Identification of a peripheral blood gene signature predicting aortic valve calcification

Donal Macgrogan, Beatriz Martínez-Poveda, Jean-Pierre Desvignes, Leticia Fernandez-Friera, Manuel José Gomez, Eduardo Gil Vilariño, Sergio Callejas Alejano, Pablo García-Pavía, Jorge Solis, Joaquín Lucena, et al.

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Donal MacGrogan[1,2,*], Beatriz Martínez Poveda[1,2], Jean-Pierre Desvignes[3], Leticia Fernandez-Friera[2,4,5], Manuel José Gomez[6], Eduardo Gil Vilariño[7], Sergio Callejas Alejano[7], Pablo Garcia-Pavia[2,8,9], Jorge Solis[10], Joaquín Lucena[11], David Salgado[3], Gwenaelle Collod-Béroud[3], Emilie Faure[3], Alexis Théron[12], Julia Torrents[13], Jean-François Avierinos[3,12], Lorena Montes[14], Ana Dopazo[7], Valentín Fuster[15,16], Borja Ibañez[2,17], Fátima Sánchez-Cabo[6], Stephane Zaffran[3] & José Luis de la Pompa[1,2,*]

[1] Intercellular Signaling in Cardiovascular Development & Disease laboratory, Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC), Melchor Fernández Almagro 3, 28029 Madrid, SPAIN
[2] Ciber de Enfermedades Cardiovasculares, Instituto de Salud Carlos III, Melchor Fernández Almagro 3, 28029 Madrid, SPAIN
[3] Aix Marseille University, MMG, INSERM U1251, Marseille, FRANCE
[4] Translational Laboratory for Cardiovascular Imaging and Therapy, CNIC, SPAIN
[5] HM Hospitales-Centro Integral de Enfermedades Cardiovasculares, Madrid, SPAIN
[6] Bioinformatics Unit, CNIC, SPAIN
[7] Genomics Unit, CNIC, SPAIN
[8] Departmento de Cardiología, Hospital Universitario Puerta de Hierro, Madrid, SPAIN
[9] Universidad Francisco de Vitoria, Pozuelo de Alarcón, Madrid, SPAIN
[10] Departmento of Cardiología, Hospital Universitario Doce de Octubre, Madrid, SPAIN
[11] Servicio de Patología Forense, Instituto de Medicina Legal y Ciencias Forenses, Sevilla, SPAIN
[12] Service de Cardiologie, Hôpital de la Timone, 13005, Marseille, FRANCE
[13] Service d'anatomie et Cytologie Pathologiques, Hôpital de la Timone, 13005, Marseille, FRANCE
[14] Hospital Clínico San Carlos, Madrid, SPAIN
[15] Cardiovascular Imaging and Population Studies Laboratory, CNIC, SPAIN
[16] Cardiology Department, Icahn School of Medicine at Mount Sinai, New York, New York, USA
[17] IIS-Fundación Jiménez Díaz Hospital Universitario, Madrid, SPAIN
* Corresponding authors

Correspondence:
José Luis de la Pompa
Intercellular Signaling in Cardiovascular Development & Disease laboratory
Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC)
Melchor Fernández Almagro 3, 28029 Madrid, SPAIN.
Tel: +34 91 4531200
Email: jlpompa@cnic.es

ABSTRACT

Calcific aortic valve disease (CAVD) is a significant cause of illness and death worldwide. Identification of early predictive markers could help optimize patient management. RNA-sequencing was carried out on human fetal aortic valves at gestational weeks 9, 13, and 22, and on a case-control study with adult non-calcified and calcified bicuspid and tricuspid aortic valves. In dimension reduction and clustering analyses, diseased valves tended to cluster with fetal valves at week 9 rather than normal adult valves, suggesting that part of the disease program might be due to re-iterated developmental processes. The analysis of groups of co-regulated genes revealed predominant immune-metabolic signatures, including innate and adaptive immune responses involving lymphocyte T cell metabolic adaptation. Cytokine and chemokine signaling, cell migration, and proliferation were all increased in CAVD, whereas oxidative phosphorylation and protein translation were decreased. Discrete immune-metabolic gene signatures were present at fetal stages and increased in adult controls, suggesting that these processes intensify throughout life and heighten in disease. Cellular stress-response and neurodegeneration gene signatures were aberrantly expressed in CAVD, pointing to a mechanistic link between chronic inflammation and biological ageing. Comparison of the valve RNA-sequencing dataset with a case-control study of whole blood transcriptomes from asymptomatic individuals with early aortic valve calcification identified a highly predictive gene signature of CAVD and of moderate aortic valve calcification in overtly healthy individuals. These data deepen and broaden our understanding of the molecular basis of CAVD and identify a peripheral blood gene signature for the early detection of aortic valve calcification.

Keywords

Human fetal valve; CAVD; inflammation; peripheral blood biomarker; gene signature

INTRODUCTION

Calcific aortic valve disease (CAVD) is caused by calcium build-up of the aortic valve leaflet and may be associated with hemodynamic obstruction, leading to different degrees of aortic stenosis (AS). Untreated severe AS leads to heart failure, with valve replacement remaining the only available treatment. Severe AS is preceded by aortic sclerosis, a subclinical form of CAVD that develops over many years without hemodynamic consequence (6). The prevalence of aortic valve disorders increases with age, with 25% of individuals >65 years with aortic sclerosis progressing to AS at a rate of 2% per year (4). Other than age, the principal CAVD risk factor for AS is a congenitally malformed bicuspid aortic valve (BAV), which is present in 0.5-2% of the general population (31). Patients with AS and a BAV are a decade younger at diagnosis than those with normal tricuspid aortic valve (TAV) morphology (2). Premature leaflet thickening and calcification in BAV patients may be caused by abnormal shear stress hemodynamics.

CAVD shares mechanistic features with atherosclerosis (6). The current accepted model implicates initial lipid oxidation, inflammatory infiltration by T-lymphocytes and macrophages, cytokine and pro-fibrotic signaling, and altered extracellular matrix (ECM) secretion, leading to ectopic bone formation and tissue mineralization (11, 22). The factors predicting transition from the "at risk" status to aortic sclerosis and subsequently to severe AS, are not known (25). Transcriptional profiling of CAVD has helped to identify hundreds of genes differentially expressed between calcified and normal valves (8). Multi-omics approaches combining transcriptomics and proteomics have provided a high-resolution expression atlas and established potential drivers of disease progression (30).

Despite this progress, thus far no medical therapy has been proven to be effective in preventing progression to severe disease, with randomized clinical trials demonstrating the lack of effect of statins on disease progression (36). It has been suggested that statin treatment would have to begin at an earlier disease stage to have a beneficial effect. Current imaging modalities, especially echocardiography, remain the gold standard for diagnosis of valve disease but are

mainly performed when symptoms appear. Non-contrast computed tomography may have significant predictive value in both asymptomatic and symptomatic patients, but remains a source of radiation exposure and may not be available in centers with limited resources. Biomarkers may complement imaging modalities to detect early changes, especially early calcification. The ideal biomarker would predict disease as well as improve prognosis, subclinical disease management, and risk stratification and reduce disease-associated morbidity and mortality. Already identified potential biomarkers include markers of myocardial injury, cardiac mechanical stretch, inflammation, and hemostasis imbalance; however, with the exception of lipoprotein(a), these are not specific to CAVD or have yet to be validated in large randomized studies (32).

The aortic valve develops from rudimentary endocardial cushions that are subsequently refined into thin leaflets well into postnatal life. This process is dependent on complex cell-ECM interactions and is modulated by hemodynamics, and its spatio-temporal dysregulation results in malformations and maladaptive remodeling (10). Several lines of evidence indicate that valve disease at any age is accompanied by some underlying structural defect (29), suggesting that latent CAVD has a developmental origin. Moreover, developmental pathways may also be re-activated during tissue repair processes in the disease setting. For example, molecular mechanisms controlling heart valve cell differentiation are shared with cartilage, tendon, and bone development (reviewed in (15), and are reactivated during endochondral ossification and mature lamellar bone formation taking place in end-stage aortic valve disease (18). Remarkably, only end-stage CAVD has been described at the molecular level, whereas the molecular underpinnings of subclinical disease are poorly understood, due in part to difficulties in accessing biological material from early lesions.

