LETTER TO THE EDITOR

THE ROLE OF INFLAMMATION IN TYPE A AORTIC DISSECTION: A PILOT STUDY

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Type A aortic dissection (TAAD) is a severe cardiovascular disease with high mortality rates. Current evidence suggests inflammation as the main mechanism of its complex pathophysiology. Accordingly, in this study the eventual presence of inflammatory cells in aorta specimens and any contribution of these cells in both apoptosis and metalloproteinase levels were assessed. The potential relationship between plasma inflammatory molecules and TAAD was also detected. In addition, implication in TAAD susceptibility of ten common and functional single nucleotide polymorphisms (SNP)s of six candidate genes (CCR5, TLR4, ACE, eNOs, MMP-9 and -2) was determined. Thus, histo-pathological and immunoistochemical aorta examination, TUNEL testing, genotyping of ten SNPs were performed. Levels of plasma inflammatory molecules were also determined using ELISA technique. A significant inflammatory infiltrate was observed in the examined aortas. Consistent with these data, significantly higher plasma levels of systemic inflammatory mediators characterized the cases. In addition, a high risk genotype significantly associated with TAAD susceptibility was identified. Thus, inflammation producing MMPs, cytokines and death mediators seem to be the shared pathological mechanism for TAAD in the population examined.

Aortic dissection (AD) is an age-dependent life-threatening cardiovascular disease, with severe morbidity and mortality (1, 2). It occurs in the sixth and seventh decades of life with 2,000 new cases each year in North America and 3,000 in Europe (3). AD is becoming an increasing medical problem because of both a high prevalence of underlying diseases (i.e. hypertension) and augmenting ageing in Western populations (3, 4). Ageing is associated with medial degeneration and is an important AD risk factor (5, 6). Hypertension represents the most common predisposing factor for AD in elderly subjects (2, 7). In fact, more than 70% of AD patients show hypertension (1, 2, 7). However, other risk factors contribute to AD onset, i.e. smoking, dyslipidemia, cocaine use, iatrogenic injury and accidents (1-3, 7). Thus, AD is a complex disease with several aetiological risk factors, such as environmental factors and lifestyle, having a strong role in its pathogenesis. Current evidence also suggests a key involvement of different genetic factors (1-3, 8). However, its pathophysiology remains unclear (1-3). A large number of speculations have been proposed. Overall impression is based on an initiating event of

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AD onset, the intimal tear, determined by subintima or media necrosis and apoptosis. It implies both separation of the aortic wall layers and a rapid propagation of AD. This condition may determine the reduction of perfusion of numerous organs with devastating complications (1-3). Recent evidence suggests inflammation as the principal mechanism of aorta AD medial degeneration (6, 9-12). In particular, it has been demonstrated that inflammatory cells, such as lymphocytes and macrophages, not only increase the expression of proteases and cell adhesion molecules, but also release reactive oxygen species. Thus, they contribute to the apoptosis of vascular smooth muscle cells (VSMCs) in the aortic artery, and finally lead to medial degradation (6, 9-12). Furthermore, this condition determines increases in systemic inflammatory molecules [i.e. Interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), metalloproteinase (MMP)s, C-reactive protein (CRP)], as detected in individuals with aortic dissection (6, 9-12).

Based on these observations, in this study the eventual presence of inflammatory cells in aorta specimens and any contribution of these cells in apoptosis, MMP-9 level and aorta wall alterations were assessed in aorta samples from patients affected by type A AD (TAAD). The relationship between levels of plasma inflammatory molecules and TAAD was also detected. Furthermore, the implication was also determined in TAAD susceptibility of ten common and functional single nucleotide polymorphisms (SNP)s of six candidate genes (CCR5, TLR4, ACE, eNOs, MMP-9 and -2) involved in innate and inflammatory responses, hypertension, production of reactive oxygen species and MMP synthesis (13). Identification of a genetic risk profile useful both in diagnostics and to develop more targeted treatment for individual patients and to suggest additional or alternative therapeutic strategies for both prevention and treatment of TAAD was also assessed. This might permit to identify potential biomarkers as risk factors for early detection of this ailment.

