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To my beloved brother Aris, the greatest supporter I could ever have in my life.

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Vascular ECs organize themselves in a single cellular layer, that is, the endothelium, at the luminal face of all blood vessels. In addition to its major function in controlling gas exchanges at the pulmonary level, the endothelium regulates the flow of circulating blood cells and of various bioactive molecules such as growth factors, coagulation proteins, lipoproteins and hormones, in particular through the presence of membrane-bound receptors. The endothelium also plays pivotal roles in the regulation of a broad range of physiological processes: it regulates hemostasis through the expression of both anti-thrombotic and prothrombotic factors; in tight cooperation with the underlying extracellular matrix and smooth muscle cells, it controls vascular tone with angiotensin I conversion and bradykinin degradation, and contributes to the metabolism of vasoactive amines. It also participates in inflammatory and immune responses through surface antigens and adhesive molecules and through the synthesis of cytokines. Moreover ECs, which are in direct contact with plasma and cellular components of blood, are targets of many endogenous molecules and xenobiotics.

Endothelial cells have an important role in inflammation since they facilitate the migration of inflammatory cells to the inflamed tissues. Importantly chronic inflammation has deleterious effects on vascular endothelium promoting premature and accelerated atherosclerosis which is not fully accounted by traditional atherosclerosis risk factors. In fact, patients with chronic inflammatory diseases such systemic lupus erythematous and inflammatory arthritides have a 2-3 fold increased risk for cardiovascular events which represents a major cause of morbidity and mortality in these diseases. Delineation of the mechanisms involved in the interaction between endothelial cells and inflammation in this context may facilitate efforts to alleviate the deleterious effects of inflammation on vascular beds.

This master's thesis has two parts. In the first part we outline the establishment of an easy and cost-effective method for isolation and culture of HUVECs to supply our future experiments on the effects of inflammation and lipids on endothelial physiology. Addition of sera from active lupus patients in these cells resulted in activation of mechanisms of cellular migration and proliferation. The second part concerns the study of gene expression of human arterial endothelial cells

after incubation with recombinant HDL, which contained apolipoprotein E3. We took advantage of data generated in Dr. Zannis Lab (Boston University, USA) and in collaboration with Dr. Sanoudou Lab (Uinersity of Athens, Greece) we performed the bioinformatic analysis and literature search for the genes identified. Following total RNA isolation and hybridization on microarrays, the bioinformatics analysis revealed changes in specific genes which could inform our experiments. More specifically 16 genes were found to be directly involved in the molecular mechanisms leading to cellular proliferation. Further experiments should be carried out to validate our finding as these genes could be promising therapeutic targets against atherosclerosis.

Τα ενδοθηλιακά κύτταρα των αγγείων οργανώνονται σε μια ενιαία κυτταρική στοιβάδα, το ενδοθήλιο, στην αυλική επιφάνεια όλων των αιμοφόρων αγγείων. Εκτός από την σημαντική λειτουργία στον έλεγχο της ανταλλαγής αερίων στους πνεύμονες, το ενδοθήλιο ρυθμίζει τη ροή των κυκλοφορούντων κυττάρων του αίματος και των διαφόρων βιοδραστικών μορίων, για παράδειγμα, των αυξητικών παραγόντων, των πρωτεινών πήξης, των λιποπρωτεινών και των ορμονών, μέσω της ύπαρξης των μεμβρανικών υποδοχέων. Το ενδοθήλιο παίζει επίσης κεντρικό ρόλο στη ρύθμιση ενός ευρέως φάσματος φυσιολογικών διεργασιών: ρυθμίζοντας την αιμόσταση μέσω της έκφρασης των αντι-θρομβωτικών και προθρομβωτικών παραγόντων. Σε συνεργασία με την υποκείμενη εξωκυττάρια ουσία και τα κύτταρα των λείων μυικών ινών, ελέγχει τον αγγειακό τόνο μέσω της μετατροπής της αγγειοτενσίνης I και της αποικοδόμησης της βραδυκινίνης και συμβάλλει στο μεταβολισμό των αγγειοδραστικών αμινών. Επίσης συμμετέχει σε φλεγμονώδεις και ανοσολογικές αποκρίσεις μέσω επιφανειακών αντιγόνων και μορίων προσκόλλησης αλλά και μέσω της σύνθεσης των κυτταροκινών. Επίσης τα ενδοθηλιακά κύτταρα είναι σε άμεση επαφή με το πλάσμα και τα κυτταρικά συστατικά του αίματος αποτελόντας στόχους πολλών ενδογενών μορίων και ξενοβιοτικών.

