Original Article

# Transcriptomic Analysis of Human Retina Reveals Molecular Mechanisms Underlying Diabetic Retinopathy in Sexually Divergent Manner

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## Abstract

**Background:** Today, retinopathy is one of the major causes of vision loss. With the increasing prevalence of obesity, diabetes, blood fat, and hypertension, the number of patients with retinopathy is increasing. Gender is an important factor in a variety of retinal diseases but has rarely been studied in clinical and biological studies. The current understanding of the effect of gender on molecular changes and pathways involved in the onset and progression of diabetic retinopathy (DR) is limited. This study aims to investigate the differences in Diabetic patients' retinal gene expression between the two sexes.

**Material and Methods:** Through reanalyzing publicly available RNA-sequencing data (GSE160306), comprised of 40 post-mortem samples from 20 patients with diabetic macular edema (DME) stage of DR. Totally 29 females and 11 males had been included in the dataset. In addition, samples include 20 DME patients and 20 age matched healthy controls. Differentially expressed genes (DEGs) between males and females were retrieved utilizing EdgeR package in R. Then the enrichment analysis was performed on the up-regulated genes using CluGO plugin in Cytoscape software.

**Reustls:** Totally, in DME stage of DR 243 genes were differentially expressed ($P < 0.05$) between males and females comprised of 196 up-regulated and 48 down-regulated genes. Most up-regulated genes were enriched in pathways involved in Osteoclast-associated receptor (OSCAR) binds collagen and Surfactant protein D (SP-D), apoptosis, and tyrosine metabolism molecular functions.

**Conclusion:** According to the results, there are genes that are differentially expressed between male and female suffering DME cases. This suggests that disease is gender dependent. In this case the molecular mechanism underlying the disease might differ in females compared to males. For instance, the de-regulated genes in females are involved in Tyrosine metabolism, which has not been observed in males' de-regulated pathways. Further experimental studies are suggested to validate these results.

**Keywords:** Diabetic Retinopathy; Damaged Macular Edema; Sex Differences; Transcriptomic Analysis.

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## Introduction

Diabetes is an important epidemic that is accompanied by mortality. One of the most common complications of this disease is diabetic retinopathy (DR). Retinopathy is a microvascular complication that develops over time [1]. Under these conditions, the capillaries become swollen and secrete fluid or impair the growth of extra blood vessels at the surface of the retina. The onset of retinopathy may be latent, but gradual decrease in vision can eventually lead to blindness [2]. This disease is the main cause of blindness in people over 20 years old [3] DR is often noticed when people face a vision problem then see a physician [3].

One in three patients with diabetes develops DR. DR stages are divided into non-proliferative, proliferative DR, and diabetic macular edema in which there is growth of new retinal blood and discharge and edema in the central retina. The severity of DR is strongly related to periods of diabetes, hyperglycemia, and hypertension. Initially, DR was thought to be a vascular disease, but recent research has shown that retinal nerve damage is also involved [2, 4] .

The Early Treatment DR Study (ETDRS) is a standardized classification scheme. ETDRS and other classification systems are the basis for understanding and management of DR [4, 5].

First stage of DR is mild non-proliferative DR. This is characterized by small swollen areas in the retinal blood vessels. A small amount of fluid can be secreted into the retina and cause macular edema. This area is near the center of the retina. The second stage is moderate non-proliferative DR. At this stage, the swelling of the small blood vessels increases and leads to the impaired blood supply to the retina. This causes blood and fluid to build up in the macula. At stage three or severe non-proliferative DR, we see a severe reduction in blood supply to the retina because of blockage of more blood vessels. Eventually, the body receives signals to start growing new blood vessels in the retina. The stage of proliferative DR is an advanced stage of the disease. New fragile blood vessels form in the retina. The high vulnerability of these arteries increases the risk of fluid leakage and various vision problems such as blur, reduced field of vision, and even blindness.

The macula sends visual signals to the brain, including colors, subtle details, and distant objects. One of the complications of DR is DME (diabetic macular edema), which affects the macula in the central part of the retina and is responsible for central vision. Retinopathy leads to DME when the retina is no longer able to absorb fluid from leaking blood vessels. This condition increases the diameter and swelling of the macula in a small or large range [1, 4].

Standing on severity of DR, laser photocoagulation together with continued diabetes checkup and eye screening is recommended for disease control. Currently, intravitreal injection of Vascular Endothelial Growth Factor (VEGF) neutralizing agents is a brand new care for retinal diseases [6, 7].

