“Lucian Blaga” University of Sibiu

DOCTORAL THESIS

(Summary)

Immunological serum markers and obesity, correlation with infant nutrition

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INTRODUCTION

The thesis is based on a two fundamental and universally accepted consensus in the medical world:

- The obesity is a growing scourge of the society;
- The human milk nutrition reduces the risk of obesity.

The obesity in children has become a public health issue – over 40 millions of scholars are obese, the observation being even more serious how much this increase is accompanied by type 2 diabetes mellitus, equally[1].

The specialists of The Center for Disease Control (CDC) and The Monitoring Center of Health and Nutrition (NHANES) drew attention to the increase of prevalence of obesity in 6-23 months of age, the tendency recorded over the past 30 years [2].

Recently, Roy et al published in 2016 the results of a study in which they concluded that the second-month of life excess weight it may be considered a risk factor for the obesity in the prescholars and even in adolescents [3].

Although there are few clinical trials focused on the inflammatory effects of the increased velocity of infant weight, all focus on a common finding: the rapid postnatal growth in the first 6-12 months of life represents a strong risk factor for metabolic disease independent of birth weight [4].

Overall, the immediate postnatal period is very clear a sensitive window for modeling the nutritional and inflammatory responses (meta-inflammation or obesity-induced inflammation, inclusively), but currently there are a few guidelines regarding the management of a rapid growth in infants [5].

Since 1999, von Kries [6] promote the role of human milk in preventing obesity, by publishing the results according to which the overweight or obesity risk (the prevalence at 5 years) descreases proportionally to the breastfeeding duration.

Although it does not exist a typification of human milk immunological composition derived from healthy mothers (conversely, it does a substantial inter~ans intra-individual variations), the research in the field of human milk nutrition have suggested the existence of a „basal” common immunological composition, which all women, regardless of their origin, can reproduce it. And these physiological variations in the immune profile of human milk just reflect the individual models in immune system of the child.

The way by which „per se" the human milk nutrition prevents obesity is correctly evaluated by the whole immunological composition of human milk.

The doctoral thesis contain 180 pages: 49 pages for literature review, 108 pages for personal research and 21 pages for bibliography. Also contain 51 tables, 11 figures and 25 graphics. Are recorded 251 references, more than 40 % belonging to the last 5 years and more than 15 % to the last 3 years.

THE LITERATURE REVIEW

The first part of the doctoral thesis is made up of 3 main chapters I which they are addressed to the theoretical point of view regarding the obesity in children and the human milk nutrition.

The **chapter 1** is meant to human milk which is describes the composition/biostructure which gives it the immunological superiority. The human milk major bioactive factors and its interrelation occupies the next subchapter. Is documented the role of human milk nutrition in the pediatric pathology, respectively the protective role, antiinfectious and anti-inflammatory ones and the energetic role, to prevent the overweight and obesity. Starting from its uniqueness, are described the determinant factors of the inter~and intra-individual variations of human milk.

In the **chapter 2,** entitled "The inflammatory immunological markers in relation to human milk", are described in detail the most important markers: interleukin-6 (IL-6) and leptin. Are also mentioned adiponectin, interleukin-8 and chemerin. Initially, the IL-6 was considered the most important marker of (neonatal) sepsis and has been widely used in this regard. More studies have shown a positive association between obesity and IL-6 levels, also a positive association between IL-6 and insulin resistance and type 2 diabetes mellitus. Unfortunately, the study in a healthy population regarding the IL-6 levels and growth in infants are limited [7, 8]. Due to its dual function, leptin links the neuro-endocrin system to the immune system. The role of leptin in immune and inflammatory response modulation has recently become more and more obvious. The increasing production of leptin during infection and inflammation suggests that leptin is a part o cytokine network which governs the inflammatory-immune response and the host defense mechanisms [9-13].

