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THE EXPRESSION OF RETINAL MICRO RNA EVOKED BY HYPERGLYCEMIA AND AFTER ADIPONECTIN TREATMENT IN HUMAN RETINAL ENDOTHELIAL CELLS

BY

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ABSTRACT

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Title: The Expression of Retinal miRNA Evoked by Hyperglycemia and After Adiponectin Treatment in Human Retinal Endothelial Cells

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Background: Diabetes mellitus is a chronic metabolic disease resulting in microvascular complications including diabetic retinopathy (DR). Adiponectin (ApN) is an adipokine hormone, and recent studies demonstrated that ApN could ameliorate critical biological process involved in the pathogenesis of DR. Micro-Ribonucleic acids (miRNAs) have been documented as novel biomarkers and are increasingly considered as molecules with significant modulatory action. **Aim:** To characterize the miRNA profile and expression in human retinal endothelial cells (HRECs) exposed to hyperglycemic conditions (HG) and illustrate the effect of adiponectin on miRNA expression and related pathways in HRECs exposed to HG. **Methods:** HRECs were treated with high glucose (30mM) for 96 hours duration followed by adiponectin (30µg/ml) for 24 h. Total RNA was extracted from HRECs. The gene panel array for both adhesion and angiogenesis molecules were performed using commercial RT2 Profiler PCR arrays. Furthermore, we utilized the small RNA sequencing for microRNA expression profiling of the HRECs. **Results:** HG treatment increases the expression of different well-known adhesion and angiogenesis genes as well as predicted miRNAs involved in these pathways, which was counteracted by ApN. RNA-Seq for miRNA profiling revealed 13 differentially

expressed miRNAs in HRECs exposed to HG. miR-146a-5p was differentially expressed in HRECs treated with ApN. Analysis pathway linked the significantly changed miRNAs induced by HG to essential pathways such as hypoxia signaling, inflammation, and oxidative stress. **Conclusion:** HG induces expression of various adhesion and angiogenesis genes. Using RNA-Seq technology can accurately identify dysregulated miRNA profiles in HG retinal cells. MiR-146a was upregulated by adiponectin which targets different pathways involved in DR genesis.

DEDICATION

To my dearest family

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AMPK	Adenosine Monophosphate-activated Protein Kinase
ApN	Adiponectin
ADGRB1	adhesion G protein-coupled receptor B1
ADIPQR	Adiponectin Receptor
AGE	Advanced Glycation End product formation
AMD	Age-related Macular Degeneration
Ang-2	Angiopoietins
BRB	Blood-Retinal Barrier
Bfgf	Basic fibroblast growth factor-2
cDNA	complementary Deoxyribo Nucleic Acid
DM	Diabetes Mellitus
DME	Diabetic Macular Edema
DR	Diabetic Retinopathy
FGF	Fibroblast Growth Factor
HG	Hyperglycemia
HRECs	Human Retinal Endothelial Cells
ICAM	Inter-Cellular Adhesion Molecule
LFA-1	Leukocyte Function-associated Antigen-1
miRNA	micro Ribo-Nucleic Acid
MMP	Matrix Metalloproteinase
mRNA	messenger Ribo-Nucleic Acid

NADH	Nicotinamide Adenine Dinucleotide
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NGS	Next Generation Sequencing
NPDR	Non-Proliferative Diabetic Retinopathy
PCK	Protein Kinase C pathway
PDGF	Platelet Derived Growth Factor
PDR	Proliferative Diabetic Retinopathy
PI3K	Phosphoinositide 3-Kinase
PKC	Protein Kinase C
PRP	Panretinal Photocoagulation
PVR	Proliferative Vitreoretinopathy
qPCR	quantitative Polymerase Chain Reaction
RISC	RNA Induced Silencing Complex
RNA	Ribo-Nucleic Acid
RIN	RNA Integrity Number
ROS	Reactive Oxygen Species
TNF	Tumor Necrosis Factor
TNFα	Tumor Necrosis Factor-alpha
TGF	Transforming Growth Factor
VCAM	Vascular Cell Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor

1. INTRODUCTION

Diabetic retinopathy (DR) is a severe vision-threating disease that results from microvascular abnormality due to hyperglycemia (HG) (1). There are two types of DR: non-proliferative diabetic retinopathy (NPDR) which is the early stage and proliferative diabetic retinopathy (PDR), which is the late stage (1). Since retinal cells have a high oxygen demand, hyperglycemia initiates the angiogenesis mechanism to increase blood flow in the retina (2). Therefore, the production of the vascular endothelial growth factor (VEGF) is augmented (3). The overexpression of VEGF and angiogenesis in retina results in the formation of atypical and dysfunctional retinal vessels. These vessels can then break and leak blood and fluid in the retinal area. Consequently, this causes macular edema, retinal detachment and then vision loss and blindness (4).

One of the central regulators of angiogenesis and response of endothelial cells to VEGF is the micro RNA (miRNA). MiRNAs have been documented as novel biomarkers and are considered as molecules with significant modulatory action in many different biologic processes (5). These are small non-coding RNA molecules that negatively regulate gene expression and therefore play a role in disease processes. Numerous studies showed the relationship between miRNA and the regulation of angiogenesis. In a recent study, low level of miR-15a in diabetic retina upregulated the expression of VEGF-A in retinal cells (6). MiR-15a was identified as a critical regulator of pro-angiogenic as well as pro-inflammatory pathways.

Currently, PDR is treated with surgical interventions and laser treatments. However, they are considered as invasive and of limited efficacy (7). Since VEGF

production is increased in DR, anti-VEGF therapy has been proven to be effective in treating pathologic neovascularization in DR (8). Similar to laser treatments, anti-VEGF could be considered aggressive, and its potential effects on the vessel as well as long-term safety are still under evaluation (9). Adiponectin (ApN) is a natural hormone produced in adipose tissue is highly expressed in healthy individuals and could be affected by pathological status. For instance, a study reported that ApN concentrations were lower in type 2 diabetes patients (10) and that the severity of DR is associated with low ApN concentrations indicating its potential role in DR pathogenesis. The data of the recent work conducted in our lab demonstrated that ApN could ameliorate critical biological process involved in the pathogenesis of DR such as the oxidative stress, barrier function, and the inflammatory pathway. Based on this evidence, we hypothesize that miRNA could be involved as critical regulators in the pathogenesis and response to adiponectin administration. This study aimed to elucidate the involvement of miRNAs in the pathogenesis of microvascular complications of the human retinal endothelial cells and response to adiponectin using in vitro cell model. This was followed by an analysis to explore their gene expression patterns. To reach this goal, we, used RNA-Seq techniques with bioinformatics, to screen for miRNA and used an RT-PCR array of angiogenesis gene panel to predict the most dysregulated miRNA involved in angiogenesis. Human Retinal Endothelial Cells (HREC) were exposed to hyperglycemia conditions for 24 hours and 96 hours followed by ApN treatment to investigate the profiling and expression of miRNAs and functional pathways involved in the pathogenesis of DR.

2. LITERATURE REVIEW

2.1 Epidemiology of Diabetic Retinopathy

Diabetes mellitus (DM) is a worldwide disease that is prevalent in about 180 million people (11). This disease is characterized by an imbalance between insulin production, insulin need and the body's ability to use the available insulin. Insulin hormone is produced by β cells of the pancreas. When β cells are attacked and destroyed by the immune system, this results in type 1 diabetes. The etiology of this attack is still unknown, yet genetic factors are believed to play a significant role. In type 2 diabetes, the pancreas produces enough insulin. However, there is an insulin resistance caused by either a receptor binding problem or a defect in insulin signaling inside the targeted cells. Both types result in high glucose in the blood (hyperglycemia). Consequently, maintained hyperglycemia leads to altered microvascular function and a broad spectrum of irreversible complications such as diabetic neuropathy (12) and DR which is considered one of the most DM serious complications (13).

DR is the breakdown of retinal vasculature due to persistent hyperglycemia (1). It is considered the leading cause of blindness and vision impairment among people worldwide. DR is ranked as the fifth most common cause of moderate to severe visual impairment (14). In 2012 a pooled analysis of 35 population studies estimated that 93 million people have diabetic retinopathy worldwide and that PDR is prevalent in 7.5% of diabetic patients (15). Therefore, screening for DR remains essential for early diagnosis and preventing blindness (16). In a recent systematic review aimed to measure the prevalence of age-related eye diseases in Iranian population, DR in at least one eye was

prevalent in 2.7% of the total participants (17). The prevalence of DR was 2.5% and 2.8% in men and women respectively. Statistics showed that the difference between genders was not statistically significant (18). The highest prevalence of DR was 21.4% and was in the age group of 70–74 years. Many studies have been performed and estimated that DR is 36.5–93.6% prevalent in type 1 diabetes in the USA and Europe (19-21). A study conducted in Italy, included 1,321 diabetic patients with DM who were examined for DR, showed that DR was prevalent in 26.2% of the participants (22). Interestingly, the results showed that DR prevalence was significantly associated with diabetes duration. For instance, PDR was more prevalent after 20 years of diabetes. In the Middle East and Asian countries, the prevalence of DR in type 2 diabetes has been intensively studied since type 1 diabetes has lower incidence in these populations. Most of these countries reported the prevalence of DR to range between 12.9–23.0% (23, 24). However, Singapore had a higher DR prevalence (33.9%) than other Asian countries (25). Moving to Gulf region, Saudi Arabia also reported a high DR prevalence (36.8%) (26).

2.2 Pathogenesis of Diabetic Retinopathy

Hyperglycemia and hypoxia are the primary triggers of changes in retinal blood flow (27-29). The Pericytes provide stability for retinal vessels and control the proliferation of endothelial cells. In DR there will be a loss of pericytes, increased vascular permeability, and endothelial dysfunction (30). Over time, there will be induced-release of different angiogenic growth factors including VEGF. VEGF has a central role in retinal neovascularization and could lead to diabetic macular edema (31). There are

three receptors for VEGF, where VEGFR1 is expressed on macrophages and monocytes, VEGFR2 is expressed in vascular endothelial cells, while VEGFR3 in lymphatic endothelial cells (32). VEGF-A stimulates the proliferation of endothelial cells through VEGFR2-induced activation of different pathways. Besides, VEGF-A promotes vascular permeability by activating its receptor on the endothelial cells through two principal mechanisms: the temporary opening of para-cellular junctions and formation of trans-cellular pores (33). Still, the signaling transduction pathway is not well understood (34). Increased vascular permeability will cause macular edema by loosening the tight junctions between the endothelial cells in the capillary's walls. As the junctions loosen, the fluids will leak out into the surrounding area causing retinal tissue to swell. In addition, the retina is protected by the inner blood-retinal barrier (BRB). During NPDR, BRB breaks-down and blood constituents leak into the eye retina (35). Over time, high blood glucose will damage the retinal blood vessels and consequently will result in blurred vision. If this condition is left untreated, DR could cause blindness (4).

DR is classified into two types: NPDR or pre-DR and PDR (36). NPDR is characterized by a low number of pericytes surrounding retinal capillaries and thickened basement membrane (1). This thickening of basement membrane may lead to the death of vascular cell and vessel instability. PDR is the more advanced stage of the disease where there will be hypoxia and formation of new fragile blood vessels (37). The leakage of these vessels in the vitreous will cloud the vision. Also, retinal detachment could occur due to the formation of scar tissue and the development of glaucoma which represent the primary cause of worldwide irreversible blindness (38). The buildup of fluid in the front part of the eye increases intraocular pressure that can damage the optic nerve (39). In

2015, a meta-analysis of 47 pooled studies reported that relative risk of glaucoma is 1.48 in DM patients compared to those without diabetes (40). Moreover, the increased relative risk of glaucoma was positively associated with the diabetes duration (41).

Hyperglycemia could contribute to both microvascular and macrovascular complications due to several biochemical changes and consequently lead to DR (42). These changes include the disruption of various metabolites production such as inflammatory cytokines, vasoactive agents, growth factor as well as adhesion molecules (43). The development of retinal leukostasis, which is the adherence of myeloid-derived cells to the retinal endothelium, will cause the secretion of proteolytic enzymes. This will result in further damage leading to capillaries occlusion and focal ischemia (44, 45). Consequently, all these disruptions will increase the blood flow and vascular permeability and finally promote inflammation and pathologic angiogenesis (46).

Hyperglycemia induces vascular damage through the activity of three major pathways: PKC pathway, advanced glycation end product formation (AGE) pathway and the hexosamine pathway. These biochemical pathways become activated in the presence of an increased level of fructose-6-phosphate and glycolytic metabolites glyceraldehyde-3-phosphate (47). Once activated several inflammatory and proangiogenic factors including VEGF, basic fibroblast growth factor-2 (bFGF), angiopoietins (Ang-2), interleukin-6 and tumor necrosis factor (TNF) will be upregulated. In addition, these pathways will result in increased inflammation, oxidative stress, and vascular dysfunction and eventually increased vascular permeability then occlusion, and local ischemia (48). As mentioned previously, ischemia will initiate the angiogenesis in the retinal cells (49).

2.3 Diabetic Retinopathy and Angiogenesis

Angiogenesis is the process of forming new blood vessels from pre-existing vessels. Various conditions could trigger angiogenesis such as tissue hypoxia (50). The process starts by vasodilation of blood vessels due to nitric oxide and an increase in vascular permeability induced by VEGF. Then, separation of pericytes and breakdown of basement membrane through the action of matrix metalloproteinases (MMPs) (51). After that, endothelial cells will migrate to the site of injury. These cells will proliferate and remodel into capillary tubes. Although angiogenesis is an essential biological process, it is a feature of various diseases such as cancer, diabetic retinopathy, glaucoma and macular degeneration (52, 53). Angiogenesis is considered the fundamental cause of more than 70 diseases (54) and DR is an ischemic complication caused by angiogenesis in the avascular area of the eye (55).

2.3.1 Signaling Pathways and Molecules Involved in Retinal Angiogenesis

Retinal angiogenesis is the final common stage of various blinding disorders (56). There are different signaling cascades of cellular and molecular processes which are involved in angiogenesis and are critical for cell growth and survival (57). Some of these pathways, such as Phosphoinositide 3-Kinase (PI3K), produce biological molecules which affect angiogenesis of retinal vessels (58, 59). The angiogenic pathway is initiated when a growth factor binds to the receptor of tyrosine kinase leading to receptor dimerization. Consequently, it activates lipid kinase PI3K which will then convert membrane lipid PIP2 to its active form PIP3 (60). As a consequence, this will initiate Akt pathway which promotes cell growth via protein synthesis and reduces cellular death by

inhibiting FOXO activity (61). The PIK3CA gene, which is a component of the PI3K pathway, is positively correlated with the expression of VEGF (62). VEGF protein stimulates migration and proliferation of endothelial cells. It promotes vasodilation by indirectly stimulating production of nitric oxide which is a potent vasodilator (63). However, over-expression of VEGF has been proved to cause diabetic macular edema (64). It is produced in the retina at a higher level than average amounts which results in the formation of weak and leaky blood vessels (65).

Increase in ROS level will cause cellular oxidative stress and leads to inflammation which is the etiology of most of the diabetic complications (66, 67). There is a cross-talk between different biochemical pathways that contribute to DR pathogenesis. Hyperglycemia causes an increased flux of Krebs cycle and increases in cytosolic Nicotinamide Adenine Dinucleotide (NADH) (68). Consequently, the number of electrons will increase in the mitochondria, and more ROS will be produced (69). ROS influence the activation of the NF-κB pathway which is activated in inflammation (70). In addition, several studies showed that adhesion molecules are upregulated during inflammation and have further impact in DR pathogenesis (71, 72). The binding of white blood cells to Inter-Cellular Adhesion Molecule 1 (ICAM-1) located on the surface of endothelial cells is a characteristic of inflammation. Several stimuli including VEGF and oxidative stress increase the expression of ICAM-1 as well as Vascular Cell Adhesion Molecule (VCAM) (73). For instance, a study conducted on diabetic mice lacking the ICAM-1 gene or its ligand did not develop leukostasis, and there were no pericyte loss, degeneration of the capillaries or increased permeability (74). As the number of adherent leukocytes increases, more damage will occur to the blood-retinal-barrier (75). A study

showed that the breakdown of blood-retinal-barrier and leukostasis significantly decreased after administration of leukocyte function-associated antigen-1 (LFA-1) antagonist in diabetic rats (76). All these alterations lead to endothelial dysregulation and progression of DR.

2.3.2 Angiogenesis Factors

The expression of various angiogenic factors could contribute to the DR pathogenesis. Basic Fibroblast growth factor (bFGF) is a retinal endothelial mitogen that stimulates endothelial proliferation cells and promotes macrophage and fibroblast migration to the affected area (77). It has been shown that stimulation of angiogenesis by FGF2 causes adverse effects, such as atherosclerosis (78). For instance, a study aimed to quantitate the level of bFGF in the vitreous of 36 patients with various retinal conditions including PDR showed that eight patients had elevated bFGF level, and of these eight patients, six had the active proliferative disease (79). Results suggested that vitreous specimens from PDR patients had increased bFGF level and this could be evidence of active proliferative retinopathy. In another study, researchers aimed to investigate the significant role of bFGF and platelet-derived growth factor (PDGF) in proliferative vitreoretinopathy (PVR) pathogenesis (80). A total of 38 vitreous specimens were included in the study. Results revealed that both cytokines concentrations were elevated in PVR. It was concluded that both bFGF and PDGF might be involved in PVR pathogenesis.

Angiopoietins are vascular growth factors that have significant role in the processes of angiogenesis (81). Until today there are four known angiopoietins (82).

Angiopoietin-1 has a role in promoting endothelial cell survival as well as in stabilizing the interaction of endothelial cells with neighboring cells (83, 84). It has been shown that angiopoietin-1 can block the effect of vascular permeability by VEGF in vivo. For instance, in a study using diabetic rat model, angiopoietin-1 reduced both blood-retinal barrier breakdown and vascular endothelial injury. Also, it suppressed diabetic retinopathy development (85). On the other hand, angiopoietin-2 is upregulated by retinal ischemia, VEGF, and hypoxia and is expressed in the angiogenic process (86-88). It helps in the development and structural maturation of the newly formed blood vessels (89).

Another growth factor that regulates cell growth and has a significant role in angiogenesis is PDGF which is involved in embryonic development, angiogenesis, cell proliferation and migration (90). Similar to VEGF, over-expression of PDGF could be related to several diseases. For instance, a study conducted in Greece aimed to elucidate the correlation between the level of PDGF isoforms in eye's vitreous with PDR (91). Results showed that levels of all PDGF isoforms in vitreous as well as VEGF were significantly higher in the PDR group compared to controls. In addition, PDGF-AA and PDGF-BB were correlated considerably with PDR severity. In another study aimed to investigate the role of PDGF-AB isoform in the angiogenetic process in 23 patients with PDR and PDGF-AB concentration was measured (92). Results showed that that PDGF growth factor has a vital role in the PDR pathogenesis and a synergistic effect in collaboration with other growth factors such as VEGF and tumor necrosis factor alpha (TNFα).

There are two classes of transforming growth factors: transforming growth factor alpha (TGFα) and transforming growth factor beta (TGF-β). They activate signaling

pathway for cell proliferation, differentiation, and development. Some literature suggests that TGF-β1 gene has a role in the development of DR through disrupting angiogenesis (93). For instance, a study showed that high TGF-β1 level is found in ischemic retinal vein occlusion (94). Netrins are a group of proteins that were first discovered in nematode (95). They are involved in axon guidance and vascular development (55). A study aimed to examine the role of VEGF and netrin-1 in the pathogenesis of retinal angiogenesis where 18 patients, 10 PDR patients, and eight non-PDR patients, were included (96). After measuring the level of VEGF and netrin-1 in the vitreous fluid and serum of the patients, it was concluded that both, VEGF and netrin-1, were elevated and could have a significant role in pathological retinal angiogenesis. Similarly, another study proved this conclusion where netrin-1 has been shown to contribute to the angiogenesis and inflammatory disease including diabetic retinopathy (55). Increased level of netrin-1 in the retina has proven to be a pro-angiogenesis factor of retinal angiogenesis under hypoxic condition (97).

2.4 MicroRNAs

2.4.1 Biology of miRNA

The interest in miRNA has grown exponentially over the last decade, and its role in human diseases is being studied intensively. miRNAs have long evaded the attention of scientific community because of their small size and how the changes in miRNAs expression are correlated with gene expression and involve both biological and pathological processes (98). miRNAs are short noncoding RNAs ranges between 18-22 nucleotide bases. Genes that code for miRNAs are contained in nucleus DNA (Figure 1).

MiRNAs are firstly transcribed by RNA polymerase II, and the product is called primary miRNA that forms a hairpin loop structure. The double-stranded miRNA will be recognized by DGCR8 (DiGeorge syndrome critical region gene 8) protein and with the association of DROSHA enzyme; primary miRNA will be cut into smaller precursor miRNA (pre-miRNA). Eventually, pre-miRNA will be exported to the cytoplasm by the help of Exportin 5 enzyme. Pre-miRNA will then be recognized by Dicer enzyme, endonuclease protein, which will cleave the stem-loop and forms a short double-stranded miRNA molecule called miR:miR*duplex. Next, an Argonaute protein, Ago II, interacts with Dicer to unwind miRNA and one strand will be released (passenger strand) and degraded. The remaining strand (guide strand) interacts with Ago II and other proteins to form RNA Induced Silencing Complex (RISC). It can be guided to its target to inactivate one or multiple genes. miRNA inactivates messenger RNAs (mRNAs) which are necessary for translating the genetic information into proteins. This inactivation could be cutting the mRNA which will be further destroyed by the cell or by inhibiting the translation. In this case, RISC complex prevents the ribosome subunits from binding. In both cases, the gene is silenced since mRNA will not be translated into a protein (99). miRNAs dysregulation can have critical consequences in the human body and causes many diseases such as heart diseases and cancer (100).

