Unraveling Divergent Gene Expression Profiles in Bicuspid and Tricuspid Aortic Valve Patients with Thoracic Aortic Dilatation: The ASAP Study

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Thoracic aortic aneurysm (TAA) is a common complication in patients with a bicuspid aortic valve (BAV), the most frequent congenital heart disorder. For unknown reasons TAA occurs at a younger age, with a higher frequency in BAV patients than in patients with a tricuspid aortic valve (TAV), resulting in an increased risk for aortic dissection and rupture. To investigate the increased TAA incidence in BAV patients, we obtained tissue biopsy samples from nondilated and dilated aortas of 131 BAV and TAV patients. Global gene expression profiles were analyzed from controls and from aortic intima-media and adventitia of patients (in total 345 samples). Of the genes found to be differentially expressed with dilation, only a few (<4%) were differentially expressed in both BAV and TAV patients. With the use of gene set enrichment analysis, the cell adhesion and extracellular region gene ontology sets were identified as common features of TAA in both BAV and TAV patients. Immune response genes were observed to be particularly overexpressed in the aortic media of dilated TAV samples. The divergent gene expression profiles indicate that there are fundamental differences in TAA etiology in BAV and TAV patients. Immune response activation solely in the aortic media of TAV patients suggests that inflammation is involved in TAA formation in TAV but not in BAV patients. Conversely, genes were identified that were only differentially expressed with dilation in BAV patients. The result has bearing on future clinical studies in which separate analysis of BAV and TAV patients is recommended.
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Online address: http://www.molmed.org
doi: 10.2119/molmed.2011.00286

INTRODUCTION

Thoracic aortic aneurysm (TAA) is a pathological condition that can eventually lead to fatal rupture or dissection of the aorta. Several cellular and molecular mechanisms have been suggested to underlie this condition and much work has been done on specific candidate genes. Nonetheless, no single pathophysiological mechanism has been found to be fully explanatory, and a likely reason for this is the heterogeneity of TAAs. Among TAA patients there is a vast overrepresentation of patients presenting with the congenital malformation bicuspid aortic valve (BAV) compared with patients with a normal tricuspid aortic valve (TAV). BAV is the most common congenital cardiovascular malformation, with prevalence of 1–2%. Patients with BAV develop TAA at a younger age than patients with TAV and their aneurysms grow faster (1–3). The purpose of this study was to investigate the gene expression profiles associated with TAA formation in patients with BAV and TAV.

Results of previous investigations have established relatively well that BAV has a sizeable heritable component (4,5), but no specific causative mutations have been identified. Genes suggested to be of specific interest in BAV include *NOTCH1* and *ACTA2*, along with those of more general TAA interest such as those encoding collagens, elastin, fibrillin (FBN1), matrix-metalloproteinases and transforming growth factor (TGF)-β (1,4). In this context, the few rare syndromic forms of TAA are of interest, because they involve known causal genes such as *FBN1* (Marfan syndrome) (6) and TGF-β receptors (Loeys-Dietz syndrome) (7). Another active point of discussion on the etiology of BAV is the question of hemodynamic alterations by the malformed aortic valve. It has been shown that BAV is associated with perturbed flow and hypothesized that this disturbance could result in disease de-

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Submitted August 4, 2011; Accepted for publication September 20, 2011; Epub (www.molmed.org) ahead of print September 27, 2011.

velopment (8). However, an alternative hypothesis is that the same genetic factors that cause BAV also lead to increased TAA risk. The latter notion is supported by the fact that aortic valve replacement does not lower the TAA risk in BAV patients (9).

Taken together, the evidence indicates that TAA is a complex disease with both heritable and environmental components. As with other such diseases it is reasonable to assume that many different pathophysiological processes can lead in the same direction and ultimately show the same clinical manifestations. The notion that different processes can lead to the same clinical outcome can also be used as a research tool. Comparison of two forms of a disease with a similar final outcome allows investigation of the hypothesis that the shared properties of the two forms are the effects of the outcome. The properties that are not shared between the two forms, however, are more likely to be founded separately at earlier stages of each disease form and can therefore be considered of causal nature. Following this line of reasoning we undertook complete gene expression profiling of BAV and TAV patients with or without dilation of the thoracic aorta. Accordingly, we proceeded to identify shared and unique gene expression properties between the aortic dilation in BAV and TAV patients.

MATERIALS AND METHODS

Sample Collection

The Advanced Study of Aortic Pathology (ASAP) biobank was generated after written informed consent from all participants had been obtained according to the declaration of Helsinki and with approval by the ethics committee of the Karolinska Institute (application number 2006/784-31/1). The study included patients undergoing aortic valve surgery and/or surgery for aortic aneurysm at the Karolinska University Hospital, Stockholm, Sweden, starting from February 13, 2007 (Table 1). Patients were classified as having either BAV or TAV and as having either dilated or nondilated thoracic aorta. The criteria for the latter were >45 mm (dilated) and <40 mm (nondilated), respectively.

