

Treatment with Angiotensin-(1–7) reduces inflammation in carotid atherosclerotic plaques

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Summary

Angiotensin (Ang)-(1–7), acting through the receptor Mas, has atheroprotective effects; however, its role on plaque vulnerability has been poorly studied. Here, we investigated the expression of the renin-angiotensin system (RAS) components in stable and unstable human carotid plaques. In addition, we evaluated the effects of the chronic treatment with an oral formulation of Ang-(1–7) in a mouse model of shear stress-determined carotid atherosclerotic plaque. Upstream and downstream regions of internal carotid plaques were obtained from a recently published cohort of patients asymptomatic or symptomatic for ischaemic stroke. Angiotensinogen and renin genes were strongly expressed in the entire cohort, indicating an intense intraplaque modulation of the RAS. Intraplaque expression of the Mas receptor mRNA was increased in the downstream portion of asymptomatic patients as compared to corresponding region in symptomatic patients. Conversely, AT1 receptor gene expression was not modified between

asymptomatic and symptomatic patients. Treatment with Ang-(1–7) in ApoE^{-/-} mice was associated with increased intraplaque collagen content in the aortic root and low shear stress-induced carotid plaques, and a decreased MMP-9 content and neutrophil and macrophage infiltration. These beneficial effects were not observed in the oscillatory shear stress-induced plaque. *In vitro* incubation with Ang-(1–7) did not affect ICAM-1 expression and apoptosis on cultured endothelial cells. In conclusion, Mas receptor is up regulated in the downstream portions of human stable carotid plaques as compared to unstable lesions. Treatment with the oral formulation of Ang-(1–7) enhances a more stable phenotype in atherosclerotic plaques, depending on the local pattern of shear stress forces.

Keywords

Angiotensin, atherosclerosis, inflammation, plaque stability

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Introduction

Atherosclerosis remains one of the leading causes of mortality in developed and developing nations (1). This elevated complication rate is mainly due to the conversion of a stable atherosclerotic plaque into an unstable plaque. These vulnerable plaques are susceptible to rupture, having dramatic consequences including acute ischaemic stroke and myocardial infarction (2, 3). In addition to systemic and environmental risk factors, the development of athero-

sclerotic plaques occurs preferentially at vessel bifurcation and curved areas of the vasculature due to the presence of low endothelial shear stress force acting locally on the arterial wall (4, 5). Recently, local low endothelial shear stress has been identified as a biomechanical determinant increasing plaque vulnerability (6). However, the molecular mechanisms underlying plaque vulnerability remain largely unknown.

The renin-angiotensin system (RAS) is known to be involved in the initiation, progression and vulnerability of the atherosclerotic

plaque (7-10). There are clear evidences that angiotensin (Ang) II, synthesised by the catalytic activity of angiotensin converting enzyme (ACE), might influence atherogenesis through the increase in vascular permeability, leukocyte infiltration and low-density lipoprotein (LDL) oxidation and uptake (11-13). On the other hand, this peptide has been also shown to increase plaque vulnerability via direct modulation on macrophage trapping, oxidative stress, and matrix metalloproteinase activation (10, 14-17).

Currently, the new counter-regulatory mediator Ang-(1-7) in the RAS has been well established (18-21). This molecule is primarily produced from Ang II by the catalytic activity of angiotensin converting enzyme 2 (ACE2) (20, 21). Its binding to Mas receptor (22), often promotes opposite actions to Ang II (18, 21). In fact, while Ang II is considered as a potent vasoconstrictor and pro-atherosclerotic mediator (7), Ang-(1-7) has been thought to induce vasoprotective and atheroprotective effects (23-25).

In the present study, we evaluated the expression of RAS components in different regions of carotid plaques from patients with severe carotid stenosis (symptomatic or asymptomatic for ischaemic stroke). Moreover, we investigated the potential protective effects of an oral formulation of Ang-(1-7) included in hydroxypropyl- β -cyclodextrin [Ang-(1-7)-CyD] (26, 27), on intraplaque histological parameters associated with vulnerability in a mouse model of shear stress-induced atherogenesis in carotid artery.

Methods

Atherosclerotic human samples and study design

Residual samples from upstream and downstream regions (considering the blood flow) of internal carotid plaques obtained from patients with high-grade internal carotid stenosis (>70% luminal narrowing) and asymptomatic (n=63) or symptomatic (n=18) for ischaemic stroke were analysed in this sub-study. The entire human cohort, belonging to the previously published unmatched case-control study (performed in 2008-2009 at San Martino Hospital, Genoa, Italy), was enrolled in this sub-study (28). No significant difference in terms of age, sex, co-morbidities, and laboratory parameters was found between asymptomatic and symptomatic patients (28).

Patients were classified as symptomatic for ischaemic stroke after the first episode of ipsilateral ischaemic stroke (focal neurologic deficit of acute onset lasting more than 24 hours (h) occurring between 30 and 10 days prior to endarterectomy). They were defined as asymptomatic when they had no history of ischaemic symptoms and in the absence of signs of cerebral necrosis at magnetic resonance imaging [MRI] with diffusion sequences. Both asymptomatic and symptomatic patients underwent carotid endarterectomy (CEA) according to the recommendations published by the Asymptomatic Carotid Surgery Trial (ACST), the European Carotid Surgery Trial (ECST) and the North American Symptomatic Carotid Endarterectomy Trial (NASCET) (29-31). Exclusion criteria were: spontaneous cerebral embolism during 30 minutes (min) preoperatively and during the dissection phase of the operation, malignant hypertension, acute coronary artery disease, any

cardiac arrhythmias, congestive heart failure (II, III and IV NYHA classes), liver or renal disorders or functional abnormalities, acute and chronic infectious diseases, autoimmune and rheumatic diseases, cancer, endocrine diseases, inflammatory bowel diseases and anti-inflammatory (other than aspirin) medications, oral anti-coagulant treatments, hormone, cytokine or growth factor therapies (28). After surgical incision, upstream and downstream portions (considering the blood flow) of human internal carotid plaque were quickly processed as previously described (28). The segments were snap-frozen in liquid nitrogen and stored at -80°C for mRNA isolation and measurements. The Medical Ethics Committee of San Martino Hospital approved the study and participants provided written informed consent. The study was conducted in compliance with the Declaration of Helsinki.

