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Asrar Rashid
Edinburgh Napier University; NMC Royal Khalifa Hospital, Abu Dhabi, United Arab Emirates

Govind Benakatti
Yas Clinic, Abu Dhabi, United Arab Emirates

Feras Al-Obeida
Zayed University

Rajesh Phatak
Pediatric Intensive Care, Burjeel Hospital, Najda, Abu Dhabi, United Arab Emirates

Zainab A. Malik
Mohammed Bin Rashid University of Medicine and Health Sciences

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Asrar Rashid, Govind Benakatti, Feras Al-Obeida, Rajesh Phatak, Zainab A. Malik, Javed Sharief, Raziya Kadwa, Wael Hafez, Mohammed Toufiq, Damien Chaussabel, Rayaz Malik, Nasir Quraishi, Syed Ahmed Zaki, Guftar Shaikh, and Amir Hussain

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A dual covariant biomarker approach to Kawasaki disease, using vascular endothelial growth factor A and B gene expression; implications for coronary pathogenesis

Asrar Rashid [a,b,*], Govind Benakatti [c], Feras Al-Obeidat [d], Rajesh Phatak [e], Zainab A. Malik [f], Javed Sharief [b], Raziya Kadwa [b], Wael Hafez [b,g], Mohammed Toufiq [h], Damien Chaussabel [h], Rayaz Malik [i,j], Nasir Quraishi [k], Syed Ahmed Zaki [l], Guftar Shaikh [m], Amir Hussain [a]

[a] School of Computing, Edinburgh Napier University, Edinburgh, UK
[b] NMC Royal Hospital, Khalifa City, Abu Dhabi, United Arab Emirates
[c] Yas Clinic, Abu Dhabi, United Arab Emirates
[d] College of Technological Innovation, Zayed University, Abu Dhabi, United Arab Emirates
[e] Pediatric Intensive Care, Burjeel Hospital, Najda, Abu Dhabi, United Arab Emirates
[f] College of Medicine, Mohammed Bin Rashid University of Medicine and Health Sciences. Dubai, United Arab Emirates
[g] Medical Research Division, Department of Internal Medicine, The National Research Centre, Cairo, Egypt
[h] The Jackson Laboratory for Genomic Medicine, Farmington, Connecticut, USA
[i] Weill Cornell Medicine-Qatar, Doha, Qatar
[j] Institute of Cardiovascular Science, University of Manchester, Manchester, UK
[k] Centre for Spinal Studies & Surgery, Queen's Medical Centre, The University of Nottingham, Nottingham, UK
[l] All India Institute of Medical Sciences, Bibinagar, Hyderabad, India
[m] Endocrinology, Royal Hospital for Children, Glasgow, UK

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ABSTRACT

Introduction: Kawasaki disease (KD) is the most common vasculitis in young children, with coronary artery lesions (CALs) and coronary aneurysms (CAAs) being responsible for most KD-related deaths.
Objective: We hypothesized that Vascular Endothelial Growth Factors (VEGFs) are pivotal in KD inflammation and coronary artery lesions. This study assessed VEGF-A and VEGF-B gene expression (GE) as potential biomarkers in KD inflammation.
Study design: We analyzed NCBI-GEO datasets, categorizing gene expression patterns as "inflammatory" or "non-inflammatory". We focused on TNF-, NFKB1, VEGF-A, and VEGF-B GEs. Datasets were filtered based on differential changes in TNF and NFKB1 levels to isolate those with inflammatory shifts.
Results: Inflammatory datasets (GSE63881, GSE73464, and GSE68004) displayed elevated TNF, NFKB1, and VEGF-A GE levels during acute KD. VEGF-B GE exhibited a distinctive trend: an initial drop and subsequent rise during recovery, a pattern that was missing in the non-inflammatory group. The treatment response was also studied, with intravenous immunoglobulin (IVIG) responders showing significant downregulation of NFKB1 GE after treatment: GSE16797 [IVIG ± methylprednisolone; $p = 8.6443\text{-}03$], GSE48498 [IVIG; $p = 6.618e\text{-}02$, infliximab; $p = 3.240e\text{-}03$], and GSE18606 [IVIG; $p = 3.518e\text{-}02$]. Considering the similar binding of VEGF-A and VEGF-B to the VEGFR1 receptor, a co-variate and inverse relationship is suggested.
Conclusion: Temporal VEGF-A, VEGF-B, and GE changes show promise as new post-inflammatory biomarkers for KD. Novelty results with the biomarker approach, with the potential for a dual temporal relationship between VEGF-A and VEGF-A. A comprehensive exploration of VEGF-A and VEGF-B genes and protein analysis in KD is warranted to understand the functional aspects of these changes and how best to utilize the pattern of changes for therapeutic benefit.

* Corresponding author. School of Computing, Edinburgh Napier University, Edinburgh, UK.
E-mail address: asrar.rashid@napier.ac.uk (A. Rashid).

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Author contributions

All authors have read and approved the manuscriptConceived and designed the experiments: AR Performed the experiments: AR Analyzed the data: AR Contributed analysis, methods, and tools: AR Wrote the first draft of the paper: AR Revised critically for importance and intellectual content: GB, FA, RP, ZAM, JS, RK, WH, MT, DC, RM, NQ, SAZ, GS, AH.

1. Introduction

Kawasaki disease (KD) is the most common vasculitis that affects young children. Coronary artery lesions (CALs) and coronary aneurysms (CAAs) are the most serious complications that cause KD-related deaths. Vascular leakage has been shown to be the key feature of early KD [1, 2] and the degree of edema as an important predictor of CALs [3]. Vascular Endothelial Growth Factor A (VEGF-A), an angioproliferative and pro-inflammatory agent known to induce hyperpermeability [4] and vascular leakage, has been reported in the pathogenesis of CAL [5–8]. VEGF levels were significantly higher in KD patients with CALs than in those without CALs [9] and were elevated in both acute and sub-acute phases [5]. In a murine model study, Lin et al. reported an association between local VEGF-A and its signaling pathways with the occurrence of CALs [10]. Interestingly, differential expression of VEGF was observed in KD-neutrophil-derived VEGF in the acute phase and in peripheral blood mononuclear cells (PBMCs) in the subacute phase, suggesting its dynamic release and correlation with sequential inflammation in KD and pathogenesis of CALs [5,11]. VEGF-A has been reported to recruit neutrophils and other cells [12,13], facilitate the influx of neutrophils and inflammatory mediators into the coronary walls, and induce vascular remodeling [5,8,14,15]. Extensive expression of VEGF is observed in smooth muscle cells in stenotic and recanalized vessels in patients with KD [16,17]. The association between VEGF-A levels and inflammation in KD is complex. Various cytokines, such as TNF, NF-kB, IL-4, IL-6, and MCP-1, have been shown to stimulate VEFG-A and vice versa [18–23]. IL-4 treatment induces VEGF release in human coronary artery endothelial and smooth muscle cells [24,25]. TNF and NFKB1, known to play a central role in KD inflammation, vasculitis, and aneurysm formation, also regulate VEGF-A release and function [26,27]. Unlike VEGF-A, a vascular survival factor VEGF-B is highly expressed in the heart [28,29], which VEGF-A is critical for vascular repair and recanalization [28–30]. VEGF-B gene therapy prevents doxorubicin cardiotoxicity [31]. Hence, VEGF-B may be a safe molecule for endothelial cells, smooth muscle cells, and pericytes [28,32]. It is most abundant in the heart and is essential for cardiac blood vessel survival. Therefore, VEGF interactions could indeed be a potential marker(s) through which to understand KD inflammation evolution and predict CALs [9,33], and also an important target for suppressing inflammation [13] to improve KD outcome.

