Chylomicron remnant metabolism in familial dyslipidemias studied with a remnant-like emulsion breath test

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Abstract **We have developed a stable isotope breath test for the assessment of chylomicron remnant metabolism and report the results from the breath test in human subjects selected for disorders of chylomicron or remnant metabolism. In type I hyperlipemia, the phenotype is extreme hypertriglyceridemia due to a lack of lipoprotein lipase activity, which causes the failure of remnant formation. The type III dyslipidemia phenotype is caused by the inefficient removal of chylomicron remnants from plasma, generally because of homozygosity for apolipoprotein E2 alleles. The breath test was predicted to be abnormal in type III hyperlipemia, whereas a priori in type I hyperlipemia defective remnant clearance was not anticipated. Subjects were injected with lipid emulsions prepared with a composition similar to normal chylomicron remnants. The emulsions contained cholesteryl ester incorporating the stable nonradioactive isotope ^{13}C in the fatty acid moiety. End exhalation breath was collected at intervals after intravenous injection of the remnant-like emulsions and analyzed for ^{13}C enrichment by isotope-ratio mass spectrometry. Compared with the group of normolipemic men, the fractional catabolic rate of remnants measured by the breath test was significantly decreased ($P = 0.006$) in subjects with type III dyslipidemia. In the group with type I hyperlipemia, the fractional catabolic rate was not different ($P = 0.233$) from the control group. Therefore, the underlying capacity for remnant catabolism was normal in this group of markedly hypertriglyceridemic subjects. By short-circuiting the step of lipolysis, the remnant-like emulsion breath test provides direct information about remnant clearance and metabolism, which should assist in investigations of postprandial lipid metabolism.**—Redgrave, T. G., G. F. Watts, I. J. Martins, P. H. R. Barrett, J. C. L. Mamo, S. B. Dimmitt, and A. D. Marais. **Chylomicron remnant metabolism in familial dyslipidemias studied with a remnant-like emulsion breath test.** ***J. Lipid Res.*** **2001.** 42: **710–715.**

Supplementary key words hyperlipidemia • isotope ratio mass spectrometry • carbon isotopes • cholesteryl ester

There is increasing need for assessment of the capacity of individual subjects to metabolize the remnants of triglyceride-rich lipoproteins because of growing awareness of the contribution of remnants to atherogenesis (1). Existing methods rely on the measurement of apoB-48 in plasma or on the postprandial changes in ingested vitamin A, but neither of these methods clearly distinguishes between defective lipolysis and problems of remnant clearance. We report here the results in selected groups of subjects of an investigation of remnant metabolism using a remnant-like emulsion breath test. The new breath test overcomes difficulties in measuring remnant metabolism in humans and is timely because the cholesterol-enriched remnants of triglyceride-rich lipoproteins play a role in accelerated atherosclerosis (2–4).

The triglyceride-rich chylomicrons secreted by the intestine and very low density lipoprotein (VLDL) secreted by the liver are substrates for lipoprotein lipase, which hydrolyzes a proportion of the particle triglyceride content to produce a remnant particle that is smaller in size, contains fewer triglycerides and phospholipids, and is relatively enriched in its content of free and ester cholesterol (5). The remnants are then removed by receptor-mediated endocytosis, normally predominantly by the liver, with small uptakes by other tissues, including arteries (6). Plasma apolipoproteins are essential for the catabolism of remnants, particularly apoCII as a cofactor for lipase action and apoE as a ligand for the removal of remnants from plasma by receptors expressed in the liver and other tissues (7).

Direct measurements of remnant metabolism in humans are difficult to make because nascent triglyceride-rich lipoproteins cannot be collected for study. Some studies have been done with plasma fractions enriched with

Abbreviations: ApoE, apolipoprotein E; CETP, cholesteryl ester transfer protein; FCR, fractional catabolic rate; VLDL, very low density lipoproteins.

