficient serum 25(OH) vitamin D levels, 3 children (12%) had vitamin D deficiency, and 1 child (4%) had elevated serum 25(OH) vitamin D levels (hypervitaminosis). The remaining 12 children (48%) had sufficient serum 25(OH) vitamin D levels.
- Among children with bronchopneumonia, 16 (38%) had insufficient serum 25(OH) vitamin D levels, while 4 (10%) had elevated serum 25(OH) vitamin D levels (hypervitaminosis). The remaining 22 children (52%) had sufficient serum 25(OH) vitamin D levels, with no cases of vitamin D deficiency identified.
- Abnormal serum 25(OH) vitamin D levels were also observed in children with acute bronchiolitis: 3 children (9%) had insufficient serum 25(OH) vitamin D levels, 1 child (3%) had vitamin D deficiency, and 14 children (44%) had elevated serum 25(OH) vitamin D levels (hypervitaminosis). The remaining 14 children (44%) had sufficient serum 25(OH) vitamin D levels.
- In the healthy control group, 1 child (3%) had vitamin D deficiency, 3 children (10%) had insufficient serum 25(OH) vitamin D levels, 8 children (27%) had elevated serum 25(OH) vitamin D levels (hypervitaminosis), and 18 children (60%) had sufficient serum 25(OH) vitamin D levels.

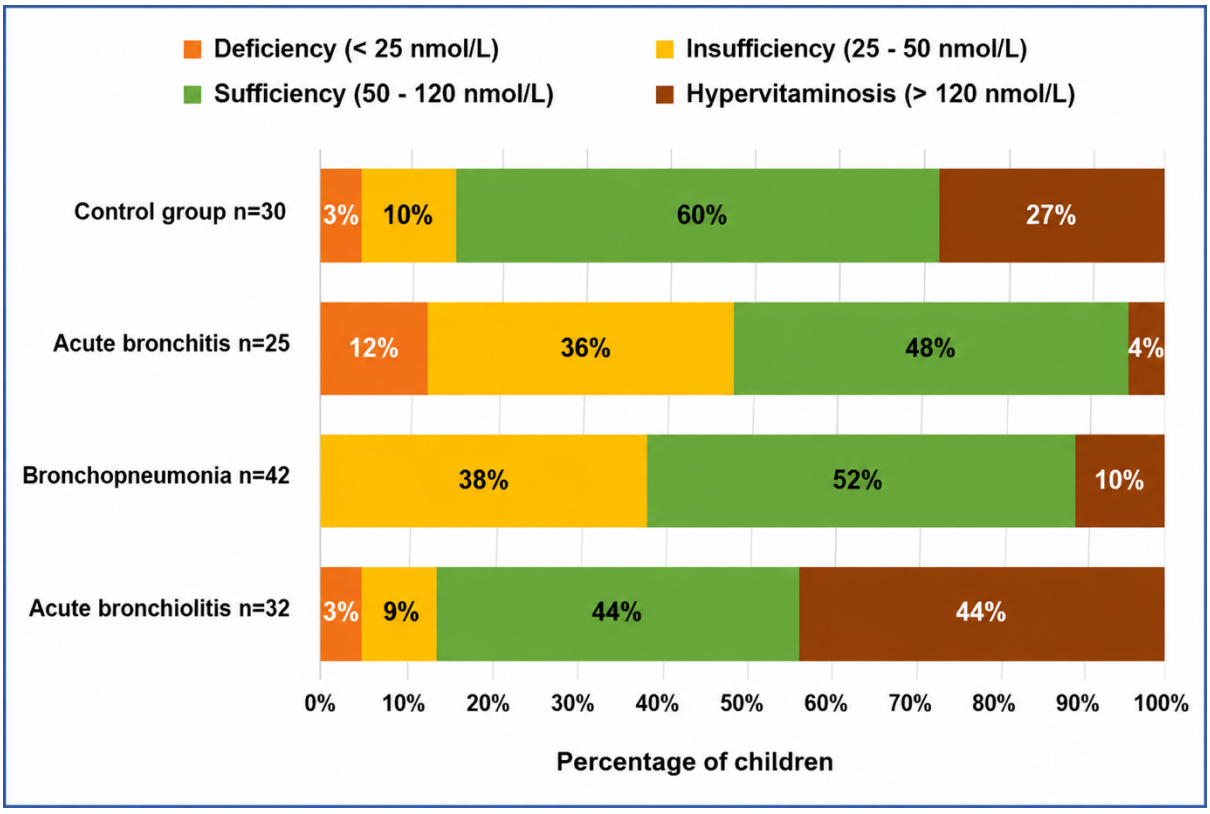


Figure 8. Distribution of the Study Population According to Serum 25(OH) Vitamin D Levels (p = 0.01).

A statistically significant difference in serum 25(OH) vitamin D levels was found among the diagnostic groups (Kruskal–Wallis test: H = 25.001, df = 3, N = 129, p < 0.001).

Subsequent pairwise comparisons using the Mann–Whitney U test demonstrated (Figure 9):

- a statistically significant difference between the healthy control group and children with bronchopneumonia (U = 399.000, z = ,àí2.639, p = 0.008);
- a statistically significant difference between the healthy control group and children with acute bronchitis (U = 229.000, z = ,àí2.468, p = 0.014);
- no statistically significant difference between the healthy control group and children with acute bronchiolitis (U = 350.000, z = ,àí1.831, p = 0.067).

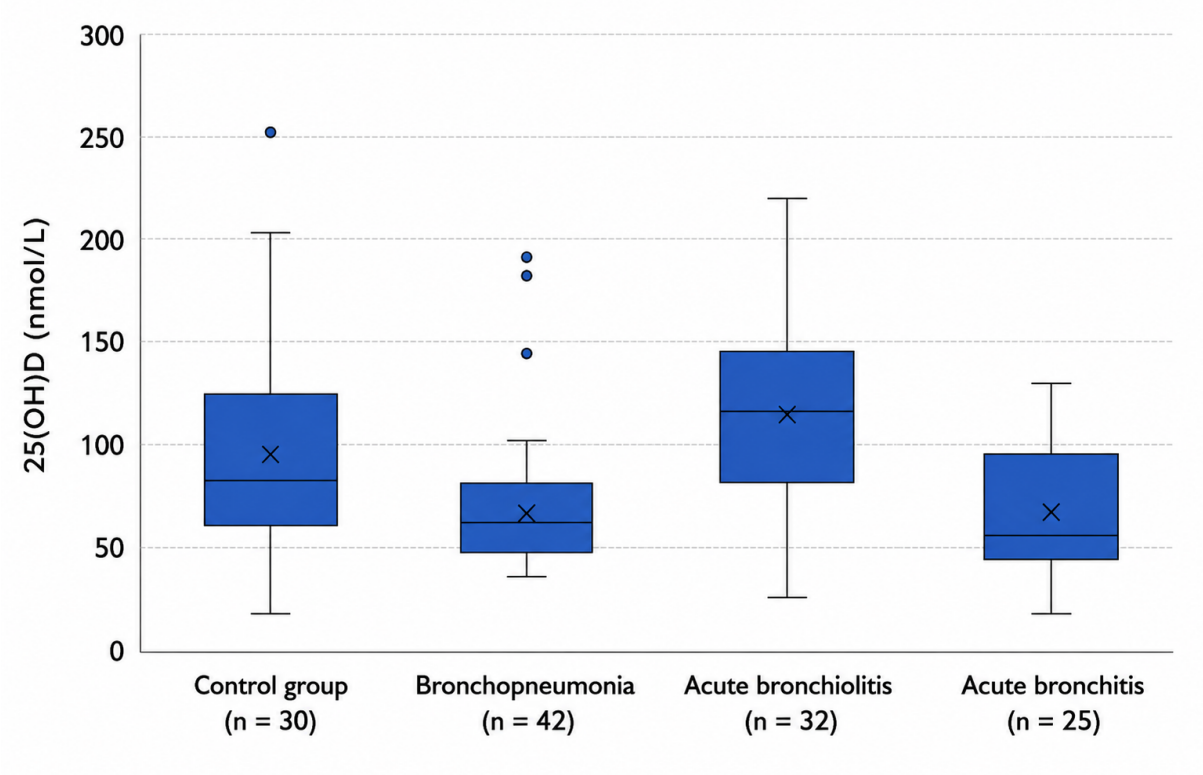


Figure 9. Comparison of Serum 25(OH) Vitamin D Levels Among the Diagnostic Groups and the Healthy Control Group.

2.4. Correlation Analysis: Association Between Serum 25(OH) Vitamin D and Parathyroid Hormone (PTH) Levels

Spearman's correlation analysis demonstrated a statistically significant association between serum 25(OH) vitamin D and parathyroid hormone (PTH) levels (r_s = −0.313, N = 123, p < 0.001). The observed correlation was negative, indicating that higher

serum 25(OH) vitamin D levels were associated with lower PTH concentrations. The strength of the correlation was weak to moderate, suggesting an association between the two variables while also indicating that additional factors contribute to the regulation of PTH.

The scatter plot illustrating this relationship (Figure 10) further confirms an inverse association between the two variables, despite the relatively wide distribution of individual values.