A clinical challenge is using conventional biomarkers, such as CRP and Pro-calcitonin, to follow the disease process and to pre-empt changes at the coronary level. Thereby allowing the potential for early appropriate anti-inflammatory measures. In recent work in sepsis, we have shown temporal changes in VEGF-A and VEGF-B gene expression (GE) against the backdrop of changes in TNF and NFKB1 GE [34, 35]. Despite reports supporting the association of VEGF-A with KD inflammation and CALs pathogenesis, dynamics of VEGF-A and VEGF-B gene expressions, that too in relation to TNF and NFKB1, have not been studied. This study aims to further important insights with respect to KD inflammation and CAL pathogenesis and present the opportunity to consider novel therapeutic targets in KD CALS prevention.

2. Material and methods

2.1. Systematic data search and pre-processing

RNA sequences and microarray genomic datasets were obtained from the NCBI Gene Expression Omnibus (GEO) dataset (http://www.ncbi.nlm.nih.gov/geo/) [36] through a systematic search strategy using the keywords 'Kawasaki Disease' [MeSH Terms] [All Fields] AND '*Homo sapiens*' [porgn] AND 'gse' [Filter]. In total, 491 datasets were obtained. Further filtering of datasets based on expressions profiling by 'array' or 'high throughput sequencing' generated 26 datasets. The array arm generated 20 datasets and the high-throughput sequencing arm generated six datasets. Of 20 datasets, after exclusions as outlined in Fig. 1, eight were eligible for final analysis (GSE68004, GSE63881, GSE16797, GSE15297, GSE48498, GSE18606, GSE109351, and GSE73464) and on the other arm, one was eligible for final analysis (GSE64486). GSE73464 consists of sub-series GSE73461, GSE73462, and GSE734639 (See Table 1). The GSE64486 dataset was considered unique as it only included samples from coronary artery tissues, whereas all others were sampled from peripheral blood monocytes (PBMC). The exclusion criteria are shown in Fig. 1. The data were normalized by logarithmic scale (log2) transformation using R-script. Further non-random selection was carried out based on 1) comparing transcriptome profiles between KD patients and controls, that is, non-KD patients with a bacterial or viral infection and healthy subjects, 2) RNA sequencing comparison of coronary artery tissue samples of KD patients and controls, and 3) comparison of KD patients between responders and non-responders before and after treatment with key drugs, such as IVIG, Methylprednisolone, and infliximab.

2.2. Gene Ontology (GO) analyses

The datasets were analyzed for differential gene expression (GE) using Qlucore Omics Explorer (QOE) version 3.7 (Qlucore AB, Lund, Sweden). Principal Component Analysis (PCA) plots allowed for two-group and multi-group comparisons and unsupervised hierarchical clustering. Every probe in the expression file was allocated to its respective HGNC (HUGO Gene Nomenclature Committee) gene symbol (s) [36]. Duplicate gene symbols (s) mapped to multiple probe IDs were collapsed via averaging. Hierarchical clustering was based on Euclidean distance and average linkage clustering. Furthermore, the data were centered to zero (mean) and scaled to a variance of 1 (one). The Student's t-test was used to compare gene expression between groups of samples. A cutoff of less than 0.05 (<0.05) was considered statistically significant for both the P-value and False discovery rate (FDR, q value). Various gene enrichment techniques were used through software solutions, including ShinyGO version 0.76[37], g-profiler version 2020[38], and GSEA provided through QOE 3.7[39, 40].

2.3. RNA-seq analysis

The GSE64486 dataset contains RNA-seq data generated from the coronary artery tissue of patients with Kawasaki Disease and controls. The paired-end RNA-seq FASTQ files for each sample within dataset GSE64486 were obtained using parallel-fastq-dump (version 0.67, fastq-dump version 2.8.0). The reads were 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 (version 0.7.17-r1188). Finally, the resulting SAM (Sequence Alignment Map) files containing the mapped reads were sorted by coordinate using Picard - SortSam (version 2.25.5). Thus, the coordinate-sorted mapped BAM (Binary Alignment Map) files were obtained per sample. These BAM files were then imported into QOE. A Gene Transfer Format (GTF) file was generated from the UCSC.edu table browser using the settings — class 'Mammal,' genome 'Human' assembly Dec.2013 (GRCh38/hg38), group 'Genes and Gene Predictions,' track 'Gencode V36,' table 'knownGene". The BAM files imported into QOE were normalized to the Trimmed Mean of the M-Values (TMM) before further analysis. The study included 11 controls; however, the gene expression data only listed 7 controls C1–C7 (C1: Enterobacter sepsis and Herpes Simplex encephalitis, C2: Pneumococcal meningitis and disseminated

