Vasoactive Endothelial Growth Factor and Heat Shock Protein Gene Expression Response in Kawasaki Disease Associated Coronary Arteritis

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Background and Aim

Coronary involvement in Kawasaki Disease (KD), whether its after SARS-CoV2 infection or not, can result in significant complications. There is the risk of aneurysm formation associated with inflammation and an unremitting fever. We wished to study the Vasoactive Endothelial Growth Factor (VEGF) and Heat Shock Response from a gene-expression perspective. Thereby aiming to furnish to insights that might be useful in the treatment of Kawasaki Disease.

Method

KD datasets based on previous work, were selected including microarray studies KD1 (GSE63881), KD2 (GSE73461), KD3 (GSE68004) and the RNAseq dataset KD4 (GSE64486) from the NCBI online repository. Based on clinical literature. HSP genes shown to be associated with angiogenesis were chosen for analysis as well as gene expression for VEFGA and VEGFB. Further in order to gain an impression of inflammatory patterns, gene expression for NFKB1 and TNF were also chosen. Tools for analysis included Gene Set Expression Analysis (GSEA). A KEGG pathway, outlining a relationship between VEGF and endothelial migration and proliferation was assumed.

Results

A KD dataset showed increased VEGFA and decreased VEGFB in acute versus convalescent samples. In all three KD datasets, HSPA1A and HSBAP1 genes were upregulated in acute versus convalescent samples. In KD4, cases of KD versus controls, VEGFB was down-regulated ($p = 4.932e-02$) and HSPBAP1 up-regulated ($p = 1.202e-03$). GSEA of KD1, KD2 and KD3, using Hallmark gene sets, suggested an inflammatory response with TNFA signaling via NFKB, IL6 JAK STAT 3 signaling, apoptosis, angiogenesis, and unfolded protein response.

Conclusions

A novel application of a model of VEGF and HSP to KD was presented. Coronary pathogenesis based on VEGF and HSP was explored. The ability to follow angiogenesis at the molecular level using a VEGF-HSP model may have therapeutic implications. Further, the significance of gene expression between VEGFA, VEGFB in KD and the relationship of HSP gene expression to angiogenesis in KD requires further study.

Key Words

Kawasaki Disease, Coronary Arteritis, Transcriptome

Introduction

Kawasaki Disease (KD) remains a leading cause of acquired aneurysms in childhood. A KD-like phenotype associated with a SARS-CoV-2, post viral condition known as Multi Inflammatory Syndrome in Children (MIS-C) is also associated with the risk of aneurysm formation. Thus, understanding the relationship between inflammation and aneurysm formation is therefore warranted. KD associated with MIS-C, also known as Paediatric inflammatory multisystem syndrome temporally associated with SARS-CoV-2 infection (PIMS-TS), changes in Vasoactive Endothelial Growth Factor (VEGF) plasma protein levels compared to controls have been documented in [1]. In KD, VEGF levels are increased in the acute stage of KD in children without coronary pathology and in the sub-acute phase in children with coronary pathology [2]. Hamamichi et al (2001)., showed that KD patients who developed coronary artery changes had increased serum VEGF levels throughout the 2 to 4 weeks after the onset of KD illness[3]. Also patients with KD has higher VEGF levels in the acute phase compared to controls, as well as children with febrile illnesses. Further, they found that VEGF production was driven by Peripheral Blood Mononuclear Cells (PBMCs), especially within monocytes. This occurred until the fourth week after the onset of KD, which followed the rise and fall of VEGF levels. Apart from the effect on coronary arteries, elevated VEGFA levels could also be associated with worsening myocardial function. In an adult cohort with ST-segment myocardial infarction, Rios-Navarro et al. (2021) showed that high VEGFA-$_{165}$b levels were associated with worse

ejection fraction and poor outcomes in conditions of reperfusion after myocardial infarction[4]. Further the interrelatioship between VEGFA and VEGFB gene expression in KD [5] from three large cohort studies has been demonstrated [6-8].

Pathognomonic of KD in the acute phase of the illness, is an un-remittent fever in the inflammatory phase of illness. During this acute phase, a prospective study showed a rise in TNF was noted during the first week after KD diagnosis[9]. Further, there was a more significant elevation in children developing coronary aneurysms compared to children with KD without coronary involvement. Thus emphasizes the importance of inflammation associated with the on-going pyrexia characteristic of KD. Yet, little is known about the relationship of fever to immune-pathogenesis in KD. Host mechanisms exist that protect during times of thermal stress. One such mechanism is that associated with heat shock protein's (HSPs). HSPs, are highly conserved proteins from across species and from an evolution perspective. However little is known about the relationship of HSPs to pathogenesis in KD. A HSP mechanism could exists that relates endothelial pathogenesis to HSPs and VEGF. For example, a human microvascular endothelial model developed using tissue engineering, suggested the importance of Heat Shock Protein (HSP) and NF-kappaB pathway activation in VEGF production and capillary blood formation[10]. In a human model elevating a lower body temperature resulted in a transient increase in mRNA gene expression of both VEGF and HSP[11]. Further, experimental studies

