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Transcriptional regulation of TIMPs in ascending aorta aneurysms

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ABSTRACT

The events that result in the establishment and progression of aortic aneurysms are complex and multifactorial. However, degradation of the extracellular matrix (ECM) of aortic tunica media appears to be a consistent histopathological and biochemical feature. An increased local expression of matrix metalloproteinases (MMPs) as well as an imbalance between MMP expression and the expression of their natural tissue inhibitors (TIMPs) have been demonstrated in dilated aortic wall. We hypothesized that a distinct MMP and TIMP expression pattern underlies the development of ascending aorta dilation. To test our hypothesis, expression levels of 10 MMPs and 4 TIMPs were assessed by real-time PCR in dilated and normal aortic tissue derived from patients that underwent elective surgical repair of ascending aorta aneurysm (AAA) and coronary artery by-pass grafting, respectively. We found no statistically significant up- or down-regulation of any individual MMP. Surprisingly, the tissue inhibitor of metalloproteinases (TIMP)-3 was significantly more expressed in dilated aortic tissue compared to control tissue, thereby reflecting an effort to counteract MMP activity. Finally, when we evaluated the MMP and TIMP co-expression pattern in normal and dilated aortic tissue, we observed that in aortic aneurysms activation of the MMP system was characterised by the co-expression of more than one proteinase and the down-regulation of TIMP-1 and -2. The latter observation is the key regulatory point that leads to ECM degradation and, subsequently, to AAA formation.

Introduction

Extracellular matrix (ECM) proteins are important structural components of the vascular wall [1]. Disturbances in the synthesis and degradation of these elements have been widely implicated in the process of cardiovascular disease [2–4].

Aneurysms of the ascending aorta (AAAs) are common degenerative diseases of the aortic wall [5]. The events that result in the establishment and progression of aortic dilation are complex and multifactorial; they involve an interaction between cellular components of the vascular wall and hemodynamic alterations, and are strongly influenced by the individuals' genetic background [2,4]. However, degradation of the ECM of aortic tunica media appears to be a consistent histopathological and biochemical feature [2,5].

Matrix metalloproteinases (MMPs) constitute a family of over 25 secreted and cell surface enzymes [6,7]. Their main function is degradation of the ECM in the process of tissue homeostasis [6,7]. MMPs are inhibited by specific endogenous inhibitors of metalloproteinases (TIMPs) comprising a family of four proteins (TIMP-1, -2, -3 and -4) [6,7]. An altered local expression of individual molecules such as MMPs or TIMPs has been shown in the dilated aortic wall [8–10]. However, it remains unclear whether increased MMP activity, decreased TIMP activity or the loss of balance between MMP expression and the expression of their natural inhibitors (TIMPs) are the key biochemical disturbances leading to the degeneration of tunica media and subsequently to aortic dilation.

We hypothesized that a distinct MMP and TIMP expression pattern underlies the development of ascending aorta dilation. To test our hypothesis, expression levels of 10 MMPs and 4 TIMPs were assessed in dilated and normal aortic tissue derived from patients that

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underwent elective surgical repair of ascending aorta aneurysm and coronary artery by-pass grafting (CABG), respectively.

Materials and methods

Patients and controls

Specimens were surgically obtained from 39 patients (median age 65, range 39-83) who underwent elective surgical repair of ascending aortic aneurysm at the involved institutes during a 24-month recruitment period (January 2006 to December 2008). Eighteen aortic tissue samples from age- and sex-matched patients that underwent CABG during the same recruitment period at the University Hospital of Heraklion, Crete, Greece comprised our control group. Tissue specimens were obtained at the time of surgery, were snap-frozen and stored at -80 °C until RNA extraction. Control samples were obtained from macroscopically healthy aortic tissue at the site of graft anastomosis. Table 1 summarises the clinical and epidemiological characteristics of patients and controls. The study was approved by the Ethics Committee of the University of Crete. All participants gave their written informed consent.

RNA extraction and reverse transcription (RT-PCR)

Total RNA and protein was extracted from each tissue sample by using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) and with the aid of a power homogenizer, according to the manufacturer's instructions. RNA concentration and purity were evaluated by a spectrophotometer. Aliquots of RNA were stored at -80 °C and -20 °C, respectively, until use.

cDNA from each sample was derived by reverse transcription of 2 μg of total RNA using the AffinityScript™ Multi Temperature cDNA synthesis kit (Stratagene, La Jolla, CA, USA). Random hexamers were used as amplification primers. To remove the RNA template, cDNA was incubated with *E. coli* RNaseH, and stored at -20°C until use.