The study included 18 TAAD patients [13 men (72%) and 5 (28%) women; mean age: 66.16±9.87] from Western Sicily enrolled at time of their admission to the Cardiac Surgery Unit of Palermo University Hospital. TAAD was diagnosed through imaging techniques. For patient selection, histopathological analyses and exclusion criteria for syndromic and familial forms (e.g. Marfan and Ehlers-Danlos syndromes) and autoimmune connective tissue disorders were utilised. Medical histories pertinent to this clinical condition were obtained from the patients' medical records. Thus, demographic and clinical features, co-morbidity conditions and pharmacological treatments were collected (Table I). The control group consisted of 128 subjects [61 (47%) men and 67 (53%) women; mean age: 61.08±5.83 years] belonging to same ethnic group as the patients, since their parents and grandparents were born in Western Sicily. Thus, a very homogenous population was enrolled. Controls were in good health according to their clinical history and blood tests (complete blood cell count, erythrocyte sedimentation rate, glucose, urea nitrogen, creatinine, electrolytes, C reactive protein, liver function tests, iron, and proteins) (Table I). Their demographic and clinical features, co-morbidity conditions and pharmacological treatments were collected (Table I). Furthermore, echocardiography imaging examinations confirmed absence of any aorta wall histopatological abnormalities in all controls. In order to detect histopathological abnormalities, control ascending aortas from 20 individuals (18 men and 2 women, mean age: 66.9±11.57) who had died from causes unrelated to aortic disease and with no sepsis at death time, as confirmed by autopsy, were also used (Table I).

Our study received approval from the local ethics committee and all participants gave their informed consent. Data were encoded to ensure patient and control protection. All measurements were performed without knowledge of the nature of material.

Aorta specimens, control aorta tissues, DNA samples of both the case subjects and 128 controls were obtained as described in the recent study by Pisano et al. (13). Furthermore, procedures, criteria, definitions and grading systems for tissue sample collection, staining, histopathological and immunohistochemical assessment, Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) testing, semi-quantitative MMP-9 evaluation and genotyping were performed as described in detail in the study of Pisano et al. (13). Information on the ten SNPs analysed was acquired

Table I. *Demographic and clinical characteristics, comorbodity conditions and pharmacological treatment of 18 patients affected by TAAD, 128 control subjects and 20 aorta controls.*

Variables	Patients	Controls	Aorta Controls
	N=18	N=128	N=20
Demographic characteristics			
Age, mean (SD)	66.2 (9.9)	61.1(5,8)	69.9 (11.57)
Body mass index, mean (SD)	29.2 (4.9)	27.9(2.9)	25.2 (1.3)
Diameter Size and dissection location			
Size (mm), mean (SD)	47.6 (6.3)	0 (0)	0(0)
Location, No (%):			
Ascending aorta	6 (33)		
Aortic bulb	1 (6)		
Ascending aorta and Aortic bulb	11 (61)		
Comorbidity conditions, No (%)			
Aortic Aneurysm Familiarity	0 (0)	0 (0)	0 (0)
Cardiovascular Ischemic Familiarity	3 (17)	34 (27)	1(5)
Smoke	7 (39)	66 (51)	3(15%)
Hypertension	9 (50)	40 (31)	1(5)
Dislipidemy	3 (17)	20 (16)	0(0)
Diabetes mellitus	3 (17)	16 (13)	0(0)
Renal failure	1 (6)	0(0)	0(0)
Bicuspid aortic valve disease	0 (0)	0(0)	0(0)
Aortic valve pathology, No (%):			
Normal	10 (56)	0 (0)	0 (0)
Prolapse	6 (33)	0 (0)	0 (0)
Vascular calcium fibrosis	2 (11)	0 (0)	0 (0)
Aortic valve disfunction, No (%):			
Normal	4 (22)	0 (0)	0 (0)
Faint incontinence	4 (22)	0 (0)	0 (0)
Moderate incontinence	3 (17)	0 (0)	0 (0)
Severe incontinence	7 (39)	0 (0)	0 (0)
Faint stenosis	0 (0)	0 (0)	0 (0)
Moderate stenosis	0 (0)	0 (0)	0 (0)
Severe stenosis	0 (0)	0 (0)	0 (0)
Atherosclerosis coronary sindrome	1 (6)	0 (0)	0 (0)
Drugs, No (%)		0 (0)	
Beta blockers	2 (11)	0 (0)	0 (0)
Central alpha-adrenergic agonists	0 (0)	0 (0)	0 (0)
Sartans	0 (0)	0 (0)	0 (0)
Calcium-channel blockers	2 (11)	21 (16)	0 (0)
ACE inhibitors	2 (11)	0 (0)	0 (0)
Antidiabetic drugs	1 (6)	0 (0)	0 (0)
Antiaggregant drugs	2 (11)	0 (0)	0 (0)
Anti-dislipidemic drugs	2 (11)	40 (31)	0 (0)
Diuretics	2 (11)	0 (0)	0 (0)

