Title: An eicosanoid protects from statin-induced myopathic changes in primary human cells

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ABSTRACT

Statins reduce plasma cholesterol levels and are effective in secondary cardiovascular disease prevention. However, statin related muscle side effects are a constant problem for patients and doctors because compliance in taking them is severely influenced by the side effects. The mechanism of statin-myopathy remains unknown.

We exposed primary human muscle cell lines (n=4) to a lipophilic (simvastatin) and a hydrophilic (rosuvastatin) statin and analyzed the transcriptome and the proteome (expressome) in an unbiased manner. Data and pathway analyses included GOrilla, Reactome and DAVID. Relevant results were confirmed by quantitative PCR and on protein level. Functional assays included proliferation and differentiation quantification of primary human muscle cells.

More than 1800 transcripts and 900 proteins were differentially expressed after exposure to statins. Simvastatin had a much worse effect on the expressome than rosuvastatin, but both statins had a severe impact on cholesterol biosynthesis, fatty acid metabolism, eicosanoid synthesis, proliferation, and differentiation of human muscle cells. Eicosanoids rescued the biological function. We also found that muscle cells were very similarly equipped for cholesterol biosynthesis than HepG2 cell.

Our data bring a new aspect to the role of skeletal muscle in cholesterol metabolism. For clinical practice, the addition of omega-3,6 fatty acids could be suitable to prevent or treat statin-myopathy.

KEYWORDS

statin, cholesterol, signal transduction, eicosanoids, human primary muscle cell, therapy

1. INTRODUCTION

Statins function by inhibiting the key reaction of cholesterol biosynthesis, the reduction of hydroxymethylglutaryl-coenzyme A to mevalonate by its reductase HMGCR. Millions of patients ingest statins for secondary prevention of cardiovascular disease. Statins have well documented side effects on skeletal muscle causing up to 70% of patients to discontinue the drug, a considerable problem for doctors and patients [1,2]. Most statin-intolerant patients complain of muscle cramps and myalgia, but asymptomatic elevation of creatine kinase and weakness may also occur. It is unknown whether the same or different mechanisms underlie the various myopathic phenotypes and how we can circumvent or treat statin-induced changes in skeletal muscle. Also, it is unclear whether lipophilic and hydrophilic statins exhibit the same effect on muscle. Removal of cholesterol might affect membrane structure and lipid rafts that are essential for cell signalling and compartmentalization of cell functions. In that case, all cholesterol-lowering agents including the new group of proprotein convertase subtilisin / kexin type 9 –inhibitors (PCSK9-inhibitors) would lead to the same spectrum of muscle complaints. Alternatively, statins might influence pathways downstream of mevalonate [3,4] causing a distinct myopathy.

Mevalonate is metabolized into squalene from which cholesterol eventually becomes synthetized [5]. Other derivatives of mevalonate, farnesyl-pyrophosphate and geranylgeranyl-pyrophosphate, are involved in isoprenoid synthesis, protein prenylation and ubiquinone synthesis. Interventions into isoprenoid synthesis has been associated with ATP depletion and DNA damage and therefore suggested for cancer therapy [6]. Although alterations in ubiquination of proteins had been an intriguing hypothesis for causing statin-induced side effects, there is no convincing evidence to support this [7,8].

In the present study, we asked whether there are key signalling pathways in muscle that are significantly affected by statins. We chose an integrated approach to study the effects of a lipophilic and a hydrophilic statin in a large number of individual primary human myoblast cell lines on molecular, cellular and functional levels. We show that statins have a significant effect on gene and protein expression profiles. A novel signal transduction pathway, modelled by us, suggests metabolic changes induced by statins, particularly, an influence on eicosanoid synthesis. Furthermore, statins directly influence the proliferative and regenerative features of skeletal muscle cells. Eicosanoid-

species substitution to statin-treated primary human muscle cells rescue these effects. Statin myopathy, therefore, appears as a distinct and potentially treatable myopathy.

2. MATERIALS AND METHODS

2.1. Human primary myoblasts and cell lines

Muscle biopsy specimens for myoblast isolation were obtained from M. vast. lat. after IRB approval (EA1/203/08 and EA2/051/10). Experiments were conducted from a total of 25 different primary human myoblast cultures (Supplemental Table S1). For all experiments, except for eicosanoid substitutions, we used primary muscle cells obtained from statin naïve individuals. Statin-myopathy patients had myalgias (>3 of 10 on a visual analoge scale) and/ or creatine kinase levels elevated to > 250 IU/l under therapy. Isolation was performed as described. [9] The percentage of fibroblasts in myoblast cultures was always below 5% as assessed by anti-desmin staining. Differentiation into myotubes was induced using Opti-MEM (Invitrogen, Germany).

2.2. Pharmacological reagents

The active form of simvastatin was purchased from ARC (Missouri, USA), rosuvastatin from LKT Laboratories (Minnesota, USA), fatty acid species from Cayman Chemical (Michigan, USA). Geranylgeranyl-pyrophosphate, mevalonolactone, and mevalonic acid were from Sigma Aldrich (Germany). (Supplemental Table S2)

2.3. Treatment of myoblast and myotubes

Human primary myoblasts were cultured in skeletal muscle growth medium (ProVitro, Berlin, Germany) in 96-well plates (3 x 10^3 cells/ well). Test substances were applied 24 h after plating (Supplemental Table S2). Every 24h, the cells were refed with fresh medium in the presence or absence of test compounds. All treatments were performed in parallel for each cell line at the same time of the day to exclude circadian rhythm effects. Proliferation was quantified by the colorimetric BrdU assay (Roche, Germany). Toxicity was assessed using the ToxiLight™ Non-destructive Cytotoxicity BioAssay Kit (Lonza, Cologne, Germany), and the Cytotoxicity Detection Kit (Roche, Germany). Amplex® Red Cholesterol Assay Kit (Invitrogen, Germany) was used to measure cholesterol. The cells were examined after 24 h, 48 h, 72 h, and 96 h. For differentiation into

myotubes, human primary myoblasts at a 70% confluence were serum starved with Opti-MEM (Invitrogen, Germany). Selected eicosanoid species (Supplemental Table S2) were added to myoblast cultures under proliferation and differentiation condition with and without statins.

2.4. Mevalonic acid in statin-treated human primary myotubes

Together with Lipidomix GmbH (Berlin, Germany), we developed a new and easy method to detect mevalonate intracellular in human primary muscle cells. Cells were lysed by three freeze and thaw cycles and mechanically using 21, 23 and 26 Gauge needles [Lysis buffer: 20 mM Tris-HCl pH 7,4; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; 1 x Complete Protease-Inhibitor (Roche, Germany)]. Protein concentration was determined using BCA kit (Biorad, Germany). After lysis, proteins were precipitated with 1/10 volume 4 M HClO4 for 10-20 min at 4°C. When pH was neutralised with 5 N KOH, samples were mixed and incubated with 1/5 volume 6 M HCl at 4°C for at least 15 h to promote conversion of mevalonate into mevalonolactone. After centrifugation at 13,000 x g for 2 min, further analysis using HPLC/MS/MS was performed by Dr. Michael Rothe at Lipidomix GmbH, Berlin, Germany. (Supplemental Table S3, Supplemental Figure S5)

2.5. RNA analyses

RNA was isolated using the NucleoSpin® RNA/Protein Kit (Marcherey-Nagel, Germany). RNA quantity and purity were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA). For qPCR, the mRNA was reverse transcribed using the QuantiTect reverse transcription kit (Qiagen, Germany). qPCR was performed according to MIQE guidelines [10] using SYBR Green I in the Mx3000P instrument (Stratagene, Germany) (Primer sequences in Supplemental Table S4).

