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Gurzeler, Erika

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Therapeutic effects of rosuvastatin in hypercholesterolemic prediabetic mice in the absence of low density lipoprotein receptor

Erika Gurzeler¹, Einari Aavik¹, Anssi Laine¹, Teemu Valkama¹, Henri Niskanen¹, Jenni Huusko¹, Minna U. Kaikkonen¹, Seppo Ylä-Herttuala¹,²,³

¹ A.I.Virtanen Institute for Molecular Sciences, Faculty of Health Sciences, University of Eastern Finland, 70210 Kuopio, Finland
² Gene Therapy Unit, Kuopio University Hospital, Kuopio, 70211 Kuopio, Finland
³ Heart Center, Kuopio University Hospital, 70211 Kuopio, Finland

Corresponding author:
Seppo Ylä-Herttuala, MD, PhD, FESC
A.I.Virtanen Institute
University of Eastern Finland
P.O.Box 1627
FI-70211 Kuopio, Finland
Tel. +358-40-3552075
E-mail: seppo.ylaherttuala@uef.fi
Abstract

Statins are effective drugs used to prevent and treat cardiovascular diseases but their effects in the absence of low density lipoprotein receptor (LDLR) and on the risk of diabetes are not yet well characterized. The aim of this study was to clarify systemic and pleiotropic effects of rosuvastatin on cardiovascular and diabetic phenotypes.

IGF-II/LDLR\textsuperscript{-/-} ApoB\textsuperscript{100/100} hypercholesterolemic prediabetic mice were used to test the effects of rosuvastatin on plasma glucose, insulin, lipids, atherosclerosis and liver steatosis. To get a more comprehensive view about changes in gene expression RNA-sequencing was done from the liver.

Rosuvastatin significantly reduced plasma cholesterol in hypercholesterolemic mice in the absence of LDLR but had no effects on atherosclerosis at aortic sinus level or in coronary arteries. Rosuvastatin also significantly reduced liver steatosis without any harmful effects on glucose or insulin metabolism. RNA-sequencing showed relatively specific effects of rosuvastatin on genes involved in cholesterol metabolism together with a significant anti-inflammatory gene expression profile in the liver. In addition, significant changes were found in the expression of Perilipin 4 and 5 which are involved in lipid droplet formation in the liver. For the first time it could be shown that Tribbles proteins are affected by rosuvastatin treatment in the hyperlipidemic mice.

Rosuvastatin had several positive effects on hypercholesterolemic mice showing early signs of diabetes, many of which are unrelated to cholesterol and lipoprotein metabolism. These results increase our understanding about the systemic and pleiotropic effects of rosuvastatin in the absence of LDLR expression.

Key words: Statins, Diabetes, Low density lipoprotein receptor, Atherosclerosis, Cholesterol metabolism, Liver
1. Introduction

Statins are inhibitors of the enzyme 3-hydroxy-3-methyl-glutaryl (HMG)-CoA reductase, which converts HMG-CoA into mevalonate, the rate-limiting step in the cholesterol biosynthesis [1-3]. Several clinical trials have shown that statins can significantly reduce plasma low density lipoprotein (LDL) cholesterol and the risk of cardiovascular events and mortality in patients with or without coronary artery disease [2,4,5]. Statins reduce intracellular cholesterol, leading to increased expression of LDL receptor (LDLR) [6] and enhanced clearance of LDL and its precursors [1,6]. However, inhibiting mevalonate synthesis is not only blocking the formation of cholesterol, leading to pleiotropic effects [6]. Statins have been associated with inhibition of cell proliferation, enhanced apoptosis, anti-inflammatory properties, improved endothelial function and modulation of angiogenesis [7,8].

Exact molecular mechanisms of those LDL-cholesterol independent effects are not yet fully understood. Most of the earlier studies investigating changes at the transcriptional level have been based on microarray analyses [9]. To our knowledge this is the first study where RNA-sequencing is used to achieve unbiased and more sensitive detection of transcripts regulated by rosuvastatin (Ros) treatment in mice.