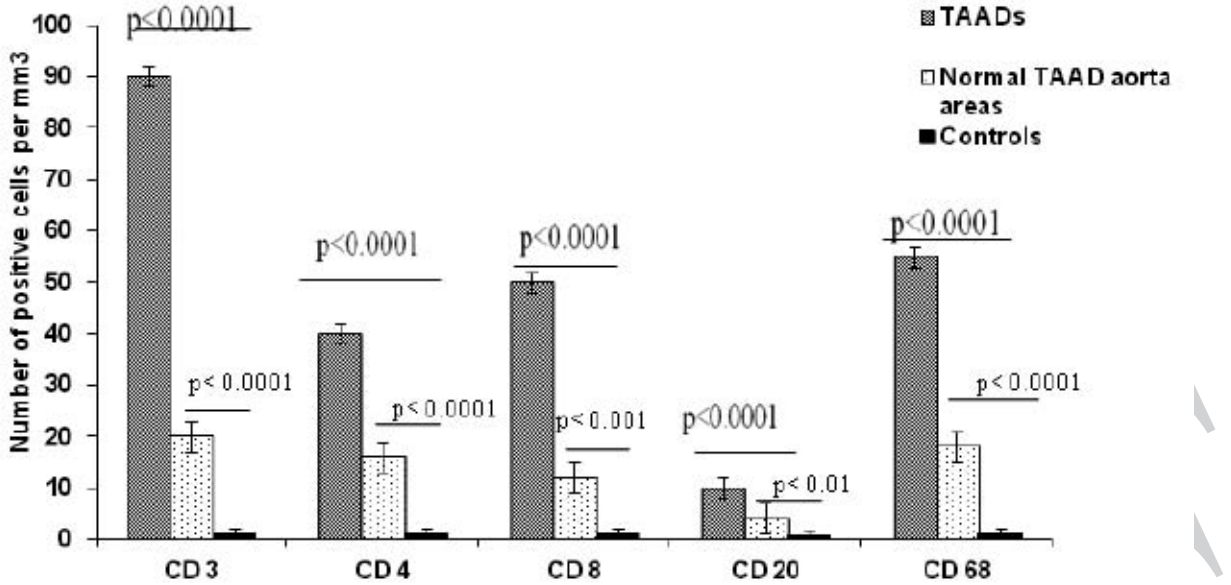

Fig. 1. *Morphometric quantization of T and B lymphocytes, T-cell subpopulations and macrophages in tissue samples of the control aortas and patients with TAAD and normal aorta TAAD areas. CD3, CD4, CD8, CD20 and CD68 positive cells in media and aventitia in 10 contiguous high-power fields (magnification 400x) were counted by two independent observers. Significant increased amounts of CD3+CD4+CD8+CD68+CD20+ cells were observed (p<0.0001, by ANOVA test).*