To gain mechanistic insight, we performed an RNA-seq analysis of human fetal and adult control and calcified bicuspid (cBAV) and tricuspid valves (cTAV). K-means clustering was used to identify dynamically regulated processes taking place simultaneously at crucial stages of

human valve development and in diseased valves. The prevailing gene signatures were associated with immune-metabolic processes and heretofore uncharacterized pathways linked to cellular stress and ageing. A search for genes shared between our gene expression datasets and datasets generated from asymptomatic subjects from the Progression of Early Subclinical Atherosclerosis (PESA) cohort (5) with aortic valve calcification and without AS identified marker genes in peripheral blood of asymptomatic individuals. When combined in quantitative RT-PCR assays, these marker genes showed good sensitivity and specificity for predicting CAVD at a subclinical stage.

RESULTS

Diseased valves are transcriptionally more similar to developing valves than to controls

Genome-wide transcriptome profiling was carried out on normal adult control valves (n=8) and severely stenotic calcified BAV (cBAV; n=5) and TAV (cTAV; n=7) valves, as well as on fetal aortic valves at gestational weeks 9 (n=4), 12-13, (n=3) and 22 (n=2) (Supplementary Table S1, see https://figshare.com/s/4357d56025cb3380a0df). These developmental timepoints correspond to mouse embryonic days (E) 13.5-17.5 (13), during which valves are remodeled by morphogenic processes leading to thinning, elongation, and formation of multi-laminar valve structure. To integrate data across studies and generalize our findings, we conducted a meta-analysis with publicly available expression data from normal adult control (n=8), cBAVs (n=10), and cTAVs (n=9) valves (8) (Figure 1A). After correcting for batch effects, Principal Component Analysis (PCA) indicated that sample differences did not correlate with batch origin (Supplementary Fig. S1A), but rather depended on disease or developmental stages (Figure 1B). The first of the PCA components (PC1) separated developing and adult valve samples according to disease severity, ranging from cTAV (right hand side) to controls (left hand side). Interestingly, fetal valves tended to cluster with diseased adult valves. There were two control samples (both from heart transplants) and one sample of embryonic samples at 9 weeks showing more heterogeneity than the rest according to the second component (y-axis) but still the range of the axis (-0.6 to 0.4) is very small, confirming the reproducibility of the samples in terms of global transcriptome. In the second PCA component (PC2), the adult control and disease samples were separated from fetal weeks 9 and 13 samples (Figure 1B). Therefore, and considering that the first PCA dimension explains 15.24% of the variance, while the second dimension explains only 7.73%, adult disease samples appeared to be more closely related to fetal than to control samples, suggesting that some of the disease pathogenesis can be ascribed to the recapitulation of developmental processes.

To further support this finding, we used Euclidean distances between overall gene expression profiles to perform hierarchical clustering (HC) of the samples (Figure 1C). Similar to what was observed in the PCA, disease samples cluster further apart from controls and appear under the same branch with embryonic samples at 9 and 13 weeks.

Clustering identifies dynamic gene expression across disease and developmental states

Differential expression analysis was initially carried out between each pair of conditions of interest (Supplementary Table S2, see https://figshare.com/s/4357d56025cb3380a0df ; 9 pairwise comparisons). However, pair-wise comparisons were not adequate to explore dynamic and coordinated gene expression profiles across different disease and developmental states. K-means analysis computing n=50 yielded dynamic expression profiles across the different conditions (Supplementary Fig. S1B, see https://figshare.com/s/d9a68e0d6faa67e4c6c3). Co-expressed genes (ie. genes undergoing similar disease/stage status expression changes) likely participate in the same process, and genes that function in the same process are regulated coordinately (20). There were 6 broad combinations of expression patterns: 1) genes down-regulated in developing valves and up-regulated in control adult valves (e.g. C34); 2) genes down-regulated in control and up-regulated in diseased valves (e.g. C7); 3) genes up-regulated in developing valves, down-regulated in adult control valves, and up-regulated in diseased valves (e.g. C45); 4) genes up-regulated in developing valves and down-regulated in adult control and disease valves (e.g. C37); 5) genes up-regulated in adult control valves and down-regulated in diseased valves (e.g. C16); and 6) genes down-regulated in developing valves, up-regulated in adult control valves, and down-regulated in diseased valves (e.g. C40) (Supplementary Fig. S1B, see https://figshare.com/s/d9a68e0d6faa67e4c6c3). Thus, part of the disease process involves a gene signature present during fetal valve development.

Comparative functional analysis identifies multiple interconnected pathways

Functional enrichment analysis of clusters with IPA identified significant associations to canonical pathways in 28 clusters (Figure 2). Subsequent unsupervised hierarchical clustering of clusters, based on the fraction of shared canonical pathways, identified 6 metaclusters (MC), termed MC1-MC6 (Figure 2). Cluster 10 did not overlap with the others in terms of functional enrichment, and was not further considered. Generally, genes highly expressed in adult disease showed low expression at gestational week 13 and vice-versa; however, there were exceptions (clusters 9, 12, 23, and 45; Figure 2). Although metaclusters were defined as non-overlapping collections of gene clusters, enriched functions could be shared between several meta-clusters. For example, canonical pathways associated to clusters 46 and 32 were shared by MC1 and MC2; those associated to cluster 12 were shared by MC2, MC3, MC4, and MC6; those to clusters 12 to 45 were shared by MC2, MC3, and MC4; and those canonical pathways enriched in clusters 5 and 45 were shared by MC4 and MC6 (Figure 2). Therefore, the biological functions represented in the different MCs are interconnected.

Innate and adaptive immune processes are present in valve disease and development

The collections of genes associated to metaclusters MC1-MC6 were reanalysed with IPA. Enriched canonical pathways associated to each MC are summarized in Figure 3. MC3 was associated to 168 pathways related to innate-adaptive immune processes (Supplementary Fig. S2, see https://figshare.com/s/d9a68e0d6faa67e4c6c3). The top 5 pathways were Th1 and Th2 Activation Pathway, TREM1 Signaling, Communication between Innate and Adaptive Immune Cells, Phagosome Formation and Dendritic Cell Maturation (Figure 3; Supplementary Fig. S3, see https://figshare.com/s/d9a68e0d6faa67e4c6c3; Supplementary Table S3, see https://figshare.com/s/4357d56025cb3380a0df). The remaining 163 enriched biological functions were also involved in critical immune-inflammatory cellular processes (Supplementary Fig. S2, see https://figshare.com/s/d9a68e0d6faa67e4c6c3; Supplementary Table S3, see

https://figshare.com/s/4357d56025cb3380a0df). Among the nine clusters combined into MC3, five of them had expression profiles characterised by increased gene expression in all adult samples, both control and CAVD (clusters 22, 9, 34, 36 and 2), whereas four clusters had increased expression in CAVD only (clusters 49, 44, 7, and 35; Figure 3). Discrete immune cell gene signatures were present at week 9 (clusters 44 and 35), week 13 (cluster 9), and week 22 (clusters 49 and 22) of development (Figure 3), but involved relatively fewer pathways than those found in control or calcified adult valves. The presence of immune and pro-inflammatory gene signatures during fetal development and in apparently healthy valves is consistent with the progressive nature of the calcified valve disease pathology.