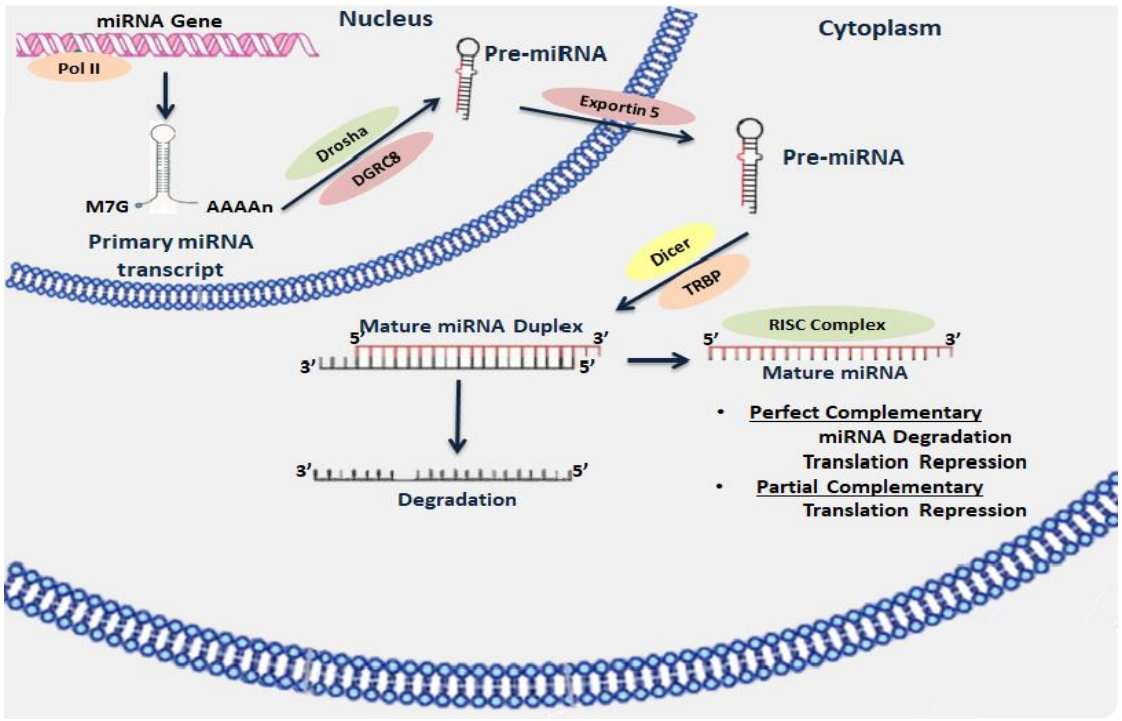


Figure 1. The biogenesis of microRNA.

2.4.2 Functions of miRNA

To date, there are more than 2,500 reported miRNAs in humans (101). miRNAs have a significant role in facilitating many biological processes and pathophysiological stress responses by regulating gene expression (102). For instance, miRNAs control a wide range of processes in cardiovascular disease such as vascular inflammation, electrical remodeling, myocardial remodeling and lipid processing (100). In addition, it can act as a biomarker for platelet reactivity (103, 104). A study conducted on zebrafish, using knockout gene strategy, revealed the role of miRNA in developmental processes. The study concluded the highly expressed miR-430 family members in zebrafish zygotic suggesting its role in development and neurogenesis (105). Similarly, another study revealed that mir-375 has a role in the secretion of hormones since it was highly expressed in the pituitary gland of zebrafish embryos (106). Further, both mir-372 and mir-373 target LATS2, which is a tumor suppressor gene, thus inducing tumorigenesis (107). These show that miRNAs have a role in physiological as well as pathological processes. Therefore, the therapeutic manipulation of miRNA is the future treatment choice for several human disorders (108).

2.4.3 MiRNA and Retinal Angiogenesis in Association to Stress in Diabetic Conditions

Recently, miRNAs have been recognized as factors that control retinal angiogenesis (109). For instance, a study conducted by Italian team identified some miRNAs that were upregulated in age-related macular degeneration (AMD) patients. Results showed that miR-146a, miR-126, miR-34a, miR-27a, miR-23a, and miR-9 were upregulated in AMD patients' serum (110). On the other hand, miR-155 was

downregulated. Similarly, another study showed that miR-126 level significantly decreased in PDR patients' serum compared to the control group (111). The study concluded that there might be an association between miR-126 and the diabetic vascular complications, particularly with PDR. Compared toNPDR and controls, the levels of miR-181c, miR-21, and miR-1179 were significantly higher in PDR patients' serum (112). Beside upregulation or downregulation of miRNAs, recent studies showed that some miRNAs could regulate pathological angiogenesis. For instance, when miR-410 interacts with 3′UTR of VEGFA mRNA, it could suppress the expression VEGF-A (113). Based on this, a study aimed to elucidate the mechanism of miR-410 targeting VEGF-A in mice model. The study showed that miR-410 was highly expressed within two weeks after birth and suppressed VEGF-A expression (113). This suggests that miR-410 could prevent retinal angiogenesis and hence a potential treatment for retinal angiogenesis in the future. CD146 is an endothelial biomarker that functions as a co-receptor to VEGF receptor 2 and is upregulated during pathological angiogenesis (114). A study showed that miR-329 inhibits CD146 and act as a novel negative regulator of angiogenesis (114). When CD146 is upregulated, it promotes tube formation and migration of endothelial cells. Results revealed that miR-329 reduced pathological angiogenesis in mice by suppressing the expression of CD146 on blood vessels. Therefore, it was proposed that miR-329 might be a future therapeutic tool to treat angiogenic diseases (114).

2.5 Adipokine, Diabetic Retinopathy, and miRNA

The adipokines are cell signaling proteins which are secreted by adipose tissue. They include metabolic regulators, angiogenic proteins and inflammatory mediators such as leptin, adiponectin, omentin, retinol, and visfatin (115). A recent study aimed to identify the correlation between serum omentin-1 concentrations and the retinal circulatory parameters in type 2 diabetic patients showed that omentin-1 concentration was positively related to the retinal blood flow (116). The study concluded that increased omentin-1 concentrations in plasma might be associated with high retinal blood flow in type 2 diabetic patients with early-stage of DR. Another study enrolled 204 diabetic patients to evaluate the omentin-1 levels in both serum and vitreous of DR patients (117). Patients were divided into four groups: control, without DR, NPDR, and PDR. Results showed that PDR patients' vitreous and serum omentin-1 levels were significantly decreased compared to all other groups. In addition, NPDR patients had reduced level of vitreous omentin-1 compared to patients without DR. Interestingly; levels of both vitreous and serum omentin-1 in the control group was significantly higher compared to the three diabetic patients' groups. It was suggested that development and severity of DR are positively correlated with the serum/vitreous omentin-1 ratio (117).

Adiponectin (ApN) is a novel modulator for vascular diseases and could be involved in the pathogenesis of DR (10). For instance; a study aimed to measure the concentration of plasma ApN in type 2 diabetic patients and to investigate the existence of an association between ApN and the severity of diabetic retinopathy (10). A total of 74 patients were involved in the study and compared with a control group consisted of 54 healthy subjects. Results revealed that ApN concentrations were significantly lower in the

diabetic patients than in control group. Moreover, there was an association between the ApN concentrations and the severity of DR. This suggests that adiponectin could be involved in the pathogenesis of DR. Similarly, another study concluded that ApN suppresses human coronary artery endothelial cells migration which is stimulated by VEGF. This shows that adiponectin may have a regulatory role in vascular processes associated with diseases including atherosclerosis and diabetes (118). Other studies showed that there is an association between the risk of obesity-related cancers such as prostate, breast, and colon cancer and decreased serum ApN levels (119, 120). Since both miRNAs and ApN level have a role in DR, the association between them could be studied to understand their effect in angiogenesis pathway.

2.6 Current Diabetic Retinopathy Treatment Options

The current treatment choices for DR are unpleasant, costly and time-consuming for the patient (121). One of the current options is laser treatment which uses high intensity light. The beam of light is directed into the eye to remove and prevent abnormal blood vessels from leaking within the eye (122). Laser treatments for diabetic eye diseases can be classified into two types: photocoagulation laser, which is also called focal laser treatment, and panretinal photocoagulation (123). Focal laser treatment is the standard option to treat diabetic macular edema (124). It slows or stops the abnormal blood vessels from leaking in the vitreous. However, the focal laser could also damage the healthy retinal tissue (125). Panretinal photocoagulation (PRP), also called scatter laser treatment, is one of the options to treat PDR (126). The doctor uses a laser to treat the peripheral retina where diabetic changes are causing damage. The procedures cause

abnormal blood vessels to shrink and prevent further growth of new blood vessels (123). However, more than one PRP procedure may be necessary. Since laser treatments cannot restore lost vision, patents should maintain regular eye examination to treat any diabetic eye changes as soon as are detected. In addition, laser treatment does not show a significant improvement in visual sharpness and does not last for an extended period (127). Therefore, other treatment tools were introduced.

VEGF protein, as mentioned previously, has a central role in the formation and healthy growth of blood vessels (3). Overexpression of VEGF causes the formation of damaged blood vessels, which leaks fluid into the eye. Anti-VEGF drugs are highly effective and target VEGF thus preventing the growth of the abnormal blood vessels (128). This decreases fluid leakage in the macula and reduces edema, thus improving vision. For instance, recent data suggest that anti-VEGF is an alternative treatment for PDR than using PRP (8). Decreased diabetic macular edema (DME) onset rate, less peripheral visual field loss and fewer vitrectomies over two years are all benefits of using anti-VEGF treatment than PRP laser (1). However, anti-VEGF drugs have a broad effect and could prevent the re-growth of healthy blood vessels in the eye (129). It is not the best choice of treatment for patients who cannot follow up and comply with regular injection regimen to prevent the recurrences of PDR. Moreover, anti-VEGF injections are invasive, and only 50% of DME patients respond effectively to the treatment (1).

Long-acting steroids are some of the potential diabetic eye treatments that have been used for DR induced by macular edema (130). Corticosteroids target the inflammatory cascade and show a significant improvement in vascular complications (131). However, this treatment is associated with limitations and adverse events including

a high incidence of cataracts and increased intraocular pressure (132). Most of the currently available therapies, including these mentioned previously, are invasive and may be failleading to blindness in the advanced stage (133). Therefore, new treatment choices are required for the early stage of DR. It was proven that ApN, among the adipokine family, has a role in regulating the retinal circulation (134). A recent study showed the significant effect of adiponectin in counteracting the effect of hyperglycemia and reducing the pathophysiological progression of DR (135). It improved the barrier dysfunction of retinal cells and reduced apoptosis, ROS production, and leukocyte adhesion in adiponectin-treated cells compared to hyperglycemic HRECs. Therefore, the effect of adipokines, especially adiponectin, in improving the altered retinal microcirculation could prevent the development and progression of DR.

Several mechanisms and pathways are involved and cause diabetic retinopathy. Therefore, we hypothesize that miRNAs may contribute to the pathogenesis of DR. The primary goal of this study is to portray the effect of ApN on miRNA expression using angiogenic gene panel, and related molecular pathways in HREC exposed to hyperglycemia. Further, to correlate such specific miRNA with the biological process involved in DR.

3. SIGNIFICANCE, HYPOTHESIS AND OBJECTIVES

The current research is significant as it helps to increase our knowledge about the role of miRNA in the pathogenesis of DR as well as the changes in HRECs exposed to diabetic conditions. This, in turn, could aid the development of a novel therapeutic approach for diabetic retinopathy. Understanding the role of miRNAs may help to identify new biomarkers to be used for DR diagnosis. One of the main challenges in developing therapy is that one miRNA has multi targets and one transcript is regulated post-transcriptionally by many epigenetic phenomena, such as methylation, and multiple miRNAs (5, 136).

Here we have identified that ApN could improve the endothelial dysfunction of HREC exposed to diabetic conditions *in vitro*. Therefore, we propose the identification of small regulatory molecules like miRNA that is differentially expressed in chronic hyperglycemia and altered by adiponectin treatment could help in understanding the pathogenesis of HG-induced endothelial dysfunction and pathways involved in such mechanisms and how can ApN improve such abnormality. We hypothesize based on previous data on HREC exposed to adiponectin, that ApN in a dose of 30ug/ml, could ameliorate the endothelial dysfunction of HREC exposed to hyperglycemia via different mechanisms. These mechanisms include: reduction of oxidative stress, anti-apoptotic, anti-inflammatory, reduction in nitric oxide production, reduce adhesion molecules such p-selectin and improve the barrier function (135). To test this hypothesis, we screened for the well-known angiogenesis and adhesion gene panels. Data obtained from the screening assay would help in the identification of the most significant miRNA involved in DR

pathogenesis. Furthermore, we used the advanced technology of small RNA sequence analysis to portray all miRNAs significantly changed in hyperglycemia and its perturbations after adiponectin application. Such RNA-sequence gave the chance to detect all possible miRNA using the latest version 22 of miRNA base, and not only selected specific panels as utilized in microarray chip techniques. Following that, we used the bioinformatics as a tool, to search for the targets (genes, and pathways) for that significant differentially expressed miRNA to highlight its role in DR pathogenesis.

The objectives of the study are the followings:

1- Screen for the genes and miRNA expressed in high glucose condition compared to normoglycemic state using in vitro model of HRECs. In subsequent experiments, exposure to glucose could be of short duration and long duration to portray and detect any change in the gene panels and predicted miRNA.

2- Screen genes and miRNA in hyperglycemic cells treated with adiponectin as a therapeutic tool to ameliorate the genesis of DR as shown in previous data. 3- Analyze the selected differentially expressed miRNA and evaluate their role by in Silico analysis to indicate its functional roles through analysis of the significant pathways involved in DR pathogenesis.

Gathering data from these objectives could help to answer the research question of the current study.

4. MATERIAL AND METHODS

4.1 Material and Reagents

In the current study, four different HRECs vials were used, representing different biological samples. Reagents used in the study are shown below in (Table 1).

Table 1: List of reagents used in the study

Item	**Company**	**Catalogue number**
Human Retinal Microvascular Endothelial Cells (HRMECs) passage	Cell System Corporation	Cat#ACBRI 181
Complete Classic Medium Kit with Serum	Cell System Corporation	4Z0-500
Culture Boost	Cell System Corporation	4CB-500
Complete Serum-Free Medium Kit with RocketFuel	Cell System Corporation	Certificate No: SF-4Z0-500-R

CSC Attachment Factor	Cell System Corporation	4Z0-210
CSC Passage Reagent Group™	Cell System Corporation	Certificate No: 4Z0-800
CSC Cell Freezing Medium	Cell System Corporation	Certificate No: 4Z0-705
BAC-OFF® ANTIBIOTIC TONIC	Cell System Corporation	4Z0-644
Trypan Blue stian (0.4%)	Gibco (Life Technologies)	15250-061
TRIZOL® reagent	Thermo Fisher Scientific, Waltham, Massachusetts, United States	Ref No: 15596026
Phosphate Buffered Saline (PBS) pH 7.4	Gibco (Life Technologies)	10-010-023
Glucose (5 and 30 mM)	Sigma	G-7021
Human gAcrp30/Adipolean	Peprotech	450-21-500UG

Table 2: List of kits

Item	Company	Catalogue number
mirVana miRNA Isolation Kit	Thermo Fisher	AM1560
miRNeasy Mini Kit	Qiagen	217004
High capacity RNA- to- cDNA kit	Applied Biosystems by thermos fisher scientific	4387406
Agilent RNA 6000 Nano Kit	Agilent Technologies, Waldbronn Germany.	5067-1511
RT^2 SYBR® Green ROX qPCR Mastermix	QIAgen	330520
TaqMan™ Gene Expression Master Mix	Applied Biosystems by thermos fisher scientific	4369016
Extracellular Matrix and Adhesion Molecules RT2 Profiler PCR Array	QIAgen	PAHS-013Z
Angiogenesis RT2 Profiler PCR Array	QIAgen	PAHS-024Z
Human Cytokine/Chemokine Magnetic Bead Panel 96 Well Plate Assay	Millipore	HCYTOMAG-60K-PX29

Table 3: List of instruments

Item	Company	Cataloug number
The Invitrogen™ Tali™ Image-based Cytometer	Life Technologies Corporation, USA.	T10796
NanoQuant Plate on TECAN Infinite 200 PRO microplate reader.	Life sciences, Switzerland	Infinite 200 PRO NanoQuant
Thermocycler (Gene Amp®, PCR System 9700)	Thermo Fisher	-
2100 Bioanalyzer system	Agilent Technologies, Waldbronn Germany	G2943CA
Mi-Seq Systems	Illumina	SY-411-9001DOC

Table 4: List of softwares

Item	Company
GraphPad Prism 7	GraphPad Software, Inc.
FastQC	Babraham Bioinformatics
DEseq2	Bioconductor
xMAP® Technology	Luminex Corporation

4.2 Study Design

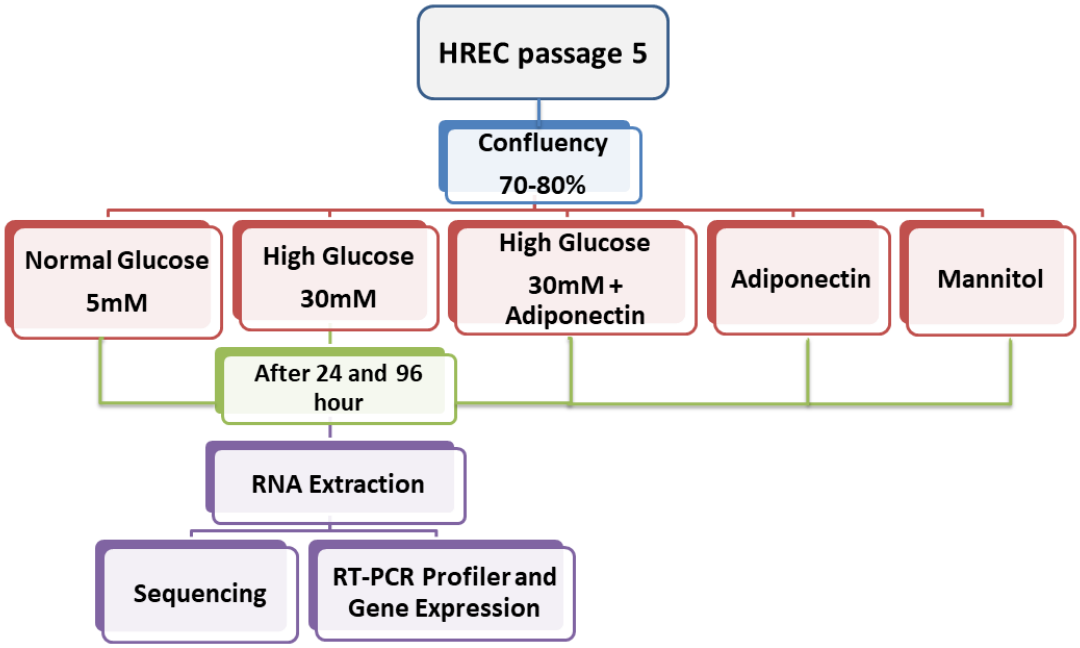


Figure 2: Study Design and workflow

4.3 Methods

4.3.1 Human Retinal Endothelial Cells Cell Culture

Cryopreserved passage 3 HRECs were rapidly thawed in a water bath at 37°C; then complete classic media was immediately added and centrifuged for five minutes at 1000 rpm. Cells were then plated in the flask after the addition of complete media with one ml of antibiotic Pen-Strep and incubated in 5% CO_2 incubator at 37°C until confluency. After 24 hours of growth, the media was changed followed by every 48 hours afterward. The cells were grown and passaged till P5-P6 and then used for the study to minimize variability.

Cell count was performed before culturing or sub-culturing HRECs using Trypan Blue. A volume of 10µl cell suspension added in to 10µl of Trypan Blue, and 15µl of this mixture was loaded on to hemocytometer and counted under a microscope. Cells were counted in the middle square as well as the four corner squares, and the below formula was used to retrieve the total number of cells:

(Total number of cells counted / 5(number of squares)) × 2(dilution factor)

×10,000

The cells have also been counted using Tali™ Image-based Cytometer. In the current study, at least three biological replicates with three independent experiments were conducted.

4.3.2 HRECs Treatment

Table 5: Groups treatment and duration. Abbreviations: s refers short exposure 24 hours, and l refers to long exposure for 96 hours

Group	Treatment	Treatment Duration
NG-s	Normal media (5mM glucose)	24 hours
HG-s	Media with 30mM glucose	24 hours
HG+ApN-s	Media with 30mM glucose and 30µg/ml of adiponectin	24 hours
ApN-s	Normal media (5mM glucose) with 30µg/ml of adiponectin	24 hours
NG-l	Normal media (5mM glucose)	96 hours
HG-l	Media with 30mM glucose	96 hours
HG+ApN-l	Media with 30mM glucose with 30µg/ml of adiponectin	96 hours

Cells were grown on a 6 well plate in complete classic media was changed after 24 hours of growth then by every 48 hours thereafter. Upon 75-85% of cell confluency, HRECs were serum starved by replacing the complete classic media with serum-free media for 6 hours. Then, HRECs were treated with different treatment groups for 24 and 96 hours. Group one is the Normal Glycemia/Control (NG) which contained standard complete media and 5 mM of glucose. Group two is high glucose (HG) where D-glucose to a final concentration of 30mM was added to regular complete media. Group three is high glucose, and adiponectin (HG+ApN) where D-glucose (30mM) and ApN (30µg/ml) were added to the complete conventional media. The last group is ApN (30µg/ml) was added to the normal complete media for 24 h. Experiments were performed with three technical replicates for each group of three different biological replicates. The following groups were studied after 24 hours and 96 hours of exposure to, normal glucose and high glucose followed by ApN administration for further 24h.

4.3.3 Gene Expression Analysis

4.3.3.1 RNA Extraction

Total RNA was extracted from treated HRECs using miRNeasy Mini Kit, Qiagen. Briefly, cells in each well were homogenized in 700µl with QIAzol lysis reagent. Cells were then incubated for 5 minutes at room temperature then transferred to microcentrifuge tubes. A 140µl total volume of chloroform was added to each microcentrifuge tube and was shaken vigorously. Microcentrifuge tubes were then

incubated for 3 minutes followed by centrifugation at 12000xg for 15 minutes at 4^{o}C. Afterward, the aqueous layer was recovered in new 1.5 ml Eppendorf tubes. A 1.5 volume of 100% ethanol was added to each tube and mix thoroughly. Then, samples were transferred to an RNeasy Mini spin column and centrifuged at ≥8000xg for 1 minute at room temperature. Series of washing steps were followed as instructed by the kit manual. After that, 60µl of RNase-free water was used to elute the RNA. Finally, RNA was quantified at absorbance 260/280 nm using NanoQuant Plate on TECAN Infinite 200 PRO microplate reader. The quality of RNA was measured using the 2100 Agilent Bioanalyzer.