from db SNP NCBI and reported in detail in the same study. Furthermore, plasma samples were also collected from all patients and controls, in a fasting state (more than 8 h without food administration from onset). From patients who underwent surgical treatment, blood samples were collected before surgery. Plasma samples were obtained from venous blood EDTA samples after a centrifugation of 3500 rpm at 4°C for 15 min immediately after collection and then stored at −80°C for further analysis. Plasma IL-6, TNF-α, Interferon (IFN)-g, MMP-2 and MMP-9 were measured by using ELISA technique, with the R&D Systems (Minneapolis, MN, USA), according to the manufacturer's instructions and our previous study (17). CRP was determined by a high-sensitivity assay using a BN II nephelometer (Dade Behring, Marburg, Germany). Detection limits were 0.7 pg/ml, 0.5 pg/ml, 0.8 pg/ml, 0.154 ng/ml, 0.156 ng/ml and 0.17 mg/l for IL-6, TNF-α, IFN-g, MMP-2, MMP-9 and CRP, respectively.

Statistical analysis was performed by using appropriate tests. Precisely, significant differences between qualitative variables were calculated by using Pearson c^2 test. Significant relationships between quantitative variables were assessed by Wilcoxon rank sum test. Furthermore, Odds Ratios (OR) with 95% Confidence Intervals (CI) and their significance were also calculated. Quantitative values of plasma inflammatory molecules were expressed as mean± SD. Categorical variables were compared by *chi*-square test or Fisher's exact test. Correlations were assessed using Spearman's rank correlation. A $P< 0.05$ was considered statistically significant. Allele and genotype frequencies were evaluated by gene count. Data were tested for goodness of fit between observed and expected genotype frequencies according to Hardy-Weinberg equilibrium, by c^2 tests. Significant differences in frequencies among groups were calculated by using c^2 test and appropriate tables (3x2, 2x2 and 3x3 tables, etc. where appropriate). Significant relationship between genetic variables and pathology risk was analysed using quasi-likely hood binomial models (13).

As reported in Fig. I, a significantly higher infiltrate of lymphocytes and macrophages in

