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Effects of a sphingolipid enriched dairy formulation on postprandial lipid concentrations.

Lena Ohlsson, PhD, Hans Burling, PhD, Rui-Dong Duan, Prof, Åke Nilsson, MD, Prof.

Affiliation

(LO, RDD, ÅN) Department of Clinical Sciences, Medicine (Gastroenterology and Nutrition), Lund University Hospital, S221 85 Lund, Sweden

(HB) Arla Foods AB, Scheelevägen 17, Lund, Sweden

Correspondence to: Lena Ohlsson, Department of Clinical Sciences, gastroenterology and Nutrition, Lund University Hospital, S-22185 Lund, Sweden. Telephone: +46-46-2220706. Fax: +46-46-184792. Lena.Ohlsson@med.lu.se. Reprints will be available in electronic but not printed form.

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The authors have no conflict of interest.

Running title: Postprandial effects of sphingolipids

Abstract

Background: The digestion of sphingolipids (SL) is slow and catalyzed by mucosal enzymes. Dietary SLs were shown to inhibit cholesterol absorption and to lower plasma cholesterol, triglycerides and hepatic fat accumulation in animal models. *Aim:* A dairy formulation based on fractionation of butter milk, which is enriched in milk polar lipids of which SL account for a large part is now available. In this study we examined if this formulation, when ingested with a standard breakfast, exerted a different influence on postprandial lipids than an equivalent control formulation lacking the polar milk lipids. *Methods:* Eighteen healthy male volunteers aged 22-65 years ingested a high fat (40 g) standard breakfast together with a milk like formulation containing 975 mg milk SL (A) or the control formulation (B). Postprandial levels of triglycerides (TG), total-, LDL- and HDL cholesterol, apolipoprotein AI, apolipoprotein B, glucose and insulin were measured 1 to 7 hours after the meal. *Results:* No difference was seen between experimental and control group in postprandial triglyceride-, insulin-, apolipoprotein A1, or apolipoprotein B levels. After one hour there was a trend of lower cholesterol concentrations in large TG-rich lipoproteins after formulation A. *Conclusion:* The SL-rich butter milk drink may affect cholesterol concentrations in TG-rich lipoproteins but has no effect on postprandial triglycerides after a breakfast with butter fat as the major lipid.

Key words: sphingomyelin, postprandial, triacylglycerol, milk polar lipids, cholesterol

Introduction

Dietary polar lipids may have beneficial health effects. Glycerophospholipids as soy lecithin lower LDL and increase HDL, although the LDL lowering effect is hard to differentiate from the effect of the high content of linoleic acid in this formulation (Knuiman et al 1989, O'Brien and Andrews 1993). Sphingolipids (SL) are a small component in the normal diet (Vesper et al 1999), and studies in which SL enriched formulations are given to humans have earlier been hard to conduct. A dairy formulation, enriched in butter milk polar lipids is now available. This formulation can be administered in a milk drink like formulation containing 2-3 times the normal daily intake of dietary SL per serving.

We showed earlier that the digestion of SL is extended and catalyzed by mucosal enzymes (Nilsson and Duan 2006). When cholesterol was given together with sphingomyelin (SM) an inhibitory effect on cholesterol absorption could be demonstrated in intact (Nyberg et al 2000) as well as lymphatic duct cannulated rats (Noh and Koo 2004) and mice, and in CaCo2 cell cultures (Eckhardt et al 2002). In apoE Leyden mice, a genetically modified animal model that reacts to dietary changes more like humans than wild type mice sphingolipids were shown to decrease plasma cholesterol and triglyceride levels when the animals were on a high fat and high cholesterol diet. The effect was shown to be linked to an inhibitory effect on cholesterol- and lipid absorption. In this experimental model the sphingolipids also decreased the accumulation of fat in the liver (Duivenvoorden et al 2006). In vitro addition of sphingolipids inhibit the action of pancreatic lipases (Patton and Carey 1981). In this study we raised the question, whether a recently developed tasty milk drink formulation, enriched in milk fat globule membrane polar lipids, influenced the postprandial lipid and lipoprotein profile. These parameters are well known indicators of cardiovascular risk and can be influenced by numerous dietary factors (Lopez-Miranda et al 2007).