2.6. RNA sequencing

RNA quality was determined using Agilent 2100 bioanalyzer (Agilent Technologies, USA). The RIN factor was >8 for all samples. cDNA libraries for paired-end sequencing were prepared using the TruSeq Stranded mRNA library preparation kit (Illumina, USA). RNA sequencing was performed at Illumina HiSeq platform (Illumina, USA).

Pre-processed raw RNA-Seq data were imported into CLC Genomics Workbench (v. 7.5.1) and processed using a standardized workflow. The workflow defines the following steps: (1) trimming

sequences (2) creating sequence quality check report (3) RNA-Seq analysis with reporting of read tracks, gene expression tracks, transcript expression tracks, fusion tracks, and un-mapped reads. All samples passed the RNA-seq quality check (Supplemental Figure S3). Trimmed reads were aligned to human reference genome (GRCh37) and mapped back to the human transcriptome (v. 19). Mapped read counts were statistically analyzed using the R packages EdgeR and DESeq for matched sample data. [11] Samples without any and with DMSO treatment were considered as reference group. Gene expression data from simvastatin and rosuvastatin treated samples with FDR<0.05 (Bonferroni) were considered as statistically significantly different from control groups. Data are stored at Gene Expression Omnibus - GSE107998.

2.7. Deep proteome analysis

Proteins were prepared according to Sapcariu et al. [12] Samples were measured by LC-MS/MS on a Q-Exactive orbitrap mass spectrometer (Thermo Scientific, Germany). For the data analysis, the MaxQuant software package version 1.4.1.2 was used, with a multiplicity of 3 for dimethylation analysis (modified epsilon amino groups of lysine plus modified N-terminal amino groups). [13] Carbamidomethylation was set as a fixed modification while oxidized methionine and acetylated N-termini were set as variable modifications. An FDR of 0.01 was applied for peptides and proteins search was performed using a human Uniprot database (August 2013). Normalized ratios of the protein groups output were used to determine proteins which are obviously regulated. (Supplemental Figure S3)

2.8. Cell quantification

Myotube fusion and maturation were quantified using an LSM 700 AxioObserver Z1 (Zeiss, Germany) and an N-Achroplan 10x/0.25 M27 objective. Image files were exported and converted in batch into black-white images with adjusted gray-scale using IrfanView (v.4.38). Separate image files for MYHCf and HOECHST 33258 were loaded into the CellProfiler software (v 2.1.1). [14,15] A pipeline for calculating multinucleated cells was used and slightly modified. [16] A myotube was defined having more than three nuclei. The fusion index was calculated by the number of nuclei in myotubes divided by the total number of nuclei counted in each image. The maturation index is described by the average number of nuclei per myotube.

2.9. Data visualization for RNA-Seq and deep proteome

For analyses with mapped count data apart from the EdgeR or DESeq package, mapped read counts were exported from CLC Genomics Workbench (v 7.5.1) and normalized for their library size using the trimmed mean of M-values normalization approach (TMM). [17] Differential gene expression data were plotted as dendogram clustered heatmaps using GENE-E (v 3.0.206). [18] Plot.ly was used to visualize quality check data. Principal component analysis was performed in discriminate gene expression data for each treatment group with ClustVis (release June 2016). [19] Venny 2.0 was used to generate the Venn diagram. [20] The Circos-Plot for omics data was generated using the Bioconductor package OmicCircos. [21]

2.10. Pathway analyses

Statistically significant, differential gene expression data from simvastatin and rosuvastatin treated myotubes and controls were further analysed with GOrilla (release March 2013), Reactome (v 53), and DAVID (v 6.7) to identify enriched GO terms and pathway clustering. [22–24] Gene lists were ranked regarding their expression value. In addition, the web-based Ingenuity pathway analysis application (IPA; Ingenuity Systems, Redwood City, CA) was used.

2.11. Statistics

All statistics for RNA-Seq and proteome data analyses were performed using RStudio (v 0.98.1006) for executing R packages (v 3.1.1). Statistical analyses for proliferation, toxicity, and qPCR data were performed with GraphPad Prism (v 7.0) using the One-way ANOVA corrected with Holm-Sidak / Dunn's method. Two-way ANOVA for multiple testing was used for all data obtained from statin, rescue experiments, and fatty acid species treated primary human muscle cells. Each comparison stands for its own. Data obtained from western blots with protein from human primary myotubes were analysed for statistical significance using two-tailed Mann-Whitney test. Values of p are shown by asterisks using the standard convention: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$

3. RESULTS

Simvastatin more than rosuvastatin extensively alters myotube expressome

The PCA analysis on transcriptome data shows grouping of statin-treated samples clearly separated from untreated and DMSO-treated samples (Figure 1A). A concern about studying primary human

samples derived from individuals of different age and gender usually has been the potentially high variation between samples. Here, however, we can show that primary human samples are very similar among various conditions (Figure 1A). We found that simvastatin had a dramatic effect on primary human muscle cells, much more so than rosuvastatin. 1807 genes (FDR<0.05) were differentially expressed after exposure to simvastatin (Figure 1B, Supplemental Table S6, Supplemental Data File 1). Simvastatin had a particularly strong impact on RNA metabolism (Table 1). The number of genes affected by rosuvastatin was only 68 (FDR<0.05) (Supplemental Table S7, Supplemental Data File 2) with a main impact on lipid and cholesterol metabolism.

Table 1. Biological process clustering. Genes and proteins differentially expressed in statin-treated human primary myotubes at mRNA and protein level were clustered into biological processes using GOrilla. The GO Term Top10 are different for each statin but similar at both expression levels. Lipid and cellular metabolic processes are most commonly modified by both statins at both expression levels. The p-values correspond to number of genes/ proteins clustered into a GO node.

Expression level	GO Term – Top 10	P-value
Simvastatin Transcriptome	• SRP-dependent cotranslational protein targeting to membrane	5.65 E-29
	• Cotranslational protein targeting to membrane	1.60 E-28
	• Protein targeting to ER	1.27 E-26
	• rRNA processing	5.10 E-26
	• Establishment of protein localization to endoplasmic reticulum	7.75 E-26
	• rRNA metabolic process	3.00 E-24
	• Protein localization to endoplasmic reticulum	4.42 E-24
	• Translational initiation	1.88 E-23
	• Nuclear-transcribed mRNA catabolic process, nonsense-mediated decay	8.48 E-23
	• Viral transcription	1.03 E-21

Simvastatin Proteome	• Negative regulation of cellular process	2.30E-04
	• Regulation of biological quality	3.91E-04
	• Signal transduction	2.69E-05
	• RNA metabolic process	7.27E-04
	• Small molecule metabolic process	5.35E-04
	• Regulation of localization	2.37E-04
	• Immune system process	4.85E-04
	• Macromolecule biosynthetic process	8.40E-04
	• Multicellular organismal process	2.72E-04
	• Positive regulation of response to stimulus	8.05E-04
Rosuvastatin Transcriptome	• Secondary alcohol biosynthetic process	3.29 E-31
	• Sterol biosynthetic process	5.28 E-30
	• Cholesterol biosynthetic process	1.47 E-29
	• Secondary alcohol metabolic process	9.54 E-27
	• Cholesterol metabolic process	6.92 E-26
	• Sterol metabolic process	1.47 E-25
	• Alcohol biosynthetic process	9.80 E-25
	• Steroid biosynthetic process	4.95 E-23
	• Organic hydroxy compound biosynthetic process	7.60 E-22
	• Regulation of cholesterol biosynthetic process	1.21 E-21
Rosuvastatin Proteome	• Small molecule metabolic process	1.73E-04
	• Oxidation-reduction process	7.32E-05
	• Small molecule catabolic process	6.13E-04
	• Cellular lipid metabolic process	9.49E-07
	• Monocarboxylic acid metabolic process	9.03E-06
	• Small molecule biosynthetic process	1.26E-05
	• Lipid metabolic process	3.11E-09
	• Regulation of lipid metabolic process	7.54E-05
	• Lipid biosynthetic process	2.90E-08
	• Organic hydroxy compound metabolic process	6.49E-08

