

Original Research - Special Issue: Intervertebral Disc Regeneration

Angiotensin II type 1 receptor antagonist Losartan inhibits TNF- α induced inflammation and degeneration processes in human nucleus pulposus cells

Babak Saravi^{1,2§}, Zhen Li^{1§}, Judith Pfannkuche^{1,2§}, Laura Wystrach², Christoph E. Albers³, Sibylle Grad¹, Mauro Alini¹, R. Geoff Richards¹, Corinna Lang^{4,5}, Norbert Südkamp², Hagen Schmal², and Gernot Lang^{2*}

¹ AO Research Institute Davos, Clavadelerstrasse 8, 7270 Davos, Switzerland

² Department of Orthopedics and Trauma Surgery, Medical Centre - Albert-Ludwigs-University of Freiburg, Faculty of Medicine, Albert-Ludwigs-University of Freiburg, Hugstetterstrasse 55, 79106 Freiburg, Germany

³ Inselspital, Department of Orthopedic Surgery, University of Bern, Freiburgstrasse 18, 3010 Bern, Switzerland

⁴ Department of Cardiology and Angiology I, Faculty of Medicine, Heart Center Freiburg University, University of Freiburg, Hugstetter Str. 55, 79106 Freiburg, Germany

⁵ Department of Medicine III (Interdisciplinary Medical Intensive Care), Medical Center, Faculty of Medicine, University of Freiburg, Hugstetter Str. 55, 79106 Freiburg, Germany

*Correspondence: Gernot.Lang, gernot.michael.lang@uniklinik-freiburg.de

§These authors contributed equally to this manuscript.

Abstract:

Our recent study detected the expression of a tissue Renin-Angiotensin System (tRAS) in human intervertebral discs (IVDs). The present study sought to investigate the impact of angiotensin II receptor type 1 (AGTR1) antagonist losartan on human nucleus pulposus (NP) cell inflammation and degeneration induced by tumor necrosis factor- α (TNF- α).

Human NP cells (4 donors, Pfirrmann grade 2-3, 30-37 years old, male) were isolated and expanded. TNF- α (10 ng/mL) was used to induce inflammation and degeneration. We examined the impact of losartan supplementation and measured gene expression of tRAS, anabolic, catabolic, and inflammatory markers in NP cells after 24 h and 72 h of exposure, respectively. T0070907, a PPAR- γ antagonist, was applied to examine the regulatory pathway of losartan.

Losartan (1 mM) significantly impaired TNF- α induced increase of pro-inflammatory (nitric oxide and TNF- α), catabolic (matrix metalloproteinases), and tRAS (AGTR1a and Angiotensin Converting Enzyme) markers. Further, losartan maintained the NP cell phenotype by upregulating aggrecan and downregulating collagen type I expression.

In summary, losartan showed anti-inflammatory, anti-catabolic, and positive phenotype modulating effects on human NP cells. These results indicate that tRAS signaling plays an important role in IVD degeneration, and tRAS modulation with losartan could represent a novel therapeutic approach.

Keywords: Intervertebral Disc, Renin-Angiotensin-System, Degeneration, Regeneration, Spine, Inflammation

1. Introduction

Low back pain (LBP) is one of the most prevalent musculoskeletal conditions and a leading cause of disability worldwide, resulting in an enormous socioeconomic burden [1]. Symptomatic intervertebral disc degeneration (IDD) is a major contributor to LBP [2]. IVD degeneration features extracellular matrix (ECM) degradation, the release of pro-inflammatory cytokines, altered spine biomechanics, angiogenesis, and neoinnervation, altogether causing discogenic pain and disability [3,4]. Pro-inflammatory cytokines regulate the expression of major catabolic enzymes, leading to progressive ECM deterioration. Several studies have shown that pro-inflammatory markers, such as tumor necrosis factor- α (TNF- α) and interleukin (IL)-6, are highly expressed in degenerated and symptomatic human IVD cells [4,5].

At present, none of the available treatment options addresses the underlying pathologic changes leading to IDD. Many symptomatic patients in the early stages of IVD degeneration do not benefit from conservative treatments and, at the same time, do not qualify for surgery [6]. Surgical techniques such as spinal fusion or total disc replacement have been established and implemented to treat symptomatic patients, although the long-term benefit compared to conservative therapies remains elusive [7–10]. In lack of a sufficient self-repair capacity of the IVD and satisfactory treatment options, new biomolecular therapies targeting the inflammatory and catabolic circles are considered lately [11]. These strategies try to reduce the inflammatory microenvironment of the IVD in order to slow down the progressive degenerative cascade [12–15].

Recently, we showed that the tissue Renin-Angiotensin System (tRAS) is expressed in human IVDs, revealing a positive correlation with pro-inflammatory cytokines and catabolic enzymes [16]. The major effector of tRAS is angiotensin 2 (AngII), which participates in pro-inflammatory pathways. The precursor protein angiotensinogen (ATG) is cleaved by the protease renin to form biologically inactive AngI, which is converted to active AngII by angiotensin-converting enzyme (ACE). RAS inhibitors belong to the most commonly prescribed drugs globally [17]. Recent research has raised evidence that ACE inhibitors could diminish TNF- α production *in vivo* and *in vitro* [18]. Price et al. demonstrated in a rodent model of rheumatoid arthritis (RA) that TNF- α release and subsequent knee joint swelling were significantly inhibited by the application of ACE inhibitors [19]. Other groups confirmed these findings, revealing beneficial anti-arthritic effects of ACE inhibitors and angiotensin II receptor type 1 blockers (ARBs) by reducing inflammation, neutrophil recruitment, hypernociception, disease activity, oxidative stress levels, and ultimately joint destruction [20–33]. Moreover, renal injury models revealed that inhibition or reduction of AngII actions via ACE inhibitors or ARBs reduced proteinuria, recruitment of pro-inflammatory cells, fibrosis, and catabolic gene expression [34–36].

We hypothesize that in human IVD cells, the expression of local RAS components might contribute to disc degeneration, inflammation, and discogenic pain. As inflammation contributes to IDD, the present study sought to investigate whether inhibition of the angiotensin II receptor type 1 (AGTR1) would have a protective effect on IVD tissue biology. Specifically, we investigated potential protective effects of angiotensin II receptor type 1 antagonist losartan on human nucleus pulposus (NP) cell inflammation and degeneration induced by TNF- α .

2. Materials and Methods

Isolation and expansion of human NP cells

Human NP waste tissue was collected from patients who underwent spinal surgeries with written consent (4 donors, male, 31-37 years old, Pfirrmann grade II). The collected NP tissue was incubated with red blood cell lysis buffer (155 mM NH_4Cl , 10 M KHCO_3 , and 0.1 mM EDTA in Milli-Q water) for 5 min on a shaker at room temperature, then washed with phosphate-buffered saline (PBS). Chopped tissue was then enzymatically digested with 0.2 % w/v pronase (Roche) in Alpha Minimum Essential Medium (α MEM, Gibco) for 1 h, followed by 65 U/mL collagenase type II (Worthington) in α MEM/10 % Fetal Calf Serum (FCS, Corning) at 37 °C for 12-14 h. A single-cell suspension was obtained by filtering through a 100 μm cell strainer. Next, cells were seeded at a density of 10,000 cells/ cm^2 and expanded with α MEM supplemented with 10 % FCS, 100 U/mL penicillin and 100 mg/mL streptomycin (1 % P/S, Gibco). Cells were cultured at a hypoxic condition of 2 % O_2 . The culture medium was changed twice a week, and cells were detached at approximately 80 % confluence using a dissociation buffer composed of 0.05 % trypsin/EDTA (Gibco) for 5 min at 37 °C. Cells were sub-cultured at a cell density of 3,000 cells/ cm^2 for expansion. Passage 1 NP cells were cryopreserved in liquid N_2 . After thawing, NP cells were expanded with high glucose Dulbecco's Minimum Essential Medium (high glucose DMEM, Sigma-Aldrich, St. Louis, MO, USA) and 10% FCS. Passage 3 NP cells were used in the present study.

Cytotoxicity test of Losartan

Cytotoxicity studies were performed to test whether losartan induced cell death of human NP cells at different doses and time points. Human NP cells were seeded in 96-well plates at a density of 2000 cells per well for cell viability analysis. Two donors with three technical replicates per donor were used for analysis. The control group was exposed to DMEM with 1% ITS+, 1% non-essential amino acids (Sigma-Aldrich, St. Louis, MO, USA), and 50 $\mu\text{g/mL}$ L-ascorbic acid 2-phosphate (Sigma-Aldrich, St. Louis, MO, USA). After 24 h, Losartan (Tocris Bioscience, Bristol, UK) was added to the treatment groups at a concentration of 100 – 250 – 500 – 750 – 1000 μM . After 24 h, 48 h, and 72 h incubation time, the cells were washed with PBS and then exposed to the Cell Titer Blue® reagent (Promega Corporation, Madison, WI, USA) diluted 1:5 in DMEM. Fluorescence intensity was determined with the Viktor 3 plate reader (Perkin Elmer, Waltham, MA, USA) after 4 h of incubation (ex/em 560/590 nm).

