Tohoku J. Exp. Med., 2017, **243**, 263-273

Integrated Bioinformatics Analysis Predicts the Key Genes Involved in Aortic Valve Calcification: From Hemodynamic Changes to Extracellular Remodeling

Mu Liu,[1, 2] Ming Luo,[3] Haoliang Sun,[3] Buqing Ni[3] and Yongfeng Shao[3]

[1]The First Medical School of Nanjing Medical University, Nanjing Medical University, Nanjing, Jiangsu, P.R. China
[2]School of the Basic Medical Sciences, Nanjing Medical University, Nanjing, Jiangsu, P.R. China
[3]Department of Cardiovascular Surgery, The First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu, P.R. China

In our aging world, increasing numbers of people are suffering from calcific aortic valve disease (CAVD). In this study, we used integrated bioinformatics analysis to predict several key genes that are involved in the initiation and progression of CAVD. Expression profiles of 15 calcific and 14 normal human aortic valve samples were generated from two gene expression datasets (GSE12644 and GSE51472). Dataset GSE26953 from the human aortic valve fibrosa-derived endothelial cells cultured under laminar or oscillatory shear stress was also evaluated. Related R packages were used to process the data. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed for functional annotation. Hub genes were identified based on the protein-protein interaction network. CAVD-related gene modules were identified by Weighted Gene Co-expression Network Analysis (WGCNA). The predicted key genes were manually reviewed. In our present work, complex connections among mechano-response, oxidative stress, inflammation and extracellular remodeling pathways in the etiology of CAVD were revealed. The key genes, thus identified, encode a transcription factor KLF2 and phospholipid phosphatase 3 (PLPP3) that are involved in mechano-responses; eNOS involved in oxidative stress; IL-8 involved in inflammation; and collagen triple helix repeat containing 1 (CTHRC1) and secretogranin II (SCG2) involved in extracellular remodeling. These gene products are predicted to play critical roles in CAVD development and progression. The present study provides valuable information for future research and drug development.

Keywords: aortic valve calcification; bioinformatics; hemodynamic environment; inflammation; oxidation
Tohoku J. Exp. Med., 2017 December, **243** (4), 263-273.

Introduction

Calcific aortic valve disease (CAVD) is the most common type of valvular disease in the modern world, causing great morbidity and mortality. CAVD affects approximately 3% of the population aged 75 years or older (Thaden et al. 2014), making it an enormous threat to public health in the aging world (d'Arcy et al. 2011). CAVD is a progressive disease (Freeman and Otto 2005). In its later stage, the fibrotic, thickened and calcific aortic valves could increase the obstruction of blood outflow, causing aortic valve stenosis (AS), which leads to significant hemodynamic deterioration. Without proper intervention, AS could cause left ventricular hypertrophy, angina pectoris, heart failure, or even death.

CAVD is a complex disease involving various mechanisms and factors, such as lipid deposition, oxidation, inflammation, and mineralization (Lindman et al. 2016). In addition, mechanical issues, such as those in the local hemodynamic environment, are thought to be a causative factor of CAVD (Gould et al. 2013). Two examples demonstrate this viewpoint. One is the susceptibility of the bicuspid aortic valve (BAV) to valve calcification (Michelena et al. 2014); BAV is a congenital valve defect in which the patient has only two aortic valves, while a normal person has three. BAVs experience more hemodynamic stress, which can increase the risk of developing CAVD (Szeto et al. 2013). The other example is the side-dependent pathological characteristics of CAVD. The fibrosa of the aortic valve, which directly faces the aorta, is the most common location for calcification, whereas the other layers of the aortic valve are relatively unaffected (Stewart et al.

Received September 13, 2017; revised and accepted November 8, 2017. Published online December 7, 2017; doi: 10.1620/tjem.243.263.
Correspondence: Professor Yongfeng Shao, M.D., Department of Cardiovascular Surgery, The First Affiliated Hospital of Nanjing Medical University, Nanjing Medical University, #300 Guangzhou Road, Gulou Dist., Nanjing, Jiangsu 210029, P.R. China.
e-mail: yongfengshao@yahoo.com

1997). This difference may be attributed to the different hemodynamic conditions (Weinberg et al. 2010). Due to its anatomic structure, the fibrosa experiences more oscillatory shear stress, pressure and stretch (Balachandran et al. 2011). Altogether, biomechanical factors are acknowledged as an initiative factor in the development of CAVD (Back et al. 2013).

Currently, no drug can prevent or halt the progression of CAVD. Aortic valve replacement (AVR) via surgical means remains the mainstream strategy (Nishimura et al. 2017). However, in addition to the high cost of the AVR surgery (McCarthy et al. 2017), complications that accompany anti-coagulation therapy, reoperation due to the lifespan of the prosthetic valves, and the higher surgical risk for gerontic patients complicate the decision to undergo surgical intervention. Therefore, a better understanding of the underlying mechanisms of CAVD and the discovery of additional key genes that may be potential therapeutic targets are required. High-throughput omics data obtained by microarray technology provide us with such an opportunity. In the past few years, several studies have reported on the gene expression profiles of human calcific aortic valves (Bosse et al. 2009; Ohukainen et al. 2015; Guauque-Olarte et al. 2016). However, due to the small sample size in these studies, repeatability and reliability are relatively poor, making it difficult to elucidate the underlying mechanisms. Several studies have performed *in vitro* evaluations of valve-derived cells that were cultured in different hemodynamic environments (Butcher et al. 2006; Holliday et al. 2011). However, the lack of cell types and poor biofidelity of the simulated 'blood flow' largely undermine the value of the data.

In the present work, we conducted an integrated bioinformatics analysis using publicly available gene expression datasets of calcific and normal human aortic valve tissues. In addition, we used a dataset containing expression profiles of *in vitro* human aortic valve endothelial cells from the fibrosa (fHAVECs) that were exposed to laminar or oscillatory shear stress for 24 hours. Batch effects were adjusted using the R package *ComBat* (Kupfer et al. 2012). To avoid shortcomings in traditional cut-off value-based enrichment analyses, Gene Set Enrichment Analysis (Subramanian et al. 2005) was employed to perform Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. Differentialy expressed genes (DEGs) were filtered out, protein-protein interaction networks were generated, and hub genes were predicted. Five common DEGs, including three DEGs exhibiting the same expression trends, appeared in both the *in vitro* study and the human valve study. To explore CAVD-related gene modules, Weighted Gene Co-expression Network Analysis (WGCNA) was performed. Genes in the CAVD-related modules were functionally annotated by GO. Publications related to the key genes revealed by the above analysis were manually reviewed and were discussed in the Discussion section.

Materials and Methods

Acquisition of microarray data

Three datasets investigating the expression profiles of calcific and normal aortic valves using microarray technology were obtained from the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/), including datasets GSE12644 (Bosse et al. 2009), GSE51472 (Ohukainen et al. 2015) and GSE26953 (Holliday et al. 2011).

First, we analyzed the combined expression profiles from GSE12644 and GSE51472, which investigated 30 human aortic valve samples (15 calcific valves versus 15 normal valves). Additionally, to study CAVD from an *in vitro* aspect, we explored expression profiles from GSE26953, which included data on human aortic valve endothelial cells (from fibrosa) that were cultured for 24 hours in different hemodynamic environments.