In the liver lipids are stored in lipid droplets (LD) [10], where the composition of lipids and proteins is determining the fate of a LD [10]. It is assumed that statins can reduce lipid accumulation in the liver, but the underlying mechanisms are not yet fully understood [11]. Perilipins, a family consisting of five members (Perilipins 1-5 (Plin1-5)), are the largest family of proteins in LD and each perilipin has a specific role in biogenesis, stabilization and degradation of LDs [10]. However, not much is known about the role of perilipins in hepatocytes [10].

Inflammation plays a key role in atherosclerosis and it increases the risk of cardiovascular diseases [12]. C-reactive protein (CRP), a classical acute phase plasma protein induced by inflammation and tissue damage, is an emerging biomarker for the risk of cardiovascular diseases and cardiovascular death [13,14]. Several large-scale randomized, controlled trials have shown that statins can reduce CRP in addition to their cholesterol lowering effects [13].

In this study we used mice overexpressing insulin-like growth factor 2 (IGF-II) with LDLR deficiency (LDLR⁻⁻) and expressing only apolipoprotein B 100 (ApoB₁₀₀/₁₀₀). These mice show early signs of diabetes [15] and have a human like lipoprotein profile [16]. Previous studies have linked new-onset diabetes to statin use.
Especially in patients with early signs of diabetes, the use of statins can increase the risk of diabetes [17]. Therefore, the mice used in this study are an ideal animal model to investigate various effects of rosuvastatin on hyperlipidemia and prediabetes, conditions commonly seen in patients.
2. Methods and Material

2.1. Animals

In this study male IGF-II/LDLR\(^{-/-}\)ApoB\(^{100/100}\) mice [15] were used. Mice were fed a regular chow diet (Harlan Teklad) or a western diet (Harlan Teklad) for two months prior to treatment. At 4 months of age, the animals were subdivided into two groups per diet and treatment groups were given rosvastatin (Crestor\(^\circ\), AstraZeneca) mixed in their food for twelve weeks according to previously tested protocol [18]. Due to preferential intake of the regular chow diet (RCD) the treatment RCD contained 0.07mg/g\(_{\text{Diet}}\) of rosvastatin (RCD-Ros) compared to the western diet (WD), where 0.1mg/g\(_{\text{Diet}}\) of rosvastatin (WD-Ros) was used. After twelve weeks animals were sacrificed and samples were taken. All animal procedures were approved by the Animal Experiment Board in Finland and carried out according to the guidelines of the Experimental Animal Committee of the University of Eastern Finland.

2.2. Metabolic analyses

Animals were fasted for 3 h prior to blood glucose measurements. Blood was taken from saphenous vein and measured with Glucometer (Ascensia Elite XL, Bayer). Plasma insulin levels were measured with an Ultra-sensitive mouse insulin ELISA kit (#90080, Chrystal Chem, Inc.). HbA1c was measured using a Mouse Hemoglobin A1c Kit (#80310, Chrystal Chem). Intraperitoneal glucose tolerance test (IPGTT) was performed on 3 h fasted mice by giving 1g/kg glucose i.p. Glucose was measured from tail vein blood at baseline and 15, 30, 60 and 120 min after injection. Intraperitoneal insulin tolerance test (IPITT) was performed by injecting 0.25IU/kg (Actrapid\(^\circ\) Penfill\(^\circ\), Novo Nordisk A/s). Blood glucose was measured from tail vein at baseline, 15, 30, 45 and 90 min after injection. Plasma lipid samples were assessed by Movet Oy Finland (Kuopio, Finland).
2.3. Echocardiography

Echocardiography measurements were performed at baseline and 6 and 12 weeks after the treatment, by using Vevo2100 Ultrasound System (FUJIFILM Visual Sonics Inc.) using a high-frequency ultrasound probe (MS400) operating at 18-38 MHs. Animals were anesthetized with isoflurane (Induction: 4.5% isoflurane, 450ml air, maintenance: 2% isoflurane, 200 ml air, Baxter International Inc.). Ejection fraction (EF), left ventricular (LV) mass, LV volume, LV anterior wall (LVAW), LV posterior wall (LVPW) and LV internal diameter (LVID) were measured from parasternal short axis M-mode. Three measurements in different breathing cycles were acquired and EF was calculated by the Vevo program with the Teichholz formula.