Transplant control samples of normal walls of the thoracic aorta were obtained from deceased subjects at the time of organ collection for heart/lung transplantation after authorization from the French National Biomedicine Agency (ethics approval PFS09-007), but were otherwise extracted and analyzed in Stockholm as described above.

Expression Analysis

A total of 345 RNA samples were analyzed by use of Affymetrix ST 1.0 exon microarrays: aortic intima-media from patients (n = 131), aortic adventitia from patients (n = 113), mammary artery intima-media from patients (n = 88) and transplant control aortic intima-media (n = 13). There was a considerable overlap of individuals in the patient groups (mammary artery and aorta). Altogether, microarray data from 192 different individuals were included in the study. All expression measurements were RMA normalized and log2 transformed. An expression cutoff of 5.5 expression units was defined, below which no gene was included in analysis (Supplementary S2).

Microarray results from aortic intima-media were confirmed by use of real-time polymerase chain reaction (PCR) on a partially independent sample set of 171 samples (127 overlap with microarray cohort). Data were analyzed according to the ΔΔCt method with normalization against the total RNA concentration as previously described (10).

Statistics

All two-sample comparisons reported were performed by use of a two-sided Student *t* test, with the assumption of unequal variance. All Student *t* test comparisons were recalculated by using linear modeling with age, sex and high-sensitivity C-reactive protein (hsCRP) as covariates, to control for confounding effects. Raw *P* values are always reported. Multiple testing adjustments of significance thresholds were done by using a false discovery rate (FDR) of 5%, as calculated by using the *qvalue* Bioconductor package with default settings (11). Gene set enrichment analysis (the GAGE algorithm) was used (12), based on functional groupings of genes defined in the gene ontology.

Immunohistochemistry

Aortas were immunohistochemically stained for CD4, CD68 and CD163 by use of standard protocols as detailed in the supplementary methods.

All supplementary materials are available online at www.molmed.org.

Table 1. Overview of the patients from which aorta samples were taken.[a]

	Nondilated BAV	Dilated BAV	Nondilated TAV	Dilated TAV
n	31	46	22	24
Female sex	26% (8)	24% (11)	27% (6)	38% (9)
History of hypertension	39% (12)	46% (21)	57% (12)	62% (15)
Aortic valve stenosis	61% (19)	70% (32)	68% (15)	4.2% (1)
Aortic valve regurgitation	45% (14)	37% (17)	41% (9)	83% (20)
Age, years	58.2 ± 12	60.7 ± 12	71.6 ± 10	61.5 ± 15
Total cholesterol, mmol/L	4.96 ± 1.1	5.07 ± 1.3	5 ± 0.98	4.79 ± 1.0
LDL cholesterol, mmol/L	3.05 ± 0.97	3.11 ± 1.0	3.29 ± 0.94	3.07 ± 0.91
Body mass index, kg/m^2	26.1 ± 4.8	26.2 ± 3.7	28 ± 4.8	26.4 ± 4.0
Maximum aorta diameter, mm	36 ± 3.0	50.3 ± 3.2	34.1 ± 3.6	53.6 ± 7.5

[a]Continuous variables are expressed as mean ± SD, categorical variables as percentage (number of subjects in group).

RESULTS

Overview of Expression Data Defines Main Groups of Interest

In investigations that involve the use of large human expression biobanks with data from diverse tissue types and patient backgrounds it is of interest to first describe the most simple and obvious differences observed. Examination of different tissue types, for example, showed that expression levels differed fundamentally even across relatively similar tissue types. For any pair-wise comparison between the four included tissue types, between 22.5% and 52% of all expressed genes showed significant differences in expression levels when we corrected for multiple testing (FDR 5%, $P <$ 0.00077). This finding is important in discussion of the proper selection of control samples, because most genes can be shown to have differential expression if sufficiently different tissue types are used as controls.

Better information can be obtained from comparisons of samples obtained from the same tissue type, for which only the clinical state of the patient was variable. Examination of expression level changes between the main clinical variables recorded showed that the most dramatic differences in aortic gene expression were observed when we compared different states of cuspidity (BAV versus TAV) and dilation (dilated versus nondilated), particularly in aortic intima-media samples. Valvular stenosis and valvular regurgitation were two other conditions for which patients often presented for surgery, but univariate analysis showed comparatively less difference in aortic gene expression between these clinical entities. Finally, it is noteworthy that hsCRP was associated with a high number of correlated gene expression levels. We took these findings into account when testing for effects of multivariate modeling. Table 2 shows the number of differentially expressed genes for all important clinical variables.