For miRNA extraction, mirVana™ miRNA Isolation Kit was used where the sample was lysed in a denaturing lysis buffer that inactivates RNases and stabilizes the RNA. Acid-Phenol: Chloroform was used to extract the lysate and removes most of the debris and cellular components. This resulted in semi-pure RNA sample that was purified using glass-fiber filter. RNA was washed several times and then eluted in a low ionic strength buffer

4.3.3.2 cDNA Preparation

High Capacity RNA to cDNA kit was used to convert the RNA to cDNA. A 1000ng of total extracted RNA was used in this process. The reaction mixture consisted of a total volume 20µl with the following volumes of each component per reaction: 10µl of 2X RT Buffer, 1.0µl of 20X Enzyme Mix, up to 9µl RNA sample and quantity sufficient to 20µl Nuclease-free water. Reverse transcription reaction was performed using Thermocycler (Gene Amp®, PCR System 9700) according to

the following cycles: incubation at 37ºC for 60 minutes, stopping the reaction by heating at 95ºC for 5 minutes followed by a hold stage at 4ºC.

4.3.3.3 Quantitative Real-time PCR

Quantitative RT-PCR was achieved using Applied Biosystem 7500 Real Time PCR system to quantitate gene expression using TaqMan Gene Expression Master Mix (Applied Biosystem, USA). A total volume of 10µl of reaction mixture consisted of different components as shown in Table 4.1.

Table 6: RT-PCR Reaction Mix

Component	**Volume per reaction (µL)**
2x Master Mix	5
20X Primer	0.5
Nuclease-free water	0.5
cDNA sample	4
Total	10

Table 7: Oligonucleotide sequences used in RT-PCR

Gene	Primer Sequence
VEGFA	IDT (Hs.PT.56a.1149801.g)
ADIPQR1	Hs01114951
ADIPQR2	Hs0022610
β-actin	Hs99999903

4.3.3.4 RT^2 Profiler PCR Arrays

RT^2 Profiler PCR Arrays are a 96-well plate that includes preloaded SYBR Green-optimized primer of the pathway and disease-specific gene panel. Using the RT^2 Profiler PCR array allows obtaining highly reliable and accurate expression analysis of the desired panel. In addition, RT^2 Profiler PCR Arrays can be modified to contain a specific panel of genes of interest tailored to the specific research interests (137).

Table 8: RT^2 Profiler PCR Arrays Reaction Mix

Component	Volume (µL)
2x RT2 SYBR Green Mastermix	1350
Nuclease-free Water	1248
cDNA sample	102
Total	2700

In this study, both human angiogenesis and extracellular and adhesion molecules PCR arrays were used to study the angiogenesis and adhesion genes in HRECs exposed to HG and thereafter by adiponectin treatment. The Human angiogenesis RT^2 Profiler PCR Array includes 84 genes related to angiogenesis. It also includes receptors, growth factors, adhesion molecules, chemokines as well as proteases and their inhibitors that have a role in the angiogenesis process. Similarly, the Human Extracellular Matrix and Adhesion Molecules RT^2 Profiler PCR Array profile 84 genes involved in cell-cell and cell-matrix interactions (138). Adhesion molecules play a role in attaching the cells to glycoproteins and proteoglycans to maintain the cell shape, structure, and function. Under the normal physiological condition, the extracellular matrix (ECM) is involved in cell growth, division, differentiation, and migration. However, pathological conditions result in remodeling of ECM and changes in the expression of cell adhesion molecules on the cell surface (139).

Human Extracellular Matrix and Adhesion Molecules RT² Profiler PCR Array contain a variety of ECM proteins such as matrix and metalloproteinases, basement membrane constituents, integrins, selectins, cell-adhesion molecule family members, and cytoskeleton bridging proteins such as catenins. The gene list of both human angiogenesis and adhesion molecules arrays are listed in Appendix 3.

4.3.4 Multiplex ELISA by Luminex 200™

Cytokines are a group of soluble proteins that mediate the communication between cells and regulate their activities (140). They also regulate a wide range of processes such as hematopoiesis, proliferation, and inflammation. Therefore, measuring cytokine concentrations is a fundamental approach to study these processes and can be used as a diagnostic tool for infection (141). Luminex technology is based on multiple analyte profiling using microsphere beads that are 5.6 microns (142). Each micro-bead has specific dye concentration resulting in the unique spectral signature which allows for identification. Therefore, several bead sets can be combined within the assay allowing for multiplexing up to 100 tests in a single reaction volume. The surface chemistry of the micro-beads allows chemical coupling of capture reagents such as oligonucleotides, receptors, antibodies or peptides. Assays are carried out in 96-well plate with up to 100 tests per well. Based on the principle of flow cytometry, high-tech fluidics will cause the suspended micro-beads to line up in a single file prior passing through the detection chamber. Therefore, each micro-bead will be measured discretely. Multiplexed results are detected using Luminex system. In the present study, Luminex was used to identify specific cytokine, IL-6, involved in inflammatory response.

Briefly, 96-well plate was washed with 200µl wash buffer and incubated 10 minutes on a shaker to clean all wells. Standard with different concentrations, control, and samples was then added to wells. Each bead vial contains specific marker/analyte of interest that need to be detected. A 150µl from each vial was added to a mixing bottle with the addition of 2550µl bead diluent in order to reach a total volume of 3000µl. Adding multiple conjugated beads to each sample helped in obtaining multiple results per sample. Beads were mixed well then pipetted (25µl) in each well with 25µl of the culture supernatant (conditioned media) and incubate at 4^{o}C overnight on shaker 500-700rpm. Next day the plate was washed without discarding or losing the beads. This is achieved by using a magnetic holder that will attract all beads and prevent their loss while washing. Conjugate was then added and incubated on a shaker for 1 hour at room temperature. This was followed by addition of Streptavidin-Phycoerythrin incubation for 30 minutes at room temperature. In order to remove unbound antibodies, the plate was placed on the magnetic holder and then washed three times. A total volume of 150 µL of Sheath Fluid was added and then the plate was run on Luminex 200™. Results were calculated by Xpotent 3.1 software and expressed in pg/ml. This technology reduces time; cost and labor effort compared with other traditional methods and has high sensitivity and specificity (143).

4.3.5 Library Preparation and miRNA Sequencing

Before sequencing it is crucial to examine the RNA quality, so as RNA degrarded not interfere with the results. A total of 1µg of total RNA was used to generate the NGS Library. miRNA was extracted from total RNA and library was prepared using NEXTflex Small RNA-Seq Kit v3 (BiooScientific). The adapter was ligated to both ends of the miRNA fragments followed by reverse transcription to cDNA. Adaptors are essential in this process since they hybridize complementary primers in the cDNA reverse transcription and hybridize to flowcell oligos prior bridge amplification during the sequencing process. Each library was quantified and run for quality control using the Agilent Bioanalyzer High Sensitivity Kit in order to avoid over clustering while sequencing. In addition, each library was normalized to 2nM, to ensure that the pool has an equal representation of each library. Then an equal volume of each normalized library was pooled into one tube for final loading into the Illumina MiSeq instrument. The adaptor sequence will hybridize with the oligonucleotides on the surface of the flow cell. DNA polymerase, as well as particular nucleotides with fluorescent terminator caps, will be added to the flow cell too. DNA polymerase will add only one nucleotide with fluorescent terminator cap each round. Once nucleotide added, fluorescent emitted will be detected by a camera and store the data as reads. Library preparation, quantification, barcoding as well as miRNA sequencing were conducted at Weill Cornell Medicine-Qatar.

Different bioinformatics softwares were used to assess the miRNA sequence as shown in the workflow (Figure 2). These include FastQC for quality control, SHRiMP for aligning the reads, and R package for analyzing the results and differential gene

expression analysis. Target genes of differentially expressed miRNAs and related pathways were identified using the gene ontology and KEGG pathway analysis.

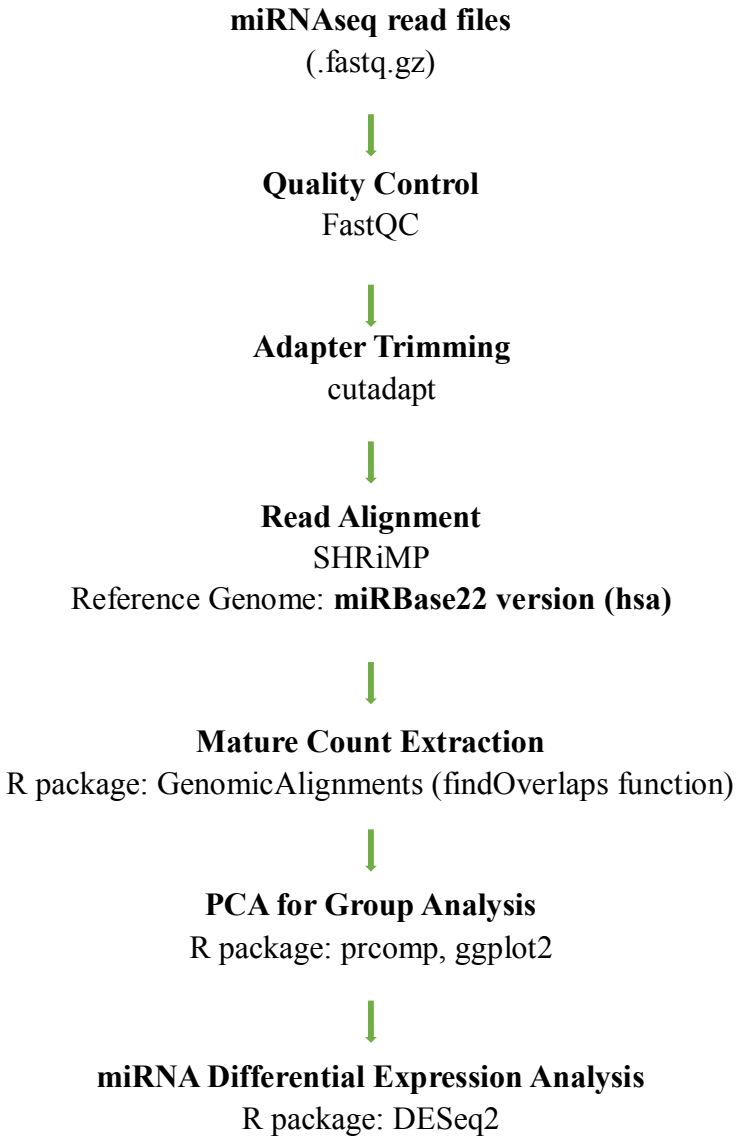


Figure 3: Workflow of miRNA sequencing analysis

4.3.6 Statistical Analysis

For in vitro studies, each experiment was independently repeated three times from three different biological samples. Therefore, values in the figures are expressed as the mean of the experiments with standard deviation. Group differences were evaluated using ANOVA followed by Tukey's post hoc test for multiple comparisons. For all analyses, two-tailed $P < 0.05$ was considered sufficient to reject the null hypothesis. Statistical analyses were conducted using GraphPad 7 for Windows (Version 6 software; San Diego, California) and Microsoft Excel 2013 (Microsoft Corp., Redmond, WA). For differential expressed miRNA, the cut off value for p-value was 0.005, and the -2 log fold change to 2 was selected.

5. RESULTS

5.1 Cell Viability and Treatment

Cell morphology, viability, and count were monitored to study the effect of hyperglycemia on HRECs and adiponectin administration on the hyperglycemic HRECs. At day 0 HRECs were seeded in 75 cm^2 flask. Using inverted microscope at power 10X cell morphology and confluency was monitored (Figure 3A and 3B).

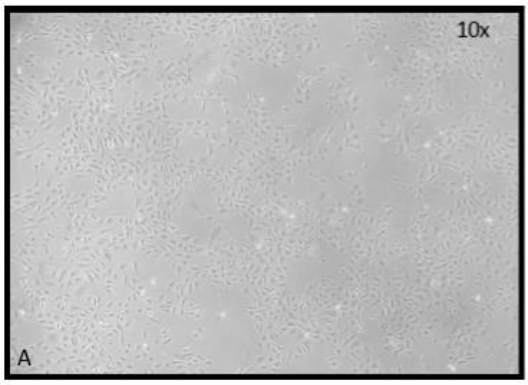


Figure 4A: Cells adherent to the culture plate confluent about 50% to

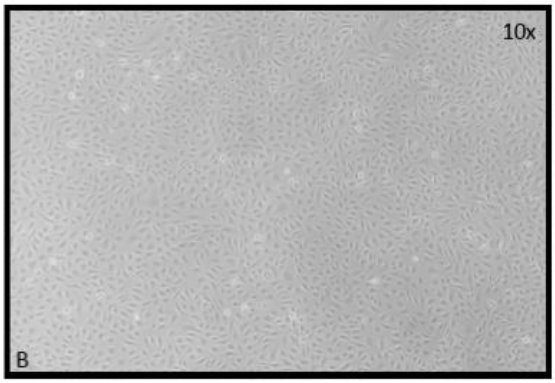


Figure 4B: Cells adherent to the culture plate with 100% confluency.

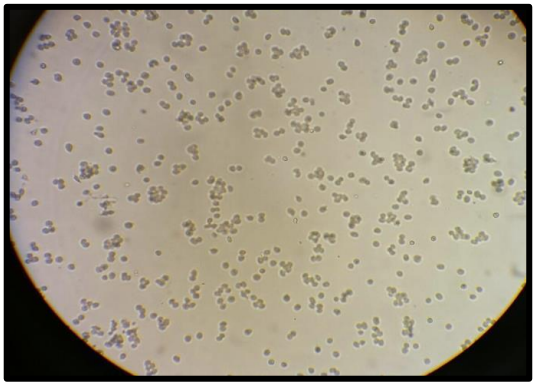
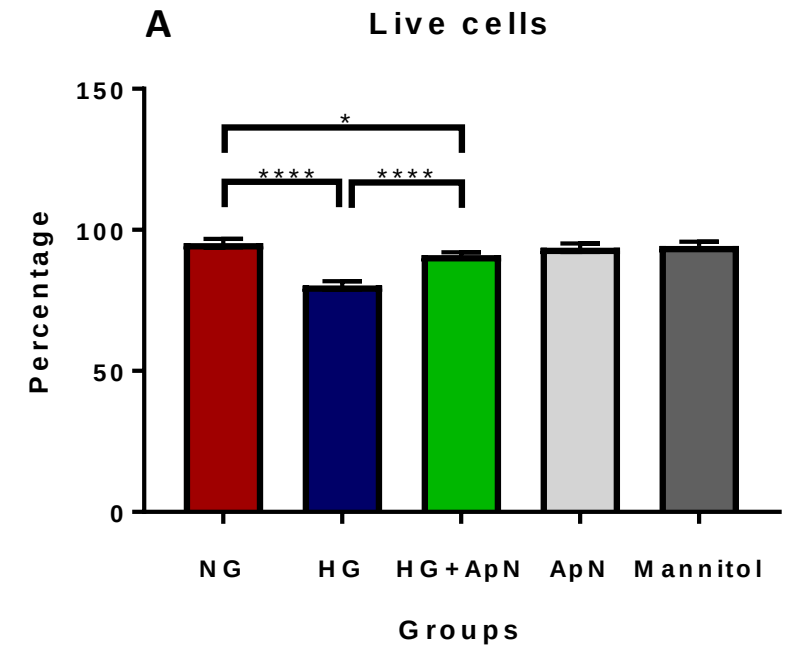


Figure 4C: Cells after trypsinazation

Cell viability is an essential parameter in verifying the health of HRECs culture and to assess the toxic effect of treatment during the experiment. This was achieved by using Tali™ Image-based Cytometer. The average cell count of all experiments, including the replicates, ranged from 2×10^6 to 4×10^6 cell/ml with average size 11-13µm. Cell viability was assessed by calculating the number of live and dead cells. Results showed that HG decreased the HRECs viability significantly compared with the normoglycemic (control) group while ApN-treated HRECs had increased the cell viability compared with HG cells. HG group had 15.8% lower cell viability compared to the normoglycemic with a significant P-value <0.0001, while HG+ApN group had 4% lower cell viability compared to the control group with a significant P-value 0.0235. Comparing HG with HG+ApN group, there was a significant decrease (12%) in cell viability in HG with P-value <0.0001. For dead cells, HG had 440% increase in the number of dead cells compared to control with a significant P-value <0.0001, and 244% increase compared with HG+ApN group with a significant P-value <0.001 (Figure 4). Furthermore, normoglycemic cells treated with adiponectin and HRECs with mannitol as an osmotic control did show any significant difference in the count of living and dead cells with two-tailed p values >0.05 (Figure 4A and 4B).


A
Live cells
150
100
50
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Percentage
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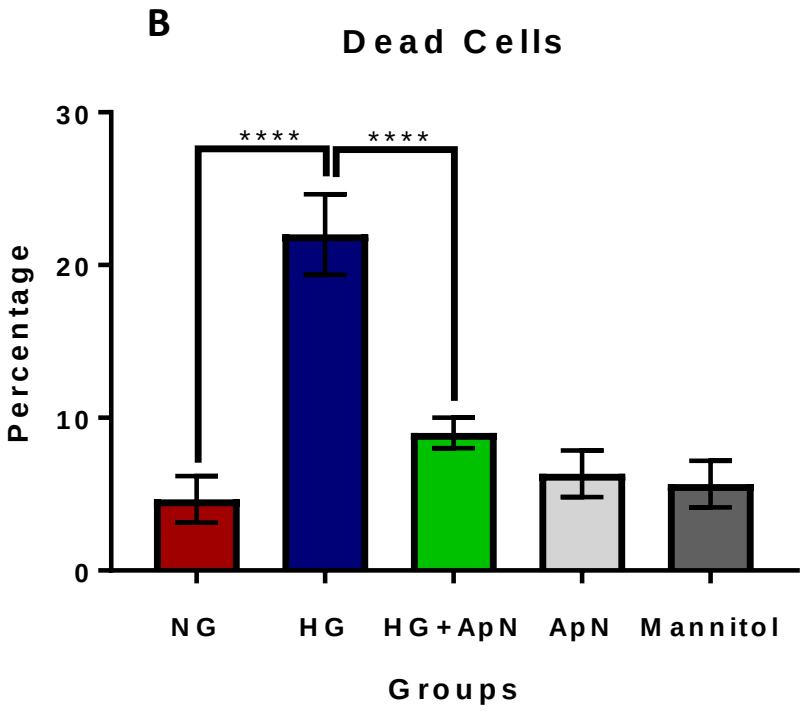
NG
HG
HG+ApN
ApN
Mannitol
Groups


B
Dead Cells
30
20
10
0
Percentage
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NG
HG
HG+ApN
ApN
Mannitol
Groups

Figure 5: Effect of high glucose and adiponectin on cell viability. Graph A represents the percentage of the live cells. Graph B represents the percentage of the dead cells. The cells treated with 5.5mM glucose, 30mM high glucose, 30mM glucose and adiponectin (30ug/ml), and normoglycemic cells treated with adiponectin (30uM) and mannitol (25mM) as an osmotic control. The data presented as means (SD) of 3-4 independent experiments, analyzed by One-way ANOVA with posthoc Tukey-Kramer multiple comparisons test between individual groups. Two-tailed p-value is significant at $p<0.05$. * $P<0.05$ and **** $P<0.0001$.

5.2 Quality of RNA and miRNA extracted from the treated HRECs

The number of cells before the extraction ranged between 1 and 1.5 million cells. After extraction, the quality of extracted RNA and miRNA was assessed before proceeding with gene expression and RNA sequencing. Such quality assays are critical in all subsequent analysis and data interpretation. The extracted RNA concentration, purity, and integrity were assessed. All samples had RNA Integrity Number (RIN) between 8.5 and 10 out of 10 and 206/280 ratio of 1.8-2.1 as shown in Figure 4. Furthermore, the concentration of miRNA and percentage in small RNA was evaluated as shown in Figure 6, indicating good percentage ranging between 20-38%.

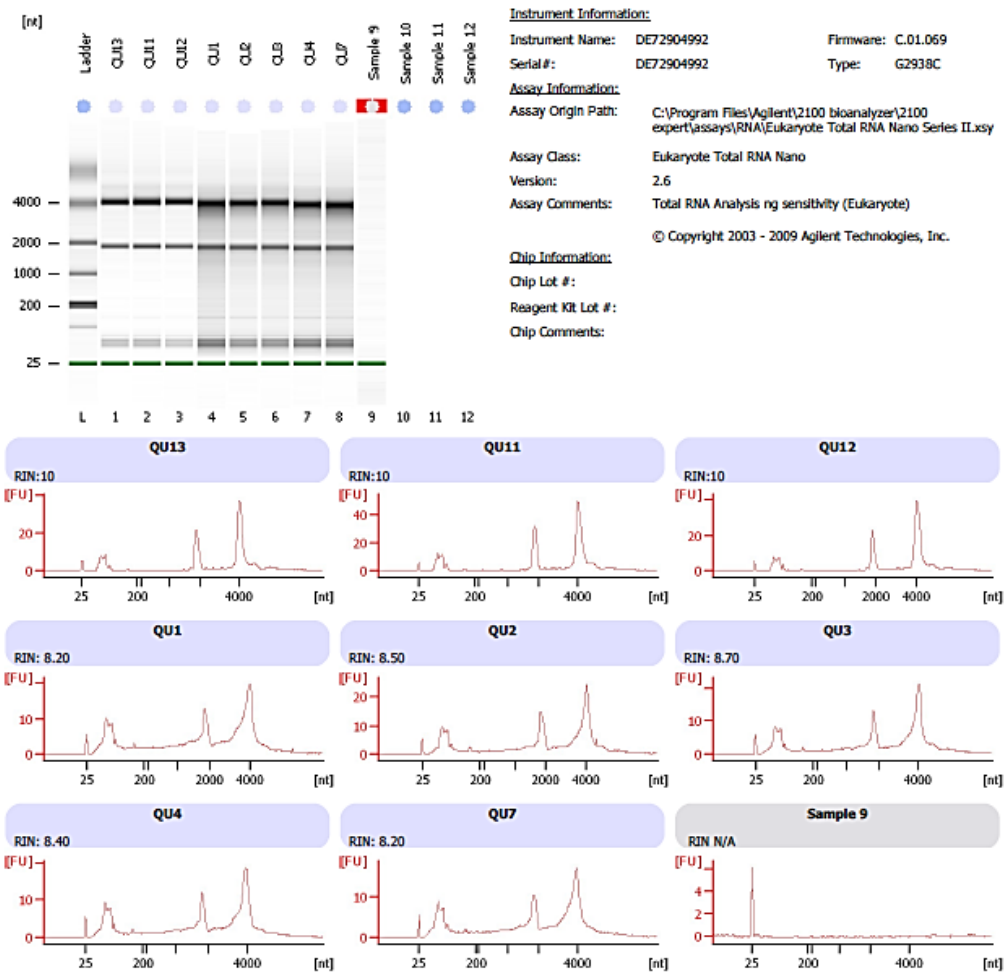


Figure 6: Total RNA quality results by 2100 Agilent Bioanalyzer. The Agilent report includes an electropherogram, RNA integrity number (RIN), and RNA concentration. RIN is a scale range between 0 to 10, where 0 represent degraded RNA sample and 10 represent highly intact RNA.