Effect of Losartan on human NP cells

Passage 3 NP cells were seeded in 12-well plates at a density of 20 000 cells/well. Overall, four donors with three replicates per donor were assessed in this experiment. Additionally, three wells per group and donor were assessed for DNA content. Cells were seeded in 2 mL DMEM with 10% FCS to allow cell attachment. On the day of seeding the 12-well plates (Day 0 (baseline)), samples were taken for normalization. After 24 h, the medium was changed to the experiment medium with six groups. The control group was cultured with 2 mL DMEM, 1% non-essential amino acids, 50 $\mu\text{g/mL}$ L-ascorbic acid 2-phosphate, and 1% ITS+. We prepared high dose (1000 μM) and low dose (100 μM) stock solutions of Losartan by dissolving 47.9 mg Losartan Pottassium and 4.79 mg, respectively, in 1mL PBS. After filtering sterile with a 0.2 μm filter, we prepared 250 μL aliquots and stored them at

-20 °C. Losartan at a concentration of 100 μ M and 1000 μ M in PBS was then added to the medium for the experiments. Human recombinant TNF- α (10 ng/mL) was added simultaneously to induce a pro-inflammatory stimulus in human NP cells.

The experimental groups were:

- Control
- Losartan 100 μ M
- Losartan 1000 μ M
- TNF- α 10 ng/mL
- TNF- α 10 ng/mL + Losartan 100 μ M
- TNF- α 10 ng/mL + Losartan 1000 μ M

After 72 h of treatment, the medium was collected and analyzed for nitric oxide (NO) content. Three wells/group were digested with 0.5mg/mL proteinase K (Sigma-Aldrich, St. Louis, MO, USA) to measure DNA content in the monolayer. Three wells/group were lysed in TRI reagent (Molecular Research Center, Cincinnati, OH, USA) and PolyAcrylCarrier (Molecular Research Center, Cincinnati, OH, USA) and stored at - 80 °C for gene expression analysis.

Pathway study with the peroxisome proliferator-activated receptor-gamma (PPAR γ) antagonist T0070907 under inflammatory conditions

To determine a potential pathway involved in the anti-inflammatory action of losartan, we conducted a pathway study with the PPAR γ antagonist T0070907 (Tocris Bioscience, Bristol, UK). Human recombinant TNF- α (10 ng/mL) was used as an inducer of inflammation. T0070907 was added to the medium to investigate if losartan's anti-inflammatory effect would be inhibited by blocking PPAR γ . DMSO was used to solubilize T0070907, and the same amount (1 μ L/mL medium) was also added to the other groups to provide equivalent culture conditions. As before, the cells were seeded on day 0 and exposed to the experimental group medium after 24 h. Gene expression analysis was performed after 24 h and 48 h of exposure, respectively. The experimental groups were:

- Control
- TNF- α 10 ng/mL
- TNF- α 10 ng/mL + T0070907 1 μ M
- TNF- α 10 ng/mL + Losartan 1 mM
- TNF- α 10 ng/mL + Losartan 1 mM + T0070907 1 μ M

Gene expression analysis

Total RNA was extracted with 1-bromo-3-chloropropane (Sigma Aldrich) followed by RNA precipitation using isopropanol and high salt precipitation solution (0.8 M sodium citrate and 1.2 M NaCl). RNA was washed with ethanol and quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc.). Reverse transcription was performed with SuperScript VILO cDNA Synthesis Kit (Life Technologies, Carlsbad, CA), followed by quantitative real-time PCR using the TaqMan™ method with 10 μ L reaction volume.

qRT-PCR was performed to assess the gene expression of matrix metalloproteinase-1 and -3 (MMP-1, -3), interleukin 6 and 8 (IL-6, -8), TNF- α , and PPAR γ as markers for inflammation and matrix

degradation. Aggrecan (ACAN), collagen I, and collagen II (COL I, II) were analyzed as markers for ECM production and cell phenotype identification. The mRNA expression levels of the following components of tRAS were also quantified: Angiotensin-Converting Enzyme (ACE), renin-like tRAS equivalent Cathepsin D, Angiotensinogen (AGT), and the Angiotensin II receptor type I (AGTR1). RPLP0 was used as a housekeeping gene in each sample (Table 1). The comparative CT method was applied for relative quantification with RPLP0 as the endogenous control.

Table 1. Characteristics of custom-designed primer-probes and Gene Expression Assays (Applied Biosystems) used for gene expression analysis

Gene acronym	Gene full name	Primer-Probe sequence catalogue number	Reporter/quencher
<i>hRPLP0</i>	Human 60S acidic ribosomal protein P0	Forward seq.: 5'-TGG GCA AGA ACA CCA TGA TG-3' Reverse primer seq.: 5'CGG ATA TGA GGC AGC AGT TTC-3'	FAM/TAMRA
<i>hACAN</i>	Human Aggrecan	Forward seq.: 5'-AGT CTT CAA GCC TCC TGT ACT CA3' Reverse primer seq.: 5'CGG GAA GTG GCG GTA ACA-3'	FAM/TAMRA
<i>hACE</i>	133 (48.90)	Hs01586213_m1	FAM/NFQ-MGB
<i>hAGTR1a</i>	Human angiotensin-II receptor type 1	Hs00258938_m1	FAM/NFQ-MGB
<i>hCTSD</i>	Human cathepsin D	Hs00157205_m1	FAM/NFQ-MGB
<i>hCOL1A1</i>	Human collagen type 1 alpha 1 chain	Forward seq.: 5'-CCC TGG AAA GAA TGG AGA TGA T-3' Reverse primer seq.: 5'ACT GAA ACC TCT GTG TCC CTT CA'3	FAM/TAMRA
<i>hCOL2A1</i>	Human collagen type 2 alpha 1 chain	Forward seq.: 5'-GGC AAT AGC AGG TTC ACG TAC A-3' Reverse primer seq.: 5'GAT AAC AGT CTT GCC CCA CTT ACC-3'	FAM/TAMRA
<i>hIL6</i>	Human Interleukin 6	Hs00985639_m1	FAM/NFQ-MGB

<i>hIL8</i>	Human Interleukin 8	Hs00174103_m1	FAM/NFQ-MGB
<i>hMMP1</i>	Human matrix-metalloproteinase 1	Hs00899568_m1	FAM/NFQ-MGB
<i>hMMP3</i>	Human matrix-metalloproteinase 3	Hs00968305_m1	FAM/NFQ-MGB
<i>hPPARγ</i>	Human peroxisome proliferator-activated receptor-gamma	Hs00234592_m1	FAM/NFQ-MGB
<i>hTNFα</i>	Human tumor necrosis factor α	Hs00174128_m1	FAM/NFQ-MGB

Biochemical analysis

DNA content was measured after overnight digestion with 0.5 mg/mL proteinase K (Sigma-Aldrich, St. Louis, MO, USA) at 56°C. DNA quantification was performed with Hoechst 33258 (Sigma-Aldrich, St. Louis, MO, USA) dye and CalfThymus DNA (Sigma-Aldrich, St. Louis, MO, USA) as standard. Nitric oxide (NO) as a marker for oxidative stress was indirectly measured by spectrophotometrical quantification of nitrite, a non-volatile breakdown product, in the sampled medium of all wells by Griess assay (Promega, Madison, WI, USA).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0 software (GraphPad Software, Inc., La Jolla, CA, USA) and Stata Statistical Software Release 15 (StataCorp. 2011, College Station, TX, USA). Data was assessed with the Shapiro-Wilk normality test. For normally distributed data, the differences were assessed using t-test or ANOVA, as appropriate. The Kruskal-Wallis test, followed by the Mann-Whitney-U-test for pairwise comparisons, was used for non-parametric testing. A two-sided p-value <0.05 was considered significant.

3. Results

3.1. Losartan does not show cytotoxic effects on human NP cells

Losartan at a concentration between 100 μ M and 1000 μ M did not significantly affect the cell viability of human NP cells (Figure 1). We observed an increase in viable cell numbers from 24 h to 72 h for the control group and all losartan groups examined. This increase in cell count was not different between different concentrations of losartan. These results indicate no cytotoxic effects of 100 μ M-1000 μ M losartan on human NP cells between 24 h and 72 h of exposure. We used losartan at a concentration of 100 μ M and 1 mM for subsequent experiments based on the cytotoxicity study results.

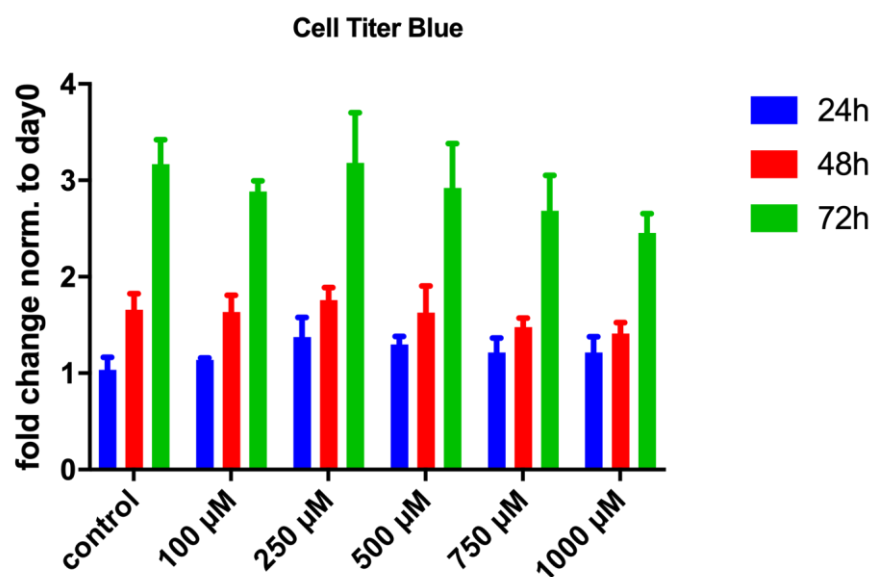


Figure 1. Relative viable cell count of human NP cells after 24 h, 48 h, and 72 h of losartan exposure. Means+standard deviations are shown. Data from 2 donors assessed in triplicates are shown (n=6).

