Retinol-Binding Protein 4 Is an Independent Factor Associated With Triglycerides and a Determinant of Very Low-Density Lipoprotein–Apolipoprotein B100 Catabolism in Type 2 Diabetes Mellitus

Bruno Vergès, Boris Guiu, Jean Pierre Cercueil, Laurence Duvillard, Isabelle Robin, Perrine Buffier, Benjamin Bouillet, Serge Aho, Marie Claude Brindisi, Jean Michel Petit

Objective—Retinol-binding protein 4 (rbp4) is an adipokine secreted by adipocytes and liver, whose levels are elevated in type 2 diabetes mellitus (T2DM). Plasma levels of rbp4 and triglycerides are strongly correlated in T2DM. However, we do not know whether this association is direct or indirect via liver fat content, and the link between rbp4 and triglyceride metabolism remains unknown.

Methods and Results—Liver fat measurement by proton spectroscopy was performed in 221 patients with T2DM, and an in vivo kinetic study with stable isotopes was carried out in 14 patients with T2DM. In multivariate analysis, triglycerides were associated positively with rbp4 (β=0.273, P<0.0001), apolipoprotein (apo) B (β=0.258, P<0.0001), and liver fat (β=0.191, P=0.002) and negatively with high-density lipoprotein cholesterol (β=−0.442, P<0.0001). rbp4 was correlated positively with apoB100 very-low-density lipoprotein (VLDL) pool (r=0.62, P=0.017) and negatively with VLDL-apoB100 total fractional catabolic rate (r=−0.66, P=0.001). In multivariate analysis, rbp4 (P=0.015), plasma triglycerides (P=0.024), and sex (P=0.026) were independently associated with VLDL-apoB100 total fractional catabolic rate. These data suggest that rbp4 may be involved in the pathophysiology of hypertriglyceridemia in T2DM by reducing VLDL catabolism. (Arterioscler Thromb Vasc Biol. 2012;32:3050-3057.)

Conclusion—In T2DM, plasma rbp4 level is associated with plasma triglycerides, independently of liver fat content. There is a strong independent negative correlation between plasma rbp4 and VLDL-apoB100 total fractional catabolic rate.

Key Words: diabetes mellitus ■ kinetic ■ liver fat ■ retinol-binding protein 4 ■ triglycerides

Cardiovascular disease is the major cause of morbidity and mortality in patients with type 2 diabetes mellitus (T2DM), and cardiovascular disease risk is 2- to 4-fold increased over nondiabetic subjects. Abnormalities of lipid metabolism, observed in T2DM, are one of the major factors contributing to vascular risk. The 2 major quantitative abnormalities of diabetic dyslipidemia are increased triglyceride and low high-density lipoprotein cholesterol (HDL-C) levels. In patients with T2DM, hypertriglyceridemia has been shown to be an independent factor associated with increased cardiovascular disease, and is likely to play a role in the residual cardiovascular risk observed in patients with T2DM treated with statins. Increased hepatic very-low-density lipoprotein (VLDL) production and reduced catabolism of triglyceriderich lipoproteins have been shown to be the kinetic abnormalities responsible for hypertriglyceridemia in T2DM. However, the precise mechanisms responsible for diabetic hypertriglyceridemia still need to be clarified, and the potential role of some adipokines in the development of increased plasma triglyceride levels, in patients with T2DM, remains unclear.

Among the adipokines, retinol-binding protein 4 (rbp4) has recently attracted much attention because of its newly demonstrated function as a potential determinant of insulin resistance, diabetes mellitus, and cardiovascular risks. Rbp4, secreted by adipocytes and liver and initially known as the specific carrier of retinol (vitamin A) in circulation, has been shown to be robustly associated with insulin resistance and lipid metabolism, suggesting that it may have other functions beyond retinol transport. Plasma levels of rbp4 are increased in mouse models of insulin resistance and T2DM. Although some human studies did not demonstrate any association between rbp4 and insulin resistance, most of them did, showing that elevated rbp4 levels in obesity impaired...
glucose tolerance and T2DM. Growing evidence suggests that rbp4 may play a more important role in lipid metabolism than in insulin resistance. During the past years, several studies have shown, in patients with T2DM, that plasma levels of rbp4 are positively correlated with triglycerides. Most of the human studies that confirmed the association between plasma rbp4 levels and insulin resistance also observed a robust association with triglycerides. Several studies have been able to show that the association between plasma rbp4 and triglycerides was independent of insulin resistance. All these data suggest that rbp4 may play a role in the pathophysiology of diabetic hypertriglyceridemia.