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remnants, but this approach is not suited for routine applications (8). An alternative strategy is to employ artificial lipoproteins that are formulated to mimic the composition and physiology of normal triglyceride-rich lipoproteins. This approach was first applied using lipid emulsion models of lymph chylomicrons (9) and has since provided important insights into chylomicron metabolism in humans (10–16).

More recently, lipid emulsion models of remnant particles have been developed (17), and these are employed in the new breath test. The remnant-like emulsions do not require lipolysis before associating with apoE, which then mediates uptake by the liver. The emulsions incorporate the stable, nonradioactive isotope ^{13}C to label the fatty acid moiety of cholesteryl esters in the remnant-like particles. After injection, the lipid emulsions rapidly gain apoE from plasma and are endocytosed by the liver. In the liver, hydrolysis of the remnant cholesteryl esters in lysosomes liberates fatty acids, which are oxidized in mitochondria to CO_2, providing a simple measure of catabolism of the remnant particles because enrichment of ^{13}C in the exhaled breath CO_2 can be directly quantitated by isotoperatio mass spectrometry without any preliminary processing or chromatography.

METHODS

Subjects

Subjects with proven types I and III hyperlipemia were studied following an intravenous injection of a remnant-like emulsion. Type I hyperlipemia was diagnosed when subjects with fasting plasma triglycerides >15 mmol/l were demonstrated to have chylomicron accumulation as well as absent post-heparin lipase activity. There were five male and four female subjects with an average age of 27 years (range 12–48 years). Type III hyperlipemia was diagnosed by ultracentrifugation of plasma lipoproteins to confirm the presence of a cholesterol-rich VLDL fraction and by genotyping apoE to confirm the presence of homozygosity for the apoE2 allele, or in two subjects the presence of one of the rare dominant mutations of apoE. There were 13 male and 4 female subjects with an average age of 53 years (range 44–65 years). All patients were receiving fat-modified diets for their hyperlipemia and were in stable body weight. A control group of subjects was recruited with an average age of 44 years (range 28–64 years) and plasma cholesterol <5.5 mmol/l and triglycerides <1.8 mmol/l. None of the control subjects had a history of cardiovascular disease. One of the control subjects but none of the type I subjects was homozygous for apoE2. General exclusion criteria were obesity (body mass index >30 kg/m^2), diabetes, proteinuria, thyroid disease, lipid-regulating therapy, or acute illness. The studies received approval from the Ethics Review Committees of Royal Perth Hospital and the University of Cape Town, and all subjects gave informed consent.

Preparation of emulsions

Uniformly labeled [^{13}C]oleic acid was purchased from Novachem Pty. Ltd. (Victoria, Australia), and cholesteryl [^{13}C]oleate was synthesized from cholesterol and [^{13}C]oleic acid as described previously (17). Briefly, equimolar amounts of uniformly labeled [^{13}C]oleic acid and cholesterol were reacted for 17 h at room temperature in dry carbon tetrachloride with 107 mol% of dicyclohexylcarbodiimide in the presence of 16 mol% N,N-dimethylaminopyridine. A 1% volume of water was added, and after stirring for 4 h, the mixture was extracted with petroleum spirits and then 2% diethyl ether in petroleum. After filtration through a small silica column, fractions shown by thin-layer chromatography to contain cholesteryl ester were combined, reduced in volume, and purified by flash chromatography on silica gel. After drying under high vacuum to constant mass, the cholesteryl (all [^{13}C])oleate was a colorless oil that crystallized (yield 80%), m.p. 45.5 to 46.5°, $[\alpha]_D^{23}$ −22.5° (c. 2.0, $CHCl_3$). Structure was confirmed by proton nuclear magnetic resonance (details available on request).

