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Effects of Long-Term High-Fat Diet and Its Reversal on Lipids and Lipoproteins Composition in Thoracic Duct Lymph in Pigs

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Background: This study was carried out to evaluate the effects of a long-term high-fat diet on lipids and lipoproteins composition in thoracic duct lymph in pigs.

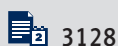
Material/Methods: We examined lymph taken from the thoracic duct from 24 female white sharp-ear pigs, divided into 3 experimental groups fed different diets for 12 months: (a) the control group, fed the standard balanced diet; (b) the HFD group, fed an unbalanced, high-fat diet, and (c) the reversal diet group (RD), fed an unbalanced, high-fat diet for 9 months and then a standard balanced diet for 3 months.

Results: Lymph analysis after 12 months of fixed diets revealed significantly higher concentration of proteins in the HFD group in comparison to the control and RD groups. Examination of lymph lipoproteins fractions showed that the high-fat diet in the HFD group in comparison to control group caused an increase in cholesterol, phospholipids, and proteins content within HDL and chylomicrons. There were also more proteins within HDL in the HFD group in comparison to the RD group and more triglycerides within chylomicrons in the HFD group in comparison to the control group.

Conclusion: A long-term high-fat diet resulted in changed structure of HDL and chylomicrons in the thoracic duct lymph. Alterations in HDL composition suggest that a high-fat diet enhances reverses cholesterol transport. Changes in chylomicrons structure show the adaptation to more intense transport of dietary fat from the intestine to the liver under the influence of a high-fat diet. Reversal to a standard balanced diet had the opposite effects.

MeSH Keywords: **Diet, High-Fat • Lipids • Lipoproteins • Lymphatic System**

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Background

A high-fat, high-carbohydrate, and low-fiber diet, known as the “Western diet” is a well-established risk factor of cardiovascular diseases. Switching to a healthy diet is known to decrease cardiovascular risk [1,2]. The lymphatic system transports lipids absorbed in the intestine and cholesterol from tissues. This latter process is known as the reverse cholesterol transport [3].

Animal and human lymph analyses showed that all blood lipoprotein classes, including chylomicrons, are present in peripheral lymph, but in amounts much lower than in the blood. Their concentrations in lymph are inversely proportional to their molecular size [4–6]. In peripheral lymph, the lymph/plasma concentration ratios of lipoproteins vary from 0.03 for VLDL (very low-density lipoprotein) to 0.2 for HDL (high-density lipoprotein) [4]. Peripheral lymph from different regions and organs exhibits different lipoprotein composition [4,7,8]. Generally, lymph from more permeable vascular beds, such as from lung, myocardium, endocrine glands, liver, and spleen, contains more lipoproteins [4,9]. The presence of lipoproteins in lymph is thought to be mostly a result of ultrafiltration, which is a passive movement across the capillary endothelium, without specific (i.e., receptor-mediated) transport processes [9–13]. Some lipoproteins, especially LDL (low-density lipoprotein), can potentially cross the endothelium via transcytosis [6,14]. Evidence for receptor-mediated transport of LDL across the endothelium has been demonstrated, but only in an *in vitro* blood–brain barrier model [15].

The size of HDL, and particularly LDL, in lymph is more heterogeneous than in plasma. Both fractions are extensively metabolically modified in the interstitial space by their interactions with cells, enzymes, and proteins transferring lipids, which changes their physical and chemical properties [8,16].

What happens to the lipoproteins within the lymph nodes remains unknown. However, it was demonstrated that under normal conditions, blood movement during passage across lymph nodes results in fluid reabsorption through the blood capillaries and, thus, in concentrating of proteins in the post-nodal lymph [17–20]. Close contact between plasma and lymph within the lymph nodes can also result in exchange processes between them [4].

Lipid compositions of pre-nodal and post-nodal lymph have been shown to be affected by several pathophysiological conditions, especially diet. However, there have been few studies on the effects of specific diet on thoracic lymph lipoprotein composition, and they have been mainly focused on the effect of dietary cholesterol. In studies by Dory et al. and Sloop et al., plasma and peripheral lymph were assessed in cholesterol-fed dogs [21,22]. Julien et al. evaluated cardiac and peripheral lymph

lipoproteins in dogs fed cholesterol and saturated fat [23]. Klein and Rudel studied the effect of a high-fat diet on thoracic duct lymph composition in non-human primates [24,25].