However, although the association between rbp4 and triglycerides, in patients with T2DM, has been found to be highly significant, some questions remain unsolved. First of all, it is not clear whether this association is direct or indirect via liver fat content. Indeed, in some animal and human studies, increased plasma rbp4 levels have been shown to be associated with nonalcoholic fatty liver disease. For instance, reduction in plasma levels of rbp4, using RNA oligonucleotide against rbp4 in high-fat diet–fed mice has been shown to reduce both plasma triglycerides and liver fat content, suggesting that the decrease in plasma triglycerides might be a result of the reduction in hepatic triglycerides. In a study performed in 75 healthy subjects, plasma rbp4 levels have been shown to be correlated with liver fat content, and it has been reported that plasma levels of rbp4 were higher in T2DM patients with nonalcoholic fatty liver disease than in T2DM patients without nonalcoholic fatty liver disease. Thus, we cannot exclude that the positive association reported between rbp4 and plasma triglycerides, in patients with T2DM, could not be as a result of the increased liver fat content observed in this population. Indeed, liver fat content has been shown to be tightly associated with plasma triglycerides in patients with T2DM, and so far, the association between plasma rbp4 and triglycerides independent of liver fat content has never been studied. Second, if a direct association between rbp4 and triglycerides does exist in patients with T2DM, the potential link between rbp4 and the metabolism of triglyceride-rich lipoproteins is unknown. To better understand a possible connection between rbp4 and triglyceride metabolism, it is important to explore the link between rbp4 and triglyceride-rich lipoprotein metabolism. Thus, to gain further insight into the relationship between rbp4 and triglycerides, in patients with T2DM, we set up a study aiming to achieve the following: (1) to analyze the specific role of rbp4 on plasma triglycerides independent of liver fat content, using an accurate assessment of liver fat content by proton magnetic resonance spectroscopy in a population of 221 patients with T2DM; and (2) to precisely determine the association between plasma rbp4 and triglyceride-rich lipoprotein kinetics with an in vivo kinetic study of lipoproteins with stable isotopes in a group of 14 patients with T2DM.

**Patients and Methods**

This prospective single-center study was approved by our regional ethics committee, and written informed consent was obtained from all patients before study inclusion.

**Patients**

We included 221 patients (121 females, 100 males) with type 2 diabetes for the first part of our study, which analyzed the specific role of rbp4 on plasma triglycerides independent of liver fat content. Patients with known liver disease, alcohol consumption >20 g/d, and those currently treated or previously treated with thiazolidinediones were excluded from the study. Because plasma levels of rbp4 have been shown to be increased in patients with overt diabetic nephropathy, we excluded from the study patients with macroalbuminuria and those with glomerular filtration rate (estimated with the Modification of Diet in Renal Disease [MDRD] equation) <60 mL/min. In addition, patients with liver fibrosis were also excluded.

For the in vivo lipoprotein kinetic study, we included 14 patients with T2DM (10 men and 4 nonmenopausal women). These patients were treated with oral glucose-lowering agents (metformin alone in 4 patients, metformin+sulfonylureas or glinides in 10 patients) and had a stable hemoglobin A1c (HbA1c) for at least 6 months. They had no overt cardiovascular disease, no macroalbuminuria, no renal impairment (glomerular filtration rate <60 mL/min), and no history of alcohol and drug abuse. No patient was treated with hypolipidemic drugs (statins, fibrates, nicotinic acid, ezitimibe, or-3) or drugs known to affect lipid metabolism (corticoids, retinoids, antiproteases, estrogens, cyclosporin, glitazones) or had been treated with such agents during the 6-month period before the study. Patients with a history of familial hyperlipidemia or plasma triglycerides >400 mg/dL (4.5 mmol/L) were excluded. Women included in the study were not using oral contraceptives.

**Liver Fat Content**

Liver fat content of the subjects was obtained using a 3.0-Tesla Magnetom TRIO TIM whole body system (Siemens, Erlangen, Germany), as previously described. Details are available in the online-only Data Supplement.

**Visceral and Abdominal Fat**

In each patient, intra-abdominal and subcutaneous fat content were assessed by magnetic resonance imaging at the level of the L4/L5 intervertebral disc.

**In Vivo Lipoprotein Kinetic Study**

**Experimental Protocol**

The kinetic study was performed for each subject in fed state with a primed bolus, followed by a 16-hour constant infusion of L-[1-13C] leucine, as previously reported. Details are available in the online-only Data Supplement.

**Isolation of Apo-B**

Apolipoprotein (apo) B100 was isolated from VLDL, intermediate-density lipoprotein (IDL), and low-density lipoprotein (LDL), as previously described. Details are available in the online-only Data Supplement.

**Determination of Leucine Enrichment by Gas Chromatograph/Combustion/Isotope Ratio Mass Spectrometry**

Amino acids were converted to N-acetyl O-propyl amino acid esters and analyzed with a Finnigan Mat Delta C isotope ratio mass spectrometer (Finnigan Mat, Bremen, Germany) coupled to an HP 5890 series II gas chromatograph (Hewlett Packard), as previously described.

