Remnant lipoproteins are related to intima-media thickness of the carotid artery independently of LDL cholesterol and plasma triglycerides

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Abstract **Remnants of triglyceride-rich lipoproteins (TRL) have been implicated in the early development of atherosclerosis. We tested this hypothesis by quantifying the plasma concentration of remnant-like particle cholesterol (RLP-C) in a cohort of healthy 50-year-old men in whom the common carotid artery intima-media thickness (CCA-IMT) was assessed by B-mode ultrasound as a surrogate marker for atherosclerosis. The subjects were given a fat-rich meal to study the generation of RLP-C during postprandial lipemia. Fasting plasma RLP-C and other major fasting plasma lipids and lipoproteins were determined twice, and the mean RLP-C concentration was strongly correlated with CCA-IMT ($r = 0.32$, $P = 0.002$). In addition, low density lipoprotein (LDL) cholesterol ($r = 0.25$, $P = 0.01$) and plasma triglycerides ($r = 0.20$, $P = 0.05$) were significantly related to CCA-IMT. Multivariate analyses showed a triglyceride-independent contribution of RLP-C to CCA-IMT. After fat intake, the median plasma RLP-C concentration was doubled after 3 h. The increase was strongly related to the postprandial generation of TRL apolipoprotein (apo)B-48, and large (S_f 60–400) TRL apoB-100. The association with CCA-IMT was somewhat stronger for the 3-h RLP-C level than for the fasting RLP-C concentration [$r = 0.27$, $P < 0.01$ (3 h) compared with $r = 0.22$, $P < 0.05$ (0 h)].—We conclude that the plasma concentration of RLP-C is related to CCA-IMT, independent of plasma triglycerides and LDL cholesterol, in a healthy middle-aged male population.**—Karpe, F., S. Boquist, R. Tang, G. M. Bond, U. de Faire, and A. Hamsten. **Remnant lipoproteins are related to intima-media thickness of the carotid artery independently of LDL cholesterol and plasma triglycerides.** ***J. Lipid Res.*** **2001.** 42: **17–21.**

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Triglyceride-rich lipoproteins (TRL) are heterogeneous and it is not likely that all species are atherogenic. Furthermore, hypertriglyceridemia may serve as a marker for clustered risk factors belonging to the insulin resistance syndrome. A better identification of the potentially atherogenic subpopulation of TRL would therefore be useful in clinical studies.

Few studies have investigated the relationship between remnant lipoprotein concentrations in plasma and early signs of atherosclerosis in healthy asymptomatic subjects. We have described the association between postprandial TRL and intima-media thickness of the common carotid artery in healthy men. First, we reported positive correlations between plasma measurements of postprandial triglycerides and common carotid artery intima-media thickness (CCA-IMT) in healthy asymptomatic subjects. In this study, whole plasma triglycerides correlated better to CCA-IMT than apolipoprotein (apo)-defined measurements of TRL (apoB-48 and apoB-100) (1). Second, we observed in a larger group of healthy subjects that postprandial triglycerides correlated with CCA-IMT independently of fasting triglycerides (2). Interestingly, the TRL apoB-48 and B-100 levels were not independent predictors of CCA-IMT in this study either. The consistency of these findings might imply that plasma triglycerides are more closely related to potentially atherogenic lipoproteins than conventional physicochemical determinations of density-defined TRL concentrations of apoB-48 and apoB-100.

It has long been argued that remnants of TRL are atherogenic, but one of the problems has been that there

Abbreviations: CCA, common carotid artery; IMT, intima-media thickness; TRL, triglyceride-rich lipoproteins; RLP-C, remnant-like particle cholesterol.

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is no consensus definition of remnant lipoproteins, nor is there a standardized method to quantify remnant lipoproteins in plasma. One attempt to solve this problem involved the use of a compositional-biological definition rather than the classic density physicochemical definition of this lipoprotein species. Several studies have used the monoclonal anti-apoB antibody JIH for this purpose, and a commercially available kit has been developed on the basis of analyzing the concentration of lipoproteins remaining unbound to the antibody (3). The initial observation showed that the properties of the JIH antibody exclude binding of chylomicrons and chylomicron remnants (4). In addition, apoB-100 containing TRL with an excess of apoE seems to be excluded. Cohn and coworkers have made thorough investigations of the compositional characteristics of the remnant-like particles (RLP) isolated with the use of the JIH antibody (5, 6). In subjects likely to have low levels of TRL remnants, lipoproteins containing neither apoB nor apoA-I contributed the cholesterol contained within the RLP fraction. It should, however, be stressed that the level of this cholesterol pool is small. However, RLP cholesterol (RLP-C) has been shown to correlate well with an increasing plasma triglyceride level (7). Taken together, detection of RLP-C, using the JIH antibody, is likely to provide a good estimate of remnant cholesterol levels in plasma.