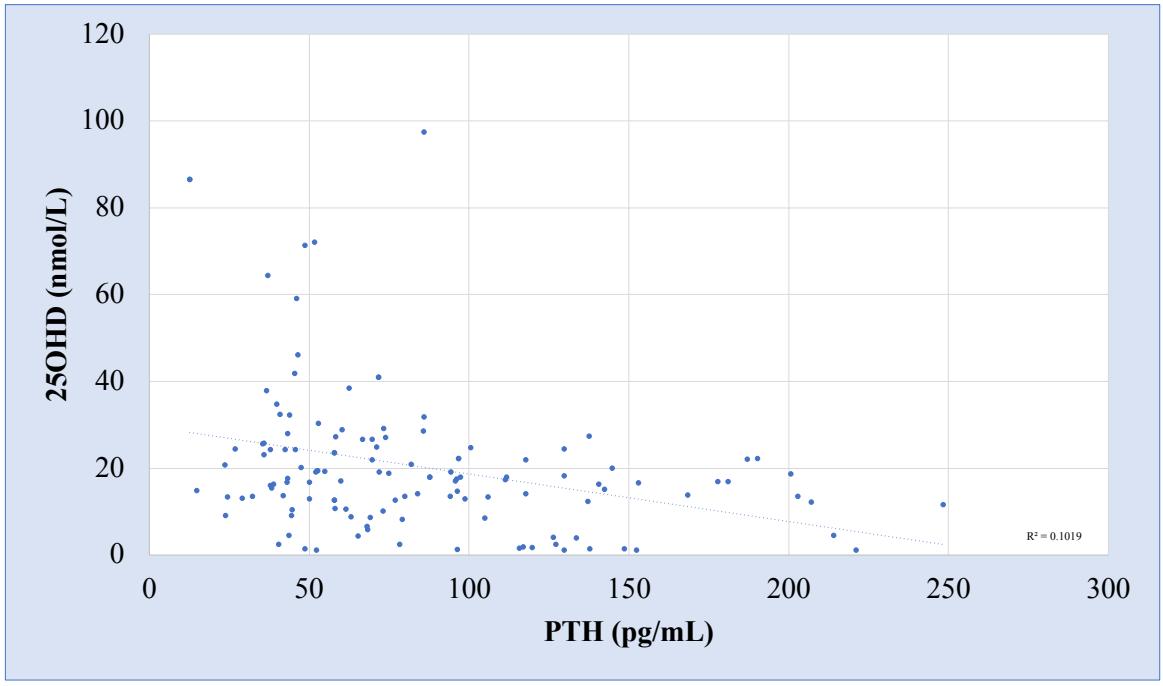


Figure 10. Scatter Plot Illustrating the Association Between Serum 25(OH) Vitamin D and Parathyroid Hormone (PTH) Levels

2.5. Comparison of Serum 25(OH) Vitamin D and Parathyroid Hormone (PTH) Levels in Children with RSV Bronchiolitis

No statistically significant difference was found between children with RSV bronchiolitis and healthy controls with respect to serum 25(OH) vitamin D levels ($U = 174.000$, $N = 44$, $z = -1.556$, $p = 0.120$).

In contrast, a statistically significant difference was observed between the two groups in parathyroid hormone (PTH) levels ($U = 391.000$, $N = 44$, $z = 3.560$, $p < 0.001$), with lower PTH levels in children with RSV infection compared with healthy controls (Table 7).

Table 7. Comparative Analysis of Serum 25(OH) Vitamin D and Parathyroid Hormone (PTH) Levels in Children with RSV Infection and Healthy Controls (Mean ± SD; Me, IQR)

Parameters	Healthy Controls (n=24)	Patients (n=20)	Statistical Significance (p-value)
25(OH)D**, nmol/L (Mean ± SD; Me, IQR)**	102.2 ± 59.082 91.1, 82.5	125.6 ± 58.847 129.9, 52.2	>0.05
PTH**, pg/mL (Mean ± SD; Me, IQR)**	25.7 ± 18.585 18.6, 13.9	8.6 ± 8.053 4.2, 15.7	**<0.001**

When vitamin D status was analyzed by category, the following findings were observed:

- A higher frequency of insufficient serum 25(OH) vitamin D levels was found among children with RSV infection compared with healthy controls (15.0% vs. 8.3%), whereas the frequency of vitamin D deficiency was similar in the two groups (5.0% vs. 8.3%).
- Sufficient serum 25(OH) vitamin D levels were observed considerably more frequently among healthy children than among children with RSV infection (54.2% vs. 25.0%).
- Children with RSV infection had a markedly higher proportion of elevated serum 25(OH) vitamin D levels (hypervitaminosis) compared with healthy controls (55.0% vs. 29.2%).

The results are presented in Figure 11.

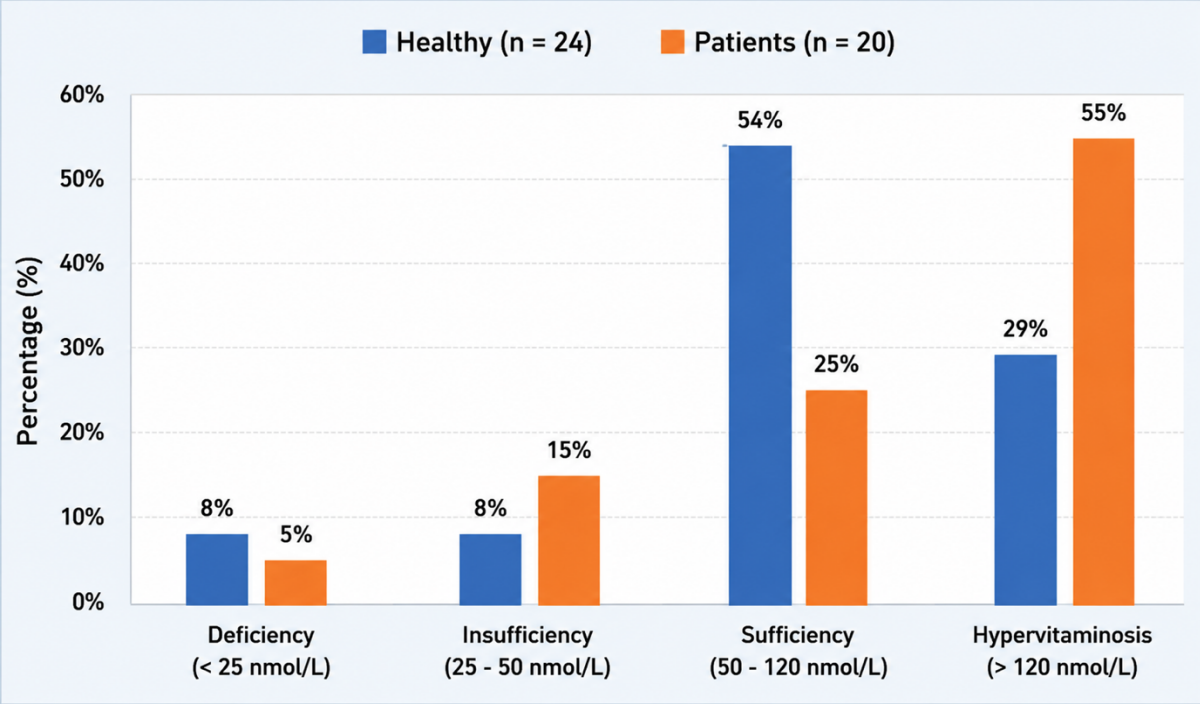


Figure 11. Distribution of Vitamin D Status in Children with RSV Infection and Healthy Controls (%).

Discussion

Acute respiratory infections (ARIs) remain one of the leading causes of morbidity and mortality worldwide. In recent years, increasing attention has been directed toward the consequences of inadequate vitamin D levels in the context of its extraskeletal (pleiotropic) effects, particularly those related to immune regulation. In this regard, vitamin D is considered to play a key role in limiting the inflammatory response. Its active metabolite, $1,25(OH)_2D$ (calcitriol), has been shown to regulate both innate and adaptive immunity by activating antimicrobial mechanisms against bacterial, viral, and fungal pathogens within cells of the innate immune system. Contemporary evidence has consistently linked low serum 25(OH)D concentrations with an increased risk of acute respiratory infections in both the general population and children. However, the evidence regarding the effectiveness of vitamin D supplementation in reducing the incidence and severity of these infections remains inconsistent across studies.

In the present study, no statistically significant differences were observed in the mean serum 25(OH) vitamin D and parathyroid hormone (PTH) levels between children with acute respiratory infections and healthy controls. Nevertheless, further analysis of vitamin D status by category demonstrated a significantly higher proportion of children with vitamin D insufficiency (25(OH)D < 50 nmol/L) among patients with ARIs. In contrast, sufficient serum vitamin D levels (>50 nmol/L) and hypervitaminosis (>120 nmol/L) were more frequently observed in healthy children. No statistically significant difference was found between the two groups regarding the prevalence of vitamin D deficiency (25(OH)D < 25 nmol/L).

The absence of a statistically significant difference in the mean serum 25(OH) vitamin D levels between children with acute respiratory infections and healthy controls is likely attributable to the distribution of individual values within both groups. The coexistence of both low and high vitamin D concentrations resulted in mutual compensation, yielding similar mean values. In contrast, categorizing vitamin D status revealed substantial differences between the groups and provided a more accurate representation of the actual distribution of serum 25(OH) vitamin D concentrations.

Evidence from both European and international studies likewise demonstrates considerable heterogeneity regarding the association between vitamin D status and acute respiratory infections in children. A systematic review and meta-analysis including 12 studies with a total of 2,279 participants reported that children with lower respiratory tract infections had significantly lower serum 25(OH) vitamin D levels than healthy controls, together with a higher prevalence of vitamin D deficiency. However, the authors emphasized the substantial

heterogeneity among the included studies with respect to age groups, diagnostic criteria, and reference thresholds used to define vitamin D status, which makes direct comparison of the findings difficult.

When comparing our findings with those of other contemporary studies, differences in the interpretation of vitamin D status and the reference values used for its classification should be taken into consideration, as these considerably limit the direct comparability of the results. For example, a retrospective study from China including 948 children aged 1–16 years, of whom 295 had acute respiratory infections, classified vitamin D status according to the Endocrine Society guidelines as follows: severe deficiency <25 nmol/L, deficiency <50 nmol/L, insufficiency <75 nmol/L, and sufficiency 75.1–120 nmol/L. Using these criteria, the authors reported a higher prevalence of vitamin D deficiency and insufficiency among children with ARIs and suggested that serum 25(OH) vitamin D concentrations below 50 nmol/L are associated with an increased risk of acute respiratory infections. Similar to our findings, they also observed statistically significant differences in vitamin D status between children with ARIs and healthy controls. However, unlike our study, they also found a statistically significant difference in the mean serum 25(OH) vitamin D levels between the two groups. Parathyroid hormone (PTH) was not assessed in the cited study.