Real-time PCR

Real-time PCR reactions were carried out using the Mx3000P Real-Time PCR system (Stratagene, La Jolla, CA, USA) with SYBR® Green I Master Mix (Stratagene, USA), according to the manufacturer's instructions. MMP and TIMP primers were designed to span at least one intron in order to avoid the amplification of contaminating genomic DNA. Primer sequences and optimal annealing temperatures are listed in Table 2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control in order to normalize MMP and TIMP mRNA expression levels. A representative pool of all samples was diluted in a series of seven 2X dilutions and used to construct a standard curve for the quantification process. Melting curves for each amplicon were generated to evaluate the specificity of the products and the results were verified by agarose gel elecrophoresis and ethidium bromide staining. Each PCR reaction included two negative controls, one with no cDNA template and one with no reverse transcription treatment. All samples were run in triplicate. Data were collected and analyzed using Mx3000P Real-Time PCR software version 2.00, Build 215, Schema 60 (Stratagene). Reproducibility of the real-time PCR results (mean value of data acquired from three independent RT-PCR experiments) for the same samples was 99%. MMP and TIMP transcription levels (normalized to GAPDH) for the pathological samples and controls were calculated using the formula: normalised sample or control $= (1 + E_{MMP\text{-}x})^{-\Delta Ct}$MMP-x/$(1 + E_{GAPDH})^{-\Delta Ct}$GAPDH. The normalized expression of each pathological sample was then divided by the mean normalized value of the controls (expression ratios). A 2-fold increased or decreased expression was considered as significant over-expression or down-regulation, respectively.

Table 1
Clinical characteristics of the patients and controls.

Characteristic	AAA patients	Controls
Median age (range)	65 (39-83)	66 (49-78)
Sex male	24 (61.5)	16 (89)
Coronary artery disease	7 (17.9)	18 (100)
Diabetes	6 (15.4)	6 (33.3)
Hypertension	28 (72)	13 (72.2)
Dyslipidemia (on lipid lowering medication)	13 (33.3)	14 (77.8)
Smoking	7 (17.9)	8 (44.4)
Aortic regurgitation (more than mild)	16 (41)	0 (0)
Bicuspid aortic valve	3 (7.7)	0 (0)
Total	39	18

Figures refer to number of patients (%) or as indicated.

Table 2
Primer sequences used for quantitative real-time RT-PCR.

Gene	Primer pair sequence (5'-3')	Annealing temperature	Product size (bp)
MMP-1	GGCCCACAAACCCCAAAAG ATCTCTGTCGGCAAATTCGTAAGC	58 °C	204
MMP-2	CACGCTGGGCCCTGTCACTCGGG GCCTCGTATACCGCATCAATC	60 °C	204
MMP-3	GATGCCCACTTTGATGATGATGAA AGTGTTGGCTGAGTGAAAGAGACC	57 °C	94
MMP-8	TGGGGCTCGCTCACTCCTC ATCAAATGTCAAACTGGGGTCAC	55 °C	192
MMP-9	TGCCCGGACCAAGGATACAGTTT AGGCCGTGGCTCAGGTTCAGG	60 °C	198
MMP-12	GGGCCCGTATGGAGGAAACA ACGGGCAAAAACCACCAAAATG	59 °C	180
MMP-14	GCCGGGGCATCCAGCAACTTTA TCCTCACCCGCCAGAACCAG	59 °C	210
ADAMTS-1	CCCGGCACTGCAAGGCGTAGG GAGGGCAGCATTGGAGGTCACTT	60 °C	283
ADAMTS-4	TGTGCGGAGGGGACGGTTCTGGT ACATCTGTGGGGGAGGGCATCA	62 °C	223
TIMP-1	CACCATGGCCCCCTTTGAGC CACGAACTTGGCCCTGATGACGA	60 °C	146
TIMP-2	CCAAAGCGGTCAGTGAGAAGGAAG GAGGAGGGGGCCGTGTAGATAAAC	60 °C	145
TIMP-3	GGCGGCAGCAGCGGCAATGAC TACCAGCTTCTTCCCCACCACCTT	60 °C	172
TIMP-4	GGCGCACCCTCAGCAGCACA GTCTGCACTGGCCGGAACTACCTT	60 °C	88
GAPDH	GGAAGGTGAAGGTCGGAGTCA GTCATTGATGGCAACAATATCCACT	60 °C	101

