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Nutritional anemia relation to changes of health indicators and fecal microbiota in infancy and early childhood

SUMMARY OF DOCTORAL DISSERTATION

Biomedical Sciences,
Medicine 06B

VILNIUS 2018

This dissertation was written between 2014 and 2018 at Vilnius University.

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Kūdikių ir mažų vaikų mitybinių mažakraujysčių ryšys su sveikatos rodiklių ir išmatų mikrobiotos pokyčiais

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ABBREVIATIONS

BM	Breast milk
IDA	Iron deficiency anemia
WHO	World Health Organization
PHC	Primary healthcare
PCoA	Principal coordinates analysis
HC	Healthy controls

DEFINITIONS

Health indicators – anthropometric measurements (birthweight, weight by age in months) and nutritional peculiarities of infants and young children.

Exclusive breastfeeding – infants receive only breast milk (BM) (directly from the breast or expressed BM) without any food or drink, not even water; except for oral rehydration solutions, medicines and vitamins under medical indications [1].

Predominant breastfeeding – infants receive BM (directly from the breast or expressed BM) as the main food source along with other drinks (water, water-based liquids, juice), except for non-human milk, formula or solid, semi-solid, or soft food [1].

Complementary feeding – infants receive solid, semi-solid or soft food and any liquids along with BM [1].

Breastfeeding on demand – breastfeeding as often and as long as the infant or child wishes [2].

Continued breastfeeding – breastfeeding along with the food appropriate for the infant's or child's age [1].

Diet diversity – consumption of four or more food groups except for BM or infant formula: vegetables and fruit, cereals (including wholegrain bread), meat, fish, eggs and dairy products (including only unsweetened products of sour milk, cottage cheese and cheese) [1].

Products with added sugar – sweetened dairy products, canned juice, cookies, sweets and chocolates.

Low birthweight – birthweight that is less than 2500 g regardless of gestational age [3].

INTRODUCTION

Anemia as a serious child health problem

Anemia is diagnosed when the number of red cells and/or hemoglobin decreases and the supply of oxygen to the tissues is disturbed [4]. Children are the most vulnerable group due to the rapid growth and high needs of micronutrients and vitamins [5]. Even though the majority of anemic patients reside in developing countries, this problem is relevant to developed countries as well [6, 7].

Nutritional anemia develops when the dietary intake of iron, folic acid, vitamin B12, copper, etc. does not meet the demands of the human body and is common in infants and young children all over the world [5, 6]. Iron deficiency is the biggest concern as it is responsible for the development of anemia in more than 50% of the cases [6]. The majority of processes in the brain are known to be dependent on iron-containing proteins. Therefore, iron deficiency disturbs myelination, neurotransmitter synthesis and metabolism, dendritogenesis and synaptogenesis in the structures of the central nervous system, which are responsible for hearing and vision, speech comprehension and usage as well as for the higher cognitive functions [8, 9]. Even though timely therapy stabilizes the iron status, anemic infants achieve lower results in cognitive tests and this disparity increases with time if a child is raised in poor social conditions [10].

Moreover, iron is essential not only for the human body, but also for gut bacteria. Inadequate nutrition may negatively impact the composition of microbiota [11]. Recently it has been established that the interaction between the host and gut microbial community plays a crucial role in human health. Microbiota's changes are related to the higher risk of chronic non-communicable diseases: allergies, obesity, atherosclerosis, ischemic heart disease [12]. Therefore, appropriate prophylaxis of nutritional anemia is essential for improving the health of infants and young children [13].

According to the World Health Organization (WHO), the most effective measures to ensure optimal nutrition of infants and young children during the first two years of life are: 1) early initiation of breastfeeding, 2) exclusive breastfeeding for 6 months, 3) promotion, protection and support of the breastfeeding on demand until 2 years of age and beyond with an adequate complementary feeding, 4) micronutrient supplementation for low-birthweight infants [5, 13]. Health care providers have the greatest impact in adjusting nutritional habits and an evidence-based everyday practice in the primary healthcare (PHC) setting is crucial [13].

Relevance and scientific novelty

Nutritional anemia in infants and young children (up to 36 months old) is a relevant problem in Lithuania (Fig. 1). According to the data of Institute of Hygiene (Vilnius, Lithuania), the incidence of anemia has more than doubled in this group from 2010 to 2014: from 16.6 to 35.8 cases per 1,000 children [14].

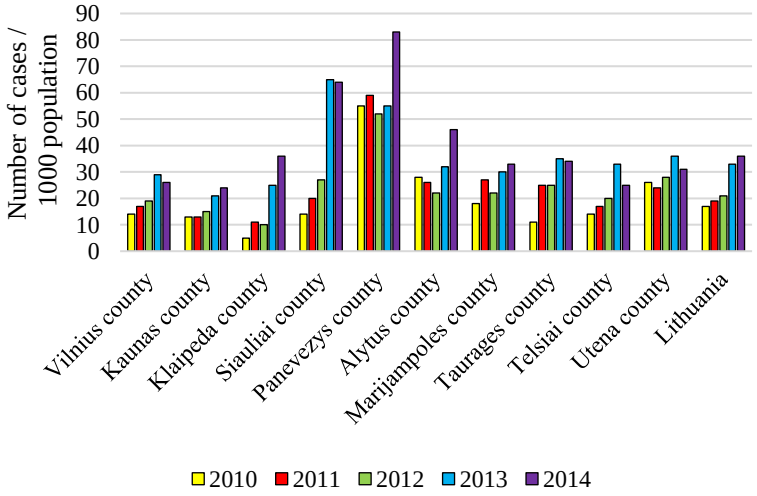

Figure 1. Incidence of nutritional anemia in infants and children under 3 years of age in Lithuania [14].

Nevertheless, previously the highest attention was paid to early diagnosis and treatment of anemia. Data on the risk factors associated with anemia in Lithuanian children is scarce [15, 16]. Therefore, this study focused on anthropometric measurements (birthweight, weight by age) and nutritional peculiarities of infants and young children diagnosed with nutritional anemia. An original, validated questionnaire was used for the first time in order to assess dietary habits of patients. This instrument could be implemented and used in the practice of primary healthcare physicians on a daily basis.

Moreover, a survey was conducted in order to assess the level of nutritional anemia prophylaxis performed by physicians in primary healthcare. The findings of the survey revealed gaps in providing infant breastfeeding and complementary feeding recommendations. It showed that nutritional anemia is a much more complex problem in Lithuania and there is a need for educating both parents and physicians. Based on the results of the study and literature, an algorithm of primary nutritional anemia prophylaxis was created. This instrument could also be implemented in the daily practice of primary healthcare physicians.

Next-generation sequencing of the V3-V4 hypervariable region of the 16S rRNA gene was used and the gut microbiome in Lithuanian infants and young children was analyzed for the first time. Next, nutritional iron deficiency anemia association with the changes of gut microbial community was identified. Worldwide, only a few studies have investigated the impact of iron deficiency and iron deficiency anemia on the intestinal microbiota in animal experiments [17, 18], *in vitro* colonic fermentation models [19, 20] and Indian women [21]. The association of mixed etiology anemia and intestinal microbiota changes in infants and school-aged children has been demonstrated in Africa [22, 23]. This study provides more insights into the development of the gut microbial community and reveals that anemia is a complex health problem. Primary nutritional anemia prophylaxis plays a crucial role in promoting the health of infants and young children.

The aim of the study

The aim of the study was to evaluate the relationship between nutritional anemia and changes of health indicators and fecal microbiota in infants and young children up to 36 months of age in Vilnius district.