**Modeling**

13C leucine enrichment was initially expressed in delta % and converted in tracer/tracer ratio before modeling. The data were analyzed with the Simulation Analysis and Modeling (SAAM) II program (SAAM Institute, Inc, Seattle, WA) using a multicompartamental model. The model chosen to describe the data is shown in Figure 1 and has already been used by others for apoB kinetic studies performed with stable isotope constant infusion.
**Statistical Analysis**

Data are reported as mean±SD. Statistical calculations were performed using the SPSS software package (Chicago, IL). For continuous variables, a Kolmogorov-Smirnov analysis was performed to test for normality. The Pearson correlation coefficients \( r \) were determined by linear regression analysis. Statistical significance of the correlation coefficients was determined by the method of Fisher and Yates. For the lipoprotein kinetic study, the Spearman correlation coefficients were used because of the smaller number of patients. Multivariate analyses were performed by stepwise multivariate linear regression. The weighted least square linear multiple regression modeling are available in the online-only Data Supplement. Direct VLDL-apoB100 fractional catabolic rate (FCR) represents direct VLDL removal from plasma (through receptor-mediated particle uptake), and indirect VLDL-apoB100 FCR represents VLDL delipidation toward IDL and LDL. Total VLDL-apoB100 FCR is the sum of direct VLDL-apoB100 FCR and indirect VLDL-apoB100 FCR.

A 2-tailed probability level of 0.05 was accepted as statistically significant.

**Results**

The main characteristics of the 221 patients are listed in Table 1. In this population, 132 (59.7%) patients were on metformin, 114 (51.6%) patients were on sulfonylurea or glinide, 6 (2.7%) patients were on \( \alpha \)-glucosidase inhibitor, and 122 (55.2%) patients were on insulin. One hundred five (47.5%) patients were treated with statin therapy. Plasma rbp4 levels were not different according to the antidiabetic treatment or the presence or absence of a statin treatment.

### Specific Association Between Plasma rbp4 and Triglycerides

Plasma triglycerides were positively correlated with liver fat content \( (r=0.369, P<0.0001) \), rbp4 \( (r=0.271, P<0.0001) \), apoB \( (r=0.438, P<0.0001) \), HbA1c \( (r=0.197, P=0.003) \), and visceral fat \( (r=0.173, P=0.001) \) and negatively correlated with age \( (r=-0.237, P=0.0003) \), HDL-C \( (r=-0.445, P<0.0001) \), and adiponectin \( (r=-0.256, P=0.0001; \text{Table } 2) \).

Liver fat content was positively correlated with triglycerides \( (r=0.369, P<0.0001) \), visceral fat \( (r=0.265, P<0.0001) \), apoB

### Table 1. Main Characteristics of the 221 Patients With Type 2 Diabetes Mellitus

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>60.7±10.2</td>
</tr>
<tr>
<td>Sex (men/women)</td>
<td>100/121</td>
</tr>
<tr>
<td>Duration of diabetes mellitus, y</td>
<td>11.9±9.9</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>34.5±6.9</td>
</tr>
<tr>
<td>Abdominal girth, cm</td>
<td>115±15</td>
</tr>
<tr>
<td>Fasting glucose, mmol/L</td>
<td>9.5±3.82</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>8.6±1.9</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>2.18±1.59</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.11±0.26</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>2.84±0.95</td>
</tr>
<tr>
<td>ApoB, g/L</td>
<td>1.01±0.27</td>
</tr>
<tr>
<td>ApoA1, g/L</td>
<td>1.44±0.28</td>
</tr>
<tr>
<td>ASAT, U/L</td>
<td>24.9±17.7</td>
</tr>
<tr>
<td>ALAT, U/L</td>
<td>40.6±29.2</td>
</tr>
<tr>
<td>GGT, U/L</td>
<td>68.3±79.0</td>
</tr>
<tr>
<td>Liver fat content, % of liver tissue weight</td>
<td>11.8±8.9</td>
</tr>
<tr>
<td>Visceral fat area, cm²</td>
<td>264±97</td>
</tr>
<tr>
<td>Subcutaneous fat area, cm²</td>
<td>388±141</td>
</tr>
<tr>
<td>Adiponectin, ng/mL</td>
<td>5.5±4.2</td>
</tr>
<tr>
<td>Rbp4, μg/mL</td>
<td>43±13</td>
</tr>
<tr>
<td>Metformin treatment, n (%)</td>
<td>132 (59.7%)</td>
</tr>
<tr>
<td>Sulfonylurea or glinide treatment, n (%)</td>
<td>114 (51.6%)</td>
</tr>
<tr>
<td>( \alpha )-glucosidase inhibitor treatment, n (%)</td>
<td>6 (2.7%)</td>
</tr>
<tr>
<td>Insulin treatment, n (%)</td>
<td>122 (55.2%)</td>
</tr>
<tr>
<td>Statin treatment, n (%)</td>
<td>105 (47.5%)</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SD. BMI indicates body mass index; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; LDL, low-density lipoprotein; Apo, apolipoprotein; ASAT, aspartate amino transferase; ALAT, alanine amino transferase; GGT, \( \gamma \)-glutamyl transferase; rbp4, retinol-binding protein 4.
Rbp4 was positively correlated with plasma triglycerides ($r=0.284$, $P<0.0001$) and visceral fat ($r=0.271$, $P<0.0001$). In multivariate analysis, rbp4 was independently and significantly associated with triglycerides ($\beta=0.284$, $P<0.0001$) and with visceral fat ($\beta=0.190$, $P=0.010$), but not with age, sex, BMI, HbA1c, or liver fat content.

