Remnant-like particle (RLP) cholesterol is an independent cardiovascular disease risk factor in women: results from the Framingham Heart Study

Judith R. McNamara a,b,c,*, Paulesh K. Shah b, Katsuyuki Nakajima d, L. Adrienne Cupples e, Peter W.F. Wilson f, Jose M. Ordovas c, Ernst J. Schaefer b,c

a Lipid Research Laboratory, New England Medical Center, 750 Washington Street, Box 216, Boston, MA 02111, USA
b Lipid Research Laboratory, Division of Endocrinology, Diabetes, Metabolism, and Molecular Medicine, New England Medical Center, Boston, MA, USA
c Lipid Metabolism Laboratory, Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA, USA
d Otsuka America Pharmaceutical Incorporated, Rockville, MD, USA
e Department of Epidemiology and Biostatistics, Boston University School of Public Health, Boston, MA, USA
f National Heart, Lung, and Blood Institute’s Framingham Heart Study, National Institutes of Health, Framingham, MA, USA

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Abstract

Remnants of triglyceride-rich lipoproteins (TRL) of both intestinal and liver origin are considered to be atherogenic, but separation of remnant lipoproteins from other TRL is difficult. An assay has been developed that allows immunoseparation of remnant-like particles (RLP) and measurement of cholesterol (RLP-C) and triglyceride (RLP-TG). We measured RLP-C and RLP-TG in fast plasma samples obtained from 1567 women participating in cycle 4 of the Framingham heart study (FHS). When values from 83 women with cardiovascular disease (CVD) were compared with the values from 1484 women without disease, concentrations in women with CVD were found to be significantly higher for both RLP-C (0.215 ± 0.102 vs. 0.186 ± 0.162 mmol/l; + 15.6%; P < 0.0001) and RLP-TG (0.319 ± 0.352 vs. 0.251 ± 0.716 mmol/l; + 27.0%; P < 0.0002). Logistic regression analysis revealed that RLP-C was significantly associated with prevalent CVD in women (P < 0.0002) after adjustment with other major risk factors. In conclusion, we have documented that RLP-C is an independent risk factor for CVD in women, and provides significantly more information than do triglycerides. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Lipoproteins; Very low density lipoproteins; Chylomicrons; Triglycerides; Apolipoprotein B; Cardiovascular disease

1. Introduction

Elevated serum triglyceride concentrations are frequently observed in patients with cardiovascular disease (CVD), and are significantly associated with CVD in most univariate analyses. Associations frequently do not remain significant, however, after adjustment with other CVD risk factors [1,2]. Triglyceride concentration represents a measure of triglyceride-rich lipoproteins (TRL), but it does not distinguish among the various subspecies of TRL, which may have varying degrees of atherogenicity. Partially hydrolyzed lipoprotein remnants of both intestinal (chylomicron) and hepatic (very low density lipoproteins (VLDL)) origin have been implicated as the subspecies that may be particularly important in potentiating CVD risk [3–9]. Studies have shown that these particles can remain in circulation for extended periods of time postprandially, even in individuals with normal fasting triglyceride concentrations [10–14].

Routine isolation and measurement of lipoprotein remnants has not been feasible, because of the difficulty in distinguishing among particles with similar apolipoprotein composition and overlapping size ranges. Although direct methods for isolation and measurement of cholesterol in low density lipoproteins (LDL), high density lipoproteins (HDL), and even lipoprotein(a)
Subjects for the study were female participants in exam cycle 4 (1987–1990) of the FHS offspring study, under an ongoing, approved protocol. In 1972 offspring of the original FHS cohort and their spouses, totaling 5124 subjects, were recruited to the Framingham offspring/spouse study. With examination cycles, occurring every 3–4 years, the number of participants at exam 4 reflects the typical number (≈4000) attending exams since the second examination cycle, with no evidence that those who attend during any given cycle are different from those who do not, with the possible exception of geographic distribution. Women at exam 4 ranged in age from 22 to 79 years (mean age, 52 years).

Information concerning smoking status, height, weight, and systolic and diastolic blood pressure (SBP and DBP, respectively) was collected at the time of examination [25]. Women reporting cigarette use within the year prior to the exam were classified as current smokers; those who reported smoking at some time in the past, but not within the previous year, were classified as former smokers; the remaining were classified as never having smoked. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters. Hypertension was defined as SBP > 140 or DBP > 90 mmHg or intake of anti-hypertensive medication. Diabetes was defined as fasting blood glucose > 6.85 mmol/l (125 mg/dl) or intake of hypoglycemic medication. Diagnosis of CVD included history of angina pectoris, myocardial infarction, stroke, or transient ischemic attack using previously published methods [25]. All informations were reviewed by a panel of physicians to determine the diagnosis.