Similar findings regarding lower serum vitamin D levels in children with respiratory infections have also been reported in a study from Nigeria, which included 250 children under 5 years of age with acute respiratory infections and 250 asymptomatic children serving as healthy controls. All ARI cases were classified as upper respiratory tract infections or lower respiratory tract infections (pneumonia and bronchiolitis). Vitamin D status was defined according to the recommendations of the American Academy of Pediatrics (AAP) and the Institute of Medicine (IOM) as follows: severe deficiency <12.5 nmol/L, deficiency 12.5–37.5 nmol/L, insufficiency 37.5–50 nmol/L, sufficiency 50–250 nmol/L, and excess 250–372.5 nmol/L. The authors reported significantly lower mean serum 25(OH) vitamin D levels in children with acute respiratory infections than in healthy controls (52.2 ± 25.6 ng/mL vs. 57.0 ± 23.9 ng/mL). Furthermore, children with lower respiratory tract infections had a higher prevalence of severe deficiency, deficiency, and vitamin D insufficiency than those with upper respiratory tract infections.

Comparable results were also reported in a study from China involving 797 children aged 3 days to 14 years with community-acquired pneumonia and 785 healthy controls. The authors classified vitamin D status using the following reference ranges: severe deficiency <25 nmol/L, deficiency 25–50 nmol/L, insufficiency 50–75 nmol/L, and sufficiency >75 nmol/L.

The mean serum 25(OH) vitamin D concentration was significantly lower in children with pneumonia than in healthy controls (19.04 ± 9.86 ng/mL vs. 31.71 ± 14.82 ng/mL). Consistent with our findings, the proportion of children with sufficient vitamin D levels was lower in the pneumonia group, whereas the prevalence of deficiency and severe deficiency was higher than among healthy controls.

A recent Bulgarian study by Rimpova et al., including 98 children with severe pneumonia and 102 healthy controls, applied different reference values for vitamin D status according to the Institute of Medicine (IOM) recommendations: sufficiency >75 nmol/L, insufficiency 50–75 nmol/L, deficiency 25–50 nmol/L, and severe deficiency <25 nmol/L. The overall mean serum 25(OH) vitamin D concentration in the study population was 52.4 nmol/L, which, according to the authors' criteria, corresponded to vitamin D insufficiency. In the control group, 50% of the children had vitamin D deficiency, whereas only 16.7% had sufficient levels and 33.3% had insufficiency. Among children with pneumonia, 33.8% had sufficient vitamin D levels, 31.7% had insufficiency, and 34.5% had deficiency. Subgroup analysis demonstrated that children with uncomplicated pneumonia more frequently had sufficient vitamin D levels (40.3%), whereas those with complicated pneumonia more commonly exhibited vitamin D deficiency (47.2%) and insufficiency (30.6%).

When interpreting these findings, it should be noted that the reference values used by the authors differ substantially from those applied in our study. In the cited study, serum 25(OH) vitamin D concentrations between 50 and 75 nmol/L were classified as vitamin D insufficiency, whereas according to the criteria used in our study, these values fall within the range of vitamin D sufficiency. Similarly, concentrations between 25 and 50 nmol/L were defined as vitamin D deficiency, while in our study this range was classified as vitamin D insufficiency, with deficiency defined only as concentrations below 25 nmol/L. If the results reported by Rimpova et al. were interpreted according to the reference values used in our study, the proportion of children with sufficient vitamin D status would be considerably higher, whereas the prevalence of vitamin D deficiency would be lower. These observations once again emphasize the difficulty of directly comparing findings across studies and highlight the importance of interpreting the results within the context of the diagnostic criteria used.

The seasonal variation in serum 25(OH) vitamin D levels observed in our study is consistent with the well-established mechanisms of cutaneous vitamin D synthesis under the influence of UVB radiation. Bulgaria is located within the temperate climatic zone between 41° and 44° north latitude, where vitamin D synthesis exhibits pronounced seasonal variation.

During late autumn and winter, the lower solar elevation substantially reduces the amount of UVB radiation reaching the Earth's surface, thereby limiting cutaneous vitamin D synthesis. This explains the lowest serum 25(OH) vitamin D concentrations observed during the winter months in our study, followed by a gradual increase throughout spring and summer. A similar seasonal pattern has been reported in other studies. For example, Li et al. found the highest serum 25(OH) vitamin D levels during the summer and the lowest during the winter in children with community-acquired pneumonia and healthy controls in China.

In our study, the summer months were characterized not only by higher mean serum 25(OH) vitamin D levels but also by greater variability in individual values, including both high and low concentrations. This likely reflects the influence not only of seasonality but also of behavioral and individual factors related to sunlight exposure. Despite the high intensity of solar radiation during summer, regions such as Southern Europe often experience very high temperatures, which may reduce the time spent outdoors and consequently decrease direct sun exposure, thereby affecting cutaneous vitamin D synthesis. Additional factors likely contributing to this variability include the use of sunscreen, differences in dietary vitamin D intake and vitamin D supplementation, as well as individual metabolic characteristics.

Our study also demonstrated differences in the distribution of vitamin D status among the individual diagnostic subgroups. The highest prevalence of vitamin D insufficiency was observed in children with bronchopneumonia and acute bronchitis, whereas children with acute bronchiolitis more frequently exhibited sufficient vitamin D levels and hypervitaminosis. Statistical analysis revealed significant differences in serum 25(OH) vitamin D levels among the diagnostic groups. Subsequent pairwise analysis demonstrated statistically significant differences between the healthy control group and children with bronchopneumonia and acute bronchitis, whereas no significant difference was found between the control group and children with acute bronchiolitis.

The absence of a statistically significant difference between children with bronchiolitis and healthy controls is likely attributable to the age characteristics of this subgroup. Patients with bronchiolitis were predominantly infants and young children younger than two years of age, in whom prophylactic vitamin D supplementation is routinely administered, particularly during the first year of life. This likely contributed to the higher serum 25(OH) vitamin D concentrations and the greater proportion of hypervitaminosis observed in this subgroup.

Our findings are partially consistent with those reported in previous studies. Similar to our results, a study conducted in India involving children with acute bronchiolitis found no

statistically significant difference in the mean serum 25(OH) vitamin D levels between patients and healthy controls.

In contrast to our findings, a study conducted in Israel reported significantly lower serum 25(OH) vitamin D concentrations in children with bronchiolitis compared with healthy controls, together with a higher prevalence of vitamin D deficiency among patients with bronchiolitis. The discrepancies between the two studies are likely attributable to differences in the reference values used to define vitamin D status, the characteristics of the study populations, and variations in prophylactic vitamin D supplementation practices. A study conducted in Indonesia among hospitalized children with acute pneumonia also reported a relatively low prevalence of vitamin D deficiency. However, the absence of a healthy control group in that study limits the possibility of direct comparisons between patients and healthy children.

The findings in the bronchopneumonia subgroup of our study are also consistent with those reported in a study from Armenia involving children with lower respiratory tract infections and healthy controls. The authors demonstrated significantly lower serum 25(OH) vitamin D concentrations in children with lower respiratory tract infections than in healthy controls, together with a higher prevalence of vitamin D hypovitaminosis among affected patients. Similar to our findings, vitamin D insufficiency was the most common abnormality, whereas severe vitamin D deficiency was relatively uncommon. Likewise, in our study, children with bronchopneumonia exhibited a high prevalence of vitamin D insufficiency, with more than one-third of the patients having serum 25(OH) vitamin D levels below 50 nmol/L, while sufficient vitamin D levels were more frequently observed in the control group. Unlike the Armenian study, however, no cases of severe vitamin D deficiency were identified among children with bronchopneumonia in our cohort. This discrepancy is likely attributable to differences in the diagnostic criteria used, the age distribution of the study populations, seasonality, and the use of prophylactic vitamin D supplementation.

Children with acute bronchitis in our study likewise exhibited a high prevalence of vitamin D insufficiency and deficiency, together with statistically significant differences compared with the healthy control group. These findings suggest that insufficient vitamin D levels may be associated not only with lower respiratory tract infections such as pneumonia but also with a broader spectrum of acute respiratory infections in childhood.

Overall, direct comparison among published studies remains challenging because of the lack of uniform diagnostic criteria for vitamin D status, differences in the age of the study

populations, seasonal variation, and variability in the use of prophylactic vitamin D supplementation.

The observed negative correlation between serum 25(OH) vitamin D and PTH levels in our study is consistent with the established physiological mechanisms through which vitamin D regulates calcium-phosphate homeostasis by suppressing PTH secretion. Lower vitamin D concentrations are associated with a compensatory increase in PTH levels, whereas sufficient or elevated vitamin D concentrations result in suppression of parathyroid hormone secretion. The inverse relationship between 25(OH) vitamin D and PTH observed in our study is also consistent with previous reports that consider PTH an indirect functional marker of vitamin D status. Nevertheless, the moderate strength of the observed correlation indicates that this relationship is not strictly linear and may be influenced by several factors, including age, vitamin D supplementation, and individual metabolic characteristics.

Published data evaluating both serum 25(OH) vitamin D and PTH levels simultaneously in children with acute respiratory infections remain limited. Within the Bulgarian pediatric population, changes in serum PTH concentrations as an indicator of vitamin D sufficiency have been investigated primarily in children with idiopathic nephrotic syndrome.

In the subgroup of children with RSV bronchiolitis, no statistically significant difference in the mean serum 25(OH) vitamin D levels was found compared with healthy controls. However, analysis of vitamin D status by category revealed several differences between the two groups. Children with RSV infection demonstrated a higher prevalence of vitamin D insufficiency and a substantially higher proportion of hypervitaminosis, whereas sufficient vitamin D levels were more frequently observed among healthy children. The prevalence of vitamin D deficiency was similar in both groups.