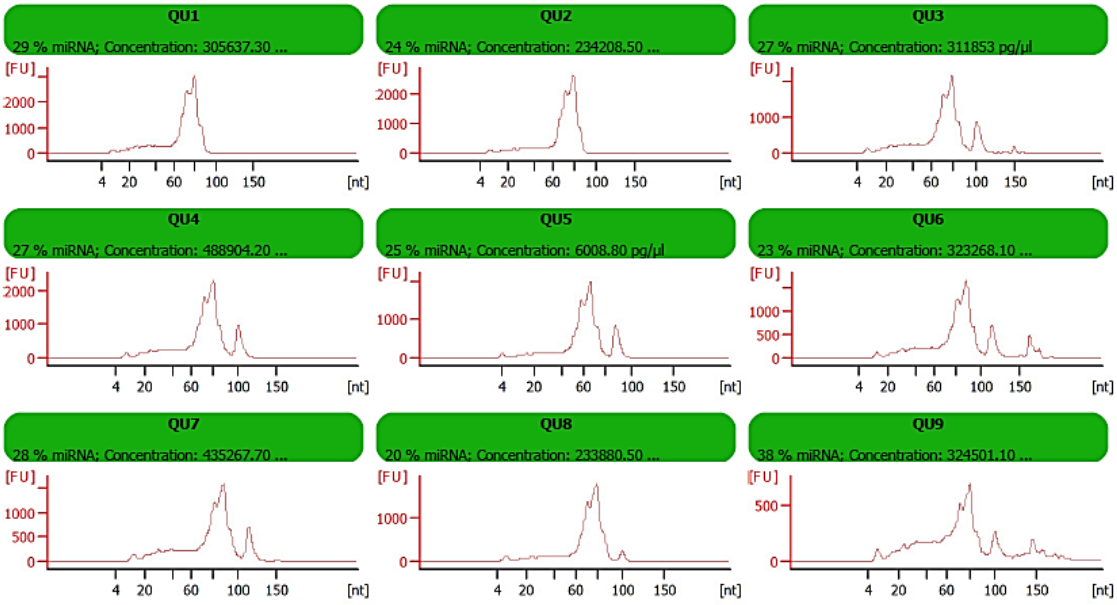


Figure 7: miRNA quality results by 2100 Agilent Bioanalyzer. The Agilent report includes an electropherogram, RNA integrity number (RIN), and RNA concentration. RIN is a scale range between 0 to 10, where 0 represent degraded RNA sample and 10 represent highly intact RNA.

5.3 Effect of Adiponectin Treatment on Adiponectin Receptors Expression

The effect of ApN on HRECs is exerted through its receptors, AdipoR1 and AdipoR2. Determining the expression of ApN receptors, using RT-PCR, could help in understanding the mechanism of ApN in DR pathogenesis. AdipoR1 was significantly decreased in HG group by 0.73 fold compared to NG group with P-value <0.001, while the expression of AdipoR1 increased significantly in HG+ApN (1.9 folds) compared to hyperglycemic cells with P-value<0.0001.Similarly, AdipoR2 showed the same expression pattern where the expression of AdipoR2 increases significantly in HG+ApN compared to hyperglycemic cells with P-value<0.001. Furthermore, HG downregulated the expression of AdipoR1 and AdipoR2 in HRECs by 0.73 and 0.65 fold change respectively. Using the qPCR, the expression of the two receptors were upregulated in HG-treated with adiponectin and, AdipoR1 mRNA expression increased by 1.86 fold changes than AdipoR2mRNA expression in ApN treated HG cells with P-value<0.0001 (Figure 7).

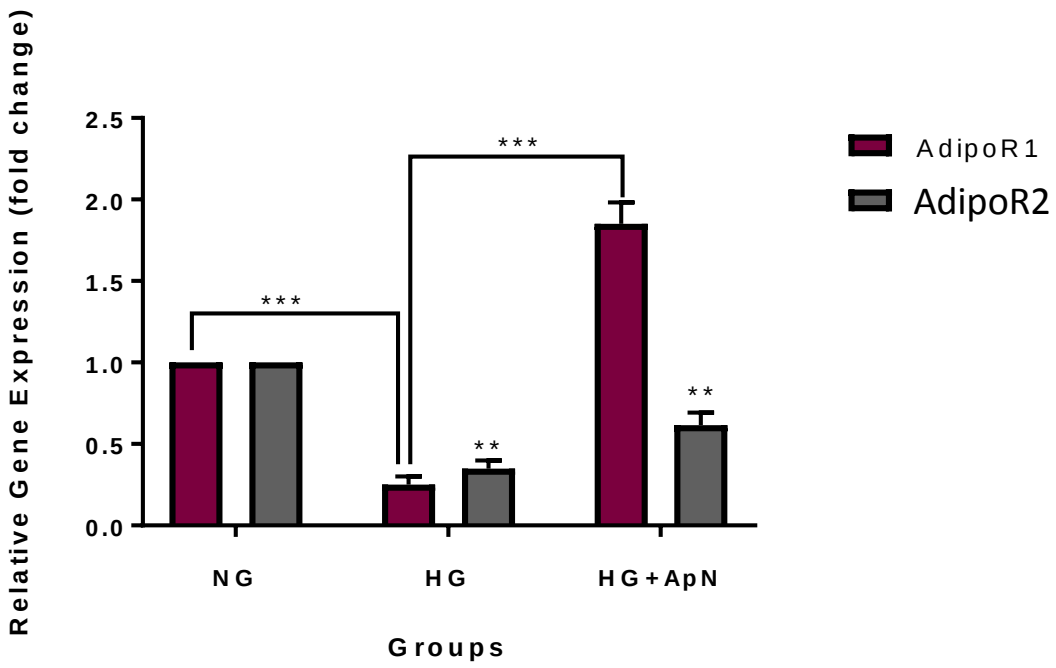


Figure 8: Gene expression analysis by RT-PCR for adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2) mRNA in HREC. HG group treated with high glucose concentration (30mM) for 24 hours and HG+ApN with both high glucose concentration (30mM) and ApN (30µg/mL). Bars show the data presented as means with a standard deviation of three independent experiments analyzed by one-way ANOVA and posthoc multiple comparison tests. Two-tailed P-value is significant at $p \leq 0.05$. For AdipoR1, *** HG is significantly different from the control group, and after ApN treatment ($P \leq 0.001$). For AdipoR2, ** both HG and HG+ApN groups are significantly different from the control group ($P < 0.01$).

5.4 Effect of Adiponectin on Inflammation and VEGF-A mRNA Expression

The expression of inflammatory biomarker IL-6 in HRECs cells under high glucose as well as adiponectin treatment conditions was examined. The level of IL-6 was significantly increased in HG group compared to NG with P-value <0.001 and decreased significantly in HG group treated with ApN compared to HG group by 1.5 folds with P-value 0.0135 (Figure 8). Also, there was a significant increase in IL-6 level in HG+ApN compared with NG (P-value 0.0018).

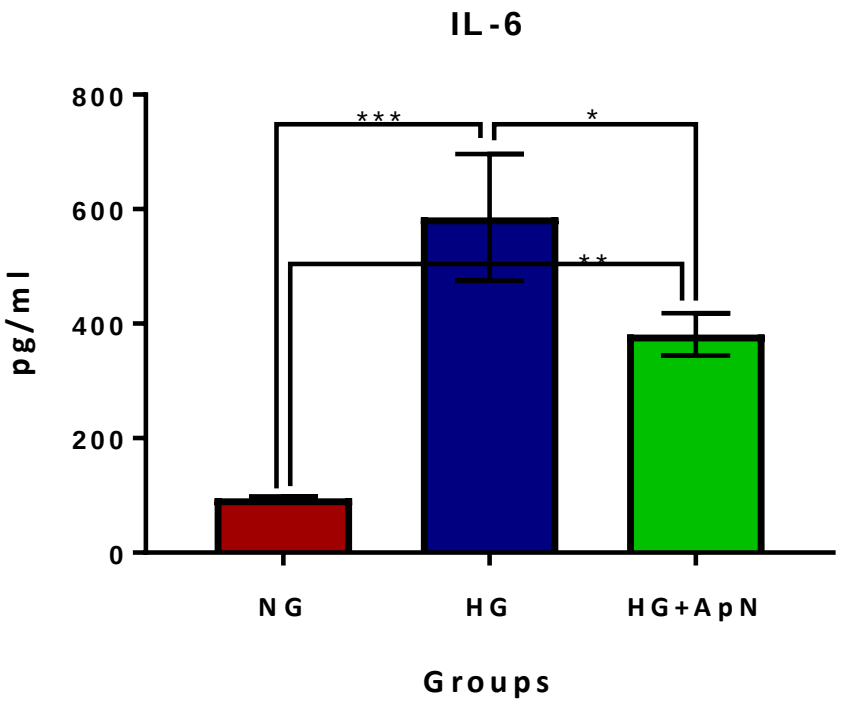


Figure 9: IL-6 cytokine in the supernatant of HRECs (conditioned media) after different treatments using multiplex ELISA. The data presented as means (SD) of 3-4 independent experiments, analyzed by One-way ANOVA with posthoc Tukey-Kramer multiple

comparisons test between individual groups. Two-tailed p-value is significant at $p<0.05$. * $P<0.05$, ** $P<0.01$and *** $P<0.001$.

The expression of VEGFA mRNA after high glucose treatment significantly increased compared to NG with (P-value 0.0025) (Figure 9). However, the VEGF-A expression significantly reduced after ApN treatment to HG cells (P-value <0.0001). Similarly, the expression was significantly decreased in HG+ApN group compared to NG (P-value 0.0031). Although ApN decreased the expression of VEGF-A in high glucose-treated cell, it increased the expression of VEGF-A in ApN-treated HRECs. However, these finding are at gene expression level and need to be examined at protein level.

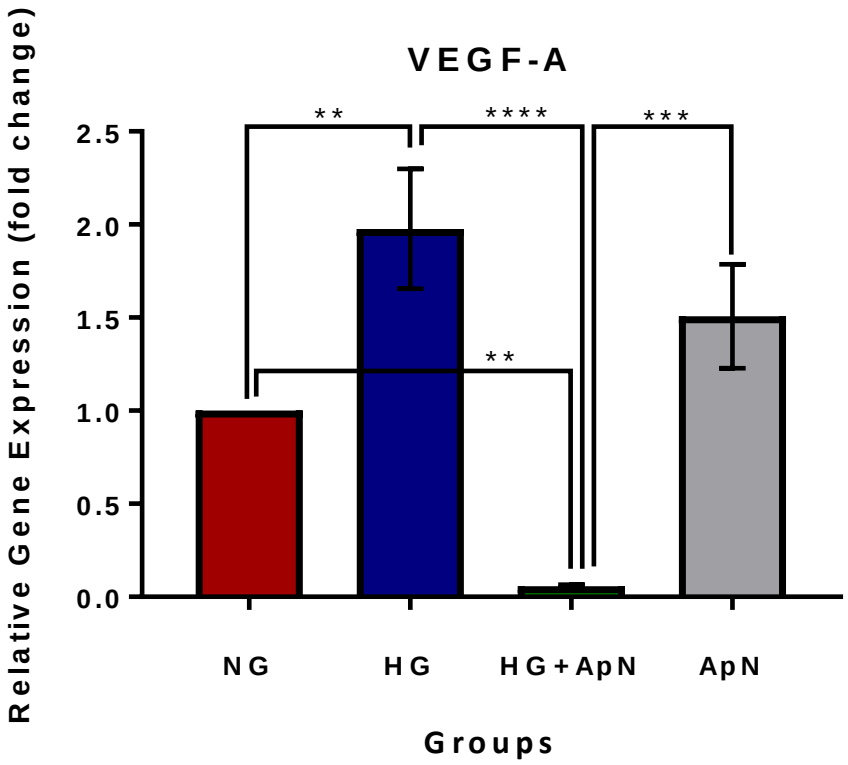


Figure 10: Gene expression analysis by RT-PCR for VEGF-A mRNA in HREC. The data presented as means (SD) of 3-4 independent experiments, analyzed by One-way ANOVA with posthoc Tukey-Kramer multiple comparisons test between individual groups. Two-tailed p-value is significant at $p<0.05$. ** $P<0.01$, *** $P<0.001$ and **** $P<0.0001$.

5.5 Analysis of miRNA Expression

5.5.1 RT-PCR Profiler

RT-PCR profiler plates were used to screen for adhesion and angiogenesis genes commercially provided by the company. The array consists of 84 genes of adhesion/angiogenesis. Using the 2 as fold regulation cut off the results of HG+ApN group was compared with HG. There was downregulation of most of the adhesion as well as angiogenesis genes (Figure 10 and Table 9). For each panel of the identified DEG, a list of miRNAs regulating the up and downregulated genes was predicted (Table 10).

A. HG group compared to HG+ApN after 24 hours treatment (Adhesion molecules gene panel)

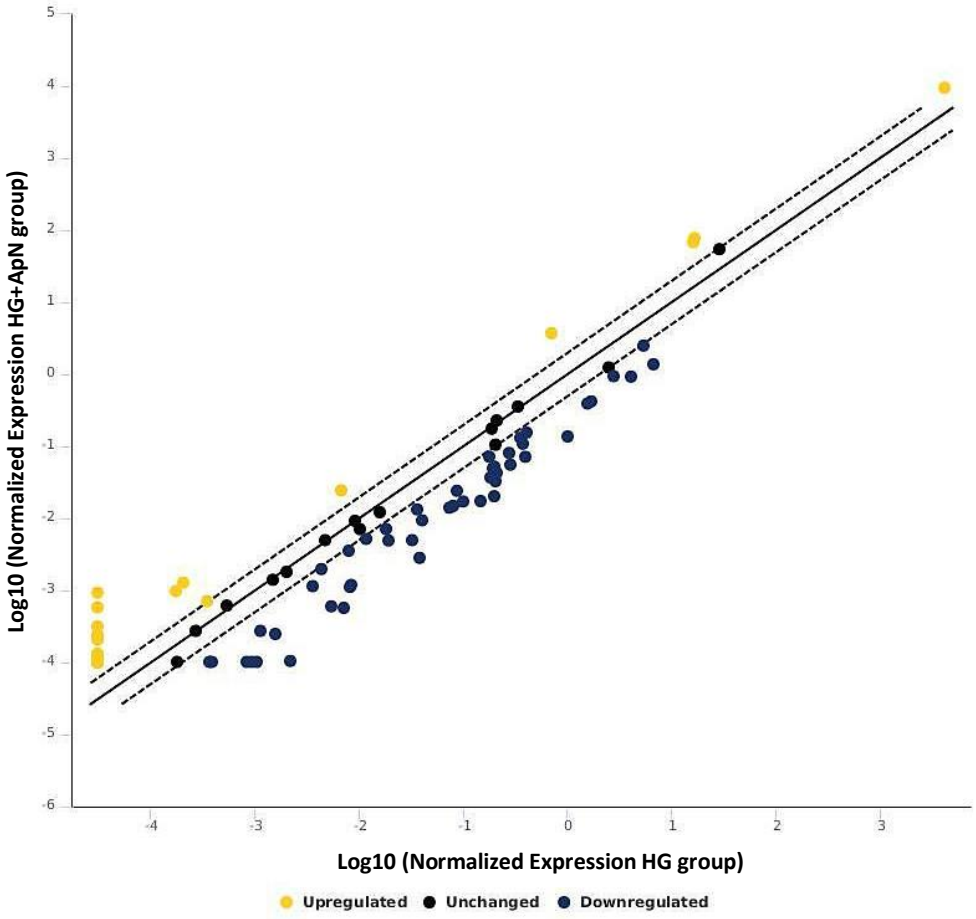


Figure 11: The scatterplot compares the normalized expression of each gene on the array between the two selected groups by plotting them against one another to visualize substantial gene expression changes. The central line indicates unchanged gene expression. The dotted lines indicate the chosen fold regulation threshold. Data points beyond the dotted lines in the upper left and lower right sections meet the selected fold regulation threshold.

Table 9: The list of the adhesion genes over and under-expressed in HG+ApN group vs. HG group

Gene	Gene ID	Fold Change
VCAM1	P19320	29.76
SELL	P14151	-20.57
ITGAL	P20701	18.60
COL8A1	P27658	-13.55
COL12A1	Q99715	-12.43
ECM1	Q16610	-10.16
ADAMTS8	Q9UP79	10.08
MMP14	P50281	-9.67
ITGB2	P05107	-9.10
THBS3	P49746	-9.04
CTNNB1	P35222	-8.33
COL16A1	Q07092	-8.27
MMP8	P22894	7.66
COL6A2	P12110	-7.28
CD44	P16070	-7.23
ITGA1	P56199	-7.00
COL1A1	P02452	6.63
TIMP1	P01033	-6.53
ITGB4	P16144	-6.42

Data represent the changes in gene expression by fold changes. The red color indicates upregulation, and the black with minus value indicates downregulation. The gene symbol name symbol and its gene ID are provided.

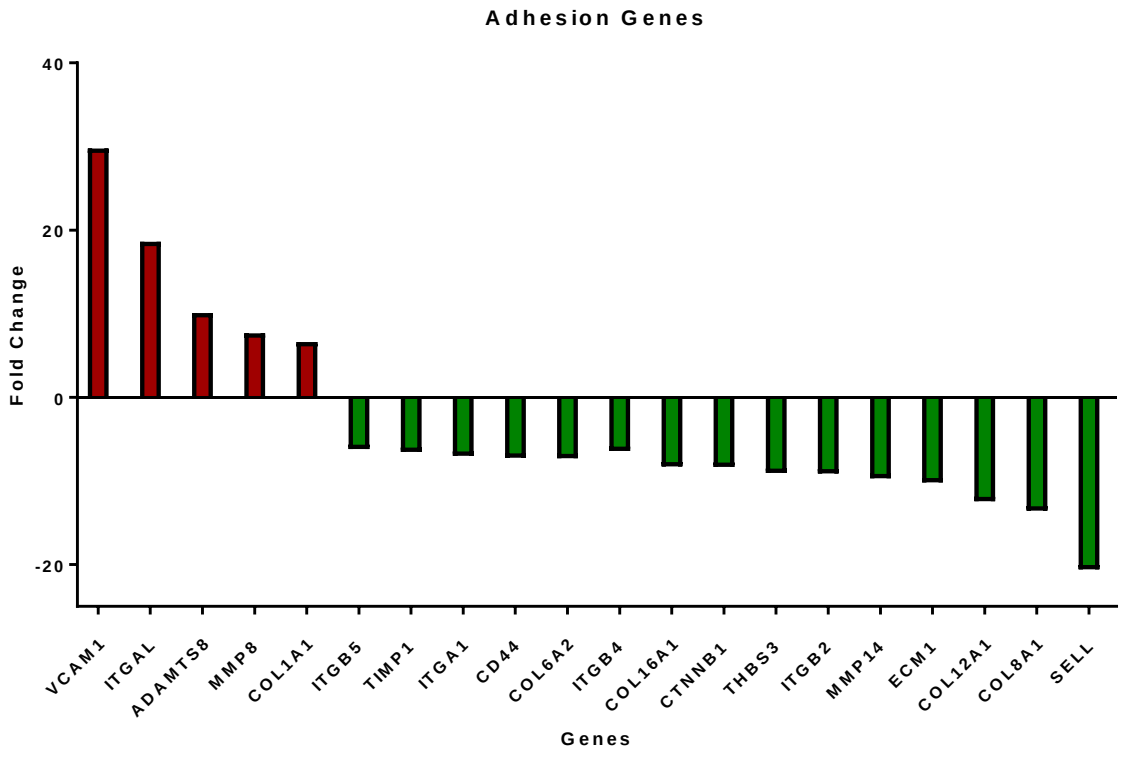


Figure 12: Adhesion genes over and under-expressed in HG+ApN group vs. HG group

Table 10: The list of miRNAs regulating adhesion genes under and over-expressed in HG+ApN vs. HG group