confirm the association of HSP to angiogenic processes [12-14]. Thus given the role of Heat Shock Proteins (HSPs) in thermal stress, the importance of HSP-associated angiogenesis in KD was hypothesized. In this study, HSPs are a group of ubiquitous chaperone proteins, providing cellular quality control and aiding the correction of nascent mis-folded proteins. HSPs genes were chosen according to their possible roles in angiogenesis. For example HSP27, also known as HSPB1, is involved with cell growth and differentiation, having both anti-oxidant and protein chaperone properties. HSPB1 is cytoprotective to various stressors, including heat, apoptosis, and schema [15]. Also, HSPA1A, a member of the HSP70 family 1A, has both angiogenic and chaperone properties, with migratory endothelial effects leading to microvessel formation[16]. Other HSPs may have undiscovered angiogenesis effects. For example, HSPBAP1 has been associated with two rare angiogenesis-related rare diseases (Orpha ID 217071 and 151)[17]. Previously we had demonstrated VEGF-A and VEGF-B gene expression patterns associated with KD datasets KD1-KD3. In order to develop VEGF-HSP understanding, a pathway diagram was adopted showing essential pathways related to angiogenesis (Figure 1). This paper aims to understand the relationship of VEGF A and B with HSPs in association with KD. Accordingly, this paper shall undertake a study of HSP and VEGF (VEGF-A and VEGF-B) gene expression in accordance with the pathway representation, focusing on VEGF associated angiogenic mechanisms. The aim is to understand the coronary artery disease development in KD.

Material and Methods

Dataset Inclusion and Search Strategy

Datasets were taken from the National Centre for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov) Gene Expression Omnibus (GEO, http://www.ncbi.nim.nih.gov/geo/). Three KD Datasets (KD1-3) were chosen as a model for VEGFA/VEGFB as shown in previous work [5] (Table 1A). Our previous work also highlighted the value of the novel KD coronary artery dataset which allowed the study of effects at the level of the coronary artery (GSE64486).

Microarray Dataset Pre-processing

All microarray datasets were pre-processed by log2 quantile normalization using R-script. GSE15297 with 41 participants was excluded due to the microarray platform not representing genes under study, namely VEGFA and VEGFB.

RNA-Seq Datasets

Initially, the paired-end RNAseq FASTQ files for each sample within the dataset GSE173317 were obtained using the tool parallel-fastq-dump (version 0.67, fastq-dump version 2.8.0). The reads were then mapped to the human reference genome (hg38 obtained from UCSC, URL: https://hgdownload.soe.ucsc.edu/goldenPath/hg38/bigZips/hg38.fa.gz) using the BWA-MEM algorithm (bwa version 0.7.17-r1188). Finally, the resulting SAM files, containing the mapped reads, were sorted by coordinate using

Picard - SortSam (version 2.25.5). Thus, the coordinate-sorted mapped BAM files were obtained per each sample.

A Gene Transfer Format (GTF) file was generated from the UCSC.edu table browser using the settings clade ‘Mammal,’ genome ‘Human’ assembly Dec.2013 (GRCh38/hg38), group ‘Genes and Gene Predictions,’ track 'Gencode V36', table ‘knownGene”. BAM files this created were analyzed in the TMM setting of QOE.

Study Design

To analyze which HSP-related genes play an essential role in KD, firstly, KD was compared to controls (two data sets). Firstly genes were elicited using bioinformatics analysis. The different subgroups within each KD were then used to understand significant gene sets. The emphasis was to understand the gene-expression patterns associated with VEGF-A, VEFG-B and HSPs. Further in order to gain an impression of inflammation patterns, gene expression for NFKB1 and TNF was also included.

Bioinformatics Software

Statistical and Gene Ontology analyses

Differential expression of the genes (DEGs) was undertaken using Qlucore Omics Explorer (QOE) version 3.1 software (Qlucore AB, Lund, Sweden). Principal Component Analysis (PCA) plots were generated from QOE. This

allowed two groups and multi-group comparison and unsupervised hierarchical clustering depicted by heat plots. mRNA array data was analyzed with Qlucore Omics Explorer version 3.7 for DEGs. To understand the genes of interest, Gene Symbols were generated for the Genes from the data to compensate for the multiple probes using an averaging method and applied according to the analysis. Hierarchical clustering was based on Euclidean distance and average linkage clustering, and all genes were centered to mean equal to zero and scaled to variance similar to one. Value q, also known as the False discovery rate (FDR), was used in the analysis. An FDR value below 0.25 was considered statistically significant. QOE was also used to generate a PCA heat plot of genes of interest.

Gene Set Enrichment Analysis

Gene Set Enrichment Analysis (GSEA) was undertaken using the QOE platform, comparing three extensive KD studies. For KD1 acute versus convalescent for KD2 and KD3 versus controls. To undertake GSEA comparison, gene sets were downloaded from the MSigDB database generating GMT files. GMT files labelled according to the specific search terms in the database, such as 'apoptosis', 'CD40,' 'VEGF' and so on .