Common GO-terms at transcriptome and proteome level influenced by both statins	**Lipid metabolic process**	8.07 E-11
	• Cholesterol metabolic process	5.65 E-10
	• Sterol metabolic process	1.65 E-09
	• Cholesterol biosynthetic process	1.37 E-08
	• Isoprenoid biosynthetic process	1.15 E-06
	• Triglyceride metabolic process	1.67 E-04
	• Fatty acid metabolic process	8.52 E-05
	• Arachidonic metabolism	3.33 E-04

When transcriptome and proteome of identical samples are compared, there may or may not be a good overlap. We found that on proteome level 3255 skeletal muscle proteins were significantly identified after exposure to statins (Supplemental Figure S4, Supplemental Data File 3). Proteins belonging to lipid metabolic, small molecule metabolic, and cellular, developmental as well as RNA metabolic processes were particularly affected (Supplemental Data File 4). A Venn diagram (Figure 1C) demonstrates that 19% of genes and proteins in simvastatin and 6% in rosuvastatin treated myotubes were significantly differentially expressed at proteome and transcriptome levels when compared to controls. The degree of similarity between RNA-Seq and proteome-data is also visualized in a circus plot (Supplemental Video) [25].

The significant effect of statins on the transcriptome and proteome of primary human muscle cells was unexpected and widespread. Because both, simvastatin and rosuvastatin, induce “statin-myopathy” we focussed our further analysis on pathways in muscle cells that were influenced by both statins. These were fatty acid metabolism, fatty acid import, and lipid metabolic processes (Table 1, Supplemental Data File 4).

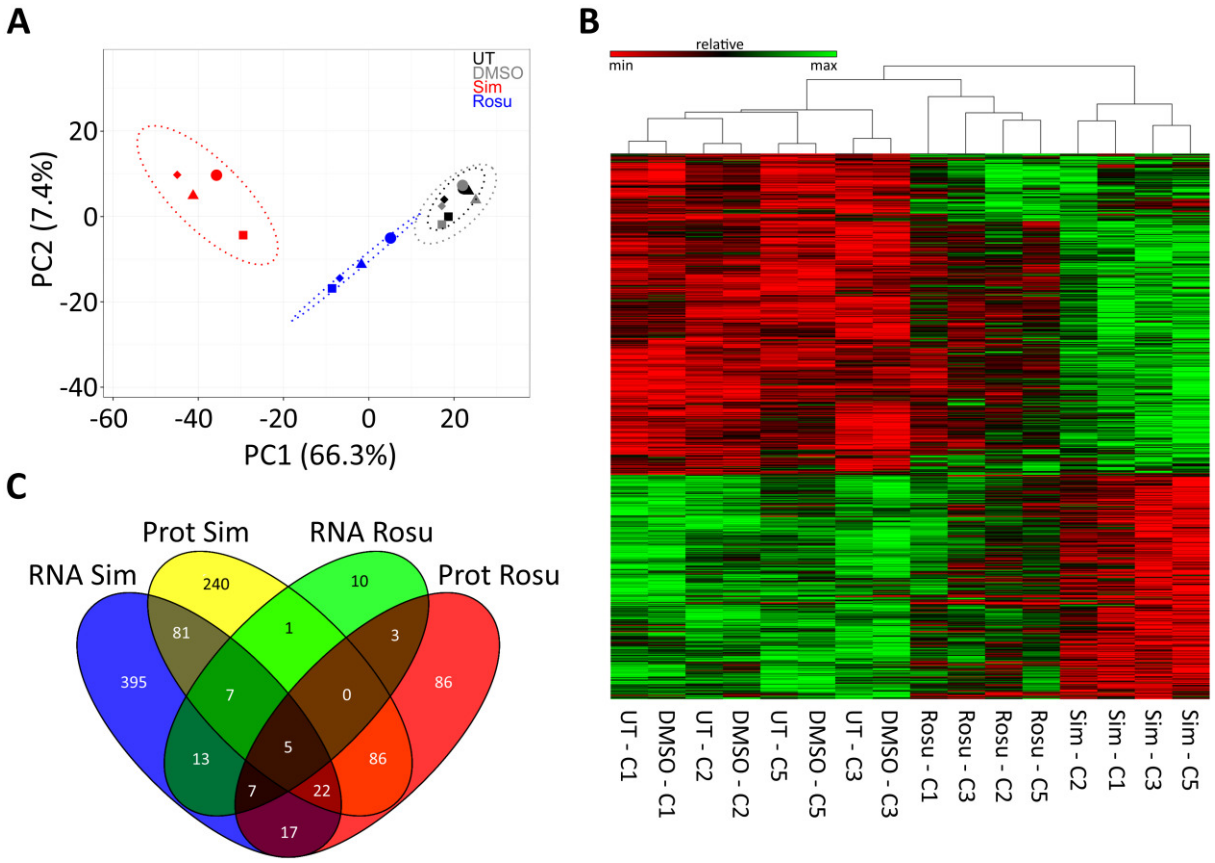


Figure 1. Gene expression profiling of primary human myotubes exposed to statins. Four primary human myotube cell lines from four different statin naïve donors were left untreated (UT), treated with DMSO, rosuvastatin (Rosu) or simvastatin (Sim). (A) Principal components analysis (PCA) of RNA-Seq samples. The PCA plot shows a clear grouping. (B) Heatmap of RNA-Seq samples represents log2 transformed normalized count data of differently expressed genes. Samples of each statin group cluster hierarchically together. Untreated and DMSO treated samples are equally clustered confirming that DMSO has no significant effect on RNA expression data. (C) Venn diagram displaying overlaps between proteome and transcriptome expression data from simvastatin and rosuvastatin groups. For cell lines used see Supplemental Table S1. See also Supplemental Figure S4 and S5 and Supplemental Data File S1 and S2.

Muscle cells possess a similar repertoire for cholesterol biosynthesis as liver

We next asked how the muscle was equipped to synthesize and metabolize cholesterol. We found key enzymes for cholesterol biosynthesis (HMGCS1, HMGCR, CYP51A1, DHCR7) as well as cholesterol transport (LDLR, ABCA1, ABCG1) expressed in human muscle cells and significantly altered by different exposure to statins. We further analysed the transcript variants of 3-hydroxy-3-methylglutaryl-

CoA reductase (HMGCR) of human muscle cells and compared them to human liver (HepG2 cells) (Figure 2A). HMGCR transcript variants (ENST00000287936 and ENST00000343975 that excludes exon 13) have an impact on cholesterol metabolism regulation and statin efficacy [26]. The transcripts in human primary myotubes and HepG2 cells were identical (Figure 2A). In our OMICs data set (Supplemental Table 8) the ENST00000343975 was also detectable at low levels. We then tested to what extent statins added to primary human muscle cells would influence cholesterol and mevalonate levels, or expression of cholesterol dependent genes (Figure 2B-D). Cholesterol and mevalonate levels in primary human myotubes were significantly lowered by statins in a concentration dependent manner. (Figure 2B and C). On mRNA level as assessed by qPCR, we found that statins increased HMGCS and HMGCR (Figure 2D), the same effects that have been described for liver [27]. Eight protein coding transcript variants are known for LDLR. In our RNASeq data set, we found six of them differentially expressed (between statin and controls) with similar fold changes (see Supplemental Table S8). ENST00000558518, ENST00000558013, ENST00000535915, and ENST00000545707 are the most supported transcript variants throughout databases. In our data set, ENST00000545707 (682 amino acids) had the highest number of uniquely mapped and total transcript reads (FPKM 7.8-19.4). This variant misses LDL receptor class A3-5 and the EGF-like 3 domain that are linked to familiar hypercholesterolemia, i.e. rs121908028, rs121908029, rs28942083, rs748944640 (UniProt identifier P01130-2). LDLR binding partner PCSK9 has only one protein coding transcript variant which is 2fold upregulated in rosuvastatin-treated human primary myotubes. Our data show, that human primary myotubes possess the same repertoire for cholesterol biosynthesis and expression regulation feedback mechanisms as liver cells.

