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Effect of classic ketogenic diet treatment on lipoprotein subfractions in children and adolescents with refractory epilepsy

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ABSTRACT

Objective: The aim of this study was to evaluate the effects of the classic ketogenic diet (KD) on low-density lipoprotein (LDL) and high-density lipoprotein (HDL) subfractions in children and adolescents with refractory epilepsy.
Methods: This prospective study recruited children and adolescents of either sex, whose epilepsy was refractory to treatment with multiple drugs. To be included, the patient had to have an indication for treatment with the KD and be treated as an outpatient. At baseline and after 3 and 6 mo of the KD, lipid profile (total cholesterol [TC], triacylglycerols [TG], LDL cholesterol [LDL-C], and HDL cholesterol [HDL-C]), apolipoproteins (apoA-I and apoB), 10 subfractions of HDL, 7 subfractions of LDL, LDL phenotype, and LDL size were analyzed using the Lipoprint system.
Results: The lipid profile components (TC, TG, LDL-C, HDL-C, apoA-I, and apoB) increased during the 3-mo follow-up, and remained consistent after 6 mo of treatment. Similarly, non-HDL-C, TC/HDL-C, LDL-C/HDL-C, and apoB/apoA-I ratios, representing atherogenic particles, significantly increased. In contrast, qualitative lipoprotein characteristics progressively changed during the follow-up period. Small LDL subfractions increased, and this profile was related with reduced LDL size (27.3 nm to 26.7 nm). The LDL phenotype became worse; 52.1% of the patients had a non-A phenotype after 6 mo of the KD. Small HDL subfractions decreased only after 6 mo of the KD.
Conclusions: KD treatment promotes negative changes in lipoprotein size and phenotype, contributing to atherogenic risk in these patients.

Introduction

Epilepsy is a neurologic disorder characterized by seizures that reflect abnormal electrical activity in one or more areas of the brain cortex [1]. The World Health Organization recently estimated that 0.7% of the population has epilepsy, representing 50 million people, with 80% living in developing countries [2].

Its etiology is associated with multiple structural and neurochemical dysfunctions, such as trauma, infectious diseases, congenital malformations, and genetic abnormalities [3]. These brain disorders result in neurobiologic, cognitive, psychological, and social consequences [1].

The majority of epilepsy treatment options is based on anti-epileptic drugs; however, 20% to 30% of children with epilepsy have seizures refractory to these drugs. For these children and adolescents, the ketogenic diet (KD) has been indicated as a coadjuvant treatment [4], with consensus regarding its benefits for epilepsy control [5].

The KD is based on high-fat, low-carbohydrate, and moderate protein content distributed in a 4:1 or 3:1 (fat: carbohydrate and protein) ratio [4]. Therefore, fat catabolism is the primary source

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of energy and responsible for the production of ketone bodies, which have considerable involvement in the therapeutic mechanisms related to seizure control [6]. In addition to the clinical benefits associated with a significant reduction in epileptic seizures, the number and quantity of required antiepileptic drugs are generally decreased when initiating the KD [4].

However, the KD is associated with side effects such as hyperuricemia, hypocalcemia, metabolic acidosis, hypercholesterolemia, kidney stones, and gastrointestinal disorders (e.g., vomiting, diarrhea, and constipation) [4]. The main clinical sign is dyslipidemia [7,8], and the primary changes in lipid metabolism are reflected as increased low-density lipoprotein cholesterol (LDL-C) and triacylglycerol (TG) levels.

Despite these recognized side effects, the literature did not describe the effect of the KD on physical lipoprotein properties in patients with refractory epilepsy. One study with healthy, normolipidemic adult men failed to report changes in TG, LDL-C, and oxidized LDL (oxLDL) levels after 6 wk on the KD [9]. However, individuals with a high proportion of small LDL particles had increased particle size related with higher LDL-1 concentrations. Compared with a low-fat diet, the use of the KD diet for 6 mo as a method for weight loss in adults resulted in reduced TG levels and increased large LDL and large HDL particles [10].

Additionally, there is a lack of prospective long-term studies that evaluate the effects of the KD on cardiovascular health in children with refractory epilepsy [11]. According to the Johns Hopkins University guidelines for dietary treatment of epilepsy in the United States, long-term changes in the lipid profile do not appear to be significant, but the influence of these changes on coronary heart disease are unknown [6].

