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# Identifying Novel Molecular Mechanisms Involved in Clotting Factor VIII (F8) Expression in Endothelial Cells as a Measure of Hemophilia Susceptibility

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**Abstract**

***Aim:*** *This study aims to identify the differential gene expression for liver and pulmonary endothelial cells, and find out genes that are upregulated and down regulated to analyze the interactions and gene enrichment.* ***Materials and methods:*** *In the present study, we have retrieved the dataset GSE139993 from NCBI Gene expression omnibus (GEO) database and compare F8 dysregulated cell type (PMEC) and F8 upregulated cell type (LSEC) to dissect the novel molecular level interactions and pathways.* ***Results:*** *A total of 11198 significantly differentially expressed genes (SG), 5 upregulated genes (UG) and 11 down regulated genes (DG) were identified. Among all the UGs and DGs, COL1A1, COL3A1, COL6A1 and POSTN were highly interconnected. Pathways identified were in accordance with the identified gene network, relating to hemophilia.* ***Conclusion:*** *Thus, we have identified that pathways and genes related to cell adhesion and platelet activity are dysfunctional in cell types with F8 under expression. This might be considered as factors that enhance the adverse effect of hemophilia in these cell types.*

**Key-words:** Hemophilia, Factor VIII, Endothelial Cells, Expression Profiling, Gene Enrichment, Novel Molecular Mechanism, Molecular Biology, Genetic Analysis, Gene Expression.

## 1. Introduction

Hemophilia is one of the most prevalent diseases across the world with India having the second-most number of patients (Kar et al. 2014). Deficiency in the production of F8 is either inherited or caused by genetic mutations. F8 has a prominent role in coagulation of blood and its deficiency leads to poor clotting of blood (Oldenburg and El-Maarri 2006). LSECs contribute to the

production of F8 as proven by earlier literature (Everett et al. 2014; Shahani et al. 2014; Fahs et al. 2014). Therapeutic approaches to treat this condition are limited and costly, which usually consist of replacement therapies or injecting F8 protein intravenously. Gene therapies provide a better cure compared to existing approaches (Peyvandi and Garagiola 2019). The data from this study can be applied to find the differences in expression levels of liver and pulmonary endothelial cells, identify the genes causing hemophilia, develop lead compounds and drug targets to treat hemophilia. This data aids in metabolomics studies by identifying the biological pathways and processes that are altered due to hemophilia (Fahs et al. 2014).

Computational biology techniques allow us to perform expression profiling, comparative analysis, identify genetic variations, analyze the genes, etc. which help us understand the underlying molecular mechanisms of genes, proteins and diseases ((Barrett et al. 2012; Edgar, Domrachev, and Lash 2002)). Expression profiling of chosen samples can be obtained through techniques such as microarray, DNA sequencing, chromatin immunoprecipitation sequencing (ChIp seq), etc. The experimental data obtained through these experiments is stored in biological databases. NCBI GEO stores gene expression datasets obtained from various computational biology techniques (Barrett et al. 2012; Edgar, Domrachev, and Lash 2002). Differentially expressed genes (DEGs) and biological pathways that might trigger hemophilia have been identified owing to the comprehensive utilization of DNA sequencing methods, data profiling and gene detection methods. Jamil M et al., gathered samples of blood from various endothelial cells to identify DEGs (Jamil et al. 2020).

Previously our team has a rich experience in working on various research projects across multiple disciplines (Sathish and Karthick 2020; Varghese, Ramesh, and Veeraiyan 2019; S.R. Samuel, Acharya, and Rao 2020; Venu, Raju, and Subramani 2019; M. S. Samuel et al. 2019; Venu, Subramani, and Raju 2019; Mehta et al. 2019; Sharma et al. 2019; Malli Sureshbabu et al. 2019; Krishnaswamy et al. 2020; Muthukrishnan et al. 2020; Gheena and Ezhilarasan 2019; Vignesh et al. 2019; Ke et al. 2019; Vijayakumar Jain et al. 2019; Jose, Ajitha, and Subbaiyan 2020). Now the growing trend in this area motivated us to pursue this project.

