Simvastatin Influences Selected Aspects of Duchenne Muscular Dystrophy Pathology in a Mouse Model

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Research

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Abstract

Background: Duchenne muscular dystrophy (DMD) is an incurable disease, caused by the mutations in the *DMD* gene, encoding dystrophin, an actin-binding cytoskeletal protein. Lack of functional dystrophin results in muscle weakness, degeneration, and as an outcome cardiac and respiratory failure. As there is still no cure for affected individuals, the pharmacological compounds with the potential to treat or at least attenuate the symptoms of the disease are under constant evaluation. The pleiotropic agents, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, known as statins, have been suggested to exert beneficial effects in the mouse model of DMD. On the other hand, they were also reported to induce skeletal-muscle myopathy.

Methods: Several methods including functional assessment of muscle function *via* grip strength measurement and treadmill test, enzymatic assays, histological analysis of muscle damage, gene expression evaluation, and immunofluorescence staining were conducted to study simvastatin-related alterations in *mdx* mice.

Results: In our study, simvastatin treatment of *mdx* mice did not result in improved running performance; however, some beneficial effect was observed when grip strength was evaluated. Creatine kinase and lactate dehydrogenase activity, markers of muscle injury, were diminished after simvastatin delivery in *mdx* mice. Nevertheless, no significant changes in inflammation, fibrosis, and necrosis were noted. Interestingly, simvastatin treatment led to the decreased mRNA level of embryonic myosin heavy chain isoform, a declined percentage of centrally nucleated myofibers, and miR-1 upregulation, suggesting an alteration in the muscle regeneration. However, similarly to the changes noticed in the expression of some angiogenic factors, the obtained results are muscle-dependent, being prominent in gastrocnemius muscle but not in the diaphragm.

Conclusion: In conclusion, we suggest that simvastatin has the potential to ameliorate selected aspects of DMD pathology; however, possible benefits still need to be thoroughly tested.

Background

Duchenne muscular dystrophy (DMD) is a progressive, severely debilitating, and lethal genetic disease caused by the mutations in the *DMD* gene, coding dystrophin, a 427 kDa actin-binding cytoskeletal protein. Dystrophin functions as a component of the large oligomeric dystrophin-glycoprotein complex (DGC), which is important for maintaining muscle fiber-extracellular matrix integrity and regulates several cellular pathways including nitric oxide (NO) production, Ca^{2+} entry, and the generation of reactive oxygen species (ROS) (1, 2). In DMD, progressive muscle weakening together with the loss of muscle mass and function is a consequence of several pathological processes including necrosis, inflammation, fibrosis, and increased oxidative stress which are results of unbalanced regenerative processes (reviewed in (3)). Recent discoveries and our research underlined also the dysregulation of angiogenesis as an additional mechanism accompanying muscle insufficiency (4–6). As the disease progresses, patients suffering

from DMD lose the ability to walk and ultimately die in the 2nd to 3rd decade of life, due to cardiac or respiratory failure (7, 8).

Taking into account the diversity of the processes which may affect DMD progression and the constant need for the development of effective therapeutics, new factors are suggested to exhibit beneficial effects on this so far incurable disease. In our previous study, we have found that lack of heme oxygenase-1 (*Hmox1*, HO-1), a heme-degrading enzyme, exerting anti-oxidant and cytoprotective activities leads to a more severe disease state. Knock-out of *Hmox1* resulted in aggravated inflammation and fibrosis as well as the impaired running capacity of dystrophic animals (9). *Hmox1* expression may be modulated by the plethora of compounds, including 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (10), commonly known as statins, discovered 40 years ago (11) and used as lipid-lowering drugs for the treatment of hypercholesterolemia and reduction of atherosclerosis. Interestingly, in 2015, Whitehead *et al*. for the first time described the protective effect of statins in dystrophic mice (12). The authors showed that simvastatin improved muscle health, reduced inflammation, oxidative stress, and increased autophagy in *mdx* animals. A few years later, the same group suggested long-term improvement in heart functions in response to simvastatin treatment (13). However, other studies performed in animal models did not confirm such favorable properties. Verhaart *et al*. found no positive outcome of simvastatin treatment on muscle function, histology, or expression of genes involved in several DMD-related processes such as inflammation, fibrosis, and oxidative stress (14). Moreover, also Finkler *et al.* did not demonstrate the salutary, anti-dystrophic impact of a different statin, rosuvastatin (15).

What is relevant, even the devastating role of statins in muscle biology has been reported. Several *in vitro* studies showed the toxic effects of those drugs on muscle cells. Importantly, concentrations of statins required to induce deleterious effects *in vitro* are far beyond the physiological range, being typically greater than 1 μM. Such concentrations are considerably (100–1000 times) higher than those found *in vivo* in humans (16). For instance, 24 h stimulation with 10 μM or 50 μM simvastatin, atorvastatin, or rosuvastatin exerted toxic effects on C2C12 myoblast cell line (17) whereas even 100 μM cerivastatin, fluvastatin, and atorvastatin were used to induce cell death and mitochondrial toxicity in L6 rat skeletal muscle cell line (18).

There are also discrepant data about the incidence of different kinds of myopathy in humans after statin therapy. Previous studies indicated a high risk of such adverse effects, e.g. showing that > 10% of statin users in the general population can be affected (19, 20). Noteworthy, a recent systematic review of clinical trials found adverse muscle symptoms only in < 1% compared with placebo controls (21). Statin-related muscle symptoms also appear to be exacerbated by several factors, including exercise (22), older age, and female sex (23). Nevertheless, published a few years ago meta-analysis by Iwere and Hewitt clearly showed, that even in aged patients (65 + years), the risk of statin-induced myopathy was comparable to placebo patients (24) which was also confirmed recently by Zhou *et al.* (25). Moreover, in September 2020, the observational analysis provided data from three large trials on 58 390 peoples treated with simvastatin for a mean of 3.4 years (HPS, SEARCH, and HPS2-THRIVE) reporting the

extremely low risk of simvastatin-induced myopathy with specific criteria for identification of the individuals at high and at lower risk (26). These data implicate that the fear of statin-caused myopathy might be in many cases overestimated. Notably, the above-mentioned risk factors for statin-induced myopathy are not relevant to boys with DMD.

Based on the published, contradictory results in the field of muscular dystrophy (5, 9) and our previous expertise in terms of the role of statins, including the angiogenesis process (27, 28), we aimed at the evaluation of the effect of simvastatin in *mdx* animals as well as tried to identify the additional molecular mechanisms responsible for the potential improvement in muscle health and function in DMD. We have found that simvastatin can ameliorate selected aspects of DMD pathology such as the elevated creatine kinase (CK) and lactate dehydrogenase (LDH) levels; however, it does not affect the important processes contributing to dystrophy progression like fibrosis, inflammation, or angiogenesis. Moreover, the simvastatin effect was, at least in our hands, muscle-specific. Therefore, we may conclude, that simvastatin treatment could be tested as a potential supportive therapy for DMD; however, it requires further, thorough investigation.

Materials And Methods

Cell culture

C2C12 murine myoblast cells were maintained in DMEM High Glucose (4.5 g/l) medium supplemented with 10% fetal bovine serum (FBS) and antibiotics: streptomycin (100 µg/ml) and penicillin (100 U/ml) (Lonza). The cells were kept at standard conditions (37 °C, 5% CO_2, 95% humidity). C2C12 myoblasts were stimulated for 24 h with 0.1 and 1 µM concentrations of simvastatin (Sigma-Aldrich).

Animals

Animal experiments were conducted in accordance with national and European legislation, after approval by the 2nd Institutional Animal Care and Use Committee (IACUC) in Kraków, Poland (approval number: 323/2018). *Mdx* mice C57BL/10ScSn-*Dmd*^{mdx}/J and control mice C57BL/10ScSnJ (WT) were purchased from the Jackson Laboratory. Mice were bred on a mixed C57BL/10ScSn and C57BL/6 × FVB background as described by us previously (9) and were housed in specific pathogen-free (SPF) conditions with water and food available *ad libitum.* Genotyping of animals was performed using PCR on the DNA isolated from the tails. Only males were used for the experiments. The first dose of simvastatin was given to the 6-week-old male littermates or age-matched mice from generation F2 to F5 and the administration was continued for 28 days until the age of 10 weeks.