Under- Expressed Genes		**Over- Expressed Genes**	
miRNA Name	**Target Genes**	**miRNA Name**	**Target Genes**
hsa-miR-1304-5p	ITGA1	hsa-miR-590-3p	VCAM1
hsa-miR-330-3p	CTNNB1	hsa-miR-23a-3p	ITGAL
hsa-miR-9-5p	ITGB4	hsa-miR-23b-3p	ITGAL
hsa-miR-519b-3p	ITGB4	hsa-miR-1178-3p	VCAM1
hsa-miR-519c-3p	ITGB4	hsa-miR-519b-3p	ADAMTS8
hsa-miR-519a-3p	ITGB4	hsa-miR-519a-3p	ADAMTS8
hsa-miR-449b-5p	COL12A1	hsa-miR-519c-3p	ADAMTS8
hsa-miR-449a	COL12A1	hsa-miR-203a	ADAMTS8
hsa-miR-34c-5p	COL12A1	hsa-miR-218-5p	COL1A1
hsa-miR-101-3p	COL12A1	hsa-miR-944	COL1A1
hsa-miR-423-5p	THBS3	hsa-miR-193a-5p	COL1A1

hsa-miR-587	COL12A1	hsa-miR-577	COL1A1
hsa-miR-34a-5p	COL12A1	hsa-miR-143-3p	COL1A1
hsa-miR-526b-5p	CTNNB1	hsa-miR-516a-3p	COL1A1
hsa-miR-374a-5p	MMP14	hsa-miR-371a-5p	COL1A1
hsa-miR-431-5p	COL12A1	hsa-miR-548d-3p	COL1A1
hsa-miR-298	COL12A1	hsa-miR-153-3p	ADAMTS8
hsa-miR-214-3p	CTNNB1	hsa-let-7g-5p	ADAMTS8, COL1A1
hsa-miR-374b-5p	MMP14	hsa-let-7i-5p	ADAMTS8, COL1A1
hsa-miR-1244	COL12A1	hsa-let-7d-5p	ADAMTS8, COL1A1

B. HG group compared to HG+ApN after 24 hours treatment (Angiogenesis panel)

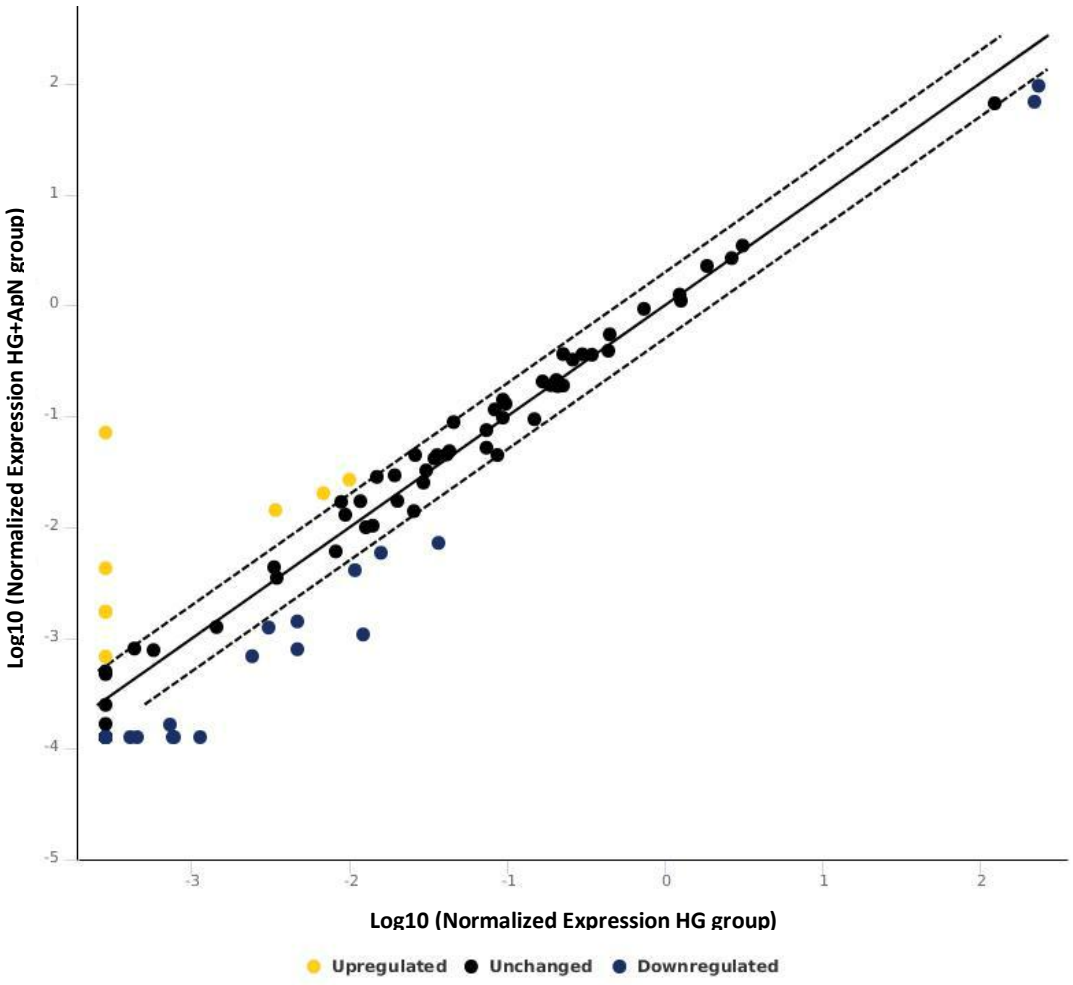


Figure 13: The scatterplot compares the normalized expression of every gene on the array between the two selected groups by plotting them against one another to visualize substantial gene expression changes. The central line indicates unchanged gene expression. The dotted lines indicate the selected fold regulation threshold. Data points beyond the dotted lines in the upper left and lower right sections meet the selected fold regulation threshold.

Table 11: Angiogenesis genes over and under-expressed in HG+ApN vs. HG group

Gene Symbol	Gene ID	Fold Regulation	Gene Symbol	Gene ID	Fold Regulation
ADAM17	P78536	-2.66	MMP9	P14780	-2.26
AGT	B0ZBE2	-2.26	NPPB	P16860	-2.26
AGTR1	P30556	-2.26	NPR1	P16066	-3.58
ALOX5	P09917	-2.26	OCLN	Q16625	-11.37
ANGPT1	Q15389	-5.93	PF4	P02776	-2.26
APOE	P02649	-5.96	PLG	P00747	-2.26
BCL2	P10415	-2.72	PTGIS	Q16647	-2.26
BCL2L1	Q07817	2.65	PTGS2	P35354	2.96
CALCA	P06881	-3.20	SELE	P16581	14.79
CCL2	P13500	248.06	SELL	P14151	-2.50
CCL5	P13501	-2.26	SELPLG	Q14242	5.99
CX3CL1	P78423	-5.08	THBD	P07204	2.36
EDN2	P20800	-2.26	TNF	P01375	-2.26
EDNRA	P25101	-6.05	VCAM1	P19320	5.97
FGF1	P05230	-2.26	KLK3	P07288	-3.53
ICAM1	P05362	4.20	IL1B	P01584	-2.26
IL11	P20809	-2.26	IL3	P08700	-8.96
F3	P13726	-2.26	FASLG	P48023	-2.26
FAS	P25445	-3.31	IL7	P13232	-2.26

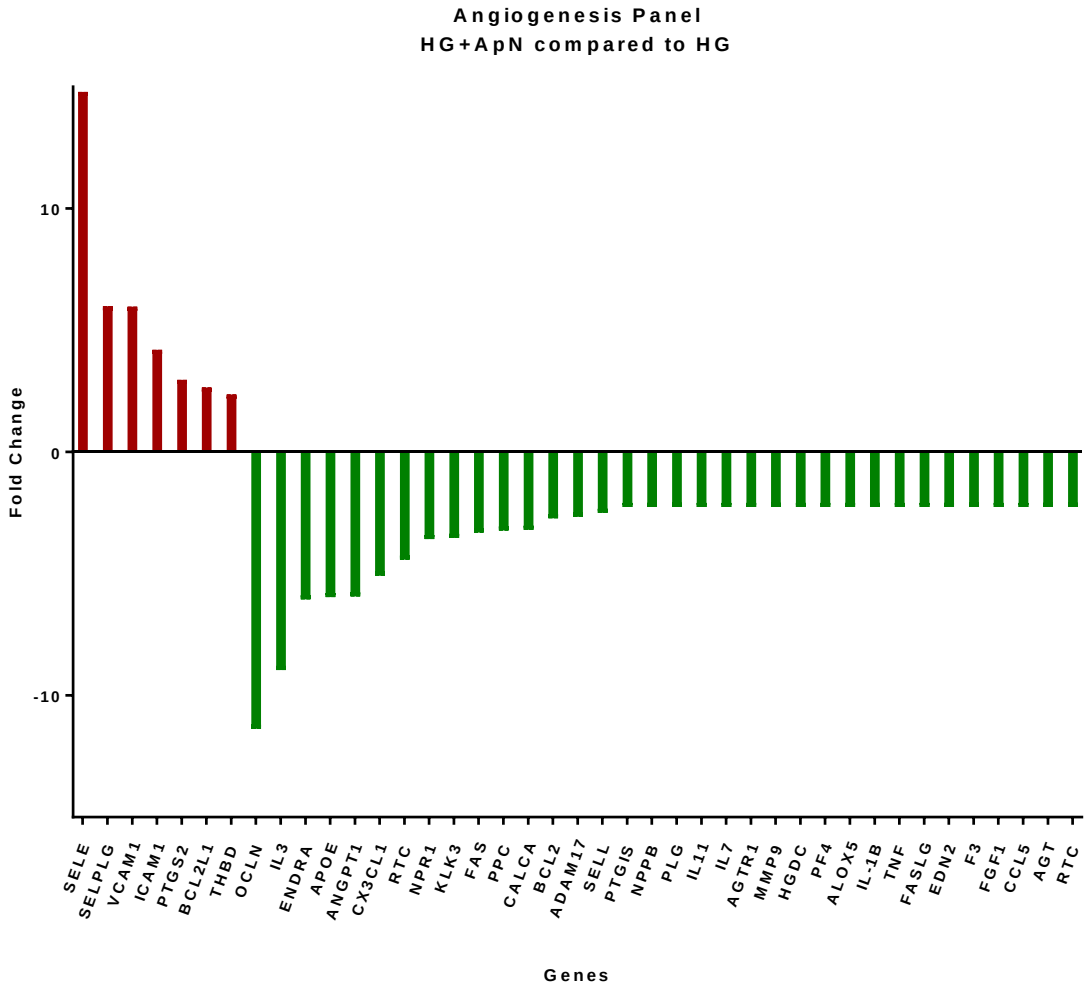


Figure 14: Angiogenesis genes over and under-expressed in HG+ApN group vs. HG group.

Table 12: miRNA regulating angiogenesis genes under and over-expressed in HG+ApN vs. HG group.

Under- Expressed Genes		Over- Expressed Genes	
miRNA Name	**Target Genes**	**miRNA Name**	**Target Genes**
hsa-miR-23a-3p	FAS	hsa-miR-374b-5p	CCL2
hsa-miR-23b-3p	FAS	hsa-miR-374a-5p	CCL2
hsa-miR-889-3p	OCLN	hsa-miR-26b-5p	PTGS2
hsa-miR-149-5p	EDNRA	hsa-miR-1297	PTGS2
hsa-miR-153-3p	ANGPT1	hsa-miR-26a-5p	PTGS2
hsa-miR-122-5p	OCLN	hsa-miR-590-3p	VCAM1
hsa-miR-767-3p	NPR1	hsa-miR-381-3p	SELE
hsa-miR-448	BCL2	hsa-miR-513b-5p	PTGS2
hsa-miR-145-5p	ADAM17	hsa-miR-300	SELE
hsa-miR-224-5p	ADAM17	hsa-miR-1305	SELE
hsa-miR-568	BCL2	hsa-miR-377-3p	SELE
hsa-miR-204-5p	ANGPT1, BCL2	hsa-miR-1178-3p	VCAM1
hsa-miR-211-5p	ANGPT1, BCL2	hsa-miR-767-5p	SELE
hsa-miR-520g-3p	ANGPT1	hsa-miR-101-3p	PTGS2
hsa-miR-98-5p	FAS	hsa-miR-1286	ICAM1
hsa-let-7g-5p	FAS	hsa-miR-576-3p	PTGS2
hsa-let-7f-5p	FAS	hsa-miR-144-3p	PTGS2
hsa-let-7c-5p	FAS	hsa-miR-1827	PTGS2
hsa-let-7i-5p	FAS	hsa-miR-143-3p	PTGS2

C. HG group compared to NG after 96 hours treatment (Angiogenesis panel)

Comparing HG group treated for 96 hours with high glucose to NG group, there was an up-regulation of many angiogenesis genes as shown the scatter plot below. A significant increase was shown in adhesion G protein-coupled receptor B1 (ADGRB1) gene as it reached 470 fold change as indicated in the heat map (Figure 15). After ApN treatment, there was a decrease in the fold change of up-regulated genes in HG group but still not as NG level (Figure 13).

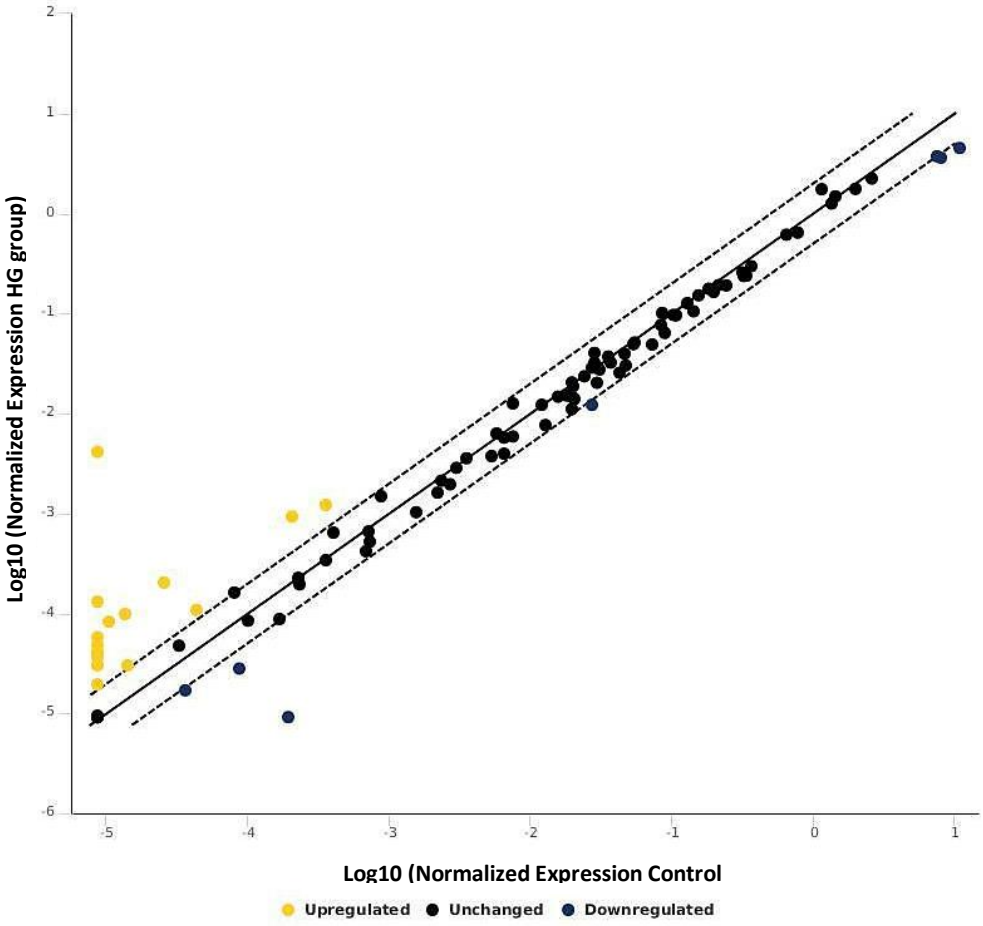


Figure 15: The scatterplot compares the normalized expression of each gene on the array between the two selected groups by plotting them against one another to quickly visualize substantial gene expression changes. The central line indicates unchanged gene expression. The dotted lines indicate the selected fold regulation threshold. Data points beyond the dotted lines in the upper left and lower right sections meet the selected fold regulation threshold.

Table 13: List of the angiogenesis genes over and under-expressed in HG group vs. NG group after 96 hours treatment

Gene Symbol	Gene ID	Fold Regulation	Gene Symbol	Gene ID	Fold Regulation
ANG	P01019	3.36	THBS2	P35442	-21.23
ADGRB1	O14514	470.51	HGF	P08581	4.65
CCL11	P51671	2.12	IGF1	P05019	2.22
COL4A3	Q01955	2.46	IL1B	P01584	-2.14
CXCL1	P09341	-2.23	PLG	P00747	4.21
CXCL9	Q07325	6.58	IFNG	P01579	3.45
EGF	P01133	4.53	PROK2	Q9HC23	-3.10
FGF1	P05230	14.93			

Data shows that most of the angiogenesis genes were upregulated in HG group compared to control. This supports that high glucose condition could induce different pathological changes including angiogenesis in HRECs.

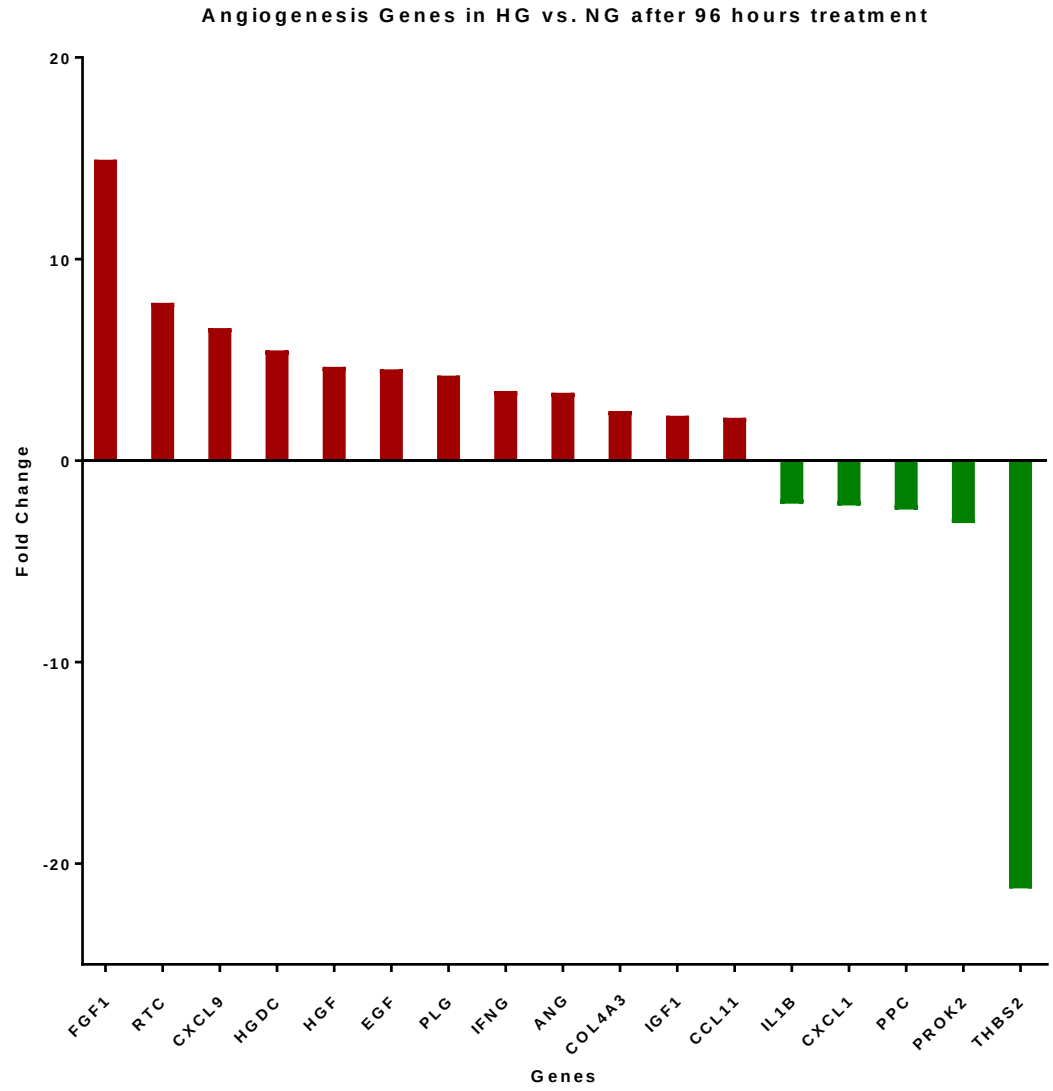
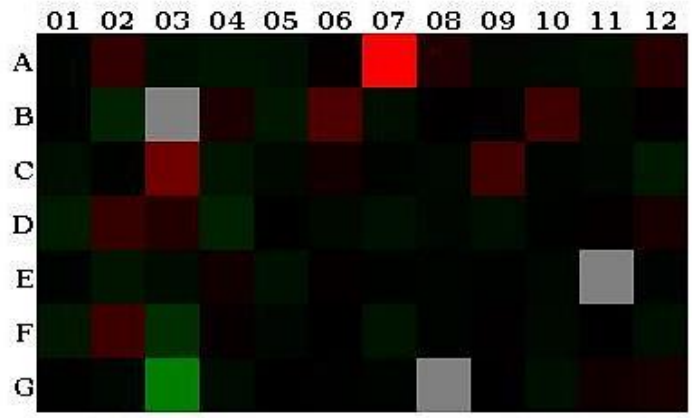
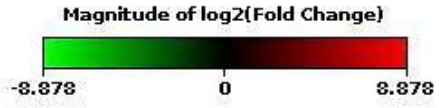


Figure 16: Angiogenesis genes over and under-expressed in HG group vs. NG group after 96 hours of treatment. Of note, ADGRB1 gene had 470.51 fold change and could not be plotted on this graph as it will shift all other genes expression down and make the graph challenging to visualize.