Nevertheless, the interactions among DEGs and the main genes engaged in hemophilia causing pathways are yet to be interpreted. Surprisingly, research has revealed that gene expression networks linked to the disease can play a part in the immune response, accentuating the biological processes and potential to treat hemophilia (Jin et al. 2009). Microarray and expression data of the genes were present in the existing research regarding the current topic. However, analysis for interactions among them and their enrichment have not been performed by anyone. Therefore, in the present study, we compared two different samples of fetal endothelial cells retrieved from GEO

database with ID GSE139993 and performed gene – gene interaction studies and gene enrichment analysis with STRING and FunRich respectively, which might be helpful to identify novel lead compounds and drug targets for hemophilia.

## 2. Materials and Methods

### 2.1 Dataset

Microarray data of gene expression profiling analysis for GSE139993 was obtained from the GEO database of NCBI. GEO database comprises public functional genomics data, obtained from various experiments such as DNA sequencing, RNA sequencing, microarray, Chromatin immunoprecipitation (ChIp) sequencing and high-throughput hybridization array (Edgar, Domrachev, and Lash 2002). GSE139993 has 32 samples with adult and fetal endothelial cells of liver, umbilical vein, microvascular endothelial cells of lung and heart, and arterial endothelial cells of the heart (Jamil et al. 2020). A total of 12 samples were taken comprising 2 groups, LSEC (6 samples) and PMEC (6 samples) shown in Table 1.

### 2.2 Identification of Significant Genes

GEO2R is an online interactive tool that compares the samples to identify DEGs. Samples from the 2 groups were analyzed using the GEO2R online tool. A total of 14086 DEGs were identified. P-value cut off was set to greater than 0.05 to identify the significant DEGs (SG).

### 2.3 Classification of Upregulated and Downregulated Genes

A cutoff of log FC $\geq 1$ and log FC $\leq -1$ was used to separate the UG and DG respectively, from the SG.

### 2.4 Gene – Gene interaction

Search Tool for the Retrieval of Interacting Genes (STRING) database was used to find any known functional gene association networks present in the GSE139993 dataset (Szklarczyk et al. 2019; von Mering et al. 2003). The SGs were given as input in STRING. The gene interactions were obtained from STRING and were incorporated into Cytoscape software to visualize the interactions among the genes.

### 2.5 Refined Gene Enrichment Analysis

Functional Enrichment was observed by performing gene ontology using FunRich (v3.1.3). The SGs were entered in FunRich to get various gene enrichment analyses, such as cellular component, molecular function, biological process, biological pathway, protein domain, site of expression, transcription factor, clinical phenotype and COSMIC (Fonseka et al. 2020).

### 2.6 Hub Genes Identification

Cytoscape software analyzes the interconnected regions called networks from the given input of genes (Shannon et al. 2003). The STRING interactions were exported from the STRING database and subjected to Cytoscape for analysis. MCODE plug-in of Cytoscape finds highly interconnected regions, known as clusters, in a network (Bader and Hogue 2003). The SGs were entered in MCODE to identify the clusters among them. The degree cutoff value in network scoring was set to 2 while the node score cutoff, K-Core and max. Depth values were set to 0.2, 2 and 100 respectively.

### 2.7 Overall Gene Enrichment

ClueGo plug-in of Cytoscape enables the search for novel markers that are potentially linked to biological pathways. It analyzes the input of genes to create an assembled network of GO and pathways by integrating GO terms with KEGG and BioCarta pathways (Bindea et al. 2009). The genes from STRING interactions were analyzed using ClueGO to identify the GO/pathway network. Ontologies and pathways selected were Chromosomal location, Molecular function, Cellular component, Biological process, Protein domains, KEGG, REACTOME pathways. The GO tree interval was set from 2 to 8, and GO term/pathway selection was set from 2 to 4% genes.

## 3. Results

### 3.1 Analysis of samples for identification of DEGs

This study contains the microarray analysis of expression profiling of genes from the GSE139993 dataset from the GEO database, provided by Jamil et al. which was presented in Table 1. The dataset comprises 32 samples, out of which 12 samples were subjected to analysis. GEO2R is used to obtain DEGs by analyzing the samples by comparing them. A total of 14086 DEGs were obtained. P – values and log FC values were calculated to identify the DEGs.

