Lanny C. Gultom*, Damayanti R. Sjarif, Herawati A. Sudoyo, Muchtaruddin Mansyur, Sri Rezeki S. Hadinegoro, Suzanna Immanuel and Mexitalia Setiawati

The role of apolipoprotein E polymorphism in improving dyslipidemia in obese adolescents following Physical Exercise and National Cholesterol Education Program Step II intervention

Abstract

Background: Lifestyle changes are important factors for managing dyslipidemia before considering blood lipid-lowering drugs. However, genetic factors can influence the response outcome.

Objective: We aimed to determine a dyslipidemia management strategy in obese adolescents.

Patients and methods: A total of 60 dyslipidemic obese adolescents received physical exercise and the NCEP step II diet for 28 days. Apolipoprotein E (apo E) genotypes and blood lipid levels were compared before and after interventions.

Results: The apo E3/E3 genotype was found to be common in all subjects. Mean levels of total cholesterol, triglycerides, and low-density lipoprotein-cholesterol (LDL-C) improved in subjects with the E3 allele after the intervention, but not the E2 allele. Total cholesterol and LDL-C, but not triglyceride levels, improved in subjects with the E4 allele.

Discussion: Apo E alleles might influence improvement in lipid profiles after diet and exercise interventions. These results could inform personalized dyslipidemia management in obese adolescents, to determine which subjects would benefit from blood lipid-lowering drugs.

Keywords: Apo E; dyslipidemia; NCEP step II diet; physical exercise.

DOI 10.1515/jpem-2014-0070
Received February 14, 2014; accepted December 8, 2014

***Corresponding author: Lanny C. Gultom,** Child Health Department of Fatmawati Hospital, Jl. RS Fatmawati, Cilandak, Jakarta Selatan, 12430, Indonesia, Phone: +62217501524, Fax: +622175911454, E-mail: lannygultom@yahoo.com

Damayanti R. Sjarif and Sri Rezeki S. Hadinegoro: Child Health Department of Cipto Mangunkusumo Hospital, Jakarta, Indonesia

Herawati A. Sudoyo: Eijkman Biology Molecular Institute, Jakarta, Indonesia

Muchtaruddin Mansyur: Community Medicine, Faculty of Medicine, University of Indonesia, Jakarta, Indonesia

Suzanna Immanuel: Clinical Pathology of Cipto Mangunkusumo Hospital, Jakarta, Indonesia

Mexitalia Setiawati: Child Health Department of Kariadi Hospital, Semarang, Indonesia

Introduction

Dyslipidemia is a lipoprotein metabolism disorder characterized by elevated levels of total cholesterol, triglycerides, and low-density lipoprotein cholesterol (LDL-C), or decreased levels of high-density lipoprotein cholesterol (HDL-C) (1). Dyslipidemia is most often caused by secondary factors, such as obesity, age, sex, race, lifestyle (diet, exercise, smoking, and alcohol), endocrine disorders (diabetes mellitus and hypothyroidism), liver and kidney diseases, and some medications (diuretics, beta-blockers, glucocorticoids, retinoic acid derivatives), with primary factors (genetics) playing a smaller role in disease development (2, 3).

Q1: The citations were revised according to the style guide (name and date has has been changed to number references). Please confirm

Encouraging lifestyle changes, including physical exercise and diet, is the cornerstone OF management strategies for dyslipidemia before considering the use of blood lipid-lowering drugs. This is because atherosclerosis begins in youth, and the initiation of atherosclerosis is related to traditional cardiovascular risk factors, including dyslipidemia (2, 4, 5). In fact, the carotid artery narrowing, which is part of atherosclerosis process and characterized by increased intima-media thickness of the carotid artery, has already been found in obese adolescents (6). One study found that exercise is beneficial in combating

Q2: Please confirm running head

dyslipidemia and protecting from atherosclerosis only when combined with a healthy diet. Plaque accumulation in the aortic arch, a marker of cardiovascular events, was reduced by diet and the effect was significantly potentiated by exercise resulting in significant plaque regression (7). Dyslipidemia management failure often results due to non-adherence to physical exercise and diet consumption programs. However, management can also fail for individuals who do make positive lifestyle changes, and genetic factors are often regarded as the cause.