Of the 2043 women who attended exam 4, plasma aliquots and complete data were available for 1614. We compared with these 1614 women who had RLP measurements performed and those 429 who were not, and found that those who measured and reported upon here were 1–2 years older, 30% more likely to have hypertension, 50% more likely to be on beta blockers, twice as likely to be treated with cholesterol-lowering drugs, and at 37% greater odds of having CVD. The women included in the study had slightly higher total cholesterol levels (5.37 vs. 5.25 mmol/l) and were more likely to be on hormone replacement therapy (12 vs. 8%). However, we found no other differences in lipid levels (LDL-C, HDL-C, or triglyceride), and no differences in the rates of smoking or diabetes. Thus, subjects measured for RLP in this study were at somewhat greater atherogenic risk than those who were not.

Statistical analyses were performed with and without the inclusion of subjects taking medications known to affect lipids (47 women), resulting in final study sets 1567 or 1614 women. Results obtained from women with documented CVD (83/92) were compared with those from women without disease (1484/1522).

2. Methodology

2.1. Study subjects

2.2. Lipoprotein analyses

Blood was drawn from each subject after a 12 h, overnight fast into tubes containing EDTA (final concentration, 0.15%). Plasma was separated by centrifugation (2500 rpm, 4°C, 20 min). Plasma lipid and lipoprotein concentrations (total cholesterol, triglycerides, and HDL-C) were measured fresh, using standard enzymatic methods, essentially as previously described [26]. LDL-C was calculated according to the formula of Friedewald et al. [27], except when triglycerides were > 4.5 mmol/l (400 mg/dl). In those cases, which included 19 of the 1567 women (1.3%), LDL-C values were omitted from the data set.

2.3. RLP analyses

RLP isolation was based on removal of apo A-I-containing particles (HDL) and most apo B-containing particles (LDL, nascent VLDL, and nascent chylomicrons), using an immunoseparation technique (Japan Immunoresearch Laboratories (JIMRO), Takasaki, Japan) previously described [21,22,28,29], which has been shown to leave particles characteristic of previously described VLDL remnants (apo B-100) and chylomicron remnants (apo B-48) in the unbound fraction [30,31]. Briefly, monoclonal antibodies to apo A-I and specific monoclonal antibodies to apo B (JI-H), which do not recognize partially hydrolyzed, apo E-enriched lipoprotein remnants, were immobilized on agarose gel. These particles have been previously characterized to contain more apo E and cholesteryl ester molecules,
and fewer triglyceride and apo C molecules than those particles bound by the antibody. These unbound particles represent approximately 10–13% of TRL in normolipidemic samples, but as much as 65% of TRL in familial dysbetalipoproteinemic (type III) samples [21]. More recently, in in vitro studies by Doi et al., the unbound RLP were shown to inhibit vasorelaxation, while bound (non-RLP) TRL produced no inhibition [32].

RLP-C and RLP-TG concentrations were measured in the FHS plasma aliquots that had been frozen at −80°C until the time of analysis. Plasma was incubated with the gel for 2 h, after which the gel, containing the bound (non-RLP) lipoproteins, was precipitated with low-speed centrifugation (5 min, 135 × g). RLP-C and RLP-TG were then measured in unbound supernatants on an Abbott Spectrum chemistry analyzer (Abbott Diagnostics, Irving, TX), using two-reagent enzymatic, colorimetric assays containing a sensitive chromophore (Kyowa Medex, Tokyo, Japan). Precision studies have yielded among-run RLP-C imprecision for two levels of RLP control over 20 runs of 9.1% at 7 mg/dl (0.18 mmol/l) and 7.3% at 24 mg/dl (0.62 mmol/l). Among-run RLP-TG imprecision for the same control was 8.3% at 22 mg/dl (0.25 mmol/l), and 5.0% at 109 mg/dl (1.23 mmol/l) [25].

2.4. Statistical analysis

Those subjects with prevalent CVD were compared with those with no evidence of CVD on a variety of known risk factors, as well as with RLP-C and RLP-TG. Student’s t-test was used to compare with the mean values of continuous measures, and a χ² statistic was calculated for categorical factors. For continuous measures, which were highly skewed, we compared those with CVD and those without, using log transformed values, although we report the untransformed means and standard deviations for each group. To further evaluate the relationship between CVD and RLP-C and RLP-TG, we established quartiles for each measure and computed age-adjusted rates for each quartile, using direct standardization and 10-year age groups (20–29, 30–39, etc.). The age distribution provided the standard distribution for these comparisons, and the Mantel extension test was employed to calculate a test of linear trend in these rates across quartiles. To adjust for known risk factors for CVD, we used logistic regression analysis with the presence or absence of prevalent CVD as the outcome. The covariates that we considered were age, definite hypertension (SBP > 140 or DBP > 90 mmHg or on hypertensive treatment), use of beta blockers, smoking status (current or former or never), prevalent diabetes, LDL-C, HDL-C, and hormone replacement therapy.