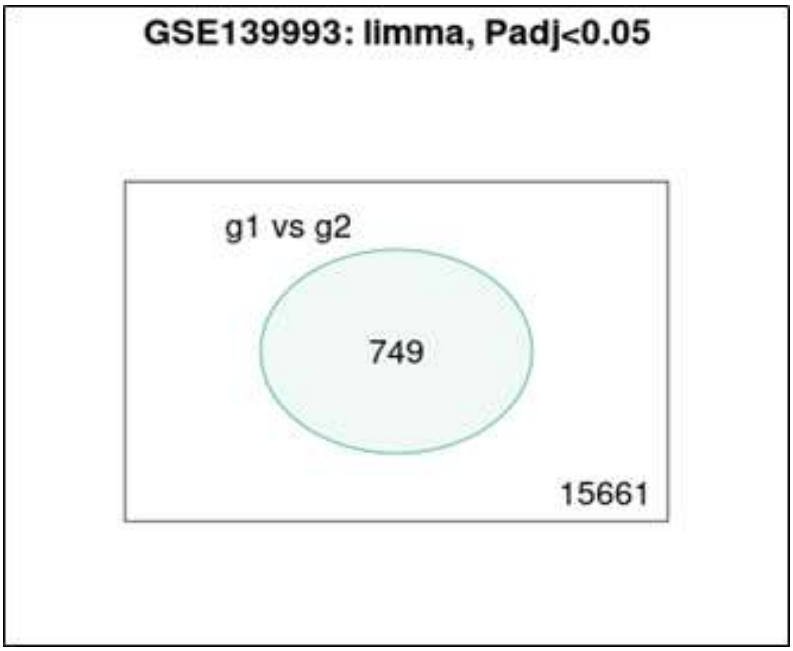
Table 1 - Retrieving Gene Expression Dataset of LSEC and PMEC from GSE139993 Dataset of NCBI GEO Database

| Group | No. of samples | Accession | Title | Source name |
|---|---|---|---|---|
| 1 | 6 | GSM4151253 | LSECs fetal 10691 rep1 | Liver sinusoidal endothelial cells |
| | | GSM4151254 | LSECs fetal 10691 rep2 | |
| | | GSM4151255 | LSECs fetal 11359 rep1 | |
| | | GSM4151256 | LSECs fetal 11359 rep2 | |
| | | GSM4151257 | LSECs fetal 11605 rep1 | |
| | | GSM4151258 | LSECs fetal 11605 rep2 | |
| 2 | 6 | GSM4151271 | HPMEC 10298 rep1 | Human pulmonary microvascular endothelial cells |
| | | GSM4151272 | HPMEC 10298 rep2 | |
| | | GSM4151273 | HPMEC 11415 rep1 | |
| | | GSM4151274 | HPMEC 11415 rep2 | |
| | | GSM4151275 | HPMEC 11844 rep1 | |
| | | GSM4151276 | HPMEC 11844 rep2 | |

### 3.2 Identification of Significant Genes

Common DEGs which were considered as significant DEGs were obtained from the Venn diagram which was a result of GEO2R analysis. The Venn diagram (Fig. 1) represents the DEGs that are common to the combination of group 1 vs group 2. These DEGs that are common to all the samples belonging to the 2 groups taken were analyzed. A total of 11198 significant DEGs were identified as common to all the samples belonging to the 2 groups analyzed.

Fig. 1 - Venn Diagram Representing the Common DEGs between Group 1 and Group 2, Out of total Genes. An Adjusted P-Value (Padj<0.05) was Used and a Total of 749 Genes were Found to be Common between the Groups. (g1: LSEC, g2: PMEC)