The apolipoprotein E (apo E) gene is located on chromosome 19 (19q13.2), and consists of 4 exons, 3 introns and 4696 base pairs, encoding a 299-amino acid protein (8). Apo E has three isoforms, namely, apo E2, E3 and E4, with the apo E3 being the wild type allele. The apo E2 allele encodes a cysteine residue at codons 112 and 158 (TGC), the apo E3 allele encodes a cysteine residue at codon 112 and an arginine residue at codon 158 (CGC), and the apo E4 allele encodes an arginine at both codons. Amino acid variation due to these polymorphisms produces three homozygous genotypes (apo E2/E2, E3/E3, and E4/E4) and three heterozygous genotypes (apo E2/E3, E2E4, and E3/E4) (9, 10).

Q3: Please note that genes are set in italic and proteins/enzymes in Roman – please indicate throughout where italics should be applied.

Apo E polymorphisms are a genetic risk factor for dementia, Alzheimer's disease, and cardiovascular disease (CVD) (11, 12). It is well established that the presence of an E4 allele is linked to higher plasma total cholesterol, triglycerides, LDL-C and lower HDL-C levels (11, 13–15), coronary artery disease (CAD), and diabetes mellitus type 2 (T2DM) (16, 17). Apo E4 allele has been found to be the prominent genetic predictors for development of CAD in the Eastern part of India (16). Meanwhile, a study in Korea found that individuals with E2/E3 genotype had lower risk for carotid plaque, but those with E3/E4 genotype had a higher risk for carotid plaque (12).

Q4: Reference 16 year is matched with ref. list. Please check and confirm.

Apo E variations also affect blood cholesterol levels differently in response to physical exercise and diet. Apo E2 and apo E3 subjects show greater improvement in lipoprotein lipid profiles following physical exercise compared with apo E4 subjects, whereas a low-fat dietary intervention tends to result in lower LDL-C and total cholesterol levels in apo E4 subjects compared with apo E3 or apo E2 subjects (8, 9, 18–21). In another study, the presence of high intake of total fat or a low ratio of polyunsaturated to saturated fatty acid causes apo E4 allele carriers to lose their protection against hypertriglyceridemia (22).

Q5: This sentence revised so as not to cause confusion with similar citations. Please check if this is appropriate in the sentence: "This study aimed to achieve the following....."

This study aimed to achieve the following (a) develop a strategy for dyslipidemia management in obese adolescents, considering the effects of apo E polymorphism and physical exercise and the National Cholesterol Education Program (NCEP) step II diet interventions for 28 days, in order to determine the need for initiating a blood lipid-lowering drug treatment regimen; (b) determine the apo E genotype profiles between dyslipidemic obese adolescents who show improved and unimproved lipid profile levels after receiving physical exercise and NCEP step II diet interventions for 28 days; (c) determine the role of apo E genotype and intervention adherence of physical exercise and NCEP step II diet for 28 days in decreasing the total cholesterol, triglycerides, and LDL-C levels, and increasing HDL-C levels in dyslipidemic obese adolescents; and (d) to confirm that the apo E2 allele does not contribute to the improvement of lipid profiles, whereas the apo E3 and E4 alleles do improve the lipid profiles in obese adolescents who receive physical exercise and NCEP step II diet interventions for 28 days.

Patients and methods

Subjects

This study used a cross-sectional design to compare lipid profiles in subjects pre- and post-intervention. All subjects were dyslipidemic obese adolescents aged 10–19 years residing in Jakarta, and were selected by consecutive sampling. Written informed consent was received from all parents before inclusion in the study. Obesity is defined when the body mass index (BMI) is greater than or equal to the 95th percentile of the BMI-CDC 2000 curve for a given age and sex (23). Dyslipidemia is confirmed when any of the 12-h fasted lipid profile levels meet the criteria of the NCEP Expert Panel on Cholesterol Levels in Children (total cholesterol, triglycerides, or LDL-C levels are borderline or high, while the HDL-C level is borderline or low (24). Exclusion criteria for participating in this study were elevated levels of serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic-pyruvate transaminase (SGPT), urea, creatinine, blood glucose and thyroid stimulating hormone (TSH), or subjects who regularly consumed alcoholic beverages or blood lipid-interfering drugs, such as diuretics, beta blockers, glucocorticoids, and retinoic acid derivatives.

Interventions

Subjects who met the inclusion criteria received physical exercise and NCEP step II diet intervention. Physical exercise was conducted at Somatokinetic Laboratory of the Sport Science Faculty of the State University of Jakarta from June 16 to July 15, 2012. All subjects performed 60 min of physical exercise three times a week, consisting of aerobic activity and muscle and bone strengthening under the supervision of a physical trainer. The NCEP step II diet consisted of a total fat content ≤30% of total daily calorie intake, saturated fatty acid content <7% total daily calorie intake and <200 mg cholesterol/day, and was followed by all subjects for 28 days in the same location. The total daily calorie intake was calculated for each subject by multiplying the required daily allowance (RDA) according to height age with the ideal body weight according to height. The provided diet

only included lunch and dinner. Breakfast was not provided because of logistical difficulties in meal provision; however, a recommended breakfast meal was designed and provided to each subject.

