# Identification of Dysregulated Pathways and key genes in Human Retinal Angiogenesis using Microarray Metadata

Umadevi Subramanian [1] and Bharanidharan Devarajan [1,*]

[1] Department of Bioinformatics, Aravind Medical Research Foundation, Madurai, India.

*Corresponding authorE-mail: bharanid@gmail.com

## Abstract

Retinal angiogenesis is a common neovascularization mechanism that causes severe irreversible vision loss in the number of retinal diseases worldwide. Patients often do not respond to the current anti-angiogenic therapies and have a vision loss. Understanding the various angiogenic pathways and factors involved in the pathogenic mechanism is vital for disease management. In this study, to identify dysregulated angiogenic pathways and specific angiogenic factors involved in vision-threatening diseases namely proliferative diabetic retinopathy (PDR), retinopathy of prematurity (ROP) and neovascular age-related macular degeneration (nAMD), we downloaded microarray metadata of samples and obtained the differentially expressed genes (DEGs) in all the disease and each disease samples compared to controls. Subsequently, we performed Gene Set Enrichment (GESA) analysis for pathways, a protein-protein interaction (PPI), and angiome network analysis using R and Cytoscape software. We identified highly enriched dysregulated pathways that were neuroactive ligand receptor interaction and cytokine-cytokine receptor interaction. The angiogenic–associated DEGs were predominately related to the cytokine-cytokine receptor interaction pathway, which we further confirmed with RNA-seq data of PDR samples. Together, our analysis of these data elucidated the molecular mechanisms of retinal angiogenesis and provided potential angiogenic targets for therapeutics.

**Keywords:** Retinal angiogenesis, Microarray-Meta data, Dysregulated angiogenic pathways, Cytokine-cytokine receptor interaction pathway

## 1. Introduction

Retinal angiogenesis is the common dysregulated pathway of numerous blinding disorders, namely, proliferative diabetic retinopathy (PDR), retinal artery (or vein) occlusion, retinopathy of prematurity (ROP), sickle cell retinopathy[1], and neovascular age-related macular degeneration (nAMD)[2]. Although there are subtle differences in the mechanism of angiogenesis, they are characterized as common neovascularization event. Angiogenesis is a physiological process through which new blood vessels formed from pre-existing vessels under hypoxic conditions[3].Hypoxia-inducible-1α drives the expression of substantial pro-angiogenic growth factors and induces endothelial cells into a tip cell phenotype. The tip cell proliferates slowly and activates matrix metalloproteinases (MMPs), plasmin, uPA, and tPA, and cleaving numerous extracellular matrix (ECM) proteins. The activated MMPs and other proteins stimulate the Notch ligand in tip cells and proliferate further to elongate a new vessel[4,5]. As the newly formed vessels mainly serve a role in a wound healing response, they usually do not restore tissue integrity, but instead, cause visual impairment[6,7].

Vascular endothelial growth factor (VEGF) is the major growth factor, induced in hypoxic conditions. VEGF family members VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PIGF), and their receptors are the validated regulators of angiogenic signaling pathway[8]. VEGF-A is the potent growth factor and mediator of intraocular neovascularization. VEGF-A stimulates not only the development of new blood vessels, but also, microaneurysm formation, capillary occlusion with ischemia, and promoting increased vascular permeability[9]. VEGF-B can regulate the function of endothelial cells. VEGF-C and VEGF-D increase vascular permeability as well as induce angiogenesis. PIGF plays a role in pathogenic angiogenesis by increasing the activity of VEGF-A[10].

The current anti-angiogenic agents, approved by the Food and Drug Administration, inhibit the VEGF pathway[8]. The anti-VEGF drugs are the molecules that target VEGF isoforms, or inhibiting VEGF receptors, or inhibiting VEGF downstream signaling[11]. About 30% of PDR patients fail to respond to initial treatment, and the majority of the responders would require multiple rounds of intravitreal injections[12]. Therefore, anti-VEGF treatment is potentially required for years, and chronic VEGF inhibition may cause side effects and drug resistance. The potential consequence of VEGF blockade is hypertension, proteinuria, impairment of wound healing/collateral vessel development, and inhibition of bone growth, infertility, inhibition of skeletal muscle, regeneration, cardiac remodeling and neuroprotective effect in the ischaemic retina[13]. Also, a dose-dependent decrease in ganglion cells was reported in rats[14].

Studies show that VEGF independent factors may stimulate angiogenesis, particularly in tumors[15]. Zhang et al. suggested identifying the angiogenic factors other than VEGF for a better therapeutic approach[16]. Gene expression studies have been widely performed to identify angiogenic factors. However, such studies will provide numerous key genes that are differentially expressed, and thus, it is a challenging task to detect reliable targets. On the molecular level, a combination of the differential gene expression (which may vary in each individual) is assumed to dysregulate the common cellular pathway[17]. Therefore, identification of the dysregulated pathways is essential for understanding disease mechanisms and the identification of targets for future application of custom therapeutic decisions[18]. The accumulation of large amounts of gene expression data in public databases provides an opportunity for identifying commonly dysregulated pathways and the gene targets that are differentially expressed[19].

In the present study, we aim to identify the dysregulated angiogenic pathways in addition to VEGF dependent angiogenic pathway in retinal diseases. To achieve our aim, we designed this study with several steps that are (i) to combine the microarray data from different reliable experiments and adjusting batch effects, (ii) to identify robust differentially expressed genes (DEGs), (iii) to conduct the pathway enrichment analysis, (iv) to identify key dysregulated angiogenic pathways and angiogenic factors through network analysis and (v) to compare and confirm the identified dysregulated angiogenic pathways and angiogenic factors with available RNA-Seq data

## 2. Materials and Methods

### 2.1. Gene expression data and preprocessing

All the microarray samples used in this study were systematically searched and downloaded from NCBI-GEO[20] after the manual curation of the sample details. Probe IDs were mapped to official gene symbols in the DAVID database[21], and non-protein coding genes, as defined by an assignment of HUGO gene symbol, were excluded from the study. In case of genes with multiple probes, gene expression level was aggregated from probeset to gene-level data by taking the maximum value across all the samples[22].

### 2.2. Missing value imputation and normalization of data

All the samples selected for the study were combined by their gene symbol using R script. The missing gene expression values were imputed by Bayesian Principal Component Analysis (BPCA) method[23], which performed well even with low entropy. Further, the whole data were quantile normalized[24] to make the distribution of values in all the samples into the

same. The outlier samples were removed based on the principal component analysis (PCA) plot. Further it was confirmed with Euclidean distance-based clustering using R script.

2.3. Identification of differentially expressed genes (DEGs) and pathway enrichment

DEGs were selected from the list by filtering, only if the moderated t-test FDR was ≤ 0.05 and the fold change (FC - a difference of the means between two groups when all the values were log2 transformed) ≥ 2 (up-regulated) or ≤ -2 (down-regulated). For the pathway analysis, Gene Set Enrichment Analysis (GSEA) proposed by Subramanian et al. implemented with GenePattern tools was performed[25]. The DEGs and FC values were regarded as a pre-ranked list, and c2.cp.kegg.v6.1 was interrogated as pathway database. We used default parameters and set the minimum gene set size 2. The output enrichment score reflects the degree to which a gene set in the pathway is over-represented at the extreme (low or high) of the entire ranked list of FC. For the pathway enrichment, normalized enrichment score (NES) was considered as a metric of influence of DEGs with p-value ≤ 0.05.

