DIURNAL VARIATIONS
IN
HUMAN TRIGLYCERIDE METABOLISM

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE AAN DE RIJKSUNIVERSITEIT TE LEIDEN, OP GEZAG VAN DE RECTOR MAGNIFICUS DR. A. A. H. KASSENAAR, HOOGLERAAR IN DE FACULTEIT DER GENEESKUNDE, VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN TE VERDEDIGEN OP DONDERDAG 23 JUNI 1983 TE KLOKKE 15.15 UUR

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STELLINGEN

1. De vorm van het 24-uurs patroon van de triglyceridenspiegel in het serum bij normale personen en patiënten met primaire hyperlipidaemie type IV is afhankelijk van de frequentie en distributie van maaltijden, maar niet van de samenstelling daarvan.
   - dit proefschrift

2. De toename van de lipoproteïnelipaseactiviteit in het plasma na heparinetoediening, die bij normale personen op een koolhydraatrijk dieet in de loop van de dag optreedt, wordt veroorzaakt door een toename van de lipoproteïnelipaseactiviteit van vetweefsel.
   - dit proefschrift

3. Patiënten met primaire hyperlipidaemie type IV, die een normale glucosetolerantie en een normaal lichaamsgewicht hebben, vertonen bij gebruik van een koolhydraatrijk dieet geen toename van de lipoproteïnelipaseactiviteit in post-heparineplasma in de loop van de dag. Dit moet worden toegeschreven aan het uitblijven van een toename van de lipoproteïnelipaseactiviteit van vetweefsel.
   - dit proefschrift

4. De veronderstelling dat insulineresistentie (definitie volgens Kahn) de lipoproteïnelipaseactiviteit van vetweefsel zou beïnvloeden, wordt zowel door onderzoek van Hansen c.s. als door onze eigen bevindingen onwaarschijnlijk gemaakt.
   - Kahn CR, Metabolism 1978; 27: 1893-1902.
   - Hansen FM c.s., Diabetologia 1983; 24: 131-135.
   - dit proefschrift

5. De bepaling van lipoproteïnelipaseactiviteit dient bij voorkeur niet te geschieden in een Tris-HCl buffer waarin tevens heparine aanwezig is.

6. Aangezien apolipoproteïne E van normale personen de lipoproteïnelipaseactiviteit stimuleert en een verhoogde frequentie van het variante apo E_4 fenotype bij patiënten met hyperlipidaemie type V geconstateerd is, verdient het aanbeveling nader onderzoek te verrichten naar het effect van apo E_4 op de lipoproteïnelipaseactiviteit.
   - Yamada N c.s., Biochem Biophys Res Comm 1980; 94: 710-715.
   - Ghiselli G c.s., Lancet 1982; 405-407.

7. Het is onjuist vrouwen tijdens de zwangerschap met insuline te behandelen, zolang zij normale glucosewaarden in het bloed hebben.

8. Beenmergtransplantatie vormt een veelbelovende therapie voor de infantiele, maligne vorm van osteopetrosis.
   - Sieff CA c.s., Lancet 1983; 437-441.

9. De bevinding dat het meerdere malen hanteren van een wegwerpspuit voor het subcutaan injiceren van insuline uit oogpunt van infectiegevaar gerechtvaardigd is, kan leiden tot een aanzienlijke kostenbesparing in de gezondheidszorg.
   - Collins BJ c.s., Lancet 1983; 559-560.

10. Het verbod een medisch beroep uit te oefenen zonder vergunning van de overheid als bedoeld in artikel 52 van de Wet voorzieningen gezondheidszorg vormt een onbehoorlijke inbreuk op het recht van vrije keuze van vestigingsplaats van de medische beroepsbeoefenaar.
    - Wet voorzieningen gezondheidszorg. Staatsblad 1982 no 563.

11. Het feit dat stellingen behorende bij een proefschrift gretiger gelezen worden dan het proefschrift doet geen recht aan de inspanningen van de auteur.

Leiden, 23 juni 1983 C. Pagano Mirani-Oostdijk

Aan mijn ouders
Aan Hanso

Cover: Study of waves by Hokusai (1760-1849), Japanese artist.

CONTENTS

ABBREVIATIONS

apo	apolipoprotein
AT	adipose tissue
d	density
FFA	free fatty acid
g	gravity
HDL	high density lipoprotein
HL	hepatic lipase
HLP	hyperlipoproteinaemia
IBW	ideal body weight
IU	international unit
K_m	Michaelis-Menten constant
LDL	low density lipoprotein
LPL	lipoprotein lipase
ns	non significant
PHLA	post-heparin lipolytic activity
SD	standard deviation
SEM	standard error of mean
SM	skeletal muscle
TG	triglyceride
VLDL	very low density lipoprotein
V_{max}	maximal reaction rate

Chapter I

GENERAL INTRODUCTION

Transport of lipids

Apart from FFA, the lipids in the blood are transported in the form of complex structures, called lipoproteins. These lipoproteins have a hydrophobic core, built up of lipids such as triglycerides and cholesterolesters, which is surrounded by a layer of the amphipathic constituents free cholesterol, phospholipids and apoproteins. In man, four major classes of lipoproteins are recognized, varying in the concentration of the various lipids and proteins and in size. The biggest lipoproteins, visible by light microscopy, are the chylomicrons. Further, in order of decreasing size, VLDL, LDL and HDL are present. The lipoproteins can be separated in several ways. Ultracentrifugation isolates them on the basis of density; electrophoresis separates them on the basis of electric charge. Table I shows compositional data of the lipoproteins, their size, density and electrophoretic properties.

The various lipoprotein classes have different functions in lipid transport (1, 2). In brief: chylomicrons are the particles which are synthesized in the intestine after consumption of fat. Via the lymphatic system they reach the bloodstream. Their function is to transport exogenous triglyceride and cholesterol to tissues. They are stepwise depleted of triglyceride by the hydrolysing action of LPL, an enzyme present at the luminal side of the vessel wall in adipose tissue and skeletal muscle. The remaining chylomicron-remnant, which is relatively rich in cholesterol, is probably taken up by the liver and further metabolized (3, 4). VLDL are predominantly synthesized by the liver and transport endogenous triglyceride, which is synthesized from glycerol and FFA. FFA are either derived from the blood or synthesized *de novo* from glucose. These particles share their removal pathway with chylomicrons (5) and finally end up as cholesterol-rich LDL. LDL transports endogenous cholesterol to peripheral cells, where the entire particle is internalized after binding to the LDL receptor. Intracellularly the cholesterol component of the LDL particle has a function in regulating cellular cholesterol synthesis (6). HDL, finally, is synthesized by the liver and intestine in the form of bilayered discs, called nascent HDL, which are composed of phospholipids, free cholesterol and apoproteins. In the blood these discs change into spherical particles by the action of lecithin-cholesterol acyltransferase (LCAT), which transfers FFA from lecithin to free cholesterol, after which the apolar cholesterolester moves to the inside of the particle. Part of the circulating HDL is derived from the catabolism of TG-rich lipoproteins. As a result of lipolysis of TG-rich particles by LPL, their core becomes smaller and fragments of the polar wall, consisting of free cholesterol, phospholipid and protein, are split off and taken up by the dense HDL subfraction, thereby inducing a shift towards less dense HDL particles (7, 8, 9). Whether this process results in the formation of new HDL particles or merely induces a shift between HDL subfractions is not entirely known (10). The function of HDL is also speculative, although a role in the clearance of excess cholesterol from the periphery has been suggested (9, 11). HDL-cholesterol is probably taken up by the liver, the adrenal glands and the ovaries by the action of the enzyme hepatic lipase, or a closely related enzyme (12).

Thus the metabolism of chylomicrons, VLDL, LDL and HDL appears to be closely interrelated.

Disturbances in the lipid transport system can be present at all levels. Fredrickson proposed a classification of the various hyperlipoproteinaemias which has been accepted worldwide (13, 14). In table II this classification is shown. Some forms of HLP, notably type II and type III and to a lesser extent also type IV, have attracted clinical attention because of their association with atherosclerosis. This thesis is concerned with lipid metabolism in normal persons and in patients with type IV HLP.

Type IV hyperlipoproteinaemia

Type IV HLP is a frequently occurring disorder. Lewis et al. reported that 14% of men and 3.5% of women aged 20-69 years had fasting serum triglyceride levels exceeding 2 mmol/l (15). Type IV HLP can either be present in a primary form (called primary endogenous hypertriglyceridaemia), or can be secondary to various illnesses, of which diabetes and chronic renal failure are the most important. Also during alcohol abuse and treatment with estrogens or glucocorticoids hypertriglyceridaemia is frequently encountered. When the degree of hypertriglyceridaemia increases, type IV HLP may develop into type V. Apart from atherosclerosis, clinical manifestations of the disease are: disseminated eruptive xanthomas, hepatosplenomegaly, lipaemia retinalis and, less often, episodes of acute abdominal pain and gouty arthritis. Hyperuricaemia was found present in one third of the patients with type IV or V HLP (16).

Pathogenesis of primary endogenous hypertriglyceridaemia

Primary endogenous hypertriglyceridaemia is frequently encountered in association with disturbances in glucose homeostasis and obesity. A number of biochemical defects has been suggested as the underlying cause of endogenous hypertriglyceridaemia; these can be classified into those that lead to increased synthesis or to decreased breakdown of TG-rich particles. A variety of methods has been developed to demonstrate these defects.

Reaven et al. consider overproduction of VLDL by the liver as a major cause for hypertriglyceridaemia (17). In studying a heterogeneous group of type IV patients, including subjects with mild diabetes and mild obesitas, they found a significant positive correlation between post-prandial insulin responses and VLDL secretion rate (18). They considered the high insulin levels observed in their subjects to be a result of insulin resistance and thought that these would increase hepatic TG synthesis from carbohydrate sources. This hypothesis is based on studies in which insulin was shown to increase VLDL synthesis from ^{14}C glucose in rat liver (19, 20). It has been criticized by others since it does not take into account that the liver may also be insulin resistant (21). Kissebah et al. have adopted the view that over-

Table I. Characteristics of the 4 major lipoprotein classes in normal human plasma.

	range of particle diameter (nm)	density (g/l)	electrophoretic mobility	triglyceride content (%)[1]	cholesterolester content (%)[1]	free-cholesterol content (%)[1]	phospholipid content (%)[1]	protein content (%)[1]	main apoproteins
Chylomicrons	75-..1000	<0.96	remain at origin	85	3	1	9	2	C,B,A
VLDL	30-80	0.96<d<1.006	pre-β	50	12	7	18	10	C,B,(E)
LDL	19-25	1.006<d<1.063	β	10	37	8	20	23	B
HDL	4-10	1.063<d<1.21	α	4	15	2	24	55	A,C, (E)

[1] lipid and protein contents are expressed in approximate percentages of total weight (1).

Table II. The hyperlipoproteinaemias.

type	chylomicrons	VLDL	LDL	floating β lipoprotein[1]
I	↑			
IIa			↑	
IIb		↑	↑	
III				↑
IV		↑		
V	↑	↑		

↑ indicates an elevation above 'normal' concentrations (1, 2).
[1] also known as 'broad β-lipoprotein'.

production of VLDL would be related to the increased FFA flux observed in type IV patients (21). They found no correlation between the TG production rate and the elevated insulin responses to oral glucose. The high FFA flux was considered to be a result of a diminished antilipolytic action of insulin due to the presence of peripheral insulin resistance. Insulin thus appears to play a role in the development of hypertriglyceridaemia. However, hyperinsulinaemia is not invariably present in this disorder (22). Many other authors have demonstrated overproduction of VLDL by various methods (23, 24) but normal production rates have also been observed (25, 26).

On the other hand, defects in the catabolism of TG-rich particles have been reported. Rössner found lower fractional catabolic rates of exogenous triglyceride after a bolus injection of Intralipid® in patients with type IV HLP than in normal subjects and attributed this to a defect in the removal mechanism of TG-rich lipoproteins (27). Boberg reported lower activities in LPL, released by an intravenous injection of heparin, in a group of patients with hypertriglyceridaemia as compared to normal subjects (28), a finding which was later confirmed by Huttunen et al. (29). Several authors observed lower LPL activities in pieces of adipose tissue from type IV patients than from normals (30, 31, 32), although normal activities have also been reported (33). A reduced LPL activity was also observed in pieces of skeletal muscle from type IV patients (32). By directly measuring the secretion of triglyceride from the splanchnic organs both Havel (34) and Boberg (35) found higher rates of synthesis in patients than in normals, although there was a considerable overlap. Since Havel found no correlation between TG secretion rate and serum TG concentration, and Boberg observed a lower fractional turnover rate of labeled VLDL in patients, they both concluded that defects in the removal mechanism were predominantly responsible for the hypertriglyceridaemia.

The conclusion which may be drawn from the apparent contradictions in many of these studies is that type IV HLP is a disorder of heterogeneous etiology. Overproduction of VLDL might lead to hypertriglyceridaemia when the normal

removal mechanism becomes overloaded, while, on the other hand, a defect in the removal system might induce hypertriglyceridaemia at a normal production level. Both defects may also occur simultaneously (34, 35, 36).

The effect of carbohydrate feeding on serum TG levels

In 1961 Ahrens et al. reported that reduction of the amount of carbohydrates in the diet lowered the level of serum TG in a group of patients with type IV HLP and that serum TG promptly rose when the carbohydrate intake was restored (37). Subsequently it was found that normal subjects also showed an increase in basal serum TG levels when their carbohydrate intake was increased. This was found to be a temporary phenomenon, which usually disappeared after a period of between 1-± 6 months (38). On feeding high carbohydrate diets to type IV patients, various authors found that, although most patients showed a moderate increase in fasting serum TG, in some of them an abnormally high increase was observed (22, 39). For this reason patients with type IV HLP were advised to keep their carbohydrate intake low.

The mechanism of this carbohydrate-induced increase in fasting serum TG appears to be two-fold. Farquhar et al. studied normal subjects and hyperlipidemics on a fat-rich as well as on a carbohydrate-rich diet and observed that the insulin response to oral glucose as well as the integrated insulin levels during the day correlated well with the degree of carbohydrate inducibility (40). They suggested that high insulin levels resulting from carbohydrate-rich food would increase hepatic TG synthesis. Also in rats carbohydrate-rich food was reported to stimulate FFA synthesis from glucose, which would lead to an increased TG production (41). On the other hand, Mancini et al. reported a lower fractional catabolic rate of Intralipid® and a lower post-heparin LPL activity on carbohydrate-rich as compared to normal food in normal subjects (42), which would indicate a reduction in TG removal.

If a permanent state of carbohydrate induction were a general cause for hypertriglyceridaemia, reduction in carbohydrate intake should normalize the disorder in all patients. However, studies of Schönfeld and Kudzma showed that restriction of carbohydrate normalized TG levels in only 1 out of 18 patients (39). This finding suggests that abnormal carbohydrate inducibility is limited to a small subgroup of type IV patients.

In this country carbohydrate-rich food is since long recommended for the dietary treatment of diabetes, since it should increase the insulin sensitivity in the peripheral tissues (43, 44). In 1979 the Committee on Food and Nutrition of the American Diabetes Association also recommended carbohydrate-rich food for diabetic patients (45). Restriction of fat intake with a concomitant increase in carbohydrate intake is nowadays considered beneficial for the whole population, including diabetics and hyperlipidaemics, in the prevention of atherosclerosis (46, 47).

Lipoprotein lipase (LPL) and methods for determining its activity

LPL, or 'clearing' factor, is the enzyme discovered by Hahn in 1943 in dogs (48). He observed that intravenous administration of heparin caused a 'clearing' of the alimentary lipaemic blood plasma. Korn in 1955 observed that the 'clearing' factor was identical to lipoprotein lipase present in heart-tissue in the rat (49). Later, LPL was also shown to be present in other tissues such as adipose tissue, skeletal muscle, lung and lactating mammary gland, and it was also found in milk (50, 51). Adipose tissue and skeletal muscle account for the major part of total LPL activity in the body (50, 51).

LPL hydrolyses triglyceride present in chylomicrons and VLDL. It is also capable of hydrolysing phospholipid (52), which explains why it can penetrate the outer shell of the lipoproteins to reach the TG-rich core. LPL requires apoprotein C-II for the full expression of its activity. In vivo apo C-II is present on the natural substrate after transfer from HDL, which serves as a reservoir for apo C-II (53). When an artificial substrate is used in vitro, apo C-II has to be added in the form of serum (54) or synthetic apo C-II. Another characteristic of LPL is the inhibition of its activity by high salt concentrations (50, 51).

LPL is synthesized in parenchymal cells. In most studies the adipocyte and the skeletal muscle cell have been used as a model. In the adipocyte the synthesis of the LPL pro-enzyme is stimulated by insulin (55, 56, 57) and glucocorticoids 50, 55). After synthesis the pro-enzyme becomes activated, possibly due to glycosylation (50, 56), but perhaps also by other factors. The next step is the transport to the outside of the cell, which is brought about by the microtubular system (58), and for which glycosylation is a requisite (50). In muscle cells (including heart cells) a similar way of LPL synthesis and secretion is believed to be present (50), but in these cells LPL synthesis seems to be unaffected (59), or inhibited (60) by insulin. Although glucagon was shown to increase muscle LPL activity in vivo (61), in vitro no such effect could be observed (59).

Once outside the cell, the enzyme is transported to the capillary endothelium, where it becomes attached to heparansulphate which lines the vessel wall (50). This is the place where LPL is believed to function in vivo. The FFA produced by hydrolysis of lipoprotein-TG are believed to be transported to the tissue cells by lateral diffusion in cell membranes (62), and glycerol is released into the blood.

Since heparin has a structure analogous to that of heparansulphate, it can release LPL from its binding site by competition (50). On this principle some methods of determining LPL activity are based. When heparin is injected intravenously, LPL appears rapidly in the plasma. This fraction is thought to represent the physiologically active fraction of the enzyme (63, 64). Up to now the fractions originating from adipose tissue and skeletal muscle cannot be separated from each other. Simultaneously with LPL another enzyme, called hepatic lipase, is released into the circulation, which is also capable of hydrolysing triglyceride from the TG-rich lipoproteins, although to a lesser extent than LPL. The total activity released by heparin is called post-heparin lipolytic activity. LPL can be separated from HL in

various ways, but the most elegant method is based on immunological differences (64). LPL can also be released from pieces of adipose tissue and skeletal muscle, again by means of heparin. These so called heparin eluates are thought to represent the physiologically active enzyme (65).

A second method of obtaining LPL from tissue pieces makes use of acetone-ether powders which are prepared from tissue specimens. To this purpose these specimens are homogenized and extracted by repeated additions of acetone and ether (66). The resulting powder is dissolved in or extracted with buffer and LPL activity can be measured. This method should determine intracellular as well as extracellular enzyme.

Outline of the present investigation

Parameters of triglyceride metabolism have mostly been studied in the fasting condition, whereas the major part of daily life is spent in the fed state. To gain insight in diurnal TG metabolism, Terpstra et al. studied the diurnal course of serum triglyceride and some lipoproteins and hormones in normal male subjects in a steady state on a carbohydrate-rich diet, at varying meal frequencies (67). We have extended these studies and in order to see whether the data were dependent on the kind of food, we compared them with those obtained in 6 normal male subjects on a fat-rich diet. Chapter 2 describes the results of this study. In Chapter 3 we report corresponding data on 3 male patients with a primary form of type IV hyperlipoproteinaemia on a carbohydrate-rich as well as on a fat-rich diet, and compare these data with those obtained in normal subjects. To avoid interference with disorders in glucose homeostasis, our patients were selected on the basis of a normal oral glucose tolerance test.

After recognition of the diurnal pattern of serum triglyceride and related parameters under standardized conditions, we have tried to determine the factors responsible for this pattern, especially those related to TG catabolism. As reported above, the enzyme LPL is responsible for the breakdown of TG-rich particles. To measure the physiologically active fraction of LPL from different tissues, we developed an assay in which heparin eluates of tissue biopsies could easily and accurately be determined. In Chapter 4 these results are given. In Chapter 5 we have tried to explain the diurnal TG pattern of normal subjects on a carbohydrate-rich diet with the help of LPL activity measurements in biopsies of adipose tissue and skeletal muscle, as well as in post-heparin plasma. We have also tried to relate changes in LPL activity to changes in insulin and glucagon levels. Besides, the impact of changes in TG levels on the other lipoproteins, especially on HDL and its subfractions, was studied. Chapter 6 gives the results of a corresponding study in patients with type IV hyperlipoproteinaemia. Data on patients were compared with those found in normal subjects and an effort was made towards explanation of their diurnal TG pattern and high TG levels.

Apart from the enzymatic aspect of TG catabolism the susceptibility to lipolysis of the TG-rich particles themselves might influence the rate of TG catabolism. In

order to observe possible changes in this susceptibility during the day, we studied serum samples, obtained from the subjects described in Chapter 5 and 6 at several times during the day, with respect to lipolysis by a standard LPL preparation. The relation of the results to the TG pattern is discussed and comparison between data of patients and normals is made in Chapter 7.

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Chapter II

INFLUENCE OF MEAL FREQUENCY ON DIURNAL LIPID, GLUCOSE AND INSULIN LEVELS IN NORMAL SUBJECTS ON A HIGH FAT DIET; COMPARISON WITH DATA OBTAINED ON A HIGH CARBOHYDRATE DIET

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ABSTRACT

Diurnal levels of serum triglyceride (TG) were measured in six normal persons consuming a fixed solid 65% fat diet under steady state conditions in a metabolic unit. The food was divided into either three or eight similar portions, differently spaced over the day and night.

The diurnal TG-profiles on this diet were practically identical to those found under comparable conditions on a 65% carbohydrate diet (1). Mean diurnal TG values did not significantly differ with varying meal frequency. Free fatty acid levels, however, were significantly higher on a high fat diet.

