Regular paper

Lower levels of Caveolin-1 and higher levels of endothelial nitric oxide synthase are observed in abdominal aortic aneurysm patients treated with simvastatin

Karolina Kowalska[1,2], Dominika E. Habrowska-Górczyńska[2], Christoph Neumayer[1], Michael Bolliger[1], Christoph Domenig[1], Agnieszka W. Piastowska-Ciesielska[2], Ihor Huk[1] and Aleksandra Piechota-Polanczyk[3]✉

[1]Department of Surgery, Division of Vascular Surgery, Medical University of Vienna, Vienna, Austria; [2]Laboratory of Cell Cultures and Genomic Analysis, Department of Comparative Endocrinology, Medical University of Lodz, Łódź, Poland; [3]Department of Medical Biotechnology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Kraków, Poland

This study was undertaken to verify whether simvastatin modulates Cav-1/eNOS expression, and if this modulation is associated with changes in pro- and anti-inflammatory cytokine and Toll-like receptor 4 (TLR4) level in abdominal aortic aneurysm (AAA). It is a 1:2 case-control study of non-statin (n=12) and simvastatin-treated patients (n=24) who underwent open AAA repair. Simvastatin treatment decreased Cav-1 (p<0.05) and increased eNOS expression (p<0.01) in the AAA wall. These changes might be dose dependent. The changes in Cav-1 and eNOS were associated with a trend towards decreased IL-6 and IL-17 concentration (p>0.05) and increased IL-10 concentration (p=0.055); however, TLR4 expression was unaffected, suggesting that simvastatin influences Cav-1 and eNOS in the AAA wall by other mechanisms. Simvastatin may modulate Cav-1 and eNOS expression in the aneurysmal wall, indicating a potentially beneficial role for statins in AAA patients.

INTRODUCTION

Abdominal aortic aneurysm (AAA) is defined as a dilatation of the aorta to a diameter greater than 50% of normal (Johnston *et al.*, 1991); it typically occurs in the infrarenal aorta (Takayama & Yamanouchi, 2013) and in most cases is associated with atherosclerosis. A recent review of pharmaceutical treatments of AAA (Kokje *et al.*, 2015) found that statins may have a beneficial effect. Statins are widely known to modulate the lipid profile, but the recent discovery of their pleiotropic effects has triggered new lines of research which indicate they may also have beneficial applications in the treatment of

AAAs (Mastoraki *et al.*, 2012). Simvastatin, a commonly-used statin, was shown to possess a substantial potential for modulating interleukins IL-4, IL-6, IL-10, and tumor necrosis factor TNF-α (Piechota-Polanczyk *et al.*, 2012), as well as the matrix metalloproteinase MMP-9 (Evans *et al.*, 2007) and nuclear factor kappa B (NF-κB) in AAAs (Piechota-Polanczyk *et al.*, 2012).

Endothelial nitric oxide synthase (eNOS) is the main producer of nitric oxide (NO) in the endothelium. Turbulent flow conditions result in its uncoupling, triggering the endothelial cell dysfunctions observed in many cardiovascular diseases (Huk *et al.*, 1997; Kanai *et al.*, 1995). Although elevated eNOS expression has a beneficial effect in the pathogenesis of AAA, its role seems to be more complex (Piechota-Polanczyk *et al.*, 2015b). Under particular conditions, eNOS uncoupling might negatively correlate with the vasodilatory effect of NO and participate in AAA formation (Gao *et al.*, 2012). In addition, the eNOS deficiency seen in atherosclerosis has been found to cause spontaneous aortic aneurysms in ApoE knock-out mice (Kuhlencordt *et al.*, 2001).

Caveolin-1 (Cav-1) is a structural protein of the caveolae known to possess a functional role in cell signalling (Kowalska *et al.*, 2016). In endothelial cells, Cav-1 participates in the regulation of eNOS signalling via modulation of eNOS activation. Cav-1 expression is positively correlated with atherosclerosis and obesity (Singh *et al.*, 2011); it has been reported to modulate MMP-2 activity in heart tissue (Chow *et al.*, 2007) and influence AAA formation through its functional relationship with angiotensin II (Ang II) receptors (Kowalska *et al.*, 2016; Takayanagi *et al.*, 2014). It might also act as an immunomodulatory agent via its action on NF-κB and TLR4 (Mirza *et al.*, 2010), and may influence the effect of simvastatin (Almansob *et al.*, 2012): simvastatin has been found to potentially downregulate Cav-1 expression and increase eNOS expression in the abdominal aorta of animals (Arora *et al.*, 2012). Nevertheless, it is not known whether statins might cause a similar effect in AAA patients.

