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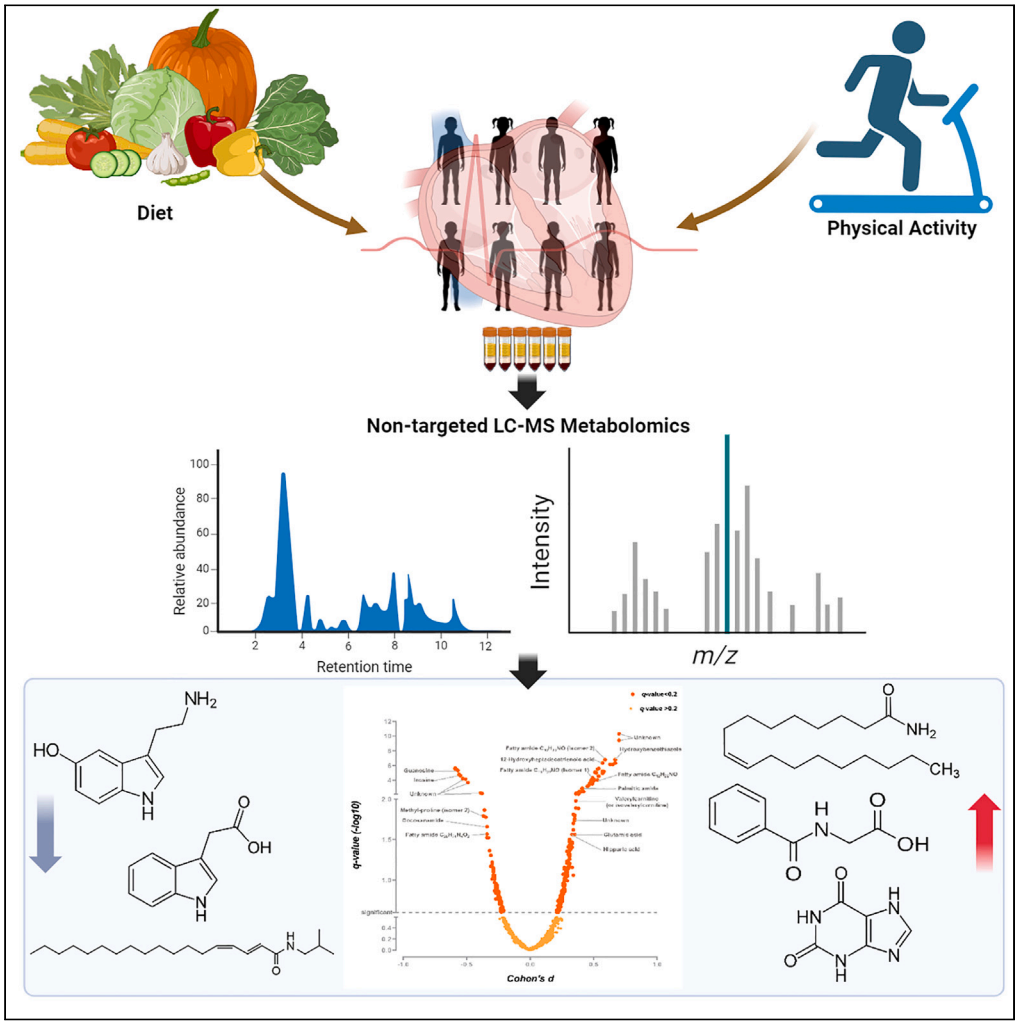

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Highlights

PANIC reveals the impact of lifestyle interventions on childhood serum metabolites

Pinpoints affected fatty amides and microbiota-derived metabolites metabolic pathways

PANIC tracks longitudinal changes over 8 years for comprehensive analysis

Early interventions can curb metabolic risks and prevent cardiometabolic diseases

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Eight-year diet and physical activity intervention affects serum metabolites during childhood and adolescence: A nonrandomized controlled trial

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SUMMARY

Long-term lifestyle interventions in childhood and adolescence can significantly improve cardiometabolic health, but the underlying molecular mechanisms remain poorly understood. To address this knowledge gap, we conducted an 8-year diet and physical activity intervention in a general population of children. The research revealed that the intervention influenced 80 serum metabolites over two years, with 17 metabolites continuing to be affected after eight years. The intervention primarily impacted fatty amides, including palmitic amide, linoleamide, oleamide, and others, as well as unsaturated fatty acids, acylcarnitines, phospholipids, sterols, gut microbiota-derived metabolites, amino acids, and purine metabolites. Particularly noteworthy were the pronounced changes in serum fatty amides. These serum metabolite alterations could represent molecular mechanisms responsible for the observed benefits of long-term lifestyle interventions on cardiometabolic and overall health since childhood. Understanding these metabolic changes may provide valuable insights into the prevention of cardiometabolic and other non-communicable diseases since childhood.

INTRODUCTION

The alarmingly high and rapidly increasing prevalence of childhood overweight and associated cardiometabolic risk factors is an important clinical, public health, and societal problem worldwide.[1–3] A major concern is that overweight and other cardiometabolic risk factors worsen since childhood and markedly increase the risk of cardiometabolic diseases in adulthood.[4–8] Pathophysiological processes underlying the development of cardiometabolic diseases begin in childhood or even during the fetal period[1,2,9] that emphasizes the need for preventing these diseases since childhood.[1,9–12]

Long-term diet and physical activity interventions have been found to decrease adiposity,[13,14] reduce insulin resistance,[15,16] and improve blood lipids[17,18] in general populations of children. However, molecular mechanisms underlying the beneficial effects of lifestyle interventions on cardiometabolic health during childhood and adolescence remain largely unknown. A nontargeted liquid chromatography-mass spectrometry (LC-MS) metabolomics analysis of blood samples is a sensitive, high-throughput method to detect endogenous metabolites and exogenous compounds[19] that can be used to reveal molecular mechanisms of the beneficial effects of lifestyle interventions on cardiometabolic health in all age groups. Diet and physical activity interventions using the nontargeted LC-MS metabolomics analysis have been shown to affect numerous blood metabolites among adults.[20–22] Part of these metabolites, such as indolepropionic acid and hippuric acid, have

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been proposed as molecular mechanisms underlying the beneficial effects of lifestyle interventions on glucose metabolism and the risk of developing type 2 diabetes.[20–22]

There are no reports on the effects of long-term lifestyle interventions on blood metabolites using the nontargeted LC-MS metabolomics analysis in general populations of children followed up until adolescence. Such scientific evidence would increase our understanding of molecular mechanisms underlying the beneficial effects of lifestyle interventions on cardiometabolic and overall health during childhood and adolescence and would thereby provide valuable insights into the prevention of cardiometabolic and other non-communicable diseases since childhood. We therefore studied for the first time the effects of a long-term diet and physical activity intervention on a wide spectrum of serum metabolites using the nontargeted LC-MS metabolomics analysis[19] in a population sample of children followed up for eight years until adolescence.

RESULTS

Baseline characteristics of children

There were no differences in baseline characteristics between the intervention group and the control group, except that the children in the intervention group consumed less high-fat milk and more low-fat milk than children in the control group (Table 1). Additionally, 98% of these children were Caucasian and 2% were Non-Caucasian.

Effects of diet and physical activity intervention on serum metabolites

The intervention had statistically significant effects on 315 molecular features over the first two years (Figure 1), and among them, 80 metabolites were annotated (Table S1). Of these 80 metabolites, 17 were affected by the intervention over two and eight years, 59 only over two years, and three only over eight years (Table 2).

The intervention had effects on 23 fatty amides (11 known, 12 unnamed) over two years (Table 2; Figure 2). Palmitic amide, linoleamide, oleamide, elaidamide, capsiamide, and myristamide decreased less in the intervention group than in the control group; palmitoleamide did not change in the intervention group but decreased in the control group; and docosanamide, erucamide, N-undecanoylglycine, and pipericine decreased in the intervention group but increased in the control group (Figure 2). The differences in all these known fatty amides, except N-undecanoylglycine, between the groups persisted until eight years (Figure 2), although the intervention effect on only myristamide remained statistically significant until then (Table 2).

The intervention affected five unsaturated fatty acids (four known, one unnamed) over the first two years (Table 2; Figure 3). 12-hydroxyheptadecatrienoic acid and hydroxyeicosatetraenoic acid decreased less in the intervention group than in the control group; hydroxyoxohexadecanoic acid increased more in the intervention group than in the control group; and oxotetradecenoic acid decreased in the intervention group but increased in the control group (Figure 3). The differences in all these known fatty acids between the groups remained until eight years (Figure 3), although the intervention effect on only 12-hydroxyheptadecatrienoic acid persisted statistically significant until then (Table 2).

The intervention had effects on four acylcarnitines (three known, one unnamed) over the first two years (Table 2; Figure 4). Octanoyl-L-carnitine, decatrienoylcarnitine, and valerylcarnitine (or isovalerylcarnitine) increased in the intervention group and decreased in the control group (Figure 4). The differences in octanoyl-L-carnitine and decatrienoylcarnitine between the groups persisted until eight years (Figure 4), although the intervention effect on only octanoyl-L-carnitine remained statistically significant until then (Table 2).

The intervention affected nine phospholipids (all nine known) over the first two years (Table 2; Figure 5). Lysophosphatidylcholine(16:0), lysophosphatidylcholine(18:2), lysophosphatidylcholine(20:2), lysophosphatidylcholine(22:5), phosphatidylcholine(38:4), and lysophosphatidylethanolamine(18:1) increased more in the intervention group than in the control group; lysophosphatidylethanolamine(16:0) decreased less in the intervention group than in the control group; and lysophosphatidylcholine(O-18:0) and phosphatidylinositol(25:0) decreased more in the intervention group than in the control group; (Figure 5). The differences in lysophosphatidylcholine(16:0), lysophosphatidylcholine(18:2), phosphatidylcholine(38:4), lysophosphatidylcholine(O-18:0), and phosphatidylinositol(25:0) between the groups remained until eight years (Figure 5), although the intervention effect on only lysophosphatidylcholine(16:0) persisted statistically significant until then (Table 2).

The intervention had effects on three sterols (two known, one unnamed) over the first two years (Table 2; Figure 5). Dehydroepiandrosterone sulfate (or epitestosterone sulfate or testosterone sulfate) and etiocholanolone sulfate (or epiandrosterone sulfate or androsterone sulfate) increased slightly less in the intervention group than in the control group (Figure 5). The differences in dehydroepiandrosterone sulfate and etiocholanolone sulfate between the groups persisted until eight years (Figure 5), although the intervention effects on these sterols did not remain statistically significant until then (Table 2).

The intervention had effects on eight gut-microbiota-derived metabolites (all eight known) over the first two years (Table 2; Figure 6). Hydroxyferulic acid, hippuric acid, indolepropionic acid, and pyrocatechol sulfate did not change in the intervention group but decreased in the control group; 3-carboxy-4-methyl-5-pentyl-2-furanpropanoic acid did not change in the intervention group but increased in the control group; *p*-cresol sulfate increased in the intervention group but decreased in the control group; indolelactic acid increased less in the intervention group than in the control group; and 3,4-dimethyl-5-pentyl-2-furanpropanoic acid decreased in the intervention group but increased in the control group (Figure 6). The differences in hydroxyferulic acid, hippuric acid, pyrocatechol sulfate, 3-carboxy-4-methyl-5-pentyl-2-furanpropanoic acid, indolelactic acid, and 3,4-dimethyl-5-pentyl-2-furanpropanoic acid between the groups remained until eight years (Figure 6), although the intervention effects on only hydroxyferulic acid, hippuric acid, and pyrocatechol sulfate persisted statistically significant until then (Table 2).