Apo E genotyping

Apo E genotyping was conducted at the Eijkman Institute for Molecular Biology, Jakarta, Indonesia. Leukocyte DNA was extracted from subjects and amplified by polymerase chain reaction (PCR) using oligonucleotide primers (forward: 5′-TAA GCT TGG CAC GGC TGT CCA AGG A-3′ and reverse: 5′-ACA GAA TTC GCC CCG GCC TGG TAC ACT GCC-3′). In addition to the buffer and nucleotide components (New England Biolabs), each amplification reaction contained 200 ng of leukocyte DNA, 40 pmol/L of each primer, 50 mM $MgCl_2$, and 0.25 µL *Taq* DNA polymerase (Eijkman Institute for Molecular Biology) in a final volume of 50 µL. Each reaction mixture was heated at 95°C for 5 min for denaturation, and subjected to two phases of amplification. The first phase of amplification consisted of 10 cycles of denaturation (95°C for 1 min), annealing (66°C for 30 s), and extension (72°C for 1 min). The second phase of amplification consisted of 25 cycles of denaturation (95°C for 1 min), annealing (60°C for 30 s) and extension (72°C for 1 min), followed by a final extension step (72°C for 5 min). We obtained approximately 600 ng of amplified apo E sequences from each amplification reaction. PCR amplification products were then purified using the QIA Quick Gel Extraction Kit Protocol (QIAGEN). After the purification process, restriction fragment length polymorphism (RFLP) was performed by adding 5 units of *Hha*I (New England Biolabs) directly to each reaction mixture for digestion of apo E sequences (120 min at 37°C). Each reaction mixture was loaded onto a 4% agarose gel treated with ethidium bromide (10 mg/mL), and then electrophoresed for 102 min under constant current (80 V). After electrophoresis, DNA fragments were visualized by ultraviolet illumination. The sizes of *Hha*I fragments were estimated by comparison with a low-molecular weight DNA ladder (New England Biolabs). A representative result of PCR-RFLP for apo E is shown in Figure 1.

Q6: Please supply manufacturer's name, city, state, country for all reagents, devices, software mentioned throughout the paper.

Ethical approval was obtained from The Ethics Committee of the Faculty of Medicine, University of Indonesia. Data were analyzed with SPSS version 17.0 using univariate and bivariate analyses (paired t-test, unpaired t-test, Wilcoxon, and Mann-Whitney).

Results

Of the 224 subjects recruited, 86 followed the blood sampling procedures and two school authorities refused to participate in the study. Among the 86 subjects, 76 (88.4%) subjects had dyslipidemia and 10 (11.6%) did not have dyslipidemia. Two out of the 76 dyslipidemic subjects also had increased levels of fasting blood glucose. Ultimately, a total of 60 of the 74 subjects completed the whole study.

Among the 60 subjects, 43 (71.7%) were male and 17 (28.3%) were female. The mean (±SD) age of the subjects was 15.63 (±2.31) years. A total of 47 (78.3%) subjects adhered to the physical exercise program, and 12 (20%) complied with the NCEP step II diet. All subjects who complied with the NCEP step II diet also adhered to the physical exercise program. Of the 48 subjects who showed non-compliance with the NCEP step II diet, 35 showed physical exercise adherence and 13 showed non-adherence to the physical exercise program (Table 1).

The apo E2/E2, E2/E3, E2/E4, E3/E3, E3/E4, and E4/E4 genotypes were found with frequencies of 0 (0%),