Post-prandial glucose and insulin responses did not significantly differ whether a high fat diet or a high carbohydrate diet was consumed.

We conclude that the composition of the diet is of little importance in determining diurnal TG patterns when the diet consists of normal food stuffs, but that these patterns are dependent on meal frequency and distribution.

INTRODUCTION

In a previous study we found that normal subjects on a fixed 65% carbohydrate diet, divided in either three or eight equivalent meals, showed wavelike diurnal serum TG patterns, with minimum values between 03.00 and 05.00 hours and maxima around 15.00 hours. Rhythmic feeding at 3 h intervals of eight equivalent meals resulted in a different pattern with lower TG values at daytime than at night (1).

In order to see whether these diurnal variations were dependent on the composition of the diet, we extended this study to one in which a solid 65% fat diet was given under comparable circumstances.

MATERIALS AND METHODS

Participants were six healthy male students, two of whom had also participated in a study on carbohydrate-rich food. They had a normal glucose tolerance and normal fasting TG values on their usual food. For 2 weeks before admission they consumed a 65% fat diet, which was continued during their stay in the metabolic unit. Informed consent was obtained.

The diet was individually adjusted in order to keep body weight constant. It comprised normal foods consisting of 65 cal% fat (one-third polyunsaturated), 23 cal% carbohydrate (one-third mono- and disaccharides) and 12 cal% protein (meat, cheese, etc.).

Food was divided into three or eight similar portions, distributed at 09.00, 13.00 and 17.00 hours (A_1 and A_2 period), every 2 h from 09.00 until 23.00 hours (B period), or evenly spaced over the day and the night (C period). Every period

lasted 6 days. Test-conditions were the same as in a previous study (1). At the sixth day of each period (A_1–B–C–A_2) we measured diurnal profiles of blood glucose, insulin, cholesterol, TG (triglyceride) and FFA (free fatty acids). In one subject the main serum lipoprotein classes were also determined. Laboratory techniques used were the glucose-oxidase method modified by Van der Slik et al. (2), the radio-immunoassay according to Berson & Yalow for insulin (3), the colorimetric method according to Giegel et al. for TG (4), the colorimetric method according to Abell et al. for cholesterol (5) and the method according to Regouw et al. for FFA (6). Lipoprotein lipids were determined gravimetrically by a combination of ultra-centrifugation, precipitation and Folch extraction (7). Statistical analysis was generally done by a multiway analysis of variance for a split-unit (split-plot) design, i.e. *P*-values for the main unit, according to Armitage (8).

RESULTS

Fig. 1 shows the diurnal course of mean TG levels (with ranges) in periods A_1, B, C and A_2 in six subjects. The diurnal TG profile is in all periods similar to that found on a carbohydrate-rich diet (1). The post-prandial peaks, more conspicuous on a fat-rich than on a carbohydrate-rich diet, are caused by chylomicrons, as can be seen from the lipoprotein profile of one subject (Fig. 2). However, the major contribution to the wavelike TG pattern is again made by VLDL-TG, as was also found in subjects on a carbohydrate-rich diet (1).

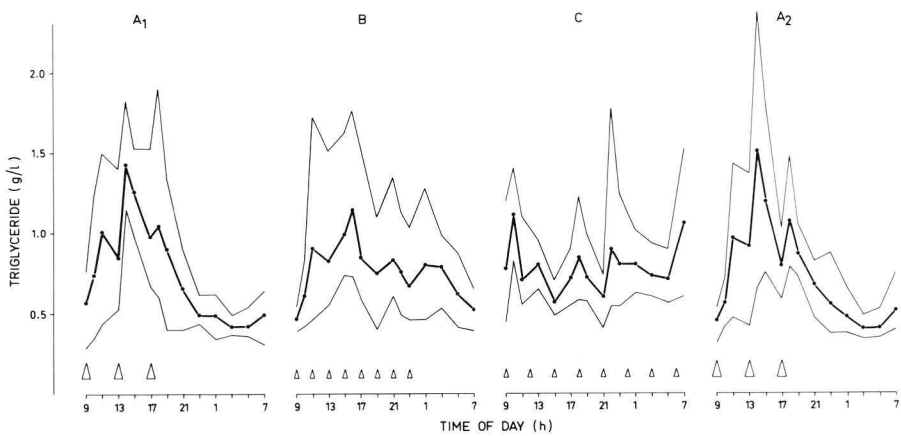

Figure 1. Diurnal course of triglyceride levels on fat-rich diet. Mean of six subjects with range. Triangles indicate timing of meals.

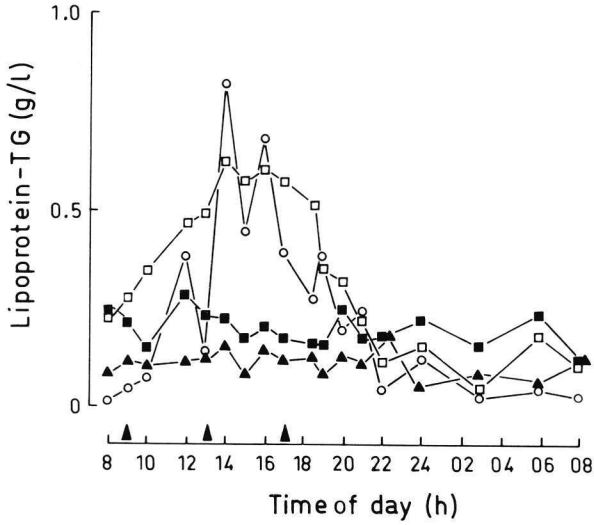

Figure 2. Diurnal course of lipoprotein-TG of one subject on a fat-rich diet. Triangles indicate timing of meals. ○, Chylomicrons; □, VLDL; ■, LDL; ▲, HDL.

No significant differences in mean diurnal TG levels in the four periods could be found ($P = 0.16$). Mean TG levels seemed to be lower than those previously found in normal subjects on a carbohydrate-rich diet (1). Because individual differences between the participants of both groups could be responsible for this, we compared the data of two subjects who participated in both dietary studies, by which we could confirm that on fat-rich food mean TG levels are lower ($P < 0.0003$ for subject I and <0.01 for subject II, standard multiway analysis of variance) than on a carbohydrate-rich diet (Table 1).

Mean FFA levels (Fig. 3) were markedly higher on fat-rich than on carbohydrate-rich diet ($P < 0.005$).

Mean cholesterol levels did not show significant diurnal fluctuations.

Post-prandial glucose and insulin increments were not significantly different from those found previously (9) in normal subjects on a carbohydrate-rich diet. In the A_1 and A_2 periods insulin but not glucose increments after breakfast are significantly greater than those after subsequent meals ($P = 0.001$), as was also seen on carbohydrate-rich food (9). In the B and C periods no such differences were apparent.

Table I. TG values (g/l) of two subjects, either on a 65% carbohydrate diet or a 65% fat diet.

		Subject I		Subject II	
	Time (hours)	Carbo-hydrate	Fat	Carbo-hydrate	Fat
A_1	09.00	1.09	0.59	0.58	0.47
	13.00	2.26	1.22	0.94	0.59
	17.00	2.67	1.18	0.76	0.76
B	09.00	0.78	0.68	0.64	0.45
	13.00	1.04	1.53	0.74	0.75
	17.00	1.05	1.01	0.80	0.77
C	09.00	2.80	1.50	0.78	0.80
	13.00	1.29	0.97	0.88	0.83
	17.00	1.86	1.22	0.70	0.86
A_2	09.00	1.93	0.50	0.66	0.44
	13.00	2.99	1.41	1.23	0.79
	17.00	3.03	1.53	0.78	0.58
		$P < 0.0003$*		$P < 0.01$*	

* Multiway analysis of variance.

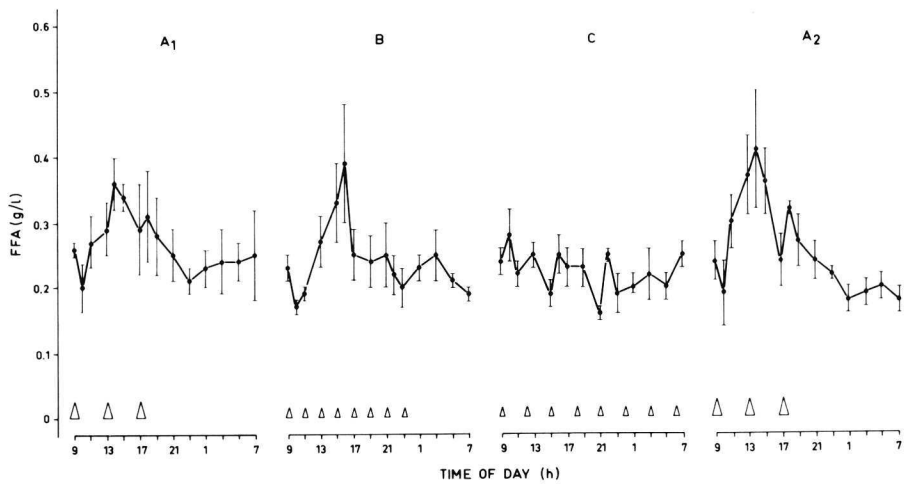

Figure 3. Diurnal course of FFA levels on fat rich diet. Mean of five subjects with error bars.

DISCUSSION

Apparently the diurnal TG pattern in normal subjects is hardly dependent on the composition of the meals, but is related to meal frequency and probably even more to meal distribution. Again, as in the first study, without food intake TG levels in the A periods show a rise early in the morning. On either fat-rich or carbohydrate-rich diet the TG profile is flattened out in the B period and even shows an inversion in the C period as compared to the A periods, although mean TG levels in the four periods are not significantly different. These results provide evidence that frequent meals decrease the amplitude of fluctuations in serum TG levels and thus might be favourable.

Our finding in two subjects having both lower fasting and post-prandial TG levels on a fat-rich than on a carbohydrate-rich diet is in accordance with Schlierf (10).

Post-prandial FFA levels are said to be the result of two opposing forces: the one decreasing because of inhibition of lipolysis by providing glucose, the other increasing as the result of hydrolysis of nutrient derived TG-rich particles. This would explain the dip in FFA following breakfast in periods A_1, B and A_2 and the rise afterwards. We also found considerably higher FFA levels on the high fat diet as compared with the carbohydrate-rich diet, which again is in accordance with data from Schlierf (10, 11).

Post-prandial glucose and insulin responses were not significantly different from those found on a carbohydrate-rich diet (9). Neither did we find significant differences in the two subjects who participated in both studies. This is contrary to the results obtained by Schlierf (12) in subjects on formula diets.

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Chapter III

DIURNAL LEVELS OF LIPIDS, GLUCOSE AND INSULIN IN TYPE IV HYPERLIPIDEMIC PATIENTS ON HIGH CARBOHYDRATE AND HIGH FAT DIET: COMPARISON WITH NORMALS

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ABSTRACT

Diurnal levels of serum triglyceride (TG), cholesterol, free fatty acids (FFA), glucose and insulin were measured in three type IV hyperlipidemic patients on a fixed solid 65% carbohydrate and a 65% fat diet when in steady state conditions in a metabolic unit. The carbohydrate-rich food was divided into either three or eight equivalent portions, differently spaced over the day and night. The fat-rich food was given in three equivalent portions only. The diurnal TG profiles on these diets showed the same characteristics as those found in normals, but increments and mean levels were considerably higher. On the carbohydrate-rich diet, mean TG levels decreased during the study. This was not seen either on the fat-rich diet or in normals. In contrast to our findings in normals, chylomicrons formed the major contribution to the serum TG pattern. FFA levels were markedly higher on the high fat than on the carbohydrate-rich diet, but not different from those in normals. Postprandial glucose responses did not differ significantly between the diets. Insulin responses were markedly higher on the carbohydrate-rich than on the fat-rich food. Glucose levels did not differ from those in normals. Insulin levels were significantly higher in the patients. Cholesterol showed minimal fluctuations, parallel to the TG pattern, which could be attributed to chylomicron cholesterol.

INTRODUCTION

Hypertriglyceridemia (HTG) may be considered a risk factor for atherosclerosis (5, 6), although recent reports have cast some doubt on this (10). An increased production of triglyceride (TG)-rich particles by the liver (11, 17), a decreased clearance of such particles (4, 9, 21) or a combination of the two (16) have been proposed as the underlying abnormality. The diagnosis of HTG usually refers to fasting TG levels. It seems unlikely, however, that only elevated fasting levels will increase the risk for atherosclerosis and that variations during the rest of the day will have no influence.

Only a few reports dealing with hypertriglyceridemic patients have rendered an account of 24-hour patterns (24, 25, 26, 27). We have recently reported diurnal levels of lipids, insulin and glucose in normals in steady state on carbohydrate- and fat-rich diets (7, 29). This study has now been extended to type IV hypertriglyceridemic patients, who were investigated in the same manner as the normals. As the patients received a carbohydrate-rich as well as a fat-rich diet, we were able to measure an overall response to food intake as well as differences between the two diets. In general, TG patterns were very similar to those observed previously in normals. The significance of this observation is discussed.

PATIENTS AND METHODS

Participants in this study were three type IV hyperlipidemic men with normal glucose tolerance tests. Clinical and biochemical data are given in Table I. They were studied first on a carbohydrate-rich and then on a fat-rich diet. The carbohydrate-rich diet contained 65 cal% carbohydrate (one third mono- and disaccharides), 23 cal% fat (one third polyunsaturated) and 12 cal% protein (meat, cheese, etc.). The fat-rich diet was composed of 65 cal% fat (one third poly-unsaturated), 23 cal% carbohydrate (one third mono- and disaccharides) and 12 cal% protein. The diet comprised only normal foods, no alcohol, and was adjusted individually in order to keep the body weight constant.

Table I. Clinical and biochemical data on the test subjects.

Subj. no.	Age (y.)	Height (cm)	Weight (kg)	Fasting TG level on 'home' diet (g/l)	Clinical signs	Caloric intake during study* (kcal/d.)
1	35	176	80.0	4.98	Coronary occlusion, myocardial infarction	3003
2	46	167	75.8	4.55	Normal ECG, gout	2669
3	28	180	97.7	7.21	Normal ECG, gout	3286

* Fixed according to intake when at home.

For two weeks before admission the patients consumed either the 65% carbohydrate diet or the 65% fat diet at home, in order to become metabolically adapted. They continued on these diets during their stay in the Metabolic Unit. Their clinical and biochemical data were the same before each admission. Informed consent was obtained.

During admission, the carbohydrate-rich diet was divided into three or eight similar portions and served at 9.00, 13.00 and 17.00 h (A1 and A2 period), every 2 hours from 9.00 until 23.00 h (B period), or evenly distributed over the day and night (C period). On the fat-rich diet, only consecutive A periods were studied. Each period lasted for 6 days. Test conditions were the same as in previous studies (7, 29). On the sixth day of each period, diurnal levels of blood glucose, insulin, cholesterol, TG, free fatty acids (FFA), and (only in period A) the main serum lipoprotein classes (chylomicrons, VLDL, LDL+HDL) were measured. Blood was taken via an indwelling catheter, which was kept patent with saline. Serum for lipid determination was kept at 4°C for a maximum of two days.

Laboratory techniques applied were the glucose-oxidase method modified by van der Slik et al. (28), the radioimmunoassay according to Berson and Yalow for insulin (3), the colorimetric method according to Giegel et al. for TG (8), the colorimetric method according to Abell et al. for cholesterol (1) and the method of

Regouw et al. for FFA (20). Lipoprotein lipids were determined gravimetrically by a combination of ultracentrifugation, precipitation and Folch extraction (18). The chylomicron fraction was defined as the lipoprotein fraction which floats after 30 min at 30 000 *g*.

Statistical analysis was performed by the one-sample Student test for differences when comparing the data on two diets in patients, and analysis of variance for randomised block design when comparing meal effects in patients. The two-sample Student test was applied when comparing data on patients and normals and analysis of variance for split-plot design when comparing meal effects between patients and normals.

RESULTS

Fig. 1 shows the diurnal course of mean TG levels. As in normals (7, 29), wave-like patterns were observed, but the amplitudes were wider and absolute values much higher.

On carbohydrate-rich food, fasting TG levels were higher than on customary 'home' diet. In the A periods, peak values occur later (at 15.00-18.00 h) than in normals (at ± 13.00 h) (29). In the B period, the TG profile seems to be more level than in the A1 period. In period C, the pattern disappears and TG levels tend to be lower at daytime than at night, as in normals (29). In the A2 period, all patients showed a decreased TG level, both fasting and postprandially, compared to the A1

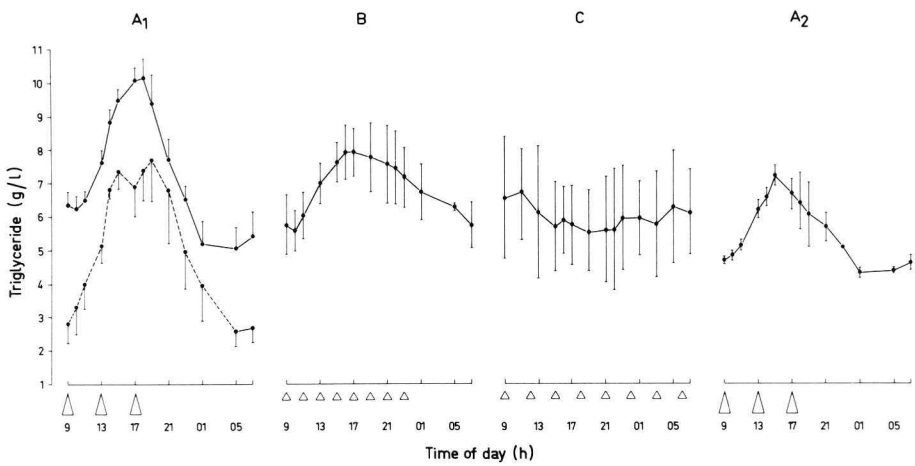

Figure 1. Diurnal course of TG levels on carbohydrate-rich (●—●) and fat-rich diet (●---●) (mean ± S.E. of three patients, in period C mean of two patients). Triangles indicate time of meals.

period ($p = 0.02$). A tendency towards this decrease, although not statistically significant in periods B and C, was found throughout the study.

On the fat-rich diet, identical TG patterns were observed during 2-4 successive A periods. The fasting TG value was lower than on customary 'home' diet, and significantly lower than on carbohydrate-rich food ($p=0.004$). The peak value again occurred later (at ± 19.00 h) than in normals (at ±14.00 h) (7) and the post-prandial increment was higher than on carbohydrate-rich diet ($p<0.05$).

Fig. 2 shows lipoprotein TG profiles of each patient on both diets. Chylomicrons contribute more than VLDL to the increase and decrease in serum TG levels.

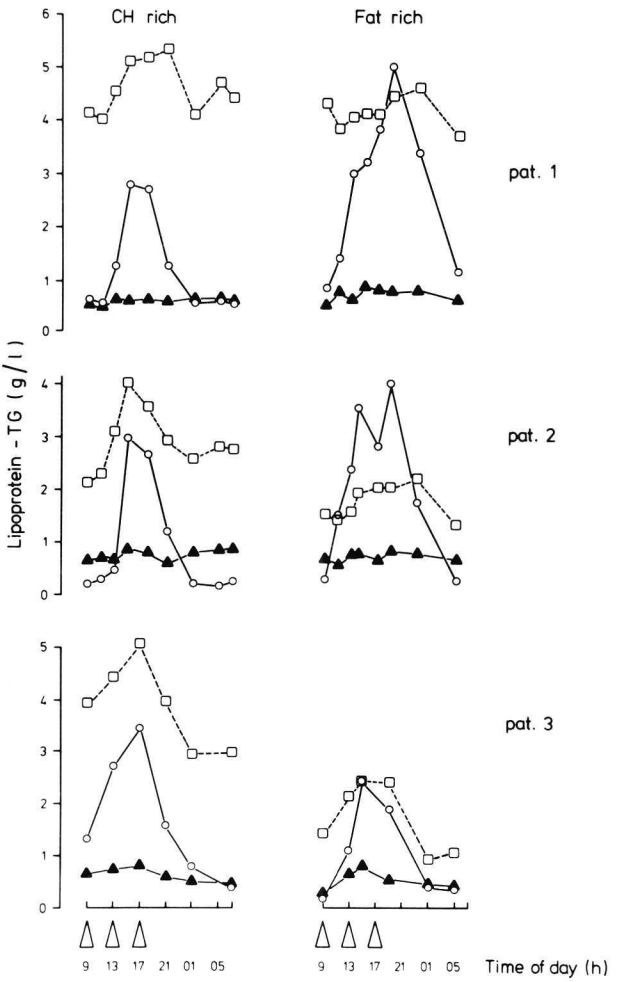

Figure 2. Diurnal course of individual lipoprotein TG levels on carbohydrate-rich (left) and fat-rich diet (right). ○ = Chylomicrons, □ = VLDL, ▲ = HDL + LDL.

Chylomicrons rise to a maximum at ± 18.00 h, after which they fall to prebreakfast levels. VLDL shows a similar, though less pronounced pattern. VLDL TG is higher on carbohydrate-rich food, whereas chylomicron TG tends to be higher on fat-rich diet. HDL and LDL TG did not show much diurnal fluctuation.

Fig. 3 shows the course of mean FFA levels on both diets in period A. They were markedly higher on fat-rich than on carbohydrate-rich food ($p<0.05$) and did not differ significantly from those in normals on both diets (7, 29).

Postprandial glucose increments and mean glucose levels (A period) did not differ significantly between diets. On carbohydrate-rich, but not on fat-rich food, the postprandial increments were more marked in patients than in normals ($p=0.02$), but the mean levels did not differ significantly.