To find out the factors independently associated with plasma triglycerides in T2DM, we performed a multivariate analysis, with triglycerides (log) as the dependent variable and age, sex, BMI, visceral fat, liver fat content, HbA1c, apoB, HDL-C, rbp4, adiponectin (log), and statin treatment as independent variables. All the variables that were significantly correlated with triglycerides in univariate analysis as potential confounding factors such as sex, BMI, and statin treatment were introduced into the statistical model. The multivariate analysis showed that HDL-C ($\beta=-0.422$, $P<0.0001$), rbp4 ($\beta=0.273$, $P<0.0001$), apoB ($\beta=0.258$, $P<0.0001$), and liver fat content ($\beta=0.191$, $P=0.002$) were independently associated with triglycerides, whereas age, sex, BMI, visceral fat, HbA1c, adiponectin, and statin treatment were not (Table 3). This statistical model explained 48% of plasma triglyceride variance.

### Table 2. Pearson Correlation Coefficients in the 221 Patients With Type 2 Diabetes Mellitus

<table>
<thead>
<tr>
<th>Variables</th>
<th>Coefficient</th>
<th>SD</th>
<th>$\beta$</th>
<th>$t$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDL-cholesterol</td>
<td>$-0.910$</td>
<td>0.125</td>
<td>$-0.442$</td>
<td>$-7.311$</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>Rbp4</td>
<td>0.011</td>
<td>0.002</td>
<td>0.273</td>
<td>4.583</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>ApoB</td>
<td>0.526</td>
<td>0.125</td>
<td>0.258</td>
<td>4.206</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>Liver fat content</td>
<td>0.011</td>
<td>0.004</td>
<td>0.191</td>
<td>3.144</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Nonsignificant variables: age, sex, BMI, visceral fat, HbA1c, adiponectin (log), and statin treatment. HDL indicates high-density lipoprotein; Rbp4, retinol-binding protein 4; ApoB, apolipoprotein B; BMI, body mass index; HbA1c, hemoglobin A1c.

Association Between Plasma rbp4 and Triglyceride-Rich Lipoprotein Kinetics: In Vivo Kinetic Study

Clinical, biological, and kinetic characteristics of the 14 patients with T2DM included in the in vivo lipoprotein kinetic study are presented in Table 4. This group of patients showed plasma triglycerides ranging from 1.20 to 4.20 mmol/L, allowing an optimal situation to analyze the potential relationship between triglyceride metabolism and plasma rbp4.

In these patients with T2DM in whom the in vivo lipoprotein kinetic study was performed, plasma rbp4 was positively correlated with plasma triglycerides ($r=0.76$, $P=0.001$) and VLDL-apoB100 pool ($r=0.62$, $P=0.017$) and negatively correlated with VLDL-apoB100 total FCR ($r=-0.72$, $P=0.004$), as shown in Figure 2, and with VLDL-apoB100 indirect FCR ($r=-0.69$, $P=0.006$). The correlation between rbp4 and VLDL-apoB100 direct FCR did not reach statistical significance ($r=-0.50$, $P=0.065$). Plasma rbp4 was not correlated with VLDL-apoB100 production rate ($r=-0.13$, $P=0.66$), IDL-apoB100 total FCR ($r=0.22$, $P=0.45$), IDL-apoB100 PR ($r=0.02$, $P=0.99$), IDL-apoB100 pool ($r=-0.16$, $P=0.64$), LDL-apoB100 PR ($r=0.14$, $P=0.63$), LDL-apoB100 FCR ($r=0.23$, $P=0.42$), or LDL-apoB100 pool ($r=-0.16$, $P=0.59$).