Actin Analysis (GSEA)

KD1-KD4 were analysed using actin related gene sets with the aim of gaining insights into the function of Actin in KD pathogenesis.

Heat Shock Proteins

For microarray analysis HSPs were chosen on the basis of a relationship to angiogenesis. This included Heat Shock Protein Beta 1 (HSPB1), HSPA1A, HSP27, and HSPBAP1. HSP Family B (Small) Member 1 (HSPB1), also known as HSP27.

Results

A. PCA Heat Maps Responses

A1. Kawasaki Disease - VEGF gene relationships

A KD dataset showed increased VEGFA and decreased VEGFB in acute versus convalescent samples (KD1, Figure 2 A & B). Then in two KD datasets versus controls (KD2: Figure 2 C & D and KD3: Figure 2 E & F). VEGFB gene expression was down-regulated in KD patients versus controls and up-regulated in KD versus controls for VEGFA gene expression.

B. HSP Response in Acute KD

In all three KD datasets, HSPA1A and HSBAP1 genes were upregulated in acute versus convalescent samples (KD1, Figure 2G i & iii), KD Versus controls (KD2, Figure 2H i & iii), and KD versus controls (KD3, Figure 2, I i & iii). KD3 was HSPB1 differentially upregulated (Figure 2, I ii) in KD versus controls; in KD1 (Figure 2,G ii) was also unregulated for HSPB1 gene expression in acute versus convalescent samples. For KD2 there was no significant change in HSPB1 gene expression.

C. Coronary Arteritis (KD4: GSE64486)

In CAD, cases of KD versus controls, VEGFB was down-regulated (p = 4.932e-02) and HSPBAP1 up-regulated (p = 1.202e-03) (Figure 3). There was no significant difference in HSP90AB1 and HSPB1 gene expression between KD and controls.

D. ACUTE KD1, KD2, and KD3 GSEA

GSEA was undertaken from three large studies (Table 3). For KD1 acute versus convalescent for KD2 and KD3 versus controls. GSEA using Hallmark gene sets suggested an inflammatory response also with TNFA signaling via NFKB, IL6 JAK STAT 3 signaling, apoptosis, angiogenesis, and unfolded protein response. Angiogenesis and Reactome signaling by VEGF. When the TNF reference gene set was used this showed

VEGF production and vascular development as well as effects noted at the endothelial level including, development, migration, and proliferation.

DISCUSSION

This study aimed to understand KD-associated inflammation through changes in gene-expression. In particular focusing on VEGF-A and VEGF-B gene expression. As discussed, an unremitting fever is pathognomonic of KD. Therefore it was hypothesized that the heat shock response could be important from a thermal stress perceptive, especially to the coronary endothelium. Thereby the aim is rot describe VEGF and HSP associated processes based upon microarray studies. We don't known the effect of HSPs in KD, thus we developed the dual fever-angiogenesis model in KD. This model seeks to explain both angiogenesis (by VEGF-based mechanisms) and the host response to fever (through HSP-based processes). To undertake the analysis, a KEGG pathway outlining a relationship between VEGF and endothelial migration and proliferation was assumed (Figure 1). In previous work, KD datasets (KD1, KD2 and KD3) representing large multi-centre studies has suggested a temporal relationship of VEGF-A and VEGF-B in KD. Thereby GSEA was applied to these datasets. In all three clinical studies, Hallmark GO pathways for VEGF production was noted. Also, the unfolded protein response pathway was significant which is a cellular stress response associated with the endoplasmic reticulum. The correction of protien folding is an important function of Heat shock Proteins in their roles as molecular chaperones, consistent with our hypothesis. Also on GSEA, significant gene sets were noted for the regulation of endothelial cell apoptosis and migration, angiogenesis and inflammation. Moreover, gene sets

associated with endothelial and muscle cell proliferation were significant. An inflammatory milieu was also suggested by the significant increase in NFKB gene expression in acute KD. Findings appropriate to the pathogenesis of KD.

Given the role of HSP in thermal injury and the function of VEGF in angiogenesis, further evidence for this dual relationship in KD pathogenesis was sort. According to KEGG annotation pathway (Figure 1), HSPB1 is a HSP related to VEGF-A present in pathways related to inflammation and endothelial migration and proliferation. Accordingly, KD study data showed that HSPB1 differentiated between acute and convalescent KD (KD1) as with KD acute versus convalescence (KD3). Soluble HSPB1 is known to be generated primarily from endothelial cells playing a vital role in regulating angiogenic balance[18]. HSBP1 achieves this through interaction with VEGF-A. HSPB1 phosphorylation by VEGF-A mediation leads to HSPB1 inhibition, thus promoting angiogenesis. Therefore suggesting HSPB1 has anti-angiogenic properties countered by VEGFA. Indeed, tumour research has shown that HSPB1 secretion is a compensatory response to excessive VEGFA production. Further insights from cancer studies suggest that HSPB1 molecule inhibits apoptosis as part of the heat shock response[19]. Therefore the value of HSPB1 is proposed as potential point of connection between VEGF and HSP gene responses. VEGFB protein is also anti-apoptotic [20], rising in the convalescent phase in KD in our analysis. Further, in our analysis, HSPB1 showed increased expression in KD compared to controls in 2 studies and VEGFB showed a significant fall in the acute phase of KD across all 3

studies. Therefore, if the genes HSPB1 and VEGFB are indicative of anti-apoptosis, then accordingly anti-apoptotic effects could be occuring at different KD time points.

