Acta Pharmacologica Sinica

www.nature.com/aps

ARTICLE

Tissue-specific relaxin-2 is differentially associated with the presence/size of an arterial aneurysm and the severity of atherosclerotic disease in humans

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Circulating or tissue-related biomarkers are of clinical value for risk stratification in patients with abdominal aortic aneurysms. Relaxin-2 (RL2) has been linked to the presence and size of arterial aneurysms, and to the extent of atherosclerosis in human subjects. Here, we assessed the expression levels of RL2 in aneurysmal (AA, $n = 16$) and atherosclerotic (ATH, $n = 22$) arteries, and established the correlation between RL2 levels and the presence/size of AA and the clinical severity of atherosclerosis. The expression levels of metalloproteinases (MMPs) and endothelial nitric oxide synthetase (eNOS) were also detected for correlations with different phenotypes of atherosclerosis and AA. Temporal artery biopsy specimens ($n = 6$) and abdominal aortic tissues harvested from accident victims during autopsy ($n = 10$) were used as controls. Quantitative tissue biomarker analysis revealed that tissue-specific RL2 was increased in patients with larger or symptomatic AA compared to subjects with atherosclerotic disease and healthy controls. In situ RL2 levels were proportional to the size and the severity of aneurysmatic disease, and were substantially elevated in patients with symptomatic aneurysm of any diameter or asymptomatic aneurysm of a diameter >350% of that of the normal artery. In contrast, tissue RL2 was inversely associated with the clinical severity of atherosclerotic lesions. Correlation between RL2 and MMP2 was different between ATH1 and ATH2, depending on atherosclerosis grade. Overall, tissue RL2 is differentially associated with discrete phenotypes of arterial disease and might exert multipotent biological effects on vascular wall integrity and remodeling in human subjects.

Keywords: arterial aneurysm; atherosclerosis; endothelial dysfunction; relaxin-2; eNOS; MMPs; biomarkers

Acta Pharmacologica Sinica (2020) 41:745–752; https://doi.org/10.1038/s41401-019-0350-5

INTRODUCTION

Abdominal aortic aneurysms (AAAs) represent the most widely studied lesions among arterial aneurysms (AAs). Although the prevalence and incidence rates of AAAs have decreased during the past two decades [1], early detection is crucial for timely and successful management because the morbidity [2] and mortality [3–6] are high in patients with a ruptured AAA (rAAA) who manage to reach the hospital alive in order to undergo emergency open or endovascular repair. Biomarkers potentially related to aneurysm formation and expansion have been investigated and reviewed twice [7, 8] in order to augment timely diagnoses, but currently, there is no clinical applicability for any of the studied biomarkers due to either no association or a weak association with the natural history of aneurysms. Our group has recently discovered a positive correlation between the serum levels of the novel biomarker relaxin-2 (RL2) and the presence/size of AAs in human subjects [9]. RL2, a nonglycosylated peptide with a structural and post-translational processing resemblance to insulin and insulin-like growth factors (ILGF), was initially identified as a reproductive hormone implicated in vasoregulation during pregnancy [10]. Ever since the discovery [11] of its receptor, known today as relaxin family peptide receptor-1 (RFXP1), research on RL2 has expanded on various tissues and systems, including cardiovascular tissues such as arteries, veins, and the atrial and ventricular myocardium [12–14].

We hypothesized [9] that serum RL2 is related to the presence and size of an AA based on the knowledge that RL2 upregulates the synthesis of matrix metalloproteinase (MMP)-2 and MMP9 [15–17], which are MMPs with a stronger association with AAs [18, 19]. Meanwhile, existing knowledge suggests that RL2 upregulates the synthesis of nitric oxide (NO) [12, 20–22]. Reduction in endothelium-derived NO is, along with oxidative stress, the common pathway of action of all major risk factors leading to endothelial dysfunction and atherosclerosis [23–27], and our group demonstrated recently [9] that serum RL2 is inversely correlated with the severity of atherosclerotic disease.