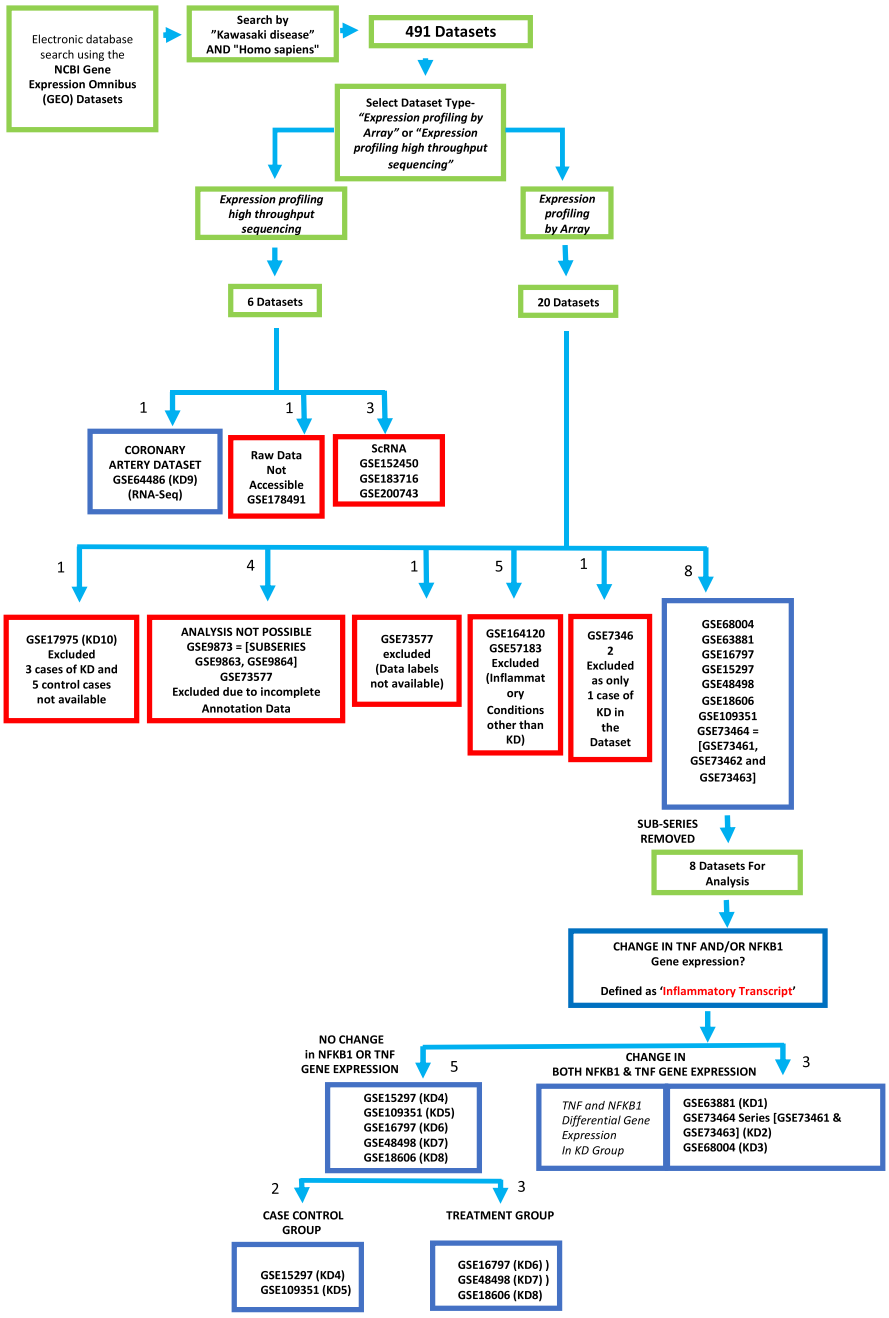

Fig. 1. Systematic Search for KD datasets from DNA Microarray and RNASeq datasets. Discriminate Molecular Mechanisms between KD positive and the Controls. Fig. 1. A systematic search for KD datasets was undertaken from the NCBI GEO database. The different stages in the filtering and selection process are shown, with the number of datasets at each filtering stage indicated next to the direction arrows. The search term "Kawasaki Disease" and "Homo Sapiens" generated 491 datasets. This was then divided into "Expression Profiling by Array (20 datasets) and Expression profiling by high throughput sequencing (6 Datasets). Of the six datasets, the datasets shown in red were excluded due to not being accessible, inadequate labeling, or entailing sc-RNA studies, or were non-KD studies, which left one dataset (GSE64486) for analysis. Of the other 20 datasets, 12 (denoted in red) were excluded due to the reasons shown in the boxes. GSE73462 was excluded because this contained only one patient with KD. Additionally, for dataset GSE73577, it was not possible to differentiate samples before and after IVIG due to data labeling issues; thus, this was also excluded from further analysis. Hence the 20 datasets were filtered down to 8 (GSE73464 is the super series containing sub-series GSE73461, GSE73462, and GSE73463). Finally, the 8 datasets were divided according to whether differential GE TNF and NFKB1 were elicited, giving three datasets (GSE63881, GSE73464, and GSE68004). For the 'No change in NFKB1' or 'TNF GE group' this was further subdivided into the 'case-control' or 'treatment' groups. In the case-control group, cases of KD were compared against controls. For the Treatment group, Responders (R) were compared against Non-Responders (NR). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

intravascular coagulation, C3: Prematurity and Serratia meningitis, C4: Meconium aspiration and pulmonary hemorrhage, C5: Developmental delay, seizures, and fever; C6: Cerebral hemorrhage and pneumonia; C7: Cholestasis, renal tubular acidosis, and agenesis of the corpus callosum). Thus, patients C1, C2, C3, and C5 were categorized as 'infected controls.'

2.4. Pathway enrichment analysis

Pathway enrichment analysis was performed using Gene Set Enrichment Analysis (GSEA) through QOE, enabling biological interpretation. Query gene sets are used from the collection of gene sets from

Table 1
Summary of KD clinical studies.

Accession number[a]	Dataset Name	Platform & Datatype	Institution	Research Facility	Study Design	n	Paper Ref
GSE63881	KD1	Illumina Human HT-12 V4.0 beadchip, Microarray	Rady Childrens Hospital San Diego	Genome Institute of Singapore	KD patients were studied in the acute versus convalescent phase of their illness.	146	[49]
GSE73464[c]	KD2	Illumina Human HT-12 V3.0 beadchip Microarray	Pediatric centers in the UK, Spain, Netherlands and the USA	Imperial College UK	GSE73461 Case-controlled study. 78 patients with KD were compared against 55 healthy controls. GSE73463 Acute patients (146) were compared against convalescent-phase samples (87)	133 233	[50]
GSE68004	KD3	Illumina Human HT-12 V4.0 beadchip Microarray	Nationwide Children's Hospital in Columbus, Children's Medical Center Dallas, Rady Children's Hospital in San Diego	BIIR Lab Dallas USA	In the case-controlled study, acute KD (n = 89) versus healthy controls (n = 37).	126	[51]
GSE15297	KD4	Stanford methodology using Ambion amplification kit followed by fluorescent labelling Microarray	Rady Children's Hospital San Diego and Children's Hospital Boston	University of California, San Diego Children's Hospital Boston and Stanford University	Case-controlled study. 23 Patients with KD, 18 febrile controls, and 10 non-febrile controls	41	[52]
GSE109351	KD5	Affymetrix Human Transcriptome Array 2.0 Microarray	Kaohsiung Chang Gung Memorial Hospital Children's Hospital Kaohsiung	Department of Medical Research Genomics and Proteomics Core Lab Kaohsiung	Case-controlled study. Comparison of healthy controls versus acute and convalescent phase KD. These groups of RNA samples were analyzed. 3 pooled acute-phase KD RNA samples and 3 convalescent-phase KD RNA samples, and 3 pooled healthy control RNA samples.	9	[53]
GSE16797	KD6	Affymetrix Human Genome U133 Plus 2.0 Array, Microarray	Kitasato University HospitalEbina General Hospital	Kitasato University Hospital or Ebina General Hospital	Compared Pre and Post Treatment in KD. The Egami scoring system was used to predict the IVIG-responsive (Group A; n = 6) and IVIG-resistant groups (Group B) before starting the commencement of treatment using. Also random allocation of the following groups was undertaken, single-IVIG treatment (Group B1; n = 6), combined therapy group (Group B2; n = 5) and IVIG-plus-methylprednisolone (IVMP).	17	[54]
GSE48498	KD7	Affymetrix Human Genome U133 Plus 2.0 Array, Microarray	Kitasato University Hospital	Agilent Technologies, Santa Clara, California, USA Compared Pre and Post Treatment in KD. Pre- and post-treatment effects	were analyzed in IFX-responsive patients (n = 8) and IVIG-responsive patients (n = 6).	14	[55]
GSE18606	KD8	Agilent G4112F Human Whole Oligo Array Microarray	Rady Children's Hospital-San Diego	Baylor Institute for Immunology Research NIAID Cooperative Centers Luminex Core Facility	Comparing responders to IVIG versus non-responders. The following groups were analyzed. 9 healthy age-appropriate controls, 12 IVIG responders, and 8 IVIG non-responders at acute and convalescent stages resulted in 48 whole blood samples.	29	[56]
GSE64486[b]	KD9	Illumina HiSeq 2000 RNA-Seq	Children's Hospital Chicago USA-wide cases	Barts & The London School of Medicine UK	Case-controlled study. Coronary artery tissue from KD (n = 8) and childhood controls (n = 11)	15	[47]

Ref – references pertaining to studies using the assigned datasets.
IVIG = Intravenous Immunoglobulin.
IFIX = Infliximab.
n = number of subjects.
Paper Ref, related to the literature referencing the associated datasets.
[a] NCBI GEO Accession number or NCBI Bio project ID.
[b] This study is from Coronary Artery Tissue; whereas all the other datasets are from whole blood.
[c] GSE73464 is the super-series for GSE73461, GSE73462 and GSE73463. GSE73462 was excluded from the analysis given that there was only 1KD patient in the dataset.

the Molecular Signature Database (MSigDB, https://gsea-msigdb.org/gsea/msigdb/collections.jsp) [39]. Gene sets were downloaded from the MSigDB database using terms representing biological molecules. Net Enrichment Score (NES) was calculated for each gene set. A highly positive NES implies an upregulated gene set, whereas a highly negative NES suggests a downregulated gene set. Permutations were calculated to estimate the p-values. The threshold for significance with respect to t-tests used to compare pathways was $p < 0.05$ and q (FDR) < 0.05.

2.5. Enrichment analysis using the ShinyGO platform

ShinyGo (version 0.77) was used for gene enrichment analysis (htt

p://bioinformatics.sdstate.edu/go/) [37]. Default settings were used with a background for all protein-folding genes in the genome with an FDR cut-off of <0.05, pathway size min 2, and max 2000 to show a maximum of 20 pathways, with 'species' set to humans. Mapping onto the Gene Ontology (GO) Biological Processes database and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, enabling biological interpretation and generating KEGG pathway diagrams, was performed.