Layout	01	02	03	04	05	06	07	08	09	10	11	12
A	AKT1 -1.12	ANG 3.36	ANGPT1 -1.39	ANGPT2 -1.57	ANGPTL4 -1.46	ANPEP 1.17	ADGRB1 470.51	CCL11 2.12	CCL2 -1.20	CDH5 -1.29	COL18A1 -1.39	COL4A3 2.46
B	CTGF -1.07	CXCL1 -2.23	CXCL10 1.04 C	CXCL5 1.98 B	CXCL6 -1.66	CXCL9 6.58 B	EDN1 -1.40	EFNA1 -1.02	EFNB2 1.04	EGF 4.53 A	ENG -1.22	EPHB4 1.04
C	ERBB2 -1.38	F3 -1.04	FGF1 14.93	FGF2 -1.65	FGFR3 -1.20	FIGF 1.58	FLT1 -1.08	FN1 -1.22	HGF 4.65	HIF1A -1.08	HPSE -1.15	ID1 -1.79
D	IFNA1 -1.92	IFNG 3.45	IGF1 2.22	IL1B -2.14	IL6 1.01	CXCL8 -1.20	ITGAV -1.50	ITGB3 -1.17	JAG1 -1.40	KDR -1.07	LECT1 1.08	LEP 1.70
E	MDK -1.11	MMP14 -1.66	MMP2 -1.36	MMP9 1.44 B	NOS3 -1.47	NOTCH4 1.08	NRP1 -1.03	NRP2 -1.10	PDGFA -1.06	PECAM1 -1.13	PF4 1.04 C	PGF -1.12
F	PLAU -1.67	PLG 4.21	PROK2 -3.10	PTGS1 1.13	S1PR1 -1.16	SERPINE1 1.02	SERPINF1 -1.51	SPHK1 -1.08	TEK 1.06	TGFA -1.20	TGFB1 -1.01	TGFB2 -1.38
G	TGFBR1 -1.06	THBS1 -1.17	THBS2 -21.23	TIE1 -1.37	TIMP1 1.02	TIMP2 -1.03	TIMP3 -1.08	TNF 1.04	TYMP -1.01	VEGFA -1.43	VEGFB 1.42	VEGFC 1.68

Figure 17: The Heat Map provides a visualization of the fold changes in expression between the selected groups for every gene in the array in the context of the array layout. The table provides the fold regulation data used for the map as well as the comments associated with each one.

Table 14: miRNA regulating angiogenesis genes under and over-expressed in HG vs. NG group

Under- Expressed Genes		Over- Expressed Genes	
miRNA Name	**Target Genes**	**miRNA Name**	**Target Genes**
hsa-miR-101-3p	PROK2	hsa-miR-590-3p	IGF1
hsa-miR-591	THBS2	hsa-miR-425-5p	IGF1
hsa-miR-182-5p	THBS2	hsa-miR-875-3p	COL4A3
hsa-miR-885-5p	PROK2	hsa-miR-1275	IGF1
hsa-miR-891b	THBS2	hsa-miR-381-3p	FGF1
hsa-miR-23a-3p	PROK2	hsa-miR-21-5p	FGF1
hsa-miR-23b-3p	PROK2	hsa-miR-590-5p	FGF1
hsa-miR-374a-5p	PROK2	hsa-miR-300	FGF1
hsa-miR-598-3p	THBS2	hsa-miR-625-5p	IGF1
hsa-miR-374b-5p	PROK2	hsa-miR-148b-3p	IGF1
hsa-miR-922	THBS2	hsa-miR-148a-3p	IGF1
hsa-miR-641	CXCL1	hsa-miR-152-3p	IGF1
hsa-miR-524-5p	THBS2	hsa-miR-125a-5p	COL4A3
hsa-miR-520d-5p	THBS2	hsa-miR-125b-5p	COL4A3
hsa-miR-30b-5p	THBS2	hsa-miR-484	FGF1
hsa-miR-30a-5p	THBS2	hsa-miR-582-5p	HGF
hsa-miR-30d-5p	THBS2	hsa-miR-519d-3p	COL4A3
hsa-miR-30e-5p	THBS2	hsa-miR-93-5p	COL4A3
hsa-miR-30c-5p	THBS2	hsa-miR-20b-5p	COL4A3
hsa-miR-96-5p	PROK2, THBS2	hsa-miR-106a-5p	COL4A3

D. HG+ApN group compared to HG after 96 hours treatment (Angiogenesis panel)

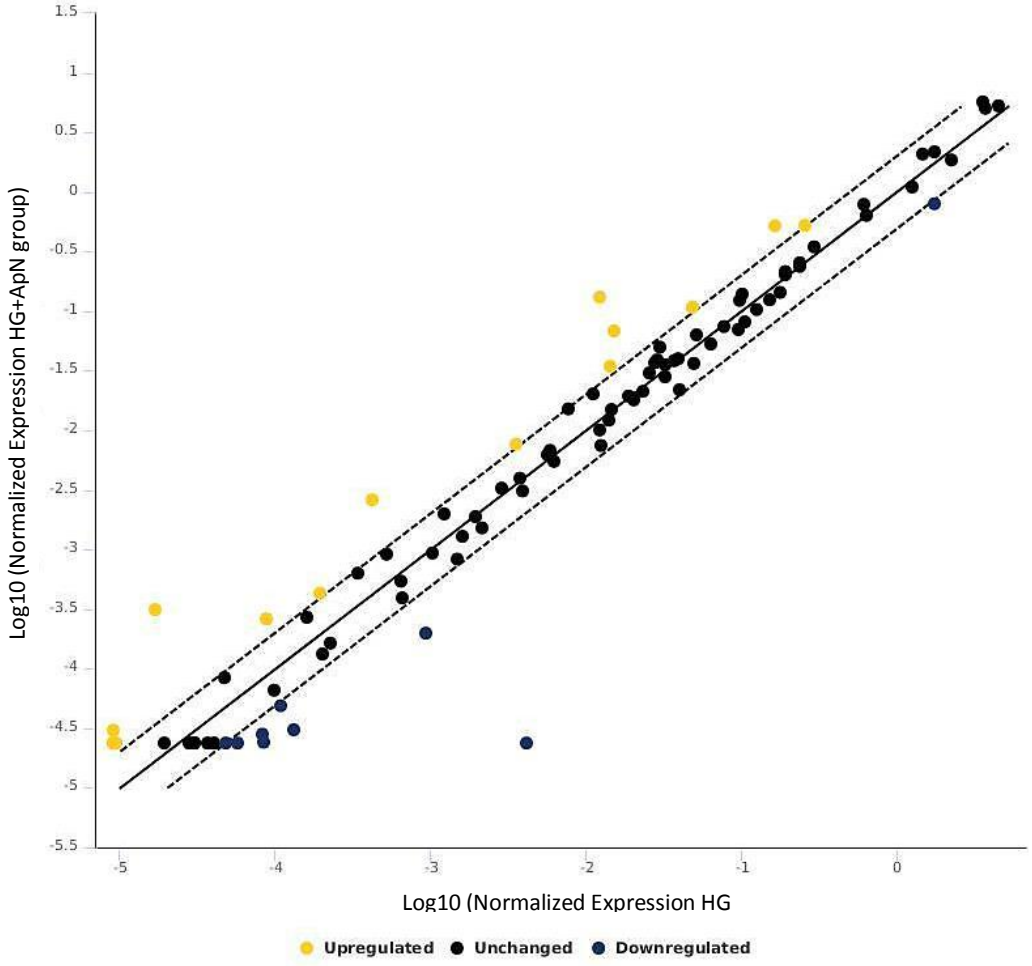


Figure 18: The scatterplot compares the normalized expression of every gene on the array between the two selected groups by plotting them against one another to quickly visualize substantial gene expression changes. The central line indicates unchanged gene expression. The dotted lines indicate the selected fold regulation threshold. Data points beyond the dotted lines in the upper left and lower right sections meet the selected fold regulation threshold.

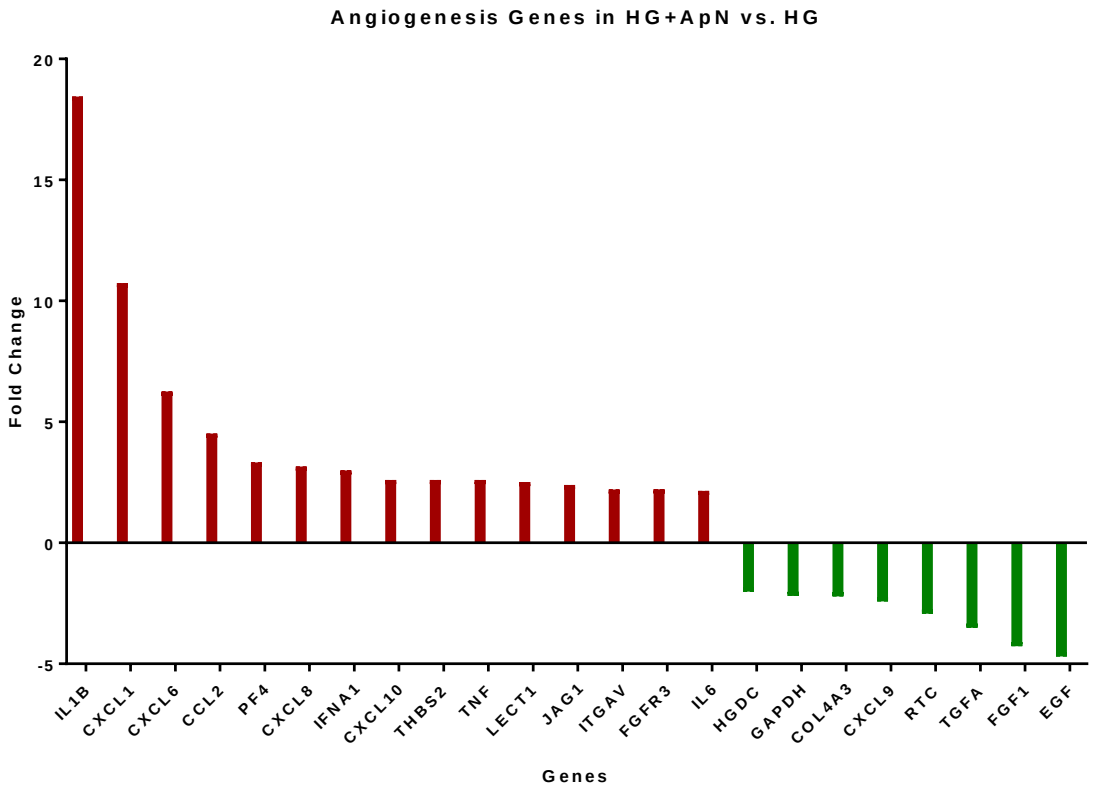
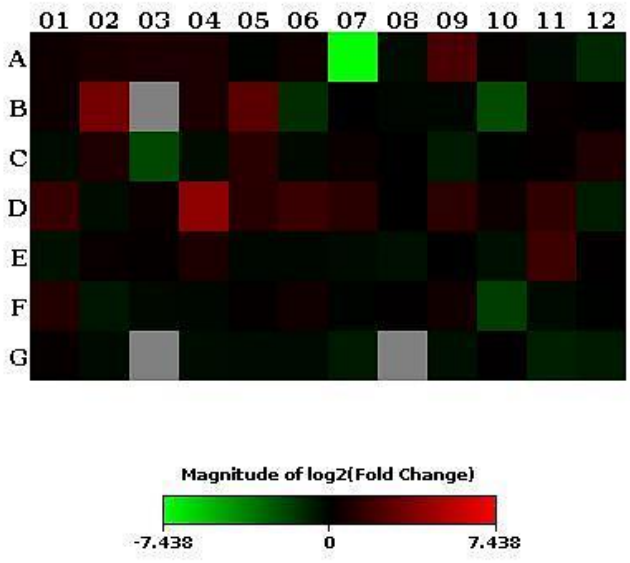


Figure 19: Angiogenesis genes over and under-expressed in HG+ApN group vs. HG group after 96 hours of treatment. ADGRB1 gene had -173.39 fold change and could not be plotted on this graph as it will shift all other genes expression down and make the graph challenging to visualize.



Layout	01	02	03	04	05	06	07	08	09	10	11	12
A	AKT1 1.34	ANG 1.64	ANGPT1 1.75	ANGPT2 1.66	ANGPTL4 -1.09	ANPEP 1.38	ADGRB1 -173.39	CCL11 -1.27	CCL2 4.52	CDH5 1.13	COL18A1 -1.21	COL4A3 -2.21
B	CTGF 1.28	CXCL1 10.74	CXCL10 2.60	CXCL5 1.67	CXCL6 6.25	CXCL9 -2.42	EDN1 -1.01	EFNA1 -1.11	EFNB2 -1.13	EGF -4.69	ENG 1.16	EPHB4 1.03
C	ERBB2 -1.25	F3 1.86	FGF1 -4.27	FGF2 -1.27	FGFR3 2.21	FIGF -1.19	FLT1 1.23	FN1 -1.01	HGF -1.72	HIF1A -1.04	HPSE 1.09	ID1 1.83
D	IFNA1 2.99	IFNG -1.27	IGF1 1.22	IL1B 18.44	IL6 2.15	CXCL8 3.15	ITGAV 2.20	ITGB3 1.01	JAG1 2.39	KDR 1.27	LECT1 2.51	LEP -1.79
E	MDK -1.40	MMP14 1.18	MMP2 1.08	MMP9 1.77	NOS3 -1.15	NOTCH4 -1.15	NRP1 -1.22	NRP2 -1.36	PDGFA 1.01	PECAM1 -1.36	PF4 3.32	PGF 1.05
F	PLAU 1.98	PLG -1.55	PROK2 -1.18	PTGS1 -1.14	S1PR1 1.10	SERPINE1 1.40	SERPINF1 -1.10	SPHK1 1.03	TEK 1.36	TGFA -3.51	TGFB1 -1.23	TGFB2 -1.04
G	TGFBR1 1.15	THBS1 -1.20	THBS2 2.60	TIE1 -1.30	TIMP1 -1.21	TIMP2 -1.23	TIMP3 -1.68	TNF 2.60	TYMP -1.38	VEGFA 1.06	VEGFB -1.84	VEGFC -1.69

Figure 20: The Heat map provides a visualization of the fold changes in expression between the selected groups for every gene in the array in the context of the array layout. The table provides the fold regulation data used for the map as well as the Comments associated with each one.

E. HG+ApN group compared to NG after 96 hours treatment (Angiogenesis panel)

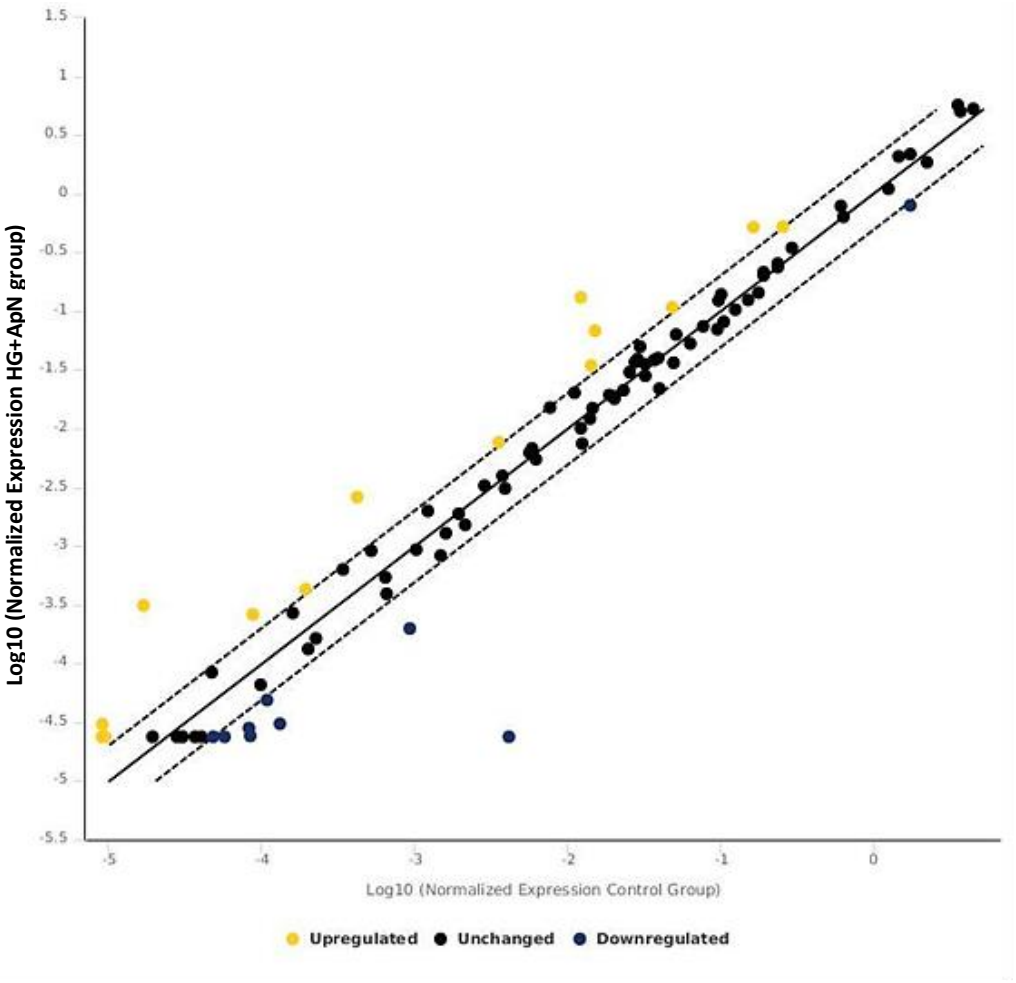


Figure 21: The scatterplot compares the normalized expression of every gene on the array between the two selected groups by plotting them against one another to quickly visualize substantial gene expression changes. The central line indicates unchanged gene expression. The dotted lines indicate the selected fold regulation threshold. Data points beyond the dotted lines in the upper left and lower right sections meet the selected fold regulation threshold.

Table 15: Angiogenesis genes under and over-expressed in HG+ApN vs. NG group

Gene symbol	Gene ID	Fold Change	Gene symbol	Gene ID	Fold Change
THBS2	P35442	-8.17	HGF	P14210	2.71
TGFA	P01135	-4.19	IFNG	P01579	2.71
PROK2	Q9HC23	-3.66	IGF1	P05019	2.71
ANG	P03950	5.50	IL1B	P01584	8.62
ADGRB1	O14514	2.71	IL6	P05231	2.18
CCL2	P13500	3.75	CXCL8	P10145	2.63
CXCL1	P09341	4.83	MMP9	P14780	2.55
CXCL10	P02778	2.71	PF4	P02776	3.47
CXCL5	P42830	3.32	PLG	P00747	2.71
CXCL6	P80162	3.75	FGF2	P09038	-2.11
CXCL9	Q07325	2.71	THBS2	P35442	-8.17
FGF1	P05230	3.50	TNF	P01375	2.71

Dara represents the angiogenesis gene expression in HRECs after 96 hours in high glucose condition followed by 24 hours ApN treatment and then compared with the control. Although many genes were still upregulated even after ApN treatment, the fold change was lower compared with HG group. This shows that ApN downregulated angiogenesis genes but still not at the control level.

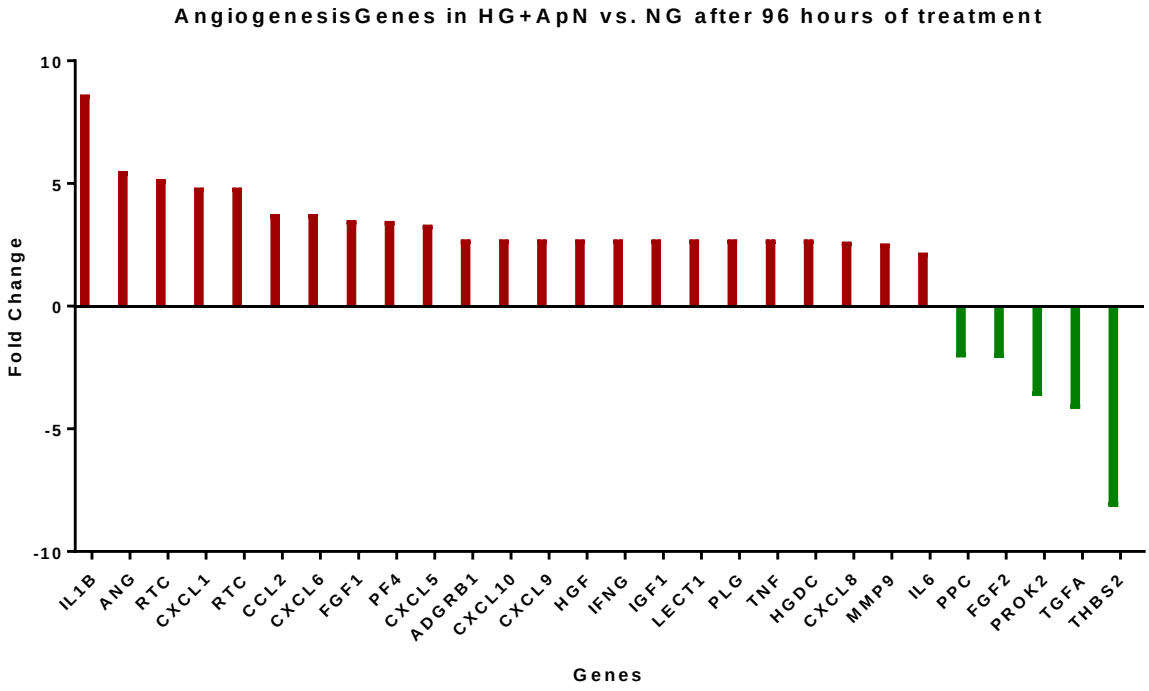


Figure 22: Angiogenesis genes over and under-expressed in HG+ApN group vs. NG group after 96 hours of treatment.