In the present study, we aimed to verify and expand the findings of our preliminary study for serum RL2 [9] by

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Received: 3 May 2019 Accepted: 12 December 2019
Published online: 5 February 2020

investigating the levels of tissue-specific RL2 in aneurysmal (AA) and atherosclerotic (ATH) arteries and establishing a correlation of tissue-specific RL2 levels with the presence/size of AA and the clinical severity of atherosclerosis. Comparisons were made between the AA and ATH artery groups and the control artery group, including temporal artery biopsy specimens (TAB) and abdominal aortic tissues harvested from accident victims during autopsy (AG). Moreover, we carried out tissue-specific measurements of MMP2, MMP9 and endothelial nitric oxide synthetase (eNOS) in the aforementioned groups of arterial specimens to investigate further correlations with different phenotypes of atherosclerosis and AA.

MATERIALS AND METHODS

Study population

In this study, a total of 53 subjects were enrolled. Our interventional groups consisted of 21 patients who had open surgery for different forms of atherosclerotic disease and 16 patients who had open surgery for an AA. These 37 patients underwent surgery at Laiko Hospital in Athens, Greece and along with 6 patients who underwent TAB in the same center were the same subjects originally recruited in our preliminary study [9]. Ten accident victims who underwent a postmortem autopsy (AG) in the Department of Anatomy, Medical School of Athens, Kapodistrian University, were further enrolled to serve as controls. The study was conducted in accordance with the Declaration of Helsinki. Apart from the accident victims, all subjects provided informed consent for the use of their clinical and laboratory results for scientific purposes. Data collection and processing remained anonymous.

Phenotypes per group

Study groups and subcategorization along with baseline characteristics have been previously described [9]. In brief, our study population was allocated to the following groups:

AA group. The AA group included patients (69.73 ± 8.69, mean ± SD; age; male sex) with AAAs ($n = 13$), thoracic aortic aneurysms (TAA) ($n = 1$), internal iliac aneurysms ($n = 1$), or popliteal aneurysms ($n = 1$). Further subcategorization with respect to the size and clinical presentation of aneurysmatic disease was implemented as described previously [9].

(1) Aneurysm group 1 (AA1) ($n = 5$; male sex): patients with an asymptomatic aneurysm of a diameter 250%–300% of that of the normal artery.
(2) Aneurysm group 2 (AA2) ($n = 4$, male sex): patients with an asymptomatic aneurysm of a diameter 300%–350% of that of normal artery.
(3) Aneurysm group 3 (AA3) ($n = 7$; male sex): patients with either a symptomatic aneurysm of any diameter or an asymptomatic aneurysm of a diameter >350% of that of normal artery.

ATH group. The ATH group included a total of 22 patients (69.52 ± 14.14, mean ± SD; age; male/female sex): 12 patients with extracranial carotid artery disease and 10 patients with peripheral arterial disease (PAD). Subsequently, we further categorized patients according to the clinical severity of underlying atherosclerosis into three groups:

(1) Atherosclerosis group 1 (ATH1, $n = 10$; male/female sex): patients with moderate functional consequences [asymptomatic internal carotid stenosis >70% or claudication (Rutherford stage 3)].
(2) Atherosclerosis group 2 (ATH2, $n = 6$; male/female sex): patients with severe functional consequences [transient ischemic attack or rest leg pain (Rutherford stage 4)].
(3) Atherosclerosis group 3 (ATH3, $n = 5$; male/female sex): patients with permanent disability [stroke or tissue loss (Rutherford stage 5–6)].

Details about diagnostic modalities and diagnostic approaches in classifying the severity of atherosclerosis can be found elsewhere [9].

TAB and AG control groups. The TAB group ($n = 6$) (69.67 ± 11.31, means ± SD; age; male/female sex) had a normal pathology report of temporal arteritis and had no history or clinical signs of AA, an extracranial carotid artery, or PAD. The AG group ($n = 10$) (43.5 ± 3.4, means ± SD; age; male/female sex) included accident victims who were autopsied within 24 h postmortem without AA found during autopsy and without a history of atherosclerotic disease. As stated in our previous report [9], the AA, ATH, and TAB study groups were well matched for age, gender, medications, medical comorbidities, smoking status, and renal and liver biochemistry. AG group subjects were substantially younger and did not have any medical comorbidities.