In the coronary artery dataset (CAD), endothelium leucocyte interaction suggested p38 gene expression upregulation in the leucocyte endothelial migration pathway. Rajashekar et al. looked into the issue of chronic inflammation and pro-inflammatory angiogenesis [21]. They showed the switching from anti-angiogenic to pro-angiogenesis of the p38 MAP kinase was associated with both TNF and oxidative stress. Thereby suggesting a point of interaction with myosin and actin, which could then be associated with coronary pathogenesis. GSEA of the KD datasets (KD1,KD2, and KD3) suggested the importance of actin-based pathways and also generated leucocyte transendothelial migration gene-set to be significant. Thus Inflammation suggested by NFKB1 gene expression, may be associated with p38 activation as a move towards structural changes which effecting the coronary endothelium. Such changes may be important in the pathogenesis leading to aneurysmal changes and associated complications, especially for children.

HSP-genes were up-regulated with unfolded protein responses noted by GSEA. Hallmark IL6 JAK STAT3 Signaling pathway and Hallmark tumour p53 (TP53, p53) Pathway were found to be significant in the three-study KD GSEA , were also genes noted to act as hubs in a cohort of critically ill Covid-19 adults [22]. Again concerning the same GSEA, p53 was noted to be

significant. Wong et al (2007)., have shown that HSP27 regulates the transcriptional activity of p53[23]. Wong et al (2017)., developed a biomarker protein panel for sepsis endotyping where a network was constructed which consisted of 18 mortality risk assessment genes related to p53[24]. Suggesting a role for p53 related cell division, metabolism and repair in septic shock.

Detailed downstream analysis for the validation of physical mechanisms is required to confirm biological processes. However, one advantage of the transcriptomic approach adopted is that it provides a systems-level view of multiple processes aiding an integrated understanding of complex biological processes [25]. Another issue in KD studies is the lack of temporal gene-sampling. Though we used extensive KD studies from whole blood data to offer insights into angiogenesis, inflammation, and VEGF effects, one is still unable to extrapolate the degree of angiogenesis at the coronary artery level, according to complete blood data. Moreover, we illustrated that gene expression of HSPBAP1 significantly differentiated KD from controls in three studies (KD1,KD2,KD3) and in acute versus convalescent KD (KD4). However, the role of the HSPBAP1 gene in angiogenesis requires further research. The difficulty of a systems based approach as offered by transcriptome wide gene expression analysis is that we are reliant on the interpretation based on current frameworks. However the KEGG VEGF-HSP pathway possibly explains the inefficacy of standard antipyretics in preventing coronary aneurysm formation. The diagram suggests that VEGF-A stimulation results in calcium and MAPK signaling to

Arachidonic Acid metabolism and PGI2 production. Given the branching nature of the pathway, infectiveness of traditional anti-inflammatory measures may be explained by not effecting the branch of the pathway which attenuates the effects of VEGFA on the endothelium (Figure 1).

Conclusion

A VEGF-HSP associated gene expression relationship was explored in this paper in relation to endothelial changes as a model for aneurysm developed in KD. HSP were chosen as their potential role in thermal injury, with an unremitting fever being pathognomonic of KD and associated with inflammation leading to coronary aneurysm development. The role of VEGFA and VEGFB subtype gene expression was described due to the potential relationship to angiogenesis. Further the importance of thermal stress pathways given the risk of anuerysm development in association with fever and inflammation was also investigated. The KEGG pathway was adopted encompassing VEGF and certain HSP proteins, namely HSPB1. This offered a relationship between the two family of proteins. This work provides a mechanism to explain the inefficacy of conventional measures for preventing angiogenesis, suggesting a novel VEGF mediated approach. Furthermore the idea of HSP being important molecules in KD because of the hyper-inflammatory state resulting in coronary artery changes is novel and requires further study. A therapuetic strategy protecting and preventing coronary artery pathogenesis in children is a potentially important way forward requiring research.

Figure 1. Corona Virus-induced VEGF mechanisms, lead to endotheliitis and coronary aneurysm formation. Host-derived HSPs and pathogenesis of CAD.