Objectives

1. Compare anthropometric measurements of anemic and healthy infants and young children.
2. Compare nutritional peculiarities of anemic and healthy infants and young children.
3. Compare the gut microbiota profile of anemic and healthy infants and young children.
4. Assess nutritional anemia prophylaxis in the primary healthcare setting.

1. MATERIALS AND METHODS

The study was carried out in the Children's Hospital (Affiliate of Vilnius University Hospital, Santaros Klinikos) in 2016-2018. The study was approved by the Vilnius Regional Bioethical Committee (2015-10-06 No.158200-15-805-317) and the State Data Protection Inspectorate (2016-03-01 No.2R-1242(2.6-1.)).

The study was divided into three parts (Fig. 2).

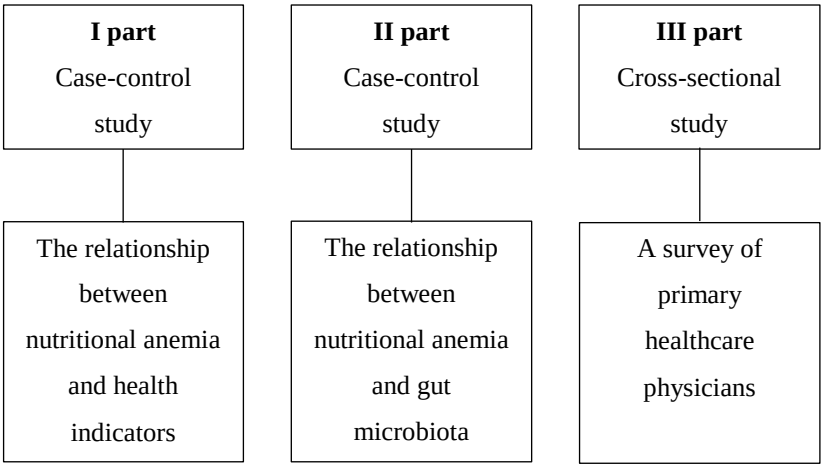

Figure 2. Parts of the study.

1.1 Part I: The relationship between nutritional anemia and health indicators

Infants and young children diagnosed with nutritional iron deficiency anemia (IDA) were enrolled in the study. Healthy children were recruited as a control group. All the subjects met the inclusion criteria: (1) up to 36 months old; (2) an otherwise healthy child and not having taken any medication for at least four weeks prior to the enrollment; (3) singleton pregnancy; (4) living in Vilnius district. The parents provided an informed consent. Detailed medical and nutritional data was collected by using a standard validated

questionnaire. IDA group was divided into two subgroups according to age: <12 months (IDA-Inf) and between 12 and 36 months (IDA-Ch).

Based on the criteria of WHO, nutritional IDA was defined as: (1) serum hemoglobin <110 g/l; (2) ferritin level <12 μg/l and/or reticulocyte-hemoglobin equivalent <28 pg; and (3) after other etiological factors of iron deficiency excluded by pediatric hematologist [4, 24]. Mild, moderate and severe IDA was defined as hemoglobin concentration 109-100 g/l, 99-70 g/l and below 70 g/l respectively [4].

1.2 Part II: The relationship between nutritional anemia and gut microbiota changes

For the microbiota research, 10 IDA patients and 10 healthy controls were enrolled. Fresh stool samples were collected from diapers and immediately frozen at -80°C until the time of processing in the laboratory (Bologna, Italy).

Bacterial DNA from fecal samples was extracted using the repeated bead-beating plus column method [25]. Final DNA concentration was measured using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The V3-V4 hypervariable region of the 16S rRNA gene was amplified using the 341F and 805R primers with added Illumina adapter overhang sequences [26]. Amplicons were purified with magnetic beads (Agencourt AMPure XP, Beckman Coulter, Brea, CA). Indexed libraries were prepared by limited-cycle PCR using Nextera technology and further cleaned up with magnetic beads (see above). Final library was pooled at equimolar concentrations (4 nM), denatured and diluted to a concentration of 6 pM. Sequencing was performed on Illumina MiSeq platform using a 2×250 bp paired-end protocol according to the manufacturer's instructions (Illumina, San Diego, CA).

Raw sequences were processed using a pipeline combining PANDAseq [27] and QIIME [28]. High-quality reads were clustered into Operational Taxonomic Units (OTUs) at 97% similarity using UCLUST [29]. Taxonomy was assigned using the RDP classifier against Greengenes database (May 2013 release). Singleton OTUs were discarded. Alpha diversity was measured by using observed OTUs, the Faith's phylogenetic diversity, Chao1 and Shannon index metrics [30]. Beta diversity was estimated by computing weighted and unweighted UniFrac distances.

1.3 Part III: A survey of primary health care physicians

An anonymous questionnaire-survey of primary healthcare physicians (family doctors and pediatricians) was conducted in Vilnius city and Vilnius district. Respondents were invited randomly and agreed to participate in the study voluntarily. All respondents were informed about the objectives of the study and the general use of the results only (preserving the anonymity and confidentiality of the respondents).

The questionnaire consisted of 19 closed, half-open and open questions to determine demographic indicators, feeding recommendations for infants and young children provided by physicians, specific IDA preventive measures applied and respondents' opinion on major obstacles for successful nutritional anemia prophylaxis.

1.4 Statistical analysis

Statistical analysis was performed using R software package (version 3.3.2). Continuous variables were provided as mean and standard deviation. Two samples were compared using Student's t-test or Wilcoxon rank-sum test based on normality. The Kruskal-Wallis rank sum test was used in order to compare three independent samples. Categorical variables were provided in absolute numbers and percentages. They were compared using Pearson's Chi-squared test.

When numbers of observations were lower than five, Fisher's exact test was applied.

For the microbiota analysis, UniFrac distances were plotted using the vegan package. Data separation in the Principal Coordinates Analysis (PCoA) was tested using a permutation test with pseudo-F ratios (function adonis). Alpha and beta diversity, taxon relative abundances were compared using Wilcoxon rank-sum test.

A p-value <0.05 was considered as statistically significant.

2. RESULTS

2.1 Part I: The relationship between nutritional anemia and health indicators

During the study period 197 children (82 females (42%)) were examined by the pediatric hematologist due to the suspicion of nutritional anemia. Of these, 131 (66 %) had secondary anemia due to the co-morbidities and were not invited to participate in the study. Overall, the study included 36 IDA infants (IDA-Inf), 23 IDA children (IDA-Ch) and 32 healthy controls (HC) (Fig. 3).