3. Results

Datasets were selected for analysis according to search, inclusion, and exclusion strategies (Fig. 1). For convenience, the final datasets were numbered serially from KD1 to KD9. The first eight (KD1-8) were microarray data of samples obtained from peripheral blood monocytes (PBMCs). These were sub-grouped based on differential expression of the pro-inflammatory genes TNF and NFKB1. The datasets with positive

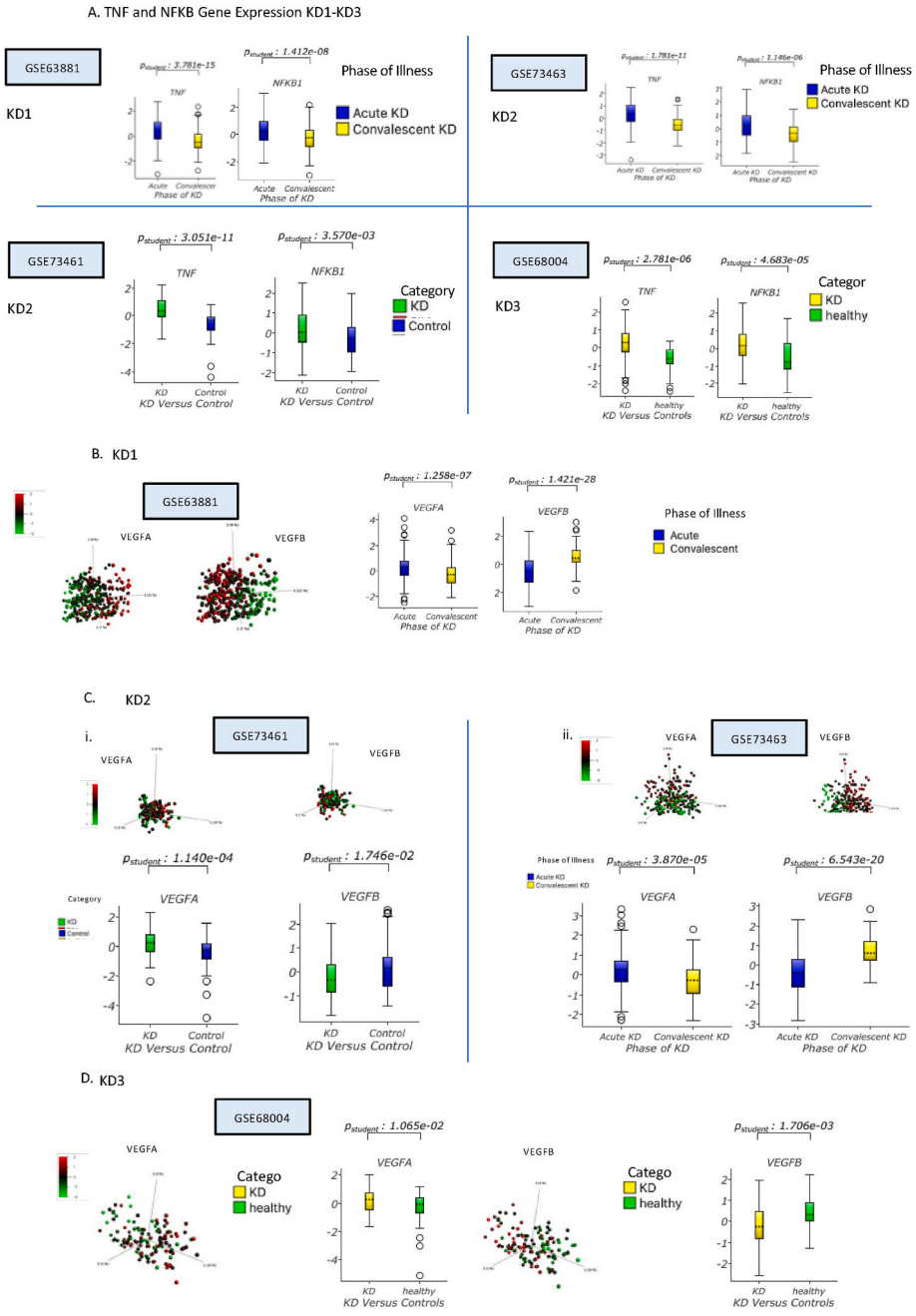

Fig. 2. TNF, NFKB1, VEGF-A, and VEGF-B Gene-Expression in the inflammatory transcriptomic group (KD1-KD3).

Fig. 2. TNF, NFKB1, VEGF-A and VEGF-B GE Studies in KD1 (GSE63881): KD2(GSE73464) and KD3(GSE68004). The relationship of VEGF-A and VEGF-B across four datasets from three studies (KD1, KD2, KD3, and KD4) is shown. PCA gene expression heat maps show the graded transition from up-regulation (red) to down-regulation (green); see key in legend. In addition, box plots show a two-group comparison using the *t*-test within the study sub-groups. Subgroups compared healthy children (controls) versus children with KD. Also, acute KD samples were compared against convalescent samples. TNF and NFKB1 gene expression patterns are also shown (Fig. 2A). TNF and NFKB1 are increased in the acute versus convalescent KD and in the comparison of KD versus controls. For KD1 (Fig. 2B) and KD2 (Fig. 2Cii): VEGF-A GE is up-regulated for acute versus convalescent samples, whereas for VEGF-B shows down-regulation in GE. For KD2(Fig. 2Ci) and KD3, VEGF-A shows significant up-regulation in children with KD versus controls, whereas, for VEGF-B, there is down-regulation in GE (Fig. 2D). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

GE for both TNF and NFKB1 were termed the 'inflammatory transcriptome group' (GSE63881:KD1, GSE73464:KD2, GSE68004:KD3), and those without these were termed the 'non-inflammatory transcriptome group' (GSE15297:KD4, GSE109351:KD5, GSE16797:KD6, GSE48498:KD7, and GSE18606:KD8). In the inflammatory transcriptome group of the three datasets, KD1 compared the GEs between acute and convalescent samples. KD2 between acute KD and both controls and convalescent samples, thus involving two sub-analyses. KD3 levels between acute KD and control samples. Similarly, in the non-inflammatory transcriptome group: KD4 compared acute KD versus febrile controls, and KD5 compared acute KD versus both controls and convalescent samples and were categorized as a 'case-control group.' The remaining three datasets, KD6, KD7, and KD8, which compared GE before and after treatment with IVIG and/or infliximab, were categorized as the 'treatment group.' KD9 (GSE64486) is an RNA-seq dataset of samples obtained from coronary artery tissues and was analyzed separately. Details of the analysis are provided below.

3.1. Differential gene expressions

The differential expression of TNF, NFKB1, VEGF-A, and VEGF-B was determined using PCA heat plots in tandem with box plots.

3.2. Inflammatory transcriptome group (KD1-KD3)

DGE showed significant upregulation of TNF, NFKB1, and VEGF-A, whereas downregulation of VEGF-B genes in all three datasets: acute versus convalescent sera in KD1 [p = 3.781e-15, p = 1.412e-08, p = 1.258e-07, p = 1.421e-28], acute KD versus controls in KD2 [p = 1.781e-11, p = 1.146e-06, p = 1.140e-04, p = 1.746e-02], acute versus convalescent sera in KD2 [p = 3.051e-11, p = 3.570e-03, p = 3.87e-05, p = 6.543e-20], and acute KD versus controls in KD3 [p = 2.781e-06, p = 4.683e-05, p = 1.065e-02, p = 1.76e-03] (Fig. 2A–D).

3.3. Non-inflammatory transcriptome group (KD4-KD8)

This group consisted of datasets with no GE positive for TNF-and NFKB1. Datasets comparing acute cases versus controls/convalescent samples were categorized as the 'case-control group' and those with pre and post-treatment comparisons as the 'treatment group. '

3.4. Case-control group: acute KD versus controls/convalescent samples (KD4 and KD5)

Differential gene expression was compared between Acute KD versus febrile and sub-class controls (adenovirus infection, drug reaction, and streptococcal infection) (KD4), and between acute KD versus convalescent KD and controls (KD5). No statistically significant changes in DGE in TNF [p = 3.860e-01, p = 9.715e-01], NFKB1 [p = 5.717e-02, p = 9.948e-01], VEGF-A [p = 8.863e-01, p = 7.722e-01], and VEGF-B[p = 7.223e-01,p = 2.458e-01] genes were found (Fig. 3Ai, Aii, & 3 B).