All contemporary treatments have a lot of imperfections take in low efficacy, and high treatment cost. These Shortcomings highlight the need to develop novel treatment alternatives. To foster the development of therapeutic strategies, exploring the pathophysiological inception and progression of DR is essential [8, 9]. Despite to the high importance of the problem, there is only one research analyzed a small set of RNA-Seq datasets for fibro vascular membranes (FVM) of PDR (proliferative diabetic retinopathy) patients [10, 11]. There is

rare disease-specific dataset for the human retina with DR. Numerous studies addressed transcriptomic analyze of the retina of animal models with DR stages. studied animal models include Streptozotocin (STZ) induced diabetic rodents [10] and oxygNA sequencing reveals retinal transcriptome changes in STZ-induced diabetic ratsen-induced retinopathy (OIR) [12]. Some researches carried on DR patients' blood and analysed small non-coding RNA [13].

Previous prospective studies have shown that risk of microvascular diseases increases on insufficient tyrosine levels in patients with diabetes [14]. Animal studies have shown that perturbing rat brain by tyrosine could stimulate dihydroxyphenylalanine synthesis [15]. Obesity, insulin resistance and pre-diabetes are factors that inhibit Tyrosine metabolism [16].

According to previous literature the incidence of type 1 diabetes is greater in males in spite of diabetes type 2 which is greater in female [17]. As mentioned earlier Type 2 diabetes is the main cause of DR [18, 19]. In this study, we performed a system biology approach in DR investigations. In this case, this study aims to investigate of molecular mechanism underlying DR in females in comparison with males. Utilizing RNA-Seq dataset of human post-mortem retinal samples (GSE160306) from patients diagnosed with DR_DME. The Differentially expressed genes (DEGs) were obtained in females compared to males. After that, gene-set enrichment analysis was performed for up-regulated genes in females with DR_DME to get an insight of DR_DME differential molecular mechanism compared with males. Eventually we found that DEGs were significantly associated in Osteoclast-associated receptor (OSCAR) binds collagen and Surfactant protein D (SP-D), apoptosis and tyrosine metabolism molecular functions in females with DR in DME stage.

## Materials and Methods

### *Data source and gene expression samples*

The expression data were downloaded from GEO (Gene Expression Omnibus, http://www.ncbi.nlm.nih.gov/geo/) under the accession number of GSE160310 [20]. The Raw data was not provided for this record and accessible data includes normalized and count matrix, which had been resulted from analysis of raw RNA-Seq data. The Illumina HiSeq 4000 (Homo sapiens) (https://www.illumina.com/) had been used for RNA-sequencing and the platform accession number is GPL20301.

Eye samples were taken from 43 posthumous human through the Iowa Lions Eye Bank (Coralville, IA) [21]. History of donors was checked and associated samples with HIV, hepatitis B or C, hospitalized isolated isolation, or neuroDEGsenerative diseases of unknown cause have been keep out [22].

### *Study Population and Demographical Information*

Samples were categorized into one of four groups: (a) diabetic with no obvious visual problem or noticeable pathology of the retina at last eye exam, (b) non-proliferative DR (NPDR) without diabetic macular edema (DME), (c) NPDR with DME, or (d) PDR and DME. Categorization of the samples was based on the early treatment DR study DR Severity Score (ETDRS-DRSS). Furthermore for each group age and gender matched samples were taken from healthy donors. None of the control group received retinal treatment. For each donor 10 samples were taken from macula region and 10 samples from retinal periphery. This study was composed of both tissues (macula/peripheral) samples. Then categories (control and DME) for males and

females were identified.

***Pre-processing and differential expressed gene analysis***

At first, genes with a mean expression value less than or equal to one were discarded. Then read counts was trimmed using upper quartile method Trimmed Mean of M-values (TMM) method [23, 24] calculated TMM(X) was normalized, using $\log_2^{x+1}$.

Then we compared gene expression of the defined disease group DME in males and females. For identifying significantly-changed transcripts; we performed differential expression gene analysis with exact test method. Only The statistically significant genes (adjusted P value <= 0.05 and Log-Fold-Change >= 2) were selected. The differential expression gene analysis and the related statistical tests were performed using EdgeR package in R.