Table 1. Baseline characteristics of children in the intervention group and the control group

	Intervention group (*N* = 296)	Control group (*N* = 194)	*p* value
Sex			0.612
Boys, *n* (%)	158 (53.4)	99 (51.0)	
Girls, *n* (%)	138 (46.6)	95 (49.0)	
Age, years	7.6 (0.4)	7.6 (0.4)	0.329
Body weight, kg	26.8 (4.7)	26.7 (5.3)	0.788
Body height, cm	128.8 (5.5)	128.5 (5.9)	0.506
Body height-SDS	0.13 (0.99)	0.11 (1.04)	0.843
BMI-SDS	−0.19 (1.04)	−0.22 (1.11)	0.717
Body weight status, *n* (%)			0.886
Normal weight	259 (87.5)	172 (88.7)	
Overweight	27 (9.1)	12 (6.2)	
Obesity	10 (3.4)	10 (5.1)	
Food consumption, g/day			
Vegetables, fruit, and berries	204 (115)	221 (119)	0.142
High-fiber (≥5%) grain products[a]	63 (39)	63 (40)	0.862
Low-fiber (<5%) grain products[b]	113 (54)	115 (51)	0.620
High-fat (60–80%) vegetable oil-based spreads	6.9 (7.4)	7.6 (8.5)	0.393
Vegetable oils	4.3 (4.5)	3.8 (3.9)	0.283
Butter and butter-based spreads	5.7 (6.9)	6.2 (7.2)	0.447
High-fat (≥1%) milk	167 (208)	220 (244)	0.025
Low-fat (<1%) milk	411 (288)	340 (288)	0.014
Red meat	56 (29)	58 (34)	0.486
Fish	15 (20)	16 (23)	0.587
Foods with high sugar content[c]	185 (136)	206 (146)	0.126
Physical activity energy expenditure, kJ × kg^{-1} × d^{-1}	101 (32)	96 (34)	0.076
Light physical activity, h/d	8.7 (1.8)	8.4 (1.8)	0.071
Moderate physical activity, h/d	1.6 (0.9)	1.5 (0.9)	0.162
Vigorous physical activity, h/d	0.4 (0.4)	0.4 (0.4)	0.716
Sedentary time, excluding sleep, h/day	3.7 (2.0)	4.0 (2.3)	0.362

The values are unadjusted means (standard deviations) and their *p* values from the independent samples t test for continuous variables and numbers (percentages) and their *p* values from the Chi-square test for categorical variables.

Data on sex, age, pubertal status, body weight, body height, body height-SDS, BMI-SDS, and body weight status were available for 296 children in the intervention group and for 194 children in the control group; on total physical activity energy expenditure for 272 children in the intervention group and for 180 children in the control group; on light, moderate, and vigorous physical activity for 258 children in the intervention group and for 167 children in the control group; on sedentary time for 256 children in the intervention group and for 167 children in the control group; and on dietary factors for 236 children in the intervention group and for 159 children in the control group.

BMI, body mass index; SDS, standard deviation score.

[a]Whole grain pasta, rice, and oatmeal.

[b]White pasta, rice, and flour.

[c]Sugar-sweetened beverages, fruit juice, candies, chocolate, added sugar, ice cream, puddings, pastries, and biscuits.

The intervention had effects on 11 amino acids (10 known, one unnamed) over the first two years (Table 2; Figure 7). Methoxybenzenepropanoic acid increased more in the intervention group than in the control group; glutamic acid did not change in the intervention group but decreased in the control group; taurine decreased less in the intervention group than in the control group; and hydroxyisoleucine increased in the intervention group but decreased in the control group (Figure 7). The difference in methoxybenzenepropanoic acid between the groups persisted until eight years (Figure 7), and the intervention effect on this amino acid remained statistically significant until then (Table 2).

The intervention affected four purine metabolites (all four known) over the first two years (Table 2; Figure 8). Guanosine and inosine decreased in the intervention group but increased in the control group, whereas xanthine and hypoxanthine decreased less in the intervention group than in the control group (Figure 8). The differences in guanosine and inosine between the groups persisted until eight years (Figure 8), and the intervention effects on these purine metabolites remained statistically significant until then (Table 2).

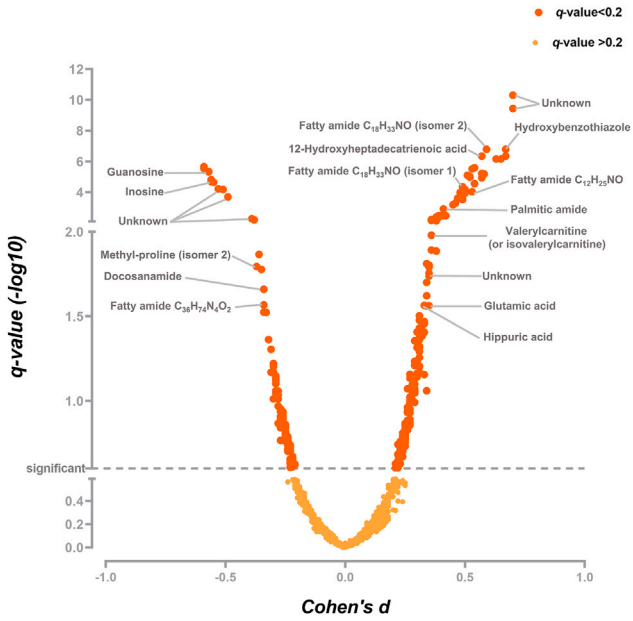

Figure 1. Effect of the diet and physical activity intervention on 315 serum molecular features over two years adjusted for age and sex, a positive value indicating a positive effect of the intervention and a negative value indicating a negative effect of the intervention

DISCUSSION

This 8-year non-randomized controlled trial in a general population of children showed that the combined diet and physical activity intervention had effects on numerous serum metabolites that could serve as molecular mechanisms underlying the beneficial impacts of lifestyle changes on cardiometabolic and overall health since childhood. The effects of the diet and physical activity intervention were most pronounced on serum fatty amides, but the intervention also affected other potentially important serum lipids, including fatty acids, acylcarnitines, phospholipids, and sterols, as well as serum gut-microbiota-derived metabolites, amino acids, and purine metabolites.

Our diet and physical activity intervention had effects on several serum fatty amides, which are bioactive lipid signaling molecules with a variety of physiological functions, including sleep induction, pain and anxiety control, eating behavior, weight control, angiogenesis, arterial dilation, as well as anti-inflammatory, neuroprotective, and anticonvulsive effects.[23,24] The functions of fatty amides in the human body depend on the length and degree of unsaturation of their acyl group.[23,24] While the exact mechanism of the biosynthesis of fatty amides remains elusive, it has been proposed that the conversion of fatty acids to their corresponding fatty amides could explain their biological activity.[25] Conversely, the degradation of fatty amides is regulated by fatty acid amide hydrolase,[26,27] which is known to hydrolyze fatty amides to their corresponding fatty acids and regulate several physiological processes in the human body.[26,27]

We observed that the diet and physical activity intervention attenuated the decrease of serum levels of several short-chain primary fatty amides with 16–18 carbons, including palmitic amide, linoleamide, oleamide, elaidamide, capsiamide, and myristamide, all of which are endogenously produced.[23,28] These findings suggest that our diet and physical activity intervention may have directly affected fatty amide metabolism by decelerating the activity of fatty acid amide hydrolase.[29] Since these fatty amides are also rich in vegetable oils,[24] the effects of our diet and physical activity intervention on the serum levels of these fatty amides could also be partly attributed to the increased consumption of vegetable oils in the intervention group.[17,30] Moreover, our intervention affected serum levels of unsaturated fatty acids by attenuating the decrease of hydroxyheptadecatrienoic acid and hydroxyeicosatetraenoic acid, increasing hydroxyoxohexadecanoic acid, and decreasing oxotetradecenoic acid. These results agree with our previous finding that the intervention increased the consumption of vegetable oil-based margarine in the same population of children.[30]

Our observation that the diet and physical activity intervention attenuated the decrease of serum levels of linoleamide is consistent with our earlier finding that the same intervention increased plasma levels of linoleic acid,[18] a potential precursor of linoleamide,[25] in the same pediatric population. Importantly, increased plasma linoleic acid has been negatively associated with the risk of coronary heart disease in prospective studies among adults.[31] Our intervention also attenuated the decrease of serum levels of hydroxyheptadecatrienoic acid, a fatty acid and a metabolite of arachidonic acid, which in turn can be derived from linoleic acid[32] and may play a role in blood clotting.[32] Moreover, our intervention attenuated the decrease of serum levels of palmitic amide, a potential product of palmitic acid. We have earlier observed that our intervention decreased plasma palmitic acid levels in the same population of children.[18] Importantly, increased plasma palmitic acid levels have been associated with increased plasma levels of LDL cholesterol, a major risk factor for coronary heart disease.[33] Our intervention also attenuated the decrease of serum levels of elaidamide, a potential product of elaidic acid. Increased plasma levels of trans fatty acids, such as elaidic acid, have been associated with increased plasma LDL cholesterol levels and systemic low-grade inflammation.[34] These observations

Table 2. Effects of diet and physical activity intervention on serum metabolites over two and eight years