ELISA

Human TIMP-3 ELISA was based on standard sandwich enzyme-linked immune-sorbent assay technology, using the TIMP3 (Human) ELISA kit (Abnova, Taipei City, Taiwan) according to the manufacturer's recommendations. Human TIMP-3-specific monoclonal antibodies were precoated onto 96-well plates. The human TIMP-3-specific detection monoclonal antibodies were biotinylated. The test samples and biotinylated detection antibodies were subsequently added to the wells followed by washing with PBS or TBS buffer. The avidin-biotin-peroxidase complex was added, and unbound conjugates were washed away with PBS buffer. HRP substrate TMB was used to visualize the HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue color product that changed to yellow after adding acidic stop solution.

Histochemical characterization of the surgical specimens

Tissue was collected at the site of the maximum diameter of the dilated aorta. Samples were fixed in formalin and embedded in paraffin. Embedded tissue was stained with eosin haematoxylin and

Table 3
Detected mRNA expression of A. MMPs and B. TIMPs in ascending aortic aneurysm tissues and control specimens.

Expressed Gene	AAAs % (N)	Controls % (N)	*P* value*
A.			
MMP-1	-	-	-
MMP-2	**46%** (18/39)	**33%** (6/18)	NS
MMP-3	**18%** (7/39)	**5%** (1/18)	NS
MMP-8	**33%** (13/39)	**39%** (7/18)	NS
MMP-9	**26%** (10/39)	**28%** (5/18)	NS
MMP-12	**15%** (6/39)	**0%** (0/18)	NS
MMP-14	**69%** (27/39)	**61%** (11/18)	NS
ADAMTS-1	**56%** (22/39)	**67%** (12/18)	NS
ADAMTS-4	**26%** (10/39)	**28%** (5/18)	NS
B.			
TIMP-1	**87%** (34/39)	**83%** (15/18)	NS
TIMP-2	**74%** (29/39)	**55%** (10/18)	NS
TIMP-3	**95%** (37/39)	**100%** (18/18)	NS
TIMP-4	**97%** (38/39)	**100%** (18/18)	NS

*x^2 test. $P<0.05$ is considered statistically significant; NS, not significant.

examined under light microscopy. Histochemical characterization was feasible in 28/39 samples.

Statistical analysis

The one-sample Kolmogorov-Smirnov test was employed to assess the normal distribution of the mRNA expression values of the genes studied. The mRNA expression of the MMPs and TIMPs in the normal and pathological sample groups, as well as in groups of different clinical and epidemiological features were compared using either parametric or non-parametric procedures where appropriate. The Pearson or Spearman rank correlation was employed to examine the pair-wise correlation of MMP and TIMP mRNA. The Chi-Square (χ^2) test was used to assess differences the mRNA expression status (over-expression or down-regulation) in MMPs and TIMPs; within or between different specimen groups. Probability values or differences less than 0.05 were considered to be statistically significant. Statistical calculations were performed using SPSS software, version 11.

Results

The mRNA expression profile of nine MMPs and four TIMPs, (namely MMP-1, -2, - 3, -8, -9 -12, -14 and ADAMTS-1, -4, as well as TIMP-1, 2, 3, 4) was assessed in 39 AAA tissue specimens. Histochemical characteristics of the AAA samples are summarized in Supplementary Table 1. Eighteen aortic tissue specimens from patients undergoing CABG were also assessed, using a quantitative real-time RT-PCR method. The ratio of the transcript levels of each MMP or TIMP versus the GAPDH mRNA levels (internal control) of the same specimen served as its normalised mRNA levels.