***Enrichment significant genes***

Gene ontology (GO) (http://geneontology.org/) and pathway enrichment analysis were done using The CluGO [25] application which is a Cytoscape [26] plugin and only terms with adjusted P value < 0.05 were considered. KEGG (https://www.genome.jp/kegg/), Reactome (https://reactome.org/) and, Wikipathway (https://www.wikipathways.org/) database were used for pathway enrichment analysis. Significance of each biological term were calculated with two-sided hyper-geometric test and adjusted P values were calculated Benjamini & Hochberg method [27-29].

## Results

***Data source and gene expression samples***

The outlier sample (which was far from others base on the hierarchical clustering of their gene expression profile) were removed. The hierarchical clustering of the samples and their related clinical information was shown in Figure-1. The number of samples in each group, along with the mean and standard deviation of their age are reported in Table 1.

**Table1 :** The number of samples in each group along with the mean and standard deviation of their age

| | Female | Male |
|---|---|---|
| Control | N =12 | N=8 |
| | 79.5 ± 3.45 | 71.75 ± 6.45 |
| DME | N=17 | N=3 |
| | 75 ± 6.20 | 81.71 ± 11.41 |

***Pre-processing and differential expressed gene analysis***

Totally, 58,051 transcripts were selected for female_DME and male_DME cases. Then transcripts with an average expression level of lower than one were removed from the expression matrix, and 32054 transcripts remained. The normalized matrix was formed and differently expressed gene analysis was done. Then, the transcripts with an adjusted P value <= 0.05 and an absolute Log Fold Change >= 2 were selected. The number of total differently expressed genes was 243 among them 48 genes were down-regulated and 196 were up-regulated.

Similarly, the DEGs analysis was performed again on the males and females from the control groups. Just as mentioned above with the mean expression level of more than or equal to 1 which consists of 31828 transcripts were selected. From the differently expressed genes results, 154 transcripts were significantly up-regulated (adjusted P value <= 0.05 and an absolute Log Fold Change >=2). Among

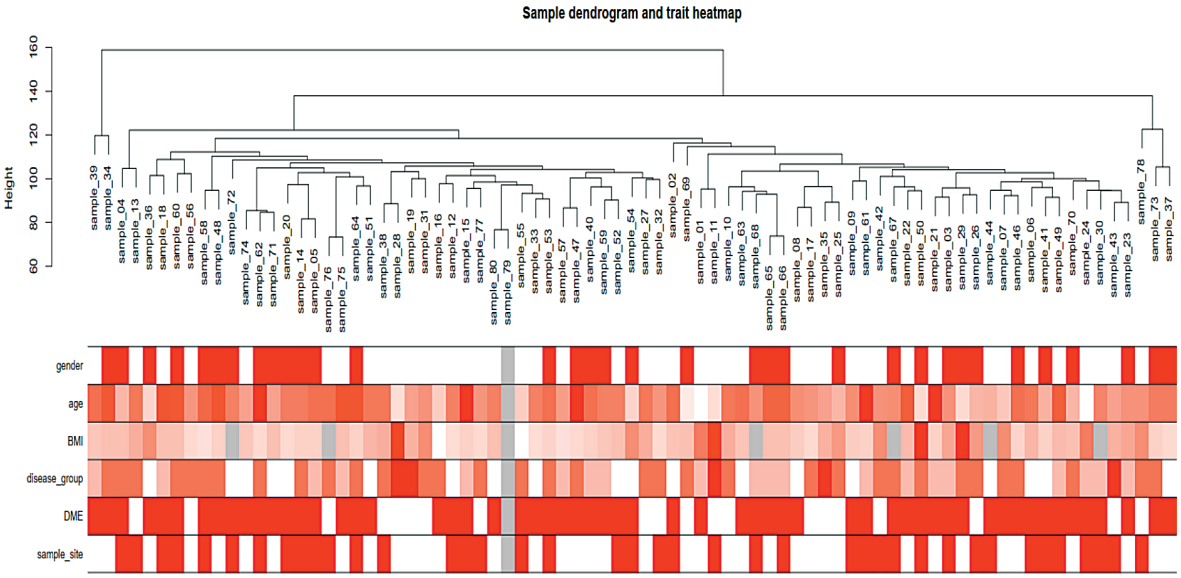


**Figure 1:** shows the hierarchical clustering of the samples. Below the figure illustrates a heat map of each sample's clinical information

the whole DEGs genes, 83 genes were up-regulated and 71 were down-regulated.