	Over 2 years			Over 8 years		
Metabolite class and metabolite	Cohen's d value	*p* value	q value	Cohen's d value	*p* value	q value
Fatty amides						
Palmitic amide	0.42	**4.72E-05**	**0.003312**	0.27	0.086009	0.634335
Linoleamide	0.34	**0.000803**	**0.027487**	0.13	0.379400	0.791612
Oleamide	0.39	**4.95E-05**	**0.003312**	0.24	0.122020	0.65383
Elaidamide	0.39	**7.06E-05**	**0.004323**	0.26	0.091874	0.634335
Capsiamide	0.31	**0.001062**	**0.033384**	0.29	0.059682	0.604171
Myristamide	0.30	**0.001326**	**0.039121**	0.34	**0.033761**	0.549486
Palmitoleamide	0.29	**0.002388**	0.058345	0.30	0.059330	0.604171
Docosanamide (Also known as behenamide)	−0.34	**0.000575**	**0.021904**	−0.18	0.075756	0.63023
Erucamide	−0.23	**0.019299**	0.186055	−0.05	0.426662	0.808154
N-undecanoylglycine	−0.25	**0.014562**	0.164838	−0.11	0.402086	0.799341
Pipericine	−0.24	**0.014515**	0.164817	−0.10	0.240331	0.714769
$C_{12}H_{25}NO$ (Unnamed)	0.52	**4.03E-08**	**9.38E-06**	0.33	**0.006042**	0.279669
$C_{13}H_{25}NO_2$ (Unnamed)	−0.24	**0.014436**	0.164807	−0.22	0.103194	0.634335
$C_{14}H_{27}NO$ (Unnamed)	0.26	**0.006679**	0.109265	0.33	**0.044912**	0.584292
$C_{15}H_{29}NO_2$, isomer 1 (Unnamed)	−0.26	**0.009505**	0.132282	−0.23	0.082521	0.632374
$C_{15}H_{29}NO_2$, isomer 2 (Unnamed)	−0.25	**0.010547**	0.138994	−0.25	0.060768	0.6057
$C_{15}H_{31}NO$ (Unnamed)	0.22	**0.018300**	0.180992	0.31	0.057012	0.603923
$C_{17}H_{33}NO$ (Unnamed)	0.28	**0.003676**	0.074847	0.31	**0.049202**	0.587594
$C_{18}H_{33}NO$, isomer 1 (Unnamed)	0.59	**2.02E-10**	**1.61E-07**	−0.13	0.333456	0.771216
$C_{18}H_{33}NO$, isomer 2 (Unnamed)	0.50	**4.6E-07**	**6.57E-05**	−0.29	**0.030991**	0.53453
$C_{20}H_{41}NO$ (Unnamed)	−0.26	**0.009002**	0.128372	−0.10	0.275258	0.735353
$C_{24}H_{47}NO$ (Unnamed)	−0.21	**0.029716**	0.236586	−0.02	0.594806	0.876836
$C_{36}H_{74}N_4O_2$ (Unnamed)	−0.34	**0.000772**	**0.027077**	−0.05	0.323621	0.762359
Fatty acids						
12-Hydroxyheptadecatrienoic acid	0.53	**9.16E-09**	3.02E-06	0.52	**5.01E-05**	**0.016065**
Hydroxyeicosatetraenoic acid	0.22	**0.023159**	0.206594	0.17	0.271163	0.731985
Hydroxyoxohexadecanoic acid	0.25	**0.010546**	0.138994	0.15	0.151161	0.681356
Oxotetradecenoic acid	−0.30	**0.002942**	0.067571	−0.21	0.061733	0.607302
$C_{23}H_{32}O_3$ (Unnamed)	0.50	**8.99E-07**	**0.000113**	0.43	**0.000895**	**0.092643**
Acylcarnitines						
Octanoyl-L-carnitine	0.30	**0.003320**	0.072076	0.25	**0.048706**	0.587594
Decatrienoylcarnitine	0.27	**0.007073**	0.113189	0.20	0.128252	0.658773
Valerylcarnitine (or isovalerylcarnitine)	0.38	**0.000283**	**0.013002**	−0.21	0.102272	0.634335
$C_{18}H_{33}NO_4$ (Unnamed)	−0.29	**0.003261**	0.071125	0.02	0.977538	0.992813
Phospholipids						
Lysophosphatidylcholine (16:0/0:0)	0.26	**0.012110**	0.148712	0.29	**0.027531**	0.514723
Lysophosphatidylcholine (18:2/0:0)	0.22	**0.032452**	0.249031	0.13	0.406376	0.801997
Lysophosphatidylcholine (20:2)	0.25	**0.015628**	0.170259	0.05	0.952588	0.988958
Lysophosphatidylcholine (22:5)	0.25	**0.013664**	0.160372	0.00	0.640688	0.894772
Lysophosphatidylcholine (O-18:0)	−0.30	**0.005550**	0.097299	−0.08	0.402046	0.799341
Lysophosphatidylethanolamine (16:0/0:0)	0.22	**0.030137**	0.238254	0.12	0.335642	0.771644
Lysophosphatidylethanolamine (18:1/0:0)	0.24	**0.017305**	0.176342	0.07	0.625537	0.887964

(*Continued on next page*)

Table 2. *Continued*

Metabolite class and metabolite	Over 2 years			Over 8 years		
	Cohen's d value	*p* value	q value	Cohen's d value	*p* value	q value
Phosphatidylcholine (38:4)	0.24	**0.015696**	0.170793	0.19	0.147586	0.679035
Phosphatidylinositol (25:0)	−0.26	**0.008136**	0.121642	−0.13	0.094216	0.634335
Sterols						
Dehydroepiandrosterone sulfate, testosterone sulfate, or epitestosterone sulfate	−0.27	**0.008756**	0.125992	−0.11	0.211960	0.701084
Etiocholanolone sulfate, epiandrosterone sulfate, or androsterone sulfate	−0.27	**0.009499**	0.132282	−0.03	0.672607	0.908648
$C_{29}H_{46}O_2$ (Unnamed)	−0.29	**0.003472**	0.073541	−0.26	**0.023883**	0.49283
Gut-microbiota-derived metabolites						
Hydroxyferulic acid	0.38	**0.000135**	**0.007188**	0.60	**5.04E-05**	**0.016065**
Hippuric acid	0.33	**0.000788**	**0.027273**	0.37	**0.033028**	0.545479
Indolepropionic acid	0.31	**0.002692**	0.062599	0.10	0.459123	0.823494
Pyrocatechol sulfate	0.21	**0.032480**	0.24905	0.37	**0.011812**	0.390785
3-Carboxy-4-methyl-5-pentyl-2-furanpropanoic acid	−0.22	**0.027754**	0.228964	−0.15	0.0874	0.634335
p-Cresol sulfate	0.30	**0.001862**	**0.048341**	0.18	0.155299	0.683875
Indolelactic acid	−0.22	**0.031549**	0.245398	−0.06	0.209306	0.699386
3,4-Dimethyl-5-pentyl-2-furanpropanoic acid	−0.29	**0.004971**	0.090299	−0.17	0.081014	0.63023
Amino acids						
$C_7H_{13}NO_2S$ (Unnamed)	−0.36	**0.000305**	**0.013616**	−0.15	0.21435	0.701084
5-Hydroxyindoleacetaldehyde	0.22	**0.029389**	0.235165	0.20	0.18859	0.694012
Glutamic acid	0.35	**0.000795**	**0.027366**	0.10	0.56367	0.861788
Hydroxyisoleucine	0.29	**0.004938**	0.090025	0.04	0.769081	0.93531
L-Norleucine	−0.22	**0.030443**	0.239552	−0.03	0.527942	0.848295
Methoxybenzenepropanoic	0.38	**0.000137**	**0.00721**	0.24	**0.037849**	0.567732
N-(3-acetamidopropyl)pyrrolidin-2-one	−0.22	**0.025631**	0.218769	0.04	0.958781	0.989583
N-acetyl-lysine	−0.12	0.232552	0.592425	−0.19	**0.004607**	**0.249653**
Phenylacetylglutamine	0.29	**0.0026**	0.061644	0.10	0.500113	0.838859
Serotonin	−0.19	0.061489	0.335538	−0.19	**0.009611**	0.354282
Taurine	0.33	**0.001084**	**0.033942**	0.18	0.084998	0.634335
Purine metabolites						
Guanosine	−0.59	**5.5E-09**	**2.19E-06**	−0.44	**0.00012**	**0.029363**
Inosine	−0.56	**7.8E-08**	**1.55E-05**	−0.43	**0.000302**	**0.051571**
Xanthine	0.32	**0.001473**	**0.041526**	0.15	0.153691	0.683875
Hypoxanthine	0.25	**0.015245**	0.168189	0.07	0.515656	0.845764
Xenobiotics						
2-Hydroxybenzothiazole or 6-Hydroxybenzothiazole	0.63	**1.38E-09**	**6.94E-07**	0.57	**2.9E-05**	**0.012061**
1,3-Diphenylguanidine	0.15	0.114729	0.451451	0.54	**0.000238**	**0.046299**
Benzyl butyl phthalate	0.39	**6.17E-05**	**0.003881**	0.48	**0.000518**	**0.067477**
Dibenzylamine	0.23	**0.018643**	0.182216	0.04	0.892708	0.976289
Naproxen	0.24	**0.019356**	0.186417	0.28	0.090874	0.634335
Tranexamic acid	−0.27	**0.009478**	0.132282	−0.02	0.706889	0.919492
$C_{17}H_{18}O_5$ (Unnamed flavonoid)	−0.24	**0.014885**	0.166525	−0.01	0.718331	0.922387

(*Continued on next page*)

Table 2. *Continued*

Metabolite class and metabolite	Over 2 years			Over 8 years		
	Cohen's d value	*p* value	q value	Cohen's d value	*p* value	q value
Methylene di-*t*-butyl-cresol	0.57	**2.35E-08**	**6.07E-06**	0.17	0.13282	0.663576
$C_7H_6O_6S$ (Unnamed phenyl sulfate)	−0.28	**0.005105**	0.091921	−0.07	0.472807	0.8284
Betonicine	−0.23	**0.028809**	0.233449	−0.28	0.096245	0.634335
Hericerin	−0.29	**0.003615**	0.074285	−0.09	0.088536	0.634335
Methyl-proline, isomer 1	−0.37	**0.000385**	**0.016050**	−0.16	0.102746	0.634335
Methyl-proline, isomer 2	−0.26	**0.009957**	0.135748	−0.23	0.142268	0.67255

The data represent the effects of the diet and physical activity intervention on 80 serum metabolites over two years and over eight years, which were analyzed using the intention-to-treat principle and linear mixed-effects models adjusting for sex and age, including main effects for time and study group × time interaction, and assuming data missing at random. The effect sizes are indicated by Cohen's d values, a positive value indicating a positive effect of the intervention and a negative value indicating a negative effect of the intervention. The statistically significant effects of the intervention are indicated by bolded *p* values of <0.05. Bolded q values (false discovery rate corrected *p* values) of <0.05 are also presented to correct the results for a multiple testing error.

together suggest that the beneficial effects of our diet and physical activity intervention on cardiometabolic health, including the attenuated increase in insulin resistance[16] and the lowered plasma levels of LDL cholesterol,[17] in the same pediatric population could be partly mediated by changes in fatty amide and fatty acid metabolism. Furthermore, our intervention attenuated the decrease of serum levels of oleamide, which is consistent with a finding that a single bout of exercise was shown to increase oleamide concentrations in the skeletal muscle of rats.[35] Finally, gut-microbiota-produced fatty amides have been shown to mediate the gut-brain feedback loop related to motivation for exercise in mice.[36]

As mentioned previously, the functions of various fatty amides depend on the length and degree of unsaturation of their acyl group.[23,24] In contrast to the effects of our diet and physical activity intervention on serum short-chain primary fatty amides, serum levels of two long-chain primary fatty amides with 20–22 carbons, docosanamide and erucamide, decreased in the intervention group but increased in the control group. These long-chain fatty amides have not been found to be biosynthesized in the human body but are exogenous compounds.[37] However, docosanoic acid and erucic acid, which are potential precursors of docosanamide and erucamide, respectively, are abundant in rapeseed oil, canola oil, and olive oil, which are common vegetable oils in Finland. Thus, one explanation for the effects of our diet and physical activity intervention on serum levels of docosanamide and erucamide may be that these fatty amides are gut-microbiota-derived metabolites[38] of dietary fats.[39]

We found that the diet and physical activity intervention increased serum levels of a few short-chain and medium-chain acylcarnitines, including valerylcarnitine (or isovalerylcarnitine), octanoylcarnitine, and decatrienoylcarnitine. Acylcarnitines transport fatty acids from the cytoplasm into the mitochondria to produce energy in beta-oxidation.[40,41] During fasting or exercise, the body may increase the breakdown of fatty acids and the production of acylcarnitines to provide additional energy for skeletal muscles.[42] Physical activity has been reported to spike up circulating short- and medium-chain acylcarnitines and thereby trigger fatty acid oxidation in adults.[43] Increased fructose intake as a result of increased fruit or fruit juice consumption has also been found to increase short-chain acylcarnitines and decrease medium- and long-chain acylcarnitines in circulation.[44,45] We have previously shown that the lifestyle intervention increased physical activity and fruit consumption in the present cohort of children[30] that may partly explain our present finding that the lifestyle intervention increased serum levels of short-chain acylcarnitines among the children.