Postprandial insulin increments were more pronounced and mean insulin levels higher on the carbohydrate-rich than on the fat-rich diet ($0.1>p>0.05$ and $p<0.02$) (Table II). Both parameters were higher in the patients than in the normals both on carbohydrate-rich ($p<0.05$) and fat-rich food ($p<0.02$ and $p<0.05$) (7, 29). Fasting insulin levels were higher on carbohydrate-rich than on fat-rich diet ($p<0.05$). They were higher in patients than in normals on carbohydrate-rich ($p<0.02$) but not on fat-rich diet (7, 29).

Total cholesterol levels did not show much diurnal variation (Fig. 4). They seemed to follow the TG pattern, as was also found in normals (29). Any fluctuations (less than 0.5 mmol/l) could be attributed mainly to cholesterol in the chylomicron fraction. On carbohydrate-rich food (period A1), mean cholesterol levels seemed to be higher than on fat-rich food, but the difference was not significant. In period A2 (carbohydrate-rich food), mean cholesterol levels were lower, but not significantly so, than in period A1.

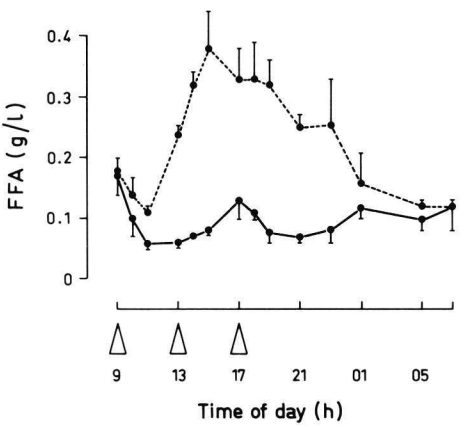

Figure 3. Diurnal course of FFA levels on carbohydrate-rich (●—●) and fat-rich diet (●---●) in period A (mean ± S.E. of three patients).

Table II. Insulin increments (μU/ml) in period A1 (initial and maximum value).

Pat. no.	Diet*	Meal 1	Meal 2	Meal 3
1	C	28->300	60-200	38-156
	F	6-140	12-66	36-104
2	C	12-160	57-114	12-64
	F	<2-25	<2-39	2-28
3	C	14-360	164-365	130-250
	F	<2-244	52-196	76-206

* C = carbohydrate-rich, F = fat-rich.

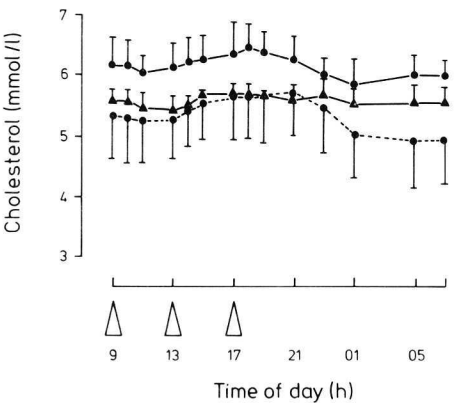

Figure 4. Diurnal course of serum cholesterol on carbohydrate-rich diet in periods A1 (●—●) and A2 (▲—▲) and on fat-rich diet in period A1 (●---●) (mean ± S.E. of three patients).

DISCUSSION

After a carbohydrate-rich diet at home during a fortnight and for four consecutive six-day periods in the Metabolic Ward, mean fasting TG levels decreased in the last three periods by nearly 2 g/l (A2 compared to A1). This differs from our findings in normals (29). This phenomenon, which is perhaps caused by adaptation to carbohydrate-rich food after an initial induction, has also been found by Antonis and Berson (2) and Lees and Fredrickson (12) in normals. Therefore we cannot say that the TG level in period B is flattened out, although it is obvious that the pattern has disappeared in period C and that, as in normals (7, 29), one can manipulate TG responses by varying frequency and distribution of meals.

On both diets, the major contribution to the pattern of serum TG levels in the patients came from chylomicrons rather than VLDL. As the separation of

chylomicrons and VLDL is based on flotation characteristics, it may well be that a proportion of the chylomicrons are big TG-rich particles of endogenous origin, which are known to appear on carbohydrate-rich food (15, 22). The finding that chylomicrons contributed mainly to the increase in serum TG in patients on both diets contrasts with findings in normals, in whom VLDL was the main contributor to the serum TG pattern (7, 29). This could point to a defect in TG clearance in the patients, as big particles like chylomicrons are usually said to be preferentially hydrolysed by lipoprotein lipase when compared with smaller particles like VLDL (13). Moreover, on the fat-rich diet the postprandial chylomicron increase (which is probably more 'diet-derived' than on the carbohydrate-rich diet) is much higher in the patients than in the normals on comparable amounts of fat (7). Redgrave and Carlson (19) also found that type IV hypertriglyceridemic patients showed higher chylomicron TG than normals after a fat load. A decreased clearance of chylomicrons, at least in the morning, could account for this. It is remarkable that the last of three identical meals does not cause as great an increase in chylomicron concentration as the preceding two. Assuming an unchanged rate of absorption, only an increased clearance at that time could account for this.

It is interesting that, whereas TG levels are much higher in the patients than in the normals, FFA levels are not, as was also found by Schlierf et al. (25, 26). Likewise, glucose levels did not differ from those of normals but then our patients were selected on the basis of normal glucose tolerance. On the other hand, mean insulin levels were higher in the patients than in the normals, which is a common finding (25, 27).

It seems that fat-rich diet leads to only slightly lower mean TG levels than six weeks on carbohydrate-rich food. On the other hand, FFA – which are a substrate for VLDL synthesis, an elevation of which might have a harmful effect on endothelial cells (14, 23) – are higher on the fat-rich diet. In view of our findings we therefore recommend a middle course and frequent meals in order to prevent high peak lipid levels.

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Chapter IV

PROCEDURE FOR THE DETERMINATION OF LIPOPROTEIN LIPASE ACTIVITY IN HEPARIN ELUATES OF HUMAN TISSUE

The activity of LPL in tissues can be determined in several ways (1, 2). When an estimate of the physiologically active fraction is required, the method of choice will be the one which determines LPL activity in heparin eluates of tissue specimens. Although in vitro heparin might release LPL also from other places than the vascular endothelium, such as for instance from the adipocyte cellmembrane, where LPL is not believed to function in vivo, this method is still favoured above the acetone-ether technique, which also measures intracellular enzyme (2, 3). This chapter describes the method of the determination of LPL activity in heparin-eluates of pieces of adipose tissue and skeletal muscle.

Tissue preparation and reference enzyme

For the development of the assay we used LPL from milk as a reference enzyme. Fresh, non-pasteurized cow's milk was centrifuged for 20 minutes at 1000 *g*, after which the cream layer was removed. The resulting skimmed milk was frozen in 0,5 ml portions at –80°C. Pieces of human adipose tissue (AT) and skeletal muscle (SM) were obtained from patients not suffering from lipid disorders, who underwent surgery for a variety of reasons and who were under general anaesthesia for not longer than 30 minutes. These patients were not heparinized, nor were they given any medication known to influence LPL. The tissue-samples were put immediately into ice-cooled saline and quickly transported to the laboratory where they were rinsed, blotted dry on filter paper, cut into pieces of appropriate size and put into screw-cap tubes, which were then placed in liquid nitrogen. In some instances fresh tissue was examined.

Substrate

Out of a variety of substrates described in the literature, we choose a substrate which had proved to be stable for at least a six-week period, and which is extensively described by Nilsson-Ehle et al. (4). It is prepared as follows: cold triolein (300 mg), glycerol-tri-1^{14}C-oleate (50 μCi) and egg lecithin (18 mg, dissolved in chloroform/methanol 1:1) as detergent were put into a glass counting vial and solvents were evaporated under a stream of N_2. Then glycerol (5 ml) was added as stabilizer. This mixture was homogenized with a Polytron PT 10-35, at setting 5 à 6 for 5 one minute-periods with one minute intervals, while on ice. The resulting emulsion became clear after some hours and could be stored at room temperature. It will be referred to as 'stock' substrate. To activate this 'stock' substrate (5) we used pooled serum obtained from fasting healthy, normolipidaemic donors, which was frozen in 1 ml portions at –20°C.

Reaction mixture and incubation procedure

Tissue pieces (varying in wet weight from 5-50 mg) were weighed in frozen condition and put into 500 μl 'elution medium'. This 'elution medium' consisted of glycine buffer (2.1 mol/l, adjusted to pH 8.3), supplemented with albumin (7.5 mg/ml, Povite, Organon) and heparin (100 IU/ml, Tromboliquine, Organon). After incubation for 50 minutes at 37° in a shaking water bath, tissue pieces were removed and samples of the 'elution medium', now containing LPL, were incubated with equal amounts of the 'substrate mixture'. This 'substrate mixture' consisted of glycine buffer (2.1 mol/l, adjusted to pH 8.3) supplemented with albumin (7.5 mg/ml), 'stock' substrate (11.2 mmol triglyceride/l) and pooled serum (15% by volume). The reaction mixture was thus finally composed of glycine buffer (2.1 mol/l, adjusted to pH 8.3), albumin (7.5 g/l), heparin (50 IU/ml), triglyceride (TG) (5.6 mmol/l) and serum (7.5% by volume). Incubations were carried out for 2 hours at 37°C in a shaking water bath, after which the reaction was stopped. When milk was used, 25 μl of skimmed milk, 1:20 diluted with distilled water (standard amount), was incubated in 275 μl reaction mixture, which consisted of equal volumes of 'elution medium' and 'substrate mixture', for 60 minutes at 37°C. To stop the reaction and to separate the FFA from the triglycerides, 3.25 ml of a mixture of methanol - chloroform - heptane (1.41:1.25:1.00 v/v/v) (6), was added to 100 or 200 μl reaction mixture, followed by addition of 1.05 ml NaOH (0,5 mol/l). After vigourous mixing the two phases were separated by centrifugation for 10 minutes at 1000 *g*. One ml of the upper phase was counted in a liquid scintillation-counter using Pico Fluor as scintillation fluid. Corrections were made, when necessary, for quenching as monitored by automatic external standardization and for the percentage of FFA recovered in the upper phase. Each assay included blank incubations without LPL. After subtraction of the radioactivity of the blank, LPL activity was calculated and expressed in mU per gram tissue, or, in case of milk, in mU per standard amount. 1 Unit (U) of enzyme activity represents the release of 1 μmol FFA per minute.

Optimal conditions for the assay of LPL

In order to find the optimal serum concentration to activate the substrate, we added increasing amounts of stock serum to the substrate mixture. The TG concentration in these experiments was 5.6 mmol/l (final concentration). Fig. 1 shows the results of these experiments. A serum concentration of 7.5% by volume (final concentration) was optimal for LPL activity from milk, adipose tissue and skeletal muscle. At higher serum concentrations LPL activity, especially from tissues, became inhibited. Routinely the serum concentration was therefore kept at 7.5% by volume (final concentration).

When milk LPL was used, the production of FFA was linear for only ± 45 minutes, as is shown in Fig. 2. This could be due to changes in substrate and/or enzyme activity. To discriminate between these two possibilities the following

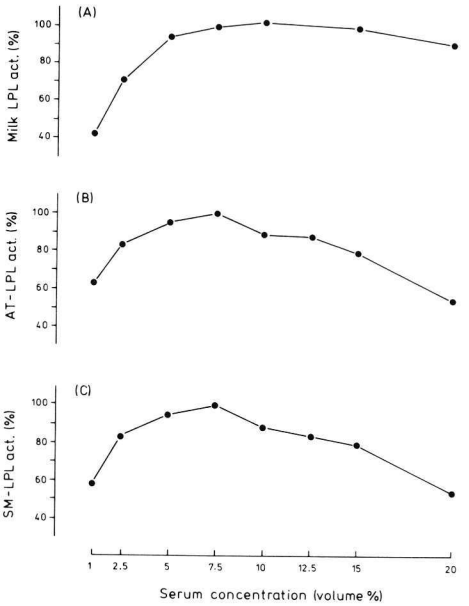

Figure 1. Effect of varying serum concentrations in the final reaction mixture (TG concentration 5.6 mmol/l) on the activity of LPL from A: skimmed milk, B: heparin eluates of adipose tissue and C: heparin eluates of skeletal muscle. Results are given in percentages of maximal LPL activity.

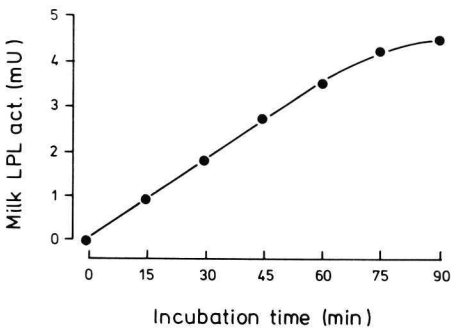

Figure 2. Release of FFA as a function of time upon incubation of milk LPL with substrate.

experiments were performed. Samples of milk LPL in the 'elution medium' and samples of the 'substrate mixture' were preincubated for varying periods at 37°C, after which substrate mixture and fresh enzyme were added respectively. Fig. 3 shows that milk LPL progressively looses activity during preincubation at 37°C, but that the substrate remains stable up to 1.5 hours. Therefore heat-instability of

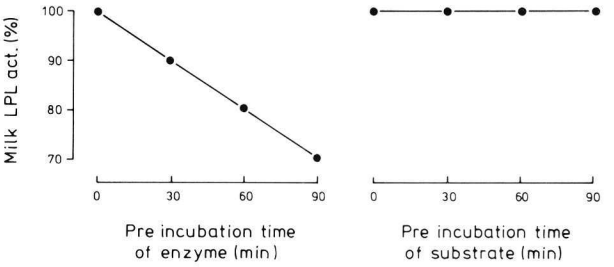

Figure 3. Effect of preincubation at 37°C of milk LPL in 'elution medium' (left) and 'substrate mixture' (right) on milk LPL activity, expressed in percentages of the activity obtained without preincubation.

the milk LPL seems to be responsible for the decline in FFA production after ± 45 minutes. In contrast, when LPL eluted from human adipose tissue or skeletal muscle was used as the enzyme, the production of FFA was linear for more than 3 hours (Fig. 4).

Fig. 5 shows the effect of substrate concentration on the lipolytic rate. It is obvious that at TG concentrations of 5.6 mmol/l, which were routinely used, the enzyme was saturated with substrate. At each TG concentration tested, the serum/TG ratio was kept constant. Michaelis-Menten constants, or K_m values, for adipose tissue and skeletal muscle were 0.40 and 0.54 mmol TG/l respectively.

Albumin (Povite), not free from FFA, was used in a concentration of 7.5 mg/ml as carrier for the FFA produced (optimal concentration, personal communication, H. Lithell). When FFA-free albumin was tested under identical conditions, the rate of lipolysis did not increase.

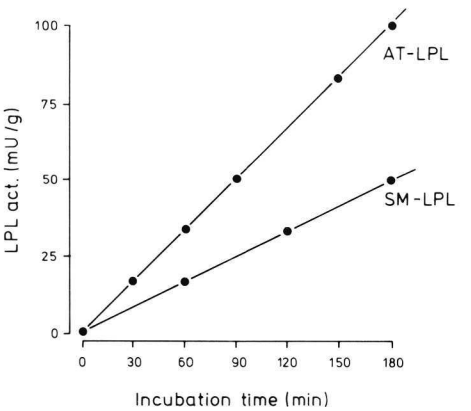

Figure 4. Release of FFA as a function of time upon incubation of heparin eluates of adipose tissue and skeletal muscle with substrate.

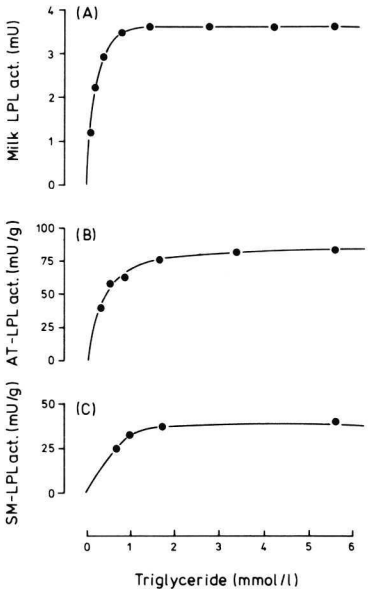

Figure 5. Effect of varying TG concentrations in the final reaction mixture on LPL activity from A: skimmed milk (mU/standard amount), B: heparin eluates of adipose tissue and C: heparin eluates of skeletal muscle.

Optimal conditions for elution of LPL from tissues

Optimal conditions for elution of LPL from tissues at 37°C were tested at various heparin concentrations, using elution times of 1 hour. Fig. 6 shows that a heparin concentration of 100 IU/ml in the elution medium yielded optimal results for both adipose and muscle tissue. At higher heparin concentrations LPL activity became inhibited. In the absence of heparin already an appreciable amount of LPL was present in the medium.

Fig. 7 shows the timespan necessary to elute the maximal amount of LPL from the tissue pieces. The major part of the enzyme activity of both tissues was released in the first 10 minutes, but up to 50 minutes the activity still slightly increased.

To ascertain that the LPL released into the medium was not originating from an intra-cellular pool, the incubations were also carried out in the presence of colchicine in a concentration of 10^{-4}M. Colchicine in this concentration should inhibit microtubular function, which is required for transport of LPL from its site of synthesis to the outside of the cell (7). No differences between incubations with or without colchicine with respect to the amount of LPL released into the medium were observed.

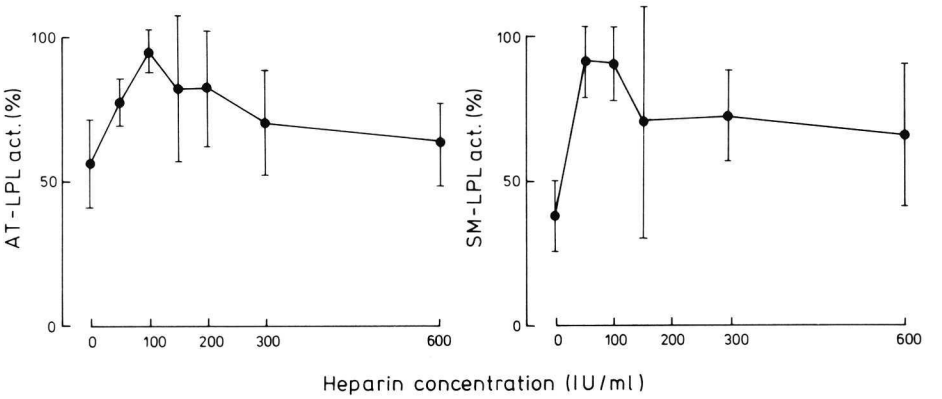

Figure 6. Effect of various heparin concentrations in the 'elution medium' upon LPL activity from adipose tissue (left) and skeletal muscle (right). Elution time is 60 minutes. Results represent mean ± SD of 6 (adipose tissue) and 3 (skeletal muscle) experiments and are expressed in percentages of maximal LPL activities.

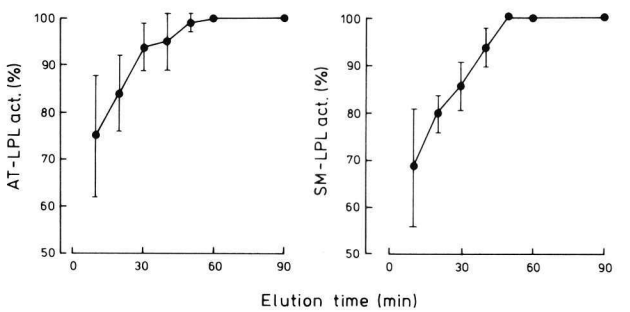

Figure 7. Effect of time upon elution of LPL from adipose tissue (left) and skeletal muscle (right). Results represent mean ± SD of 6 (adipose tissue) and 3 (skeletal muscle) experiments and are expressed in percentages of maximal activity. Heparin concentration: 50 IU/ml (final concentration).

Fig. 8 shows that the amount of FFA released was linearly related to the wet weight of the tissue pieces. Up to 50 mg of adipose tissue and 60 mg of muscle, corresponding to the amounts of tissue which can be obtained by needle biopsy (Chapter V and VI), could accurately be measured.

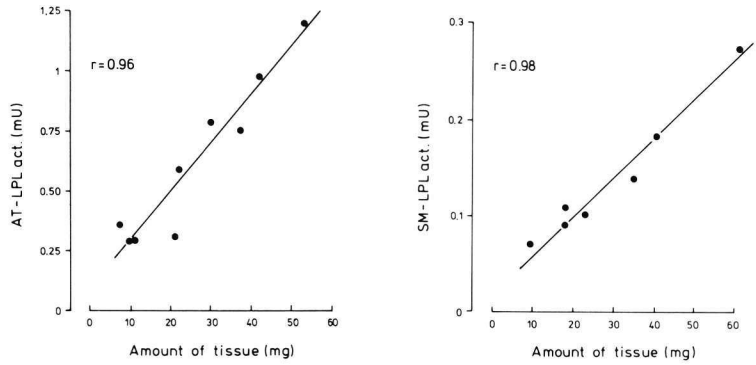

Figure 8. Release of FFA as a function of the wet weight of tissue upon incubation of heparin eluates of adipose tissue (left) and skeletal muscle (right) with substrate.

Characteristics of LPL activity

When serum was omitted from the reaction mixture, AT-LPL activity amounted to 43% and milk LPL activity to 25% of the activity obtained at optimal serum concentrations. High concentrations of NaCl (3 mol/l) inhibited the LPL activity in both systems by 75 and 90% respectively.