To find differently expressed genes in male/female DME patients, we used results of our first differently expressed genes analysis between male-DME and female-DME groups. The second differently expressed genes analysis between male-control and female-control groups is used for revealing that first DEGS gained genes are not also DEGS in control groups. Volcano plot of DEGs is given in figure 2. Table-2 shows the top 5 up and down-regulated genes.

### *Gene Set Enrichment Analyses*

DEGs analysis of mRNA in human retinal samples provided us a unique opportunity to investigate gender-specific gene transcription in diabetic eye retinopathy in DME stage. To understand the biological functions and pathways that the differently expressed genes (DME male/female patients) are involved in the gene set enrichment analysis was performed. During enrichment analysis, the gene ontology (GO) consisting of biological process, molecular function analysis and pathways were investigated.

The results of enrichment analysis showed that some of differently expressed genes like AOC3, AOC2, ADH1C, ADH1B, DCT, TYR are significantly associated with tyrosine metabolism pathway in women. IRF4, IGF2, PMAIP1, GZMB, TP73 genes are significantly associated in apoptosis pathway. Other gained pathways include Osteoclast-associated receptor (OSCAR) binds collagen and Surfactant protein D (SP-D). Figure 3 illustrate Pie plot of significant biological process. Figure 4 also illustrates significant pathways and molecular function network for DEGs.

## Discussion

As mentioned earlier, the incidence of DR is growing in spite of all recent advances in diabetes diagnosis and treatment [30]. Deeper understanding of molecular mechanism underlying DR might help us to find diagnostic, prognostic or therapeutic

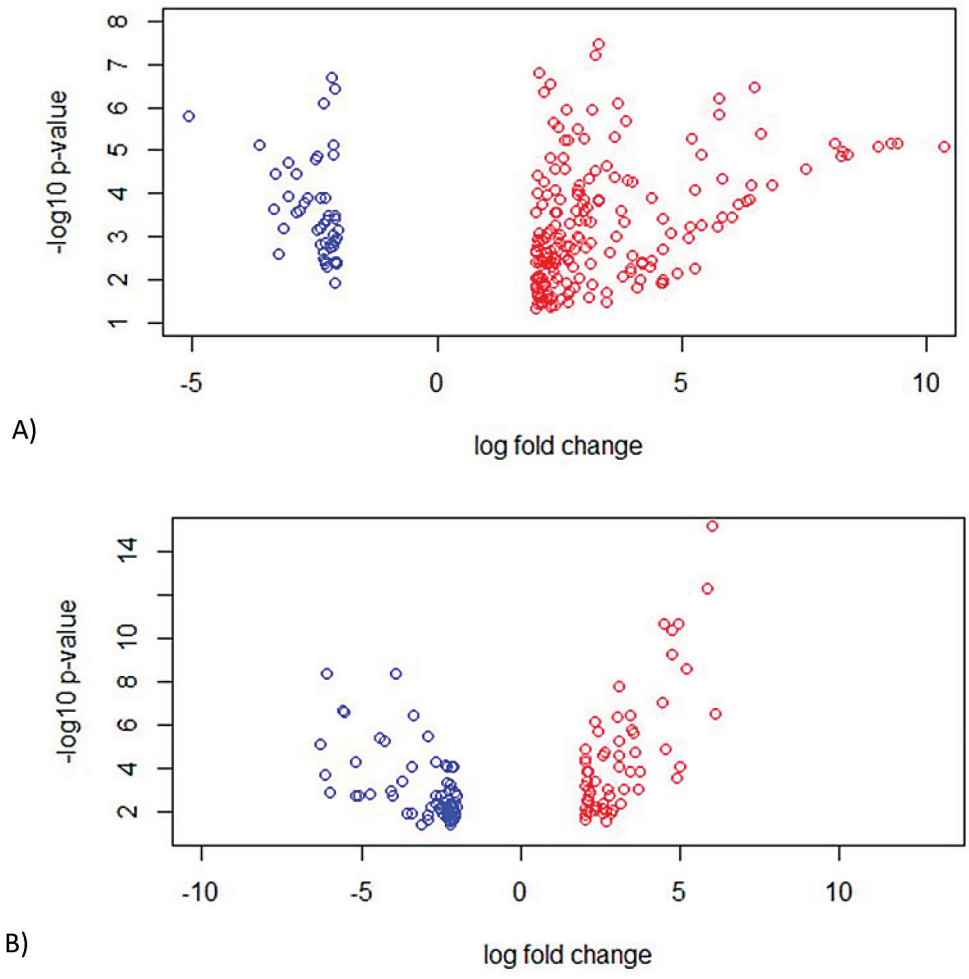


**Figure 2:** Volcano plot for differently expressed genes. A) DME_female and DME_male DEGs, B) control_female and control_male DEGs