These observations of the number of differentially expressed genes roughly

Table 2. Number of differentially expressed genes with important clinical variables.[a]

	Aorta intima-media (n = 131)	Aorta adventitia (n = 113)	Mammary artery intima-media (n = 88)
Cuspidity, (BAV/TAV)[b]	786	8	28
CRP, mg/L[c]	572	674	3
Dilation (yes/no)[b]	508	247	6
Aortic stenosis (yes/no)[b]	237	6	15
Sex (female/male)[b]	144	66	198
Body surface area, m^2[c]	36	49	141
Relative with aortic aneurysm (yes/no)[b]	34	47	99
Age, years[c]	95	24	63
Systolic blood pressure, mmHg[c]	74	7	9
Diastolic blood pressure, mmHg[c]	11	44	34
Aortic regurgitation (yes/no)[b]	35	2	8
History of hypertension (yes/no)[b]	17	1	10
Relative with valvular heart disease (yes/no)[b]	6	14	16
Serum cholesterol, mmol/L[c]	9	3	0
Regular smoker (yes/no)[b]	4	3	0
Serum triglycerides, mmol/L[c]	0	1	1

[a]Significance threshold at FDR 0.05, $P < 0.00077$. The P values in these tables are calculated in a univariate fashion on all 11611 expressed genes.
[b]Two-group categorical data for which a Student t test was used to compare the two groups.
[c]Quantitative data for which a Pearson correlation was used to compare gene expression levels.

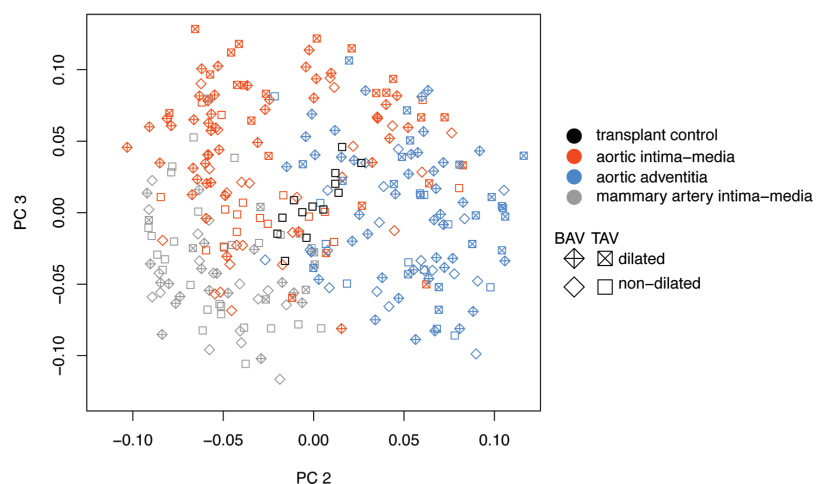

Figure 1. Principal component analysis based on gene expression of all 11,611 expressed genes in all samples. The purpose of this analysis is to give an overview of the data distribution in general and to specifically evaluate the use of the different control groups. Not surprisingly, the analysis shows separation mostly between different tissue types. Importantly, the transplant control samples roughly cluster in the middle, indicating their general similarity with nondilated intima-media control samples. Within tissue types the different patient groups are scattered randomly, indicating that regulation of only a smaller amount of genes reflect the difference between patient groups.

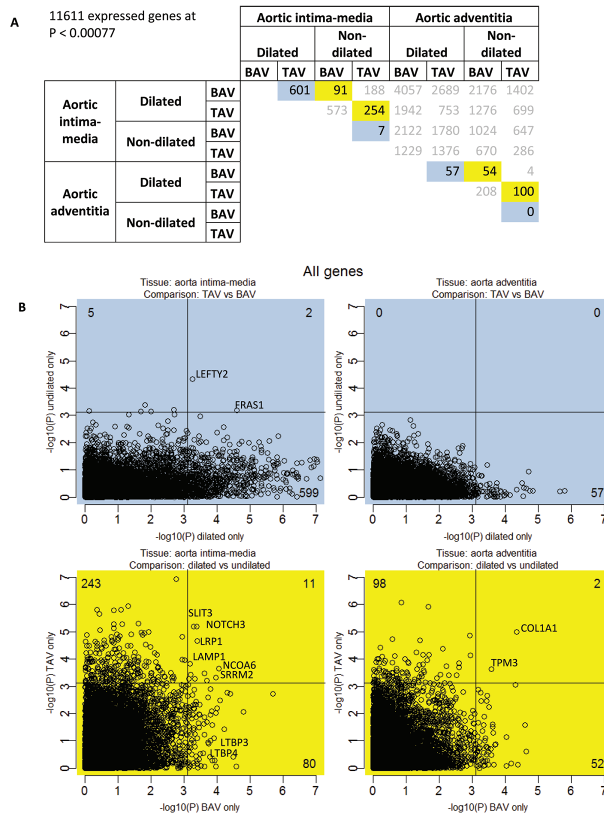

Figure 2. (A) The number of differentially expressed genes in different subgroups of patients. Blue background indicates a TAV to BAV comparison. Yellow background indicates a dilated to nondilated comparison. Grey text indicates a tunica change. Differential expression was defined as a difference in expression level at a FDR 5%, corresponding to $P <$ 0.00077 for all expressed genes. (B) Distribution of the raw P values and the false discovery rate cutoff. Each plot shows different tissue and different blue and yellow comparisons as indicated in (A). Each axis of a plot compares a different subset of patients. A complete overview of the differentially expressed genes can be found in Supplementary S4 and S5.