The chapter 3 approaches the obesity and the low-grade inflammation. The worldwide prevalence of the child obesity has greatly increased over the last 3 decades [14-16]. This observation is even more serious as it has been found the growth alike of type 2 diabetes mellitus in children [17, 18]. Some studies have shown a positive correlation between

seric levels of IL-6 and obesity and glucose intolerance, similar in the adult and pediatric population [19-21]. Although these studies do not refer to the physio-pathological aspect of the results, the association of the circulating IL-6 with anthropometric and metabolic markers link to insulin resistence indicates the participation of this cytokine to the insulin resistence since childhood and continues throughout adulthood. Most of the literature brings clear evidence that leptin from human milk is positive correlated with maternal body mass index (BMI). Of all identified bioactive compounds in human milk, the leptin provides the clearest indication that maternal BMI is positive associated with leptin human milk concentration. The relation study of human milk leptin concentration and 6 weeks, 4, 12 and 24 months maternal BMI has highlighted the inverse correlation between the two parameters just at 4 month of age [22, 23].

Other two chapters are intended to other factors which influenced the obesity in children: the maternal obesity during pregnancy and the genetic ones. Finally, are listed the cardio-vascular complications of the obesity.

PERSONAL RESEARCH. The premises of the doctoral thesis are focused on two fundamental and universally accepted consensus in the medical world:

- the obesity is a growing scourge of the society;
- the human milk nutrition reduces the risk of obesity.

The motivation of the thesis starts from the need of the inflammatory status evaluation in overweight and obese intants. As long as between obesity and the human milk nutrition there is a closely connection, than it needs a permanent reconsideration.

The main objectives are:

1. **the identification of an inflammatory markers possibly correlated with the 2-23 months of age children obesity;**
2. **the profile inflammatory markers depending on nutrition, at 2-23 months of age children, with human milk nutrition (breastfed) or other type of milk than human (no breastfed).**

Following the immunologic evaluation, this research is also proposing its outline of immune profile of 2-23 months of age child, overweight or obese, with human milk nutrition or other type of milk than human.

The secondary objectives are highlighted on the evidence of:

1. the influence of demographic factors (age, gender, environmental origin, parity, socio-economic conditions) on nutrition type and their impact on nutritional status;

2. the influence of maternal weight before, during and immediate after pregnancy on nutritional status of child;
3. effects of human milk nutrition time and the moment of diversification on nutritional status;
4. the effects of early infancy antibiotic use on nutritional status;
5. the influence of nutrition type and the nutritional status on some biological parameters (hemoglobin, glicemia).

The research is based on a prospective study which include a study group – 2-23 months of age children, breastfed, and a control group, 2-23 months of age children, no breastfed, admitted in clinic through continued hospitalization or one-day-hospitalization, for a condition which does not interfere with acute infectious pathology. The research was conducted in January 2016-December 2017 period.

Were included 84 subjects. The study group included 45 subjects (breastfed), and the control group 39 subjects (no breastfed). The age of subjects ranged between 2 to 23 months.

The research inclusion criteria were:

- the age, 2-23 months,
- the human milk nutrition or the other type of milk than human,
- the adequate diversification,
- the absence of an infectious event at the moment of evaluation,
- the absence of antibiotic therapy in the last 7 days, both child and mother,
- the absence of a chronic disease, both child and mother,
- the absence of endocrine disease, other causes of obesity than nutritional one, inclusively, in whole family,
- the absence of chronic medication, both in child and mother.

The research exclusion criteria were:

- age < 1 month and > 23 months,
- an infectious event in the moment of evaluation,
- antibiotic therapy 7 days prior the study admittance, both child and mother,
- chronic disease, endocrine one inclusively, except nutritional obesity,
- chronic medication, both child and mother,
- hemolysed or hyperbilirubinemic serum, who can interfere with the laboratory parameters results.

In the **study group** were included all the 2-23 months subjects, breastfed from birth until the moment of the study, with or without diversification (complete or not) depending on age.