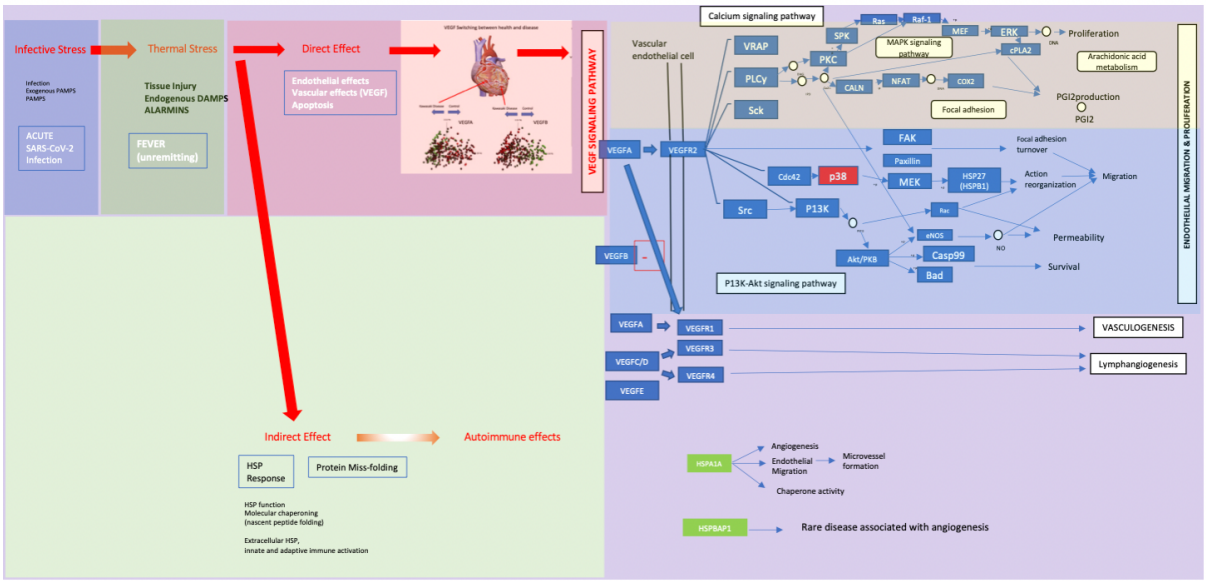


Figure 1. Gene expression changes in VEGFA and VEGFB are inversely related, suggesting a counter-regulatory fashion. The inflammatory cascade results in endothelial migration and dysangiogensis causing coronary arteritis and aneurysm development. (Fever and biomarker curves to be added to the diagram). Also, include extracellular leakage due to cell injury and necrosis. Inflammation -> autoimmunity. 1. Protein malformation 2. Antigen mimicry (BCG) 3. The HSPBAP1 gene is related to angiogenesis. HSPA1A - angiogenesis regulatory properties.

VEGF effect via the PI13k-Akt signaling pathway causes multiple effects including endothelial migration and angiogenesis [26]. This pathway potentially explains the reason why anti-inflammatory don't influence endothelial effects and associated inflammation leading to effects such as coronary artery development. Anti-inflammatory drugs such as acetaminophen and non-steroidal medication are acting on the separate PGI2 pathway affecting COX-2-based mechanisms. The VEGFA-VEGFR2 mechanism is a separate entity.