Cell migration and DNA replicative pathways are re-activated in CAVD

Metaclusters MC4 and MC6 were enriched in genes belonging to 202 and 23 IPA pathways, respectively (Supplementary Fig. S4 and S6A, see https://figshare.com/s/d9a68e0d6faa67e4c6c3; Supplementary Table S3, see https://figshare.com/s/4357d56025cb3380a0df). In general, genes associated to these clusters have increased expression during development (notably at week 13), decreased in adulthood and re-activated in CAVD. Therefore, of the 7 clusters associated with these two MCs, six were associated with more elevated gene expression in the diseased state (clusters 25, 23, 5, 45, 17, and 21), whereas only one was associated with lower expression (cluster 40; Figure 3).The top five pathways in MC4 were Actin Cytoskeleton Signaling, Molecular Mechanisms of Cancer, Axonal Guidance Signaling, Epithelial Adherens Junction Signaling and Integrin Signaling (Figure 3, Supplementary Fig. S5, Supplementary Table S3, see https://figshare.com/s/4357d56025cb3380a0df). The remaining 197 IPA pathways, represented components of fundamental cell and developmental signaling pathways (Supplementary Fig. S4, see https://figshare.com/s/d9a68e0d6faa67e4c6c3; Supplementary Table S3, see https://figshare.com/s/4357d56025cb3380a0df). The top five pathways in MC6 were Role of

CHK Proteins in Cell Cycle Checkpoint Control, Sumoylation Pathway, Cell Cycle Control of Chromosomal Replication, Cell Cycle: G2/M DNA Damage Checkpoint Regulation, Role of BRCA1 in DNA Damage Response (Figure 3, Supplementary Fig. S6B, see https://figshare.com/s/d9a68e0d6faa67e4c6c3, Supplementary Table S3, see https://figshare.com/s/4357d56025cb3380a0df). The remaining 18 IPA pathways were involved in similar processes (Supplementary Fig. S6A, see https://figshare.com/s/d9a68e0d6faa67e4c6c3; Supplementary Table S3, see https://figshare.com/s/4357d56025cb3380a0df). Cellular proliferation and migration may be part of the ongoing immune-inflammatory response associated with processes represented in MC3.

Decreased OXPHOS and ribosomal biogenesis in CAVD

Genes in MC2 were significantly associated to 83 IPA pathways (Supplementary Fig. S7, see https://figshare.com/s/d9a68e0d6faa67e4c6c3; Supplementary Table S3, see https://figshare.com/s/4357d56025cb3380a0df), the top 5 of which were Mitochondrial Dysfunction, EIF2 Signaling, Oxidative Phosphorylation, Sirtuin Signaling Pathway (Supplementary Fig. S8A-C), Regulation of eIF4 and p70S6K Signaling (Supplementary Fig. S8D,E see https://figshare.com/s/d9a68e0d6faa67e4c6c3; Supplementary Table S3, see https://figshare.com/s/4357d56025cb3380a0df). The remaining 78 pathways in MC2 were also implicated in energy production and protein metabolism (Supplementary Fig. S7, see https://figshare.com/s/d9a68e0d6faa67e4c6c3; Supplementary Table S3, see https://figshare.com/s/4357d56025cb3380a0df), showing strong enrichment in genes for nuclear encoded subunits of the electron transport chain complexes and ribosomal biogenesis (Supplementary Fig. S8A-C, see https://figshare.com/s/d9a68e0d6faa67e4c6c3; Supplementary Table S3, see https://figshare.com/s/4357d56025cb3380a0df). Other gene signatures were AMP-activated protein kinase (AMPK), ER stress (Unfolded protein response), oxidative stress

response (NRF2-mediated Oxidative Stress Response), cellular longevity (Telomerase Signaling), and neurodegenerative disease (Huntington's Disease Signaling; Supplementary Fig. S7, see https://figshare.com/s/d9a68e0d6faa67e4c6c3; Supplementary Table S3, see https://figshare.com/s/4357d56025cb3380a0df), suggesting the modulation of cellular stress responses, and age-related disease gene signatures. Of the nine clusters in MC2, six had, on average, decreased expression in disease conditions (clusters 24, 31, 32, 16, 41, and 1), while one had increased expression in the diseased state (cluster 12) and three were unchanged (clusters 31, 26, and 37; Figure 3). In general, MC2 transcripts had peak expression at week 13, with lower expression at the other developmental stages (Figure 3), suggesting that metabolic processes are dynamically regulated during aortic valve development. Overall, this analysis indicates that CAVD is associated with decreased OXPHOS metabolism and altered proteostasis.

Stress response gene signatures are altered in CAVD

MC1 and MC5 did not overlap significantly with any of the other MCs but included similar biological processes. Genes in MC1 were associated to 7 pathways (Figure 3; Supplementary Fig. S9A, see https://figshare.com/s/d9a68e0d6faa67e4c6c3; Supplementary Table S3, see https://figshare.com/s/4357d56025cb3380a0df), the top 5 being Sphingosine-1-phosphate Signaling, Endocannabinoid Developing Neuron Pathway, Amyloid Processing, Huntington's Disease Signaling, Dermatan Sulfate Degradation (Figure 3; Supplementary Fig. S9B, see https://figshare.com/s/d9a68e0d6faa67e4c6c3; Supplementary Table S3, see https://figshare.com/s/4357d56025cb3380a0df). The expression of MC1 genes was relatively increased in adult controls and decreased in CAVD (Figure 3). MC5 was enriched in a single pathway, Unfolded protein response (Figure 3; Supplementary Fig. S9C, see https://figshare.com/s/d9a68e0d6faa67e4c6c3; Supplementary Table S3, see https://figshare.com/s/4357d56025cb3380a0df). MC5 genes were expressed at decreased levels in controls and increased in CAVD (Figure 3). These data suggest that chronic immune-

inflammatory processes are linked to cellular stress and age-related disease in calcific aortic valve pathology.

Identification of circulating biomarkers predicting CAVD

To identify potential biomarkers useful for predicting CAVD, a sub-set of genes from the aortic valve study was compared with whole blood RNA-Seq data from asymptomatic individuals with aortic valve calcification, detected by non-contrast computed tomography in the *Progression of Early Subclinical Atherosclerosis* (PESA) study (5). PESA participants undergo simultaneous cardiac calcium scoring and blood sampling. Out of the 455 PESA participants for which RNA-seq data was available in visit 2 (ie. three years after recruitment), 59 had evidence of valve calcification and 52 of theses had sufficient integrity for qPCR analysis. None of them had valvular stenosis/regurgitation, evidencing an early stage of the disease. Their transcriptome was compared to the transcriptome of PESA individuals without valvular calcification (n=395). From the whole set of genes identified as differentially expressed ($p<0.05$, Supplementary Table S5, see https://figshare.com/s/4357d56025cb3380a0df), six were selected that showed a disease-specific pattern in the RNA-seq data of human aortic valves (see Methods for selection criteria; Supplementary Fig. S1, see https://figshare.com/s/d9a68e0d6faa67e4c6c3). Five of these genes were related to adaptive innate and adaptive responses (*CD28, ITK, PAG1, NLRC5, and ASCC3*) and a sixth was related to the WNT pathway (*LINS1*) (Supplementary Table S6, see https://figshare.com/s/4357d56025cb3380a0df). The expression of the six genes was measured in whole blood (WB) on 104 PESA participants [52 cases: asymptomatic individuals with calcification of the aortic valve (PESA-SD); 52 controls: individuals without calcification (PESA-CTL)] and 12 patients with CAVD recruited at Monteprincipe Hospital (WB-CAVD) by qRT-PCR and correlated with the degree of aortic valve calcification (Figure 4A, Supplementary Table S4, see https://figshare.com/s/4357d56025cb3380a0df). Considering all conditions, the 6

genes showed some degree of correlation when considered individually ($P \leq 0.27$; Figure 4A, Supplementary Table S7, see https://figshare.com/s/4357d56025cb3380a0df). Given that, *NLRC5* and *PAG1* were also associated with total calcification of the coronaries (Supplementary Fig. S10A, see https://figshare.com/s/d9a68e0d6faa67e4c6c3, Supplementary Table S7, see https://figshare.com/s/4357d56025cb3380a0df) and were therefore excluded as potential specific markers of aortic valve calcification.

To determine the utility of *ASCC3*, *ITK*, *CD28* and *LINS1* as potential markers of valve calcification, a ROC curve was built to represent the predictive value of the model to classify calcific disease patients (WB-CAVD) vs PESA-SD individuals and vs PESA-CTL, yielding areas under the curve (AUCs) of 0.97 and 0.91 respectively (Figure 4B). An additional model was built to assess the power of the gene signature to distinguish between individuals without calcification (PESA-CTL) from those with subclinical disease (PESA-SD; AUC=0.71).