Reproducibility of the assay

The mean ± SD of the coefficient of variation for AT-LPL activity was 12.2% ± 5 (n = 8). Since different tissue pieces might contain varying amounts of connective tissue, which do not contribute to LPL activity, we also expressed AT-LPL activity per gram total lipid of the tissue pieces. As the correlation coefficient between total lipid weight and wet weight was 0.99, this implies that expression of AT-LPL activity per gram lipid does not result in a lower coefficient of variation.

The coefficient of variation for SM-LPL activity was 8% when activities of more than 33 mU/g and 25% when activities of less than 10 mU/g were found.

The coefficient of variation for the milkstandard was 8% over a 7 month period.

Recovery of FFA in the upper phase

To check the recovery of FFA in the upper phase after liquid-liquid partition of lipids, a known amount of ^{3}H-oleic acid was added to the samples before lipid partition. In twenty assays the upper phase contained 61% ± 2 (mean ± SD) of total FFA.

Ranges of LPL activity in human adipose tissue and skeletal muscle

In adipose tissue obtained from various localisations from both male and female patients at surgery, LPL activities varying from 18 to 200 mU/g were found. These activities are in good agreement with those reported by Lithell (8) and Taskinen (9). Since the AT-LPL activities from various sites of the body are not the same (10), we have used tissue from identical sites in our clinical studies (Chapter V and VI).

The LPL activity of skeletal muscle depends on the amount of red muscle fibres (11). We therefore estimated the proportion of red and white fibres by microscopic examination of histochemically coloured muscle slices (12). We found lower activities (3.3-6.6 mU/g in muscles with a low percentage of red fibres (<30%) as compared to muscles with higher percentages of red fibres (>60%, activities 16.7 to 50 mU/g). Two pieces of the vastus lateralis muscle, showing ± 65% of red fibres, had activities of 16.7 and 25 mU/g respectively.

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Chapter V

DIURNAL CHANGES IN SERUM TRIGLYCERIDES AS RELATED TO CHANGES IN LIPOLYTIC ENZYMES, (APO)LIPOPROTEINS AND HORMONES IN NORMAL SUBJECTS ON A CARBOHYDRATE-RICH DIET

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ABSTRACT

Normal subjects in steady state on a carbohydrate-rich diet (three equivalent meals a day at 9.00, 13.00 and 17.00 h), show a wave-like serum triglyceride (TG) pattern with a top at 14.00 h. Post-heparin lipoprotein lipase (LPL) activity increased from a mean value of 49 mU/ml ± 13 (SD) in the fasting state to 127 mU/ml ± 18 in the fed state ($p<0.005$). This was due to an increase in adipose tissue LPL activity which was at 16.30 and 21.30 h significantly higher than basal levels (128.3 ± 81.5 and 87.7 ± 23.2 versus 43.3 ± 9.3 mU/g, mean ± SD; $p<0.05$ and $p<0.01$ respectively). Skeletal muscle LPL activity was low (5.8 mU/g ± 2.3, mean ± SD) and showed no diurnal change. The observed changes in TG hydrolysing capacity in the course of the day might explain the TG pattern.

High density liproprotein (HDL) subfractions HDL_2 and HDL_3 were separated by density gradient ultracentrifugation and had mean hydrated densities of 1.088 and 1.135 g/ml respectively. While HDL_2 showed no diurnal change, HDL_3-cholesterol – and phosphlipid significantly increased during the day ($p<0.005$ and <0.001 respectively), reaching their highest levels in the evening. Since the rise in HDL_3-lipids follows the fall in serum TG, this provides further indication that the metabolism of these fractions is mutually related.

INTRODUCTION

Hypertriglyceridaemia has since long been considered a risk factor for atherosclerosis (1, 2), although some investigators do not find an increase in coronary heart disease (CHD) in hypertriglyceridaemic patients (3). Whatever the cause of this discrepancy, it is becoming clear that an efficiently functioning triglyceride (TG) metabolism is not only important in itself, but also in relation to the metabolism of other plasma lipoproteins, especially the high density lipoproteins (HDL).

Recently we studied diurnal TG metabolism in normal subjects in a steady state on varying diets (4, 5). We found that the frequency and distribution of the meals, rather than the composition, were responsible for the characteristic diurnal TG pattern and suggested a role for feeding-related hormones. In the present study we have tried to find an explanation for the diurnal TG pattern by investigating one aspect of TG catabolism. Since lipoprotein lipase (LPL) is the enzyme responsible for the breakdown of TG-rich particles, we measured LPL activity during the day in biopsies from adipose tissue and skeletal muscle as well as in post-heparin plasma.

We also studied the diurnal profiles of other plasma lipid parameters, with special emphasis on changes in HDL and its subfractions HDL_2 and HDL_3. HDL, and especially its lighter subfraction, is considered to be a negative risk factor for the development of atherosclerosis (6), but the mechanism is still poorly understood. Knowledge of the diurnal behaviour of this lipoprotein might lead to a better understanding of its physiological role.

MATERIAL AND METHODS

Design of the study

Participants in this study were 5 normolipaemic healthy male students, whose data are given in Table I. They were studied on a carbohydrate-rich diet, which contained 65 energy % carbohydrate (one third mono- and disaccharides), 23 energy % fat (one third poly-unsaturated) and 12 energy % protein. The diet comprised only normal foods, excluded alcohol and was individually adjusted in order to keep body weight constant. Two weeks prior to admission the participants followed this diet at home in order to become metabolically adapted. The diet was continued during their stay in the metabolic ward, where it was divided into three equivalent meals, given at 9.00, 13.00 and 17.00 h. On the sixth day after their admission, when a steady state had been reached (4), four biopsies of adipose tissue were taken from the abdominal wall by means of needle aspiration at 8.30, 12.30, 16.30 and 21.30 h, and two muscle biopsies from the left and right m. vastus lateralis with the Bergström needle (7) at 8.30 and 16.30 h. Via an indwelling catheter, placed in a forearm vein and kept patent with saline, blood was obtained throughout this day for lipid and hormone determinations. The next morning, at 8.15 h, 100 IU heparin (Tromboliquine, Organon, Oss, Holland)/kg body weight were intravenously (i.v.) administered. After 20 and again after 45 minutes blood was taken for determination of post-heparin LPL activity and hepatic lipase (HL) activity. As an i.v. injection with heparin disturbs the metabolic steady state, the second heparin injection was given some days later (usually on day 12) at 16.15 h when the same parameters were determined in the fed state.

The protocol of the study was approved by the Committee on Medical Ethics, University Hospital, Leiden. All participants gave their written informed consent.

Table I. Clinical and biochemical data of test subjects.

Subjects	Age (years)	IBW[1] (%)	Fasting serum TG (mmol/l)		Fasting HDL-cholesterol (mmol/l)	
			'home' diet	CH[2]-rich diet	'home' diet	CH[2]-rich diet
RW	22	107	1.02	2.36	1.28	0.98
NH	22	90	0.81	1.16	1.68	0.91
MS	22	94	1.10	1.74	1.71	1.33
CJ	32	109	1.56	1.75	1.19	1.01
CB	38	107	1.07	1.40	1.18	1.11

[1]% IBW, percent of Ideal Body Weight according to Metropolitan Life Insurance Tables.
[2]CH = carbohydrate.

Assay of lipoprotein lipase activity in biopsies

The biopsies were immediately washed in saline, blotted dry on filter paper, put into screw-cap tubes and placed in liquid nitrogen. All specimens from one person were analysed on the same day within a month. LPL was eluted from the specimen during a 50 minutes incubation at 37°C in 500 μl buffer A: a glycine buffer (2.1 mol/l, adjusted to pH 8.3 with NaOH 1 mol/l), supplemented with albumin (7.5 mg/ml, Povite, Organon, Oss, Holland) and heparin (100 IU/ml, Tromboliquine). Stock substrate emulsion was prepared according to Nilsson-Ehle et al. (8) with a specific activity of 50 μCi glycerol-tri-(1-^{14}C)oleate (The Radiochemical Centre, Amersham, UK) per 300 mg triolein (Fluka, Buchs, Switzerland). From this stock emulsion the substrate mixture was prepared by dilution with buffer A without heparin, to produce a final TG concentration of 5.6 mmol/l. Pooled fasting serum, obtained from healthy normolipaemic donors, was added for optimal activation of the substrate (final concentration 7.5 vol%).

Three 100 μl samples of the LPL eluate were incubated with 100 μl substrate mixture. After incubation for 2 h at 37°C, during which period the release of free fatty acids (FFA) was linear, the reaction was terminated by adding 3.25 ml of a mixture of methanol-chloroform-heptane 1.41:1.25:1.00 (v/v/v) (9), followed by 1.05 ml NaOH 0.5 mol/l. After separation of the two phases by centrifugation, 1 ml of the upper phase was counted in a liquid-scintillation counter (Packard model B 2450-08, Brussels, Belgium), using Pico Fluor (Packard) as scintillation fluid.

Each assay series included blank incubations without tissue samples and incubations with a standard LPL sample derived from skimmed bovine milk, kept frozen at −80°C. Corrections were made (i) for quenching, as monitored by automatic external standardization; (ii) for the percentage of FFA recovered in the upper phase, as checked by double label incubations in each assay and (iii) for deviation from the mean of the milk standard. The mean ± SD of the coefficients of variation for adipose tissue LPL activity was 12.2% ± 5 (n=8); the coefficient of variation for skeletal muscle was 8% when activities of more than 33 mU/g and 25% when activities of less than 10 mU/g were found.

The amount of adipose tissue obtained from one biopsy (20-80 mg wet weight) usually permitted incubation in triplicate; muscle pieces (8-30 mg) were incubated single or in duplicate.

As muscles with varying amounts of red and white fibres exhibit different LPL activities (10), we examined the distribution of these fibres in each muscle biopsy. For this purpose microscopic slices were cut from each piece of muscle, which were then histochemically coloured (11). No difference was found in percentages of red and white fibres between the left and right side muscle, nor between the subjects.

Assay of Post Heparin Lipolytic Activity (PHLA)

The blood samples were immediately put on ice. Plasma was separated in a refrigerated centrifuge and stored at −80°C until assay. Extrahepatic (lipoprotein) lipase and hepatic lipase were determined by means of the immunochemical method as described by Huttunen et al. (12).

Hormone, lipid, lipoprotein and apoprotein determinations

During the day blood was taken for determination of various lipid parameters and hormones. Insulin was measured by radioimmunoassay (RIA) using a standard of human insulin (NOVO, Copenhagen, Denmark) according to Berson and Yalow (13). Glucagon was, after extraction of serum with ethanol, determined by RIA, using a commercial kit (NOVO). Serum TG was measured according to Giegel et al. (14) and free fatty acids (FFA) according to Regouw et al. (15).

Lipoproteins were separated by sequential ultracentrifugation in a MSE 25 ultracentrifuge (Measuring and Scientific Equipment Ltd., London, UK) with a 6×14 ml swing-out rotor adapted for 5 ml. Three ml saline was carefully layered in the tube over 2 ml serum. Chylomicrons were separated after spinning for 30 min at 30000 *g*. After replenishment with saline to 5 ml, very low density lipoproteins (VLDL) were obtained by pipetting the upper ± 2.5 ml after a run of 17 h at 100000 *g*. To the remaining part heparin (5%)-manganese chloride (2 mmol/l) was added, which precipitates lipoproteins containing apo B, and leaves HDL in the supernatant. HDL subfractions were separated by density gradient ultracentrifugation according to Terpstra et al. (16) with slight modifications: 3.6 ml serum was brought up to $d = 1.25$ g/ml with solid KBr, after which 50 mg sucrose and 50 μg Coomassie Brilliant Blue were added. After mixing, 1 ml KBr solution $d = 1.21$ g/ml was then carefully layered, followed by 3 ml NaCl solution $d = 1.10$, 2 ml NaCl solution $d = 1.019$ and 2 ml distilled water. Samples were centrifuged for 18 h at 284000 *g* in a MSE 75 ultracentrifuge with a 3×14 ml swing-out rotor. HDL_2 and HDL_3 became then visible as separate bands. The tube was sliced between the two bands and the HDL_2 and HDL_3 fraction were collected. The mean hydrated density of the HDL_2 fraction was 1.088 g/ml and of the HDL_3 fraction 1.135 g/ml, as measured with a DMA 45 density meter (Mettler/Paar, Graz, Austria). The density of the layer between these two bands was 1.10 g/ml.

In each lipoprotein fraction we determined TG, cholesterol according to Abell et al. (17) and phospholipids (Phospholipids B-test Wako, Wako Pure Chemical Industries Ltd., Osaka, Japan). Protein was measured according to Lowry et al. (18).

All apoproteins were determined by radial immuno-diffusion. Apo A-I and A-II were measured as described by Albers et al. (19) and apo B as described by Havekes et al. (20). For the determination of apo E the serum samples were diluted 5-10 fold in urea (final concentration 4 mol/l) prior to application to the agarose plate containing anti apo E antiserum (2 vol%).

Statistical analysis

All diurnal changes were statistically evaluated by a two-way analysis of variance, supplemented by Scheffé's analysis (21), unless otherwise indicated. Correlations were determined by linear regression analysis.

RESULTS

Diurnal lipids, lipoproteins and apoproteins

All subjects showed an increase in fasting serum TG and a decrease in HDL-cholesterol after switching from their 'home' diet to the carbohydrate-rich diet (see Table I). Fig. 1 shows the diurnal course of serum TG, VLDL-TG and chylomicron-TG. As described earlier (4), the serum TG level rose after breakfast from 1.68 mmol/l (SEM 0.18) at 9.00 h to a maximum of 2.61 mmol/l (SEM 0.34) at about 14.00 h. Then the level fell till about 5.00 h the next morning, after which it rose again to the initial value. Both VLDL and chylomicrons contributed to the increase and decrease in the pattern, but VLDL made the greatest contribution.

No diurnal fluctuations were observed in the LDL fraction (mean LDL-cholesterol 3.32 mmol/l, SEM 0.36, data not shown).

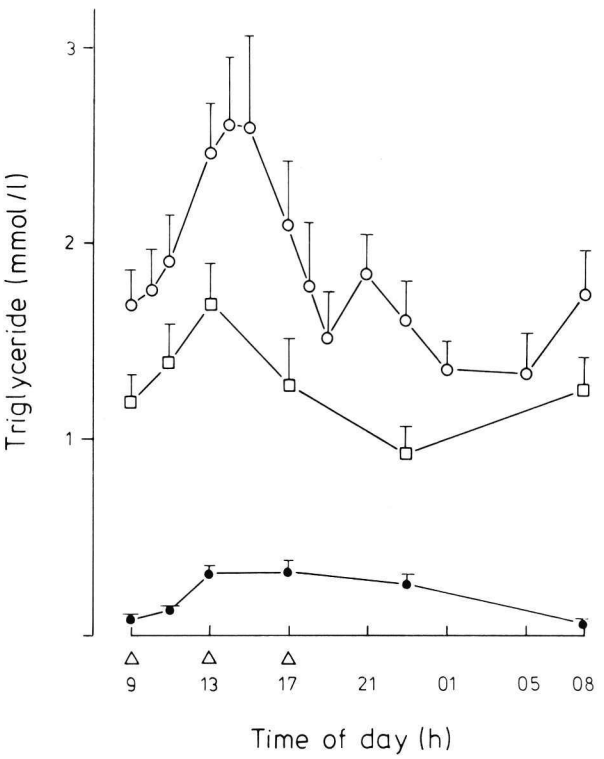

Figure 1. Diurnal course of serum TG (○—○), VLDL-TG (□—□) and chylomicron-TG (●—●). Mean of 5 subjects with SEM. Triangles indicate time of meals.

In Fig. 2 the diurnal course of HDL is shown. It appeared that no diurnal changes occur in HDL-cholesterol levels ($p=0.79$). HDL-phospholipid seemed to increase in the course of the day, reaching its highest levels in the evening ($p=0.07$).

Fig. 3 gives the diurnal course of HDL_2 and HDL_3. The HDL_2 subfraction showed no diurnal change, neither in cholesterol and phospholipid, nor in total protein (mean fasting value 0.48 g/l, SEM 0.13, data not shown) or total mass (mean fasting value 0.92 g/l, SEM 0.17, data not shown). HDL_3, however, significantly increased in both its cholesterol and phospholipid moiety ($p<0.005$ and <0.001 respectively), reaching its highest levels in the evening. HDL_3-protein (mean fasting value 1.75 g/l, SEM 0.32) and HDL_3-total mass (mean fasting value 2.66 g/l, SEM 0.37) did not change significantly ($p=0.87$ and $p=0.42$ respectively, data not shown).

Fig. 4 shows the diurnal course of FFA. The high fasting level decreased after the first meal, to remain low during daytime, except for a slight increase in the afternoon. Thereafter the level returned to high levels in the post-absorptive state.

Fig. 5 shows the diurnal course of the apoproteins A-I, A-II, B and E in serum. Both apo A-I and apo B levels increased in the afternoon to reach their highest levels in the evening ($p<0.0001$ and $p<0.005$ respectively). Apo A-II and apo E levels did not change significantly during the day ($p=0.11$ and $p=0.10$ respectively).

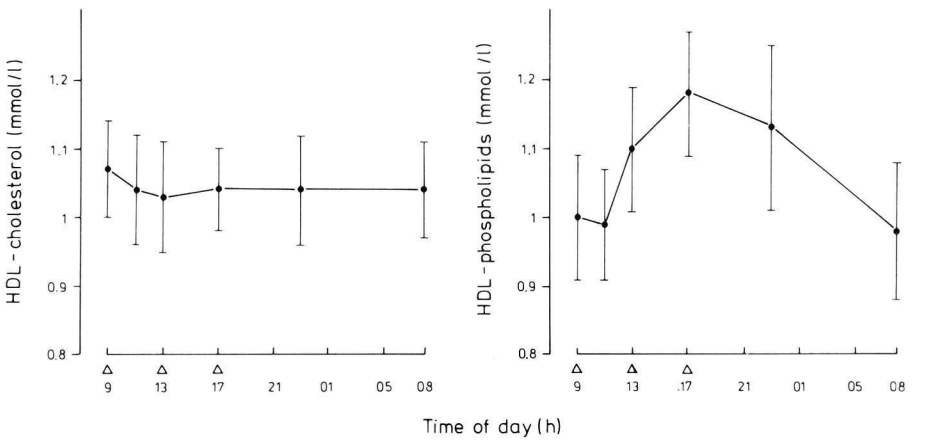

Figure 2. Diurnal course of HDL-cholesterol (left) and HDL-phospholipids (right). Mean of 5 subjects ± SEM. Triangles indicate time of meals.

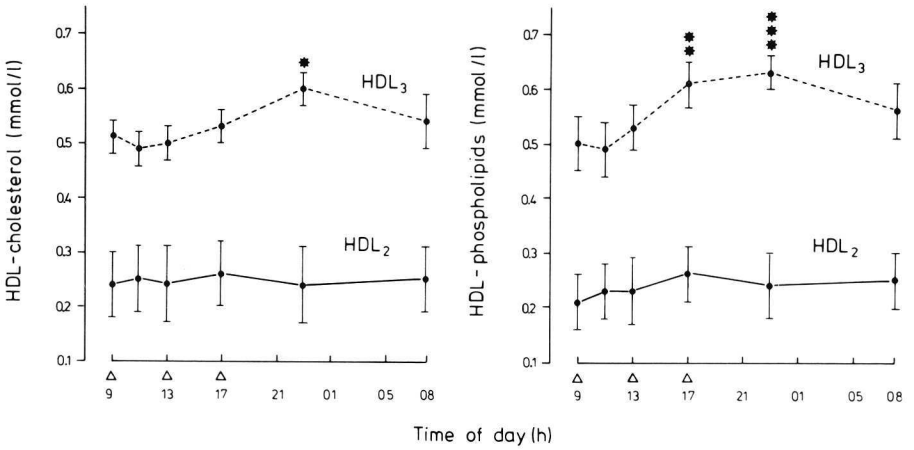

Figure 3. Diurnal course of HDL_2- (solid lines) and HDL_3- (dotted lines) cholesterol (left) and phospholipids (right). Mean of 5 subjects ± SEM. Triangles indicate time of meals.

*significantly different from 11.00 h, $p<0.02$

** and *** significantly different from 09.00 and 11.00 h, $p<0.005$ and <0.001 respectively (method of Scheffé).

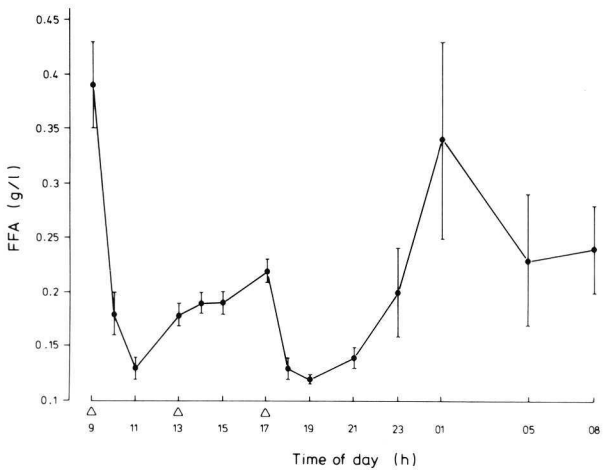

Figure 4. Diurnal course of serum FFA. Mean of 5 subjects ± SEM. Triangles indicate time of meals.