In the **control group** were included all the 2-23 months subjects, no breastfed, respectively other type of milk than human, adapted to age (except, delactosed formula, hypoallergenic or hydrolysated protein), from birth, with or without diversification (complete or incomplete) depending on age.

The method of evaluation consisted in anamnestic, clinical and biological evaluations.

The weight measurement was performed using the electronic scales, calibrated for each determination. For weight percentiles have been used the growth charts, 0-36 months, boys and girls, CDC 2000 [24].

The levels dosing of **reactive ultrasensitive C protein (hs-PCR)** was used the immunoturbidimetric method, with the expectant normal values < 0,5 mg/dL [25].

For **leptin** was performed the immunoenzymatic assay (EIA technique). For the child younger than 24 months (no CDC recommendation regarding the use of BMI as a reporting value), the reference values depend on age groups. However, the reference values are relative because they are influenced by the laboratory-specified method of work [25].

The used methosd for **IL-6** was immunochemical with chemiluminiscent detection (ECLIA), with the refecence value < 7 pg/mL [25].

The statistical methods were: mean, median, standard deviation (SD), Chi-Square test, Spearman correlation coefficient, Student T test or Mann-Whitney test, Kolmogorov-Smirnov test, the classification tree [26].

The results and the discussions followed the course of the used methodology of research, starts from comments of the results of research parameters, to comparative analysis of the parameters ans, finally, to statistical correlations between these.

The whole study group was split, according to nutrition type, in:

- **study group**, 45 subjects, 2-23 months of age, breastfed from birth to the moment of study, without diversification (2-6 months subjects), incomplete diversification (7-12 months subjects) and complete diversification (13-23 months subjects).
- **control group,** 39 subjects, 2-23 months of age, no breastfed from birth to the moment of study, without diversification (2-6 months subjects), incomplete diversification (7-12 months subjects) and complete diversification (13-23 months subjects).

The age distribution was no equilibrated, homogeneous, the number of 2-12 months cases from control group being significantly lower (p = 0,004). Instead, the **gender distribution** was equilibrated (1:1), but the male subjects with other type milk than human nutrition were significantly more than female subjects with human milk nutrition.

The other type of milk than human nutrition was more frequent in urban **environment**.

The **socio-economic conditions** contributed to the preliminary conclusions according to which the majority of subjects with good or very good socio-economic conditions were breastfed, compared with those with medium or poor conditions.

The parity did not bring the significant results (Chi-Square = 7.878, p = 0.096).

The maternal obesity offered a very significant results (OR = 6.400, 95%CI:2.415-16.959, p = 0.000), in the sense that the majority of subjects coming from obese mothers have received the other type of milk than human, just as the majority of subjects coming from not obese mothers have received human milk.

About **pathologic personal history** was proven once again the anti-infectious role of human milk; the majority of subjects feeded with other type of milk than human presented a pathologic personal history, compared with those with breastfed, the difference being very significant (OR = 3.667, 95%CI:1.277-10.527, p = 0.013).

More cures of antibiotic were significant frequently in control group (Chi-Square = 14.920, p = 0.005) [27].

At the first reading of biological results, in the whole study group was found that the mean of IL-6 values was higher than normal; the mean values of leptin coincided with the upper limit of normal values, and the mean of hs-PCR was slightly below the lower limit of normal. The median of values was below the upper limit of normal for IL-6, leptin and hs-PCR.

The comparative analysis of the parameters used in research

Was found that the association between leptin and maternal obesity have a strong significance, in the sense of subjects coming from obese mothers and fed with other type of milk than human presented a higher values of leptin (p = 0,029).

No association between leptin and infectious events, neither in study and control groups (p0,07, respectiv 0,501).

Was found that the association between IL-6 and maternal obesity did not presented any statistical significance (p = 0,592), although the IL-6 values were significant higher in control group than in study group (p = 0,024).

The values of IL-6 were strong and significant associated with the infectious events in the personal history of subjects from control group (p = 0,003).