Analysis was carried out with different perspectives, differential gene expression and pathway regulation between retinal angiogenesis (case) samples and controls, case and tissue (retina, and RPE/C) specific controls, cases and disease (dry AMD, non-Proliferative Diabetic Retinopathy - NPDR, and Diabetic Mellitus - DM) specific control samples, disease specificity (nAMD, PDR, ROP) and control samples as given in figure 1. Among the DEGs, the angiogenesis-related genes were identified by GO term in GSEA and the list is given by Chu et al.[26]. Further, the STRING database was used to retrieve the predicted interactions for the identified DEGs and graphically represented using Cytoscape 3.2.0.

2.4. RNA-Seq analysis

The available RNA-Seq samples related to this study in NCBI-SRA are PDR (Accession No.: SRP097696) and whole retinal (SRP119766) control samples. They were downloaded by SRA Toolkit, aligned to human reference genome hg38 using CLC Genomics Workbench 12.0, quantile normalized in R, and analyzed using EdgeR.

## 3. Results

3.1. Standardization of gene expression data

The analysis was primarily done with 243 control samples, 95 normal retinal control (GSE60436 (3 samples), GSE28133 (19), GSE29801 (55), GSE32614 (12), GSE41019 (3), GSE57864 (3)), 138 normal RPE/C control (GSE18811 (31), GSE29801 (96), GSE43257 (2), GSE50195 (7), GSE5741 (2)), and 32 disease cases includes, 16 (8 retina, and 8 RPE/C) nAMD (Accession No.: GSE29801), 6 fibrovascular membrane of PDR (GSE60436), and 10 retinal microvascular endothelial cells of ROP (GSE5946) samples. The studies with disease

specific control samples include, 64 dry AMD retina (GSE29801 (61), GSE17938 (3)), 9 dry AMD RPE/C (GSE50195), 6 neural retinal NPDR (GSE53257) and 6 neural retinal DM (GSE53257) samples. After preprocessing and merging of data, 15.75% of missing data were imputed. All the samples which contain 18621 genes were quantile normalized (Figure S1). The 318 control samples (95 retina, 138 RPE/C) used in the primary study were obtained from different technical platforms. Eventhough they were quantile normalized, to reduce the bias the outlier samples have to be removed. In order to this, all the disease samples and control samples were subjected to PCA (Figure S2). The disease samples were retained, the control samples which were scatterd/ outgrouped were removed, and clustering tree was construted with remaining samples. This process was iteratively done once observed the clear clustering of case and control samples (Figure S3). This results to 31 retina and 34 RPE/C control samples.

3.2. Dysregulated pathways of all the cases with all normal control samples

One thousand three hundred eighty-three genes displayed differential expression with two-fold change (FDR $\leq 0.05$) in all the cases compared to normal control samples (Figure S4). Of these, 852 were downregulated and 531 upregulated. Gene set enrichment analysis (GSEA) with respect to pathways showed that 237 DEGs were associated with 22 different dysregulated pathways (significant with $p \leq 0.05$, NES of pathways are given in Table 1). We then analyzed DEGs that are associated with angiogenesis. We identified 127 angiogenic-associated enriched DEGs, of which 31 were based on gene ontology (GO) (NES: 1.74 with $p = 0.01$) and 96 DEGs were based on the literature. The highly enriched pathways with their significantly altered DEGs and their association with angiogenesis were graphically represented in Figure 2.

The neuroactive ligand receptor interaction pathway was the highly dysregulated pathway with the normalized enrichment score (NES) of 3.15, and 15 DEGs were identified in this pathway. Of these, 11 DEGs were unique to this pathway and while, 4 DEGs GRIN1, CHRM2, GRIN2C and GRIN2D were connecting other pathways. Only UTS2R was found to be angiogenic-associated (Figure 2).

3.2.2. Cytokine-cytokine receptor interaction pathway

Cytokine-cytokine receptor interaction pathway, next to the neuroactive ligand receptor interaction pathway, had enrichment score 2.55 (Table 1). We identified 25 DEGs (Figure 2 and S5) in this pathway. Among these, down-regulated VEGFA, CCL3, EGFR, PDGFC and up-regulated genes VEGFB, VEGFC, CX3CL1, ACVRL1, CCL23, CCL24, CCL26, OSM, and TNFRSF25 genes were found to be angiogenic-associated. Of interest, we identified that

three of the angiogenic-associated genes PDGFC, CXCL1, and EGFR were also involved in the other pathways, as shown in Figures 2 and S5.

3.2.3. Other pathways with DEGs of angiogenic association

In addition to highly enriched cytokine-cytokine receptor interaction and Neuroactive ligand receptor interaction pathways, we showed 13 other pathways with angiogenic-associated DEGs. Regulation of the actin cytoskeleton pathway has ten angiogenic DEGs; Adherens junction and Epithelial cell signaling in Helicobacter pylori infection have seven angiogenic DEGs; tight junction has six angiogenic DEGs. Three angiogenic DEGs were involved in Alzheimer's disease and arrhythmogenic right ventricular cardiomyopathy ARVC pathways and 2 DEGs were in endocytosis and pathogenic Escherichia coli infection pathways. Five pathways had one DEG, namely, aminoacyl tRNA biosynthesis (YARS), Huntington's disease (BAX), p53 signaling pathway (BAX), ribosome (RPL29) and Vibrio cholerae infection (GNAS).

The angiogenic-associated DEGs that were involved in two enriched pathways include ADAM10, BAX, CXCL1, ITGAV, JAM3, NRAS, and PDGFC. CTNNA1, MAPK1, and RAC1 were involved in three enriched pathways; RHOA in four pathways; EGFR and CDC42 were involved in more than five pathways. Despite the above, 92 angiogenic-associated DEGs were not involved in any of the enriched pathways. For instance, we identified the SEMA3B gene with high fold change of 5.154 and not involved in any of the enriched pathways.

3.2.4. Angiogenic interactome of DEGs through protein-protein interaction (PPI)

In order to identify the interactome of angiogenic-associated DEGs, their protein-protein interaction (PPI) partners and pathways associated with them are connected as network and graphically illustrated in Figure 3. PPP2CA showed the highest connectivity, which is associated with the tight junction pathway, and it mostly connected to the ribosome pathway. Followed by PPP2CA, RHOA and EGFR showed more interacting partners and pathways.

3.2.5 Pathway specificity analysis

To examine the specificity of enriched pathways (Table 1), we performed a similar analysis using two sets of control samples, one from the retina and one from RPE/Choroid samples (Figure 1). Compare to enriched pathways obtained using all control samples; we identified that almost all the enriched pathways were enriched either using retina or RPE/Choroid or both except Cysteine and methionine metabolism and Vibrio cholerae infection. However, there are other pathways were enriched in the two control data sets that were not enriched in all control data as shown in Supplementary Table S1. Nevertheless, the

enriched pathways, as shown in Table 1 is not affected by the two different retina and RPE/Choroid control data sets, not random, although they influence the pathway enrichment analysis.

3.2.6 Disease-specific analysis

Furthermore, we performed disease-specific pathway enrichment analysis whether a single disease influences the angiogenic-associated pathways and genes. The heatmap of the dysregulated pathways with respect to disease specificity is illustrated in Figure 4. The highly enriched pathways Neuroactive ligand receptor interaction pathway and cytokine-cytokine receptor interaction pathway was not affected by the specific disease. The cytokine-cytokine receptor interaction pathway with highly angiogenic-associated DEGs was graphically illustrated as a heat map in Figure 5. Many angiogenic-associated genes (mentioned previously) showed similar patterns of fold change in all three retinal diseases. For example, VEGFA gene was downregulated in three all retinal diseases individually and all three together. We also performed the disease-specific comparative analysis using different control data set (Supplementary Table S2). When PDR samples compared with NPDR and DM samples, we observed the almost same pattern of dysregulated pathways. However, we did not observe similar pattern when we compared dry AMD with AMD samples.