Simvastatin treatment

An activation procedure was based on the published protocol (29). Briefly, 4 mg of simvastatin (Sigma-Aldrich) was dissolved in 200 µl of ethanol. Then 300 µl of 0.1 N NaOH was added to the solution and subsequently incubated at 50 °C for 2 h. The pH was brought to 7.2 by HCl, and the concentration of the

stock solution was adjusted to 2 mg/ml. The stock solution was kept at 4 °C. For 28 days both WT and *mdx* mice received 10 mg/kg body weight (BW)/day via oral gavage. Mice of both genotypes were randomly separated into the vehicle (solvent)- and simvastatin-treated groups. The administered dose was chosen based on the literature data (12).

Grip strength assay

Forelimb grip strength was assessed one day before the first dose, at day 15, and one day after the last administration, using a grip strength meter with a triangular pull bar (Ugo Basile) as described earlier (30, 31). The measurements were repeated 3 times with a 1 min break in between. The results were calculated as an average from 3 measurements, normalized to BW, and expressed as N/kg BW.

Treadmill test

The treadmill exhaustion test was performed after the last dose of simvastatin using the Exer-3/6 (Columbus Instruments) at 15 degrees downhill by the investigator blind to the mice genotype. We employed the protocol described previously (9) with modification. Briefly, after 3 daily acclimation sessions of 15 min at 8 m/min and one day at 20 m/min, 10-week-old male mice were subjected to an exhaustion treadmill test. Mice were warmed up at 5 m/min for 5 min before the test. For the test, mice ran on the treadmill at 5 m/min for 2 min, 7 m/min for 2 min, 8 m/min for 2 min, 10 m/min for 5 min, and 12 m/min for 15 min. Afterward, speed was increased by 1 m/min to a final speed of 20 m/min. Exhaustion was defined by the inability of the animal to remain on the treadmill despite stimulation by gentle touching.

Blood cell count

The blood was collected directly from the *vena cava* to the EDTA-coated tubes and analyzed using scil Vet abc (Horiba). The total number of white blood cells (WBC) and the percentage of granulocytes, monocytes, and lymphocytes among WBC, was calculated in 10-week-old mice treated with vehicle and simvastatin.

Histological and immunofluorescent analysis of the muscles

For histological assessment of inflammation (hematoxylin and eosin, H&E), fibrosis (Masson's trichrome), vessels staining (CD31/α-SMA), necrosis and evaluation of the muscle fiber's size (IgG/IgM/IgA/laminin staining), and centrally nucleated fibers (CNF) abundance, muscles were collected and pre-treated with OCT medium (Leica) for few minutes directly after collection. Afterward, they were transferred to new, OCT-containing tubes, frozen in liquid nitrogen-cooled isopentane, and stored at -80 °C. Then, 10 µm thick sections were cut on a cryotome (Leica), placed on the previously coated with poly-L-lysine slides, air-dried for at least 2 h, and kept at -20 °C for further analyses. H&E staining and Masson's trichrome were performed on the 4% buffered formalin-fixed (pH 7.4) frozen sections. For **H&E**, tissue sections were incubated in Mayer's hematoxylin (Sigma-Aldrich) for 12 min, rinsed with tap water (15 min), and stained for 1.5 min in 0.1% eosin solution (96% EtOH and distilled water, 7:3) (Sigma-Aldrich). After the staining,

the sections were incubated in increasing concentrations (70%, 96% (× 2), 99.8% (× 2)) of aqueous EtOH (POCH), then 2 times in xylene (Sigma-Aldrich) and sealed in Histofluid medium (Chemilab). **Masson's trichrome** (Trichrome Stain (Masson) Kit, Sigma-Aldrich) was performed following the manufacturer protocol. After the staining, the sections were incubated in increasing concentrations (70%, 96% (× 2), 99.8% (× 2)) of aqueous EtOH, then 2 times in xylene and sealed in Histofluid medium (Chemilab). Analyses were conducted according to our previous studies (4, 9, 32) after taking pictures of the whole tissues. The assessment of inflammation and fibrosis extent was conducted using arbitrary units, respectively: 0 - no signs of inflammation/collagen deposition; 1 - any sign of leukocyte infiltration and myofiber swelling/collagen deposition; 2 - visible inflammation, myofiber swelling, and rhabdomyolysis/collagen deposition; 3 - signs of inflammation, myofiber swelling, and rhabdomyolysis which take around half of a field of view/collagen deposition takes up around half of the field of view; 4 - a substantial part of the muscle in the field of view is infiltrated and degenerated/collagen deposition takes the substantial part of the field of view. The analysis of CNF indicating the level of regeneration was performed based on H&E staining; 10–15 pictures/tissue were randomly taken and the percentage of CNF among all fibers was calculated.

Immunofluorescent staining of **CD31/α-SMA** positive vessels was performed as described by us previously with slight modifications (33). Primary antibodies: rabbit anti-human α-SMA (Abcam, ab5694) and rat anti-mouse CD31 (BD Pharmingen, 550274) were used followed by the incubation with secondary antibodies: goat anti-rat Alexa Fluor 488 (for detection of α-SMA) and goat anti-rabbit Alexa Fluor 568 (for detection of CD31). Pictures of the whole tissue were taken and CD31/α-SMA positive vessels were analyzed quantitatively per muscle area. The results were presented as a number of vessels per area. **Necrosis** was assessed by the immunofluorescent staining of the IgG/IgM/IgA (goat anti-mouse IgG, IgM, IgA Alexa Fluor 488 antibody, Thermo Fisher Scientific) with laminin α2 (rabbit anti-mouse antibody, Abcam, ab11576; secondary antibody: goat anti-rabbit Alexa Fluor 568) and showed as a percentage of necrotic fibers in the stained muscle. Evaluation of the **muscle cross-sectional area (CSA) and the mean fiber area** were determined by semi-automatic muscle analysis using segmentation of histology (SMASH) (34) based on immunofluorescent staining of laminin.

The stainings were visualized under Nikon Eclipse T*i* fluorescent microscope. All histological assessments were analyzed by the investigator blind to the mice group using ImageJ software. If necessary, the brightness and/or contrast were adjusted to all of the pictures equally.

Determination of serum CK and LDH concentrations

To estimate the activity of CK and LDH diagnostic Liquick Cor-CK and Liquick Cor-LDH kits were used, respectively, according to the manufacturer protocols (Cormay). Blood was collected from *vena cava* and was allowed to clot at room temperature for 30 min and then centrifuged at 4 °C for 10 min at 2000 *g*. The assay was performed using a clear serum, without the signs of hemolysis. The absorbance values were then converted to CK and LDH (U/l).

RNA isolation, reverse transcription (RT), and quantitative real-time PCR (qRT-PCR)

Collected muscles were protected in RNA*later* (Sigma-Aldrich), snap-frozen in liquid nitrogen, and stored at -80 °C for downstream analyses. RNA was isolated as in our previous study (9) using the Chomczynski-Sacchi method (35). Its quality and concentration were determined by NanoDrop Spectrophotometer (Thermo Fisher Scientific). qRT-PCR was performed as described previously (9) using StepOne Plus Real-Time PCR (Applied Biosystems - Thermo Fisher Scientific) and SYBR Green PCR Master Mix (Sigma-Aldrich), specific primers (listed in Table 1), and cDNA obtained in the RT reaction with recombinant M-MuLV reverse transcriptase (Thermo Fisher Scientific). *Eef2* was used as a housekeeping gene. LNA miRCURY RT-PCR Kit and miRCURY LNA SYBR PCR Kit (Qiagen, Hilden, Germany) were applied for miRNAs determination. The expression of miR-1, miR-133a, and miR-206 (miRCURY LNA™ miRNA PCR Assays) was normalized to the constitutive SNORD68 gene (miRCURY LNA™ miRNA PCR Assay). Relative quantification of gene expression was calculated based on the comparative C_t method ($\Delta C_t = C_{t\ \text{gene of interest}} - C_{t\ \textit{Eef2/SNORD68}}$) and presented as the relative expression in comparison to vehicle-treated animals.