Given this background, the goal of this study was to evaluate the effects of the classic KD on LDL and HDL subfractions in children and adolescents with refractory epilepsy.

Materials and methods

Study design and participants

This prospective study was conducted from June 2012 to March 2014 at the Brazilian Reference Center–Instituto da Criança do Hospital das Clínicas da Faculdade de Medicina da USP. Children and adolescents of both sexes, aged 1 to 19 y, with epileptic seizures, independent of the etiology, refractory to multiple drugs, and for whom KD was indicated were included. Given the primary study focus on lipid metabolism, patients receiving hormone replacement therapy, using lipid-lowering drugs, or those who had diabetes mellitus, hypothyroidism, hyperthyroidism, or Down syndrome were excluded.

Each patient participated in three measurements: baseline, which was the period after the decision that treatment was indicated but before the start of the KD; time 1, which occurred after 3 mo of KD treatment; and time 2, which occurred after 6 mo of KD treatment. The guardians responsible for the children provided written, informed consent. The study was approved by the Ethic Committees (No. 0068012.1.0000.5421).

Dietary protocol

The guidance and dietary planning protocol followed the recommendations of the classic KD, and the caloric intake was adjusted for ideal body weight according to the body mass index (BMI) *Z* score [12,13]. The diet was introduced without fasting, and the children and adolescents were followed as outpatients. Patients started treatment with a diet of 2:1 fat to carbohydrate and protein, and the proportion was increased weekly until it reached 4:1. To evaluate the adherence to the KD, ketosis levels were monitored using ketone bodies in urine (Accu-chek/Keto-diabur test, Roche, Switzerland) and plasma (Ranbut kit, Randox Laboratories Limited, United Kingdom).

Anthropometric data

Weight and height were used to calculate BMI. The BMI *Z* scores for age (ZBMI/A) were obtained based on the growth curves recommended by the World Health Organization [12,13].

Blood sample collection

At baseline, time 1, and time 2, blood samples were collected in EDTA tubes after a 12-h fast. To preserve the plasma samples (obtained at 3000*g* and 4°C for 15 min), the protease inhibitors aprotinin (10 μg/mL), benzamidine (10 μM), and phenylmethanesulfonyl fluoride (5 μM), and the antioxidant butylated hydroxytoluene (100 μM) were added. Plasma was stored at −80°C until the analytical procedures.

Lipid and apolipoproteins

Plasma total cholesterol (TC), HDL-C, LDL-C, and TG were measured using enzymatic and colorimetric methods and standard protocols (Roche Diagnostics GmbH®, D-68298 Mannheim). The apolipoproteins apoA-I and apoB were determined using the immunoturbidimetric method with apoA-I and apoB kits (Randox Laboratories Limited, United Kingdom).

Lipoprotein size and concentration

The LDL subfractions, HDL subfractions, and mean LDL particle size were determined from plasma using the Lipoprint system (Quantrimetrix). This method uses linear electrophoresis on a nondenaturing polyacrylamide gel to separate and quantify lipoprotein subfractions. LDL subfractions were identified according to the size of and concentration in each particle (LDL-1 to LDL-7). LDL-1 and LDL-2 were large particles, and LDL-3 to LDL-7 were small particles. The LDL sizes were classified as phenotype A (≥26.8 nm), intermediate phenotype (26.51–26.79 nm), or phenotype B (≤26.5 nm). The intermediate phenotype and phenotype B were collectively considered the non-A phenotype. Ten subfractions were identified for HDL (HDL-1 to HDL-10), which were combined as follows: large (HDL-1 to HDL-3), intermediate (HDL-4 to HDL-7), and small (HDL-8 to HDL-10) HDL particles.

Statistical analysis

Continuous variables are reported as mean and SD. Chi-square tests were used to compare categorical variables (age groups, sex, and race), and comparisons of changes over time (phenotype A and non-A phenotype) were conducted using the McNemar test. SPSS version 20.0 (IBM Corp., Armonk, NY, USA) was used to perform these analyses.

Changes in the variables associated with the lipid profile and lipoprotein subfractions were analyzed using the generalized estimated equation (GEE) method using a Gaussian distribution and gamma with link function, inverse, and identity. Models were adjusted for sex, age, and total antiepileptic drug (AED) dose (mg) for all three measurement periods. Adjustment for the latter was performed because previous studies suggested a positive association between AED use and cholesterol metabolism [14]. Because the anthropometric variables did not change during the study period, the models were not adjusted for these variables. These comparisons were conducted using intention-to-treat analysis, and the analyses were performed using R software, version 3.0.2. The significance level for all analyses was $P < 0.05$.