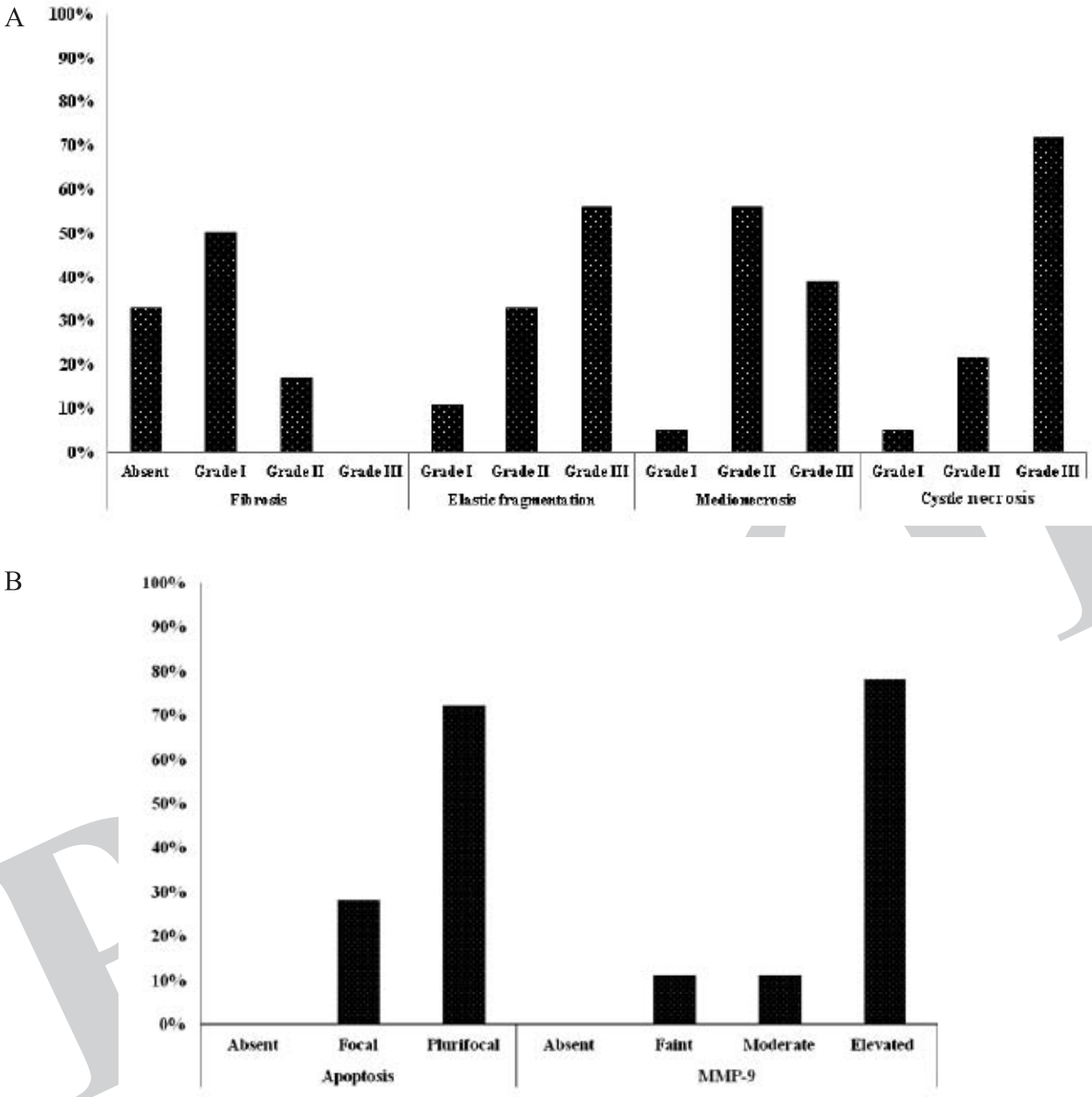

Fig. 2. *Aorta wall abnormalities in tissue aorta samples. **A)** Elastic fragmentation, medio-necrosis, cystic necrosis, medial fibrosis essentially of grade II and III were found. **B)** Focal and plurifocal apoptosis of VSCM cells were also found in all tissue aorta samples from patients. In addition, moderate and elevated MMP-9 amounts were observed in 2 and 14 cases respectively, and faint quantity in only 2 cases.*

tissue aorta wall samples from TAAD patients compared with both control aortas and normal areas from the same TAAD tissues was evidenced by immunoistochemical investigation. Interestingly, the infiltration of inflammatory cells was particularly considerable in the vasa vasorum of adventitia from the aorta patient samples. In contrast, a very small infiltrate of these cells was observed in control aortas, which appears to be less significant in respect to that found in normal aorta TAAD areas. $CD20^{+}$

Table II. *Genotype distributions and allele frequencies of -786 eNOs, D/I ACE, -735C/T MMP-2, -1562C/T SNPs in 18 patients and 128 matched controls [2x2 comparisons between the different groups with odd ratio (OR) and 95% confidence interval].*

Candidate genes	Reference SNP number	Candidate SNPs	Patients (n=18)		Matched controls (N=128)		P (3x2, 2x2 table)	OR (95% CI)
eNOS	rs2070744	-786T/T	10	55%	81	63%	0.006	
		-786T/C	3	17 %	42	33%		
		-786C/C	5	28%	5	4%		
		-786T	23	64%	204	80%	0.03	2.6 (2.1-3.95) p=0.03
		-786C	13	36%	52	20%		
ACE	rs1799752	I/I	2	11%	70	55%	0.001	
		D/I	1	5%	1	1%		
		D/D	15	84%	57	44%		
		I	5	14%	141	55%	0.000008	7.6 (2.8-20.1) p<0.0001
		D	31	86%	115	45%		
MMP2	(rs 2285053)	-735C/C	13	72%	123	96%	0.0002	
		-735C/T	4	22%	5	4%		
		-735T/T	1	6%	0	0%		
		-735C	30	83%	251	98%	0.0001	10.4 (2.8-30.1) p<0.0007
		-735T	6	17%	5	2%		
MMP-9	(rs3918242)	-1562C/C	10	55%	116	90%	0.0001	
		-1562C/T	5	28%	9	8%		
		-1562T/T	3	17%	3	2%		
		-1562C	25	70%	241	94%	0.000005	16.4 (3.4-28-9) p<0.00001
		-1562T	11	30%	15	6%		