Table 1
The sequences of primers used for the determination of gene expression by qRT-PCR.

Gene	*Sequence 5'-3'*
Ang 1	*F: CAGTGGCTGCAAAAACTTGA*
	R: TGGGCCATCTCCGACTTCAT
Col1a1	*F: CGATCCAGTACTCTCCGCTCTTCC*
	R: ACTACCGGGCCGATGATGCTAACG
Cxcl12	*F: CTAGTCAAGTGCGTCCACGA*
	R: CCCACCACTGCCCTTGCATG
Eef2	*F: TCAGCACACTGGCATAGAGG*
	R: GACATCACCAAGGGTGTGCA
Hmox1	*F: CCTCACTGGCAGGAAATCATC*
	R: CCTCGTGGAGACGCTTTACATA
Kdr	*F: CGGCCAAGTGATTGAGGCAG*
	R: ATGAGGGCTCGATGCTCGCT
Mmp11	*F: CAGATTTGGTTCTTCCAAGG*
	R: AGATCTTGTTCTTCTCAGGAC
Myh3	*F: TCTAGCCGGATGGTGGTCC*
	R: GAATTGTCAGGAGCCACGAA
Spp1	*F: CCATCTCAGAAGCAGAATCTCCTT*
	R: GGTCATGGCTTTCATTGGAATT
Tgfb1	*F: GGATACCAACTATTGCTTGAG*
	R: TGTCCAGGCTCCAAATATAG
Vegfa	*F: ATGCGGATCAAACCTCACCAA*
	R: TTAACTCAAGCTGCCTCGCCT

Enzyme-linked immunosorbent assay (ELISA)

The fragments of muscles were snap-frozen in liquid nitrogen, homogenized in 1% Triton X-100 in PBS using TissueLyser (QIAGEN), and centrifuged (7 000 *g*, 10 min, 4 °C). The protein lysates were collected, and total protein concentration was determined by bicinchoninic acid (BCA, Sigma-Aldrich) assay. 100 µg of protein lysate was used to determine the level of vascular endothelial growth factor (VEGF), fibroblast

growth factor-2 (FGF2), endoglin (CD105), and stromal cell-derived factor-1 (SDF1) according to the vendor's instructions (R&D Systems). To assess the level of osteopontin (OPN), 750 times-diluted mouse serum was subjected to the test and the concentration was quantified based on the absorbance values according to the manufacturer's protocol (R&D Systems).

Statistical analyses

Data are presented as mean ± SEM. Differences between groups were tested for statistical significance using the one-way ANOVA followed by Tukey's post-hoc test or the unpaired 2-tailed Student's *t*-test (when statin treatment was evaluated in *mdx* mice only); $p \leq 0.05$ was considered significant. The outliers were identified based on Grubb's test.

Results

Simvastatin treatment decreases CK and LDH activities in mdx mice

Administration of simvastatin for 28 days in a dose of 10 mg/kg BW/day by oral gavage caused a slight drop in the BW both in WT and *mdx* mice, the effect visible after the delivery of the first few doses. No other prominent changes in the mice's behavior were observed. The initial drop in the body weight did not exceed an alarming percentage or aggravated further in the following days. Moreover, animals started to gain weight over time in a similar matter to the vehicle group (Fig. 1A). The blood cell analysis did not reveal any detrimental effect of statin treatment measured at the end of the experiment (Fig. 1B-E). Despite no apparent changes in the number of white blood cells (WBC) (Fig. 1B), a significant rise in the percentage of granulocytes (Fig. 1C) and monocytes (Fig. 1D) was noted at the expense of the lymphocytes (Fig. 1E) in the vehicle and simvastatin-treated *mdx* mice in comparison to appropriate WT.

One of the hallmarks of DMD is the elevated level of LDH and CK, markers of muscle damage (36). Accordingly, the activity of CK (Fig. 1F) and LDH (Fig. 1G) was potently increased in the dystrophic animals, whereas it dropped in *mdx* mice treated with simvastatin. No disturbing changes as the result of simvastatin were apparent in WT animals, hence for clarity we next focused on the investigation of simvastatin effectiveness predominantly in dystrophic individuals.

Simvastatin treatment fails to improve the exercise capacity of the mdx mice but significantly augments forelimb grip strength

To assess the functional effect of statin treatment we carried out two types of tests. Conducted after the last dose of statin administration treadmill experiment did not show any difference in the running capacity of *mdx* mice when compared to the vehicle-treated mice (Fig. 2A). However, the rate of the

increase in forelimb grip strength was higher for dystrophic animals treated with simvastatin in comparison to the vehicle group - the tendency was visible already on day 15 and sustained till day 29, after the last dose of the treatment (Fig. 2B).

Simvastatin treatment does not affect inflammation and necrosis of the muscles in dystrophic animals

H&E staining did not reveal any effect of simvastatin on inflammation, regardless of the type of the analyzed muscle (Fig. 3A, B). Accordingly, the expression of an inflammatory gene, heme oxygenase-1 (*Hmox1)* was affected by the treatment neither in gastrocnemius nor in the diaphragm (Fig. 3C). The muscle necrosis, as assessed by the immunofluorescent staining of the IgG/IgM/IgA, the membrane-impermeable markers, was also not attenuated in *mdx* mice upon simvastatin administration (Fig. 3D).

Simvastatin does not reduce fibrosis in dystrophic animals

In dystrophic muscles, collagen deposition was clearly visible; however, the simvastatin treatment did not attenuate fibrosis as shown by semi-quantitative analysis of trichrome staining both in gastrocnemius (Fig. 4A) and in diaphragm (Fig. 4B) muscles. Of note, the mRNA (*Spp1* gene) and protein level of OPN, one of the markers of fibrosis, was not affected by statin treatment (Fig. 4C - E). Furthermore, the expression of other fibrotic factors, including transforming growth factor-beta 1 (*Tgfb1*) and matrix metalloproteinase 11 (*Mmp11*) was unchanged in both analyzed muscles (Fig. 4F, G). Although there was a significant decrease in collagen type I alpha 1 chain (*Col1a1)* in the gastrocnemius muscle (Fig. 4F) no such effect was found in the diaphragm (Fig. 4G).

Simvastatin treatment influences muscle regeneration

Based on the laminin staining of the muscle, we were able to evaluate the size of the fibers, which appeared to be larger in the gastrocnemius muscle of dystrophic animals upon simvastatin treatment (Fig. 5A, B). Importantly, also the expression of embryonic myosin heavy chain isoform *Myh3*, encoding eMyHC especially relevant in the matter of muscle regeneration (37), was perceived to be declined in gastrocnemius muscle (Fig. 5C). Simultaneously, the percentage of CNF was lower when simvastatin-treated *mdx* mice were compared to the vehicle group (Fig. 5D). Interestingly, increased during muscle regeneration, the protein level of FGF2 (38), was reduced in gastrocnemius muscle of simvastatin-receiving animals (Fig. 5E). As microRNAs, especially so-called myomiRs, play an important role in muscle regeneration (39) we decided to check the expression of the three, most commonly used miRNAs: miR-1, miR-133a, and miR-206. Results for simvastatin treated *mdx* animals shown a significant upregulation of miR-1, a prominent rising tendency in miR-133a, and no difference in miR-206 (Fig. 5F). Notably, no changes in fiber size and CNF were noticed in the diaphragm (Fig. 5G-I).