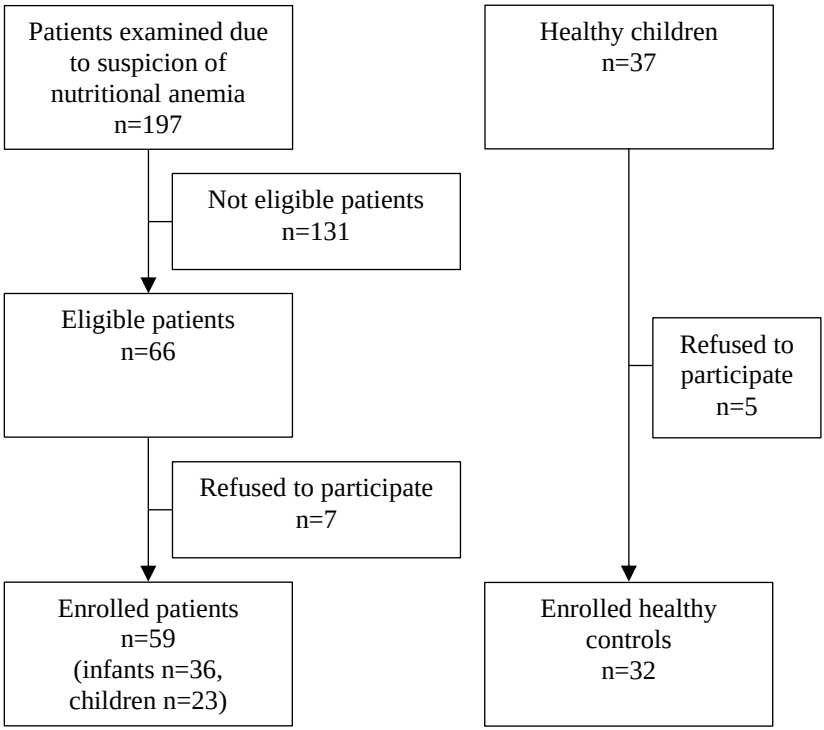

Figure 3. Flow chart of the study population.

Nutritional IDA was diagnosed at the average age of 8±2 months in the IDA-Inf group and 17±5 months in the IDA-Ch group. There were four infants (11%) whose diagnosis had been established at the age of 3-5 months. Basic characteristics of the study participants are given in Table 1. The parental age, education and a prevalence of anemia in pregnancy did not differ among the groups.

Table 1. Basic characteristics of the study population.

	IDA-Inf (n=36)	**IDA-Ch (n=23)**	**HC (n=32)**
Age	8 ± 2	17 ± 5	14 ± 8
Hemoglobin, g/l*	96 ± 13	99 ± 9	120 ± 7
Mild anemia	19 (52 %)	14 (61 %)	
Moderate anemia	15 (42 %)	8 (35 %)	
Severe anemia	2 (6 %)	1 (4 %)	
Male*	25 (70 %)	12 (52 %)	11 (34 %)
First child in a family	23 (64 %)	12 (52 %)	18 (56 %)
Vaginally delivered	28 (78 %)	16 (70 %)	24 (75 %)
Gestational age, weeks*	38 ± 3	39 ± 2	40 ± 1
Premature infants*	11 (31 %)	2 (9 %)	1 (3 %)
Birthweight, g*	2844 ± 580	3417 ± 453	3621 ± 428
Low birthweight newborns*	7 (22 %)	1 (5 %)	0
Age when birthweight was doubled, months*	3 ± 1	4 ± 1	6 ± 2
Received prophylactic iron supplementation	7 (21 %)	1 (5 %)	3 (12 %)

*Statistically significant with $p<0.05$

More than 70% of the study participants were delivered vaginally. Mothers reported that newborns were placed in a skin-to-skin contact (IDA-Inf n=20 (56%), IDA-Ch n=14 (61%), HC n=24 (75%), p>0.05), but only for a very short period of time. Skin-to-skin contact lasting more than 60 minutes was uncommon in the study cohort (IDA-Inf n=1 (5%), IDA-Ch n=2 (14%), HC n=2 (8%), p>0.05). About 50% of the mothers reported that the newborns had been put to the breast within one hour after the birth (IDA-Inf n=18 (50%), IDA-Ch n=12 (52%), HC n=22 (69%), p>0.05). There were more newborns separated from their mothers during day-time (and night-time as well) in the IDA-Inf group compared to the IDA-Ch and HC groups (IDA-Inf n=22 (61%), IDA-Ch n=8 (35%), HC n=8 (25%), p<0.05).

Healthy children were fed human milk directly from the breast more often than others during the stay in a postnatal ward, but these rates decreased when they were discharged from hospital and returned home (Fig. 4). More than one third of the newborns were additionally given formula or glucose solution prior to the discharge from hospital (IDA-Inf n=15 (42%), IDA-Ch n=8 (35%), HC n=11 (34%), p>0.05). Nevertheless, only three mothers reported medical indications for supplementation. The most common reasons of this practice were "low milk production" and "an irritable newborn".

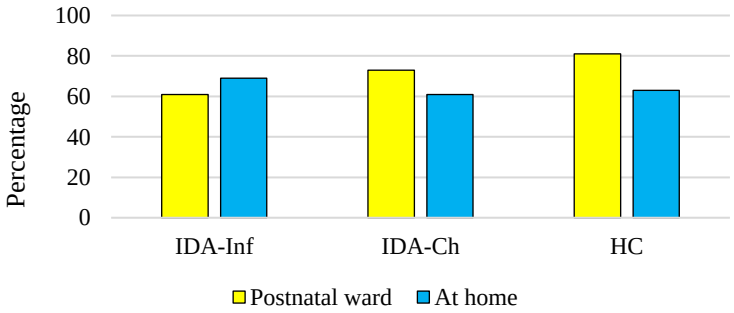

Figure 4. Distribution of the study participants based on the feeding directly from the breast during the stay in a postnatal ward and after returning home.

More than half of the mothers received gifts for artificial infant feeding in facilities providing maternity and newborn care, in PHC institutions (IDA-Inf n=23 (64%), IDA-Ch n=13 (57%), HC n=23 (72%), p>0.05). Parents of IDA infants and children used these gifts more often than the parents of healthy children (IDA-Inf n=16 (70%), IDA-Ch n=11 (85%), HC n=11 (48%), p>0.05).

Most of the children were breastfed on demand day and night (IDA-Inf n=26 (72%), IDA-Ch n=19 (83%), HC n=24 (75%), p>0.05). Nevertheless, the rates of exclusive breastfeeding in the first month of life were 50-75% (Fig. 5).

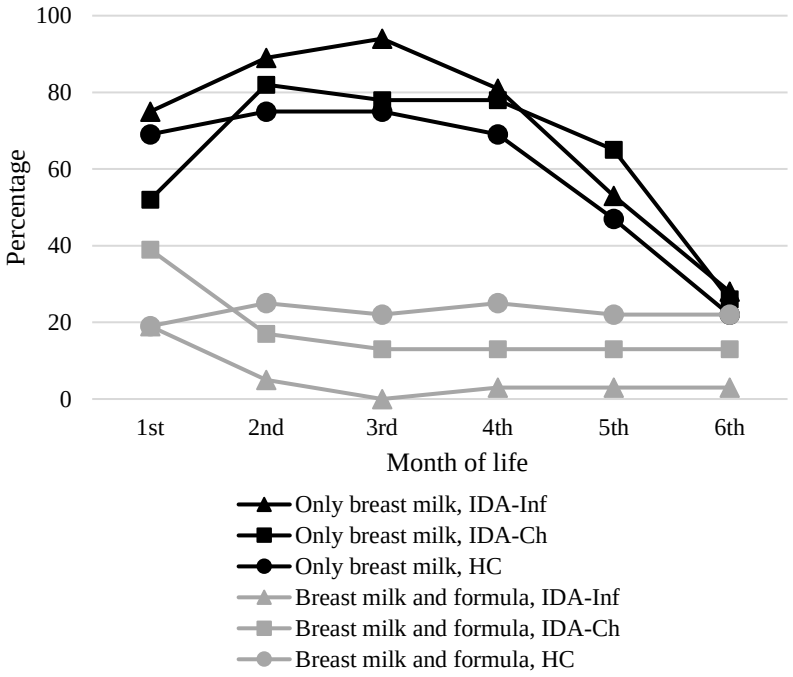

Figure 5. Distribution of the study participants based on exclusive breastfeeding during the first six months of life.