Tissue collection and preservation

Arterial specimens from all subjects were collected with an aseptic technique at room temperature. In the AA group, part of the aneurysmatic sac, consisting of all arterial layers, was collected during open repair of the aneurysm. In the ATH group, only the atherosclerotic plaque was collected during carotid endarterectomy or the lower extremity revascularization procedure. In the TA group, part of the temporal artery, consisting of all arterial layers, was collected during TAB. In the AG group, part of the infrarenal abdominal aorta, consisting of all arterial layers, was collected during autopsy. The samples were flushed with normal saline and immediately stabilized with a commercially available tissue reagent (Allprotect Tissue Reagent, QIAGEN GmbH, D-40724 Hilden, Germany). Stabilized tissues were transported within the time and temperature limits set by the manufacturer and stored at −20 °C until analysis was performed.

Analysis of atherosclerotic and aneurysm tissues

Immunohistochemical staining. Ten serial paraffin sections of 5 μm thickness along the arterial specimens were mounted on polylysine-coated microscope slides and allowed to dry overnight, pending immunohistological staining. Sections were deparaffinized in xylene and a series of graded ethanol and finally stained with the appropriate antibodies (a) rabbit anti-human Relaxin-2 (Meridian, Life Science, Inc., UK) (working concentration: 5 μg/mL), (b) rabbit anti-human MMP2 (MBL, USA) (working concentration: 15 μg/mL), (c) rabbit anti-human MMP9 (Thermo Fisher Scientific, USA) (working concentration: 10 μg/mL), (d) eNOS (Thermo Fisher Scientific, USA) (working concentration: 2 μg/mL). The Zytochem Plus Detection Kit (Germany) was used for the development as described by the manufacturer.

qRT-PCR. Total RNA was isolated from acquired samples using the Tri Reagent (Sigma, Saint Louis, MO, USA), according to the manufacturer's protocol [28]. cDNA was synthesized by RT (M-MLV, reverse transcriptase; Sigma), and real-time quantitative polymerase chain reaction was performed by using SYBR Green (Invitrogen, Life Technologies, NY, USA), according to the manufacturer's protocol. The following primers synthesized by Eurofins Genomic (Ebersberg Germany) were used: (a) RL2: Forward: 5′-AGAAATTGTGCCATCCTTCATC-3′, Reverse: 5′-AGGGTTA ACTTCAGCTCCTGTG-3′; (b) MMP2: Forward: 5′-ATGACGATGAGCTA TGGACCTT-3′, Reverse: 5′- CTGTTGTACTCCTTGCCATTGA-3′; (c) MM P9: Forward: 5′-ACTTTGACAGCGACAAGAAGTG-3′, Reverse: 5′-GGC ACTGAGGAATGATCTAAGC-3′; (d) eNOS: Forward: 5′-CATCACCAG

GAAGAAGACCTTT-3′, Reverse: 5′-ATACAGGATTGTCGCCTTCACT-3′; (e) β-actin: Forward: 5′-GATCAAGATCATTGCTCCTCCT-3′, Reverse: 5′-ATACTCCTGCTTGCTGATCCAC-3′.

The thermal cycling protocol was as follows: 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and then 60 °C for 1 min. Negative PCR controls were run to verify the absence of genomic DNA contamination (no reverse transcription control). Fluorescence was recorded at regular intervals following the 60 °C annealing/extension segment of the PCR, and real-time data showing the relative fluorescence versus cycle number were analyzed. Because of the paucity of good internal PCR controls for tissue specimens, RL2, MMP2, MMP9, and eNOS expression (for consistency of measurement) was determined from a ΔCt value [expression = 2(−ΔΔCt)] where ΔCt was derived for each individual specimen and calculated by subtracting the mean Ct value for all targets measured from the individual Ct value of a given PCR target, as previously described. The results were then reported as the mean ± SEM for each peptide (RL2, MMP2, MMP9, and eNOS) measured in tissue.

Statistical analysis

Data are presented as the mean ± standard deviation (mean ± SD). Statistical significance between groups was calculated using Tukey's multiple comparison test. Correlation between measured parameters was assessed by Pearson analysis. Differences were considered significant when a two-tailed $P < 0.05$ was calculated. All statistical calculations were performed using GraphPad Prism, version 4.03 (GraphPad, Inc., CA, USA).