3.2. Effect of losartan on human NP cells proliferation and NO release

The different experimental media did not alter the DNA content of the human NP cells after 72 h of exposure. As expected, we observed a significant increase in NO release from the cells of the experimental groups containing TNF- α ($p < 0.001$). Losartan addition did not significantly affect NO release than the TNF- α only group after 72 h of exposure (Figure 2).

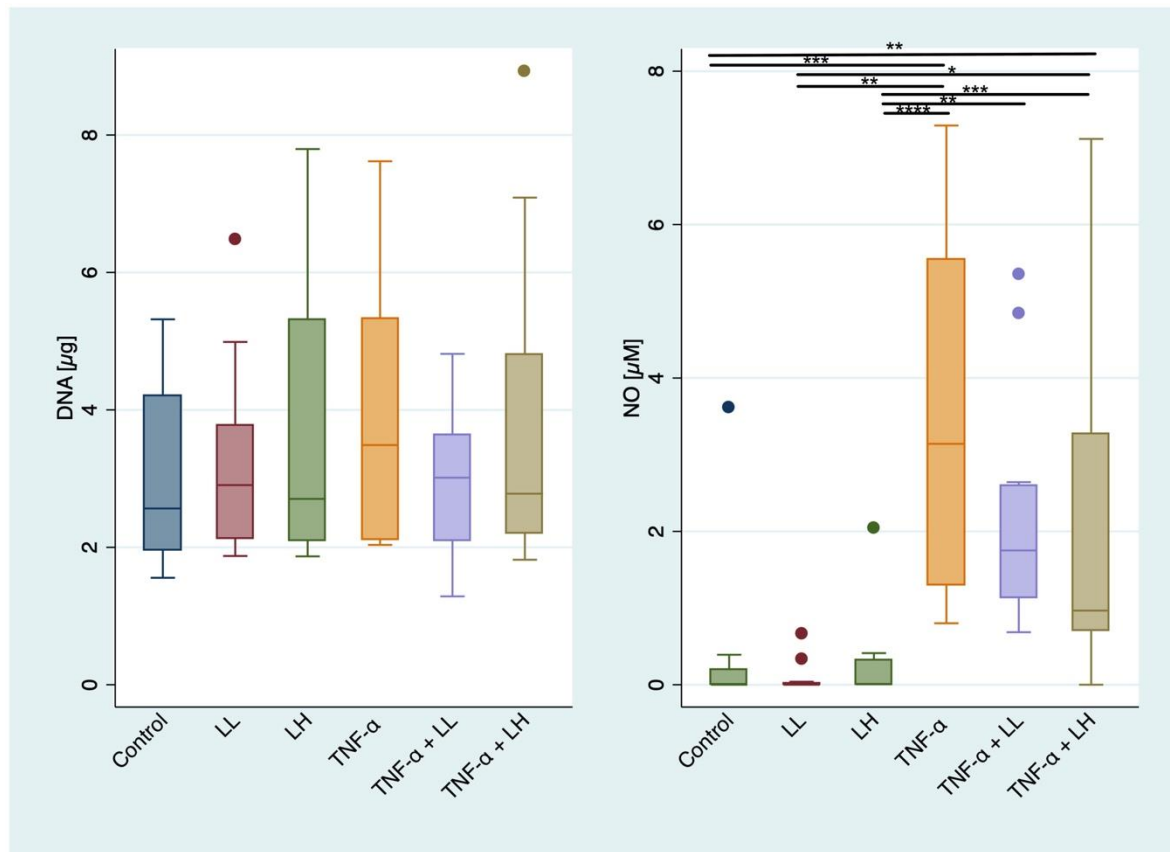


Figure 2. Effect of losartan and TNF- α on DNA content of human NP cells (A) and NO concentration in conditioned medium (B). Median and interquartile ranges (IQR) are shown. Dots represent outliers. Data from 4 donors assessed in triplicates are shown (n=12).

LL: Losartan 100 μ M; LH: Losartan 1000 μ M. *p<0.05; **p<0.01, ***p<0.001, ****p<0.0001.

3.3. Losartan positively modulates the phenotype of human NP cells under a pro-inflammatory condition

A significant upregulation of the pro-inflammatory cytokines IL-6 and IL-8 mRNA expression was observed under inflammatory conditions, which was unaltered by supplementation with losartan (Figure 3A). Gene expression of TNF- α was upregulated following the addition of TNF- α (p<0.01), a trend that could be decreased by the addition of 1000 μ M losartan (p<0.01). Further, we found that the combination of 1000 μ M losartan (p<0.001) and 100 μ M Losartan (p<0.01) with TNF- α induced increase of PPAR γ gene expression compared to the control group, indicating that the angiotensin II receptor type 1 was involved in the regulation of PPAR γ expression through AGTR1 in inflammatory settings. TNF- α significantly increased pro-inflammatory tRAS markers ACE (p<0.05) and AGTR1 (p<0.01). This effect was downregulated by blocking the AGTR1 receptor with 1000 μ M losartan, which also downregulated AGT expression (TNF- α +Losartan *vs.* Control, p<0.01), indicating that this upregulation of pro-inflammatory tRAS markers was mediated through the AGTR1 receptor under inflammatory situations (Figure 3B). TNF- α also upregulated the expression of Cathepsin D (p<0.01), which was not altered by losartan addition. TNF- α induced upregulation of MMP-3 showed decreasing trends upon the addition of 1000 μ M losartan, indicating an antidegenerative effect of high doses losartan. However, this finding failed to reach significance (p=0.2676) for the comparison TNF- α *vs.* TNF- α + 1000 μ M Losartan. Interestingly, when TNF- α was present, the addition of losartan

significantly upregulated the gene expression of ACAN (TNF- α + 1000 μ M Losartan *vs.* TNF- α $p<0.05$) and downregulated collagen I expression (TNF- α + 1000 μ M Losartan *vs.* TNF- α , $p<0.05$), indicating a positive phenotype modifying effect of losartan under inflammatory conditions (Figure 3C).



Figure 3. Gene expression of inflammatory (A), tissue remodeling (B), and tRAS markers (C) among the experimental groups. Gene expression was normalized to Day 0 (baseline) values. Data from 4 donors assessed in triplicates are shown (n=12).

#: compared to control; +: compared to Losartan 100 μ M; †: compared to Losartan 1000 μ M; ‡: compared to TNF- α ; §: compared to TNF- α + Losartan 100 μ M; * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

3.5. Pathway study in human NP cells treated with the PPAR γ antagonist T0070907 under inflammatory conditions

As we found potential interactions between AGTR1 inhibition and PPAR γ gene expression changes, we evaluated whether losartan could directly interact with the PPAR γ receptor. Therefore, we

examined the impact of an additional group containing the PPAR γ antagonist T0070907 on respective genes after 24 (Figure 4) and 72 h (Figure 5) of exposure. For the inflammatory markers IL-6 ($p < 0.01$) and TNF- α ($p < 0.05$), a significant upregulation of gene expression could be observed in the inflammatory groups compared to the control group after 24 h, confirming the findings of the previous experiments (Figure 3). In accordance with the previous experiments, this increase was not attenuated by losartan for IL-6. For TNF- α , a marked decrease was observed, which barely missed statistical significance (TNF- α vs. TNF- α + Losartan, $p = 0.0883$). The suppression of TNF- α induced inflammation by the PPAR γ antagonist T0070907 was not significant. A noticeable trend could be observed in the gene-expression analysis of the catabolic marker MMP1. Here, significant upregulation was observed between the control group and inflammatory groups (Control vs. TNF- α $p < 0.0001$), which could be decreased by the addition of losartan (TNF- α vs. TNF- α + Losartan $p < 0.0001$). This effect was reversed with T0070907 in the medium (TNF- α + Losartan vs. TNF- α + Losartan + T0070907 $p < 0.0001$), indicating a partial agonism and anti-degenerative effects of losartan via the PPAR γ receptor in human NP cells. TNF- α also led to a significant increase of ACAN. However, we did not observe any differences for the other experimental groups. Further, losartan reduced collagen I gene expression in TNF- α treated NP cells (TNF- α vs. TNF- α + Losartan $p < 0.001$). T0070907 intervention seemed to attenuate this effect of losartan; although the difference was not significant ($p = 0.1990$).

Concerning the expression of the tRAS genes, we observed an upregulation of the gene expression of AGTR1a, ACE, AGT, and Cathepsin D in inflammatory conditions (control vs. TNF- α : ACE $p < 0.0001$, AGT $p < 0.0001$, AGTR1a $p < 0.01$; Figure 4). Losartan significantly reduced the upregulation of tRAS genes, which was reversed by the PPAR γ antagonist T0070907 for ACE (TNF- α + Losartan vs. TNF- α + Losartan + T0070907 $p < 0.01$), Cathepsin D (TNF- α + Losartan vs. TNF- α + Losartan + T0070907 $p < 0.01$) and AGT (TNF- α + Losartan vs. TNF- α + Losartan + T0070907 $p < 0.001$; Figure 4). This trend seemed to be consistent for the angiotensin II receptor type 1, although not significant ($p = 0.4913$).