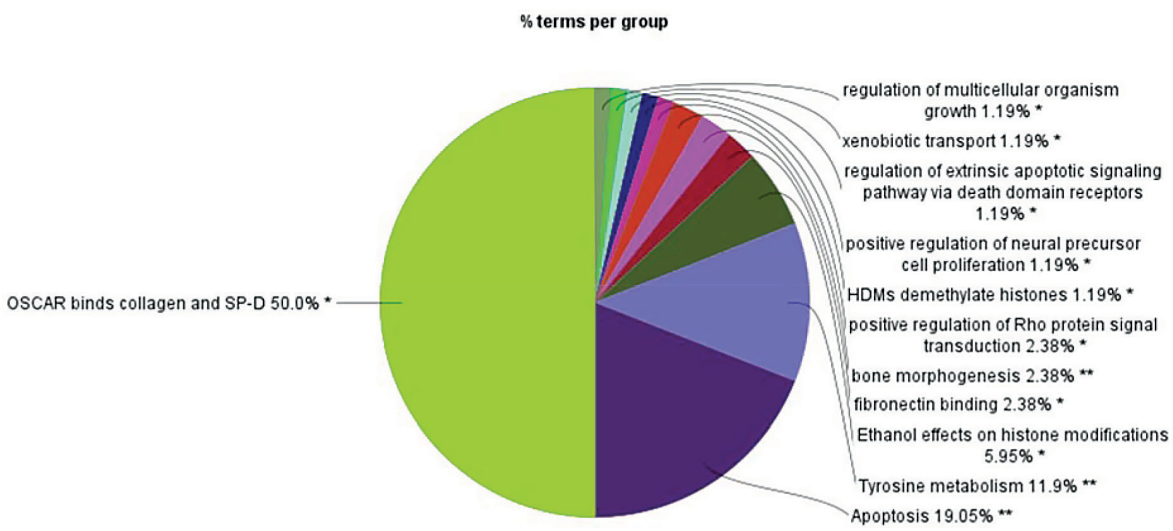


**Figure 3:** Pie plot of associated differently expressed genes pathways for DME_female patients

**Table 2:** Top ten up and down-regulated genes were mentioned in the table

| | Ensembl ID | logFC | logCPM | PValue | FDR | Adjusted_ PValue | Gene_symbole |
|---|---|---|---|---|---|---|---|
| Up-regulated DEGs | ENSG00000012817 | 13.02615 | 4.361591 | 2.37E-06 | 0.001895 | 2.84E-05 | KDM5D |
| | ENSG00000067048 | 12.62191 | 3.958664 | 7.91E-07 | 0.0011 | 1.69E-05 | DDX3Y |
| | ENSG00000114374 | 12.62177 | 4.719804 | 4.84E-06 | 0.002691 | 4.17E-05 | USP9Y |
| | ENSG00000131002 | 11.84282 | 3.184551 | 2.54E-06 | 0.001895 | 2.84E-05 | TXLNGY |
| | ENSG00000183878 | 11.53315 | 3.894582 | 2.50E-06 | 0.001895 | 2.84E-05 | UTY |
| | ENSG00000129824 | 11.43504 | 2.780016 | 2.77E-06 | 0.002017 | 2.96E-05 | RPS4Y1 |
| | ENSG00000233864 | 11.28188 | 2.628853 | 1.10E-06 | 0.001208 | 1.92E-05 | TTTY15 |
| | ENSG00000188770 | 10.37013 | 3.465483 | 8.35E-06 | 0.003668 | 5.27E-05 | OPTC |
| | ENSG00000067646 | 9.403586 | 1.748554 | 6.65E-06 | 0.003185 | 4.68E-05 | ZFY |
| | ENSG00000012817 | 13.02615 | 4.361591 | 2.37E-06 | 0.001895 | 2.84E-05 | KDM5D |
| Down-regulated DEGs | ENSG00000259647 | -5.08576 | -2.88257 | 1.56E-06 | 0.001392 | 2.26E-05 | AC111152.2 |
| | ENSG00000257392 | -3.62932 | -0.90201 | 7.59E-06 | 0.003474 | 5.04E-05 | AC126177.3 |
| | ENSG00000229389 | -3.33238 | -2.41752 | 0.000223 | 0.02793 | 0.000610102 | AC111152.1 |
| | ENSG00000215203 | -3.31801 | -3.02848 | 3.34E-05 | 0.009473 | 0.000149139 | GRXCR1 |
| | ENSG00000213158 | -3.22802 | -0.79711 | 0.002433 | 0.100812 | 0.003788545 | GAPDHP36 |
| | ENSG00000162763 | -3.13258 | -2.91606 | 0.000662 | 0.049491 | 0.001392665 | LRRC52 |
| | ENSG00000233868 | -3.05378 | -2.94024 | 0.000116 | 0.019931 | 0.000384459 | AC009302.1 |
| | ENSG00000255502 | -3.03561 | -2.53496 | 1.96E-05 | 0.006682 | 9.84E-05 | AP003730.2 |
| | ENSG00000250612 | -2.88578 | -3.18622 | 0.000262 | 0.030856 | 0.000678002 | AC114786.2 |
| | ENSG00000259647 | -5.08576 | -2.88257 | 1.56E-06 | 0.001392 | 2.26E-05 | AC111152.2 |