Lipid mixtures containing triolein (135 mg), phosphatidylcholine (75 mg), cholesteryl [^{13}C]oleate (70 mg), and cholesterol (24 mg) (all more than 99% pure) were emulsified by sonication for 1 h in 8.5 ml of 2.2% glycerol in water. After sonication, the emulsion mixture was centrifuged at 3,000 rpm for 10 min to remove titanium fragments and then filtered through a 0.22-μm filter into sterile vessels. Emulsion preparations were tested for sterility and pyrogenicity. The emulsion preparation (~14 ml) was placed into a sterile syringe and stored frozen at −20°C. Emulsions were thawed at 4°C and warmed to room temperature just before use. The remnant-like emulsion particles were of average diameter [55 ± 3 nm (n = 40)] measured by negative-stain electron microscopy. The composition of the injected remnant-like emulsion was (% by weight, n = 4) triolein, 55.9 ± 1.6; cholesteryl oleate, 8.2 ± 0.7; cholesterol, 7.7 ± 0.7; and phosphatidylcholine, 28.2 ± 1.8.

Breath test clinical procedure

Two baseline (zero-time) breath samples were collected from subjects who had fasted for 14 h overnight. Subjects were injected intravenously with the remnant-like emulsion. Breath samples were obtained by having the subjects exhale fully through a drinking straw and into a tube. Alveolar air was sampled, and the tube was immediately capped. Breath samples were obtained at intervals over 24 h (10, 20, 30, 40, and 50 min and then 1, 2, 3, 4, 4.5, 5, 5.5, 6, 6.5, 7, 8, 9, 10, 11, 12, 21, and 24 h after injection). Subjects sat quietly during the first 8 to 9 h of the study, after which they consumed a light meal and resumed normal daily activities, excluding strenuous exertion. The CO_2 in the exhaled breath samples was analyzed by isotope-ratio mass spectroscopy using a Finnigan BreathmatPlus machine (Thermoquest Systems Pty. Ltd, Sydney, Australia). The ratio of $^{13}CO_2/^{12}CO_2$ was referenced to Peedeebelimnite standard values, and the delta unit value was calculated using the Breathmat software. The delta units reference a sample of limestone, a standard in the ^{13}C isotope ratio field, and basal (non-enriched) values correspond to approximately 1% ^{13}C, with small variations depending on the proportions of diet deriving from C3 or C4 photosynthesis (see Discussion).

Plasma lipid and apolipoprotein analysis

Plasma lipids were measured by automated hospital routines according to standard procedures. ApoB-48 was quantitated as previously described (18). Briefly, chylomicrons and their remnants were isolated from plasma by ultracentrifugation. ApoB-48 was separated from other proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to membranes that were incubated with a rabbit antibody to apoB, then reacted with anti-rabbit IgG (horseradish peroxide conjugated) and enhanced chemiluminescence reagent (Amersham). After development, the apoB-48 bands were quantified by densitometry and standardized against purified apoB-48 protein of known mass. The inter- and intra-assay variabilities were 9% and 5%, respectively.

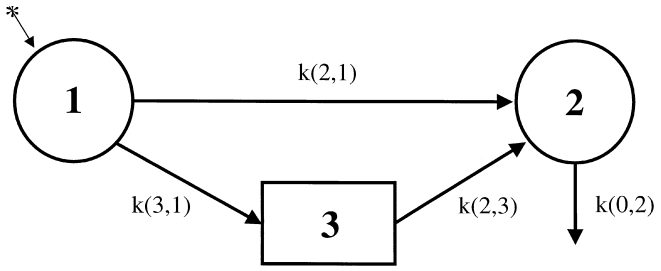

Fig. 1. Representation of the compartmental model used to fit the breath test enrichment data. The plasma compartment into which the ^{13}C-labeled material is injected is compartment 1. Compartment 2 represents the breath, which is sampled during the course of the study, and compartment 3 represents the main pathways for clearance and metabolism of the injected label within the body before the appearance of $^{13}CO_2$ in the breath.