Simvastatin treatment does not affect vascularization in dystrophic muscles

Recent discoveries underline the role of dysregulation of angiogenesis in DMD pathology (4, 5, 31). In our previous studies, we have found concentration- and cell-type dependent effect of statins on VEGF synthesis and overall angiogenic activity (27, 40, 41). However, in the C2C12 mouse myoblast cell line simvastatin at the physiologically relevant concentrations (0.1-1 μM) did not affect *Vegfa* (Supplementary Fig. 1A). Interestingly, *in vivo*, the decrease in *Vegfa* and also kinase insert domain receptor (*Kdr)* expression after simvastatin delivery was found in gastrocnemius of *mdx* mice, indicating the possible anti-angiogenic effect (Fig. 6A). Nevertheless, such alterations were not perceived for other angiogenic genes, such as angiopoietin-1 (*Ang1)* and C-X-C motif chemokine 12 (*Cxcl12)*, also known as gene coding stromal cell-derived factor 1 (SDF1) (Fig. 6A). Importantly, VEGF (Fig. 6B), as well as SDF1 (Fig. 6C) and endoglin (CD105) (Fig. 6D) protein level was also unaffected. Furthermore, no effect of statin treatment on the analyzed factors was detected in diaphragm muscle, both on mRNA and protein level (Fig. 6E-H). Moreover, when the number of $CD31^+$/α-SMA^+ vessels was evaluated in gastrocnemius muscle, no differences were noted after statin delivery (Fig. 6I). Interestingly, a significant rise in the number of $CD31^+$/α-SMA^+ vessels was observed in the diaphragm, once again showing discrepancies between various muscles (Fig. 6J).

Discussion

Despite many years of intensive and profound studies, DMD remains an incurable disease. Newest, most promising therapies, using the latest advances in genetic modification, namely CRISPR/Cas9 technology, are still far from clinical introduction and acceptance. Thus, glucocorticoids, with prednisolone and deflazacort being the most commonly used, still serve as a gold standard therapy for patients suffering from DMD. Unfortunately, except for undoubtful beneficial effects in e.g. prolonging ambulation, their daily administration was shown to exert many side effects leading to, among others, osteoporosis, diabetes, or muscle atrophy (42). As there is a constant need to investigate novel strategies, which could at least attenuate the severity of the disease, many researchers focus not only on new drug discoveries but also repurposing of the already existing ones.

HMG-CoA reductase inhibitors, commonly known as statins, seem to be the perfect choice for such investigation. Despite the still ongoing discussion regarding statin-induced myopathy, myositis, and rhabdomyolysis (16, 20, 22, 43) more and more studies describe that the benefits of treatment outweigh the possible risks which, of note, are usually not relevant to DMD boys (25, 26). In our study, no further deterioration in inflammation, fibrosis, and necrosis was visible as the consequence of simvastatin delivery to dystrophic animals. Importantly, no elevation in, strongly associated with statin-induced myopathy, CK level (43), indicated no additional damage to the muscle after one month of simvastatin administration. Moreover, no significant or alarming systemic changes were observed in regards to the total WBC or distinguished subpopulation e.g. granulocytes, lymphocytes, and monocytes, at the end of the experiment.

In contrast to the previous discussion about the deleterious muscle-related alterations, over recent years, several studies demonstrated the positive effects of statins on overall skeletal muscle health, including

their anti-inflammatory and anti-fibrotic properties (44, 45). In the present study we have found that among various processes contributing to dystrophy progressions, simvastatin can ameliorate, in a muscle-type specific manner, only selected aspects of DMD pathology influencing regeneration markers without any effect on inflammation, fibrosis, or angiogenesis. Interestingly, Whitehead *et al.*, already in 2015 showed the protective influence of simvastatin in dystrophic animals (12). However, the recent publication by Verhaart *et al.* described the lack of the effect of this statin and even put into question whether presented by Whitehead *et al.* high level of simvastatin (obtained after administration in the food and drink) in the blood of the animals was possible to be obtained (14). When different statins were investigated by other groups, results were also inconclusive. It was shown that pravastatin, another FDA-approved cholesterol-lowering drug, can be considered in DMD therapy as it can upregulate utrophin A expression via eEF1A2 (46). Utrophin A, which is an autosomal homolog of dystrophin, is suggested to functionally compensate for dystrophin loss in DMD muscles (47). On the other hand, Finkler *et al.* demonstrated no beneficial effects of rosuvastatin. Moreover, a visible accretion of inflammation was remarked upon treatment (15). Such discrepancies in the obtained results might be related to several divergences in the applied methodology, including age and background of the mice, type and dose of the statin that was used, route of administration, and length of time the drug was given to the animals. Nevertheless, so diverse strategies give an undoubtful chance to investigate the effects of statins from different perspectives and various stages of mice development and disease progression. In our study, the dose of simvastatin was 10 mg/kg BW. In that matter, the applied approach was similar to the one used by other groups (12, 14). Moreover, despite the most promising results were obtained by Whitehead *et al.* when simvastatin was provided in food and water (12), we strongly believe that oral gavage administration is more relevant, giving us the opportunity to more precisely control the given dose. Additionally, literature data confirm this method of statin delivery to be more effective in the manner of the obtained in the blood simvastatin concentration (14). Notable, although the effect of simvastatin treatment on *mdx* mice was not as profound as in Whitehead *et al.* (12) study, we did remark some interesting changes.

We observed not only typical for dystrophic animals and described in our previous papers (4, 5, 9), elevation in the most commonly acknowledged muscle damage markers: CK and LDH activity level, but also a significant decrease in given parameters in *mdx* mice upon simvastatin treatment, what is in line with work by Whitehead *et al*. (12). Such an outcome might suggest lower degeneration of the muscle fibers. However, no alterations in typical muscle degeneration markers, namely necrosis, and inflammation (48), allow us to confirm such a hypothesis. In contrast to the results obtained by Whitehead *et al.*, which showed visibly reduced inflammatory cell infiltration and a diminished number of CD68 macrophages (12), we did not observe the anti-inflammatory potential of simvastatin when the histological assessment of gastrocnemius and diaphragm muscles was performed. Moreover, the expression of the *Hmox1* gene, coding anti-oxidant, and cytoprotective HO-1 enzyme was also not affected by the treatment. Similar results were obtained by Verhaart *et al.* (14), who reported no effect on inflammation even with the prolonged by two months, in comparison to us, time of drug administration. Furthermore, in opposition to the published data suggesting the anti-fibrotic role of simvastatin in *mdx*

animals (12, 13), we did not observe any effect of the treatment on fibrosis, neither in histological assessment of collagen deposition nor expression of fibrosis-related genes. Those observations were confirmed by the evaluation of OPN expression, a recently described biomarker of DMD associated with regeneration, inflammation, and fibrosis (49, 50), which was also not affected by simvastatin.

On the other hand, interesting results were obtained in our study concerning muscle regeneration. When gastrocnemius muscle was investigated we noticed a significant increase in the mean myofiber size. Together with histological assessment of the CNF, a decrease in the expression of *Myh3*, which is important in muscle repair (51), and downregulation of highly expressed during regeneration, FGF2 protein (38), we might imply normalization of the regenerative process in investigated muscle. We suggest that a decline in the number of CNF and *Myh3* mRNA level could be related to faster maturation of the regenerating fibers and as a result, loss of the related to that process markers (48). Moreover, a significant rise in muscle-specific miR-1 and increasing tendency in miR-133a, so-called myomiRs, might indicate more efficient muscle regeneration, as it was demonstrated that such upregulation promotes muscle differentiation and therefore, improves muscle repair (52, 53). Interestingly, no effect of simvastatin on dystrophic muscle regeneration was demonstrated by Whitehead *et al.* and Verhaart *et al.* (12, 14). Moreover, it needs to be emphasized that such alterations were noticed only in the gastrocnemius muscle. When we analyzed the diaphragm, we did not observe any changes. It shows that, at least in our hands, simvastatin treatment-related outcomes are strongly muscle type-specific. In order to better understand the complex mechanisms exerted by statins, their effects on various cell types including muscle satellite cells (mSC), crucial for muscle regeneration (54), should be investigated.

Enlargement in muscle fiber size together with improved muscle regeneration could further explain observed by us amelioration in the forelimb grip strength. Interestingly, we described that even though in both vehicle and simvastatin groups strength was augmented, the rate of the improvement was significantly higher in drug-treated *mdx* mice. Interestingly, when a measurement of the specific force of the muscle was performed by Whitehead *et al*. (12) and Verhaart *et al.* (14), they showed significant improvement and lack of any effect, respectively. As overall mice performance in a treadmill test, influenced not only by muscle strength but also by the respiratory and cardiovascular systems, was not altered by the treatment, we might speculate that obtained by us effect might not be strong enough to cause systemic changes in the mice.