Results

Of the 52 patients who attended the group meeting, 38 children and adolescents were eligible (Fig. 1). The KD was not indicated for four patients because they had significant seizure control; another five were not included because they had diabetes mellitus, hypothyroidism, Down syndrome, or a combination of these diseases. An additional five patients refused KD treatment because they claimed they did not have good family and economic support. After baseline, 12 patients ceased the KD, and another 3 patients ceased the KD after 3 mo, resulting in 23 patients who completed the study.

The average age of the included patients was 6.5 y (1.3–18 y). Of these, 7 were adolescents (27%), and 19 were children (73%; $P = 0.001$). Twenty of the patients were male (76.9%), whereas 6 were girls (23.1%; $P = 0.006$), and the proportion of each race did not differ significantly (white: 65.4%; non-white: 34.6%; $P = 0.117$). The etiology of epilepsy was heterogeneous, including genetic syndromes (e.g., GLUT-1 deficiency) and electro-clinical syndromes; the etiology for 35% of the children and adolescents was unknown (data not shown). Although weight and height

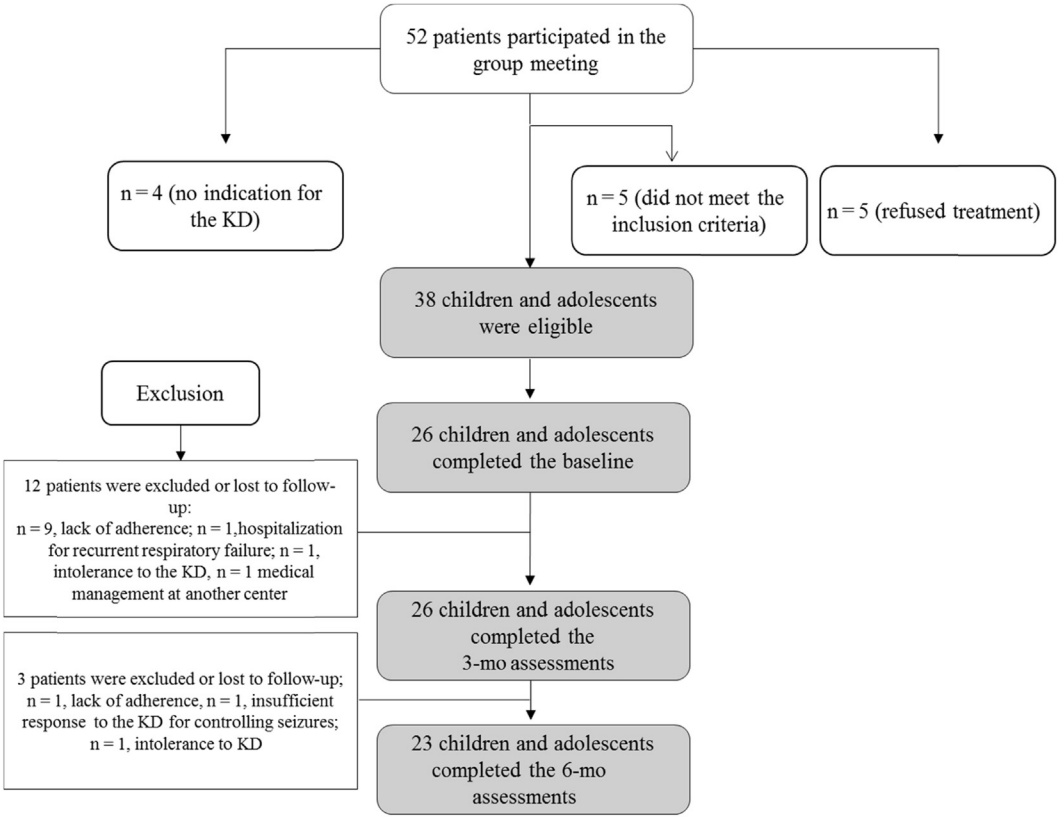

Fig. 1. Flowchart of patient inclusion, exclusion, and loss to follow-up. KD, ketogenic diet.

significantly increased over time, BMI and ZBMI/A remained stable (Table 1).