follow what could be expected when taking tissue morphology into account: comparisons of different tissues showed a high degree of difference, whereas comparisons of different states of the same tissue showed less difference, with the degree of difference being dependent on the profoundness of the state change. This concept is more concisely visualized in the principal components analysis in Figure 1. Here the clustering of different color-coded tissues indicates the clear tissue differences, whereas for dilated and nondilated aortas from BAV and TAV patients the separation is less evident. Some separation of dilated aortic intima-media from nondilated aortic intima-media samples is visible in the upper left corner, indicating that this tissue contains the largest dilation-dependent gene expression differences. Importantly, the investigated control tissues from transplant controls (black), mammary artery intima-media (faded gray) and aortic intima-media from nondilated TAV patients generally clustered together but separated from dilated aortic tissue

Gene Expression Profiles Reveal Similar and Divergent Types of TAA

Because of the above findings we undertook all further analyses separately by tissue type, always comparing the four patient groups of nondilated TAV, dilated TAV, nondilated BAV and dilated BAV. Transplant control samples and mammary artery samples were included only as extra controls in the downstream analysis presented later. Comparing these four main patient groups, we found the number of differentially expressed genes presented in Figure 2.

Dilation was accompanied by more pronounced differences in gene expression in aortic intima-media samples from TAV patients than from BAV patients, and most of the difference between BAV and TAV patients was found in the aortic intima-media samples obtained from patients with dilated aorta. In contrast, virtually no difference was observed between nondilated aortas from patients in the two categories. Of the genes differentially expressed with dilation, only 13 (<4%) were common between the BAV and TAV patient groups (yellow fields in Figure 2 and Supplementary S5). This result was not confounded by age, sex or hsCRP effects, because the results illustrated in Figure 2 were similar after correction for these variables. In the aorta intima-media the age, sex and hsCRP-corrected result for TAV versus BAV was 713 (in dilated) and 1 (in nondilated) and for dilated versus nondilated 98 (in BAV) and 106 (in TAV).

Genes found to be differentially expressed between BAV and TAV patients included *FRAS1* and *LEFTY2*, which were consistently regulated regardless of dilation status. Genes that were differentially expressed between BAV and TAV patients are shown in the blue fields of Figure 2 (and Supplementary S5). Genes that were differentially expressed between dilated and nondilated patients are shown in the yellow fields of Figure 2. Several genes were differentially expressed with dilation in both BAV patients and in TAV patients. These genes included *LRP1*, *LAMP1*, *NOTCH3*, *NCOA6* and *SLIT3*, which were used for further validation performed using real-time PCR (Table 3).

Pathway Analysis Shows Unique and Shared Pathways in Aortic Dilation in BAV and TAV

In the BAV and TAV groups we furthermore investigated which pathways

Table 3. Differently expressed genes between dilated and nondilated aortic intima-media of both BAV and TAV patients.[a]

		Dilated versus nondilated							
		Microarray				Real-time PCR			
		BAV only		TAV only		BAV only		TAV only	
Symbol	Gene name	*P*	Fold change	*P*	Fold change	*P*	Fold change	*P*	Fold change
EHD2	*EH-domain containing 2*	6.4E-04	1.3	3.7E-04	1.4	2.4E-02	1.5	2.4E-04	1.8
LRP1	*Low density lipoprotein-related protein 1*	4.0E-04	1.3	2.2E-05	1.4	8.3E-02	1.4	4.8E-02	1.9
LY6G5B	*Lymphocyte antigen 6 complex*	2.4E-04	1.3	3.4E-04	1.4	5.8E-02	1.2	2.4E-03	1.8
LAMP1	*Lysosomal-associated membrane protein 1*	6.7E-04	1.2	1.5E-04	1.3	5.9E-02	1.3	1.6E-02	1.9
MAVS	*Mitochondrial antiviral signaling protein*	7.1E-04	1.2	5.4E-04	1.2	3.7E-02	1.4	6.1E-03	1.7
MLL2	*Mixed-lineage leukemia 2*	4.8E-04	1.2	5.2E-04	1.3	3.9E-02	1.4	1.5E-04	2.8
NOTCH3	*Notch homolog 3*	4.2E-04	1.4	6.4E-06	1.7	1.8E-02	1.5	1.1E-02	2.0
NCOA6	*Nuclear receptor coactivator 6*	8.9E-05	1.3	2.3E-04	1.4	2.5E-01	1.2	8.3E-03	2.1
SRRM2	*Serine/arginine repetitive matrix 2*	1.1E-04	1.3	4.7E-04	1.4	3.7E-02	1.5	8.8E-06	2.4
SLIT3	*Slit homolog 3*	5.1E-04	1.4	6.4E-06	1.9	1.5E-03	1.6	2.0E-03	1.9
TSHZ3	*Teashirt zinc finger homeobox 3*	2.0E-04	1.4	6.1E-04	1.5	1.9E-01	1.2	5.5E-04	2.2