Microarray data processing

For datasets GSE12644 and GSE51472, which were generated using the *Affymetrix Human Genome U133 Plus 2.0 Array*, original data (.CEL files) were downloaded and preprocessed using the *RMA* algorithm. The systematic error introduced by the different time and location of the experiments is referred to as 'batch effects'. Because three batches existed among these two datasets, the R language package *ComBat* (Kupfer et al. 2012) was used to adjust for batch effects (Chen et al. 2011). For dataset GSE26953, which was generated using the *Illumina HumanHT-12 V3.0 expression beadchip*, the online analysis tool GEO2R was employed. Differentially expressed genes (DEGs) were discovered by the *limma* package. An adjusted P value < 0.05 and $|\log_2 FC| > 1$ were set as cut-off criteria. All codes were run under the R environment version 3.4.1.

Enrichment analysis using the Gene Set Enrichment Analysis method

GO enrichment analysis and KEGG pathway enrichment analysis were performed using the Gene Set Enrichment Analysis method (GSEA Version 3.0; Broad Institute, Cambridge, USA) (Subramanian et al. 2005) with MSigDB Version 6.0. Official protocols were utilized. Briefly, further P values were estimated by 1,000 permutations. Permutation type was set as 'phenotype' for human samples and 'gene set' for the *in vitro* experiments. Gene sets with an FDR < 25% and a nominal P value < 0.05 were considered to be significantly enriched pathways/terms.

Protein-protein interaction (PPI) network construction

We used the Search Tool for the Retrieval of Interacting Genes (STRING) (Szklarczyk et al. 2015) database (https://string-db.org) to generate a PPI network. The minimum required interaction score was 0.4 (medium confidence). Network data were loaded into *Cytoscape* software (version 3.5.1), and hub genes were calculated by applying *Cytohubba* (Chin et al. 2014) using the *MCC* algorithm.

Weighted Gene Co-expression Network Analysis (WGCNA)

To explore genes and gene modules related to CAVD, WGCNA was performed on the expression profiles of human samples (Langfelder and Horvath 2008). Briefly, 3,000 genes with the highest variance among samples were selected. According to the network topology, the soft threshold power was set to 12 in order to create a nearly scale-free network. Gene co-expression adjacency matrices were calculated and transformed into the topological overlap matrix (TOM). The dynamic tree cut algorithm was applied to screen the

modules, with the minimum module size being 30. GO enrichment analyses were used to functionally annotate the CAVD-related gene modules. Genes with the highest Gene Significance (GS) in modules with the highest and lowest correlation coefficients were regarded as significantly associated with CAVD, and related publications were manually reviewed.

Results

Processing the microarray data

To determine potential outliers, Pearson correlation coefficients between every two samples were calculated, and their distance matrix was visualized in the cluster dendrogram (Fig. 1A). Principal component analysis (PCA) was performed, and the plot is displayed in Fig. 1B. As revealed by these figures, samples were divided into two main categories, except GSM317342. Therefore, this sample was excluded in the following analysis. Box plots indicated that the preprocessing successfully normalized the expression profiles to a comparable level (Fig. 1C, D).

Identification of differentially expressed genes (DEGs)

Data from GSE12644 and GSE51472 on a total of 29 human aortic valve samples (15 calcific valves versus 14 normal valves) were preprocessed and compared, revealing 140 up-regulated and 68 down-regulated genes. *MMP12* was the most up-regulated gene in calcific valves (fold change = 18.71), while *ADIPOQ* was the most down-regulated gene (fold change = 0.23).

By analyzing gene expression profiles of human aortic valve endothelial cells from the fibrosa (fHAVECs) that were exposed to laminar or oscillatory hemodynamic environments in GSE26953, we identified 150 DEGs (52 up-regulated and 98 down-regulated).

The Venn diagram (Fig. 2A) reveals that 5 genes (*PLAU, PLPP3, ID1, KIT* and *HMOX1*) were differently expressed in both the *in vitro* experiments and human samples. Their fold changes are shown in Fig. 2B. Publications related to these DEGs were manually reviewed and will be discussed in the Discussion section.

GO enrichment analysis

GO enrichment analysis was performed using the GSEA method. The top 10 enriched terms were determined. In human samples (Fig. 3A), GO terms related to the lymphocyte-mediated immune response were substantially up-regulated in calcific valves. In the *in vitro* experiments (Fig. 3B), GO terms related to skeletal muscle cell differentiation were significantly up-regulated in the

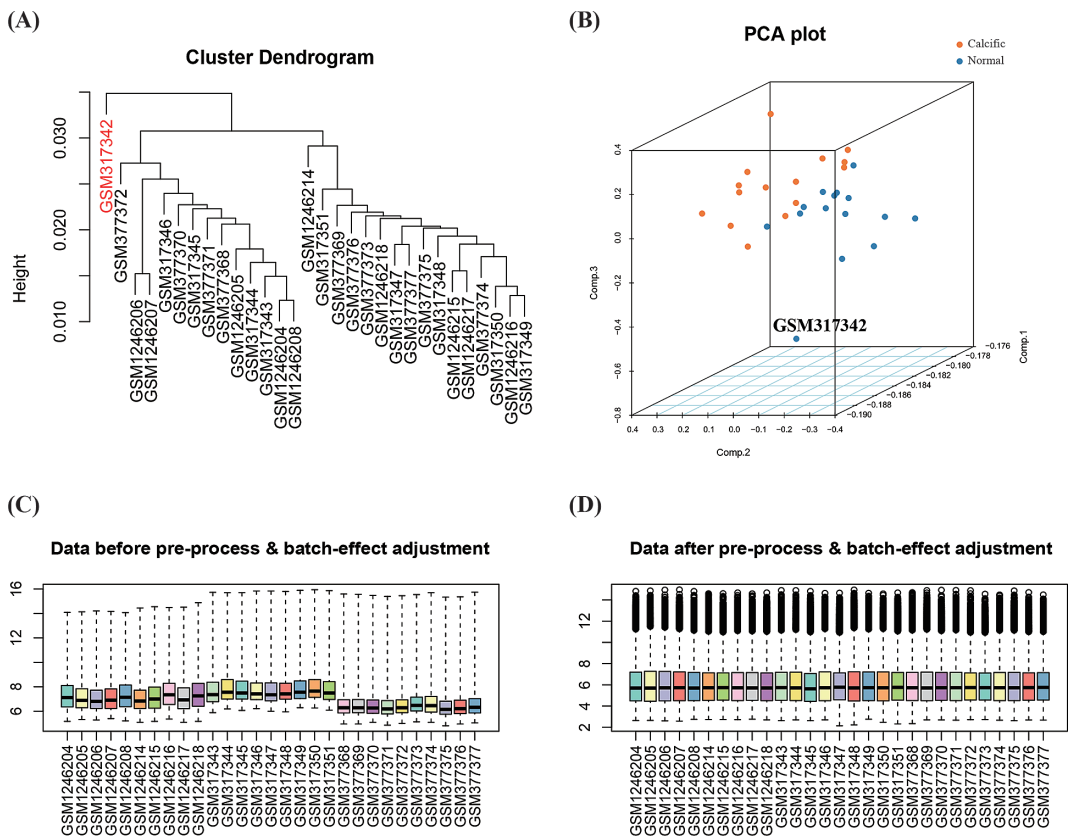

Fig. 1. Detection of outliers and batch-effects adjustment.
GSM317342 was identified as an outlier sample according to (A) Cluster dendrogram of Pearson correlation coefficients and (B) Principal component analysis plot. As revealed by box plot (C), three batches exist among the original data and samples are not comparable. As revealed by box plot (D), preprocess and batch-effects adjustment successfully normalized the expression profiles to a comparable level.