The shift toward higher serum 25(OH) vitamin D concentrations observed in children with RSV bronchiolitis is likely explained by the age characteristics of this subgroup. These patients were predominantly infants and young children, in whom prophylactic vitamin D supplementation is administered more intensively, particularly during the first year of life. This likely accounts for the high prevalence of hypervitaminosis and the absence of a statistically significant difference in mean serum 25(OH) vitamin D levels compared with healthy controls.

Our findings are partially consistent with a study conducted in Tashkent in 2022, in which 51.28% of children with acute bronchiolitis had sufficient vitamin D levels (>75 nmol/L), 20.51% had vitamin D insufficiency (52.5–72.5 nmol/L), and 28.2% had vitamin D deficiency (<50 nmol/L). It should be noted, however, that viral pathogens were not

investigated in that study and that the reference values used to classify vitamin D status differed from those applied in our investigation.

Comparable findings regarding the proportion of children with sufficient vitamin D levels have also been reported in a study from Turkey, in which 52.2% of patients with acute bronchiolitis had normal serum 25(OH) vitamin D concentrations (>50 nmol/L). However, the authors reported a higher prevalence of vitamin D insufficiency (29.7%) and deficiency (18%) than that observed in our study.

In contrast to our findings, a study from China published in 2023 demonstrated significantly lower serum 25(OH) vitamin D concentrations in children with RSV-associated acute respiratory infections compared with healthy controls.

Of particular interest was the statistically significant reduction in PTH levels observed in children with RSV infection compared with healthy controls. This finding is likely related to the higher serum 25(OH) vitamin D concentrations and the greater prevalence of hypervitaminosis in the RSV subgroup, which may have resulted in suppression of parathyroid hormone secretion. These findings are consistent with the physiological inverse relationship between 25(OH) vitamin D and PTH that was also demonstrated in the overall study population.

3. Results and Discussion for Objective 3

To determine the serum concentrations of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α during acute respiratory infections.

The analysis demonstrated a statistically significant difference in TNF-α levels between children with acute respiratory infections and healthy controls ($t = 2.023$, $df = 87.867$, $p = 0.046$).

No statistically significant differences were observed between patients and healthy controls with respect to IL-1β or IL-6 levels (Table 8).

Table 8. Comparative Analysis of Serum IL-1β, IL-6, and TNF-α Levels in Children with Acute Respiratory Infections and Healthy Controls (Mean ± SD; Me, IQR)

Parameters	**Patients (n=99)**	**Healthy Controls (n=30)**	**Statistical Significance (p-value)**
IL-1β (Mean ± SD; Me, IQR)	8.6 ± 4.055 9.1, 5.2	8.8 ± 3.520 8.40, 4.11	>0.05
IL-6 (Mean ± SD; Me, IQR)	12.7 ± 21.038 4.5, 9.9	24.0 ± 46.251 6.2, 17.9	>0.05
TNF-α (Mean ± SD; Me, IQR)	3.6 ± 3.174 2.0, 2.6	2.6 ± 1.773 2.0, 0.6	<0.05

To identify statistically significant differences among the individual diagnostic groups and the healthy control group, the Kruskal–Wallis non-parametric test was applied (Table 9).

Statistically significant differences were found among the groups with respect to:

- IL-1β: $W = 47.674$, $df = 3$, $N = 129$, $p = 0.000$;
- IL-6: $W = 14.489$, $df = 3$, $N = 129$, $p = 0.002$;
- TNF-α: $W = 46.928$, $df = 3$, $N = 129$, $p = 0.000$.

Table 9. Serum IL-1β, IL-6, and TNF-α Levels According to Diagnostic Group (Mean ± SD; Me, IQR)

Clinical Parameters	Healthy Controls (n = 30)	Bronchiolitis (n = 32)	Bronchopneumonia (n = 42)	Acute Bronchitis (n = 25)	Statistical Significance (p-value)
IL-1β, **pg/mL**	8.0 ± 2.796 8.2, 3.8	10.9 ± 1.985 10.7, 2.0	9.7 ± 3.651 8.9, 5.9	3.9 ± 2.649 2.7, 5.4	<0.001

IL-6, **pg/mL**	25.9 ± 51.67 5.0, 15.7	11.7 ± 16.142 4.8, 9.3	5.7 ± 7.325 4.2, 2.2	25.7 ± 33.418 14.5, 17.8	<0.01
TNF-α, **pg/mL**	2.6 ± 1.556 2.0, 0.5	1.5 ± 0.268 1.5, 0.4	4.7 ± 4.037 2.3, 5.9	4.2 ± 2.085 4.0, 4.1	<0.001

3.1. Comparison of Serum IL-1β Levels Among the Diagnostic Subgroups and the Healthy Control Group

Pairwise comparisons using the Mann–Whitney U test demonstrated the following (Figure 12):

- no significant difference in serum IL-1β levels between the healthy control group and children with bronchopneumonia ($p = 0.332$);
- significantly higher IL-1β levels in children with acute bronchiolitis ($U = 251.000$, $p = 0.001$);
- significantly lower IL-1β levels in children with acute bronchitis ($U = 95.000$, $p < 0.001$).

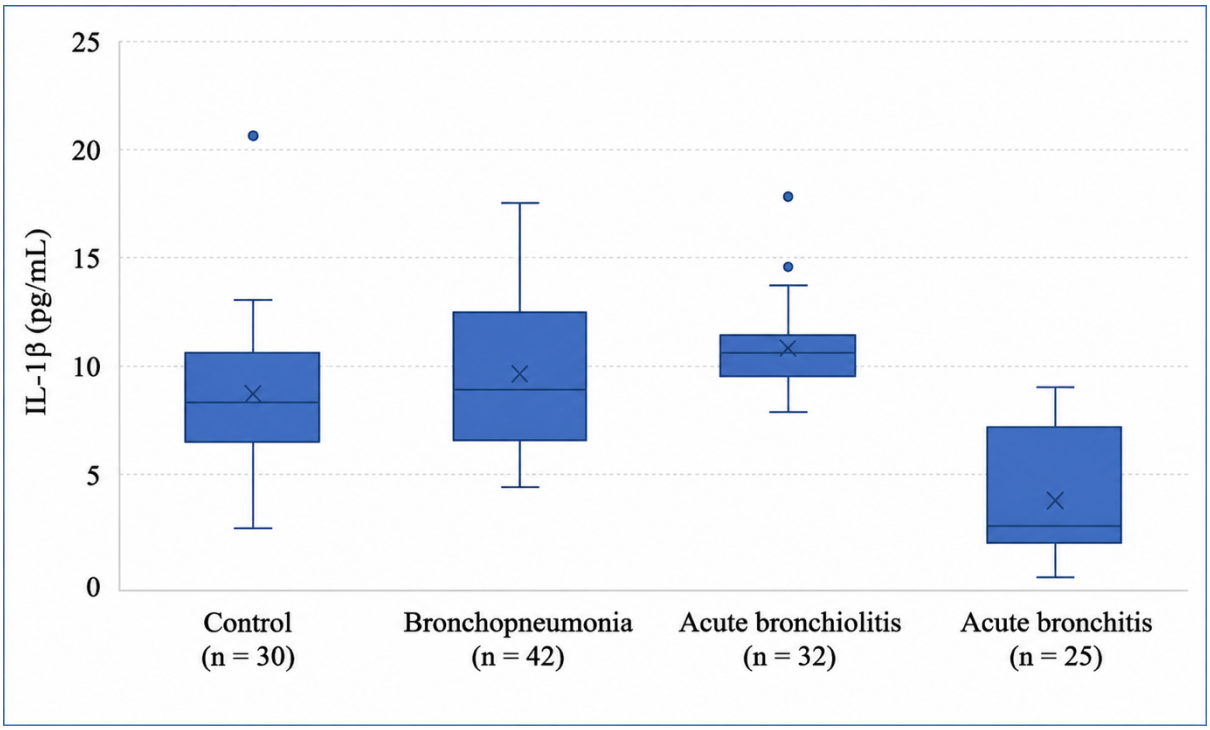


Figure 12. Comparison of Serum IL-1β Levels Among the Diagnostic Groups (pg/mL)

3.2. Comparison of Serum IL-6 Levels Among the Diagnostic Subgroups and the Healthy Control Group

Pairwise comparisons using the Mann–Whitney U test demonstrated the following (Figure 13):

- a statistically significant difference between the healthy control group and children with bronchopneumonia ($U = 428.000$, $z = -2.308$, $p = 0.021$);

- no statistically significant difference between the healthy control group and children with acute bronchiolitis ($U = 446.500$, $z = -0.472$, $p = 0.637$);
- no statistically significant difference between the healthy control group and children with acute bronchitis ($U = 318.000$, $z = -0.964$, $p = 0.335$).