RESULTS

Immunohistochemical tissue biomarkers-qualitative analysis

Tissue biomarkers are presented in Fig. 1. Our qualitative analysis showed that MMPs and eNOS were colocalized to residual elastic fiber fragments in aneurysmal tissue and in atherosclerotic plaques. Interestingly, we found that Relaxin-2 lined endothelial cells (ECs) in all groups; the tunica media was composed of alternate layers of elastic lamellae in both AA and ATH. Metalloproteinase staining was also found in endothelial cells of both AA and ATH. We also found thickening of the tunica media marked with disarrangement of smooth muscle cells (in all groups). In both the AA and ATH groups, we observed an accumulation of amorphous material and plasma membrane rupture, while in the TAB group, MMP staining showed a diffusion of the endothelial lining bound by tight junctions. Finally, eNOS staining showed features of endothelial cell vacuolation (all groups) and plasma membrane rupture in the AA group.

Expression tissue mRNA levels of RL2, MMP2, MMP9, and eNOS in all groups

In all three ATH subgroups, the RL2 level was significantly higher than those of MMP2, MMP9, and eNOS ($P < 0.05$) (Fig. 2). Similarly, RL2 tissue levels were increased in comparison to MMP2, MMP9, and eNOS levels ($P < 0.05$). In TAB patients, RL2 tissue levels were increased compared to those of MMPs and eNOS ($P < 0.05$ for all). There was no significant difference between RL2 and MMPs and eNOS in the AG group. All data of the mRNA tissue levels are presented in Supplementary Table 1 for the AA and ATH groups and Supplementary Table 1 for the TAB and AG groups.

Differences in tissue RL2, MMPs, and eNOS mRNA levels across increasing severities of aneurysmatic and atherosclerotic disease

Across increasing severity of the ATH group, MMP2, MMP9, and eNOS expression decreased in patients with a more severe presentation of atherosclerotic disease ($P < 0.05$) (Fig. 2a). In contrast, within the aneurysmatic disease (subgroup AA1 to AA3), RL2, MMP2, and MMP9 as well as eNOS tissue levels increased ($P < 0.05$) (Fig. 2b).