2.4. Histology

Mice were sacrificed 12 weeks after the treatment and the tissues were perfused with phosphate buffered saline (Dulbecco’s Phosphate Buffered Saline, D8537, Sigma Aldrich), followed by 4h fixation in 4% paraformaldehyde in 7.5% sucrose. Samples were subsequently transferred to 15% sucrose and processed within the following days.

For histological analysis, 5µm thick paraffin sections were used. Sinus level analysis was done from serial sections (every 25µm) stained with hematoxylin-eosin and lesions were analyzed with ImageJ Fiji analysis software [19] from 40x magnified pictures. Coronary artery lesions were analyzed from hematoxylin-eosin stained sections at the branching point between ascending aorta and coronary artery. Lesion size was analyzed as described above. Steatosis was quantified from hematoxylin-eosin stained liver sections with ImageJ Fiji from five different sections at a magnification of 400x. To quantify liver fibrosis, the sections were stained with Picro-Sirius Red (ab150681, Abcam) and quantified as above mentioned. All images were taken with Nikon Eclipse Ni microscope with a Ds-Ri2 camera (Nikon Instruments Europe BV).

2.5. RNA-sequencing library preparation and data analysis

RNA-Seq libraries were prepared as described [20], except that RNA was isolated from snap frozen livers by using Trizol reagent (RNA/DNA/Protein Isolation Reagent, Molecular Research Center Inc) and ribosomal
RNA was removed by using RiboZero Gold kit (#MRZG12324, Illumina). Final libraries were amplified using 13-14 cycles and were sequenced for 50 cycles using Illumina HiSeq 2000 at EMBL Genomics Core Facility (Heidelberg, Germany). Processing of sequencing reads was carried out as described[21], except that data was mapped to mouse genome (version mm9) using tophat v2.0.7 [22]. Differentially expressed genes were identified by quantifying exonic reads with analyzeRepeats.pl tool included in HOMER 4.7 software followed by edgeR analysis [23] thresholds of FDR < 0.05, reads per kb per million reads (RPKM) > 0.5 and fold change 2. Venn diagram was made using Venny2.1 program and gene ontology analysis was performed using GO Enrichment Analysis [24-26].

RNA-seq data access:

The RNA-seq data can be found under GEO accession number GSE111125

(Reviewers can access the data from link: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE111125 Enter token ubatoeumdfqjjuv into the box)

2.6. Protein Measurements

Plasma CRP and Serum amyloid A (Saa) levels were measured with Enzyme linked immunoabsorbent Kits (Boster Biological Technologies; Tridelta Development Ltd., respectively) according to provided protocols. Total proteins were isolated from snap frozen liver tissue samples using T-PER lysis buffer (Thermo Scientific), containing PhosSTOP (Roche) and cOmplete Ultra Tablet Mini EASY Pack (Roche). Protein concentration was measured with BCA Kit (Pierce® BCA Protein Assay Kit, Thermo Scientific) and 20 or 50 µg were used for electrophoresis and transferred to 0.2 µm nitrocellulose membrane (Trans-Blot Turbo, BioRad Laboratories Inc.). Membranes were blocked in 5% skim milk in TBS-Tween and incubated with the following antibodies overnight: Plin4 (Everest Biotech, Ramona, USA), Plin5 (Thermo Fisher Scientific), Trib2 (Cell Signalling Thechnologys), Trib3 (Thermo Fisher Scientific), pAkt, mTOR, pmTOR, Erk1/2 and pErk1/2 (Cell Signalling Thechnologys) with corresponding secondary antibodies. Proteins were detected with ChemiDoc XRS (BioRad) by using ECL-Plus kit (Pierce ECL Plus, Thermo Scientific). Stain-free gels were
UV activated for 2.5 min before blotting and total protein amount was measured with ChemiDox XRS before ECL detection, specific bands were normalized to total protein using the ImageLab software (BioRad).