Methods

The test drink A used in this study was based on processed and enriched butter milk and contains 4,0 % Lacprodan PL-20 powder from Arla Foods Amba. This powder contains 20 % (w/w) milk phospholipids originating from the serum phase of butter oil production. The test drink (A) contains 700 mg SM, 180 mg glucoceramides, and 95 mg gangliosides (mainly GD3). Total amount of SL was 975 mg in one portion of 340 ml. A placebo drink (B) was made by 5,82 % skim milk powder containing 37% protein in which the phospholipids have been replaced by 2/3 of egg PL isolate by Fresenius GmbH and 1/3 butter oil. The placebo drink contains 119 mg of SL per portion. The cholesterol content was adjusted to be the same in both drinks, i.e. 11 mg per 100 mL or 38 mg per serving. Based on dry weight both formulations contained 58,5 % milk protein and 6,6 % lactose as major constituents. The drinks has been subjected to heat treatment at 143° C for 6 s and poured into Tetra Brick containers under aseptic conditions. The sterility of the drink has been verified by a certified bacteriological control laboratory. Test and placebo drink was manufactured at Trenums Industries, Tingsryd, Sweden, under supervision of Arla-experts. Trenums is an approved facility for manufacturing of food items. The drinks were stored in refrigerator and it is recommended to shake it a little before consumption.

Twenty male healthy volunteers in the age of 21-65 years, mean 39 years of age and mean BMI 24.3 were recruited through announcements on local intranet and advertisement in different work places in Lund. Exclusion criteria were abnormal fasting blood glucose levels, and hyperlipidemia in addition to signs of liver or kidney disease or inflammatory activity as indicated by raised C-reactive protein, liver tests (bilirubin, alkaline phosphatase, gammaglutamyltranspeptidase and aminotransferases), and creatinine levels. All participants

gave their written informed consent prior to the study. The study was registered and approved by the Regional Human Ethics Committee of Lund-Malmö, Sweden.

The subjects were fed a standard breakfast containing the milk drink or the control formulation in a randomized crossover design and the wash out period between trial days was 10-14 days. The breakfast consisted of one 275 g portion of semolina pudding (8,0 g fat, 1029 KJ), 28 mL cream (10,1 g fat, 392 KJ), 20 g butter (16,2 g fat, 596 KJ), one slice of bread (0,8 g fat, 298 KJ), 17 g liver paste or 15 g cheese (4,0 g fat, 202-214 KJ) and 16 g blackberry jam (128 KJ) (table 1). Coffee or tea was served, without sugar, and the cream could be taken with these drinks or with the semolina pudding and blackberry jam. The breakfast except the test drinks thus contained 2645-2657 KJ (637-640 Kcal) and 39,1 g fat of which about two thirds was milk fat. The total cholesterol intake from the breakfast is 130 mg without the milk drink.

Blood samples were taken immediately before the breakfast, when the subjects were fasting since 10 o'clock the night before, and after 1, 3, 5 and 7 hours. The samples were analyzed for triglycerides, cholesterol, blood glucose, insulin, HDL and LDL cholesterol, apoAI and apoB, liver tests, C-reactive protein, hemoglobin, white blood cell count and platelet count. Baseline concentrations of plasma lipids before each trial are shown in table 2. All analyses were done at the Department of Clinical Chemistry, University Hospital of Lund, Sweden using the accredited routine methods developed by Roche Diagnostics (MSDS — COBAS Integra® — General Chemistry). Two mL EDTA-plasma were frozen for other analyses if motivated.

Participants conducted food registrations in the form of diaries over four days before experiments were performed and the intake was analyzed by the use of Dietist XP software with the Swedish National Food Administration database (20080306) (Kost och Näringsdata,

Sweden). Dietary intakes were related to recommended Swedish dietary guidelines (SNF 2005).