3.3. Pathway analysis using RNA-seq data

We further extended our analysis with the RNA-sequencing data set in order to show whether the pathway enrichment analysis is biased with microarray data. We compared nine PDR samples with 13 normal whole retina samples, and we identified 5816 upregulated and 2031 downregulated genes. The supplementary table 3 shows the dysregulated pathways with the NES score. The graft versus host disease pathway was enriched with the highest NES (4.15); however, it did not include the angiogenic DEGs. As expected, the Cytokine-cytokine receptor interaction pathway with NES of 3.35 is the most dysregulated in the retinal angiogenesis included with seventeen angiogenic-associated DEG. Among the DEGs, ACVRL1, CCL19, CCL23, CXCL1, IL10RA, IL18RAP, IL1R2, IL3RA, IL4R, OSM, TNFRSF25 and TNFRSF4 were observed with similar expression as that of the results from microarray data analysis. Notably, of the VEGF family, only VEGFC was observed with significant upregulation with 1.3 fold change.

## 4. Discussion

Neovascularization by dysregulation of angiogenic pathways is the major cause of vision loss in many retinal diseases. The purpose of this study is to identify dysregulated angiogenic pathways in addition to VEGF dependent angiogenic pathways and specific angiogenic

236 factors involved in vision-threatening retinal diseases, include PDR, ROP and nAMD. We
237 thought that the most feasible approach to this end would be to combine all the expression
238 data from retinal diseases with angiogenesis, to identify common dysregulated angiogenic
239 pathways. We showed that VEGF independent pathways are highly enriched by the
240 differentially expressed genes (DEGs) of the retinal angiogenesis samples. The two highly
241 enriched pathways are neuroactive ligand receptor interaction and cytokine-cytokine
242 receptor interaction pathways (Figure 4). Among the several DEGs involved in neuroactive
243 ligand receptor interaction pathway, though our angiome-network showed no
244 angiogenic-associated genes, ADRA1B [27], CHRM2[28], DRD4[29], GALR3[30], GLP2R[31],
245 GRIN2C[32], GRIN2D[33], HTR5A[34], P2RY2[35], UTS2R[36] DEGs have been reported to be
246 involved in angiogenesis. The neuroactive ligand receptor interaction pathway has been
247 reported to be the most significant pathway for cancers and angiogenesis and is also closely
248 connected with the poor prognosis of cancer[37–41].

249 Based on the angiome-network with enriched DEGs, we report that the
250 cytokine-cytokine receptor interaction pathway is the highly dysregulated angiogenic
251 pathway enriched with fifteen angiogenic-associated DEGs (Supplementary Figure S5)
252 followed by regulation of actin cytoskeleton, adherens junction and epithelial cell signaling
253 in *Helicobacter pylori* infection. Jennifer et al. [42] experimentally identified novel
254 VEGF-independent cytokines and their association with PDR, suggesting the association of
255 cytokines in angiogenesis. In this study, we have shown that ten angiogenic-associated
256 cytokines were significantly up-regulated in the dysregulated cytokine-cytokine receptor
257 interaction pathway. Interestingly, VEGFA along with other angiogenic factors CCL3,
258 EGFR and PDGFC are significantly down-regulated, suggesting that VEGF independent
259 cytokines play a vital role in retinal angiogenesis. Apart from the ten up-regulated
260 angiogenic-associated genes, with widespread literature survey, we account that CCL19[43],
261 IL18RAP[44], IL1R2[45], LTB[46], IL10RA[47], CNTF[48] up-regulated genes may contribute to the
262 dysregulation of angiogenesis. Therefore, we report that cytokine-cytokine receptor
263 interaction pathway is the common dysregulated pathway in retinal angiogenesis as
264 previously stated in the animal models of retinal angiogenesis[49].

265 Of interest, to support that cytokine-cytokine receptor interaction pathway as common
266 dysregulated angiogenic pathways in retinal diseases, we constructed a regulatory pathway
267 (figure 6) with observed DEGs in this pathway and literature support. First, we exhibit that
268 the VEGF pathway is downregulated by showing the downregulation of VEGFA gene
269 expression (Figures 5 and 6) and upregulation of CCL19 with CCR7 in retinal angiogenesis.

The CCL19 and CCR7 have been reported to regulate the VEGF pathway negatively[50–52]. Additionally, the DEGs CCL3[53] and EGFR[54] are reported to induce VEGFA expression, which is downregulated in this study. We observe the downregulation of other DEGs c-Met, RAS, ERK, Elk1 and STAT3 that could affect the VEGFA dependent angiogenesis[52]. The upregulated DEGs ITFRSF25, TNFRSF4, CCL23, CCL26, and CX3CL1 of cytokine-cytokine interaction pathway is reportedly activating PI3K/AKT pathway[55–59], which in turn induces the angiogenesis through activating mTOR[54] and NFKB pathway[60]. The upregulated DEGs IL18RAP and CXCL1has shown to modulates the NFKB and thus induces the angiogenesis. Furthermore, the upregulated DEG ACVRL1 is reported to promote endothelial cell proliferation by activating SMAD1[61] and coexpression of IL12 with CCL19 reported to regulates the JAK/STAT signaling[62], may help to mediate the angiogenesis indirectly[61–63]. We also observe that the other dysregulated pathways (Table 1) include aminoacyl tRNA biosynthesis, pathogenic Escherichia coli infection signaling, epithelial cell signaling in Helicobacter pylori infection and cysteine and methionine metabolism pathway, reported to trigger VEGF dependent angiogenic pathway[64–66], are negatively enriched. We also support the fact that VEGF-independent angiogenic pathway in retinal angiogenesis using the PPI network. Most of the PPI partners of VEGFA namely EGF, FLT1, HIF1A, IGF1, KDR, PIK3CA, TGFB1, THBS1 are downregulated in this study. However, we have observed that the upregulation of VEGFB and VEGFC. However, comparing the expression VEGFB and VEGFC in each disease and results of RNA-seq analysis, we have identified that VEGFC expression is consistent, individually significant upregulation was observed in PDR (more than two FC).

Further, to identify the specific angiogenic factors that are related to the cytokine-cytokine receptor interaction pathway, we have examined the DEGs in the disease-specific data. The fold change pattern of DEGs related to cytokine-cytokine receptor interaction pathway is following the same pattern. However, among DEGs, CCL19, CCL26, CCL24, CXCL1, ACVRL1, OSM, IL4R, CCL23and IL18RAP are significantly upregulated genes; while, VEGFA, EGFR, and IFNGR2 are downregulated genes in all three retinal diseases. The DEGs CCL14, CNTF, CX3CL1, IL10RA, IL15RA, IL1R2, IL3RA, LTB, PDGFC, TNFRSF25 and TNFRSF4 are upregulated genes in any of two retinal diseases. Further, when we compared the results with DEGs of cytokine-cytokine receptor interaction pathway enriched using RNA-Seq data, we have observed more than twelve DEGs that have similar expression viz ACVRL1, CCL19, CCL23, CXCL1, IL10RA, IL18RAP, IL1R2, IL3RA, IL4R, OSM, TNFRSF25 and TNFRSF4 , suggesting their role in angiogenesis

304 invariably. Furthermore, we have observed that CXCL1 is the major hub of upregulated
305 DEGs based on the protein-protein interactions with the angiome-pathway network (figure
306 3), and thus it would be a potential target. In addition to CXCL1, based their angiogenic role
307 (Figure 6), the PPI network (figure 3) and the RNA-seq data results, we highlight that
308 VEGFC, ACVRL1, CCL23, CX3CL1 and TNFRSF25 are the notable targets.