Animal model

To induce a vulnerable atherosclerotic plaque we used the mouse model of shear stress-induced atherogenesis and plaque vulnerability developed by the research group of Prof. R. Krams (4). In this model, specific patterns of shear stress are applied to the wall of the carotid artery of apolipoprotein E deficient (ApoE^{-/-}) mice. The composition of atherosclerotic plaques is determined by the distinct patterns of shear stress. Plaques induced by low shear stress have histological features of vulnerable plaques, whereas plaques developed by vortexes of oscillatory shear stress display a more stable phenotype (4, 32, 33).

ApoE^{-/-} mice in a C57BL/6J background were obtained from Jackson Laboratories. Animals (15-20 weeks of age) were fed for 11 weeks of the experimental period with a Western-type diet (15% cocoa butter and 0.25% cholesterol, Diet W; abDiets, Netherlands). After a two-weeks period of Western diet, specific patterns of shear stress were applied to the right carotid artery by the placement of a cast around the vessel (4). The cast consists of two longitudinal halves of a cylinder with a cone-shaped lumen. The geometry of the cast was developed to produce vortices downstream, therefore exposing the artery wall to oscillations in shear stress (OSS) in the downstream region and a low shear stress (LSS) in the region upstream the blood flow.

The surgical procedure of cast implantation was performed as previously described (32). Briefly, the animals were anaesthetised by 2-3% isoflurane inhalation, and the anterior cervical triangles were accessed by a sagittal anterior neck incision. Both halves of the cast were placed around the right common carotid artery and fixed with a suture. During surgery, adequacy of anaesthesia was monitored by careful visual and tactile control of mouse consciousness (changes in breathing rate and volume, heart rate, sweating and tearing). After closing the wounds, the animals were allowed to recover. Nine weeks after surgery, the animals were euthanised to collect tissue and serum samples. During the last three weeks before euthanizing (from week 6 to 9 of cast implantation), mice were orally treated with Ang-(1-7)-CyD (equivalent of 30 $\mu\text{g}/\text{kg}/\text{day}$ of Ang-(1-7) for five consecutive days per week given by gavage) (27) or respective vehicle control (hydroxypropyl β -cyclodextrin). Then, mice were euthanised (ketamine 100 mg/kg, xylazine

Gene	Function	Nucleotide sequence	Size (bp)	Accession number
AGT	Fw	5'- aactatctccccggaccatc -3'	117	NM_000029
	Rv	5'- tcagctcggtgagcagaat -3'		
	probe	5'- FAM-acctgaccatgccccactggt-BHQ1 -3'		
AT1	Fw	5'- cgcggtttgatatttgaca -3'	132	NM_000685
	Rv	5'- atcacctgggtcgaattgtt -3'		
	probe	5'- FAM-tgatctaaaatggctgggtttttatctgaa-BHQ1 -3'		
ACE	Fw	5'- ctaagcggcagcagtacaac -3'	94	NM_000789
	Rv	5'- cagcaggtggcagctctt -3'		
	probe	5'- FAM-agcaggatctactccaccgaag-BHQ1 -3'		
ACE2	Fw	5'- tgctatgtgaggcaag -3'	92	NM_021804
	Rv	5'- cccacatatccaagcaaa -3'		
	probe	5'- FAM-tgatgaatgcctatcctctctatcagctc-BHQ1 -3'		
MAS	Fw	5'- gaaccacgaacatctcaact -3'	140	NM_002377
	Rv	5'- aagcacaggaaccagaggag -3'		
	probe	5'- FAM-acgcctcagtcgggaatgcac-BHQ1 -3'		
REN	Fw	5'- ggctgt ttgattatgctggaag -3'	79	NM_000537
	Rv	5'- tattcttgcctcccaggtg -3'		
	probe	5'- FAM-ccctacactcccagatctcttc-BHQ1 -3'		
RPS13	Fw	5'-cgtcccacttggtgaag-3'	90	NM_001017
	Rv	5'-ccgatctggaaggagtaagg-3'		
	probe	5'- FAM-acatctgacgacgtgaaggagcagatt-BHQ1 -3'		

Table 1: Human primers and probes used for real-time PCR.

AGT – Angiotensinogen; AT1 – Angiotensin II receptor type 1; ACE – Angiotensin converting enzyme; ACE2 – Angiotensin converting enzyme 2; MAS – Angiotensin-(1–7)/Mas receptor; REN – Renin; RPS13 –House-keeping gene.

10 mg/kg) and blood/tissue samples were collected. Serum glucose, triglycerides, total cholesterol, LDL cholesterol and high-density lipoprotein (HDL) cholesterol were routinely measured and expressed as mmol/l. After collection, the tissues were removed and snap-frozen in liquid nitrogen and stored at -80°C for protein measurements or frozen in cryoembedding medium for histological analysis. This animal study was approved by local ethics committee and Swiss Regulatory Authorities (authorisation number 2026.2) and it is according with to the Guide for the Care and Use of Laboratory Animals of United States National Institutes of Health.

Real-time RT-PCR

Total mRNA was isolated with Tri-reagent (MRC Inc.) from upstream or downstream specimens of human carotid plaques. Reverse transcription was performed using the ImProm-II Reverse Transcription System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Real-time PCR (StepOne Plus, Applied Biosystems, Foster City, CA, USA) was performed with the ABsolute™ QPCR Mix (ABgene, Epsom, UK).

Specific primers and probes (► Table 1) were used to determine the mRNA expression of renin, angiotensinogen (AGT), ACE,

ACE2, Mas and AT1. The fold change of mRNA levels was calculated by the comparative C_t method. The measured C_t values were first normalised to the RPS13 internal control, by calculating a delta C_t (ΔC_t). This was achieved by subtracting the RPS13 C_t values from the gene of interest C_t value. A delta delta C_t ($\Delta\Delta C_t$) was calculated by subtracting the designated baseline control group ΔC_t value from the study group ΔC_t values. The $\Delta\Delta C_t$ was then plotted as a relative fold change with the following formula: $2^{-\Delta\Delta C_t}$.

