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"IMMUNOLOGICAL MARKERS OF RESPIRATORY INFECTIONS IN BREASTFED INFANTS VERSUS FORMULA FED INFANTS"

SUMMARY OF THE PhD THESIS

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INTRODUCTION

Upper respiratory tract infections (URIs) are among the top of pediatric pathologies in our country. Moreover, according to World Health Organization (WHO) data, and worldwide, this pathology ranks first among the causes of morbidity and mortality in infants and young children. If we start from the classical interpretation of the pathogenesis of infections, we can state that their signs and symptoms in the human body are the direct consequence of microbial replication and the degree of cytotoxicity of pathogens. Although these mechanisms are important to understand the further development of respiratory infections, much of the current scientific research has focused on immunological responses to infections and their role in the pathogenesis of the disease. As a result, in modern medicine, there is an increasing tendency towards the evaluation of the immune response in order to establish the diagnosis of infectious diseases. The approach of the immunological diagnosis is supported by recent concerns of researchers who are making considerable efforts to find and validate new biological markers that reflect the immune response of the human body to the contact with microbial agents. In recent years, we have witnessed a breakthrough in computerization and technology processes, both in clinical medicine and especially in paraclinical specialties, which have raised the possibilities of medical diagnosis. The current development of new technologies in the field of biomedical sciences allowed the revolution of the laboratory diagnosis by obtaining accurate and precise outcomes for the analytical tests, regardless of their complexity.

All these aspects of acute respiratory infections and the complexity of the immunological diagnosis have been intertwined with the results of scientific research that revealed the existence of some correlations between the “immune system in breast milk” and the immune status of the breastfed infant with a view to outline the premises of the present paper. It aims at providing new arguments for strengthening the scientific basis that supports the idea of “transferring high specific protection” from the mother to the breastfed infant by assessing the production of cytokines in acute respiratory infections in infants naturally fed versus infants with artificial nutrition. The involvement of some cytokines in the immunopathogenetic mechanisms of respiratory infections, the significant part they play in immunomodulation and immunoprotection, and the fact that breast milk cytokines remain immunologically active in the infant’s organism are very strong arguments for their use as biological markers for the assessment of the immune response of the infant to respiratory infections.

The paper is structured in 9 chapters, includes 217 bibliographic notes, and offers in the first part a wide range of topical information and results of certain literature studies related to the subject chosen, while the second part of this doctoral thesis presents the results of my personal scientific investigations, illustrated with the help of an iconography comprising 82 tables, 32 figures and 54 charts, which complete and sustain the text, and prefigure the final conclusions.

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With all my love, I dedicate this doctoral work to my father's memory, Nicolae Popa.

Keywords: respiratory infections, infants, natural feeding, formula feeding, immune response, immune markers, cytokines, interleukin 2, interleukin 8

GENERAL PART

The first part of the paper is made up of four chapters, in which the current state of knowledge regarding the subject chosen is presented. Thus, **the first chapter** presents the current aspects of respiratory infections in infants, both internationally and at national level. Numerous studies show that infant respiratory diseases rank first in the pediatric diagnosis hierarchy, and of the upper respiratory tract infections (URIs), pneumonia and bronchiolitis are major causes of hospitalization in infants under 1 year of age with increased prevalence during winter. According to World Health Organization (WHO) data, respiratory infectious pathology is the leading cause of morbidity and mortality among infants and young children, especially in the underdeveloped or developing countries [1-13]. Also, in the first chapter, I have elaborated on some etiological considerations of acute respiratory infections. These have revealed that the involvement of viral agents in the etiology of acute respiratory infections – URIs is more frequent, that most of the time, pathological agents of bacterial origin are responsible for coinfections or superinfections and are predominantly associated with an unfavourable evolution of the disease, up to fatal complications, especially in infants. Furthermore, in the etiological picture of acute respiratory infections, the central element is the respiratory syncytial virus (RSV) [14-25]. The first chapter also includes aspects related to the risk factors for URIs, to the need to keep under control the risk factors for good a respiratory prophylaxis. Thus, breastfeeding and adequate parenting (limiting exposure of the infant to pollutants such as cigarette smoke) can be education methods meant to combat modifiable risk factors [25-45].