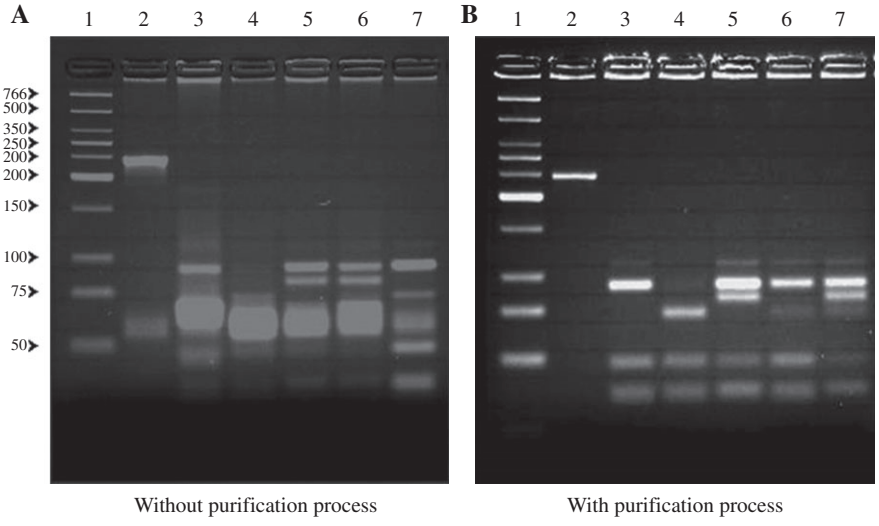

Figure 1 Polymerase chain reaction-restriction fragment length polymophism (PCR-RFLP) of apolipoprotein E.
(A) PCR-RFLP of apo E gene without purification process shows a dimer (55 bp). (B) PCR-RFLP of apo E gene with purification process show no dimer and fragment of 38 38, 48, 72, 83, and 81 base pairs (bp). Lane 1: marker, lane 2: control, lane 3: apo E3/E3, lane 4: apo E4/E4, lane 5: apo E2/E3, lane 6: apo E3/E4, lane 7: apo E2/E4.

Table 1 Subjects' characteristics.

Characteristic	Mean (SD)	n (%)
Sex (n=60)		
Male		43 (71.7)
Female		17 (28.3)
Age, years (n=60)	15.63 (2.31)	
Physical exercise adherence (n=60)		
Yes		47 (78.3)
No		13 (21.7)
Compliance with NCEP step II diet (n=60)		
Yes		12 (20)
No		48 (80)
Compliance with NCEP step II diet (n=12)		
Physical exercise adherence		12 (100)
Non-physical exercise adherence		0 (0)
Non-compliance of NCEP step II diet (n=48)		
Physical exercise adherence		35 (72.9)
Non-physical exercise adherence		13 (27.1)

SD, standard deviation.

Table 2 Dyslipidemia profile improvement after physical exercise and NCEP step II diet interventions based on apo E genotype.

Dyslipidemia improvement	E2/E2 n=0	E2/E3 n=3	E2/E4 n=1	E3/E3 n=44	E3/E4 n=9	E4/E4 n=3	Total n (%)
Total cholesterol							
Yes	0	3	1	39	6	2	51 (85)
No	0	0	0	5	3	1	9 (15)
Triglycerides							
Yes	0	2	0	23	3	0	28 (46.7)
No	0	1	1	21	6	3	32 (53.3)
LDL-C							
Yes	0	3	1	30	4	2	40 (66.7)
No	0	0	0	14	5	1	20 (33.3)
HDL-C							
Yes	0	0	0	8	3	0	11 (18.3)
No	0	3	1	36	6	3	49 (81.7)

Table 3 Effects of genotype, physical exercise, and NCEP step II diet on changes in the mean level of blood lipids.

Factor	Mean level changes (SD), mg/dL	Mean level changes (SD), mg/dL	p-Value
Apo E genotype	E3/E3 (n=44)	Non-E3/E3 (n=16)	
Total cholesterol[a]	16.5 (22.4)	17.1 (19.9)	0.917
Triglycerides[a]	14.6 (42.8)	5.7 (26.4)[b]	0.697
LDL-C[a]	12.3 (19.5)	16.6 (17.4)	0.440
HDL-C[a]	7.1 (5.0)	7.4 (4.6)	0.831
Physical exercise adherence	Yes (n=47)	No (n=13)	
Total cholesterol[a]	16.4 (22.4)	17.8 (19.4)	0.833
Triglycerides[c]	10.6 (34.7)	4.1 (56.3)	0.881
LDL-C[a]	12.8 (19.0)	15.5 (19.2)	0.649
HDL-C[a]	7.2 (4.9)	7.1 (4.9)	0.952
Compliance of NCEP step II diet	Yes (n=12)	No (n=48)	
Total cholesterol[a]	17.3 (25.2)	16.5 (20.9)	0.902
Triglycerides[a]	12.6 (35.1)	8.4 (41.3)	0.362
LDL-C[a]	15.1 (21.0)	13.0 (18.6)	0.734
HDL-C[a]	5.5 (3.9)	7.6 (5.0)	0.191

[a]independent t-test; [b]increase; [c]Mann-Whitney test. SD, standard deviation. Mean changes were calculated as: blood lipid level post-intervention – pre-intervention.