All of the patients used at least two AEDs, with valproate (62%) and clobazam (54%) being the two most commonly used. After 6 mo of the KD, 68% of the children and adolescents had a >50% reduction in seizures; although the remaining 32% had a <50% reduction in seizures, they were not excluded from the study because the family and health team decided that the general clinical profile and quality of life improved after the KD (data not shown).

Excessive ketonuria levels were noted for all of the patients after the KD was increased to a 3:1 ratio. Except for one patient, urinary ketosis was stable (>150 mg/dL) only after introduction of the KD with a higher proportion of fat (4:1). This profile was confirmed by a significant increase in β-hydroxibutyrate levels compared with baseline (baseline = 0.69; time 2 = 3.06 mmol/L; $P < 0.001$): 296% (time 1) and 343% (time 2).

Table 1
Anthropometric characteristics of children and adolescents with refractory epilepsy, based on measurement period during a ketogenic diet intervention

Variables	Baseline	3 mo	6 mo	*P* value
	n = 26	n = 26	n = 23	
Weight, kg	25.7 (16.4)	29.6 (22.1)*	29.7 (21.9)*	**0.015**
Height, cm	116.5 (24.2)	118.3 (23.9)*	119 (24.2)*,†	**<0.001**
BMI, kg/m^2	19 (5.5)	18.7 (6)	18.3 (5.9)	0.219
ZBMI/A	0.93 (1.5)	0.61 (1.8)	0.39 (1.7)	0.148

BMI, body mass index; ZBMI/A, *Z* score of BMI for age
Values expressed as mean (SD). Comparisons along time evaluated by the generalized estimation equations adjusted for total dosage of antiepileptic drugs
Bold values indicate the differences during follow-up time
* Significantly different from baseline.
† Significantly different from 3 mo ($P < 0.05$).

The concentrations of TC, TG, LDL-C, HDL-C, apoA-I, apoB, and non-HDL-C and the TC/HDL-C, LDL-C/HDL-C, and apoB/apoA-I ratios increased significantly during the follow-up period. The HDL-C/apoA-I and LDL-C/apoB ratios remained unchanged. Although the TG/HDL-C ratio was 37% higher at time 2 than at baseline, this was not considered significant (Table 2). There was a progressive and significant trend for lipid biomarkers to increase (Fig. 2). Although the values were higher at time 2 than at baseline, they were similar to those at time 1, except for TG levels.

The LDL subfractions progressively increased during the intervention. After 3 mo of treatment with KD, the percentage of large LDL particles significantly increased; after 6 mo, the percentage had decreased but remained higher than that at baseline. Conversely, the percentage of small LDL subfractions continuously and progressively increased over time and was twice as high at time 2 than at time 1. The percentage of small HDL particles had significantly decreased at time 2. At both times 1 and 2, significant changes in LDL were related with the reduced size of LDL particles (Fig. 3). Additionally, a negative effect on the LDL phenotype was observed; phenotype B increased to 30.7% and 52.7% of patients at 3 mo and 6 mo, respectively (Fig. 4).

Discussion

Although the present results confirmed the positive clinical benefits of the KD for the treatment of refractory epilepsy, they highlighted, for the first time, the negative effects of this diet on the qualitative aspects of lipoproteins. Small LDL particles migrate more easily to the subendothelial layer, where they are more susceptible to oxidation and other modifications,

Table 2
Lipid profile, lipoprotein subfractions, and LDL size in children and adolescents with refractory epilepsy, based on measurement period during a ketogenic diet intervention