Since the core of this study is the immunological aspects of respiratory infections in infants fed naturally versus those artificially fed, **the second chapter** presents the immunological mechanisms involved in the pathogenesis of respiratory infections in infants. This chapter is structured in two subchapters. The first subchapter reveals the general notions of immunity (definition, immune system functions, types of immune response), as well as aspects of the

complex organization of the immune system to create an optimal framework for the development of immune mechanisms, and to elaborate the innate, nonspecific immune response, respectively the acquired specific immune response, with its two components, cellular immune response and humoral immune response [48-56]. The second subchapter describes the correlations between immunological mechanisms and pathogenesis of respiratory infections using the RSV model, an experimental model that served researchers to study the multiple effects of viral activity on both the respiratory tract and the human immune system. This subchapter represents basically a synthesis of the numerous studies that have brought about the most eloquent scientific data, for a better understanding of the immune response to URIs of viral etiology in infants who, although protected by the presence of RSV-specific maternal antibodies, represent, however, the most immunologically immature category, and therefore the most susceptible to contacting viral infections. In the description of the innate or non-specific immune response in infants with RSV, I have revealed both the involvement of cellular components and cytokines, highlighting the interleukin 8 (IL-8) intervention in chemotaxis and phagocytosis, central processes of innate immunity. Also, in describing the acquired or specific immune response from RSV infection in infants, I highlighted both the involvement of cellular and humoral components, pointing to the immunomodulatory cytokine interference in Th1/Th2 response differentiation, as well as the involvement of interleukin 2 (L-2) in the Th1-type immune response [77-130].

Chapter three is dedicated to cytokines. In the first part, it contains a general characterization of cytokines, their definition and classification, and in the second part, there are presented the functional aspects of interleukin 2 (IL-2) and interleukin 8 (IL-8), the two cytokines that constituted the diagnostic markers at the heart of the immunological evaluation of the infants under study [48-77].

Chapter four covers general aspects of laboratory diagnosis in acute respiratory infections, highlighting the major role of acute phase proteins in the diagnostic algorithm of inflammatory response in respiratory infections, as well as the current directions of laboratory assessment for immunological markers. If inflammatory markers, such as C-reactive protein (CPR), procalcitonin (PCT), erythrocyte sedimentation rate (ESR), or total leukocyte count outline an inflammatory context without specificity for acute respiratory infections, current scientific research has shown increases, more or less specific, of some immunological markers, such as

cytokines, in URIs. The production of cytokines after stimulation by viral, bacterial, fungal antigens can be measured by a variety of methods: ELISA (Enzyme-Linked ImmunoSorbant Assay), flow cytometry (determination of intracellular cytokines), polymerization reaction in real time (real time PCR) for the expression of cytokines by mRNA, multiplex polymerase chain reaction (PCR) assays [131-147].

PERSONAL RESEARCH PART

Chapter five prefaces the actual personal research by presenting the elements of the current scientific context that have led to the development of the motivation of research. This chapter includes aspects related to the concept of "immune system in breast milk", challenges, imperatives, and evidence, from the outline of this concept to its current evolution. Starting from the concept that bioactive factors in breast milk constitute a "true immune system" that complements the immaturity of the new-born's adaptive immune system, as well as the insufficient development of the components of the innate immune system, these have been classified according to several criteria: chemical nature, their functions, the mechanism of action within the immune response. The role of bioactive factors in breast milk on the infant's immune system has been proven by numerous studies. Some of these have highlighted the significant part of cytokines in breast milk in immunomodulation and immunoprotection. Scientific evidence has strengthened the concept of "immune system in breast milk" and highlighted its correlations with the immune status of the breastfed infant [151-172, 202].