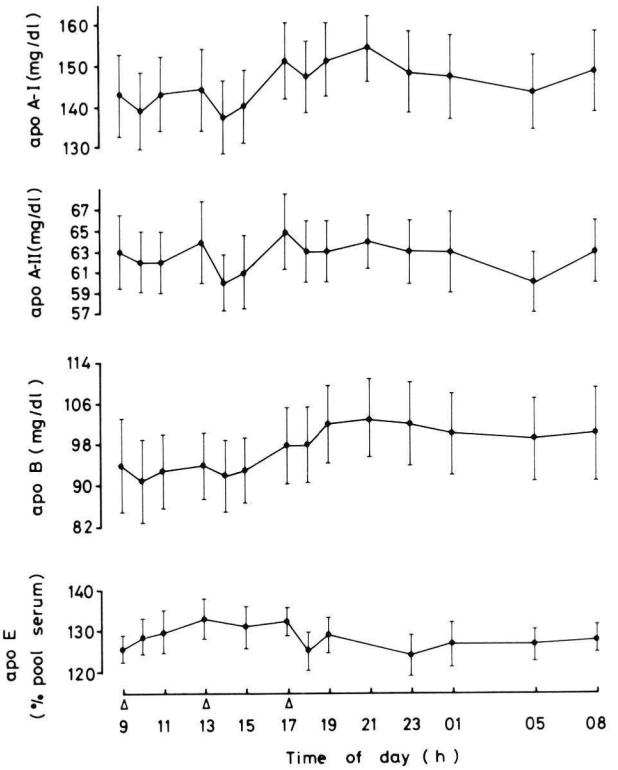

Figure 5. Diurnal course of the apoproteins A-I, A-II, B and E in serum. Mean of 5 subjects ± SEM. Triangles indicate time of meals.

Diurnal lipolytic enzyme activities

Fig. 6 shows the activities of LPL in the biopsies. Adipose tissue LPL (AT-LPL) activity did not change till 12.30 h, but increased in the afternoon to reach at 16.30 h significantly higher levels than at 8.30 h ($p<0.05$). At 21.30 h the activity was still elevated above basal values ($p<0.01$). Skeletal muscle LPL (SM-LPL) activity was rather low on this diet and did not change during the day.

In Table 2 PHLA-values in the fasting (8.15 h) and in the fed (16.15 h) state are given. All subjects showed an increase in LPL activity from the fasting towards the fed state. HL activity did not change.

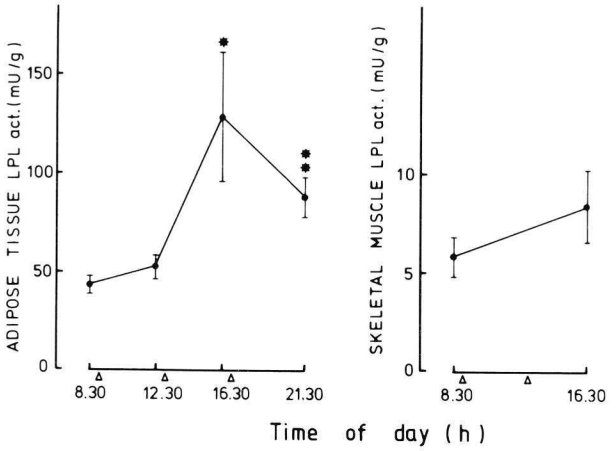

Figure 6. Diurnal course of adipose tissue LPL activity (left) and skeletal muscle LPL activity (right). Mean of 5 subjects ± SEM (NB: AT-LPL at 21.30 h: mean of 4 subjects). Triangles indicate time of meals.

* significantly different from 8.30 h (method of Scheffé, $p<0.05$).

**significantly different from 8.30 h (paired Student's t-test, $p<0.01$; method of Scheffé, $p=0.36$).

Table II. Post-heparin lipolytic activity (PHLA) in the fasting and the fed state.

LPL and HL activities (in mU/ml) were determined in plasma after intravenous injection of 100 IU heparin/kg body weight in the fasting state (day 7, 8.15 h) and the fed state (± day 12, 16.15 h).

Subjects	LPL activity[1]		HL activity[2]	
	fasting	fed	fasting	fed
RW	32	119	571	579
NH	39	153	355	348
MS	48	143	223	214
CJ	59	112	327	330
CB	67	106	528	510
mean ± SD	49 ± 13	127 ± 18*	401 ± 130	396 ± 131

[1]mean value of activity in plasma 20 and 45 minutes after heparin.

[2]activity in plasma 20 minutes after heparin.

*significantly different from fasting values, $p<0.005$ (paired Student's t-test).

Hormonal variations

Since LPL activity is said to be sensitive to hormonal variation, we measured insulin and glucagon levels immediately before and every 30 minutes up to two hours after each meal. Fig. 7 shows the diurnal course of mean serum insulin and glucagon levels. As reported previously (22), insulin exhibited a three peak pattern with higher responses during the first than during subsequent meals. Glucagon decreased after each meal, falling to almost undetectable levels after the third meal,

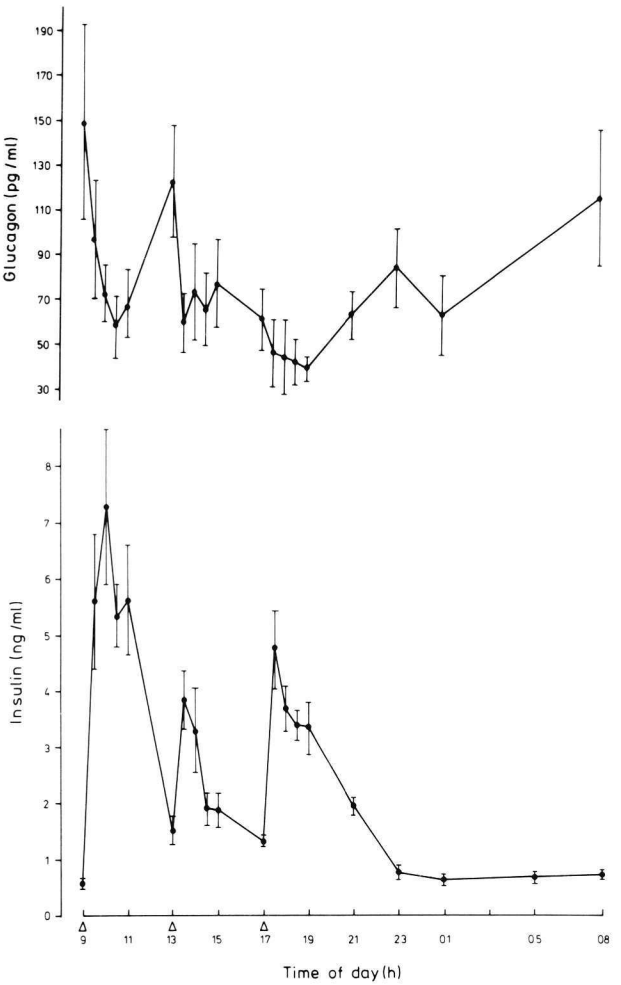

Figure 7. Diurnal course of serum glucagon (upper part) and serum insulin (lower part). Mean of 5 subjects ± SEM. Triangles indicate time of meals. 1 mg insulin = 27.3 U.

and rose again in the post-absorptive period. Significant correlations between hormonal parameters and LPL activity were only found for the amount of insulin secreted after meals (expressed as the area under the insulin profile from 9.00 to 17.00 h) and the increase of AT-LPL activity (at 16.30 h) above basal values ($r=0.88$, $p<0.025$). No such correlation existed between these areas and the increases of PH-LPL activity in plasma from the fasting towards the fed state ($r=0.68$, $p>0.05$).

DISCUSSION

Although our subjects were probably in a state of carbohydrate-induction during the test period, as their fasting serum TG was higher and their HDL-cholesterol level was lower than on 'home' diet, we consider them to be in a steady state during the admission period as was demonstrated previously (4).

They again show the well-defined TG pattern on a regimen of three equivalent meals per day at 9.00, 13.00 and 17.00 h (4), as a result of changing ratios of influx and efflux during the day. The rapid fall of serum TG in the course of the day, in spite of the meals at 13.00 and 17.00 h, made us wonder whether the efflux of TG would increase in the course of the day. In an attempt to study this, we indirectly estimated efflux by determining LPL activity. We measured this activity in two ways: in plasma after an intravenous injection with heparin and, as this method does not discriminate between tissue origin, also in biopsies from adipose tissue and skeletal muscle. Post-heparin LPL activity should represent the total amount of functionally active enzyme (12, 23). To measure tissue LPL activity we preferred heparin-eluates of tissue to acetone-ether powders, since the former method measures functionally active enzyme, whereas in the latter non-functionally active enzyme is also included (24, 25).

The observation that AT-LPL activity increases in the course of the day is in agreement with the findings of others (25, 26). The increase shows a lag-time of at least four hours, as was also observed by Pykälistö et al. (25). Although mean AT-LPL activity slightly decreased from 16.30 h to 21.30 h, it is elevated above basal values in the late afternoon and early evening.

Skeletal muscle LPL activity was found to be rather low on this diet. A possible explanation for this might be that, on a carbohydrate-rich diet, muscles preferentially use glucose (27). To our knowledge only two studies have dealt with changes in SM-LPL activity during the day. Lithell found a decrease in SM-LPL activity in the course of the day when subjects were studied on non-specified meals under non-steady state conditions (26). Taskinen on the other hand observed an increase in SM-LPL activity in the course of the day when subjects were studied before and after a glucose-infusion of 5 h duration (28). We did not find significant changes in SM-LPL activity during the day, but this could be due to the low sensitivity of the assay in this low range.

LPL activity of both tissues is known to be influenced by hormones. AT-LPL

activity rises due to the action of insulin (25, 29). We were able to demonstrate a significant positive correlation between the magnitude of the insulin response to meals and the increase in AT-LPL activity, which is in agreement with Pykälystö et al. (25). Factors responsible for variations in SM-LPL activity are not so well defined: insulin might either increase (30) or decrease (31) SM-LPL activity. A stimulating effect of glucagon on SM-LPL is also described (32). We found no significant correlations between hormonal parameters and SM-LPL activity.

In this study fasting PH-LPL activity was significantly lower than in a group of subjects on normal food (unpublished obervation, H. Jansen), as was also found by Mancini et al. (33). This is probably caused by the low SM-LPL activity observed, and might contribute to the carbohydrate-induced increase in fasting TG. In the course of the day PH-LPL activity was found to increase several fold, which can only be attributed to the increase in AT-LPL activity. The total TG hydrolysing capacity in the afternoon, is, therefore, higher than in the fasting state, which might explain the diurnal TG pattern, i.e. the decrease in serum TG in the afternoon before the last meal. As HL activity does not change during the day, it is unlikely to play a major role in the development of the TG pattern. This is in agreement with the view of Jansen et al. (34) and strenghtened by the observation that HL preferentially acts on phospholipids present in LDL and HDL (35).

Apart from its possible role in TG efflux, glucagon is also reported to act on other plasma lipids (36). It should stimulate TG lipolysis in adipose tissue, which might contribute to explain the high levels of FFA in the fasting state when glucagon levels are high and insulin levels low. These high FFA levels are probably substrate for the VLDL synthesis which is observed between 05.00 and 08.00 h. Immediately after the first meal FFA levels drop, probably due to the fall in glucagon and the rise in insulin. Whether glucagon has any direct (inhibitory) effect on TG synthesis, independent of its effect on FFA levels (as stated by Heimberg, 37), could not be ascertained by our experiments.

LDL-cholesterol did not change in the course of the day. Since the assay for serum apo B is based on the entry of non-VLDL particles into the agarose gel (20), we were surprised to find an increase in serum apo B from $\pm$ 15.00 h onwards till late in the evening. This increase might be due to small partly lipolysed particles which can enter the gel, but which are however not observed in either the VLDL or the LDL fraction on ultracentrifugation (the LDL fraction should include remnant particles with our method). Another posibility might be that in the course of the day LDL becomes apo B enriched.

Serum Apo A-I rises after fat-ingestion as a result of intestinal chylomicron synthesis and subsequent transfer of the apoprotein to HDL (38). We did not observe apo A-I increases after each meal, but it is known that on carbohydrate-rich food chylomicron synthesis is low. The increase of apo A-I in the afternoon coincides with the increase of HDL_3 lipids and is probably related to it. Diurnal changes in serum apo A-II were not found, which seems to agree with findings of Kay et al. on fat-rich food (39).

Triglyceride metabolism is related to HDL metabolism at several levels. During

lipolysis of TG-rich particles surface remnants are transferred to the HDL fraction. These surface remnants consist of free cholesterol, phospholipid and protein, and are considered to be building blocks for new HDL. When produced in vitro during lipolysis of VLDL, in the absence of HDL, they appear in the density range 1.04-1.21 g/ml (40); when HDL_3 in present they fuse with HDL_3 to produce HDL_2-like particles (41). Also in vivo the HDL_2 subfraction rises after initiating TG hydrolysis (42, 43). However, the terms HDL_2 and HDL_3 do not always apply to the same HDL subfractions, because identical methods of ultracentrifugation are not always used. Traditionally HDL_2 and HDL_3 are isolated by analytical ultracentrifugation between *d* 1.063-1.125 and 1.125-1.210 g/ml respectively. When using density gradient ultracentrifugation two (16, 44) or three (45, 46) subfractions can be isolated, the difference being probably due to the use of swing-out or fixed angle rotors. After density gradient ultracentrifugation of our prestained serum samples, we observed two visible HDL bands, with densities of 1.088 (HDL_2) and 1.135 (HDL_3) g/ml. This is in accordance with the findings of Terpstra et al. (16) and Cheung and Albers (44), who also found a density of 1.100 g/ml between the two fractions. When during lipolysis newly formed 'HDL_2-like' particles would have a mean hydrated density of 1.110 g/ml as stated by Patsch (41), they are probably the same particles which were isolated by Anderson in his intermediate HDL subfraction (designated subclass II, (45)), and they would enter our HDL_3-subfraction. This is probably the reason why we observe diurnal changes in our HDL_3, but not in our HDL_2 subfraction. These changes are not reflected in the total HDL fraction (Fig. 2). Moreover, there is a difference in lipid concentration between the sum of both subfractions (Fig. 3) and the total HDL fraction (Fig. 2), which is probably due to the different isolation techniques employed. Total HDL might be overestimated, since it might contain particles called very high density lipoproteins which after density gradient ultracentrifugation are found in the $d>1.21$ g/ml fraction; HDL subfractions, isolated by density gradient ultracentrifugation, might be damaged due to the forces applied (47) and will then be underestimated.

We observed the highest level of the HDL_3 subfraction in the evening, at a time when a great amount of TG-rich particles have been broken down. Our results therefore seem to be consistent with the hypothesis of others that there is a precursor-product relationship between TG-rich and high density lipoproteins (41, 48).

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Chapter VI

DIURNAL CHANGES IN SERUM TRIGLYCERIDES AS RELATED TO CHANGES IN LIPOLYTIC ENZYMES, (APO)LIPOPROTEINS AND HORMONES IN PATIENTS WITH TYPE IV HYPERLIPIDAEMIA ON A CARBOHYDRATE-RICH DIET: COMPARISON WITH NORMAL SUBJECTS

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ABSTRACT

Diurnal parameters of triglyceride metabolism were investigated in 5 type IV hyperlipidaemic patients on a high carbohydrate diet and compared with those of normal subjects studied previously. Post-heparin lipoprotein lipase (LPL) activity was not different from normals in the fasting state (53 ± 16 vs 49 ± 13 mU/ml, mean ± SD), but failed to show the normal increase towards the fed state (16.30 hour) as a result of an absence of the normal increase in adipose tissue (AT) LPL activity. Also in the fasting state was AT-LPL activity lower than in normal subjects (20 ± 10 vs 43 ± 9 mU/g, mean ± SD, $p<0.01$). Skeletal muscle LPL activity was not different from that in normals (5 ± 2 vs 6 ± 2 mU/g, mean ± SD). The nonoccurrence of the normal increase in AT-LPL activity might contribute to the hypertriglyceridaemia. Concentrations of HDL and its subfractions were lower than in normals. Patients showed a smaller increase in HDL_3-phospholipid in the course of the day than normals and an absence of the normal increase in HDL_3-cholesterol and serum apo A-I despite comparable decreases in TG levels and similar diurnal activities of hepatic lipase. The possibility of a defect in the lipid transfer process is discussed.

INTRODUCTION

Type IV hyperlipidaemic patients are characterized by a high level of triglyceride(TG)-rich lipoproteins and a low concentration of high density lipoproteins (HDL) (1, 2). Since a high level of HDL is considered to be a negative risk factor for the development of atherosclerosis (1), it is possible that, apart from the possible atherogenic effect of TG-rich lipoproteins (3, 4) or their remnants (5), this low HDL concentration may also contribute to the increased risk for atherosclerosis in these patients. Evidence is accumulating that the metabolism of the TG-rich lipoproteins and HDL is closely related at several stages (6, 7). The importance of a well-functioning TG metabolism, therefore, extends beyond that of the TG-rich lipoprotein fraction itself.

Increased production of TG-rich particles by the liver (8, 9), impaired removal of these particles (10, 11, 12) or a combination of these two mechanisms (13, 14) have been proposed as a cause for endogenous hypertriglyceridaemia. Most of these studies were, however, performed in the fasting state, while not much attention was given to the fed state, in which the main part of daily life is spent. We have recently found that in type IV hyperlipidaemic patients the diurnal TG patterns are comparable to those of normal subjects, but have a greater amplitude and reach the maximum later in the afternoon (15-17). In another study, we have attempted to explain the diurnal TG pattern in normal subjects by studying the diurnal course of lipoprotein lipase (LPL) activity (18). We now have extended this study to type IV hyperlipidaemic patiens and, by comparing their data with those of the normal subjects, we attempt to account for the differences in the diurnal TG pattern between the two groups.

We also studied the diurnal course of other parameters which influence TG metabolism, in particular the hormones insulin and glucagon and various lipids. In view of the relation between TG and HDL metabolism, we placed emphasis on changes in total HDL and its subfractions in order to find an explanation for the low HDL levels observed in type IV patients.

MATERIAL AND METHODS

Design of the study

Participants in this study were 5 male patients with a primary form of type IV hyperlipidaemia. Thyroid, liver and renal functions were normal, diabetes mellitus could be excluded (normal oral glucose tolerance test) and no drugs known to affect carbohydrate and lipid metabolism were used. Clinical and biochemical data of the patients are given in Table I. Lipid parameters in relatives were not studied. The patients were investigated when in a steady state on a carbohydrate-rich diet composed of 65 energy % carbohydrate (1/3 mono- and disaccharides), 23 energy % fat (1/3 poly-unsaturated) and 12 energy % protein. The protocol they were submitted to was identical to that described previously for five normal test subjects (18). Briefly, for two weeks prior to admission to the metabolic ward the patients consumed the above mentioned diet, which was individually adjusted, at home. It was continued during their stay in the hospital, where it was divided into three equivalent meals consumed at 09.00, 13.00 and 17.00 h. On the sixth day of their admission, four biopsies of adipose tissue were taken from the abdominal wall and two muscle biopsies from the left and right m. vastus lateralis. Blood was obtained throughout this day for determination of various lipids and hormones. At day 7, in the fasting state (8.15 h), the first heparin injection (100 IU/kg body weight) was intravenously administered for the determination of post-heparin lipolytic activity. The second heparin injection, in the fed state (16.15 h), was given a few days later when the steady state had been reestablished. The protocol was approved by the Committee on Medical Ethics, University Hospital, Leiden. All participants gave their written informed consent. Laboratory determinations were performed as previously described (18).

Statistical analysis

Diurnal changes were evaluated by a two-way analysis of variance, supplemented by the analysis of Scheffé (19). For all other data, the unpaired Student's t-test was used, unless otherwise indicated.

Table I. Clinical and biochemical data of test subjects.

Subjects	Age (years)	IBW[1]	Fasting serum TG (mmol/l)		Fasting HDL-cholesterol (mmol/l)		Clinical signs
			'home' diet[2]	CH[3]-rich diet	'home' diet[2]	CH[3]-rich diet	
MS	48	125	9.5	7.7	0.94	0.48	gout
PF	30	112	11.2	6.8	0.61	0.69	coronary infarction history of xanthomatosis
HN	38	128	8.6	4.7	0.78	0.77	history of xanthomatosis
HvdM	37	110	6.2	4.2	0.78	0.66	history of xanthomatosis
JN	43	108	6.0	4.6	0.79	0.57	——

[1]% IBW, percent of Ideal Body Weight according to Metropolitan Life Insurance Tables.
[2]Mean of 2 values on occasions within 2 months preceding the study.
[3]CH = carbohydrate.

RESULTS

The average age of the patients was somewhat older than that of the group of normal subjects previously studied (39.2 years ± 6.8 vs 27.2 ± 7.4, mean age ± SD, $p<0.05$) (18). They were also slightly more overweight than the normal subjects as judged from their greater deviation from IBW (116.6% ± 9.2 vs 101.4% ± 8.73, mean ± SD, $p<0.05$). However, when the amount of body fat as a percentage of body weight was calculated by means of skinfold measurements (20) and K_{40} measurements (21), patients did not differ from the normal subjects (skinfold measurement: 20.2% ± 2.4 vs 15.6% ± 4.5; K_{40} measurement: 24.8% ± 10 vs 18.7% ± 7, mean ± SD). Changing from their 'home' diet to the carbohydrate-rich one caused a slight decrease in their fasting serum TG levels as well as in their serum cholesterol and HDL-cholesterol levels (Table I).