The frequency of exclusive breastfeeding increased in all the groups during the second and third months of life (most notably in the IDA-Inf group), but declined significantly later on. Only several infants were exclusively breastfed for the whole period of six months in a study cohort (IDA-Inf n=6 (18%), IDA-Ch n=3 (13%), HC n=5 (16%), p>0.05). The average duration of exclusive breastfeeding was 3±2 months and it did not differ among the groups.

About half of the mothers encountered breastfeeding problems (IDA-Inf n=13 (36%), IDA-Ch n=11 (48%), HC n=17 (53%), p>0.05). There were slightly more children receiving formula in the group of healthy controls (IDA-Inf n=1 (3%), IDA-Ch n=3 (13%), HC n=7 (22%), p=0.05) (Fig. 5).

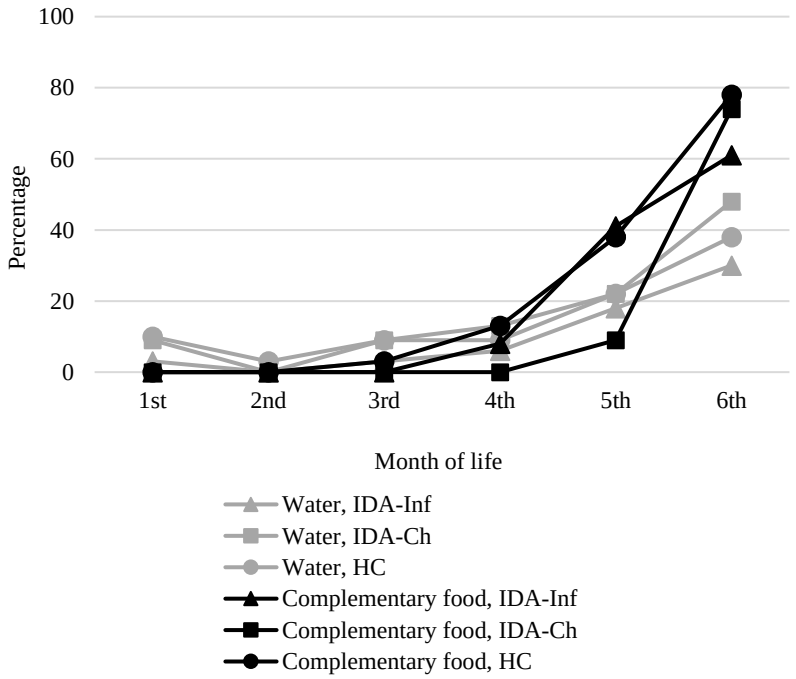

Figure 6. Distribution of the study participants based on the consumption of water and complementary food during the first six months of life.

At the beginning of the fifth month of life, water was introduced to the diet of increasingly more participants. More than one third of them were given water in the sixth month of life (IDA-Inf n=10 (30%), IDA-Ch n=11 (48%), HC n=12 (38%), $p<0.05$) (Fig. 6).

The consumption of complementary food significantly increased during the fifth month of life, especially in the IDA-Inf and HC groups (IDA-Inf n=14 (42%), IDA-Ch n=2 (9%), HC n=12 (38%), $p<0.05$). Nevertheless, the proportion of the study participants receiving complementary food at the age of six months did not differ among the groups (IDA-Inf n=20 (61%), IDA-Ch n=17 (74%), HC n=25 (78%), $p>0.05$) (Fig. 6).

Vegetables were introduced first and were followed by fruit and berries, cereals, meat, fish, eggs and dairy products. The IDA children were given meat later than the other study participants (IDA-Inf at 6±1 months, IDA-Ch at 7±1 months, HC at 6±1 months, $p<0.05$).

The consumption of unmodified cow's milk was uncommon in the cohort, but an increasing consumption of products with added sugar was noticed in the second half of infancy (Fig. 7). Every third participant consumed products with added sugar in the seventh month of life and almost all the IDA and healthy children consumed them in the twelfth month. This was the least important problem in the IDA infants group and the difference was statistically significant in the twelfth month of life.

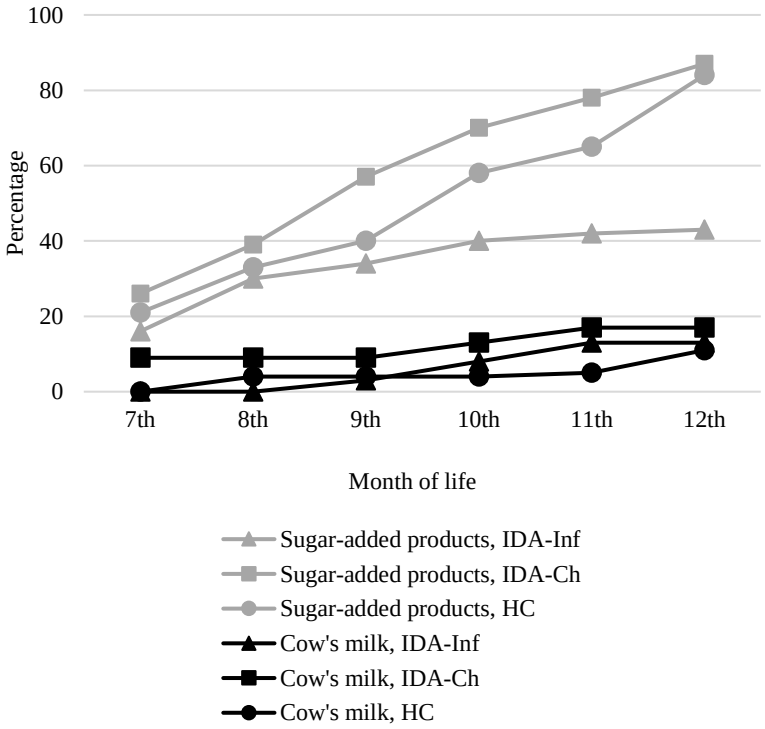

Figure 7. Distribution of the study participants based on the consumption of products with added sugar and cow's milk during the second six-month period of life.

At the age of 6-11 months all the IDA infants continued to be breastfed, but three IDA children (13%) and four healthy controls (13%) were already weaned ($p<0.05$). Diet diversity increased during the second half-year period of life in all the groups, but was the lowest in the IDA infants, both at 6-8 and 9-11 months of age ($p<0.05$) (Fig. 8).

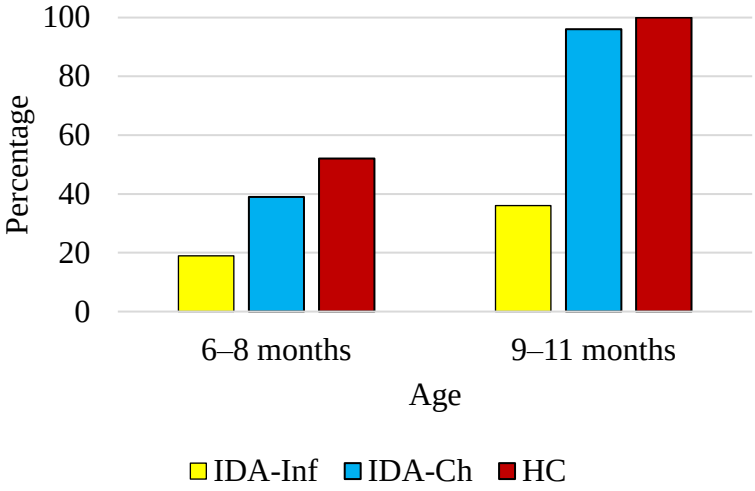

Figure 8. Distribution of the study participants based on the diversity of diet during the second six-month period of life.