To expand already described knowledge we decided to investigate also a different aspect of DMD progression – angiogenesis alterations, especially because our previous studies revealed a decrease in a major pro-angiogenic factor, VEGF, both at mRNA and protein level in skeletal muscles of dystrophic animals in comparison to wild-type counterparts (4, 5, 31). Importantly, improvement of endothelial function and vasculoprotective action are well-recognized statin effects (55). Our previous experiments clearly showed that statins regulate angiogenesis. We have demonstrated that atorvastatin at the pharmacologically relevant concentration (100 nM) enhanced the expression of endothelial nitric oxide synthase (eNOS) in human microvascular endothelial cells (HMEC-1). Moreover, atorvastatin prevented the hypoxia-induced decline in eNOS expression (28). The regulation of several angiogenic factors was

observed by us after statin stimulation in human umbilical vein endothelial cells (HUVEC) but these effects may be also cell- and dose-dependent (27, 41). Moreover, accelerated vascularization upon simvastatin treatment was also demonstrated in models of peripheral ischemia and corneal neovascularization (56). In the present study, despite significant changes in some of the tested angiogenic genes in gastrocnemius muscle, no complementary effects were noticed on the protein level. Although a decrease in *Vegfa/Kdr* signaling might indicate the anti-angiogenic effect of simvastatin, neither VEGF protein nor abundance of CD31/α-SMA double-positive blood vessels were observed. Despite the elevated number of vessels in the diaphragm, no other investigated factors were affected, rather suggesting no profound effect on angiogenesis as the result of simvastatin administration. Noteworthy, it again shows that simvastatin might not be influencing various muscles in the same manner.

Conclusion

In conclusion, we suggest that simvastatin has the potential to positively influence selected aspects of DMD pathology. Despite muscle-dependent changes and the requirement for further research, improvement in forelimb grip strength with decreased CK and LDH activity and ameliorated muscle regeneration, allow us to consider simvastatin as a potential drug in combined therapies for DMD.

Abbreviations

α-SMA – Alpha-smooth muscle actin; ***Ang1*** – Angiopoietin 1; **BCA** – Bicinchoninic acid; **BW** – Body weight; **CD105** – cluster of differentiation 105, Endoglin; **CD31** – Cluster of differentiation 31; **CD68** – Cluster of Differentiation 68; **CK** – Creatine kinase; ***Col1a1*** – Collagen type I alpha 1; **CNF** – Centrally nucleated fibers; **CSA** – Cross-sectional area; ***Cxcl12*****, SDF-1** – C-X-C motif chemokine ligand 12, stromal cell-derived factor 1; **DGC** – Dystrophin-glycoprotein complex; **DMD** – Duchenne muscular dystrophy; **DNA** – Deoxyribonucleic acid; **EDTA** – Ethylenediamine tetraacetic acid; ***Eef2*** – Eukaryotic elongation factor 2; **ELISA** – Enzyme-linked immunosorbent assay; **eNOS** – Endothelial nitric oxide synthase; **EtOH** – Ethanol; **FBS** – Fetal bovine serum; **FGF2** – fibroblast growth factor-2; **H&E** – Hematoxylin and eosin; **HMEC-1** – Human microvascular endothelial cells; **HMG-CoA** – 3-hydroxy-3-methylglutaryl coenzyme A; ***Hmox1,*** **HO-1** – Heme oxygenase-1; **HUVEC** – Human umbilical vein endothelial cells; **IACUC** – Institutional Animal Care and Use Committee; **IgA** – Immunoglobulin A; **IgG** – Immunoglobulin G; **IgM** – Immunoglobulin M; ***Kdr*** – Receptor for VEGF (VEGF-R2); **LDH** – Lactate dehydrogenase; **miR-1** – MicroRNA-1; **miR-133a** – MicroRNA-133a; **miR-206** – MicroRNA-206; ***Mmp11*** – Matrix metalloproteinase 11; **mSCs** – Muscle satellite cells; ***Myh3*****, eMyHC** – Myosin heavy chain 3, embryonic myosin heavy chain; **NO** – Nitric oxide; **OCT** – Optimal cutting temperature compound; **PBS** – Phosphate-buffered saline; **PCR** – Polymerase chain reaction; **qRT-PCR** – Quantitative PCR; **RT** – reverse transcription; **ROS** – Reactive oxygen species; **SEM** – Standard error of the mean; **SMASH** – Semi-automatic muscle analysis using segmentation of histology; ***SNORD68*** – Small Nucleolar RNA, C/D Box 68; **SPF** – Specific pathogen-free;

Spp1*, OPN** – Secreted phosphoprotein 1, osteopontin; ***Tgfb1 – Transforming growth factor beta 1; **WBC** – White blood cells; **WT** – Wild type; ***Vegfa*, VEGF** – Vascular endothelial growth factor A

Declarations

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Authors' contributions

OM performed the research, acquired and analyzed the data, wrote the first version of the manuscript; PP performed the research, acquired and analyzed the data, contributed to manuscript writing; KK performed the research, acquired and analyzed the data; JD discussed the data and edited the manuscript; AŁ supervised the study, designed the research, performed the research, interpreted the data, contributed to manuscript writing. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were conducted in accordance with national and European legislation, after approval by the 2nd Institutional Animal Care and Use Committee (IACUC) in Kraków, Poland (approval number: 323/2018).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Figures

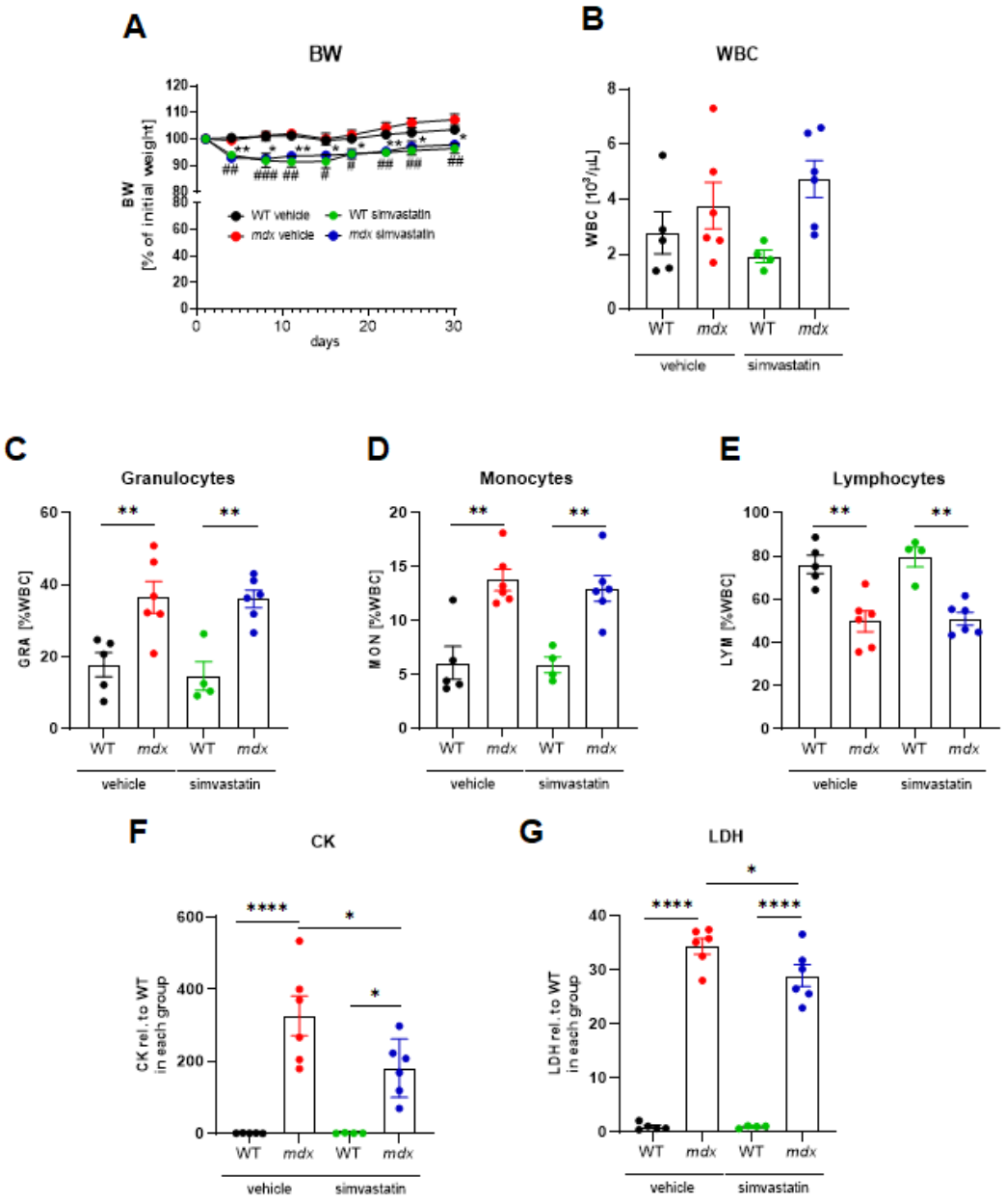

Figure 1.