The aim of the study presented here was to determine whether simvastatin treatment changes the Cav-1/eNOS ratio in tissues of AAA patients. To verify the mechanism behind those changes, measurements were taken of the tissue levels of pro-inflammatory IL-6 and IL-17 and of anti-inflammatory IL-10, as well as the TLR4 receptor, which are known to regulate Cav-1 expression (Jones *et al.*, 2001; Weiss *et al.*, 2015).

MATERIALS AND METHODS

Patients. This study comprised 120 patients who underwent open AAA repair between September 2009 and December 2012 at the Department of Surgery, Medical University of Vienna, as previously described (Piechota-Polanczyk *et al.*, 2015a).

From that cohort, a group was formed of patients treated with simvastatin or who had taken no statins for at least six months before the AAA repair procedure. The patients were then matched according to the AAA diameter. Finally, 12 "non-statin" patients and 24 simvastatin-treated patients were chosen to study the effects of simvastatin on Cav-1 and eNOS in AAA.

The following patients were excluded: those who had taken statins other than simvastatin; those who had taken non-steroidal anti-inflammatory drugs, except aspirin, in the medication list; those with chronic diseases such as liver diseases, inflammatory diseases or malignant diseases; those who were recreational drug users; those who were abusing alcohol.

Patient data was prospectively collected after obtaining written informed consent, and aneurysm wall tissue was harvested during surgery for retrospective analysis. Patients were matched in a two (simvastatin) to one (non-statin) ratio by age, sex, and AAA diameter (in the listed order). Twelve non-statin patients (10 men, two women) were included in the control group, and 24 patients who had received simvastatin (22 men, two women) in their medical history (20–40 mg daily for a minimum of six months) were included to the simvastatin group. AAA diameter was measured with pre-operative computed tomography angiography.

The study was approved by the local institutional ethics committee (EC 294/2009) at the Medical University of Vienna.

Tissue harvesting. The AAA samples were collected as described previously (Piechota-Polanczyk *et al.*, 2013). After collection, the samples were immediately frozen in liquid nitrogen and stored at –80°C.

Enzyme-linked Immunosorbent Assays (ELISA) for eNOS, IL-6, IL-10, IL-17. First, 30 mg samples of frozen aortic wall were grinded and subjected to protein extraction with the Mammalian Cell Lysis Kit according to the manufacturer's instructions (Sigma-Aldrich, USA). Following this, ELISA kits were used to assay the eNOS, IL-10, IL-17 (R&D Systems, USA) and IL-6 (eBioscience, USA) content, according to the manufacturer's instructions. The samples were diluted where necessary. Absorbance was read at 450 nm (VICTOR3 1420, Perkin Elmer, USA). All assays were performed in duplicate.

Real-time quantitative polymerase chain reaction (RT-qPCR) for Cav-1, eNOS, TLR4. As with the protein analysis, 30 mg of frozen aortic wall was used for RNA extraction with RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA). RT-qPCR was conducted on an Applied Biosystems® 7500 Real-Time PCR Systems (Thermo Fisher Scientific) using a Power SYBR® Green PCR Master Mix according to the manufacturer's instructions (Thermo Fisher Scientific). Primers were designed using Primer3 version 0.4.0 software (http://frodo.wi.mit.edu/). *hEF-2*: Sense primer: 5'GAGATCCAGTGTCCAGAGCAG3', Antisense primer: 5'CTCGTTGACGGGCAGATAGG3'; *Cav-1*: Sense primer: 5'AGTGCATCAGCCGTGTC-TATTCCA3', Antisense primer: 5'TCTGCAAGTT-GATGCGGACATTGC3'; *TLR4*: Sense primer: 5'CA-

GAGTTTCCTGCAATGGATCA3', Antisense primer: 5'GCTTATCTGAAGGTGTTGCACAT3'; *eNOS*: Sense primer: 5'CCCGCTTCCTGTTTCTTAGT3', Antisense primer: 5'GGCACAGTCCCTTATGGTAAA3'. Eukaryotic translation elongation factor 2 (*hEF-2*) was used as a reference gene for analysis. qPCR data was analyzed according to the Roche algorithm (Pfaffl *et al.*, 2002). In order to avoid the detection of non-specific products for each reaction, melting curve analysis was performed. The relative expression of *Cav-1*, *TLR4* and *eNOS* was expressed as a ratio of the gene of interest to a reference gene for each sample. All experiments were run in triplicate.