When comparing the diet diversity of the IDA children and healthy controls in the second and third year of life, it was found that every third child consumed products from four or more food groups every day (IDA-Ch n=6 (28%), HC n=4 (24%), p> 0.05). Most children received vegetables, fruit and berries and cereals every day. About half of the study participants consumed meat and drank unmodified cow's milk daily. There were no statistically significant differences among the groups.

Although the consumption of cow's milk did not differ among the groups, there were two IDA children (9%) who consumed more than 400 ml of cow's milk (aged 13 and 23 months) and one IDA child (4%) who consumed about 2000 ml of cow's milk (aged 32 months).

2.2 Part II: The relationship of nutritional anemia and gut microbiota changes

The study included ten patients with nutritional iron deficiency anemia (IDA-gr) (6-32 months of age) and ten healthy children (HC-gr) (7-34 months of age). The main characteristics of the participants are given in Table 3. The IDA group had a significantly lower level of hemoglobin and a lower birthweight. All subjects were vaginally delivered.

Table 3. Basic characteristics of the study population.

	IDA-gr (n=10)	**HC-gr (n=10)**
Hemoglobin, g/l*	102 ± 5	123 ± 8
Age, months	13 ± 8	16 ± 8
Gestational age, weeks	38 ± 2	40 ± 1
Birthweight, g*	3252 ± 613	3808 ± 309

*Statistically significant with $p<0.05$

All the study participants were exclusively breastfed for 4±2 months and introduced to plant-based products at 5±1 months of age, followed by animal-based products at 6±1 months of age with no differences among the groups. Most of the participants were still breastfed at the moment of enrollment (IDA-gr n=8 and HC-gr n=7, $p>0.05$). The proportion of children consuming meat (IDA-gr n=7 and HC-gr n=10, $p=0.06$) and fish (IDA-gr n=5 and HC-gr n=10, $p<0.05$) was lower in the IDA group.

Alpha diversity metrics did not significantly differ among the groups (Fig. 9). The Principal Coordinates Analysis (PCoA) based on the weighted and unweighted UniFrac distances showed distinct microbial signatures in the IDA patients and in the healthy controls (Fig. 10).

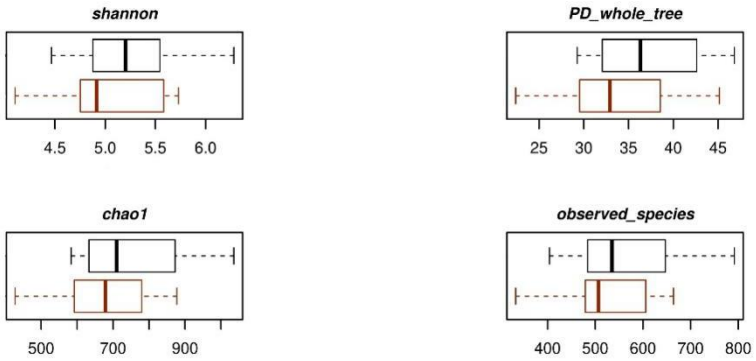

Figure 9. Microbiota alpha diversity metrics in the iron deficiency anemia patients (red) and in the healthy controls (black).

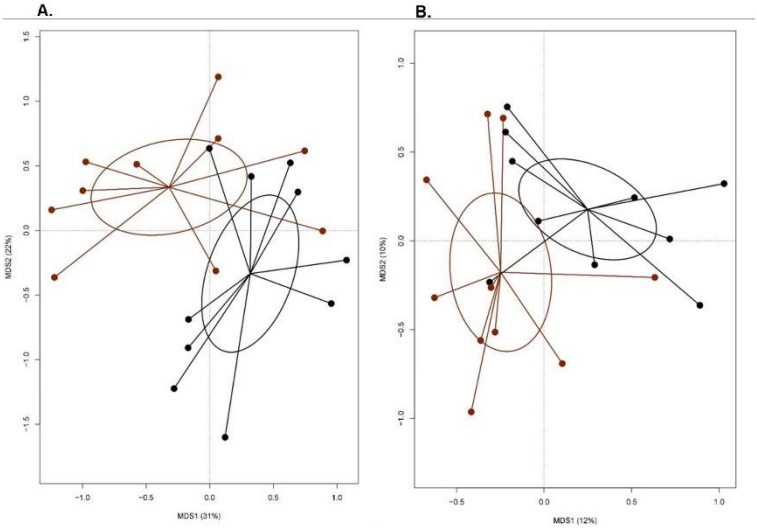

Figure 10. PCoA based on the unweighted (A) and weighted (B) UniFrac distances of the microbiota in the iron deficiency anemia patients (red) and in the healthy controls (black).

At the phylum-level, *Firmicutes* (mean relative abundance, 54.0%), *Actinobacteria* (23.4%) and *Bacteroidetes* (14.2%) dominated and accounted for about 90% of the gut microbiota community in the entire cohort. Although statistical significance was not achieved, microbial community of the IDA patients was enriched in *Bacteroidetes* (IDA-gr vs HC-gr, 18.8% vs 9.5%), *Proteobacteria* (5.3% vs 3.9%) and depleted in *Actinobacteria* (19.3% vs 27.5%), *Verrucomicrobia* (0.8% vs 3.0%) (Fig. 11).

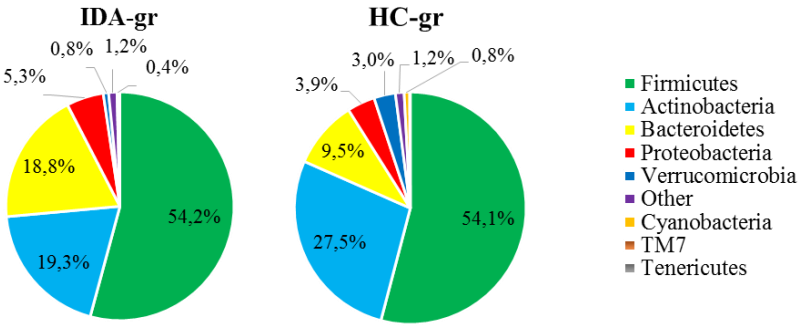

Figure 11. Relative abundances of phylum-level taxa in the iron deficiency anemia patients (IDA-gr) and in the healthy controls (HC-gr).

At the family level (Fig. 12), a decreased relative abundance of *Coriobacteriaceae* was revealed in the IDA patients (IDA-gr vs HC-gr, 3.5% vs 8.8%, $p<0.05$). These changes were associated with the depletion of the *Collinsella* (*C. aerofaciens*) genus. Enrichment of *Veillonellaceae* (IDA-gr vs HC-gr, 13.7% vs 3.6%, $p<0.05$) was determined by the over-representation of *Veillonella* genus (*V. parvula* and *V. dispar* species). Furthermore, bacterial community of the IDA patients was enriched in *Enterobacteriaceae* (IDA-gr vs HC-gr, 4.4% vs 3.0%, $p<0.05$), thus resulting in a significantly decreased *Bifidobacteriaceae* to *Enterobacteriaceae* ratio (IDA-gr vs HC-gr, 19.5 vs 93.3, $p<0.05$).

Decreased relative abundances of *Dorea* and *Dialister* bacteria were found in the IDA patients. *Clostridium neonatale* was detected in this group only.