2.7. Statistics

GraphPad Prism5 was used for statistical analysis. As indicated Student’s t-test, one-way ANOVA or two-way ANOVA with Bonferroni post-hoc-test was applied. Values are shown as mean ± standard error of the mean (SEM). P < 0.05 was considered as statistical significant. The following symbols are used in figures and tables:

* p < 0.05, ** p <0.01, *** p < 0.001.
3. Results

3.1. Rosuvastatin does not affect weight, glucose, HbA1c levels or glucose metabolism

Ros treatment did not modify glucose levels significantly when compared to control animals on the same diet. Six weeks after the treatment, mice fed with RCD showed significantly lower values of HbA1c compared to RCD-Ros fed mice. This difference, however, disappeared after 12 weeks of treatment. Animals fed with WD did not show any significant changes in the HbA1c levels between control and Ros treated groups. By the end of the study, RCD animals had similar weights as animals fed with WD (Table 1).

<table>
<thead>
<tr>
<th>Chow Diet</th>
<th>Baseline</th>
<th>3 weeks</th>
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<th>9 weeks</th>
<th>12 weeks</th>
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<tr>
<td></td>
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<td>Rosuvastatin</td>
<td>Control</td>
<td>Rosuvastatin</td>
<td>Control</td>
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<tr>
<td>Weight [g]</td>
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<td>31,9 ± 0,8</td>
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<td>Glucose [mmol/l]</td>
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<td>7,4 ± 0,3</td>
<td>7,0 ± 0,3</td>
<td>6,9 ± 0,4</td>
<td>10,7 ± 0,7</td>
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<td>HbA1c [%]</td>
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<td>-</td>
<td>4,4 ± 0,3**</td>
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<td>Rosuvastatin</td>
<td>Control</td>
<td>Rosuvastatin</td>
<td>Control</td>
</tr>
<tr>
<td>Weight [g]</td>
<td>37,8 ± 1,5</td>
<td>37,7 ± 1,1</td>
<td>37,6 ± 1,5***</td>
<td>29,8 ± 0,8</td>
<td>37,9 ± 1,4**</td>
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<tr>
<td>Glucose [mmol/l]</td>
<td>10,8 ± 0,3</td>
<td>10,1 ± 0,4</td>
<td>8,5 ± 0,5</td>
<td>7,3 ± 0,6</td>
<td>9,5 ± 0,6</td>
</tr>
<tr>
<td>HbA1c [%]</td>
<td>4,9 ± 0,2*</td>
<td>4,4 ± 0,1</td>
<td>-</td>
<td>-</td>
<td>3,8 ± 0,2</td>
</tr>
</tbody>
</table>
Table 1: Metabolic parameters of IGF-II/LDLR\textsuperscript{-/-}ApoB\textsuperscript{100/100} mice at baseline and during rosuvastatin treatment compared to placebo treated mice. Values are mean ± SEM. Statistical analysis was performed with Two-way ANOVA. Weight and Glucose n=7-12/group, HbA1c n=6-7/group.

While in RCD fed animals insulin levels increased significantly over time compared to baseline levels, insulin levels decreased significantly in WD-Ros mice (Figure 1A).

To investigate the effect of Ros on glucose and insulin regulation in IGF-II/LDLR\textsuperscript{-/-}ApoB\textsuperscript{100/100} mice, IPGTT and IPITT tests were performed at baseline and 6 and 12 weeks after the treatment. The area under the curve (AUC) showed no changes irrespective of the diets or treatment (Figure 1 B, C).
Figure 1: Glucose metabolism of IGF-II/LDLR<sup>−/−</sup> ApoB<sup>100/100</sup> mice. Fasted insulin plasma levels (A) IPGTT and calculated AUC (B), IPITT and calculated AUC (C). Results are shown as mean ± SEM. One-way ANOVA with Bonferroni post-test was used for Insulin level and AUC comparison. Insulin levels n=4-7/group, IPGTT and IPITT n=5-11/group.