The first chapter of personal research also discusses the benefits of breastfeeding on the immune system development and anti-infectious defense in infants and young children, as well as aspects of the natural dynamics of immune benefits of breast milk, in the context of promoting sensitization or immunological tolerance for the infant fed with breast milk. This chapter also encompasses the chart of the "entero-broncho-mammary pathway", based on literature data, according to which this secretory IgA B lymphocyte circuit, together with the mucosal immune system, represent the means of transferring the specific high-level protection from the mother to the child through breastfeeding. The described evidence, reinforced by the results of the research on the immune benefits of breast milk in anti-infectious defense, in the immunological development of the infant and the young child, opens up new perspectives in monitoring the

health of the infant, leading the current diagnostic trends towards increasing the use of immunological markers [154-183, 202].

Chapter six presents the premises, objectives, materials and methodology of the personal research. **The premises of the research** are, in fact, a synthesis of all the aspects presented in chapter five, prefacing the personal part of the thesis, with the current scientific data, necessary for the creation of the motivational research background. Starting from the research premises, the following clear objectives were set:

I) Immunological evaluation of infants with URIs and finding some correlations between serum levels of IL-2 and IL-8 cytokines with serum or plasma levels of the other inflammatory and immunological parameters in the studied groups;

II) Comparing the immune status of breastfed infants with that of infants fed with various milk formulas according to gender and age groups;

III) A more accurate correlation of immunological parameters with the type of infant's milk diet regarding the more frequent types of URIs;

IV) Completion of scientific evidence advocating the transfer of cytokine dosages from the research compartment to the paraclinical diagnosis compartment;

V) Obtaining arguments in favour of alignment of serum IL-2 and IL-8 cytokine doses in the immunological marker package of laboratory diagnosis for URIs;

VI) Contribution to the setting of the baseline values for serum IL-2 and IL-8 cytokines by their serum dosages in control group subjects;

VII) Highlighting a correlation between the risk factors represented by the harmful parents' behaviours (smoking during pregnancy and lactation) and accelerating inflammatory processes.

Research material: To achieve the objectives aimed at, I have conducted a prospective randomized study, which took place over a period of 12 months (January 2014 - January 2015) in the Pediatric Clinic of the Pediatric Clinical Hospital of Sibiu. I included infants **aged 1-22 months** who met the criteria established for the investigated group, respectively for the control group. The study group consisted of **86 subjects** divided into:

A) ***Research group*** included ***54 infants*** selected according to the following criteria:

Inclusion criteria:

- Presence of signs and symptoms for acute respiratory infections on admission;
- Laboratory tests suggestive of a positive inflammatory profile;
- Milk as the basic diet;

Exclusion criteria:

- Signs and symptoms suggestive of acute infections with other localization than the respiratory one;
- The presence of chronic diseases in the personal medical history of the mother and infant;
- Existence of malformations of the respiratory and cardiovascular systems in the baby;
- Corticosteroid or immunosuppressive treatments (mother, baby) prior to admission;
- Hemolyzed or lipemic biological samples.

B) ***The control group*** *comprised* ***32 infants*** *selected based on the following criteria:*

Inclusion criteria:

- Absence of signs and symptoms for URIs or with other localization;
- Negative inflammatory profile, supported by laboratory tests;
- Milk as the basic diet;

Exclusion criteria:

- Signs and symptoms suggestive of URIs or with other localization;
- Laboratory tests suggestive of a positive inflammatory profile;
- Presence of chronic diseases in the personal medical history of the mother and infant;
- Existence of malformations of the respiratory and cardiovascular systems in the baby;
- Corticosteroid or immunosuppressive treatments (mother, baby) prior to admission;
- Hemolyzed or lipemic biological samples.

Both the investigated group and the control group were subdivided into 2 groups, depending on the type of milk diet, i.e. natural food (**LM/MLM**) and artificial food (**LP/MLP**):

- **Research group: 28** naturally fed infants (**LM**) and **26** artificially fed infants (**LP**);
- **Witness group: 16** naturally-fed infants (**MLM**) and **16** artificially-fed infants (**MLP**).