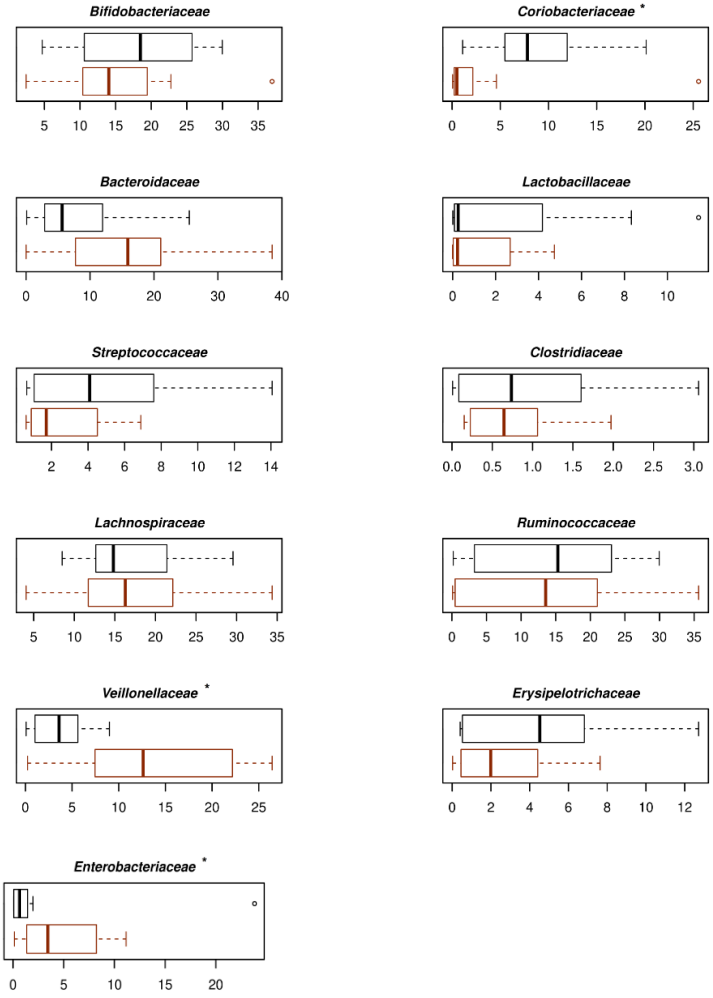

Figure 12. Relative abundances of the family-level taxa in the iron deficiency anemia patients (red) and in the healthy controls (black). *Statistically significant with $p<0.05$

2.3 Part III: A survey of primary healthcare physicians

Fifty-nine respondents participated in the survey, the majority were family doctors (n=43, 73%). The average age of the subjects was 37±10 years.

Fifty-six (95%) respondents answered the questions about the infant feeding recommendations they give to the families in the first six months after the child's birth. Although most of them would support exclusive breastfeeding for four or six months (n=51 (91%) and n=43 (77%) respectively), some of them would consider giving an infant water during illness or start complementary feeding when an insufficient weight gain or anemia is diagnosed. The remaining physicians (n=13, 23%) would always recommend mixed feeding or complementary feeding before the age of six months. Overall, there were 38 respondents who would consider starting complementary feeding before the age of six months. They would give vegetables (n=33, 87%), grains (n=17, 45%), fruit or berries (n=12, 32%) and meat (n=7, 18%).

After starting complementary feeding, the majority of the physicians would recommend continued on-demand breastfeeding (n=53, 90%), but breastfeeding until 24 months of age and beyond would be encouraged and supported by less than half of the respondents (n=20, 34 %). Therefore, exclusive breastfeeding until six months of age and continued on-demand breastfeeding until 24 months and beyond with adequate complementary food would be recommended only by some physicians (n=6, 10%).

Only 24 respondents (41%) would prescribe prophylactic iron supplements for premature infants, 6 (10%) – for the low birthweight infants and 11 (19%) – for the infants who get insufficient amount of iron with food.

Nevertheless, the respondents mentioned the following problems as major obstacles for the efficient IDA prophylaxis: the absence of reimbursable liquid iron supplements for infants and young children (n=30, 51%); too little time allocated for patient consultation (n=26,

44%); parents not following provided recommendations (n=24, 41%); lack of scientific information on infant and young children NA prophylaxis strategies for physicians (n=22, 37%).

3. DISCUSSION

3.1 Part I: The relationship between nutritional anemia and health indicators

In Lithuania, this is the first study focusing on the anthropometric measurements of infants and young children, nutritional peculiarities from the first days of life and their link to nutritional IDA. By comparing three groups of subjects we defined the main risk factors associated with IDA in the first or in the second and third years of life.

The IDA infants group was significantly different from the other two groups, i.e. their gestational age and birthweight were lower compared to the rest of the subjects and they doubled their birthweight as early as being three months old. The body iron stores directly depend on the gestational age and birthweight; therefore, premature and low birthweight infants experience a greater IDA risk [31-34]. Nevertheless, patients in the IDA-infant group did not receive prophylactic iron supplementation and, in accordance with the studies conducted in other European countries, developed IDA anemia [32, 35, 36]. On the other hand, the IDA-infants doubled their birthweight earlier than the other subjects. Faster weight gain is known to increase the IDA risk [31, 37]. Thus, it can be concluded that the nutritional anemia in the IDA infants was associated with the lower body iron stores and the higher needs for rapid growth.

The IDA children group was less different from the healthy controls: all were born mature and had normal birthweight. Nevertheless, they grew faster and doubled their birthweight earlier than the healthy children. It was demonstrated once again that children who gain weight more rapidly have higher nutritional iron needs and are at a greater risk of developing IDA [31, 37].

In addition, there were more boys than girls among the children diagnosed with IDA. According to the literature, their iron status is more affected by growth velocity and nutritional habits due to hormonal differences [33]. Thus, boys experience a greater IDA risk [16, 37, 38].

According to the WHO, exclusive breastfeeding for the first six months should be protected, promoted and supported in order to ensure the optimal nutrition of the infant [39]. Implementation of the Baby-friendly Hospital Initiative is one of the most effective measures to increase exclusive and continued breastfeeding rates [40]. In accordance with the data of the World Breastfeeding Initiative Trends Report in Lithuania [41], it was found that the facilities providing maternity and neonatal care did not implement all the Baby-friendly Hospital Initiative practices. Only half of the newborns were placed in a skin-to-skin contact for a very short period of time and were breastfed within the first hour after the birth. Supplementation with formula or glucose solution without any medical indications was common in the cohort. Moreover, violations of the International Code of Marketing of Breast-milk Substitutes were detected in the birth units and primary health care institutions. All these circumstances are known to negatively impact the success and duration of breastfeeding [40].

Indeed, only one third of the subjects were exclusively breastfed at the age of six months and only one fifth were exclusively breastfed for the entire six-month period. These results are in line with the national reports: only 35.4% of infants were exclusively breastfed until six months of age in 2016 [42]. Even worse rates of exclusive breastfeeding are noticed in the Latvia (16.4%) and Estonia (26.4%) [43, 44]. This is of particular concern as sub-optimal duration of exclusive breastfeeding increases the risk of infections and inflammation which may disturb iron homeostasis in the body [45-47]. On the other hand, no relationship was established between IDA and the duration or frequency of exclusive breastfeeding. This is in line with systematic reviews [45].

We noticed that more healthy controls were supplemented with formula during infancy compared to the IDA subjects. It is a known fact that formula fed infants have greater body iron stores and experience a lower IDA risk [33, 34, 48]. Unfortunately, excess iron intake in iron-replete infants is associated with decreased linear growth, increased incidence of infectious diseases and altered homeostasis of other microelements in the body [49].