Oil Red O staining

Eleven sections per mouse carotid LSS and OSS and five sections per mouse aortic sinus were stained with Oil Red O, as previously described (34). Sections from carotid and aorta were counterstained with Mayer's hemalun and rinsed in distilled water. Quantifications were performed with MetaMorph software. Data were calculated as ratio of stained area on total lesion area.

Immunohistochemistry in mouse carotid plaques and mouse aortic sinus

Mouse carotid plaque samples processing were performed as previously described (32). Briefly, the carotid artery was macroscopi-

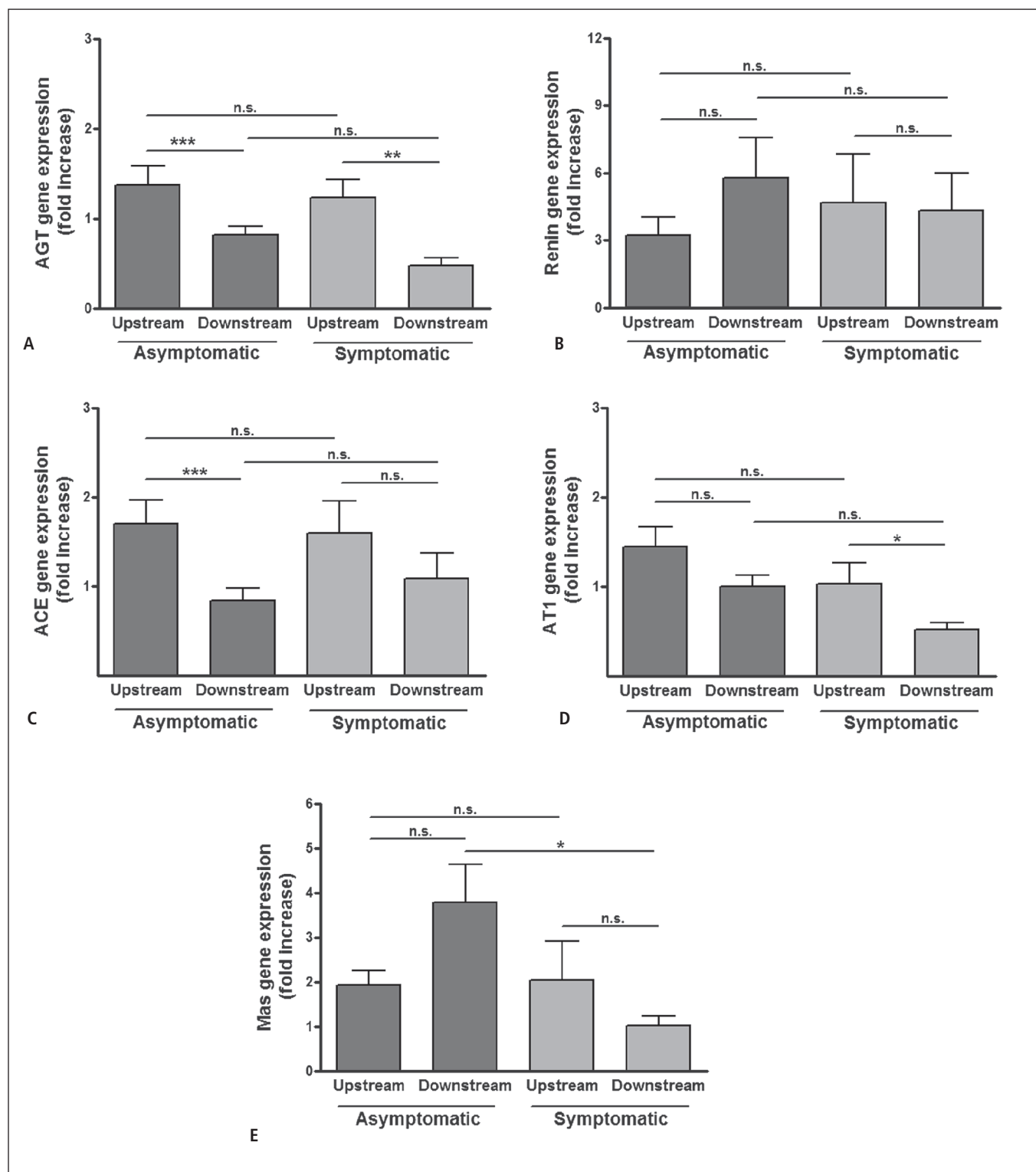


Figure 1: Expression of the Mas receptor gene is increased in the human stable plaques. Intraplaque mRNA expression of AGT (A), renin (B), ACE (C), AT1 receptor (D) and Mas receptor (E) in patients asymptomatic or symptomatic for ischaemic stroke. Relative expression normalised to house-

keeping gene was calculated with the comparative Ct method and shown as fold change of mRNA levels. n.s.: non-significant, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Data were expressed as mean \pm SEM (asymptomatic patients: $n = 63$; symptomatic patient: $n = 18$).

Table 2: Mouse serum lipid profile at sacrifice.

Serum lipid profile (mmol/l)	Vehicle-treated mice (n=15)	Ang-(1-7)-CyD-treated mice (n=15)	P-value
Total Cholesterol	23.57 ± 1.84	23.44 ± 1.10	0.9496
LDL*-cholesterol	20.30 ± 1.63	20.24 ± 1.08	0.9746
HDL+-cholesterol	4.22 ± 0.29	4.09 ± 0.18	0.7075
Triglycerides	0.86 ± 0.06	0.90 ± 0.07	0.7119
Fatty Free Acid	0.72 ± 0.09	0.68 ± 0.06	0.7223

Data are expressed as mean ± SEM. P-value calculated according to unpaired t-test. *LDL: low-density lipoprotein. +HDL: high-density lipoprotein.

cally cut in the upstream and downstream regions of the cast device (LSS and OSS regions, respectively), and then frozen in OCT medium. LSS and OSS portions from all mice were serially cut in 5 µm transversal sections. Eleven sections per staining (separated by 45 µm from each other) were used. Transversal sections of 5 µm of the aortic sinus were serially cut in through the heart and aortic roots. For each aortic sinus, five sections (separated by 50 µm from

each other) were assessed for each staining. The sections were fixed in acetone and immunostained with specific anti-mouse CD68 (macrophages, dilution: 1:400; ABD Serotec, Düsseldorf, Germany), anti-mouse Ly-6B.2 (neutrophils, dilution: 1:50; ABD Serotec), and anti-mouse MMP-9 (dilution: 1:60; R&D Systems, Abigdon, UK). Quantifications were performed with MetaMorph software. Data were calculated as percentages of stained area on total lesion area (macrophages and MMP-9) or number of infiltrating cells on mm² of lesion area (neutrophils).