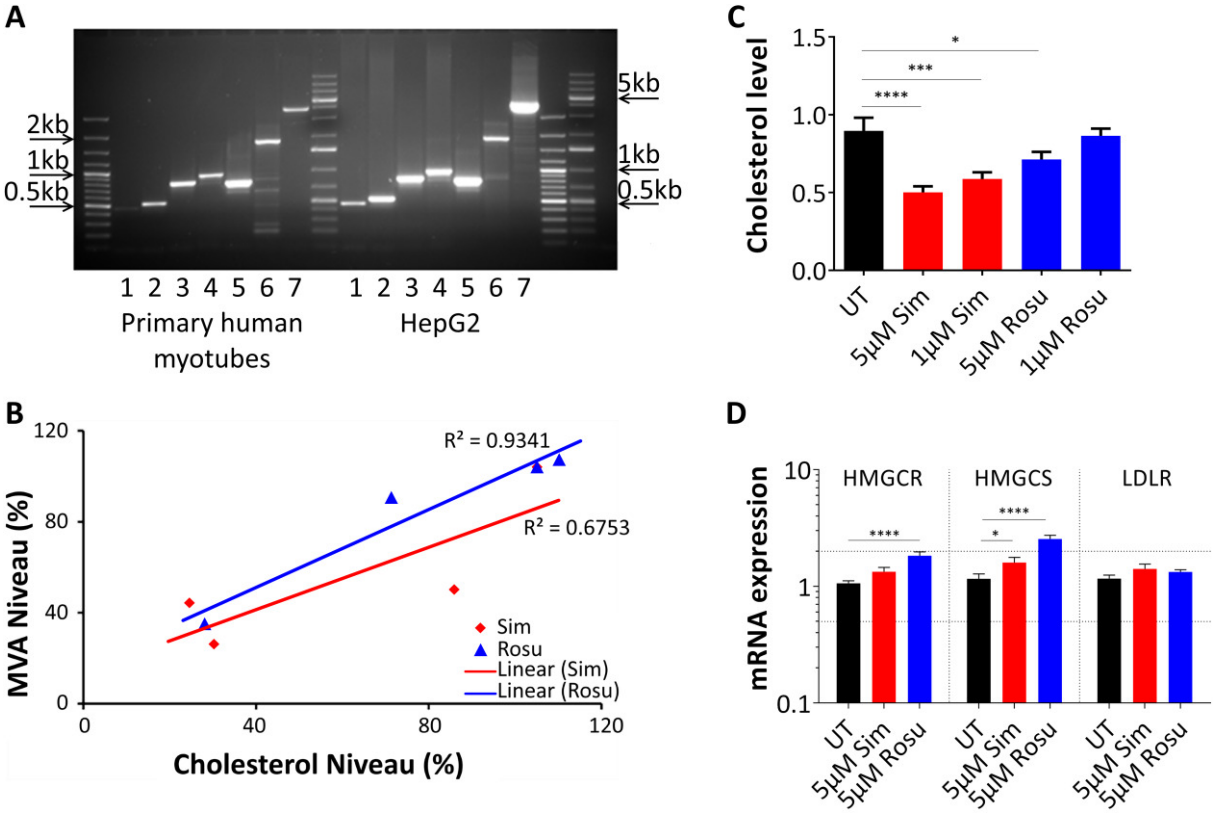
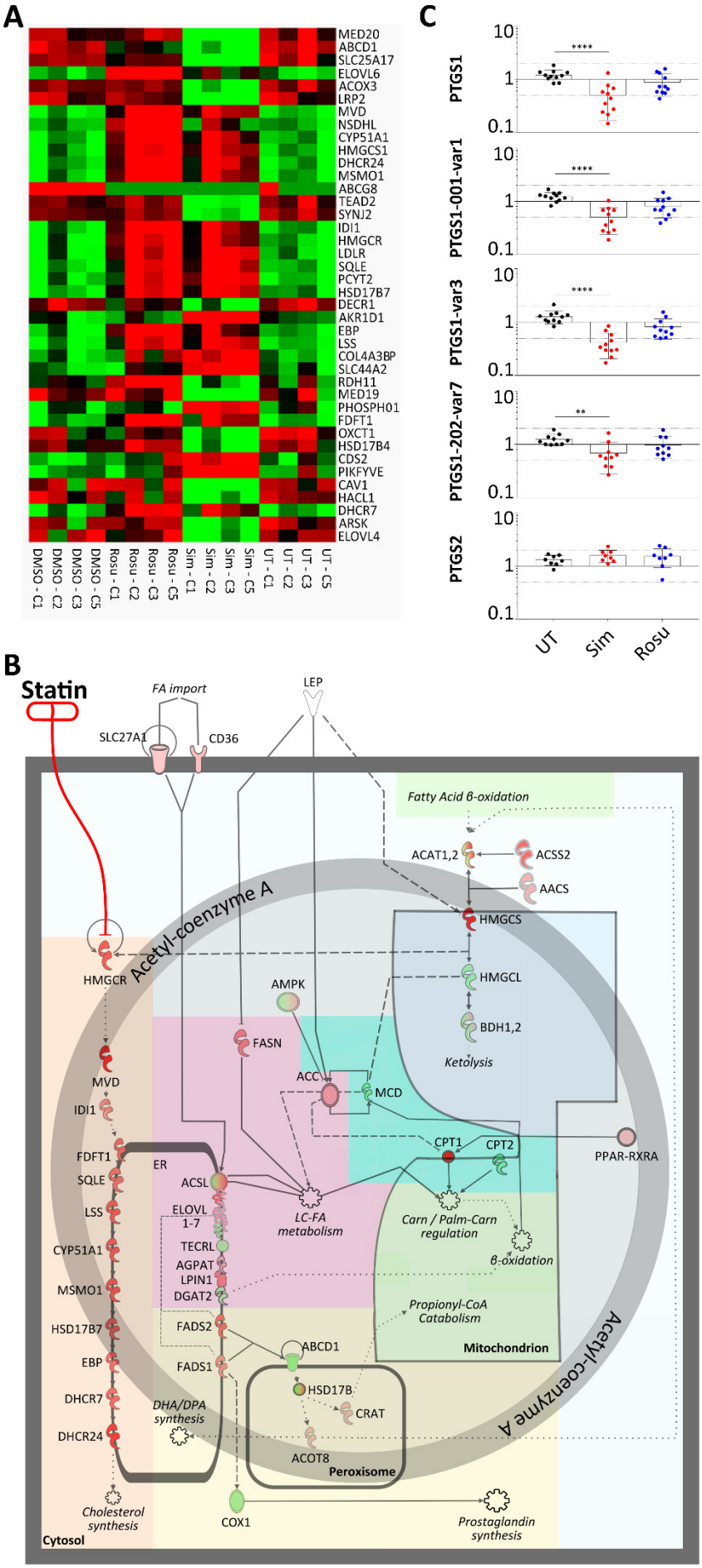