Western blot for Cav-1, eNOS, TLR4. A 30 μg sample of protein was separated electrophoretically and transferred onto polyvinylidene fluoride membranes (PVDF) by wet transfer (Bio-Rad Laboratories, USA). Membranes were then blocked in 5% fat-free milk for one hour prior to an overnight incubation at 4°C with a primary rabbit anti-Cav-1 polyclonal antibody, rabbit anti-NOS3 polyclonal antibody and goat anti-TLR4 polyclonal antibody (dilution 1:200; Santa Cruz, respectively) diluted in 1% fat-free milk. Mouse anti-GAPDH monoclonal antibody was used as a reference (dilution 1:1 000; Santa Cruz).

Next, the membranes were washed three times for 15 minutes with Tris-buffered saline-Tween 20 (TBST) buffer, and incubated for one hour with secondary antibodies conjugated with alkaline phosphatase: anti-rabbit IgG, anti-mouse IgG, anti-goat IgG (dilutions 1:15 000; Sigma-Aldrich). The membranes were washed three times in TBST buffer, and the bands visualized using Novex®AP Chromogenic Substrate (Thermo Fisher Scientific). A densitometric analysis of protein expression levels was then conducted using ImageJ version 1.34 software (http://rsbinfo.nih.gov/ij/; USA). The results were calculated as the ratio of Cav-1, TLR4 and eNOS expression to the GAPDH expression. All experiments were conducted in triplicate for all patients.

Statistical analysis. Continuous demographic and biochemical data is presented as median, minimum and maximum, or mean ±S.E.M. demographic categorical data was described with absolute frequencies and percentages. Comparisons between groups were performed using the Kruskal-Wallis test (or non-parametric Mann-Whitney U-test) and $\chi 2$ test depending on the normality of distribution. Two-way analysis of variance and the Dunns *post hoc* test were used to calculate differences. To calculate correlations, Spearman's rank correlation coefficient (r) test was used. Grubbs' test was performed to identify statistically significant outliers ($p<0.05$), and these were excluded from the statistical analysis of the results (GraphPad Prism software).

RESULTS

Patients' characteristics

The non-statin and simvastatin patient data is presented in Table 1. The median aneurysm diameter was 56 mm (49–65 mm) in the non-statin group and 55.5 mm (48–120 mm) in the simvastatin group. The patients receiving statins had significantly lower total cholesterol ($p=0.026$) and LDL cholesterol levels ($p=0.012$). No differences in C-reactive protein (CRP), fibrinogen, creatinine, haemoglobin, or leukocyte levels were noticed (all $p\geq0.05$).

Table 1. Patient characteristics.
Patient characteristics are presented in the table. Data is presented as frequencies or median (minimum-maximum). Statistical significance for binary variables was assessed using generalized linear models, while metric values were analysed using linear mixed regression models. P-value $p < 0.05$ was considered as statistically significant and is indicated in bold.

	Non-statin (n=12)	Simvastatin (n=24)	P value
Age (range)	69 (50–75)	68 (55–80)	1.000
AAA diameter [mm] (range)	56 (49–65)	55.5 (48–120)	0.710
Body mass index [kg/m2] (range)	27.5 (23–37)	26.0 (22.0–34.5)	0.282
Coronary artery disease	4/12 (33%)	5/24 (21%)	0.815
Cerebrovascular artery disease	8/12 (67%)	8/24 (33%)	0.239
Hypertension	10/12 (83%)	21/24 (87%)	0.867
Nicotine	8/12 (67%)	16/24 (67%)	0.780
CRP [mg/dl] median (range)	0.35 (0.03–3.0)	0.33 (0.06–3.28)	0.804
Cholesterol [mg/ml] median (range)	236.5 (143.0–267.0)	192.5 (110.0–264.0)	0.026
HDL [mg/ml] median (range)	46.0 (32.0–59.0)	47.0 (29.0–75.0)	0.679
LDL [mg/dl] median (range)	141.7 (79.2–181.0)	110.0 (56.0–169.0)	0.012
Creatinin [mg/dl] median (range)	1.15 (0.76–1.48)	1.1 (0.78–4.0)	0.703
Haemoglobin [g/dl] median (range)	14.0 (13.0–15.7)	13.6 (12.4–18.0)	1.000
Leucocytes [mln/ml] median (range)	8.0 (6.0–11.0)	7.2 (5.3–13.0)	0.251
Fibrinogen [mg/dl] median (range)	391.0 (260.0–549.0)	347.0 (213.0–581.0)	0.136

Simvastatin decreases Cav-1 expression and dose-dependently increases eNOS in AAA wall

eNOS and CAV-1 expression was evaluated on the protein and mRNA level. As Cav-1 is present at high levels in most of the cells, Western blot was sufficient to evaluate its level.