VLDL-apoB100 total FCR was positively correlated with HDL-C ($r=0.57$, $P=0.03$) and negatively with triglycerides ($r=-0.78$, $P=0.001$) and plasma rbp4 ($r=-0.72$, $P=0.004$).

To analyze whether the association between plasma rbp4 and total VLDL-apoB100 FCR was independent, we performed a multivariate analysis with VLDL-apoB100 total FCR as the dependent variable and age, sex, HbA1c, triglycerides, rbp4, BMI, homeostasis model assessment of insulin resistance, and HDL-C as independent variables. The multivariate analysis showed that total VLDL-apoB100 FCR was independently associated with plasma rbp4 ($\beta=-0.465$, $P=0.015$), plasma triglycerides ($\beta=-0.414$, $P=0.024$), and sex.
We find in patients with T2DM, in univariate analysis, a positive correlation between plasma triglycerides and rbp4. Such positive correlation has already been reported in several studies.\textsuperscript{10,11,14} As previously reported by other groups, we observe, in univariate analysis, a significant positive correlation between plasma triglycerides and liver fat content.\textsuperscript{19,20,25,26} In most of these studies, the association between liver fat content and triglycerides has been studied with qualitative (nonalcoholic fatty liver disease or not) or semiquantitative (steatosis grade) evaluation methods.\textsuperscript{19,20} In the present study, we confirm this association with liver fat content as a continuous variable precisely measured by proton magnetic resonance spectroscopy.

Patients with T2DM show increased liver fat content compared with nondiabetic individuals.\textsuperscript{20,26} For instance, in the Edinburgh Type 2 Diabetes study, performed in 939 patients with T2DM, hepatic steatosis was present in 57% of the participants.\textsuperscript{19} Because of the positive correlation between triglycerides and liver fat content, in T2DM, because rbp4 is synthesized not only in adipocytes but also in liver and because a possible association between rbp4 and liver fat content has been suggested in some animal\textsuperscript{16} and human studies,\textsuperscript{17,18} it is important to analyze more precisely the relationship between rbp4 and liver fat and to look whether the positive association between rbp4 and triglycerides is independent or not of liver fat content. We show in our population, using a multivariate analysis, that rbp4 and liver fat content are both independently associated with plasma triglycerides in patients with T2DM. Thus, we are able to clarify the link between rbp4 and triglycerides in T2DM and to demonstrate that the association between plasma rbp4 and triglycerides is independent of liver fat content.

Interestingly, we did not find any significant association between plasma rbp4 and liver fat content. Circulating rbp4 has been shown to correlate positively with nonalcoholic fatty liver disease in some studies,\textsuperscript{17,18} but not others.\textsuperscript{27,28} However, most of those studies have been performed in limited numbers of subjects. Our study, performed in a large population of patients with T2DM using a precise evaluation of liver fat content by proton magnetic resonance spectroscopy, does not find any significant association between plasma rbp4 and liver fat content. This indicates that the link between rbp4 and liver fat content is not likely to be significant in T2DM.

On the contrary, plasma rbp4 level was positively correlated with visceral fat, suggesting that increased plasma rbp4 in T2DM may originate mainly from visceral adipose tissue. Our results are in line with those from Klöting et al,\textsuperscript{29} who reported a 2-fold increased expression of rbp4 mRNA in the visceral adipose tissue in a group of individuals with T2DM or impaired glucose tolerance.

In our present study, rbp4 was not correlated with HDL-C. A negative correlation between rbp4 and HDL-C has been reported in 1 study\textsuperscript{10} and in a subgroup of 25 patients with apoAV TC heterozygote,\textsuperscript{14} but not in 2 other studies performed in large populations.\textsuperscript{11,30} Our data and those from the previous large studies indicate that rbp4 is not likely to be an important factor influencing HDL-C level in patients with T2DM.

### Discussion

In the present study, we show for the first time, using a very precise method to measure liver fat content (proton magnetic resonance spectroscopy) in a significant population of 221 patients with T2DM, that plasma rbp4 level is associated with plasma triglycerides, independently of liver fat content. This reinforces the idea of a robust and independent link between rbp4 and triglycerides. In addition, we show that rbp4 is significantly and inversely associated with VLDL-apoB100 total FCR, independently of plasma triglycerides, indicating a tight association between rbp4 and VLDL catabolism. All these data indicate that rbp4 may be an important factor to be considered in the pathophysiology of hypertriglyceridemia in T2DM.

We find in patients with T2DM, in univariate analysis, a positive correlation between plasma triglycerides and rbp4. Such positive correlation has already been reported in several studies.\textsuperscript{10,11,14} As previously reported by other groups, we observe, in univariate analysis, a significant positive correlation between plasma triglycerides and liver fat content.\textsuperscript{19,20,25,26} In most of these studies, the association between liver fat content and triglycerides has been studied with qualitative (nonalcoholic fatty liver disease or not) or semiquantitative (steatosis grade) evaluation methods.\textsuperscript{19,20} In the present study, we confirm this association with liver fat content as a continuous variable precisely measured by proton magnetic resonance spectroscopy.