Figure 2. Statin effect in primary human myotubes on mevalonate and cholesterol dependent gene expression level. (A) HMGCR transcript variants amplified for exon 1-20 in primary human myotubes and liver HepG2 cells. Primary human muscle cells and HepG2 cells share the identical HMGCR transcripts. Unspecific bands in lane 6 were sequenced. [1, Exon1-5; 2, Exon 3-8; 3, Exon 6-11; 4, Exon 9-14; 5, Exon 12-17; 6, Exon 15-20; 7, Exon 1-20] (B) Mevalonate levels in human primary myoblasts after 72h statin treatment at different concentrations. Mevalonate levels correlate to cholesterol levels. Data were normalized to total protein level and relativized to DMSO treated samples. (n=3) (C) Relative cholesterol levels were determined under delipidated medium conditions in primary human myoblasts after 72 h statin treatment at different concentrations. (D) Relative mRNA expression of HMGCR, HMGCS, and LDLR were examined in human primary myoblasts undergoing differentiation under statin treatment (5 µM) for 72 h-96 h. The results were normalized to two reference genes and calculated relatively to DMSO-treated samples. For cell lines used see Supplemental Table S1. [UT, untreated (black); Sim, simvastatin (red); Rosu, rosuvastatin (blue); HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; HMGCS, 3-hydroxy-3-methylglutaryl-CoA synthase 1; LDLR, low density lipoprotein receptor]

Statins interfere with fatty acid metabolism

To further analyze pathways significantly affected by both statins, we used Gene Ontology enRIchment anaLysis (GOrilla) and the Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis (Supplemental Data File 4). Within the gene ontology terms *fatty acid metabolism, fatty acid import, and lipid metabolic processes*, we identified significant effects on genes involved in lipid, fatty acid, and arachidonic/ eicosanoid metabolism as well as on retinoic acid response of the retinoid X receptor's (RXR) pathway and carnitine shuttling (Figure 3A). Based on these findings, we qualitatively analysed genes and proteins with known or predicted interactions to HMGCR and included a quantitative level for pathway modelling. We modelled a regulatory network showing the statin-induced alterations on HMGCR-associated pathways in human muscle (Figure 3B, Supplemental Table S5). Among the general impact of statins on acetyl-CoA dependent metabolic processes we found particularly interesting upregulation of fatty acid desaturases. Fatty acid desaturases catalyze precursor molecules for prostaglandin and eicosanoid synthesis, molecules involved in mediating pain.

Indeed, we found that a number of genes involved in eicosanoid synthesis, such as the ATP-binding cassette sub-family D member 1, the elongation of very long chain fatty acids 4 and 6 as well as the aldo-keto reductase family 1 member D1, fatty acid desaturase, and phospholipase A2 group III were significantly different expressed (Figure 3A,B). Significant reduction of PTGS1 (Figure 3C), the key enzyme in ω-3 and ω-6 fatty acid metabolism converting eicosapentaenoic acid and arachidonic acid to eicosanoids including prostaglandins, could indicate a disturbance in eicosanoid biosynthesis.


A
MED20
ABCD1
SLC25A17
ELOVL6
ACOX3
LRP2
MVD
NSDHL
CYP51A1
HMGCS1
DHCR24
MSMO1
ABCG8
TEAD2
SYNJ2
IDI1
HMGCR
LDLR
SQLE
PCYT2
HSD17B7
DECR1
AKR1D1
EBP
LSS
COL4A3BP
SLC44A2
RDH11
MED19
PHOSPH01
FDFT1
OXCT1
HSD17B4
CDS2
PIKFYVE
CAV1
HACL1
DHCR7
ARSK
ELOVL4
DMSO - C1
DMSO - C2
DMSO - C3
DMSO - C5
Rosu - C1
Rosu - C2
Rosu - C3
Rosu - C5
Sim - C1
Sim - C2
Sim - C3
Sim - C5
UT - C1
UT - C2
UT - C3
UT - C5
C
PTGS1
PTGS1-001-var1
PTGS1-var3
PTGS1-202-var7
PTGS2
10
1
0.1
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UT
Sim
Rosu
B
Statin
FA import
LEP
SLC27A1
CD36
Fatty Acid ß-oxidation
ACAT1,2
ACSS2
AACS
HMGCS
HMGCL
BDH1,2
Ketolysis
Acetyl-coenzyme A
HMGCR
AMPK
FASN
ACC
MCD
MVD
IDI1
FDFT1
SQLE
LSS
CYP51A1
MSMO1
HSD17B7
EBP
DHCR7
DHCR24
ER
ACSL
ELOVL 1-7
TECRL
AGPAT
LPIN1
DGAT2
FADS2
FADS1
LC-FA metabolism
CPT1
CPT2
PPAR-RXRA
Carn / Palm-Carn regulation
ß-oxidation
Propionyl-CoA Catabolism
Mitochondrion
ABCD1
HSD17B
CRAT
ACOT8
Peroxisome
DHA/DPA synthesis
Cholesterol synthesis
Cytosol
COX1
Prostaglandin synthesis

Figure 3. Statin influence on lipid metabolic processes and on PTGS1 and PTGS2 mRNA expression in human myotubes. (A) RNA expression of human primary myotubes treated with statins compared to controls. Heatmap displays gene enrichment in lipid metabolic processes at RNA level. (Green – downregulated; Red – upregulated) (B) Summary of statin-induced alterations in muscle lipid metabolism. Statin-induced changes in cholesterol biosynthesis (orange) are a direct effect of HMGCR inhibition. This also affects HMG-CoA synthesis and ketone body metabolism (blue) and especially Ac-CoA. Ac-CoA directly affects fatty acyl and triglyceride biosynthesis (magenta) and impairs eicosanoid synthesis (yellow). Also, Ac-CoA influences carnitine-palmitoyl transferases (turquois) and mitochondrial beta oxidation (green). See also Supplemental Table S5. (C) Transcript variant expression study of PTGS1 and PTGS2 in human primary myotubes statin-treated for 96h. For PTGS1, we analyzed three different variants. PTGS1 variant 3 shows the lowest expression values (mean fold change = 0.41). Thus, it mainly reflects the low expression level detected for all variants See Supplemental Table S4 for PCR primer details. For cell lines used see Supplemental Table S1. [UT, untreated (black); Sim, 5 µM simvastatin (red); Rosu, 5 µM rosuvastatin (blue); Ac-CoA, acetyl coenzyme A; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; PTGS, prostaglandin-endoperoxide synthase (**** $p<0.0001$)]

Eicosanoid AL-8810 reverses the effect off simvastatin on primary human muscle cells

If an impairment in eicosanoid biosynthesis would be responsible for statin related side effects on muscle, reconstitution with eicosanoids might be beneficial. We first identified parameters on cellular level that would be possible to quantify and measured the effect of statins on proliferation and differentiation in primary human muscle cells. Myoblast proliferation decreased in response to both, simvastatin and rosuvastatin, in a concentration-dependent manner (Figure 4A, Supplemental Figure S1B). We found no evidence for statin toxicity (Supplemental Figure S1A) or oxidative stress as measured by nitric oxide concentration (Supplemental Figure S2A,B). Rescue experiments with mevalonate and geranylgeranyl pyrophosphate demonstrate that the effect on proliferation was indeed statin-specific. (Supplemental Figure S1B). In addition, both statins markedly delayed myoblast differentiation as quantified by fusion index, and by myosin heavy chain expression on mRNA and protein level (Figure 4B and C).