This model was then validated in whole blood samples of an independent cohort of CAVD patients (n=16), together with samples from 25 of the 52 PESA-SD individuals and 35 of the 52 PESA-CTL individuals taken three years after visit 2 (Figure 4C). The model achieved AUCs of 0.90 and 0.88 to discriminate CAVD patients from PESA-SD and PESA-CTL individuals, respectively. The AUC of the model to distinguish PESA-SD from PESA-CTL was again 0.71 (Figure 4C). Thus, our model identifies a gene expression signature in blood that is highly predictive of valve disease and of aortic valve calcification levels in otherwise healthy individuals.

DISCUSSION

This study provides, for the first time, a comprehensive molecular profile of shared and unique cellular activities taking place in aortic valve development and CAVD. The transcriptome of adult disease samples appeared to be more closely related to fetal week 9 than to control samples or weeks 13 and 22, suggesting that some of the disease pathogenesis might be ascribed to the recapitulation of processes taking place in early development. The overall cBAV and cTAV gene expression profiles were essentially identical, confirming previous findings suggesting that valve calcification depends on the same biological pathways, regardless of valve morphology (8, 26). Therefore, the mechanisms leading to BAV formation are independent of those leading to BAV calcification; in other words, the cause of the embryonic defect is distinct from the resulting calcification process.

Functional analysis provided compelling evidence that innate and adaptive immune interactions take place in the aortic valve throughout life (Supplementary Fig. S10B 13, see https://figshare.com/s/d9a68e0d6faa67e4c6c3). The fetal immune cell gene signature in MC3 was notably simpler than in adult control or CAVD samples, with fewer immune cell gene expression signatures present at gestational weeks 9 (3/9 clusters), 13 (1/9 clusters), or 22 (3/9 clusters) than in adult controls (5/9 clusters) or in CAVD (9/9 clusters), suggesting that aortic valve immune cell enrichment in the aortic valve increases over time. Moreover, the presence of only one immune cell gene signature at 13 weeks (cluster 9), compared with three at 9 and 22 weeks of gestation, implies that the immune landscape is dynamically regulated in developing valves. These observations are consistent with previous findings of multiphasic changes in shear stress-response, cell-ECM interaction and adaptive immunity pathways from week 14 to week 22 of human aortic valve development (7).

The predominant T cell gene signature in the RNA-seq dataset strengthens the notion that unresolved immune-inflammatory response is a major CAVD component (17). This association is well established, with early immunohistological findings indicating the presence of T

lymphocytes in immune infiltrates (23, 24). More recent studies have revealed the existence of T cell infiltration in BAV and TAV calcific disease, consisting of non-specific chemokine driven polyclonal T cell recruitment, and memory-effector CD8+ T cell clonal expansions (34, 35), supporting the interpretation of an antigen-induced immune response. Moreover, this antigen-driven response involved circulating expanded CD8+ and, to a lesser extent, CD4+ T cell clones between circulating blood and valve tissues suggesting a systemic disease (34). The gene expression signatures of several other immune-competent cells, including B-lymphocytes, macrophages, and dendritic cells were also present, confirming previous characterization of cellular infiltrates in CAVD (33). Together with a body of literature suggesting a tight inflammatory cell-valve interstitial cell interaction in CAVD (11, 17), our data are consistent with the notion that unresolved inflammation ultimately drives myo-fibroblast and osteoblast-like differentiation and leads to heterotopic bone formation. Therefore, targeted therapeutic immunosuppression of calcification might be approachable by better characterizing the various infiltrating immune-cell subtypes.

Functional analysis revealed that OXPHOS metabolism was decreased in CAVD, with coordinated expression reduction of multiple nuclear genes encoding subunits of mitochondrial electron transport chain complexes I, III, IV and V. These changes might be part of ongoing adaptive metabolic reprogramming during T lymphocyte activation (27), when upon encountering an antigen, T cells proliferate and acquire new functions, switching energy metabolism from OXPHOS to glycolysis. However, both increased and decreased expression of glycolytic pathway components were present, suggesting that decreased OXPHOS metabolism cannot be ascribed solely to T cell activation. Moreover, adult aortic valves showed markedly reduced transcript levels of multiple subunits of the small and large protein ribosomal complexes (RPS and RPL respectively) and eukaryotic translation initiation factors (EIF1-4), possibly reflecting co-ordinate dysregulation of OXPHOS activity and ribosomal biogenesis (19). This occurred in uncalcified adult valves as well (clusters 31, 37, and 12), suggesting declining

function with age. In contrast signatures for cell death and apoptosis (for example in MC3) and, energy-demanding cell processes such as proliferation, DNA repair, and cell movement (in MC4 and MC6) were increased, possibly reflecting cellular heterogeneity within the calcified valve.

Decreased mitochondrial metabolism and protein translation may be a manifestation of altered physiological stress responses that occur during ageing (30), which is also related to the inability to maintain adaptability to stress and re-establish homeostasis in response to environmental perturbations. Thus, multiple stress-response gene signatures were present in MC2 and to a lesser extent in MC1 and MC5 (Supplementary Fig. S10B, see https://figshare.com/s/d9a68e0d6faa67e4c6c3). These included SIRT, a highly conserved family of NAD-dependent deacetylases, linking transcriptional regulation to mitochondrial function, stress resistance, metabolism, cell survival and organismal longevity (3) and mTOR (Mammalian target of rapamycin), a nutrient sensing system which regulates protein synthesis, to promote cell growth and proliferation (19). Moreover, deficiency of the unfolded protein response (UPR) pathway, which is activated by misfolding of newly synthesized proteins as part of the ER stress response (9) contributes to inflammation, disease and ageing. Gene signatures including amyloid processing and Huntington's disease signaling were also featured implying shared calcific valve and neurodegenerative disease mechanisms (Supplementary Fig. S10B, see https://figshare.com/s/d9a68e0d6faa67e4c6c3). Therefore, chronic inflammation drives metabolic stress and biological ageing in CAVD.

Given the phenomenon of cellular trafficking of T cells between the diseased valve tissue, and circulating blood (34), we reasoned that aortic valve and circulating blood from CAVD patients share common genes that would be absent in subclinical individuals. In the PESA study we identified a subpopulation of asymptomatic individuals in this cohort with > 1 valve calcification detected by imaging. Therefore, we searched for circulating biomarkers by comparing the CAVD transcriptomes and whole blood from datasets of the subclinical PESA study population, and identified six differentially expressed genes, four of which were found to

predict subclinical and overt valve disease. Therefore *ASCC3, ITK, CD28,* and *LINS* were specific to aortic valve calcification, whereas *PAG1* and *NLRC5* were also associated with coronary artery calcification, suggesting that valve and vascular calcification proceed by shared and separate mechanisms. These mechanisms have yet to be examined, but noteworthy is that five of six genes belong to T cell activation and NF-kB pathways. This gene signature could be used in combination with high-resolution imaging for the early diagnosis of valve calcification.

Study Limitations. A limitation of our study is the low sample numbers especially for week 22 samples (n=2). Investigational studies involving human fetal issue are limited by ethical and legal considerations on medical abortion. Nevertheless, our bioinformatics analysis showed that the week 22 samples clustered together and separated well from the other groups by PCA and we are confident of the robustness of the results involving this group. Moreover, differential expression analysis was performed using limma, a statistical method developed ad-hoc for RNA-seq data with low sample size (28).

METHODS

Study population and aortic valve collection.

Fetuses. Aortic valves from human fetuses were obtained from electively- (weeks 9; n=4 and week 12/13; n=3) or medically (week 22; n=2) -terminated pregnancies after written informed consent in concordance with French legislation (PFS14-011) and prior protocol approval from the "Agence de la biomédecine" (Supplementary Table S1, see https://figshare.com/s/4357d56025cb3380a0df). Aortic valves were recognized by their anatomical landmarks under the microscope and leaflets were isolated with minimal aortic wall contamination Samples were immediately flash frozen in liquid nitrogen. The investigation conformed to the principles outlined in the *Declaration of Helsinki*.