Figure 2. VEGFA, VEGFB, HSP's, TNF, NFKB1 Gene-Expression in KD datasets

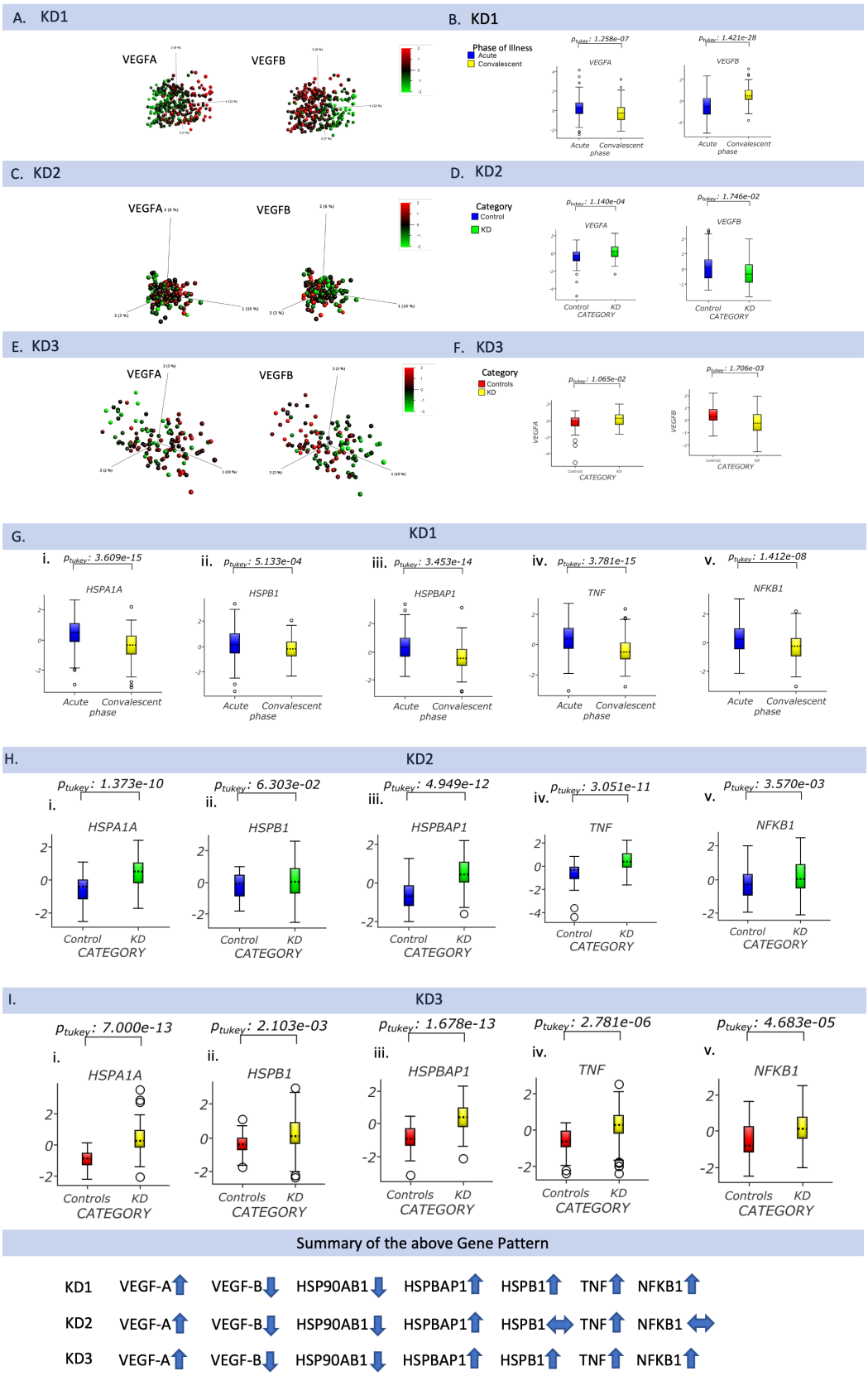
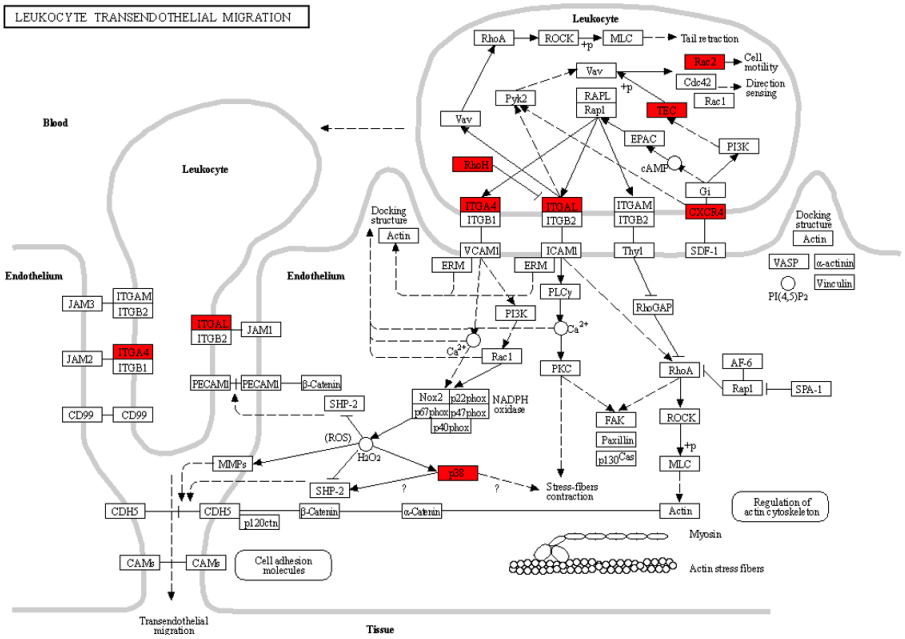