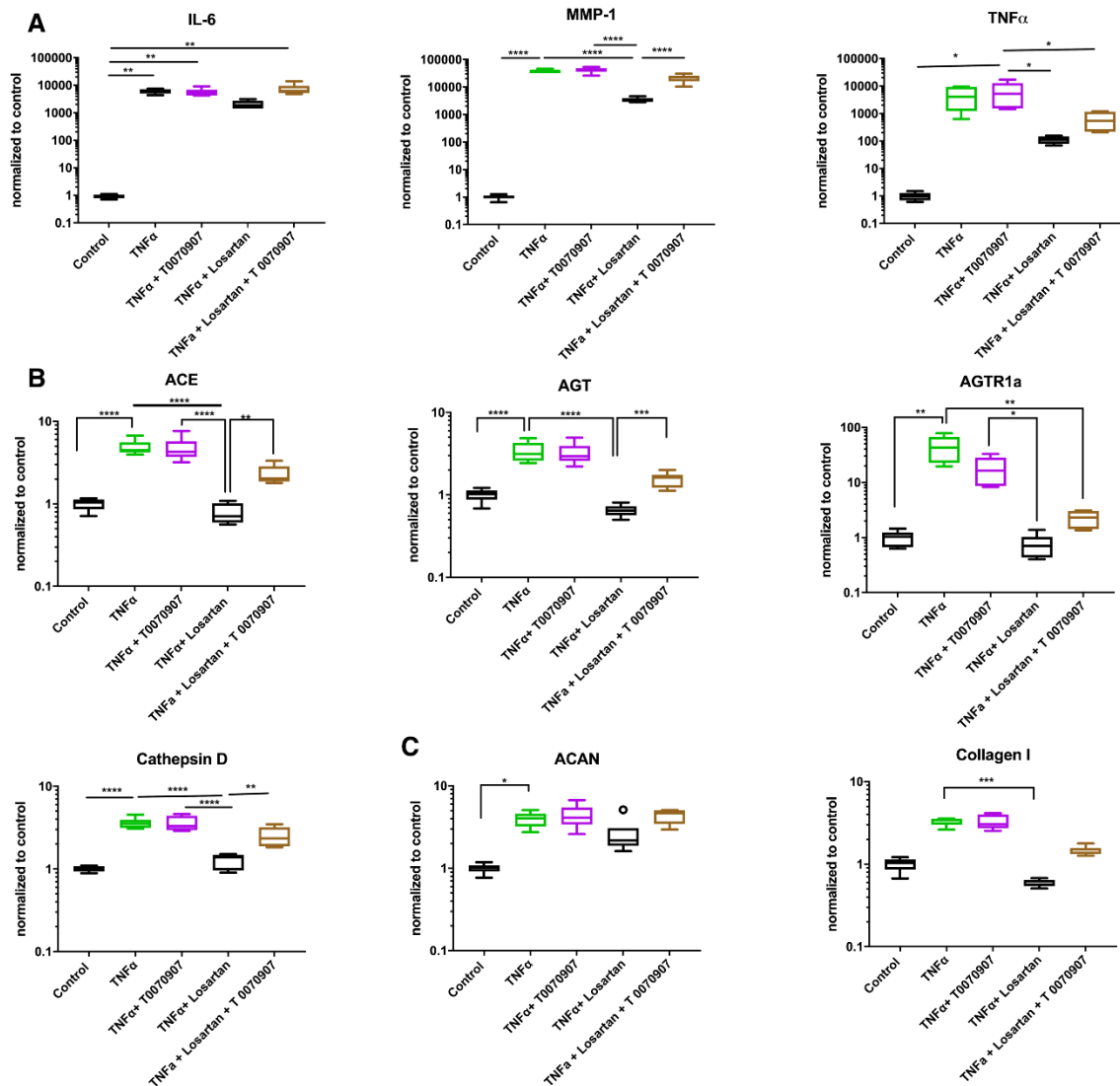


Figure 4. Influence of the PPAR γ pathway on losartan induced gene expression changes of inflammatory and tissue degeneration markers (A), tRAS markers (B), and cell phenotype markers (C) in human NP cells after 24 h of intervention. Gene expression was normalized to Day 0 (baseline) values. Data from 2 donors assessed in triplicates are shown (n=6); *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Figure 5 illustrates the gene expression results of the experimental groups after 72 h of exposure to the experimental groups medium. There seemed to be a lower effect of all interventions on the examined genes with an increase of experimental duration. In contrast to the results after 24 h of exposure, we observed a downregulation of ACAN for the TNF- α group, which was neutralized in the TNF- α + Losartan group. PPAR γ antagonist T0070907 increased the gene expression for the tRAS genes cathepsin D (TNF- α + Losartan vs. TNF- α + Losartan + T0070907, p<0.01) and ACE (TNF- α + Losartan vs. TNF- α + Losartan + T0070907, p<0.001), but not AGT and AGTR1 after 72 h of exposure. Further, T0070907 also reversed the anti-inflammatory effects of losartan with respect to TNF- α expression-after 72 h of exposure (p<0.05).

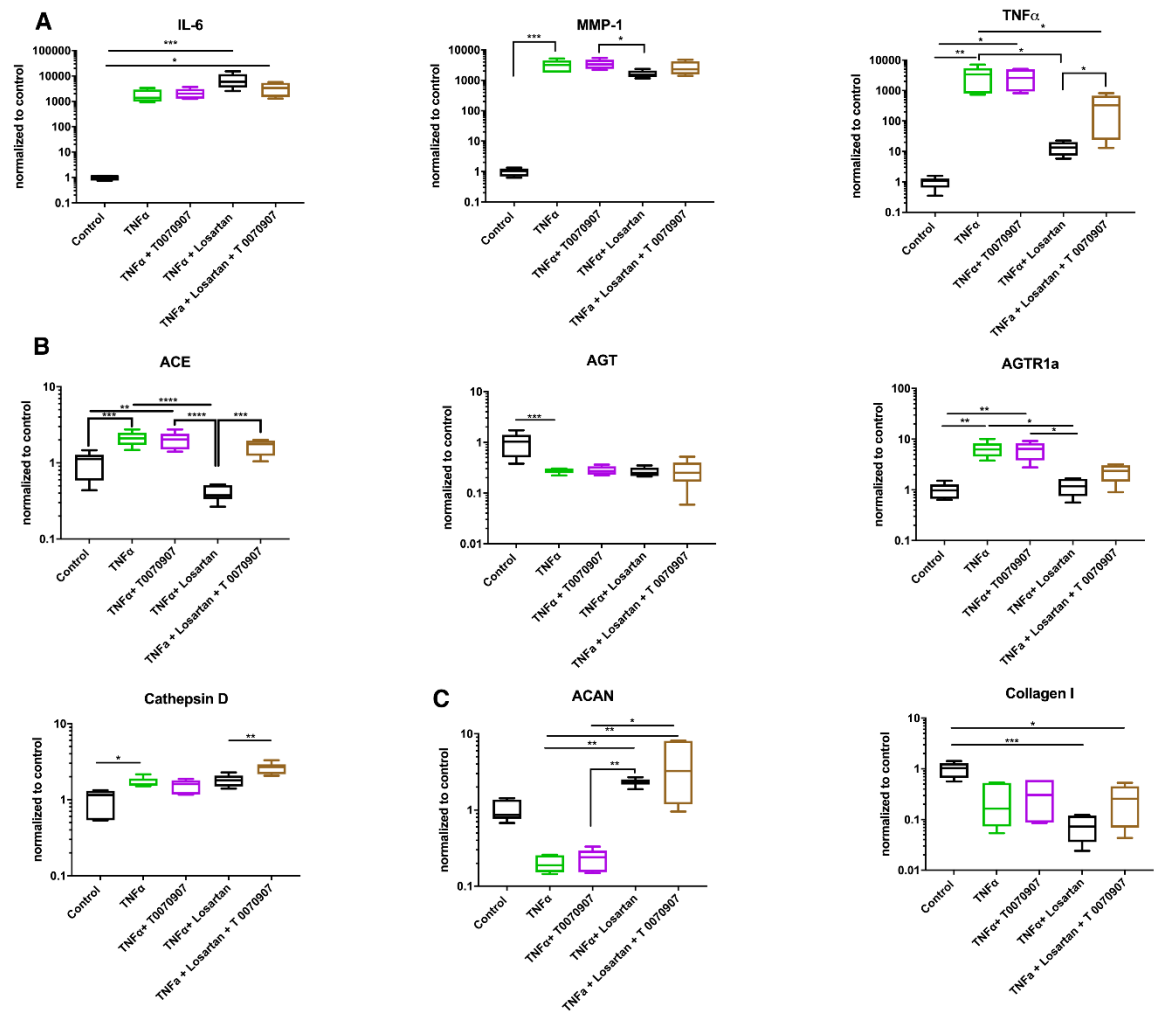


Figure 5. Influence of the PPAR γ pathway on losartan induced gene expression changes of inflammatory and tissue degeneration markers (A), tRAS markers (B), and cell phenotype markers (C) in human NP cells after 72 h of intervention. Gene expression was normalized to Day 0 (baseline) values. Data from 2 donors assessed in triplicates are shown (n=6); *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001

4. Discussion

The present study sought to investigate the protective effect of the AGTR1 antagonist losartan on human NP cell inflammation and degeneration induced by TNF- α . Outcomes revealed that TNF- α induced the expression of pathologic tRAS molecules and led to pro-inflammatory and catabolic effects in human NP cells. Inhibition of AGTR1 with losartan could partly inhibit the inflammatory and catabolic interaction. Therefore, present results suggest that TNF- α induced disc degeneration may partially be mediated through AGTR1 signaling. Treatment with AGTR1 antagonist losartan could inhibit the TNF- α induced degenerative state and maintain the NP cell phenotype.

In accordance with previous studies, we found that losartan interacted with the PPAR γ pathway [37]. Inhibition with the PPAR γ inhibitor T0070907 partly abolished the effects of losartan on nucleus pulposus cells. This implies that this interaction contributes to losartan's anti-inflammatory effects, as also suggested by Price et al. [19]. Our results also indicate that losartan has an impact on the gene expression of extracellular matrix related components. Losartan has been reported to affect TGF-beta expression and reduce collagen I production in human fibroblasts [38]. Additionally, the interaction of losartan with TGF-beta signaling was confirmed by other workgroups [39].