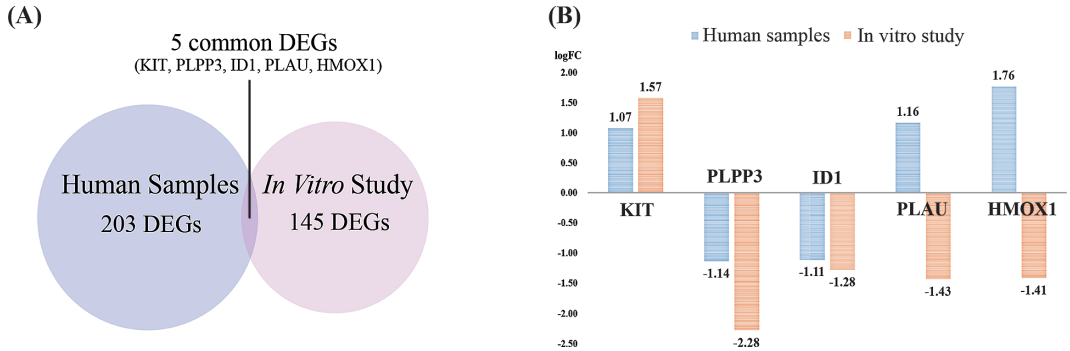

Fig. 2. Identification of DEGs in both the *in vitro* study and human samples.
(A) Venn diagram revealed that 5 genes were differently expressed both in the *in vitro* study and human samples. (B) $\log_2$(fold change) of five DEGs appealed in both studies (*PLAU*, *PLPP3*, *ID1*, *KIT*, *HMOX1*). Positive value indicated up-regulation in calcific samples/oscillatory shear.

fHAVECs exposed to an oscillatory hemodynamic environment, whereas GO terms related to the regulation of wound healing and regulation of IL-8 production were significantly down-regulated.

KEGG pathway enrichment analysis

KEGG analyses were performed using the GSEA method. The top 10 significantly enriched pathways were identified. The leukocyte transendothelial migration pathway was considerably enriched among human calcific valves (Fig. 3C). The DNA replication pathway was significantly up-regulated while the steroid biosynthesis pathway was significantly down-regulated among the fHAVECs exposed to an oscillatory hemodynamic environment (Fig. 3D).

Construction of the PPI network and identification of hub genes

PPI networks of DEGs were constructed using the STRING database. Using the *Cytohubba* application in the *Cytoscape* software, hub genes were determined and visualized (Fig. 4A, B). As revealed in these figures, *CXCL8* (also known as *IL-8*) was identified as the hub gene of CAVD with the highest prediction score (Fig. 4A). Meanwhile, the gene *NOS3* (commonly known as *eNOS*) was recognized as the hub gene with the highest prediction score among DEGs identified in the *in vitro* experiments (Fig. 4B).

WGCNA

Genes that interact tend to show similar expression patterns. Therefore, we constructed a co-expression network to screen gene modules with similar expression profiles. Six gene modules with similar expression patterns were identified by WGCNA. As summarized in Table 1, four of these modules had a P value < 0.05 and were functionally annotated by GO terms, with the lowest P values in three categories: Cellular Component (CC), Molecular Function (MF), and Biological Process (BP). Genes with the highest GS in modules with the highest and lowest correlation coefficients were as follows: *CTHRC1* (from module 'blue', GS = 0.92, fold change = 3.71) and *SCG2* (from module 'turquoise', GS = 0.93, fold change = 4.44). They were regarded as CAVD-related key genes, and related publications were manually reviewed and are discussed in the Discussion section.

Discussion

As life expectancy increased and lifestyles shifted, CAVD has placed a heavy burden on the healthcare system and poses great challenges to public health, thus requiring more in-depth research. Currently, no medical therapies have been proven to be effective in preventing or halting the development of CAVD. This shows the importance of exploring the key genes in CAVD, which may be drug targets. CAVD involves complex biological processes (Lindman et al. 2016). Moreover, the microenvironment around the calcific lesions, including a series of bio-chemical and biomechanical factors as well as several pathways, also contributes to the initiation and progression of CAVD.

The overload of local hemodynamic stress has been regarded as a causative factor in the development and progression of CAVD (Balachandran et al. 2011; Back et al. 2013; Gould et al. 2013; Wang et al. 2014). GSE26953 datasets containing expression profiles of fHAVECs exposed to laminar/oscillatory shear stress were combined with the integrated expression profiles of human samples. By comparing DEGs in both the *in vivo* and *in vitro* gene expression profiles, we discovered 5 common DEGs and explored the influence of local hemodynamic changes on valve calcification.

PLPP3, also known as *PPAP2B*, encodes phospholipid phosphatase 3, which catalyzes the dephosphorylation of lipid substrates, and was identified as a down-regulated gene in both human calcific aortic valve samples (fold change = 0.45) and *in vitro* samples (fold change = 0.21). The *PLPP3* gene has been shown by genome wide association studies (GWAS) to harbor risk loci for coronary artery disease (Mehta 2011). Risk alleles were further shown to be associated with lower endothelial *PLPP3* expression (Erbilgin et al. 2013). Recently, Busnelli et al. (2017)