One of the burning issues is whether quantification of RLP-C is adding information about the plasma level of potentially atherogenic lipoprotein levels that cannot be obtained by an alternative and easier method, for example, by quantification of plasma triglycerides. We therefore determined plasma RLP-C concentrations in a cohort about whose members we already had information concerning the relationship between CCA-IMT and fasting, as well as postprandial, plasma triglycerides (2), to analyze if additional information could be obtained by quantification of RLP. We hypothesized that accurate determination of potentially atherogenic remnant lipoproteins would be better correlated with CCA-IMT than plasma triglycerides.

MATERIALS AND METHODS

Subjects and protocol

Ninety-six healthy 50-year-old white men participated in the study. They were randomly selected and recruited through a population survey of all residents in the greater Stockholm area as described (2). Participants were all homozygous for the *apoE3* allele. They came for two visits: the first one was a screening visit that included fasting plasma blood sampling, blood and the second visit, after 1 to 3 months, included an oral fat tolerance test of a mixed-meal type. The study was approved by the local Ethics Committee of the Karolinska Hospital, and all participants gave informed consent to the study.

Blood sampling and analytical procedures

Venous blood was drawn into precooled disodium-ethylenediaminetetraacetic acid Vacutainer tubes. Plasma was recovered within 30 min after a low speed centrifugation at 4°C. Phenylmethylsulfonyl fluoride and aprotinin (final concentrations of 10 μM and 28 μg/ml, respectively) were added to samples taken in connection with the oral fat tolerance test to inhibit degradation of apoB. Major fasting plasma lipoprotein lipids were determined after the combination of preparative ultracentrifugation and precipitation (8). Samples taken in connection with the oral fat tolerance test were subjected to density gradient ultracentrifugation to isolate subfractions of TRL in which the concentrations of apoB-100 and apoB-48 were determined by analytical sodium dodecyl sulfate-polyacrylamide gel electrophoresis (9). The RLP-C concentration was determined by a novel immunoseparation technique (Jimro-II), made available by the Japanese Immunoresearch Laboratories Company, Gunma, Japan. Frozen plasma stored at −70°C was used and the time elapsed between harvesting plasma and analysis of samples was less than 2 years. A total of 5 μl of plasma was mixed with 300 μl of lipoprotein separation medium consisting of a Sepharose gel suspension to which monoclonal antibodies directed against apoB-100 (JIH) and apoA-1 had been attached. The separation medium was gently mixed for 120 min and thereafter allowed to settle for another 15 min. The cholesterol content (RLP-C) was assayed enzymatically in the supernatant with an autoanalyzer (Cobas-Mira+; Roche, Rahway, NJ). Two quality control plasmas were included in all runs, at a low level (mean, 7.7 mg/dl) and a high level (mean, 24.9 mg/dl). The coefficients of variation for the analysis were 7.7% at the low level and 6.6% at the high level. Samples were analyzed in duplicate. All samples derived from the same subject were run at the same time.

Quantification of CCA-IMT

Measurement of CCA-IMT was done according to the European Lacidipine Study on Atherosclerosis ultrasound protocol (10). The ultrasound equipment included a 2000 II sa (Biosound, Indianapolis, IN) with an 8-MHz high resolution annular array scanner. The scans were recorded on video tapes and interpreted by a person, unaware of the characteristics of the study subjects, at the Center for Medical Ultrasound, Division of Vascular Ultrasound Research (Wake Forest University, Winston-Salem, NC).