Sirius Red staining

Sections of mouse carotid LSS and OSS, and aortic sinus were rinsed with water and incubated with 0.1% Sirius red (Sigma Chemical Co, St Louis, MO, USA) in saturated picric acid for 90 min. Sections were rinsed twice with 5% acetic acid in water for 10 seconds (sec), then immersed in absolute ethanol three times before clearing in xylene twice and cover-slipping. The sections were then photographed with identical exposure settings under ordinary polychromatic light microscopy. Total collagen quantifications were performed with MetaMorph software. Data were calculated as percentages of stained area on total lesion area.

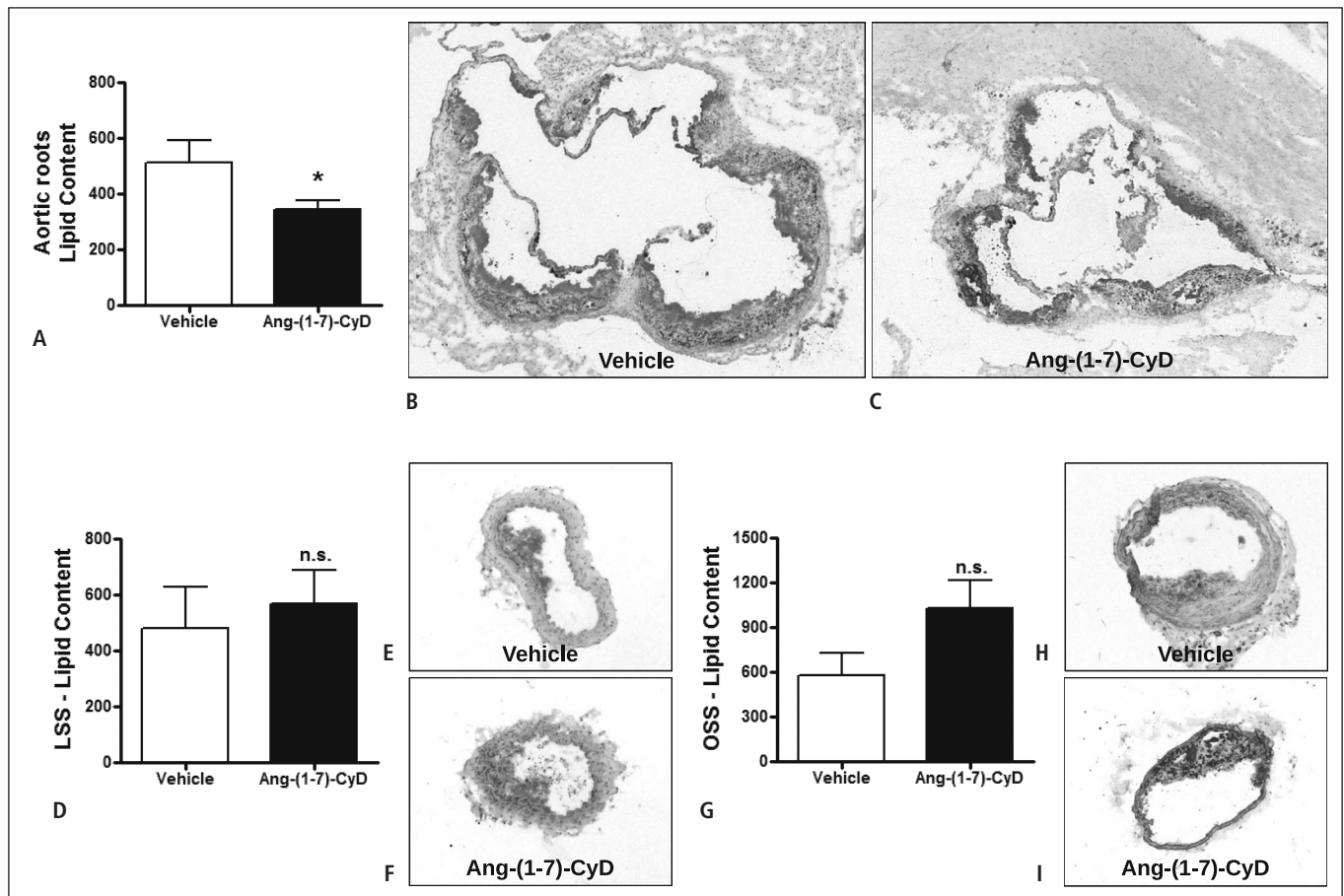


Figure 2: Treatment with Ang-(1-7)-CyD reduced the lipid content in aortic root plaques, but not in plaques developed at both LSS and OSS regions. Quantification and representative microphotographs lipid staining in: mouse aortic root (A-C), carotid LSS region (D-F) and carotid OSS regions (G-I). n.s.: non-significant, * p<0.05. Data were expressed as mean ± SEM (n=15 per group).

Human vascular endothelial cells (HECV) culture and flow cytometry analysis of ICAM-1

HECV (1.6×10^4 cells/well) were plated in six-well plates and allowed to adhere overnight. Cells were then treated with control medium (CTL) or Ang-(1-7) (at 10^{-8} to 10^{-6} M) (27, 35, 36) in the presence or absence of 20 ng/ml of human recombinant TNF-alpha (R&D Systems Ltd) for 3 h. Floating and adhering cells were collected from culture wells by repeatedly washing and by trypsinisation. Subsequently, cells were stained with propidium iodide ([PI], Sigma Aldrich) to determine cells viability, or anti-human ICAM-1/CD54-Phycoerythrin (Pharmingen, San Jose, CA, USA) accordingly to the manufacturer's instructions. The samples were analysed by flow cytometry with a FACS Calibur (Becton Dickinson, San Diego, CA, USA). Ten thousand events for each sample were acquired. Results were expressed as mean fluorescence intensity.