In addition to the effects of our diet and physical activity intervention on serum levels of several fatty amides, acylcarnitines, and fatty acids, the lifestyle intervention also affected serum levels of many phospholipids and sterols, which provides evidence for the broad impacts of our lifestyle intervention on lipid metabolism in a general population of children. These findings on lipid metabolism are also consistent with our earlier observations dealing with the beneficial effects of our diet and physical activity intervention on diet quality,[27,46] plasma fatty acids,[18] and plasma LDL cholesterol[17] among children. Moreover, the present observation that serum dehydroepiandrosterone sulfate, measured by non-targeted LC-MS metabolic profiling, increased less in the intervention group than in the control group agrees with our recent finding, using a targeted LC-MS analysis, that the same diet and physical activity intervention attenuated the increase of serum dehydroepiandrosterone and dehydroepiandrosterone sulfate in both sexes and also attenuated the increase of serum androstenedione and testosterone and delayed pubarche in boys.[47]

Our diet and physical activity intervention had effects on serum levels of several gut-microbiota-derived metabolites, which are chemically phenolic acids, amino acids, or fatty acids. The intervention attenuated the decrease of hydroxyferulic acid, hippuric acid, indolepropionic acid, and pyrocatechol sulfate, increased *p*-cresol sulfate, and attenuated the increase of indolelactic acid. Urinary hippuric acid has been proposed as a biomarker for fruit and vegetable consumption in children[48] and for polyphenol intake in adults.[49] Higher serum level of hippuric acid has been associated with a lower fasting blood glucose concentration and higher insulin secretion among adults.[20] A higher dietary intake of fiber has been associated with a higher serum level of indolepropionic acid[50] that has been related to higher insulin secretion and a lower risk of type 2 diabetes among adults with impaired glucose tolerance.[22,50] We have earlier found that the diet and physical activity intervention increased vegetable consumption and fiber intake[30,46] and attenuated the increase of insulin

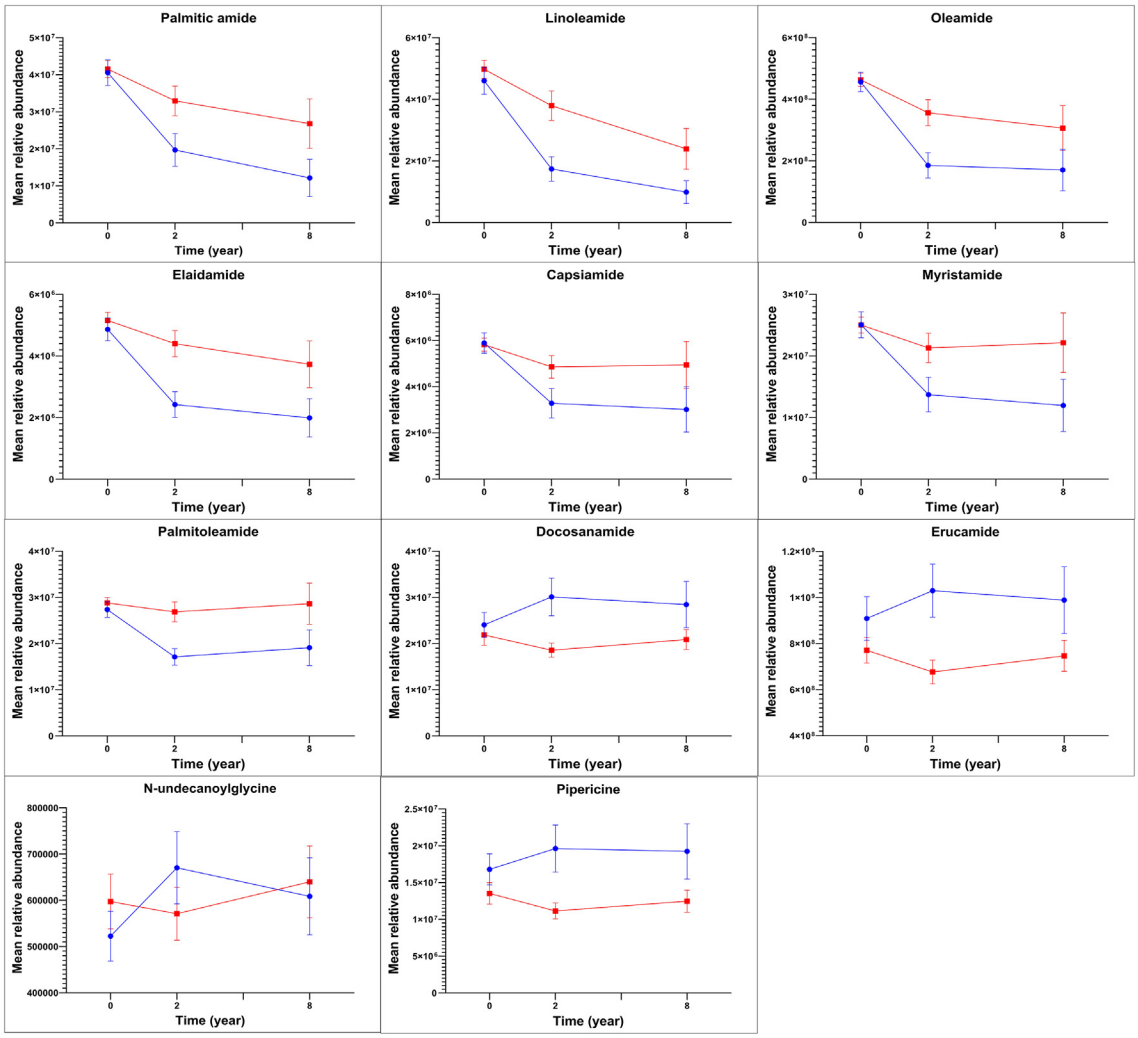

Figure 2. Mean relative abundances of serum fatty amides in the intervention group (red lines) and in the control group (blue lines) at baseline, 2-year follow-up, and 8-year follow-up examinations

Changes in the means are presented with 95% confidence intervals.

resistance[16] in the present cohort of children. All these observations together suggest that gut-microbiota-derived metabolites, such as hippuric acid and indolepropionic acid, could mediate the beneficial effects of improved diet quality on glucose metabolism among adults and children.

Moreover, our intervention decreased serum levels of two furan fatty acids, 3,4-dimethyl-5-pentyl-2-furanpropanoic acid and 3-carboxy-4-methyl-5-pentyl-2-furanpropanoic acid, which have not been reported to be endogenous compounds but have been found in fish and regarded as biomarkers for fish consumption.[51–53] However, as we have previously observed no difference in fish consumption between the intervention group and the control group,[30] eating fish is unlikely to explain the intervention effects on these furan fatty acids. Instead, furan fatty acids have been suggested to be derived from gut microbiota,[51] and the effects of our lifestyle intervention on these furan fatty acids could be partly due to changes in gut microbiota. In fact, a recent study found that about 75% of microbiome and metabolome features that distinguish individuals with ischemic heart disease from healthy individuals after controlling for medications and lifestyle factors are present in those exhibiting dysmetabolism, suggesting that major alterations of the gut microbiome and metabolome might begin long before the clinical onset of ischemic heart disease.[54]

We found that the diet and physical activity intervention had effects on serum levels of several amino acids, for example by attenuating the decrease of taurine, increasing hydroxyisoleucine, and preventing the decrease of glutamic acid. Taurine has been shown to slow aging process and increase in response to physical activity.[55] Four-hydroxyisoleucine has been found to inhibit obesity-related insulin resistance in adipocytes and hepatocytes by reducing inflammation and regulating the state of M1/M2 macrophages.[56] Glutamic acid is metabolized in the human body into glutamate, which is among key neurotransmitters in the central nervous system and has been suggested to be important for

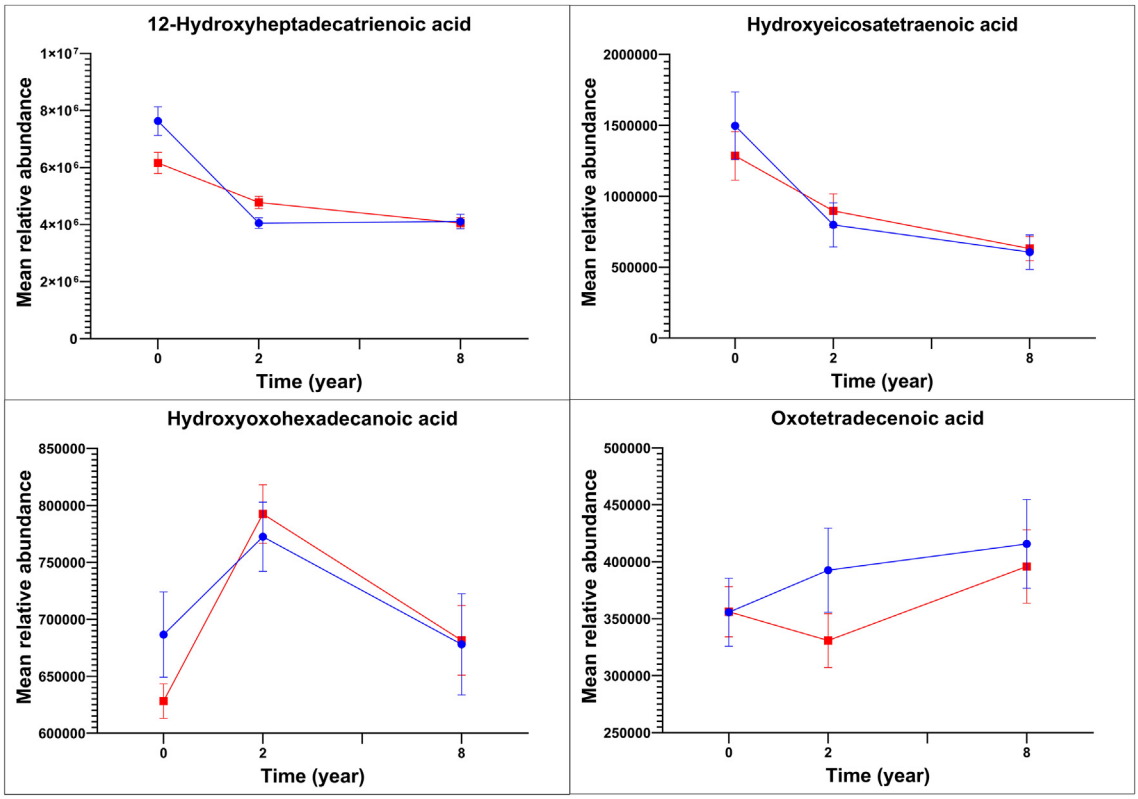

Figure 3. Mean relative abundances of serum fatty acids in the intervention group (red lines) and in the control group (blue lines) at baseline, 2-year follow-up, and 8-year follow-up examinations

Changes in the means are presented with 95% confidence intervals.

learning and memory.[57] Therefore, these amino acids may be molecular mechanisms underlying the beneficial effects of lifestyle changes on cardiometabolic and brain health in our pediatric population.[16,58]

Our diet and physical activity intervention affected purine metabolism by decreasing serum levels of guanosine and inosine and by increasing serum levels of hypoxanthine and xanthine. Purine metabolism is essential for adequate adenosine triphosphate (ATP) production for energy homeostasis, for example during strenuous exercise.[59] High-intensity speed-power exercise training has been shown to activate the purine salvage pathway,[60] in which hypoxanthine and guanine are converted to inosine monophosphate and guanosine monophosphate for adenine nucleotide resynthesis.[59] However, our findings suggest that the diet and physical activity intervention, which did not include strenuous exercise, may not have activated the purine salvage pathway but the purine degradation pathway. This is because our intervention increased serum levels of hypoxanthine, which inhibits the purine salvage pathway and activates liver xanthine oxidase through the purine degradation pathway.[61] In the purine degradation pathway, accumulated adenosine monophosphate (AMP) is degraded to inosine and hypoxanthine that are further metabolized to xanthine and uric acid by xanthine dehydrogenase and xanthine oxidase.[62] It would have been useful to measure serum uric acid and xanthine oxidase activity, as increased serum uric acid and xanthine oxidase activity have been

