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Protein Intake in Infancy and Carotid Intima Media Thickness at 5 Years – A Secondary Analysis from a Randomized Trial

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Abstract

Background: Nutrition in childhood has an influence on the cardiovascular function later on in life. European Childhood Obesity Project is a multicenter, randomized clinical intervention trial examining the effect of early protein intake on later health outcomes, particularly adiposity and related disorders. The aim of the study was to examine the effect of nutritional intervention – different protein intake in infancy on carotid intima-media thickness (cIMT) at 5 years. The association of cardiovascular risk factors with cIMT was also assessed. **Methods:** Healthy term formula-fed infants in five European countries were enrolled either to the higher (HP) or to the lower (LP) protein group. Observational group consisted of breastfed infants. Plasma insulin, glucose, lipid profile, IGF-1, apolipoprotein A1 and B were measured as well as anthropometric parameters of parents and a child, blood pressure and physical activity. **Results:** No difference in cIMT between HP and LP group was observed. Insulin, HOMA-IR index and total IGF-1 were positively associated with cIMT but after adjustment for confounders only an inverse association between ApoA1 and positive between ApoB/ApoA1 and cIMT were significant. **Conclusion:** High versus low protein intake in infancy does not influence cIMT at 5 years. cIMT in healthy children at 5 years is associated with their apolipoprotein profile.

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Introduction

Arteriosclerotic alterations progress from childhood over to adulthood. The association to multiple risk factors like unfavorable lipid profile, body weight, blood pressure and diabetes is well studied in adults but less is known about this in children [1].

Arteriosclerosis has a long, asymptomatic, preclinical period. Pathological changes in the arteries of children develop decades before clinical manifestation of the disease. Some authors suggest that specific types of lesions occurring in younger ages (fatty streaks) are transformed into another type of lesions (fibrous plaques) in young adults and middle-aged persons, while others demonstrate that the progression from fatty streaks to plaque might be arrested in childhood and does not begin to a significant extent until after puberty in males and after menopause in females [2, 3].

Autopsy studies on arterial specimens of human subjects have shown that fatty streaks can be found even in the aortas of 3-year-old children [4]. In The Bogalusa Heart Study coronary artery changes were found in children as early as in the first decade of life [5]. These findings have been confirmed later in other populations, and extended to arteriosclerosis at different vascular sites [6].

Nutrition in childhood has an influence on the risk of arteriosclerosis independently of the known risk factors in adults [7]. In an observational study of 306 children at 5 years of age in the Netherlands, carotid intima media (cIMT) was significantly thicker in children who had been exclusively breastfed in infancy for 3–6 months [8]. In adults several dietetic intervention studies improving the serum lipid profile by diet have shown an association with reduced severity of coronary heart disease [9]. However, there is a lack of intervention studies in early childhood with subsequent follow-up into later ages.

In the European Childhood Obesity Project (CHOP) we demonstrated that higher protein intake during the first year of life may increase the risk of overweight and obesity later at the age of 6 [10, 11]. Obesity is known to be associated with several cardiovascular risk factors including dyslipidemia, hyperinsulinemia, hypertension and early arteriosclerosis in adults and children [12, 13].

Evelein et al. demonstrated in healthy young children that excess early postnatal weight gain, which was also observed in CHOP, leads to thicker arterial walls at 5 years of age [14].

In addition, the different protein content in study formulas used in the project was compensated by total fat to achieve their identical energy contents, which might independently affect long-term arteriosclerotic risk.

Finally, insulin-like growth factor 1 (IGF-1), which was increased in infants from 'higher protein' intake group, may also affect the arteriosclerotic process by exerting an antiapoptotic effect on vascular smooth muscles end endothelial cells [15, 16].

The main purpose of the study reported herein was to examine the effect of the early nutritional intervention on carotid intima media thickness – a marker for subclinical arteriosclerotic disease – at five years of age. The secondary aim was to analyze the relationship between risk factors for cardiovascular disease and carotid intima-media thickness.