The present study was performed to evaluate the effects of a long-term high-fat diet and its reversal on lipids and lipoproteins distribution in thoracic duct lymph in pigs. This study complements a recently published paper in which we evaluated zoometric, cardiovascular, and metabolic parameters in response to long-term HFD feeding [23].

The porcine model was used in our study because in pigs, as in humans, a high-fat diet leads to the features of metabolic syndrome, and there are many similarities between the chemical and physical properties of lipoproteins in pigs and humans [27, 28].

Material and Methods

Animals and sample collection

The study was performed with 24 female Polish Landrace pigs (also known as white domestic sharp-ear pigs). The following inclusion criteria had to be met: body weight of 40 kg, normal clinical condition in physical examination, normal blood count, and normal serum concentrations of total protein, albumin, electrolytes, creatinine, and urea.

The pigs were housed in a single room, in 3 pens (every group in a separate pen), in which the temperature was maintained at 18–20°C and humidity was 60–75%. All pigs had free access to drinking water. The pigs were allocated into 3 groups: 1) Group 1 was the control group, which for 12 months was fed a standard, commercial, balanced low-fat diet that provided all the nutritional needs of young pigs. After reaching 60 kg in weight, the pigs were fed the diet for adult sows, with total energetic value of 2100 kcal/kg and which contained the following nutritional ingredients: proteins (14.7%), fat (3.1%), crude protein (4.7%), dry mass (90.44%), ash (6.06%), NaCl (0.5%), Ca (1.05%), P (0.77%), lysine (0.62%), methionine (0.24%), cystine (0.3%), threonine (0.48%), and tryptophan (0.183%) and the following vitamins: A (13243 IU/kg), D₃ (2000 IU/kg), E (81.65 mg/kg), B₁ (4.11 mg/kg), B₂ (7.16 mg/kg), PP (50.22 mg/kg), B₅ (24.29 mg/kg), B₆ (6.11 mg/kg), and B₁₂ (36 µg/kg). The pigs from the control group had limited access to food, in which caloric intake was calculated to be proportional to pig weight, but the maximum of calories allowed per day was 4200 kcal.

2) Group 2 was fed an unbalanced, high-fat diet (HFD) with 3200 kcal/kg energy content. This diet had a 5-fold higher fat content (by adding beef tallow), 13% higher protein content, and 4% higher sugar content than the diet fed to

the control group. The access to food was not limited in this group, and they consumed up to 4 kg of feed/pig/day (12 800 kcal/pig/day).

3) Group 3 was the reversal diet group (RD), which was fed an unbalanced HFD for 9 months (as described for group 2), and then, for the next 3 months, was fed a standard balanced diet (as described for group 1).

Body weight in all pigs was examined at the beginning of the experiment and every 3 months, and serum lipids were examined in all pigs at the end of the study. After 12 months, all animals were euthanized with an intravenous injection of pentobarbital (0.6 ml/kg; Morbital® Biowet, Puławy, Poland) after a 24-h fast. The thoracic duct lymph was obtained via direct puncture.

The study was approved by the First Local Ethics Committee for Animal Experimentation of the Institute of Immunology and Experimental Therapy in Wrocław, Poland (approval No. 23/2009). The study protocol adhered to the Guide for the Care and Use of Laboratory Animals, 8th edition, developed by the National Institute of Health (<http://www.ncbi.nlm.nih.gov/books/NBK54050/>).