Data analysis

Mann-Whitney U nonparametric test was used for comparisons between mRNA expression within human plaques (asymptomatic vs symptomatic and upstream vs downstream), between parameters of mouse plaque vulnerability in Ang-(1-7)- and vehicle-treated mice. One-way ANOVA was used for multiple group comparison, and the unpaired Student t-test was used for two group comparison for *in vitro* results. Statistical analysis was performed using GraphPad Prism software (GraphPad, San Diego, CA, USA). A value of $p < 0.05$ was considered significant and the results are expressed as mean \pm SEM.

Results

RAS components are differently expressed in human carotid plaques

We observed that the AGT gene, the precursor of the RAS cascade, was highly expressed in the entire cohort, indicating a potential intraplaque modulation of RAS. However, no difference in the expression of AGT gene between the asymptomatic and symptomatic patients was shown (► Figure 1A). Nevertheless, AGT expression was reduced in the downstream regions as compared to upstream portions in both asymptomatic and symptomatic patients (► Figure 1A). Renin mRNA was also highly expressed in human plaques. Anyway, no significant differences were observed between the asymptomatic and symptomatic patients, or between the upstream and downstream plaque portions (► Figure 1B).

Considering the intraplaque mRNA expression of ACE and AT1 receptor, no difference was observed between asymptomatic and symptomatic patients. However, ACE mRNA levels were decreased in the downstream portions of asymptomatic carotid plaques as compared to upstream (► Figure 1C). On the other hand, AT1 receptor mRNA was reduced in the downstream portions of symptomatic plaques as compared to upstream (► Figure

1D). The mRNA expression of ACE2 was very weak in all human plaques, being detected only in few samples with no significant differences among the groups (data not shown). Conversely, the Mas receptor mRNA was highly expressed within human carotid plaques. In particular, the expression of this receptor was significantly increased in the downstream portions of the asymptomatic patients as compared to the corresponding regions of the symptomatic patients (► Figure 1E). No statistical differences were observed between upstream and downstream regions in both asymptomatic and symptomatic patients (► Figure 1E).

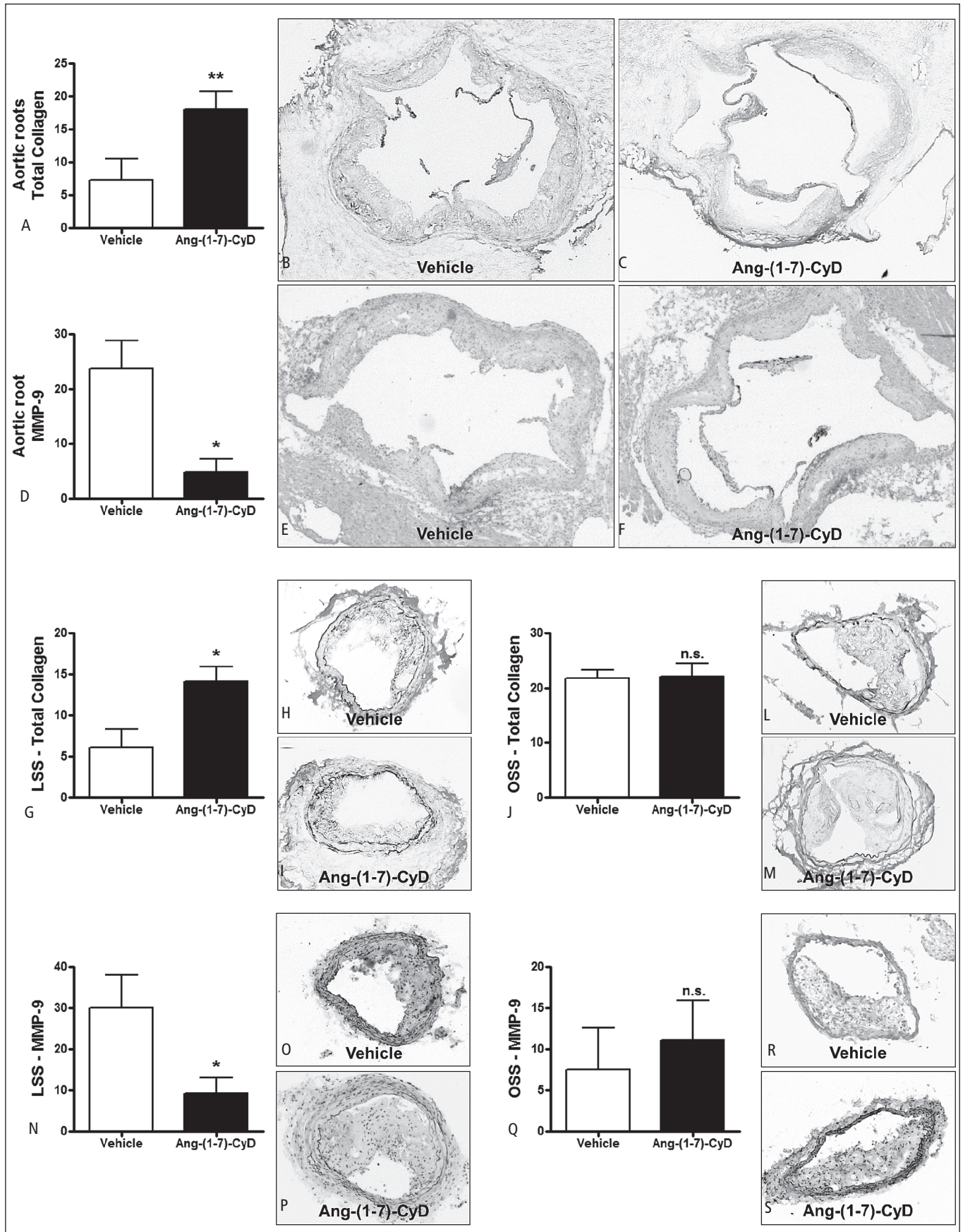
Treatment with Ang-(1-7)-CyD improves histological parameters of intraplaque vulnerability

Since the expression of Mas was increased in the downstream portions of human more stable plaques (asymptomatic patients), we sought to investigate the potential benefits of a three-week oral treatment with the Ang-(1-7)-CyD as compared to the corresponding vehicle (CyD) on mouse aortic roots and carotid plaque vulnerability and serum lipid profile. Treatment with Ang-(1-7)-CyD did not affect serum lipid profile as compared to control vehicle (► Table 2). Conversely, treatment with Ang-(1-7)-CyD reduced the lipid content within aortic root plaques (► Figure 2A-C). However, no change on lipid deposition was observed in both LSS (► Figure 2D-F) and OSS regions (► Figure 2G-I). Interestingly, treatment with Ang-(1-7)-CyD markedly increased the collagen content in the aortic root (► Figure 3A-C) and LSS-induced plaques (► Figure 3G-I), but not in the OSS-induced plaques (► Figure 3J-M) as compared to control vehicle. Accordingly, MMP-9 intraplaque content was decreased in aortic roots (► Figure 3D-F) and LSS-induced plaques (► Figure 3N-P) of Ang-(1-7)-CyD-treated mice, but not within OSS-induced plaques (► Figure 3Q-S).