309 In conclusion, the cytokine-cytokine receptor interaction pathway is highlighted as the
310 common contributor for the retinal angiogenesis in PDR, ROP and nAMD through
311 microarray metadata analysis. Further, several DEGs in the cytokine-cytokine receptor
312 interaction pathway are identified as potential angiogenic factors that may cause the retinal
313 angiogenesis. However, as only a few studies have been published related to the highlighted
314 VEGF-independent retinal angiogenic pathways and genes, we hope that this work will lead
315 future investigations into mechanisms of retinal angiogenesis, as well as identification of
316 potential drug target.

317 **References**

318 1. Aiello, L. M. Perspectives on diabetic retinopathy. *Am. J. Ophthalmol.* (2003).
319 doi:10.1016/S0002-9394(03)00219-8

320 2. Das, A. & McGuire, P. G. Retinal and choroidal angiogenesis: Pathophysiology and
321 strategies for inhibition. *Progress in Retinal and Eye Research* (2003).
322 doi:10.1016/j.preteyeres.2003.08.001

323 3. Folkman, J., Watson, K., Ingber, D. & Hanahan, D. Induction of angiogenesis during
324 the transition from hyperplasia to neoplasia. *Nature* (1989). doi:10.1038/339058a0

325 4. Dulak, J., Józkowicz, A. & Łoboda, A. *Angiogenesis and vascularisation: Cellular
326 and molecular mechanisms in health and diseases*. *Angiogenesis and Vascularisation:
327 Cellular and Molecular Mechanisms in Health and Diseases* (2013).
328 doi:10.1007/978-3-7091-1428-5

329 5. Neve, A., Cantatore, F. P., Maruotti, N., Corrado, A. & Ribatti, D. Extracellular matrix
330 modulates angiogenesis in physiological and pathological conditions. *BioMed
331 Research International* (2014). doi:10.1155/2014/756078

332 6. Andreoli, C. M. & Miller, J. W. Anti-vascular endothelial growth factor therapy for
333 ocular neovascular disease. *Current Opinion in Ophthalmology* (2007).

doi:10.1097/ICU.0b013e3282f0ca54

7. Schlingemann, R. O. & Witmer, A. N. Treatment of retinal diseases with VEGF antagonists. *Progress in Brain Research* (2009). doi:10.1016/S0079-6123(09)17517-9

8. Ferrara, N., Hillan, K. J., Gerber, H. P. & Novotny, W. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. *Nature Reviews Drug Discovery* (2004). doi:10.1038/nrd1381

9. Hoeben, A. *et al.* Vascular endothelial growth factor and angiogenesis. *Pharmacological Reviews* (2004). doi:10.1124/pr.56.4.3

10. Usui, Y. *et al.* Angiogenesis and Eye Disease. *Annu. Rev. Vis. Sci.* (2015). doi:10.1146/annurev-vision-082114-035439

11. Giet, M., Henkel, C., Schuchardt, M. & Tolle, M. Anti-VEGF Drugs in Eye Diseases: Local Therapy with Potential Systemic Effects. *Curr. Pharm. Des.* (2015). doi:10.2174/1381612821666150225120314

12. Mirshahi, A. *et al.* Bevacizumab-augmented retinal laser photocoagulation in proliferative diabetic retinopathy: A randomized double-masked clinical trial. *Eur. J. Ophthalmol.* (2008).

13. Simó, R. & Hernández, C. Intravitreous anti-VEGF for diabetic retinopathy: Hopes and fears for a new therapeutic strategy. *Diabetologia* (2008). doi:10.1007/s00125-008-0989-9

14. K., N. *et al.* Vascular endothelial growth factor-A is a survival factor for retinal neurons and a critical neuroprotectant during the adaptive response to ischemic injury. *American Journal of Pathology* (2007).

15. Bałan, B. J. & Słotwiński, R. VEGF and tumor angiogenesis. *Central-European Journal of Immunology* (2008).

16. Zhang, F. *et al.* VEGF-B is dispensable for blood vessel growth but critical for their survival, and VEGF-B targeting inhibits pathological angiogenesis. *Proc. Natl. Acad. Sci. U. S. A.* (2009). doi:10.1073/pnas.0813061106

17. Kim, Y. A., Wuchty, S. & Przytycka, T. M. Identifying causal genes and dysregulated

pathways in complex diseases. *PLoS Comput. Biol.* (2011). doi:10.1371/journal.pcbi.1001095

18. Ahn, T., Lee, E., Huh, N. & Park, T. Personalized identification of altered pathways in cancer using accumulated normal tissue data. in *Bioinformatics* (2014). doi:10.1093/bioinformatics/btu449

19. Liu, K. Q., Liu, Z. P., Hao, J. K., Chen, L. & Zhao, X. M. Identifying dysregulated pathways in cancers from pathway interaction networks. *BMC Bioinformatics* (2012). doi:10.1186/1471-2105-13-126

20. Barrett, T. *et al.* NCBI GEO: Archive for functional genomics data sets - Update. *Nucleic Acids Res.* (2013). doi:10.1093/nar/gks1193

21. Huang, D. W., Sherman, B. T. & Lempicki, R. A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* (2009). doi:10.1038/nprot.2008.211

22. Galavotti, S. *et al.* The autophagy-associated factors DRAM1 and p62 regulate cell migration and invasion in glioblastoma stem cells. *Oncogene* (2013). doi:10.1038/onc.2012.111

23. Oba, S. *et al.* A Bayesian missing value estimation method for gene expression profile data. *Bioinformatics* (2003). doi:10.1093/bioinformatics/btg287

24. Bolstad, B. M., Irizarry, R. A., Åstrand, M. & Speed, T. P. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* (2003). doi:10.1093/bioinformatics/19.2.185

25. Subramanian, A. *et al.* Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci. U. S. A.* (2005). doi:10.1073/pnas.0506580102

26. Chu, L. H., Rivera, C. G., Popel, A. S. & Bader, J. S. Constructing the angiome: A global angiogenesis protein interaction network. *Physiol. Genomics* (2012). doi:10.1152/physiolgenomics.00181.2011

27. Tressel, S. L. *et al.* Pharmacology, biodistribution, and efficacy of GPCR-based pepducins in disease models. *Methods in molecular biology (Clifton, N.J.)* (2011).

doi:10.1007/978-1-60761-919-2_19

28. Pacini, L. *et al.* M2muscarinic receptors inhibit cell proliferation and migration in urothelial bladder cancer cells. *Cancer Biol. Ther.* (2014). doi:10.4161/15384047.2014.955740

29. Campa, D. *et al.* Polymorphisms of dopamine receptor/transporter genes and risk of non-small cell lung cancer. *Lung Cancer* (2007). doi:10.1016/j.lungcan.2006.11.007

30. Mansouri, S. *et al.* GalR3 activation promotes adult neural stem cell survival in response to a diabetic milieu. *J. Neurochem.* (2013). doi:10.1111/jnc.12396

31. Romero, G., Von Zastrow, M. & Friedman, P. A. Role of PDZ Proteins in Regulating Trafficking, Signaling, and Function of GPCRs. Means, Motif, and Opportunity. in *Advances in Pharmacology* (2011). doi:10.1016/B978-0-12-385952-5.00003-8

32. Shang, Y., Zhang, J. & Huang, E. J. HIPK2-mediated transcriptional control of NMDA receptor subunit expression regulates neuronal survival and cell death. *J. Neurosci.* (2018). doi:10.1523/JNEUROSCI.3577-17.2018

33. Ferguson, H. J. M. *et al.* Glutamate dependent NMDA receptor 2D is a novel angiogenic tumour endothelial marker in colorectal cancer. *Oncotarget* (2016). doi:10.18632/oncotarget.7812

34. Zamani, A. & Qu, Z. Serotonin activates angiogenic phosphorylation signaling in human endothelial cells. *FEBS Lett.* (2012). doi:10.1016/j.febslet.2012.05.047

35. Ding, M. *et al.* Enhancer RNA – P2RY2e induced by estrogen promotes malignant behaviors of bladder cancer. *Int. J. Biol. Sci.* (2018). doi:10.7150/ijbs.27151

36. Chu, L. H., Lee, E., Bader, J. S. & Popel, A. S. Angiogenesis interactome and time course microarray data reveal the distinct activation patterns in endothelial cells. *PLoS One* (2014). doi:10.1371/journal.pone.0110871

37. Huan, J. *et al.* Insights into significant pathways and gene interaction networks underlying breast cancer cell line MCF-7 treated with 17β-Estradiol (E2). *Gene* (2014). doi:10.1016/j.gene.2013.08.027

38. Wei, P., Tang, H. & Li, D. Insights into Pancreatic Cancer Etiology from Pathway Analysis of Genome-Wide Association Study Data. *PLoS One* (2012).