[a] *P* values and fold changes are given both for expression microarray measurements and for real-time PCR measurements.

were associated with dilation. This investigation was done with a gene set enrichment analysis for all comparison combinations shown in Figure 2. With the gene set enrichment analysis method we investigated whether differential expression of predefined functional groupings of genes is higher than expected by chance. This higher gene set enrichment score was demonstrated for the overlapping gene sets designated cell adhesion, extracellular region and extracellular space, for the overlapping gene sets immune response and inflammatory response, and for the cellular component group integral to plasma membrane. Supplementary S3 shows the gene set enrichment scores for all significantly associated gene sets, but the findings are more clearly illustrated in Figure 3. Here the number of genes that were significantly differentially expressed in the representative gene sets extracellular region and immune response are shown, with significance thresholds modified to reflect a smaller multiple-testing burden.

This figure shows that when we analyzed the differential expression of genes associated with dilation (yellow fields) of the extracellular region set, a higher percentage of genes showed significant differential expression than observed when we analyzed all expressed genes together. This result was demonstrated both in both aortic adventitia and aortic intima-media, and in both BAV and TAV patients. The immune response set of genes was also overrepresented as significantly changed in several comparisons. It is noteworthy that the cuspidity change (blue fields) of the table reveals that in aortic intima-media almost half of all immune response genes were differentially expressed between dilated BAV and dilated TAV patients.

Immune Response Gene Expression is Upregulated in the Media of Dilated Aorta in TAV Patients

To further investigate the directions and magnitudes of change in the identi-

			Aortic intima-media				Aortic adventitia			
			Dilated		Non-dilated		Dilated		Non-dilated	
			BAV	TAV	BAV	TAV	BAV	TAV	BAV	TAV
11611 expressed genes at P < 0.00077	Dilated	BAV		4.4%	0.8%	1.5%		0.5%	0.4%	0.0%
		TAV			4.4%	2.0%			1.6%	0.8%
	Non-dilated	BAV				0.1%				0.0%
		TAV								
457 extracellular region genes at P < 0.0052	Dilated	BAV		21%	4.6%	9.0%		5.7%	5.5%	0.7%
		TAV			18%	11%			12%	6.1%
	Non-dilated	BAV				0.4%				0.0%
		TAV								
85 immune response genes at P < 0.0082	Dilated	BAV		46%	2.4%	18%		2.4%	13%	0.0%
		TAV			39%	9.4%			17%	4.7%
	Non-dilated	BAV				1.2%				0.0%
		TAV								

Figure 3. The percentage of differentially expressed genes on comparison of different subgroups of patients and different subsets of genes. Differential expression is defined as a difference in expression level at a false discovery rate of 5%, and *P* values are as noted in the table. The top section compares all 11,611 expressed genes, and the two following sections show the extracellular region and immune response subsets, respectively. These gene sets were selected because of their identification using the GAGE algorithm (Supplementary S3). The effect of changing to similar gene sets (for example, from immune response to inflammatory response) is negligible. Lists of which genes are differentially expressed in each set and plots comparing distribution of the raw *P* values similar to the plots in Figure 2B are provided in Supplementary S4 and S5, respectively.

