Influence of rosuvastatin dose on total fatty acids and free fatty acids in plasma
Correlations with lipids involved in cholesterol homeostasis

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Abstract
This study investigates for the first time the influence of four doses of rosuvastatin on total fatty acids (TFA) and free fatty acids (FFA) in human plasma and correlates their changes in concentration with changes in the concentration of other lipids involved in cholesterol homeostasis.

This study was a placebo-controlled, randomized, double-blind, crossover experiment. The study used a single group of 16 men and consisted of 5 treatment periods lasting 4 weeks each with placebo and 4 doses of rosuvastatin (5, 10, 20, and 40 mg). Each subject changed 5 medical treatments and received in each new treatment different tablets of rosuvastatin or placebo compared to those taken in previous treatments, in a random order. Between treatment periods there was a wash-out period of 2 weeks, without treatment.

Changes in TFA and FFA were significant compared to placebo and between different doses of rosuvastatin. We found a continuous logarithmic decrease in levels of TFA, FFA, low-density lipoprotein (LDL)-cholesterol, total cholesterol, triglycerides, phospholipids, and apolipoprotein B-100, and a continuous increase in levels of high-density lipoprotein (HDL)-cholesterol and apolipoprotein A-1 by increases the dose of rosuvastatin. Analysis of the correlation of TFA and FFA with the main lipids and lipoproteins in cholesterol homeostasis indicated a linear regression with high correlation coefficients and all P-values were less than .05 level.

The concentrations of TFA and FFA are significantly influenced by the dose of rosuvastatin. They are strongly correlated with those of other lipids and lipoproteins involved in cholesterol homeostasis. The mechanisms of cholesterol homeostasis regulation are involved in changing the concentrations of TFA and FFA.

Abbreviations: CVD = cardiovascular disease, FAs = fatty acids, FFA = free fatty acids, HDL = high-density lipoprotein, HMG-CoA = 3-hydroxy-3-methylglutaryl-coenzyme A, LDL = low-density lipoprotein, SD = standard deviation, TFA = total fatty acids.

Keywords: cholesterol homeostasis, free fatty acid, plasma lipids, rosuvastatin, total fatty acids

1. Introduction
Among the large number of risk factors associated with atherosclerosis, blood lipid levels are important. Analysis of lipids in atheroma reveals the presence of cholesteryl esters, triglycerides, and oxidative derivatives from cholesterol and fatty acids (FAs).[1,2] The high level of low-density lipoprotein (LDL) cholesterol in plasma, particularly in small and dense form, is a risk factor for atherosclerosis and implicitly for cardiovascular disease (CVD).[3] Cholesterol esters with polyunsaturated fatty acids were in higher concentration in atherosclerotic plaques compared to plasma.[1] These polyunsaturated fatty acids are very sensitive to oxidation by free radical species, and the resulting lipid peroxide radical may be relevant in atherogenesis.[4] It has been found that high amounts of free fatty acids (FFA) in plasma have contributed to an increased risk of CVD and mortality.[5,6] FAs have different behavior in the pathologies of atherosclerosis. For example, among saturated FAs, only a high concentration of palmitic acid increased mortality in patients with CVD.[7] High levels of trans FAs have been associated with coronary heart disease.[6]

Reducing high plasma lipids significantly reduced the risk of atherosclerosis in people with and without pre-existing CVD.[8] High lipids levels (hyperlipidemia) can be treated with diet and medication. Treatment depends on many factors, and the first to consider is the degree of hyperlipidemia. There are different classes of drugs available to lower lipid levels, but statins are the most important class.[8]

Rosuvastatin is known as a highly effective statin for lowering LDL-cholesterol, being structurally similar to 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA), which is a precursor in cholesterol biosynthesis. Rosuvastatin is unique because it
contains a polar group of methyl sulfonamide that leads to a specific interaction with HMG-CoA reductase.\[19\] In addition to reducing LDL-cholesterol level, rosuvastatin also decreased the levels of total cholesterol, apolipoprotein B-100, triglycerides, phospholipids, free fatty acids, and increased apolipoprotein A-1 and high density lipoprotein (HDL)-cholesterol.\[10-12\]