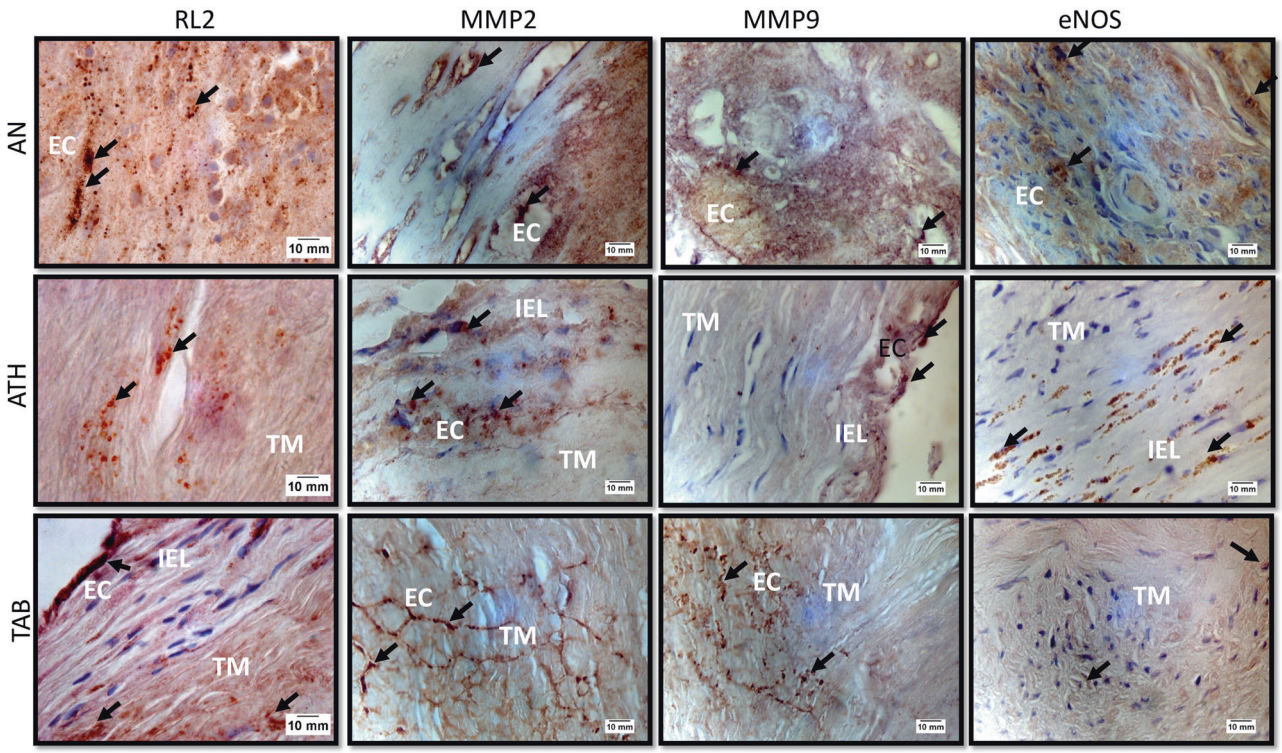

Fig. 1 Immunohistochemistry staining of specimens from patients with an aneurysm, patients with atherosclerosis and patients under investigation for temporal arteritis. Representative immunopositive images from the 3 groups with AA and ATH; TAB showed a brown color for Relaxin 2, MMP2, MMP9 and eNOS. IEL inner elastic lamina, EC endothelial cells, TM tunica media.

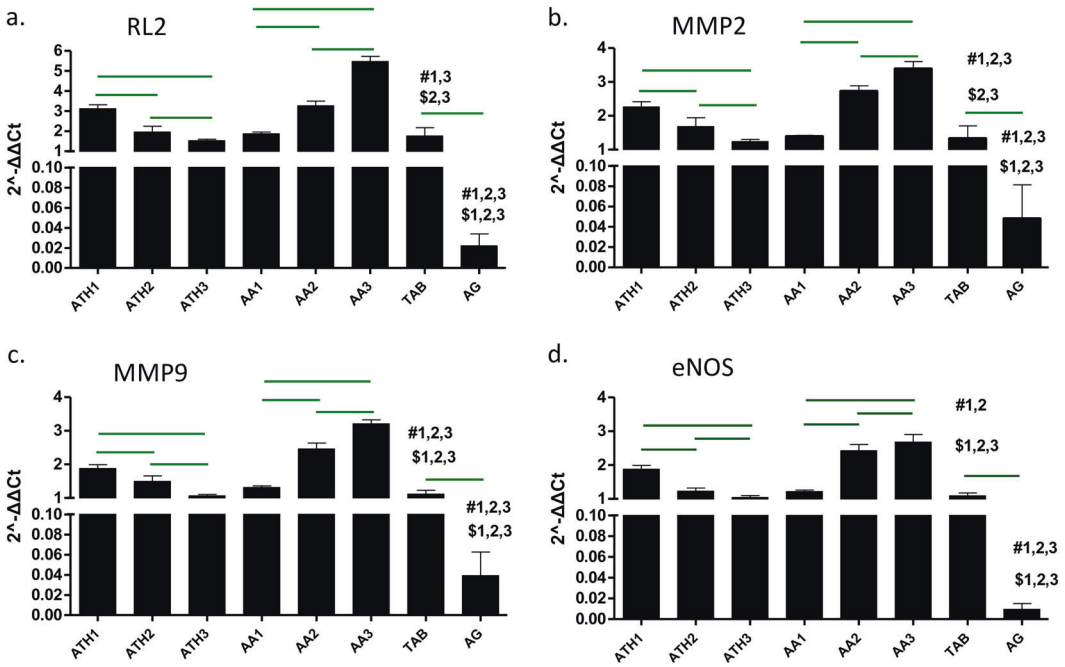

Fig. 2 mRNA tissue levels of all subgroups in (a) RL2; (b) MMP2; (c) MMP9; (d) eNOS. The green line indicates a significant difference between subgroups (ATH 1,2,3; AA 1,2,3) and groups (TAB, AG). # indicates a significant difference between TAB or AG and ATH; $ indicates a significant difference between TAB or AG and AA.

Comparison of RL2, MMPs, and eNOS mRNA levels between all study groups

RL2, MMPs, and eNOS mRNA levels were substantially higher in the AA group than in the ATH, TAB, and AG groups (Supplemental graphs a, b, c and d). There were no significant differences between RL2, MMP2, and eNOS levels in the ATH and TAB groups. Only MMP9 was significantly higher in the ATH group than in the TAB group. The AG group had significantly lower levels of RL2, MMPs, and eNOS mRNA than the other groups had.