Methods

Study Design
The detailed design of the CHOP study was published previously [10]. Briefly, it is a double-blind, randomized, multicenter, prospective nutritional intervention trial in infants, conducted in Belgium, Germany, Italy, Poland and Spain. Children were enrolled during the first eight weeks of life. Formula-fed (FF) infants were randomized in two intervention groups and breastfed (BF) infants were included as a reference group. All children were followed-up to the age of six years.

The study was approved by the ethic committees of all study centers. Written informed parental consent was obtained for each infant.

Intervention
The intervention consisted of two sets of infant and follow-on formulas with either higher (2.05 and 3.2 g/dl; HP) or lower (1.25 and 1.6 g/dl; LP) protein content given to the infants during the first year of life. All formulae complied with the 1991 EU Directive on Infant and Follow-on Formulae [17]. Identical energy density was achieved by adapting the difference in protein content with fat. BF infants had to be exclusively breastfed for at least three months.

Study Population
Apparently healthy, singleton, term infants born between October 1st 2002 and July 31st 2004, were recruited in the 11 sites of the study centers in Germany (Munich and Nuremberg), Belgium (Liege and Brussels), Italy (4 sites in Milano), Poland (Warsaw) and Spain (Reus and Tarragona). At recruitment, breastfeeding was promoted and supported. A total of 1,678 infants (540 LP, 550 HP, 588 BF) were enrolled in the original study (median age at the baseline visit was 16 days). Clinical characteristics of all study participants at study entry were published previously [10].

Data Collection
Birth weight and length were obtained from hospital data. Afterwards all anthropometric measures were taken by a trained study personnel at the baseline visit, at three, six, twelve, 24 months of age, and biannually until the age of six years following standard operating procedures based on the World Health Organization (WHO) Growth Multicentre Reference study [18].

cIMT was measured during the visit at 5 years of age. All children coming to the study visit were offered to have cIMT evaluated but not all families accepted an additional and time-consuming procedure. The combined thickness of the intimal and medial arterial wall components of the common carotid artery was determined with a linear transducer by a trained member of the medical staff. Subjects were examined in a supine position with a slightly overextended neck in a two-dimensional presentation of the longitudinal view of the vessel. cIMT of the far wall was measured 1–2 cm below the bifurcation of the common carotid artery at the late diastolic phase. At least one image for the right and one for the left artery from each subject was recorded, saved in JPG format, and transferred to the Children's Memorial Health Institute in Warsaw, Poland.

The following ultrasound devices were used in the different sites:
– Belgium: Siemens Sonoline Antares, Siemens Medical Solution USA, Mountain View, Calif., USA, 5.0–10.0 MHz (Brussels); ATL Philips HDI 5000, Bothell, Wash., USA, 5.0–12.0 MHz (Liege);
– Germany: Philips iE33, Philips GmbH, Hamburg, Germany, 11–13 MHz;
– Italy: GE Logiq 9, GE Healthcare Medical System, Milwaukee, Wisc., USA, 10–12 MHz;
– Poland: Vivid Pro 7, GE Vingmed Ultrasound, Horten, Norway, 8–13 MHz;
– Spain: Acuson Antares System, Siemens Medical Solutions USA, Mountain View, Calif., USA, 5–10 MHz (Reus); Acuson Sequoia 512 TM, Siemens Medical Solutions USA, Mountain View, Calif., USA, 8–15 MHz (Tarragona).

Measurements with a manual cursor placement method were performed by a blinded, trained physician on the pictures from all centers. Especially dedicated software was created to firstly, calibrate the ruler based on two points with 1 cm distance visible on the scale of the picture; secondly, to perform three measurements on each picture and calculate a mean value for the right and left artery. For analysis, the sum of left and right cIMT divided by two, alternatively left or right measurement in case of only one good quality picture, was used.

At the same visit at 5 years, blood pressure was measured by an oscillometric technique with Dinamap Procare blood pressure monitor (GE Medical Systems, Freiburg, Germany).