3 (5%), 1 (1.7%), 44 (73.3%), 9 (15%) and 3 (5%), respectively. The allele frequencies of apo E2, E3, and E4 were 0.033 (3.3%), 0.833 (83.3%) and 0.133 (13.3%), respectively. indicating that the three alleles are not equally distributed (χ^2=76.1, p<0.001. The dyslipidemia improvement profile after physical exercise and NCEP step II diet stratified by apo E genotype is shown in Table 2. Improvement of total cholesterol, triglycerides, LDL-C, and HDL-C levels after receiving the interventions was found in 51 (85%), 28 (46.7%), 40 (66.7%) and 11 (18.3%) subjects, respectively. Table 3 shows that apo E genotype, physical exercise adherence, and compliance with the NCEP step II diet did not play a role in the mean changes in blood lipid profiles after interventions (p>0.05). Meanwhile, physical exercise and NCEP step II diet did influence the mean blood lipid levels depending on apo E allele, as shown in Table 4.

Discussions

The improvement of total cholesterol and LDL-C levels found in this study following diet and exercise intervention were also reported in previous studies (25–32), even though the duration of the current study was relatively shorter at only 28 days. This study showed that management of dyslipidemia is very difficult; only 12 subjects complied to both the physical exercise and NCEP Step II diet programs during the study, even though diet and exercise facilities were provided (Table 1). The difficulty of dyslipidemia management and the high proportion of adolescents with dyslipidemia encountered in this study illustrate the low level of understanding and importance of dyslipidemia in adolescents, which can be a trigger of atherosclerosis development in adulthood. This situation

Table 4 Influences of physical exercise and NCEP step II diet interventions on the mean levels of blood lipids based on the apo E allele.

Apo E allele	Mean (SD) levels before interventions, mg/dL	Mean (SD) levels after interventions, mg/dL	p-Value
Apo E2 allele (n=4)			
Total cholesterol[a]	129.3 (19.1)	119.5 (16.0)	0.203
Triglycerides[a]	83.8 (15.4)	90.5 (18.0)	0.692
LDL-C[a]	84.5 (18.9)	73.0 (18.6)	0.114
HDL-C[a]	38.5 (6.4)	35.5 (3.8)	0.134
Apo E3 allele (n=56)			
Total cholesterol[a]	175.4 (30.3)	159.6 (24.7)	0.000
Triglycerides[b]	119.6 (57.0)	109.2 (56.8)	0.047
LDL-C[a]	122.5 (25.2)	110.0 (21.0)	0.000
HDL-C[a]	45.9 (9.8)	38.8 (8.3)	0.000
Apo E4 allele (n=13)			
Total cholesterol[a]	191.6 (29.8)	171.5 (29.1)	0.005
Triglycerides[a]	113.2 (54.7)	121.9 (53.2)	0.258
LDL-C[a]	136.5 (30.9)	117.7 (29.5)	0.003
HDL-C[a]	50.9 (5.5)	41.9 (4.8)	0.000

[a]Dependent t-test, [b]Wilcoxon test. SD, standard deviation.

increases the difficulties that clinicians will face in managing dyslipidemia in obese adolescents.

In addition, we found that improvements in total cholesterol and LDL-C levels could occur in a relatively short period of time (28 days) if intervention with the NCEP Step II diet and physical exercise is conducted as soon as possible. This considerably shortens the duration of dyslipidemia management compared with the current algorithm (33, 34). Current algorithms emphasize that the time interval needed to effectively evaluate the blood lipid profile is at least 3 months. For example, blood lipid-lowering drugs will not be provided until a dyslipidemic obese adolescent shows an LDL-C level maintained at ≥130 mg/dL for at least 6 months. In the present study, more than 50% of the subjects did not show improvement in HDL-C and triglyceride levels, which may have been due to the short duration of intervention in this study (28 days). Hence, longer interventions might be needed to induce changes in triglycerides and HDL-C. Leon and Sanchez (35) found that regular physical exercise must be carried out for at least 8 weeks in order to observe increases in HDL-C levels. Furthermore, maintained physical exercise is required to induce significant reductions in triglycerides (36). Finally, we did not find an effect of specific apo E genotypes on improving lipid profile levels, because the apo E3/E3 genotype was most common in subjects with both improved and unimproved lipid profiles (Table 2).

It was not possible to conduct multivariate analyses to determine the role of factors or combination of factors that contribute to the changes of lipid profile levels, because the data did not follow the normal distribution required for linear analyses. Therefore, we could not derive a quantitative model to predict the changes in lipid profile levels (Table 3). This may have been due to the fact that the distributions of each apo E genotype, physical exercise adherence, and compliance with the NCEP step II diet were not evenly spread across subjects. Therefore, in future studies, a larger and more diverse study population is required to confirm the relationships among these factors.