Statistics

Significance of differences between the results obtained with the A and the B formulation were examined using paired ANOVA multiple measures. Bonferroni post hoc test was carried out when significant differences between diets were found. The calculation of incremental area under curve (iAUC) and AUC were done using the trapezoid rule. The Students paired t-test was used to calculate if there were any difference between cholesterol in TG-rich lipoproteins after formulation A and B after one hour since this was the time for the highest lipoprotein output. All statistical analyses and calculations of iAUC were performed with GraphPad Prism (version 5.00, GraphPad software, San Diego, Ca USA).

Results

Characteristics of the plasma lipid levels from 18 of the 20 participants in fasting state are shown in table 2. One did not fulfill the study and values from one participant were excluded due to protocol deviation. The fasting values for this subject was 7,5 mmol/l TG and 13 mIU/L insulin probably due to heavy eating and drinking later than 10 pm. The study was participant blinded and randomized with regard to the order in which formulation A and B were taken. We received registration of food intake before each trial from nine individuals but only eight participants are included in table 3. We excluded the registration from the person who deviated from the protocol and presented elevated fasting TG levels mentioned above.

The effects on postprandial parameters of the two lipid formulations are shown in Figure 1. As shown in Figure 1a the postprandial triglyceride responses were almost identical after formulations A and B. The concentrations were highest after 1 hour and then declined. After 7 hours they were slightly lower than the fasting values. In case of total cholesterol the postprandial concentrations were slightly lower after formulation A, but the differences were small and not statistically significant (Figure 1b). There were no statistically significant difference between the A and the B formulation in insulin and glucose levels (Figure 1c and d). The postprandial decreases in HDL- and LDL cholesterol 1 and 3 hours after the B formulation exceeded those obtained with formulation A (Figure 1 e and f). At the later time intervals, HDL cholesterol was higher after the A than after the B formulation but the difference was, however, not significant with paired ANOVA multiple measures. To estimate the postprandial concentrations of cholesterol in TG-rich lipoproteins we subtracted both LDL and HDL-cholesterol from total cholesterol (Figure 2a). We observed a 30 % lower iAUC with formulation A but the difference was not statistically significant. At one hour postprandially, the time point at which the intestinal output of TG-rich lipoproteins is

culminating, there was a trend ($p = 0,063$) that cholesterol concentrations in TG-rich lipoproteins was lower after formulation A. Since the intestine is a source of apolipoprotein A1 and B48 we measure the postprandial concentrations of the individual proteins as well as the ratio. The apo B/ApoA1 ratio reflects to some extent the concentration of small dense LDL (figure 2b). In this study the Apo B and apo A1 (individual concentrations not shown), or the apoB/apoA1 ratio did not differ postprandially between the formulations. The ingestion of the meal took about 20 minutes and the measurements were performed one hour after the end of the meal. The iAUC for the different parameters were not significantly different between formulation A and B (Table 4).

Discussion

This paper shows that a dairy formulation containing 975 mg sphingolipids, of which 700 mg is sphingomyelin, and polar dairy glycerophospholipids as well, did not influence the postprandial triglyceride increase from a high fat breakfast. There was a trend that cholesterol concentrations in TG-rich lipoproteins (total cholesterol excluding HDL and LDL) was lower after formulation A. It is possible that a larger study could have presented a true difference but with present data we do not consider this as proof of a SL-mediated inhibition of cholesterol absorption.