Laboratory procedures

Blood samples

Venous peripheral blood samples were taken from pigs after 24-h fasting, using the Sarstedt S-Monovette system (Sarstedt AG & Co., Nümbrecht, Germany). Blood samples were centrifuged (1000×g for 15 min at 4°C). Serum samples were frozen at -80°C and stored until analysis. Total serum cholesterol (TC) and triglyceride (TG) levels were evaluated using enzymatic tests (Spinreact, Spain). Cholesterol concentration in HDL fraction was also determined using enzymatic tests from Spinreact, but after precipitation of very low-density lipoprotein (VLDL) and LDL fractions with the use of Quantolip tests (Immuno AG). In this method, a solution of polyethylene glycol (A-PEG 20 000, 95 g/L in 0.1 mmol/L sodium buffer, pH 6.5) leads to precipitation of VLDL and LDL fractions. After centrifugation, the entire HDL fraction is in the supernatant. Concentrations of TC, TG, and HDL were determined on a Beckman DU-650 spectrophotometer. LDL values were calculated using the Friedwald formula: $LDL = TC - (HDL + TG/5)$.

Lymph samples

Samples of lymph were taken from the thoracic duct, then frozen at -80°C and stored until analyses. Collected samples were used for evaluation of lipids and lipoproteins distribution. Lipoprotein fractions from lymph samples were isolated by sequential density-gradient ultracentrifugation,

as previously described [29]. The following lymph lipoprotein fractions were isolated: chylomicrons with fraction of $d < 1.006$ g/ml, which corresponded to the density range of VLDL; fraction of $1.006 < d < 1.063$ g/ml, which corresponded to the density range of LDL; and fraction of $1.063 < d < 1.21$ g/ml, which corresponded to the density range of HDL. Triglycerides, cholesterol, phospholipids, and lipoprotein fractions in lymph were analyzed using enzymatic methods (Biolabo, France). The concentrations of proteins in lymph were measured using a BC assay kit (Protein Assay Kit, Optima, Interchim, France).

Statistical analysis

Values of weight and serum lipids are presented as mean and \pm standard deviation [\pm SD]. Non-normally distributed values are presented as median with interquartile range (Q1–Q3). In comparisons of 2 groups (HFD+RD vs. control), we use the *t* test for statistical analysis of normally distributed values (weight after 6 and 9 months), and the non-parametric Mann–Whitney test was used for statistical analysis of non-normally distributed values (weight after 3 months). Differences among the 3 groups were calculated using ANOVA for normally distributed values (i.e., weight after 12 months, serum lipids, lymph/serum LDL ratio, and HDL: LDL ratio in lymph) and the Kruskal–Wallis test was performed for non-normally distributed values (i.e., lipids, proteins, phospholipids and lipoproteins in lymph, lymph/serum HDL ratio, and HDL: LDL ratio in serum). In the cases of statically significant results, post hoc analysis was performed. Differences in lipoproteins fractions in lymph between the study groups were assessed using the Kolmogorov–Smirnov test followed by an unpaired *t* test for normally distributed values or the Mann–Whitney *U* test for data that had a non-Gaussian distribution (GraphPad Software, CA, USA). The correlations between the study parameters were evaluated using Spearman's rank correlation test. In all calculations, $p \leq 0.05$ was considered as statistically significant.

Results

Weight and serum lipids profile in the study groups

Table 1 presents the results of changes in pigs' mean weights in the study groups. The pigs in the HFD group had a higher mean weight, but the differences among groups were not statistically significant. Table 2 shows serum lipid profiles in all the study groups. There were no statistically significant differences among the study groups.

Lipids, proteins, phospholipids, and lipoproteins in lymph

Lymph analysis revealed significantly higher levels of proteins in the HFD group in comparison to the RD and control groups.

Table 1. Changes in pigs' weight (means [±SD]) in the study groups during experimental diets.

		Control (n=8)	HFD+RD (n=16)	Statistics p value
Mean weight [kg] [±SD]	At the beginning	40	40	–
	After 3 months	129.13 [±10.01]	133.06 [±13.91]	0.430
	After 6 months	197.5 [±20.21]	202.88 [±19.96]	0.550
	After 9 months	234.25 [±29.42]	240.31 [±27.49]	0.620
		HFD (n=8)	RD (n=8)	
After 12 months	242 [±28.39]	263.75 [±21.95]	242 [±17.32]	0.120

Table 2. Lipids concentrations (means [±SD]) in serum after 12 months of experimental diets in the study groups.