Targeting the tissue renin-angiotensin system may have significant therapeutic potential in modulating the metabolism of the degenerative IVD, which may potentially translate into a reduction of discogenic pain.

The tissue Renin-Angiotensin Systems – AngII as a pro-inflammatory and catabolic hormone

The renin-angiotensin-system (RAS) plays an important role in the induction and progression of tissue injuries in the cardiovascular system [40]. During previous years, a diversity of locally acting renin-angiotensin systems were identified in multiple tissues such as skin, kidney, liver, vulva, gastrointestinal tract, nervous system, lymphatic tissue, and bone, revealing participation in inflammatory and nociceptive processes [41,42]. While AngII is well accepted as a classical cardiovascular hormone inducing vasoconstriction, the latest findings also demonstrated inflammatory cells to express RAS components generating AngII, inducing axon sprouting and nerve growth [43–47]. Morimoto et al. and Price et al. were the first to describe local renin-angiotensin systems and their contribution to inflammation in the rat musculoskeletal system [19,48]. Recent research supports the hypothesis that the RAS and specifically AngII may be considered as a locally-acting modulator of tissue homeostasis and regeneration, especially in the cardiovascular and nervous systems [49]. Furthermore, AngII induces the production of pro-inflammatory markers, such as IL-6, TNF- α , and adhesion molecules, and functions as a true pro-inflammatory mediator that regulates inflammation, growth, and fibrosis [50–54].

In our previous work, we confirmed the expression of AngII in tissue samples of degenerated and symptomatic discs by gene expression analysis and immunohistochemistry [16]. Disc tissue samples that expressed more tRAS factors revealed significantly higher gene expression levels of pro-inflammatory (IL-6 and TNF- α) and catabolic genes (ADAMTS 4 and 5), indicating that tRAS contributes to the inflammatory processes operant in IDD. These findings were supported by the fact, that the gene expression of the NP phenotype, such as ACAN and COL2, was reduced in increased tRAS expressing discs. Furthermore, disc tissues with highly positive tRAS expression revealed lower glycosaminoglycan (GAG)/DNA ratios, implying the accelerated state of catabolism due to IDD.

Our present work indicates that AngII has the potential to influence IVD matrix degradation, as the inhibition of its receptor, AGTR1, led to significant changes in the gene expression of relevant

extracellular matrix genes. The presence and contribution of ACE and AGTR1 to inflammatory processes have recently been demonstrated in synovium samples from RA patients [19,55,56]. Furthermore, our findings are supported by previous work showing increased tRAS activity found in blood monocytes, nodules, synovial fluid, and synovial tissue of patients with RA [57–59]. Price and coworkers analyzed the protective potential of losartan in rats having RA [19]. Chronic joint inflammation was induced by intraarticular and periarticular injection of Freund's complete adjuvant of heat-killed *Mycobacterium tuberculosis* into the knee joint. Acute joint inflammation was induced by intraarticular injection of λ -carrageenan and kaolin. Western blot analysis and immunohistochemistry reflected the elevated concentrations of AngII protein and AGTR1 in synovium from animals with acute and chronic joint inflammation. Losartan substantially reduced joint swelling and suppressed TNF- α generation in a dose-dependent manner. Morimoto et al. examined the biological role of AngII in rat annulus fibrosus cells in monolayer culture by real-time polymerase chain reaction [48]. Stimulation of rat IVD cells with AngII increased the mRNA expression of ADAMTS-5 significantly, indicating extracellular matrix degradation.

In summary, along with other recent investigations on musculoskeletal tissues, our present work provides strong evidence that factors of the renin-angiotensin system are likely to contribute to the inflammatory processes that are operant in IDD and therefore provide a novel therapeutic target to combat the disabling symptoms of active discopathy.

Anti-inflammatory therapies via RAS inhibition – a potential target in IDD?

Recent data underline that RAS inhibitors have anti-inflammatory effects in various tissues and organs and have beneficial effects in inflammatory musculoskeletal diseases [20–23,25–33,60]. Fukuzawa and coworkers demonstrated that oral administration of ACE inhibitors in mice reduced LPS induced TNF- α production, though high concentrations were needed to reach a meaningful effect [18]. In the kidney, RAS inhibitors completely suppressed LPS-induced IL-6 and TNF- α mRNA levels [61]. Renal AngII is a key factor in mediating various components of the immune and inflammatory responses and acts as a pro-inflammatory agent [54,62]. This suggests that the administration of RAS inhibitor in therapeutic dosages to humans with hypertension may also suppress cytokines levels. The angiotensin-converting enzyme inhibitor Enalapril inhibits AngII synthesis from AngI and may suppress pro-inflammatory cytokine production, as previously shown in vitro [63,64]. Besides this, Captopril is additionally known to have antirheumatic effects comparable to D-penicillamine [20,65,66]. Therefore, Captopril was considered a valuable drug in patients with hypertension and RA, especially due to the favorable benefit/risk ratio as Captopril lacks serious side effects [66]. Cardoso et al. demonstrated that losartan, but not Enalapril and Valsartan, inhibits the expression of IFN- γ , IL-6, IL-17F, and IL-22 in PBMCs from RA patients suggesting losartan could be a better option for hypertension treatment in RA patients [67].

Our results reflect previous findings that found downregulations of inflammatory markers in inflammatory cell culture models after AGTR1 blockade or knockdown [68,69]. In contrast to our experiments, these studies used lipopolysaccharides to stimulate inflammation in the respective cell cultures and might not be fully comparable. However, our workgroup recently validated the superior potential of TNF- α -induced inflammatory cell culture models to examine degenerative disc diseases

[14]. Further, we showed that PPAR γ pathway stimulation through losartan has protective effects on human NP cells for the first time. Losartan seems to exhibit at least some of its effect through the PPAR γ pathway, as the inhibition of PPAR γ partly diminished the protective effects of losartan. In accordance with the present results, several studies reported that losartan could get internalized through the AGTR1 receptor and act as a partial PPAR γ agonist [68,70,71]. Noticeably, the PPAR γ effects of ARBs might be too small regarding the reachable tissue levels to exert a significant anti-inflammatory effect. Therefore, new drug delivery methods, the development of new ARBs with more potent PPAR γ activation properties, or other PPAR γ agonists might be needed if PPAR γ stimulation is the target of interest [72]. PPAR γ activation properties of ARBs can be more seen as a beneficial effect in addition to the inhibition of the pathological tRAS arm. Overall, more details about the interactions of the tRAS, PPAR γ pathway, and ARBs in human IVD cells are warranted in the future.

Interestingly, inhibition of AGTR1 receptors through losartan reduced TNF- α -induced ACE and AGTR1 upregulation. The upregulation of ACE through AGTR1 was already shown before by Koka et al. [73]. They also could inhibit this upregulation through losartan addition, which is compliant with our data. Notably, the inflammatory environments stimulate ACE and AGTR1 expression, as seen in our data, which indicates stimulation of the pathological arm of the tRAS in inflammatory settings. This was also shown by Takeshita et al., who suggested a previously unknown cross-talk between the TNF- α and the tRAS [74]. Therefore, the reproducible induction of the pathological arm of the tRAS by TNF- α for all examined donors in the present work suggests an important role in inflammatory and degenerative processes in IVDs. As shown by our data (Figure 3), losartan is especially effective in inflammatory settings, supporting its therapeutic potential in degenerative disc diseases.

Strengths and Limitations

The present study is associated with several strengths. The present experiments are the first pathway studies of the tRAS for IVD cells. As we used human NP cells from patients' IVDs in this study, these findings could directly impact clinical and therapeutical strategies in contrast to preclinical studies in other species that often need to be verified in humans first. We provided novel evidence that treatment with losartan suppresses pro-inflammatory and degenerative responses to inflammatory stimuli in human NP cells. We further revealed an interaction of the tRAS with the PPAR γ pathway, which could be of potential interest for future pathway studies.

Some limitations need to be addressed to interpret the results adequately. These studies were conducted in vitro with 2-dimensional monolayer cell culture, and in vivo interactions can not be perfectly simulated with this methodological approach and the concentrations used for the intervention arms. The concentrations of losartan chosen in this experiment was based on previous in vitro pathway studies with other human cell types, as evidence for intervertebral disc cells is scarce [68,75,76]. Therefore, the concentration in our cell cultures might not reflect the available tissue concentrations in humans after usual orally available doses of losartan. However, the present preclinical experiments help to understand the role of the tRAS and its inhibition in human NP cells

and support future planning of in vivo studies that will need to be conducted to confirm these results. Furthermore, we observed differences in outcome effects sizes regarding the two timepoints, indicating that genes might be differently affected by tRAS modulation depending on exposure time. For example, tRAS gene expressions (ACE, AGT, CTSD, AGTR1) seem to be more affected by 24 h of losartan exposure than 72 h. Future studies could include more time points and genes to ensure not to overlook effects on relevant genes. Moreover, other therapeutic approaches of tRAS modulation, such as comparison of RAS inhibitors and stimulation of the protective tRAS arm, could be conducted and compared to evaluate the best therapeutic approach. Finally, an expansion of methodological techniques to quantify and visualize receptor expression changes is recommended and already planned by our group to characterize the nature of tRAS in the IVD.