Was found that the values of hs-PCR from control group were very significant higher than study group, without any relation with provenance from obese mothers (p = 0,000).

The values of hs-PCR were not significant associated with infectious events (p = 0,837).

The comparative analysis of weight, leptin, IL-6 and hs-PCR values in the study and control groups revealed the following:

- both, mean and median weight values from control group were significant higher than study group (p = 0,001);
- both, mean and median leptin, IL-6 and hs-PCR values were higher in the control group; however, the difference did not presented any significance (p = 0,716, for leptin; p = 0,080, for IL-6; p = 0,138, for hs-PCR);
- the same trend was found on hemoglobin and glicemia values; the parameters were evaluated to demonstrate the antianemic role of human milk, respectively the relation of glicemia with obesity-induced metabolic syndrome (p = 0,032) [28], as a secondary objectives

The statistical correlation between clinical and biological parameters in the whole study group, in the study group and control group.

To reveal more constent significance of research paramaters were used the results of **correlation coefficient** between those parameters.

✓ <u>on the whole study group</u>,

- *the age* was strong and positive correlated with weight (p = 0,000), negative and weak, but with statistical significance (p = 0,000) with *leptin*, and positive and weak, with statistical significance (p = 0,001), with *hs-PCR*;
- *the weight* was negative and weak correlated with leptin, but with statistical significance (p = 0,009), negative and moderate, with statistical significance (p = 0,000), with *glicemia*;
- *leptin* was correlated weak and negative with hs-*PCR* (p = 0,028);
- *IL-6* was correlated positive and weak, but with statistical significance (p = 0,000) with *hs-PCR*; negative and weak, but with statistical significance (p = 0,000) with *hemoglobin*;

- ✓ <u>on the study group</u>,
  - *the weight* was correlated negative and moderate, with statistical significance (p = 0,000) with *leptin*, negative and weak with IL-6 (p = 0,021);
  - *leptin* was correlated positive and moderate, with statistical significance (p = 0,000), with *IL-6*;
- ✓ <u>on the control group</u>,
  - *IL-6* was correlated positive and strong with *hs-PCR* (p = 0,000).

The **correlation coefficients** did not bring the positive arguments regarding the correlation between inflammatory markers and obesity in the study groups. Otherwise, the literature specifies that from statistical point of view a great values variability of the study parameters could prevent the establishment of a robust correlations.

The only compliant correlations were:

- IL-6 was positive correlated with hs-PCR, especially in control group;
- leptin was positive correlated with IL-6, in study group.

For this reason a superior analysis has been made, that is the composition of classification tree.

The **classification tree** of the nutrition type, the clinical parameter (weight) and biological parameters (leptin, IL-6 and hs-PCR) allowed the configuration of immune profile, the classification tree meeting a very great grade of accuracy (92,9 %):

- ✓ of the overweight/obese subject, making the main objective of the doctoral thesis
  - breastfed
    - with higher values of leptin and
    - normal values of hs-PCR,
  - not breastfed
    - with 3 posibilities
      - higher values of hs-PCR, or
      - normal values of hs-PCR and higher values of leptin, or
      - normal values of hs-PCR, normal values of leptin and higher values of IL-6; the result is acceptable if refer to literature data according to which IL-6 is the more potent marker of low-grade inflammation;

- ✓ of the normal weight subject, al subiectului cu greutate normală, making the main objective of the doctoral thesis
    - breastfed
        - normal values of leptin; the result is expected, since leptin is given two circumstances of intervention in weight regulation (as hormone and as cytokine, involved in low-grade inflammation);
    - not breastfed
        - normal values of hs-PCR, leptin and IL-6.