Our results indicated that simvastatin treatment modulates Cav-1 expression at the gene and protein level in the AAA wall (Fig. 1). After simvastatin treatment, expression of the Cav-1 gene and protein was approximately half that (45%) of the non-treated group (p=0.033, Fig. 1). Additionally, a daily dose of 20 mg simvastatin significantly decreased Cav-1 protein expression ($p<0.05$) when compared to controls; however, no dose-dependent effect was visible.

In the caveolae, Cav-1 blocks the action of eNOS by binding to it. A decrease in Cav-1 was accompanied by a significant increase in eNOS: $p<0.01$ and $p<0.001$ for 20 mg and 40 mg of simvastatin, respectively (Fig. 2B). No significant change in *eNOS* expression was observed at the gene level (Fig. 2A).

A statistically significant correlation was observed between the expression of Cav-1 and eNOS in simvastatin-treated patients (p=0.042, Table 2) No other correlation between Cav-1, eNOS and cytokines was observed.

Simvastatin's influence on Cav-1 and eNOS is not associated with changed cytokine levels in AAA wall

To confirm whether a decrease in Cav-1 and an increase in eNOS is associated with changes in the pro- or anti-inflammatory cytokine profiles, the levels of IL-6, IL-17 and IL-10 were analyzed (Fig. 3). Lower levels of IL-6 tended to be observed in patients with higher doses of simvastatin (40 mg), although this relationship was not significant (448.3±72.37 pg/ml in control vs.

293.8±120.7 pg/ml in simvastatin, p=0.055) (Fig. 3A). Likewise, there was a tendency towards a lower IL-17 content following simvastatin treatment (37% reduction): 60.21±16.12 pg/ml in controls *vs.* 38.31±7.624 pg/ml in the simvastatin group (Fig. 3B). In addition, the simvastatin group demonstrated a strong, dose-dependent tendency towards increasing IL-10 levels (Fig. 3C): Simvastatin at a dose of 40 mg reduced the IL-10 level by 40% when compared to controls (p=0.055).

Simvastatin treatment does not influence TLR4 expression in the AAA wall

Changes in Cav-1 are known to be associated with TLR4 stimulation (Wang *et al.*, 2009). Therefore, in this

Table 2. Correlations of Cav-1, eNOS and cytokines in the simvastatin study group.
The correlation of the expression of Cav-1 assessed by Western blot, eNOS expression assessed by ELISA, *Cav-1* and *eNOS* gene expression assessed by RT-qPCR and cytokine level assessed by ELISA, were made with Spearman's correlation test calculated for the study group (simvastatin). $P<0.05$ was considered as statistically significant and is marked in bold, vs., versus, R_s-Spearman rank correlation coefficient.

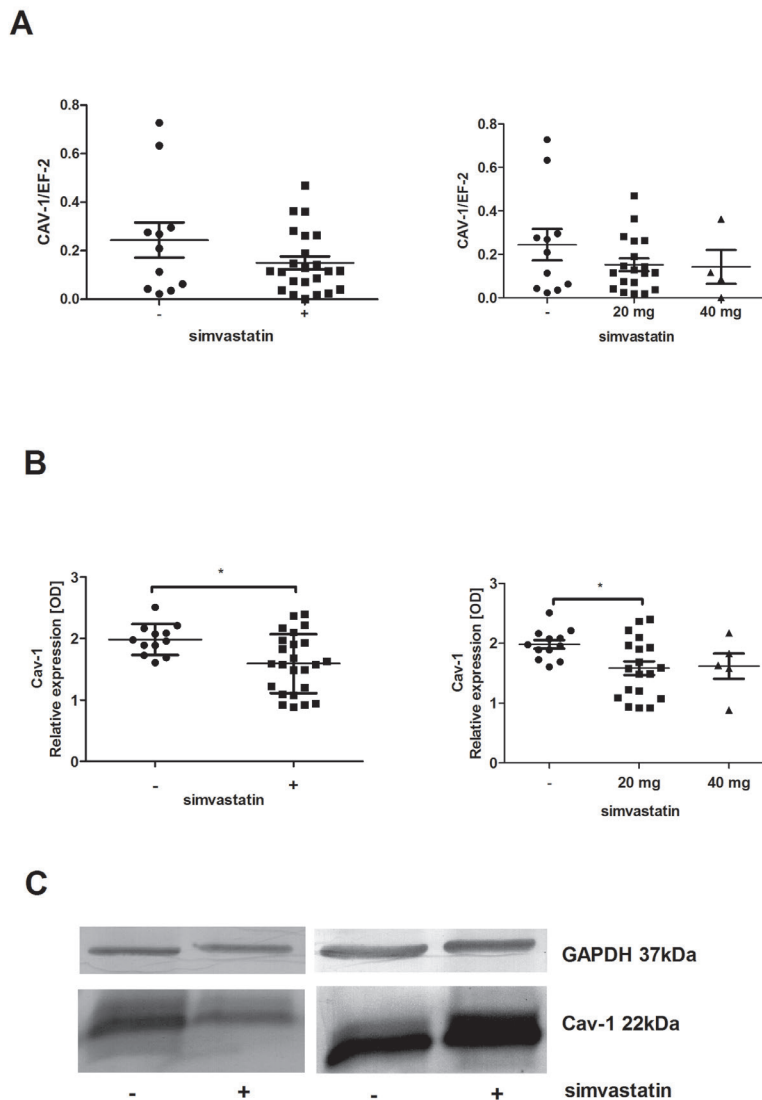|	R_s	P value
Cav-1 *vs.* eNOS	−0.1544	0.4583
IL-6 *vs.* eNOS	−0.09	0.6688
IL-10 *vs.* eNOS	0.09652	0.6537
IL-17 *vs.* eNOS	−0.04162	0.8434
Cav-1 vs. eNOS	0.2243	0.3035
IL-6 *vs.* Cav-1	−0.1157	0.5905
IL-10 *vs.* Cav-1	0.1937	0.3759
IL-17 *vs.* Cav-1	0.1726	0.4199