Cholesterol levels depend on age, weight, and gender and tend to increase with age. There are established threshold concentrations for LDL-cholesterol, HDL-cholesterol, total cholesterol, and triglycerides above which the risk of CDV is high.\[16\] Increasing the dose of statin lowers blood LDL cholesterol level.\[11\] In addition, it should be noted that statins also have multiple sides effects that are dose-dependent.\[13\]

High dosages of statins administrated to animals for long periods of time introduced changes in cell morphology and necrosis in cells. These side effects observed in animals were generated by the drug at high dosage levels, which are not used in human. However, several studies have shown that statins are associated with modest but notable adverse effects especially in elderly patients at the maximal recommended dosages of statins.\[14,15\]

The studies presented above indicate that high concentrations of total fatty acids (TFA) and free fatty acids (FFA) in the blood are associated with an increased risk of CVD.\[5-7\] Therefore, it is very important to know the influence of statin dose on decreasing the concentration of TFA and FFA in order to optimize the effects of statins.

There is no article to examine the influence of increasing statin doses on FFA and TFA. Dose-dependent effects of rosuvastatin on total cholesterol, apolipoprotein B-100, triglycerides, phospholipids, apolipoprotein A-1 and HDL-cholesterol were already done, but no information is available on the influence of rosuvastatin dose on TFA and FFA in plasma.

This study investigates for the first time the influence of 4 doses of rosuvastatin on the concentration of TFA and FFA and the correlation of their changes in concentration with changes in the concentration of other lipids in men with hypercholesterolemia.

2. Methods

2.1. Subjects

This study included 16 Caucasian men aged >65 years with hypercholesterolemia defined as LDL-cholesterol >363 mmol/L (140 mg/dl) and total plasma cholesterol >5.18 mmol/L (200 mg/dl). The subjects with any form of cardiovascular disease, diabetes, renal or hepatic diseases, autoimmune disorders, smoking, and inflammatory diseases were excluded. Subjects did not take other lipid-lowering medications, any regular medications, or herbal supplements that could interfere with statins. All subjects gave informed written consent before participating in this study.

2.2. Study design and clinical information

The study was approved by the Research Ethics Committee of the University of Medicine and Pharmacy of Timisoara (No. 44/2019) and was conducted in accordance with the ethical regulations imposed by the Declaration of Helsinki (1975), as revised in 2008(5).

This study was a placebo-controlled, randomized, double-blind, crossover experiment involving a single group of subjects. When choosing the size of the study, it was taken into account that it is a preliminary crossover experiment with a single group of subjects. The study began with a 1-week diet period for stabilizing study parameters. At the end of this period, measurements were made for the baseline values. The study consisted of 5 treatment periods lasting 4 weeks each in which subjects received different sequences of treatments. Between treatment periods there was a wash-out period of 2 weeks, when subjects did not receive any treatment in order to eliminate the effects of previous treatment. Subjects were randomized to the first 4-week treatment period in which they received placebo tablets or tablets from 1 of the doses of rosuvastatin (5, 10, 20, or 40 mg). This was followed by a 2-week wash-out period. The study continued with another 4 treatment periods of 4 weeks each, in which each subject changed medical treatment and received in each new treatment different tablets of rosuvastatin or placebo compared to those taken in previous treatments, in a random order. After each of these treatment periods, followed a wash-out period of 2 weeks without treatment. At the end of this study, each subject received tablets of each commercial dose of rosuvastain (5, 10, 20, and 40 mg) and placebo. A wash-out period of 2 weeks proved to be sufficient to eliminate the effect of the previous dose of rosuvastatin.\[10,16\] The change in LDL-cholesterol concentration can reach a stable steady state in a 2-week treatment period with rosuvastatin.\[17\] For safety, we chose 4 weeks for the treatment period. Oral administration of rosuvastatin was given daily at bedtime, in a single dose, without any change during this study period.