Social economics, family medical history, physical activity of the study children, and maternal behavior during pregnancy were assessed by specially designed questionnaires.

At 5½ years a fasted venous blood sample was drawn from all children that agreed for the procedure. Serum samples were stored at –70°C and transported on dry ice to the central laboratory (The Children's Memorial Health Institute, Warsaw, Poland) for analysis of IGF-axis parameters, apolipoprotein A1 (ApoA1) and apolipoprotein B (ApoB). The variables of the IGF-axis were measured with the use of immunoradiometric assay (Diagnostic Systems Laboratories Inc., Webster, Tex., USA). Serum insulin levels were quantified using immunoradiometric assay (DiaSource, Nivelles, Belgium). Fasting insulin and glucose was used to estimate insulin resistance by the homeostasis model of assessment of insulin resistance (HOMA-IR index) [19]. ApoA1 and ApoB were determined by an automated immunoturbidimetric method using polyclonal antisera (Orion Diagnostica, Espoo, Finland). Glucose, total cholesterol, high-density lipoprotein cholesterol –

HDL cholesterol, low-density lipoprotein cholesterol – LDL cholesterol and triglycerides (TG) were analyzed in the respective laboratories of the local study centers. An enzymatic method was used for determination of total cholesterol, HDL cholesterol, LDL cholesterol and TG. An enzymatic reference method with hexokinaze or indirect potentiometry (Reus, Spain) was used for glucose measurement.

Statistical Methods

Symmetric continuous data is presented as the mean ± standard deviation (SD), skewed continuous and count data as median and inter quartile range [IQR]. Comparisons between the feeding groups were performed using Student's t test, Wilcoxon rank sum tests or χ^2-tests, respectively. Spearman correlation coefficients between cIMT values and selected risk factors were calculated and tested for significance. Linear regression models on log-transformed cIMT measurements were applied to adjust for possible confounders (country, gender, type of feeding and smoking of the mother during pregnancy). The study had a sufficient power (80%) to detect a difference in cIMT between the two formula groups.

Data management was carried out with SAS version 9.2 (SAS Institute Inc., Cary, N.C., USA) and statistical analysis with R 2.10.1 (The R Foundation for Statistical Computing).

Results

Of the randomized 1,090 formula-fed children, 528 (48%) were followed until 5 years of age. Dropout numbers and the reasons for dropout were not significantly different between the two randomized groups. Additional 16% (n = 178) of all randomized, formula-fed children were excluded for noncompliance, illness or medication. Loss to follow-up was higher in the observational breast-fed group (fig. 1). The overall mean age at the 5 years visit was 5.03 (±0.05).

Characteristics of the population at 5 years of age differed slightly from the original study population, that is, children of parents with higher educational background were more likely to stay in the study [10]. Breastfed infants differed from formula-fed in terms of socioeconomic status, prevalence of pregnancy smoking, and parental BMI. However, no differences between the two randomized formula groups were observed.

Comparison of the subgroup of children who underwent cIMT measurement – 52% (n = 403) with those who did not give their consent – 48% (n = 368) did not demonstrate any significant differences in educational status of parents, smoking behavior, parental and child BMI, blood pressure, physical activity, insulin, glucose or lipid parameters (data not shown).