Τα ενδοθηλιακά κύτταρα έχουν ένα σημαντικό ρόλο στη φλεγμονή, δεδομένου ότι διευκολύνουν τη μετανάστευση των φλεγμονωδών κυττάρων στους φλεγμαίνοντες ιστούς. Η χρόνια φλεγμονή έχει επιφέρει σημαντικές επιπτώσεις στο αγγειακό ενδοθήλιο οδηγώντας σε πρώιμη και επιταχυνόμενη αθηροσκλήρωση που δεν εξηγείται πλήρως από τους παραδοσιακούς παράγοντες κινδύνου της αθηροσκλήρωσης. Οι ασθενείς με χρόνιες φλεγμονώδεις ασθένειες όπως Συστηματικό Ερυθηματώδη Λύκο και φλεγμονώδεις αρθρίτιδες έχουν 2-3 φορές αύξημένο κίνδυνο για καρδιαγγειακά συμβάματα που αντιπροσωπεύει μια σημαντική αιτία νοσηρότητας και θνησιμότητας στις ασθένειες αυτές. Περαιτέρω κατανόηση των μηχανισμών που εμπλέκονται στην αλληλεπίδραση μεταξύ των ενδοθηλιακών κυττάρων και της φλεγμονής σε αυτό το πλαίσιο μπορεί να διευκολύνει τις προσπάθειες για αναχαίτηση των τοξικών επιδράσεων της φλεγμονής στο αγγειακό δίκτυο.

Η παρούσα διατριβή αποτελείται από δύο μέρη. Στο πρώτο μέρος

περιγράφεται η ανάπτυξη μιας απλής και οικονομικά αποδοτικής μεθόδου για την απομόνωση και ανάπτυξη των πρωτογενών ανθρώπινων ενδοθηλιακών κυττάρων HUVECs για την κάλυψη των μελλοντικών μας πειραμάτων σχετικά με τις επιπτώσεις της φλεγμονής και των λιπιδίων στην φυσιολογία του ενδοθηλόυ. Επώαση των κυττάρων με ορό ασθενών με ενεργό ΣΕΛ οδήγησε σε ενεργοποίηση των μηχανισμών της κυτταρικής μετανάστευσης και πολλαπλασιασμού. Το δεύτερο μέρος αφορά στην μελέτη της γονιδιακής έκφρασης ανθρώπινων αρτηριακών ενδοθηλιακών κυττάρων μετά από επώαση με ανασυνδυασμένη HDL, η οποία περιείχε απολιποπρωτεΐνη Ε3. Σε συνεργασία με το εργαστήριο του Καθηγητή Β. Ζαννή (Πανεπιστήμιο Βοστώνης, ΗΠΑ) από όπου και αντλήσαμε δεδομένα και σε συνεργασία με το εργαστήριο της Επ. Καθηγήτριας Δ. Σανούδου (Πανεπιστήμιο Αθηνών) πραγματοποίησαμε βιοπληροφορική ανάλυση, αναζήτηση και μελέτη της βιβλιογραφίας για τα προσδιοριζόμενα γονίδια. Μετά την απομόνωση RNA και υβριδισμό σε μικροσυστοιχίες, η βιοπληροφορική μας ανάλυση αποκάλυψε αλλαγές σε συγκεκριμένα γονίδια τα οποία θα μπορούσαν να τροφοδοτήσουν περαιτέρω τα πειράματα μας. Πιο συγκεκριμένα 16 γονίδια βρέθηκαν να εμπλέκονται άμεσα στους μοριακούς μηχανισμούς που οδηγούν σε κυτταρικό πολλαπλασιασμό. Περαιτέρω πειράματα χρειάζεται να διεξαχθούν προς επικύρωση των αποτελεσμάτων μας, καθώς τα γονίδια αυτά θα μπορούσαν να αποτελούν υποσχόμενους θεραπευτικούς στόχους κατά της αθηροσκλήρωσης.