The research methodology aimed at performing a comparative analysis of the immune status of breastfed infants versus infants fed with milk formulas, including:

- Anamnestic and clinical diagnostic data recorded in the observation sheets of the patients who met the criteria for inclusion in the study;
- Usual laboratory investigations: Blood count, C-reactive protein, Sideremia, Proteinemia, Serum Protein Electrophoresis, Serum Immunogram, Complement C3 Fraction.
- Special laboratory investigations: IL-2 and IL-8 interleukins serum levels measurements, for which ELISA research kits were used, which were not provided with recommendations for reference value ranges. Therefore, following the analysis of the results of studies that made cytokine dosages on healthy subjects, mostly performed on adult subjects, I considered as increased values for interleukin 2 (IL-2), all values obtained > **15 µg/ml,** and for interleukin 8 (IL-8), all values **> 35 pg/ml.**

Ethical aspects of research: To achieve this research, the ethical aspects of the Helsinki Declaration have been observed. For each of the patients included in the study, informed parental consent was obtained because the subjects ranged from 1-22 months of age. Upon admission, the consent of the parents was documented by the signature on the acceptance form, a form approved by the Ethics Committee of the Sibiu Pediatric Hospital. Procedures have been observed to minimize the risks of blood collection, including interrogation in relation to the presence of chronic or transmissible diseases in personal or family medical history. The subject's confidentiality has also been observed.

Statistical data analysis: For the statistical processing of the data, the Excel 2010 database was used, which allowed the application of the SPSS statistical program, version 21. There were applied methods of inferential statistics, both of the type of parametric tests, which gave valid results based on the mean of the variable, as well as non-parametric tests, median-based tests. The following tests were used: t-Student (T-TEST), Chi square test (CHI-TEST), ANOVA,

Kolmogorov-Smirnov, Mann-Whitney test, Kruskal-Wallis, multiple regression (using the stepwise method). A statistically significant difference was considered to be a $p < 0.05$ value.

Chapter seven is the most extensive part of the thesis and presents **the results of personal research,** while **chapter eight** presents the discussions on research results, the complex analysis of the data obtained, compared with the data from the literature. Below, there are the more important aspects of the results and discussions of my personal research part.

Regarding the results of the analysis performed for the general, demographic and clinical characterization of the groups under study, the following are worth mentioning:

- the non-homogeneous distribution of the subjects in the groups: 63% for the research group, 37% for the control group;
- statistically significant differences ($p = 0.05$) between the mean age of the subjects in the study group (10.8 months) and the mean age of the subjects in the control group (7.4 months);
- relatively homogeneous distribution by gender, both in the research group (44% males / 56% females) and in the control group (56% males / 44% females);
- in the investigated group, which was divided into three groups, depending on the diagnosis of the respiratory disease, the most common diagnosis was pneumonia (54%), followed by the diagnosis of URI (28%), while the diagnosis of bronchiolitis was the most rare (18%);
- from the point of view of the presence of the personal history of respiratory infections, the distribution between the two groups was uniform (over 50% of the subjects of the investigated group, respectively of the witness group presented personal history of respiratory infections);
- after the separation of the studied groups (the investigated group and the control group) in 4 different groups according to the exposure to the intra-family smoking (smoking mother, smoking father, both parents smokers, no exposure), the analysis of the distribution of the subjects revealed that in the research group, the largest number of infants were exposed to intra-family smoking (35% with smoking father, 17% with smoking mother, 22% with both parents as smokers), and in the control group the subjects without exposure to intra-family smoking represented more than half (56%) of the total number of subjects.