Variables	Baseline	3 mo	6 mo	*P* value
	n = 26	n = 26	n = 23	
TC, mg/dL	156 (28)	242 (85)*	230 (81)*	<**0.001**
TG, mg/dL	81 (37)	103 (49)*	119 (88)*	**0.003**
LDL-C, mg/dL	91 (21)	160 (75)*	152 (70)*	<**0.001**
HDL-C, mg/dL	48 (13)	61 (19)*	58 (17)*	**0.001**
apoA-I, mg/dL	119 (17)	143 (34)*	140 (30)*	<**0.001**
apoB, mg/dL	89 (15)	139 (55)*	138 (59)*	<**0.001**
Non-HDL-C	107 (24)	181 (86)*	172 (83)*	<**0.001**
TC/HDL-C	3.5 (1.4)	4.3 (2.0)*	4.4 (2.6)*	**0.012**
LDL-C/HDL-C	2.1 (1.1)	2.9 (1.7)*	2.9 (2)*	**0.026**
TG/HDL-C	1.9 (1.7)	1.9 (1.4)	2.6 (3.2)	0.284
HDL-C/apoA-I	0.4 (0.1)	0.4 (0.1)	0.4 (0.1)	0.340
LDL-C/apoB	1.0 (0.2)	1.1 (0.2)	1.1 (0.1)	0.073
apoB/apoA-I	0.8 (0.2)	1 (0.4)*	1 (0.6)*	**0.002**
Large LDL, %	44 (12.5)	87.5 (40.9)*	71.9 (27.2)*,†	<**0.001**
Small LDL, %	1 (1.2)	9.2 (10.2)*	17.3 (22.8)*,†	<**0.001**
LDL size, nm	27.3 (1.8)	27 (3.3)*	26.7 (5.8)*,†	<**0.001**
Large HDL, %	27.3 (7.7)	29.4 (9)	30.4 (10)	0.530
Intermediate HDL, %	51.9 (6.5)	47.3 (6.9)	50.8 (7.5)	0.050
Small HDL, %	20.7 (6.6)	23.3 (8.4)	18.8 (8.4)†	**0.033**

apo, apolipoprotein; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triacylglycerols
Comparisons based on time were evaluated using generalized estimation equations adjusted for sex, age, and total dosage of antiepileptic drugs. Values are expressed as mean and SD
Bold values indicate the differences during follow-up time
* Significantly different from baseline.
† Significantly different from 3 mo ($P < 0.05$).

intensifying the atherogenicity of this lipoprotein [15]. It is currently accepted that these particles have a greater affinity for proteoglycans in the arterial wall and a higher percentage of glycated apoB [16]. Regarding these aspects, previous in vitro studies have shown that small LDL particles had a lower affinity for B/E receptors than large LDL particles [17], contributing to increased hepatic clearance of large LDL particles. Contrary, small LDL particles remain in plasma longer and are highly susceptible to migration to the arterial wall, favoring the oxidative process and uptake by macrophage-expressed scavenger receptors.

The increase in the percentage of small LDL particles and the reduction in LDL size were significant. Despite multiple pathways for small LDL particle generation, hypertriglyceridemia is probably the most important in biology system. High TG levels represent a potent, positive stimulus for cholesterol ester transfer protein activation [18], which promotes cholesterol transfer from HDL to apoB-rich lipoproteins and generation of small LDL particles. This process also increases hepatic lipase activity, which induces LDL hydrolysis, contributing to the generation of small, dense LDL particles [19].

LDL quality is associated with a phenotype in which the LDL size determines a more or less atherogenic profile. Consensus is lacking for a cutoff point of the phenotype of LDL [20]; however, studies have found associations between phenotype B or non-A phenotype and a smaller LDL size. Regarding the cutoff point (<26.8 nm) adopted in the present study, the results confirm that the KD contributed to atherogenic non-A phenotype in a time-dependent manner (30.7% after 3 mo and 52.1% after 6 mo).

At the same time, the relationship between HDL size and cardiovascular risk remains controversial. In the present study, the HDL subfractions were less modified by the KD than the LDL subfractions. Only the percentage of the small HDL particles was reduced after 6 mo of the intervention.

Although large HDL particles are more competent in reverse cholesterol transport [21], the smaller HDL particles exhibit greater antioxidant activity and antiinflammatory action, preventing oxidative modification of LDL in the subendothelial layer. The results of the present study suggest that KD treatment has a bimodal effect on lipoprotein size; small HDL subfractions decreased, whereas small LDL particles increased. This profile was reinforced by the negative effect on lipoprotein size promoted by TG levels. KD treatment was associated with increased TG levels, reduced HDL-C levels, and, consequently, a tendency for a high ratio of TG to HDL-C. The atherogenic role of LDL is well established [22], but reduction in HDL size can be analyzed using atherogenic (reverse cholesterol transport) and nonatherogenic (antioxidant and antiinflammatory) functions. Regarding the latter contradictory role, the negative changes in LDL particles remain a major modification observed in children and adolescents treated with the KD.