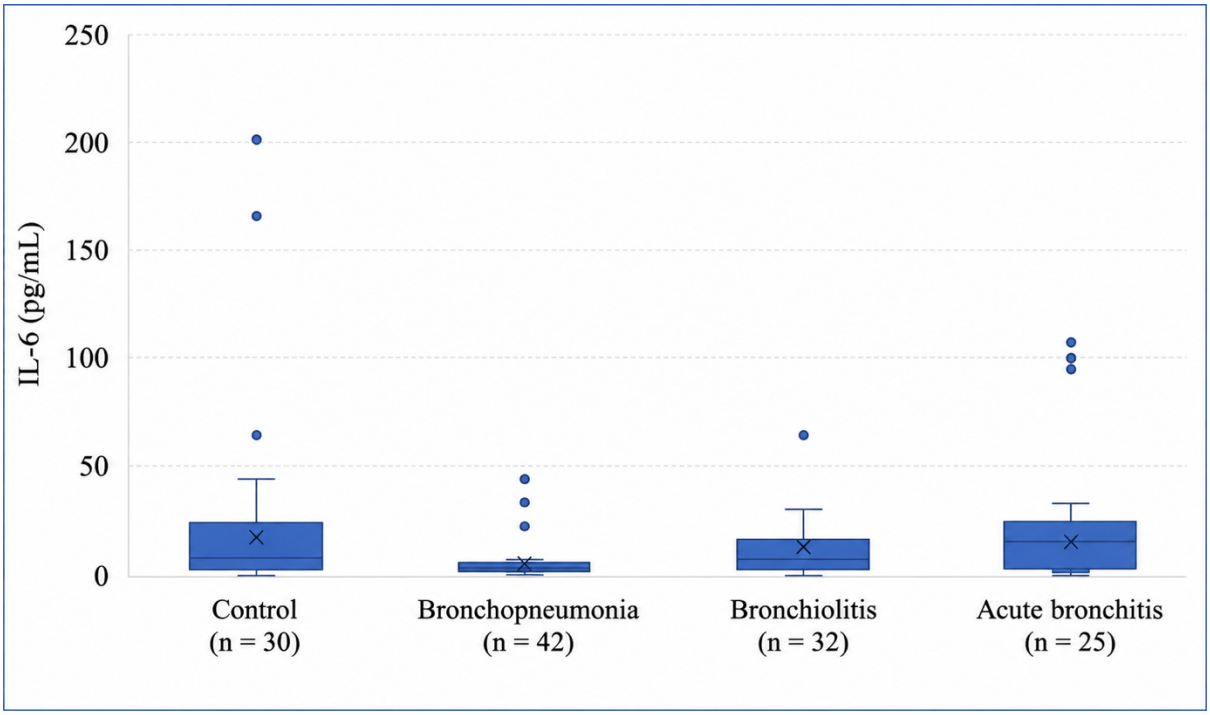


Figure 13. Comparison of Serum IL-6 Levels Among the Diagnostic Groups (pg/mL)

3.3. Comparison of Serum TNF-α Levels Among the Diagnostic Subgroups and the Healthy Control Group

Pairwise comparisons using the Mann–Whitney U test demonstrated the following (Figure 14):

- no statistically significant difference between the healthy control group and children with bronchopneumonia ($U = 480,000$, $z = -1.715$, $p = 0.086$);
- significantly lower TNF-α levels in children with acute bronchiolitis ($U = 106.000$, $z = -5.281$, $p < 0.001$);
- significantly higher TNF-α levels in children with acute bronchitis ($U = 198.500$, $z = -2.989$, $p = 0.003$).

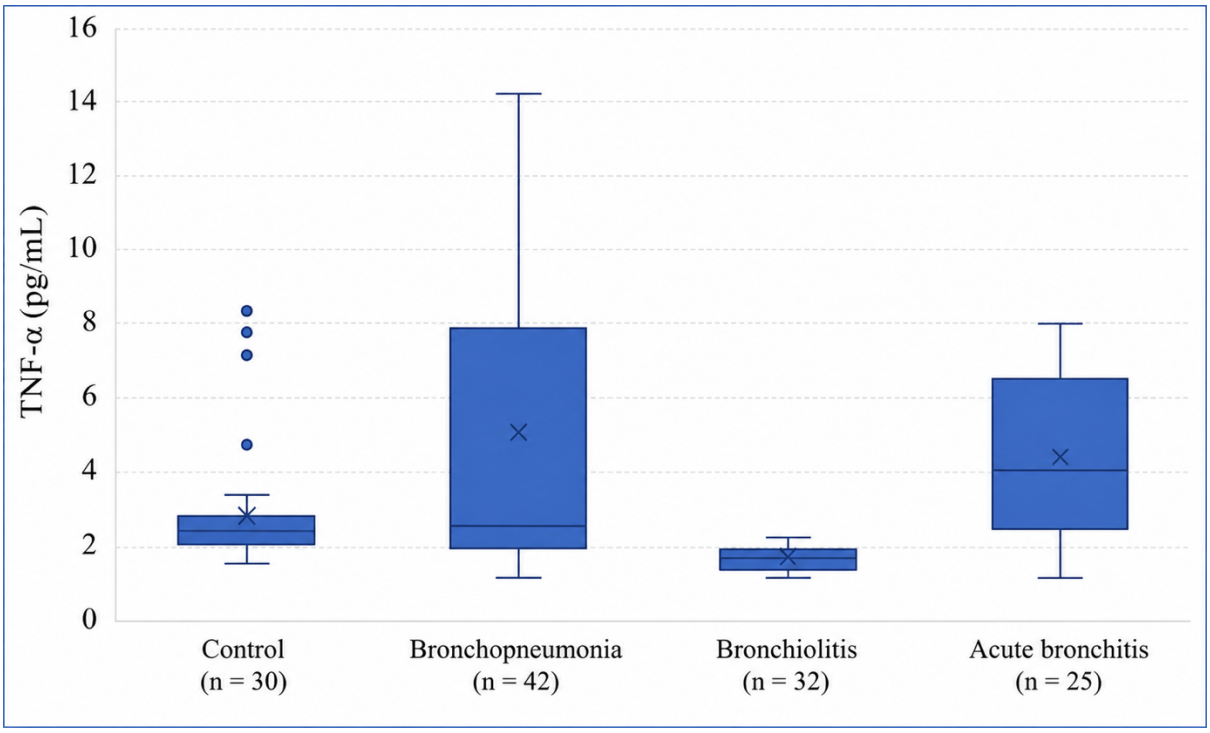


Figure 14. Comparison of Serum TNF-α Levels Among the Diagnostic Groups (pg/mL)

3.4. Analysis of Serum Pro-inflammatory Cytokine Levels (IL-1β, IL-6, and TNF-α) in the Subgroup of Children with RSV Infection

The analysis demonstrated statistically significant differences between children with RSV infection and healthy controls with respect to:

- IL-1β (U = 121.000, N = 44, z = -2.807, p = 0.005);
- TNF-α (U = 409.500, N = 44, z = 4.007, p < 0.001).

No statistically significant difference was observed between the two groups with respect to IL-6 ($U = 278.500$, $N = 44$, $z = 0.908$, $p = 0.364$) (Table 10).

Higher IL-1β levels were observed in children with RSV infection than in healthy controls, whereas TNF-α levels were lower in the RSV group. IL-6 levels did not differ significantly between the two groups.

Table 10. Comparative Analysis of Serum IL-1β, IL-6, and TNF-α Levels in Children with RSV Infection and Healthy Controls (Mean ± SD; Me, IQR)

Parameters	Overall (n = 44)	Healthy Controls (n = 24)	Patients (n = 20)	Statistical Significance (p-value)
IL-1β, **pg/mL (Mean ± SD; Me, IQR)**	9.9 ± 2.994 10.2, 3.2	9.0 ± 3.543 8.4, 3.9	11.0 ± 1.712 10.7, 1.7	<0.01

IL-6**, pg/mL (Mean ± SD; Me, IQR)**	13.5 ± 25.403 5.1, 12.3	18.2 ± 33.230 7.7, 17.2	7.9 ± 7.859 4.3, 1.8	>0.05
TNF-α**, pg/mL (Mean ± SD; Me, IQR)**	2.1 ± 1.555 1.7, 0.6	2.7 ± 1.942 1.9, 0.6	1.5 ± 0.285 1.5, 0.4	<0.001

Discussion

Analysis of the serum concentrations of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α revealed differences in the cytokine profile between children with acute respiratory infections (ARIs) and healthy controls. Comparison of the overall group of children with ARIs and the healthy control group demonstrated a statistically significant difference only in TNF-α, with higher serum levels observed in children with acute respiratory infections. No statistically significant differences were found between patients and healthy controls with respect to IL-1β or IL-6.

Analysis of the individual diagnostic subgroups revealed statistically significant differences for all three cytokines. The highest serum IL-1β levels were observed in children with acute bronchiolitis, whereas children with acute bronchitis had significantly lower levels than healthy controls. No statistically significant difference in serum IL-1β levels was found between children with bronchopneumonia and healthy controls.

With respect to IL-6, a statistically significant difference was observed only between children with bronchopneumonia and healthy controls. No significant differences in serum IL-6 levels were found in children with bronchiolitis or acute bronchitis compared with healthy children.

The analysis of TNF-α demonstrated a distinct distribution pattern among the diagnostic groups. Children with acute bronchitis exhibited significantly higher serum TNF-α levels than healthy controls, whereas children with bronchiolitis had significantly lower levels. No statistically significant difference was observed between children with bronchopneumonia and healthy controls.

In the subgroup of children with RSV infection, statistically significant differences were observed for IL-1β and TNF-α. Children with RSV bronchiolitis exhibited higher serum IL-1β levels and lower TNF-α concentrations than healthy controls. No statistically significant difference in IL-6 levels was detected between the two groups. These findings indicate distinct pro-inflammatory cytokine profiles among the different clinical forms of acute respiratory infections in childhood.

The role of pro-inflammatory cytokines in pediatric bronchopulmonary diseases has been discussed in a review article from Uzbekistan addressing the pathogenetic role of

microcirculatory disturbances in bronchiolitis, bronchitis, and pneumonia. The authors emphasize the importance of IL-1β, IL-6, and TNF-α in the development of endothelial dysfunction, increased vascular permeability, and impaired microcirculation during acute respiratory infections. According to the authors, these inflammatory mediators contribute to endothelial activation, increased expression of adhesion molecules, and the development of microthrombosis, which may contribute to a more severe clinical course of bronchopulmonary diseases.

Regarding children with acute bronchiolitis, our findings are partially consistent with those reported by Cortegano et al., who investigated pro-inflammatory cytokine levels in nasal lavage fluid (NLF) from children with mild-to-moderate bronchiolitis not requiring hospitalization. Despite the substantial differences between the two studies—including the methodology used (local cytokine assessment in nasal lavage fluid versus serum measurements in our study) and the clinical characteristics of the patients—the findings are noteworthy. The authors reported increased IL-1β, IL-6, and TNF-α levels in children with bronchiolitis compared with healthy controls. Furthermore, children with RSV bronchiolitis exhibited lower IL-1β and IL-6 levels than those with bronchiolitis caused by other respiratory viruses.