Detailed clinical characteristics of the subgroup with ultrasound measurement are listed in table 1.
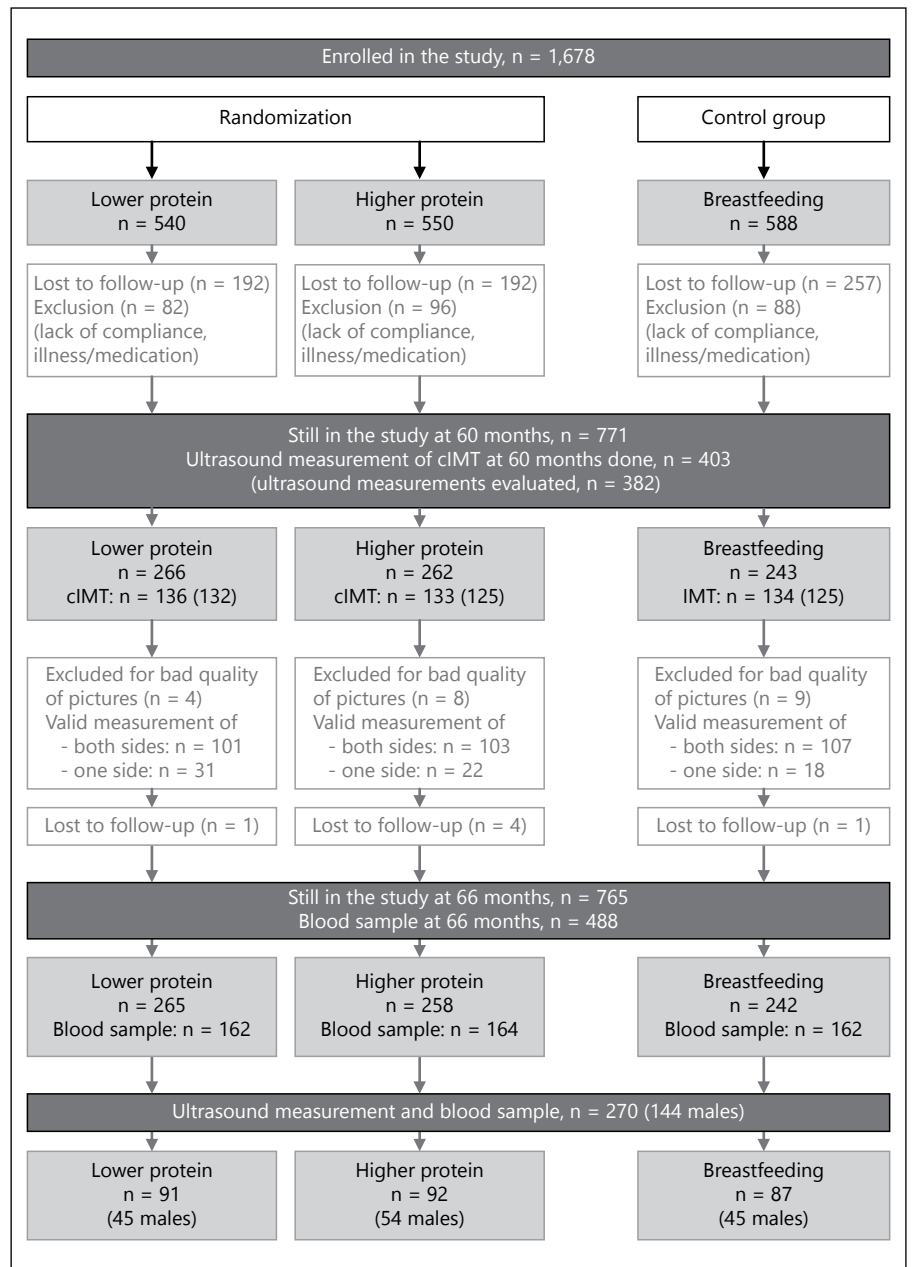
Fig. 1. Randomization, allocation and study participation.

Comparison by Feeding Groups

Table 2 summarizes cIMT measurements by feeding groups. No significant differences in cIMT between the formula feeding groups were found. There was no difference between formula fed and breastfed children, either.

Associations with Risk Factors

We found an association between cIMT and total IGF-1 (R = 0.12; p = 0.04), insulin (R = 0.16; p = 0.01) and HOMA-IR index (R = 0.16; p = 0.01).

There was also an inverse association between cIMT and ApoA1 (R = –0.16; p = 0.01) and a positive association with ApoB (R = 0.13; p = 0.02) and ApoB/ApoA1 ratio (R = 0.15; p = 0.01) (table 3). The association between ApoA1, ApoB/ApoA1 ratio and cIMT was significant also after adjustment for country, gender and feeding group.