The statistical analysis at the level of the four resulting groups (LM, LP, MLM, MLP), after the subdivision of the research group and of the control group, according to the different milk diet of the infants revealed:

- Statistically significant differences ($p = 0.005$) between the mean age of the infants in the investigated group of the naturally fed infants (LM) and the witness group of the breastfed infants (MLM);
- There were no statistically significant differences between the two genders, in terms of average age, in any of the four groups (LM, LP, MLM, MLP);
- Regarding the distribution of subjects in the four different groups in terms of smoking exposure, i.e. non-exposed and the three categories of exposed (smoking mother, smoking father, both parents smokers), there were statistically significant differences ($p = 0.04$) between the examined groups (LM, LP, MLM, MLP);
- There were no statistically significant differences regarding the presence of respiratory diseases between the two groups of investigation (LM and LP), although pneumonia was more frequent in both groups (60.71% in the LM group and 46.15% in the LP group), and the number of bronchiolites was noticeably lower among the breastfed infants (10.71%).
- The distribution according to the presence/absence of personal history of respiratory infections showed statistically significant differences ($p = 0.004$) among the studied groups, with a higher presence of personal history of respiratory infections in infants fed with various milk formulas (LP, MLP), compared to breastfed infants (LM, MLM), both among the research groups (39.29% for LM and 69.23% for LP) and among the control groups (31.25% for MLM and 81.25% for MLP);
- Regarding the statistical analysis of the usual biological parameters taken into consideration in the studied groups, I recorded the following results:
    - The comparative analysis of the variables that quantified the C-reactive protein (CRP) showed statistically significant differences ($p < 0.0001$) globally, among the four groups, and according to the gender of the patients, it revealed statistically significant differences only for the male gender ($p = 0.021$), in whom the highest mean value of CRP was also obtained, in the LM group (breastfed boys had a more pronounced acute phase reaction);

- There were no statistically significant differences in the differences between the variables that quantified plasma hemoglobin, globally and for females, but there were differences ($p = 0.04$) in the male gender, between the investigated group of artificially fed infants (LP) and both witness groups: MLM and MLP;
- The statistical analysis of the mean values of sideremia revealed significant differences between the investigated groups (LM, LP) and the control groups (MLM, MLP), both globally and in each of the two genders, and the mean values of sideremia were significantly higher in male infants fed with breast milk, both in the investigated group (LM) and in the control group (MLM);
- The statistical analysis of the variables that quantify the total leukocyte count revealed significant differences ($p < 0.001$) among the four groups, both globally and by gender.
- The statistical analysis for total serum protein did not show significant differences between the groups, globally or by gender; Of the electrophoretic fractions of the proteins, the alpha fraction ($\alpha 2$), although presenting mean values within the reference range, the differences were significant globally, both in males and in females, between LM versus MLM, LM vs. MLP, LP versus MLM, and LP versus MLP groups;
- Regarding the statistical analysis of serum immunoglobulins, there were significant differences between groups, globally for IgA ($p = 0.005$), IgM ($p = 0.003$), IgG ($p = 0.014$), for females for IgA ($p = 0.046$), IgG ($p = 0.05$), in males for IgM ($p = 0.001$).
- The statistical analysis for the complement C3 fraction did not reveal significant differences between the four groups, either globally or by gender, but statistically significant differences ($p = 0.011$) were found in the control group of artificially fed infants (MPL) between genders, with higher values for the female gender.

❖ Immunological evaluation of infants from the studied groups using the already validated laboratory parameters was supplemented by the IL-2 and IL-8 interleukins dosing; The statistical analysis and the study of the correlations between interleukins and other analysed variables revealed the following aspects:

- For both interleukins, there were statistically significant differences between the investigated and control groups (LM versus MLM, and LM vs. MLP, but also LP vs.

MLM, LP vs MLP), both globally and per gender ($p < 0.0001$); Mean values of both IL-2 and IL-8 were much higher in the LM group than in the LP group;