421 doi:10.1371/journal.pone.0046887

422 39. Myers, J. S., von Lersner, A. K. & Sang, Q. X. A. Proteomic upregulation of fatty acid
423 synthase and fatty acid binding protein 5 and identification of cancer- and
424 race-specific pathway associations in human prostate cancer tissues. *J. Cancer* (2016).
425 doi:10.7150/jca.15860

426 40. Shi, Z. *et al.* Exploring the key genes and pathways of osteosarcoma with pulmonary
427 metastasis using a gene expression microarray. *Mol. Med. Rep.* (2017).
428 doi:10.3892/mmr.2017.7577

429 41. Wei, L., He, F., Zhang, W., Chen, W. & Yu, B. Bioinformatics analysis of microarray
430 data to reveal the pathogenesis of diffuse intrinsic pontine glioma. *Biol. Res.* (2018).
431 doi:10.1186/s40659-018-0175-6

432 42. Bromberg-White, J. L. *et al.* Identification of VEGF-independent cytokines in
433 proliferative diabetic retinopathy vitreous. *Investig. Ophthalmol. Vis. Sci.* (2013).
434 doi:10.1167/iovs.13-12518

435 43. Yan, X. J. *et al.* Identification of outcome-correlated cytokine clusters in chronic
436 lymphocytic leukemia. *Blood* (2011). doi:10.1182/blood-2011-03-342436

437 44. Benevides, L. *et al.* IL17 promotes mammary tumor progression by changing the
438 behavior of tumor cells and eliciting tumorigenic neutrophils recruitment. *Cancer Res.*
439 (2015). doi:10.1158/0008-5472.CAN-15-0054

440 45. Mar, A. C. *et al.* Interleukin-1 receptor type 2 acts with c-Fos to enhance the
441 expression of interleukin-6 and vascular endothelial growth factor A in colon cancer
442 cells and induce angiogenesis. *J. Biol. Chem.* (2015). doi:10.1074/jbc.M115.644823

443 46. Wang, D. & DuBois, R. N. Measurement of Eicosanoids in Cancer Tissues. *Methods*
444 *in Enzymology* (2007). doi:10.1016/S0076-6879(07)33002-4

445 47. Zadka, Ł. *et al.* The expression of IL10RA in colorectal cancer and its correlation with
446 the proliferation index and the clinical stage of the disease. *Cytokine* (2018).
447 doi:10.1016/j.cyto.2018.04.030

448 48. Li, R., Wen, R., Banzon, T., Maminishkis, A. & Miller, S. S. CNTF mediates
449 neurotrophic factor secretion and fluid absorption in human retinal pigment

epithelium. *PLoS One* (2011). doi:10.1371/journal.pone.0023148

49. Recchia, F. M., Xu, L., Penn, J. S., Boone, B. & Dexheimer, P. J. Identification of genes and pathways involved in retinal neovascularization by microarray analysis of two animal models of retinal angiogenesis. *Investig. Ophthalmol. Vis. Sci.* (2010). doi:10.1167/iovs.09-4006

50. Matsumura, A. *et al.* HGF regulates VEGF expression via the c-Met receptor downstream pathways, PI3K/Akt, MAPK and STAT3, in CT26 murine cells. *Int. J. Oncol.* (2013). doi:10.3892/ijo.2012.1728

51. Ueda, M. & Saji, H. Radiolabeled probes targeting hypoxia-inducible factor-1-active tumor microenvironments. *Scientific World Journal* (2014). doi:10.1155/2014/165461

52. Xu, Z. *et al.* CCL19 suppresses angiogenesis through promoting miR-206 and inhibiting Met/ERK/Elk-1/HIF-1α/VEGF-A pathway in colorectal cancer. *Cell Death Dis.* (2018). doi:10.1038/s41419-018-1010-2

53. Liao, Y. Y. *et al.* CCL3 promotes angiogenesis by dysregulation of miR-374b/ VEGF-A axis in human osteosarcoma cells. *Oncotarget* (2016). doi:10.18632/oncotarget.6708

54. Karar, J. & Maity, A. PI3K/AKT/mTOR Pathway in Angiogenesis. *Front. Mol. Neurosci.* (2011). doi:10.3389/fnmol.2011.00051

55. Schreiber, T. H. & Podack, E. R. Immunobiology of TNFSF15 and TNFRSF25. *Immunol. Res.* (2013). doi:10.1007/s12026-013-8465-0

56. Song, J., So, T. & Croft, M. Activation of NF-κB1 by OX40 Contributes to Antigen-Driven T Cell Expansion and Survival. *J. Immunol.* (2008). doi:10.4049/jimmunol.180.11.7240

57. Jeong, W., Bae, H., Lim, W., Bazer, F. W. & Song, G. Differential expression and functional roles of chemokine (C-C motif) ligand 23 and its receptor chemokine (C-C motif) receptor type 1 in the uterine endometrium during early pregnancy in pigs. *Dev. Comp. Immunol.* (2017). doi:10.1016/j.dci.2017.07.007

58. Nakayama, T. *et al.* Eotaxin-3/CC Chemokine Ligand 26 Is a Functional Ligand for

CX3CR1. *J. Immunol.* (2010). doi:10.4049/jimmunol.0904126

59. Gregg, B., Lumeng, C. N. & Bernal-Mizrachi, E. Fractalkine signaling in regulation of insulin secretion. *Islets* (2014). doi:10.4161/isl.27861

60. Jo, S. Y. *et al.* CT perfusion can predict overexpression of CXCL8 (Interleukin-8) in head and neck squamous cell carcinoma. *Am. J. Neuroradiol.* (2013). doi:10.3174/ajnr.A3610

61. Kritharis, A., Al-Samkari, H. & Kuter, D. J. Hereditary hemorrhagic telangiectasia: Diagnosis and management from the hematologist's perspective. *Haematologica* (2018). doi:10.3324/haematol.2018.193003

62. Kochumon, S. *et al.* Adipose tissue expression of CCL19 chemokine is positively associated with insulin resistance. *Diabetes. Metab. Res. Rev.* (2019). doi:10.1002/dmrr.3087

63. Wang, H., Lafdil, F., Kong, X. & Gao, B. Signal transducer and activator of transcription 3 in liver diseases: A novel therapeutic target. *International Journal of Biological Sciences* (2011). doi:10.7150/ijbs.7.536

64. Mirando, A. C., Francklyn, C. S. & Lounsbury, K. M. Regulation of angiogenesis by aminoacyl-trna synthetases. *International Journal of Molecular Sciences* (2014). doi:10.3390/ijms151223725

65. Cane, G. *et al.* Up-regulation of intestinal vascular endothelial growth factor by Afa/Dr diffusely adhering Escherichia coli. *PLoS One* (2007). doi:10.1371/journal.pone.0001359

66. Liu, N. *et al.* Helicobacter pylori promotes angiogenesis depending on Wnt/beta-catenin-mediated vascular endothelial growth factor via the cyclooxygenase-2 pathway in gastric cancer. *BMC Cancer* (2016). doi:10.1186/s12885-016-2351-9

**Acknowledgments:** The authors are thankful to the Science and Engineering Research Board, Govt. of India (PDF/2016/001448), for financial support. The authors thank Prof. VR. Muthukkaruppan for the helpful discussion.

