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EARNEST

EARly Nutrition programming- long term follow up of Efficacy and Safety Trials and integrated epidemiological, genetic, animal, consumer and economic research

Instrument: Integrated Project

Thematic Priority 5.4.3.1: Food Quality and Safety

Final public report on activity: 1.1.1

Follow-up studies in the NUHEAL cohorts in Hungary, Spain and Germany (UNI PECS, LMU-MUENCHEN, UGR)

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The NUHEAL Study

Healthy women in European usually meet their increased energy and protein needs during pregnancy. However, the relative increase of reference intakes is far higher for a number of micronutrients as compared to energy. The n-3 LC-PUFA status depends mainly on the maternal consumption of fatty sea fish. However, food habits vary widely and many pregnant women do not consume appreciable amounts of sea fish.

Study design

Alternative sources of n-3 LC-PUFA are fish oil preparations that can be consumed as part of supplements. To test the feasibility, safety and efficacy of such an approach, an European multicenter, randomized, double blind, placebo controlled study was designed. Spain, Germany and Hungary were chosen as participating countries since they represent European areas with traditionally high, medium, and low consumption of sea fish. We hypothesized that FO supplementation either with or without additional 5-MTHF will increase maternal plasma DHA and EPA, which should lead to increased fetal n-3 LC-PUFA availability and potentially to improved pregnancy outcome. To test these hypotheses maternal and neonatal DHA plasma levels were quantified and pregnancy outcomes monitored. Primary outcome variable in infants was the relative content of DHA in plasma phospholipids in cord blood. Secondary outcomes included APGAR, umbilical pH, birth length and birth weight.

The study was conducted as a randomized, double-blind, placebo controlled clinical trial with 2 x 2 factorial design to assess the effects of increased intake of FO, 5-MHTF, both FO and 5-MTHF or placebo from the 22nd week of gestational age (GA) until delivery on pregnancy and birth outcomes. Pregnant women received milk based supplements either with a fish oil (FO) preparation (DHA: 500 mg/day + EPA: 150 mg/day), 5-methyl-tetra-hydrofolic acid (5-MTHF: 400 µg/day), both, or placebo from the 22nd week of GA up to the delivery, together with vitamins/minerals in amounts meeting the recommendations during pregnancy for European mothers *(Krauss-Estchman S, et al. 2007).*

There were no differences concerning baseline characteristics between intervention groups**.** Basal dietary intake of DHA of the participating women was similar in all intervention groups at the 20th and the 30th week of pregnancy. Potential cofounders were assessed by means of standardized questionnaires containing information on children's health status. The study population did not differ from the not tested population concerning maternal parity, BMI, smoking habits, obstetrical risk factors, complications at parturition or perinatal morbidity.

At the 20th week of gestation maternal baseline relative levels (wt %) of AA, DHA and the AA/DHA ratio did not differ significantly between the 4 intervention groups. DHA relative levels in plasma and erythrocyte PL were in general higher in the FO and FO+5-MTHF groups compared to the placebo and 5-MTHF groups at the 30th week of pregnancy and at delivery as well as in cord blood. No significant differences between 5-MTHF supplemented groups and those that did not receive 5-MTHF with respect to fatty acid percentages in maternal or cord plasma or erythrocyte PL were found**.** AA relative concentrations in plasma and erythrocyte PLs were similar between intervention groups *(Escolano MV, et al. Clin Nutr 2010).*

The follow-up of the NUHEAL study is still ongoing under the NUTRIMENTHE EU FP7 Project, up to 9.5y.

The NUHEAL Follow-up Study (EARNEST Theme 1: Activity 1.1.1.)

1. DATA OBTAINED FROM THE DIFFERENT NEUROPSYCHOMOTOR TESTS PASSED:

1.1. HEMPEL AND TOUWEN TESTS (4 & 5.5 years old)

The present study was conducted to assess the long term effects of DHA supplementation to pregnant women during the second half of pregnancy and infants during the first 6 months of postnatal life on later neurologic development of children NUHEAL project (QRLT-1999-00888). The follow-up of these children were done up to 6.5y within the EARNEST EU FP6 Programme.