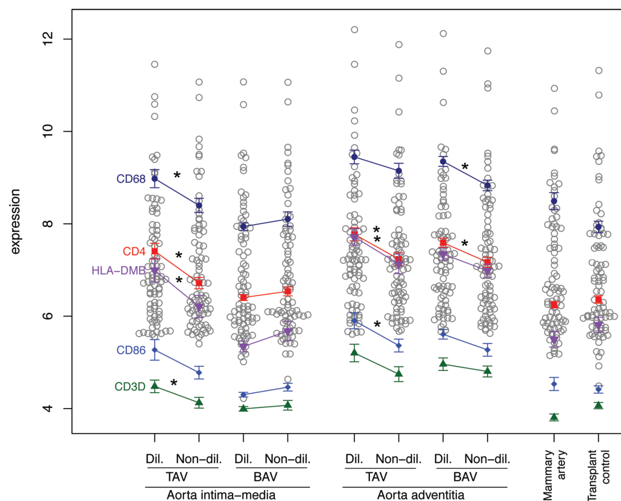

Figure 4. Mean expression of immune response genes by patient group and tissue type. Each grey circle shows the mean of the expression of 1 of the 85 expressed genes from the immune response gene set. Common cell type markers are highlighted in black filled circles: *CD68*, *CD4*, *HLA-DMB*, *CD8* and *CD3D*. Error bars indicate standard error of the mean; *comparison significant at $P < 0.05$. All highlighted genes are significantly differentially expressed between transplant controls and aorta intima-media from dilated TAV patients. Supplementary material S6 shows other well-known immune response genes that follow the same pattern of expression: *CD163, CD3E, CD302, CXCR4, HLA-DOA* and *IL1B*, as well as immune response genes that do not change between these patient groups: *IL13, IL17F, IL17A, IL9, IL4, IL5* and *FOXP3*. Likewise the plot for extracellular region genes is found here.

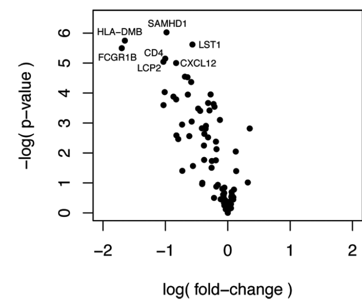

Figure 5. Differential immune response gene expression in dilated aortic intima-media. Comparison is made between dilated TAV (n = 23) and BAV (n = 47) patients. The y-axis indicates significance of difference and the x-axis indicates the magnitude and direction of change—negative values show overexpression in TAV patients and positive in BAV patients. Each dot is a gene, so a point in the upper left corner indicates a gene that is significantly overexpressed in the dilated aortic intima-media of TAV patients. A version of the figure with further labelling of gene names, as well as additional comparisons is found in Supplementary S7.

fied gene sets, we proceeded with a combination of plotting of relative expression and volcano plots (Figures 4 and 5, and Supplementary S6 and S7). Plotting of the mean expression levels of the immune response genes in all patient groups showed that these genes consistently had higher expression in the aortic intima-media of dilated TAV patients. In contrast, the aortic media of dilated BAV patients showed unchanged or even decreased immune response gene expression. In the aortic adventitia the immune response genes generally showed higher expression, but with less difference between BAV and TAV patients. The immune response gene expression profile of nondilated TAV patients matches that of other types of controls such as mammary artery media and transplant. These results are shown in Figure 4. In this figure, as well as in the Supplementary S6, a number of established cell markers have been highlighted. From these it can be learned that classic T-cell and macrophage markers such as *CD4*, *CD3* and *CD68*/*CD163* follow the pattern of most of the immune response genes. Other markers for specific cell subtypes, such as *FOXP3* (Treg cells), *IL17* (Th17 cells), *IL4*, *IL5* and *IL13* (Th2 cells), had low expression levels and no change of expression levels according to cuspidity or dilation status. Although we measured gene expression, the most likely interpretation of the data is that the aortic intima-media of TAV patients is infiltrated by Th1 cells and macrophages after it has dilated. This change is illustrated in Figure 5. A similar infiltration was not observed in BAV patients.

Cell Markers of Inflammation Are More Prominent on the Media of Dilated Aorta in TAV Patients

The observed mRNA expression level changes of the immune response genes were validated in an expanded cohort of 171 aorta intima-media samples by using real-time PCR. Two known immune response genes, CD4 and CIITA, were measured and both showed eight- to ninefold higher expression ($P < 0.001$) in dilated TAV patients compared with dilated BAV patients. In comparison with nondilated patients the pattern was similar to the low expression shown for microarray data in Figure 4.

The immune response change was qualitatively investigated on the protein level by using immunohistochemical analysis as shown in Figure 6. Here we observed infiltration of $CD4^+$, $CD68^+$ and $CD163^+$ cells only in the media of dilated aortas from TAV patients. This protein level change of inflammatory markers has previously been noted (13). Consistent with Figure 4 there are generally higher levels of $CD4^+$, $CD68^+$ and $CD163^+$ cells in aortic adventia. However, no clear differences between BAV and TAV patients were observed; hence, adventitial inflammation does not satisfy our hypothesis regarding differences between patient groups and causality.

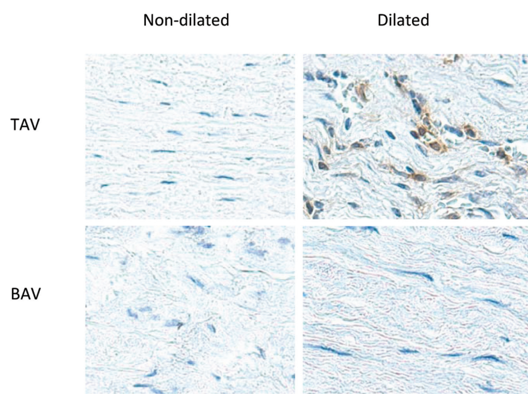

Figure 6. Immunohistochemical stainings showing CD4 in the aortic media of nondilated or dilated BAV or TAV patients. Images showing additional zoom levels as well as CD68 and CD163 stainings are available in Supplementary S8.