**Author Contributions:** Conceptualization, U.S., methodology, U.S., writing—original draft preparation, U.S., Investigation, B.D., writing—review and editing, B.D., supervision, B.D.

**Disclosure:** The authors declare no conflict of interest.

**Table(s)**

**Table 1.** List of dysregulated pathways enriched in retinal angiogenic disease samples compared to normal control samples.

| Dysregulated pathway | Normalized enrichment score | p value |
|---|---|---|
| Neuroactive ligand receptor interaction | 3.101 | 0.000 |
| Cytokine cytokine receptor interaction | 2.553 | 0.000 |
| Arachidonic acid metabolism | 1.931 | 0.007 |
| Aminoacyl tRNA biosynthesis | -1.572 | 0.042 |
| Tight junction | -1.631 | 0.042 |
| Pathogenic Escherichia coli infection | -1.646 | 0.032 |
| Cysteine and methionine metabolism | -1.667 | 0.035 |
| Vibrio cholerae infection | -1.753 | 0.018 |
| Endocytosis | -1.768 | 0.014 |
| Regulation of actin cytoskeleton | -1.872 | 0.012 |
| Epithelial cell signaling in helicobacter pylori infection | -1.873 | 0.016 |
| Arrhythmogenic right ventricular cardiomyopathy | -1.886 | 0.011 |
| p53 signaling pathway | -1.892 | 0.008 |
| Protein export | -1.909 | 0.004 |
| Lysosome | -1.925 | 0.004 |
| Adherens junction | -2.040 | 0.002 |
| Spliceosome | -2.094 | 0.004 |
| Alzheimers disease | -2.108 | 0.000 |
| Parkinsons disease | -2.324 | 0.002 |

| | | |
|---|---|---|
| Huntingtons disease | -2.494 | 0.000 |
| Oxidative phosphorylation | -2.579 | 0.000 |
| Ribosome | -4.047 | 0.000 |