Neurological assessment

At 4 years of age, children were neurologically examined according to Hempel test. The Hempel assessment is organized into 5 functional domains: *fine motor function, gross motor function, posture and muscle tone, reflexes and visuomotor behaviour*. The findings of the Hempel examination result in a clinical classification consisting of the following categories: a) neurologically normal; b) simple MND; c) complex MND; and d) major neurological dysfunction. Neurological findings can be also summarized with the neurological optimality score (NOS) by assessing performance on 56 representative items of the neurological examination. The fluency subscore was also calculated; it consists of 15 items of the NOS focused on the fluency of motor behaviour.

Neurological assessment at 5½ years of age was performed according to Touwen test. The examination is organized into eight functional domains: *posture and muscle tone, reflexes, involuntary movements, coordination and balance, fine manipulative ability, associated movements, sensory deficits and cranial nerve function.* The examination results in a clinical classification: a) neurologically normal; b) simple MND; c) complex MND; d) definitively abnormal neurological condition. The NOS of the Touwen assessment consists of 64 items with age specific criteria for optimality.

Findings in the children at 4y and 5.5y *(Escolano MV, et al. J Nutr, 2010)*

- None of the children had a definitely abnormal neurological condition. No significant differences in clinical neurological condition between the 4 intervention groups were found.
- AA levels in maternal plasma at the 20th week of gestation and in maternal erythrocyte PC at the 30th week of pregnancy were higher in normal children compared to children with MND at 5½ years of age; however, after adjustment for confounders no significant association between AA and neurological classification was observed.
- Children classified as optimal at 5½ *had significantly higher DHA percentages in cord blood plasma PL* than those classified as suboptimal. DHA relative concentrations in maternal erythrocyte PL at delivery were higher and the AA/DHA ratios lower in optimal children at 5½ compared to the suboptimal. After adjustment for confounders the association between cord blood and maternal DHA levels at delivery and the occurrence of optimality at 5½ years of age *remained significant.*

- **Concluding remarks**
- The present randomized multicenter trial showed neither beneficial nor harmful effects of maternal FO supplementation during the second half of pregnancy on long term neurologic development of children.
- Higher DHA levels in fetal and maternal blood during the course of pregnancy were related to a better performance on neurological examinations of the children at 5½ years of age.

1.2. THE KAUFMAN TEST (6.5 y)

The present study approaches the effects of n-3 LC-PUFA supplementation to pregnant women during the second half of pregnancy and to the infants in the first 6 months of life on long term neurological condition of the children at 6 ½ years of age.

Study participants (270 pregnant women) where again approached and asked to participate in the neurological assessment of their children at 6½ years of age and 161 complied with the request in the 3 Centers involved LMU, UNIPECS and UGR. Finally 154 were assessed with the Kaufman Assessment Battery for Children (Kaufman-ABC) at a mean age of 79.16±12.68 months.

Assessment of cognitive function **(Kaufman-ABC)**

The Kaufman-ABC test is designed for children between 2.5 and 12.5 years of age and measures intelligence and achievement. Neurological findings are summarized with four scores: Sequential Processing Scale, Simultaneous Processing Scale, Achievement Scale (not used in the present study) and Mental Processing Composite (MPC). The Sequential Processing Scale primarily measures short-term memory and consists of subtests evaluating children's ability to solve problems that require the arrangement of stimuli in sequential order. The Simultaneous Processing Scale examines *problem-solving skills* that require processing of many stimuli at once. The Sequential and Simultaneous Processing Scales are combined to form the MPC, which is a global measure of the child's cognitive ability and is considered equivalent to the intelligence quotient. Raw scores are transformed into standard scores with a mean of 100 and a standard deviation of 15 and percentile scores.