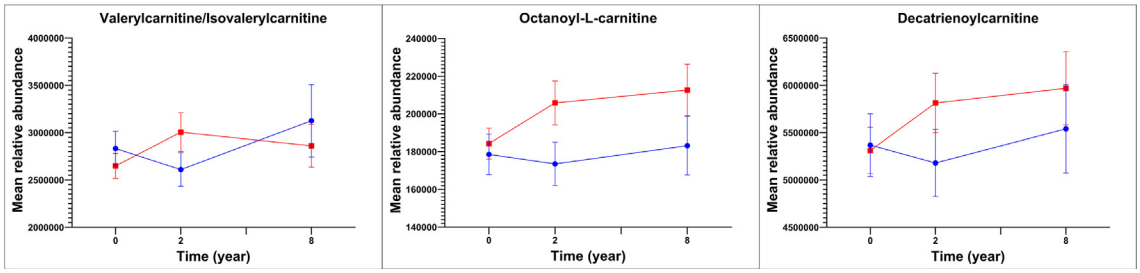

Figure 4. Mean relative abundances of serum acylcarnitines in the intervention group (red lines) and in the control group (blue lines) at baseline, 2-year follow-up, and 8-year follow-up examinations

Changes in the means are presented with 95% confidence intervals.

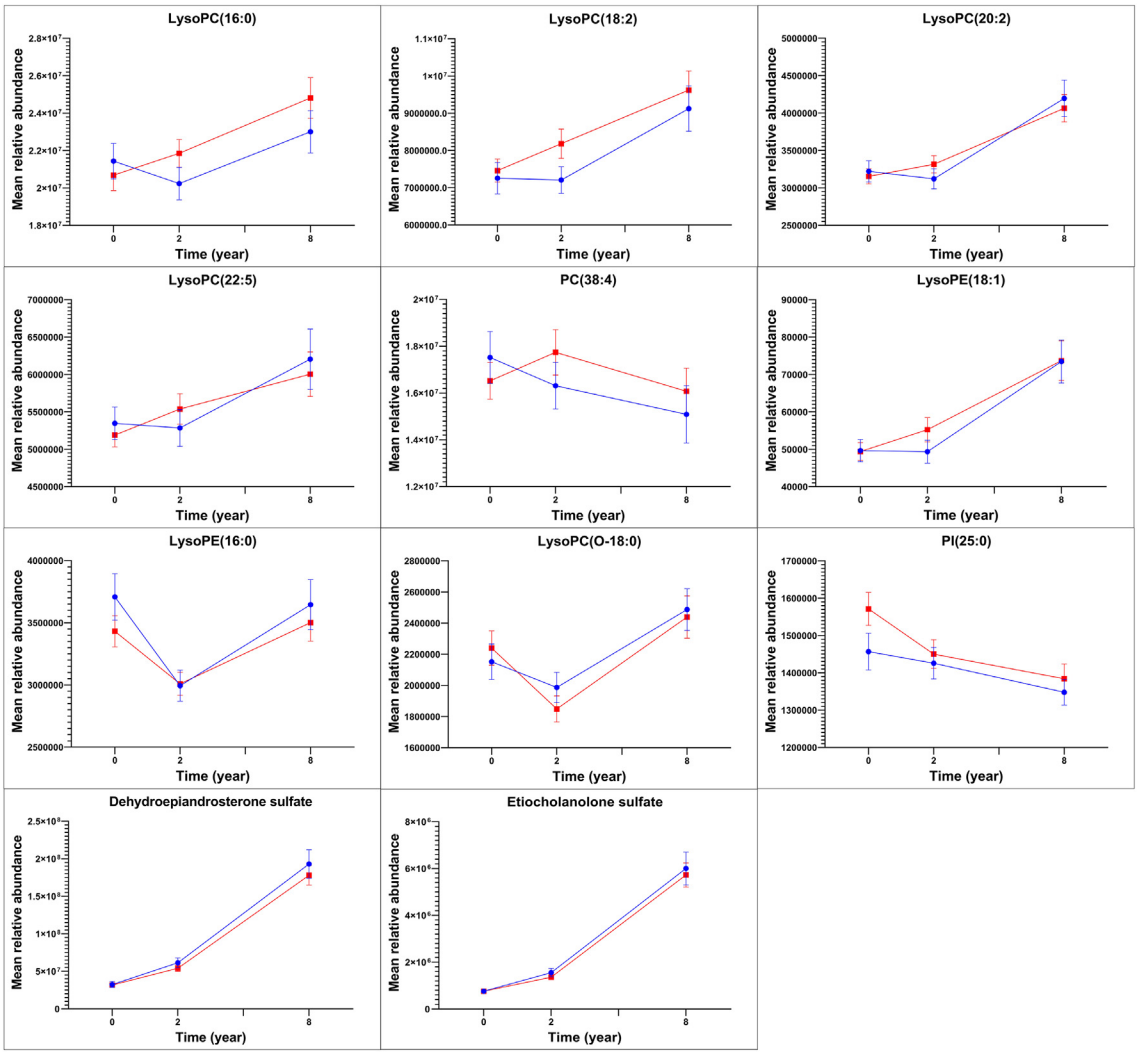

Figure 5. Mean relative abundances of serum phospholipids and sterols in the intervention group (red lines) and in the control group (blue lines) at baseline, 2-year follow-up, and 8-year follow-up examinations

Changes in the means are presented with 95% confidence intervals.

associated with obesity, liver dysfunction, dyslipidemia, insulin resistance, and endothelial dysfunction.[63,64] Finally, gut microbiota has been found to affect purine metabolites, especially guanosine, inosine, and hypoxanthine,[65] that could partly explain the effects of our lifestyle intervention on purine metabolites.

In conclusion, this long-term diet and physical activity intervention study demonstrates that the nontargeted metabolomics analysis of serum samples using the LC-MS method increases our understanding of possible mechanisms underlying the beneficial effects of lifestyle interventions on cardiometabolic and overall health during childhood and adolescence. Additional long-term diet and physical activity intervention studies are warranted to provide further evidence for the beneficial effects of lifestyle changes on cardiometabolic and other health outcomes and to reveal blood metabolites mediating these health effects using the LC-MS metabolomics and other omics analyses. These studies will provide valuable insight into the prevention of cardiometabolic and other non-communicable diseases since childhood and will support evidence-informed health-policy making to promote child and adolescent health by prioritizing pediatric programs aimed at fostering healthy eating and regular physical activity, making the living environment more motivating for healthy lifestyles, and incentivizing children, adolescents, and their caregivers to make healthy choices in their daily lives.

Limitations of the study

We had for the first time an opportunity to study the long-term effects of a diet and physical activity intervention on a wide spectrum of serum metabolites using the nontargeted metabolomics analysis by the sensitive LC-MS method in a general population of children followed up until adolescence and including 3 time points in the statistical analyses. The advantage of studying children instead of adults is that it decreases

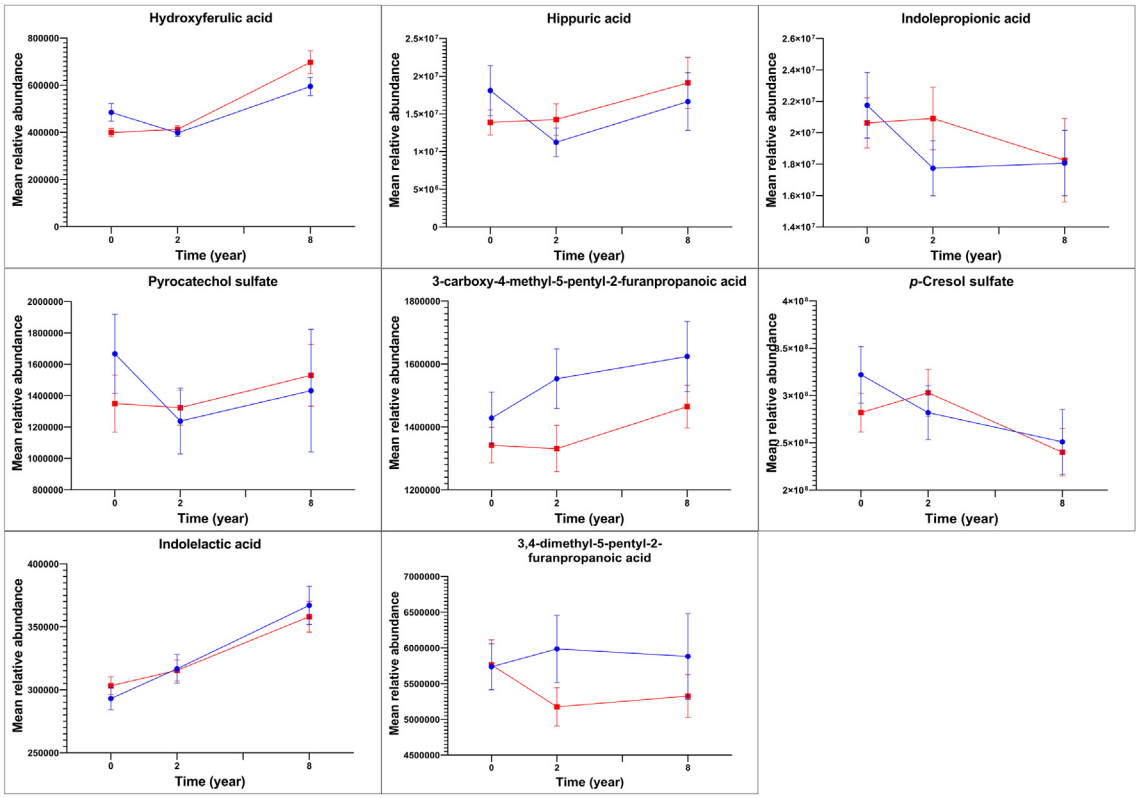

Figure 6. Mean relative abundances of serum gut-microbiota-derived metabolites in the intervention group (red lines) and in the control group (blue lines) at baseline, 2-year follow-up, and 8-year follow-up examinations

Changes in the means are presented with 95% confidence intervals.

confounding by chronic diseases, medications, smoking, and alcohol consumption all of which may be reflected by serum metabolome. A limitation of our study is that we did not randomly allocate the children into the intervention group and the control group. However, we analyzed the data using linear mixed-effects models that allowed us to control for confounding by possible differences in serum metabolites at baseline. Puberty is a potential confounding factor in studies among children, as it markedly affects metabolism. However, puberty is unlikely to be a confounding factor in our study as we included only prepubertal children at baseline in the statistical analyses, and there was no difference in the occurrence of puberty between the intervention group and the control group during the study.[16] The drop-out rate in both groups was also exceptionally low until two years, and given the long follow-up period from prepuberty to adolescence, the drop-out rate remained relatively low until eight years, and there was no selective dropping out in either group over eight years. Many effects of the diet and physical activity intervention on serum metabolites found over the first two years were no longer statistically significant after eight years, although small differences in these metabolites between the groups remained. This is likely to be explained by a modest intensity of the lifestyle intervention after the first two years and a limited statistical power in the statistical analyses due to a reduced number of participants attending the 8-year follow-up examinations. Finally, it would have been useful to have data on fecal microbial composition to provide a more comprehensive insight into the effects of diet and physical activity intervention on gut-microbiota-derived metabolites during childhood and adolescence.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
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  - Data and code availability
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
  - Study design and participants
  - Diet and physical activity intervention
  - Assessment of diet and physical activity