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### Table 4. Clinical and Biological Characteristics and VLDL–, IDL–, LDL–ApoB100 Kinetic Data of the 14 Patients With Type 2 Diabetes Mellitus

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patients With Type 2 Diabetes Mellitus (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>56.9±9.3</td>
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<tr>
<td>Sex (men/women)</td>
<td>10/4</td>
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<tr>
<td>BMI, kg/m(^2)</td>
<td>9.0±4.4</td>
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<td>HbA1c, %</td>
<td>31.96±4.26</td>
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<tr>
<td>Fasting glucose, mmol/L</td>
<td>10.10±3.11</td>
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<tr>
<td>Triglycerides, mmol/L</td>
<td>2.70±0.93</td>
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<td>HDL-cholesterol, mmol/L</td>
<td>1.06±0.28</td>
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<tr>
<td>LDL-cholesterol, mmol/L</td>
<td>3.61±1.16</td>
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<tr>
<td>ApoB, g/L</td>
<td>1.23±0.27</td>
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<tr>
<td>Rbp4, µg/mL</td>
<td>42±16</td>
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<tr>
<td>Adiponectin, ng/mL</td>
<td>5.8±2.2</td>
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<tr>
<td>HOMA-IR</td>
<td>4.1±1.9</td>
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<tr>
<td>VLDL-apoB100 pool, mg</td>
<td>719±382</td>
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<td>IDL-apoB100 pool, mg</td>
<td>551±256</td>
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<td>LDL-apoB100 pool, mg</td>
<td>3227±757</td>
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<tr>
<td>VLDL-apoB100 total FCR, pool/d</td>
<td>4.46±1.70</td>
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<tr>
<td>VLDL-apoB100 direct FCR, pool/d</td>
<td>1.40±1.28</td>
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<tr>
<td>VLDL-apoB100 indirect FCR, pool/d</td>
<td>3.06±0.75</td>
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<td>IDL-apoB100 total FCR, pool/d</td>
<td>2.85±1.77</td>
</tr>
<tr>
<td>IDL-apoB100 direct FCR, pool/d</td>
<td>0.30±0.68</td>
</tr>
<tr>
<td>IDL-apoB100 indirect FCR, pool/d</td>
<td>2.55±1.85</td>
</tr>
<tr>
<td>LDL-apoB100 FCR, pool/d</td>
<td>0.49±0.15</td>
</tr>
<tr>
<td>VLDL-apoB100 PR, mg/kg per day</td>
<td>32.43±16.71</td>
</tr>
<tr>
<td>IDL-apoB100 PR, mg/kg per day</td>
<td>14.86±6.58</td>
</tr>
<tr>
<td>LDL-apoB PR, mg/kg per day</td>
<td>16.29±3.28</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ApoB, apolipoprotein B; Rbp4, retinol-binding protein 4; VLDL, very-low-density lipoprotein; IDL, intermediate-density lipoprotein; FCR, fractional catabolic rate; PR, production rate. HOMA-IR, homeostasis model assessment of insulin resistance calculated as glucose (mmol/L)×plasma insulin (mU/L)/22.5.

\(β=0.399, P=0.026\). The statistical model used explained 79% of total VLDL-apoB100 FCR variance.
In the present study, we clearly show that liver fat content and rbp4 are 2 independent factors associated with hypertriglyceridemia in patients with T2DM. The association between increased liver fat content and hypertriglyceridemia in T2DM has been reported in many studies, and kinetic studies from Adiels et al have shown that overproduction of fatty acids to the liver that increases the cytosolic triglyceride pool has been shown to be correlated with VLDL secretion. Patients with T2DM show increased flux of free fatty acids to the liver that increases the cytosolic triglyceride storage pool and promotes VLDL production by reducing posttranslational degradation of apoB and by increasing microsomal triglyceride transfer protein expression. In addition, animal studies indicate that increased hepatic free fatty acid and hepatocyte lipid content promote de novo lipogenesis through endoplasmic reticulum stress. We show that increased rbp4 is another factor associated with hypertriglyceridemia. It has been suggested by Takebayashi et al that the relationship between triglycerides and rbp4 could be mediated by steatosis. However, we demonstrate, in our study, that this association is independent of liver fat content. In addition, the results of our multivariate analysis show a positive association between plasma rbp4 and plasma triglycerides, in patients with T2DM, which is independent not only of liver fat content but also of HDL-C or factors associated with insulin resistance, such as visceral fat or adiponectin. All these data suggest that rbp4 may play a direct role in the development of hypertriglyceridemia in patients with T2DM. This is in line with animal studies that have shown that reduction of several genes involved in the triglyceride metabolism, such as those of enzymes implicated in β-oxidation of fatty acids or of apoC-III, which is an inhibitor of the lipoprotein lipase. At the level of peripheral tissues, rbp4 may act by binding to cell surface receptors triggering a janus tyrosine kinase/signal transducer and activator of transcription (JAK/STAT) cascade that regulates the expression of genes involved in lipid metabolism. However, so far, we do not have any clear indication on the precise mechanism(s) that may explain the association between rbp4 and the catabolism of VLDL, and further studies are needed to get further insight into a possible direct effect of rbp4 on triglyceride metabolism in humans.