Nevertheless, the results of this study suggested that the apo E allele might have played a role in determining the extent of lipid profile improvements in dyslipidemic obese adolescents who received physical exercise and NCEP step II diet interventions for 28 days (Table 4). The exercise and diet interventions did not influence improvements in total cholesterol, triglycerides, and LDL-C levels in subjects with the apo E2 allele. By contrast, total cholesterol, triglycerides, and LDL-C levels were improved following the interventions in subjects with the apo E3 allele. Finally, subjects with the E4 allele showed a mixed response; total cholesterol and LDL-C levels improved, but there was no change in triglyceride levels following diet and exercise interventions.

Based on these results, we propose an algorithm for dyslipidemia management in obese adolescents as shown in Figure 2. In this algorithm, interventions are given for 28 days to dyslipidemic obese adolescents who show increased levels of total cholesterol, triglycerides, or LDL-C. The subject receives 60 min of physical exercise consisting of aerobic activity and muscle and bone strengthening under supervision of a trainer three times in a week, and an NCEP step II diet consisting of total fat content ≤30% of total calories, saturated fatty acids <7% of total daily calories, and <200 mg cholesterol/day. The interventions should be continued if blood lipid profiles improve, such as reductions in total cholesterol, triglycerides, and LDL-C levels. If no such improvements are evident, apo E genotyping should be performed. If the subject carries the apo E2 allele, the interventions should be continued and blood lipid-lowering drugs must be administered. If the subject has the apo E4 allele, the triglycerides level should be closely monitored, and interventions should be continued if the subject shows a reduction in triglycerides level, whereas if triglyceride levels are increased, blood lipid-lowering drugs should be introduced while continuing the diet and exercise programs.

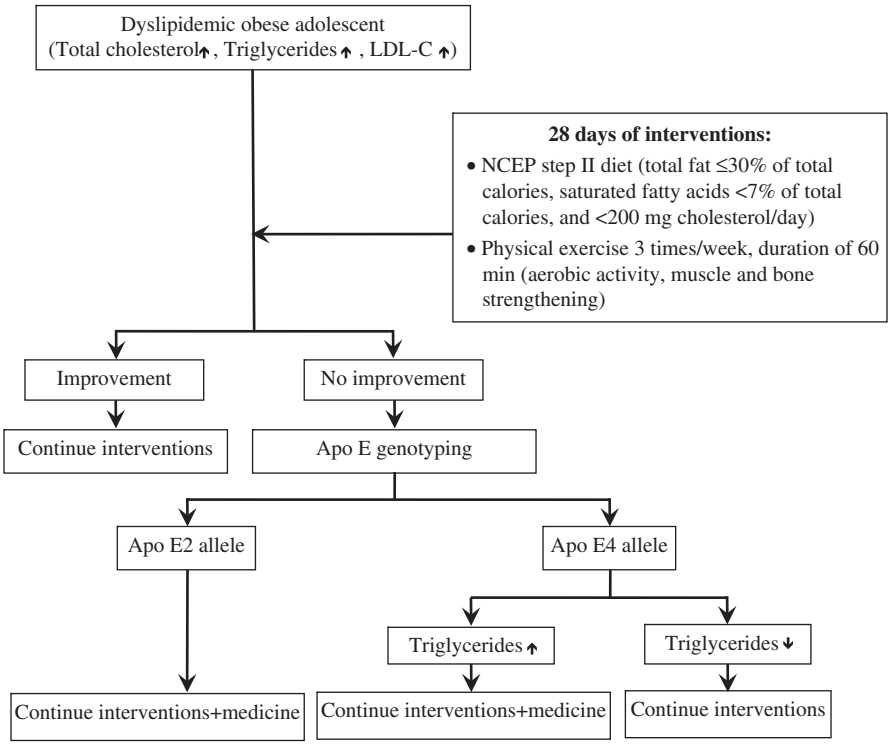

Figure 2 Algorithm of dyslipidemia management in obese adolescents.

Conclusion and recommendations

Together, these results suggest a new model for clinical dyslipidemia management in obese adolescents. Treatment with blood lipid-lowering drugs should be initiated when dyslipidemia persists in obese adolescents who have received physical exercise and NCEP step II diet interventions for at least 28 days. Further research is needed to determine the effects of blood lipid-lowering drugs with respect to different apo E alleles in obese adolescents whose dyslipidemia does not improve even after 28 days of diet and exercise interventions. Therefore, we strongly suggest that parents and school authorities continuously monitor subjects who show improved blood lipid profiles until they reach a BMI that is appropriate for their age. To accomplish this, the role of the family and school authorities involved in student health (unit kesehatan sekolah/UKS) should be increased, in order to help detect and prevent the occurrence of obesity at an early stage.