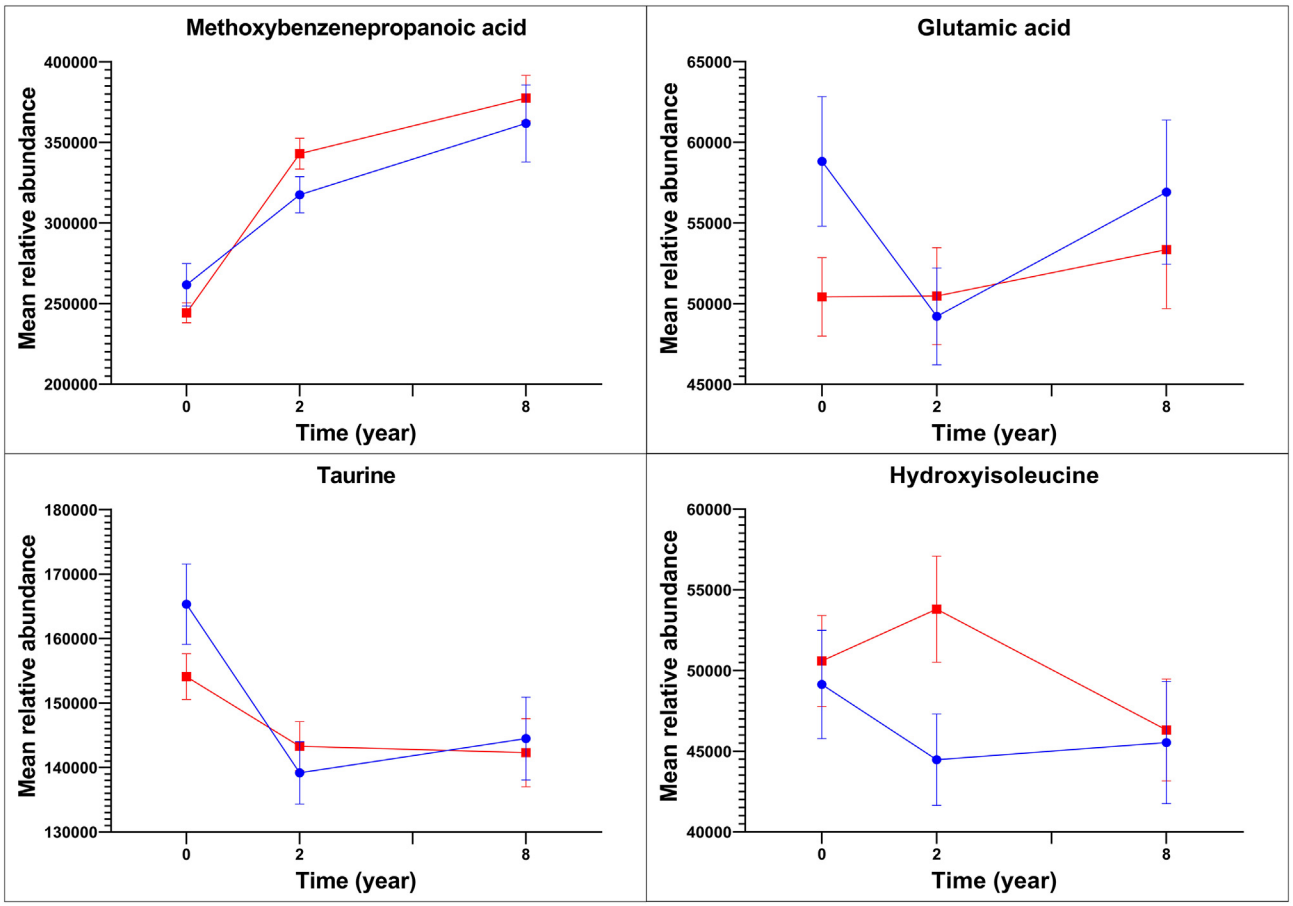

Figure 7. Mean relative abundances of serum amino acids in the intervention group (red lines) and in the control group (blue lines) at baseline, 2-year follow-up, and 8-year follow-up examinations

Changes in the means are presented with 95% confidence intervals.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.110295.

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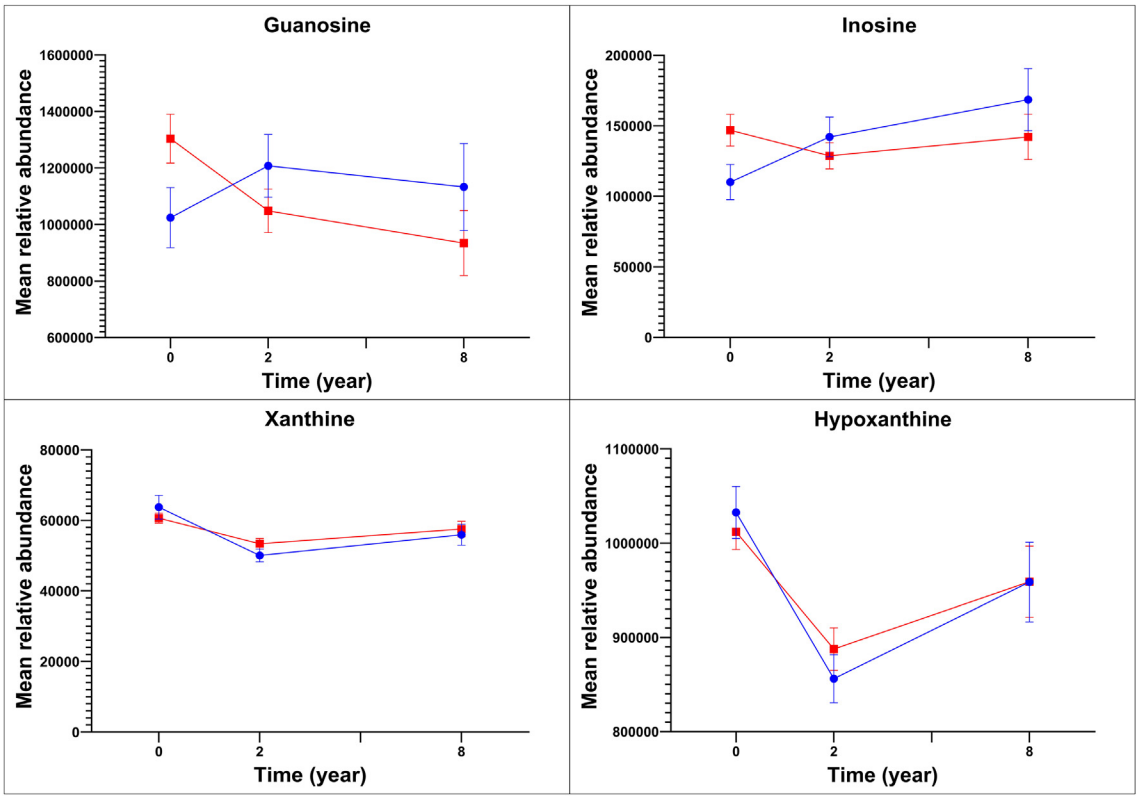

Figure 8. Mean relative abundances of serum purine metabolites in the intervention group (red lines) and in the control group (blue lines) at baseline, 2-year follow-up, and 8-year follow-up examinations

Changes in the means are presented with 95% confidence intervals.

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Graphical abstract was created with www.BioRender.com.

AUTHOR CONTRIBUTIONS

T.A.L. as the principal investigator of the PANIC study has access to all of its data and takes responsibility for the integrity of the data and the accuracy of the data analyses. I.Z., A.M.E., A.K., J.V., M.L., T.S., E.A.H., N.L., S.S., U.S., S.A., K.H., and T.A.L. contributed to the acquisition of the data for the current work. T.A.L., K.H., and M.K. secured funding for the work. T.A.L. and K.H. conceptualized and designed the current work and contributed equally to it as senior authors. A.K., R.H., and S.M. performed statistical analyses of the work. I.Z., T.A.L., and K.H. drafted the manuscript, and all other authors critically revised the manuscript for its intellectual content. T.A.L., K.H., M.K., M.L., and S.A. provided their administrative, technical, or material support for the work.

DECLARATION OF INTERESTS

K.H., V.M.K., and A.K. are affiliated with Afekta Technologies Ltd Afekta Technologies Ltd. - Metabolomics service company.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Sera from the participants	University of Eastern Finland	Statements 69/2006 and 422/2015
Chemicals, peptides, and recombinant proteins		
Methanol LC/MS Grade	Optima™, Fisher Chemical	10767665 (Cas 67-56-1)
Acetonitrile LC/MS Grade	VWR Chemicals	83640.320 (Cas 75-05-8)
Formic acid LC/MS Grade	Optima™, Fisher Chemical	10596814(Cas 64-18-6)
Ammonium formate LC/MS Grade	VWR Chemicals	84884.180 (Cas 540-69-2)
Water Milli-Q	N/A	N/A
Deposited data		
PANIC study and its variables	Home - Physical Activity and Nutrition in Children (panicstudy.fi)	N/A
Metabolomics data	The annotated metabolite dataset is publicly available as of the date of publication within Table S1	N/A
Software and algorithm		
MS-DIAL (version 4.80)	Tsugawa et al.[66], http://prime.psc.riken.jp/compms/msdial/main.html	N/A
R (version R 3.6.0)	R Core Team[67]	N/A
Others		
RP column (Zorbax Eclipse XDBC18, 2.1 × 100mm, 1.8 μm, Agilent Technologies, Palo Alto, CA, USA)	UHPLC, manufactured by Agilent Technologies.	N/A
HILIC, BEH Amide 1.7 μm, 2.1 × 100 mm, Waters Corporation, Milford, MA, USA	Acquity UPLC, Waters Corporation.	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to the lead contact, Timo A. Lakka (timo.lakka@uef.fi).

Materials availability

This study did not yield new unique reagents.

Data and code availability

- The PANIC study data are not publicly available due to research ethical reasons and as the owner of the data is the University of Eastern Finland and not the research group. Information about the PANIC study and its variables, including those used in the present paper, are described at Home - Physical Activity and Nutrition in Children (panicstudy.fi). The annotated metabolite dataset is publicly available as of the date of publication within Table S1.
- This study did not report the original script code. The workflow of data analysis is described in an earlier publication[19] and the package is available at https://github.com/antonvsdata/notame.
- Any additional information on the PANIC study is available on a reasonable request.