Table 16: miRNA regulating angiogenesis genes under and over-expressed in HG+ApN vs. NG group

Under- Expressed Genes		Over- Expressed Genes	
miRNA Name	**Target Genes**	**miRNA Name**	**Target Genes**
hsa-miR-1178-3p	TGFA	hsa-miR-590-3p	IGF1
hsa-miR-591	THBS2	hsa-miR-374a-5p	CCL2
hsa-miR-148a-3p	TGFA	hsa-miR-374b-5p	CCL2
hsa-miR-148b-3p	TGFA	hsa-miR-425-5p	IGF1
hsa-miR-152-3p	TGFA	hsa-miR-1323	CXCL6
hsa-miR-101-3p	PROK2, TGFA	hsa-miR-548o-3p	CXCL6
hsa-miR-182-5p	THBS2	hsa-miR-206	IGF1
hsa-miR-885-5p	PROK2	hsa-miR-1	IGF1
hsa-miR-891b	THBS2	hsa-miR-613	IGF1
hsa-miR-205-5p	TGFA	hsa-miR-1275	IGF1
hsa-miR-942-5p	TGFA	hsa-miR-21-5p	FGF1
hsa-miR-23b-3p	PROK2, TGFA	hsa-miR-381-3p	FGF1
hsa-miR-374a-5p	PROK2, TGFA	hsa-miR-590-5p	FGF1
hsa-miR-23a-3p	PROK2, TGFA	hsa-miR-300	FGF1
hsa-miR-598-3p	THBS2	hsa-miR-625-5p	IGF1
hsa-miR-374b-5p	PROK2, TGFA	hsa-miR-148b-3p	IGF1
hsa-miR-922	THBS2	hsa-miR-148a-3p	IGF1
hsa-miR-301b	TGFA	hsa-miR-152-3p	IGF1
hsa-miR-301a-3p	TGFA	hsa-miR-484	FGF1
hsa-miR-140-3p	TGFA	hsa-miR-582-5p	HGF

5.5.2 RNA Sequencing

Before analyzing the sequencing results, the quality of run should be evaluated. Using FastQC software, the average of Q score across all bases was 36 which indicate approximately 99.99% accuracy of base calling (Figure 23 and 24). After checking the quality of sequencing, trimming of the reads was done to remove adapter sequences, any ambiguous nucleotides and restricting the fragment size to miRNA size (Figure 25). Principal Component Analysis (PCA) is a method for compressing a broad set of data into a smaller set that captures the essence of the original data. The graph usually shows the clustering of cells' transcriptions with a similar profile. In our study, most of the groups with the same transcription profile clustered together (Figure 26) except for one control group that was an outlier and showed different transcription. This could be due to biological variability between samples. Therefore, for all comparisons involving control groups, the sample NC1 was excluded because it clustered with HG samples on the PCA plot (Figure 26). For comparisons that involved replicates, differentially expressed miRNAs were found using DEseq2 (R package). Comparison of differentially expressed miRNAs from different groups of treated HRECs revealed 14 miRNAs, of which 12 were up-regulated and two down-regulated (Table 17 and Figure 27).

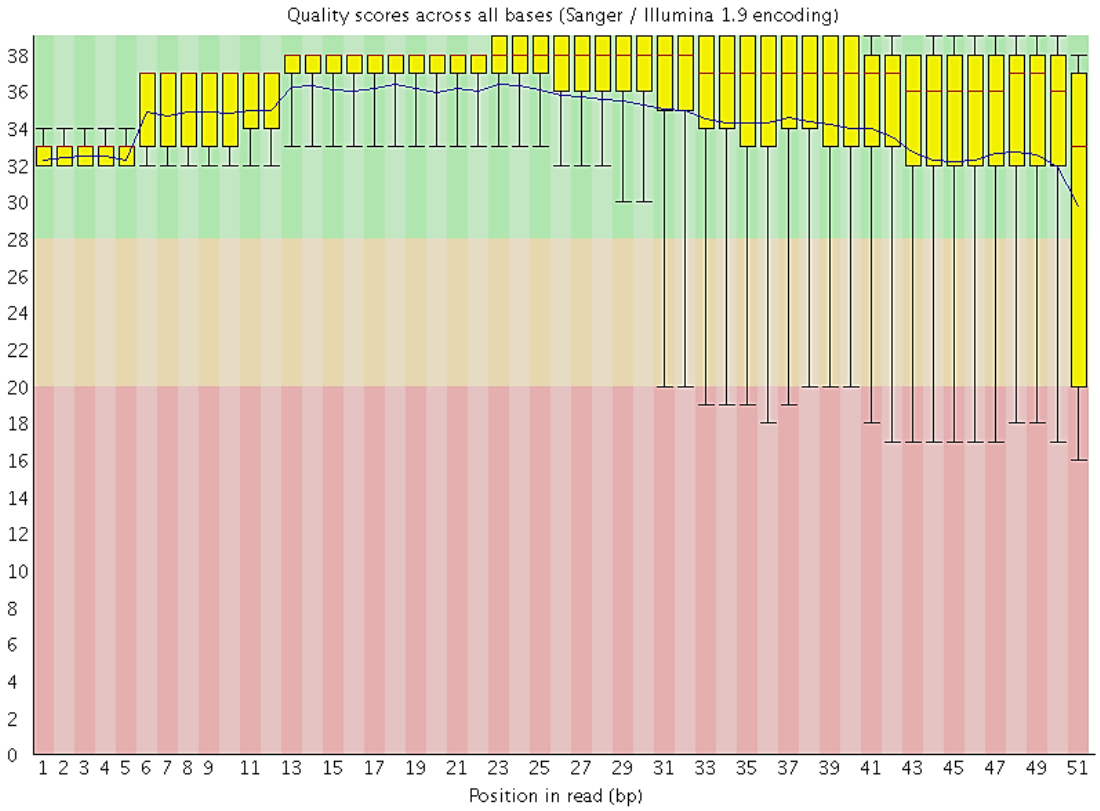


Figure 23: Quality scores (Phred score) across all bases. All bases had a score of 30 and above which indicate 99.9% accuracy of base calling during the sequencing.

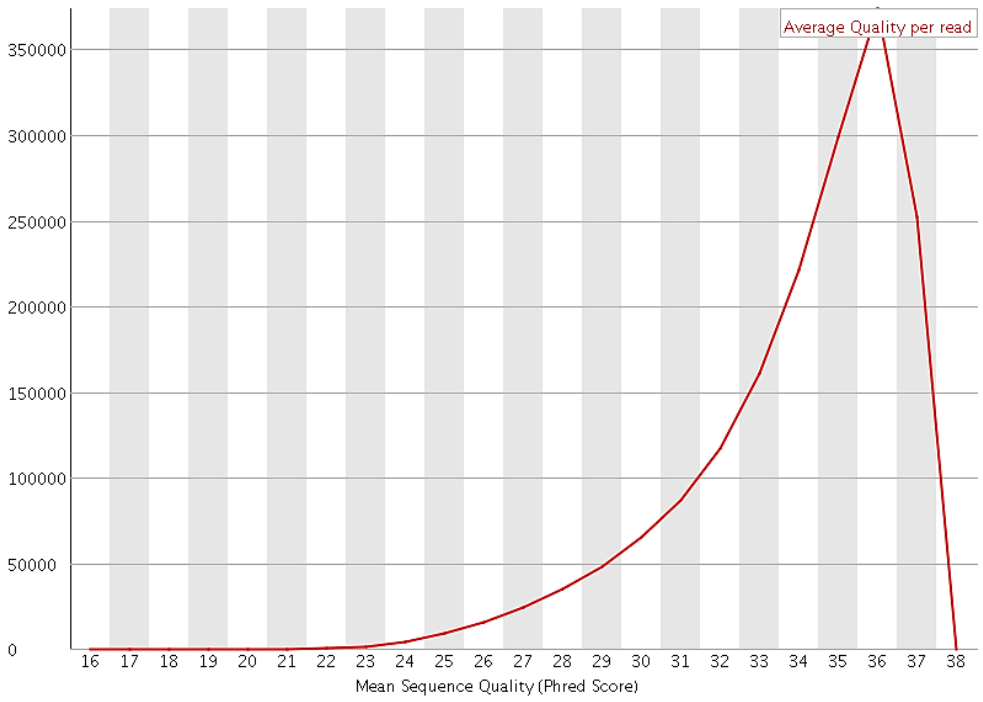


Figure 24: Quality scores (Phred score) distribution over all sequences. The average score is 36 which indicate 99.99% accuracy of base calling during the sequencing.

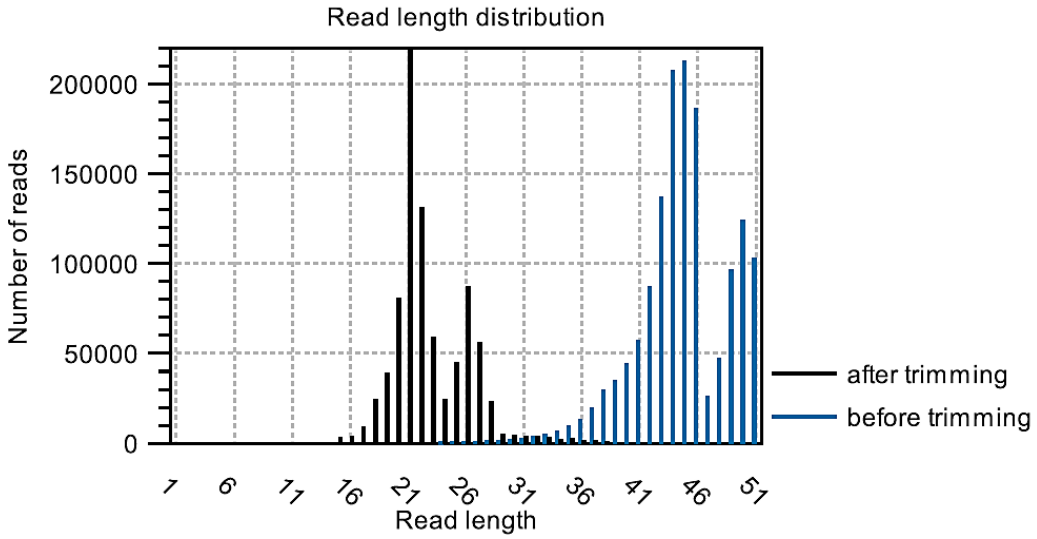


Figure 25: Reads length before and after trimming

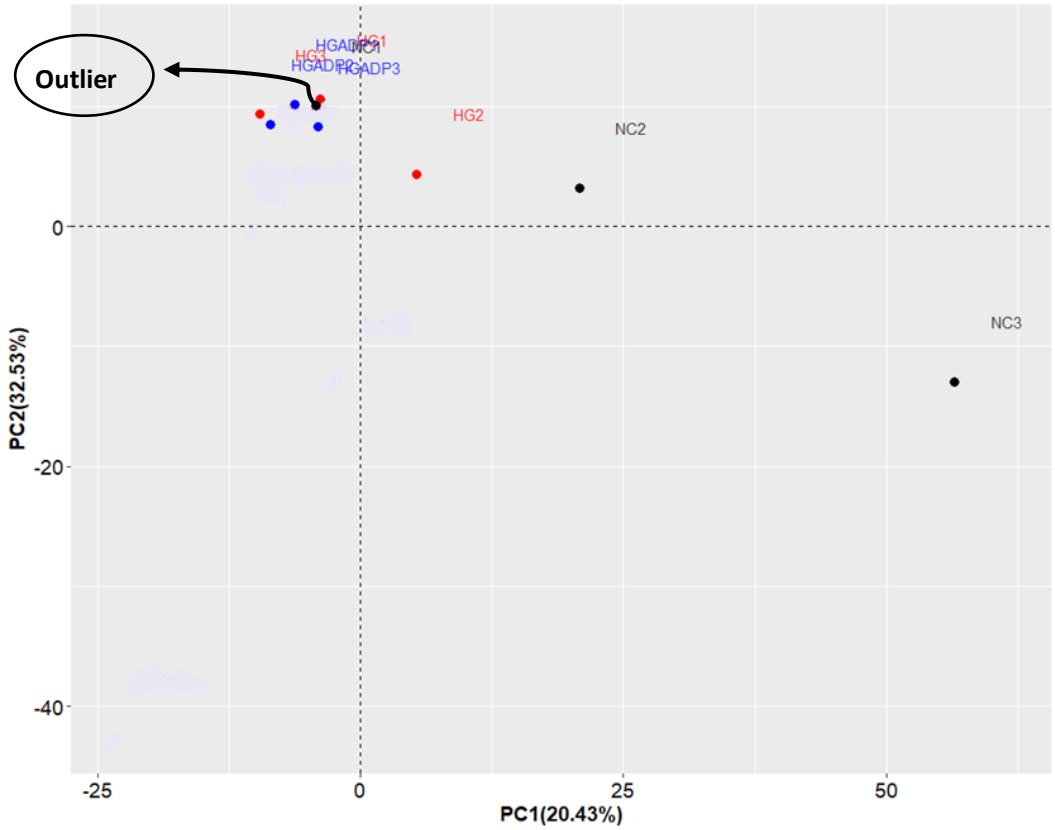


Figure 26: Principal Component Analysis of all samples. It is a statistical procedure used to visualize the distribution of the samples and whether is an outlier.

Table 17: Significantly differentially expressed miRNAs (P-value < 0.05; regulated greater than 2-fold change or less than -2) due to hyperglycemia (96 hours, 30mM glucose) compared to normoglycemia (NG).

miRNA	P-value	Fold change	Style
hsa-let-7a-5p	8.47E-08	2.30	Up
hsa-let-7c-5p	9.91E-05	1.64	Up
hsa-let-7e-5p	5.78E-05	1.60	Up
hsa-let-7f-5p	2.72E-07	2.34	Up
hsa-let-7g-5p	1.45E-05	1.74	Up
hsa-miR-224-5p	8.00E-06	1.79	Up
hsa-miR-26b-5p	2.37E-05	1.66	Up
hsa-miR-30c-5p	5.56E-05	1.67	Up
hsa-miR-493-5p	2.43E-05	2.13	Up
hsa-miR-543	3.17E-05	2.18	Up
hsa-miR-887-3p	0.000132989	-1.91	Down
hsa-miR-98-5p	3.72E-06	2.14	Up
has-miR-27a	5.82E-03	-5.00	Down

Table 18: Significantly differentially expressed miRNAs (P-value < 0.05; regulated greater than 2-fold change or less than -2) due to high glucose (96 hours, 30mM glucose) compared to ApN treatment (24 hours, 30µg/ml).

miRNA	P-value	Fold change	Style
hsa-miR-146a-5p	3.88E-05	1.16	Up (solely detected in ApN treated cells)

Data are presented as fold changes, with P-values (P-value < 0.05; regulated greater than 2-fold change or less than -2) for miRNA. A total of 13 miRNAs were dysregulated in HG group compared to the control while one miRNA, miR-146a-5p, was solely detected in ApN treated HRECs.

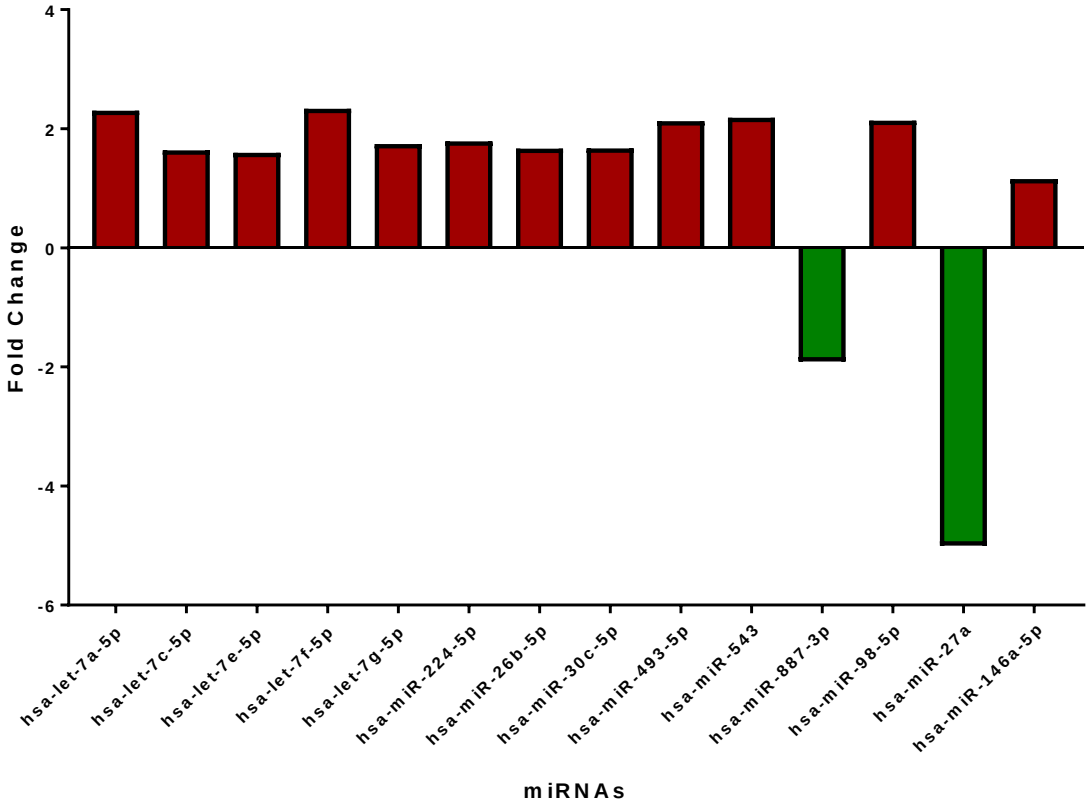


Figure 27: Significantly differentially expressed miRNAs (P-value < 0.05; regulated greater than 2-fold change or less than -2) due to hyperglycemia and ApN treatment (24 hours, 30µg/mL).

After normalization of raw sequencing data, analyzing them and performing differential gene expression comparison of miRNAs from different treated groups, related pathways of the dysregulated miRNAs were determined. This was achieved by searching various databases, such as miRsystem and Target-scan, and the target genes are summarized in Table 18.

Table 19: Summary of the miRNAs' targeted pathways and genes in HG vs.NG.

miRNAs	Targeted Pathway	Target Genes	EnterzID
hsa-miR-27a	Cytokine receptor interaction	EGFR	1956
		LIFR	3977
	Chemokine signaling pathway	GNB5	10681
	Insulin signaling pathway	FOXO1	2308
		MKNK2	2872
		RPTOR	57521
		MYO1C	4641
	Gluconeogenesis	ALDH9A1	223
	Pyruvate metabolism	ALDH9A1	223
	Metabolic pathways	ALDH9A1	223
		PFAS	5198
		GPAM	57678

	Angiogenesis	APC	324
	Cadherin signaling pathway	EGFR	1956
	Interleukin signaling pathway	MKNK2	2872
	Oxidative stress response	DUSP5	1847
		MKNK2	2872
		NFE2L2	4780
		SP1	6667
	Matrix Metalloproteinases	MMP13	4322
	TNF alpha Signaling Pathway	FADD	8772
		CUL1	8454
hsa-let-7a-5p	Gluconeogenesis	ALDH7A1	501
	Metabolic pathways	ADSL	158
		AMPD2	271
		AOC2	314
		DAD1	1603
		LTA4H	4048
		MTHFD1	4522
	Cytokine receptor interaction	BMPR1B	658
		EGFR	1956
		IL6	3569
	Chemokine signaling pathway	HRAS	3265
		NFKB1	4790
	VEGF signaling pathway	HRAS	3265

		KRAS	3845
		NRAS	4893
	Cell adhesion molecules CAMs	CD276	80381
	Adipocytokine signaling pathway	NFKB1	4790
	Type II diabetes mellitus	PIK3R1	5295
hsa-let-7c-5p	Gluconeogenesis	LDHA	3939
	Metabolic pathways	CHST6	4166
		NME4	4833
		PTGS1	5742
	Cytokine receptor interaction	ACVR1B	91
		MPL	4352
	Chemokine signaling pathway	FOXO3	2309
		GSK3A	2931
		NRAS	4893
	VEGF signaling pathway	PTGS2	5743
		PTK2	5747
hsa-let-7e-5p	Metabolic pathways	AMPD2	271
		POLR3D	661
		COX10	1352
		FDPS	2224
		GLUL	2752
	Cytokine receptor interaction	TNFRSF1A	7132

	Chemokine signaling pathway	CRK	1398
		PTK2	5747
		RAP1A	5906
	VEGF signaling pathway	PTK2	5747
	Type I diabetes mellitus	HLA-C	3107
	Type II diabetes mellitus	IRS4	8471
		IRS2	8660
	Cell adhesion molecules CAMs	CLDN4	1364
		HLA-C	3107
hsa-let-7f-5p	Gluconeogenesis	ALDH7A1	501
	Metabolic pathways	ATP5B	506
		CYP19A1	1588
		FDPS	2224
		GLUL	2752
	Cytokine receptor interaction	EDA	1896
		IL13	3596
hsa-let-7g-5p	Metabolic pathways	RRM2	6241
		RPL12	6136
	Cytokine receptor interaction	IL13	3596
	Chemokine signaling pathway	KRAS	3845
	VEGF signaling pathway	KRAS	3845
hsamiR-224-5p	Metabolic pathways	ARSB	411

	Cytokine receptor interaction	CD40	958
		PDGFRB	5159
		CXCR4	7852
	Chemokine signaling pathway	CDC42	998
		CXCR4	7852
	VEGF signaling pathway	CDC42	998
	Cell adhesion molecules CAMs	CD40	958
hsa-miR-26-5p	Gluconeogenesis	PDHA2	5161
		PFKP	5214
	Metabolic pathways	NAT1	9
		ACADM	34
		AGL	178
		AK4	205
	Cytokine receptor interaction	CXCL9	4283
		IL12A	3592
	Chemokine signaling pathway	ARRB1	408
		FOXO3	2309
		NFKB1	4790
	VEGF signaling pathway	PPP3R1	5534
		PTGS2	5743
	Cell adhesion molecules CAMs	CDH1	999
		CDH5	1003
	Type II diabetes mellitus	INSR	3643
		PIK3CG	5294

	Type I diabetes mellitus	FASLG	356
		CD28	940
		IL12A	3592
hsa-miR-30c-5p	Gluconeogenesis	PKM	5315
	Metabolic pathways	DDOST	1650
		HPRT1	3251
		MGAT2	4247
	Cytokine receptor interaction	CSF1	1435
	Chemokine signaling pathway	PIK3R2	5296
		MAP2K1	5604
	VEGF signaling pathway	RAC1	5879
		RAC3	5881
	Cell adhesion molecules CAMs	CDH1	999
		ITGA4	3676
	Type II diabetes mellitus	ITGA4	3676
		PIK3R2	5296
		PKM	5315
		SOCS1	8651
hsa-miR-887-3p	Metabolic pathways	MTHFD1L	25902
hsa-miR-98-5p	Metabolic pathways	MCAT	27349
	Cytokine receptor interaction	FAS	355
		CXCL2	2920
		IL6	3569
	Chemokine signaling pathway	CCL5	6352

		CXCL2	2920
	VEGF signaling pathway	NRAS	4893
	Type II diabetes mellitus	HK2	3099
		IRS2	8660
	Type I diabetes mellitus	FAS	355
hsa-miR-146a-5p	Cytokine receptor interaction	FAS	355
		CXCR4	7852
		IL8	3576
	Chemokine signaling pathway	CCR9	10803
		NFKB1	4790
		CXCR4	7852
	Cell adhesion molecules CAMs	ITGB2	3689
		L1CAM	3897
	Adipocytokine signaling pathway	NFKB1	4790
	Type I diabetes mellitus	FAS	355

6. DISCUSSION

Diabetic retinopathy (DR) is one of the chronic complications of diabetes that causes breakdown of retinal vasculature due to persistent hyperglycemia. It is considered the leading cause of vision loss and blindness among people worldwide (1). DR is ranked as the fifth most common cause of moderate to severe visual impairment and characterized by endothelial cell dysfunction, the breakdown of the BRB, and retinal neovascularization. Therefore, practical therapeutic approaches are required to control the progression of DR. Understanding the molecular mechanisms including the expression of the genes and their regulatory molecules such as miRNA and biological pathways in diabetic conditions, could help to reveal the pathogenesis of this complicated process.

miRNAs are small non-coding RNA molecules that negatively regulate the gene expression and therefore play a crucial role in disease processes. They have been documented as novel biomarkers, and numerous studies showed the relationship between miRNA and the regulation of different pathways including angiogenesis (144). Furthermore, ApN is a hormone produced in human adipose tissue, and the data demonstrated that ApN could ameliorate critical biological process involved in the pathogenesis of DR in HRECs (135). It acts as an anti-inflammatory, insulin-sensitizing and suppresses the formation of neovascularization (145). Recent studies showed that ApN regulates the expression of different inflammatory as well as angiogenesis and adhesion genes including IL-6, VEGF-A, ICAM-1, and integrity of the BRB in the diabetic retina. However, ApN therapeutic mechanism is not well-understood. One of the regulators that could have a role in the ApN action is miRNA. Based on this we aimed to

investigate the effect of hyperglycemia on miRNA in vitro using HRECs and the impact of ApN on miRNA expression and related molecular pathways in HRECs exposed to the hyperglycemic conditions. The current study establishes some significant findings that help in describing the role of hyperglycemia and ApN in expressing different miRNAs in HRECs. These findings are discussed in the following paragraphs.