Earlier animal experiments demonstrated both an inhibition of cholesterol absorption with sphingomyelin (Eckhardt et al 2002, Noh and Koo 2004, Nyberg et al 2000) and a cholesterol- and triglyceride lowering effect of several sphingolipids in apoE Leyden mice fed a cholesterol- and lipid rich diet which were fed 0,2-1 % of the respective sphingolipid in the diet for four weeks (Duivenvoorden et al 2006). Recently, milk polar lipids were shown to reduce hepatic steatosis and hyperlipidemia in C57BL/6 mice (Wat et al 2009). Since the reason behind the effects in rodents has not been clarified, any explanation for the lack of effect on postprandial lipids in humans must be speculative. Indirect measurements of triglyceride fatty acid absorption in the ApoE Leyden mice suggested that 1% phytosphingosine decreased fatty acid absorption after a fat bolus dose (Duivenvoorden et al 2006). Such an effect might be exerted at the level of intestinal lipolysis (Patton and Carey 1981), via an effect of the sphingoid bases in the mucosal cells (Garmy et al 2005) or on the postprandial fatty acid metabolism. Whatever the mechanism, it may obviously be influenced by dose of sphingolipids as well as the amount of fat and cholesterol given, and other dietary conditions. The dose of sphingolipids given in this study i.e. 975 mg should be in a range within which effects might be realistically expected, but which may still be insufficient.

Furthermore the effects seen in the apoE Leyden mice may require the exposure to sphingolipids over longer time periods, whereas this study examines acute effects only.

As a general feature HDL- and LDL-cholesterol decreases initially after dietary lipid intake by the action of cholesterol ester transfer protein (CETP) which transfer cholesterol ester from both HDL and LDL to TG-rich lipoproteins during the postprandial phase. In this study HDL decreased less with formulation A, and it is therefore difficult to conclude, whether dietary SL cause inhibition of cholesterol absorption or influence the CETP mediated transfer of cholesterol ester from other lipoprotein classes. We also do not know at present if this difference is due to the presence of sphingolipids in A or is caused by other components of milk fat globule membranes. For instance the presence of milk polar lipids in the diet might increase the formation of polar lipid surface coat material which is transferred to HDL as the triglyceride rich lipoproteins are metabolized by lipoprotein lipase. An earlier study showed that feeding soy lecithin with a triglyceride rich meal decreased the average size of postprandial lipoproteins and thus the amount of polar surface component per mass of triglyceride (Beil and Grundy 1980). Soy phosphatidylcholine was also shown to increase the levels of HDL₂ levels (O'Brien and Andrews 1993), i.e. the most phospholipid-rich HDL sub fraction. Further studies including the size spectrum of triglyceride rich lipoproteins, activity of CETP and lipid transfers after ingestion of polar milk lipids are necessary to explain the cholesterol findings in this study.

The relation between glucose-, insulin action and ceramide in diabetes and obesity is important (Summers 2006). We have, however, neither found any differences in insulin or glucose levels in this study, and so far, nor been able to demonstrate any change in plasma sphingolipids after feeding the milk sphingolipids (unpublished).

Diet registrations revealed no significant difference between the intakes before the two occasions, indicating that no false negative findings were caused by change in diet.

In summary, we found no immediate significant effect of milk polar lipids on postprandial plasma lipid levels. Thus, the result from this relatively small study on healthy individuals does not support our hypothesis that milk polar lipids affect our plasma lipids and lipoprotein profile. However, the formulation enriched in milk polar lipids was well tolerated and suitable for further human studies e.g. in diabetic and obese subjects with high fasting and postprandial triglyceride levels and low HDL, in which beneficial effects are plausible.

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Legend to figures

Figure 1. Normalized postprandial plasma lipids, lipoproteins, insulin and glucose concentrations presented as mean \pm SEM from 18 male subjects after intake of 350 ml SL-enriched milk drink (A) or an equivalent placebo drink (B) together with a standardized breakfast. A) plasma triacylglycerol, b) total plasma cholesterol, c) insulin, d) glucose, e) HDL, f) LDL.

Figure 2. a) Cholesterol in TG-rich lipoproteins in mmol/L calculated by subtracting values for HDL and LDL from total cholesterol. b) Normalized postprandial apolipoprotein ratio of ApoB/ApoA1. Both a and b are presented as mean \pm SEM from 18 male subjects after intake of 350 ml SL-enriched milk drink (A) or an equivalent placebo drink (B) together with a standardized breakfast.

Table 1. Macro- and micro nutrient composition of the test meal.