Figure 1

Simvastatin treatment does not affect WBC abundance but leads to decreased CK and LDH activity. (A) Changes in the BW over the time of simvastatin treatment; n=5-7/group. Results presented as a percentage of initial weight (mean ± SEM); * $p \leq 0.05$, ** $p \leq 0.01$, WT simvastatin vs WT vehicle; # $p \leq$

0.05, ## p ≤ 0.01; ### p ≤ 0.001, mdx simvastatin vs mdx vehicle. (B) Unchanged number of WBC in the peripheral blood; n=5-6/group. Increased percentage of (C) granulocytes and (D) monocytes in dystrophic animals compared to the WT mice without the effect of simvastatin. (E) Diminished percentage of lymphocytes in dystrophic animals compared to the WT mice without simvastatin-related changes. (F) Increased serum activity of CK in mdx animals with a concomitant decrease in simvastatin-treated dystrophic mice; n=5-6/group. (G) Increased serum LDH activity in mdx mice declined after 28 days of simvastatin administration; n=5-6/group. Results shown as a mean ± SEM; * p ≤ 0.05, ** p ≤ 0.01, **** p ≤ 0.0001.

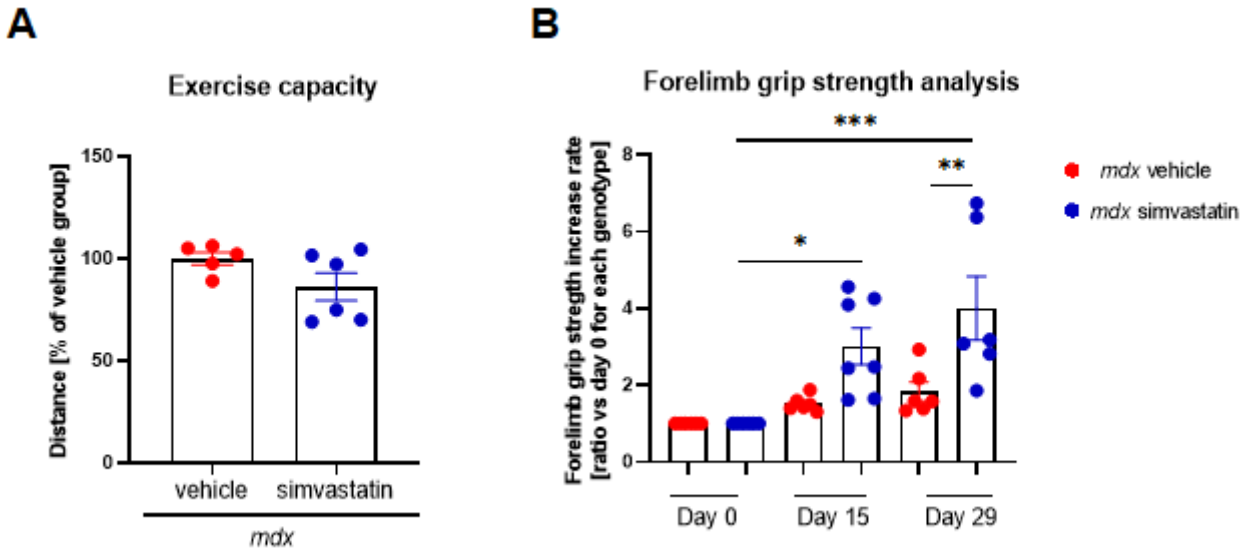

Figure 2

The rate of forelimb grip strength increase is elevated in dystrophic animals upon simvastatin treatment. (A) Downhill running treadmill test presented as the percentage of the running distance compared to vehicle-treated animals; n=5-6/group, mean ± SEM. (B) Forelimb grip strength analysis shown as a strength increase rate at day 15 and day 29 of statin delivery in comparison to day 0; n=5-7/group, mean ± SEM; * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001.

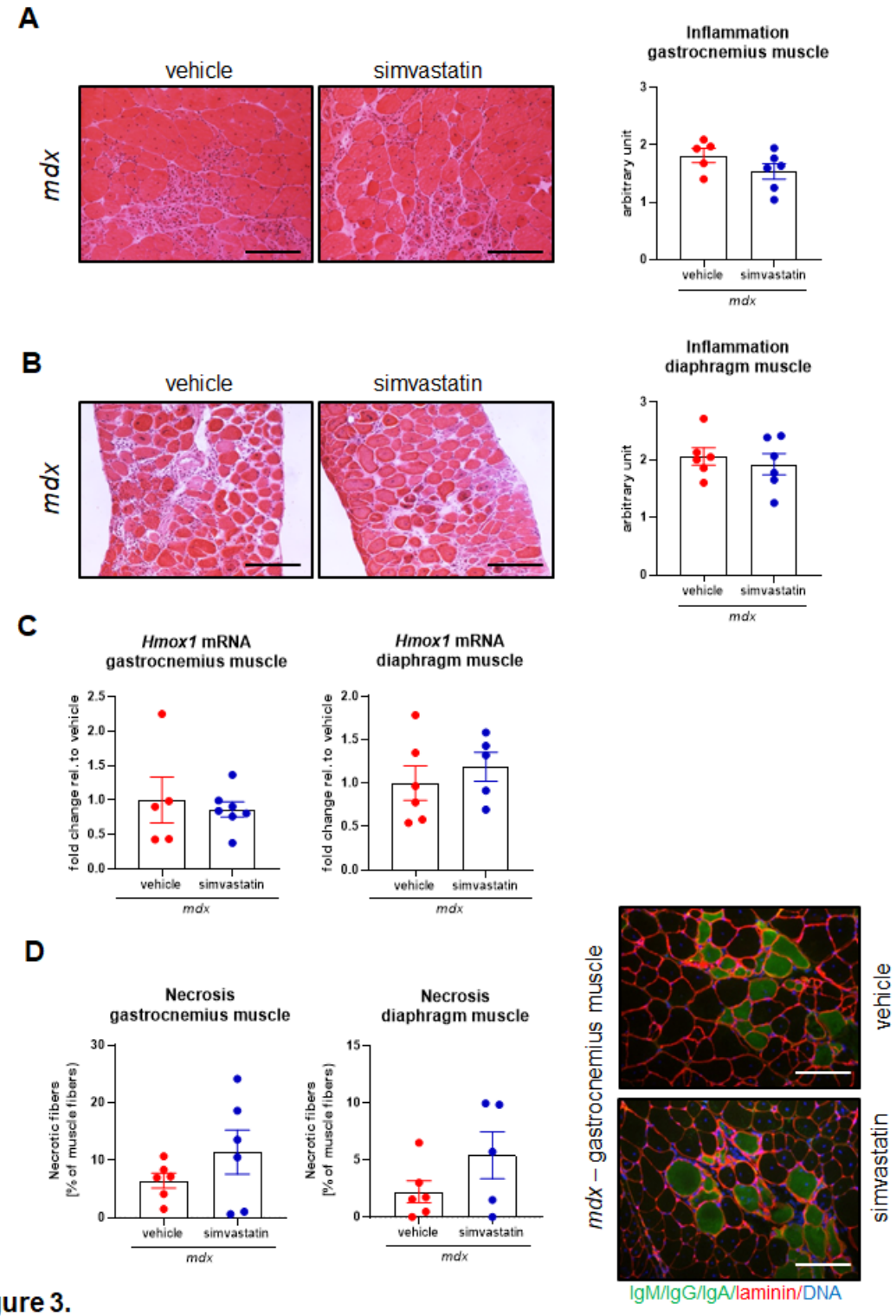

Figure 3.