Participants agreed with the lifestyle change, in terms of diet and physical activity. All of them followed the same usual diet throughout the study. They completed a questionnaire about personal medical histories and a comprehensive physical examination, including vascular risk factors as hypertension, hypercholesteremia, cardiovascular disease, diabetes, etc.

2.3. Laboratory analyses

The variables of this study are the concentration of FAs from total plasma lipids and total plasma FFA, total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, phospholipids, apolipoprotein A-1, and apolipoprotein B-100.

Blood samples for laboratory analyses were collected at room temperature into commercially tubes after an overnight fast of more than 10 hours and were centrifuged at 2500×g for 15 minutes. Plasma samples were aliquoted and stored at -70°C. Blood samples analyses were performed at the end of placebo and statin treatments.

Phospholipids were assayed by standard enzymatic-spectrophotometric method with a commercial kit (Wako Diagnostics, Osaka, Japan). Apolipoprotein A-1 and apolipoprotein B-100 were determined by a nephelometric turbidity method using a commercial Siemens ProSpec Analyzer (Siemens Co., Marburg, Germany). Total cholesterol, triglycerides, LDL-cholesterol, and HDL-cholesterol were assayed by routine laboratory techniques using standard enzymatic-spectrophotometric methods (Roche Diagnostics, Basel, Switzerland). FAs analysis was performed by gas chromatography-mass spectrometry of their corresponding fatty acid methyl ester. All FAs from plasma lipids were methylated in 15 minutes at room temperature with methyl iodide in solution of dimethyl sulfoxide and by addition of solid sodium hydroxide.\[18\] FFA were selectively methylated in 1 minute at room temperature with methyl iodide in a solution of dimethyl sulfoxide and in the presence of anhydrous potassium carbonate.\[19\]
2.4. Statistical analysis

The variables of the statistical analysis are expressed numerically as mean ± standard deviation (SD). All measured values were entered into the study. Paired t test for dependent means was used for statistical analysis, because we compare the means of 2 sets of values that are directly related to each other. We have pairs of observed values: one before and another after treatment. All statistical analyses were two-tailed test and the confidence interval was 95% for the mean changes. Statistical P values greater than .05 significance level indicate that no effect was statistically observed. All statistical analyses were conducted with Statistical Test Calculator online version 2018 from Social Science Statistics (https://www.socsicostatistics.com/tests/) and Microsoft Excel 2016 (Microsoft Corp. USA).

3. Results

The recruited subjects were Caucasian men over 65 years of age with the following baseline characteristics: total cholesterol 6.17 mmol/L, free fatty acids 3.44 nmol/L. Subjects had a high baseline plasma level of triglycerides. In this short-term treatment, all doses of rosuvastatin were well tolerated and no side effect was observed.

Table 1 shows the absolute concentration of lipid variables measured in treatment with different doses of rosuvastatin and the corresponding changes compared to placebo expressed as a mean of the percentage changes. Compared with placebo results, all doses of rosuvastatin significantly reduced TFA, FFA, total cholesterol, LDL-cholesterol, apolipoprotein B-100, triglycerides, phospholipids, and significantly increased HDL-cholesterol and apolipoprotein A-1.

Table 2 shows the P value of changes between different doses of rosuvastatin. It can be seen that there are 2 situations. The first is between the 2 neighboring doses (5 mg vs 10 mg, 10 mg vs 20 mg, and 20 mg vs 40 mg), when the changes were borderline significant and some were insignificant. The second was for larger differences between doses, when the changes were significant with 2 exceptions: HDL-cholesterol and triglycerides. The highest significance was between 5 mg and 40 mg.