- Regarding the study of independent prediction factors, globally, for the various multiple regression variables (stepwise method), the results of this study reveal that acute respiratory infections are independent prediction factors for both IL-2 and IL-8 interleukins values ($p < 0.0001$), as well as for C-reactive protein ($p = 0.0014$);
- In the group of naturally-fed infants (LM, MLM), only acute respiratory infections were found to be independent prediction factors for the CRP value ($p = 0.018$), and of IL2 ($p = 0.0013$), IL8 ($p = 0.002$) interleukins;
- In the group of artificially fed infants (LP, MLP), one prediction factor for several variables, and several prediction factors for a variable have been highlighted:
    - The prediction factor of URIs for the CRP ($p=0.03$) and IL-8 ($p=0.01$) variables;
    - Intra-family smoking ($p=0.0083$) and IgA ($p=0.0005$) were prediction factors for variable IL-2;
    - Complement C3 faction had the most prediction factors: age ($p=0.0011$), exposure to smoking ($p=0.018$), IgM ($p=0.0033$), and IgG ($p=0.012$) immunoglobulins;
- The assessment of the quality and the diagnostic value of the analysed parameters in terms of performance criteria represented by sensitivity and specificity (AUROC calculation) revealed that, globally:
    - for IL-2 values ≥ 17.2 pg /ml, the ability of this variable to identify patients with URIs is characterized by 100% sensitivity and specificity;
    - for IL-8 values ≥ 9.9 pg /ml, performance criteria for URIs predictive ability were 100% sensitivity and 96.87% specificity;
    - -for CRP values ≥ 7 mg /dl, the sensitivity was 66.67% and the specificity was 100%.
- At the level of the studied groups, there were significant positive correlations ($p <0.0001$) between the two IL-2 and IL-8 interleukins, but also between them and the analysed quantitative parameters, with different correlation coefficient values: CRP, IgA IgM, IgM, IgG immunoglobulins, total leukocyte count, neutrophil count and

lymphocyte count, alpha protein electrophoretic fraction; although there were statistically significant correlations between CRP and the two IL-2 and IL-8 interleukins, the results of this study showed a low degree of positive association between these variables (non-homogeneous graphical distribution of correlation points);

- The results of the study showed a positive correlation between intra-family smoking exposure and the IL-2 ($p = 0.0006$) and IL-8 ($p = 0.047$) quantification variables; The highest values for the variables that quantify IL-8 and IL-2 were recorded in the most exposed infants with both parents smokers, results similar to those of recent studies that demonstrated that "tobacco causes acceleration of inflammatory processes by increasing the expression of inflammation mediators, and an increase in inflammatory serum markers"; Concerning the intricate influence of the beneficial factor represented by breast milk with the destructive factor represented by tobacco, it should be mentioned that the results for the variables that quantify IL-2 and IL-8 were higher in the research group of naturally fed infants (LM) compared with the investigated group of the artificially fed infants (LP) within all infant groups exposed to intra-family smoking;
- In the LM research group, the results of the specific immune response analysis from the acute respiratory infections, by quantification of IL-2 values, have shown to be approximately 10 times higher in the case of more severe respiratory infections (pneumonia); Regarding the quantification of the non-specific immune response from URIs, by the IL-8 dosing results, in both investigated groups (LM, LP), globally, between the IL-8 quantitative variables, according to the respiratory infections, statistically non-significant differences were reported; It should be noted that a higher degree of positive correlation has been found among the IL-8 values and the severity of respiratory infections in the research group of naturally-fed infants (LM) as compared to its level in the investigated group of infants fed with various milk formulas (LP);
- Personal pathological respiratory antecedents are a poor predictor for the interpretation of cell-specific immune response through IL-2 and the non-specific immune response quantified by IL-8;
- Because the prevalence of Hyper IL-2 (all values that exceeded 15 pg /ml) in both investigated groups (LM, LP) was 100%, for all age groups, it can be said that for the

investigated groups (LM, LP), the specific immune response to respiratory infections, quantified by interleukin 2 (IL-2) was independent of the infant milk diet; However, in the control group of artificially fed infants (MLP), there were differences between the age groups for hyper IL-2 prevalence. These results support the idea according to which, in healthy infants (in the absence of a diagnosis of respiratory infection or of other localization) the serum concentration of IL-2 may vary depending on the milk diet, and that in case of feeding with various milk formulas, there could be a non-infectious antigen stimulation that could influence the immune response, especially because "during childhood, there is a fine balance between tolerance and immunological sensitization" [158, 173-176, 202];