To evaluate the relationship between CVD and RLP, we performed several analyses with variable definitions of the remnant variables, (1) using the actual measured RLP-C or RLP-TG concentration as a continuous variable on a log scale; (2) a set of three measures indicating quartiles 2, 3 and 4; and (3) using only the quartile 4 as a dichotomous measure. These various definitions were used to evaluate whether the relationship between CVD and the lipid measures was linear on a log odd scale, as using the actual measurement assumes. In some analyses, National Cholesterol Education Program (NCEP) cutpoint measures were also used for LDL-C, HDL-C, and triglyceride, in order to compare with the effects of elevated remnant lipoproteins to that of other elevated lipids as stipulated by NCEP guidelines. Evaluation of triglycerides, using the quartile 4 as a dichotomous measure, was also used.

3. Results

Comparisons were performed between the 1484 FHS offspring women with no evidence of CVD and the 83

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Units</th>
<th>No CVD</th>
<th>CVD</th>
<th>P</th>
<th>Age adjusted P</th>
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<td>Number</td>
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<td>83</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Age</td>
<td>Years</td>
<td>50.9 ± 9.9</td>
<td>60.4 ± 7.1</td>
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<td>Triglycerides</td>
<td>mmol/l</td>
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<tr>
<td></td>
<td>mg/dl</td>
<td>12.2 ± 1.18</td>
<td>1.59 ± 0.92</td>
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<td>0.0154b</td>
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<tr>
<td>RLP-C</td>
<td>mmol/l</td>
<td>107.8 ± 104.2</td>
<td>140.9 ± 81.5</td>
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<tr>
<td></td>
<td>mg/dl</td>
<td>7.20 ± 6.25</td>
<td>8.32 ± 3.94</td>
<td>0.0001b</td>
<td>0.0196b</td>
</tr>
<tr>
<td>RLP-TG</td>
<td>mmol/l</td>
<td>0.251 ± 0.716</td>
<td>0.319 ± 0.352</td>
<td>0.0002b</td>
<td>0.0174b</td>
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<tr>
<td></td>
<td>mg/dl</td>
<td>22.2 ± 63.3</td>
<td>28.2 ± 31.1</td>
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<td>RLP-C &gt; 75th percentile</td>
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<td>23.5%</td>
<td>51.8%</td>
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<tr>
<td>RLP-TG &gt; 75th percentile</td>
<td></td>
<td>24.0%</td>
<td>43.2%</td>
<td>0.001</td>
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</table>

* BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; RLP-C, remnant-like particle cholesterol; RLP-TG, remnant-like particle triglyceride.

b Measures are not normally distributed and were log-transformed for statistical analysis.
women diagnosed with prevalent CVD. Prior to performing statistical analyses, standard log transformation was imposed on the non-linear variables, RLP-C, RLP-TG, and triglyceride (Table 1). However, while triglyceride was made linear by the transformation, RLP-C and RLP-TG remained non-linear, indicating a threshold effect. In addition, the relationship between RLP and age-adjusted rates for CVD was also non-linear (Table 2). It was therefore decided that statistical analyses would be performed using dichotomous cut-points for all lipid variables. Since cutpoints based on the approximate, 75th percentile of the population are used as part of the NCEP Adult Treatment Panel recommendations, and are also generally used by physicians to determine the need for treatment; it appears to be a reasonable approach for those measures, and avoids the problems of non-linearity associated with RLP measures. Therefore, NCEP cutpoints were used for LDL-C [≥ 4.1 mmol/l (160 mg/dl)], HDL-C [< 0.90 mmol/l (35 mg/dl)], and TG [> 2.25 mmol/l (200 mg/dl)]; 75th percentile cutpoints were used to discriminate RLP-C and RLP-TG. Since the NCEP cutpoint for triglycerides is higher than the 75th percentile for the FHS women, the 75th percentile for triglycerides was also used in a separate analysis. Comparisons to either exclude or include individuals on lipid-lowering medication, with adjustment for medication effects, were virtually identical, and therefore only results from comparisons where those individuals were excluded are provided.