Figure 3

Simvastatin treatment does not attenuate inflammation and necrosis in mdx mice. Representative pictures of H&E staining of (A) gastrocnemius muscle and (B) the diaphragm with semi-quantitative analysis of inflammation; scale bar: 100 μm; mean ± SEM; n=5-6/group. (C) Unaffected by simvastatin treatment expression of Hmox1 gene in gastrocnemius and diaphragm muscles, presented as a mean ± SEM, qRT-PCR. (D) Necrosis assessment using immunofluorescent staining of IgM/IgG/IgA binding

(green) with laminin (red) and its calculation presented as a mean ± SEM, indicating no differences between groups; n=5-6/group; scale bar: 100 µm.

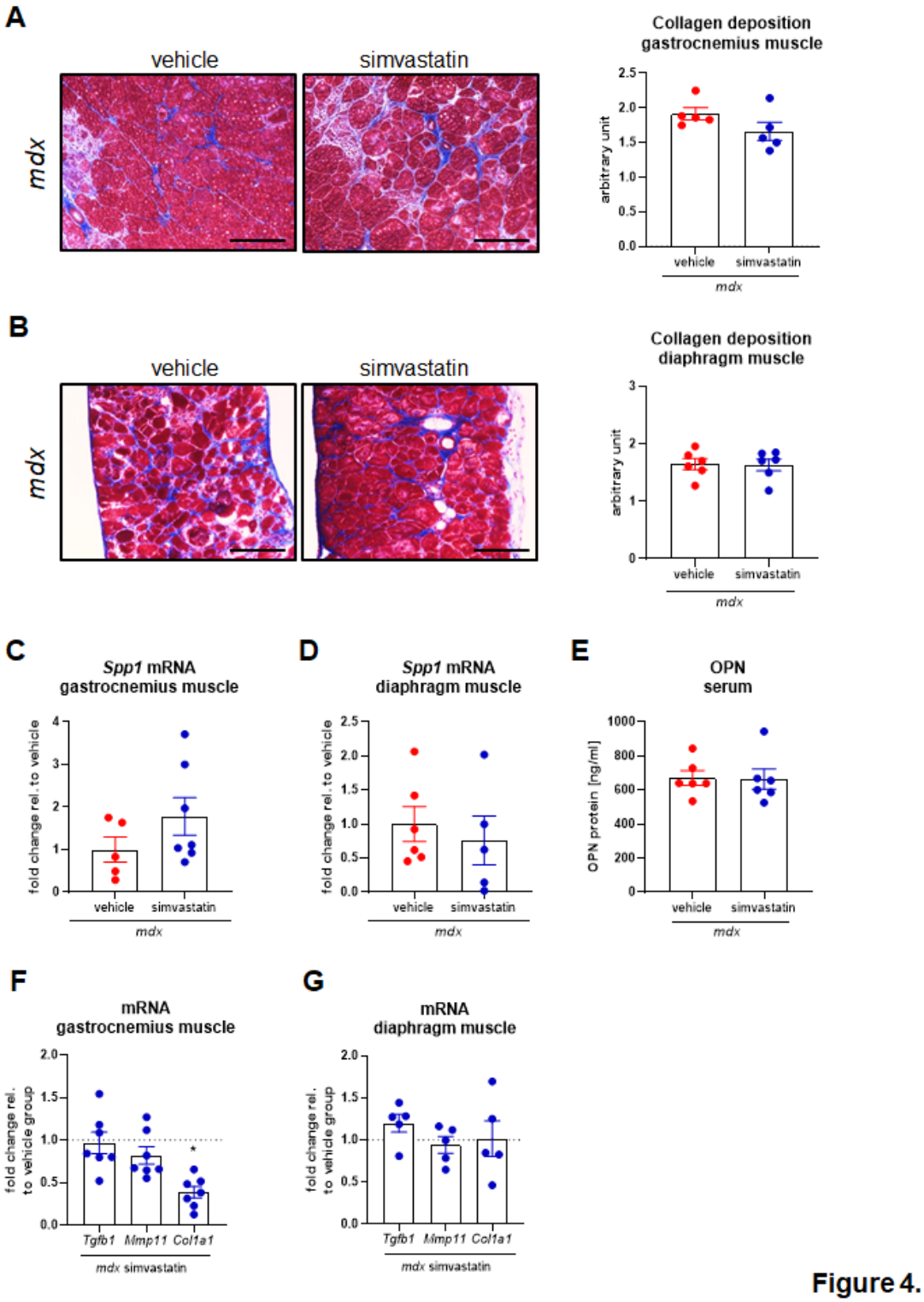

Figure 4.

Figure 4

Simvastatin treatment has no impact on fibrosis in dystrophic animals. Representative photos of Masson's trichrome staining with semi-quantitative analysis of collagen deposition showing no changes in the extent of fibrosis in (A) gastrocnemius and (B) diaphragm of simvastatin-treated animals; scale

bar: 100 μm; n=5-6/group. Unaffected by simvastatin treatment expression of Spp1 gene in (C) gastrocnemius and (D) diaphragm muscles, presented as a mean ± SEM, qRT-PCR. (E) No changes in the protein level of serum marker of fibrosis, OPN, n=6/group, mean ± SEM, ELISA. Unchanged expression of fibrotic markers: Tgfb1 and Mmp11, in (F) gastrocnemius and (G) diaphragm of mdx mice after statin administration and a significant decrease in Col1a1 mRNA in (F) gastrocnemius muscle, but not in (G) the diaphragm; n=5-7/group, vehicle level marked with the dotted line; qRT-PCR. Data are presented as mean ± SEM; * $p \leq 0.05$.