- The prevalence of Hyper IL-2 in the study groups (LM, LP) was 100% for all categories of acute respiratory infections studied (URIs, bronchiolitis, pneumonia), which would justify considering IL-2 as a possible immunological marker in the direct diagnosis of acute respiratory infections, but with no importance in the differential diagnosis of URIs;
- The prevalence of Hyper IL-8 (all values exceeding 35 pg/ ml) in the study groups (LM, LP) and per age groups ranged from a minimum of 62.5% in the LP group, in the age group between 6-11 months, and a maximum of 100% in the LM group, in the age group over 18 months. According to these results, it can be said that the non-specific immune response in respiratory infections, quantified by IL-8, is dependent of age and of milk diet of infants;
- The increased prevalence of Hyper IL-8 in acute upper respiratory infections in infants is a proof for the use of this chemokine, as a marker for the positive diagnosis, but the poor correlation of Hyper IL-8 with the degree of severity of the respiratory infections did not allow the outlining of the arguments needed for using IL-8 as a differential diagnosis marker in acute respiratory infections. The lack of these arguments may be due, on the one hand, to the moderate number of the analysed cases and the very short half-life ($T_{1/2}$) for IL-8, whose serum concentration rapidly increases in acute infections and, on the other hand, to the personal socio-demographics of subjects included in the study [68, 69, 73, 76];
- The results of the comparative analysis between the prevalence of the elevated values of the two studied interleukins (IL-2 and IL-8), depending on gender and type of acute respiratory infection, revealed significant differences only for IL-8.

Chapter nine presents the **final conclusions** of the research, after the analysis of groups and all the correlations established between them, depending on the variables chosen:

1. The breastfed infants in the investigated group registered a statistical significant difference in terms of the mean age from those in the control group;
2. In both groups, pneumonia was the most common, but the number of bronchiolitis was noticeably lower among the breastfed infants.
3. Both in the investigated groups (LM, LP) and in the case of control groups (MLM, MLP), statistically significant differences were recorded due to a significantly higher presence of the respiratory pathological antecedents in infants fed with various milk formulas compared to breastfed infants.
4. Among the four groups examined (LM, LP, MLM, MLP), there were statistically significant differences in intra-family smoking exposure according to the distribution of infants within the groups corresponding to the four categories.
5. The serum concentration of C-reactive protein (CPR) was statistically significantly different between the investigated groups (LM, LP) and the control groups (MLM, MLP), globally, and by gender, only in the males.
6. The mean values of total leukocyte count were significantly higher in infants with acute upper respiratory tract infections in the investigated groups (LM, LP), compared to the values recorded for controls (MLM, MLP).
7. At the level of the investigated groups (LM, LP), there was a slight increase in the mean neutrophil count, more pronounced in the subjects with artificial food (LP), and an average value of leucocytes higher in the breastfed infants groups (LM).
8. At the level of the entire group, the mean value obtained for the lymphocyte weight in the total leukocytes ranged within the age range, but there were statistically significant differences between the investigated groups (LM, LP) and the control groups (MLM and MLP), globally and in females.
9. The statistical analysis of the mean values of sideremia revealed significant differences between the investigated groups (LM, LP) and the control groups (MLM, MLP), both globally and in each of the two genders. The low values of sideremia in both investigated groups (LM, LP) express the likelihood of the occurrence of iron deficiency anemia in an infectious context.
10. The statistical analysis for total serum protein did not reveal significant differences between groups, either globally or by gender. For *the α2 fraction,* the differences were statistically

significant between the groups to be investigated and the control groups in both genders, suggesting a higher diagnostic value in infant respiratory infections.