Serum lipids parameters [mg/dl]	Control (n=8)	HFD (n=8)	RD (n=8)	Statistics (ANOVA) p value
Total cholesterol	75.89 [±10.84]	76.46 [±15.53]	75.46 [±6.96]	0.980
Triglycerides	30.46 [±24.50]	44.14 [±29.68]	44.19 [±19.05]	0.460
HDL	29.99 [±5.65]	30.11 [±9.02]	25.93 [±3.92]	0.370
LDL	39.81 [±8.41]	37.52 [±6.49]	40.70 [±7.53]	0.690

Because of the high heterogeneity of the results, there were no other statistically significant differences among the study groups in other parameters. However, the differences in concentrations of total cholesterol and HDL fraction between the HFD group and control group were close to the statistical thresholds of significance. Table 3 presents the concentrations of lipids, proteins, phospholipids, and lipoproteins in lymph and HDL: LDL ratios in lymph and in serum after 12 months of administering different diets in the study groups.

Composition of lipoproteins fractions in lymph

Analysis of lipoproteins fractions in lymph (Table 4) showed an increase in cholesterol, phospholipids, and proteins content within chylomicrons and HDL in the HFD group in comparison to the control group. There were also more triglycerides within chylomicrons in the HFD group in comparison to the control group, and there were more proteins within HDL in comparison to the RD group.

Relations between serum and lymph lipid concentrations

The high-fat diet seemed to have stronger effect on the concentration of HDL and LDL in the lymph in comparison to their concentrations in serum. There was no statistically significant correlation between HDL in serum and in lymph or between LDL in serum and in lymph in any study group. Table 5 presents lymph/serum ratios of HDL and LDL in our groups.

There were strong positive correlations ($p < 0.05$) between HDL and LDL concentrations in lymph in the HFD and RD groups (in the HFD group: $r = 0.88$; in the RD group: $r = 0.93$) and between triglycerides and chylomicrons in lymph in the same groups (in the HFD group: $r = 0.91$; in the RD group: $r = 0.91$). These differences were not statistically significant in the control group.

Discussion

The concentration of proteins in lymph after 12 months of fixed diets was significantly greater in the HFD group in comparison to the RD and control groups. Differences in total cholesterol and in HDL concentrations in lymph in the HFD group in comparison to the control group were close to the conventional level of statistical significance (Table 3). Analysis of lipoproteins fractions in lymph indicated that the higher protein concentration in the HFD group resulted from its increase within HDL and chylomicrons (Table 4).

The concentration of HDL in human peripheral lymph has been estimated in previous studies at about 15–20% of its plasma concentration [30–33]. In our study, HDL lymph/serum ratios in the control and RD groups were 13% and 14%, respectively, and in the HFD group the ratio was 23% (Table 5). Furthermore, the HDL: LDL ratio in lymph and the HDL: LDL ratio in serum in the control group were similar (0.83 and 0.85, respectively) (Table 3). This observation is consistent with earlier studies performed on sheep lung lymph [8] and on dog and pig cardiac

Table 3. Concentrations of lipids, proteins, phospholipids, and lipoproteins in lymph and HDL:LDL ratios in lymph and serum after 12 months of experimental diets in the study groups.

Parameters [mg/dl]	Control (n=8) median (Q1–Q3)		HFD (n=8) median (Q1–Q3)		RD (n=8) median (Q1–Q3)		Statistics (Kruskal-Wallis test) p value
Total cholesterol	61.62	(55.11–77.00)	128.78	(87.80–164.20)	97.35	(52.89–109.55)	0.058
Triglycerides	137.17	(115.21–216.50)	262.08	(219.21–637.29)	386.47	(119.45–423.28)	0.180
Proteins	52.66#	(46.46–56.02)	61.36#,#	(56.88–68.02)	49.89##	(39.81–56.17)	0.008*
Phospholipids	0.78	(0.65–0.79)	1.26	(1.03–1.71)	1.19	(0.56–1.29)	0.130
Chylomicrons	3.36	(1.69–4.31)	35.89	(6.05–96.23)	16.68	(2.41–44.37)	0.120
VLDL	27.72	(22.56–41.26)	34.68	(10.40–50.00)	39.90	(33.21–49.92)	0.680
LDL	5.33	(2.66–11.18)	8.24	(5.08–14.67)	7.89	(6.01–9.37)	0.490
HDL	4.41	(2.85–6.07)	8.01	(5.37–18.87)	6.25	(3.30–9.19)	0.098
HDL: LDL ratio in lymph	0.83	(0.72–1.70)	1.23	(0.86–1.41)	0.73	(0.56–0.84)	0.160
HDL: LDL ratio in serum	0.85	(0.53–0.95)	0.87	(0.64–1.02)	0.58	(0.55–0.70)	0.350