Comparisons to RLP-C, RLP-TG, and triglyceride concentrations for FHS offspring women with and without diagnosed CVD are shown in Table 1. All three measures were significantly higher in women with CVD than in those without disease. The percentage of women with CVD who had RLP-C and RLP-TG concentrations above the 75th percentile of the population [28] was also significantly higher. Other parameters that were significantly higher ($P < 0.01$) in women with prevalent CVD were age, BMI, SBP, glucose, total and LDL-C, and triglyceride, while HDL-C was significantly lower; measures other than RLP-C and RLP-TG have been previously reported [33,34]. After age-adjustment, significant differences ($P < 0.05$) remained for RLP-C, RLP-TG, BMI, SBP, triglycerides, and HDL-C. Histories of beta blocker use, hypertension, and diabetes also occurred significantly more frequently in those with prevalent CVD than in those without diagnosed disease ($P < 0.01$), but a history of cigarette smoking was not significant.

Prevalence rates for CVD increased dramatically in the upper quartile of remnant lipoprotein levels among women (Table 2), with an age-adjusted estimate of 84.6 per 1000 women in the upper quartile of RLP-C compared with the estimates of 31.5–50.2 per 1000 for the lower three quartiles, and 77.3 per 1000 for RLP-TG versus 38.3–47.2 per 1000. These results suggest that

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Association of CVD prevalence with RLP-C and RLP-TG concentrations based on quartiles*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RLP-C</strong></td>
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<tr>
<td>Mean concentration</td>
<td>CVD number</td>
</tr>
<tr>
<td>Totals</td>
<td>0.13 ± 0.01</td>
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<tr>
<td>Quartile 1</td>
<td>0.15 ± 0.00</td>
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<tr>
<td>Quartile 2</td>
<td>0.17 ± 0.01</td>
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<tr>
<td>Quartile 3</td>
<td>0.29 ± 0.29</td>
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<tr>
<td><strong>P value</strong></td>
<td></td>
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<tr>
<td><strong>RLP-TG</strong></td>
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</tr>
<tr>
<td>Mean concentration</td>
<td>CVD number</td>
</tr>
<tr>
<td>Totals</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>Quartile 1</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>Quartile 2</td>
<td>0.19 ± 0.02</td>
</tr>
<tr>
<td>Quartile 3</td>
<td>0.59 ± 1.35</td>
</tr>
<tr>
<td><strong>P value</strong></td>
<td></td>
</tr>
</tbody>
</table>

*RLP-C, remnant-like particle cholesterol; RLP-TG, remnant-like particle triglyceride; mean concentration, mean plasma concentration (mmol/l); CVD number, number of subjects in each quartile diagnosed with CVD; total number, total number of subjects in each quartile; age adjusted rate, age adjusted rate of CVD in each quartile.
the primary risk of remnant lipoproteins lie in the upper quartile.

In univariate correlations (Table 3), log-transformed RLP-C and RLP-TG were significantly associated with all other measures of CVD risk ($P < 0.0001$). Not surprisingly, the highest $r$-values were seen for the correlations between RLP and triglycerides ($r = 0.8$).

Results for logistic regression analyses are presented in Table 4. Since the age-adjusted prevalence rates indicate a non-linear relationship between RLP and CVD, we report analyses for the dichotomous measure representing the upper gender-specific quartile. The results indicated that RLP-C levels in the upper quartile were significantly associated with prevalent CVD ($P = 0.0015$), with an odds ratio (OR) of 2.3 (95% confidence limits, 1.4–3.8), after adjustment for age, hypertension, smoking, diabetes, LDL-C, HDL-C, β blockers, and replacement hormones. In comparable models after adjustment for the same covariates, the association for RLP-TG with CVD was weaker (OR = 1.6, 95% confidence limits; 0.9–2.6, $P = 0.09$), while the association for total triglycerides with CVD was not statistically significant, either as the NCEP cutpoint, or as the 75th percentile. We did not evaluate the relationship of RLP-C, RLP-TG, and total triglycerides in the same model because these variables are highly correlated.

There were 839 postmenopausal women in the study, representing 56.5% of the total number of women. Within the postmenopausal group, there were 67 with established CVD. Therefore, 8.0% of postmenopausal women had CVD, but 80.7% of the 83 women with CVD were postmenopausal. Results of logistic regression analysis for postmenopausal women were similar to those for the entire female cohort (RLP-C > 75th percentile, $P = 0.0024$; RLP-TG > 75th percentile, $P = 0.12$).