Validation of Previous Studies

Finally we sought to replicate findings from previous microarray studies on TAA. We verified that *TNC* (*tenascin C*) and *SPP1* (*osteopontin*) showed significant differential expression in dilated aortas when we compared TAV (n = 24) and BAV (n = 47) patients, as has been previously reported (14). Both *TNC* and *SPP1* had higher expression in TAV (P = 0.00045 and P = 0.0000471, respectively). Of the 11,611 expressed genes they ranked as the 455th and 178th, respectively, of the most significantly differentially expressed genes. Also in dilated aortic media it could be verified that *FBN1* was differentially expressed between BAV and TAV (15). *FBN1* showed overexpression in TAV samples that was significant at P = 0.00047 (rank 469th of all expressed genes).

We could only partially verify the metallothionein findings by Phillipi *et al.* (16). The two metallothionein genes *MT1G* and *MT1H* had lower expression (P = 0.00224 and P = 0.0318, respectively) in aortic intima-media from dilated BAV samples (n = 47) compared with control samples (n = 13). However, none of the remaining metallothionein genes described (*MT1A*, *MT1E*, *MT1F*, *MT1L* and *MT1X*) had significantly lowered expression, neither in dilated BAV samples nor in dilated TAV samples. This finding was also observed when only samples from male patients were investigated and when all samples were included. In adventitial samples only the expression of *MT1G* was significantly lower than in control samples (P = 0.04). Several other genes of interest from Table 2 in Phillipi *et al.* (16) did, however, show a pattern of downregulation compared with control samples. These genes included *CD163*, *IL1R1*, *TIMP4* and *TMOD1*, which all had higher expression in controls at P < 0.05. Likewise, the MMP overexpression in dilated aorta could be replicated.

DISCUSSION

Here we describe the gene expression profiling of a large number of aortic and mammary artery samples taken from individuals at different stages in the development of TAA. We investigated the molecular pathophysiology of aortic dilation and found that many genes show differential expression. To discover the genes of importance in aneurysmal disease, we focused on the specific comparison of aortic dilation in BAV patients versus aortic dilation in TAV patients. As discussed in the Introduction, there is an increased incidence of TAA formation in BAV patients compared with individuals with a normal tricuspid valve, and our results further support the existence of fundamental differences in pathophysiology. Any shared gene expression patterns can therefore be hypothesized to be the result of the vastly altered tissue state after aortic dilation. Conversely, any gene expression pattern unique to either BAV or TAV patients can be hypothesized to be etiologically important in the development of dilation in this particular patient group.

Generally, the overlap between genes differentially expressed with dilation in BAV and TAV patients was low. When we compared dilated versus nondilated aortic intima-media samples, only 11 genes were differentially expressed both in TAV patients and in BAV patients. This small number of genes is in contrast with the hundreds of genes that were differentially expressed with aorta dilation either only in BAV or only in TAV patients. This finding underscores the fundamental molecular differences between the two forms of dilation and highlights why accurate classification is important in the search for underlying causes of disease. It also has a bearing on future clinical studies, in which separate analysis of BAV and TAV patients is highly recommended. In contrast, in the nondilated state comparatively little difference was displayed between BAV and TAV gene expression profiles. Provocatively, this finding corresponds poorly with a model of BAV disease solely mediated by lifelong alteration of flow patterns, which should show stronger contrast to the more recent valve disorders of the nondilated TAV patients investigated.

A common expression trait in both patient groups included cell adhesion and extracellular region genes. These gene sets were identified by using pathway-centric gene set enrichment analysis. Because dilation primarily is a process involving degradation of structural proteins, this finding was not unexpected. Furthermore, because degradation is a feature commonly seen in BAV and TAV patients, the extracellular region genes were not surprisingly found to be differentially expressed in both patient groups and both tunicas, as a reflection of a shared pathogenesis of the two disease forms. Of note, the specific extracellular region genes that were differentially expressed when BAV and TAV patients were compared were nonetheless

different between the two groups (Supplementary S5).

In contrast to cell adhesion and extracellular region genes, the immune response genes showed a clear expression profile difference between BAV and TAV patients. Further analysis showed that this difference was observed because of a general overexpression of immune-related genes in the dilated aortic intima-media of TAV patients compared with the dilated aortic intima-media of BAV patients. This difference was observed both in comparison to nondilated TAV patients and comparison to the transplant control aortas or mammary artery intima-media samples.