5. Conclusions

TNF- α induced the expression of tRAS molecules and led to pro-inflammatory and catabolic effects in human NP cells. Inhibition of the angiotensin II receptor type 1 with losartan could inhibit this inflammatory and catabolic response. Further, we uncovered an interaction of losartan with the PPAR γ pathway in human NP cells. These results demonstrate that TNF- α induced disc degeneration may partially be mediated through AGTR1 signaling. Treatment with AGTR1 antagonist losartan could inhibit the TNF- α induced degeneration process and maintain the NP cell phenotype. The inhibition of the pathological tRAS pathway with angiotensin II receptor type 1 blockers could be a novel therapeutic strategy for discogenic back pain caused by intervertebral disc degeneration.

Author Contributions: Conceptualization, Zhen Li, Sibylle Grad, Mauro Alini, R. Geoff Richards, Hagen Schmal and Gernot Lang; Data curation, Babak Saravi, Zhen Li, Judith Pfannkuche and Laura Wystrach; Formal analysis, Babak Saravi, Zhen Li, Judith Pfannkuche and Laura Wystrach; Investigation, Sibylle Grad, Mauro Alini, Corinna Lang, Norbert Südkamp, Hagen Schmal and Gernot Lang; Methodology, Zhen Li, Judith Pfannkuche, Christoph E. Albers, Sibylle Grad and Gernot Lang; Project administration, Zhen Li, Sibylle Grad, Mauro Alini, R. Geoff Richards, Norbert Südkamp, Hagen Schmal and Gernot Lang; Supervision, Christoph E. Albers, Mauro Alini, R. Geoff Richards, Norbert Südkamp and Gernot Lang; Validation, R. Geoff Richards and Corinna Lang; Visualization, Babak Saravi; Writing – original draft, Babak Saravi and Gernot Lang; Writing – review & editing, Babak Saravi, Zhen Li, Judith Pfannkuche, Laura Wystrach, Christoph E. Albers, Sibylle Grad, Mauro Alini, R. Geoff Richards, Corinna Lang, Norbert Südkamp, Hagen Schmal and Gernot Lang.

Funding: This study was funded by the German Spine Society (DWG), German Arthritis Foundation (DAH), the Foundation for the Promotion of Alternate and Complementary Methods to Reduce Animal Testing (SET) under the project InflammoDisc [number 59], AO Foundation, and AOSpine International. GL was supported by the Berta-Ottenstein-Programme for Advanced Clinician Scientists, Faculty of Medicine, University of Freiburg. The article processing charge was funded by the Baden-Württemberg Ministry of Science, Research and Art, and the University of Freiburg in the funding program Open Access Publishing.

Conflicts of Interest: The authors declare no conflict of interest.

Data Sharing Statements: Datasets are available on request. The raw data and all related documents supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

References

1. Vos, T.; Abajobir, A.A.; Abate, K.H.; Abbafati, C.; Abbas, K.M.; Abd-Allah, F.; Abdulkader, R.S.; Abdulle, A.M.; Abebo, T.A.; Abera, S.F.; et al. Global, regional, and national incidence, prevalence, and years lived with disability for 328 diseases and injuries for 195 countries, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. *The Lancet* **2017**, *390*, 1211–1259, doi:10.1016/S0140-6736(17)32154-2.
2. Battié, M.C.; Joshi, A.B.; Gibbons, L.E. Degenerative Disc Disease: What is in a Name? *SPINE* **2019**, *44*, 1523–1529, doi:10.1097/BRS.0000000000003103.
3. Adams, M.A.; Roughley, P.J. What is Intervertebral Disc Degeneration, and What Causes It?: *Spine* **2006**, *31*, 2151–2161, doi:10.1097/01.brs.0000231761.73859.2c.
4. Risbud, M.V.; Shapiro, I.M. Role of cytokines in intervertebral disc degeneration: pain and disc content. *Nat Rev Rheumatol* **2014**, *10*, 44–56, doi:10.1038/nrrheum.2013.160.
5. Takahashi, H.; Suguro, T.; Okazima, Y.; Motegi, M.; Okada, Y.; Kakiuchi, T. Inflammatory Cytokines in the Herniated Disc of the Lumbar Spine: *Spine* **1996**, *21*, 218–224, doi:10.1097/00007632-199601150-00011.
6. Mannion, A.F.; Impellizzeri, F.M.; Leunig, M.; Jeszenszy, D.; Becker, H.-J.; Haschtmann, D.; Preiss, S.; Fekete, T.F. EUROSPINE 2017 FULL PAPER AWARD: Time to remove our rose-tinted spectacles: a candid appraisal of the relative success of surgery in over 4500 patients with degenerative disorders of the lumbar spine, hip or knee. *Eur Spine J* **2018**, *27*, 778–788, doi:10.1007/s00586-018-5469-4.
7. Wahood, W.; Yolcu, Y.U.; Kerezoudis, P.; Goyal, A.; Alvi, M.A.; Freedman, B.A.; Bydon, M. Artificial Discs in Cervical Disc Replacement: A Meta-Analysis for Comparison of Long-Term Outcomes. *World Neurosurgery* **2020**, *134*, 598–613.e5, doi:10.1016/j.wneu.2019.10.032.
8. Gangl, M. Spezifischer Kreuzschmerz – die erste Leitlinie. *Manuelle Medizin* **2020**, *58*, 46–52, doi:10.1007/s00337-019-00630-z.
9. Findlay, C.; Ayis, S.; Demetriades, A.K. Total disc replacement versus anterior cervical discectomy and fusion: a systematic review with meta-analysis of data from a total of 3160 patients across 14 randomized controlled trials with both short- and medium- to long-term outcomes. *The Bone & Joint Journal* **2018**, *100-B*, 991–1001, doi:10.1302/0301-620X.100B8.BJJ-2018-0120.R1.
10. Petersen, T.; Laslett, M.; Juhl, C. Clinical classification in low back pain: best-evidence diagnostic rules based on systematic reviews. *BMC Musculoskelet Disord* **2017**, *18*, 188, doi:10.1186/s12891-017-1549-6.
11. Rustenburg, C.M.E.; Faraj, S.S.A.; Ket, J.C.F.; Emanuel, K.S.; Smit, T.H. Prognostic factors in the progression of intervertebral disc degeneration: Which patient should be targeted with regenerative therapies? *JOR Spine* **2019**, *2*, doi:10.1002/jsp2.1063.
12. Smith, L.J.; Silverman, L.; Sakai, D.; Le Maitre, C.L.; Mauck, R.L.; Malhotra, N.R.; Lotz, J.C.; Buckley, C.T. Advancing cell therapies for intervertebral disc regeneration from the lab to the clinic: Recommendations of the ORS spine section. *JOR Spine* **2018**, *1*, e1036, doi:10.1002/jsp2.1036.

13. Buckley, C.T.; Hoyland, J.A.; Fujii, K.; Pandit, A.; Iatridis, J.C.; Grad, S. Critical aspects and challenges for intervertebral disc repair and regeneration-Harnessing advances in tissue engineering. *JOR Spine* **2018**, *1*, e1029, doi:10.1002/jsp2.1029.
14. Li, Z.; Gehlen, Y.; Heizmann, F.; Grad, S.; Alini, M.; Richards, R.G.; Kubosch, D.; Südkamp, N.; Izadpanah, K.; Kubosch, E.J.; et al. Preclinical ex-vivo Testing of Anti-inflammatory Drugs in a Bovine Intervertebral Degenerative Disc Model. *Front. Bioeng. Biotechnol.* **2020**, *8*, 583, doi:10.3389/fbioe.2020.00583.
15. Du, J.; Pfannkuche, J.; Lang, G.; Häckel, S.; Creemers, L.B.; Alini, M.; Grad, S.; Li, Z. Proinflammatory intervertebral disc cell and organ culture models induced by tumor necrosis factor alpha. *JOR Spine* **2020**, doi:10.1002/jsp2.1104.
16. Li, Z.; Wystrach, L.; Bernstein, A.; Grad, S.; Alini, M.; Richards, R.; Kubosch, D.; Südkamp, N.; Izadpanah, K.; Kubosch, E.; et al. The tissue-renin-angiotensin-system of the human intervertebral disc. *eCM* **2020**, *40*, 115–132, doi:10.22203/eCM.v040a07.
17. Stagnitti, M.N. Trends in Utilization and Expenditures of Prescribed Drugs Treating Diabetes, Hypertension, and High Cholesterol for Persons under Age 40 in the U.S. Civilian Noninstitutionalized Population, 2000 and 2010. In *Statistical Brief (Medical Expenditure Panel Survey (US))*; Agency for Healthcare Research and Quality (US): Rockville (MD), 2001.
18. Fukuzawa, M.; Satoh, J.; Sagara, M.; Muto, G.; Muto, Y.; Nishimura, S.; Miyaguchi, S.; Qiang, X.L.; Sakata, Y.; Nakazawa, T.; et al. Angiotensin converting enzyme inhibitors suppress production of tumor necrosis factor- α in vitro and in vivo. *Immunopharmacology* **1997**, *36*, 49–55, doi:10.1016/S0162-3109(96)00160-9.
19. Price, A.; Lockhart, J.C.; Ferrell, W.R.; Gsell, W.; McLean, S.; Sturrock, R.D. Angiotensin II type 1 receptor as a novel therapeutic target in rheumatoid arthritis: In vivo analyses in rodent models of arthritis and ex vivo analyses in human inflammatory synovitis. *Arthritis Rheum* **2007**, *56*, 441–447, doi:10.1002/art.22335.
20. Agha, A.M.; Mansour, M. Effects of Captopril on Interleukin-6, Leukotriene B₄, and Oxidative Stress Markers in Serum and Inflammatory Exudate of Arthritic Rats: Evidence of Antiinflammatory Activity. *Toxicology and Applied Pharmacology* **2000**, *168*, 123–130, doi:10.1006/taap.2000.8985.
21. Dalbeth, N. The non-thiol angiotensin-converting enzyme inhibitor quinapril suppresses inflammatory arthritis. *Rheumatology* **2005**, *44*, 24–31, doi:10.1093/rheumatology/keh398.
22. Flammer, A.J.; Sudano, I.; Hermann, F.; Gay, S.; Forster, A.; Neidhart, M.; Künzler, P.; Enseleit, F.; Périat, D.; Hermann, M.; et al. Angiotensin-Converting Enzyme Inhibition Improves Vascular Function in Rheumatoid Arthritis. *Circulation* **2008**, *117*, 2262–2269, doi:10.1161/CIRCULATIONAHA.107.734384.
23. Liu, H.-M.; Wang, K.-J. Therapeutic effect of Captopril on rheumatoid arthritis in rats. *Asian Pacific Journal of Tropical Medicine* **2014**, *7*, 996–999, doi:10.1016/S1995-7645(14)60175-9.
24. Miyagi, M.; Ishikawa, T.; Kamoda, H.; Suzuki, M.; Murakami, K.; Shibayama, M.; Orita, S.; Eguchi, Y.; Arai, G.; Sakuma, Y.; et al. ISSLS Prize Winner: Disc Dynamic Compression in Rats Produces Long-Lasting Increases in Inflammatory Mediators in Discs and Induces Long-Lasting Nerve Injury and Regeneration of the Afferent Fibers Innervating Discs. *Spine* **2012**, *37*, 1810–1818, doi:10.1097/BRS.0b013e31824ffac6.
25. Tang, Y.; Hu, X.; Lu, X. Captopril, an angiotensin-converting enzyme inhibitor, possesses