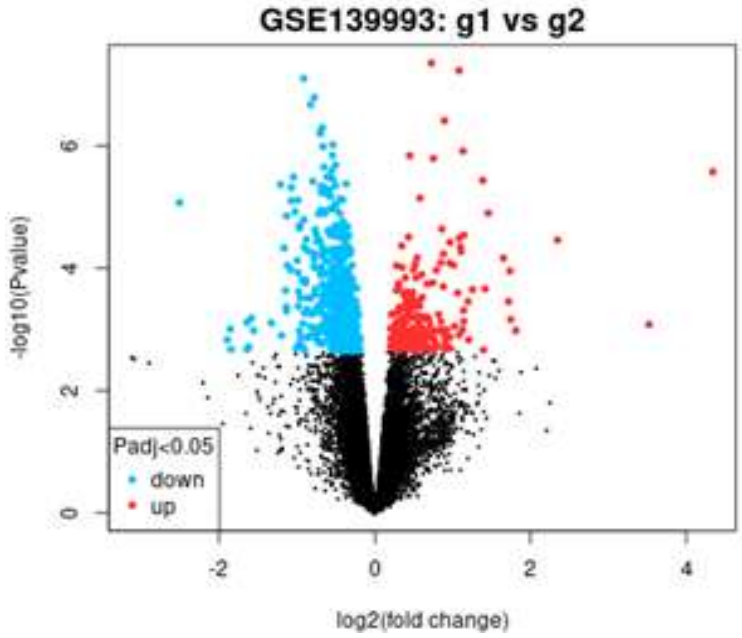

### 3.3 Classification of Upregulated and Downregulated Genes

A cutoff of log FC ≥ 1 and log FC ≤ −1 was used to separate the upregulated and downregulated DEGs respectively, from the significant DEGs (Fig. 2). A total of 5 UG and 8 DG were identified which are mentioned in Table 2.

Table 2 - Upregulated (UG) and Downregulated (DG) Genes Identified Using the Appropriate log FC cutoff (log FC ≥ 1 and log FC ≤ −1).

| ID | UG/DG | Entrez ID | Gene symbol | Gene title |
|---|---|---|---|---|
| ILMN_1773079 | UG | 1281 | COL3A1 | Collagen type III alpha 1 chain |
| ILMN_2150856 | UG | 5055 | SERPINB2 | Serpin family B member 2 |
| ILMN_2171384 | UG | 6374 | CXCL5 | C-X-C motif chemokine ligand 5 |
| ILMN_1701308 | UG | 1277 | COL1A1 | Collagen type I alpha 1 chain |
| ILMN_1732151 | UG | 1291 | COL6A1 | Collagen type VI alpha 1 chain |
| ILMN_1670490 | DG | 10630 | PDPN | Podoplanin |
| ILMN_1800642 | DG | 5649 | RELN | Reelin |
| ILMN_2170814 | DG | 27074 | LAMP3 | Lysosomal associated membrane protein 3 |
| ILMN_2157099 | DG | 8900 | CCNA1 | Cyclin A1 |
| ILMN_1790761 | DG | 10631 | POSTN | Periostin |
| ILMN_2196328 | DG | 10631 | POSTN | Periostin |
| ILMN_1678841 | DG | 10537 | UBD | Ubiquitin D |
| ILMN_1805807 | DG | 7781 | SLC30A3 | Solute carrier family 30 member 3 |

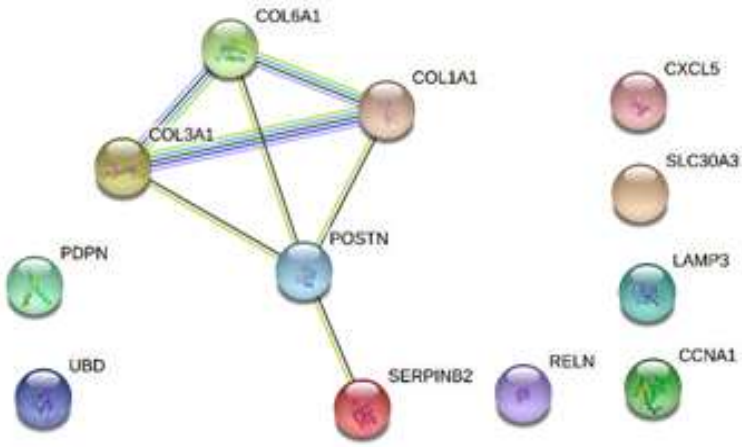
Fig. 2 - Volcano Plot for Differentially Expressed Genes (DEGs) in LSEC vs PMEC from the GSE139993 Dataset. Black Dot Represents Commonly Expressed Genes. Blue Dots Represent Genes that are Overexpressed in LSEC and Red Dots are Genes Overexpressed in PMEC