MMP transcript levels

MMP-1 exhibited an absence of mRNA expression, while MMP-3 as well as ADAMTS-4 displayed a limited mRNA expression in both AAA samples as well as in controls. MMP-2, -8, -9, 12, 14 and ADAMTS-1 exhibited detectable transcript levels in a range of 15-69%

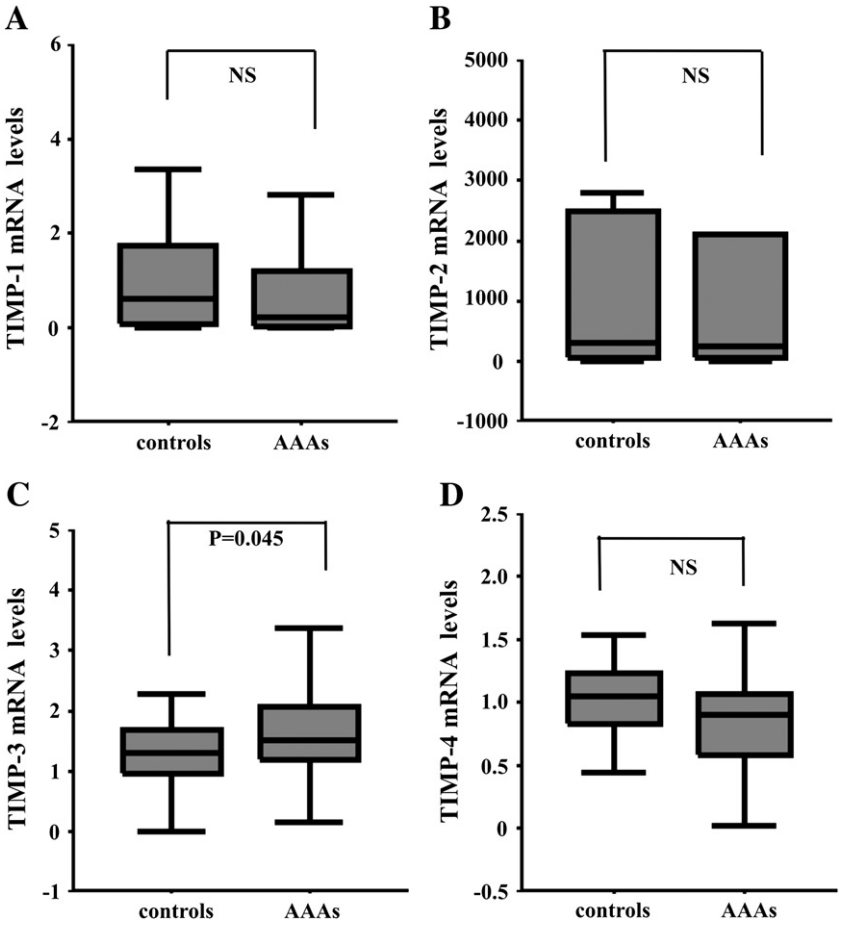

Fig. 1. Normalized transcript levels of A. TIMP-1, B. TIMP-2, C. TIMP-3 and D. TIMP-4 in AAAs and control specimens. TIMP-3 mRNA expression levels were significantly elevated in aortic aneurysms (1.74 ± 0.15) compared to controls (1.25 ± 0.15) ($P = 0.045$, t-test). Lower TIMP-1 and -4 transcript levels were observed in AAAs compared to controls, however the difference was not statistically significant.

of the aneurysm tissue samples and in a range of 0-33% of the control specimens, respectively, as shown in Table 3A. Notably, MMP-12 mRNA was present only in aneurysm specimens (15%). All MMPs, however, displayed similar mRNA expression levels in the pathological as well as the control specimens.

TIMP transcript levels

TIMP-1, -2, -3 and -4 mRNA expression was observed in the majority of the AAA (ranging from 75% to 97%) and control specimens (ranging from 55% to 100%), as shown in Table 3B. Interestingly, TIMP-3 mRNA expression levels were significantly elevated in aortic aneurysms (mean ± SEM: 1.74 ± 0.15) compared to controls (1.25 ± 0.15) (P = 0.045, t-test) (Fig. 1A). Accordingly, protein levels of TIMP-3 -assessed by ELISA- were significantly correlated with the mRNA expression levels of TIMP-3 assessed by real-time PCR (Pearson's r = 0.481, p = 0.023). Lower TIMP-1 and -4 transcript levels were observed in AAAs (0.90 ± 0.23 and 0.86 ± 0.07, respectively) compared to controls (1.84 ± 0.91 and 1.02 ± 0.11, respectively). However, the difference was not statistically significant (Fig. 1B, C). The differences in the ratio of MMPs to their natural tissue inhibitors (TIMPs) between the AAA and control tissues were further assessed. Every MMP/TIMP grouping was tested in the AAA and control tissue. No statistical significant correlations were obtained.