#, ## Differences between values that were statistically significant; * p statistically significant (p<0.05).

Table 4. Differences in lipoproteins fractions in lymph between the study groups. Only the values with P-values <0.05 are presented.

Fractions (means)	Groups	Lipoproteins			
		Chylomicrons	VLDL	LDL	HDL
Cholesterol	C vs. HFD	0.10 vs. 0.59	ns	ns	0.06 vs. 0.12
	C vs. RD	ns	ns	ns	ns
	HFD vs. RD	ns	ns	ns	ns
Triglycerides	C vs. HFD	0.24 vs. 1.92	ns	ns	ns
	C vs. RD	ns	ns	ns	ns
	HFD vs. RD	ns	ns	ns	ns
Phospholipids	C vs. HFD	0.08 vs. 0.49	ns	ns	0.08 vs. 0.17
	C vs. RD	ns	ns	ns	ns
	HFD vs. RD	ns	ns	ns	ns
Proteins	C vs. HFD	0.13 vs. 0.67	ns	ns	0.10 vs. 0.30
	C vs. RD	ns	ns	ns	ns
	HFD vs. RD	ns	ns	ns	0.30 vs. 0.12

ns – statistically not significant.

Table 5. Lymph/serum ratios of HDL and LDL after 12 months of fixed diets in the study groups.

parameters [mg/dl]	Control (n=8) mean value	HFD (n=8) mean value	RD (n=8) mean value	Statistics p value
Lymph/serum HDL ratio	0.13	0.23	0.14	0.270
Lymph/serum LDL ratio	0.23	0.27	0.26	0.910

lymph [7]. It was documented that the HDL/LDL ratio in lymph and the HDL/LDL ratio in plasma are the same in individual species [7]. However, in our study, the HDL: LDL ratio in lymph and the HDL: LDL ratio in serum in the HFD group were quite different (1.23 and 0.87, respectively) (Table 3).

HDL is involved in reverse cholesterol transport, carried from cells to the liver [3]. Free cholesterol from peripheral cells is transferred to HDL in the interstitial space, which starts the process of reverse cholesterol transport [3,4,34,35]. It has been demonstrated that the average size of HDL in lymph is larger than the average size of corresponding HDL in plasma, and that HDL in lymph contains more free cholesterol and phospholipids and has a higher cholesterol/protein ratio than HDL in plasma [30,36,37]. The higher HDL: LDL ratio in lymph in comparison to the HDL: LDL ratio in plasma and the increased cholesterol, phospholipids, and proteins content within HDL in lymph in our HFD group suggest that a high-fat diet enhances reverse cholesterol transport. Switching to a low-fat diet, as in the RD group, seems to have the opposite effect.