	Breakfast meal: 275 g semolina pudding, 28 mL cream, 20 g butter, 1 slice of bread, 17g liver pat�e, 16 g blackberry jam	Test drink/ placebo (in 350 ml)
KJ/Kcal	2683/643	807/193
Protein (g)	16.9	8,19
Fat (g)	39.4	3.85
Carbohydrates (g)	54.3	31.2
Calcium (mg)	315	130/271
Fiber (g)	3.4	0
Cholesterol (mg)	99.3	38

Table 2

Fasting concentrations in all participants (n=18) before ingestion of formulations A and B.

Postprandial values are presented in Figure 1 a-h as differences from the fasting values.

	Formulation A	Formulation B
Triglycerides		
(mmol/L)	0.97 ± 0.37	1.07 ± 0.39
Cholesterol		
(mmol/L)	4.35 ± 0.93	4.42 ± 0.82
LDL-cholesterol		
(mmol/L)	2.85 ± 0.80	2.91 ± 0.76
HDL-cholesterol		
(mmol/L)	1.31 ± 0.19	1.32 ± 0.18
Apolipoprotein B		
(g/L)	0.74 ± 0.21	0.74 ± 0.21
Apolipoprotein A1		
(g/L)	1.36 ± 0.16	1.35 ± 0.12
ApoA1/ApoB		
(g/L)/(g/L)	0.55 ± 0.15	0.55 ± 0.16
Blood glucose		
(mmol/L)	4.98 ± 0.52	5.12 ± 0.37
Insulin		
(mIU/L)	4.27 ± 1.90	4.78 ± 1.77

Table 3. Amounts of nutrient intake of participants (n=8) before each test day. Values are mean of 8 subjects and four days intake \pm SD.

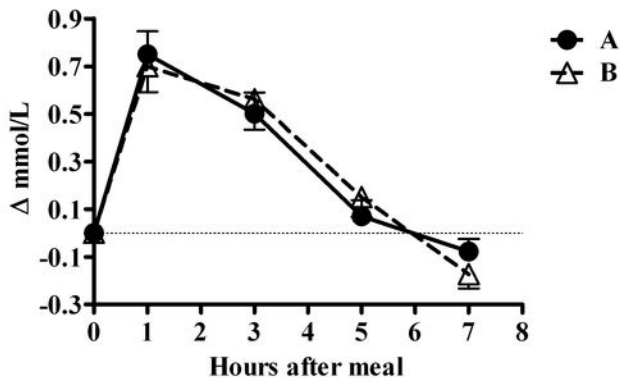
	Before intake of formula A	Before intake of formula B
Energy intake		
(kJ)	10828 \pm 2846	9731 \pm 2666
Fat intake		
(g)	102.7 \pm 35.3	91.5 \pm 38.7
Saturated fatty acids		
(g)	40.1 \pm 17.1	37.6 \pm 16.7
Monoenoic fatty acids		
(g)	38.5 \pm 15.5	32.2 \pm 15.5
Polyenoic fatty acids		
(g)	14.7 \pm 4.6	11.2 \pm 4.8
Cholesterol intake		
(mg)	429.1 \pm 194.7	394.8 \pm 200
Fiber		
(g)	19.7 \pm 6.4	19.9 \pm 7.8
Calcium		
(mg)	928 \pm 424	1092 \pm 607
Fat intake the day before		
(g)	112.3 \pm 65.9 ¹	107.1 \pm 73.6 ¹
Energy intake the day before (KJ)		
	11591 \pm 116.6 ¹	10792 \pm 108.7 ¹

¹ Intake the day prior to trial. Mean of 8 subjects.