Patients. The CAVD study population consisted of BAV (n=5) or TAV (n=7) patients referred for aortic valve or aortic root surgery at Monteprincipe Hospital and Hospital Clínico San Carlos (Madrid, Spain; Supplementary Table S1, see https://figshare.com/s/4357d56025cb3380a0df). Patients were prospectively recruited between December 2009 and February 2013. Patients with a history of rheumatic heart disease, infective endocarditis, or connective tissue disorders were excluded. To determine the morphology and functional state of the aortic valve, all patients underwent clinical evaluation and preoperative 2-dimensional echocardiography according to the *American Heart Association* and the *American College of Cardiology* guidelines (21). The aortic valve morphology and the presence of valvular degeneration, including the extent of leaflet calcification, thickening, prolapse, and/or redundancy, were documented at surgery. Calcification was graded as mild (1/3 leaflet area affected) or severe (2/3 leaflet area affected). Immediately following surgical removal, a portion of the aortic valve leaflet was placed in sterile saline buffer and stored at -80°C until processing. Control valve samples were obtained at autopsy of individuals without cardiac problems who suffered a traumatic death (n=6) or at time of transplantation from heart transplant receptors with normal aortic valves (n=2). All patients or close relatives (for autopsy controls) gave written informed consent, and the study was approved

by the Health Service Ethics Committees (Comité Ético de Investigación Clínica) of participant centers. The investigation conformed to the principles outlined in the *Declaration of Helsinki*.

Whole-blood samples. Genes of interest were quantified by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in whole-blood (WB) samples from age-matched case-control males (n=104) of the Progression of Early Subclinical Atherosclerosis (PESA). Subclinical cases (PESA-SD) were defined as participants with aortic-valve calcification score >1, as assessed by the Agatston method (1). Controls (PESA-CTL) had no calcification of the aortic valves or coronary arteries. Patients with aortic valve stenosis and moderate to severe calcification (WB-CAVD; n=12) were recruited from Monteprincipe Hospital (Supplementary Table S4, see https://figshare.com/s/4357d56025cb3380a0df). For validation, an independent cohort of mainly severe aortic valve stenosis patients (n=16) was obtained from the MRVALVE study. Whole blood was collected in PAXGene tubes (BDBiosciences) and stored at -80^{o}C before processing.

RNA isolation

For each valve, total RNA from a single aortic valve cusp was extracted using the TRIzol LS (Invitrogen, Carlsbad, CA, USA) method and digested with RNase free DNase I (Invitrogen). For PESA and CAVD validation total RNA was using either the PAXgene blood mRNA kit (PreAnalytiX, Hombrechtikon, Switzerland) for manual isolation or the QIAsymphony PAXgene blood RNA kit (PreAnalytiX) for automated isolation using a QIAsymphony SP liquid handling robot (Qiagen, Venlo, Netherlands). RNA purity, concentration, and integrity were assessed by Nanodrop 2000 spectrophotometry (Thermo Scientific) and automated electrophoresis in a 2100 Bioanalyzer (RNA6000 Nano LabChip; Agilent). Samples with a RIN > 6 were selected for reverse transcription.

RNA-seq data generation

RNA-seq was performed on DNase I-treated RNA samples with a RIN > 7. RNA-seq libraries were created using the TruSeq Stranded Total RNA with Ribo-Zero Gold Prep Kit (Fetal

samples) or TruSeq™ RNA Sample Prep Kit v2 (Patient samples; Illumina, Diego, CA). Final cDNA libraries were checked for quality and quantified in the 2100 Bioanalyzer (Agilent). The libraries were loaded in the flow cell at 8 pM, and clusters were generated in Cbot and sequenced as 50bp single-end reads on a Hiseq 2500 instrument (adult samples) or 75 base single-end reads on a Genome Analyzer IIx instrument (fetal samples; Illumina, Diego, CA). Image analysis and base calling were performed using RTA and CASAVA.

Bioinformatics analysis of RNA-Seq.

Data preprocessing

FASTQ files were pre-processed with cutadapt (16) to eliminate Illumina adapter remains and reads were aligned to the human reference transcriptome (GRCh38.78) using RSEM 1.2.31 (14). Raw data from 27 RNA-seq samples from GSE76718 were downloaded from GEO and pre-processed and aligned using the same pipeline. Control samples were present in all experiments, allowing for batch correction of the data using ComBat (12). Data were normalized using TMM with log-ratio trimming=0.3 and sumTrim=0.05 and voom-transformed. Only genes with at least 1 cpm in at least 10 samples were considered for further analysis.

Differential Expression Analysis

Differential expression between groups was performed using mixed models with eBayes, as implemented in the Bioconductor limma package (28). A gene was classified as differentially expressed if its Benjamini-Hochberg adjusted *P*-value was < 0.05 in any of the contrasts under study (Supplementary Table S2, see https://figshare.com/s/4357d56025cb3380a0df).

Clustering and functional analyses

To identify groups of genes with similar expression profiles across the six conditions under consideration, k-means clustering was performed with the R ComplexHeatmap package on all expressed genes (19,962 genes). The Elbow method was used to estimate the optimal number of clusters, which was set as k=50. The number of genes per cluster was between 215 and 680. Ingenuity Pathway Analysis (IPA, Qiagen) was then used to identify significant associations

between canonical pathways and clusters. Twenty-eight clusters, out of the original collection of 50, were found to be associated to at least one canonical pathway with Benjamini-Hochberg (B-H) adjusted *P*-value < 0.05 (Supplementary Table S3, see https://figshare.com/s/4357d56025cb3380a0df). Concordance between canonical pathway profiles was then used to identify groups of clusters that could be associated to similar functions. One cluster (cluster 10) did not share any enriched pathway with any other cluster and was not considered further. The remaining 27 clusters were grouped into six meta-clusters, using the fraction of shared pathways as distance measure. Meta-clusters contained from 332 to 3304 genes (Figure 2). IPA was used again to re-annotate meta-clusters (Figure 3).

Data availability

Data are deposited in the NCBI GEO database under accession number GSE148219.

qRT-PCR for validation studies in whole blood

Selection of candidate genes for qRT-PCR. We used whole blood transcriptomics data generated in the PESA study to identify genes that might potentially serve as predictors of CAVD. The criteria to select these candidate genes were: (i) Genes differentially expressed between individuals with (n=59) and without (n=396) calcification of the aortic valve (p-value<=0.05) but not differentially expressed between individuals with and without calcification in the coronaries (p-value>0.05; Supplementary Table S5, see https://figshare.com/s/4357d56025cb3380a0df). (ii) Aortic valve- specific differentially expressed genes with a top 10% correlation between their expression level and the level of calcification of the aortic valves. (iii) Their expression levels in the RNA-seq samples of the aortic valves were either up-regulated in disease v.s. no disease samples or they were upregulated in early embryonic valve samples and in the diseased samples but not in the control samples. From the 10 genes fulfilling the three criteria, 6 were selected based on their presumptive function (Supplementary Table S6, see https://figshare.com/s/4357d56025cb3380a0df).

qRT-PCR. RNA samples from PESA and CAVD patients (75ng) was reverse transcribed using the High Capacity cDNA Reverse Transcription kit with RNase Inhibitor (ThermoFisher Scientific). Gene expression was quantified by qPCR using TaqMan probes (ThermoFisher Scientific; Supplementary Table S8) in 52 case-control pairs from the PESA study (case: positive calcification of the aortic valve) and in 12 CAVD patients from Monteprincipe Hospital. Each reaction was performed in triplicate and the final reaction volume was set to 10µl. qPCRs were conducted in 384-well plates (Cat. no. 4344345; Applied Biosystems) using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Each well included 3µl (2.25 ng) cDNA, 5µl 2x TaqMan Universal Mater Mix (ThermoFisher Scientific), 0.5µl 20x TaqMan assay (ThermoFisher Scientific), and 1.5µl H_2O. The cycling protocol was 10 min at 95°C followed by 60 cycles of 15 s at 95°C and 60 s at 60°C. Baseline and threshold were set automatically in SD S 2.3 (ThermoFisher Scientific) to calculate Cq values. The Cq values were then imported into qBasePlus (Biogazelle, Zwijnaarde, Belgium) to calculate relative quantities normalised to *GAPDH*. Data were scaled per sample to eliminate potential biases.