General introduction on endothelium

1.1 The normal endothelium

The endothelium, a vital component of the vascular wall, comprises 10^{13} endothelial cells and covers approximately 7 m^2 in an average adult (1). These endothelial cells provide the physical interface between blood and surrounding tissue, regulate nutrient and blood component traffic, and participate in many physiologic events such as hemostasis, inflammation, and angiogenesis (1–4).

Endothelial cells are able to inhibit the coagulation of the blood in multiple levels. First, by gathering phosphatydilserine residues in the internal membrane surface of their cellular membrane thus blocking their exposure to the coagulation factors. Also they block the platelet's activation by expressing enzymes that degradate ATP and ADP, suspend thrombin and the exposure of the subendothelial collagen. Furthermore, by releasing Nitric Oxide (NO) and prostacyclins (PGI_2), endothelial also block the platelet's activation.

Endothelial cells bind and maybe synthesize inhibitors of the VIIa/Tissue Factor (TF) pathway in the mechanism of coagulation. By activating Protein C and secreting Protein S they can block the activated coagulation factors. Finally, by expressing heparin and tissue plasminogen activator, the EC can activate antithrombin III and eventually participate in the lysis of thrombus (5-6).

Endothelial cells play an important role on the diffusion of various molecules from the blood stream to the tissues as well as from cell-to-cell. Certain junction proteins (Vascular Endothelial Cadherin, Ve-Cadherin), similar to other junctional proteins of epithelial cells allow the transfer of liquids and macromolecules in the tissue (7-8).

The regulation of the vascular tone is also under the control of the endothelial cells. The most powerfull vasodilator is Nitric Oxide (NO) synthesized by endothelial Nitric Oxide Synthase (eNOS or NOS3) which converts arginine into citruline, releasing NO. Nitric Oxide release, leads in cGMP production and activation of Protein Kinase G, culminating eventually in the relaxation of smooth muscular cells of vessels (9).

Inflammatory response can also be modified by the endothelial cells at the level of the expression of adhesion molecules such as Vascular Cell Adhesion Molecule 1 (VCAM-1) and Intercellular Adhesion Molecule (ICAM-1) and E-selectin. Wiebel-Palade bodies are the storage granules of endothelial cells containing von Willebrand factor and P-selectin and thus play a dual role in hemostasis and inflammation. Additional Wiebel-Palade bodies contain major chemokines such as Inteleukin-8, eotaxin-3, endothelin-1, osteoprotegerin, angiopoietin-2, the tetraspanin CD63/lamp3 and α-1,2-fucosyltransferase VI (10).

Another important aspect of the endothelial cells is their role in immune response. Cultured human endothelial cells can activate naïve CD4+ T cells to produce certain chemokines. To do so, an interaction via the MHC and T cell receptor complex is needed along with the presence of certain co-stimulatory factors (CD58) (11).

1.2 Pathophysiology of endothelium

Endothelial dysfunction is mainly caused by endothelial damage and the endothelial cells' activation. This activation includes a series of changes in endothelial cells gained after inflammation trigerring (12). Endothelial cells play a central role in acute and adaptive immune response orchestrating immune response at various levels (13).

The production of vasoactive mediators, especially prostacyclin, from the arterioral endothelial cells increases locally the blood flow and allows easier transport

of leukocytes at the site of inflamation. Proinflammatory cytokines such as the TNF and IL -1 induce the expression from the endothelial cells of Cycloxygenase (Cox -2), leading to the production of prostacyclins from arachidonic acid that is released from the membrane phosphatidylcholine.

The activated ECs synthesize and present in their membrane surface the tissue factor (TF), which triggers the cataract of thrombosis, a protective function against dissemination of microbes, entrenching locally the site of microbial invasion.

Any dysfunction to the above mechanisms can lead in leukocyte aggravation and increased vascular permeability (13). Additionaly any exposure to the platelets, of the subendothelial collagen leads in the activation of the coagulation cataract.

Endothelial cells die mainly an apoptotic death. Apoptosis is a term used to distinguish from the cellular necrosis, which is characterized by extensive cellular edema of certain tissues. In apoptosis, the activation of certain caspases takes place that leads in proteolysis of basic cellular membranes and in a condensed nucleus due to the fragmentation of DNA.