On the contrary, no association was observed between cIMT and anthropometric parameters of neither the parents nor the child. Blood pressure, glucose, total choles-

Table 1. Population characteristics

	Higher protein (HP)		Lower protein (LP)		Breastfed (BF)	
	n	mean ± SD; median [IQR]; n (%)	n	mean ± SD; median [IQR]; n (%)	n	mean ± SD; median [IQR]; n (%)
Mother's BMI, kg/m^2	133	24.7±4.9	135	23.9±4.3	134	22.9±4.3**
Father's BMI, kg/m^2	132	26.6±3.7	136	26.2±4	133	25.7±3.4
Smoking during pregnancy, n (%)	133	42 (31.6)	135	48 (35.6)	134	13 (9.7)**
Birth weight, kg	133	3.3±0.4	136	3.3±0.3	134	3.4±0.3
Birth length, cm	132	51.1±2.8	135	50.6±2.5	134	51±2.6
Weight at 5 years, kg	127	20.1±3.6	133	19.5±2.6	128	19.2±2.9**
Height at 5 years, cm	125	110.4±4.6	133	110.8±4.2	128	110±4.8
BMI at 5 years, kg/m^2	124	16.4±2.1	133	15.9±1.6*	128	15.8±1.5**
Overweight, %	124	14 (11.3)	133	13 (9.8)	128	12 (9.4)
Obesity, %	124	8 (6.4)	133	6 (4.5)	128	3 (2.3)
Systolic/diastolic blood pressure, mm Hg	113	99.3±11/58.4±7.7	119	99±10.2/58.5±7.7	117	98.6±10.3/58.2±7.8
High-normal blood pressure, %	113	9 (8.0)	119	10 (8.4)	117	8 (6.8)
Hypertension, %	113	11 (9.7)	119	11 (9.2)	117	9 (7.7)
IGF-1, ng/dl	103	118 [83.8; 156.5]	104	116 [88.2; 145.2]	104	115.5 [88.4; 154.2]
Insulin, µIU/ml	103	6 [4.5; 7.6]	104	5.6 [4.4; 7.5]	104	5.5 [4.5; 7.1]
Total cholesterol, mg/dl	104	166 [150; 181.2]	104	171.5 [150; 186.2]	105	158 [142; 170]**
LDL cholesterol, mg/dl	102	103 [86.5; 114]	103	101 [887; 117.5]	104	91 [78.8; 107.5]**
HDL cholesterol, mg/dl	103	53 [46; 62]	104	53 [44; 61]	104	52 [45; 59]
Triglyceride, mg/dl	104	52.5 [38; 77.5]	104	50.5 [42.8; 73.8]	105	54 [43; 70]
ApoB, g/l	103	0.9 [0.7; 1.1]	104	0.9 [0.7; 1.1]	104	0.8 [0.6; 1]
ApoB/ApoA1	103	0.6 [0.5; 0.9]	104	0.6 [0.5; 0.8]	104	0.6 [0.5; 0.8]
ApoA1, g/l	103	1.4 [1.3; 1.6]	104	1.4 [1.3; 1.5]	104	1.4 [1.2; 1.6]

* p < 0.05 for HP vs. LP; ** p < 0.05 for formula fed vs. breastfed.

Table 2. cIMT by feeding group

	Higher protein (HP)		Lower protein (LP)		p value HP vs. LP	Breastfed (BF)		p value formula fed vs. BF
	n	cIMT (mm) median [IQR]	n	cIMT (mm) median [IQR]		n	cIMT (mm) median [IQR]	
All	125	0.38 [0.32; 0.45]	133	0.39 [0.33; 0.5]	n.s.	125	0.4 [0.34; 0.49]	n.s.
Boys	72	0.38 [0.32; 0.45]	61	0.4 [0.33; 0.5]	n.s.	62	0.4 [0.34; 0.49]	n.s.
Girls	53	0.37 [0.32; 0.45]	72	0.38 [0.32; 0.48]	n.s.	63	0.39 [0.34; 0.48]	n.s.

n.s. = Not significant.

terol, HDL-cholesterol, LDL-cholesterol and TG, which are all associated to cIMT in adulthood, showed no correlation at the age of 5 years.

cIMT measures did not significantly vary neither between boys and girls nor between the children of smoking and non-smoking mothers.