The main clinical outcomes of atherosclerosis (myocardial infarction and stroke) usually occur in adults; however, the development of atherosclerosis begins early in life, and its progression is directly associated with lipoprotein levels [23]. Based on the recommendation of the American Academy of Pediatrics, 38% and 40% of children and adolescents in the present study had hypercholesterolemia (TC ≥200 mg/dL) and hypertriglyceridemia, respectively, at 3 mo [24]; the percentage of the children and adolescents with hypertriglyceridemia increased to 48% at 6 mo.

The association between lipoprotein concentrations and development of atherosclerosis in children and adolescents was reported previously in a study that evaluated autopsy results for 204 individuals aged 2 to 39 y [25]. Fatty streaks and fibrous plaques were present in the coronary arteries and the aorta of 28% of children and adolescents aged 2 to 15 y. In the same study, the atherosclerotic lesions were significantly correlated with TC, LDL-C, and TG concentrations [25].

In addition to lipids, apolipoproteins are associated with cardiometabolic risk and CVDs. ApoB, the main protein in LDL, is a strong indicator of the number of circulating atherogenic

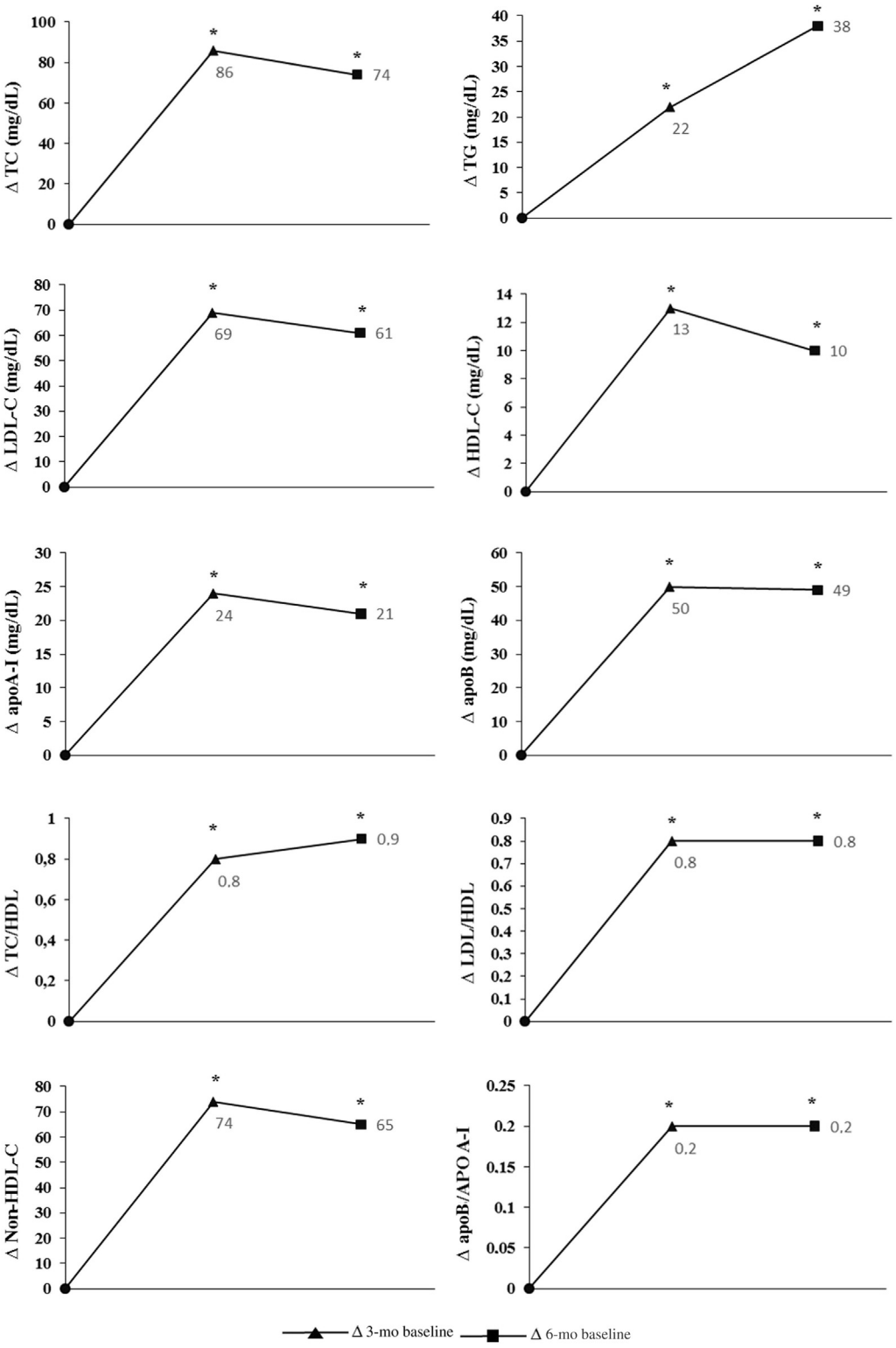

Fig. 2. Changes in the classic lipid profile in children and adolescents with refractory epilepsy, based on measurement period during a ketogenic diet intervention. Comparisons based on time were evaluated using GEE adjusted for sex, age, and total dosage of antiepileptic drugs. Values are expressed as mean and SD. *Significantly different from baseline ($P < 0.05$). apo, apolipoprotein; GEE, generalized estimation equations; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triacylglycerols.