11. In the group of artificially fed infants with different milk formulas, humoral immune response was more pronounced in the female gender, both for the investigated group (LP) and for the MLP control group. Serum complement component C3 revealed a more intense nonspecific immune response for the artificially fed female infants in the control group (MLP).
12. Determination of the serum concentration of IL-2 and IL-8 has an originality note under the conditions in which these immunological markers do not yet have validated methods of laboratory determination.
13. The assessment of the acquired specific immunity by the statistical analysis of the results obtained for IL-2 dosing highlights the fact that in acute respiratory infections in females, there appears to be a better expressed cellular immune response than in males.
14. The assessment of non-specific innate immunity by statistical analysis of the results obtained for IL-8 dosing, allows stating the following:
    - Naturally fed infants develop an ampler nonspecific innate immune response.
    - In females, the non-specific immune response is better expressed
    - In the LM, MLM groups, only URIs were prediction factors for CRP, IL2, IL8.
15. In the group of artificially fed infants (LP, MLP), several independent prediction factors were highlighted for the value of a variable; the CRP and IL-8 variables had only URI as independent predictive factors, IL-2 had intra-family smoking and IgA as predictive factors, and the C3 complement fraction had the most prediction factors i.e. age, smoking exposure, immunoglobulin M and G values.
16. For the entire study group, statistical analysis of the quantitative variables revealed positive correlations for almost all variables studied, respectively negative correlations between lymphocytes weight and IL-2, IL-8, CRP. We have found the lack of significant correlations between the monocyte weight and the variables studied.
17. For the ability to identify patients with URIs, CRP has a good indication as a diagnostic marker for URIs in infants with a 70% sensitivity and 100% specificity, and with a better performance at the level of the groups made up of breast-fed infants (LM and MLM)

compared to that registered in the groups including infants fed with various milk formulas (LP and MLP);

18. IL-2 and IL-8 have shown maximum performance to identify patients with URIs (100% sensitivity and specificity of at least 97%), with a higher prediction capacity than CRP; the sensitivity and specificity of the IL-2 and IL-8 dosing tests did not show any differences according to the infants' milk diet, performance being maximum in all four groups (LP, LM, MLM, MLP)
19. Intra-family exposure to cigarette smoke correlates positively with serum levels of IL-2 and IL-8, especially for both smoking parents.
20. In the study group of breastfed infants (LM), there has been recorded a cell-specific immune response measured by IL-2 values, 10-fold more intense in the case of severe URIs (pneumonia).
21. In both investigated groups (LM, LP), globally, there were statistically significant differences between the values of the variables that quantify IL-8 according to the type of respiratory infections.
22. In the investigated group of breastfed infants (LM), between IL-8 values and the severity of respiratory infections, I found a higher degree of positive correlation compared to the group of infants fed with milk formulas (LP).
23. Personal history of respiratory infections is a poor predictor in the interpretation of the specific cellular immune response quantified by IL-2 and of the non-specific innate response quantified by IL-8.
24. For the investigated groups (LM, LP), the specific immune response in respiratory infections, quantified by IL-2, was independent of the infant's milk diet.
25. IL-2 may be considered as a possible immunological marker in the direct diagnosis of acute respiratory infections, but unimportant in the differential diagnosis of URIs.
26. Nonspecific immune response in URIs, quantified by IL-8, is dependent on age and milk diet of infants.
27. The low degree of correlation of elevated IL-8 levels with the severity of respiratory infections has not allowed outlining the necessary arguments for the use of IL-8 as a differential diagnosis marker in acute respiratory infections.

28. The prevalence of elevated IL-2 levels by gender and type of URI was independent of these factors, while the prevalence of elevated IL-8 levels was influenced by both gender and by the type of URI.

The originality and innovative contributions of the thesis are:

- The objectives of the thesis are not found in another study, namely IL-2 and IL-8 dosing in parallel to breastfed infants, respectively infants fed with milk formulas in the context of the diagnosis of acute respiratory infections.
- The kits used to determine serum concentrations for cytokines have been designed for determinations in the field of research without recommendations for the reference intervals, so that after analyzing the results of the literature studies, we have set our own values for the comparative analyses, contributing to the establishment of reference values, and their use in shaping the immunological profile in acute respiratory infections in children.
- Opening up several future research directions, such as the cytokine study in dynamics, correlation with a certain etiological diagnosis of viral or bacterial infection.