We then tested six different eicosanoid species (Supplemental Table S2) for their effect on proliferation and differentiation of primary human muscle cells during statin exposure. We identified

AL-8810, an 11β-fluoro analog of prostaglandin 2α, but not prostaglandin 2α, to significantly reverse the effect of simvastatin on proliferation differentiation (Figure 4D). AL-8810 had no effect on rosuvastatin treated myotubes (Supplemental Figure S4A-C). 5,6-EET, another arachidonic acid metabolite, increased proliferation independently from the statin effect (8Z,14Z). EDA, found in human milk, showed a negative trend on proliferation without impact on myotube fusion. Other eicosanoids, such as 12-HETE, did not have a significant effect on statin-treated primary human muscle cells.

Thus, eicosanoid species influence human primary muscle cell proliferation and fusion. Depending on statin type, eicosanoids have potential to ameliorate statin-induced changes in human primary myotubes.

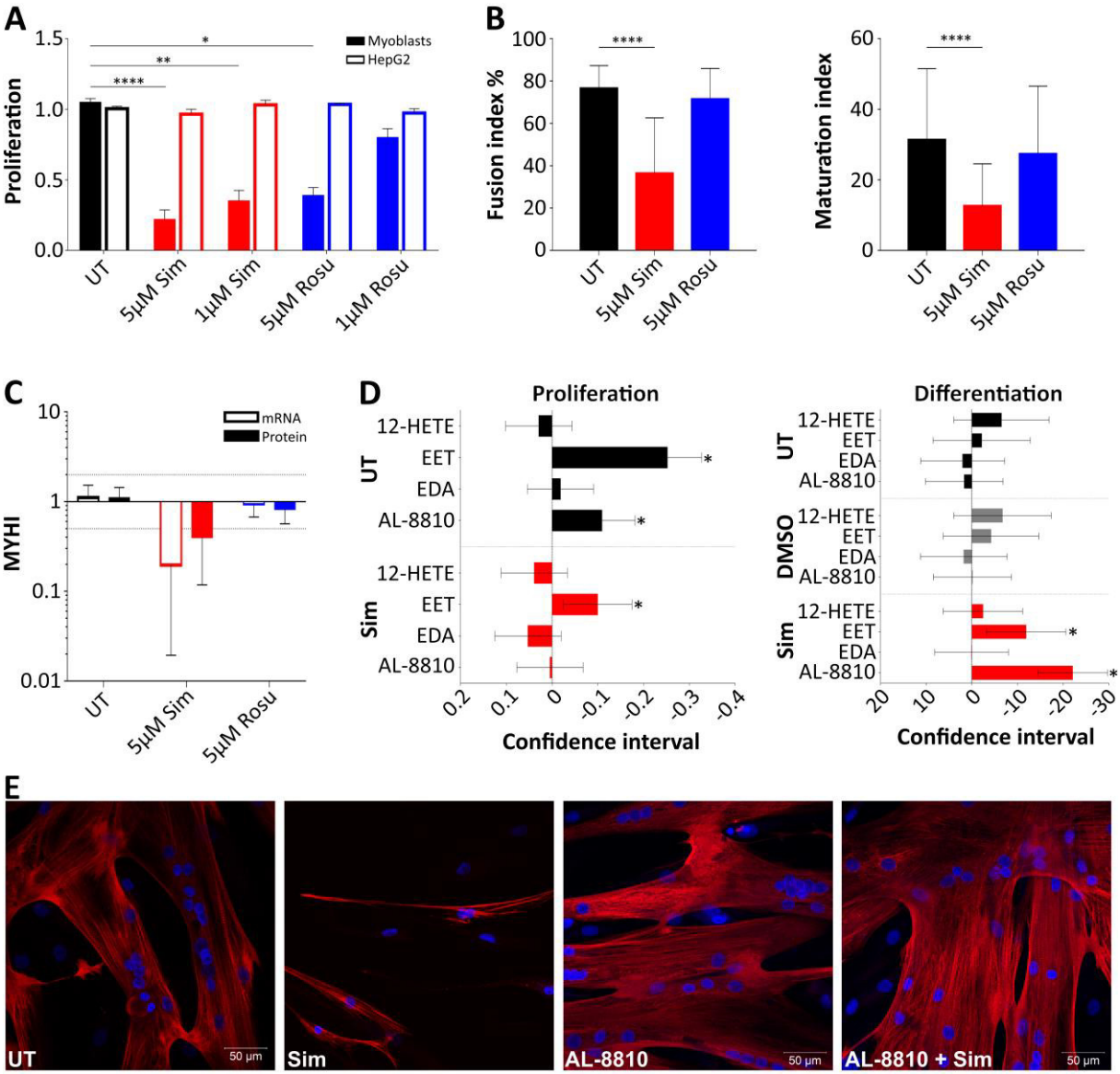

Figure 4. Statin influence on fatty acid metabolism of human myotubes. (A) Cell proliferation (BrdU) test: Primary human myoblasts and liver HepG2 cells were treated with statins for 72 h. At 5µM

concentration, statins negatively influence human myoblast but not HepG2 proliferation. The proliferation data were relatively calculated to DMSO-treated myoblasts to introduce the effect of DMSO. (B) Differentiation of primary human myoblasts under statin treatment for 96 h. Differentiation of human myoblasts into myotubes is delayed under treatment with 5μM simvastatin or 5μM rosuvastatin. Myotube fusion and maturation index (number of nuclei per myotube) were determined by counting MYHI (red) positively immunolabelled myotubes and HOECHST (blue) stained nuclei using the cell image analysis software CellProfiler v.2.1.1. (n=2-5) (C) MYHI mRNA and protein expression were examined in myotubes treated with 5 μM statin for 84 h to 96 h under differentiation condition relative to DMSO-treated samples- Dashed lines represent 2 fold and 0.5 fold expression changes. (D) Proliferation of human myoblasts under simvastatin and/or eicosanoid species treatment relative to DMSO. (5)6-EET ameliorates proliferation for simvastatin-treated samples. (95% confidence interval). (D,E) Differentiation of human myoblasts into myotubes under treatment with simvastatin and/or fatty acids. Myotube fusion index was determined by counting MYHI (red) positively immunolabelled myotubes and HOECHST (blue) stained nuclei using the cell image analysis software CellProfiler v.2.1.1. (E) Human statin-treated myotubes regain normal morphology when the eicosanoid AL-8810 is added to the medium. See also Supplemental Figure S5. For cell lines used see Supplemental Table S1. [UT, untreated (black); Sim, simvastatin (red); Rosu, rosuvastatin (blue); MYHI, myosin heavy chain 1]

4. DISCUSSION

In our comprehensive expressome analysis, molecular and cellular assessment of muscle related statin effects we discovered that statins have profound and unexpected effects on the transcriptome and proteome of primary human muscle cells. Some cellular functions such as RNA synthesis and turnover were much more influenced by simvastatin than by rosuvastatin, but cholesterol and fatty acid metabolism as well as eicosanoid synthesis pathway were severely affected irrespective of the class of statin that was delivered. The phenotype could be rescued by addition of eicosanoids.