particles [26]. In contrast, apoA-I, the major protein component of HDL, is an antiatherogenic biomarker [27]. The significant increase in LDL-C in the present study mirrored the increase of ~55% in total apoB concentration at times 1 and 2. Therefore, KD provides a positive stimulus for synthesis of new LDL particles and enrichment of the cholesterol content in this lipoprotein.

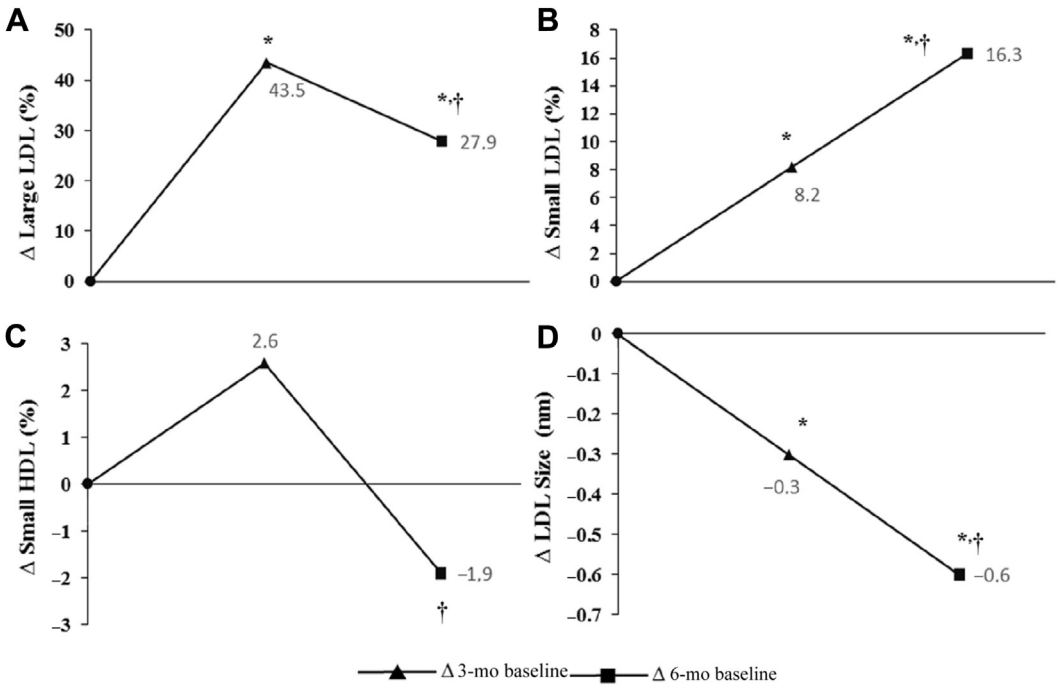

Fig. 3. Changes in low-density lipoprotein (LDL) cholesterol subfractions and size in children and adolescents with refractory epilepsy, based on measurement period during a ketogenic diet intervention. Comparisons based on time were evaluated using GEE adjusted for sex, age, and total dosage of antiepileptic drugs. Values are expressed as mean and SD. GEE, generalized estimation equations; HDL, high-density lipoprotein; LDL, low-density lipoprotein. *Significantly different from baseline. †Significantly different from 3 mo ($P < 0.05$).

Despite the very well-established adverse effects (AEs) of KD on the lipid profile [7,8,28], the effect of the KD on lipoprotein subfractions and atherogenic phenotype was not previously studied in the context of epilepsy. Our results confirm previous findings and present the tendency of the lipid profile of patients with epilepsy to achieve a "plateau effect" after 6 mo of KD treatment. However, small LDL particles progressively increased. Therefore, the study do not question the clinical relevance of KD but highlight for the first time the importance of monitoring the size of lipoproteins.