CONCLUSIONS

1. The nutrition with other type of milk than human (control group) prevailing, with statistical significance, in urban environment.
2. The distribution on environmental origin was not homogeneous, the difference being significant, with predominance of urban not breastfed subjects and rural breastfed subjects.
3. The breastfed was associated with good and very good socio-economic conditions for the majority of subjects.
4. The maternal obesity was significant correlated with the type of nutrition: the majority of subjects not breastfed coming from obese mothers and the majority of breastfed subjects coming from non-obese mothers.
5. Regarding the frequency of infectious events, the majority of subjects not breastfed presented a pathologic personal history, with great significance.
6. The more cures of antibiotic were significant more frequent in control group.
7. The mean IL-6 values was higher than upper limit of normal values; for leptin – coincided with the upper of normal; for hs-PCR – slightly below to the lower limit of normal.
8. Both, mean and median for leptin, IL-6 and hs-PCR from control group were higher than those from study group.
9. The median for leptin, IL-6 and hs-PCR was below the upper limit of normal in study group.
10. The leptin values were associated with maternal obesity, but not with infectious events.
11. In the whole study group leptin was negativ and weak correlated with hs-PCR.
12. In the study group leptin was positive and moderate correlated with IL-6.

13. The IL-6 values were associated with maternal obesity, but without any statistical significance and were very strong associated with infectious events.
14. The IL-6 was negative and weak correlated with hemoglobin in the whole study group, and positive and weak with hs-PCR, but with statistical significance.
15. The IL-6 was negative and weak correlated with weight in the whole study group, and positive and strong with hs-PCR in control group.
16. The values of hs-PCR were significant higher in control group, but without any relationship with maternal obesity.
17. Both, weight mean and median in control group were significant higher.
18. The weight was correlated, in the whole study group:
    - negative and weak with leptin, with statistical significance,
    - negative and moderate with glicemia, with statistical significance,
    - negative and weak with IL-6, with statistical significance.
19. The age was correlated, in the whole study group:
    - strong and positive with weight,
    - weak and negative with leptin,
    - weak and positive with hs-PCR.
20. The immunologic profile for 2-23 months child, overweight/obese, breastfed, assume higher values for leptin and normal values for hs-PCR.
21. The immunologic profile for 2-23 months child, overweight/obese, not breastfed, assume:
    - higher values for hs-PCR, or
    - normal values for hs-PCR and higher values for leptin, or
    - normal values for hs-PCR, normal values for leptin, and higher values for IL-6.
22. The immunologic profile for 2-23 months child, with normal weight, breastfed assume normal values for leptin.
23. The immunologic profile for 2-23 months child, with normal weight, not breastfed, assume normal values for hs-PCR, normal values for leptin, and normal values for IL-6.

Finally, the chapter 9 include : personal contributions, the limits of the study and recommendations.

The **personal contribution**, in fact the success of the doctoral thesis, reside in identification of the inflammatory immunological markers correlated with obesity in 2-23 months children.

Even if the statistical processing has not always succeeded to provide the semnificative data, the mean and median of the values have demonstrated the major role of human milk in reducing the risk of obesity. The advanced statistical methods managed the outline of the immunologic profile of the 2-23 months subject, breastfed or not, with proper diversification.

The values variability of the parameters, and the multitude of the used parameters, on the other hand, as otherwise similar findings from literature, have generated the less robust results, outlining with the relatively small number of cases what we might call the **limits of the study.**

Starts from the study results, further research will be able to answer the questions: when starts the child obesity and what are the most important markers for highlighting the inflammation and the obesity-induced complications?

Each stage of the study, with its achievements an limits, it might be good for further research.

Thereby, for perspective studies, the **recommendations** will be the following:

- the structure of the study according to age groups (0-4 months, 5-8 months, 9-12 months, 24 months, 6 years, 12 years);
- the methodology of the study to be completed with additional biological parameters (immunoglobulin, lipidic profile).

At the moment, even the determinations of reactive ultrasensitive C protein, interleukin-6, and leptin, may seem daunting and expensive, for the further research and pediatric practice the benefits can exceed the costs.

From this perspective, the immunological profile of 2-23 months children, overweight or obese, breastfed or not, is an important step in the diagnostic and treatment management of obesity in childhood.

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