3.2. WD-Ros significantly reduced total cholesterol levels

To verify if cholesterol levels decline despite of the LDLR deficiency, total cholesterol was measured. We could show that mice fed with WD-Ros showed significantly lower total cholesterol levels 12 weeks after the...
treatment compared to baseline values and when comparing WD-Ros treated mice to WD control (Figure 2 B). Similar trends were seen when animals were fed with RCD and in triglyceride levels (Figure 2 A, C, D). We further investigated whether Ros was able to reduce atherosclerosis in LDLR\(^{-/-}\)ApoB\(^{100/100}\) mice. Lesion areas in aortic sinuses and coronary arteries showed no significant reduction between control and Ros groups (Figure 2 E, F). These results indicate that lesions in mice fed WD are already quite progressed and cannot be reduced within the treatment period.

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Figure 2: Lipid measurements of IGF-II/LDLR\(^{-/-}\)ApoB\(^{100/100}\) mice. Total plasma cholesterol of animals fed with RCD (A) and WD (B) before treatment and 12 weeks after (A). Triglyceride plasma levels of animals fed with RCD (C) and WD (D) before treatment and 12 weeks after (A). Quantification of aortic sinus level lesion area.
areas (E). Quantification of coronary artery lesions (F). Representative pictures are shown on the right panel. Values are shown as a mean ± SEM and statistical analysis was performed by using one-way ANOVA with Bonferroni post-test for lipid measurements and Student’s t-test for lesion measurements. Plasma levels n=7-12/group, Aortic sinus lesion area and stenosis n=7-10/group.

3.3. Rosuvastatin significantly reduced liver steatosis and lipid droplet size in WD animals

To analyze if Ros affects liver steatosis and fibrosis, hematoxylin-eosin and picro-sirius red stainings were performed. We demonstrate that steatosis was significantly reduced, to a similar level as in RCD fed mice, in animals fed with WD-Ros. No changes were seen in mice fed with RCD or RCD-Ros (Figure 3 A, D). Similar results were seen when LD sizes were investigated (Figure 3 B, D). However, fibrotic area increased significantly in animals treated with WD-Ros compared to control animals (Figure 3 C, D).

Figure 3: Liver histology of IGF-II/LDLR<sup>−/−</sup>ApoB<sup>100/100</sup> mice. Quantification of steatosis in the liver when animals were fed RCD and WD 12 weeks after treatment (A). Liver droplet size quantification of animals fed RCD and WD 12 weeks after treatment (B). Quantification of fibrotic area in the liver of animals fed RCD and WD 12 weeks after treatment (C). Representative pictures of hematoxylin-eosin for steatosis analysis and Picro
Sirius Red staining used for fibrosis analysis (D). Results are shown as mean ± SEM. Statistical analysis was performed by using one-way ANOVA with Bonferroni post-test, n= 8-11/group.

3.4. RNA-seq analysis showed the specificity of rosvastatin treatment and plasma analysis underlined the anti-inflammatory properties of statins

RNA-seq was used to investigate the genome-wide transcriptional effects of Ros in the liver. In RCD-Ros fed mice compared to control mice, expression of 19 genes was significantly changed, of which 16 genes were up- and three were down-regulated. In WD-Ros group, 216 genes were differentially regulated compared to WD control animals. Of these 128 genes were down- and 88 were up-regulated (Table1S). Only eight genes were differentially regulated independently of the diet (Fig.4 A). The low number of affected genes together with the volcano plots (Fig. 4 D) emphasizes the specificity of statin treatment. Gene ontology studies revealed that the commonly regulated genes mainly belong to cholesterol pathways. When mice were fed with RCD, mainly genes involved in lipid metabolism were affected by Ros treatment. When animals were fed with WD, genes involved in lipid metabolism and inflammation were differentially regulated upon Ros treatment (Fig. 4 B and C).