Previous studies have shown that particle size represents a threefold increase in cardiovascular risk, independent of other lipid parameters [29,30]. In a number of individuals with coronary artery disease and normal serum cholesterol concentrations, atherogenicity was directly related with particle size, suggesting the importance of clinical studies to monitor not only the classic lipid profile but also nontraditional risk factors [31].

In a recent study that evaluated vascular function using carotid ultrasound [32], initial alterations in lipids, apoB, and arterial function within the first year of KD treatment appeared to be reversible and not significant after 24 mo of treatment; however, the authors did not describe whether the classic KD was used or whether there was any change in the fatty acid composition. Furthermore, this relationship was not assessed using nontraditional biomarkers.

The changes observed in the lipid profile with epilepsy are associated not only with the KD but also the simultaneous effect of drugs used during treatment. For example, valproic acid, carbamazepine, and phenytoin promote dyslipidemia by stimulating cytochrome P450 [33].

According to one study, the AEs of the KD are still being compiled and should be of scientific interest [34]. In 2011, one group of researchers described a case of a 6-y-old boy who developed an anaphylactic reaction to eggs after using the modified Atkins diet to treat epilepsy; the boy had to stop the diet after remaining seizure free for 6 mo [35]. Therefore, it is important for pediatric neurologists and dietitians to recognize the side effects, treatment safety, and scientific basis of the KD [34].

Dyslipidemia and changes in the qualitative aspects of lipoproteins are a synergistic side effect of the global treatment of refractory epilepsy, which must be monitored in these patients. For this, we propose strategies to maintain the clinical efficacy, while preventing acute and chronic adverse responses to the KD. First, use a modified KD, with fewer atherogenic fatty acids. Second, initiate coadjuvant KD treatment early to reduce the

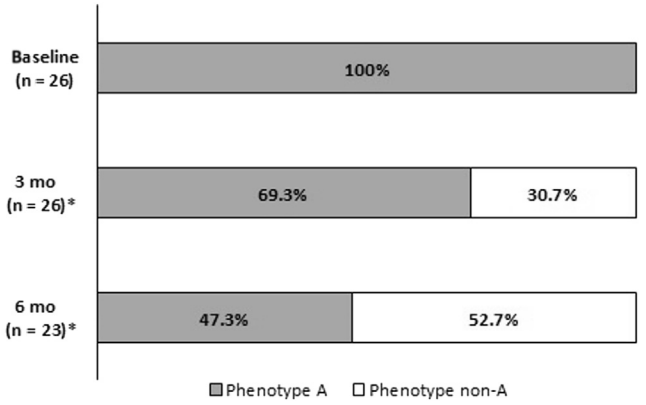

Fig. 4. Changes in phenotype in children and adolescents with refractory epilepsy, based on measurement period during a ketogenic diet intervention. Comparisons along time evaluated using the McNemar test. *Significantly different from baseline ($P < 0.05$).

number and quantity of AEDs that show dyslipidemic action. Finally, include lipoprotein size in routine laboratory examinations in addition to the classic lipid profile.

This study had certain limitations. The small sample size could decrease the statistical power to detect differences during the follow-up; however, considering that the prevalence of refractory epilepsy is low (20–30%) within the 0.5% to 1% of all epilepsy cases, the sample size characterizes the epidemiologic profile of this disease. Additionally, the observed differences confirm the effect of the KD on lipid profile and lipoprotein size. Because the study design did not include a control group, we could not establish a causal relationship for the changes. Furthermore, the clinical response to the KD needs to be interpreted with caution because the etiologic origin of epilepsy is complex; additional variables might need to be evaluated regarding the response to the KD and its side effects.

Conclusion

Treatment using the KD had a negative effect on the lipid profile and contributed to a more atherogenic phenotype that involved generation of small LDL subfractions and smaller HDL size. However, we do not believe that the KD should be contraindicated for refractory epilepsy, and its clinical relevance should continue to be encouraged. However, results of the present study suggest that additional studies should be conducted that include a longer follow-up, cardiometabolic risk biomarkers, and an atherosclerotic subclinical analysis (i.e., particle size, oxidized LDL, and carotid ultrasound).

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