biomarkers. Although, wide variety previous studies have been performed for investigation of DR molecular mechanism, there's still lack of information about the differences of the disease occurrence between males and females [31]. In this case, by analyzing publicly available RNA-sequencing data set (GSE160310) we found DEGs in males compared to females. Up regulated genes was utilized for enrichment analysis. As the results of enrichment analysis shows the most of up-regulated genes take part in pathways involving in Osteoclast-associated receptor (OSCAR) binds collagen and Surfactant protein D (SP-D), apoptosis and tyrosine metabolism molecular functions. OSCAR costimulates osteoclastogenesis

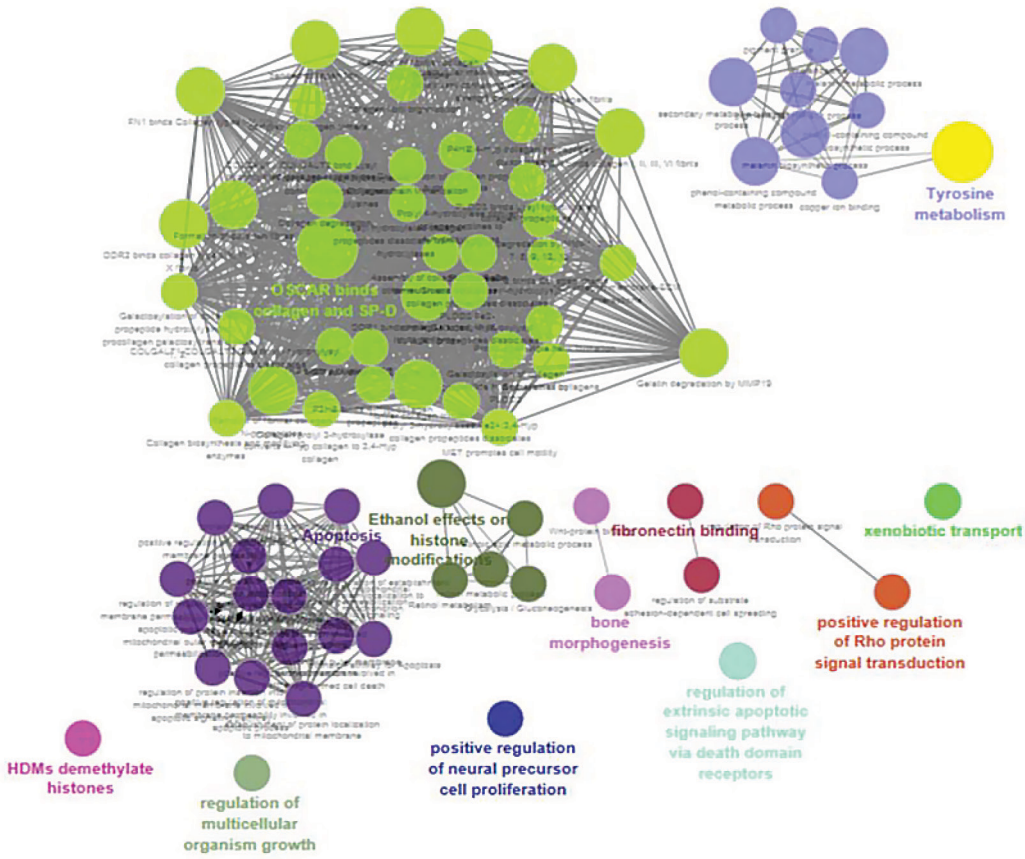


**Figure 4:** biological function and pathway of DME_female and DME_male differently expressed genes

via signaling through the ITAM-harboring adaptor protein Fc receptor gamma (FCRG) [32]. OSCAR is considered as a collagen receptor. It is highly expressed by preosteoclasts [33]. The expression of OSCAR has been also reported in endothelial cells [34]. Since, DR is a microvascular complication they might be involved in this defect.

SP- A and D belong to the "c-type lectins" supper family [35]. They participate in pulmonary immunity [35]. The expression of these molecules has also been reported in other organs aside from pulmonary system including vaginal and amniotic fluid, the gastrointestinal tract, renal system and finally the ocular system [36, 37]. Early retinal DEGs was observed to be associated with the several mutations in in SP-A and SP-D [37]. On the other hand the loss of SP-A in retinal and Retinal and Müller cell was reported to attenuate neovascularization in the oxygen-induced retinopathy mouse model [29]. Therefore, SP-A is reported as a potential biomarker of retinal inflammation during neovascularization.

One of the other prominent characteristics of DR progression is cell death. According to previous observations, a diabetic environment can lead to cell death induction in several retinal cell types. Among all cell death mechanisms identification of apoptosis in diabetic retinal cell was the main emphasis of the most previous literature [38-40].

The final results of this project were also showed the significant association between

tyrosine metabolism and DR-DME risk. The increased DR risk was observed only in female patients. Our study revealed that Risk of DR could be controlled by tyrosine control in women patients.