In the current study, the exposure of HRECs to high glucose (30mM) causes a significant reduction in the cell viability, whereas administration of ApN to hyperglycemic HRECs counteract such effect and maintains the cell viability and promotes a significant decrease in cell death. Several studies supported this finding and are consistent with our results. For instance, a study conducted on HRECs cultured with increasing glucose concentrations for different time intervals showed a decrease in cell viability. A concentration of 30mM of glucose reduced the viability of HREC in a dose-dependent manner (146). This phenomenon was observed in other cell types as well as in animal models. A study conducted on retinal endothelial cells of Rhesus monkey showed that cell viability significantly differed after 24 hours of different glucose treatments. Cells treated with normal glucose concentration, 5.5mM, had an increase in cell viability in contrast to cells treated with 30mM of glucose which had 20% decrease in cell viability (147). In addition, a study conducted on rat retinal capillary endothelial cells proved that cells treated with 30mM of glucose for 48 and 72 hours showed a significant decrease in cell viability compared to retinal cells treated with 5.5mM glucose (148).

Cells treated with ApN showed enhanced cell viability compared to cells treated with high glucose. For instance, a recent study on NRK-52E cell line indicated that exposure to high glucose results in a significant decrease in cell viability. This can be

partially prevented by ApN since it decreases the cells apoptosis by activating activated protein kinase (AMPK) enzyme (149). Similarly, a study examined the effect of ApN on porcine aortic endothelial cells cultured in standard and high glucose conditions. Results showed that ApN increased the cell viability in both normoglycemic and hyperglycemic conditions (150). This, in turn, could improve the retinal cell function and BRB in DR (135).

APN performs various biologic actions through its receptors. The data of the current study demonstrated that Both ApN receptors, AdipoR1 and AdipoR2 mRNA expression were significantly downregulated in HRECs cultured in high glucose condition. Receptors expression significantly increased in ApN-treated HRECs. However, the expression was significantly upregulated after treating cells with ApN. Moreover, ApNR1 expression was higher compared to ApNR2. These results are inconsistent with other findings where the level mRNA of ApNR1 and ApNR2 was evaluated (151). The study by Hsu et al. on Retinal cells of STZ-induced diabetic rats showed that the levels of ApNR1 and ApNR2 mRNA were significantly higher compared to control group (151). Another study performed on type 1 diabetes human retina and mouse model concluded that the expression of ApNR1 in the retina was significantly elevated compared to control, while ApNR2 expression remained unchanged (152). Therefore, ApNR1 may have a significant role in the pathological process of diabetic retina while ApNR2 has a minor role (153). Such difference could be justified due to the species specificity and the type of animal model used to induce diabetes which is different than human retinal cells. Such reduction in expression of the adiponectin receptors in response to HG observed in our study, may contribute to decreased

adiponectin effect in HRECs. Since the expression of ApN receptors increases after ApN treatment, it can exert its therapeutic effect in HRECs via its receptors which can enhance the endothelial dysfunction.

The expression of VEGF-A and IL-6 increased in HRECs under high glucose condition and remarkably decreased in ApN-treated cells. VEGF-A and IL-6 share some common characteristics since both are induced by hyperglycemia and hypoxia as well as playing a significant role in vascular permeability, inflammation, and angiogenesis (154). In the current study hyperglycemia significantly increased the expression of VEGF-A compared to normoglycemic conditions in HRECs and reached approximately 2 fold change. This increase was counteracted by ApN where the VEGF-A expression significantly decreased in ApN-treated cells (P-value <0.0001). Several studies have documented a substantial rise in vitreous VEGF levels in PDR (155-158). However, ApN could decrease the expression of VEGF. For instance, a study conducted on human retinal pigment epithelial cell lines (ARPE-19) revealed that cells treated with ApN had lower VEGF mRNA expression. In addition, there was a negative correlation between ARPE exposed to ApN and level of VEGF. Therefore, demonstrating the protective effect of ApN in diseases associated with angiogenesis (159).

Interestingly, cells treated with only ApN showed a high expression of VEGF-A. In different tissues, ApN could act as an angiogenic factor to form new blood vessels. For instance, a study examined the level of ApN and VEGF-A in human chondrosarcoma tissues showed a strong correlation between the high level of adiponectin expression and VEGF-A expression. ApN induces the expression of VEGF-A in human chondrosarcoma cells and consequently promotes angiogenesis (160). However, the case is different in

retinal endothelial cells. The increased expression of VEGF-A occurs at the gene level but not at the protein level. Although the expression level of VEGF-A mRNA is elevated, by ApN administration, still such phenomenon is not well understood yet, and there is a suppression at protein level of VEGF-A in HRECs. This was proven by a study stating that ApN significantly increased the production of different proteins, including VEGF, in osteoblasts but was not the same case in endothelial cells (161).

Since hyperglycemia induces inflammation, it was expected that IL-6, a pro-inflammatory cytokine, to be elevated in HG group compared to control. Numerous studies showed a significant increase in IL-6 level in diabetic patients (162-164). Since ApN acts as an anti-inflammatory molecule, cells treated with ApN showed a significantly lower level of IL-6 compared to cells under hyperglycemic conditions. Studies showed that patients who have metabolic syndrome with a high level of IL-6, TNF-α, and hsCRP concentrations had lower ApN concentrations compared with control (165). Moreover, ApN anti-inflammatory actions are exerted by suppressing the binding of NF-kB to the DNA, and therefore the level of NF-kB target genes, including IL-6, will be suppressed (166). This indicates the therapeutic effect of ApN in improving the retinal cell function in DR. By suppressing NF-kB signaling leukocyte adhesion in HRECs will be suppressed, hence, decreasing the inflammation and endothelial dysfunction in HRECS, especially after the addition of ApN to HG cells.

Hyperglycemia and ApN treatment affect the expression of multiple adhesion and angiogenesis genes in HRECs. The RT-PCR profiler plate consisted of 84 different genes that were commercially provided. Comparing the adhesion gene panel of HG and HG+ApN groups, revealed that five genes were upregulated in HG+ApN group

compared to HG and 15 adhesion genes were downregulated. When examining HRECs treated for 24 hours for angiogenesis gene panel, the results showed that seven genes were upregulated, and 34 genes were downregulated in HG cells treated with adiponectin (HG+ApN) compared to the cells exposed to high glucose (HG). However, when HRECs were exposed for high glucose for a longer duration for 96 hours, and then we examined them using the same angiogenesis panel, the data revealed that nine genes were upregulated and nine genes were downregulated in HG+ApN compared to HG. One of the distinctive results in the angiogenesis panel is the expression of adhesion G protein-coupled receptor B1 (ADGRB1) which had a 470-fold increase in HG compared to control. After exposing HRECs to ApN treatment, the fold changed of ADGRB1 mRNA expression decreased significantly to -173.39. Searching databases, ADGRB1 is a brain-specific angiogenesis inhibitor 1 (167). However, no studies have been done on human retinal endothelial cells so far, and further studies are needed to explore its role in DR genesis. The results of the adhesion gene panel showed that the four most upregulated mRNA were of the following genes; VCAM1, ITGAL, ADAMTS8, and MMP8, while most downregulated mRNA are for the following four genes SELL, COL8A1, COL12A1, and ECM-1.

The results of the angiogenesis panel in HG cells exposed for short time (24 hours) and longer time (96 hours) was inconsistent and showed some variations. For 24 hours exposure to HG followed by adiponectin treatment, the four most upregulated genes were SELE, SELPLG, VCAM-1, and ICAM-1 and the four most downregulated genes were OCLN, IL-3, APOE, and ANGPT1. For the prolonged time of HG exposure (96 hours) followed by ApN treatment demonstrated that the four most upregulated genes

were IL-1B CXCL1, CXCL6, and CCL2 while the four most downregulated genes were EGF, FGF-1, TGFA, and CXCL9. These genes have chemokine and growth factor activities. Table 18 shows the aforementioned genes and the coded proteins. This proves that ApN could have a role in suppressing the expression of many adhesion molecule genes and therefore reduces the leukostasis and inflammation in retinal cells.

Table 20: Most up and downregulated adhesion and angiogenesis genes in HRECs treated cells for 24 and 96 hours (RT-PCR profiler results)

24-hour HG exposure and treatment		96-hour HG exposure and treatment		24-hour HG exposure and treatment		96-hour HG exposure and treatment	
Upregulated genes	**Protein code**	**Upregulated genes**	**Protein code**	**Downregulated genes**	**Protein code**	**Downregulated genes**	**Protein code**
VCAM-1	Vascular cell adhesion protein 1	IL-1B	Interleukin-1 Beta	SELL	L-selectin	EGF	Pro-epidermal growth factor
ITGAL	Integrin alpha-L	CXCL1	Growth-regulated alpha protein	COL8A1	Collagen alpha-1(VIII) chain	FGF-1	Fibroblast growth factor 1
ADAMTS8	A disintegrin	CXCL6	C-X-C motif	COL12A1	Collagen	TGFA	Protransformi

	and metalloproteinase with thrombospondin motifs 8		chemokine 6		alpha-1(XII) chain		ng growth factor alpha
MMP8	Neutrophil collagenase	CCL2	C-C motif chemokine 2	ECM-1	Extracellular matrix protein 1	CXCL9	C-X-C motif chemokine 9
SELE	E-selectin	PF4	Platelet factor 4	OCLN	Occludin	ADGRB1	Brain-specific angiogenesis inhibitor 1
SELPLG	P-selectin glycoprotein ligand 1	CXCL8	Interleukin 8	IL-3	Interleukin-3	GAPDH	Glyceraldehyde-3-phosphate dehydrogenas

							e
ICAM-1	Intercellular adhesion molecule 1	IFNA1	Interferon, alpha 1	APOE	Apolipoprotein E	COL4A3	Collagen, type IV, alpha 3 (Goodpasture antigen)

miRNAs are commonly dysregulated in pathological conditions including DR. Here we present the miRNAs expressed under high glucose state and after ApN treatment in HRECs using the small RNA sequence technique. In the current study, a total of 14 miRNAs were differentially expressed ($P<0.05$) in HRECs under different conditions. Other studies showed higher numbers of differentially expressed miRNAs; however, those studies were conducted on diabetic rats rather than human cells using both RNA sequence and microarray (152, 168). Another reason could be that the number of detected miRNA in retinal endothelial cells is considerably lower than miRNAs in total retina differences in cellular composition complexity (152).

Comparing the current results of RT-PCR profiler with the present results of RNA sequencing analysis with bioinformatics, demonstrated that there were no common miRNAs identified between the two approaches. This shows the limitation of RT-PCR profiler plates pre-coated with specific known genes since it restricts the screening to particular genes and prevents the discovery of new miRNAs beyond the customized assay. For instance, a study was performed to evaluate different bioinformatics methods to study miRNAs (169). It was suggested that there are some challenges in this field since, for example, conventional microarray probes could miss miRNAs of the same family and varies by few nucleotides. Another challenge is the cross-hybridization between similar miRNAs sequences which could affect the outcome. Therefore, in the current study, we used different approaches to screen, compare, and validate the miRNA results.

Different databases were used to search for the gene targets of differentially expressed miRNAs, obtained by small-RNA-Seq. Results showed that most of them were

involved in various common pathways including cytokine-cytokine receptor interaction, chemokine signaling pathway, VEGF signaling pathway, cell adhesion molecules, and diabetes mellitus. Others had distinctive involvement in other pathways (table 18). For instance, gene ontology results for miR-27 showed that it is involved in angiogenesis, cadherin signaling pathway, oxidative stress response as well as gluconeogenesis. This was supported by various studies since miR-27a expression was upregulated by high glucose and targeted the PPARγ gene in streptozotocin-induced diabetic rats (170). Moreover, a study concluded that miR-27a might have an association with type 1 and 2 diabetes (171, 172). Similarly, a recent study showed that glucose intolerance in mice resulted from miR-let-7 overexpression despite the increase in insulin secretion (173).

Out of the 14 differentially expressed miRNAs, 13 were expressed in HRECs exposed to hyperglycemic condition for 96 hours as mentioned previously. However, the last miRNA, miR-146a-5p, was differentially and solely expressed in HRECs under high glucose and then treated with ApN. Searching for target genes and pathways for has-miR 146-5P, showed that it has similar involvement as other differentially expressed miRNAs and targets the cytokine-cytokine receptor interaction pathway, chemokine signaling pathway, cell adhesion molecules, and diabetes mellitus. Overexpression of VEGF was associated with the downregulation of miR-146a-5p whereas miR-146a-5p regulates many genes transcript levels including NF-kB and VEGF in diabetic retinopathy (174, 175). Moreover, diabetic streptozotocin rat's retinal endothelial cells showed a decrease in miR-146a expression which was proven to regulate the expression of fibronectin. Increase in the production of extracellular matrix proteins and fibronectin is one of DR dysfunctions that are induced by hyperglycemia (176). Therefore, miR-146a injection

could counteract the high effect of glucose and decrease the level of fibronectin in diabetes (177). In a recent study where human retinal endothelial cells were used to study the role of miR-146a in attenuating the inflammatory pathways induced by hyperglycemia (178). Results indicated that miR-146a decreased the elevation of glucose-induced TNFα and inhibited the activation of NF-κB to protect the retinal cells. Therefore, miR-146a could be a potential therapeutic approach for DR targeting TNFα and NF-κB inflammatory pathways. Such data could highlight the role of adiponectin in ameliorating the HG conditions which pertain to DR pathogenesis. These could include inflammatory pathways, adhesion molecules and, angiogenesis as shown in these data.

The following study has some limitations that could be addressed and improved in the future. For example, only IL-6 was assessed as an inflammatory cytokine due to limited technical facilities. Moreover, assessment of adhesion molecules like VCAM-1 and ICAM-1 as well as other antioxidants would strengthen the findings of the study. Also, due to limited time and facilities, RT-PCR profiler conducted twice as a screening tool, and no P-value was obtained for the expressed genes. Repeating this screening three times would give more validated and significant results than just screening once. miRNAs identified in the current study need to be validated at qPCR level. Once validated, possible target genes of the verified miRNAs could be identified and then and conduct Western blots to show a reduction in corresponding protein levels. Also, transfecting cells with antagomirs, to block miRNAs; showing that protein expression level can be rescued is further validations of the miRNA-gene target connection.

In conclusion, a total of 14 dysregulated miRNAs in diabetic human retinal endothelial cells were identified in this study. MiRNAs targets and pathways were

determined using bioinformatics analysis and different databases. It was noticed how miRNA could affect multiple genes through different pathways. This, in turn, could a potential approach for using miRNAs as therapeutic targets for DR. Adiponectin could improve the dysfunction of HREC through different mechanisms through the upregulation of the AdipR1, improving the viability and moreover the regulation of mir-146a which target different pathways involved in DR genesis.

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APPENDIX

Appendix A: RT^2 Profiler PCR Arrays

Gene list of human endothelial cell biology array.

Angiogenic Growth Factors

Growth Factors & Receptors: ANG, ANGPT1, ANGPT2, ANPEP, TYMP, FGF1, FGF2 (BFGF), FIGF (VEGFD), FLT1 (VEGFR1), JAG1, KDR (VEGFR3), NRP1, NRP2, PGF, VEGFA, VEGFB, VEGFC.

Cell Adhesion Molecules: ADGRB1 (BAI1), COL4A3, CXCL8, NRP1, NRP2.

Extracellular Matrix (ECM) Molecules: ANGPTL4, F3, PECAM1, PF4, PROK2, SERPINE1 (PAI-1), SERPINF1.

Other Angiogenic Factors: HIF1A, NOS3 (eNOS), SPHK1.

Cytokines: CCL11 (eotaxin), CCL2 (MCP-1), CXCL1 (GRO1, GROa, SCYB1), CXCL10 (INP10), CXCL5 (ENA-78, LIX), CXCL6 (GCP-2), CXCL9 (MIG), EDN1, IFNA1, IFNG, IL1B, IL6, MDK, TNF.

Growth Factors & Receptors: CTGF, EFNA1, EFNB2, EGF, EPHB4, FGFR3, HGF, IGF1, ITGB3, PDGFA, S1PR1, TEK (TIE-2, TIE2), TGFA, TGFB1, TGFB2, TGFBR1

(ALK5).

Cell Adhesion Molecules: CCL11 (eotaxin), CCL2 (MCP-1), CDH5, COL18A1, CTGF, ENG (EVI-1), ERBB2 (HER-2, NEU), FN1, ITGAV, ITGB3, S1PR1, THBS1 (TSP-1), THBS2.

Extracellular Matrix (ECM) Molecules: LECT1, LEP (leptin), MMP14, MMP2, MMP9, PLAU (UPA), PLG, TIMP1, TIMP2, TIMP3.

Other Angiogenic Factors: AKT1, HPSE, ID1, NOTCH4, PTGS1 (COX1), TIE1.

Gene list of cell adhesion molecules

Transmembrane Receptors

CD44, CDH1 (E-Cadherin), HAS1, ICAM1, ITGA1, ITGA2, ITGA3, ITGA4 (CD49D), ITGA5, ITGA6, ITGA7, ITGA8, ITGAL, ITGAM, ITGAV, ITGB1, ITGB2, ITGB3, ITGB4, ITGB5, MMP14, MMP15, MMP16, NCAM1, PECAM1, SELE, SELL (LECAM-1), SELP, SGCE, SPG7, VCAM1.

Cell-Cell Adhesion

CD44, CDH1 (E-Cadherin), COL11A1, COL14A1, COL6A2, CTNND1, ICAM1, ITGA8, VCAM1.

Cell-Extracellular Matrix (ECM) Adhesion

ADAMTS13, CD44, ITGA1, ITGA2, ITGA3, ITGA4 (CD49D), ITGA5, ITGA6, ITGA7, ITGA8, ITGAL, ITGAM, ITGAV, ITGB1, ITGB2, ITGB3, ITGB4, ITGB5, SGCE, SPP1, THBS3.

Other Cell Adhesion Molecules

CNTN1, COL12A1, COL15A1, COL16A1, COL5A1, COL6A1, COL7A1, COL8A1, VCAN, CTGF, CTNNA1, CTNNB1, CTNND2, FN1, KAL1, LAMA1, LAMA2, LAMA3, LAMB1, LAMB3, LAMC1, THBS1 (TSP-1), THBS2, CLEC3B, TNC, VTN.

Extracellular Matrix (ECM) Molecules Basement Membrane Constituents

COL4A2, COL7A1, LAMA1, LAMA2, LAMA3, LAMB1, LAMB3, LAMC1, SPARC.

Collagens & ECM Structural Constituents: COL11A1, COL12A1, COL14A1, COL15A1, COL16A1, COL1A1, COL4A2, COL5A1, COL6A1, COL6A2, COL7A1, COL8A1, FN1, KAL1.

Extracellular Matrix (ECM) Proteases

ADAMTS1, ADAMTS13, ADAMTS8, MMP1, MMP10, MMP11, MMP12, MMP13, MMP14, MMP15, MMP16, MMP2, MMP3, MMP7, MMP8, MMP9, SPG7, TIMP1.

Extracellular Matrix (ECM) Protease Inhibitors: COL7A1, KAL1, THBS1 (TSP-1), TIMP1, TIMP2, TIMP3.

Other Extracellular Matrix (ECM) Molecules

VCAN, CTGF, ECM1, HAS1, SPP1, TGFBI, THBS2, THBS3, CLEC3B, TNC, VTN.

Appendix B: Box plot for normalized sequenced data