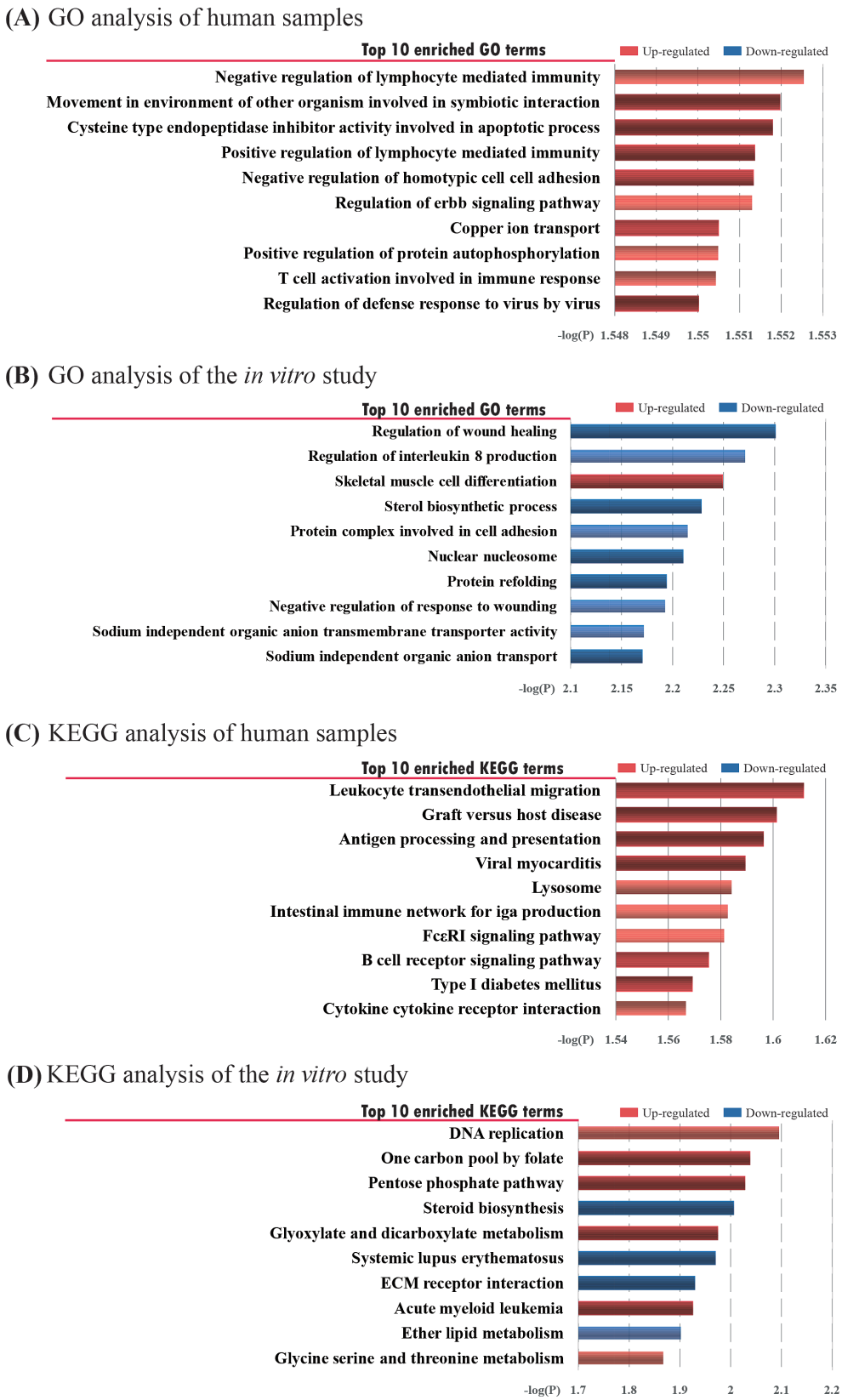

Fig. 3. Top 10 enriched terms/pathways revealed by GO and KEGG analysis using GSEA.
Top 10 enriched terms/pathways revealed by (A) GO enrichment analysis in human calcific aortic valves. (B) GO enrichment analysis in the *in vitro* study. (C) KEGG enrichment analysis in human calcific aortic valves. (D) KEGG enrichment analysis in the *in vitro* study. The horizontal axis refer to the -log(*P* value) of each enriched term.

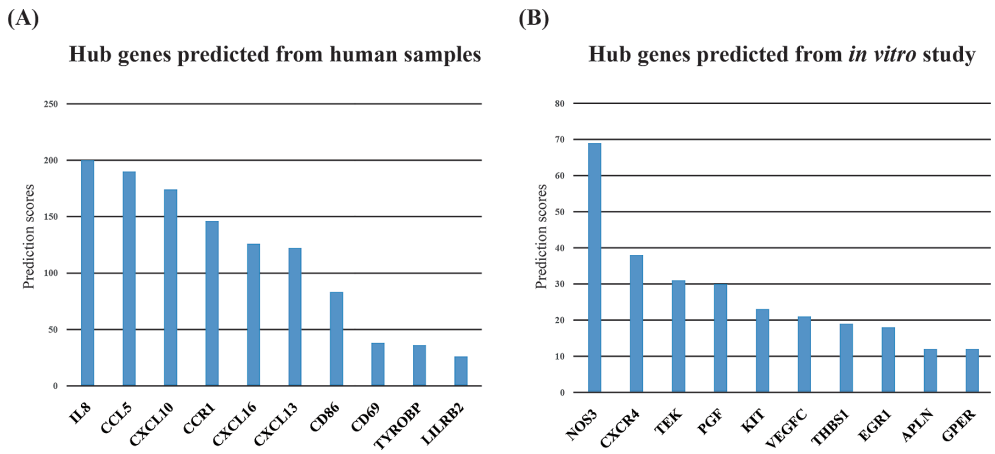

Fig. 4. Figures demonstrate 10 hub genes with the highest prediction scores.
Top 10 hub genes were predicted from (A) human calcific aortic valves and (B) the *in vitro* study of fHAVECs that were exposed to laminar or oscillatory shear stress for 24 hours. The vantical axis refer to the prediction scores.

Table 1. CAVD-related gene modules identified by WGCNA.

Module name	Correlation coefficient	*P* value	Modules' functional annotation by Gene Ontology terms		
			BP	CC	MF
blue	–0.84	**< 0.001**	Cell Adhension	Extracellular Region Part	Growth Factor Binding
turquoise	0.82	**< 0.001**	Immune Response	Extracellular Region	Carbohydrate Binding
green	0.54	**0.002**	Smooth Muscle Contraction	Smooth Muscle Contractile Fiber	Cytoskeletal Protein Binding
brown	–0.51	**0.004**	Vasculature Development	Extracelluar Space	Sphingolipid Binding
yellow	–0.32	0.09		(Not Applicable)	
grey	–0.13	0.5			

BP, Biological Process; CC, Cellular Component; MF, Molecular Function.
Significant *P* values ($P < 0.05$) were marked in **Bold** text.

revealed that deletion of hepatic *PLPP3* altered plasma lipid composition and worsened atherosclerosis in apoE$^{-/-}$ mice. In the present work, we predicted that PLPP3 might participate in the development of CAVD in a mechano-sensitive manner. Interestingly, Wu et al. (2015) found that *PLPP3* was a key gene in atherosclerosis due to its role in regulating endothelial responses to hemodynamic environments. Furthermore, *PLPP3* expression can be promoted by the flow-sensitive transcription factor KLF2 (Wu et al. 2015), which, according to our study, was down-regulated in the *in vitro* experiments (fold change = 0.21). Normally, KLF2/PLPP3 maintains the formation of anti-flow endothelial cell alignment and contributes to the anti-inflammation/adhesion phenotype of endothelial cells by inhibiting lysophosphatidic acid (LPA) and the downstream LPA receptor (LPAR) signaling pathway (Wu et al. 2015). Interestingly, oxLDL-derived LPA was recently shown to promote the progression of AS through the LPAR1/NF-κB pathway (Nsaibia et al. 2017). Thus, down-regulation of KLF2/PLPP3 may contribute to LPA overproduction and activation of downstream procalcification pathways. Given the similarities between CAVD and atherosclerosis (Sathyamurthy and Alex 2015), PLPP3 and its downstream pathways, particularly those related to lipid peroxidation, may be important in regulating the biomechanical response. This hypothesis requires additional experimental studies to confirm.

The importance of ID1, otherwise known as 'inhibitor of DNA binding-1', in angiogenesis is well described (Wang et al. 2011). ID1 promotes endothelial progenitor cell proliferation via the PI3K/Akt/NFκB/Survivin pathway (Li et al. 2012). As endothelial progenitor cells are essential for endothelial repair (Kirton and Xu 2010), the down-regulation of *ID1* (fold change = 0.46 in human samples; fold change = 0.41 in *in vitro* experiments) many weaken the stability of aortic valves and may contribute to endothelial damage, inflammatory cell infiltration and valve calcification.

KIT, which encodes the human homolog of the proto-oncogene *c-kit*, is a transmembrane receptor for stem cell factors and is generally regarded as a cardioprotective gene (Yang et al. 2013). *KIT* was up-regulated in human calcific valves (fold change = 2.09) and was predicted to be one of the hub genes in the *in vitro* experiments (Fig. 4B). Up-regulation of the 'protective' gene *KIT* in calcific sam-

ples casts doubt on whether it functions as a protective gene in CAVD. Additionally, as *KIT* has been widely investigated as an oncogene, we hypothesize that anti-cancer drugs targeting KIT or its related pathways may be protective against CAVD.

PLAU and *HMOX1* expression showed discrepancies between the *in vivo* data and the *in vitro* data (Fig. 2B). *PLAU* was up-regulated among human samples (fold change = 2.24) but was down-regulated in the *in vitro* study (fold change = 0.37). *PLAU* encodes urokinase-type plasminogen activator (u-PA), which is associated with extracellular proteolysis and angiogenesis (Stojkovic et al. 2014). Angiogenesis, a feature of inflammation and extracellular remodeling, might be cardioprotective in myocardial infarction. However, it may contribute to the progression of CAVD and, therefore, may be a key step in valve calcification (Weiss et al. 2013). Because *PLAU* was down-regulated in cultured endothelial cells under oscillatory shear stress conditions, the elevation in *PLAU* expression in calcific aortic valves might be caused by other cell types.