Another form of cellular death is autophagy, in which cells that lack nutrients, degradate their cellular organelles encompassing them in membranes leading them for further destruction into lysosomes. Although this mechanism is considered as mechanism of survival, it can lead also to a caspase-independent cellular death (14).

Endothelial cell death can be either apoptotic, necrotic or due to autophagy. Oxidative stress is a strong cause of endothelial cell death. The basic stimulus is ROS production from the macrophages and neutrophils under the inflammatory process leading in vascular remodelling. In pathological states such as hypertension, heart failure and cardiac hypertrophy, the endogenously produced ROS causes cellular death leading to endothelial dysfunction and atherosclerosis (15, 16).

Additional forms of metabolic stress such as hyperglycemia, hyperlipidemia, and hypoxia interfere with the PI3K - Akt molecular pathway and decreased insulin and eNOS activity leading to cellular dysfunction and apoptosis (17,18,19).

As it has already been stated, the endothelial cells participate in inflammation, either acute or chronic. Acute inflammation that can activate naïve ECs is divided in type 1, taking place wuthin hours which is gene-independent and type 2, which is a relatively slower procedure and it, depends on expression of specific genes (20).

Type 1 activation is mediated after a ligand –GPCR (G Protein Coupled Receptors) interaction. GPCR's may be histamine receptors that lead in elevation of the intracellular calcium through the activation of PLCb and triphosphate inositol release but also through the activation of RHO GPC. The process is active for no longer than 20 minutes due to receptor's desensitization.

Type 2 endothelial activation is more extensive and is mediated mainly through TNF a and the IL -1, produced mainly by activated leukocytes. Stimulation by these two mediators leads finally to the activation of two transcription factors, NF-kB and AP1 that induce proinflammatory response through new genes transcription.

In inflammation there is distinct adherence molecules at the surface of ECs. VCAM -1 and ICAM -1 along with the release of certain chemokines such as CCL 2 facilitate the shift from neutrophil infiltration to monocyte-rich infiltration within hours. Further endothelial damage leads to further inflammation, especially at the capillaries (20). TNF and IL -1 along with IFN-γ, induce further the inflammatory process and lead in major endothelial damage and apoptosis mainly through caspase 8.

In the case of chronic inflammation, the T helper 1 immune response induces the attraction of IFN-γ producing T – cells; ECs express the chemokine CXCL 10 and through the CXCR 3 receptor further Th_1 cells are recruited (21). This is a positive feedback mechanism that facilitates Th_1 response. On the other hand, in

Th2 response (release of chemokines IL -4, IL -5 and / or IL 13), ECs respond by expressing chemokine CCL 26 and the expression of molecules such as the VCAM-1 that facilitate the local attraction of Th2 adhesion cells as well as eosinophils (21).

The net result after endothelial damage, is the release in the blood circulation of certain microparticles the so-called endothelial microparticles (EMPs) originating from activated or apoptotic EC's. Importantly, there is also release of detached endothelial cells in the blood stream; these are called Circulating Endothelial Cells (CECs) and are detached from the vascular wall as a consequence of the endothelial damage (22,23).

Recent studies suggest that the bone marrow of the adult is a source of the Endothelial progenitor Cells (EPC's), a population of cells that is capable of forming new vessels. They can circulate in the blood stream, repopulate and differentiate into mature endothelial cells. These cells are believed to be recruited after endothelial damage and play a crucial role in vascular remodelling after an endothelial damage (24,25).

It is evident that the recruitment of the EPC's is the last step after endothelial damage. Increased levels of EMP's and CEC'c along with decreased levels of EPC's are an indirect evidence of failure of compensation after endothelial damage (26,27,28).

1.3 Cell Migration and Cell Proliferation

Cell migration is an essential cellular function critical in embryonic development as well as in wound healing, nervous and immune systems formation and function. Any dysfunction in in the cell migration could lead in immune deficiency, congenital malformations (eg. patent foramen ovale) and inflammatory diseases.

Crucial steps in a cell movement are the polarization, protrution and adhesion. All of them are complex processes at the molecular level and the full understanding remains a scientific challenge.

Many cells move in diverse types of interconnected groups, rather than migrating individually. Some cells, such as trunk neural crest cells and mouse embryonic germ cells, maintain loose connections. Keratinocytes stay tightly connected in a sheet as they fill in a wound. During development of cellular tubes, such as blood vessels, distal cells lead while towing the rest of the cells behind them. Various and complex signaling pathways mediate the communication between migrating border cells and their microenvironment, as well as proteins involved in the mechanics of movement.