One limitation of our study may be that the number of patients included in the kinetic study is not very high. One must consider that kinetic studies in humans, because of a very heavy workload, are usually performed in a limited number of patients. This is particularly true for stable isotopes with the use of high-sensitivity isotope ratio mass spectrometer.
allowing accurate estimation of isotopic enrichment and compartmental modeling, which represents the goal standard for the analysis of lipoprotein kinetic studies.\textsuperscript{23,41} Such in vivo kinetic studies with stable isotopes performed in limited number of subjects provide accurate and reliable data.\textsuperscript{5,24,41–43} As far as our present kinetic study is confirmed, we think that the strong negative correlation between rbp4 and VLDL catabolism is likely to explain the positive association between rbp4 and triglycerides. This negative correlation between plasma rbp4 and triglycerides in patients with T2DM. These data suggest that rbp4 may be involved in the pathophysiology of hypertriglyceridemia in T2DM by reducing VLDL catabolism.

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Disclosures

None.

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Bruno Vergès, Boris Guiu, Jean Pierre Cercueil, Laurence Duvillard, Isabelle Robin, Perrine Buffier, Benjamin Bouillet, Serge Aho, Marie Claude Brindisi and Jean Michel Petit

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Liver fat content

Briefly, sagittal, coronal and axial slices through the right lobe of the liver were acquired and a 27-cm$^3$ spectroscopic volume of interest was positioned on segment VII. Single-voxel MR spectroscopic data were acquired by using seven breath-hold point-resolved spatially localized spectroscopic pulse sequences (repetition time, 1500 msec; eight acquisitions; and 2048 data points over 1250 Hz spectral width) with echo times of 30, 40, 50, 60, 80, 100, and 135 msec to measure T2 relaxation times of water and methylene. To minimize T1 effects, we acquired a free-breathing point-resolved spatially localized spectroscopic pulse sequence (4000/30; 32 acquisitions; 2048 data points over 1250 Hz spectral width; acquisition time, 4 min). For each of the two voxel placements, automated optimization of gradient shimming followed by manual adjustment of central frequency was performed, and water line widths of 40–50 Hz were obtained. The Java-based MR user interface spectroscopic analysis package was used for the time-domain analysis. Metabolite signals were analyzed using the Advanced Magnetic Resonance (AMARES) fitting algorithm within jMRUI, which enables the inclusion of a large amount of prior knowledge. We measured the water peak at 4.7 ppm and the methylene peak at 1.3 ppm. Spectra were used only if homogeneity after shimming was better than 0.45 ppm, measured as the full width at 50% peak height. Peak integrals were quantified by fitting to a Gaussian line shape. Finally, liver fat content (as a percentage) was calculated as previously reported. \textsuperscript{1} \textsuperscript{1}H-MRS has proven to be a very sensitive non invasive method to detect liver fat content and has shown to correlate well with liver biopsy results \textsuperscript{1,2}

Supplemental Material II

In vivo lipoprotein kinetic study

*Experimental protocol*

Food intake, with a leucine poor diet (1700 Kcal.day\(^{-1}\), 55% carbohydrates, 39% fats and 7% proteins), was fractionated in small equal portions which were provided every 2 hours, starting 6 hours prior to the tracer infusion up to the end of the study, in order to avoid important variations in apolipoprotein plasma concentration, as previously performed by our group\(^1\) and others \(^2\). To determine the kinetic of apoB100, the subjects received an intravenous injection of a 0.7 mg.kg\(^{-1}\) bolus of L-[\(^{1-13}\)C]leucine (99% \(^{13}\)C, Eurisotop, Saint Aubin, France) immediately followed by a 16-hour constant infusion at 0.7 mg.kg\(^{-1}\).h\(^{-1}\). Blood samples were collected at hours 0, 0.25, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 14, 15 and 16. Serum was separated by centrifugation and stored at -80°C.