As described above, comparison of children with acute bronchiolitis and healthy controls in our study demonstrated higher serum IL-1β, lower TNF-α, and no statistically significant difference in IL-6 levels. Similarly, children with RSV bronchiolitis showed higher IL-1β and lower TNF-α concentrations than healthy controls.

Recent evidence suggests that IL-6 may serve as a potential prognostic biomarker in RSV infections. A review by Vázquez et al., summarizing current knowledge on the role of cytokines in RSV infection, reported that elevated IL-6 levels are associated with a more severe clinical course and poorer prognosis in RSV bronchiolitis. The review summarizes several studies demonstrating that increased IL-6 concentrations in children with RSV infection correlate with more pronounced clinical symptoms, a higher risk of hospitalization, greater need for oxygen therapy, and more severe bronchiolitis.

The absence of statistically significant differences in serum IL-6 levels among children with bronchiolitis and RSV bronchiolitis in our study is likely explained by the predominantly moderate disease severity observed in the study population (60–66%) and the relatively small proportion of patients with severe bronchiolitis (10–13%).

In the bronchopneumonia subgroup, significantly lower serum IL-6 levels were observed compared with healthy controls, whereas IL-1β and TNF-α did not differ

significantly. Previous studies have demonstrated that the severity of pneumonia is associated with both the concentration and profile of circulating cytokines. A prospective study including 1,886 patients with pneumonia showed that elevated serum IL-6 concentrations were associated with more severe disease and an increased risk of mortality. Similar findings have been reported in children with severe pneumonia requiring mechanical ventilation, in whom significantly higher serum IL-6 levels were observed compared with healthy controls, whereas TNF-α levels did not differ significantly.

In our study, all children with bronchopneumonia had moderate disease severity, which likely explains the lower IL-6 levels compared with healthy controls and the absence of statistically significant differences in TNF-α. This interpretation is further supported by a systematic review and meta-analysis demonstrating that elevated IL-6 levels are characteristic primarily of severe community-acquired pneumonia in children and may serve as a potential biomarker of disease severity.

With regard to acute bronchitis, published data describing the cytokine profile in the pediatric population remain limited. Yusupova and Alieva investigated serum IL-1β, IL-6, and TNF-α levels in children with acute bronchopulmonary diseases and reported increased concentrations of all three cytokines in children with acute bronchitis compared with healthy controls. The cytokine response was more pronounced in pneumonia and in the more severe forms of the disease.

In contrast to these findings, our study demonstrated a statistically significant increase only in TNF-α among children with acute bronchitis, whereas IL-1β levels were lower and IL-6 levels did not differ significantly from those of healthy controls. These discrepancies are likely attributable to differences in the study populations, patient age, and disease severity. In our cohort, children with acute bronchitis predominantly had mild clinical disease, which likely explains the absence of a pronounced systemic pro-inflammatory response. This is consistent with previous reports indicating that serum levels of pro-inflammatory cytokines correlate with the severity of the infectious process and are generally higher in more severe forms of acute respiratory disease.

4. Results and Discussion for Objective 4

To investigate the relationship between vitamin D status, pro-inflammatory cytokine levels, and the clinical course of acute respiratory infections in children.

4.1. Correlation Analysis to Assess the Relationship Between Serum 25(OH) Vitamin D Levels and Pro-inflammatory Cytokines

A Spearman correlation analysis was performed to evaluate the relationship between serum 25(OH) vitamin D levels and pro-inflammatory cytokines, both in the overall study population and within the individual diagnostic subgroups.

The overall correlation analysis demonstrated statistically significant associations between (Table 11):

- 25OHD и IL-1β (r_s=0.211, N=129, p=0.016);
- 25OHD и TNF-a (r_s= -0.360, N=129, p=0.000).

No statistically significant correlation was found between 25(OH)D and IL-6 (r_s= -0.014, N=129, p=0.879).

The correlation between 25(OH) vitamin D and IL-1β was positive, indicating that higher vitamin D levels were associated with a tendency toward higher IL-1β concentrations.

In contrast, the correlation between 25(OH) vitamin D and TNF-α was negative, indicating that higher vitamin D levels were associated with lower TNF-α concentrations.

Table 11. Correlation Between Serum 25(OH) Vitamin D, IL-1β, IL-6, and TNF-α Levels in the Study Population (Spearman rs)

		25OHD	IL-1β	IL-6	TNF-α
25OHD	Correlation Coefficient (r_s)	1,000	,211*	-,014	-,360**
	Sig. (2-tailed)	.	,016	,879	,000
	N (брой пациенти)	129	129	129	129
IL-1β	Correlation Coefficient (r_s)	,211*	1,000	-,221*	-,131
	Sig. (2-tailed)	,016	.	,012	,139
	N (брой пациенти)	129	129	129	129
IL-6	Correlation Coefficient (r_s)	-,014	-,221*	1,000	,344**
	Sig. (2-tailed)	,879	,012	.	,000
	N (брой пациенти)	129	129	129	129
TNF-α	Correlation Coefficient (r_s)	-,360**	-,131	,344**	1,000
	Sig. (2-tailed)	,000	,139	,000	.
	N (брой пациенти)	129	129	129	129
* $p<0.05$					
** $p<0.01$					

Evaluation of the correlation coefficients using a five-point scale (Table 12) demonstrated:

- a weak positive correlation between 25(OH)D and IL-1β;
- a moderate negative correlation between 25(OH)D and TNF-α.

Table 12. Five-Point Scale for the Interpretation of Correlation Strength

Strength of Correlation	Correlation Coefficient (*r*)
Weak	up to 0.30
Moderate	0.31–0.50
Substantial	0.51–0.70
Strong	0.71–0.90
Very Strong	>0.90

The correlation analysis within the individual diagnostic groups demonstrated the following (Table 13):

- Bronchopneumonia (n = 42)

- a statistically significant positive correlation between TNF-α and IL-1β (rs = 0.523, p < 0.001), indicating a substantial association;
- a statistically significant positive correlation between TNF-α and IL-6 (rs = 0.409, p = 0.007) , indicating a moderate association.

- Acute bronchitis (n = 25)

- TNF-α and IL-1β showed a statistically significant negative substantial correlation (rs = -0.606, p = 0.001);
- TNF-α and IL-6 showed a statistically significant positive substantial correlation (rs = 0.633, p = 0.001);
- IL-6 and IL-1β showed a statistically significant negative substantial correlation (rs = -0.645, p = 0.001).

- Healthy control group (n = 30)

- TNF-α and IL-6 showed a statistically significant positive moderate correlation (rs = 0.389, p = 0.034).

- No statistically significant correlations were found between 25(OH) vitamin D and the pro-inflammatory cytokines in any of the diagnostic groups. Likewise, no statistically significant correlations were observed in the acute bronchiolitis subgroup.

Table 13. Correlations Between Serum 25(OH) Vitamin D, IL-1β, IL-6, and TNF-α Levels Within the Individual Diagnostic Groups (Spearman rs)

Diagnosis		25OHD	IL-1-b	IL-6
Bronchiolitis (n = 32)	IL-1-b	0,147		
	IL-6	- 0,182	0,100	
	TNF-a	- 0,167	0,225	0,287
Bronchopneumonia (n = 42)	IL-1-b	- 0,091		
	IL-6	- 0,215	0,149	
	TNF-a	- 0,203	0,523***	0,409**
Acute Bronchitis (n = 25)	IL-1-b	0,095		
	IL-6	- 0,237	- 0,645**	
	TNF-a	0,000	- 0,606**	0,633**
Healthy Controls (n = 30)	IL-1-b	0,095		
	IL-6	0,288	- 0,332	
	TNF-a	- 0,119	0,090	0,389*
* *p<0.05*				
** *p<0.01*				
*** *p<0.001*				

4.2. Correlation Analysis to Assess the Relationship Between Serum 25(OH) Vitamin D Levels, Pro-inflammatory Cytokines, and the Clinical Severity of Acute Respiratory Infections

Because variability in clinical disease severity was observed only among patients with acute bronchiolitis, the correlation analysis between disease severity, serum 25(OH) vitamin D levels, and pro-inflammatory cytokines was performed exclusively in this group. In contrast, disease severity was homogeneous in patients with bronchopneumonia and acute bronchitis—being uniformly moderate in bronchopneumonia and mild in acute bronchitis—therefore, a similar correlation analysis was not applicable.

Among patients with acute bronchiolitis, no statistically significant correlations were found between clinical disease severity and serum levels of 25(OH) vitamin D, IL-1β, IL-6, TNF-α, or C-reactive protein (CRP). However, a statistically significant positive correlation was observed between IL-6 and CRP levels (rs = 0.487, p = 0.005), indicating coordinated activation of the inflammatory response in these patients. The remaining correlations between the investigated variables did not reach statistical significance (Table 14).

Table 14. Correlation Analysis Assessing the Relationship Between Serum 25(OH) Vitamin D Levels, Pro-inflammatory Cytokines, and Clinical Severity of Acute Bronchiolitis

		Clinical Severity	25OHD	IL-1-b	IL-6	TNF-a	CRP
Clinical Severity	Correlation Coefficient	1,000	-,053	,271	,143	,149	,179
	Sig. (2-tailed)	.	,773	,134	,435	,417	,335
	N	32	32	32	32	32	31
25OHD	Correlation Coefficient	-,053	1,000	,147	-,182	-,167	-,313
	Sig. (2-tailed)	,773	.	,423	,318	,362	,087
	N	32	32	32	32	32	31
IL-1-b	Correlation Coefficient	,271	,147	1,000	,100	,225	-,032
	Sig. (2-tailed)	,134	,423	.	,588	,216	,864
	N	32	32	32	32	32	31
IL-6	Correlation Coefficient	,143	-,182	,100	1,000	,287	0,487**
	Sig. (2-tailed)	,435	,318	,588	.	,111	0,005
	N	32	32	32	32	32	31
TNF-a	Correlation Coefficient	,149	-,167	,225	,287	1,000	,099
	Sig. (2-tailed)	,417	,362	,216	,111	.	,597
	N	32	32	32	32	32	31
CRP	Correlation Coefficient	,179	-,313	-,032	0,487**	,099	1,000
	Sig. (2-tailed)	,335	,087	,864	0,005	,597	.
	N	32	32	32	32	32	32
** p<0.01 level							

4.3. Analysis of the Subgroup of Children with RSV Infection

The overall correlation analysis demonstrated statistically significant associations between the following variables (Table 15):

- 25(OH)D and IL-1β – a positive moderate correlation (rs = 0.329, p = 0.029);
- 25(OH)D and TNF-α – a negative moderate correlation (rs = -0.326, p= 0.031);
- IL-6 and TNF-α – a positive moderate correlation (rs = 0.466, p= 0.001).