3.5. Treatment group: responders versus non-responders (KD6-KD8)

DGE in TNF, NFKB1, VEGF-A, and VEGF-B genes was compared between responders(R) and non-responders (NR) before and after treatment with key medications, such as IVIG, methylprednisolone, and infliximab. Responders were defined based on various clinical and laboratory parameters, such as achievement of defervescence, subsidence of rash, improved inflammatory marker profiles, and echocardiographic findings. However, studies have not reported a timeline for diagnosis, treatment, and sampling.

Compared to non-responders, responders showed significant downregulation of NFKB1 genes after treatment with KD6 [IVIG ± methylprednisolone; p = 8.6443-03], KD7 [IVIG; p = 6.618e-02, infliximab; p = 3.240e-03], and KD8 [IVIG; p = 3.518e-02]. TNF was downregulated in non-responders [p = 1.013e-03] and VEGF-B was upregulated in both responders and non-responders [p = 3.541e-02, p = 8.375e-03] post-IVIG treatment in KD8. No significant changes were observedfound in VEGF-A gene expression (Fig. 4A–C).

3.6. Coronary artery dataset (GSE64486:KD9)

GSE64486, an RNA-Seq dataset, from coronary tissue of KD patients and controls showed no significant change in the DGE of TNF [p = 1.458e-01], NFKB1[p = 3.978e-01], and VEGF-A [p = 9.892e-01]. However, VEGF-B gene expression was downregulated in KD versus control samples [p = 4.932e-02](Fig. 5A–D) (see Fig. 6).

3.7. Pathway enrichment analysis

Pathway enrichment analysis by GSEA using Biocarta and Gene Ontology (GO) pathways allowed the representation of gene sets of significance (Table 2). The GSEA profiles of patients was compared with those of the controls. The thresholds of $p < 0.05$ and $q < 0.05$ were considered statistically significant. This showed significant upregulation of genes of various immune and inflammatory profiles: upregulation of interleukin 1, 6, and 8; TNF receptor binding; vascular adhesion molecules; leucocyte tethering and rolling molecules; vascular endothelial cell adhesion; cell-cell adhesion; and vascular extravasation molecules. Fig. 5E shows the enrichment analysis of the coronary artery dataset by ShinyGo compared to the controls, showing differential gene expression for pathways related to adhesion, lymphocyte activation, white cell migration, signalling, cell stimulation, migration, and proliferation.

4. Discussion

This study explored the dynamics of differential gene expression of VEGF-A and VEGF-B in conjunction with TNF-and NFKB1 in Kawasaki disease in children with KD. We analyzed eight microarray datasets (KD1-8) of peripheral blood samples and one RNA-seq dataset (KD9) of post-mortem coronary artery tissue, yielding 148 samples. After appropriate filtering, the data was divided into 'inflammatory' and 'non-inflammatory' transcriptome groups, with the latter further sub-categorized into 'treatment' and 'case-control' groups based on study characteristics. This study revealed a novel gene expression pattern, which has not been previously reported. The inflammatory group (KD1-3) demonstrated significant differential changes, with upregulation of TNF and NFKB1 genes, which corresponded with that of VEGF-A. Our findings align with those of Hamamichi et al. (2001), who reported that elevated VEGF-A in acute KD(5) and circulating levels of VEGF-A levels were associated with the development of coronary aneurysms [6,7]. A temporal shift in VEGF expression has been reported, switching from neutrophil-derived cells in the acute phase to peripheral blood mononuclear cells (PBMCs) derived during convalescence. This mirrors the sequential inflammation in KD, where neutrophils are the most crucial cells during the acute phase and PBMCs during convalescence, characterized by chronic inflammation, healing, and vascular remodeling. Maeno et al. reported higher VEGF levels in KD patients with coronary artery lesions (CALs) than those without, with levels rising in the subacute phase [11]. They also observed that serum VEGF levels correlated with serum CRP levels, a clinical inflammation marker supporting the role of VEGF in inflammation, as VEGF-A has been reported to act with other cytokines such as NFKB1, IL-6, and IL-1β in the inflammatory cascade [41,42].

However, the role of VEGF-B in KD has not been previously described. The upregulation of VEGF-B genes in the convalescent phase highlights their role in the post-inflammatory phase. The countereffects of VEGF-A, which has proangiogenic and proinflammatory effects, and VEGF-B, which has vascular and cardioprotective effects, have been reported [28,31,32]. VEGF-B is highly expressed in the heart [28,29]. Studies have shown that VEGF-B inhibition can limit coronary tube