Mathematical modeling of breath enrichment data

A compartmental model describing the appearance of labeled CO_2 in breath was developed using the SAAM II program (SAAM Institute, Seattle, WA). The model was developed assuming that the fractional rate constants [k(i,j)] were time invariant and first order. The model used to fit the tracer data is shown in **Fig. 1**.

Compartment 1 of the model represents the plasma compartment into which the labeled remnant-like emulsion is injected. Material in compartment 1 can be lost from plasma to compartment 2 or 3. Compartment 2 represents labeled CO_2 in breath and is sampled during the course of the study. The primary pathway for the clearance of emulsion and the appearance of labeled CO_2 is via compartment 3. This compartment may include the processes associated with uptake and hydrolysis of the labeled oleate. The compartmental model was fitted to the observed $^{13}CO_2$ breath data, and estimates of the fractional catabolic rate (FCR) were determined as the sum of the two rate constants out of compartment 1 [k(2,1) + k(3,1)].

Statistical analysis

All analyses were carried out using the Statistical Packages for Social Studies. Group variables were compared by two-tailed unpaired Student's *t*-test. Results with *P* values <0.05 were considered statistically significant.

RESULTS

Plasma lipids, lipoproteins, and apoB-48

Plasma triglycerides, total cholesterol, high-density lipoprotein (HDL) cholesterol, and apoB-48 measurements are summarized in **Table 1**. Compared with the group of normolipemic men, the group with type I hyperlipemia showed markedly increased plasma triglyceride concentrations ($P < 0.001$) and decreased HDL cholesterol ($P < 0.001$), whereas total plasma cholesterol was not significantly different, although the variance was larger in this group. In the group with type III hyperlipemia, plasma triglycerides and total cholesterol were both significantly increased ($P < 0.001$), whereas plasma HDL cholesterol was moderately decreased ($P < 0.01$). Measurements of plasma apoB-48 contents were markedly increased ($P < 0.001$) in both hyperlipemic groups compared with controls.

Breath test results

Figure 2 shows the patterns of enrichment of expired CO_2 with ^{13}C in the breath of the normolipemic, type I hyperlipemic, and type III hyperlipemic subjects. Fig. 2A shows that, in the normolipemic controls subjects, the enrichment increased rapidly by an average of more than 6 units to reach a peak approximately 5 h after injection of the emulsion, followed by a progressive slow decline except for a discontinuity at about 10 h toward pre-injection values over the ensuing 18 h. Fig. 2B shows that the initial starting enrichment value was approximately 2 units higher in the type I subjects (**Table 2**). Nevertheless, in the group with type I hyperlipemia there was significant enrichment, rising to a peak approximately 4 units above baseline about 5 h after injection of the emulsion. Fig. 2C shows that the responses of the group with type III hyperlipemia were reduced and that the relative enrichment with ^{13}C increased less than 2 units from baseline over the course of the study.

The rate constants of the model used to derive the fractional clearance rates are shown in Table 2. Only k(3,1) shows a significant difference from other groups for type III hyperlipidemia ($P < 0.005$), and this rate constant is the principal determinant of the FCR of the remnant-like emulsion (see Methods). The FCR of the injected remnant-like emulsion calculated from the model are shown in **Table 3**. Compared with the group of normolipemic men, the group with type I hyperlipemia showed a small decrease in FCR, but the difference was not significant, as shown by the considerable overlap in confidence intervals with the control group. In contrast, the group with type III hyperlipemia showed a significant decrease in FCR, with no overlaps in confidence intervals compared with controls or with the type I subjects ($P = 0.006$).