Statistics

Skewed values were log-transformed before statistical testing. The fasting plasma lipoprotein lipids and the fasting plasma RLP-C were measured on two occasions and the mean of these values was used for studying associations. Using the average of several determinations has been recommended for variables normally showing a large intraindividual variability such as plasma triglycerides and RLP-C (11). Univariate associations between lipid variables and IMT were analyzed by calculating Pearson correlation coefficients. Variables that were significant in this step were then entered into a multivariate analysis in which adjustment for cumulative tobacco consumption was made.

RESULTS

Fasting plasma concentrations of RLP-C and major lipoproteins

Fasting plasma concentrations of major lipids and lipoproteins in this cohort have been shown previously (2). In essence, the major proportion of subjects is normolipidemic as a consequence of the random population-based recruitment of healthy 50-year-old men. Median and interquartile range (IQR) levels for plasma concentrations of cholesterol and triglycerides were 5.38 (4.63–5.86) and

1.08 (0.80–1.68) mM, respectively. Fasting plasma lipids and RLP-C were measured on two occasions within 3 months in each subject and the median and IQR of the average individual RLP-C values from the two measurements were 0.19 (0.16–0.31) mM. The RLP-C values showed a skewed distribution. There was a strong linear relationship with fasting plasma triglycerides measured on the same occasion ($r = 0.83$, $P < 0.001$).

The fasting plasma RLP-C level was strongly associated with the concentration of apoB-48 and apoB-100 in the S_f 60–400 fraction ($r = 0.73$, $P < 0.001$ and $r = 0.75$, $P < 0.001$, respectively). The corresponding associations for apoB-48 and apoB-100 in the S_f 20–60 fraction were $r = 0.64$ ($P < 0.001$) and $r = 0.68$ ($P < 0.001$). As the fasting plasma concentration of apoB-48-containing lipoproteins is low and cannot explain the fasting concentration of RLP-C, we looked specifically for determinants of RLPs within the endogenous TRL. It seemed as if the relative proportion of large very low density lipoprotein (VLDL) was a major determinant of RLP-C, as the ratio between S_f 60–400 apoB-100/total TRL apoB-100 was associated with RLP-C ($r = 0.55$, $P < 0.001$), whereas the the corresponding relation with S_f 20–60 apoB-100/total TRL apoB-100 was not significant ($r = 0.18$, NS).

Postprandial concentrations of RLP-C

The median and IQR of RLP-C in the fasting state was 0.18 (0.13–0.30) mM, increased to 0.38 (0.28–0.57) mM at 3 h, and reverted to 0.27 (0.18–0.51) mM after 6 h (**Fig. 1**). The fasting plasma concentration of RLP-C seemed to predict the postprandial increase in RLP-C. The average RLP-C concentration was doubled between 0 and 3 h irrespective of the basal level, indicating that a high basal level also predicted a larger postprandial increase in RLP-C. Typically, the median fasting RLP-C concentration in subjects above the 90th percentile increased from 0.47 to 0.97 mM at 3 h, whereas the corresponding values for subjects below the 10th percentile increased from 0.09 to 0.17 mM.

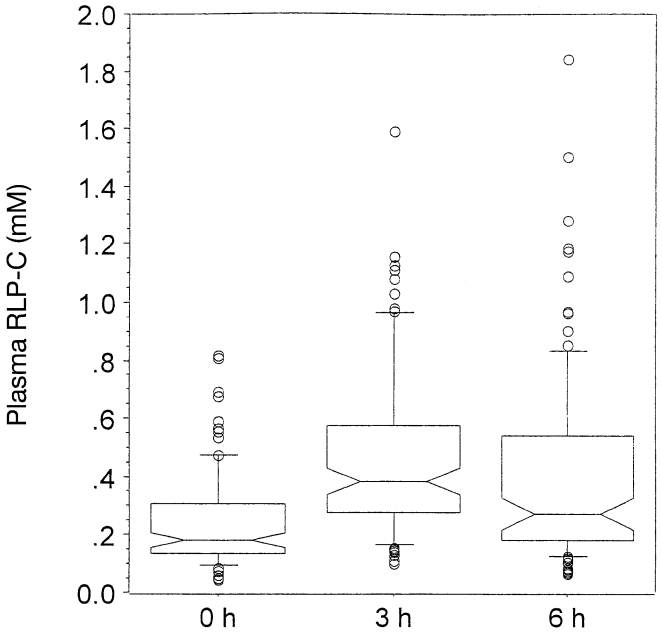

Fig. 1. Box plot of plasma concentrations of remnant-like particle cholesterol (RLP-C) before and after intake of the mixed meal. The box indicates the median and the interquartile range. The upper and lower error bars indicate the 90th and 10th percentile, respectively. The corners of the pointed indentations indicate the upper and lower 95th confidence limit about the median.