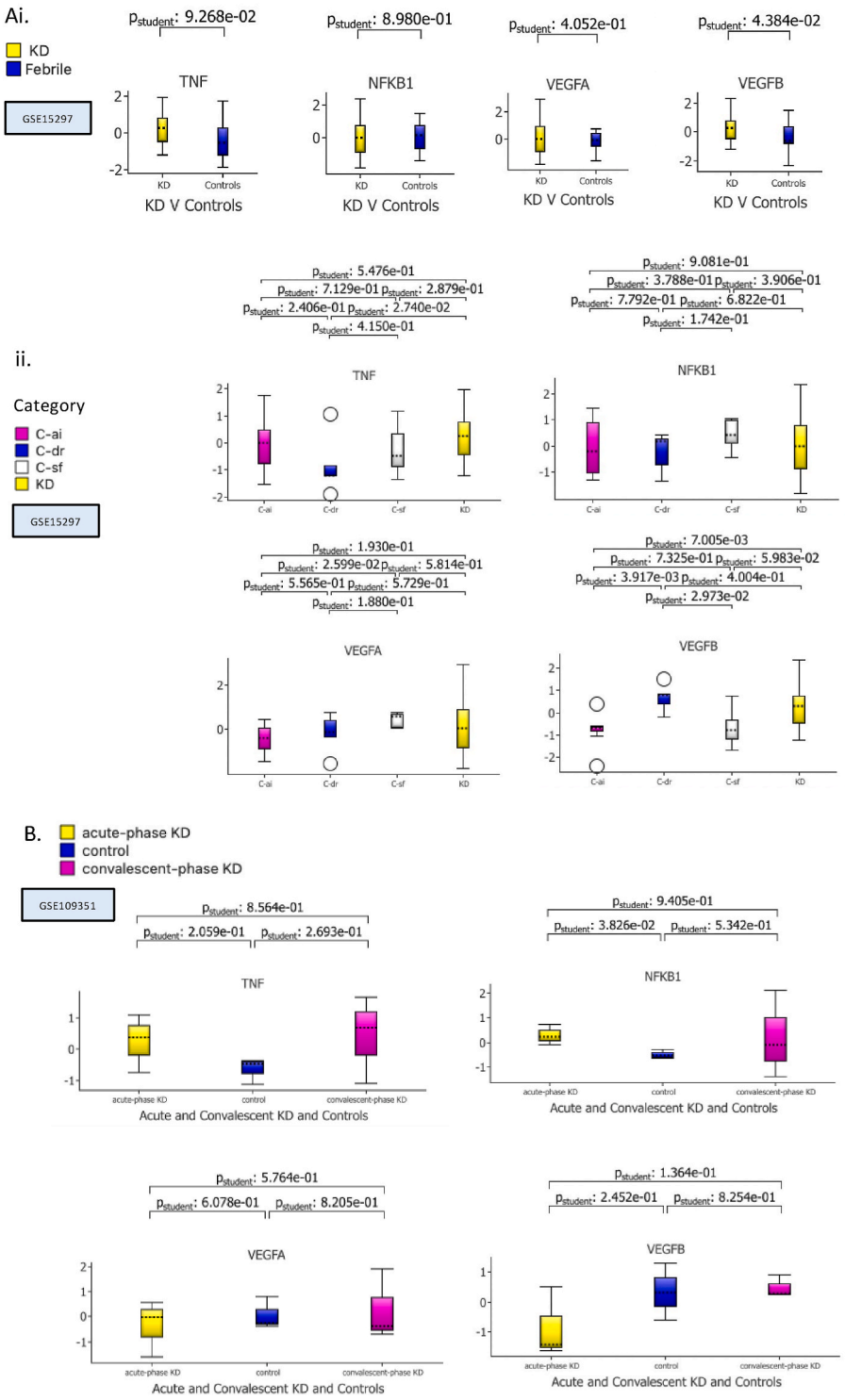

Fig. 3. Transcriptomic Analysis of Case-control Datasets in KD studies (KD4-KD5).
Fig. 3. Two datasets are shown KD4:GSE15297 (Fig. 3A) and KD5:GSE109351 (Fig. 3B). KD4 compared KD (23 patients) versus febrile (18 patients) (Fig. 3Ai) and KD (23 patients) versus Control subjects by illness [C-ai: adenovirus infection (n = 8), C-dr: drug reaction (n = 5) and C-sf: streptococcal infection (n = 5)] (Fig. 3Aii). For KD versus febrile controls. For KD4 in both groups (Fig. 3Ai and Figure Aii), no statistically significant change in GE was elicited in the four genes analyzed, including TNF [p = 3.860e-01,9.958e01,4.619e-01,9.884e-01], NFKB1 [p = 5.717e-02,6.267e-01, 2.996e-01, 7.334e-01], VEGF-A [p = 8.863e-01,8.752e-01, 4.550e-01,9.988e-01], and VEGF-B[p = 7.223e-01,5.623e-01, 8.214e-01,9.991e-01] genes. KD5 compares acute KD (3 patients), convalescent KD (3 patients), and controls (3 patients) (Fig. 3B). In KD5, there was no significant difference in GE between acute KD versus convalescent KD samples or between either acute KD or convalescent samples and controls.

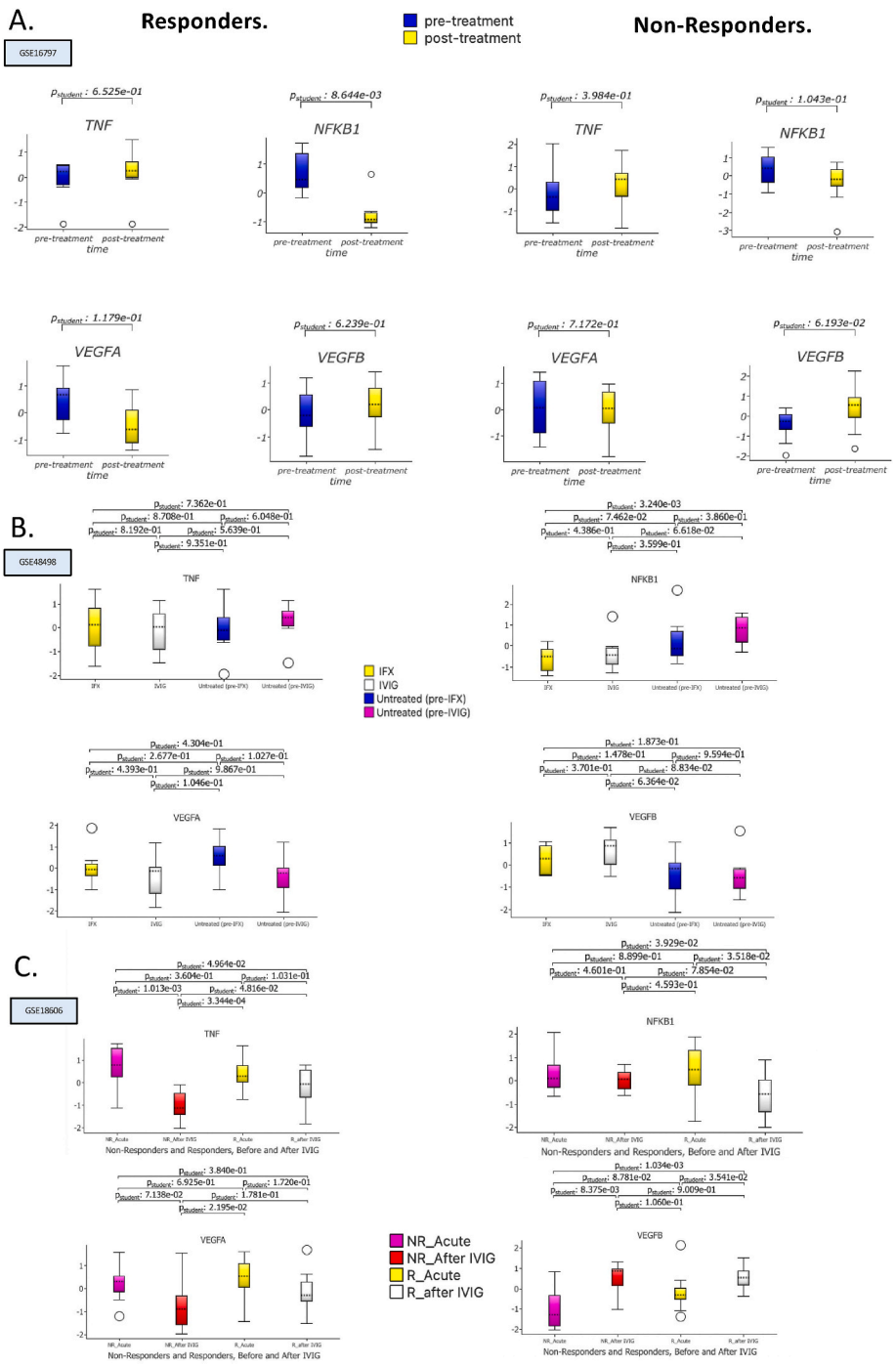

Fig. 4. KD treatment profiles showing Non-Responders and Responders to immune modulation therapies (KD6-KD8).

Fig. 4. Gene expression (GE) studies for KD patients show treatment profiles for KD6(GSE16797:Fig. 4A), KD7 (GSE48498:Fig. 4B), and KD8 (GSE18606:Fig. 4C). GE was compared for TNF, NFKB1, VEGF-A, and VEGF-B for all of the datasets. KD6 compared pre-treatment versus post-treatment of patients with KD(n = 17 in each group)(Fig. 4A). When IVIG-responsive (IVIG-R) pre-treatment (6 patients) was compared to IVIG-R post-treatment (6 patients), there was a significant down-regulation of NFKB1 GE (p = 8.644e-03), in contrast, this change in GE was not present in the IVIG-non responsive (IVIG-NR) group. There was no change in TNF, VEGF-A, and VEGF-B GE in either the IVIG-R or IVIG-NR groups.

In KD7, patients receiving Infliximab (IFX) (n = 8) were derived from the IVIG Non-Responders (NR) (n = 8); the Responder (R) group (n = 6) consisted of the pre-intravenous immunoglobulin (IVIG) group (n = 6), and Responder post-IVIG group (6 patients). Thus the IFX group was compared against the IVIG R group, untreated (pre-IFX) and untreated (pre-IVIG) (Fig. 4B). There was no difference in the different subgroups with regard to TNF, VEGF-A, and VEGF-B GE. For NFKB1, the only difference was downregulation in the IFX group when compared to the untreated (pre-IVIG group).

KD8 consisted of whole blood samples from healthy age-appropriate controls (n = 9), Intravenous Immunoglobulin (**IVIG**), Non-Responders (**NR**)(n = 8), and IVIG Responders (R) patients (n = 12). NR patients before and after IVIG were compared to Responders before and after IVIG (Fig. 4C). TNF GE showed a significant fall in GE in the NR group pre and post-IVIG (p = 1.013e-03) but not in R. For NFKB1 and VEGF-A GE, there was no difference between R and NR. VEGF-B GE showed significant up-regulation (p = 8.375e-03) in NR and in R (p = 3.541e-02) after IVIG.