mRNA levels and clinical features of MMPs and TIMPs

Transcriptional down-regulation of TIMP-1 and -2 is depicted in aortic aneurysm tissues with a diameter greater than 55 mm. Specifically, significantly lower TIMP-1 and -2 mRNA levels were observed in tissue specimens of patients with an aneurysm diameter greater than 55 mm (P = 0.013 and 0.014, Mann-Whitney test, respectively) (Fig. 2). Sex was found to be significant only in the case of TIMP-1 in that male patients displayed higher mRNA levels (mean ± SEM: 1.01 ± 0.31) compared to females (0.55 ± 0.41) (P = 0.043, Mann-Whitney test).

No correlation was observed between the degree of aortic regurgitation or the presence of bicuspid aortic valve disease and the transcript levels of any of the MMPs and TIMPs included in the present study. Furthermore, patients' smoking habits, lipid lowering therapy or the presence of disorders such as diabetes, coronary artery disease and hypertension were not found to be associated with the transcript levels of any of the MMPs and TIMPs tested.

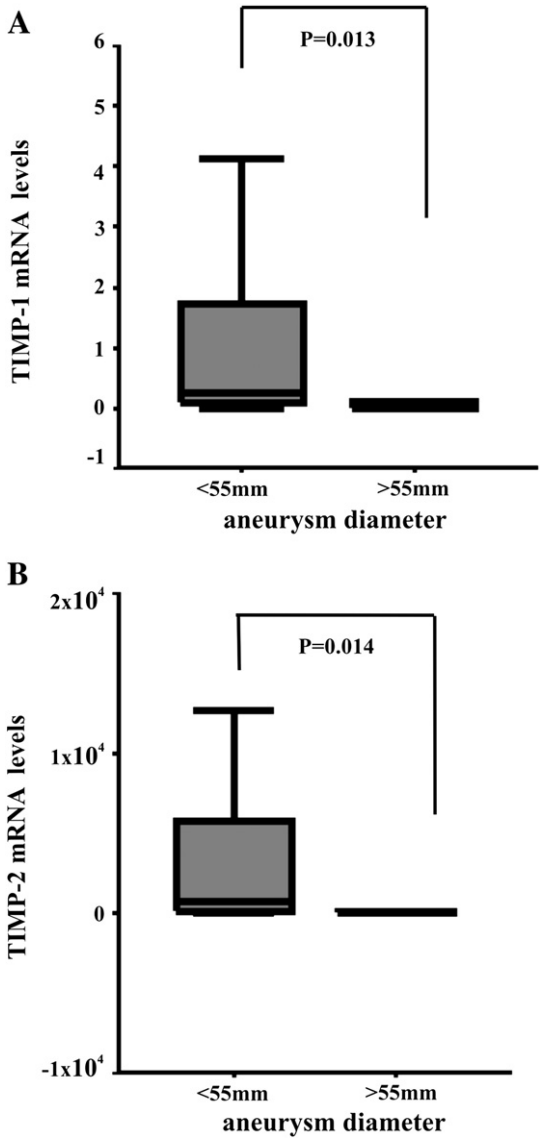

Fig. 2. Normalized transcript levels of A. TIMP-1 and B. TIMP-2 in AAAs in respect of aneurysm diameter. Significantly lower TIMP-1 and TIMP-2 mRNA levels were observed in tissue specimens of patients with aneurysm diameter higher than 55 mm (P = 0.013 and 0.014, Mann-Whitney test respectively).

MMP mRNA expression profile

As mentioned in paragraph *3.1*, the MMPs included in the present study (except for MMP-1 mRNA that was not expressed at all in aortic tissues) did not exhibit detectable transcript levels in all aneurysm or control samples. Therefore, we initially evaluated the mRNA co-expression profile of MMPs in the aneurysm and control tissues based on whether their mRNA was expressed or not in each sample.

The only significant correlation observed in the control tissues was that of MMP-9 and ADAMTS-4 mRNA expression (P = 0.009, Pearson) (Table 4A).