*Isolation of apo-B*

VLDL (density (d) < 1.006 g.ml\(^{-1}\)), IDL (1.006 < d< 1.019) and LDL (1.019 < d < 1.063) were isolated from plasma by sequential flotation ultracentrifugation, using a 50.4 rotor in a L7 apparatus (Beckman Instruments, Palo Alto, USA). IDL and LDL fractions were then dialyzed against a 10 mmol.l\(^{-1}\) ammonium bicarbonate buffer pH 8.2 containing 0.01% EDTA and 0.013% sodium azide. VLDL, IDL and LDL fractions were delipidated with diethylether-ethanol and apoB100 from each lipoprotein fraction was isolated by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (3%). After staining with Coomassie blue R-250, apoB100 bands were excised from the polyacrylamide gels and hydrolyzed in 6 M HCl at 110°C for 16 h under nitrogen vacuum. Samples were then centrifuged to remove polyacrylamide. Supernatants were then lyophilized in a Speed Vac (Savant Instrument, Farmingdale, New York, USA). Lyophilized samples were dissolved in 50% acetic acid and applied to an AG-50W-X8 200-400 mesh cation exchange resin (Bio-
Rad, Richmond, USA) and aminoacids were recovered by elution with 4N NH$_4$OH.

**Modeling**

$^{13}$C leucine enrichment was initially expressed in delta % and converted in tracer/tracee ratio prior to modelling. The data were analyzed with the Simulation Analysis And Modeling (SAAM) II program (SAAM Institute, Inc., Seattle, WA) using a multicompartmental model. The model chosen to describe the data is shown in Figure 1 and has already been used by others for apoB kinetic studies performed with stable isotope constant infusion. Development of compartmental models in stable isotope experiment has been previously described in details. A forcing function was used to drive the appearance of leucine tracer into the different lipoprotein fractions. Because labeling of intracellular and plasma leucine is not always similar, we used VLDL-apoB100 plateau enrichment as the forcing function to estimate the intrahepatic leucine precursor pool. The delay compartment accounted for the time required for the synthesis and secretion of apoB100 into the plasma. Plasma VLDL-apoB100 kinetic data are represented by compartments 1 and 2, and plasma IDL-apoB100 kinetic data are represented by compartments 11 and 12. Compartment 21 represents plasma LDL apoB100. Compartments 2 and 12 turned over more slowly than did compartments 1 and 11, respectively. These compartments were required for optimal model fit of the data.

As the experiment was performed in the steady state, fractional synthetic rate equaled fractional catabolic rate (FCR).

- Direct VLDL-apoB100 FCR and indirect VLDL-apoB100 FCR from VLDL to IDL or LDL, expressed in pool.day$^{-1}$ were calculated as follows:
  - Direct VLDL-apoB100 FCR = $M2 \frac{k(0,2)}{(M1 + M2)}$
  - Indirect VLDL-apoB100 FCR→IDL/LDL = $M1[ k(11,1) + k(21,1)]/( M1 + M2)$
where $k(i,j)$ is the fractional transfer coefficient from compartment $j$ to $i$, and $M_j$ represents the apoB mass (expressed as concentration per liter of plasma) of compartment $j$.

Total VLDL-apoB100 FCR is the sum of direct VLDL-apoB100 FCR and indirect VLDL-apoB100 FCR→IDL/LDL.

- Direct IDL-apoB100 FCR and indirect IDL-apoB100 FCR from IDL to LDL were calculated as follows:
  - Direct IDL-apoB100 FCR = $M_{12} k(0,12)/(M_{11} + M_{12})$
  - Indirect IDL-apoB100 FCR→LDL = $M_{11} k(21,11)/( M_{11} + M_{12})$

Total IDL-apoB100 FCR is the sum of direct IDL-apoB100 FCR and indirect IDL-apoB100 FCR→LDL

- LDL-apoB100 FCR = $k(0,21)$

Production rates (PR) of the apoB100 in each lipoprotein fraction were normalized to body weight and calculated as follows:

$$PR = \text{apoB100 FCR (for each lipoprotein fraction)} \times \text{apoB100 pool size /body weight},$$

where apoB100 pool size is calculated by multiplying the apoB100 concentration, in the lipoprotein fraction (VLDL, IDL or LDL) by the estimated plasma volume (4.5% of body weight). In obese subjects (BMI ≥ 30), a correction of plasma volume was performed as previously reported by many authors $^7,8$. The plasma volume was modified by multiplying by a correction factor to take into account the decrease in relative plasma volume associated with an increase in body weight $^8$.

The Akaike Information Criterion (AIC) was used to compare different models and the model with the lowest AIC value was chosen. Moreover, the physiological plausibility of the model has been verified $^9$. The goodness of fit of the model was assessed by the analysis of the residuals with the runs test $^5$.