TABLE 1. Plasma lipid, lipoproteins, and apolipoprotein B-48 (apoB-48) contents in the control subjects and in patients with familial hyperlipemia

Subjects	n	Triglycerides	Cholesterol	HDL Cholesterol	ApoB-48
		mmol/l	*mmol/l*	*mmol/l*	*mg/l*
Normolipemic	16	1.1 ± 0.07	5.0 ± 0.17	1.4 ± 0.10	6.9 ± 1.08
Type I hyperlipemic	9	23.7 ± 3.14[a]	6.5 ± 0.90	0.4 ± 0.03[a]	59.7 ± 9.91[a]
Type III hyperlipemic	17	4.2 ± 0.72[a]	7.7 ± 0.36[a]	1.0 ± 0.09[b]	32.9 ± 4.49[b]

Results are means ± SEM. Significant differences from normolipemic control subjects are indicated ([a] $P < 0.001$ and [b] $P < 0.01$).

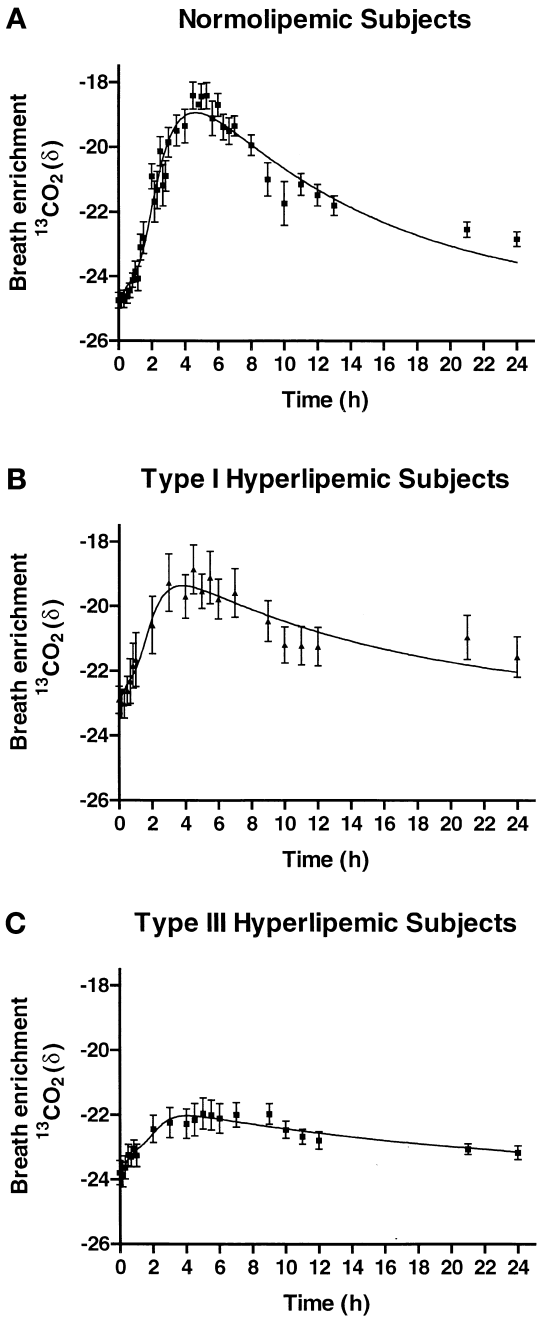

Fig. 2. The patterns of enrichment of the breath CO_2 with ^{13}C in the control normolipemic subjects (A), in hypertriglyceridemic subjects with type I hyperlipemia (B), and in subjects with hypertriglyceridemia and hypercholesterolemia due to type III hyperlipemia (C). The data are means with standard error bars, and the line is that fitted by the compartmental model to the averaged data.

DISCUSSION

We recently developed remnant-like emulsions, which, when injected into mice, became associated with apoE and were rapidly taken up by the liver. Incorporated [^{13}C]cholesteryl oleate was metabolized and appeared as $^{13}CO_2$ in expired breath (19, 20). In the present study, remnant catabolism using the breath test was measured in groups of subjects with familial hyperlipemia. The breath test was severely impaired in subjects with type III dyslipidemia, which we expected because of the known association with defective remnant clearance in the type III phenotype (4). Cardiovascular disease risk is increased in type III dyslipidemia, consistent with the involvement of abnormal remnant clearance.