515

## Figures

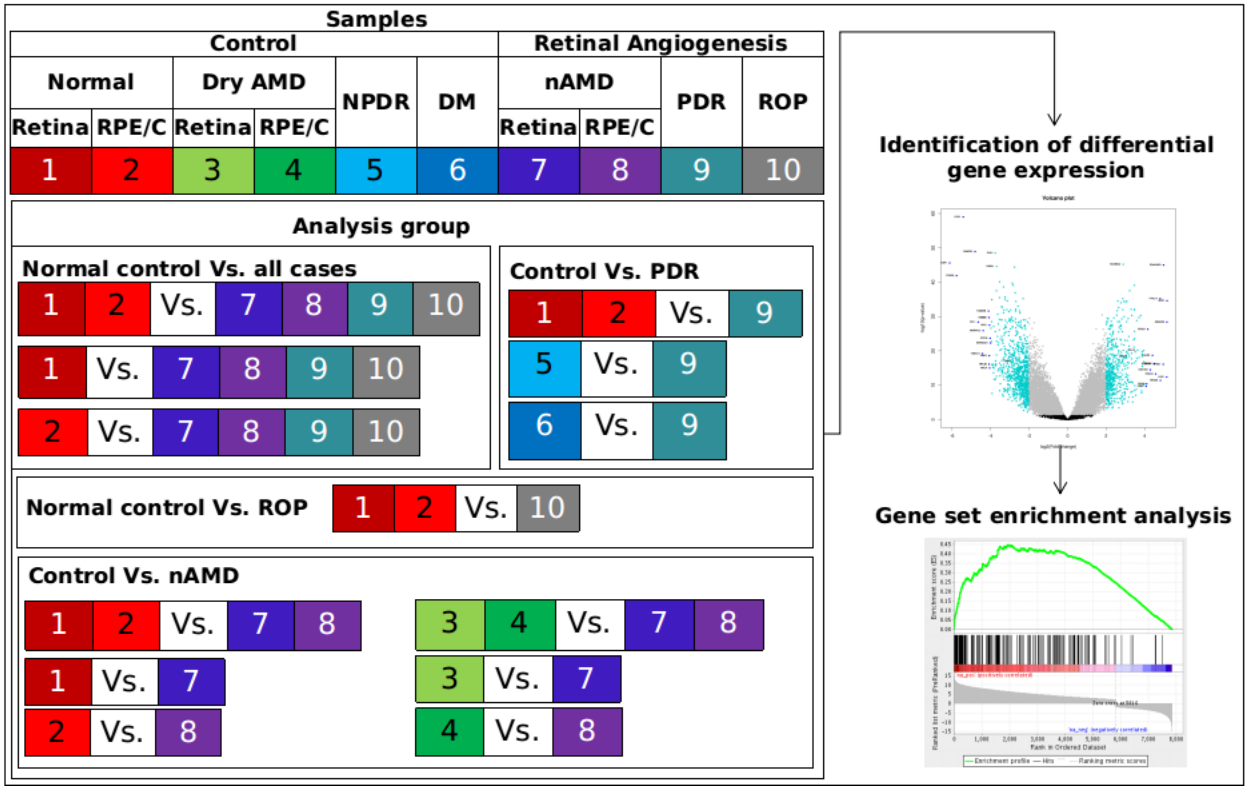


**Figure 1.** Schema of the workflow - samples, analysis groups used, and the methods followed

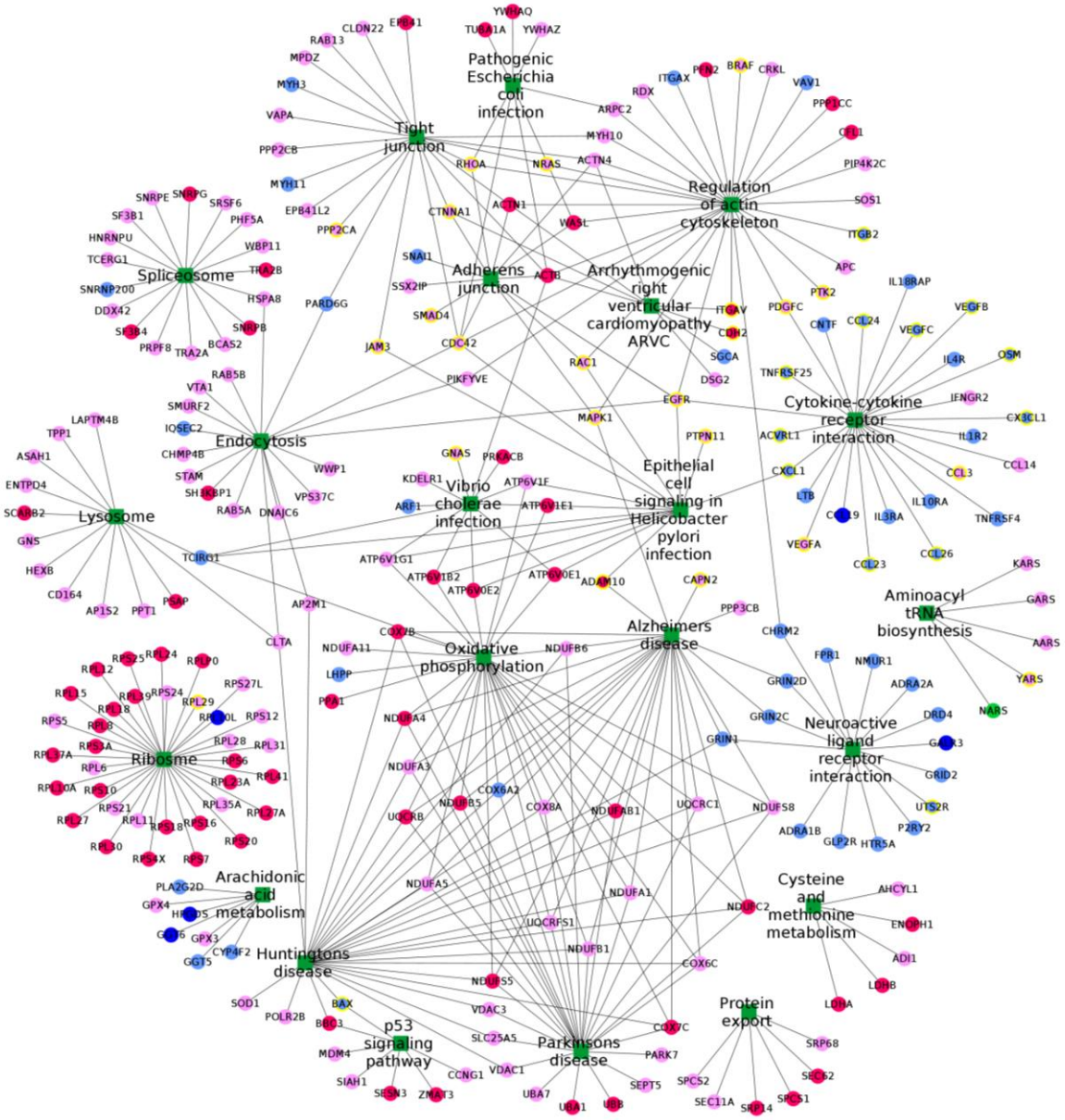


**Figure 2**. Dysregulated pathway interaction network for the deferentially expressed genes (DEGs). The green node represents the dysregulated pathways, pink represents DEGs with fold change between -3 and -2, red represents FC ≤ -3, pale blue represents FC between 2 and 3, dark blue represents FC ≥ 3. The round edged square shape of node represents pathways, circle represents DEGs, and yellow circle represents DEGs associated with angiogenesis GO.

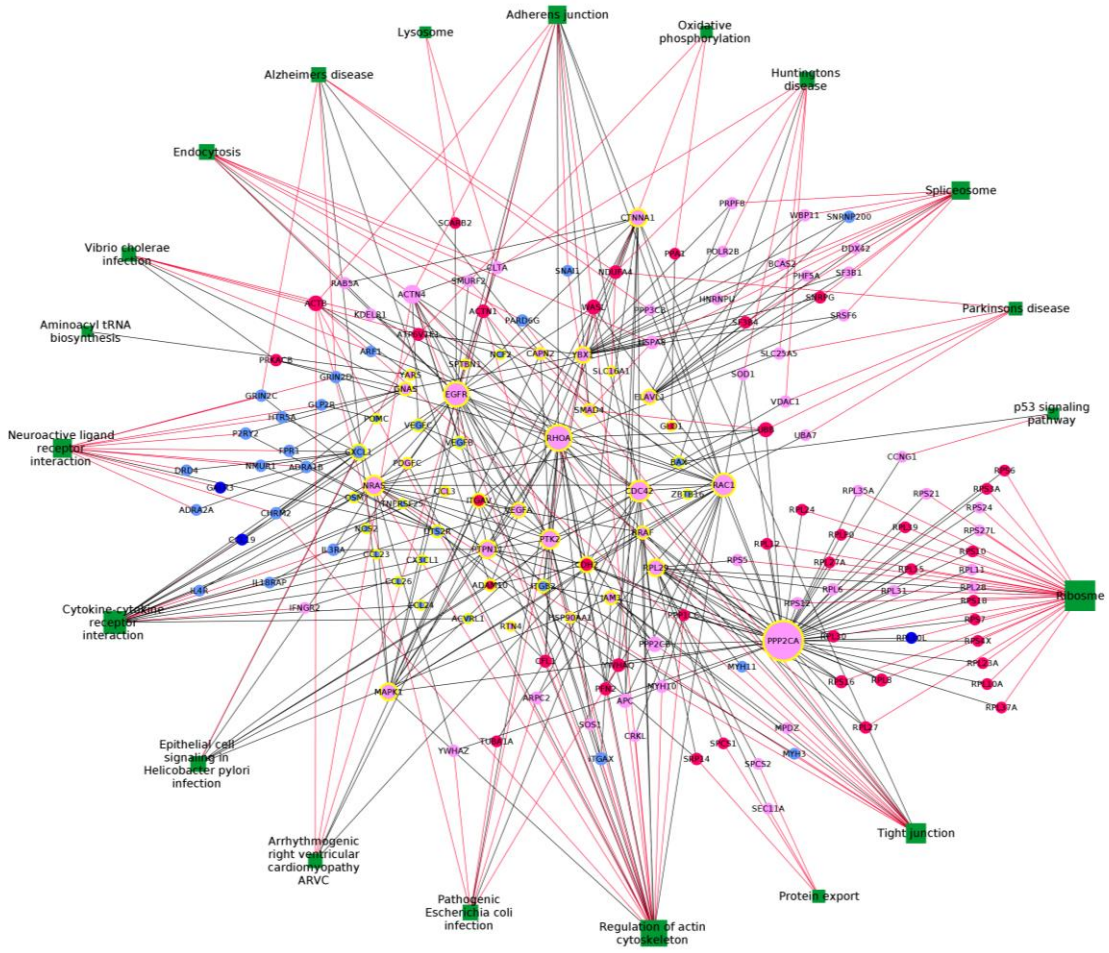


**Figure 3.** PPI network of core angiogenesis GO related differentially expressed genes and their PPI partners with the interaction of dysregulated pathways. The green node represents the dysregulated pathways, pink represents DEGs with fold change between -3 and -2, red represents FC ≤ -3, pale blue represents FC between 2 and 3, dark blue represents FC ≥ 3. The square shape of node represents pathways, circle represents DEGs, and yellow circle represents DEGs associated with angiogenesis GO. Edges red in colour represents the interaction between the pathway and the PPI partners of the DEGs which associated with angiogenesis. The size of the node corresponds to the degree i.e. number of edges linked to that node.