Cell proliferation is controlled by growth factors that bind to cell surface receptors which connect to signaling molecules. These molecules activate transcription factors which bind to DNA to modulate the production of proteins, resulting in cell division. Dysfunction of any step in this regulatory cascade causes abnormal cell proliferation, an underlying cause of many human pathological conditions, most notably cancer and aging. Defining mechanisms responsible for alterations in cell cycle progression is crucial to understanding many human diseases, most notably cancer.

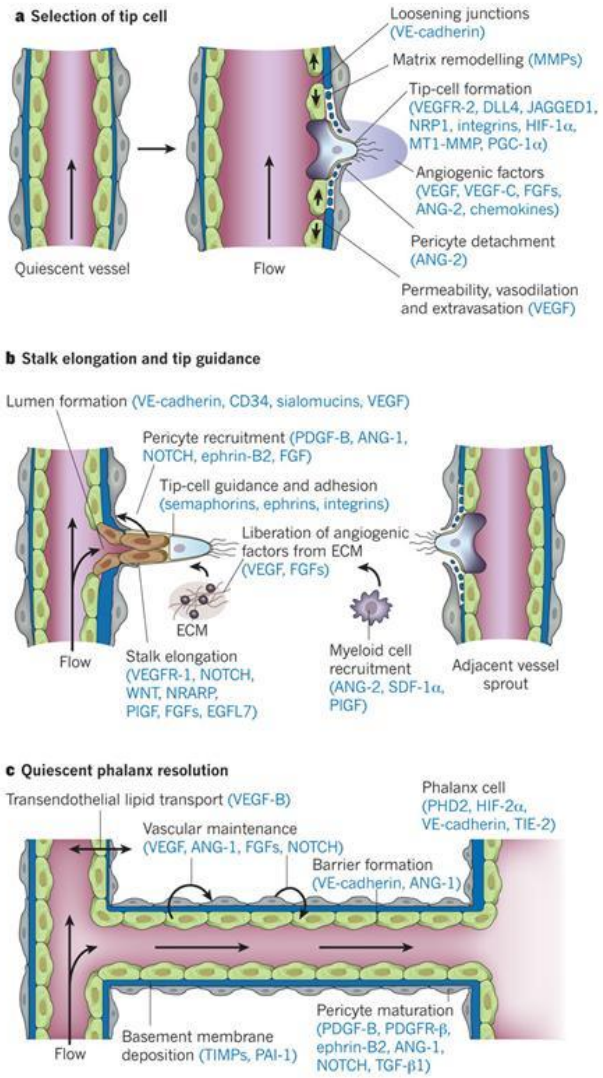
Cell proliferation assays have been widely used to assess cell-cycle regulatory

factors such as growth factors, cytokines, mitogens, and drugs. These assays have evolved from classical [3H]-thymidine incorporation, to 5'-bromo-2'-deoxy-uridine (BrdU) incorporation, to WST-1, WST-8, MTT, or XTT methods.

1.4 Normal Angiogenesis and Vasculogenesis

The term angiogenesis is referred to the formation of new vessels from pre-existing ones and includes the migration, the differentiation of EC's and eventually the reformation of a vessel (29,30,31).

When a naïve EC detects a certain signal such as the VEGF, VEGF-C, ANG-2, FGFs or chemokines that are released by a hypoxic, inflamed or malignant cell, the pericytes detach from the basal membrane of the vessel through the action of the matrix metalloproteinases (MMPs). The ECs relax their connections and the maternal vessel allege. The VEGF increases the penetrability of endothelial cells and allows the extravasation of plasma proteins and the creation of an extracellular matrix scaffold. In response to the integrin stimulation, the ECs migrate in this surface. For the final formation of a vascular tube the ECs migrate massively to the tip of the formation tube and elongation is taking place. This process depends on signals from VEGFRs, Notch, neuropilines (NRPs) and Notch ligands, Dll-4 and Jagged-1, as it is shown in the picture below.