The initial starting enrichment value was approximately 2 units higher in the type I subjects, probably reflecting the very low fat diets consumed by this group. Endogenous ^{13}C enrichment varies between food groups (21, 22) and therefore is affected by dietary sources. The discontinuity at about 10 h seen in all groups of subjects corresponds to the resumption of normal daily activities and the consumption of a light meal. The breath test depends not only on plasma removal of the emulsion remnant particles into the liver for catabolism but also on the subsequent metabolism of fatty acids hydrolyzed from the emulsion cholesteryl ester. Changes in fluxes of fatty acids in the liver could therefore possibly confound the interpretation of the enrichment data. Fatty acid kinetics have been found to be different from controls in patients with familial combined hyperlipidemia (23, 24) in association with impaired postprandial lipid metabolism. We are unaware of parallel findings in subjects with type I or type III dyslipidemia. Whereas the findings of the present study are clearly consistent with the present interpretation, it is still possible that changes in fatty acid flux contributed to the result. Nevertheless, the findings are consistent with other evidence that remnant clearance is abnormal in type III hyperlipemia, whereas a priori there is no reason to anticipate abnormal remnant clearance in type I hyperlipemia.

An uncertainty with the interpretation of the breath test data is the potential influence of the pool size and flux rate of the fatty acid oxidation pathway. For example, if a patient were depleted in hepatic glycogen and the insulin-glucagon ratio was low, more of the ^{13}C fatty acid would be expected to be oxidized instead of re-esterified. Further direct experimentation is needed to determine whether this phenomenon could influence the breath test after an overnight fast, but preliminary findings in a series of normolipidemic patients tested in our laboratory either fasting or after a standard meal do not show any difference.

Another currently unresolved issue is the potential confounding influence of cholesteryl ester transfer protein (CETP). It cannot be excluded that the emulsion cholesteryl ester was transferred to other plasma lipoproteins before catabolism. There is no evidence that differences in CETP activity are associated with type I or type III hyperlipemia, yet the expansion of pool size in acceptor lipoproteins possibly enhanced transfer. An argument against this possibility is that the breath test was not different from controls in the type I subjects. What are lacking are deterministic studies of postprandial lipid metabolism in individuals selected for high or low CETP activity. Inhibitors of CETP have recently become available (25), and their use may help to resolve this issue.

Whether plasma triglyceride concentrations predict cardiovascular disease has been a contentious issue, perhaps because the remnants of the triglyceride-rich lipoproteins

TABLE 2. Basal ^{13}C enrichments and model rate constants in the control subjects and in patients with familial hyperlipemia

Subjects	Normolipemic (n = 16)	Type I Hyperlipemic (n = 9)	Type III Hyperlipemic (n = 17)
Basal enrichment (delta units)	−24.7 ± 0.28	−22.9 ± 0.42[a]	−24.1 ± 0.29
k(2,1) (h^{-1})	0.024 ± 0.006	0.022 ± 0.007	0.008 ± 0.005
k(3,1) (h^{-1})	0.139 ± 0.023	0.089 ± 0.030	0.030 ± 0.020[b]
k(2,3) (h^{-1})	7.308 ± 1.748	8.435 ± 2.331	6.277 ± 1.696
k(0,2) (h^{-1})	1.363 ± 0.263	1.416 ± 0.351	0.977 ± 0.255

Results are means ± SEM. Significant differences from other groups by analysis of variance with Bonferroni comparisons of means are indicated ([a] $P < 0.04$ and [b] $P < 0.005$).

are pro-atherogenic, whereas the primary particles, such as chylomicrons, are not (26, 27). Whereas hypertriglyceridemia is generally associated with the increased risk of cardiovascular disease, this is not true for subjects with type I hyperlipemia where undegraded chylomicrons persist in the circulation but the risk of cardiovascular disease is not increased. In the present study, the subjects with type I hyperlipemia were extremely hypertriglyceridemic. However, the breath test was not different from normolipemic control subjects. Therefore, despite the lack of lipoprotein lipase activity, the underlying capacity for remnant catabolism was normal in this group of subjects with severe hypertriglyceridemia.