### 3.4 Gene – Gene Interaction

The UGs and DGs were combinedly given as input to the STRING database, in the multiple proteins input box by selecting *Homo sapiens* as the organism. 4 interactions were identified among the given input of genes as seen in Fig. 3. This tells us that COL1A1, COL3A1, COL6A1, SERPINB2 and POSTN genes have known genetic interactions such as co-occurrence across genomes and putative homologs.

Fig. 3 - Gene – Gene Interaction Analysis of UGs and DGs Combined, Performed Using STRING Database





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### 3.5 Refined Gene Enrichment Analysis

FunRich analysis of the common genes identified 8 genes to be located in the extracellular region, 2 genes involved in extracellular matrix structural constituent, 4 genes involved in Immune response, 4 genes involved in Integrin family cell surface interaction pathway, 9 genes are present in the signal peptide domain, 11 genes are expressed in the plasma, 4 genes are related to transcription factor – nuclear factor 1 C (NFIC), set of 2 genes each present in head and neck, and eyes; 14 genes are prone to somatic mutations in the large intestine. This data has been represented in table 3. The gene enrichment analysis identified that obtained differential gene expression of COL3A1; SERPINB2; CXCL5; COL1A1; COL6A1; PDPN; RELN; LAMP3; CCNA1; POSTN; UBD; SLC30A3 genes were involved in the risk of pathogenesis of cancer of the large intestine, and were involved in the risk of suffering from hemophilia.

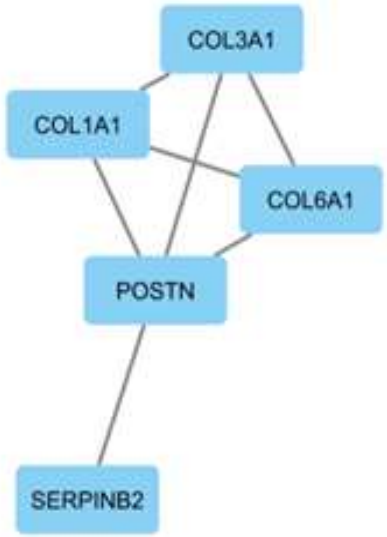
Table 3 - Gene Enrichment Analysis of UG (Upregulated) and DG (Downregulated) Using FunRich

| **Analysis** | **Analysis detail** | **No. of genes in the dataset** |
|---|---|---|
| Cellular component | Extracellular region | 6 |
| Molecular function | Extracellular matrix structural constituent | 3 |
| Biological process | Protein metabolism, cell growth/maintenance, cell communication, signal transduction | 3 each |
| Biological pathway | Integrin family cell surface interactions, Beta1 integrin cell surface interactions | 3 each |
| Protein domain | Signal peptide | 7 |
| Site of expression | Plasma | 11 |
| Transcriptio n factor | NFIC | 4 |
| Clinical phenotype | Autosomal dominant, Cardiovascular, Ears, Face, Feet, Growth, Head and Neck, Heart, Mouth, Prenatal Manifestations, Respiratory, Skeletal, Skin, Skin/Nails/Hair | 3 each |
| COSMIC | Large intestine | 12 |

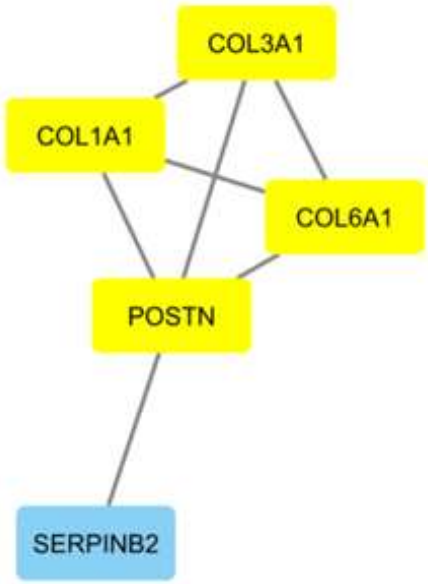
### 3.6 Hub Genes Identification

The HUB gene identification using Cytoscape and MCODE analysis produced COL1A1, COL3A1, COL6A1, SERPINB2 and POSTN genes as highly interacting gene clusters (Fig. 4).