Fig. 1 shows the diurnal course of mean serum TG, chylomicron-TG and very

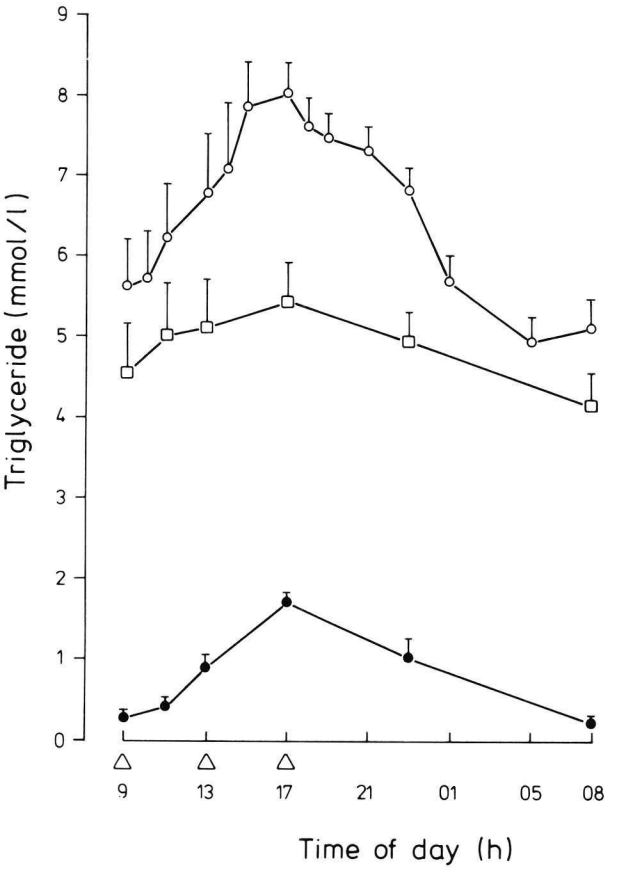

Figure 1. Diurnal course of serum TG (○—○), VLDL-TG (□—□) and chylomicron-TG (●—●). Mean of 5 patients with type IV hyperlipidaemia ± SEM. Triangles indicate time of meals.

low density lipoprotein (VLDL)-TG. Whereas in normal subjects serum TG reached a top at 14.00 h (18), patients showed an increase until 17.00 h. The absolute increase was greater in patients than in normal subjects and chylomicrons made the greatest contribution.

No diurnal fluctuation was observed in the low density lipoprotein (LDL) fraction as was also found in normal subjects (18). Mean diurnal LDL-cholesterol was significantly lower than in the normal subjects (2.28 mmol/l ± 0.4 vs 3.32 mmol/l ± 0.8, mean ± SD, $p<0.05$, data not shown).

Fig. 2 shows the diurnal course of HDL. As in normals (18), HDL-cholesterol showed no significant diurnal variation ($p=0.14$), in contrast to HDL-phospholipid which significantly increased, reaching its highest levels in the evening ($p<0.001$).

Fig. 3 shows the diurnal course of the HDL subfractions with respect to their cholesterol and phospholipid moieties. HDL_2- and HDL_3-cholesterol showed no diurnal changes ($p=0.9$ and $p=0.25$, respectively), but HDL_2- and HDL_3-phospholipid increased significantly in the course of the day, reaching their highest levels in the evening ($p<0.02$ and $p<0.03$, respectively). In normal subjects we previously observed diurnal increases in HDL_3-cholesterol and -phospholipid (18). The protein content of HDL_2 and HDL_3 (mean fasting value 0.17 g/l, SEM 0.02 and 0.96 g/l, SEM 0.04, respectively) did not show significant diurnal changes ($p=0.15$ and $p=0.33$, respectively) as was also observed in normal subjects (18). The concentration of HDL and its subfractions was clearly much lower than that of the normal subjects in each constituent measured.

Fig. 4 shows the diurnal course of the apolipoproteins A-I, A-II, B and E in serum. Unlike normals (18), patients did not show a significant change in apo A-I levels in the course of the day ($p=0.14$). As in normals (18), no significant diurnal change in apo A-II levels ($p=0.07$) was found. Statistical analysis of apo B revealed

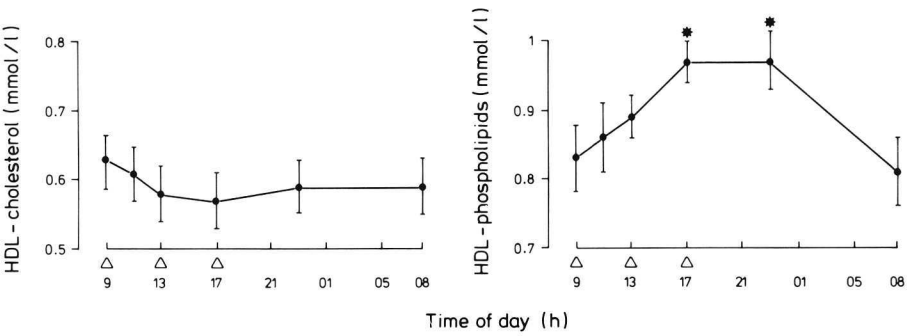

Figure 2. Diurnal course of HDL-cholesterol (left) and HDL-phospholipids (right). Mean of 5 patients ± SEM. Triangles indicate time of meals.
*significantly different from 9.00 h, $p=0.0058$ (method of Scheffé).

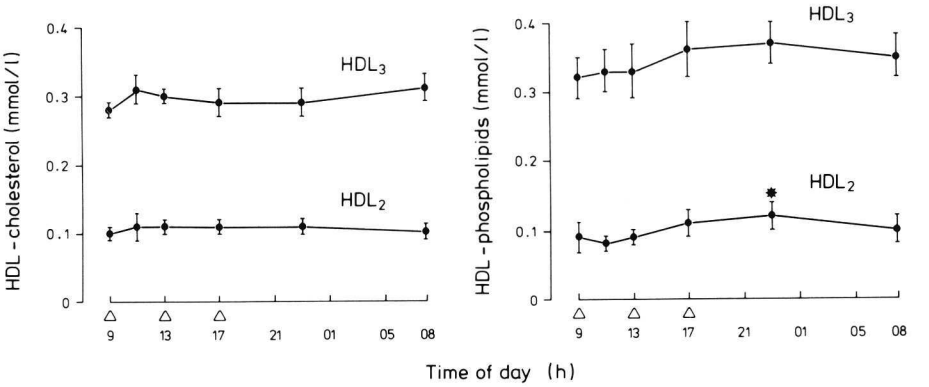

Figure 3. Diurnal course of HDL_2- and HDL_3-cholesterol (left) and -phospholipids (right). Mean of 5 patients ± SEM. Triangles indicate time of meals.
*HDL_2-phospholipids at 23.00 h significantly different from 11.00 h, $p<0.05$ (method of Scheffé).

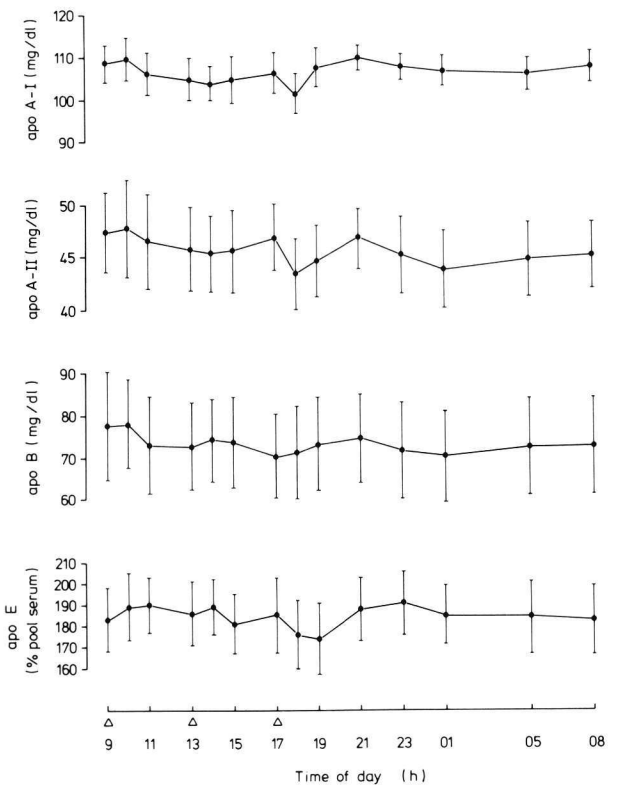

Figure 4. Diurnal course of the apoproteins A-I, A-II, B and E in serum. Mean of 5 patients ± SEM. Triangles indicate time of meals.

a very slight, but significant diurnal change ($p<0.005$) which appeared to depend on a decrease in the first half of the day, whereas normal subjects showed an increase in the afternoon (18). Apo E levels also showed a slight diurnal change ($p=0.019$). Mean diurnal levels of apo A-I, A-II and B were lower than in normal subjects, whereas apo E levels were higher.

Fig. 5 shows the diurnal course of free fatty acids (FFA). As described previously, the level decreases after breakfast, remains low during daytime except for a slight increase in the afternoon, and increases again to initial values in the postprandial state. The pattern was neither quantitatively nor qualitatively different from that in normal subjects ($p=0.42$).

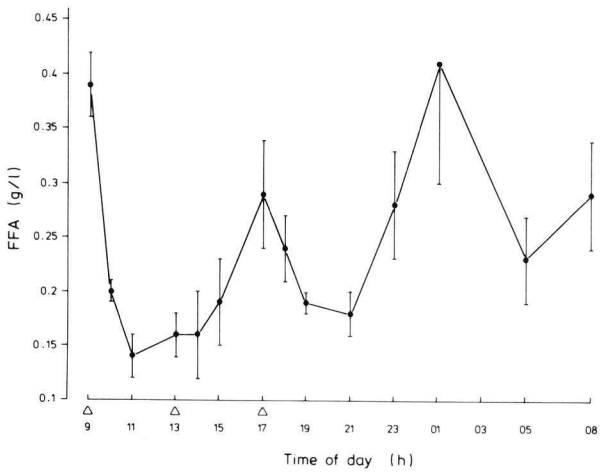

Figure 5. Diurnal course of serum FFA. Mean of 5 patients ± SEM. Triangles indicate time of meals.

Fig. 6 shows the diurnal course of LPL activity in the biopsies. Mean adipose tissue LPL (AT-LPL) activity did not change significantly in the course of the day ($p=0.67$). This is in striking contrast with observations in normal subjects, who showed a significant increase (18). Moreover, fasting values of AT-LPL activity were significantly lower than in normal subjects ($p<0.01$). When individual data are considered, one patient (MS) does show an increase in AT-LPL activity in the course of the day, while this activity was unchanged in the other four patients. Fasting skeletal muscle LPL (SM-LPL) activity was not significantly different from that in the normal subjects ($p=0.48$) (18). Three patients showed no change from 8.30 to 16.30 h as was also observed in the normal subjects. Data for two patients at 16.30 h are lacking.

Post-heparin lipolytic activity (PHLA) in the fasting (8.15 h) and in the fed state (16.15 h) is shown in Table II. LPL activity did not show a significant increase from the fasting towards the fed state ($p=0.26$, paired Student's t-test), which again is in

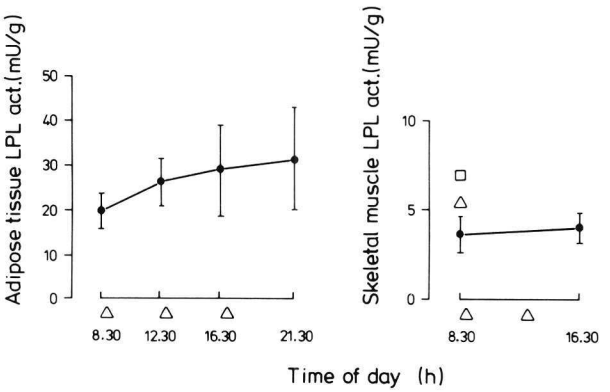

Figure 6. Diurnal course of adipose tissue LPL activity (left) and skeletal muscle LPL activity (right). Mean of 5 patients ± SEM for AT-LPL activity and mean of 3 patients ± SEM with two single values at 8.30 h for SM-LPL activity. Triangles indicate time of meals.

Table II. Post-heparin lipolytic activity (PHLA) in the fasting and the fed state.

LPL and HL activities (in mU/ml) were determined in plasma after intravenous injection of 100 IU heparin/kg body weight in the fasting state (day 7, 8.15 h) and the fed state (± day 12, 16.15 h).

Subjects	LPL activity[1]		HL activity[2]	
	fasting	fed	fasting	fed
MS	39	53	547	422
PF	73	44	290	292
HN	27	51	357	293
HvdM	57	79	306	357
JN	29	38	373	357
mean ± SD	45 ± 20	53 ± 16	375 ± 102	344 ± 54

[1]mean value of activity in plasma 20 and 45 minutes after heparin.
[2]activity in plasma 20 minutes after heparin.

striking contrast with the data for the normal subjects, who showed a significant increase (18). Fasting LPL activity was not significantly different from that in the normal subjects ($p=0.76$). Hepatic lipase (HL) activity was not significantly different from that in normals ($p=0.75$) and did not show a change from the fasting towards the fed state ($p=0.38$, paired Student's t-test).

Blood glucose concentrations were determined twenty times daily and did not differ from those in normal subjects ($p=0.32$, data not shown).

Fig. 7 shows the diurnal course of mean insulin and glucagon levels. Insulin increases after the first meal were clearly higher than after the others, as was

previously observed (15, 18). Insulin levels did not differ from those in normal subjects (p=0.39). Glucagon showed a pattern similar to what was observed in the normal subjects and no difference in glucagon levels was observed between the two groups (p=0.44). No correlations between these hormonal parameters and LPL activity could be found in the patients.

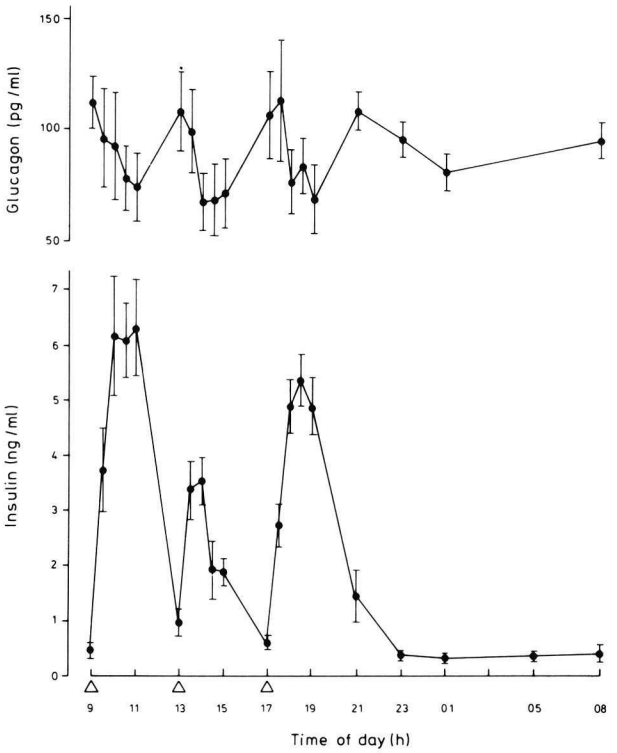

Figure 7. Diurnal course of serum insulin (lower part) and serum glucagon (upper part). Mean of 5 subjects ± SEM. Triangles indicate time of meals.
1 mg insulin = 27.3 U.

DISCUSSION

Patients with type IV hyperlipidaemia show diurnal serum TG patterns which are quite similar to those of normal subjects despite major differences in their lipolytic (TG hydrolysing) capacity during the day. In normal subjects, we have previously found an increase in total lipolytic capacity (determined as post-heparin LPL activity) from the fasting towards the fed state, which was thought to be due to an increase in AT-LPL activity (18). The patients described here do not show a

significant increase in post-heparin LPL activity in the course of the day; this seems to agree well with the observation that four patients did not show an increase in their AT-LPL activity in the course of the day. Why one patient failed to show an increase in total LPL activity notwithstanding an increase in AT-LPL activity is not known. Our findings concerning diurnal AT-LPL activity in type IV patients differ from those of Goldberg et al., who observed no differences between type IV patients and normal subjects in AT-LPL activity on fat-free formula diets (22). Also Pykälistö et al., on administering fat-free formula diets, observed an increase in AT-LPL activity from the fasting towards the fed state in type IV patients (23). Taskinen and Nikkilä noted a smaller increase in AT-LPL activity during a 6 hour infusion of a standard amount of glucose in obese type IV patients as compared with normals, but the response in obese normolipidaemics was similar (24). Perhaps their findings were related to the rather non-physiological administration of the nutrients, while our patients consumed normal food.

The observation that fasting levels of AT-LPL activity were significantly lower in patients than in normal subjects is in agreement with the findings of others (25, 26). Since our patients were not obese and therefore were unlikely to have had enlarged fat cells, we suppose that fasting AT-LPL activity when expressed per adipocyte will also be lower than in normals, which would be in agreement with the data of Larsson et al. (27) and Taylor et al. (28).

Few reports deal with SM-LPL activity. Taskinen et al. have reported lower SM-LPL activities in type IV patients as compared to those in normal subjects (26). We found no differences between patients and normals, but both our groups had rather low SM-LPL activities, possibly due to the carbohydrate-rich diet consumed (18). The low SM-LPL activity is probably responsible for the low fasting post-heparin LPL activity, since in the fasting state muscle enzyme is predominantly released in the early phase after heparin injection (29).

AT-LPL activity is stimulated by insulin (23, 30). Taylor et al. (28) and Taskinen and Nikkilä (24) have suggested that in type IV patients low AT-LPL activity and its impaired response to food intake might be a result of insulin resistance. Unlike many other type IV patients (9), ours do not differ from normal subjects in the amounts of insulin secreted or in blood glucose concentrations. Neither were diurnal FFA levels different from those in normal subjects. The existence, therefore, of insulin resistance in our patients, as far as the glucose decreasing and the antilipolytic action of insulin are concerned, seems to be unlikely. Moreover, glucagon responses were also within the normal range, which suggests a normal insulin sensitivity at the pancreatic level (31). Perhaps, the defect in AT-LPL may be due to factors other than insulin action, e.g., defects in the release mechanism as suggested by Taylor et al. (28).

The decrease in serum TG which had already begun before the absorption of the third meal cannot be explained by a change in total LPL activity in the course of the day. It is possible that the TG-rich particles themselves changed in their suitability to serve as a substrate for LPL in the course of the day and hence determined the TG pattern; studies on this subject are presently in progress.* Apart from this,

*See chapter VII.

changes in TG synthesis in the course of the day may have also determined the TG pattern in our patients. Since the concentration of FFA is lower in the afernoon and early evening than in the fasting state, TG synthesis at this time might be less than in the morning. The nonoccurrence of the normal increase in LPL activity may explain the differences in the diurnal TG pattern between patients and normal subjects, i.e., the greater amplitude and the delay in reaching the maximum values. Although the low LPL activity in the afternoon is sufficient to provide a reasonable TG clearance, it fails to reduce the wave-like TG pattern to a normal level. We therefore feel that the absence of the normal increase in LPL activity, as caused by a defect in AT-LPL, is likely to play an important role in the occurrence of the high TG levels.

As previously reported, serum apo B primarily reflects LDL apo B (32) and probably also apo B from partly lipolysed VLDL-particles (18). The absence in our patients of the observed increase in apo B in normal subjects in the course of the day might be a result of the low LPL activity in the afternoon.

Concentrations of total HDL, HDL subfractions and their constituents were lower than in normal subjects, which is in accord with data of others (2). The reason for this observation is not quite clear: both a decreased synthesis (33) and an increased clearance are reported (33-35). A portion of the HDL fraction results from lipolysis of TG-rich particles, as fragments of the polar wall of these particles fuse with dense HDL material to form less dense (d=1.110 g/ml) HDL (36). As a result of our isolation procedure, we measure these newly formed particles in the HDL_3 fraction (18). We did note an increase in the phospholipid moiety of the HDL_3 fraction in the course of the day, as was previously observed in our normal subjects, but the mean increase between 11.00 and 23.00 h was lower in patients (0.04 mmol/l) than in normal subjects (0.14 mmol/l). This is also reflected in a smaller increase in total HDL-phospholipid in patients as compared with normal subjects. Considering the decrease in serum TG up to 23.00 h, which was slightly greater in the patients than in the normal subjects, we would have expected a similar or perhaps an even greater increase in the HDL_3-phospholipid fraction in the patients, especially since the activity of hepatic lipase, which is thought to clear HDL (37), did not differ between our two groups. The absence in our patiens of the normal increase in apo A-I and HDL_3-cholesterol levels in the course of the day also indicates that HDL formation from TG-rich lipoproteins might be different in patients from that in normals. Observations of Chung et al. also point to a possible defect in this shuttle (38). Whether these findings can account for the low concentration of HDL in type IV patients remains to be confirmed.

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Chapter VII

DIURNAL COURSE OF THE SUITABILITY OF SERUM SAMPLES OBTAINED FROM NORMAL SUBJECTS AND FROM PATIENTS WITH TYPE IV HYPERLIPIDAEMIA TO SERVE AS SUBSTRATE FOR LIPOPROTEIN LIPASE

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ABSTRACT

Serum samples obtained at various times of the day from 4 normal subjects and 5 patients with type IV hyperlipidaemia on a carbohydrate-rich diet, were incubated with milk lipoprotein lipase (LPL) to investigate whether triglyceride (TG)-rich particles differ in their suitability to serve as a substrate for LPL at various times during the day and between normals and patients. Mean K_m and V_{max} of this reaction increased in patients between 9.00 and 13.00 h ($p=0.016$ and 0.015, respectively) and decreased again thereafter, while no diurnal change was observed in normals ($p=0.31$ and 0.27, respectively). At 13.00 and 23.00 h patients showed a higher mean K_m ($p<0.02$) and at 23.00 h a higher mean V_{max} ($p<0.03$) than did normals. Rearrangement of the data revealed that in neither group was a diurnal difference in reaction velocity apparent at TG concentrations occurring in vivo and that patients had significantly lower reaction velocities than normals only at 13.00 h at TG concentrations below 3 mmol/l. This indicates that a defect in particle behaviour is not likely to play a role in the development of hypertriglyceridaemia.