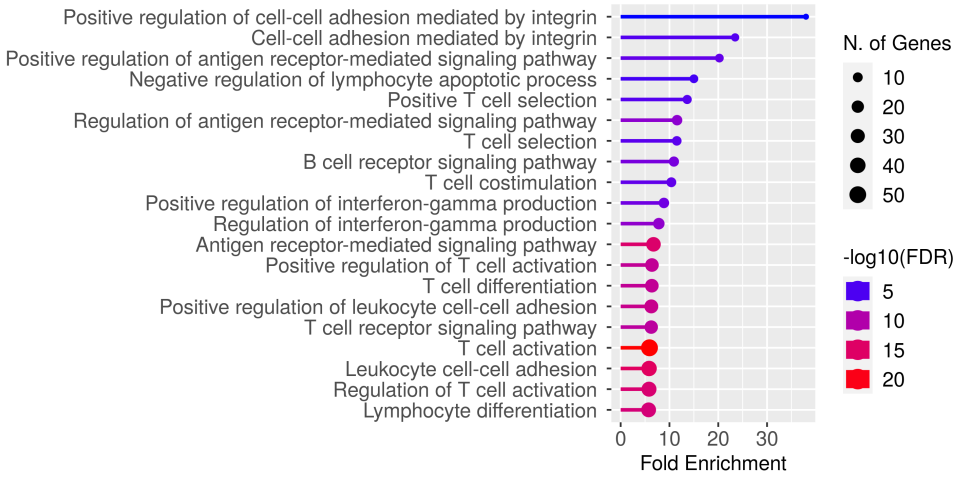
Figure 2. Kawasaki Dataset PCA heat map gene expression and box plots shown, KD1 (Figure 2A &B), KD2 (Figure 2C & D) and KD3 (Figure 2E & F). For KD1 acute versus convalescent samples, KD2 and KD3 (KD versus control samples), there is a significant difference with downregulation of VEGFB, HSPA1A, HSPBAP1, TNF and NFKB1 gene expression. HSPB1 has no significant difference in gene expression for KD2, but for KD1 HSPB1 is increased in acute versus convalescent samples and for KD3 HSPB1 is increased in KD versus control samples. For KD1 acute versus convalescent samples, KD2 and KD3 (KD versus control samples), there is significant upregulation in VEGFA gene expression. KD1, KD Acute versus convalescent samples, VEGF A is up-regulated (p=1.258e-07). VEGF B is down-regulated (p=1.42e-28), HSP90AB1 down-regulated (p=1.054e-06), HSPBAP1 up-regulated (p=3.453e-14). KD2, KD Versus Control, VEGFA up-regulated (p=1.140e-04), VEGFB down-regulated (p=1.746e-02), HSP90AB1 down-regulated (p=1.647e-07), HSPBAP1 up-regulated (p=4.949e12). KD3, KD versus Control; VEGFA up-regulated (p=1.140e-04), VEGFB down regulated (p=1.746e-02), HSP90AB1 down-regulated (p=1.647e-07), HSPBAP1 up-regulated (p=4.949e-12). [A-F Adapted from Rashid et al., (2022) [5]].

Figure 3. Gene expression in Coronary artery KD versus controls

A.



B.



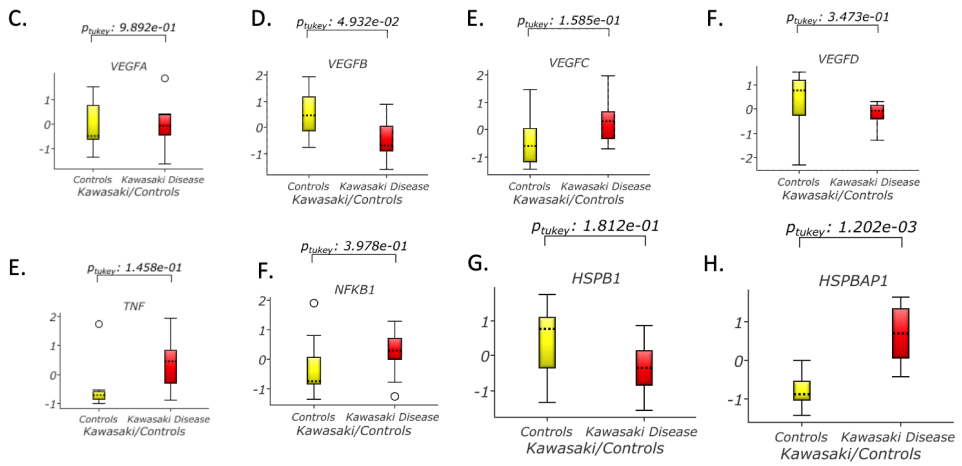


Figure 3. Coronary artery gene expression from the KD4 dataset patients with KD versus controls. t-Test Kawasaki Disease versus control (p=0.001, q=0.12) elicited 527 genes which then underwent gene enrichment. This led to the KEGG diagram using Pathfind (**A**) being generated which provides network visualisation of the enriched GO molecular component terms in the form of a network. This shows the leucocyte endothelial interaction with the enriched genes from our analysis shown in red. Gene enrichment from the gene list also allowed mapped to the GO bar-chart (**B**).Which also exemplifies leucocyte adhesion as well as other pathways related to innate and adaptive immunity. Negative regulation of lymphocyte apoptotic process is noted.

TABLE 1A: SUMMARY OF KD Clinical DATASETS

Accession number*	Platform	Named		Disease	STUDY DESIGN	Subject Number	Paper Reference
GSE63881	Illumina HumanHT-12 V4.0 beadchip	KD Levine Microarray		KD1	KD Cases prior and after treatment	146	{Hoang, 2014 2320}
GSE73461	Illumina HumanHT-12 V3.0 beadchip	KD Imperial Microarray		KD2	Disease versus Control	606	{Wright, 2018 #2323}
GSE68004	Illumina HumanHT-12 V4.0 beadchip	KD Biomarker Microarray		KD3	Disease versus Control	162	{Jaggi, 2018 #2321}
GSE64486 PRJNA 271099	Illumina HiSeq 2000	RNA-Seq		KD4 Barts	coronary artery tissue from KD (n=8) and childhood controls (n=7)	15	{Rowley, 2015 #2394}