Findings

- There were no statistical differences in the Kaufman-ABC scores at 6 ½ years of age between the 4 intervention groups**.**
- The groups FO and FO+5-MTHF and the group placebo and 5-MTHF were similar with respect to their basal clinical and socio-demographic characteristics and LC-PUFA (wt %) in cord blood. No statistically significant differences between both FO supplemented groups pooled together and the 5-MTHF and placebo groups pooled together too.
- No significant bivariate correlations between intelligence scores and fatty acid percentages in umbilical plasma or erythrocyte PLs were found.
- There were *significant bivariate correlations* between the MPC score at 6½ years of age and DHA percentage concentrations in maternal erythrocyte PE at the 20^{th} and 30^{th} week of pregnancy. The AA/DHA ratio in maternal erythrocyte PE at the 20^{th} week of pregnancy was also significantly correlated with the MPC. After the stepwise multiple regression including confounding factors as co-variates, these correlations were not confirmed at 6½ years of age.

- Children with MPC scores over 50th percentile had mothers with significantly higher DHA percentage concentrations in *erythrocyte PE and PC* during the course of pregnancy.
- Adjustment for confounders in the stepwise logistic regression**,** showed that children whose mothers had higher DHA percentages in erythrocyte PE at delivery were more likely to have a MPC score over the 50th percentile. Likewise higher AA/DHA ratio in maternal erythrocyte PE at delivery was associated with a MPC scores under the 50th percentile.

Concluding remarks

- Our results do not provide evidence for a beneficial effect of n-3 LC-PUFA supplementation during the second half of pregnancy and the first months of postnatal life on cognitive performance at 6½ years of age.
- Prenatal DHA status seems to have subtle positive effects on neurodevelopment, but further research is needed to assess whether these effects are maintained beyond early infancy.
- The optimal doses for efficacy at different developmental stages also require consideration.

1.3. CHILD BEHAVIOR CHECKLIST (CBCL) TEST

The Child Behavior Checklist (CBCL) is a standardized instrument that provides a parental report of the extent of a child's behavioral problems and social competencies. This assessment test can also be used for diagnosing children who suffer from ADD and ADHD. The version used of this test in the NUHEAL Follow-up project is suited for individuals within the age range of 18 months to 5 years, and can measure behaviors that demonstrate hyperactivity, bullying, aggression, defiance, and violence.

The CBCL can be self-administered or administered by an interviewer. It consists of 118 items related to behavior problems which are scored on a 3-point scale ranging from not true to often true of the child. There are also 20 social competency items used to obtain parents' reports of the amount and quality of their child's participation in sports, hobbies, games, activities, organizations, jobs and chores, friendships, how well the child gets along with others and plays and works by him/herself, and school functioning.

Preliminary findings:

Within the NUHEAL Follow-up project we passed this test to 149 children from the 3 countries involved, Germany, Hungary and Spain at 5.5 y. No statistical differences in the children classified as normal, border-line or pathological between the different supplemented groups were observed. The results are currently analyzed and a manuscript is planned to be prepared very soon.

2. ANALYSIS OF NUTRITIONAL INTAKE & ANTHROPOMETRY AT 4 YEARS OF AGE IN A PROSPECTIVE COHORT: THE NUHEAL STUDY

Seafood consumption during childhood may be beneficial for child neurodevelopment, perhaps via mechanisms involving docosahexanoic acid (DHA) and ecoisapentaenoic acid (EPA), found at high concentrations especially in fatty fish.

The dietary intake was evaluated in a total of 160 children at 4 years, born from the mothers participant in the NUHEAL study. The dietary intake assessment was done *using a food*

frequency questionnaire (FFQ) adapted for each country. Spanish food tables were used for nutrients and University of Granada data for fatty acids composition.

Findings:

- The Spanish children showed a higher energy, protein, carbohydrate and total lipids intake that those from Germany or Hungary**.** Saturated, monounsaturated and polyunsaturated fatty acids (PUFAs) including DHA and EPA (P=0.000), as well as cholesterol, animal protein and iron intake were higher in the Spanish children.
- Folate intake was significantly higher in Hungarian children.
- The German children had the lowest mean intake of carbohydrate and PUFAs.
- The BMI of Spanish children were higher than German and Hungarian**.**
- No statistical differences were shown between the four intervention groups in any country neither depending on nutrient intake, nor in the anthropometry parameters.
- No statistical differences in nutrient intake between female and male children at 4y were shown**.**

Concluding remarks:

- Spanish Andalusian children show a different pattern of dietary intake from German or Hungarian children.
- Spanish children have a higher mean intake of DHA and EPA, a dietary intake of cholesterol, and a higher rate of obesity than the other European children studied.
- Further analysis will be developed in order to clarify the differences in growth between the three EU countries related to diet and other factors, and the impact on neurodevelopment.