Figure 1. Simvastatin influence on the Cav-1 gene (A) and protein (B) expression.
The results from representative Western blots are presented in panel (**C**). Gene expression was evaluated with RT-qPCR, whereas protein expression was evaluated by the Western blot technique. Results are presented as mean ±S.E. *$p<0.05$; Cav-1, caveolin-1; EF-2, elongation factor 2; O.D., optical density.

study we examined whether simvastatin treatment influences the observed changes in Cav-1 and eNOS by influencing the TLR4 expression. However, simvastatin was not found to have any significant effect on TLR4 (Fig. 4).

DISCUSSION

Our study demonstrates that simvastatin treatment decreases Cav-1 level but significantly increases eNOS expression in patients with AAA. At rest, eNOS is inactive and anchored to the caveolae by Cav-1. A recent study found an elevated Cav-1 gene and protein expression to be associated with impaired eNOS activity in cardiovascular diseases (Ricchiuti *et al.*, 2011). In addition, Cav-1 and eNOS gene polymorphisms have been identified in patients with arterial diseases, suggesting that these genes play a role in the development of intima thickness (Testa *et al.*, 2012). Cav-1 was also proposed to play a critical role in a mouse model of AAA formation, as Cav-1 knockout

mice did not develop Ang II-induced AAA (Takayanagi *et al.*, 2014); the authors suggest that Cav-1 may induce an increase in the level of the ADAM17 metalloprotease, which further activates TNF-α and epithelial growth factor receptor, leading to AAA development (Takayanagi *et al.*, 2014).

In addition, a decrease in Cav-1 and an increase in eNOS has been reported in endothelial tissue rather than in smooth muscle cells in rabbits on a high-fat diet following simvastatin treatment (Arora *et al.*, 2012); however, no change in eNOS phosphorylation was detected, suggesting a possible role might be played by other mechanisms related to the antioxidative role of statins or accumulation of cGMP, which is an indicator of eNOS-dependant NO bioavailability (Meda *et al.*, 2010; Piechota-Polanczyk *et al.*, 2012).