* NCBI GEO Accession Numer or NCBI Bioproject ID

Supplement TABLE 2: GSEA summary of three datasets

REFERENCE GENE SETS	GENE SETS	KD3			KD2			KD1		
		NES	p-Value	FDR	NES	p-Value	FDR	NES	p-Value	FDR
APOPTOSIS	BIOCARTA_FAS_PATHWAY	1.62	0.01	0.03	NS	NS	NS	NS	NS	NS
APOPTOSIS	BIOCARTA_AKT_PATHWAY	1.68	0.02	0.02	1.8	0.01	0.02	NS	NS	NS
APOPTOSIS	GOBP_INTRINSIC_APOPTOTIC_SIGNALING_PATHWAY	1.65	0	0.02	1.72	0	0.02	1.66	0	0.049
APOPTOSIS	GOBP_EXTRINSIC_APOPTOPIC_SIGNALING_PATHWAY	2.12	0	0.00	1.86	0	0.01	1.78	0.004	0.03
HALLMARK	HALLMARK_TNFA_SIGNALING_VIA_NFKB	2.42	0	0.00	2.24	0	0.00	2.13	0	0.004
HALLMARK	HALLMARK_IL6_JAK_STAT3_SIGNALING	2.39	0	0.00	2.32	0	0.00	2.18	0	0.006
HALLMARK	HALLMARK_APOPTOSIS	1.98	0	0.00	1.93	0	0.00	1.83	0.002	0.001
HALLMARK	HALLMARK_ANGIOGENESIS	2.01	0	0.00	1.91	0	0.00	1.91	0.004	0.007
HALLMARK	HALLMARK_P53_PATHWAY	1.89	0	0.01	1.77	0.004	0.01	1.77	0.008	0.02
HALLMARK	HALLMARK_UNFOLDED_PROTEIN_RESPONSE	-2.01	0.002	0.00	-1.72	0.02	0.03	NS	NS	NS
HALLMARK	HALLMARK_INFLAMATTORY_RESPONSE	2.45	0	0.00	2.26	0	0.00	2.06	0	0.003
VEGF	KEGG_VEGF_SIGNALING_PATHWAY	2.02	0	0.00	1.62	0.01	0.03	1.85	0	0.04
VEGF	REACTOME_SIGNALING_BY_VEGF	1.97	0	0.00	1.65	0.1	0.03	1.6	0.03	0.12
VEGF	WESTON_VEGFA_TARGETS	NS	NS	NS	1.41	0.03	0.10	1.35	0.07	0.21
TNF	GO_ENDOTHELIAL_CELL_DEVELOPMENT	2.01	0	0.00	1.63	0.008	0.02	1.6	0.03	0.04
TNF	GO_ENDOTHELIAL_CELL_MIGRATION	2.17	0	0.00	1.66	0.002	0.02	1.6	0	0.04
TNF	GO_ENDOTHELIAL_CELL_PROLIFERATION	2.06	0	0.00	1.8	0	0.01	1.69	0	0.02
TNF	GO_ANGIOGENESIS_INVOLVED_IN_WOUND_HEALING	1.85	0.004	0.00	1.75	0.002	0.01	1.59	0.03	0.04
TNF	GO_BLOOD_VESSEL_ENDOTHELIAL_CELL_MIGRATION	1.77	0	0.01	1.27	**0.05**	0.15	1.38	0.03	0.11
TNF	GO_MUSCLE_CELL_PROLIFERATION	1.99	0	0.00	1.78	0	0.01	1.79	0	0.001
TNF	GO_VASCULAR_ENDOTHELIAL_GROWTH_FACTOR_PRODUCTION	1.63	0.02	0.12	2.01	**0**	0.00	2.07	0	0.001
TNF	GO_POSITIVE_REGULATION_OF_DNA_BINDING_TRANSCRIPTION_FACTOR_ACTVITY	1.63	0.004	0.12	2.04	**0**	0.00	1.79	0.002	0.01
TNF	GO_REGULATION_OF_VASCULAR_ASSOCIATED_SMOOTH_MUSCLE_CELL_PROLIFERATION	NS	NS	NS	1.71	0.006	0.01	1.75	0	0.01
TNF	GO_REGULATION_OF_VASCULAR_DEVELOPMENT	NP	NP	NP	NP	NP	NP	NP	NP	NP
TNF	GO_VASCULATURE_DEVELOPMENT	NP	NP	NP	1.71	0.006	0.01	1.8	0	0.01
TNF	GO_POSITIVE_REGULATION_OF_CELL_DIFFERENTIATION	NP	NP	NP	1.75	0	0.01	1.61	0.01	0.04

Table 2. NES= Net Enrichment score, p-Value and FDR = false discovery rate, NS = Not significant. NP = Not Present (after doing the GSEA calculation this did not appear on the list of genes tested by GSEA). KD1 GSE 63881 (LEVINE) GSEA according to the 'Phase' of illness, t-test, 171 acute versus 170 convalescent samples. KD 2 GSE 73461 (Imperial) t-test, according to 'Category' (78 KD cases were compared against 55 (afebrile) controls). KD 3 GSE 68004 Biomarker contains cKD 76, inKD 13, and Controls 37. cKD and inKD combined to KD category. Then KD (89) was compared against controls (37) according to 'Category' in the dataset.

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