Table 15. Correlations Between Serum 25(OH) Vitamin D, IL-1β, IL-6, and TNF-α Levels in Children with RSV Infection and Healthy Controls (n = 44)

		CRP	25OHD	IL-1β	IL-6	TNF-α
CRP	Correlation Coefficient	1,000	-,158	,181	-,170	-,130
	Sig. (2-tailed)	.	,306	,240	,271	,399
	N	44	44	44	44	44
25OHD	Correlation Coefficient	-,158	1,000	,329*	-,056	-,326*
	Sig. (2-tailed)	,306	.	,029	,719	,031
	N	44	44	44	44	44
IL-1β	Correlation Coefficient	,181	,329*	1,000	-,053	-,072

	Sig. (2-tailed)	,240	,029	.	,732	,644
	N	44	44	44	44	44
IL-6	Correlation Coefficient	-,170	-,056	-,053	1,000	,466**
	Sig. (2-tailed)	,271	,719	,732	.	,001
	N	44	44	44	44	44
TNF-α	Correlation Coefficient	-,130	-,326*	-,072	,466**	1,000
	Sig. (2-tailed)	,399	,031	,644	,001	.
	N	44	44	44	44	44
* *p< 0.05 level*						
** *p< 0.01 level*						

Correlation analysis performed exclusively in children with RSV infection demonstrated the following:

- a statistically significant positive correlation between IL-6 and TNF-α (rs = 0.544, p = 0.013), indicating a substantial association;
- the remaining correlations did not reach statistical significance (Table 16, Figure 15).

Table 16. Correlations Between Serum 25(OH) Vitamin D, IL-1β, IL-6, and TNF-α Levels in Children with RSV Infection

		25OHD	IL-1β	IL-6	TNF-α
25OHD	Spearman's rho	1,000	,148	-,384	-,235
	Sig. (2-tailed)	.	,533	,094	,318
	N	20	20	20	20
IL-1β	Spearman's rho	,148	1,000	,180	,403
	Sig. (2-tailed)	,533	.	,447	,078
	N	20	20	20	20
IL-6	Spearman's rho	-,384	,180	1,000	0,544*
	Sig. (2-tailed)	,094	,447	.	0,013
	N	20	20	20	20
TNF-α	Spearman's rho	-,235	,403	0,544*	1,000
	Sig. (2-tailed)	,318	,078	0,013	.
	N	20	20	20	20
* *p<0.05 level*					

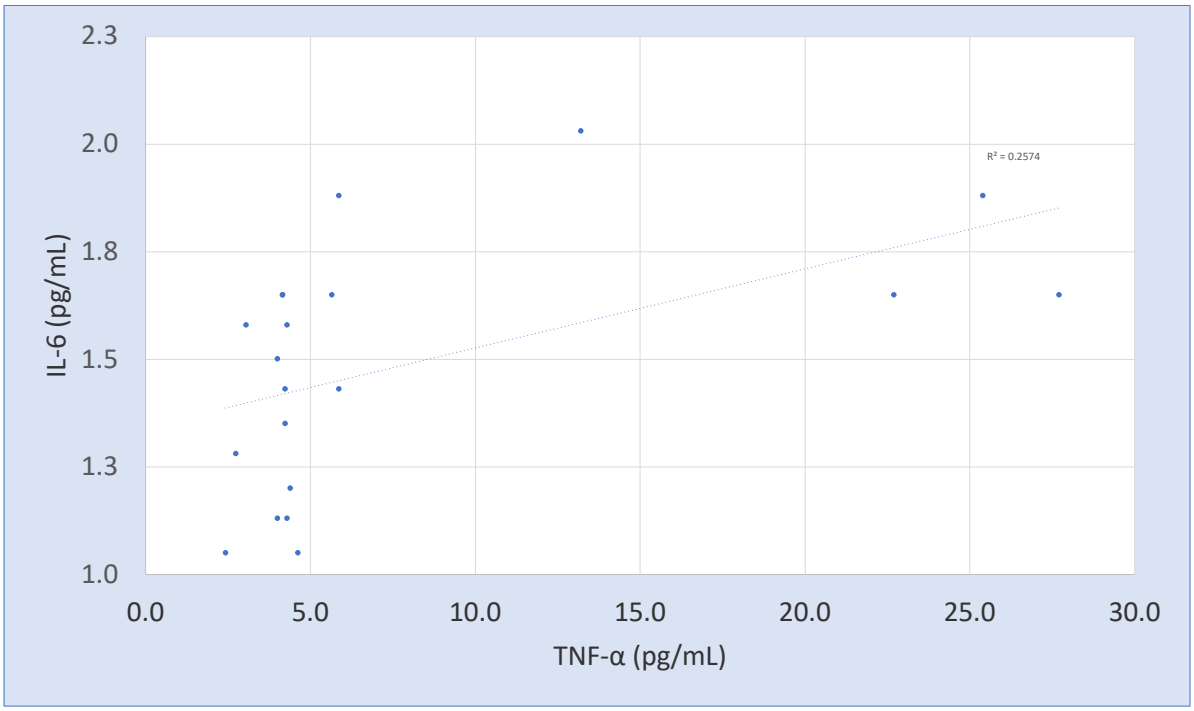


Figure 15. Scatter Plot Illustrating the Relationship Between IL-6 and TNF-α in Patients with RSV Infection

Among patients with RSV bronchiolitis, no statistically significant correlations were found between clinical disease severity and serum levels of 25(OH) vitamin D, IL-1β, IL-6, TNF-α, or C-reactive protein (CRP) (Table 17). All observed correlation coefficients were weak and did not reach statistical significance ($p > 0.05$). No association was identified between disease severity and these biomarkers, which is likely attributable to the relatively small sample size and the complex, multifactorial nature of the disease.

Table 17. Correlation Analysis Assessing the Relationship Between Serum 25(OH) Vitamin D Levels, Pro-inflammatory Cytokines, CRP, and Clinical Severity of Acute RSV Bronchiolitis

		Clinical Severity	25OHD	IL-1β	IL-6	TNF-α	CRP
Clinical Severity	Correlation Coefficient	1.000	0.136	0.264	0.146	0.149	-0.107
	Sig. (2-tailed) (p-value)	.	0.566	0.261	0.538	0.530	0.653
	N	20	20	20	20	20	20

Discussion

To address Objective 4, a correlation analysis was performed to evaluate the relationship between serum 25(OH) vitamin D levels, pro-inflammatory cytokines, and the clinical course of acute respiratory infections in children.

In the overall study population, statistically significant associations were identified between serum 25(OH) vitamin D levels and some of the investigated pro-inflammatory cytokines. A weak positive correlation was observed between 25(OH) vitamin D and IL-1β, indicating a tendency for IL-1β concentrations to increase with higher vitamin D levels. In addition, a moderate negative correlation was found between 25(OH) vitamin D and TNF-α, suggesting that higher vitamin D levels were associated with lower TNF-α concentrations. No statistically significant association was observed between serum 25(OH) vitamin D levels and IL-6.

No statistically significant associations between 25(OH) vitamin D and the investigated pro-inflammatory cytokines were demonstrated in any of the individual diagnostic subgroups. However, distinct interaction patterns among the cytokines themselves were identified. In children with bronchopneumonia, statistically significant positive correlations were observed between TNF-α and IL-1β, as well as between TNF-α and IL-6, suggesting coordinated activation of the inflammatory response in this patient group. In children with acute bronchitis, statistically significant correlations were found among all investigated pro-inflammatory cytokines. Negative correlations were observed between TNF-α and IL-1β, and between IL-6 and IL-1β, whereas a substantial positive correlation was identified between TNF-α and IL-6. In the healthy control group, only a moderate positive correlation between TNF-α and IL-6 was observed. No statistically significant correlations among the pro-inflammatory cytokines were found in patients with acute bronchiolitis.

Because variability in clinical severity was present only among patients with acute bronchiolitis, the relationship between disease severity, serum 25(OH) vitamin D levels, and pro-inflammatory cytokines was evaluated exclusively in this subgroup. In contrast, disease severity was homogeneous in patients with bronchopneumonia (moderate severity) and acute bronchitis (mild severity), making a similar correlation analysis in these groups inappropriate. Among patients with acute bronchiolitis, no statistically significant correlations were found between clinical disease severity and serum levels of 25(OH) vitamin D, IL-1β, IL-6, TNF-α, or C-reactive protein (CRP). The only statistically significant finding was a positive correlation between IL-6 and CRP, indicating coordinated activation of the inflammatory response in children with acute bronchiolitis. The remaining correlations did not reach statistical significance.

In the subgroup of children with RSV infection, the overall correlation analysis demonstrated statistically significant associations between 25(OH) vitamin D and selected pro-inflammatory cytokines. A moderate positive correlation was observed between 25(OH) vitamin D and IL-1β, while a moderate negative correlation was identified between 25(OH) vitamin D and TNF-α. In addition, a moderate positive correlation was found between IL-6 and TNF-α. When the analysis was restricted to children with RSV infection only, a statistically significant positive correlation with substantial strength remained between IL-6 and TNF-α, whereas the remaining correlations did not reach statistical significance. Similarly to the findings in the overall bronchiolitis group, no statistically significant relationship was observed between clinical disease severity and serum levels of 25(OH) vitamin D, IL-1β, IL-6, TNF-α, or CRP in patients with RSV bronchiolitis. All observed correlations were weak and failed to reach statistical significance. No association was identified between disease severity and the investigated laboratory biomarkers, which is likely attributable to the limited sample size and the complex pathophysiology of the disease. These findings suggest that the inflammatory response during RSV infection is characterized by coordinated activation of pro-inflammatory cytokines rather than by a direct relationship with serum vitamin D levels or disease severity.