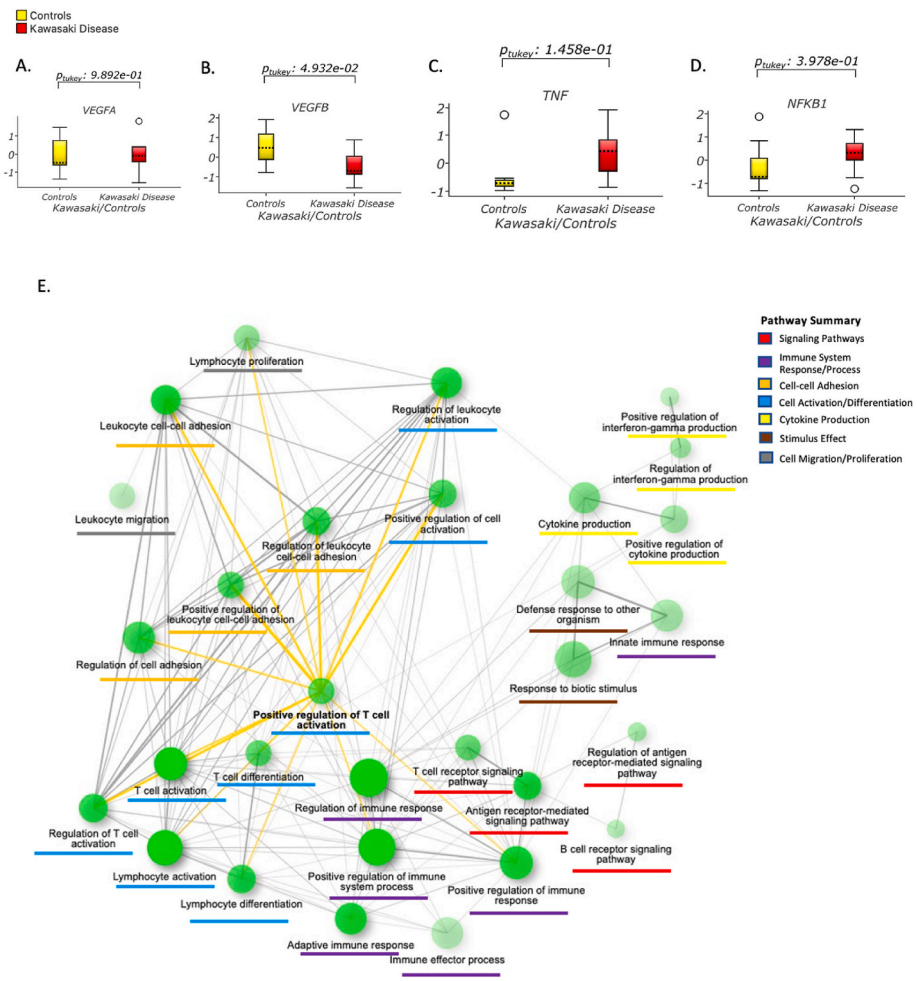

Fig. 5. Coronary arteritis in Kawasaki disease compared to controlled patients from deceased samples.
Fig. 5. Using the childhood coronary artery dataset **KD9** (GSE64486). Gene expression box plots are shown **(A–D)**, only VEGFB significantly up-regulated compared to controls. *t*-Test Kawasaki Disease versus control ($p = 0.001$, $q = 0.12$) elicited 527 genes which then underwent enrichment and the GO network generated. The SHINYGO network enrichment pattern is shown **(E)** illustrating the GO terms as shown, including antigen receptor-mediated signaling pathway, lymphocyte and leucocyte activation as well as others.

formation [30]. VEGF-A and VEGF-B exhibit a close molecular relationship and act through the same VEGF receptor [43]. Jin et al. (2018) reported a counter-regulatory role of VEGF-A and VEGF-B in adipose tissue differentiation [44].

NFKB1 downregulation may be crucial in the control of inflammation [45]. This was observed in responders in the noninflammatory treatment group. However, investigating the impact of treatment, the pre-and post-treatment analyses revealed inconsistent patterns of gene expression across the R and NR groups. This suggests that the expression of these cytokines, including VEGFs, is not necessarily linearly correlated. Temporal non-linear expression of NFKB1 and TNF genes during the course of KD is well known [46]. Persistent VEGF elevation or increasing levels during the subacute or convalescent phase after IVIG treatment have been shown to be predictors of CALs development [9]. Conversely, VEGF-B gene downregulation in the coronary dataset (GSE64486) from end-stage KD samples could indicate subsided inflammation, consistent with KD's natural history of KD [47]. Time-trajectory-based sampling based on the evolution of KD inflammation is required to better understand the variations in gene expression, both in relation to each other and with the advancement of inflammation.

Enrichment analysis of inflammation-related pathways identified inflammation and cytokine profiles associated with KD inflammation and VEGF-A function. It demonstrated upregulation of interleukins 1, 6, and 8, which are known to induce the secretion and release of VEGF-A (18, 19, 20, 21, 22, 23), TNF receptor binding linked to TNF signaling pathways, and cytokines involved in chemotaxis and vascular leakage. These include leucocyte tethering and rolling molecules, vascular adhesion molecules, vascular endothelial cell adhesion, cell-cell adhesion, and extravasation molecules, which are induced by VEGF-A to induce local inflammation, vascular leakage, and vasculitis [5,12–15, 27]. Lin et al. (1992)[48] reported increased IL-6 and IL-8 levels during the first week of KD, differentiating patients with and without coronary artery aneurysms.

The limitations of our analysis include the lack of clinical details, such as clinical information on children with coronary artery lesions, which would best be included in prospective studies. Furthermore, gene expression data are widely utilized to understand disease patterns and processes. Gene expression is one of the many aspects that contribute to the complex mechanisms that regulate host/pathogen interactions and disease progression. However, we have described a similar pattern of gene expression in sepsis (accepted for publication in the 'Shock' Journal) and in this study across large datasets. This highlights the importance of our findings. Additionally, different studies cannot standardize covariates such as age, sampling time points, and treatment status, which are crucial factors in gene expression studies. Although a cross-platform or meta-analysis was beyond the scope of this study, similar patterns across large multicenter studies suggest that our normalization