Figure 1 shows the graphical variation of the mean percent changes of total fatty acids, free fatty acids, total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, phospholipids, apolipoprotein A-1, and apolipoprotein B-100 depending on the dose of rosuvastatin. As can be seen, the variation of these parameters as a function of rosuvastatin dose is not linear. A non-linear function was used to match the data. The function that best describes these changes was a natural logarithm. For total cholesterol the equation was y = –5.996ln(x) – 19.741 (R² = 98.97%), for LDL-cholesterol was y = –6.352ln(x) – 32.805 (R² = 99.78%), for HDL-cholesterol was y = 1.421ln(x) + 4.383 (R² = 99.79%), for triglycerides was y = –3.082ln(x) – 11.206 (R² = 99.73%), for phospholipids was y = –3.37ln(x) + 17.238 (R² = 98.49%), for Apolipoprotein B-100 was y = –5.624ln(x) – 28.302 (R² = 99.72%), for apolipoprotein A-1 was y = 1.2624ln(x) + 1.788 (R² = 98.62%), for total fatty acids was y = –4.731ln(x) – 16.035 (R² = 99.85%), and for free fatty acids was y = –2.678ln(x) – 13.372 (R² = 99.31%). R² is the coefficient of determination. The higher the R², the better the function corresponds to the experimental values. The values of R-squared are between 98.49% and 99.85%, which indicates that changes in lipid concentration are very well described by these logarithmic equations. The linear equations had lower values for R-squared, being between 89.19% and 97.15%.

Table 3 shows the linear regression equations, the correlation coefficients (r) and statistical P values, which are correlation analysis parameters of TFA and FFA with the main lipids and lipoproteins in cholesterol homeostasis. The linear function is characterized by the slope and the intercept that can be easily obtained from the equation. The dependent variable Y is TFA and FFA and the independent variable X is a lipid from cholesterol homeostasis. With this linear regression equation, we can predict changes in TFA and FFA concentrations using changes in the concentration of other lipids at different doses of rosuvastatin. Changes in the concentration of TFA have the best correlation in the following order according to the correlation coefficient: total cholesterol, triglycerides, HDL-cholesterol, LDL-cholesterol, apolipoprotein B-100, phospholipids and apolipoprotein A-1. For FFA this order is: Apolipoprotein B-100, LDL-cholesterol, triglycerides, phospholipids, total cholesterol, HDL-cholesterol, triglycerides, phospholipids, total cholesterol, HDL-cholesterol, apolipoprotein A-1, and apolipoprotein B-100 depending on the dose of rosuvastatin.

Table 1: Effect of rosuvastatin dose on the concentration of the measured variables in the study.

| Variable                  | Placebo | 5 mg          | 10 mg         | Rosuvastatin | 20 mg         | 40 mg         |
|---------------------------|---------|---------------|---------------|--------------|---------------|---------------|
| Free fatty acids, μmol/L  | 32.79± 0.33 | 32.44±0.36   | 29.52±0.38   | 41.49±0.40  | 47.52±0.30   | 56.12±0.33    |
| Total fatty acids, μmol/L | 29.35± 0.34 | 29.32±0.36   | 26.84±0.30   | 39.16±0.30  | 52.20±0.30   | 53.48±0.33    |
| Triglycerides, mmol/L     | 3.92± 2.79 | 3.81±2.79    | 2.79±2.79    | 3.81±2.79   | 3.81±2.79    | 3.81±2.79     |
| Phospholipids, mmol/L     | 5.51± 1.64 | 5.39±1.64    | 5.10±1.64    | 5.10±1.64   | 5.10±1.64    | 5.10±1.64     |
| HDL-cholesterol, mmol/L   | 10.5± 1.87 | 10.5±1.87    | 10.5±1.87    | 10.5±1.87   | 10.5±1.87    | 10.5±1.87     |
| Total cholesterol, mmol/L | 4.07± 163.11| 4.07±163.11  | 4.07±163.11  | 4.07±163.11 | 4.07±163.11  | 4.07±163.11   |
| Apolipoprotein B-100, mmol/L| 65.03±48.79| 65.03±48.79  | 65.03±48.79  | 65.03±48.79 | 65.03±48.79  | 65.03±48.79   |
| Apolipoprotein A-1, mmol/L| 37.17±3.49 | 37.17±3.49   | 37.17±3.49   | 37.17±3.49  | 37.17±3.49   | 37.17±3.49    |

*P < .05.
**P < .001.
***P < .0001.
****P < .000001.
*****P < .0000001.
******P < .00000001.