Further, we investigated the effect the Ang-(1-7)-CyD treatment on intraplaque infiltration of inflammatory cells. Treatment with Ang-(1-7)-CyD markedly reduced the accumulation of macrophages and neutrophils within aortic root plaques (► Figure 4A-C and D-F, respectively) as compared to control vehicle. In plaques induced by LSS, the Ang-(1-7)-CyD treatment reduced neutrophil (► Figure 4N-P), but not macrophage infiltration (► Figure 4G-I). Treatment with Ang-(1-7)-CyD did not modify the intraplaque infiltration of these inflammatory cells in OSS regions (► Figure 4J-M and Q-R).

Treatment with Ang-(1-7) does not affect ICAM-1 expression on HECV *in vitro*

As expected, TNF-alpha significantly increased the expression of ICAM-1 on HECV. Co-incubation with Ang-(1-7) did not affect ICAM-1 upregulation induced by TNF-alpha (► Figure 5A). In the absence of TNF-alpha, Ang-(1-7) did not produce any effect on ICAM-1 expression on HECV (► Figure 5A). Importantly, treatment with Ang-(1-7) did not induce any significant effect on HECV apoptosis (► Figure 5B).



Discussion

Human study

It is largely known that the “systemic” RAS is involved in the initiation, progression and vulnerability of atherosclerotic plaques (7, 8). In the present study, we observed that AGT and renin, the precursor substrate and the primary enzyme, respectively, from the RAS peptidergic cascade, were highly expressed locally within human plaques. These data suggest an intense modulation of the RAS components within the inflamed atherosclerotic tissue via potential paracrine and/or autocrine actions. Interestingly, we found that the expression of the AGT, ACE and AT1 receptor genes was up regulated in the upstream regions of the plaques as compared to the downstream, indicating that the RAS might be differentially influenced by the distinct patterns of shear stress. In line with these results, a recent study showed that oscillatory flow induces a time-dependent increase in the expression of the AT1 receptor on endothelial cells exposed to a laminar flow (37). In the same study, *in vivo* data showed that the expression of the AT1 receptor was increased on the endothelial cells lining on the inner curvature of mouse aortic arch (area associated with the initiation of atherosclerosis), but it was almost absent on the outer curvatures (37). This study mainly addressed the early changes induced by wall shear stress on isolated endothelial cells and on the endothelium of plaque-prone area. To our knowledge, our work is the first to address the expression of RAS components on human atherosclerotic plaques associated to different pattern of shear stress. At present, our data reveal the expression of the RAS genes in the whole plaque biopsies and therefore the cellular source of each component is not yet known. Considering that no additional histological material was available from our human cohort, we could not address this point with co-localisation assays and, thus, this point represents an important limitation of our study.

While the involvement of the Ang II/AT1R axis in the occurrence of atherosclerotic plaque instability has already been addressed (8), less is known about the ACE2/Ang-(1-7)/Mas receptor axis. The ACE2 is reported as the major enzyme responsible for Ang-(1-7) formation (38, 39). However, this peptide might be generated by additional pathways involving prolylcarboxypeptidase, prolylendopeptidase or neutral endopeptidase (20). Campbell et al. reported that in human coronary vessels Ang-(1-7) is mainly formed by prolylendopeptidase (40), indicating that the main pathway of Ang-(1-7) formation may vary depending on the vascular bed. In our study, we detected a very weak expression level of the ACE2 gene in human plaques. The mRNA was detected in

only few samples, with no significant difference between groups. However, due to its multiple pathways of formation, it was not possible to estimate the local level of Ang-(1-7). Differently from our results, Sluimer et al. showed that ACE2 mRNA is expressed in both early and advanced human carotid atherosclerotic lesions (41). In their work, the quantification of immunoreactivity analysis for ACE2 showed no significant differences during all stages of atherosclerosis. Therefore, also this aspect requires further confirmation.

Ang-(1-7) acting through Mas receptor often promotes opposite effects to the Ang II, thereby promoting many beneficial cardiovascular outcomes (18-21, 42). In our study, we found that the expression of the Mas receptor mRNA was very high in all plaque, with a significant increase in the downstream portion of asymptomatic patients as compared to corresponding regions of symptomatic patients. We have previously shown that plaques from the asymptomatic patients exhibited a more stable phenotype when compared to the plaques from the symptomatic patients (32). Thus, the increased expression of Mas receptor in the downstream portion of asymptomatic patients may suggest a potential protective role of this receptor on plaque vulnerability. To better investigate this potential role, we treated ApoE^{-/-} mice submitted to carotid low shear stress-induced vulnerable plaques, with a novel oral formulation of Ang-(1-7) that has been shown to potently activate the Mas receptor (26, 27).