Plain water and complementary food were introduced at the average age of four months in all the cohort. It was noticed that a smaller number of the IDA children consumed complementary food at this point of time, but there was no statistical difference at the age of six months. Taking into account that the first complementary food types are poor in iron, it remains doubtful whether this difference played a role in the development of anemia in this group [50]. It is possible that a later introduction of meat could be a stronger factor increasing the risk of IDA [51].

Consumption of unmodified cow's milk in the first year of life was uncommon in the cohort, but a sharp increase in the consumption of products with added sugar was found to be a new emerging problem. Usually these products are of high energy value, resulting in a higher weight gain and, in turn, in higher micronutrients needs. On the other hand, children that eat these products receive less iron, vitamin E, niacin, calcium and zinc [52, 53]. It may be speculated that the consumption of products with added sugar could increase the risk of nutritional anemia: compared to the healthy controls, the IDA children doubled their birthweight earlier (depleted body iron stores), received meat later (did not increase body iron stores) and their diet was complemented with non-nutritious snacks. One study that was conducted on preschool children in Lithuania found that almost 70% of children aged between12 and 36 months consume products with added sugar [54].

Furthermore, diet diversity was lowest in the IDA infant group (particularly at the age of 9-11 months) and contributed to the development of IDA. Nutritional needs increase during the second half

of infancy, but infants are able to eat only small amounts of food [55]. First complementary food types are poor in microelements and vitamins and therefore only a diverse diet can meet the nutritional needs of a fast-growing body [50, 56]. On the other hand, the IDA infants had the lowest body iron stores at birth and it cannot be excluded that they were already iron deficient at the introduction of complementary food. Previously it was found that iron deficiency is associated with infant feeding difficulties [57, 58].

A low diet diversity was also found in the second and third years of life after assessing the diet of the IDA children and healthy controls. Although there were no statistically significant differences among the groups, these children were more vulnerable and any other risk factors might have a relatively higher significance in pathogenesis of nutritional anemia [5].

Consistent with other studies, consumption of unmodified cow's milk was associated with IDA development in the second or third year of life in our cohort [33, 59]. Cow's milk contains only small amounts of iron, inhibits iron absorption from other food types and often leads to occult bleeding from the intestine [60].

The major limitation of this study is a small sample size. Nevertheless, important gaps were revealed in making diagnosis of IDA in a primary healthcare setting. First, the diagnosis of IDA in some patients was rejected by pediatric hematologist after evaluating the complete blood count and additional blood tests. It may be speculated that complete blood test results were misinterpreted by primary healthcare physicians. Finally, we had only 197 patients with a confirmed diagnosis of anemia. Nevertheless, almost 70% of them had anemia due to co-morbidities and were not eligible for the study. Therefore, it cannot be excluded that the data provided by the Institute of Hygiene on the incidence of nutritional anemia in infants and young children is not accurate due to diagnostic errors and incorrect diagnosis coding according to the International Classification of Diseases. Detailed large-scale research is required in order to evaluate the problem of nutritional anemia in Lithuanian children. Only a

detailed analysis of the results would help to effectively address this problem in Lithuania.

3.2 Part II: The relationship between nutritional anemia and gut microbiota changes

Worldwide, little is known about how iron deficiency and iron deficiency anemia impact the development of the gut microbial community.

First, it was found that the gut microbial community in the cohort consisted of *Firmicutes* (with the most abundant *Ruminococcaceae, Lachnospiraceae* and *Streptococcaceae* families), *Actinobacteria* (dominated by *Bifidobacteriaceae*) and *Bacteroidetes* (dominated by *Bacteroidaceae*). These results were in line with the peculiarities of microbiota development in infants and children living in Europe [61-63], thus it was possible to continue further analysis.

The study included iron deficient anemic, but otherwise healthy children; nevertheless, significantly increased relative abundance of *Enterobacteriaceae* and decreased *Bifidobacteriaceae/ Enterobacteriaceae* ratio in the IDA patients was observed. Similar changes were observed not only *in vitro* and *in vivo* models [17, 19, 20], but also in populations of African infants and school-aged children with a high prevalence of infections and systemic inflammation [22, 23]. On the one hand, an increased abundance of *Enterobacteriaceae* is also associated with inflammatory bowel diseases. On the other hand, an enrichment in bifidobacteria and butyrate producers is observed in remission [64-66]. It cannot be ruled out that the unfavorable gut microbial community in our cohort could also induce local inflammatory response of the host and, in turn, interfere with iron absorption and homeostasis.

Furthermore, signs of a profound dysbiosis were observed in the *Veillonellaceae* family. The IDA children had an increased abundance of *V. parvula* and *V. dispar* and decreased levels of *Dialister*. Recently, over-representation of *Veillonellaceae* was associated with

the increased bronchial asthma risk at five years of age and a deep ulceration in Crohn's disease [65, 67]. Moreover, an increased relative abundance of *Veillonella* genus was observed in feces of children with type 1 diabetes and *Dialister* genus was completely absent before the onset of type 1 diabetes in infants [68, 69]. Taking into account this data, we may speculate that the IDA children can experience a greater risk of autoimmune diseases in the future.

A significantly decreased abundance of *Coriobacteriaceae* family (*Collinsella aerofaciens* in particular) was found. These changes are also observed in inflammatory bowel diseases [66]. On the other hand, diminished level of *Bifidobacterium* and *Collinsella* at the age of six months is related to obesity at the age of 18 months [70]. Thus, IDA in infancy can increase the risk of metabolic diseases later in childhood.

Furthermore, a significantly decreased relative abundance of *Dorea* (butyrate producing bacteria) in the IDA children was revealed. Surprisingly, *Clostridium neonatale* was observed only in the IDA group. The latter bacteria was previously isolated only from newborn feces [71].

The findings of this study suggest that iron deficiency anemia is associated with a significant dysbiosis of the gut microbiota. In agreement with the previous studies, it was revealed that iron deficiency induced an overgrowth of *Enterobacteriaceae* family and, in turn, decreased the *Bifidobacteriaceae/Enterobacteriaceae* ratio [17-20, 22, 23]. Most significant changes associated with iron deficiency anemia in infancy and early childhood were observed in the lactic acid producing bacterial community.

The study was conducted at a single point in time ant it remains unclear when exactly the changes in the gut microbiota occurred: did dysbiosis disrupt the host's ability to absorb iron from food and caused the anemia or, on the contrary, were the gut bacterial community changes induced by anemia? It is possible that unfavorable changes in the microbiota result in the competition for iron between the host and the bacteria. On the other hand, the most recent study shows that

siderophores produced by the commensal *E. coli* are necessary for iron absorption by the host cells [72]. The present study provides more questions than answers and further research is needed to learn more about the role of the gut microbiota on the host's iron homeostasis.

Moreover, it was noticed that iron deficiency anemia is associated with unfavorable changes of the gut microbial community. It cannot be excluded that patients will experience a greater risk of autoimmune, allergic and metabolic diseases in the future. Further longitudinal research is needed in order to assess the relationship between iron deficiency anemia and these chronic non-communicable diseases.

3.3 Part III: A survey of primary healthcare physicians

Although exclusive breastfeeding for six months has been promoted in Lithuania since 2003, only 50% of the physicians recommended this practice in the previous decade [73, 74] and only 80% of the respondents in this study. This is alarming as there is a direct relationship between the practice of primary healthcare and the success of breastfeeding. According to a recent study, infants who attend a breastfeeding promoting clinic are breastfed six months longer [75]. Unfortunately, breastfeeding and infant feeding topics are covered poorly in the medical study programs and there is a lack of continued training services for physicians [41].