HMOX1, which encodes heme oxygenase 1, was up-regulated among human samples (fold change = 3.38) but was down-regulated in the *in vitro* study (fold change = 0.38). *HMOX1* is regarded as an antioxidative and atheroprotective gene (Pechlaner et al. 2015) and has been reported to be involved in hemodynamics-related arterial remodeling (Freidja et al. 2011). Although *HMOX1* was up-regulated in calcific valve samples, its down-regulation in aortic valve endothelial cells under disturbed blood flow conditions may still contribute to oxidation, inflammation and calcification.

In summary, these five genes may play hemodynamics-related roles in the development of CAVD. However, considering the relatively short stimulation time in the *in vitro* study (24 hours), these genes may play important roles in early biological changes of CAVD, making them potential drug targets for disease prevention. Their exact roles in the etiology of valve calcification should be explored in future studies. At the same time, we may discover that these hemodynamics-related genes are inextricably linked with oxidative stress, inflammation and extracellular remodeling.

Oxidative stress has been shown to participate in the development of CAVD (Miller et al. 2008; Kennedy et al. 2009; Li and Förstermann 2013) and be associated with local hemodynamic changes (Richards et al. 2013). NOS3, commonly known as eNOS, has long been known for its protective effects against aortic valve inflammation and calcification (Clapp et al. 2004; Kennedy et al. 2009). Low expression of eNOS can reduce the cellular NO level, thus contributing to oxidative stress and facilitating eNOS uncoupling, which will, in turn, increase the generation of reactive oxygen species (ROS) and promote further oxidative damage to the endothelium (Miller et al. 2008; Li and Förstermann 2013).

In the present work, *NOS3* was down-regulated in the *in vitro* study following exposure to a disturbed hemodynamic environment (fold change = 0.42). *NOS3* was also identified as a hub gene that responds to hemodynamic changes (Fig. 4B). According to the literature, *NOS3* is a mechano-sensitive gene that is regulated by the flow-sensitive KLF2 transcription factor (SenBanerjee et al. 2004; Richards et al. 2013). The findings suggest that there is a close relationship between hemodynamics and endothelial oxidative stress in the development of CAVD.

Considering that both *PLPP3* and *NOS3* are positively regulated by the flow-sensitive transcription factor KLF2, we searched the KEGG database for *KLF2* to determine related pathways in different hemodynamic environments. '*Fluid shear stress and atherosclerosis pathway*' (ko05418) revealed that under normal laminar shear stress, KLF2 is produced via the MEP2K5/MEPK7/MEF2 pathway and can reduce the expression of the procalcification gene *NF-κB*. The '*FoxO signaling pathway*' (ko04068) revealed that *KLF2* transcription is promoted by Smad3/4 and deacetylated FoxO but is inhibited by FoxG1. However, expression differences in the above genes were revealed in neither the human samples nor the *in vitro* study. Therefore, the upstream pathway that regulates *KLF2* expression and plays an important role in the etiology of CAVD is still unknown.

Although disrupted lipid metabolism and deposition contribute to the development of atherosclerosis and although CAVD resembles atherosclerosis in many ways (Sathyamurthy and Alex 2015), in the present work, neither GO terms nor KEGG pathways related to lipid metabolism were among the top-ranked lists in human samples. Moreover, statins (inhibitors of HMG Co-A reductase and reduces low-density lipoprotein cholesterol) have been shown to be unable to halt the progression of CAVD in recent randomized clinical trials (Chan et al. 2010). Similarly, in our study, as suggested by the KEGG analysis (Fig. 3D), the steroid biosynthesis pathway was significantly down-regulated in the fHAVECs exposed to oscillatory shear stress. Therefore, lipid production may not be a major concern in CAVD progression, whereas lipid retention and modification may play key roles in CAVD etiology.

Lipid peroxidation may be one of the underlying lipid modification processes during valve calcification, as valve mineralization is strongly promoted by oxidized low-density lipoprotein (LDL) and its derived reactive lipid species (Cote et al. 2008; Nadlonek et al. 2013). Recently, oxidized phospholipids were recognized as a critical factor in the development and progression of CAVD (Yeang et al. 2016; Kamstrup et al. 2017). Furthermore, oxidized phospholipid binding with apo(a) could induce pro-inflammatory IL-8 expression (Scipione et al. 2015). Considering the formation of oxidized phospholipids by lipid peroxidation (Greig et al. 2012) and the distinct regulatory functions of different oxidized phospholipids species on inflammation (Freigang 2016), it may be helpful to reveal the impact of oxidative stress on the profiles of oxidized phospholipids and the

exact roles of specific oxidized lipid metabolites in the initiation and progression of CAVD.

Inflammation is closely related to oxidative stress and endothelial dysfunction and is thought to be the precursor of aortic valve calcification (Raggi 2015; Siti et al. 2015). Both the results of our study and the literature indicate that inflammation plays an important role in aortic valve calcification.

The leukocyte transendothelial migration pathway and GO terms regarding the leukocyte-mediated immune response were significantly enriched among human calcific valves, as revealed by our analysis. Endothelial adhesion molecules such as VCAM-1 (fold change = 2.88 among human samples) contribute to the initiation of leukocyte infiltration and inflammation. Adhesion molecules like VCAM-1 have also been reported to be overexpressed in altered hemodynamic environments (Sucosky et al. 2009). Given the above facts, the initiation of inflammation may be inextricably linked with the local hemodynamic environment.

CXCL8 (also known as IL-8), a pro-inflammatory cytokine that is up-regulated (fold change = 2.11) in calcific aortic valves, belongs to the CXC chemokine family and is often regarded as a mediator of the inflammatory response (Russo et al. 2014). In the present work, *IL-8* was identified as the hub gene in CAVD with the highest score among human samples (Fig. 4A). Meanwhile, GO terms related to the regulation of IL-8 production were significantly down-regulated in cultured fHAVECs exposed to an oscillatory hemodynamic environment. Moreover, as suggested by the literature, oxidized phospholipids bind with apo(a) to induce *IL-8* expression (Scipione et al. 2015). These findings indicate that dysregulation of IL-8 production is a key node in the complex network connecting the local hemodynamic environment, oxidative stress and inflammatory response.

Extracellular remodeling is a key process in CAVD (Chen and Simmons 2011) and has long been regarded as a result of inflammation (Kaden et al. 2005). Matrix metalloproteinases (MMPs) can contribute to the initial loss of collagen, damages in endothelial integrity, extracellular matrix remodeling, exacerbating inflammation and, finally, inducing valve calcification (Nissinen and Kahari 2014; Pasipoularides 2016). As indicated by our study, *MMP12* was the most up-regulated gene in calcific valves (fold change = 18.71); *MMP1, 7, 9* and *13* were all up-regulated DEGs, while *TIMP* (tissue inhibitor of metalloproteases) was significantly down-regulated.

CTHRC1, also known as 'collagen triple helix repeat containing 1', was up-regulated in calcific valves and was predicted to be highly associated with CAVD by WGCNA. CTHRC1 has been reported to be associated with several cancers and promote the expression of *MMP9* in an ERK-dependent manner (Kim et al. 2014). CTHRC1 inhibits Smad2/3 activation and reduces collagen deposition; thus, it is a key gene in vascular remodeling (LeClair and Lindner 2007). CTHRC1 has been widely studied in cancer biology and shown to play an important role in cardiovascular diseases, but its role in CAVD remains unclear.