All values are expressed as mean ± SD.
and apolipoprotein A-1. The correlation coefficients have very close values, being higher than 0.99. Only the correlation between FFA and apolipoprotein A-1 has a correlation coefficient of 0.984. The $P$ values indicate that all correlations were significant.

4. Discussion

We evaluate information on the effect of 4 doses of rosuvastatin on the concentration of TFA and FFA in subjects with hypercholesterolemia. No such study has been done so far regarding TFA and FFA. We demonstrate that their changes in concentration are strongly correlated with changes in the concentration of total cholesterol, LDL-cholesterol, triglycerides, phospholipids, apolipoprotein B-100, and apolipoprotein A-1.

Previous studies on the dose-dependent effect of rosuvastatin and other statins are primarily focused on total cholesterol, LDL-cholesterol, triglycerides, phospholipids, apolipoprotein B-100, and apolipoprotein A-1. However, these variations are not continuous from dose to dose in these articles. For example, HDL and apolipoprotein A-1 have different variations from the 5 mg dose to the 10 mg dose. Discrepancies may be caused by the heterogeneity of the subjects chosen for the study. In our study, we had a homogeneous group of Caucasian men and we found a continuous decrease of the levels of TFA, FFA, total cholesterol, LDL-cholesterol, triglycerides, phospholipids, and apolipoprotein B-100, and a continuous increase of HDL-cholesterol and apolipoprotein A-1 levels by increases the dose of rosuvastatin.

Rosuvastatin is known as a synthetic drug, which can inhibit HMG-CoA reductase that convert HMG CoA to mevalonate in the cholesterol biosynthesis. The effect is a reduction in the amount of cholesterol synthesized, as well as other end products in this synthesis process. Cholesterol concentration is tightly regulated through the homeostatic mechanisms in a dynamic state of equilibrium through a feedback control that acts at transcriptional and posttranscriptional levels.

| Variable                  | $P$ value |
|---------------------------|-----------|
| Free fatty acids, $\mu$mol/L | <.01      |
| Total fatty acids, $\mu$mol/L | <.01      |
| Triglycerides, mmol/L     | <.05      |
| Phospholipids, mmol/L     | <.05      |
| LDL-cholesterol, mmol/L   | <.05      |
| HDL-cholesterol, mmol/L   | <.05      |
| Total cholesterol, mmol/L | <.05      |
| Apolipoprotein B-100, nmol/L | <.01    |
| Apolipoprotein A-1, nmol/L | <.01    |

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**Figure 1.** Variation of percentage changes in total fatty acids (TFA), free fatty acids (FFA), total cholesterol (TCh), LDL-cholesterol (LDL), HDL-cholesterol (HDL), triglycerides (TG), phospholipids (PL), apolipoprotein A-1 (Apo A), apolipoprotein B-100 (Apo B) as a function of rosuvastatin dose.
homeostasis is achieved through a variety of mechanisms,\cite{1} such as biosynthesis, intestinal absorption, uptake through LDL receptors, transport to peripheral cells by LDL, and reverse cholesterol transport to the liver for recycling and degradation. These regulatory mechanisms are involved several proteins, which activate all genes involved in the metabolism of cholesterol and all lipids that participate in cholesterol homeostasis.\cite{2,3} The most important regulatory elements are HMG-CoA reductase and the biosynthesis, because the transport of hepatic FAs to peripheral cells is coordinated feedback control of hepatic cholesterol and FAs biosynthesis, the concentration of TFA and FFA. Practically, it is a strong coordinated feedback control of hepatic cholesterol and FAs biosynthesis, because the transport of hepatic FAs to peripheral tissues and cells can be done together with cholesterol as complex particles of lipoproteins.