Our findings indicate that simvastatin has a dose-dependent influence on eNOS but not on Cav-1. Patients treated with 40 mg of simvastatin demonstrated a significantly higher eNOS protein expression than those
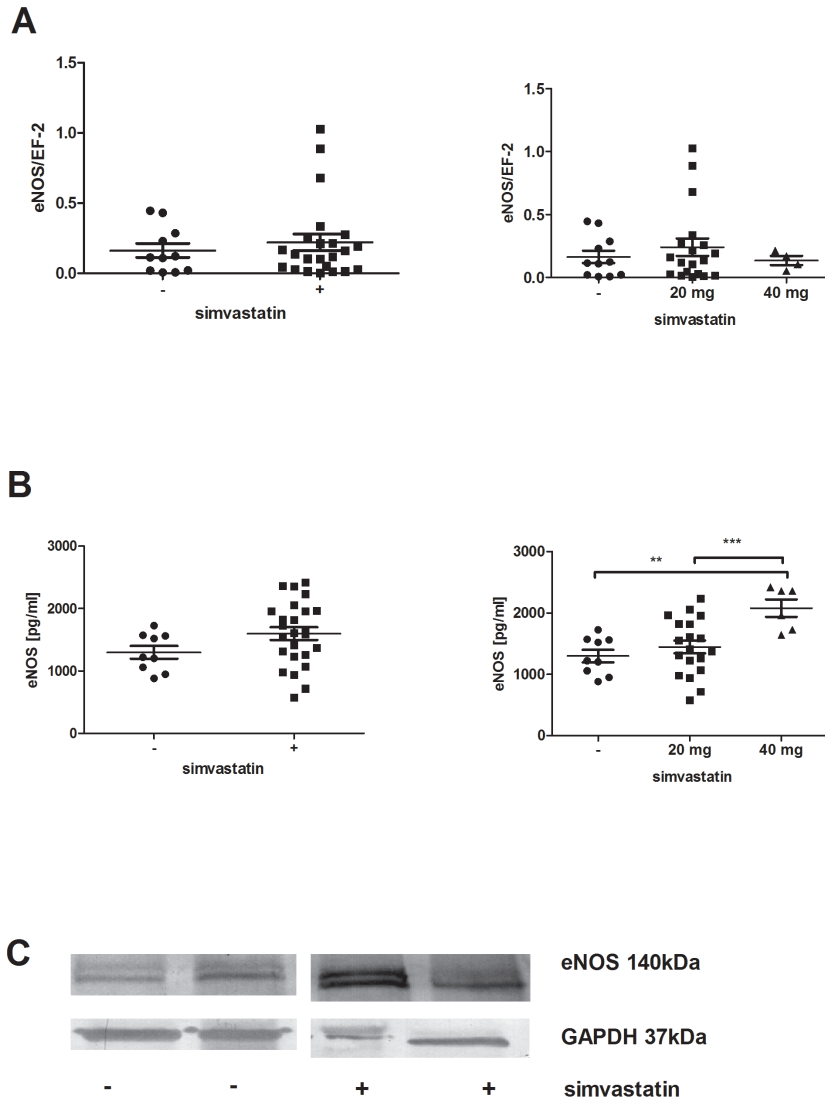
A

B

C

eNOS 140kDa

GAPDH 37kDa

−   −   +   +   simvastatin

Figure 2. Simvastatin influence on the eNOS gene (A) and protein (B) expression indicated a dose-dependent increase in eNOS.
The results from a representative Western blot are presented in panel (**C**). The result were obtained by RT-qPCR, ELISA and Western blot techniques and are presented as mean ±S.E. **$p<0.01$; ***$p<0.001$; eNOS, endothelial nitric oxide synthase; EF-2, elongation factor 2; O.D., optical density.

treated with 20 mg. Previous studies have also found statins to have a similar effect on endothelial cells, where simvastatin induced eNOS phosphorylation in a dose-dependent manner, leading to further activation of Akt and AMPK (Hou *et al.*, 2015). Those two intracellular proteins regulate eNOS activity (Ching *et al.*, 2011; Sun *et al.*, 2006).

However, the lack of dose dependence observed in our study for Cav-1 may be partially associated with the low number of patients treated with 40 mg of simvastatin, as a partial trend was visible. Nonetheless, it should be stated that the effect of statins strongly depends on the cell type and the type of statin used. For example, simvastatin at a dose of 10 μM increases eNOS and [NO]/[NOO⁻] balance in human umbilical endothelial cells to the same extent as atorvastatin used at half of that dose (5 μM) (Heeba *et al.*, 2007). Furthermore, statins may have an anti- or pro-oxidative effect depending on their concentration and the type of cells on which they are acting (Loboda *et al.*, 2006). The effect

of simvastatin may also vary depending on the administered dose. For instance, studies on tumor vascularization found mice treated with a low dose of simvastatin (0.2 mg/kg) to have augmented eNOS phosphorylation when compared to mice treated with a higher dose (10 mg/kg); however, the higher dose was also associated with a significantly lower level of oxidative stress (Chen *et al.*, 2013). Therefore, both the type and dose of statin may influence the results associated with the effect of simvastatin on Cav-1 and eNOS obtained in the study presented here.