Discussion

Main Findings

To our knowledge this is the first report from randomized nutritional intervention trial in healthy, adequate for gestational age infants focused on subclinical arterioscle-

Table 3. Spearman correlations of cIMT with potential arteriosclerotic risk factors

	All		Boys		Girls	
	n	Spearman correlation R	n	Spearman correlation R	n	Spearman correlation R
Perinatal factors						
Mother's BMI, kg/m^2	381	0.00	195	0.03	186	−0.04
Father's BMI, kg/m^2	380	−0.08	194	−0.12	186	−0.04
Birth weight, kg	382	−0.06	195	−0.14	187	0.02
No. of cigarettes/day during pregnancy	379	−0.05	193	−0.07	186	−0.03
Lifestyle						
Hours spent playing outside	308	0.05	154	0.08	154	−0.02
Hours spent watching TV/playing video games	296	−0.08	148	−0.14	148	−0.02
Child's physiological markers at 5 years						
Weight, kg	367	0.01	186	−0.04	181	0.07
Height, cm	365	0.07	184	0.01	181	0.13
BMI, kg/m^2	364	−0.06	184	−0.08	180	−0.04
Systolic blood pressure, mm Hg	334	0.03	168	−0.01	166	0.07
Diastolic blood pressure, mm Hg	334	0.06	168	0.03	166	0.07
Child's biochemical markers at 5½ years						
IGF-1, ng/dl	297	0.12*	156	0.15	141	0.11
Total cholesterol, mg/dl	298	−0.04	154	0.01	144	−0.08
LDL cholesterol, mg/dl	294	−0.04	151	0.00	143	−0.09
HDL cholesterol, mg/dl	296	0.08	152	0.07	144	0.08
Triglyceride, mg/dl	298	−0.06	154	−0.06	144	−0.06
Insulin, µIU/ml	297	0.16*	156	0.19*	141	0.13
HOMA-IR index	290	0.16*	151	0.22*	139	0.09
ApoB, g/l	297	0.13*	156	0.15	141	0.11
ApoA1, g/l	297	−0.16*, §	156	−0.16	141	−0.17*
ApoB/ApoA1	297	0.15*, §	156	0.15	141	0.14

* $p < 0.05$; § $p < 0.05$ adjusted for country, gender, feeding group.

rosis in early childhood. Although at 5 years of age no differences in cIMT between the HP and LP group could be observed it cannot be excluded that longer time is needed for the development of detectable cIMT diversity.

At the same age of 5 years an association between cIMT and several risk factors was evaluated. Child's and parental anthropometrics, life style/environmental factors and blood pressure were not correlated with cIMT. Nevertheless, ApoA1 and ApoB/ApoA1 ratio were significantly associated with cIMT while lipoprotein cholesterol was not.

Comparison with Other Studies and Implications

We did not confirm the observation from Wheezing-Illnesses-Study-Leidsche-Rijn (WHISTLER) birth cohort study, that demonstrated a difference in cIMT between breastfed and formula-fed children. However, it is worth noting, that the breastfed group was not randomized [8].

The present study also did not confirm any long-term association of pregnancy smoking with carotid intima media thickness at 5 years. Such a relationship could be expected based on other studies, which demonstrated significantly increased aortic IMT in neonates whose mothers smoked during pregnancy [20]. It might be that ultrasound measurement of the aorta in newborns does not represent the situation in other arteries, especially at a later age.

Similarly, no association of cIMT was found with physical activity in our study, although in older Japanese children, aged 6–14, the lifestyle assessed by questionnaires (hours of TV watching, hours of sleeping) had an impact on cIMT [21].

Also obesity was reported by a number of observational studies to be associated with increased cIMT [22]. In our analysis no association with child's anthropometry

was detected, but contrary to other studies our cohort consisted of healthy, adequately-nourished children, with low prevalence of obesity (4.5% obese; 10.2% overweight) [23].