Comparison of RL2, MMPs, and eNOS mRNA levels between subcategorized study groups

In the ATH subgroups, further statistical analysis (Fig. 2a–d) revealed that RL2, MMPs, and eNOS mRNA levels were significantly higher in the ATH1 and ATH2 subgroups than in the TAB group, with the exception of RL2 mRNA levels in the ATH2 subgroup, which were not significantly different from TAB. mRNA levels were lower or not significantly different in patients with clinically severe atherosclerotic disease (ATH3 subgroup) compared to the TAB group. Finally, the AG group had significantly lower mRNA levels than all subgroups had.

Further statistical analysis revealed that only in arterial specimens from larger or symptomatic aneurysms (AA2 and AA3 groups) were the RL2, MMPs, and eNOS mRNA levels consistently higher than those in the ATH subgroups or in the TAB and AG groups. Specimens from smaller aneurysms (AA1 group) had significantly higher RL2, MMPs, and eNOS mRNA levels only compared to the levels in patients with clinically severe atherosclerotic disease (ATH3 subgroup) and to the levels in the AG group and generally had significantly lower or not significantly different levels compared to those in less clinically advanced atherosclerotic disease (ATH1 and ATH2 subgroups) and in the TAB group.

Correlation between RL2 and MMPs and eNOS in the AA and ATH groups

In patients with mild atherosclerosis (group ATH1), RL2 was positively correlated with eNOS and inversely correlated with MMP2, whereas it was positively associated with MMP2 and eNOS in moderate atherosclerotic disease (ATH2) (Table 1, upper right). In severe atherosclerosis (ATH3), no correlations were found between RL2 and MMP2, MMP9, and eNOS (Table 1, upper right). With respect to AAs, RL2 was positively associated with eNOS and inversely correlated with both MMP2 and MMP9 only in patients with mild dilatation (AA1 group). In contrast, no significant correlations of RL2 were found in patients with moderate and severe arterial aneurysmatic disease (AA2 and AA3) (Table 1, lower left). Furthermore, RL2 was positively correlated with MMP2, MMP9, and eNOS in the TAB group. In the AG group, RL2 did not correlate with either MMPs or eNOS ($P > 0.05$).

DISCUSSION

The principal finding of this study is that tissue-specific RL2 is increased in patients with a larger or symptomatic AA in comparison to subjects with atherosclerotic disease and to healthy controls. Notably, in situ RL2 levels were proportionally increased according to the size and severity of aneurysmatic disease and were substantially elevated in patients with a symptomatic aneurysm of any diameter or an asymptomatic aneurysm of a diameter >350% of that of the normal artery in comparison to (a) patients with asymptomatic or smaller AAs, (b) subjects with atherosclerosis, and (c) control subjects. On the other hand, an inverse association of tissue RL2 with the clinical severity of atherosclerotic lesions was observed. These findings are generally consistent with our previously published data on serum RL2 [9], and to the best of our knowledge, this is the first report on the correlation of tissue-specific RL2 mRNA levels in human subjects with aneurysms and atherosclerosis. Further to our previous work, our current study indicated that tissue MMPs and eNOS fluctuate with exactly the same pattern as RL2 in various phenotypes of both aneurysmatic and atherosclerotic disease, and importantly, RL2 was not related to MMPs in moderate and severe aneurysmatic and atherosclerotic disease, suggesting an independent role of this multipotent molecule on vascular wall integrity and remodeling.

Table 1. Correlation matrix of assessed biomarkers tissue mRNA levels in each aneurysm and atherosclerosis group separately, depending on the severity of atherosclerosis.

AA Down left / ATH Upright

	RL2 -1	MMP2 -1	MMP9 -1	eNOS -1
RL2 -1		−0.523	0.16	0.514
MMP2 -1	−0.65		−0.318	−0.369
MMP9 -1	−0.69	0.34		0.283
eNOS -1	0.74	−0.223	−0.171	

AA Down left / ATH Upright

	RL2 -2	MMP2 -2	MMP9 -2	eNOS -2
RL2 -2		0.924	0.172	0.493
MMP2 -2	−0.114		0.206	0.595
MMP9 -2	0.4	−0.216		0.732
eNOS -2	0.05	−0.451	0.9	