Measurements of the concentrations of remnants in plasma are possible but are technically difficult because of the low concentrations in plasma of remnants compared with other lipoproteins (28, 29). In the past, postprandial studies using vitamin A have led to useful insights relating defective remnant clearance to increased atherosclerosis, but the procedures are cumbersome, not practical for routine screening, and perhaps unreliable (30). Other potential markers for abnormal remnant metabolism are increased plasma contents of apoB-48 and the remnant-like particle cholesterol (RLP-C) test (28, 29, 31, 32). However, both the RLP-C test and measurements of apoB-48 lack specificity for distinguishing defects in lipolysis from defects in remnant clearance, shown clearly by the present study, where apoB-48 was increased in both type I and type III hyperlipemic subjects (Table 1), whereas the breath test was abnormal only in type III subjects (Table 2). Furthermore, for a rapid catabolic-pathway-like remnant clearance and metabolism, a functional test, such as the breath test, is desirable. The new breath test is simple to administer, and clinical studies are now in progress to determine its value in measuring the effects of therapeutic interventions.

Analysis of the breath enrichment data by compartmental modeling is an important aspect of the method. One of the advantages of the modeling is that the need for correction of emulsion dose for body size is obviated because the fractional clearance rate is independent of individual variation in this regard. The only restriction in metabolic terms is that the subjects must not undertake physical activity during the test, to prevent artifactual dilution of label due to accelerated oxidation of unlabeled substrate.

The specificity of the breath test for detecting defective remnant clearance in type III dyslipidemia testifies that the breath test may be used to distinguish hypertriglyceridemic individuals at increased risk of cardiovascular disease from other individuals where a lipolysis defect may cause hypertriglyceridemia without increased risk of cardiovascular disease. This may be particularly relevant to the one third of patients with coronary disease who have increased triglyceride and normal cholesterol levels (33). Additional studies with the breath test as the endpoint are ongoing, testing the ability of various interventions to enhance remnant catabolism. Studies in progress using the breath test include the effects of abdominal obesity and type II diabetes on postprandial lipid metabolism. The breath test can be carried out repeatedly in intervention trials, for example to assess the impact on remnant clearance of up-regulation of clearance pathways with lipid-lowering drugs. The breath test has also been useful in many studies of mice, testing the effects of a number of obesity models and also of gene knockout and transgenic manipulations (20).

The future usefulness of the breath test in clinical investigation will depend on whether it identifies individuals at increased risk of cardiovascular diseases in the absence of other conventional risk factors and whether it allows the assessment of therapeutic interventions that decrease cardiovascular risk. The breath test may prove easier to administer and interpret than presently available chromatographic methods of measuring clearance of postprandial lipids, particularly as stable isotope measurements become more routine.

TABLE 3. Fractional catabolic rate of chylomicron remnant-like emulsion calculated from ^{13}C breath data in the control subjects and in patients with familial hyperlipemia

Subjects	n	Mean Pools/Day	SEM	95% Confidence Intervals
Normolipemic	16	3.91	0.93	1.93–5.96
Type I hyperlipemic	9	2.67	0.38	1.79–3.55
Type III hyperlipemic	17	0.91[a]	0.20	0.48–1.34

[a] Significant difference from normolipemic control subjects; $P < 0.005$.

The clinical application of the breath test was supported by the University of Western Australia and by a grant from the Lotteries Commission of Western Australia. Therapeutic interven-

tion trials using the breath test have been supported by grants from Merck Sharpe & Dohme and Bristol Myers Squibb. The assistance of the research nurses and laboratory staff in Perth and Cape Town is particularly acknowledged.

Manuscript received 12 June 2000, in revised form 14 November 2000, and in re-revised form 21 December 2000.

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