Although increased blood pressure is a known cardiovascular risk factor [24] we did not find an association between cIMT and blood pressure; however, our cohort consisted of younger children than those from other reports [25]. An elevated blood pressure above 95th percentile, according to Neuhauser et al. was found in 8.8% of our subjects. In 7.8% of subjects blood pressure was in a high-normal range [26].

Several observational studies demonstrated an association between insulin resistance and carotid intima-media thickness [22]. In the present study, insulin and HOMA-IR index were significantly associated with cIMT although the association was not significant after adjustment for confounders.

Similarly, our study demonstrated a positive association of total IGF-1 with cIMT at 5 years. This association disappeared after adjustment for confounders but a potential clinical significance of this finding is interesting. Infants with higher IGF-1 concentration in infancy tend to have lower IGF-1 in late adolescence [27], while population studies in adults suggest that lower IGF-1 levels are associated with increased risk of ischemic heart disease and stroke. Direct mechanisms of such a relation need to be elucidated [28].

Although HDL cholesterol was not associated with cIMT, ApoA1 showed inverse association and the ApoB/ApoA1 ratio was positively associated with cIMT. This may indicate the role of lipid disorders in arteriosclerosis at such an early age, as ApoA1 (HDL component) is responsible for cholesterol clearance from arteries and ApoB is the protein component of LDL, chylomicrons and very low-density lipoproteins. Contrary to apolipoproteins, the lipoprotein cholesterol was measured in local laboratories. Despite strict laboratory quality control, higher variability of this parameter should be considered. Another difference involving methodological issues is the possibility to use non-fasting blood samples, which is allowed only for apolipoprotein measurements [29].

Epidemiologic studies and clinical trials have demonstrated that the ApoB/ApoA1 ratio, representing the balance between proatherogenic and antiatherogenic lipoproteins, is a better predictor of cardiovascular risk in adults than any of the cholesterol indexes [30, 31]. Juonala et al. suggests that apolipoproteins might have additional value over conventional lipid measurements in pediatric cardiovascular risk assessment [29].

Strengths and Limitations

The strength of our study is its experimental design with randomization, early nutritional intervention and long-term follow-up of a large, cross-country cohort of healthy children. However, the long follow-up was associated with significant cohort attrition. Our drop-out ratio was similar to several other cohorts reported by different authors and suggested as acceptable in the context of long-term follow up studies [32]. Loss to follow up is inevitable with time, even with the best study design and conduct.

Because of a long distance between study centers, no standard inter-center variability analysis was possible to perform. According to an existing evidence, the reproducibility of cIMT ultrasound measurement is quite similar across several studies. With the use of standardized protocols and equipment, the measurement error in the cIMT measurements is estimated for 10–20% [33]. Despite detailed standard operating procedures, common training sessions for sonographers from all study centers, and final measurements by one trained person, the authors are aware of a bias during image acquisition. However, as children from the high- and low-protein group were randomly distributed, even assuming that there is possible systematic error among study centers, the difference between formula groups should be possible to identify.

Median values of cIMT measured in our cohort were slightly greater, than the reference values for 6 years old children published by Doyon et al. [34].

Due to the methodological issues described earlier, such as lack of an inter-center variability control and a non-standard method of the final measurement, with manual ruler calibration, our results, although sufficient for cIMT comparison between groups within the study may not be adequate for comparison with other studies and reference values.

Conclusions

Despite existing evidence of the impact of high vs. low early protein intake on increased risk of childhood obesity any longer-term effects on cIMT cannot be detected at 5 years. We speculate that our subjects might be too young to detect a significant change in cIMT in response to an early nutritional intervention. Pubertal development as well as an accumulation of environmental factors over time might play a role in the development of cIMT diversity.

However, even at this young age in healthy children an association between apolipoprotein profile and carotid intima media thickness can be observed.

Disclosure Statements

Acknowledgments

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