INTRODUCTION

Type IV hyperlipidaemia is a common lipid abnormality, which in some studies is associated with an increased risk for atherosclerosis (1, 2). Overproduction of triglyceride (TG)-rich particles by the liver (3, 4) or gut, defective removal of such particles from the circulation (5-7) or a combination of the two (8, 9) have been proposed as the underlying abnormality. A faulty removal mechanism can be the result of either a defect in the enzyme lipoprotein lipase (LPL), which is responsible for the breakdown of TG-rich particles, or a defect in the substrate. Several investigators have indeed reported reduced LPL activities in type IV patients (10-13) but not much attention has been paid to the TG-rich particles themselves.

We recently studied diurnal TG metabolism in normal subjects and patients with type IV hyperlipidaemia (14-16) when in a steady state. Under identical circumstances, the patients exhibited a similar wave-like diurnal TG pattern as did the normal subjects, which differed from the normal pattern mainly in having a greater amplitude and in reaching the maximum value later in the afternoon. In an attempt to find an explanation for the occurrence of the diurnal patterns in the two groups as well as for the observed differences between these groups, we subjected normal subjects and patients with type IV hyperlipidaemia to a study in which various parameters of TG catabolism were investigated. Our results concerning diurnal LPL activity determination have been published elsewhere (17,*). Apart from this, properties of TG-rich particles themselves might also change in the course of the day and hence influence the development of the TG pattern, since it is reported that different TG-rich particles display differences in their suitability to serve as a substrate for LPL, i.e., the larger particles (chylomicrons) should be a better substrate than the smaller ones (VLDL) due to a lower K_m and a higher V_{max} of the

*See chapter VI.

former (18, 19). We have therefore now studied the suitability of TG-rich particles to serve as a substrate for LPL at various times of the day in patients as well as in normal subjects. In addition, the data of the two groups were compared in order to detect possible differences.

One factor which might influence this 'suitability' is the amount of the apoproteins C present on the particle. Apo C-II is an important activator of LPL (20), whereas apo C-III (C-III_0, C-III_1, C-III_2) is reported to inhibit it (21). The C-II/C-III ratio is considered by some authors to determine the extent of TG hydrolysis (22). To relate changes in particle function to these biochemical parameters, this ratio was determined on TG-rich particles.

MATERIALS AND METHODS

Design of the study

Participants in this study were four normolipidaemic healthy male students and five male patients with the primary form of type IV hyperlipidaemia whose data are given in Table I. They were studied on a carbohydrate-rich diet which contained 65 energy % carbohydrate (one third mono- and disaccharides), 23 energy % fat (one third polyunsaturated) and 12 energy % protein. The diet was comprised of only normal foods, included no alcohol and was individually adjusted to keep body

Table I. Clinical and biochemical data of test subjects.

	Age (years)	IBW[1] (%)	Fasting serum TG (mmol/l) on 'home' diet	Clinical signs
Normals				
NH	22	90	0.81	—
CJ	32	109	1.56	—
CB	38	107	1.07	—
PdB	23	98	1.04	—
Patients				
MS	48	125	9.5	gout
PF	30	112	11.2	coronary infarction, history of xanthomatosis
HN	38	128	8.6	history of xanthomatosis
HvdM	37	110	6.2	history of xanthomatosis
JN	43	108	6.0	—

[1] % IBW, percent of Ideal Body Weight according to Metropolitan Life Insurance Tables.

weight constant. Two weeks prior to admission to the hospital participants consumed this diet at home, in order to become metabolically adapted. The diet was continued during their stay in the metabolic ward, where it was divided into three equivalent meals given at 9.00, 13.00 and 17.00 h. On the sixth day after their admission, when a steady state had been reached (14), blood was obtained throughout the day for determination of lipids, apoproteins and the suitability of TG-rich particles to serve as a substrate for LPL.

Enzyme preparation

Lipoprotein lipase was prepared from fresh, nonpasteurized cow's milk (obtained from a local farmer). After centrifugation for 25 minutes at 1000 *g*, the cream was removed and the skimmed milk dialysed for 20 h against 0.02 M sodium citrate buffer (pH 7.0) containing 0.1 M NaCl (23). Thereafter, the material was further dialysed against distilled water for 7 h, followed by dialysis against saline (0.15 M NaCl) for 17 h (24). Each dialysis was performed at 4°C and in the presence of 0.001% β-mercaptoethanol. The final dialysate was centrifuged for 10 minutes at 1000 *g* to remove heavy insoluble material, after which cold acetone (1/3 of the dialysate volume) was added dropwise while stirring continuously. After 15 minutes, the precipitate was collected by centrifugation (7 minutes at 1000 *g*), placed on a Büchner funnel and washed with ice-cold distilled water and ether. The resulting acetone-ether powder was diluted with distilled water to a concentration of 2 mg/ml and kept frozen at –90°C in small portions until required for use. When tested against an artificial triglyceride emulsion (25) it showed the characteristics of LPL activity, i.e., dependence on serum for full activity and inhibition by high salt concentrations.

Reaction mixture

Serum from each subject, obtained at six time points per test day, was incubated with milk LPL. These sera were never stored for more than 48 h and were kept meanwhile at 4°C. In order to obtain kinetic parameters for the enzymatic reaction, four serum (substrate) concentrations of each time point were tested. For this purpose, the sera were diluted 1:0.5, 1:1 and 1:2. Lipoprotein depleted serum (LPDS) was used as the diluting agent so as to keep the concentration of all serum ingredients other than lipoproteins in the assay constant. LPDS was obtained by ultracentrifugation of serum from normal subjects by d=1.21 g/ml, followed by extensive dialysis against phosphate buffered saline. Reactions were carried out at 37°C in a 50 mM Tris HCl buffer, pH 7.4. The final volume was 236.8 μl, which was composed of 25 μl of diluted enzyme, 11.8 μl buffer and 200 μl of serum or serum dilutions. LPL was appropriately diluted (usually 1:60-1:25 of the stock concentration) in order to limit the hydrolysis of TG to within 10%. Under these conditions, the FFA production increased linearly for a duration of up to 60 minutes. Samples

were taken at 20-minute intervals for up to 60 minutes, after which they were placed on ice to stop the reaction. Determination of free fatty acids (FFA) was started within 40 minutes.

Free fatty acid determination

FFA were determined by means of an enzymatic/colorimetric method as recently described (26). All reagents used were obtained in a kit (NEFA-C-test Wako, Wako Pure Chemical Industries, Ltd., Osaka, Japan). In contrast to the original method, the reaction volume was reduced: 40 μl of the test material were incubated with 120 μl of reagent A and 240 μl of reagent B, which necessitated an incubation of 60 minutes for test material with reagent A. During these incubation procedures, LPL action was found to be completely inhibited. After this process, the incubation samples were put on a microtiter plate and measured for absorbance at 550 nm in a Titertek Multiskan (Flow Laboratories, Ayrshire, Scotland).

Enzyme kinetics

For each sample, Lineweaver-Burke plots were constructed by means of linear regression analysis. More than ninety percent of the correlation coefficients were greater than 0.95. K_m is expressed as mmol TG/l and V_{max} as mmol FFA/l/h at a standard enzyme concentration.

Apoprotein determination

The ratio of the apoproteins C-II and C-III was determined on very low density lipoproteins, which were isolated by selective precipitation with Mg-heparin according to Uterman et al. (27). They were separated by polyacrylamide slab gel electrophoresis in urea containing buffers which were prepared according to Kane et al. (28). Protein bands were stained with Fast Green FCF (29) and quantified by densitometric scanning using a Shimadzu CS 910 dual wave length TLC-scanner (Tokio, Japan) with 640 and 750 nm as sample and reference wave lengths respectively. No correction for possible differences in chromogenicity between the different apoproteins was made. The densitometric peak responses were quantitated by automatic integration and ratios of the apoproteins C-II and C-III calculated.

Statistical analysis

Diurnal changes were evaluated by means of analysis of variance, supplemented by the method of Scheffé (30). In all other cases, the Wilcoxon one and two-sample tests were applied.

RESULTS

Mean Michaelis-Menten constants, or K_m values, of the reaction between serum and standard LPL are shown for both groups in Fig. 1. Although normal subjects tended to show an increase from 9.00 to 13.00 h, the K_m did not change significantly during the day ($p=0.31$). The patients, however, did show a significant increase in K_m from 9.00 to 13.00 h ($p=0.016$), after which the K_m decreased to again reach a slight insignificant peak ($p=0.52$) at 23.00 h. As can be seen from Table II, patients have significantly higher K_m values than normal subjects only at 13.00 and 23.00 h.

Fig. 2 shows the mean maximal reaction rates (V_{max}) of both groups. The V_{max} of the normal subjects tended to show an increase from 9.00 to 13.00 h, but statistical analysis revealed no significant diurnal change ($p=0.27$). Patients showed a significant increase in V_{max} from 9.00 to 13.00 h ($p=0.015$), after which it decreased again; a second peak at 23.00 h was not different from that at 9.00 h ($p=0.76$). From Table II it can be seen that patients showed significantly higher V_{max} values than normals only at 23.00 h.

From these data we may conclude that TG-rich particles of normal subjects did not change in their suitability to serve as a substrate for LPL during the day, since no significant diurnal change in K_m or V_{max} occurred. On the contrary, TG-rich particles of patients did however show a diurnal change in K_m and V_{max}, which was apparent between 9.00 and 13.00 h. In order to understand the significance of this finding in patients for the in vivo situation, the data were rearranged and comparison was made between mean reaction velocities at 9.00 and 13.00 h at substrate concentrations which were in the in vivo range. Fig. 3 shows these data at

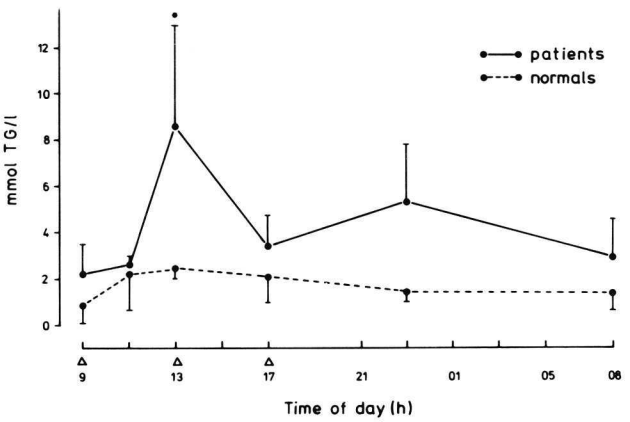

Figure 1. Diurnal course of Michaelis-Menten constants (K_m) of the reaction between serum and standard LPL. Mean of 4 normal subjects and 5 patients ± SD, respectively. Triangles indicate time of meals.

*significantly different from 9.00 h ($p=0.016$, method of Scheffé).

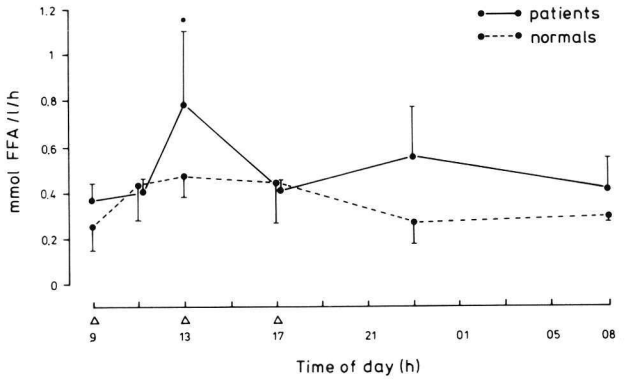

Figure 2. Diurnal course of V_{max} of the reaction between serum and standard LPL. Mean of 4 normal subjects and 5 patients ± SD, respectively. Triangles indicate time of meals.
*significantly different from 9.00 h (p=0.015, method of Scheffé).

Table II. Mean K_m and V_{max} (± SD) of the reaction between serum and standard LPL.

	K_m (mmol TG/l)			V_{max} (mmol FFA/l/h)		
	normals	patients		normals	patients	
9.00 h	0.82 ± 0.71	2.20 ± 1.28	ns	0.25 ± 0.10	0.37 ± 0.06	ns
11.00 h	2.21 ± 1.54	2.61 ± 0.28	ns	0.43 ± 0.15	0.40 ± 0.06	ns
13.00 h	2.47 ± 0.42	8.60 ± 4.30	p<0.02	0.47 ± 0.09	0.78 ± 0.32	ns
17.00 h	2.09 ± 1.09	3.43 ± 1.31	ns	0.44 ± 0.17	0.41 ± 0.04	ns
23.00 h	1.44 ± 0.39	5.31 ± 2.49	p<0.02	0.27 ± 0.10	0.55 ± 0.22	p<0.03
8.00 h	1.39 ± 0.77	2.99 ± 1.63	ns	0.29 ± 0.02	0.41 ± 0.14	ns

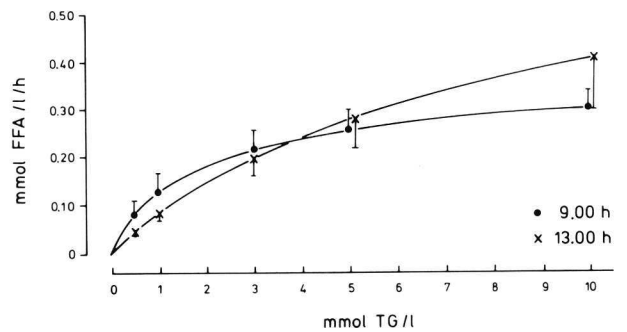

Figure 3. Velocities of the reaction between serum of patients obtained at 9.00 h and 13.00 h and standard LPL at varying TG concentrations. Mean of 5 patients ± SD, respectively.

TG concentrations of 0-10 mmol/l, which were obtained by substituting several TG concentrations in the individual mathematical functions. Only at low TG concentrations (0.5 and 1.0 mmol/l) did the velocity of the reaction between serum obtained at 13.00 h and LPL seem to be lower than that of serum obtained at 9.00 h ($p=0.06$). At TG concentrations occurring in vivo, no differences were apparent between the reaction velocities of serum at the two time points, which implies that also in patients there was no difference in the suitability of TG-rich particles to serve as substrate for LPL in the course of the day.

Differences in reaction-kinetics between patients and normals were apparent at 13.00 and 23.00 h. We have related the observed findings to the in vivo situation in a similar way to that described above. Fig. 4 shows mean reaction velocities of serum obtained at 13.00 h and LPL for both groups at TG concentrations of up to 10 mmol/l. Only at low TG concentrations (below 3.0 mmol/l) were TG-rich particles from patients a less effective substrate than those of normal subjects. Fig. 5 shows the same data for serum obtained at 23.00 h. No significant difference at the TG concentrations substituted was observed between the two groups at this time point.

Fig. 6 shows the ratio of the apolipoproteins C-II and C-III on TG-rich particles in the course of the day in both groups. No diurnal change was observed in either group (normals $p=0.33$; patiens, $p=0.80$) nor were there differences between the groups.

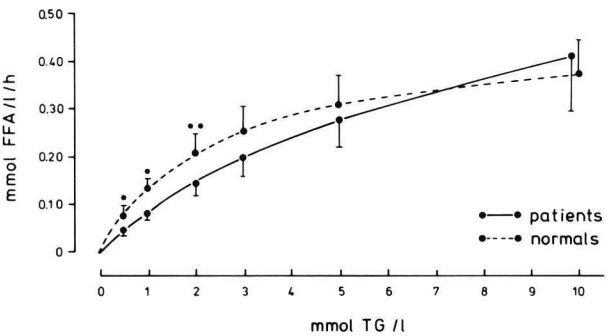

Figure 4. Velocities of the reaction between serum of patients and normal subjects obtained at 13.00 h and standard LPL at varying TG concentrations. Mean of 4 normal subjects and 5 patients ± SD, respectively.
* $p<0.02$.
** $p<0.05$.

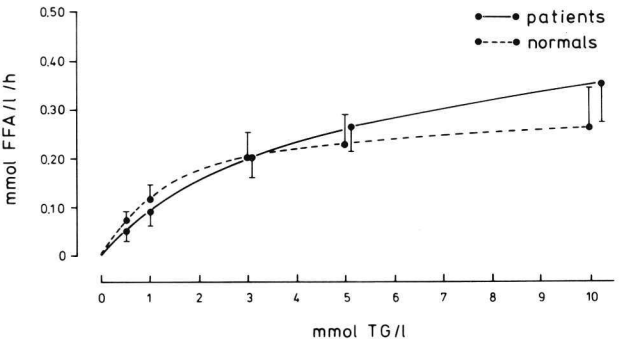

Figure 5. Velocities of the reaction between serum of patients and normal subjects obtained at 23.00 h and standard LPL at varying TG concentrations. Mean of 4 normal subjects and 5 patients ± SD, respectively.

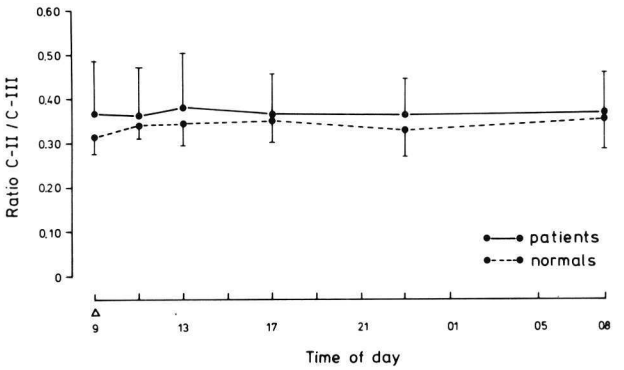

Figure 6. Diurnal course of the ratio C-II/C-III on TG-rich particles of normal subjects and patients. Mean of 4 normal subjects and 5 patients ± SD, respectively. Triangles indicate time of meals.

DISCUSSION

For details on the diurnal course of serum TG and its main contributors chylomicron-TG and VLDL-TG, we refer to previous articles (17,*). In this study we have investigated whether there are differences in the suitability of TG-rich particles to serve as a substrate for LPL (i) at various times in the course of the day and (ii) between normal subjects and patients with type IV hyperlipidaemia. For this purpose we used whole serum instead of isolated TG-rich lipoproteins as a substrate for LPL. Since LPL has a much higher affinity for the TG-rich lipoproteins than for the other lipoproteins (18, 19), the first will be selectively

*See chapter VI.

attacked. In this way our method approaches the in vivo condition and lacks the disadvantage of damage to lipoproteins when isolated by ultracentrifugation (31).

Concerning the first question, we observed diurnal changes in the kinetic parameters K_m and V_{max} in the patient group and a trend towards a similar change in normal subjects. These changes probably reflect changes in the composition of the particles, which at any time point are the result of the continuously changing processes of TG synthesis and TG breakdown. However, although K_m and V_{max} change, the TG-rich particles do not vary at different times of the day in their suitability to serve as a substrate for LPL at the TG concentrations which prevail in vivo. The influence of the higher K_m, which indicates a lower affinity for the enzyme, is apparently nullified by the simultaneous increase in V_{max}. Our findings might therefore indicate that neither in normal subjects nor in patients is the TG pattern a result of diurnal variations in particle behaviour. Variations in lipolytic activity or in TG synthesis in the course of the day, as reported by us elsewhere (17,*), are apparently the main factors in determining the TG pattern.

The finding that particle behaviour does not differ between time points at which varying concentrations of chylomicrons prevail contrasts with the view that chylomicrons should be a better substrate for LPL than VLDL (18, 19). However, a considerable proportion of these 'chylomicrons' in both of our groups of subjects, but especially in the patients, will be large liver-derived VLDL particles which are known to occur on a carbohydrate-rich diet (32). Although the density and diameter of these particles may come close to those of 'real' chylomicrons, there are a number of differences in the composition of lipids and apoproteins (33) which may account for differences in kinetic behaviour. Perhaps this accounts for the disagreement of our results with the literature. It is also possible that our findings are due to the use of whole serum instead of isolated lipoproteins.

As to the second question, we noted a trend towards higher K_m values during the entire day in the patients as compared with the normal subjects, which reached a significant difference at 13.00 and 23.00 h. As shown, the only observed defect in the particles of patients was present at 13.00 h at low TG concentrations. The influence of the higher K_m on the reaction velocity of patients particles at 23.00 h was abolished by the simultaneous increase in V_{max}. As to the defect in particle behaviour, our observations are roughly in agreement with those of Chung et al., who reported a diminished TG hydrolysis of isolated VLDL particles from type IV patients as compared with those of normals at VLDL-TG concentrations ranging from 0.79-2.82 mmol/l (34). However, they used VLDL-particles obtained from fasting subjects, while our observations concern nonfasting whole serum. It is unclear why in our hands the defect is limited to particles obtained in the nonfasting state. Since the defect is not observed throughout the day, it is not likely to represent a major factor in the pathogenesis of hypertriglyceridaemia.

The significance of the C-II/C-III ratio on TG-rich particles as a regulator of the extent of TG hydrolysis seems to be somewhat controversial in the literature. Eisenberg et al. (35) and Catapano et al. (36) observed no change in this ratio on TG-

*See chapter VI.

rich particles during hydrolysis of up to 60% of the original TG concentration. They therefore concluded that this ratio was not important as a determinant for hydrolysis. Others, on finding a lower ratio in the heavier VLDL subfraction, concluded the opposite (22, 37). Carlson and Ballantyne (22) and Catapano (38) observed a lower C-II/C-III ratio on TG-rich particles of type IV patients as compared with those of normal subjects and thought that this might contribute to the development of hypertriglyceridaemia. Our results indicate that since no difference in this ratio is found between normal subjects and patients in spite of differences in K_m and V_{max}, it does not seem to determine TG hydrolysis. Moreover, the lack of change in ratio during the day in patients despite diurnal changes in K_m and V_{max} further supports this view.