Fig. 4 - HUB Genes Identification Using (a) Cytoscape Analysis and (b) MCODE Analysis was Performed to Identify the Gene Network from STRING Interactions




(a)



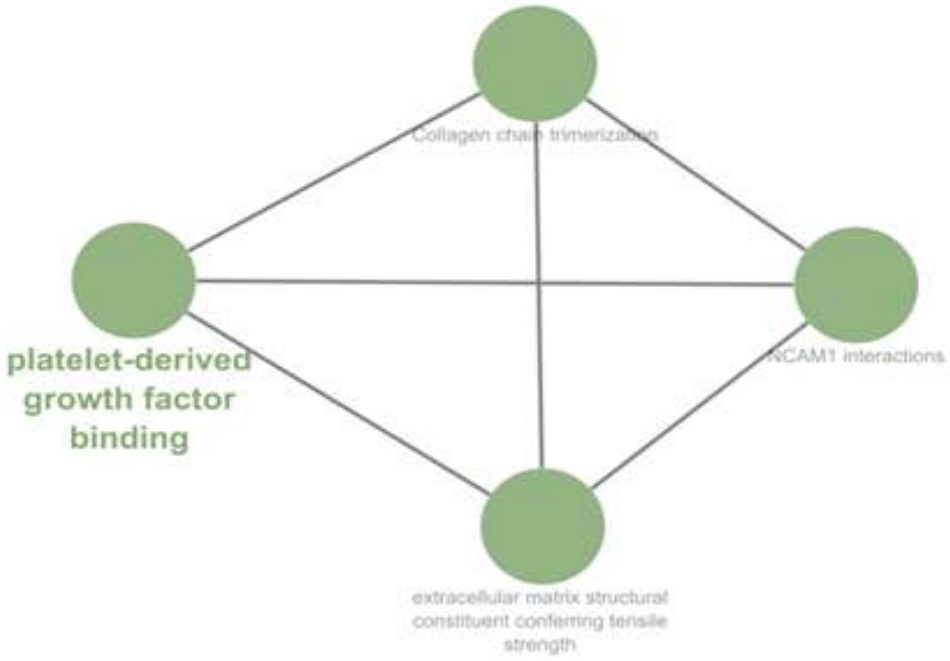

(b)

### 3.7 Overall Gene Enrichment

From the given input of the marker list of genes from STRING interactions, ClueGo plugin has found out the biological pathways that are linked to the input genes. The GO and pathway enrichment analyses of the hub genes depicted that they were mainly enriched in platelet-derived

growth factor binding, collagen chain trimerization, NCAM1 interactions and extracellular matrix (ECM) structural constituent conferring tensile strength pathways which are linked to the markers given as input, which is represented in (Fig. 5). This depicts that the genes were enriched in crucial pathways that are mostly related to platelet-derived growth factor binding.

Fig. 5 - ClueGo Analysis Showing the Potential Biological Pathways that are Linked to Markers/Genes Given as Input




## 4. Discussion

The microarray data of the GSE139993 gene expression profiling was retrieved from the gene expression omnibus (GEO) database and the samples were analyzed using GEO2R to identify the differentially expressed genes (DEGs) among adult and fetal human LSEC and PMEC. A total of 11198 significantly differentially expressed genes (SG), 5 upregulated genes (UG) and 8 downregulated genes (DG) were identified from the GEO2R analysis from the pool of genes which did not show difference in LogFC. COL1A1, COL3A1, COL6A1, SERPINB2 and POSTN genes shown genetic interactions in STRING and Cytoscape whereas COL1A1, COL3A1, COL6A1 and POSTN genes were identified as a cluster by MCODE. ClueGo has identified platelet-derived growth factor binding, collagen chain trimerization, NCAM1 interactions and extracellular matrix (ECM) structural constituent conferring tensile strength pathways to be associated with the gene network.