- chondroprotective efficacy in a rat model of osteoarthritis through suppression local renin-angiotensin system. *International Journal of Clinical and Experimental Medicine* **2015**, *8*, 12584–12592.
26. Fahmy Wahba, M.G.; Shehata Messiha, B.A.; Abo-Saif, A.A. Ramipril and haloperidol as promising approaches in managing rheumatoid arthritis in rats. *European Journal of Pharmacology* **2015**, *765*, 307–315, doi:10.1016/j.ejphar.2015.08.026.
 27. Guerra, G.C.B.; de Menezes, M.S.S.; de Araújo, A.A.; de Araújo Júnior, R.F.; de Medeiros, C.A.C.X. Olmesartan Prevented Intra-articular Inflammation Induced by Zymosan in Rats. *Biological & Pharmaceutical Bulletin* **2016**, *39*, 1793–1801, doi:10.1248/bpb.b16-00296.
 28. Sagawa, K.; Nagatani, K.; Komagata, Y.; Yamamoto, K. Angiotensin receptor blockers suppress antigen-specific T cell responses and ameliorate collagen-induced arthritis in mice. *Arthritis Rheum* **2005**, *52*, 1920–1928, doi:10.1002/art.21040.
 29. Refaat, R.; Salama, M.; Abdel Meguid, E.; El Sarha, A.; Gawayed, M. Evaluation of the effect of losartan and methotrexate combined therapy in adjuvant-induced arthritis in rats. *European Journal of Pharmacology* **2013**, *698*, 421–428, doi:10.1016/j.ejphar.2012.10.024.
 30. Silveira, K.D.; Coelho, F.M.; Vieira, A.T.; Barroso, L.C.; Queiroz-Junior, C.M.; Costa, V.V.; Sousa, L.F.C.; Oliveira, M.L.; Bader, M.; Silva, T.A.; et al. Mechanisms of the anti-inflammatory actions of the angiotensin type 1 receptor antagonist losartan in experimental models of arthritis. *Peptides* **2013**, *46*, 53–63, doi:10.1016/j.peptides.2013.05.012.
 31. Wang, D.; Hu, S.; Zhu, J.; Yuan, J.; Wu, J.; Zhou, A.; Wu, Y.; Zhao, W.; Huang, Q.; Chang, Y.; et al. Angiotensin II type 2 receptor correlates with therapeutic effects of losartan in rats with adjuvant-induced arthritis. *J. Cell. Mol. Med.* **2013**, *17*, 1577–1587, doi:10.1111/jcmm.12128.
 32. Queiroz-Junior, C.M.; Silveira, K.D.; de Oliveira, C.R.; Moura, A.P.; Madeira, M.F.M.; Soriani, F.M.; Ferreira, A.J.; Fukada, S.Y.; Teixeira, M.M.; Souza, D.G.; et al. Protective effects of the angiotensin type 1 receptor antagonist losartan in infection-induced and arthritis-associated alveolar bone loss. *J Periodontal Res* **2015**, *50*, 814–23, doi:10.1111/jre.12269.
 33. Perry, M.E.; Chee, M.M.; Ferrell, W.R.; Lockhart, J.C.; Sturrock, R.D. Angiotensin receptor blockers reduce erythrocyte sedimentation rate levels in patients with rheumatoid arthritis. *Annals of the Rheumatic Diseases* **2008**, *67*, 1646–1647, doi:10.1136/ard.2007.082917.
 34. Ruiz-Ortega, M.; Lorenzo, O.; Suzuki, Y.; Rupérez, M.; Egido, J. Proinflammatory actions of angiotensins. *Current Opinion in Nephrology and Hypertension* **2001**, *10*.
 35. Wolf, G.; Neilson, E.G. Angiotensin II as a renal growth factor. *J. Am. Soc. Nephrol.* **1993**, *3*, 1531–1540.
 36. Mezzano, S.A.; Ruiz-Ortega, M.; Egido, J. Angiotensin II and Renal Fibrosis. *Hypertension* **2001**, *38*, 635–638, doi:10.1161/hy09t1.094234.
 37. Schupp, M.; Janke, J.; Clasen, R.; Unger, T.; Kintscher, U. Angiotensin Type 1 Receptor Blockers Induce Peroxisome Proliferator-Activated Receptor- γ Activity. *Circulation* **2004**, *109*, 2054–2057, doi:10.1161/01.CIR.0000127955.36250.65.
 38. Diop-Frimpong, B.; Chauhan, V.P.; Krane, S.; Boucher, Y.; Jain, R.K. Losartan inhibits collagen I synthesis and improves the distribution and efficacy of nanotherapeutics in tumors. *Proceedings of the National Academy of Sciences* **2011**, *108*, 2909–2914, doi:10.1073/pnas.1018892108.
 39. Cohn, R.D.; van Erp, C.; Habashi, J.P.; Soleimani, A.A.; Klein, E.C.; Lisi, M.T.; Gamradt, M.; ap Rhys, C.M.; Holm, T.M.; Loeys, B.L.; et al. Angiotensin II type 1 receptor blockade attenuates TGF- β –

induced failure of muscle regeneration in multiple myopathic states. *Nat Med* **2007**, *13*, 204–210, doi:10.1038/nm1536.

40. Namsolleck, P.; Recarti, C.; Foulquier, S.; Steckelings, U.M.; Unger, T. AT₂ Receptor and Tissue Injury: Therapeutic Implications. *Curr Hypertens Rep* **2014**, *16*, 416, doi:10.1007/s11906-013-0416-6.

41. Patil, J.; Schwab, A.; Nussberger, J.; Schaffner, T.; Saavedra, J.M.; Imboden, H. Intraneuronal angiotensinergic system in rat and human dorsal root ganglia. *Regulatory Peptides* **2010**, *162*, 90–98, doi:10.1016/j.regpep.2010.03.004.

42. Paul, M.; Poyan Mehr, A.; Kreutz, R. Physiology of Local Renin-Angiotensin Systems. *Physiological Reviews* **2006**, *86*, 747–803, doi:10.1152/physrev.00036.2005.

43. Hoch, N.E.; Guzik, T.J.; Chen, W.; Deans, T.; Maalouf, S.A.; Gratzke, P.; Weyand, C.; Harrison, D.G. Regulation of T-cell function by endogenously produced angiotensin II. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* **2009**, *296*, R208–R216, doi:10.1152/ajpregu.90521.2008.

44. Jurewicz, M.; McDermott, D.H.; Sechler, J.M.; Tinckam, K.; Takakura, A.; Carpenter, C.B.; Milford, E.; Abdi, R. Human T and Natural Killer Cells Possess a Functional Renin-Angiotensin System: Further Mechanisms of Angiotensin II-Induced Inflammation. *JASN* **2007**, *18*, 1093–1102, doi:10.1681/ASN.2006070707.

45. Chakrabarty, A.; Blacklock, A.; Svojanovsky, S.; Smith, P.G. Estrogen Elicits Dorsal Root Ganglion Axon Sprouting via a Renin-Angiotensin System. *Endocrinology* **2008**, *149*, 3452–3460, doi:10.1210/en.2008-0061.

46. Côté, F.; Do, T.H.; Laflamme, L.; Gallo, J.-M.; Gallo-Payet, N. Activation of the AT₂ Receptor of Angiotensin II Induces Neurite Outgrowth and Cell Migration in Microexplant Cultures of the Cerebellum. *J. Biol. Chem.* **1999**, *274*, 31686–31692, doi:10.1074/jbc.274.44.31686.

47. Gendron, L.; Côté, F.; Payet, M.D.; Gallo-Payet, N. Nitric Oxide and Cyclic GMP Are Involved in Angiotensin II AT₂ Receptor Effects on Neurite Outgrowth in NG108-15 Cells. *Neuroendocrinology* **2002**, *75*, 70–81, doi:10.1159/000048222.

48. Morimoto, R.; Aakeda, K.; Iida, R.; Nishimura, A.; Tsujii, M.; Obata, S.; Kasai, Y.; Uchida, A.; Sudo, A. Tissue renin-angiotensin system in the intervertebral disc. *Spine* **2013**, *38*, E129-136, doi:10.1097/BRS.0b013e31827b8c89.