The increased inflammatory state during WD could be verified by plasma analysis. As expected, plasma CRP values were significantly increased in animals fed with WD compared to RCD. Furthermore, a significant reduction in plasma CRP was observed in animals treated with WD-Ros compared to WD control animals. A similar effect was seen in plasma Saa values. The increased levels of Saa during WD could be significantly diminished by Ros treatment (Figure 4 E). These results clearly emphasize the anti-inflammatory effects of statins.

With RNA-seq we were able to identify two groups of genes, Plin and Tribbles (Trib) which are affected by Ros treatment. It is known that Plin5 plays an important role in controlling lipolysis, the role of Plin4, in contrast, is not very well understood [27]. Here we show that in Ros treated mice, Plin4 was downregulated independently of the diet while Plin5 only showed a small decrease in WD-Ros. Investigation of the protein level showed that Plin4 was increased in WD fed mice when compared to RCD. However, no difference between control and Ros treated animals was seen (Figure 4 F). Plin5 exhibited very low protein expression
level in liver, which is in line with the sequencing data. When animals were treated with RCD-Ros a significant increase in Plin5 was visible, but no difference, however, was seen in WD groups (Figure 4 F).

Figure 4: RNA-seq analysis of livers. Differentially regulated genes, when comparing rosuvastatin treated animals with controls when animals were fed with WD and RCD (A). Gene ontology analysis showed that differentially regulated genes were mainly involved in stilbenoid, cholesterol and acute phase response (B). Heat map showing differentially regulated genes between rosuvastatin and control treatment with RCD or WD (C). Volcano Plots of RNA-seq results from RCD and WD when comparing control diet to rosuvastatin
treatment (D). Plasma values of CRP and Saa of animals 12 weeks after treatment (E). Western blot analysis of Plin4 and Plin5 (F). Heat map was created with Excel2016. Statistical analysis: values are shown as a mean ± SEM and statistical analysis was performed by using one-way ANOVA with Kruksal-Wallis post-test for Western Blot data and Student’s t-test for plasma measurements. RNA-seq n= 4-6/group, CRP and Saa analysis n=8-11, Western Blot n=8/group.

Trib proteins, the second identified group of proteins by RNA-seq, are well-conserved proteins regulating different cellular processes [28]. Both Trib2 and Trib3 were upregulated independently of the diet at RNA level. Further investigation of the protein level showed that Trib2 expression did not change significantly with Ros treatment. Unlike Trib2, expression level of Trib3 protein was increased in RCD groups compared to WD animals. WD-Ros treatment decreased the expression of Trib3 significantly compared to WD control diet. Trib proteins are signaling mainly through mitogen-activated protein kinase (Mapk) and Pi3k (phosphoinositide 3-kinase) pathways [29]. Therefore, downstream signaling of Trib3 was studied in more detail. No changes were seen in pAkt between placebo and Ros treated animals (Figure 5 A). Thus, Trib3 downregulation did not affect phosphorylation of Akt. However, pmTOR was significantly increased in WD-Ros mice, while mTOR levels did not differ (Figure 5 A, C and D). Thus, statin treatment might activate phosphorylation of mTOR in mice fed with WD. Erk1/2 was increased with Ros treatment both during RCD and WD but no changes in phosphorylation of Erk1/2 were detected (Figure 5 D and E).
Figure 5: Downstream signaling of Akt: Quantification of Western Blot analysis: of pAkt (A), mTOR (B), pmTOR (C), Erk1/2 (D), pErk1/2 (E) in the liver. Expression levels of pAkt, mTOR, pmTOR, Erk1/2 and pErk1/2 determined by Western Blot (F) Values are shown as a mean ± SEM and statistical analysis was performed by using Student’s t-test, n= 7-8/group.