Overall, the results indicate that although associations between vitamin D and certain pro-inflammatory cytokines were identified in the overall study population, these relationships were not maintained within the individual diagnostic groups. In contrast, stronger and disease-specific associations were observed among the pro-inflammatory cytokines themselves, suggesting that the inflammatory response is more closely related to the underlying pathophysiological mechanisms of each disease than to serum vitamin D concentrations.

The presence of statistically significant correlations between 25(OH) vitamin D and selected pro-inflammatory cytokines in the overall study population, but not within the individual diagnostic groups, is likely explained by the greater variability of data in the combined sample, which included both children with acute respiratory infections and healthy controls. Combining heterogeneous groups with different ages, clinical diagnoses, and immune response characteristics increased the range of observed values and, consequently, the likelihood of detecting statistically significant associations. Conversely, analyses within the individual diagnostic subgroups involved smaller sample sizes and more homogeneous clinical characteristics, thereby reducing statistical power and limiting the detection of significant correlations. Furthermore, differences in the pathophysiological mechanisms

underlying the individual diseases may explain the absence of a universal relationship between vitamin D and inflammatory biomarkers.

Studies investigating the relationship between vitamin D status and pro-inflammatory cytokines in children with acute respiratory infections remain limited. Most published studies have focused on the effects of vitamin D supplementation on immune responses in healthy children. A randomized controlled trial involving healthy children aged 4–11 years evaluated the effects of vitamin D supplementation on vitamin D status and related health outcomes. The investigators also assessed several inflammatory biomarkers, including TNF-α, IL-1β, and IL-6, to determine the potential influence of vitamin D on innate and adaptive immune responses. Children were classified as having vitamin D insufficiency at serum concentrations of ≤50 nmol/L and vitamin D sufficiency at ≥50 nmol/L. Following supplementation, no participants remained vitamin D deficient, and children with sufficient baseline vitamin D levels exhibited higher TNF-α concentrations in the supplementation group than in the placebo group. The authors suggested that this finding may reflect maintenance of a more balanced Th1/Th2 immune profile in children with better vitamin D status. Nevertheless, they emphasized that evidence regarding the effects of vitamin D on pro-inflammatory cytokines in children remains inconsistent, particularly with respect to IL-6.

Similar findings have been reported in healthy young children living at 45.5° north latitude, where serum 25(OH) vitamin D concentrations above 75 nmol/L were associated with significantly higher circulating levels of IL-6 and TNF-α. The authors proposed that vitamin D concentrations above this threshold may contribute to maintaining a balanced immune response.

In contrast, Hauger et al. demonstrated that winter vitamin D_3 supplementation at a dose of 10 μg/day, maintaining serum concentrations around 60 nmol/L, did not result in significant changes in markers of the innate immune response, including IL-6, in healthy children living at northern latitudes.

The findings of the present study are consistent with this heterogeneity in the published literature. In the overall study population, a moderate negative correlation was observed between 25(OH) vitamin D and TNF-α, supporting the established immunomodulatory properties of vitamin D and its ability to suppress the production of certain pro-inflammatory cytokines. In contrast, no statistically significant relationship was found between 25(OH) vitamin D and IL-6, whereas the positive correlation with IL-1β was weak. The absence of consistent associations between vitamin D and individual cytokines within the diagnostic

subgroups is likely attributable to both the limited sample size and the complex, multifactorial nature of the inflammatory response in acute respiratory infections. Comparable conclusions have been reported in a systematic review and meta-analysis evaluating the effects of vitamin D supplementation on immune responses in respiratory infections and inflammatory conditions. The authors demonstrated a statistically significant reduction in CRP following vitamin D supplementation but found no significant effect on IL-6 concentrations.

Regarding the absence of a correlation between the clinical severity of acute bronchiolitis and serum levels of 25(OH) vitamin D, IL-1β, IL-6, TNF-α, and CRP, the present findings are consistent with part of the available literature. Similar to our results, Golan-Tripto et al. and Mittal et al. reported no statistically significant association between serum vitamin D concentrations and disease severity in children with acute bronchiolitis.

Overall, both the available literature and the findings of the present study indicate that the relationship between vitamin D status and pro-inflammatory cytokines during childhood is complex and remains incompletely understood. These associations are likely influenced by multiple factors, including patient age, disease severity, infectious etiology, seasonal variation, the degree of vitamin D insufficiency, and individual immune response characteristics.

V. CONCLUSIONS

1. Children with acute respiratory infections exhibited differences in vitamin D status, with lower serum 25(OH) vitamin D levels occurring more frequently in acute bronchitis and bronchopneumonia.

2. Acute respiratory infections in childhood are associated with alterations in the pro-inflammatory cytokine profile, while the individual clinical entities, including RSV bronchiolitis, demonstrate distinct patterns of immune response.

3. The observed associations between serum 25(OH) vitamin D levels and pro-inflammatory cytokines support the role of vitamin D in the regulation of the inflammatory immune response during childhood.

4. No association was found between serum 25(OH) vitamin D levels, pro-inflammatory cytokines, and the clinical severity of acute respiratory infections. These findings do not support the routine use of vitamin D supplementation solely to modify the inflammatory response or reduce disease severity and further confirm the complex, multifactorial interaction between vitamin D status, immune response, and the clinical manifestations of acute respiratory infections.

VI. CONTRIBUTIONS

Contributions Novel for Bulgaria

1. A comprehensive assessment of serum 25(OH) vitamin D, parathyroid hormone (PTH), and the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α was performed in children with acute respiratory infections and healthy controls.
2. Distinct characteristics of vitamin D status and cytokine profiles were identified among the different clinical forms of acute respiratory infections in childhood.
3. The relationships between serum 25(OH) vitamin D levels and the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α were analyzed in children with acute respiratory infections.

Scientific and Theoretical Contributions

1. The present study expands current knowledge regarding the relationship between vitamin D status and the inflammatory response in children with acute respiratory infections.
2. The findings provide a basis for future clinical, epidemiological, and interventional studies aimed at further elucidating the role of vitamin D in the inflammatory response and the clinical course of acute respiratory infections in childhood.

VII. APPENDICES
Appendix 1

1.1. List of Scientific Publications Related to the Dissertation

1. Stoykova G., Shentov B. (2023). Study on 25(OH)D Status in Hospitalized Children with Acute Respiratory Infections – Preliminary Results. Journal of Biomedical and Clinical Research, 16(2), 131–135. https://doi.org/10.2478/jbcr-2023-0017

2. Petkova G.S., Mineva E.N., Petkova M.P. (2024). Study on Correlation Between Vitamin D Status and Pro-inflammatory Cytokines in Children with RSV Bronchiolitis. World Journal of Biology Pharmacy and Health Sciences, 20(2), 321–329. https://doi.org/10.30574/wjbphs.2024.20.2.0882

3. Petkova G.S., Mineva E.N., Botsova V.T. (2024). Clinical Study of Vitamin D Levels in Hospitalized Children with Acute Respiratory Infections. Pediatric Reports, 16(4), 1034–1041. https://doi.org/10.3390/pediatric16040088

1.2. List of Scientific Conference Presentations Related to the Dissertation

1. Poster presentation: Vitamin D Deficiency in Children with Acute Respiratory Infections. Stoykova G., Shentov B., Botsova V. Presented at the 15th National Congress of Pediatrics with International Participation, 23–26 September 2021, Borovets, Bulgaria.

2. Poster presentation: Preliminary Results of a Clinical Study of Vitamin D Levels in Hospitalized Children with Acute Respiratory Infections. Stoykova G., Botsova V., Shentov B. Presented at the 8th Veliko Tarnovo National Scientific Pediatric Conference “From Symptom to Diagnosis – Clinical Cases”, 9–11 June 2023, Arbanasi, Bulgaria.

3. Poster presentation: Preliminary Results of a Clinical Study of Vitamin D Levels in Hospitalized Children with Acute Respiratory Infections. Stoykova G., Botsova V., Shentov B. Presented at the 25th Jubilee National Conference for Pediatricians and

General Practitioners with International Participation, 23–26 May 2024, Hotel Majestic, Sunny Beach, Bulgaria.

4. Poster presentation: Preliminary Results of a Clinical Study of Vitamin D Levels in Hospitalized Children with Acute Respiratory Infections. Stoykova G., Botsova V., Shentov B. Presented at the 9th Veliko Tarnovo National Scientific Pediatric Conference “From Symptom to Diagnosis – Clinical Cases”, 31 May–2 June 2024, Arbanasi, Bulgaria.

5. Poster presentation: Results of a Clinical Study of Vitamin D Levels in Hospitalized Children with Acute Respiratory Infections. Stoykova G., Botsova V., Shentov B. Presented at the Jubilee Scientific Conference “50 Years of Medical University – Pleven”, 1–3 November 2024, Pleven, Bulgaria.

1.3 Participation in Research Projects

1. Research Project No. 8/2021: Assessment of Vitamin D Status in Children with Acute Respiratory Infections.
   Principal Investigator: Assoc. Prof. P. Laleva, MD, PhD.
   Host Institution: Department of Pediatrics, University Hospital “Dr. Georgi Stransski”, Pleven, Bulgaria.

2. Research Project No. 21/2022: Immunological Aspects of Vitamin D Deficiency in Children with Acute Respiratory Diseases.
   Principal Investigator: Prof. Adelaida Ruseva, MD, PhD.
   Host Institution: Department of Pediatrics, University Hospital “Dr. GeorgiStransski”, Pleven, Bulgaria.
   Funding: Medical University – Pleven.