49. Unger, T.; Steckelings, U.M.; Santos, R.S. dos *The protective arm of the renin angiotensin: functional aspects and therapeutic implications*; 2015; ISBN 978-0-12-801485-1.

50. Han, Y.; Runge, M.S.; Brasier, A.R. Angiotensin II Induces Interleukin-6 Transcription in Vascular Smooth Muscle Cells Through Pleiotropic Activation of Nuclear Factor-κB Transcription Factors. *Circulation Research* **1999**, *84*, 695–703, doi:10.1161/01.RES.84.6.695.

51. Moriyama, T.; Fujibayashi, M.; Fujiwara, Y.; Kaneko, T.; Xia, C.; Imai, E.; Kamada, T.; Ando, A.; Ueda, N. Angiotensin II stimulates interleukin-6 release from cultured mouse mesangial cells. *J. Am. Soc. Nephrol.* **1995**, *6*, 95–101.

52. Ruiz-Ortega, M.; Ruperez, M.; Lorenzo, O.; Esteban, V.; Blanco, J.; Mezzano, S.; Egido, J. Angiotensin II regulates the synthesis of proinflammatory cytokines and chemokines in the kidney. *Kidney International* **2002**, *62*, S12–S22, doi:10.1046/j.1523-1755.62.s82.4.x.

53. Ekholm, M.; Kahan, T.; Jörneskog, G.; Bröijersen, A.; Wallén, N.H. Angiotensin II infusion in man is proinflammatory but has no short-term effects on thrombin generation in vivo. *Thrombosis*

Research **2009**, *124*, 110–115, doi:10.1016/j.thromres.2008.12.040.

54. Ekholm, M.; Kahan, T.; Jörneskog, G.; Brinck, J.; Wallén, N.H. Haemostatic and inflammatory alterations in familial hypercholesterolaemia, and the impact of angiotensin II infusion. *J Renin Angiotensin Aldosterone Syst* **2015**, *16*, 328–338, doi:10.1177/1470320315575848.

55. Walsh, D.A.; Mapp, P.I.; Wharton, J.; Polak, J.M.; Blake, D.R. Neuropeptide degrading enzymes in normal and inflamed human synovium. *Am. J. Pathol.* **1993**, *142*, 1610–1621.

56. Walsh, D.A. Angiotensin converting enzyme in human synovium: increased stromal [125I]351A binding in rheumatoid arthritis. *Annals of the Rheumatic Diseases* **2000**, *59*, 125–131, doi:10.1136/ard.59.2.125.

57. Goto, M.; Fujisawa, M.; Yamada, A.; Okabe, T.; Takaku, F.; Sasano, M.; Nishioka, K. Spontaneous release of angiotensin converting enzyme and interleukin 1 beta from peripheral blood monocytes from patients with rheumatoid arthritis under a serum free condition. *Annals of the Rheumatic Diseases* **1990**, *49*, 172–176, doi:10.1136/ard.49.3.172.

58. Goto, M.; Sasano, M.; Fuzisawa, M.; Okabe, T.; Nishizawa, K. Constitutive production of angiotensin converting enzyme from rheumatoid nodule cells under serum free conditions. *Annals of the Rheumatic Diseases* **1992**, *51*, 741–742, doi:10.1136/ard.51.6.741.

59. Veale, D.; Yanni, G.; Bresnihan, B.; FitzGerald, O. Production of angiotensin converting enzyme by rheumatoid synovial membrane. *Annals of the Rheumatic Diseases* **1992**, *51*, 476–480, doi:10.1136/ard.51.4.476.

60. Shi, Q.; Abusarah, J.; Baroudi, G.; Fernandes, J.C.; Fahmi, H.; Benderdour, M. Ramipril attenuates lipid peroxidation and cardiac fibrosis in an experimental model of rheumatoid arthritis. *Arthritis Res Ther* **2012**, *14*, R223, doi:10.1186/ar4062.

61. Niimi, R.; Nakamura, A.; Yanagawa, Y. Suppression of Endotoxin-Induced Renal Tumor Necrosis Factor- α and Interleukin-6 mRNA by Renin-Angiotensin System Inhibitors. *Jpn.J.Pharmacol* **2002**, *88*, 139–145, doi:10.1254/jjp.88.139.

62. Rüster, C.; Wolf, G. Angiotensin II as a Morphogenic Cytokine Stimulating Renal Fibrogenesis. *JASN* **2011**, *22*, 1189–1199, doi:10.1681/ASN.2010040384.

63. Schindler, R.; Dinarello, C.A.; Koch, K.-M. Angiotensin-converting-enzyme inhibitors suppress synthesis of tumour necrosis factor and interleukin 1 by human peripheral blood mononuclear cells. *Cytokine* **1995**, *7*, 526–533, doi:10.1006/cyto.1995.0071.

64. Peeters, A.C.T.M.; Netea, M.G.; Kullberg, B.J.; Thien, T.; Van Der Meer, J.W.M. The effect of renin-angiotensin system inhibitors on pro- and anti-inflammatory cytokine production. *Immunology* **1998**, *94*, 376–379, doi:10.1046/j.1365-2567.1998.00524.x.

65. Popa, C.D.; van Riel, P.L.C.M. The Use of Captopril in Rheumatoid Arthritis: Combining Treatment Targets! *Canadian Journal of Cardiology* **2013**, *29*, 639.e13, doi:10.1016/j.cjca.2013.02.017.

66. Martin, M.F.R.; McKenna, F.; Bird, H.A.; Surrall, K.E.; Dixon, J.S.; Wright, V. CAPTOPRIL: A NEW TREATMENT FOR RHEUMATOID ARTHRITIS? *The Lancet* **1984**, *323*, 1325–1328, doi:10.1016/S0140-6736(84)91821-X.

67. Cardoso, P.R.G.; Matias, K.A.; Dantas, A.T.; Marques, C.D.L.; Pereira, M.C.; Duarte, A.L.B.P.; Rego, M.J.B. de M.; Pitta, I. da R.; Pitta, M.G. da R. Losartan, but not Enalapril and Valsartan, Inhibits the Expression of IFN- γ , IL-6, IL-17F and IL-22 in PBMCs from Rheumatoid Arthritis Patients. *TORJ* **2018**, *12*, 160–170, doi:10.2174/1874312901812010160.

68. An, J.; Nakajima, T.; Kuba, K.; Kimura, A. Losartan inhibits LPS-induced inflammatory signaling through a PPAR γ -dependent mechanism in human THP-1 macrophages. *Hypertens Res* **2010**, *33*, 831–835, doi:10.1038/hr.2010.79.
69. Wong, M.H.; Chapin, O.C.; Johnson, M.D. LPS-Stimulated Cytokine Production in Type I Cells Is Modulated by the Renin–Angiotensin System. *Am J Respir Cell Mol Biol* **2012**, *46*, 641–650, doi:10.1165/rcmb.2011-0289OC.
70. Schupp, M.; Lee, L.D.; Frost, N.; Umbreen, S.; Schmidt, B.; Unger, T.; Kintscher, U. Regulation of Peroxisome Proliferator–Activated Receptor γ Activity by Losartan Metabolites. *Hypertension* **2006**, *47*, 586–589, doi:10.1161/01.HYP.0000196946.79674.8b.
71. Villar-Cheda, B.; Costa-Besada, M.A.; Valenzuela, R.; Perez-Costas, E.; Melendez-Ferro, M.; Labandeira-Garcia, J.L. The intracellular angiotensin system buffers deleterious effects of the extracellular paracrine system. *Cell Death Dis* **2017**, *8*, e3044–e3044, doi:10.1038/cddis.2017.439.
72. Fujimura, A.; Ushijima, K.; Ando, H. Does the PPAR γ -activating property of telmisartan provide a benefit in clinical practice? *Hypertens Res* **2013**, *36*, 183–183, doi:10.1038/hr.2012.189.
73. Koka, V.; Huang, X.R.; Chung, A.C.K.; Wang, W.; Truong, L.D.; Lan, H.Y. Angiotensin II Up-Regulates Angiotensin I-Converting Enzyme (ACE), but Down-Regulates ACE2 via the AT1-ERK/p38 MAP Kinase Pathway. *The American Journal of Pathology* **2008**, *172*, 1174–1183, doi:10.2353/ajpath.2008.070762.
74. Takeshita, Y.; Takamura, T.; Ando, H.; Hamaguchi, E.; Takazakura, A.; Matsuzawa-Nagata, N.; Kaneko, S. Cross talk of tumor necrosis factor- α and the renin–angiotensin system in tumor necrosis factor- α -induced plasminogen activator inhibitor-1 production from hepatocytes. *European Journal of Pharmacology* **2008**, *579*, 426–432, doi:10.1016/j.ejphar.2007.11.016.
75. Soldner, A.; Benet, L.Z.; Mutschler, E.; Christians, U. Active transport of the angiotensin-II antagonist losartan and its main metabolite EXP 3174 across MDCK-MDR1 and Caco-2 cell monolayers: Active transport of losartan and EXP 3174. *British Journal of Pharmacology* **2000**, *129*, 1235–1243, doi:10.1038/sj.bjp.0703150.
76. Chang, L.-T.; Sun, C.-K.; Chiang, C.-H.; Wu, C.-J.; Chua, S.; Yip, H.-K. Impact of simvastatin and losartan on antiinflammatory effect: in vitro study. *J Cardiovasc Pharmacol* **2007**, *49*, 20–26, doi:10.1097/FJC.0b013e31802ba4ec.