Carmeliet P and Rakesh JK. Nature 2011;473:298-307

Figure 1. Steps in vasculogenesis

Close to the tip cell, the stalk cells act as augmenter cells in the formation and the elongation of the tube, under the stimulation of Notch. The marginal positioned cells receive the environmental signals of direction like efrines and semaforins while cells of trunk release molecules such as EGFL 7 in extracellular matrix in order to transport territorial information regarding the place of neighbouring cells so as to is elongated the trunk. Protease inhibitors (TIMPs, PAI -1) lead in the final formation of

the basic membrane and restore the cellular connections in order to allow the ideal distribution of blood flow. The vessels degenerate if they are do not receive blood (32).

1.5 Vascular morphogenesis (Vasculogenesis)

Although controversial, the repair of vascular or expanding adult pathological vessels can be done by attracting cells of the bone marrow and / or endothelial progenitor cells in the vascular wall. Progenitor cells can be settled within the existing endothelium and collateral vessels containing the majority of blood volume in ischemic tissue are expanding with different mechanisms that include the recruitment and activation of myeloid cells (33).

EPCs are mobilized from the bone marrow, enter the circulation as circulating EPCs and migrate to areas of injury. The strongest factor that mobilizes EPCs from the bone marrow and the direct areas of damage is the VEGF (34,35). Other factors that play a positive role in the mobilization of EPCs from the bone marrow are cytokines such as G-CSF and GM-CSF402, the estrogens, the erythropoietin and statins in a manner independent of their lipid-lowering action (36,37,38).

The prevailing theory is the mobilization of endothelial progenitor cells from the bone marrow in response to signals of ischemia or hypoxia by a mechanism that involves the eNOS and NO production which activates the MMP-9 and releases the cells from the marrow stroma. The cells enter the circulation and migrate to areas of damage driven by a slope of growth factors and chemiotactic substances which direct them at the site of interest.

1.6 Vascular damage in systemic lupus erythematosus (SLE)

Systemic lupus erythematosus (SLE) is a systemic disease of autoimmune etiology, involving multiple genetic and environmental factors. Affects 0.05% of the Western population, particularly young women, and is characterized by the production of autoantibodies against the nucleus. The immune response in SLE is characterized by increased B-cell activation, which are stimulated by autoantigens through both T cell-dependent and independent mechanisms. Recently, special emphasis is given to the role of innate immunity in the pathogenesis of the disease. The innate immunity can induce adaptive immunity, which uses mechanisms of the first against autoantigens. Nephritis is the most important and frequent cause of mortality in patients with SLE and is characterized by the formation and deposition of immune complexes in the kidney that trigger mechanisms of inflammation by attracting leukocytes and the stimulation/filtration of the renal cells.

SLE affects practically all systems of the human body, including the kidneys, CNS, cardiovascular and musculoskeletal system. Patients' symptoms vary in their clinical features, prognosis and response to therapy. The phenotype of the disease is the result of a wide range of changes in gene expression that occur not only in the affected tissues but also at effector cells.

The etiology of SLE is not yet fully understood and it is believed that the disease occurs as a result of complex interactions between multiple genetic, hormonal and environmental factors. Inherited as a multigene disease, currently there is limited information on the genetic loci involved in the development and progression of SLE. Many genetic factors have been associated with the development of SLE, which was confirmed by several studies done to date. Most significant correlation found in locus of human leukocyte antigen (HLA). Specifically, the allele HLA-DR2 and B8-DR3 haplotype have been associated with the disease, and the complement components C2 and C4a (chromosome 6) C1r and C1s (chromosome 12). Other genes that have been reported occasionally is the mannose binding protein-2 (MBL2) and the IL-1, IL-6, IL-10, Bcl-2, APT1LG1 and ADPRT 1.

Early vascular damage in SLE occurs in both the macrovascular and the microvascular network. The endothelial dysfunction is present even when the disease is in remission. Carotidic atherosclerotic plaques are detected in up to 21% of patients with SLE under 35 years of age, rising to almost 100% in patients older than 65 years of age and also premature atherosclerosis of the aorta is detected (39,40).

Risk factors for atherosclerosis in SLE include a combination of the 'traditional' risk factors from the Framingham Study and 'non-traditional' risk factors.