3. Allergy Parameters *(Krauss-Etschmann S, et al. J Allergy Clin Immunol. 2009;123(5):1176-8)*

We wished to analyze potential associations between current use of room disinfectants in private homes and respiratory, cutaneous, and allergic symptoms in young children. From 270 women completing the NUHEAL intervention study, 176 offspring attended the clinic at the age of 4y for a medical and neurologic examination as part of the main trial. During this visit, parents completed a standardized questionnaire, which included questions from the International Study of Allergy and Asthma in Childhood and the Influences of Lifestyle-Related Factors on the Immune System and the Development of Allergies in Childhood study on respiratory, cutaneous, and allergic symptoms, as well as physician-diagnosed allergic disease. In particular, we asked the following question: ‘‘Do you use room disinfectants or sprays (daily, at least once per week, occasionally, never)?’’.

Main Findings

- From the total of 176 children (106 Spanish, 45 German, and 25 Hungarian infants), 101 were exposed to room disinfectants. While half of the Hungarian children were exposed to disinfectants, Spanish children were exposed with significantly higher frequency and German children with significantly lower frequency, which is in line with previous publications. Room disinfectants were used daily or almost daily in 14.8%, at least once per week in 33%, occasionally in 9.7%, and never in 42.6% of households of all participants.
- The likelihood for treatment of atopic eczema was increased in children exposed to room disinfectants.

- When we stratified the children for the presence or absence of maternal allergies, increased effect estimates were observed for selfreported asthma ever in offspring from allergic mothers.
- An increased likelihood was observed for self-reported atopic eczema ever, diagnosed atopic dermatitis, and rash ever or rash in the past year in offspring from non allergic mothers.
- This indicates that children at risk for allergies respond differently to room disinfectants compared with children with average risk for allergies. The presence of allergic disease in a family member might also influence cleaning strategies.

Conclusion:

- Our study supports that exposure to conventional household cleaning products represents a risk factor for obstructive airway disease in children with pre-existing risk for allergic disease.
- Future preventive strategies might aim to restrict use of common room disinfectants.

4. Cheek cell phospholipid fatty acid analysis during the follow up of the NUHEAL subjects

The method applied for the analysis of cheek cell fatty acid composition did not permit to determine the full fatty acid spectrum, but the ratios of the major long chain polyunsaturated fatty acids docosahexaenoic acid (22:6), dihomo-gamma-linolenic acid (20:3) and arachidonic acid (20:4) to linoleic acid (18:2) could reliably be determined. The validity of this approach has been demonstrated by high correlations of these ratios between cheek cells and red blood cells in samples collected from adults. The results of the analysis of the samples available until the EARNEST project end (Oct 2010) are reported in the table below.

Ratios of docosahexaenoic acid (22:6), dihomo-gamma-linolenic acid (20:3) and arachidonic acid (20:4) to linoleic acid in cheek cell phospholipids collected from NUHEAL participants at the ages of 4, 5.5 and 6.5 years.

age	18:1/18:2	18:2/20:3	18:2/20:4	18:2/22:6
4.0 (n=67)	2.06±0.66	17.1±7.1	5.72±3.14	23.5±12.9
5.5 (n=17)	1.75±0.37	19.2±6.1	6.55±2.67	32.6±2.3
6.5 (n=47)	1.86±0.38	13.9±6.3	5.66±7.34	22.9±14.3

Laboratory analyses are not yet completed, because not all samples have been transported to the laboratory at Munich. Nevertheless, the table with the available data indicates that there is considerable variation between subjects, which indicates the importance of including a measure of the actual fatty acid status (as determined via cheek cell analysis), in addition to the classification into intervention or control groups, as a confounding factor into the statistical evaluation of the neurological examinations.