The 0 to 3-h increase in RLP-C was strongly associated with the corresponding increase in TRL apoB-48 ($r = 0.68$, $P < 0.001$), whereas the increase in TRL apoB-100 was only weakly associated with the change in RLP-C concentration (NS). This was largely dependent on the fact that the 0- to 3-h increase in S_f 60–400 apoB-100 was significantly and positively associated with the change in RLP-C ($r = 0.40$, $P < 0.001$), whereas the 0- to 3-h change in S_f 20–60 apoB-100 concentration was not ($r = -0.05$, NS).

Relationship between RLP-C and CCA-IMT

Fasting plasma RLP-C ($P = 0.002$), plasma triglycerides ($P = 0.05$), and low density lipoprotein (LDL) cholesterol ($P = 0.01$) were all significantly associated with CCA-IMT, whereas VLDL and high density lipoprotein (HDL) cholesterol were not (**Table 1**). These univariate relationships were all based on the mean of two lipid and lipoprotein determinations performed 1 to 3 months apart in each subject. As the oral fat challenge was performed only once, duplicate measurements for the postprandial RLP-C concentrations were not obtained and the associations between CCA-IMT and postprandial RLP-C determinations were therefore based on the single fasting RLP-C determination performed on samples taken in connection with the test meal. The association between CCA-IMT was somewhat stronger using the 3-h RLP-C level than the fasting RLP-C concentration [$r = 0.27$, $P < 0.01$ (3 h) compared with $r = 0.22$, $P < 0.05$ (0 h)]. The association between CCA-IMT and the 6-h RLP-C value was similar to that of the fasting sample ($r = 0.21$, $P < 0.05$).

Multiple stepwise linear regression analysis was performed on the fasting plasma variables to analyze the independent relationships of RLP-C, LDL cholesterol, and plasma triglycerides to CCA-IMT (**Table 2**). HDL and VLDL cholesterol were not included in the analyses as they did not show significant associations in the univariate analysis.

TABLE 1. Univariate correlation coefficients between fasting plasma lipids and lipoproteins and CCA-IMT

	CCA-IMT	
	r	*P*
Triglycerides	0.20	0.05
Cholesterol		
VLDL	0.15	0.13
LDL	0.25	0.01
HDL	−0.07	0.47
RLP	0.32	0.002

Data were analyzed using Pearson correlation coefficients. Values are based on the mean of two determinations made on plasma samples drawn on separate occasions within 3 months. Triglycerides, VLDL cholesterol, and RLP cholesterol were log-transformed prior to statistical computations.

TABLE 2. Multiple stepwise regression analysis of the relationships of plasma lipids and lipoproteins to CCA-IMT

	Step 1	F to Remove	Step 2	F to Remove	Step 3	F to Remove	Step 4	F to Remove	Step 5	F to Remove
RLP-C	0.33	10.8	0.21	4.10	0.33	10.83	0.26	5.67	0.43	4.14
Plasma triglycerides	−0.06	—	0.27	6.73	—	—	−0.10	—	−0.21	0.9
LDL-C	0.16	—	0.18	—	0.16	—	0.17	2.53	0.19	3.11
Multiple R^2		0.11		0.11		0.11		0.11		0.14

Step 1. Multiple stepwise linear regression (MSLR), adjustment for smoking.
Step 2. MSLR, adjustment for smoking and plasma triglycerides.
Step 3. MSLR, adjustment for smoking; plasma triglycerides not included in the analysis.
Step 4. MSLR, adjustment for smoking and LDL cholesterol (LDL-C).
Step 5. MSLR, adjustment for smoking, plasma triglycerides, and LDL-C.