Various factors regulate the changes in Cav-1 and eNOS, including cytokines. However, our results indicate that simvastatin treatment had no effect on the expression of pro-inflammatory IL-6, IL-17 or anti-inflammatory IL-10, three cytokines which play a key role in the development of AAA (Johnston *et al.*, 2013; Jones *et al.*, 2001). Despite this, previous studies have found that pro-inflammatory cytokines, especially IL-6, are associated with AAA formation and that lack of Cav-1 decreases
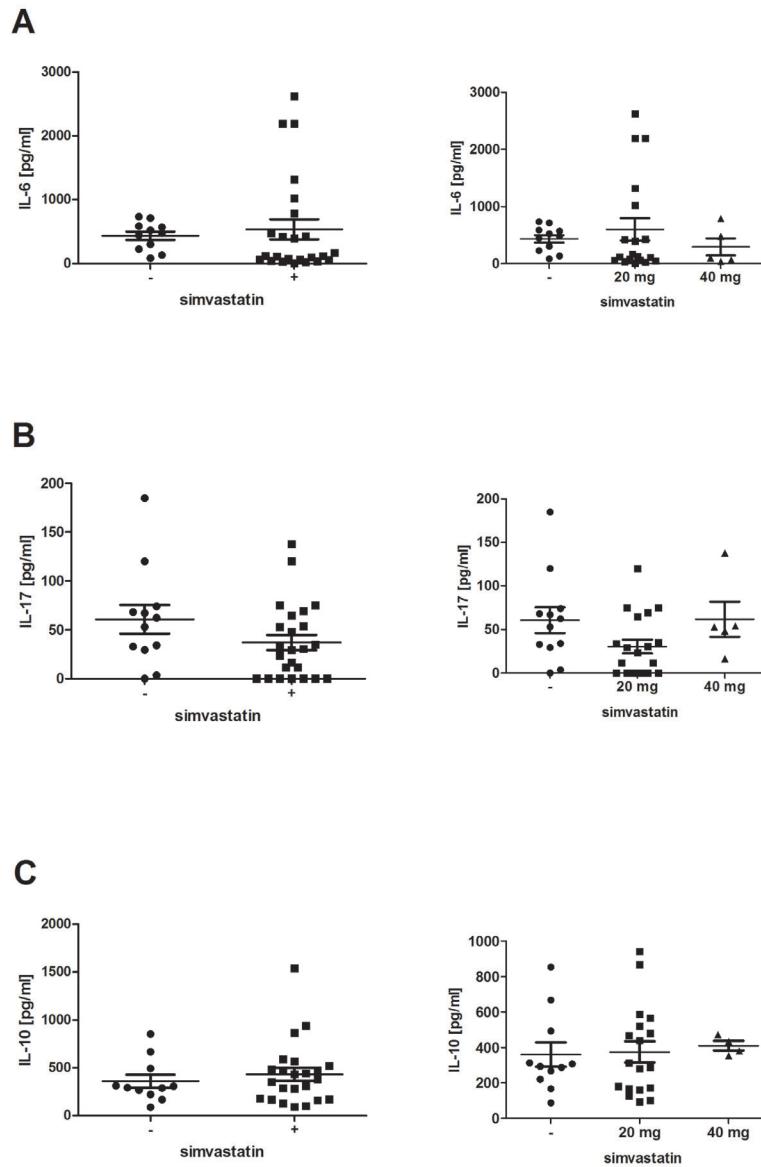
A

B

C

Figure 3. Simvastatin influence on IL-6 (A) IL-17 (B) and IL-10 (C) indicated no influence of simvastatin on those cytokines.
Results are presented as mean ±S.E. in pg/ml after adjustment to protein concentration obtained by ELISA tests. IL, interleukin.

IL-6 level in the vascular media layer (Takayanagi *et al.*, 2014). In addition, a negative correlation has been observed between Cav-1 and IL-17 in mice with ulcerative colitis (Weiss *et al.*, 2015).

Additionally, the results of clinical trials indicate that patients subjected to non-coronary artery cardiac surgery demonstrated a reduced inflammatory response following perioperative, but not postoperative, treatment with simvastatin (20 mg); this was reflected by lower IL-6, IL-8 and Cav-1 levels but higher eNOS expression and NO production. Increased eNOS levels were associated with higher Akt phosphorylation, while decreased Cav-1 was accompanied by reduced phosphorylation of p38 of MAPK in the heart tissue (Almansob *et al.*, 2012). Therefore, the influence of simvastatin on Cav-1 and eNOS in acute inflammatory response in these states may be associated with changes in pro-inflammatory cytokine levels.