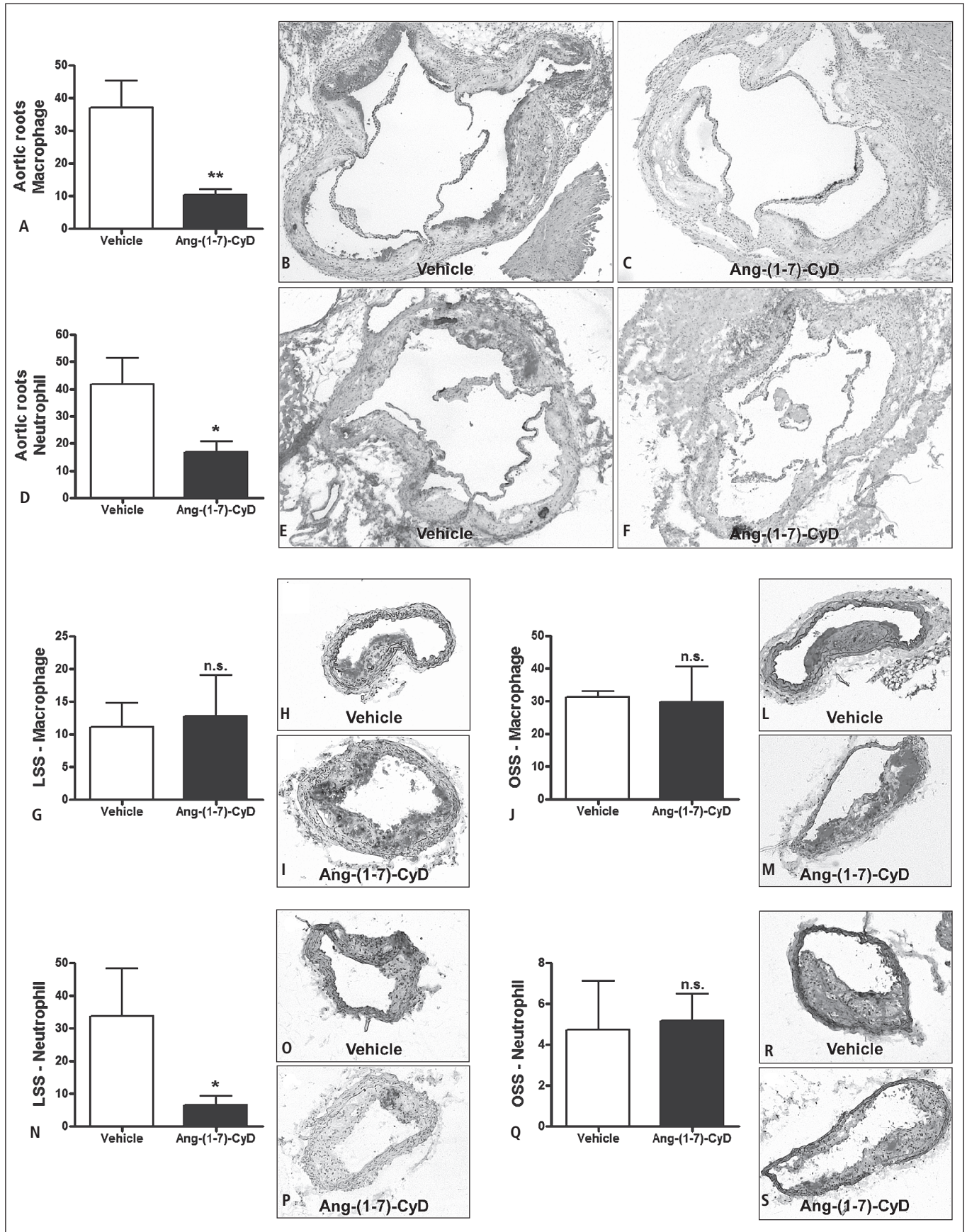
Animal study

The importance of wall shear stress to the etiology of atherosclerosis is known since more than two decades (43). However, only recently it was developed an appropriate animal model to study the cause-effect relationship between the patterns of wall shear stress and plaque formation (4). The cast model of shear stress-induced atherogenesis and plaque vulnerability described by Cheng et al. (4) has a further advantage that it allows the induction of plaques with distinct phenotypes in the same carotid artery. While low shear stress (LSS) fields promote the formation of larger plaques with a more vulnerable phenotype, oscillatory shear stress (OSS) induces more stable plaques (4, 32). More recently, data from this model and also from other human studies (6, 32, 44) identified LSS as a key biomechanical mediator in the conversion of atherosclerotic lesion from stable to unstable plaque. By using this animal model, we observed that Ang-(1-7)-CyD increased the stability of LSS-induced plaques, but not OSS plaques, suggesting that the Ang-(1-7) treatment inhibits the conversion of stable plaques to vulnerable plaques by OSS.

The potential atheroprotective effect of the Ang-(1-7) was demonstrated for the first time in 2010 (23). Tesanovic et al. showed that the long-term infusion of the Ang-(1-7) promoted a reduction on plaque size and improved the vascular endothelial function of ApoE^{-/-} mice (23). They suggested that these effects were mediated by an increase in the expression of eNOS and by an improvement on vascular oxidative stress, involving a complex interaction of both Mas and AT2 receptors (23). In line with these results, AVE-0991, a non-peptide synthetic Mas agonist, inhibited athero-

Figure 3: Ang-(1-7)-CyD increased the collagen content and decreased MMP-9 expression in aortic root and LSS-induced plaques.

Quantification and representative microphotographs of collagen staining in: mouse aortic root (A-C), carotid LSS region (G-I) and carotid OSS regions (J-M). Quantification and representative microphotographs of MMP-9 immunostaining in: mouse aortic root (D-F), carotid LSS region (N-P) and carotid OSS regions (Q-S). * p<0.05 and ** p<0.01. Data were expressed as mean ± SEM (n=15 per group).



genesis in ApoE^{-/-} mice, which was blocked by the selective Mas antagonist A-779, evidencing the protective effect of Mas receptor (24). In both studies, there were no data available regarding the potential protective role of Ang-(1-7) on the plaque phenotype. Here, we observed that the Ang-(1-7)-CyD treatment not only reduced lipid content in the aortic root plaques, but also increased collagen content, decreased MMP-9 expression and reduced neutrophil and macrophage infiltration, resulting in plaques with a more stable phenotype. The Ang-(1-7)-CyD treatment was also effective on carotid plaques. However, as plaque stabilisation occurred exclusively in plaques developed by LSS, but not by OSS, we can hypothesise that still unknown haemodynamic dependent-mechanisms might influence Ang-(1-7) efficacy. In this line, a previous work has shown that overexpression of ACE2, the precursor enzyme of Ang-(1-7) peptide, stabilises atherosclerotic plaque in aorta from rabbits, by reducing macrophage infiltration and lipid deposition and by increasing collagen content (45). However, this work did not clarify the distinct roles of Ang II and Ang-(1-7) in the beneficial actions of ACE2 overexpression, since this enzyme increases the Ang-(1-7) levels, but concomitantly decreases the levels of Ang II.