3.5. Rosuvastatin treatment did not affect cardiac parameters

To evaluate if Ros treatment influenced cardiac performance, echocardiography was performed at baseline and 6 and 12 weeks after the treatment. Ros treatment, however had no significant effect on the cardiac performance, neither when animals were fed with RCD nor with WD (Table 2).
<table>
<thead>
<tr>
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<th>12 weeks</th>
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<td></td>
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<td>Control</td>
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<tr>
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<td>57,8 ± 1,6</td>
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<td>117,8 ± 7,3</td>
<td>120 ± 4,9</td>
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<tr>
<td>LV Vol; d [µl]</td>
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<tr>
<td>LVAW; d [mm]</td>
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<td>LVID; d [mm]</td>
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<td>0,87 ± 0,0</td>
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Table 2: Echocardiographic measurements of IGF-II/LDLR-/-ApoB100/100 mice at baseline and at 6 and 12 weeks after rosuvastatin treatment. Values are mean ± SEM. Statistical analysis was done with two-way ANOVA with repeated measures with Bonferroni post-test, n=8-11/group.
4. Discussion

This study was performed to investigate the specificity and molecular mechanisms of statin treatment in hyperlipidemic prediabetic mice. Statin treatment can increase the risk of type 2 diabetes, especially in people with impaired glucose metabolism [30,31]. Hence, we closely monitored changes in glucose metabolism. In this mouse model, Ros treatment did not affect glucose metabolism negatively. On the contrary, there was a clear trend towards a better glucose tolerance in mice treated with WD-Ros compared to mice fed with WD. LDLR–/ApoB100/100 is a commonly used mouse model for human familial hypercholesterolemia and atherosclerosis [32]. Although the main mechanism of statins to lower cholesterol is via LDLR upregulation, it has been shown that statins can reduce total cholesterol even if LDLR is absent or defective by decreasing ApoB production [33,34]. Also, it has been shown that in patients with nonalcoholic steatohepatitis the absolute rate of ApoB-100 synthesis is reduced [35]. These findings support the view that in animals lacking LDLR, ApoB reduction is the main mechanism for the decrease in total cholesterol.

We showed that total plasma cholesterol levels were reduced with statin treatment, especially when animals were fed WD. However, RNA-seq analysis did not reveal any specific mechanisms behind this reduction. No changes were seen in RNA expression levels of lipoprotein receptors, such as Ldlr related protein 1 (Lrp), Very low-density lipoprotein receptor (Vldlr) or Scavenger receptor class B 1 (Scarb1). Also, ApoB or microsomal triglyceride transfer protein (Mtp) did not change significantly with Ros treatment. Although animals fed with WD-Ros lost weight in the beginning of the study, at 12 weeks after the treatment, the weight was not anymore significantly different and therefore not responsible for the changes in cholesterol levels.

Recently Pei Yu et al.[36] showed that in ApoE−/− mice lacking Scarb1, Ros treatment reduced atherosclerosis in aortic sinuses and coronary arteries without lowering total cholesterol levels. In our study, statin treatment was not able to reduce aortic sinus lesions or coronary artery stenosis, showing that if lesions are already advanced, their reduction is unlikely by statin treatment in animals lacking LDLR. In contrast to stenosis progression, Ros was able to significantly reduce steatosis in livers of WD fed animals compared to control treated mice. Additionally, liver LD decreased significantly in size. Surprisingly, animals fed with WD-Ros showed increased levels of fibrosis in their livers which is in line with RNA-seq results where increased levels of fibrotic genes such as collagen type 1 alpha 1 chain (Col1a1) were visible. However, Okada et al.[37] showed that Ros inhibited liver fibrosis in a rat model of high-fat and high-cholesterol diet. In more detail,
they could show that transforming growth factor-β (Tgf-β), connective tissue growth factor (Ctgf) and Col1a1 mRNA levels were downregulated in the liver after Ros treatment. In contrary, our results show that Tgf-β and Ctgf expression levels were not changed but Col1a1 was significantly upregulated. However, differences in species and animal models may explain these different findings.