cell infiltrate was less represented in three groups, even if significant differences were observed by comparing the three cohort tissues. Immunostaining with CD68 antibody also indicated that macrophages were prevalently present in aortas from TAAD patients in respect to control aortas and normal areas from the same TAAD tissues.

Different microscopic aorta wall abnormalities were also found in aorta samples from 18 TAAD patients and control aorta tissues. In particular, elastic fragmentation, medio-necrosis, cystic necrosis, medial fibrosis essentially of grade II and III were observed (Fig. 2 A). Focal and plurifocal apoptosis of media VSCM cells was essentially assessed in all tissue aorta samples from patients and control aortas. The MMP-9 semi-quantification also evidenced moderate and elevated MMP-9 amounts in 2 and 14 cases and faint only in 2 cases, respectively, as reported in Fig. 2 B. In addition, the evaluation of correlations between tissue aorta wall alterations and some risk factors (such as hypertension, body mass index, smoking and age) demonstrated positive

correlations. In particular, positive correlations were detected between inflammatory infiltrate, elastic fragmentation, apoptosis and hypertension (r=0.497, p=0.0001, n=18 for inflammatory infiltrate; r=0.267, p=0.03 n=18 for elastic fragmentation; r=0.342, p=0.006, n=18 for apoptosis).

As shown in Table II, significant differences both in genotype distributions and allele frequencies were assessed only for -786T/C eNOs, D/I ACE, -735C/T MMP-2, -1562 C/T MMP-9 SNPs. In addition, using quasi-hood binomial statistical models we found that D/D ACE and -1562T/T MMP-9 genotypes are independent risk factors for TAAD (p=0.001). In contrast, no significant differences were observed either in genotype distribution or allele frequencies of +896A/G TLR-4 SNP and Δ32CCR5 deletion between patients and controls, even if a trend was observed for +896A/G TLR4 SNP (p=0.059 data not shown). Furthermore, we examined the eventual relationship between D/D, -735T/T, -786C/C and -1562 C/T genotypes and mortality. Interestingly, 8 of 15 patients (53% and equal to 44% of all patients) with D/D genotype died, while none of the 3 patients with D/I and I/I genotypes died. By comparing these data, no significant difference was found (8 *vs* 7 and 0 *vs* 3, table 2x2 Fisher's exact test). Furthermore, 5 of the 8 dead patients (equal to 33% of the 15 patients and 28% of all patients) were also carriers of -735T/T MMP-2, -1562T/T MMP-9 and -786T/T eNOs genotypes. In searching a high genetic risk profile, we also assessed the frequency of DACE/-735TMMP-2/-1562TMMP-9/ -736CeNOs high risk genotype in patients and controls. By comparing this frequency with that of other combinations, the frequency of this high risk genotype was significantly overrepresented in patients compared to controls (p=0.001 by c^2 test, 2x2 table; data not shown). Patients with high risk genotype showed significant associations with microscopic aorta wall alterations compared to controls (p=0.02 for elastic fragmentation of grade II and III; p=0.002 for cystic necrosis; p=0.01 plurifocal apoptosis; p=0.003 for severe MMP-9 amount and p=0.03 for inflammatory infiltrate, by c^2 test and appropriate tables, data not shown). In addition, they also had a significant association with hypertension (p=0.01, by c^2 test and appropriate tables, data not shown). Interestingly, 9 of these patients were D/D carriers.