Statistical analysis

A linear mixed model was used to assess correlation between the expression of each gene in WB and the degree of calcification in the aortic valve for each disease group (PESA-SD, WB-CAVD) separately. Visualization was performed using the multiplot function and the ggplot2 R package. All samples were jointly analyzed using a linear mixed model with the origin of the samples as random effect. The same analysis was performed to assess the correlation of the gene expression with calcification of the coronaries. Four genes which showed no correlation with the amount of calcium in the coronaries were selected to build a gene expression signature using a multinomial model with interaction for the prediction of the three groups of individuals with different disease severity WB-CAVD>PESA-SD>PESA-CTL. To quantify the predictive power

of the gene signature between different groups of individuals a logistic regression model was fit to the data and the corresponding ROC curves were built using the ROCR R package.

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Author contributions

Donal MacGrogan, PhD: Design of work: Substantial; interpretation of data: Substantial; Writing: original draft: Lead; Writing: review & editing: Lead. final approval of the version to be published.

Beatriz Martínez Poveda, PhD: Investigation: Lead; Methodology: Substantial; final approval of the version to be published.

Jean-Pierre Desvignes, MD: Investigation: Supporting; final approval of the version to be published.

Leticia Fernandez-Friera, MD, PhD: Data curation: Substantial; Formal analysis: Substantial; Investigation: Supporting; final approval of the version to be published.

Manuel José Gomez, PhD: Formal analysis: Substantial; Visualization: Substantial; final approval of the version to be published.

Eduardo Gil Vilariño, BSc: Investigation: Supporting; final approval of the version to be published.

Sergio Callejas Alejano, BSc: Investigation: Supporting; final approval of the version to be published.

Pablo Garcia-Pavia, MD, PhD: Investigation: Supporting; final approval of the version to be published.

Jorge Solis, MD, PhD: Investigation: Supporting; final approval of the version to be published.

Joaquín Lucena, MD, PhD:Investigation: Supporting; final approval of the version to be published.

David Salgado, PhD: Data curation: supporting; Formal analysis: supporting; Investigation: supporting; final approval of the version to be published.

Gwenaelle Collod-Béroud, PhD: Formal analysis: Supporting; Investigation: Supporting; final approval of the version to be published.

Emilie Faure, PhD: Formal analysis: Supporting; Investigation: Supporting; final approval of the version to be published.

Alexis Théron, MD: Investigation: Supporting; final approval of the version to be published.

Julia Torrents, MD: Investigation: Supporting; final approval of the version to be published.

Jean-François Avierinos, MD: Investigation: Supporting; final approval of the version to be published.

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Ana Dopazo, PhD: Investigation: Supporting; Supervision: Supporting; final approval of the version to be published.

Valentín Fuster, MD, PhD: Investigation: Supporting; Resources: Supporting; final approval of the version to be published.

Borja Ibañez, MD, PhD: Investigation: Supporting; Writing – review & editing: Supporting; final approval of the version to be published.

Fátima Sánchez-Cabo, PhD: Formal analysis: Equal; Writing – review & editing: Supporting; final approval of the version to be published.

Stephane Zaffran, PhD: Investigation: Supporting; Supervision: Supporting; Writing –review & editing: Supporting; final approval of the version to be published.

José Luis de la Pompa, PhD: Conceptualization: Lead; Funding acquisition: Lead; Project administration: Lead; Resources: Lead; Supervision: Equal; Visualization: Equal; Writing – review & editing: Supporting.

Competing interests

The authors declare no competing interests.

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FIGURE LEGENDS

Figure 1. Aortic valve collection summary, bioinformatics pipeline and mapping of sample batches and biological conditions in gene expression profile-based principal component analysis (PCA) plots. (*A*) Study outline summarizing aortic valve collection. During the study period, aortic valve leaflets were collected from human fetuses at gestational week (week) 9 (n=4), 12-13 (n=3), and 22 (n=2), adult controls (n=8) and patients with calcified BAV (cBAV; n=5) or TAV (cTAV; n=7). Samples were processed and subject to RNA-seq to characterize their gene expression profiles. Complementary expression data from control, cBAV and cTAV samples was downloaded from GEO (GSE76718). The resulting data set collection was analyzed with a bioinformatics pipeline that included preprocessing of fastq files with cutadapt, alignment against a human transcriptome reference and estimation of gene expression levels with RSEM, and differential expression testing with limma, considering that samples could be ascribed to five experimental batches. Expression profiles were clustered using k-means to obtain a collection of 50 clusters, which were then annotated with the Canonical Pathway feature of IPA. Twenty-seven clusters associated to at least one canonical pathway (with Benjamini-Hochberg adjusted p-value < 0.05) were grouped into six meta-clusters on the basis of concordant annotations. Meta-clusters were finally re-annotated with IPA. (*B*) Hierarchical clustering of the samples overall gene expression based on Euclidean Distance (*C*) Gene expression profile PCA plot mapping of biological conditions. Sample points are colored according to fetal developmental stage or adult disease state (no disease, n=16; cBAV, n=15 and cTAV, n=16). In PC1, control samples separate from fetal samples and cBAV or cTAV samples. In PC2, early-stage fetal samples (weeks 9 and 13) separate from adult control and disease samples. Percentages on axis labels denote the amount of variance explained by each principal component.

Figure 2. Concordance analysis of k-means clustering. K-means-based gene clusters (see Supplementary Fig. S 1B, see https://figshare.com/s/d9a68e0d6faa67e4c6c3) were grouped into metaclusters according to the agreement of their enriched canonical pathways. The white to violet squares in the central heatmap represent the percentage (from 0 to 100) of Canonical Pathways (CP) shared between each pair of 50 k-means clusters for which at least one enriched and shared term was found (k=27 out of the original 50 clusters). Hierarchical clustering of the concordance matrix identified six meta-clusters (MC1-6) The heatmap on the left represents the mean gene expression values for each cluster across biological conditions, on a normalized scale (from -2 to 2, for values below and above the mean, respectively). The dendrogram at the top represents functional relatedness between clusters, which was used to group them into meta-clusters. Meta-clusters are identified with colored boxes (MC1 to MC6, in red, blue, green, purple, orange and yellow, respectively) and represent groups of clusters associated to similar functions.

Figure 3. Functional analysis of meta-clusters. The six gene metaclusters (MC1-6) described in Figure 2 were annotated with IPA. Barplots on the right-hand side represent enrichment values for the top five IPA Canonical Pathways associated with each meta-cluster (MC), selected according to enrichment significance and having an adjusted Benjamini-Hochberg $P<0.05$. Meta-clusters are identified with colored boxes (MC1 to MC6, in red, blue, green, purple, orange and yellow, respectively) and represent groups of clusters associated to similar functions. Central boxes list all the genes associated with the selection of pathways associated with each metacluster. The heatmap on the left represents normalized expression values for individual genes across biological conditions, on a normalized scale (from -2 to 2, for values below and above the mean, respectively).

Figure 4. A gene expression signature predictive of CAVD. (A) Correlation of qPCR data (y-axis) vs aortic-valve calcification levels (x-axis) for the 6 selected genes in whole blood (WB). Each point represents an individual´s level of gene expression (y-axis) and its aortic valve calcification (x-axis). Dots are colored based on the group to which the individual belongs: PESA-CTL (n=52), PESA-SD (n=52) or WB-CAVD (n=12). Solid lines represent the linear model fit for PESA-SD and WB-CAVD patients. Shaded regions represent the confidence of the linear regression estimate for each calcium level. (B) Receiver operating characteristic (ROC) curve of multivariate logistic regression model predicting CAVD (WB-CAVD) vs preclinical aortic-valve calcification (PESA-SD; thick discontinuous line, AUC=0.97) vs valve calcification (PESA-CTL; thick solid line, AUC=0.91). A third ROC curve for the prediction of valve calcification (PESA-SD) vs no calcification (PESA-CTL; thin solid line, AUC=0.71) is shown. The discontinuous diagonal line indicates a fifty-fifty percent chance to predict each group. (C) Follow-up of the model in PESA subgroups after 3 years. Validation in an independent group of CAVD patients (WB-CAVD, n=16) vs a PESA-SD subgroup (n=25; thick discontinuous line, AUC=0.90) vs a PESA-CTL subgroup (n=35; thick solid line, AUC=0.88). A third ROC curve for the prediction of PESA-SD (n=25) vs PESA-CTL (n=35; thin solid line, AUC=0.71) is shown. The thin discontinuous diagonal line indicates a fifty-fifty percent chance to predict each group.