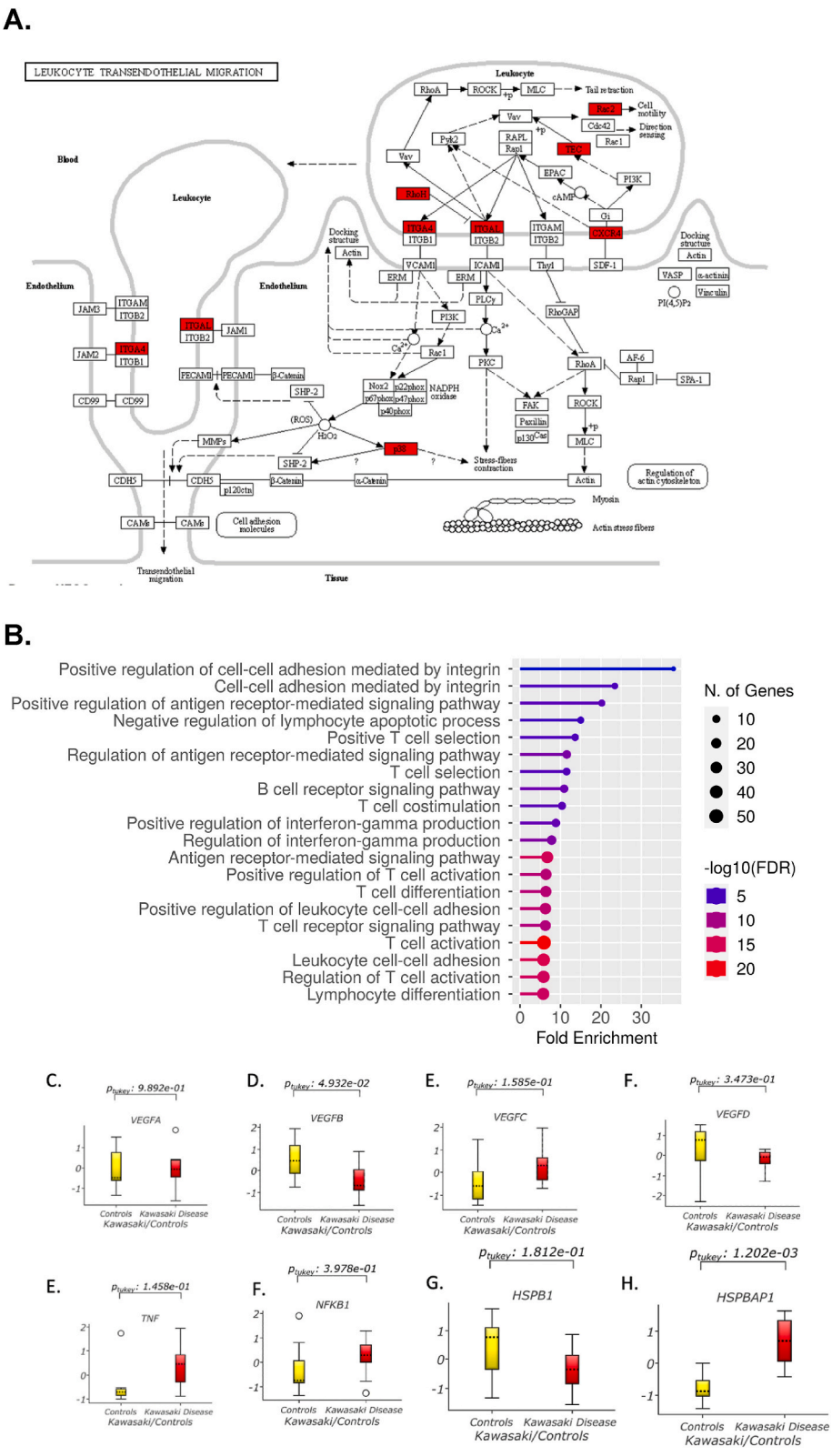

Fig. 6. Gene expression in Coronary artery KD versus controls

Fig. 5. Coronary artery gene expression from the KD9 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Pathway enrichment analysis (GSEA).

Pathway	NES	p	q
Biocarta Cytokine Production	1.71	0.006	0.11
GO B Cell Mediated Immunity	2.2	0	0
GO Cellular Extravasation	1.65	0.04	0.16
GO Cytokine Metabolic Process	1.75	0.04	0.09
GO Defence Response to Bacterium	2.06	0	0.003
GO Humoral Immune Response	2.08	0	0.002
GO Immunoglobulin Production	2.07	0	0.002
GO Leucocyte Adhesion to Vascular Endothelial Cell	1.72	0.04	0.1
GO Leucocyte Tethering or Rolling	1.76	0.006	0.1
GO Positive Regulation of Cell Cell Adhesion	1.7	0.03	0.11
GO Positive Regulation of Cytokine Production	1.59	0.049	0.15
GO Positive Regulation of Interferon Gamma Production	1.84	0.008	0.05
GO Positive Regulation Interleukin 1 Production	1.71	0.04	0.1
GO Positive Regulation Interleukin 6 Production	1.74	0.04	0.1
GO Positive Regulation Interleukin 8 Production	1.62	0.03	0.14
GO Tumor Necrosis Factor Receptor Binding	1.62	0.03	0.15

Table 2. Coronary artery dataset KD9 (GSE 64486) underwent GSEA of Cases versus controls. Variables were identified according to the averaging of their Genes Symbols. Elicited GO pathways are shown. The gene set used was TNF GMT Reference for GSEA.
*Biocarta Cytokine Production Involved in Inflammatory Response.

and processing pipeline was effective. Historically, KD studies have been primarily cross-sectional in design, making it challenging to account for temporal relationships. A longitudinal approach could be beneficial for future research, with additional clinical parameters highly desirable. Furthermore, a significant limitation of KD research is that analyzing coronary artery levels of VEGF-A and VEGF-B is only possible from the extracted tissue. In situ gene expression studies of the coronary arteries during KD are not technically feasible. Differences in gene expression results between large KD observational studies and smaller sample size studies may be due to data normalization and pre-processing or the timing of cross-sectional sampling relative to inflammation levels. We also explored the differences in gene expression between IVIG Responders (R) and non-responders (NR). One dataset showed changes in TNF and NFKB1 gene expression in R, whereas another suggested an increase in VEGF-B gene expression in R and NR. Moreover, the KD data used in this study were obtained from studies that did not have standardized temporal milestones for blood collection. Studies only compared before and after treatment response, acute versus convalescence, and controls versus cases, without using standard time points. Consequently, sampling bias may arise when blood is collected from patients with subsided inflammation. Future studies should address these limitations to provide more robust and accurate insights into KD's molecular mechanisms underlying KD.

Further research is needed to elucidate the feedback loops that affect gene expression and protein production, particularly the VEGF-A/VEGF-B interactions. Understanding this dual relationship may have implications for more accurate biomarker identification in KD and assessment of changes in the coronary artery. Considering the advancements achieved through the pharmacological actions of VEGF-A alone, understanding the VEGF-A/VEGF-B duality may provide valuable insights for enhancing biomarker accuracy and therapeutic approaches in KD. Moreover, practical considerations are required to understand how gene expression patterns can be translated to the bedside. One option might be to evaluate temporal VEGF-A and VEGF-B protein levels.

5. Conclusion

In conclusion, this study represents an inaugural examination of the intricate interplay between VEGF-A and VEGF-B gene expression and their correlation with TNF and NFKB1. The differential gene expression (DGE) patterns discerned between the inflammatory and non-inflammatory cohorts provide compelling evidence of the intricate linkage between TNF, NFKB1, and VEGF-A gene expression. VEGF-A is of paramount significance during the acute pro-inflammatory stage of Kawasaki disease (KD), whereas VEGF-B is a key player in the subsequent post-inflammatory phase. Temporal fluctuations in gene expression highlighted the potential of employing a synergistic VEGF-A/B strategy for KD biomarker identification. Moreover, these findings underscore the potential applicability of interventions targeting VEGF-A modulation in the context of KD. For a more comprehensive understanding, future investigations should prioritize time-trajectory-based sampling, accounting for pertinent clinical covariates. Such endeavors will be instrumental in elucidating the dynamics between VEGF-A and VEGF-B, and their correlative associations with TNF and NFKB1. The ultimate objective is to identify novel biomarkers and therapeutic avenues, with a focus on mitigating the risk of Coronary Artery Lesions (CALs) in patients with KD.

Data availability

The datasets selected for analysis in this study were obtained from gene expression microarray data from public repositories. Readers can access the data from the online European Bioinformatics Information database as a part of the European Molecular Biology Laboratory (https://www.ebi.ac.uk) and the National Library of Medicine GEO Dataset Repository https://www.ncbi.nlm.nih.gov). There were no restrictions on access to data from the aforementioned repositories.

Research and Ethics Committee

The paper was waived from review by the hospital Central Scientific Committee and the Research and Ethics Committee, given the secondary analysis of data from primary studies.

Funding statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations and Acronyms

BAM	Binary Alignment Map
CALs	Coronary Artery Lesions/aneurysms

DGE	Differential Gene Expression
FDR	False discovery rate
GE	Gene Expression
GSEA	Gene Set Expression Analysis
GO	Gene Ontology
IVIG	Intravenous Immunoglobulin
KD	Kawasaki Disease
KEGG	Kyoto Encyclopedia of Genes and Genomes
NFKB1	Nuclear Factor κB subunit 1
PCA	Primary Component Analysis
PBMC	Peripheral Blood Monocytes
QOE	Qlucore Omics Explorer
R	Responders
NR	Non-Responders
TMM	Trimmed Mean of the M-Values
TNF	Tumor Necrosis Factor
VEGF-A	Vascular Endothelial Growth Factor A
VEGF-B	Vascular Endothelial Growth Factor B

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