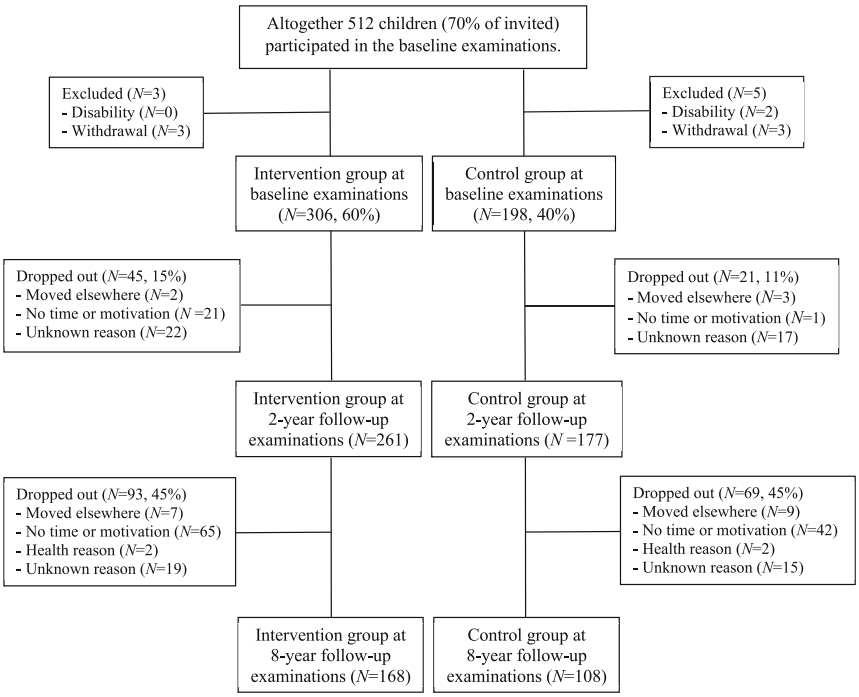
EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Study design and participants

The Physical Activity and Nutrition in Children (PANIC) study is an 8-year, nonrandomized, controlled trial to investigate the long-term effects of a combined diet and physical activity intervention on cardiometabolic risk factors in a general population of children from the city of Kuopio, Finland (NCT01803776).[16–18,30,46] The Research Ethics Committee of the Hospital District of Northern Savo approved the study protocol

in 2006 and 2015 (Statements 69/2006 and 422/2015). The caregivers gave their written informed consent, and the children provided their assent to participation. Moreover, the caregivers and adolescents gave their written informed consent before the 8-year examinations. The PANIC study has been carried out in accordance with the principles of the Declaration of Helsinki as revised in 2008.

We have described the study design, the study setting, the recruitment of participants, the inclusion and exclusion criteria, the reasons for dropping out, and the assessments in detail previously[16–18,46] and also provide this information in below figure. In brief, we invited 736 children 6–9 years of age who started the first grade in 16 primary schools of the city of Kuopio in 2007–2009 to participate in the study. Altogether, 512 children (248 girls, 264 boys) who accounted for 70% of those invited, accepted the invitation and attended the baseline examinations between October 2007 and December 2009. We excluded six children from the study at baseline either owing to their physical disabilities that could hamper participation in the intervention or withdrawal of the families because they had no time or motivation to attend the study. We also excluded data from two children whose parents or caregivers later withdrew their permission to use these data in the study. The final PANIC study sample thus included 504 children at baseline.



Flowchart of the Physical Activity and Nutrition in Children (PANIC) study

We allocated the 504 children from nine schools to a combined diet and physical activity intervention group (306 children, 60%) and the children from seven schools to a control group (198 children, 40%) to avoid contamination in the control group by local or national health promotion programs that could have been initiated in the study region during the study. We also proportionally matched the intervention and control groups according to the location of the schools (urban vs. rural) to minimize sociodemographic differences between the groups. We included more children in the intervention group than in the control group because of a larger number of dropouts expected in the intervention group and to retain sufficient statistical power for comparison between the groups. The children, their parents or caregivers, or people carrying out the examination visits or doing the measurements were not blinded by the group assignment.

Of all 504 children who participated in the baseline examinations, 438 (87%) attended the 2-year examinations, and 277 (55%) attended the 8-year examinations. At the 2-year examinations, 261 children were from the intervention group (85%) and 177 children were from the control group (89%). At the 8-year examinations, 169 (55%) adolescents were from the intervention group and 108 (55%) adolescents were from the control group. Those who participated in the 2-year or 8-year examinations did not differ in age, body height standard deviation score (SDS), body mass index (BMI) SDS, or the distribution of sex or study groups at baseline from those who dropped out over two or eight years.

We excluded 14 children who had entered clinical puberty by the baseline examinations from the present statistical analyses to minimize possible confounding by metabolic changes caused by puberty. After excluding these children, the number of participants in these statistical analyses was 490 (257 girls, 233 boys) at the baseline examinations, 424 (219 girls, 205 boys) at the 2-year examinations, and 270 (122 girls, 148 boys) at the 8-year examinations.

Diet and physical activity intervention

The goals of the individualized and family-based diet and physical activity intervention were to 1) decrease the consumption of significant sources of saturated fat and particularly high-fat dairy and meat products, 2) increase the consumption of significant sources of unsaturated fat and particularly high-fat vegetable oil-based margarines, vegetable oils, and fish, 3) increase the consumption of vegetables, fruits, and berries, 4) increase the consumption of significant sources of fiber and particularly whole grain products, 5) decrease the consumption of significant sources of sugar and particularly sugar-sweetened beverages, sugar-sweetened dairy products, and candy, 6) decrease the consumption of significant sources of salt and the use of salt in cooking, 7) increase total physical activity by emphasizing its diversity, 8) decrease total and particularly screen-based sedentary behavior, and 9) avoid excessive energy intake. The goals of the intervention were based on the Finnish nutrition and physical activity recommendations, which are in accordance with the corresponding international recommendations.

We have described the contents of the individualized and family-based diet and physical activity intervention in detail earlier.[16–18,30,46] In brief, the intervention during the first two years consisted of six intervention visits that occurred 0.5, 1.5, 3, 6, 12, and 18 months after the baseline examinations.[16,17,30] Each intervention visit included 30–45 min of dietary counseling and 30–45 min of physical activity counselling for the children and their parents or caregivers in our research facility at the University of Eastern Finland. The children and their parents or caregivers received individualized advice from a clinical nutritionist and a specialist in exercise medicine on how to improve diet, increase physical activity, and decrease sedentary time of the children in everyday conditions. Each visit had a specific topic on diet, physical activity, and sedentary behavior according to the intervention goals and included practical tasks on these topics for the children. The children and their parents or caregivers also received fact sheets on diet, physical activity, and sedentary time, as well as verbal and written information on opportunities for exercising in the city of Kuopio. Some material support was also given for physical activity, such as exercise equipment and allowance for playing indoor sports. Of the 306 children in the intervention group who attended the baseline examination, 266 (87%) participated in all six visits, 281 (92%) in at least five visits, and 295 (96%) in at least four visits in our research facility. The children in the intervention group, particularly those who did not attend organized sports or exercise, were also encouraged to participate in after-school exercise clubs organized at the nine schools by trained exercise instructors of the PANIC study. The children in the control group were not allowed to attend these exercise clubs to avoid a non-intentional intervention in the control group. Altogether, 254 (83%) of the 306 children in the intervention group participated in at least one of the after-school exercise clubs at the schools, and 124 (41%) participated at least once a month.

After the 2-year follow-up examinations, the diet and physical activity intervention was mild and was continued until the 8-year examinations.[46] The intervention included individualized diet and physical activity counseling sessions 3, 5, 6, and 7 years after baseline and a group-based counseling session at schools 4 years after baseline. The participants were able to attend the counseling sessions occurring 3, 5, 6, and 7 years after baseline with or without their parents, who were given individualized counseling in a separate room. Each of these counseling sessions had a specific topic on diet, physical activity, and sedentary behavior according to the intervention goals. The group-based counseling session at schools 4 years after baseline included an active lesson with practical tasks.

The children and their parents in the control group received general verbal and written advice on health-improving diet and physical activity at baseline, but they were not given active intervention.

Assessment of diet and physical activity

We have described the assessment of dietary factors and physical activity in detail earlier.[16,17,30,46] In brief, the consumption of food and drinks were assessed using 4-day food records. The caregivers recorded all food and drinks consumed by their children at baseline and 2-year examinations, whereas the adolescents recorded their food and drink consumption at the 8-year examinations. We calculated food consumption and nutrient intake using the Micro Nutrica dietary analysis software, Version 2.5, which is based on detailed information about the nutrient content of foods in Finland and other countries.[68,69] Moreover, the clinical nutritionists updated the software by adding new food items and products with their actual nutrient content based on new data in the Finnish food composition database (https://fineli.fi/fineli/fi/index) or received from the producers. We assessed total physical activity energy expenditure, light, moderate, and vigorous physical activity, as well as total sedentary time using individually calibrated combined heart rate and body movement monitoring.[16] Average total physical activity energy expenditure was calculated in kJ × kg^{-1} daily. Light, moderate, and vigorous physical activity were defined as time spent at intensity >1.5 and ≤4.0 metabolic equivalents (METs), >4.0 and ≤7.0 METs, and >7.0 METs, respectively, by defining one MET as an energy expenditure of 71 J × kg^{-1} × min^{-1} or oxygen uptake of 3.5 mL × kg^{-1} × min^{-1}. Total sedentary time was defined as time spent at intensity ≤ 1.5 METs excluding sleep.

Assessment of body composition and puberty

We assessed body height using a wall-mounted stadiometer and body weight using the InBody 720 bioelectrical impedance device (Biospace, Seoul, South Korea), with the weight assessment integrated into the system, in the morning after the participants had fasted for 12 h.[16] We computed age- and sex-standardized BMI-SDS using Finnish ref.[70]

We defined overweight and obesity using the International Obesity Task Force criteria, corresponding to an adult BMI cut-point of 25 for overweight and 30 for obesity.[71] Research physicians carried out a medical examination, assessed pubertal status, and defined clinical puberty using stages described by Tanner as breast development stage ≥2 for girls and testicular volume ≥4 mL assessed using an orchidometer for boys.[72,73]

METHOD DETAILS

Sample preparation for metabolomics analyses

We have described the metabolomics analysis and the identification of serum metabolites in detail earlier.[19] In brief, we performed the non-targeted LC-MS metabolomics analysis of fasting serum samples at the Metabolomics Center, Biocenter Kuopio, Finland. Fasting serum samples were randomized before the LC-MS analysis. After the samples were thawed entirely in ice bath for 3 h, 100 μL of each sample was mixed with 400 μL of acetonitrile and centrifuged (2500 rpm, 4°C, 5 min) on a 96-well filter plate (Captiva ND Plate 0.2 μm PP, Agilent Technologies, USA) to precipitate proteins from the sample.[19] The samples were pipette mixed with four pipette strokes to thoroughly precipitate serum proteins. The samples were centrifuged (700 × g for 5 min, 4°C) and the supernatants were collected to a 96-well plate (96 DeepWell PP Plate, Thermo Fisher Scientific Nunc, Rochester, NY, USA) which was covered (96 Well Cap Natural, Thermo Fisher Scientific Nunc A/S, Roskilde, Denmark). A small portion, approximately 2 μL, was extracted from half of the randomly selected protein precipitated study samples. This extracted portion was then pooled together to create a quality control (QC) sample, specifically for within-batch QC. During the analysis, these QC samples were injected at the beginning of the sequence to equilibrate the analytical platform and then after every 12th sample throughout the analysis. In addition, a solvent blank was prepared and injected at the beginning of the sequence. For between-batch QC, a composite between-batch QC sample was created by combining individual within-batch QC samples from five batches.

Liquid chromatography - Mass spectrometry analyses

The LC-MS analysis for the non-targeted metabolite profiling was carried out at the LC-MS metabolomics center (Biocenter Kuopio, University of Eastern Finland, Finland). To meet the wide diversity of sample components, all samples were analyzed using two different chromatographic techniques, i.e., reversed phase and hydrophilic interaction chromatography (HILIC) for amphiphilic and hydrophilic metabolites, respectively. In addition, the data were acquired in both electrospray ionization (ESI) polarities, i.e., ESI positive (ESI+) and ESI negative (ESI-).