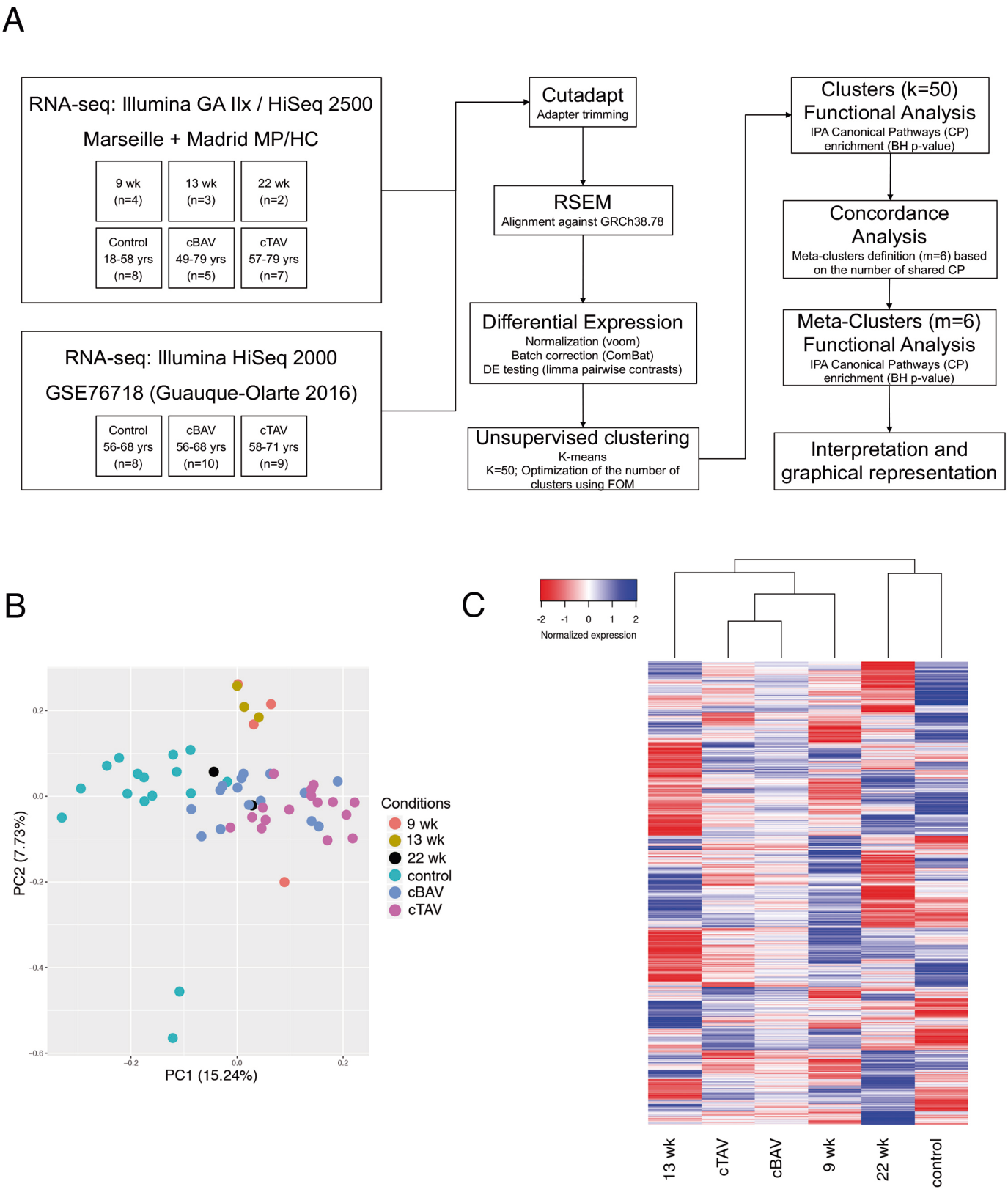
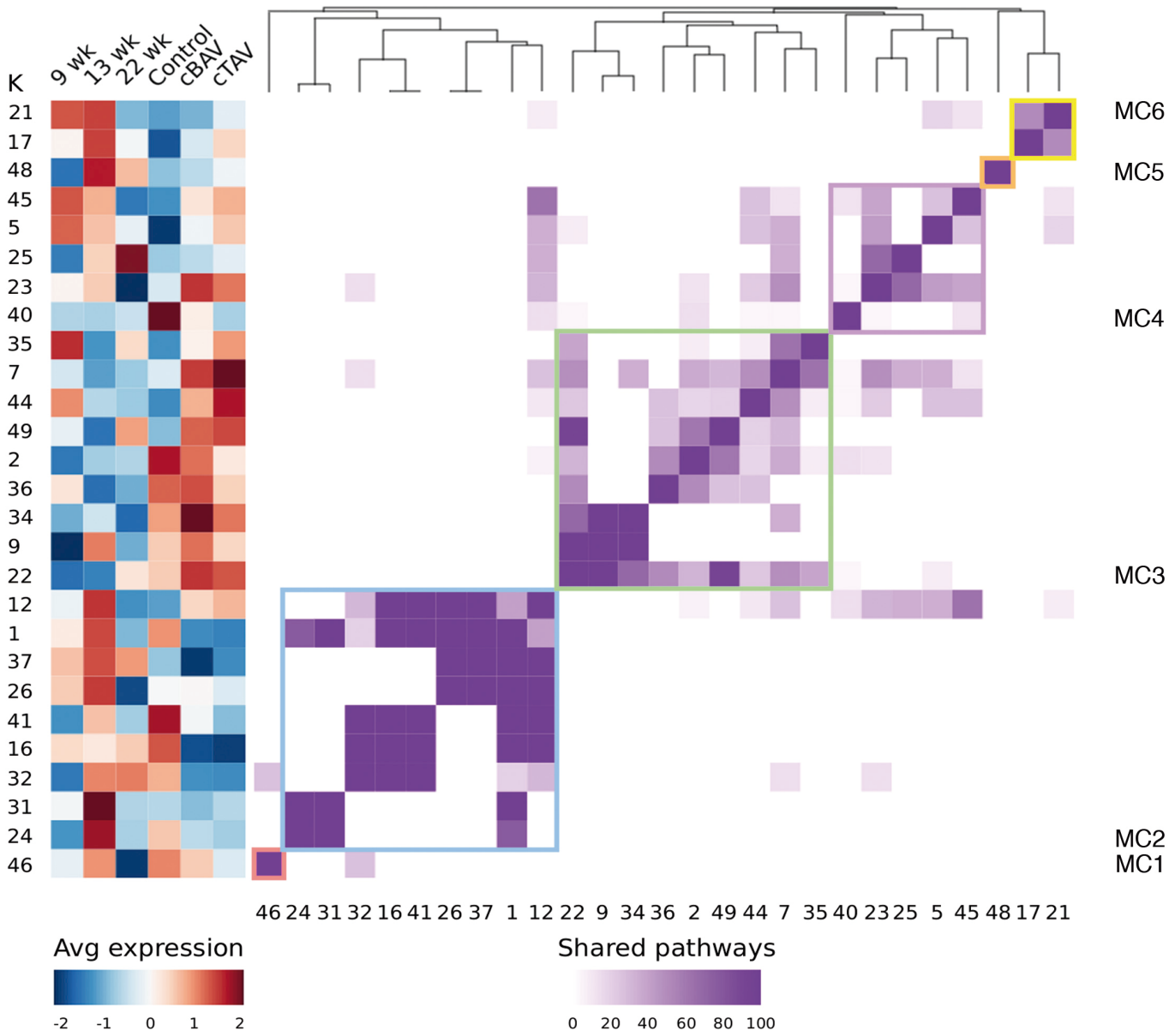