In the aneurysm tissues, however, multiple positive correlations were observed. Specifically, the expression status (expression or not) of MMP-9 was positively correlated with that of MMP-2, -3, -12, -14 and ADAMTS-4 (P < 0.001, P = 0.010, P = 0.042, P = 0.009, P < 0.001, Spearman, respectively). ADAMTS-4 mRNA expression status was also positively correlated with that of MMP-2 and -3 (P < 0.001 and P = 0.042, Spearman, respectively), while the MMP-2 mRNA expression status was marginally not significantly associated with that of MMP-14 (P = 0.056, Spearman) (Table 4B).

mRNA co-expression analysis pair wise of MMPs and TIMPs

A significantly different mRNA co-expression pattern was displayed in the AAA and control tissues.

Specifically, a significantly positive correlation was observed between TIMP-3 and -4 mRNA levels (P = 0.010, Pearson correlation) in the control group. TIMP-2 transcript levels were found to be positively correlated with those of MMP-14 (P = 0.049). Finally, a significant positive co-expression was observed between ADAMTS-1 and MMP2 mRNA levels (P = 0.002, Pearson correlation) (Table 5A).

On the other hand, the mRNA co-expression pattern was significantly altered in AAA specimens since only the positive correlation of TIMP-3 and -4 is maintained (P = 0.010, Pearson) and all other former correlations are abolished while new ones take place. In detail, TIMP-3 transcript levels were now found to be positively correlated with those of MMP-8 (P = 0.032, Pearson), while MMP-2 mRNA levels were positively correlated with ADAMTS-1 (P < 0.001, Spearman). Interestingly, TIMP-1 mRNA is co-expressed with TIMP-2 (P = 0.001, Spearman), but negatively correlated with TIMP-3 and -4 (P = 0.028 and P = 0.025, Spearman, respectively). Furthermore, a

Table 4
Spearman correlation tables demonstrating the co-expression profile of MMPs based on their expression status (expression or not) in A. control tissues and B. ascending aortic aneurysm tissues (AAAs).

A. Control specimens

		MMP-3	*MMP-2*	*MMP-8*	*MMP-9*	*MMP-14*	*ADAMTS-4*
MMP-3	*rho*	1.000					
	P						
MMP-2	*rho*	-0.141	1.000				
	P	0.541					
MMP-8	*rho*	-0.175	0.372	1.000			
	P	0.447	0.097				
MMP-9	*rho*	0.316	0.000	0.277	1.000		
	P	0.163	1.000	0.224			
MMP-14	*rho*	0.235	0.030	0.037	0.337	1.000	
	P	0.306	0.897	0.872	0.135		
ADAMTS-4	*rho*	-0.125	0.141	0.252	0.553**	0.362	
	P	0.589	0.541	0.270	**0.009**	0.106	1.000

MMP-12 mRNA is not expressed in the control specimens, **Correlation is significant at the 0.01 level (2-tailed).

B. ascending aortic aneurysm tissues (AAAs)

		MMP-3	*MMP-2*	*MMP-8*	*MMP-9*	*MMP-12*	*MMP-14*	*ADAMTS-4*
MMP-3	*rho*	1.000						
	P							
MMP-2	*rho*	0.258	1.000					
	P	0.099						
MMP-8	*rho*	-0.141	-0.198	1.000				
	P	0.372	0.209					
MMP-9	*rho*	0.392*	0.565**	-0.147	1.000			
	P	**0.010**	**0.000**	0.354				
MMP-12	*rho*	0.000	0.059	-0.043	0.315*	1.000		
	P	1.000	0.711	0.787	**0.042**			
MMP-14	*rho*	0.121	0.297	-0.017	0.399*	0.088	1.000	
	P	0.445	**0.056**	0.914	**0.009**	0.578		
ADAMTS-4	*rho*	0.315*	0.688**	-0.103	0.655**	0.066	0.109	
	P	**0.042**	**0.000**	0.517	**0.000**	0.676	0.494	1.000

*Correlation is significant at the 0.05 level (2-tailed), **Correlation is significant at the 0.01 level (2-tailed).

number of negative correlations were noted between TIMP-2 and -4 and a group of MMPs. In detail, TIMP-2 mRNA levels are inversely correlated with those of MMP-8 and MMP-12 ($P = 0.001$ and $P < 0.001$, Spearman, respectively), while TIMP-4 transcript levels are negatively correlated with those of MMP-12 ($P = 0.004$, Pearson) (Table 5B).