Although only several respondents would always recommend giving water to infants under six months of age, this practice was common in the previous decade and was promoted by almost 30-50% of physicians [73, 74, 76]. Nevertheless, it was noticed that some of the respondents would recommend giving water to the infant during illness or hot weather. This means that this practice could be more prevalent than it was observed in this study and reach even 30% as was published earlier [75].

About 25% of physicians would recommend complementary feeding before the infant reaches six months old. Another recent study

also reveals significant differences in the clinical practice: early complementary feeding is promoted by 3-33% of physicians [75].

The majority of the respondents would recommend continued breastfeeding until the age of 12 months, but only one third would recommend it until the age of 24 months. These results are similar to the ones published in the past decade and may be related to healthcare professionals' insufficient knowledge of the significance and benefits of continued breastfeeding for children [73, 76].

Particular attention should be given to the fact that early complementary feeding is recommended for nutritional anemia prophylaxis in the cohort. However, first complementary food types are poor in vitamins and microelements and their bioavailability is lower than that in breast milk [50]. Thus, prophylactic supplementation with microelements is recommended for infants in high-risk groups (mainly, low birthweight infants) [5]. Unfortunately, less than 50% of the respondents would prescribe prophylactic iron supplements for premature infants; less than 10% – for low birthweight infants. Only 20% of the physicians would recommend iron supplementation for infants and children who get insufficient amounts of iron from food. This data reveals significant gaps in primary healthcare practice.

It is interesting to note that, according to the subjective assessment by the respondents, the greatest obstacles in the prophylaxis of nutritional anemia are external reasons, such as the absence of reimbursable liquid iron supplements for infants and small children, too little time allocated for patient consultation, parents not following provided recommendations. However, it was found that due to the lack of knowledge or other reasons, only few physicians adhere to the WHO recommendations, i.e. recommend exclusive breastfeeding until the age of six months, followed by frequent breastfeeding up to the age of 24 months or longer while feeding an age-appropriate complementary food. Most often it is argued that The European Society for Pediatric Gastroenterology, Hepatology and Nutrition recommends complementary feeding from four months of age.

However, this Society highlights that “exclusive breastfeeding until 6 months of age is a desirable goal” and complementary feeding should not be introduced before the age of four months or delayed beyond six months [77, 78].

In conclusion, our survey of parents and healthcare professionals revealed that no favorable conditions for proper nutrition of the infants and young children were created in healthcare facilities: principles of Baby-Friendly Hospital Initiative were not implemented or supervised; breastfeeding was not protected, promoted or supported in maternity units and parents were given inappropriate complementary feeding recommendations. Nevertheless, it was noticed that the physicians were very interested in breastfeeding and complementary feeding. This could serve as a basis for a fast and effective implementation of measures of evidence-based nutritional anemia prophylaxis.

CONCLUSIONS

1. Low birthweight and early doubling of the birthweight was associated with nutritional iron deficiency anemia.
2. Low diet diversity increased the risk of developing nutritional iron deficiency anemia in infancy and a later introduction of meat, consumption of unmodified cow's milk and products with added sugar increased the risk of developing nutritional iron deficiency anemia in second and third years of life.
3. Nutritional iron deficiency anemia is associated with unfavorable gut microbiota changes: a decreased *Bifidobacteriaceae/ Enterobacteriaceae* ratio and relative abundance of *Coriobacteriaceae,* increased *Veillonellaceae* relative abundance.
4. Primary healthcare physicians do not apply all nutritional anemia prophylactic measures in their practice and the greatest problems are a lack of breastfeeding promotion, protection and support; inappropriate complementary feeding recommendations and gaps in the use of specific prophylactic measures.

RECOMMENDATIONS

The algorithm of prophylaxis is provided in Figure 13. In order to implement these measures, it is necessary to educate primary healthcare physicians on lactation and breastfeeding and to update their knowledge on complementary infant feeding and iron deficiency anemia.

The results of the gut microbiota analysis support the need for further studies in order to determine whether the changes observed remain after treating iron deficiency anemia and whether the risk of chronic non-communicable diseases increases.

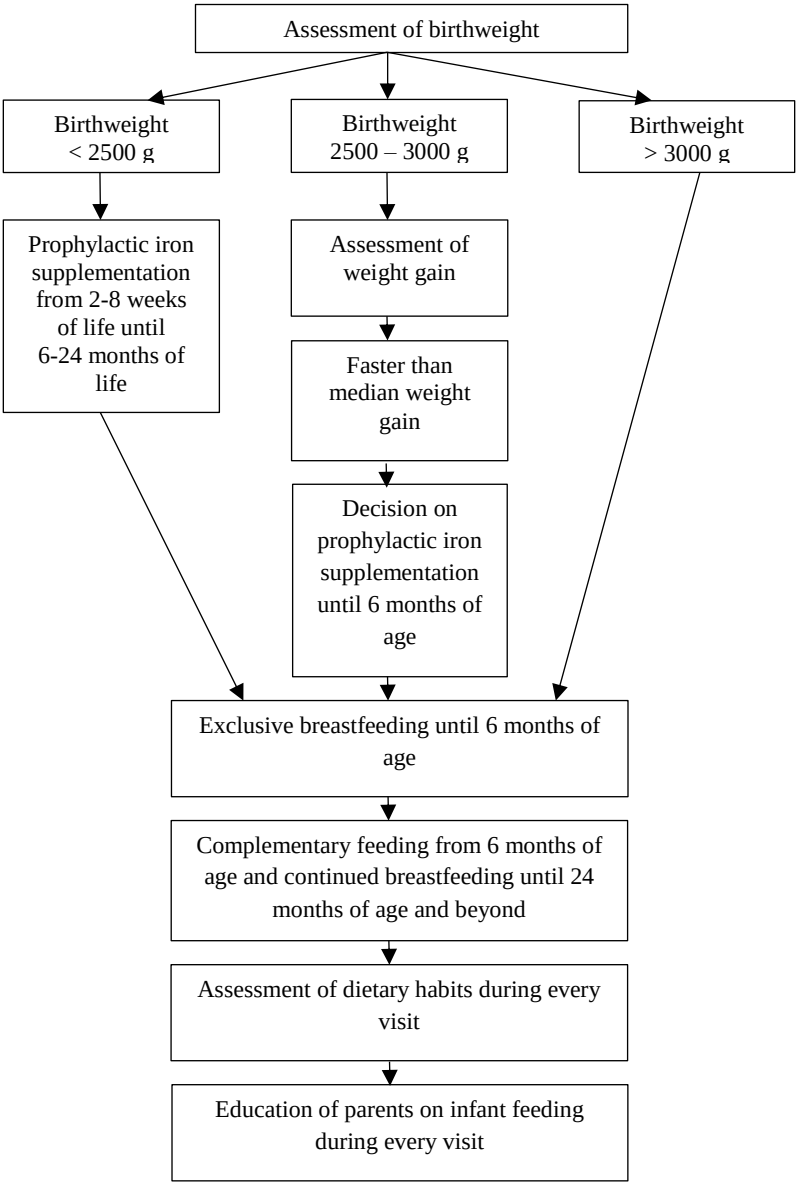

Figure 13. Algorithm for prophylaxis of nutritional iron deficiency anemia in infants and young children.

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