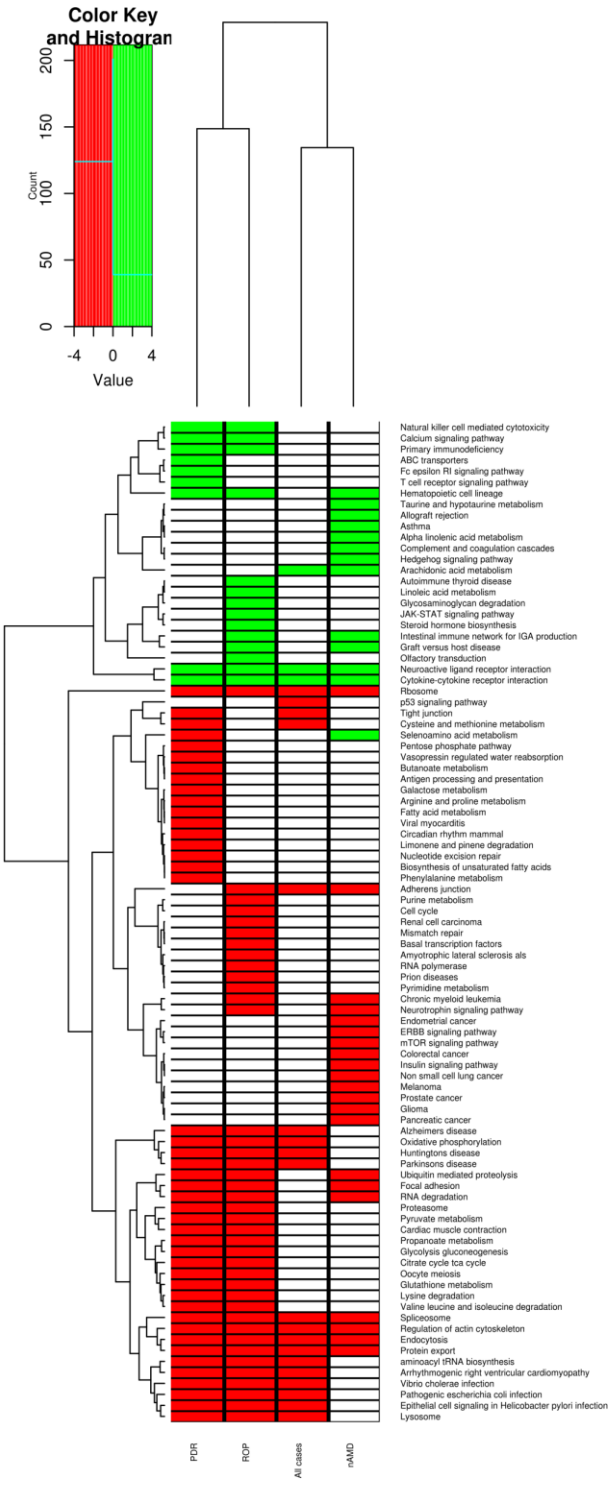


**Figure 4.** Clustering annotation with heat map of dysregulated pathways in retinal angiogenesis related diseases

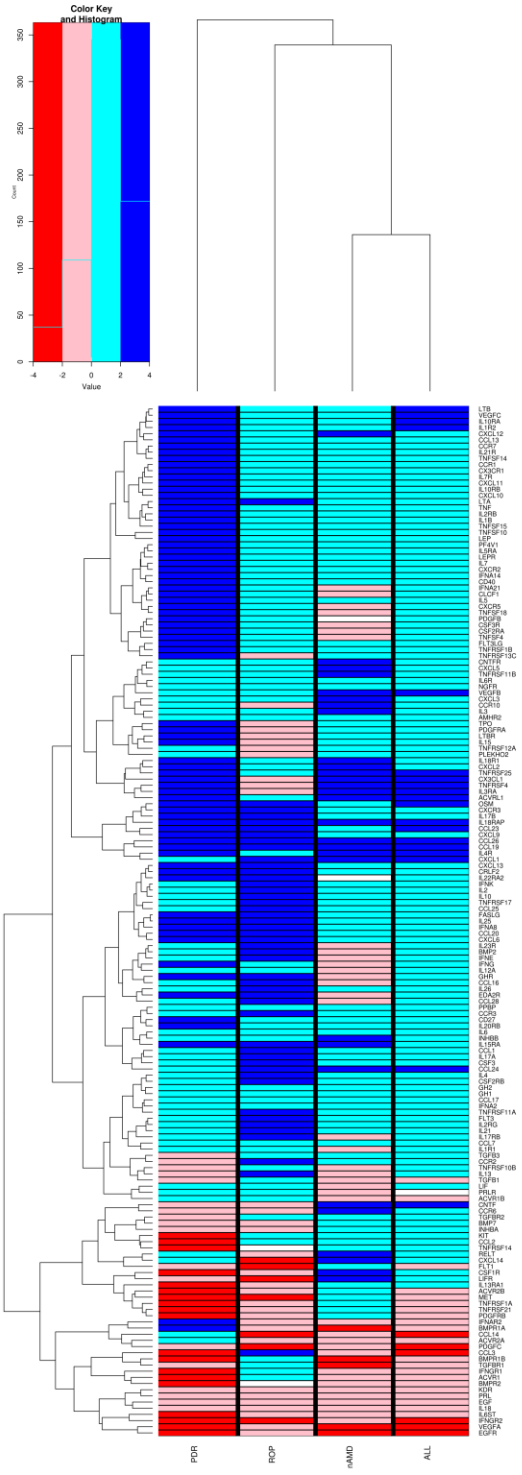


**Figure 5**. Heat map of DEGs in cytokine-cytokine receptor interaction pathway in retinal angiogenic related diseases

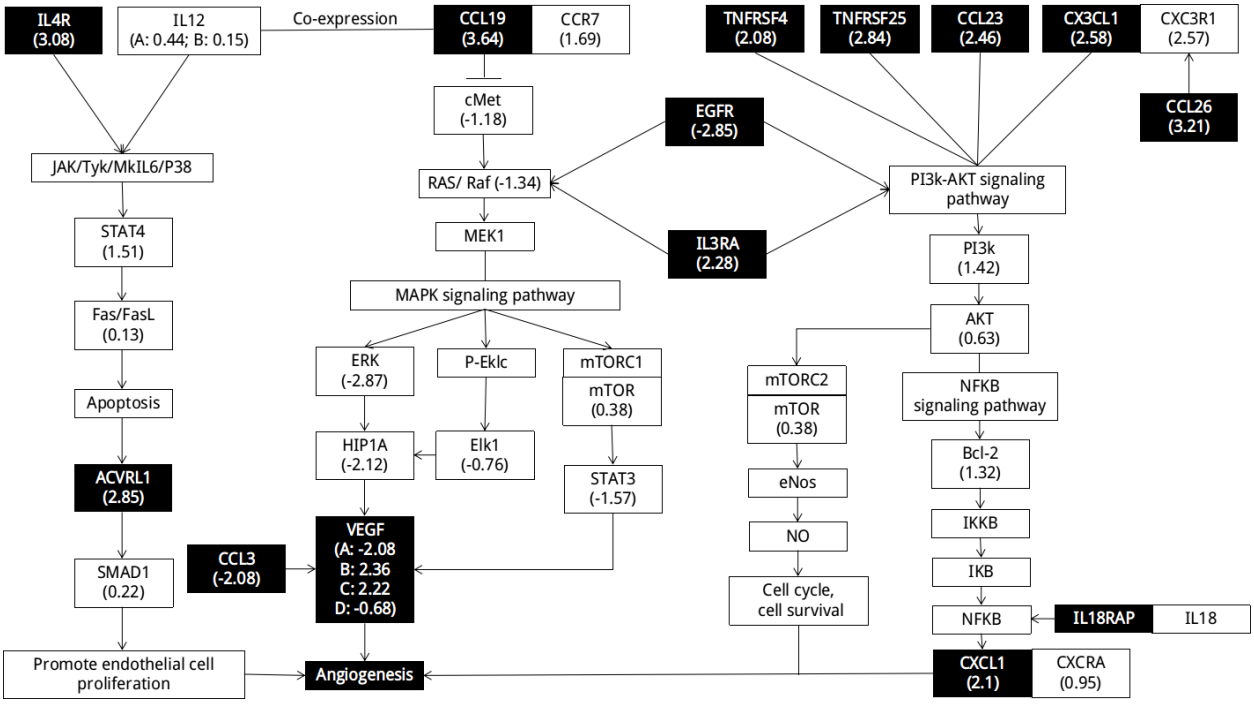


**Figure 6.** Regulatory role of DEGs in cytokine-cytokine receptor interaction pathway. Dark boxes indicate the DEGs identified in the retinal angiogenesis samples used in this study.