Discussion

The breakdown of collagen and elastic fibres of the aortic wall is considered to be the culprit biochemical process leading to the formation and rupture of AAAs [2,11]. This theory was mostly supported by the fact that certain enzymes, destructive to ECM proteins and mostly members of the MMP family, have been localized within aneurysmal arteries [8–10]. Moreover, the balance between MMPs, especially that of MMP-2 and-9, and their natural inhibitors has also been shown to govern matrix remodelling and is believed to play a central role in the pathogenesis of AAAs [12–16]. Studies in animal models have further supported the role of MMPs in aortic aneurysm formation. Mice deficient of MMP-9 or MMP-2 proved to be resistant to aneurysm induction compared to their corresponding wild-type background mice [17,18].

However, regulation of MMP activity is complex. Matrix metalloproteinases are synthesised by a variety of cell types, are secreted as pro-MMPs and can be activated by a number of proteinases including other MMPs [6,7]. Moreover, MMP activity can be restrained by the natural inhibitors (TIMPs). Thus, it is more likely that a particular expression pattern of MMPs/TIMPs rather than up- or down-regulation of an individual molecule forms the biochemical micro-environment that leads to the degradation of the aortic ECM. In order for such a distinct expression pattern to be revealed a large number of MMPs and/or TIMPs have to be screened in aortic tissue.

This is the first time that the mRNA expression and co-expression profile of a wide range of metalloproteinase and related molecules were assessed in dilated aortic tissues. An interesting initial observation was the lack of expression of MMP-1, in both AAA and control tissue. In accordance with our finding, previous studies have reported low expression levels of MMP-1 in the aorta, indicating a rather low significance of MMP-1 in the process of vascular ECM degradation [9]. We further observed that MMPs included in the present study did not exhibit detectable transcript levels in all aneurysm- or control-samples. This could be partly due to the relative heterogeneity of our sample, the complex pathophysiology of the disease or the different stages of the disease, which are not necessarily reflected by the aneurysm size. However, even in this heterogeneous transcription pattern, it was clear that when the MMP pathway is activated more than one molecule is expressed. This expression was reflected by the multiple co-expression correlations detected in the AAA tissue. Notably, the only significant transcriptional correlation observed in the control tissues was that of MMP-9 and ADAMTS-4.

We further managed to identify a distinct expression portrait of MMPs and TIMPs in AAAs. The most important feature of the revealed pattern was that in AAA tissue, MMP-activation is accompanied by the down-regulation of TIMPs (especially TIMP-1 and 2). The latter observation supports the notion that the primary alteration in the biochemical chain that leads to ECM degradation is the down-regulation of TIMP-1 and -2. This hypothesis is further supported by the observation that TIMP -1 and -2 had a lower expression in larger aneurysms (>55 mm). In contrast, TIMP-3 was over-expressed in AAA probably in an effort to counteract increased MMP activity (positive feedback). The latter observation was verified by assessment of the protein levels of TIMP-3. Indeed, a significant correlation was observed between mRNA and protein levels of TIMP-3 in the AAA tissue. Notably, the overexpression of TIMP-3 in AAA tissue was the

Table 5
Correlation tables demonstrating the pair-wise co-expression profile of TIMPs and MMPs mRNA in A. control tissues and B. ascending aortic aneurysm tissues (AAAs).

		TIMP-1	*TIMP-2*	*TIMP-3*	*TIMP-4*	*MMP-2*	*MMP-8*	*MMP-9*	*MMP-14*	*ADAMTS-1*
A. Control specimens										
TIMP-1	*CC*	1.000								
	P									
TIMP-2	*CC*	-0.278	1.000							
	P	0.504								
TIMP-3	*CC*	-0.357	0.150	1.000						
	P	0.192	0.678							
TIMP-4	*CC*	-0.419	0.224	0.590**	1.000					
	P	0.120	0.534	**0.010**						
MMP-2	*CC*	-0.485	-0.320	0.708	0.538	1.000				
	P	0.408	0.599	0.115	0.271					
MMP-8	*CC*	0.680	-0.834	0.193	-0.044	-1.000x	1.000			
	P	0.093	0.166	0.679	0.926					
MMP-9	*CC*	0.022	0.543	0.266	0.684	1.000x	0.507	1.000		
	P	0.972	0.635	0.666	0.203		0.662			
MMP-14	*CC*	-0.117	0.879*	0.522	0.009	1.000x	-0.434	-0.033	1.000	
	P	0.731	**0.049**	0.099	0.979		0.390	0.967		
ADAMTS-1	*CC*	-0.138	-0.362	0.207	-0.006	0.987*	0.585	-0.999x	0.208	
	P	0.686	0.339	0.519	0.985	**0.002**	0.300		0.621	1.000

CC: Correlation coefficient, corresponding to Pearson correlation values; *Correlation is significant at the 0.05 level (2-tailed); **Correlation is significant at the 0.01 level (2-tailed). x: limited number of cases.
MMP-12 mRNA is not expressed in the control specimens.