AA Down left / ATH Upright

	RL2 -3	MMP2 -3	MMP9 -3	eNOS -3
RL2 -3		0.069	−0.042	−0.04
MMP2 -3	−0.127		0.015	0.378
MMP9 -3	0.324	0.07		0.249
eNOS -3	0.261	−0.43	−0.345	

Down-left illustrated the AA groups. Upright illustrated the ATH groups.
−1 represents the AA1 or ATH1 group; −2 represent the AA2 or ATH2 group; −3 represent AA3 or ATH3 group.
R values indicate Pearson's correlation coefficients.
Significant association between two tissue biomarkers is presented with green color; no statistical significance presented with red color; while the minus symbol (−) in green boxes indicates an inverse association.
AA aneurysm group, *ATH* atherosclerosis group, *RL2* relaxin-2, *MMP* matrix metalloproteinase, *eNOS* endothelial nitric oxide synthetase

AAs are characterized by structural alterations of the vascular wall resulting, in part, from the degradation of collagen and elastin fibers. These changes are associated with inflammatory infiltrates [29] and excessive production of MMPs [30, 31], which are assumed to orchestrate the widespread matrix destruction. An association between AA and MMPs has been described in both AAA [32, 33] and PAD [34, 35]. MMP transcripts expressed physiologically at low levels increase rapidly in the presence of inflammation-associated vascular remodeling. Notably, increased expression of MMPs has been observed by other authors in human aneurysm tissue [31] and has been linked to extracellular matrix degradation and increased risk of rupture in response to distending intra-arterial pressure [36, 37]. Previous studies have shown a complementary role of MMP2 and MMP9 in aneurysmal dilatation. In brief, MMP2 primarily acts as a collagenase that initiates cleavage of the collagen triple helix, and subsequently, single chains are subject to degradation by MMP9 [38, 39]. In our study, we found that increased expression of both MMPs is related to a more severe phenotype of AA disease, even though previous studies have reported contradicting conclusions on the clinical utility of circulating plasma [40] and serum [41] levels of MMP2 and MMP9. In addition, we observed an inverse correlation of RL2 with MMPs in the early stages of AA. However, the association of MMPs with RL2 in advanced stages was not noted; this suggests that further mechanisms are involved.

Endothelial cells express eNOS to serve as an important source of NO, a potent vasodilator and inhibitor of inflammation, platelet aggregation, and smooth muscle cell proliferation [42]. Interestingly, eNOS gene polymorphisms have been associated with vascular diseases [43]; two recent meta-analyses have identified the T786C polymorphism of eNOS as a predictor for the development of intracranial aneurysms in the cerebral vascular system [44, 45], and various polymorphisms of eNOS have been linked to AAA [46–48]. Our findings of increased eNOS levels in larger aneurysms are in accordance with a recent animal study [49], in which increased eNOS activity reduced smooth muscle α-actin and upregulated MMPs during flow-induced intracranial aneurysm formation. However, experiments in mice have shown that eNOS deficiency results in an increased incidence of AAA formation [40], and Aoki et al. [50] showed that eNOS suppresses cerebral aneurysm formation by reducing hemodynamic stress to arterial walls. Currently, there is no clinical utility of eNOS as a biomarker for predicting the natural history of human aneurysms, which are a multifactorial disease, and the contradicting evidence suggests that eNOS may play a lesser role in aneurysm formation and progression than other factors.

Destabilization of plaques is implicated as a clinical manifestations of atherosclerotic disease [51, 52]. Among various proteinases, MMP2 and MMP9 have been shown to be the predominant ones secreted by T lymphocytes and macrophages [53, 54] across atherosclerotic plaque development [55]. MMP2 is constitutively expressed in all human vascular cells [56], but plaques express increased MMP2 compared to that in normal vessels, especially unstable ones [57]. Overall, MMP2 levels are increased in patients with atherosclerotic [58, 59] PAD, especially in combination with type II diabetes [60] or acute coronary syndromes [61, 62]. Additionally, circulating MMP9 levels are increased in patients with atherosclerosis [62], acute coronary syndromes [62, 63], and type II diabetes [64]. There is also substantial evidence for a link between heightened MMP9 and plaque vulnerability [65, 66] through facilitation of basement membrane breakdown, smooth muscle cell migration, plaque neovascularization, and inflammatory cell infiltration [67, 68].