We were surprised to find a dramatic effect of statins on cholesterol and eicosanoid synthesis in primary human muscle cells. We investigated the cellular repertoire of muscle cells to synthesise cholesterol and compared it to human liver cells (HepG2). We found that muscle and liver share the same repertoire for cholesterol biosynthesis (i.e. HMGCS1, HMGCR, CYP51A1, DHCR7) and cholesterol transport (i.e. ABCA1, ABCG1, LDLR, SCARB1), possibly pointing towards a so far underestimated role of skeletal muscle in cholesterol homeostasis. [28] It might be possible that muscle synthesizes cholesterol to the same or a larger degree as liver. The human body consists of more than 25% muscle mass, whereas the liver only makes up 2-3%. If cholesterol synthesis is an integral part of muscle function, it would be interesting to speculate whether the cholesterol is produced to support the liver to meet all demands or whether skeletal muscle only produces cholesterol for its own needs without secreting it into the circulation. Alternatively, our findings may not be physiologically relevant because cultured primary cells could adapt their metabolism in response to altered needs *in vitro*. However, HepG2, a hepatic cancer cell line and the source of many analyses that moulded our present understanding about cholesterol metabolism, is also prone to *in vitro* artefacts as well as alterations due to its malignant transformation. It will be the role of future experiments employing *in vivo* microdialysis [30] together with metabolomic plus lipidomic analysis of dialysates under defined challenging conditions that will provide the necessary answers.

Negative effects of statins on skeletal muscle have been extensively reported before. However, the published results differ in almost all aspects, particularly in regard of the pathomechanism of statin-effects on muscle. Different cell types, different organisms and small sample sizes contributed to poor comparability of studies. Most experimental data were obtained in non-human (C2C12) and/or malignant cell lines. [31,32]

We found that genes involved in cholesterol pathway like HMGCR, HMGCS1, LDLR, and PCSK9 are increased under statin treatment in muscle cells. [31] If HMGCS1 is increased and HMG-CoA cannot be reduced any more due to the effect of statins, HMG-CoA will increase and accumulate. Therefore, HMG-CoA might reconvert into ketone synthesis, namely acetoacetyl-CoA and Ac-CoA. As muscle is not known to be able to produce keton bodies (whereas liver would be) increased Ac-CoA would shuttle into the tricarboxylic acid cycle, carnitine-palmitoyl-transferases as well as fatty acid and triglyceride biosynthesis (Figure 5). All pathways appear affected by statins in muscle as we find increased protein levels of Ac-CoA carboxylase alpha (1.3-1.5fold), a very prominent upregulation of carnitine-palmitoyl-transferases 1 (CPT1A) (4.8-12fold), and an influence on key enzymes that catalyze the transformation of palmitic acid into the various fatty acids (Suppl. Table 5).

Multiple metabolic effects are possible and are intermingled between each other. In rodents, high carnitine-palmitoyl-transferases 1 levels have been shown to induce mitochondrial overload by acyl-carnitine accumulation with excess ß-oxidation leading to insulin resistance [33]. In rat hepatocytes, high Ac-CoA concentrations trigger insulin resistance *in vivo* [34]. An effect on human muscle cells can only be speculated on but could hint towards a mechanism for an increased incidence of type 2 diabetes after statin exposure. [35,36]

We concentrated our further investigation on the formation of arachidonic acid and eicosapentanoic acid, the precursor of prostaglandins (PG) and eicosanoids. These molecules have multiple effects including influences on cell proliferation and differentiation [37] and being pain mediators, a frequent complaint of statin-users. The degree of statin influence on prostaglandin and eicosanoid synthesis was striking: Fatty acyl and triglyceride biosynthesis involves LPIN1, ACSL, ligases (ACSL), elongases (ELOVL), de-/carboxylases (AGPAT, DGAT2), and desaturases (FADS) (see Supplemental Table 5 for expression details). Fatty acid desaturases FADS1 and FADS2 catalyze the formation of arachidonic acid and eicosapentanoic acid. PTGS1 (COX1) as the next important enzyme synthesizing eicosanoids and prostaglandins also was significantly downregulated. PTGS is interesting because nonsteroidal anti-inflammatory drugs such as ibuprofen inhibit PTGS and influence prostaglandin levels. We show here that a natural occurring epoxy-fatty acid, AL-8810, positively influences statin-induced effects on muscle cell proliferation and differentiation. We believe that these findings also translate into the clinic because it has recently been described that eicosapentaenoic and docosahexaenoic acids added to statin therapy to prevent enlargement of carotid plaques unexplainable also significantly reduced severe and less severe musculoskeletal side

effects [38]. Our study provides an explanation and would support a notion that omega-3 fatty acids should be added to statin therapy either as a prevention or a treatment of statin myopathy.

AUTHOR CONTRIBUTIONS

S.A.G, O.P., S.P., N.J., K.S., G.P., G.D. performed experiments and analysed data. U.G., E.S-T. recruited patients. W.K. performed genetic analysis. S.A.G., E.S-T., S.S. designed and organized the study and analysed data. S.A.G. and S.S. wrote the manuscript.

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5. REFERENCES

[1] J.D. Cohen, E.A. Brinton, M.K. Ito, T.A. Jacobson, Understanding Statin Use in America and Gaps in Patient Education (USAGE): an internet-based survey of 10,138 current and former statin users, J.Clin.Lipidol. 6 (2012) 208–215.

[2] R. Collins, C. Reith, J. Emberson, J. Armitage, C. Baigent, L. Blackwell, R. Blumenthal, J. Danesh, G.D. Smith, D. DeMets, S. Evans, M. Law, S. MacMahon, S. Martin, B. Neal, N. Poulter, D. Preiss, P. Ridker, I. Roberts, A. Rodgers, P. Sandercock, K. Schulz, P. Sever, J. Simes, L. Smeeth, N. Wald, S. Yusuf, R. Peto, Interpretation of the evidence for the efficacy and safety of statin therapy, Lancet. 388 (2016) 2532–2561.

[3] P.D. Thompson, G. Panza, A. Zaleski, B. Taylor, Statin-Associated Side Effects, J. Am. Coll. Cardiol. 67 (2016) 2395–2410. doi:10.1016/j.jacc.2016.02.071.

[4] H. Knoblauch, V. Schoewel, W. Kress, A. Rosada, S. Spuler, Another side to statin-related side effects, Ann.Intern.Med. 152 (2010) 478–479.

[5] S.Z. Bathaie, M. Ashrafi, M. Azizian, F. Tamanoi, Mevalonate Pathway and Human Cancers, Curr.Mol.Pharmacol. 10 (2017) 77–85.

[6] C. Martín Sánchez, J.M. Pérez Martín, J.-S. Jin, A. Dávalos, W. Zhang, G. de la Peña, J. Martínez-Botas, S. Rodríguez-Acebes, Y. Suárez, M.J. Hazen, D. Gómez-Coronado, R. Busto, Y.-C. Cheng, M.A. Lasunción, Disruption of the mevalonate pathway induces dNTP depletion and DNA damage., Biochim. Biophys. Acta. 1851 (2015) 1240–53. doi:10.1016/j.bbalip.2015.06.001.

[7] A. Gupta, P.D. Thompson, The relationship of vitamin D deficiency to statin myopathy, Atherosclerosis. 215 (2011) 23–29.

[8] D.A. Bookstaver, N.A. Burkhalter, C. Hatzigeorgiou, Effect of coenzyme Q10 supplementation on statin-induced myalgias, Am.J.Cardiol. 110 (2012) 526–529.

[9] A. Marg, V. Schoewel, T. Timmel, A. Schulze, C. Shah, O. Daumke, S. Spuler, Sarcolemmal repair is a slow process and includes EHD2, Traffic. 13 (2012) 1286–1294.

[10] S.A. Bustin, V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, J. Vandesompele, C.T. Wittwer, The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments, Clin.Chem. 55 (2009) 611–622.

[11] M. Lohse, A.M. Bolger, A. Nagel, A.R. Fernie, J.E. Lunn, M. Stitt, B. Usadel, RobiNA: a user-friendly, integrated software solution for RNA-Seq-based transcriptomics, Nucleic Acids Res. 40 (2012) W622–W627.