SCG2, or secretogranin II, is a protein belonging to the chromogranin/secretogranin family of neuroendocrine secretory proteins. *SCG2* was significantly up-regulated in calcific valves and was predicted to be a CAVD-related gene by WGCNA. Cleavage of SCG2 produces the active peptide secretoneurin, which could induce coronary angiogenesis in myocardial infarction by enhancing VEGF signaling in endothelial cells (Albrecht-Schgoer et al. 2012). As angiogenesis may contribute to CAVD progression (Weiss et al. 2013), it is necessary to ascertain the exact role of SCG2 in CAVD etiology.

In conclusion, we used integrated bioinformatics analysis to identify several key genes that are involved in the development and progression of CAVD, including *KLF2*, *PLPP3*, *ID1*, *KIT*, *PLAU* and *HMOX1*, which are involved in mechano-responses; *eNOS*, which is involved in oxidative stress; *VCAM-1*, *MMP*s and *IL-8*, which are involved in inflammation; and *CTHRC1* and *SCG2*, which are involved in extracellular remodeling.

Furthermore, we predicted that mechano-response, oxidative stress, inflammation and extracellular remodeling pathways jointly form an indivisible, sophisticated network that underlies CAVD development: Aberrant mechanical stress inhibits flow-sensitive transcription factors (such as KLF2), thereby initiating the development of CAVD. Aberrant mechanical stress can lower the expression of anti-oxidation genes (such as *PLPP3* and *eNOS*), causing eNOS uncoupling and oxidative stress. Oxidative stress promotes endothelial damage by the peroxidation of lipid metabolites, activating several procalcification pathways (such as the LPA/LAPR1/NF-κB pathway), damaging the integrity of the endothelium and promoting the expression of several inflammatory mediators (such as VCAM-1 and IL-8). Inflammation, which is characterized by the infiltration of leukocytes, can escalate oxidative stress and extracellular matrix remodeling (mediated by gene products, such as MMPs, CTHRC1 and SCG2). These all promote the procalcification phenotype shift in the valvular microenvironment and can further exacerbate CAVD progression.

This study had several limitations, mainly because of the certain degree of disconnection between the *in vitro* study and human samples. On one hand, the 24-hour treatment time in the fHAVEC experiments was relatively short and may not allow the observation of long-term changes following exposure to a chronically altered hemodynamic environment. On the other hand, unlike the *in vitro* experiments using cultured endothelial cells, human valve samples contain more than just the endothelium. The other cell types present in the sample may dilute the expression values of some genes. Additionally, all human samples evaluated in both datasets were derived from male patients. Considering that sex-dependent pathology exists in CAVD (Simard et al. 2017), it would be worthwhile to include

more female-derived samples in future research. Although some limitations exist, this study still provides additional insight into the complex mechanisms of CAVD, which still require further research to confirm and explore.

Acknowledgments

This work was supported by project BK20151590 of the *National Natural Science Foundation of Jiangsu Province*. We would like to thank Dr. Na Qin from the Professor Hong-bing SHEN's laboratory at the School of Public Health and Miss Tong Jin from the Department of Bioinformatics at the School of Basic Medical Sciences for their suggestions regarding this study.

Conflicts of Interest

The authors declare no conflict of interest.

References

Albrecht-Schgoer, K., Schgoer, W., Holfeld, J., Theurl, M., Wiedemann, D., Steger, C., Gupta, R., Semsroth, S., Fischer-Colbrie, R., Beer, A.G., Stanzl, U., Huber, E., Misener, S., Dejaco, D., Kishore, R., et al. (2012) The angiogenic factor secretoneurin induces coronary angiogenesis in a model of myocardial infarction by stimulation of vascular endothelial growth factor signaling in endothelial cells. *Circulation*, **126**, 2491-2501.

Back, M., Gasser, T.C., Michel, J.B. & Caligiuri, G. (2013) Biomechanical factors in the biology of aortic wall and aortic valve diseases. *Cardiovasc. Res.*, **99**, 232-241.

Balachandran, K., Sucosky, P. & Yoganathan, A.P. (2011) Hemodynamics and mechanobiology of aortic valve inflammation and calcification. *Int. J. Inflam.*, **2011**, 263870.

Bosse, Y., Miqdad, A., Fournier, D., Pepin, A., Pibarot, P. & Mathieu, P. (2009) Refining molecular pathways leading to calcific aortic valve stenosis by studying gene expression profile of normal and calcified stenotic human aortic valves. *Circ. Cardiovasc. Genet.*, **2**, 489-498.

Busnelli, M., Manzini, S., Hilvo, M., Parolini, C., Ganzetti, G.S., Dellera, F., Ekroos, K., Janis, M., Escalante-Alcalde, D., Sirtori, C.R., Laaksonen, R. & Chiesa, G. (2017) Liver-specific deletion of the *Plpp3* gene alters plasma lipid composition and worsens atherosclerosis in apoE$^{-/-}$ mice. *Sci. Rep.*, **7**, 44503.

Butcher, J.T., Tressel, S., Johnson, T., Turner, D., Sorescu, G., Jo, H. & Nerem, R.M. (2006) Transcriptional profiles of valvular and vascular endothelial cells reveal phenotypic differences: influence of shear stress. *Arterioscler. Thromb. Vasc. Biol.*, **26**, 69-77.

Chan, K.L., Teo, K., Dumesnil, J.G., Ni, A. & Tam, J.; ASTRONOMER Investigators (2010) Effect of Lipid lowering with rosuvastatin on progression of aortic stenosis: results of the aortic stenosis progression observation: measuring effects of rosuvastatin (ASTRONOMER) trial. *Circulation*, **121**, 306-314.

Chen, C., Grennan, K., Badner, J., Zhang, D., Gershon, E., Jin, L. & Liu, C. (2011) Removing batch effects in analysis of expression microarray data: an evaluation of six batch adjustment methods. *PLoS One*, **6**, e17238.

Chen, J.H. & Simmons, C.A. (2011) Cell-matrix interactions in the pathobiology of calcific aortic valve disease: critical roles for matricellular, matricrine, and matrix mechanics cues. *Circ. Res.*, **108**, 1510-1524.

Chin, C.H., Chen, S.H., Wu, H.H., Ho, C.W., Ko, M.T. & Lin, C.Y. (2014) *cytoHubba*: identifying hub objects and sub-networks from complex interactome. *BMC Syst. Biol.*, **8** Suppl 4, S11.

Clapp, B.R., Hingorani, A.D., Kharbanda, R.K., Mohamed-Ali, V., Stephens, J.W., Vallance, P. & MacAllister, R.J. (2004) Inflammation-induced endothelial dysfunction involves reduced nitric oxide bioavailability and increased oxidant stress. *Cardiovasc. Res.*, **64**, 172-178.

Cote, C., Pibarot, P., Despres, J.P., Mohty, D., Cartier, A., Arsenault, B.J., Couture, C. & Mathieu, P. (2008) Association between circulating oxidised low-density lipoprotein and fibrocalcific remodelling of the aortic valve in aortic stenosis. *Heart*, **94**, 1175-1180.

d'Arcy, J.L., Prendergast, B.D., Chambers, J.B., Ray, S.G. & Bridgewater, B. (2011) Valvular heart disease: the next cardiac epidemic. *Heart*, **97**, 91-93.

Erbilgin, A., Civelek, M., Romanoski, C.E., Pan, C., Hagopian, R., Berliner, J.A. & Lusis, A.J. (2013) Identification of CAD candidate genes in GWAS loci and their expression in vascular cells. *J. Lipid Res.*, **54**, 1894-1905.

Freeman, R.V. & Otto, C.M. (2005) Spectrum of calcific aortic valve disease: pathogenesis, disease progression, and treatment strategies. *Circulation*, **111**, 3316-3326.