Indeed, in our current study, increased levels of MMP2 and MMP9 were related to the severity of atherosclerotic disease, while the RL2 level was inversely associated with atherosclerotic burden. Our group has recently shown serum RL2 to be increased in the early clinical stages of atherosclerosis and decreased in more advanced stages of atherosclerotic disease [9], and it was hypothesized that RL2 upregulation represents a form of protective mechanism; however, it was unclear whether this early increase was actually beneficial. The inverse correlation of RL2 with MMP2 in mild atherosclerosis and the positive association with eNOS in moderate atherosclerosis is suggestive of a beneficial effect. Enhanced NO bioavailability through eNOS upregulation and decreased expression of H2S generating enzymes might increase plaque stability and integrity [69, 70]. It remains unclear why this protective mechanism is ameliorated in advanced stages of atherosclerosis, but our findings provide further evidence of potential therapeutic use of RL2 in atherosclerotic disease, as other investigators have recently demonstrated [71].

There is a good size of evidence that RL2 increases NO synthesis in both an acute and a delayed fashion, although the actual mechanism and whether this is related directly or indirectly to RFXP1 is not clear [72, 73]. The activity and expression of not only eNOS [74–77] but also inducible-NOS (iNOS) [74, 76, 78–84] and neuronal-NOS (nNOS) is increased by RL2. Due to the production of NO and activation of guanyl cyclase, NOS activation by RL2 leads to an increase in cyclic guanyl monophosphate (cGMP) [78, 79, 83, 85]. Based on activation of NOS and cGMP, NO production by RL2 can be further enhanced by an increase in eNOS activity by Akt (Protein Kinase B) phosphorylation [86], upregulation of iNOS activity following stimulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-

controlled transcription [79, 81], and finally by increased activity of eNOS, following activation of the endothelin B (ET_B) receptor by increased activity of MMP2 and MMP9 by RL2 [15]. Interestingly, inhibition of NF-κB-mediated transcription reduces the RL2-related increase in MMP9 expression and activity [86], suggesting a relationship between the effects of RL2 upon MMPs and NO [12]. Furthermore, RL2 can decrease the expression of tissue inhibitors of MMPs (TIMPs) [87–89], and recently, it was shown that the known pathways of RL2/RFXP1 signaling [including activation of phosphoinositide 3-kinase (PI3K), Akt, protein kinase C (PKC)-ζ, and extracellular signal-regulated protein kinases 1 and 2 (ERK1/2)] are connected with the increased expression of mRNA for MMP9 [4]. These insights could be useful for investigating the utility of RL2 in the clinical setting [12].

Certain limitations should be acknowledged in our study. We enrolled four relatively small groups of patients, which might have hampered the identification of associations. Furthermore, our study is limited by the fact that the AG group was not matched with other groups for age and comorbidities, while in the ATH group, subjects presented with heterogeneous arterial pathologies. These limitations warrant caution when interpreting the results and drawing conclusions. In our study, we included data from autopsy subjects, as Gupta et al. [90] have shown that under conditions similar to ours, autopsy specimens are reliable for conduction molecular estimations. Importantly, no definite conclusions can be made regarding the underlying mechanisms of RL2 in vascular diseases. Contemporary literature does not provide mechanistic data on possible RL2 effects on AAs. Consequently, the potential applicability of RL2 as a novel therapeutic target merits further investigation. A postulation may be advanced that RL2 represents a feedback protective mechanism to counteract an ongoing detrimental process.

In conclusion, tissue-specific RL2 is higher in patients with an AA, showing a positive relationship with the size of the aneurysmatic dilatation. Levels of RL2 are also inversely correlated with the severity of atherosclerotic disease. Future studies with larger cohorts are warranted to verify and expand our results with the ultimate aim to reveal possible etiopathogenetic backgrounds of atherosclerosis and aneurysm formation.

AUTHOR CONTRIBUTIONS

KP wrote the paper and designed the study; AK performed the research; GG and NP analyzed the data; C. Kontogiannis, C. Kourek, KSM and DVC wrote the paper; and IK and SG designed the study.

ADDITIONAL INFORMATION

The online version of this article (https://doi.org/10.1038/s41401-019-0350-5) contains supplementary material, which is available to authorized users.

Competing interests: The authors declare no competing interests.

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