Age and use of beta blockers were the two most significant variables associated with CVD; current smoking was also significant. Introduction of beta blockers into the model also accounted for a significant decrease in magnitude of the association of RLP-C with prevalent CVD, a phenomenon we have previously observed in comparisons to other potential risk factors, i.e. β blockers alter lipoprotein composition [35,36]. Since β blockers are commonly prescribed for CVD patients, they represent an important confounder, frequently overlooked in statistical evaluations of CVD associations. However, after adjustment for age and beta blocker use, as well as other known risk factors, RLP-C was still highly significant.

### 4. Discussion

Cardiovascular disease remains the leading cause of death and disability in USA [37]. Because many patients die suddenly of CVD, outside the hospital, the identification of individuals at high risk continues to be important for prevention. Major CVD risk factors identified by the NCEP Adult Treatment Panel include age, gender, hypertension, cigarette smoking, diabetes mellitus, family history of premature CVD, increased LDL-C, and decreased HDL-C [37].
Potential additional lipoprotein risk factors that have been proposed include increased Lp(a) [36,38–40], the presence of small dense LDL [41–45], triglycerides [23,24,44–51], and RLP-C and RLP-TG concentrations [50,52–54]. Difficulties with total triglyceride measurement as a risk factor include biologic variability and differences in triglyceride content among TRL subspecies. These sources of variability may account for the lack of consistent finding for triglycerides as an independent CVD risk factor [23,24,45–51]. Significant associations between triglycerides and CVD have been observed more frequently in women, however, than in men [23,24,47]. LDL particle size also has been not shown clearly to be an independent risk factor [42–45].

TRL remnants have been proposed as a CVD risk factor for many years [3], but they have been difficult to isolate due to their size heterogeneity, which overlaps with other TRL, and the fact that they contain the same lipid and apolipoprotein components. Methods of separation have previously been limited to ultracentrifugation and chromatography. Separation methods based on size and density have necessarily resulted in a heterogeneous population of particles in most cases. In addition, the procedures required to isolate remnants by these methods generally precluded large studies.

Previous work by us and by others using the immunoseparation method have shown associations between RLP concentrations and CVD risk factors [50,52–55]. Preliminary comparisons to a combined group of CVD patients from FHS and New England Medical Center indicated that RLP-C and RLP-TG were significantly higher in male and female CVD patients than in FHS controls [50]. Studies by Takeichi et al. have shown significantly higher levels of RLP-C and RLP-TG in victims of sudden cardiac death than in individuals who died from sudden non-cardiac causes of death [52,54]. A small prospective study in patients with coronary artery disease has also shown that patients in the highest tertile for RLP-C had the greatest number of secondary events over a 3-year period [55]. In addition, studies involving individuals who are at particular risk for CVD, namely those with diabetes mellitus and those with type III hyperlipoproteinemia, have shown similar increases in RLP-C and RLP-TG concentrations [53,56–58].

The current study included approximately 1500 well-characterized FHS female participants. All CVD diagnoses were carefully adjudicated, and all subjects were free-living at the time of exam, eliminating potential confounders associated with hospitalization [59,60]. Since this was a prevalence study, the likelihood exists that the most severely affected had been placed on drug therapy, while others had made significant changes to modify their risk factors. Those individuals with LDL-C concentrations above, or HDL-C concentrations below, the NCEP cut points, and not on therapy, would have been relatively few, thereby minimizing lipoprotein differences between individuals with and without prevalent CVD. The fact that RLP-C was still significant in women, however, even under these circumstances, indicates a potentially very important analyte for assessing risk.

From the results of the study, it appears that measurement of RLP-C may be of particular benefit to women, as part of a risk assessment panel. Risk factors in women have previously been shown to differ somewhat from those in men [23,61,62]. Diabetes mellitus, for example, is associated with a higher relative risk of CVD in women than in men; diabetes is also associated with increased triglycerides; and triglycerides have been shown to be a stronger CVD risk factor in women, than in men [23,61,62]. Postmenopausal status is also associated with increased triglycerides and with increased risk of CVD. In the current FHS prevalence analysis of exam 4, postmenopausal women represented 56.5% of women, but represented 80.7% of women with CVD. And while triglyceride > 2.25 mmol/l (200 mg/dl) or > 75th percentile was not independently significant when placed in the model, RLP-C was highly significant in women as a whole and also in the postmenopausal subgroup. And while we cannot exclude colinearity in these results among RLP and triglycerides, we have observed that the OR for RLP-C in women was greater than for either RLP-TG or total triglyceride. This suggests that RLP-C had a more directed association with CVD than do the other measures, neither of which is statistically significant.

In conclusion, we have documented that RLP-C is an independent risk factor for CVD in women, and provides significantly more information than do triglycerides.

Acknowledgements


References