Freidja, M.L., Toutain, B., Caillon, A., Desquiret, V., Lambert, D., Loufrani, L., Procaccio, V. & Henrion, D. (2011) Heme oxygenase 1 is differentially involved in blood flow-dependent arterial remodeling: role of inflammation, oxidative stress, and nitric oxide. *Hypertension*, **58**, 225-231.

Freigang, S. (2016) The regulation of inflammation by oxidized phospholipids. *Eur. J. Immunol.*, **46**, 1818-1825.

Gould, S.T., Srigunapalan, S., Simmons, C.A. & Anseth, K.S. (2013) Hemodynamic and cellular response feedback in calcific aortic valve disease. *Circ. Res.*, **113**, 186-197.

Greig, F.H., Kennedy, S. & Spickett, C.M. (2012) Physiological effects of oxidized phospholipids and their cellular signaling mechanisms in inflammation. *Free Radic. Biol. Med.*, **52**, 266-280.

Guauque-Olarte, S., Droit, A., Tremblay-Marchand, J., Gaudreault, N., Kalavrouziotis, D., Dagenais, F., Seidman, J.G., Body, S.C., Pibarot, P., Mathieu, P. & Bosse, Y. (2016) RNA expression profile of calcified bicuspid, tricuspid, and normal human aortic valves by RNA sequencing. *Physiol. Genomics*, **48**, 749-761.

Holliday, C.J., Ankeny, R.F., Jo, H. & Nerem, R.M. (2011) Discovery of shear- and side-specific mRNAs and miRNAs in human aortic valvular endothelial cells. *Am. J. Physiol. Heart Circ. Physiol.*, **301**, H856-867.

Kaden, J.J., Dempfle, C.E., Grobholz, R., Fischer, C.S., Vocke, D.C., Kilic, R., Sarikoc, A., Pinol, R., Hagl, S., Lang, S., Brueckmann, M. & Borggrefe, M. (2005) Inflammatory regulation of extracellular matrix remodeling in calcific aortic valve stenosis. *Cardiovasc. Pathol.*, **14**, 80-87.

Kamstrup, P.R., Hung, M.Y., Witztum, J.L., Tsimikas, S. & Nordestgaard, B.G. (2017) Oxidized Phospholipids and Risk of Calcific Aortic Valve Disease: The Copenhagen General Population Study. *Arterioscler. Thromb. Vasc. Biol.*, **37**, 1570-1578.

Kennedy, J.A., Hua, X., Mishra, K., Murphy, G.A., Rosenkranz, A.C. & Horowitz, J.D. (2009) Inhibition of calcifying nodule formation in cultured porcine aortic valve cells by nitric oxide donors. *Eur. J. Pharmacol.*, **602**, 28-35.

Kim, H.C., Kim, Y.S., Oh, H.W., Kim, K., Oh, S.S., Kim, J.T., Kim, B.Y., Lee, S.J., Choe, Y.K., Kim, D.H., Kim, S.H., Chae, S.W., Kim, K.D. & Lee, H.G. (2014) Collagen triple helix repeat containing 1 (CTHRC1) acts via ERK-dependent induction of MMP9 to promote invasion of colorectal cancer cells. *Oncotarget*, **5**, 519-529.

Kirton, J.P. & Xu, Q. (2010) Endothelial precursors in vascular repair. *Microvasc. Res.*, **79**, 193-199.

Kupfer, P., Guthke, R., Pohlers, D., Huber, R., Koczan, D. & Kinne, R.W. (2012) Batch correction of microarray data substantially improves the identification of genes differentially expressed in rheumatoid arthritis and osteoarthritis. *BMC Med. Genomics*, **5**, 23.

Langfelder, P. & Horvath, S. (2008) WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics*, **9**, 559.

LeClair, R. & Lindner, V. (2007) The role of collagen triple helix repeat containing 1 in injured arteries, collagen expression, and transforming growth factor *β* signaling. *Trends Cardiovasc. Med.*, **17**, 202-205.

Li, H. & Förstermann, U. (2013) Uncoupling of endothelial NO synthase in atherosclerosis and vascular disease. *Curr. Opin. Pharmacol.*, **13**, 161-167.

Li, W., Wang, H., Kuang, C.Y., Zhu, J.K., Yu, Y., Qin, Z.X., Liu, J. & Huang, L. (2012) An essential role for the Id1/PI3K/Akt/NFkB/survivin signalling pathway in promoting the proliferation of endothelial progenitor cells in vitro. *Mol. Cell. Biochem.*, **363**, 135-145.

Lindman, B.R., Clavel, M.A., Mathieu, P., Iung, B., Lancellotti, P., Otto, C.M. & Pibarot, P. (2016) Calcific aortic stenosis. *Nat. Rev. Dis. Primers*, **2**, 16006.

McCarthy, F.H., Savino, D.C., Brown, C.R., Bavaria, J.E., Kini, V., Spragan, D.D., Dibble, T.R., Herrmann, H.C., Anwaruddin, S., Giri, J., Szeto, W.Y., Groeneveld, P.W. & Desai, N.D. (2017) Cost and contribution margin of transcatheter versus surgical aortic valve replacement. *J. Thorac. Cardiovasc. Surg.*, doi: 10.1016/j.jtcvs.2017.06.020. [Epub ahead of print].

Mehta, N.N. (2011) A genome-wide association study in europeans and South asians identifies 5 new Loci for coronary artery disease. *Circ. Cardiovasc. Genet.*, **4**, 465-466.

Michelena, H.I., Prakash, S.K., Della Corte, A., Bissell, M.M., Anavekar, N., Mathieu, P., Bosse, Y., Limongelli, G., Bossone, E., Benson, D.W., Lancellotti, P., Isselbacher, E.M., Enriquez-Sarano, M., Sundt, T.M. 3rd, Pibarot, P., et al. (2014) Bicuspid aortic valve: identifying knowledge gaps and rising to the challenge from the International Bicuspid Aortic Valve Consortium (BAVCon). *Circulation*, **129**, 2691-2704.

Miller, J.D., Chu, Y., Brooks, R.M., Richenbacher, W.E., Pena-Silva, R. & Heistad, D.D. (2008) Dysregulation of antioxidant mechanisms contributes to increased oxidative stress in calcific aortic valvular stenosis in humans. *J. Am. Coll. Cardiol.*, **52**, 843-850.

Nadlonek, N.A., Lee, J.H., Weyant, M.J., Meng, X. & Fullerton, D.A. (2013) ox-LDL induces PiT-1 expression in human aortic valve interstitial cells. *J. Surg. Res.*, **184**, 6-9.

Nishimura, R.A., Otto, C.M., Bonow, R.O., Carabello, B.A., Erwin, J.P. 3rd, Fleisher, L.A., Jneid, H., Mack, M.J., McLeod, C.J., O'Gara, P.T., Rigolin, V.H., Sundt, T.M. 3rd & Thompson, A. (2017) 2017 AHA/ACC Focused Update of the 2014 AHA/ACC Guideline for the Management of Patients With Valvular Heart Disease: A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. *Circulation*, **135**, e1159-e1195.

Nissinen, L. & Kahari, V.M. (2014) Matrix metalloproteinases in inflammation. *Biochim. Biophys. Acta*, **1840**, 2571-2580.

Nsaibia, M.J., Boulanger, M.C., Bouchareb, R., Mkannez, G., Le Quang, K., Hadji, F., Argaud, D., Dahou, A., Bosse, Y., Koschinsky, M.L., Pibarot, P., Arsenault, B.J., Marette, A. & Mathieu, P. (2017) OxLDL-derived lysophosphatidic acid promotes the progression of aortic valve stenosis through a LPAR1-RhoA-NF-*κ*B pathway. *Cardiovasc. Res.*, **113**, 1351-1363.