To investigate transcriptional changes of Ros treatment, RNA-seq from the livers was performed. Only 19 genes in the RCD group and 216 genes in the WD group were altered with WD-Ros compared to control animals, which supports the specificity of Ros at the molecular level. As shown by Wang et al. [38] high fat diet has a large impact on hepatic gene regulation. They identified 160 genes which were differentially expressed when high fat diet was compared to a regular chow diet. Twenty-five of those genes were found to be differentially regulated also in our study. These include genes affecting inflammatory and oxidative stress responses, such as C-X-C motif chemokine ligand 1 (Cxcl1) and Saa3 which were upregulated during WD in Wang et al. study. In our study those genes were clearly decreased upon Ros treatment, which is in line with the anti-inflammatory effects of statins. On the other hand, genes involved in lipid metabolism, such as Cyp51, lanosterol synthase (Lss) and NAD(P) dependent steroid dehydrogenase-like (Nsdhl) were all downregulated in Wang et al. report. When our hyperlipidemic prediabetic mice were fed WD-Ros, however, those genes were significantly upregulated, showing that statins influenced lipid metabolism also at the transcriptional level.

JUPITER Trial demonstrated that Ros treatment has a beneficial effect on cardiovascular events by lowering CRP values even if LDL cholesterol levels are below current treatment goals [39]. Later, CANTOS Trial showed that reducing inflammation decreased the risk of cardiovascular events independently of lipid-levels [40]. In this study, we could show that CRP levels decreased significantly in mice fed with WD-Ros compared to WD diet control mice. In addition to CRP also Saa has been shown to be a good predictor for cardiovascular events [41]. Similar to CRP, hepatic Saa increases as a response to inflammation and statin treatment can lower Saa plasma levels [41,42]. In our study, both Saa1 and 2 RNA expression in the liver was significantly downregulated with WD-Ros. Further, we could confirm that Saa1 plasma levels were significantly reduced in animals fed with WD-Ros compared to animals fed with the control diet. RNA-seq results showed, that especially in animals fed with WD, Ros had a beneficial effect on inflammatory genes. The expression of genes involved in cholesterol biosynthesis, such as Hmger, mevalonate kinase (Mvk), squalene epoxidase (Sqle),
Cyp51 and stearoyl-Coenzyme A desaturase 2 (Scd2) were affected during both diets. Results show that the anti-inflammatory property of Ros is more pronounced in animals fed with WD. Previously, we have shown that statins have significant anti-inflammatory effects on macrophages [43].

Trib genes are highly conserved genes, which regulate inflammatory signaling pathways through Mapk and Pi3k pathways [29]. Trib family members are differentially expressed in various tissues but exact mechanisms and contribution to inflammatory diseases are not fully understood [29]. It has been shown that Trib3 plays an important role in glucose and lipid metabolism in the liver by inhibiting Akt/protein kinase B [44]. On the contrary, Weismann et al.[45] showed that Trib3 affects lipid metabolism in the liver via PPAR-γ mechanism independent of PI3K. We could show for the first time that Trib genes are affected by Ros treatment in mice lacking the Ldlr gene. In our study the protein levels of Trib2 and Trib3 were downregulated in animals treated with WD-Ros, while Ros treatment did not affect the expression of these proteins in mice fed with RCD. We could not see any changes in downstream signaling pathways, such as phosphorylation of Akt, mTOR or Erk1/2 apart from a slight but significant increase in pmTOR in animals treated with WD-Ros. We also identified Plins to be differentially regulated by statin treatment. We saw a significant downregulation of Plin4 RNA levels by Ros in animals fed with WD.

In conclusion, we show that Ros acts at the transcriptional level specifically in the liver on lipid and inflammatory pathways without affecting glucose metabolism or cardiac performance. We could also show that in LDLR<sup>−/−</sup>ApoB<sup>100/100</sup> mice, total cholesterol and liver steatosis could be reduced with Ros treatment. Plin4 and Trib2/3 likely play important roles as molecular mediators in the liver after Ros treatment.
Conflict of interest

None

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Author contributions

E.G designed the study, collected data, performed histological analysis and wrote the manuscript. A.L and T. V carried out the animal work and helped with histological analysis. J.H helped with animal work. E.A, H.N and M.U.K carried out RNA-seq and helped with analysis. S.Y.-H designed the study and revised the manuscript.
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