Liquid chromatography - Mass spectrometry analyses of amphiphilic metabolites

The analysis of amphiphilic metabolites was carried out using an ultra-high performance liquid chromatography (Vanquish Flex UHPLC system, Thermo Scientific, Bremen, Germany) coupled online to a high-resolution mass spectrometry (HRMS, Q Exactive Classic, Thermo Scientific, Bremen, Germany). Samples were analyzed using a reversed phase technique. The sample solution (2 μL) was injected onto a RP column (Zorbax Eclipse XDBC18, 2.1 × 100mm, 1.8 μm, Agilent Technologies, Palo Alto, CA, USA) that was kept at 40°C. The mobile phase, delivered at 400 μL/min, consisted of water (eluent A) and methanol (eluent B), both containing 0.1% (v/v) of formic acid. The following gradient profile was used: 0–10 min: 2 to 100% B, 10–14.50 min: 100% B, 14.50–14.51 min: 100 to 2% B; 14.51–20 min: 2% B. The sample tray was at 10°C during these analyses. Mass spectrometry was equipped with a heated ESI. The positive and negative ionization modes were used to acquire the data in centroid mode. The following ESI source settings were utilized; spray voltage 3.0 kV for positive and negative ionization modes, sheath gas (20), auxiliary gas (5), and sweep gas (1) (flow rates as arbitrary units for ion source). The capillary temperature and the probe heater temperature were set to 350°C and 400°C, respectively. The S-lens Radio Frequency (RF) level was set to 50 V. A full scan range from 120 to 1,200 (m/z) was used with the resolution of 70,000 (m/Δm, full width at half maximum at 200 u). The injection time was set to 200 ms, and Automated Gain Control (AGC) was targeted at 1,000,000 ions.

For product ion scan (MS/MS) experiments, the Q-Exactive spectrometer was used with the same source parameters and chromatography conditions as described above. Two scan events were used: (a) an MS scan with a mass resolution power, AGC target and maximum injection time set to 70,000 (m/Δm, full width at half maximum at 200 u), 1,000,000 ions and 100 ms, respectively, and (b) an MS/MS scan (in HCD mode) at a normalized collision energy ranging from 20 to 40%, depending on the molecule, with a mass resolution power, AGC target, maximum injection time, isolation window set to 17,500 (m/Δm, full width at half maximum at 200 u), 50,000, 50 ms and 1.5 m/z, respectively. Loop count was 3, apex trigger 0.2 to 3 s, and dynamic exclusion 15 s. The data for MS/MS experiments was collected in profile mode. The detector was calibrated before the sample sequence and subsequently operated at high mass accuracy (<2 ppm). Continuous mass axis calibration was performed by monitoring reference ions m/z 214.08963 in the positive ionization mode.

Liquid chromatography - Mass spectrometry analyses of hydrophilic metabolites

For the analysis of hydrophilic compounds, an ultra-high performance liquid chromatography (1290 LC system, Agilent Technologies, Waldbronn, Karlsruhe, Germany) coupled online to a high-resolution mass spectrometry (HRMS, 6540 UHD accurate-mass quadrupole-time-of-flight (qTOF) mass spectrometry, Agilent Technologies, Waldbronn, Karlsruhe, Germany) was used. Samples were analyzed using a hydrophilic interaction chromatography technique (HILIC). The sample solution (2 μL) was injected onto a column (HILIC, Acquity UPLC BEH Amide 1.7 μm, 2.1 × 100 mm, Waters Corporation, Milford, MA, USA) that was kept at 45°C. Mobile phases, delivered at 600 μL/min, consisted of 50% (v/v) (Eluent A) and 90% (v/v) (Eluent B) acetonitrile, respectively, both containing 20 mM ammonium formate (pH 3). The following gradient profile was used: 0–2.5 min: 100% B, 2.5–10 min: 100% B → 0% B, 10–10.1 min: 0% B → 100% B; 10.1–12.5 min: 100% B. The total runtime was 12.5 min. The sample tray was at 10°C during these analyses.

Mass spectrometry was equipped with a heated ESI source, operated in both positive and negative ionization modes. The source used the following conditions: drying gas temperature 325°C and a flow of 10 L/min, sheath gas temperature 350°C and a flow of 11 L/min, nebulizer pressure 45 psi, capillary voltage 3500 V, nozzle voltage 1000 V, fragmentor voltage 100 V, and skimmer 45 V. Nitrogen was used as the

instrument gas. For data acquisition, a 2 GHz extended dynamic range mode was used in both positive and negative ion modes from 50 to 1600 (m/z). The data were collected in the centroid mode at an acquisition rate of 2.5 spectra/s (i.e., 400 ms/spectrum) with an abundance threshold of 150.

For automatic data dependent MS/MS analyses, the precursor isolation width was 1.3 Da, and from every precursor a scan cycle of 4 most abundant ions were selected for fragmentation. These ions were excluded after two product ion spectra and released again for fragmentation after a 0.25 min hold. Precursor scan time was based on ion intensity, ending at 20,000 counts or after 300 ms. Product ion scan time was 300 ms. Collision energies were 10, 20, and 40 V in subsequent runs.

The TOF was calibrated before the sample sequence and subsequently operated at high accuracy (<2 ppm). Continuous mass axis calibration was performed by monitoring two reference ions from an infusion solution throughout the runs. The reference ions were m/z 121.050873 and m/z 922.009798 in the positive mode and m/z 112.985587 and m/z 966.000725 in the negative mode.

Both high resolution mass spectrometries were equipped with a heated electrospray source, and the data were acquired in positive and negative ionization modes. Data-dependent MS/MS data were acquired at the beginning and end of the analysis from the quality control samples.

Metabolomics data analyses

Data preprocessing

Data matrix generation was performed separately for each of the four analytical modes. The molecular features were obtained using the MS-DIAL software[66] and the parameters are included in the Table S2. The data were preprocessed using the "notame"[19] pipeline in R software (R Core Team, https://www.R-project.org). The main parts of this prepossessing procedure are drift correction and quality assessment, wherein the molecular features were corrected for the drift pattern caused by the LC-MS procedures, and feature quality was assessed based on the quality control samples.[19] The samples were measured in five analytical batches in the LC-MS analysis, and the preprocessing was performed within-batch followed by between-batch correction using the respective QC samples (see chapter "sample preparation for metabolomics analyses").

The data was preprocessed with the following protocol: A) Data points with a value of zero were marked as NA and B) the features were flagged (i.e., considered bad quality) if their occurrence was less than 30% in QC samples. C) Next, the molecular features were corrected for the drift pattern caused by the LC-MS procedures. Within the function, the features were first log-transformed, and then a regularized cubic spline regression line was fitted separately for each feature based on the quality control samples. The smoothing parameter was chosen from an interval between 0.5 and 1.5 using leave-one-out cross-validation to prevent overfitting (Figures S1–S4). Lastly, the abundances were transformed back to the original scale after the drift correction. D) The quality of features was further assessed and the features remained unflagged (i.e., considered good quality) if either of the following criteria was met; 1) non-parametric relative standard deviation of QC samples (RSD_r = 1.4826 × MAD(QC)/median(QC)), where MAD = median absolute deviation) were below 30% and non-parametric D-ratio (D_ratio_r = MAD(QC)/MAD(biological)) were below 60%, or 2) relative standard deviation of QC samples (RSD = SD(QC)/mean(QC)), non-parametric relative standard deviation of QC samples, and variation of the QC samples compared to the biological samples (D_ratio = SD(QC)/SD(biological)) were below 20%, 20%, and 20%, respectively. E) Missing values were imputed using random forest imputation in three phases. First, only the good quality features (i.e., nonflagged) were imputed to prevent the flagged features from affecting the imputation. Thereafter, if more than 80% of the values of a feature were missing, it was imputed with an arbitrary raw abundance of "1". Lastly, the rest of the flagged metabolic features were imputed with random forest as described by Stekhoven and Buhlmann.[74] In brief preprocessing steps were A) Mark NA B) Flag detection C) Drift correct D) Flag quality E) Impute.

After the preprocessing protocol, all batches of a single analytical mode were combined, and the median of each quality metric across batches was chosen as an overall quality metric.[19] The preprocessed data from the four analytical modes (i.e., HILIC positive, HILIC negative, RP positive, and RP negative) were combined into one data matrix. As the same QC sample was used for all analytical modes as well as in all the batches, the variation between batches was normalized according to the QC samples (Figures S1–S4).

Selection of molecular features for identification of compounds

The effect of intervention on the serum metabolites was assessed by statistical evaluation (see chapter "sample size calculations and statistical analyses"). The molecular features that passed the notame quality assessment and were statistically affected by the intervention with p-values <0.05 (q-values <0.2) were subjected to metabolite identification. In addition, the molecular features were filtered based on their analytical characteristics, focusing on molecular features with MS/MS data available, signal-to-noise ratios >5, retention time between 0.8 and 13 min, and raw signal abundance >10,000 and 200,000 in the Q-TOF-MS and Orbitrap data, respectively.

Identification of compounds

The selected molecular features were identified following the 4-level metabolite annotation scheme proposed by the Metabolomics Standards Initiative.[75] The MS/MS fragmentation patterns were compared with candidate molecules from databases (METLIN, MassBank of North America (MoNA), Human Metabolome Database (HMDB), LIPID MAPS), earlier published literature, and in-house pure compound library of over 1800 authenticated standards, which includes retention time, mass-to-charge ratio (m/z), and chromatographic data (including MS/MS spectral data) for all molecules present in the library.

QUANTIFICATION AND STATISTICAL ANALYSIS

Sample size calculations and statistical analyses

We have described the sample size calculations for the PANIC study earlier.[16] Sample size calculations for the PANIC study were based on the effects of a dietary intervention on fasting serum insulin and Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) among children in the Special Turku Coronary Risk Factor Intervention Project (STRIP).[15,76] Because of a larger number of children in the PANIC study than in the STRIP study, we approximated a slightly smaller difference for the change in fasting serum insulin and HOMA-IR of 0.3 SD between the intervention group (60% of children) and the control group (40% of children) with a power of 80% and a two-tailed *p*-value for the difference between the groups of 0.05, allowing for a 20% loss to follow-up or missing data. However, these calculations did not allow for non-independence within schools, and therefore the power could be lower than 80%. According to these calculations, we resulted in a sample size of at least 275 children in the intervention group and at least 183 children in the control group at baseline.

We analyzed the intervention effects on serum metabolites using the intention-to-treat principle and linear mixed-effects models adjusting for sex and age, including main effects for time and study group × time interaction, and assuming data missing at random. We considered results with a *p*-value of <0.05 statistically significant and also used false discovery rate corrected *p*-values (*q*-values) to control the results for a multiple testing error and help interpret the statistical significance of the results.[77] We used Cohen's d values to assess effect sizes[78] and select serum molecular features for the identification of metabolites. We analyzed the data using the R software (R Core Team, 2021) and the IBM SPSS Statistics software (IBM Corp., Armonk, NY, USA).

ADDITIONAL RESOURCES

The Research Ethics Committee of the Hospital District of Northern Savo approved the study protocol in 2006 and 2015 (Statements 69/2006 and 422/2015). The caregivers gave their written informed consent, and the children provided their assent to participation. Moreover, the caregivers and adolescents gave their written informed consent before the 8-year examinations.