Finally, we wanted to verify if simvastatin treatment may change the TLR4 expression, which has been

shown to regulate Cav-1 in cells (Wang *et al.*, 2009). However, no difference in the TLR4 gene or protein expression was observed in the AAA tissue of the analysed groups. Hence, our results are in line with Azor *et al.* (Azor *et al.*, 2011) who found that simvastatin or lovastatin administration had no influence on the TLR4 level in chronic idiopathic urticaria. In contrast, Moutzouri and coworkers (2012) report that a high dose of simvastatin (40 mg) or the combination of a low dose of simvastatin/ezetimibe (10/10 mg) reduces TLR2 and TLR4 membrane expression in patients with hypercholesterolemia treated with lipopolysaccharide (LPS). Also, at a dose of 80 mg/day for four days, simvastatin reduced the influence of LPS-induced elevated TLR2 and TLR4 level on circulating monocytes and suppressed the activity of TNF-α and monocyte chemoattractant protein-1 (Niessner *et al.*, 2006). The protective effect of statins on TLR4 receptors may be associated with the attenuation of the TLR4/NF-kB-mediated inflam-
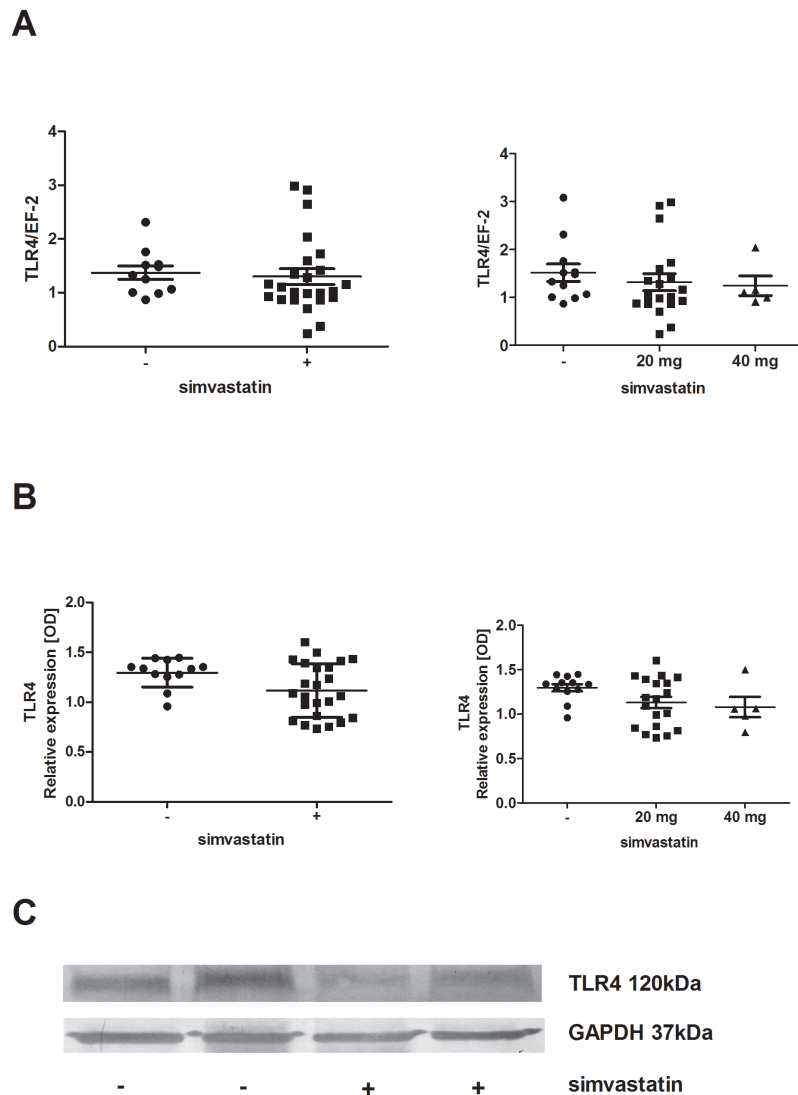
A

B

C

Figure 4. Simvastatin influence on the TLR4 gene (A) and protein (B) expression indicated no influence of the statin on the TLR4 expression.
The results from a representative Western blot are presented in panel (**C**). The results were obtained by RT-qPCR, ELISA and Western blot technique and are presented as mean ± S.E. TLR4, toll-like receptor type 4; EF-2, elongation factor 2; O.D., optical density.

matory response, as shown for murine models of brain injury and lung inflammation (Chen *et al.*, 2009; Shao *et al.*, 2007).

To summarise, our findings indicate that patients displayed a significantly lower Cav-1 and higher eNOS expression in AAA tissue following simvastatin treatment. Therefore, simvastatin treatment may affect the Cav-1 and Cav-1/eNOS ratio in the AAA wall, suggesting that statins may play a potentially beneficial role in AAA patients. Nevertheless, further studies are necessary to verify the exact mechanisms behind those observations.

Acknowledgements

Conflicts of Interest

The authors declare no conflicts of interest.

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