Gene set expression analysis revealed no clearly identifiable gene ontology set of genes that could be clearly identified as unique for the BAV patient group. However, 132 individual genes (80 + 52; Figure 2, yellow fields, lower right quadrants) were differentially expressed with dilation in BAV patients, but not in TAV patients. Genes on this list include *PLXNA1*, *EP400* and *BAT2*, which have previously been discussed in connection with BAV and TAA (14,17). Furthermore, the TGF-β binding proteins *LTBP3* and *LTBP4* were observed to be highly specific for dilation in BAV patients only. Recent studies from our group have suggested that decreased inclusion of an alternatively spliced form of the *FN1* gene, secondary to differences in the TGF-β signaling pathway, is a likely important factor for aortic dilation in BAV patients (18). Another target of interest could be the BAV-dilation specific *ADAMTSL1* gene, because this would not be the first *ADAMTS* gene to be implicated in cardiovascular disease (19). A full list of genes is available in the supplementary materials (S4-S5), and because of the experimental setup of comparing dilation in BAV and TAV, these genes are hypothesized to be involved at a more causal level of dilation in BAV patients.

Individual genes that were common to dilation regulation in both BAV and TAV patients are given in Table 3. In these 11 genes, one trend seems to be endocytosis and autophagy. It was recently demonstrated that *LRP1* can stimulate *NOTCH3*-driven trans-endocytosis (20). Likewise, *LAMP1* is a lysosomal membrane protein involved in autophagy (21). In fact, TGF-β plays a role in all these pathways; it downregulates *NOTCH3* signaling to promote *SMC* gene expression (22), regulates the initiation of autophagy (23) and is a ligand for *LRP1* (24). Because all these genes are common to dilation regulation for both BAV and TAV patients, one may speculate that induction of autophagic and endocytotic signaling could ultimately orchestrate the degeneration in vascular smooth muscle cell and extracellular matrix seen in both patient groups.

As we explain in the next section on study limitations, however, we chose not to venture far into speculative assumptions on individual genes, but instead present the full data set for subsequent use in candidate-gene–driven research. To this end, lists of genes that were differentially expressed within significance thresholds set for immune response, extracellular region, and all expressed genes are found in Supplementary S4.

Limitations

One limitation of the study concerns the use of healthy patient material. It is not ethically possible to obtain aorta samples from truly healthy individuals, so the comparisons in this study are implicitly made with either nondilated aortas from TAV patients (who nonetheless suffer from valve disease), mammary artery tissue from the same patients (which is a different tissue type altogether) and from transplant donors who are generally outside of clinical control. In addition, in the study we focused on mRNA expression levels and included only smaller-scale protein level investigations. The reason for this strategy was that high-throughput RNA technologies presently are at a more advanced level, but nonetheless this focus may have affected our conclusions. A final issue to be addressed is validation of results. The results presented here were well validated with real-time PCR. However, in addition to technical validation, a problem with microarray expression studies can be biological validation because of the lack of consistency between different studies. Without access to raw data from other studies, we are limited to presenting our findings as is. To further clarify the problem, however, we analyzed differentially expressed genes from several previous studies of different stages of aortic aneurysm formation (14–16). Overall, these findings were significant in the present investigation as well; in most instances the previously identified genes were found to be significantly differentially expressed. Typically the expression levels for these genes ranked among the 500 most significantly changed gene expression levels in this study. However, these ranking levels also highlight the problems of focusing on only the few most differentially expressed genes in any single study, including this one.

CONCLUSIONS

In summary, our data show large differences between the processes of aorta dilatation in BAV and TAV patients. This finding indicates that aortic dilation in BAV and TAV patients arises from different etiologies. Pathway analysis suggests that aortic dilation in BAV is a process without immune response involvement, whereas aortic dilation in TAV has immune response involvement, possibly at an earlier pathological stage in TAV patients. This conclusion is based on the design of our study, in which two disease forms with a similar endpoint were compared. Although TAA formation ultimately seems to be a disease of the connective tissue and the cell adhesion machinery, the cause of connective tissue dysfunction in TAV patients is likely to be linked to the immune response, particularly the macrophage and Th1-mediated components. In contrast, the expression levels of the immune response genes were either unchanged or decreased compared with controls in BAV patients.

Consequently, the main points that follow from our study are that aortic dilation in BAV and TAV patients is associated with very different gene expression profiles and is therefore likely to reflect different etiologies. Although gene expression changes for cell adhesion and extracellular region genes were exhibited in aortic dilation in both TAV and BAV patients, only aortic dilation in TAV patients was accompanied by a general upregulation of immune response genes. The full gene expression data set that is released here represents a powerful resource for future investigations into TAA formation.

ACKNOWLEDGMENTS

This study was supported by the Swedish Research Council (12660), the Stockholm County Council, the Swedish Heart-Lung foundation, the European Commission (FAD, Health-F2-2008-200647), DASTI (Danish Agency for Science, Technology and Innovation) and a donation by Fredrik Lundberg.

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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