The association between TFA and FFA changes with changes in total cholesterol, LDL-cholesterol, HDL-cholesterol, phospholipids, apolipoprotein triglyceride B-100, apolipoprotein A-1 was suggested by linear regression analysis. High values of correlation coefficients demonstrate a close relationship between TFA and FFA with total cholesterol, LDL-cholesterol, HDL-cholesterol, phospholipids, apolipoprotein B-100 triglycerides, apolipoprotein A-1. All these correlations were very significant. This suggests that changes in TFA and FFA concentrations with increasing rosuvastatin dose depend on corresponding degree of changes in lipid concentrations involved in cholesterol homeostasis. Due to this high correlation, we can use the linear regression functions presented in Table 3 to predict the TFA and FFA values from the values of other lipids.

This study may have some limitations, given the relatively small number of male subjects, a small age range, and short-term treatment. The first limitation was reduced by using a crossover experiment with 5 treatment periods and a homogeneous group of Caucasian men. A 4-week short-term treatment with rosuvastatin was selected because it has been experimentally found that in 2 weeks, the change in LDL-cholesterol levels can reach a steady state. Although increasing the duration of the treatment period would be unlikely to change the results of our study, an evaluation over a longer duration of treatment is needed. Future studies should include a larger sample size and may be extended to other age groups, different populations or ethnic groups, and women, in order to further strengthen our findings. This study should be analyzed with these limitations and cannot be considered to have general applicability.

5. Conclusions

Our findings showed that concentrations of TFA and FFA are significantly influenced by rosuvastatin doses. The regulatory mechanisms of cholesterol homeostasis are involved in changing the concentrations of TFA and FFA. The concentrations of TFA and FFA are strongly correlated with those of other lipids and lipoproteins involved in cholesterol homeostasis. These results suggest that rosuvastatin is directly involved in blocking cholesterol synthesis and is indirectly involved in changing the concentration of TFA and FFA, as well as other lipids and lipoproteins in cholesterol homeostasis. Our findings show that the dose-dependent effects of rosuvastatin treatment on the concentration of TFA and FFA can be a source of beneficial consequences if we take into account their involvement in

Table 3
Correlation parameters of TFA and FFA with lipids and lipoproteins involved in cholesterol homeostasis.

| Independent variable (X) | TFA                   | FFA                   |
|--------------------------|-----------------------|-----------------------|
|                          | Linear regression     | Correlation coefficient (r) | P value | Linear regression     | Correlation coefficient (r) | P value |
| Total cholesterol        | Y = 1.71X + 2.57      | 0.9997                | <.0001 | Y = 62.4X + 295.86   | 0.9976                | <.0001 |
| LDL-cholesterol          | Y = 1.69X + 5.52      | 0.9983                | <.0001 | Y = 61.8X + 399.83   | 0.9997                | <.0001 |
| HDL-cholesterol          | Y = –58.8X + 59.25    | -0.9991               | <.0001 | Y = –1418.9X + 2361.4| -0.9952               | <.0001 |
| Triglycerides            | Y = 20.5X – 6.25      | 0.9994                | <.0001 | Y = 754.6X – 31.13   | 0.9995                | <.0001 |
| Phospholipids            | Y = 5.9X – 1.32       | 0.9979                | <.0001 | Y = 216.5X + 149.23  | 0.9989                | <.0001 |
| Apolipoprotein B-100     | Y = 0.027X + 4.47     | 0.9982                | <.01   | Y = 0.996X + 361.44  | 0.9999                | <.0001 |
| Apolipoprotein A-1       | Y = -1.87X + 82.7     | -0.9914               | <.0001 | Y = -68.16X + 3208   | -0.9841               | <.0001 |
atherosclerosis through their high sensitivity to free radical oxidation. Future studies on individual fatty acids are warranted if we consider that individual fatty acids have different effects in atherosclerosis.

**Author contributions**

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