After adjustment for cumulative smoking, RLP-C was the single variable entering the equation and explained up to 11% of the variation of CCA-IMT within the group. As plasma triglycerides and RLP-C were closely associated, the triglyceride variable was also forced into, or removed entirely from, the multivariate model. When plasma triglycerides were forced into the equation, RLP-C was included in the multivariate model, showing a triglyceride-independent contribution of RLP-C to CCA-IMT. When plasma triglycerides were left out, LDL cholesterol still did not enter the equation. Finally, both plasma triglycerides and LDL cholesterol were forced into the equation. RLP-C still entered the multivariate model and all variables together explained 14% of the variation in CCA-IMT.

In these statistical computations the average of two measurements was used for LDL-C, RLP-C, and plasma triglycerides. The reason for this was to bring down the "noise" of RLP-C and plasma triglycerides. If single measurements were used, either from the first or the second visit, they were still significantly related to CCA-IMT, but in multivariate analyses LDL turned out to be the only variable significantly related to CCA-IMT. Accordingly, plasma triglycerides and RLP-C gained in strength in relation to CCA-IMT when the average of two measurements was used in contrast to LDL-C, which is known to have less intraindividual variation.

DISCUSSION

The present study is the first to show an association in healthy humans between a surrogate marker for early atherosclerosis and a marker for remnants of TRL that is independent of plasma triglycerides and LDL cholesterol. This study therefore lends support to the notion that there is a subpopulation within the TRL fraction that is atherogenic and that this subpopulation can be identified by quantification of RLP-C. However, RLP do not seem to be a homogeneous population of lipoproteins. In subjects with low plasma triglycerides, a major proportion of the normally low level of RLP-C is attributable to particles less likely to be remnants of TRL as they are devoid of apoB (6). The RLP-C concentration increased after fat intake, and this increase was observed even in subjects with low fasting plasma RLP-C or triglyceride levels, indicating that postprandial lipemia is a condition in which remnant lipoproteins are generated in all subjects. As further evidence that a proneness to accumulation of remnant lipoprotein cholesterol is linked to development of early atherosclerosis, we found that the correlation between RLP-C and CCA-IMT was at least as strong, if not stronger, in the postprandial state.

The increase in RLP-C after fat intake correlated with the accumulation of apoB-48 in the TRL fraction, but also with the increase in large apoB-100 TRL particles. We have previously shown that when large VLDL (apoB-100 particles) accumulate in postprandial plasma they acquire an altered lipid and apolipoprotein composition (12). The most prominent features are cholesterol enrichment and an increased apoE and apoC-I content. This compositional profile fits well with the lipoprotein ligand exclusion of apoB described for the JIH antibody used in the RLP assay (4). As a major proportion of the RLP-C is accounted for by apoB-100 particles, we suggest that the increased postprandial concentration of RLP-C is to a significant degree due to accumulation of endogenous (apoB-100) TRL and that the determination of RLP-C should not be seen as a marker for chylomicron remnant cholesterol only. In a previous study comparing the pattern of TRL in the postprandial state in subjects with and without manifest coronary atherosclerosis, the postprandial accumulation of endogenous TRL proved to be the best discriminator between the groups (13). Normotriglyceridemic and hypertriglyceridemic patients with coronary atherosclerosis exhibit abnormal accumulation of endogenous TRL in the postprandial state compared with matched healthy control subjects. Furthermore, TRL particles that accumulate in postprandial plasma of patients with manifest coronary atherosclerosis seem to carry an excess of apoC-I and apoE (14).

Associations between cardiovascular disease and plasma triglycerides are often fairly weak and it has been argued that the association is underestimated for statistical reasons, the main one being the imprecision by which plasma triglycerides are determined (15). One of the reasons for the imprecision is the high day-to-day variation. In the present study we had two independent determinations of lipid and lipoprotein variables and the average of these two determinations was used to study the correlation with the CCA-IMT. We suggest that such an approach may in-

crease the likelihood of detecting independent associations between triglyceride-related metabolic risk factor variables and surrogate variables for atherosclerosis.

The present work indicates that quantification of plasma RLP-C might be useful to assess cardiovascular risk over and above measurements of LDL cholesterol and plasma triglycerides. Previous markers of remnant lipoproteins have all involved complicated analytical procedures, normally including ultracentrifugation, which is not feasible in a routine clinical chemistry laboratory setting. This procedure for quantification of the plasma concentration of RLP-C uses plasma and involves only two steps; that is, immunoseparation and enzymatic determination of cholesterol.

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