Of note, in order to clarify the cellular mechanism by which Ang-(1-7) reduced the inflammatory cell infiltration within atherosclerotic plaques, we attempted to evaluate the effects of the peptide on adhesion molecule expression on cultured HECV. We observed that co-incubation with Ang-(1-7) did not change ICAM-1 expression on both untreated and TNF-alpha-stimulated HECV, suggesting that the protective action of this peptide on atherosclerotic plaque phenotype might be not related to reduction on adhesion molecule expression on endothelial cells. Importantly, treatment with Ang-(1-7) did not induce any effect on HECV apoptosis, suggesting that doses selected for these experiments were not toxic for HECV.

Clinical perspectives and conclusions

The present study shows that the RAS components are differently expressed in atherosclerotic plaques associated with distinct shear stress patterns. Importantly, the Mas receptor is upregulated in downstream portions of human stable plaques, suggesting a protective effect of this receptor against plaque vulnerability. An oral chronic treatment with the Ang-(1-7)-CyD (a Mas receptor agonist) in ApoE^{-/-} mice enhances a stable phenotype in atherosclerotic plaques depending on the local patterns of shear stress.

The Ang-(1-7)-CyD formulation used here, not only allows the administration of the Ang-(1-7) orally, but also promotes a sus-

Figure 4: Treatment with Ang-(1-7)-CyD reduced the accumulation of macrophages and neutrophils in the vulnerable plaques. Quantification and representative microphotographs of staining for macrophages in: mouse aortic root (A-C), carotid LSS region (G-I) and carotid OSS regions (J-M). Quantification and representative microphotographs of staining for neutrophils in: mouse aortic root (D-F), carotid LSS region (N-P) and carotid OSS regions (Q-S). * p<0.05 and ** p<0.01. Data were expressed as mean ± SEM (n=15 per group).

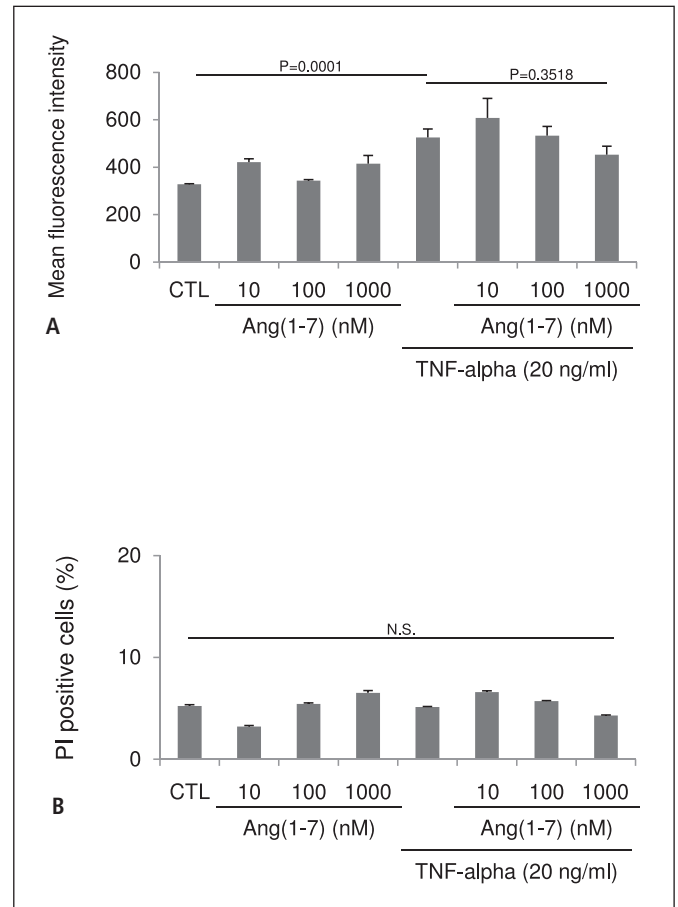


Figure 5: Treatment with Ang-(1-7) does not affect adhesion molecule expression and apoptosis in HECV. Cells were incubated for 3 h with control medium (CTL), the indicated concentrations of Ang-(1-7) with or without 20 ng/ml TNF-alpha. Then, flow cytometry analysis of ICAM-1 and PI on HECV was performed. A) Data are expressed as mean ± SEM of mean fluorescence intensity (n=9-13). B) Data are expressed as mean ± SEM of percentages of PI-positive cells (n=3). n.s.: non-significant.

tained increase in the Ang-(1-7) serum levels (27, 42). Since this peptide has a short plasmatic half-life of 10-15 sec (46), this characteristic is essential for the action of the Ang-(1-7). Cyclodextrin inclusion compounds are largely used by the pharmaceutical industry, since they promote enhancement of drug stability, absorption across biological barriers and gastric protection against digestive enzyme degradation (42, 47). It has been suggested that the Ang-(1-7)-CyD inclusion compound passes intact through the gastrointestinal tract until it entirely reaches the colon; in sequence, the colon-microflora breaks the cyclodextrin into small saccharides, delivering the free form of the peptide which is absorbed (27, 47). Thus, only the free form of Ang-(1-7) is absorbed. In the present study, we did not address the pharmacokinetics of Ang-(1-7)-CyD; however, previous studies showed that the increase in Ang-(1-7) plasma levels by Ang-(1-7)-CyD sustains for 24 h (48) and produced actions dependent on the activation of receptor Mas (27). Moreover, studies using the same dosage and posology of the

What is known about this topic?

- Carotid atherosclerotic plaque inflammation and vulnerability is influenced by both systemic and local inflammation.
- Serum and intraplaque inflammatory biomarkers have been investigated as potential targets to reduce plaque vulnerability.

What does this paper add?

- The Mas receptor (a new component of the renin-angiotensin system) is up regulated in human stable carotid plaques.
- Treatment with the Mas receptor agonist Angiotensin (1–7) reduces atherosclerotic inflammation in mouse plaques.

present study showed effective actions in different pathological conditions (35, 36). Therefore, our results may shed some lights on the use of Ang-(1-7)-CyD a new pharmacological approach for potentially stabilising atherosclerotic plaques.

Conflicts of interest

None declared.

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