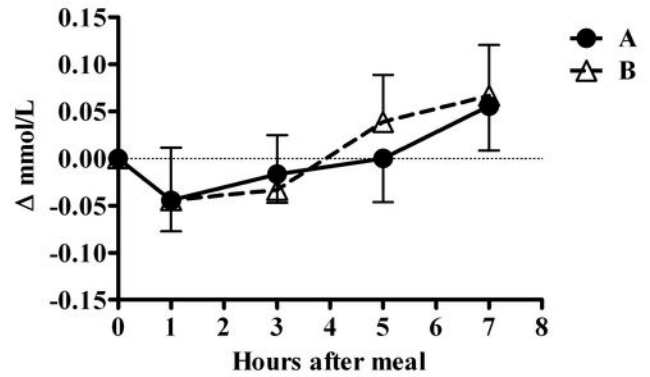
Table 4. The iAUC for normalized values from 0h to 7 hrs for formula A and B and different plasma components. The values are mean and 95% CI, n = 18

	Formula A	Formula B	Statistic analysis
	Total Peak area (95 % CI)	Total peak area (95% CI)	(Students paired t- test p>0.05 = n.s)
TG	2.41 (1.71-3.12)	2.69 (1.87-3.51)	0.35
Insulin	18.11 (12.63-23.60)	17.17 (11.93-22.42)	0.74
Glucose	0.71 (-0.09-1.50)	0.57 (0.04-1.11)	0.71
Cholesterol	0.39 (0.17-0.60)	0.52 (0.12-0.93)	0.52
HDL	0.15 (0.05-0.25)	0.12 (0.02-0.21)	0.66
LDL	0.15 (0.06-0.24)	0.19 (0.03-0.35)	0.69
Cholesterol in TG- rich lipoprotein	1.61 (0.87-2.36)	2.10 (1.42-2.77)	0.27
ApoA1	0.12 (0.05-0.18)	0.13 (0.05-0.22)	0.82
ApoB/ApoA1	0.21 (-0.17-0.56)	0.01 (0.00-0.22)	0.30
ApoB	0.04 (0.02-0.07)	0.04 (-0.00-0.09)	0.99