The relatively high concentration of LDL in the lymph from the thoracic duct in all our study groups is of some interest. The LDL concentration in human lymph obtained from the dorsum of the foot was estimated to be 10% that of plasma [10,12]. It was also demonstrated that after intravenous infusion of ^{131}I -labeled LDL into humans, the concentration of LDL radioactivity in the lymph was 1: 10 that in plasma [13]. In our study, the lymph/serum LDL ratio was 23% in the control group, 27% in the HFD group, and 26% in the RD group. Lymph in the thoracic duct is generally thought to be the mixture of post-nodal lymph and chyle from the intestine. Recent studies have shown that the lymph in the thoracic duct also comes from the hepatic lymphatic system [38]. The large pore structure of liver capillary endothelium probably allows particles the size of LDL to pass through and enter the hepatic lymphatic system [38]. In addition, we proposed the hypothesis that some LDL might also pass through the adventitia of large blood vessels to their adventitial lymphatic vessels. The number of adventitial lymphatic vessels increases with the progression of atherosclerosis, as assessed by intima-media thickness (IMT) [39]. Moreover, theoretically, there is also the possibility that LDL is produced by conversion of chylomicrons and VLDL in the thoracic duct [40]. However, analysis of cholesteryl ester compositions and apoprotein B electrophoretic mobility in chylomicrons and VLDL from thoracic duct in non-human primates and their plasma LDL concentrations have suggested that intestinal chylomicrons and VLDL probably are not transformed to plasma LDL particles [24].

The importance of the presence of LDL in lymph has not been determined. LDL lipoproteins are generally thought to transport cholesterol toward peripheral cells. However, it was

demonstrated that LDL, like HDL, also undergoes significant modification within interstitial spaces and LDL in lymph has increased cholesterol content in comparison to LDL in plasma [8,34]. Thus, although LDL can accept only the minor portion of free cellular cholesterol, it has been suggested that LDL is also involved in reverse cholesterol transport, a process usually explained by HDL passage [4,34,35], and this is supported by the strong positive correlations between HDL and LDL concentrations in lymph in the HFD and RD groups, not present in the control group.

The concentrations of triglycerides, VLDL, and chylomicrons in lymph from the thoracic duct were also relatively high in our study groups, taking into account the 24-h fasting. The concentrations of triglycerides and chylomicrons in lymph were especially high in the HFD and RD groups. The mean concentrations of triglycerides in lymph were over 6 times higher than in plasma in these groups. The large size of triglycerides-carrying lipoproteins is the major factor limiting their ability to cross the endothelium barrier [6, 10, 41]. The high level of chylomicrons in lymph and its strong positive correlation with triglycerides concentrations in our study suggest that triglycerides in lymph mostly come from the intestines. We speculate that the higher levels of chylomicrons and triglycerides in the HFD and RD groups are the result of altered gastrointestinal motility under the influence of a high-fat diet [42] or by changed permeability of lymphatic vessels for lipids within the intestine in the HFD and RD groups. The 24-h fasting was probably not enough time to absorb fat from the gut in these groups.

The biological task of VLDL is to transport lipids from the liver to fatty tissue. We assume that VLDL in lymph in our study came mostly from the hepatic lymphatic system. The greater concentrations of cholesterol, triglycerides, phospholipids, and proteins within chylomicrons in our HFD group in comparison to the control group probably reveal characteristic changes in chylomicrons structure due to the need to transport more dietary fat from the intestine to the liver under the influence of the high-fat diet. Klein and Kugel found no dietary cholesterol-induced differences in the apoprotein patterns of chylomicrons and VLDL in thoracic duct lymph, but the high-cholesterol diet was administered intraduodenally at a constant rate during 36 h in their study [24]. We presume that 36 h might be not enough time to evoke adaptation to a high-fat diet through increase in the amount of proteins within lipoproteins in lymph.

We are aware of some limitations of our study. Firstly, the small number of pigs in the study groups might have resulted in failure to find significant differences. Secondly, lymph in the thoracic duct is the mixture of lymph from peripheral regions, the intestine, and the liver, which makes it difficult to interpret lipoprotein sources and the changes under high-fat diet conditions, even with lymph sampling after 24-h fasting. Therefore,

further studies are needed to analyze the effects of specific diets on lymph from different parts of the lymphatic system (e.g., comparison of pre- and post-nodal lymph), and further research is warranted.

Conclusions

In conclusion, the long-term HFD significantly changed the structure of HDL and chylomicrons in the thoracic duct lymph. These alterations suggest that a high-fat diet enhances reverse cholesterol transport and, on the other hand, results in adaptation to more intense transport of dietary fat from the

intestine to the liver. Reversal to a standard balanced diet has the opposite effects.

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Conflict of interest

None.

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