In metabolism pathway of tyrosine, the precursor of melanin pigments, is catabolized and converted into a wide range of biologically important molecules. Tyrosine metabolism can lead to the production of hormones such as thyroxine and triiodothyronine and can be used to produce neurotransmitters such as L-DOPA, dopamine, adrenaline or noradrenaline [41]. Also, tyrosine can be catabolized to fumarate and acetate [22].

Tyrosine also plays an important role in the synthesis of thyroid hormones. Thyroid hormones triiodothyronine (T3) and thyroxin (T4) are released from the thyroid gland and regulate the body's metabolism. These hormones are produced by the follicular cells of the thyroid gland through the process of thyroperoxidase. This action converts active tyrosine on thyroglobulin to iodine. In mammals, tyrosine can be derived from phenylalanine. Phenylalanine is an essential amino acid, while tyrosine can be produced in the body [41].

Hui-Huan [42] found that a deficiency of pure tyrosine and phenylalanine increased the risk of DR by reviewing the results of 1,032 patients. The results of their study suggest that low levels of plasma phenylalanine and tyrosine independently and simultaneously increase the risk of DR in type 2 diabetes. In addition, the presence of low-grade phenylalanine and tyrosine renal insufficiency increases the risk of DR. Their research opened new hypotheses for scientists to study a new important pathway that links neuro-degeneration to microvascular impairment.

## Conclusion

The incidence of type 2 diabetes is higher in females and there is strong relation between type 2 diabetes and the DR. Thus, women are at more risk for DR. No previous study mentioned gender as a factor to control and treat this disease. Therefor we performed an analysis to investigate molecular mechanism of DR in females that are different from the males. To this end, we found some specific pathways which have been observed only in female suffering DR. for instance tyrosine metabolism. The results of enrichment analysis showed that some of differently expressed genes like AOC3, AOC2, ADH1C, ADH1B, DCT, and TYR are significantly associated with tyrosine metabolism pathway in women. IRF4, IGF2, PMAIP1, GZMB, TP73 genes are significantly associated in apoptosis pathway. Controlling tyrosine level in females might control symptoms and development of macula damage. Further prospective cohort studies are needed to confirm these findings.

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**Footnotes and Financial Disclosures**

**Conflict of interest:**

The authors have no conflict of interest with the subject matter of the present manuscript.