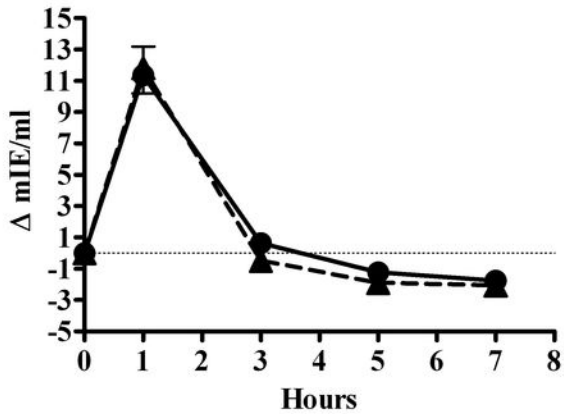
a) Plasma triacylglycerol



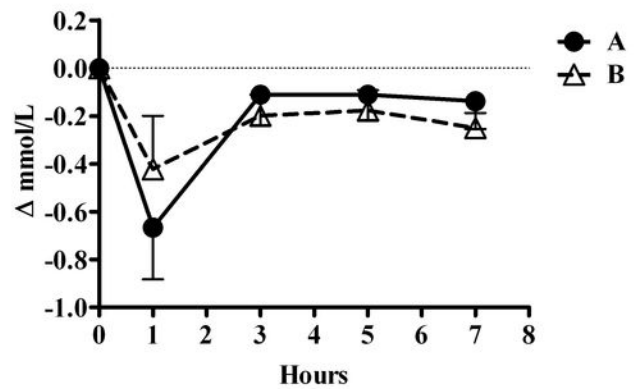
b) Total cholesterol



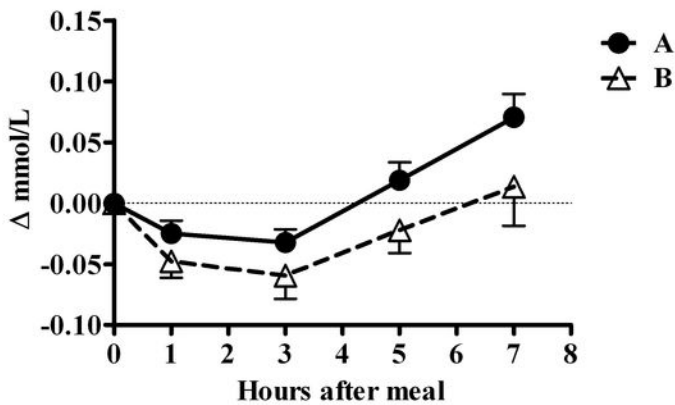
c) Insulin



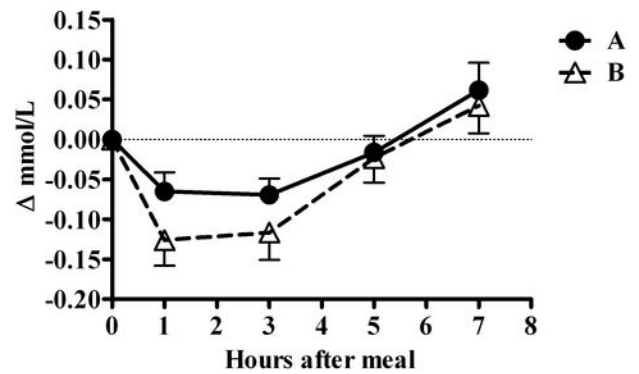
d) Glucose



e) HDL

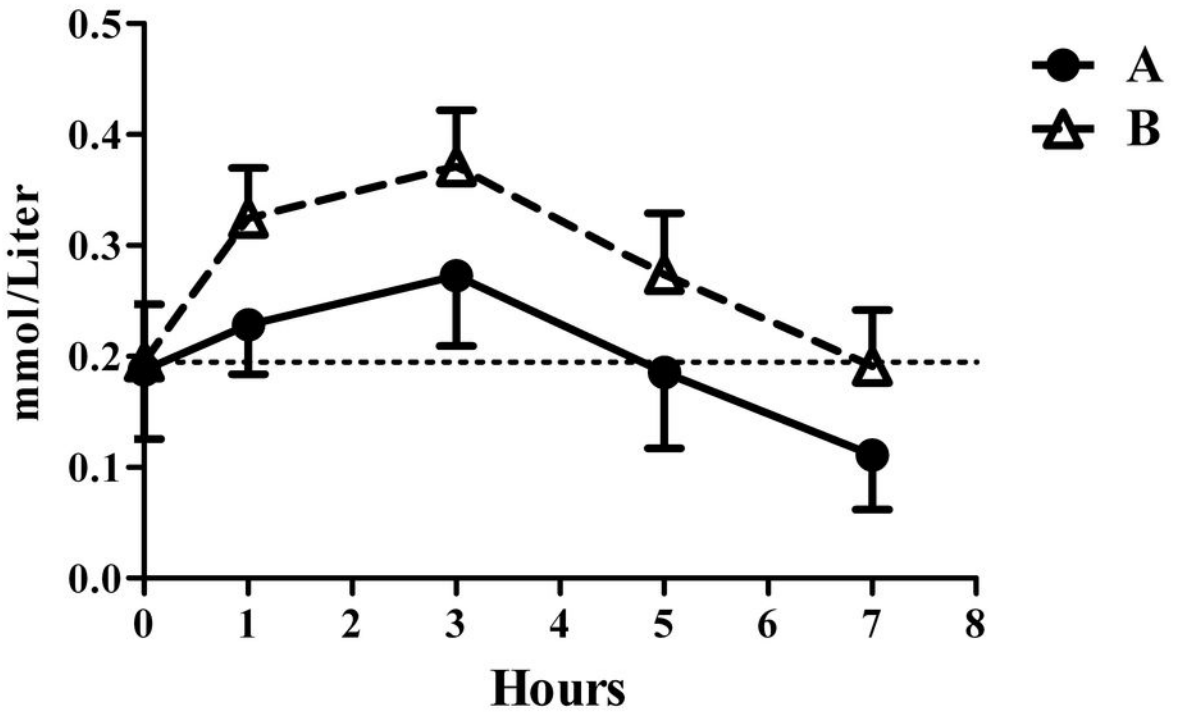


f) LDL



a)

Cholesterol (LDL and HDL excluded)



b)

ApoB/ApoA1