Ohukainen, P., Syvaranta, S., Napankangas, J., Rajamaki, K., Taskinen, P., Peltonen, T., Helske-Suihko, S., Kovanen, P.T., Ruskoaho, H. & Rysa, J. (2015) MicroRNA-125b and chemokine CCL4 expression are associated with calcific aortic valve disease. *Ann. Med.*, **47**, 423-429.

Pasipoularides, A. (2016) Calcific Aortic Valve Disease: Part 1: molecular Pathogenetic Aspects, Hemodynamics, and Adaptive Feedbacks. *J. Cardiovasc. Transl. Res.*, **9**, 102-118.

Pechlaner, R., Willeit, P., Summerer, M., Santer, P., Egger, G., Kronenberg, F., Demetz, E., Weiss, G., Tsimikas, S., Witztum, J.L., Willeit, K., Iglseder, B., Paulweber, B., Kedenko, L., Haun, M., et al. (2015) Heme oxygenase-1 gene promoter microsatellite polymorphism is associated with progressive atherosclerosis and incident cardiovascular disease. *Arterioscler. Thromb. Vasc. Biol.*, **35**, 229-236.

Raggi, P. (2015) Inflammation and calcification: the chicken or the hen? *Atherosclerosis*, **238**, 173-174.

Richards, J., El-Hamamsy, I., Chen, S., Sarang, Z., Sarathchandra, P., Yacoub, M.H., Chester, A.H. & Butcher, J.T. (2013) Side-specific endothelial-dependent regulation of aortic valve calcification: interplay of hemodynamics and nitric oxide signaling. *Am. J. Pathol.*, **182**, 1922-1931.

Russo, R.C., Garcia, C.C., Teixeira, M.M. & Amaral, F.A. (2014) The CXCL8/IL-8 chemokine family and its receptors in inflammatory diseases. *Expert Rev. Clin. Immunol.*, **10**, 593-619.

Sathyamurthy, I. & Alex, S. (2015) Calcific aortic valve disease: is it another face of atherosclerosis? *Indian Heart J.*, **67**, 503-506.

Scipione, C.A., Sayegh, S.E., Romagnuolo, R., Tsimikas, S., Marcovina, S.M., Boffa, M.B. & Koschinsky, M.L. (2015) Mechanistic insights into Lp(a)-induced IL-8 expression: a role for oxidized phospholipid modification of apo(a). *J. Lipid Res.*, **56**, 2273-2285.

SenBanerjee, S., Lin, Z., Atkins, G.B., Greif, D.M., Rao, R.M., Kumar, A., Feinberg, M.W., Chen, Z., Simon, D.I., Luscinskas, F.W., Michel, T.M., Gimbrone, M.A. Jr., Garcia-Cardena, G. & Jain, M.K. (2004) KLF2 Is a novel transcriptional regulator of endothelial proinflammatory activation. *J. Exp. Med.*, **199**, 1305-1315.

Simard, L., Cote, N., Dagenais, F., Mathieu, P., Couture, C., Trahan, S., Bosse, Y., Mohammadi, S., Page, S., Joubert, P. & Clavel, M.A. (2017) Sex-related discordance between aortic valve calcification and hemodynamic severity of aortic stenosis: is valvular fibrosis the explanation? *Circ. Res.*, **120**, 681-691.

Siti, H.N., Kamisah, Y. & Kamsiah, J. (2015) The role of oxidative stress, antioxidants and vascular inflammation in cardiovascular disease (a review). *Vascul. Pharmacol.*, **71**, 40-56.

Stewart, B.F., Siscovick, D., Lind, B.K., Gardin, J.M., Gottdiener, J.S., Smith, V.E., Kitzman, D.W. & Otto, C.M. (1997) Clinical factors associated with calcific aortic valve disease. *J. Am. Coll. Cardiol.*, **29**, 630-634.

Stojkovic, S., Kaun, C., Heinz, M., Krychtiuk, K.A., Rauscher, S., Lemberger, C.E., de Martin, R., Groger, M., Petzelbauer, P., Huk, I., Huber, K., Wojta, J. & Demyanets, S. (2014) Interleukin-33 induces urokinase in human endothelial cells: possible impact on angiogenesis. *J. Thromb. Haemost.*, **12**, 948-957.

Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M.A., Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S. & Mesirov, J.P. (2005) Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci. USA*, **102**, 15545-15550.

Sucosky, P., Balachandran, K., Elhammali, A., Jo, H. & Yoganathan, A.P. (2009) Altered shear stress stimulates upregulation of endothelial VCAM-1 and ICAM-1 in a BMP-4- and TGF-*β*1-dependent pathway. *Arterioscler. Thromb. Vasc. Biol.*, **29**, 254-260.

Szeto, K., Pastuszko, P., del Alamo, J.C., Lasheras, J. & Nigam, V. (2013) Bicuspid aortic valves experience increased strain as compared to tricuspid aortic valves. *World J. Pediatr. Congenit. Heart Surg.*, **4**, 362-366.

Szklarczyk, D., Franceschini, A., Wyder, S., Forslund, K., Heller, D., Huerta-Cepas, J., Simonovic, M., Roth, A., Santos, A., Tsafou, K.P., Kuhn, M., Bork, P., Jensen, L.J. & von Mering, C. (2015) STRING v10: protein-protein interaction networks,

integrated over the tree of life. *Nucleic Acids Res.*, **43**, D447-452.

Thaden, J.J., Nkomo, V.T. & Enriquez-Sarano, M. (2014) The global burden of aortic stenosis. *Prog. Cardiovasc. Dis.*, **56**, 565-571.

Wang, G., Qiu, J., Hu, J., Tang, C. & Yin, T. (2011) Id1: a novel therapeutic target for patients with atherosclerotic plaque rupture. *Med. Hypotheses*, **76**, 627-628.

Wang, H., Leinwand, L.A. & Anseth, K.S. (2014) Cardiac valve cells and their microenvironment—insights from in vitro studies. *Nat. Rev. Cardiol.*, **11**, 715-727.

Weinberg, E.J., Mack, P.J., Schoen, F.J., Garcia-Cardena, G. & Kaazempur Mofrad, M.R. (2010) Hemodynamic environments from opposing sides of human aortic valve leaflets evoke distinct endothelial phenotypes in vitro. *Cardiovasc. Eng.*, **10**, 5-11.

Weiss, R.M., Miller, J.D. & Heistad, D.D. (2013) Fibrocalcific aortic valve disease: opportunity to understand disease mechanisms using mouse models. *Circ. Res.*, **113**, 209-222.

Wu, C., Huang, R.T., Kuo, C.H., Kumar, S., Kim, C.W., Lin, Y.C., Chen, Y.J., Birukova, A., Birukov, K.G., Dulin, N.O., Civelek, M., Lusis, A.J., Loyer, X., Tedgui, A., Dai, G., et al. (2015) Mechanosensitive PPAP2B Regulates Endothelial Responses to Atherorelevant Hemodynamic Forces. *Circ. Res.*, **117**, e41-e53.

Yang, J., Zhou, Y., Liu, B., Wang, H. & Du, X. (2013) Trop2 plays a cardioprotective role by promoting cardiac c-kit+ cell proliferation and inhibition of apoptosis in the acute phase of myocardial infarction. *Int. J. Mol. Med.*, **31**, 1298-1304.

Yeang, C., Wilkinson, M.J. & Tsimikas, S. (2016) Lipoprotein(a) and oxidized phospholipids in calcific aortic valve stenosis. *Curr. Opin. Cardiol.*, **31**, 440-450.