Figure_1_MacGrogan_et_al



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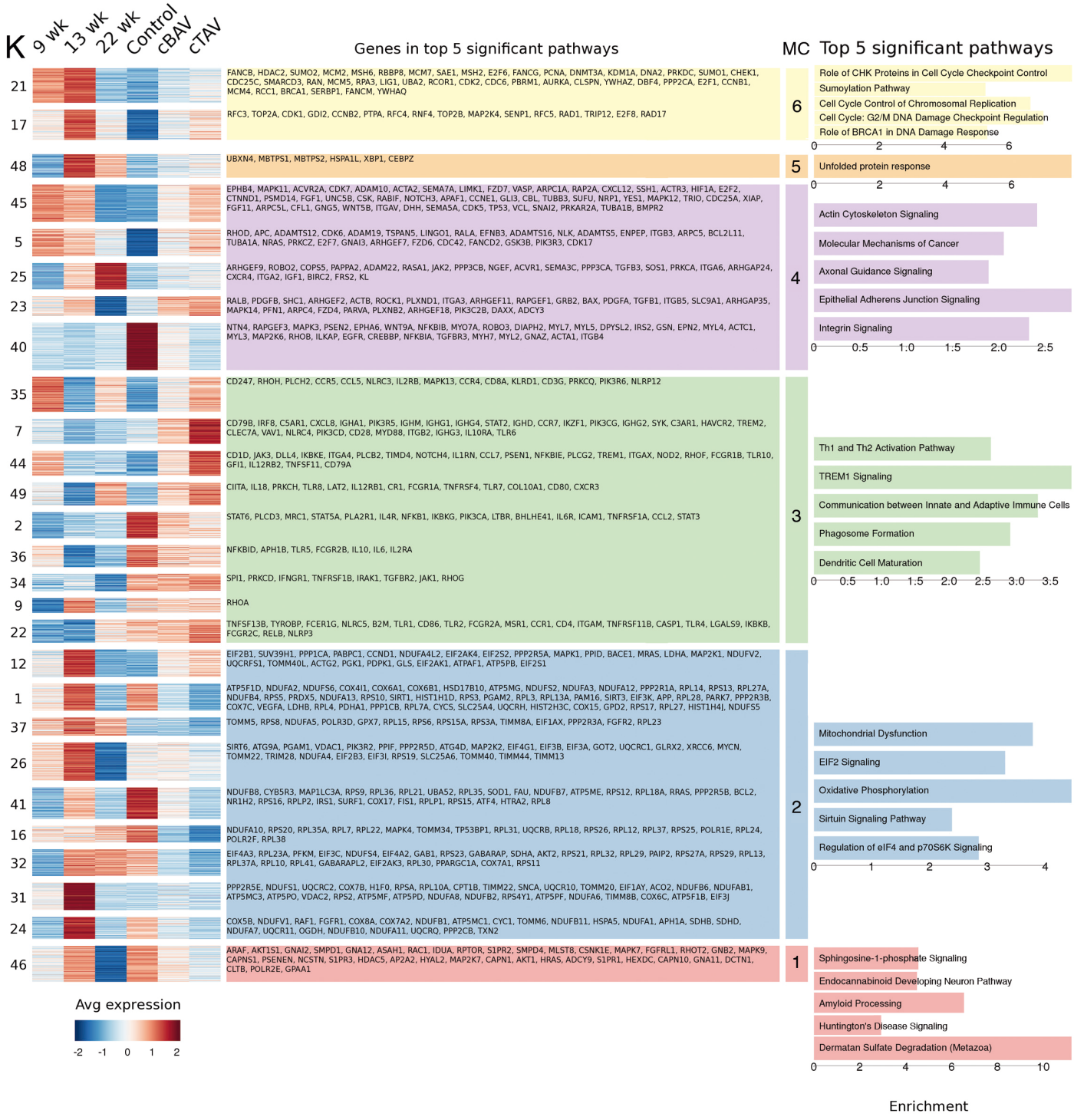

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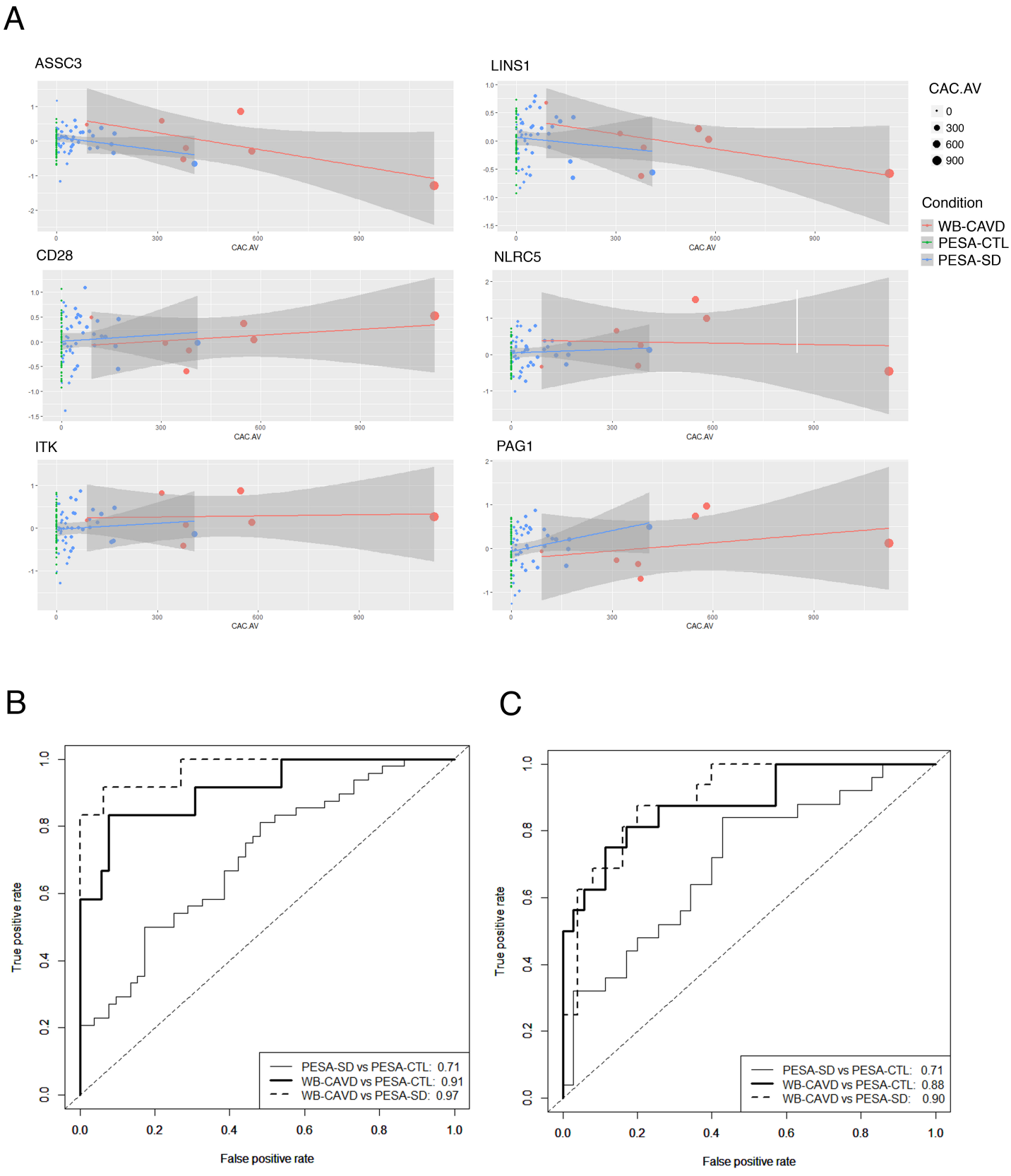

Figure 4_MacGrogan et al