Furthermore, governments are urged to participate in reducing the prevalence of obesity by (a) increasing programs that encourage physical exercise and reducing excessive calorie intake in obese children, and (b) by developing a specific program based on the continuum of care concept, such as providing facilities for physical exercise, creating an educational curriculum that includes a sufficient component of physical education, restricting the promotion of junk food in the media, labeling the nutrient ingredients in each food product, and promoting healthy lifestyles that include regular physical exercise and a diet composed of balanced nutrients.

References

1. US Preventive Services Task Force. Screening for lipid disorders in children: US Preventive Services Task Force Recommendation Statement. Pediatrics 2007;120:e215–9.
2. Garg A, Simha V. Update on dyslipidemia. J Clin Endocrinol Metab 2007;92:1581–9.
3. Stamford BA, Moffatt RJ. Lipids and health: past, present, and future. In: Moffatt RJ, Stamford B, editors. Lipid metabolism and health. Boca Raton: Taylor and Francis Group, 2006:1–11.
4. Franklin BA, Durstine JL, Roberts CK, Barnard RJ. Impact of diet and exercise on lipid management in the modern era. Best Pract Res Cl En 2014:28:405–21.
5. McGill HC Jr, McMahan CA, Gidding SS. Preventing heart disease in the 21st century: implications of the pathobiological determinants of the atherosclerosis in youth (PDAY) study. Circulation 2008;117:1216–27.
6. Hariyanto D, Madiyono B, Sjarif DR, Sastroasmoro S. Hubungan ketebalan tunika intima media arteri carotis dengan obesitas pada remaja. Sari Pediatri 2009;11:159–66.