The Gene ontology (GO) annotations of COL1A1 include identical protein binding and platelet-derived growth factor binding; COL3A1 include integrin binding and SMAD binding; COL6A1 include platelet-derived growth factor binding; POSTN include heparin binding and cell adhesion molecule binding; SERPINB2 include serine-type endopeptidase inhibitor activity and takes part in blood coagulation and signalling pathways. From the GO annotations and MCODE analysis of the genes (Fig. 5), we can say that COL1A1, COL3A1, COL6A1 and POSTN genes are highly interconnected to each other which justifies the MCODE analysis obtained. Parallelly, the ClueGo analysis was also in accordance with the other analyses as it shows that platelet-derived growth factor binding, collagen chain trimerization, NCAM1 interactions and extracellular matrix (ECM) structural constituent conferring tensile strength pathways were associated with the network of genes.

The F8 gene is involved in collagen chain trimerization pathway and formation of fibrin clot pathway according to the data available from gene cards (Stelzer et al. 2016). COL1A1, COL3A1, COL6A1 genes were observed to be involved in collagen chain trimerization pathway and SERPINB2 was involved in formation of fibrin clot pathway, proving their relevance to the F8. The analyses performed identified the DEGs, biological pathways and their interactions which are related to F8 production that can be targeted for developing therapeutic approaches for treating hemophilia.

The network of interacting genes obtained from STRING and Cytoscape analyses has shown that they are related to the production of F8 factor by LESCs whereas some studies point out the connections of these genes to other diseases/disorders. An extremely rare haplotype in the upstream regulatory (5-prime) region of COL1A1 genesis linked to reduction of bone quality and fracture of the hip (Jin et al. 2009). COL3A1 gene is susceptible to Chinese sporadic intracranial aneurysm (Hua et al. 2008). COL6A1 is related to diffuse idiopathic skeletal hyperostosis and ossification of the posterior longitudinal ligament according to a study conducted by Tsukaharaet. al. (Tsukahara et al. 2005). POSTN was observed to be expressed by the mesenchymal stromal cells in vitro (Coutu et al. 2008). SERPINB2 regulates phagocytosis and migration of macrophages as a response by the immune system in kidney injury (Sen et al. 2020). However, the association of these genes to the production of F8 was not available in existing literature and was discovered through the analyses performed in this study.

Our institution is passionate about high quality evidence based research and has excelled in various fields ((Vijayashree Priyadharsini 2019; Ezhilarasan, Apoorva, and Ashok Vardhan 2019; Ramesh et al. 2018; Mathew et al. 2020; Sridharan et al. 2019; Pc, Marimuthu, and Devadoss 2018; Ramadurai et al. 2019). We hope this study adds to this rich legacy.

The major limitation of our study is that the bioinformatics data derived from the tools needs to be confirmed by wet lab experiments to prove actual gene co-expression and interaction in predicted pathways.

From this study, it is evident that these pathways can be targeted and the underlying mechanisms of the hub genes can be studied further with keeping in mind the enrichment analysis obtained with FunRich. This study provides a path towards discovering potential leads for the treatment of hemophilia and aids in comprehending the underlying novel molecular mechanisms.

## 5. Conclusion

LESCs produce F8 which when produced in inadequate amounts leads to poor clotting of blood. The gene enrichment analysis manifested that most of the genes were present at the extracellular region; the highest number of genes were expressed in the plasma; many genes were involved in cell communication and metabolism processes along with various other enrichment analyses. This study has identified 5 upregulated and 8 downregulated genes, their interaction and the pathways related to the DEGs from pathway enrichment analysis. Indeed, the enrichment study showed pathways that were closely linked to haemophilia. This study provides a path towards discovering potential leads for the treatment of hemophilia and aids in comprehending the underlying novel molecular mechanisms.

## Declarations

### Conflict of Interest

The authors of this paper declare no conflict of interest.

### Author Contribution

Author DV was involved in data collection, data analysis, manuscript writing. Author MD was involved in conceptualization, guidance and critical review of manuscript.

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