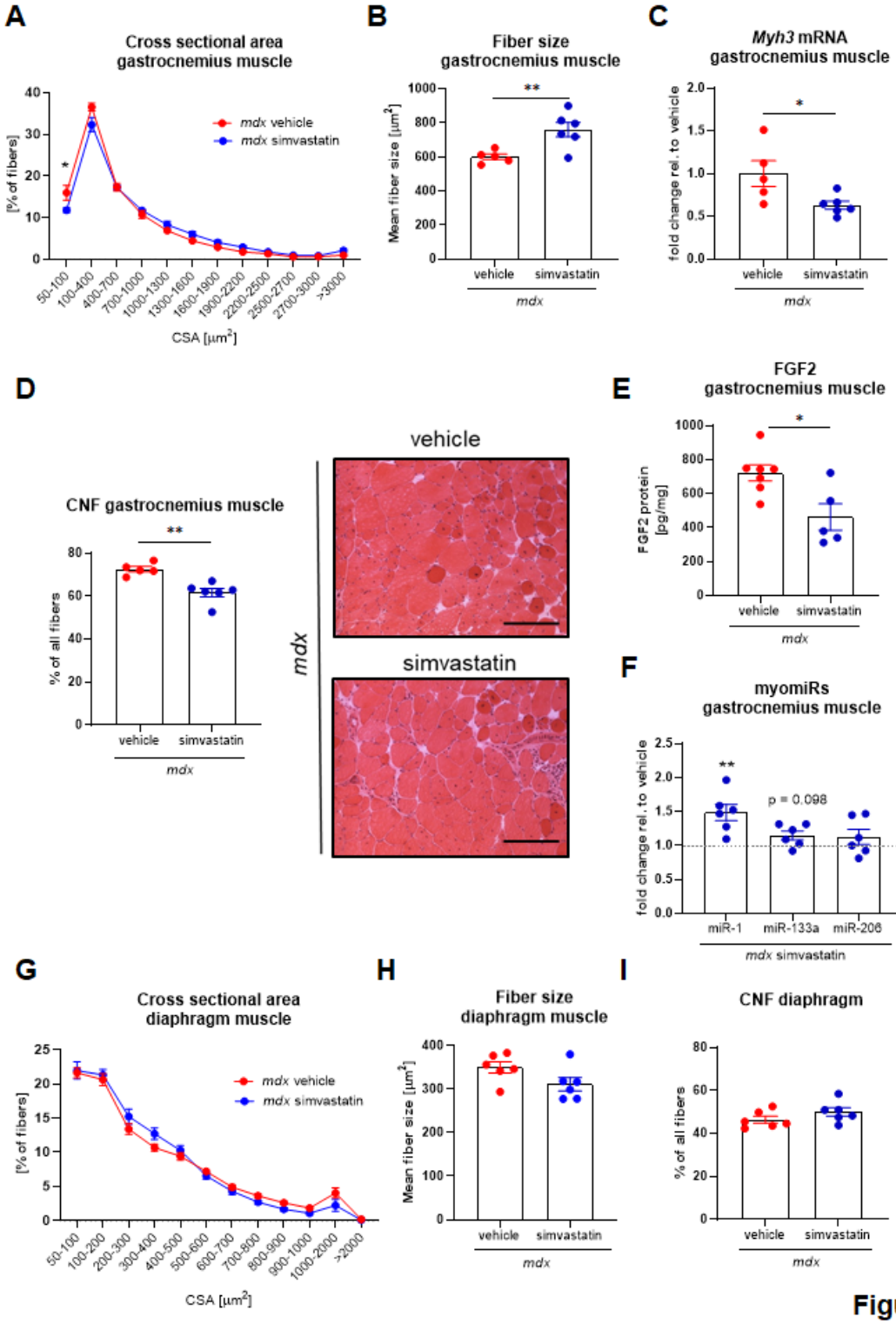

Figure 5.

Figure 5

Simvastatin treatment affects several aspects of muscle regeneration in mdx mice. (A, B) Quantification of muscle fiber size based on laminin staining (not shown) indicating no effect of simvastatin treatment; n=5-6/group; mean ± SEM; * $p \leq 0.05$, ** $p \leq 0.01$. (C) Decreased expression of myosin heavy chain isoform-coding gene: Myh3, presented as mean ± SEM; * $p \leq 0.05$, qRT-PCR. (D) Semi-quantitative and qualitative analysis of CNF performed based on H&E staining showing a drop in CNF number in mdx animals treated with simvastatin when compared to the vehicle group; scale bar: 100 μm; n=5-6/group; mean ± SEM; ** $p \leq 0.01$. (E) The diminished protein level of FGF2 in gastrocnemius muscle of dystrophic animals upon simvastatin treatment; n=5-7/group; presented as mean ± SEM; * $p \leq 0.05$, ELISA. (F) myomiRs: miR-1, miR-133a and miR-206 expression upon simvastatin treatment in gastrocnemius muscle of mdx animals presented as mean ± SEM; vehicle level marked with the dotted line; ** $p \leq 0.01$, LNA qRT-PCR. (G-H) No changes in diaphragm muscle fiber size and (I) percentage of CNF were observed in statin-treated mdx mice; presented as mean ± SEM.

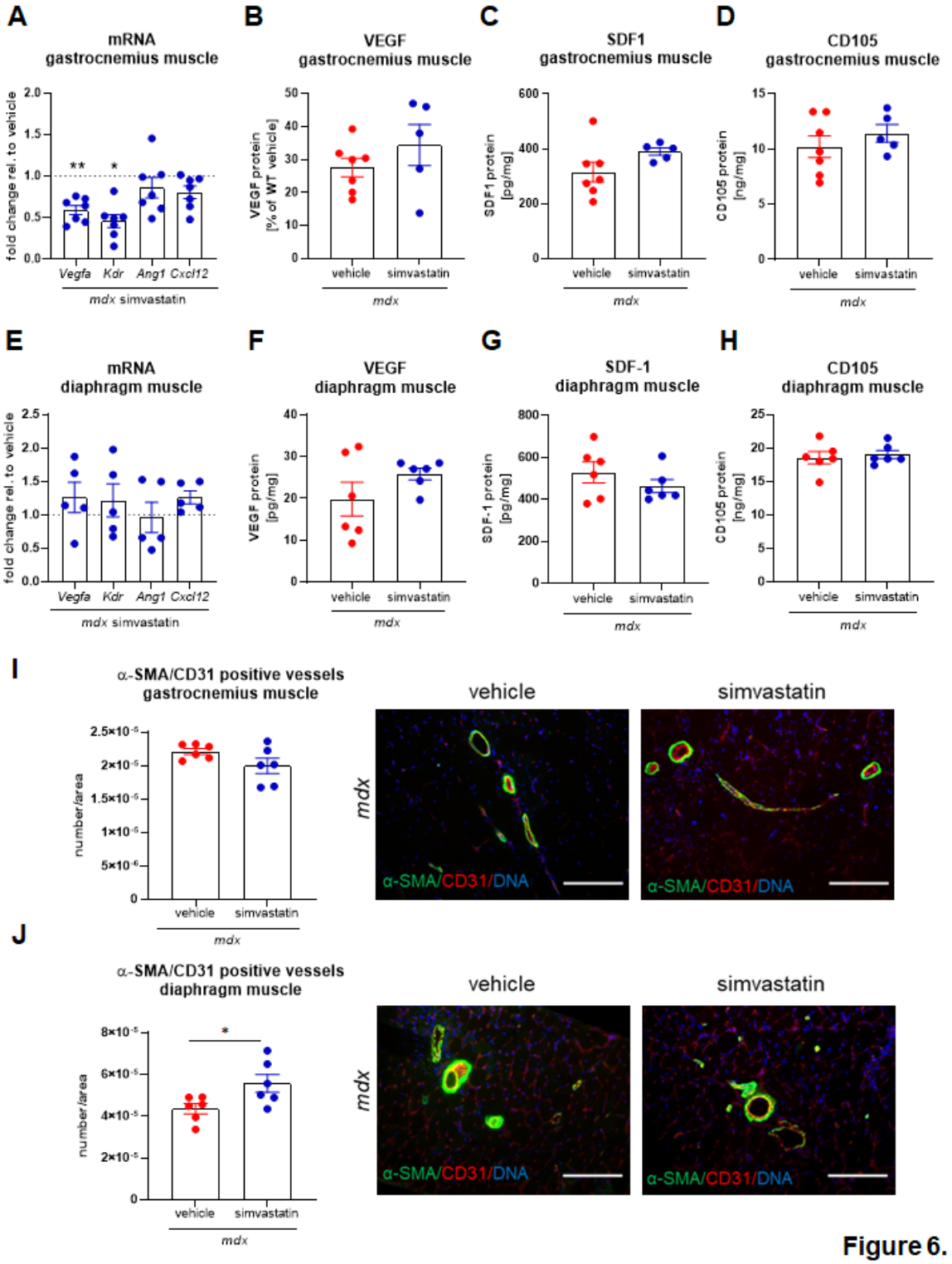

Figure 6.

Figure 6

Simvastatin treatment does not influence the angiogenic markers in dystrophic animals. (A) Decreased mRNA level of angiogenesis-related Vegfa and Kdr in gastrocnemius muscle of simvastatin-treated mdx mice and no changes in both Ang1 and Cxcl12; n=7/group; presented as mean ± SEM; vehicle level marked with the dotted line; * $p \leq 0.05$, ** $p \leq 0.01$, qRT-PCR. The unaffected protein level of (B) VEGF, (C) SDF1, and (D) CD105 in gastrocnemius muscle of statin-receiving animals; n=5-7/group; presented as

mean ± SEM, ELISA. Lack of the simvastatin effect on angiogenesis-related factors on (E) mRNA (Vegfa, Kdr, Ang1, and Cxcl12; vehicle level marked with the dotted line) and protein (F-H) (VEGF, SDF1, and CD105) level in diaphragm muscle; n=5-6/group; presented as mean ± SEM; qRT-PCR, ELISA. Semi-quantitative and qualitative analysis of blood vessels performed based on CD31/α-SMA double staining showing no difference in (I) gastrocnemius muscle of mdx animals treated with simvastatin when compared to the vehicle group and significant increase in (J) diaphragm muscle; n=6/group; presented as mean ± SEM; scale bar: 100 μm; * $p \leq 0.05$.

Supplementary Files

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- SupplFigure1statins01.01.21final.pptx