7. Cesar L, Suarez SV, Adi J, Adi N, Vazquez-Padron R, et al. An essential role for diet in exercise-mediated protection against dyslipidemia, inflammation and atherosclerosis in ApoE$^{-/-}$ mice. PLoS One 2011;6:e17263.
8. Walden CC, Hegele RA. Apolipoprotein E in hyperlipidemia. Ann Intern Med 1994;120:1026–36.
9. Ye SQ, Kwiterovich PO. Influence of genetic polymorphisms on responsiveness to dietary fat and cholesterol. Am J Clin Nutr 2000;72(Suppl):1275S–84S.
10. Elliott R, Ong TJ. Nutritional genomics. Brit Med J 2002;324:1438–42.
11. Haan MN, Mayeda ER. Apolipoprotein E genotype and cardiovascular diseases in the elderly. Curr Cardio Risk Rep 2010;4:361–8.
12. Shin MH, Choi JS, Rhee JA, Lee YH, Nam HS, et al. APOE polymorphism and carotid atherosclerosis in Korean population: the Dong-gu Study and the Namwon Study. Atherosclerosis 2014;232:180–5.
13. Ariza MJ, Sanchez-Chaparro MA, Barón FJ, Hornos AM, Calvo-Bonacho E, et al. Additive effects of LPL, APOA5 and APOE variant combinations on trygliceride levels and hypertriglyceride level and hypertriglyceridemia: results of the ICARIA genetic sub-study. BMC Med Genet 2010;11:66.
14. Povel CM, Boer JM, Imholz S, Dollé ME, Feskens EJ. Genetic variants in lipid metabolism are independently associated with multiple features of the metabolic syndrome. Lipids Health Dis 2011;10:118.
15. Boulenouar H, Benchekor SM, Meroufel DN, Hetraf SA, Djellouli HO, et al. Impact of APOE gene polymorphisms on the lipid profile in an Algerian population. Lipids Health Dis 2013;12:155.
16. Biswas S, Ghoshal PK, Halder B, Ganguly K, DasBiswas A, et al. Apolipoproteins AI/B/E gene polymorphism and their plasma levels in patients with coronary artery disease in a tertiary care-center of Eastern India. Indian Heart J 2013;65:658–65.
17. Chaudhary R, Likidlilid A, Peerapatdit T, Tresukosol D, Srisuma S, et al. Apolipoprotein E gene polymorphism: effects on plasma lipids and risk of type 2 diabetes and coronary artery disease. Cardiovasc Diabetol. 2012;11:36.
18. Hagberg JM, Wilund KR, Ferrell RE. APO E gene and gene-environment effects on plasma lipoprotein-lipid levels. Physiol Genomics 2000;4:101–8.
19. Taimela S, Lehtimaki T, Porkka K, Rasanen L, Viikari J. The effect of physical activity on serum total and low-density lipoprotein cholesterol concentration varies with apolipoprotein E phenotype in male children and young adults: the cardiovascular risk in young Finns study. Metabolism 1996;45:797–803.
20. St.-Amand J, Prud'Homme D, Moorjani S, Nadeau A, Tremblay A, et al. Apolipoprotein E polymorphism and the relationships of physical fitness to plasma lipoprotein-lipid levels in men and women. Med Sci Sports Exerc 1999;31:692–7.
21. Ferreira AP, Ferreira CB, Brito CJ, Souza VC, Córdova C, et al. The effect of aerobic exercise intensity on attenuation of post-prandial lipemia is dependent on apolipoprotein E genotype. Atherosclerosis 2013;229:139–44.
22. Paula RS, Souza VC, Benedet AL, Souza ER, Toledo JO, et al. Dietary fat and apolipoprotein genotypes modulate plasma lipoprotein levels in Brazilian elderly women. Mol Cell Biochem 2010;337:307–15.
23. Barlow SE and the Expert Committee. Expert committee recommendations regarding the prevention, assessment, and treatment of child and adolescent overweight and obesity: summary report. Pediatrics 2007;120:S164–92.
24. Expert Panel on Integrated Guidelines for Cardiovascular Health and Risk Reduction in Children and Adolescents. Expert Panel on integrated guidelines for cardiovascular health and risk reduction in children and adolescents: summary report. Pediatrics 2011;128(Suppl):213S–56S.
25. Yu-Poth S, Zhao G, Etherton T, Naglak M, Jonnalagadda S, et al. Effects of the National Cholesterol Education Program's step I and step II dietary intervention programs on cardiovascular disease risk factors: a meta-analysis. Am J Clin Nutr 1999;69:632–46.
26. Stefanick ML, Mackey S, Sheehan M, Ellsworth N, Haskell WL, et al. Effects of diet and exercise in men and postmenopausal women with low levels of HDL cholesterol and high levels of LDL cholesterol. N Eng J Med 1998;339:12–20.
27. Varady KA, Jones PJ. Combination diet and exercise interventions for the treatment of dyslipidemia: an effective preliminary strategy to lower cholesterol levels? J Nutr 2005;135:1829–35.
28. Miller ER, Erlinger TP, Young DR, Jehn M, Charleston J, et al. Results of the diet, exercise, and weight loss intervention trial (DEW-IT). Hypertension 2002;40:612–8.
29. Andersen RE, Wadden TA, Bartlett SJ, Zemel B, Verde TJ, et al. Effects of lifestyle activity vs structured aerobic exercise in obese women. J Am Med Assoc 1999;281:335–40.
30. Wilund KR, Ferrell RE, Phares DA, Goldberg AP, Hadberg JM. Changes in high-density lipoprotein-cholesterol subfractions with exercise training may be dependent on cholesteryl ester transfer protein (CETP) genotype. Metabolism 2002;51:774–8 (Abstract).
31. Williams PT, Krauss RM, Stefanick ML, Vranizan KM, Wood PD. Effects of low-fat diet, calorie restriction, and running on lipoprotein subfraction concentracions in moderately overweight men. Metabolism 1994;43:655–63.
32. Niebauer J, Hambrecht R, Velich T, Hauer K, Marburger C, et al. Attenuated progression of coronary artery disease after 6 years of multifactorial risk intervention: role of physical exercise. Circulation 1997;96:2534–41.
33. NCEP expert panel on blood cholesterol levels in children and adolescents. National Cholesterol Education Program (NCEP): highlights of the report of the expert panel on blood cholesterol levels in children and adolescents. Pediatrics 1992;89:495–501.
34. Holmes KW, Kwiterovich PO. Treatment of dyslipidemia in children and adolescents. Curr Cardiol Rep 2005;7:445–56.
35. Leon AS, Sanchez OA. Response of blood lipids to exercise training alone or combined with dietary intervention. Med Sci Sports Exerc 2001;33:502–15.
36. Angelopoulos TJ. Obesity, lipoproteins, and exercise. In: Dalam: Moffatt RJ, Stamford B, editors. Lipid metabolism and health. Boca Raton: Taylor and Francis Group, 2006:173–82.

Q7: As per journal style 5 author et al. only allowed. Please check and confirm 5 author et al. for ref. 20

Q8: Please confirm the ref. 36