[12] S.C. Sapcariu, T. Kanashova, D. Weindl, J. Ghelfi, G. Dittmar, K. Hiller, Simultaneous extraction of proteins and metabolites from cells in culture, MethodsX. 1 (2014) 74–80.

[13] J. Cox, M. Mann, MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification, Nat.Biotechnol. 26 (2008) 1367–1372.

[14] M.A. Bray, M.S. Vokes, A.E. Carpenter, Using CellProfiler for Automatic Identification and Measurement of Biological Objects in Images, Curr.Protoc.Mol.Biol. 109 (2015) 14.

[15] A.E. Carpenter, T.R. Jones, M.R. Lamprecht, C. Clarke, I.H. Kang, O. Friman, D.A. Guertin, J.H. Chang, R.A. Lindquist, J. Moffat, P. Golland, D.M. Sabatini, CellProfiler: image analysis software for identifying and quantifying cell phenotypes, Genome Biol. 7 (2006) R100.

[16] D. Logan, Calculating multinucleated cells in muscle fibers, (2016). http://forum.cellprofiler.org/t/calculating-multinucleated-cells-in-muscle-fibers/2108.

[17] M.D. Robinson, D.J. McCarthy, G.K. Smyth, edgeR: a Bioconductor package for differential expression analysis of digital gene expression data, Bioinformatics. 26 (2010) 139–140.

[18] J. Gould, GENE-E is a matrix visualization and analysis platform designed to support visual data exploration, (2016). http://www.broadinstitute.org/cancer/software/GENE-E/index.html.

[19] T. Metsalu, J. Vilo, ClustVis: a web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap, Nucleic Acids Res. 43 (2015) W566–W570.

[20] J.C. Oliveros, Venny. An interactive tool for comparing lists with Venn's diagrams., Http://bioinfogp.cnb.csic.es/tools/venny/index.html. (2015). http://bioinfogp.cnb.csic.es/tools/venny/index.html.

[21] Y. Hu, C. Yan, C.H. Hsu, Q.R. Chen, K. Niu, G.A. Komatsoulis, D. Meerzaman, OmicCircos: A Simple-to-Use R Package for the Circular Visualization of Multidimensional Omics Data, Cancer Inform. 13 (2014) 13–20.

[22] G. Dennis Jr., B.T. Sherman, D.A. Hosack, J. Yang, W. Gao, H.C. Lane, R.A. Lempicki, DAVID: Database for Annotation, Visualization, and Integrated Discovery, Genome Biol. 4 (2003) 3.

[23] E. Eden, R. Navon, I. Steinfeld, D. Lipson, Z. Yakhini, GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists, BMC.Bioinformatics. 10 (2009) 48.

[24] I. Vastrik, P. D'Eustachio, E. Schmidt, G. Gopinath, D. Croft, B.B. de, M. Gillespie, B. Jassal, S. Lewis, L. Matthews, G. Wu, E. Birney, L. Stein, Reactome: a knowledge base of biologic pathways and processes, Genome Biol. 8 (2007) R39.

[25] B. Schwanhausser, D. Busse, N. Li, G. Dittmar, J. Schuchhardt, J. Wolf, W. Chen, M. Selbach, Global quantification of mammalian gene expression control, Nature. 473 (2011) 337–342.

[26] M.W. Medina, R.M. Krauss, The role of HMGCR alternative splicing in statin efficacy, Trends Cardiovasc. 19 (2009) 173–177.

[27] A. Leszczynska, M. Gora, D. Plochocka, G. Hoser, A. Szkopinska, M. Koblowska, R. Iwanicka-Nowicka, M. Kotlinski, K. Rawa, M. Kiliszek, B. Burzynska, Different statins produce highly divergent changes in gene expression profiles of human hepatoma cells: a pilot study, Acta Biochim. 58 (2011) 635–639.

[28] H.H. Wang, G. Garruti, M. Liu, P. Portincasa, D.Q.-H. Wang, Cholesterol and Lipoprotein Metabolism and Atherosclerosis: Recent Advances In reverse Cholesterol Transport, Ann. Hepatol. 16 (2017) 21–36. doi:10.5604/01.3001.0010.5495.

[29] A. Karatasakis, B.A. Danek, J. Karacsonyi, B. V Rangan, M.K. Roesle, T. Knickelbine, M.D. Miedema, H. Khalili, Z. Ahmad, S. Abdullah, S. Banerjee, E.S. Brilakis, Effect of PCSK9 Inhibitors on Clinical Outcomes in Patients With Hypercholesterolemia: A Meta-Analysis of 35 Randomized Controlled Trials, J. Am. Heart Assoc. 6 (2017) e006910. doi:10.1161/JAHA.117.006910.

[30] M. Boschmann, S. Engeli, K. Dobberstein, P. Budziarek, A. Strauss, J. Boehnke, F.C.G.J. Sweep, F.C. Luft, Y. He, J.E. Foley, J. Jordan, Dipeptidyl-peptidase-IV inhibition augments postprandial lipid mobilization and oxidation in type 2 diabetic patients., J. Clin. Endocrinol. Metab. 94 (2009) 846–52. doi:10.1210/jc.2008-1400.

[31] J.S. Burg, P.J. Espenshade, Regulation of HMG-CoA reductase in mammals and yeast, Prog.Lipid Res. 50 (2011) 403–410.

[32] P.J. Mullen, B. Luscher, H. Scharnagl, S. Krahenbuhl, K. Brecht, Effect of simvastatin on cholesterol metabolism in C2C12 myotubes and HepG2 cells, and consequences for statin-induced myopathy, Biochem.Pharmacol. 79 (2010) 1200–1209.

[33] T.R. Koves, J.R. Ussher, R.C. Noland, D. Slentz, M. Mosedale, O. Ilkayeva, J. Bain, R. Stevens, J.R. Dyck, C.B. Newgard, G.D. Lopaschuk, D.M. Muoio, Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance, Cell Metab. 7 (2008) 45–56.

[34] R.J. Perry, J.P. Camporez, R. Kursawe, P.M. Titchenell, D. Zhang, C.J. Perry, M.J. Jurczak, A. Abudukadier, M.S. Han, X.M. Zhang, H.B. Ruan, X. Yang, S. Caprio, S.M. Kaech, H.S. Sul, M.J. Birnbaum, R.J. Davis, G.W. Cline, K.F. Petersen, G.I. Shulman, Hepatic acetyl CoA links adipose tissue inflammation to hepatic insulin resistance and type 2 diabetes, Cell. 160 (2015) 745–758.

[35] D.J. Betteridge, R. Carmena, The diabetogenic action of statins - mechanisms and clinical implications, Nat.Rev.Endocrinol. 12 (2016) 99–110.

[36] S.J. Wakil, L.A. Abu-Elheiga, Fatty acid metabolism: target for metabolic syndrome, J.Lipid Res. 50 Suppl (2009) S138–S143.

[37] K.M. Jansen, G.K. Pavlath, Prostaglandin F2alpha promotes muscle cell survival and growth through upregulation of the inhibitor of apoptosis protein BRUCE, Cell Death.Differ. 15 (2008) 1619–1628.

[38] A. Alfaddagh, T.K. Elajami, H. Ashfaque, M. Saleh, B.R. Bistrian, F.K. Welty, Effect of Eicosapentaenoic and Docosahexaenoic Acids Added to Statin Therapy on Coronary Artery Plaque in Patients With Coronary Artery Disease: A Randomized Clinical Trial, J. Am. Heart Assoc. 6 (2017) e006981. doi:10.1161/JAHA.117.006981.