B. Ascending aortic aneurysm tissues (AAAs)											
TIMP-1	*CC*	1.000									
	P										
TIMP-2	*CC*	-0.590a**	1.000								
	P	**0.001**									
TIMP-3	*CC*	-0.383a*	-0.307a	1.000							
	P	**0.028**	0.119								
TIMP-4	*CC*	0.384a*	-0.112a	0.418b*	1.000						
	P	**0.025**	0.572	**0.010**							
MMP-2	*CC*	-0.040a	-0.017a	-0.152a	-0.143b	1.000					
	P	0.874	0.948	0.560	0.570						
MMP-8	*CC*	-0.406a	-0.867a**	0.595b*	0.298b	0.086a	1.000				
	P	0.191	**0.001**	**0.032**	0.322	0.872					
MMP-9	*CC*	-0.200a	0.200a	-0.135b	0.053b	0.000	1.000bx	1.000			
	P	0.580	0.580	0.711	0.884	1.000					
MMP-12	*CC*	-0.200a	-1.000a**	-0.405b	-0.946b*	1.000x	0.119b	-1.000bx	1.000		
	P	0.800	**0.000**	0.426	**0.004**		0.924				
MMP-14	*CC*	-0.342a	-0.053a	-0.104a	0.084a	0.071	-0.400a	0.297a	0.200a	1.000	
	P	0.094	0.819	0.615	0.676	0.800	0.286	0.405	0.747		
ADAMTS-1	*CC*	0.014a	0.033	-0.052a	-0.035	0.777**	-0.030a	0.055a	0.400a	0.06a	
	P	0.943	0.875	0.786	0.851	**0.000**	0.934	0.881	0.600	0.977	1.000

CC: Correlation coefficient, wherein a: Spearman correlation *rho* values; b: Pearson correlation values; **Correlation is significant at the 0.05 level (2-tailed); ***Correlation is significant at the 0.01 level (2-tailed), x: limited number of cases.

only statistically significant correlation between an individual molecule's expression profile and AAA. TIMP-3 and -4 and the co-expression pattern of MMPs in AAA tissue were similar to that observed in the control tissue. Another strong correlation regarding the expression profile of MMPs in AAA tissue was observed between MMP-2 and -9. This observation is in accordance with previous results in murine models that reported on the co-operative action of MMP-2 and -9 in the process of aortic dilation [17].

The study, however, has certain limitations. The most important limitation is the lack of histochemical characterization of the surgical specimens. More information on the pathophysiology of AAA formation would have been derived if the MMP expression pattern had been associated with histochemical alterations of the tunica media. Another important limitation is the appropriateness of control tissues. Coronary artery disease involves many common biochemical pathways and various common predisposing factors, such as

hypertension, with the development of AAAs. Thus, control tissue cannot be considered normal tissue. It should be noted that although the sample size of the present study is one of the largest in literature, it nevertheless is insufficient to establish negative associations. Thus, it is plausible that other undetectable associations still exist.

In conclusion, we did not reveal a statistically significant up-or down-regulation of any individual MMP in AAA tissue. Of note is that the tissue inhibitor of metalloproteinases (TIMP)-3 was significantly more expressed in dilated aortic tissue compared to non-dilated tissue probably in an effort to counteract MMP activity. Activation of the MMP system when observed – more commonly on AAA tissue - was characterised by the up-regulation of more than one proteinase and the down-regulation of TIMP-1 and -2. The latter observation is the key regulatory point leading to ECM degradation and, subsequently, to AAA formation.

Supplementary materials related to this article can be found online at doi:10.1016/j.thromres.2010.08.015.

Conflict of interest statement

All authors state that they do not have any financial interests or connections, direct or indirect that might raise the question of bias in the present work.

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