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SUMMARY AND DISCUSSION

This thesis is concerned with metabolic studies on human triglyceride metabolism. As described in Chapter I, the TG-rich lipoproteins, i.e., the chylomicrons and VLDL, are responsible for the transport of exogenous respectively endogenous triglyceride to the various tissues. The metabolism of these lipoproteins is closely related to that of the other lipoproteins. One of the main reasons why attention is focussed on disturbances in TG metabolism which lead to an increase in the VLDL concentration is the frequent association with coronary heart disease. Whether the high concentration of the TG-rich particles or their remnants is responsible for this, or whether the accompanying low HDL concentration is the major cause, is not known. The relation between atherosclerosis and endogenous hypertriglyceridaemia, however, is less well established than that between this disorder and hypercholesterolaemia.

Many internal and external factors, especially food intake and physical activity, are known to influence TG metabolism. It thus seems likely that diurnal fluctuations may occur in serum TG levels, which implies that studies on this subject should cover a 24 hour timespan. Therefore our test subjects were studied for 24 hours after being brought into a steady state.

In Chapter II and III the diurnal course of serum TG and the TG-rich lipoproteins is described in normal subjects and patients with type IV hyperlipidaemia at various meal frequencies. In order to examine the influence of the nature of the food on the TG levels, these studies were carried out on a carbohydrate-rich (65 energy % carbohydrate) as well as on a fat-rich (65 energy % fat) diet. For both groups it was concluded that not the nature of the food, but the frequency and the distribution of meals were mainly responsible for the diurnal TG pattern. We did notice, however, quantitative differences regarding serum TG levels between both diets: in both groups the TG level on the carbohydrate-rich diet was higher than that on the fat-rich diet.

Type IV hyperlipidaemic patients with a normal glucose tolerance showed diurnal wave-like TG patterns which were quite similar to those of normal subjects. Whereas normal subjects, on a regimen of 3 equivalent meals at 9.00, 13.00 and 17.00 h, showed an increase in serum TG from 9.00 h onwards until 14.00 h, followed by a decrease to below starting values in the early morning and a subsequent increase to 9.00 h, patients only differed in this pattern in showing a higher TG level and a greater amplitude and in reaching the maximum later in the afternoon.

In order to understand the development of the TG patterns and to account for the hypertriglyceridaemia in the patients, subsequent studies were carried out as to the factors determining TG catabolism. The enzyme lipoprotein lipase, which is mainly synthesized in adipose tissue and skeletal muscle and functions at the

luminal surface of the endothelium in these tissues, is responsible for the hydrolysis of TG-rich lipoproteins. The physiologically active fraction of total LPL can be measured in plasma after intravenous administration of heparin. No differentiation as to tissue origin can be made in this way and therefore an assay was developed in which the physiologically active fraction of the LPL activity from tissue specimens could be measured. In Chapter IV this assay is extensively described. LPL was subsequently measured in 5 normal subjects (Chapter V) and 5 type IV hyperlipidaemic patients with a normal glucose tolerance (Chapter VI) on a carbohydrate-rich diet. Adipose tissue was obtained from the abdominal wall; skeletal muscle from the musculus vastus lateralis. Normal subjects showed a 2½ fold increase in post-heparin LPL activity in the afternoon (16.30 h) as compared to the fasting state (8.30 h), whereas patients, who in the fasting state had similar activities as normals, failed to show an increase in the course of the day. The increased post-heparin LPL activity in the normal subjects appeared to result from an increase in AT-LPL activity between 12.30 h and 16.30 h. A significant positive correlation was found between the increase in AT-LPL activity (between 8.30 and 16.30 h) and the amount of insulin secreted in this timespan. Despite diurnal insulin levels, which were not significantly different from those in normal subjects, 4 out of 5 patients did not show any increase in AT-LPL activity in the course of the day. Moreover their fasting AT-LPL activity was lower than in normal subjects. SM-LPL activity was similar in the two groups and did not show a diurnal change. No correlations could be detected between the concentrations of the hormones insulin and glucagon on the one hand and SM-LPL activity on the other.

Apart from the enzymatic aspect of TG catabolism, the susceptibility to lipolysis of the TG-rich particles might also influence TG catabolism. In Chapter VII we show the results of an investigation in which serum, obtained at several times during the day from the subjects just mentioned, was incubated with a standard LPL and the rate of lipolysis measured. It appeared that the suitability of TG-rich particles to serve as a substrate for LPL did not change in the course of the day in the two groups. Particles of patients were a less effective substrate than those of normals only at one time point (13.00 h) of the day.

Referring to these findings, we propose the following explanation for the diurnal TG pattern. Early in the day TG synthesis exceeds TG catabolism; a considerable VLDL synthesis, reflected in the increase in serum TG already before breakfast and presumably resulting from the high fasting FFA levels, together with the influx of TG from breakfast, are probably responsible for this. The decrease in serum TG in normal subjects at 14.00 h was initially attributed to the increase in LPL activity. However, the decrease in serum TG in patients, although occurring later (17.00 h), can neither be explained by a diurnal change in LPL activity, nor by a diurnal change in the susceptibility to lipolysis of the TG-rich particles. This leaves a diurnal change in VLDL synthesis as the major determinant of the diurnal TG pattern. The low FFA concentration in the afternoon as compared to the fasting state, probably a result of the antilipolytic action of insulin, makes a lower VLDL synthesis in the afternoon /evening as compared to the fasting state plausible. Since

the diurnal FFA pattern as well as its concentration were in normal subjects not different from those in patients, this theory might also apply to the normal subjects. Although the final explanation for the decrease in serum TG in the two groups must apparently be sought in a diurnal change in VLDL synthesis, the increase of LPL activity in the course of the day in normal subjects is likely to account for their earlier decrease in serum TG as compared to patients.

We consider the nonoccurrence of the normal increase in AT-LPL activity in the course of the day an important factor in the pathogenesis of hypertriglyceridaemia in our patients. Since a defective lipolysis of TG-rich particles of patients as compared to those of normal subjects was only apparent at one time point of the day, it is not likely that defects in the substrate are the major cause for the disorder. Neither do we consider an increased VLDL production by the liver to account for the hypertriglyceridaemia in our patients, since diurnal FFA and insulin levels, an increase of which is held to be responsible for an increased VLDL production (Chapter I), did not differ between patients and normal subjects.

As already mentioned, the metabolism of HDL is narrowly related to that of the TG-rich lipoproteins, since among others part of the HDL fraction is derived from surface fragments which are liberated from the TG-rich lipoproteins during lipolysis. In patients lower concentrations of HDL and its subfractions were observed than in normal subjects. It was remarkable that whereas normal subjects showed an increase in the concentration of serum apo A-I, HDL_3-cholesterol and -phospholipid in the course of the day until 23.00 h, following the decrease in TG in the afternoon, patients showed no diurnal change in HDL_3-cholesterol and apo A-I levels and a smaller increase in HDL_3-phospholipid in the same period notwithstanding a similar decrease in serum TG until 23.00 h. Since the activity of hepatic lipase, the enzyme responsible for the catabolism of HDL, was similar in the two groups, we initially concluded that a defect in the transfer process of surface fragments might explain these findings. Perhaps, however, we should consider the total amount of TG which is broken down between $\pm$ 14.00 h and 23.00 h. This might in normals be greater than in patients because of the higher LPL activity in this period in the former and hence result in a greater increase in the HDL_3-lipid fraction and serum apo A-I. If so, the smaller diurnal variations in the HDL fraction in patients as compared to normal subjects might directly be related to the defect in adipose tissue LPL. Whether this would account for the low HDL concentrations in patients remains to be answered.

SAMENVATTING EN DISCUSSIE

In dit proefschrift worden metabole studies beschreven, die tot doel hebben meer inzicht te verkrijgen in de triglyceridenstofwisseling bij de mens. Zoals in hoofdstuk I vermeld is, hebben de TG-rijke lipoproteïnen chylomicronen en VLDL een belangrijke functie in het transport van exogeen respectievelijk endogeen triglyceride naar de verschillende weefsels. Daarnaast is het metabolisme van deze lipoproteïnen nauw gerelateerd aan dat van de andere lipoproteïnen. Eén van de voornaamste redenen waarom er veel aandacht geschonken wordt aan de ontsporing van het normale triglyceridenmetabolisme, die tot een verhoging van de VLDL concentratie leidt, is de associatie met hart- en vaatziekten. Of de hoge concentratie van de TG-rijke deeltjes of hun 'remnants' in dit opzicht de belangrijkste boosdoener is, òf de lage concentratie van het HDL, die bij deze afwijking gevonden wordt, is niet bekend. Overigens is het verband tussen atherosclerose en endogene hypertriglyceridaemie minder duidelijk dan dat tussen deze afwijking en hypercholesterolaemie.

Het triglyceridenmetabolisme is gemakkelijk te beïnvloeden door tal van interne en externe factoren, waarbij vooral voedselopname en lichaamsbeweging een rol spelen. Het lijkt dus aannemelijk, dat er gedurende de dag en nacht verschuivingen op kunnen treden in dit metabolisme en dat de bestudering hiervan een etmaal zou moeten duren. Om bovengenoemde redenen zijn bij onze proefpersonen, nadat zij in een metabole evenwichtssituatie gebracht waren, 24-uurs studies gedaan.

In de hoofdstukken II en III hebben wij allereerst getracht een indruk te geven van het beloop van de concentratie van TG en de TG-rijke lipoproteïnen in het serum gedurende een dag bij normale proefpersonen en patiënten met type IV hyperlipidaemie op een regime van verschillende voedingsschema's. Om de invloed van de aard van de voeding op de TG-spiegels te bestuderen zijn deze studies zowel verricht met een koolhydraatrijk (65 energie % koolhydraat) als met een vetrijk (65 energie % vet) dieet. Voor beide groepen proefpersonen werd geconcludeerd, dat niet de aard van de voeding, maar de frequentie en distributie van de maaltijden voornamelijk van invloed zijn op de vorm van het 24-uurspatroon van het TG-gehalte in het serum. Bij gebruik van de verschillende diëten werden wel kwantitatieve verschillen ten aanzien van het TG-gehalte opgemerkt: bij beide groepen speelde het TG-patroon zich op het koolhydraatrijke dieet op een hoger niveau af dan op het vetrijke dieet. Ook waren bij beide groepen op het vetrijke dieet de FFA-spiegels hoger dan op het koolhydraatrijke dieet, terwijl, alleen bij patiënten, de insulinespiegels op het vetrijke dieet lager waren dan op het koolhydraatrijke dieet.

Bij patiënten met type IV hyperlipidaemie en een normale glucosetolerantie traden TG-patronen op, die veel gelijkenis vertoonden met die van normale proef-

personen. Op een regime van 3 equivalente maaltijden per dag, die om 9.00, 13.00 en 17.00 uur genuttigd werden, vertoonden normale proefpersonen vanaf 9.00 uur een stijging van het serum TG tot omstreeks 14.00 uur, die gevolgd werd door een daling tot onder de uitgangswaarde in de vroege ochtend, waarna weer een stijging tot de uitgangswaarde optrad. Bij patiënten bleven de verschillen in dit patroon ten opzichte van dat bij de normale proefpersonen beperkt tot het optreden van een hogere TG-concentratie, een grotere amplitude en het op een later tijdstip bereiken van de topwaarde.

Om een nader inzicht in het totstandkomen van de genoemde TG-patronen te verkrijgen en om de oorzaak van de hoge TG-spiegels bij de patiënten te achterhalen werd vervolgens nader onderzoek verricht naar factoren die het TG-catabolisme bepalen. Het enzym lipoproteïnelipase, dat voornamelijk in vet- en spierweefsel gesynthetiseerd wordt en zijn werking uitoefent aan de luminale zijde van het endotheel in deze weefsels, is verantwoordelijk voor de afbraak van TG-rijke lipoproteïnen. De fysiologisch werkzame fractie van het totale lichaams-LPL is te verkrijgen door intraveneus heparine toe te dienen en het hierdoor vrijgekomen enzym in het plasma te meten. Op deze wijze kan echter geen onderscheid worden gemaakt tussen de verschillen in weefselherkomst van het LPL. Daarom werd een bepaling opgezet, die tot doel had de fysiologisch werkzame fractie van het LPL te meten in stukjes vet- en spierweefsel. In hoofdstuk IV is deze bepaling beschreven. Vervolgens werd LPL volgens beide methoden in de loop van de dag gemeten bij een 5-tal normale proefpersonen (Hoofdstuk V) en een 5-tal patiënten met type IV hyperlipidaemie (Hoofdstuk VI), die een normale glucosetolerantie hadden. Deze proefpersonen werden ditmaal alleen bestudeerd op een koolhydraatrijk dieet, dat in 3 equivalente maaltijden (9.00, 13.00 en 17.00 uur) verdeeld was. Vetweefsel werd verkregen uit de abdominale subcutis, spierweefsel uit de musculus vastus lateralis. Het bleek, dat normale proefpersonen in de middag (16.30 uur) een ongeveer 2½ maal zo hoge LPL-activiteit in het serum na heparinetoediening vertoonden dan in de nuchtere toestand, terwijl deze activiteit bij de patiënten, hoewel in nuchtere toestand gelijk aan die bij normale proefpersonen, geen verandering gedurende de dag te zien gaf. De stijging van de LPL-activiteit in het serum na heparine in de loop van de dag bleek bij de normale proefpersonen te berusten op een toename van de LPL-activiteit van vetweefsel, die tussen 12.30 en 16.30 uur op gang kwam. Er bleek een statistisch significante positieve correlatie te bestaan tussen de toename van de vetweefsel-LPL-activiteit tussen 8.30 en 16.30 uur en de hoeveelheid insuline die in die tijd geproduceerd werd. Ondanks insulinespiegels die niet van die bij normale proefpersonen verschilden vertoonden 4 van de 5 patiënten geen toename van de vetweefsel-LPL-activiteit gedurende de dag. Bovendien lagen de waarden van deze activiteit in nuchtere toestand bij de patiënten significant lager dan bij de normale proefpersonen. De activiteit van spier-LPL was voor beide groepen niet verschillend en veranderde niet in de loop van de dag. Er bleken geen correlaties te bestaan tussen de concentraties van de hormonen glucagon en insuline enerzijds en spier-LPL-activiteit anderzijds.

Naast het enzymatische aspect van de TG-afbraak kan ook de 'geschiktheid' van

de TG-rijke deeltjes om als substraat voor LPL te dienen van invloed zijn op de afbraak. In hoofdstuk VII hebben wij getracht een indruk van deze 'geschiktheid' te geven door serum, verkregen op verschillende tijdstippen van de dag van de zojuist genoemde proefpersonen als substraat aan te bieden aan een standaard LPL-preparaat en de lipolyse te meten. Bij beide groepen bleek de 'geschiktheid' van TG-rijke deeltjes om als substraat voor LPL te dienen gedurende de dag niet te veranderen. Bij de patiënten waren de TG-rijke deeltjes op één tijdstip van de dag (13.00 uur) een minder geschikt substraat voor LPL dan die van normale proefpersonen.

Gebruik makend van deze gegevens menen wij, dat het 24-uurs patroon van het TG-gehalte in het serum op de volgende wijze verklaard kan worden. Bij beide groepen overheerst in het begin van de dag de aanmaak over de afbraak; een aanzienlijke VLDL-synthese, die weerspiegeld wordt in een toename van de TG-concentratie al voor het ontbijt en die waarschijnlijk het gevolg is van de hoge FFA-concentratie op dat moment, te zamen met de influx van TG ten gevolge van de eerste maaltijd, zijn hier waarschijnlijk verantwoordelijk voor. De daling van het TG-gehalte in het serum, die bij de normale proefpersonen omstreeks 14.00 uur inzette, werd aanvankelijk door ons gezien als een gevolg van de toegenomen LPL-activiteit. De daling van het serum-TG bij de patiënten, die weliswaar later (17.00 uur) inzette, blijkt echter noch te verklaren met een verandering in de LPL-activiteit gedurende de dag, noch met een verandering in de 'geschiktheid' van de TG-rijke deeltjes, zodat een verandering in de VLDL-synthese gedurende de dag de voornaamste verklaring lijkt te vormen. De lagere FFA-concentraties in de middag ten opzichte van de nuchtere toestand, waarschijnlijk veroorzaakt door een door insuline geïnduceerde remming van de lipolyse, maken aannemelijk dat de VLDL-synthese in de middag/avond lager kan zijn dan in de nuchtere toestand. Aangezien echter zowel het 24-uurs patroon als de concentratie van FFA bij de normale proefpersonen niet verschillen van die van patiënten, veronderstellen wij dat ook normale proefpersonen een lagere VLDL-synthese in de middag t.o.v. de ochtend vertonen. Bij beide groepen moet de verklaring voor de daling van het TG-gehalte in het serum dus waarschijnlijk gezocht worden in een verandering van de VLDL-synthese gedurende de dag. Echter, de toename van de LPL-activiteit in de loop van de dag bij de normale proefpersonen is waarschijnlijk wèl verantwoordelijk voor het feit, dat de daling van het TG-gehalte in het serum bij hen al op een eerder tijdstip inzet dan bij de patiënten.

Wij menen dat het uitblijven van de normale stijging van de LPL-activiteit in het plasma na heparinetoediening in de loop van de dag, die het gevolg is van het uitblijven van de normale toename van de activiteit van vetweefsel-LPL, een belangrijke rol speelt in de pathogenese van de hypertriglyceridaemie bij onze patiënten. Aangezien TG-rijke deeltjes van patiënten maar op één tijdstip van de dag een minder goed substraat voor LPL bleken te zijn dan die van normale proefpersonen, is het niet waarschijnlijk dat defecten in het substraat zelf een rol spelen. Evenmin hebben wij reden om een verhoogde VLDL-productie door de lever als oorzaak voor de hypertriglyceridaemie aan te nemen, aangezien wij geen ver-

schillen vonden tussen patiënten en normale proefpersonen in de spiegels van FFA en insuline gedurende de dag. In de literatuur wordt immers een verhoging van deze spiegels verantwoordelijk gesteld voor een verhoogde VLDL-productie (hoofdstuk I).

Zoals reeds vermeld, is het metabolisme van HDL nauw gerelateerd aan dat van de TG-rijke lipoproteïnen; o.a. wordt een deel van de HDL-fractie gevormd door fragmenten van de polaire mantel van de TG-rijke lipoproteïnen die tijdens lipolyse door LPL afgesplitst worden. Bij patiënten werd een veel lagere concentratie van de HDL-fractie en haar subfracties gevonden dan bij normale proefpersonen. Opmerkelijk was dat terwijl normale proefpersonen een stijging van de concentratie van HDL_3-cholesterol, HDL_3-phospholipiden en serum apo A-I tot 23.00 uur vertoonden, die volgde op de daling van het TG-gehalte in de middag, er bij de patiënten in dezelfde periode geen stijging van de HDL_3-cholesterol en serum apo A-I concentratie en een veel geringere stijging van de HDL_3-phospholipidenconcentratie optrad dan bij de normale proefpersonen. Wanneer wij de daling van het TG gehalte in het serum in aanmerking nemen, lijkt er tot 23.00 uur bij de patiënten en de normale proefpersonen ongeveer evenveel TG te verdwijnen. Aangezien er bovendien geen verschil bestond in de activiteit van het hepatisch lipase bij beide groepen, het enzym dat verantwoordelijk wordt gesteld voor de afbraak van HDL, hebben wij aanvankelijk geconcludeerd dat er mogelijk een defect in de overdracht van mantelfragmenten naar de HDL-fractie aanwezig zou zijn bij de patiënten dat de genoemde verschillen t.o.v. normalen zou verklaren. Misschien moeten wij echter de totale hoeveelheid TG die tussen $\pm$ 14.00 en 23.00 uur wordt afgebroken in beschouwing nemen. Deze zou bij de normale proefpersonen wel eens groter kunnen zijn dan bij de patiënten tengevolge van de hogere LPL-activiteit in deze periode bij de eersten, hetgeen dan zou kunnen resulteren in een sterkere stijging van de HDL_3-lipiden en het serum apo A-I. In dit geval zouden de geringere veranderingen in de HDL-fractie gedurende de dag bij de patiënten rechtstreeks een gevolg kunnen zijn van het defect in vetweefsel-LPL. Of dit tevens een verklaring vormt voor de lage HDL-concentraties bij patiënten zal moeten blijken.

CURRICULUM VITAE

De schrijfster van dit proefschrift werd geboren op 8 september 1954 te Rotterdam. Van 1966 tot 1971 bezocht zij de dependance van het Rotterdamsch Lyceum te Krimpen a/d IJssel, waar zij in 1971 het diploma HBS-B behaalde. In datzelfde jaar werd de studie Geneeskunde aan de Rijksuniversiteit te Leiden aangevangen. In 1974 werd het kandidaatsexamen afgelegd. In 1976 behaalde zij het doctoraal-examen en op 26 mei 1978 legde zij het artsexamen af.
In de jaren 1973-1975 was zij student-assistent op het Anatomisch-Embryologisch Laboratorium van de Rijksuniversiteit Leiden.
Vanaf september 1978 tot heden is zij aangesteld als wetenschappelijk ambtenaar, ten laste van het Praeventiefonds, aan de Rijksuniversiteit Leiden en werkzaam in de Diabeteswerkgroep (hoofd: Prof. Dr. J. Terpstra) van de afdeling voor Stofwisselingsziekten en Endocrinologie (hoofd: Prof. Dr. D. Smeenk), Academisch Ziekenhuis Leiden.

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