Consistent with histological, immunoistochemical and genetic data, significant differences in IL-6, TNF-α, IFN-g, CRP, MMP-2 and-9 plasma levels were detected between TAAD patients and controls (12.66±2.1 *vs* 3.1±0.99, p<0.001; 16.78±1.2 *vs* 7.1±2.2, p<0.0001; 12.13±1.7 *vs* 2.1±0.5 p<0.0001; 14.66±3.2 *vs* 4.6±1.67, p<0.0001; 56.8±3.8 *vs* 12.54±1.6, p<0.0001; and 59.7±3.7 *vs* 11.7±2.6, p<0.0001, respectively).

The results of this study indicated that inflammatory cellular infiltration characterizes the medial degeneration of aorta wall from aorta samples of hypertensive and old TAAD patients examined. It was distributed and mainly observed in the walls of the vasa vasorum of adventitia, suggesting the possible migration of inflammatory cells from these blood vessels into the aorta media. Other studies demonstrated analogous data (6, 9-12). In addition, focal and plurifocal apoptosis of media VSMC cells were also detected in all tissue aorta samples from patients compared with control aortas. Thus, apoptotic death should seem to contribute to the reduction of cellularity and impaired matrix homeostasis. On the other hand, elevated MMP-9 amounts and elastic fragmentation, medionecrosis, cystic necrosis, medial fibrosis essentially of grade II and III were also detected in the major number of aorta samples from patients than control aortas. Positive correlations between these aorta alterations and hypertension were also detected. Based on these data, we likely suppose a key role of inflammation in determining medial degeneration by affecting primarily components of extracellular matrix and media VSCM cells. Consistent with these data, significant higher plasma levels of MMP-2 and MMP-9, CRP, IL-6, TNF-α and INF-g were also observed in TAAD patients than in controls. In addition, the significant levels of INF-g and CD4+ and CD8+cells led us to suppose that TAAD pathogenesis may be prevalently characterized by Th1immune responses.

Furthermore, we also highlighted a key of some genetic variants (-786T/C eNOs, D/IACE, -1562 C/T MMP-9, -735C/T MMP-2 SNPs) of eNOs, ACE, MMP-9 and -2 genes in TAAD susceptibility. These SNPs are well known for their capacity to modulate local and systemic inflammatory degree, tissue injury, hypertension and vascular matrix homeostasis (13-17). In addition, a higher frequency of this risk

genotype, DACE/-735TMMP-2/-1562TMMP-9/-736CeNOs, was detected in cases than in controls. It was significantly associated with tissue aorta wall abnormalities observed and hypertension. Thus, our suggestion is that this genotype might determine in carriers alteration of endothelial and VSMC media cells, to improve hypertension and consequently the degree of inflammation and a very high fragility of aorta wall, and increase the susceptibility of onset of this disease. On the other hand**,** we observed in the normal aorta areas from the same TAAD patient's specimens higher significant amounts of inflammatory/immune cells in respect to control aortas. In addition, this might suggest a strong role of genetic factors in TAAD development and progression and justify the wide inter-individual variation in TAAD susceptibility. Thus, it could be hypothesized that inter-individual variation in TAAD is the result of genetic variability able to modify the rate of biological vascular ageing, blood pressure, degree of inflammation and tissue injury. On the other hand, we recently suggested that biological age rather than chronological age is a better predictor of vascular risk (18). Accordingly, we propose that the determination of DACE/-735TMMP-2/-1562TMMP-9/-736CeNOs high risk genotypes might contribute to predict the TAAD onset and to identify potential targets for therapeutic intervention and as possible biomarkers for TAAD.

However, our study presents some limitations, such as a very small number of patients in respect to controls. In order to support specific clinical recommendations clinical data are also necessary. In addition, no activity and levels of ACE and eNOs were evaluated in our study to confirm their significant associations and higher overexpression observed in 18 cases studied. Thus, further and larger studies are necessary to validate our findings, and particularly to confirm whether these SNPs likely affect the prediction of this aorta ailment and are associated with more severe aorta wall abnormalities by performing histological and immunohistochemical assessments and gene expression analysis.

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