The classical risk factors are widely used to identify people at increased risk for developing atherosclerosis and should be taken into account when assessing a patient with lupus. Patients with SLE have an increased frequency of traditional risk factors associated with early vascular damage. Correlation has been reported between smoking and arterial thrombotic events. Hypertension is associated with increased cardiovascular risk in SLE patients and dyslipoproteinemia (characterized by elevated levels of VLDL, high triglyceride levels, high or normal LDL and low HDL disorders) in combination with dysfunction of chylomicrons formation have also been reported. Changes in function of lipoproteins, including high Lpa and oxidized LDL, also have been described in patients with SLE. HDL has also been reported to exhibit proinflammatory properties and is accompanied by elevated levels of oxidized LDL. Several metabolic disorders that also predispose to atherosclerosis in the general population are also present at increased frequency in patients with S.L.E. like hyperomocystinemia and resistance to insoulin (41,42,43,44,45,46,47,48).

One possible mechanism proposed in SLE vascular damage is the imbalance between endothelial damage and repair. Increased endothelial damage considered as an increase in circulating apoptotic endothelial cells, high levels of which correlate strongly with endothelial dysfunction, production of prothrombotic tissue factor, constitute a putative biomarker for future atherosclerosis. Various soluble adhesion molecules as VCAM-1, ICAM and E-selectin released after endothelial injury, are elevated in SLE and are associated with increased levels of calcifications in the coronary arteries as well as inflammatory activation of metalloproteinases associated with the presence of carotid plaques. These findings suggest that chronic activation of the endothelium and inflammation are important factors for atherosclerotic pathology.

Despite extensive endothelial cell death recorded in SLE, a phenomenon that would normally mobilize an enhanced vascular repair, the later is significantly impaired in lupus. Patients exhibit a reduced number of bone marrow and circulating endothelial progenitor cells of the angiogenic myeloid series. Thus patients with SLE exhibit defective repair of damaged endothelium, leading to the establishment of an environment that induces the development of atherosclerotic plaque (46,47,48).

Patients with SLE exhibit high levels of circulationg IFNa. The IFNa induces apoptosis in EPCs/CECs and removes the myeloid cells to non-angiogenic phenotypes like mature dendritic cells (49). Although it is likely that the reduction in IL-1 and the increase of its inhibitor receptor represents a phenotype that protects the vascular system, a profile that enhances cytokine antiangiogenic responses could be regarded as pathologic and accelerates atherosclerosis in S.L.E. Indeed, vascular activation in conjunction with increased levels of type I interferons can lead to endothelial damage flushes followed by defective repair.

The proinflammatory cytokine TNF-a appears to play an important role in the initiation and expansion of atherosclerotic lesions. TNF-a induces the expression of adhesion molecules on the surface of ECs and induces the expression of proteins that induce chemoattraction of monocytes and T-lymphocytes to the vascular wall. In SLE, the levels of TNF-a are elevated in serum and correlated positively with the calcifications in the coronary arteries. However, the precise role of this cytokine in SLE vasculopathy remains unclear (50,51).

Activation of the complement by immune complexes (ICs) in SLE induces the expression of adhesion molecules in ECs such as VCAM-1 and E-selectin. These molecules may enhance the recruitment of neutrophils and endothelial damage. High levels of ICs against oxLDL/β2GPI have been reported in SLE and with a positive correlation with various cardiovascular risk factors (52,53).

The oxidative burden is a well-confirmed factor for endothelial damage and typically is higher in SLE. Patients are characterized by high levels of active oxygen radicals and nitrite that modify proteins in combination with the presence of antibodies produced against the modified proteins, make an environment that predisposes to the development atherosclerosis. Homocysteine, a molecule which

increases free oxygen radicals in the circulation is increased in SLE (53). The presence AECA (Anti Endothelial Cell Antibodies) has also been reported as a common finding in both SLE and in other autoimmune diseases (54).

1.7 Vascular damage in other autoimmune diseases

Increased levels of CECs have been also reported in rheumatoid arthritis. CECs were isolated by total peripheral blood using immunomagnetic beads and CD 146 marker and were examined under a fluorescence microscope. Increased number of cells was recorded without any correlation with endothelial dysfunction or other biochemical indeces of inflammation in plasma (55).

In systemic sclerosis the measurement of CECs and EPCs, were found to be increased and were correlated with the clinical progression of the disease (56). A recent study demonstrated increased circulating EPC levels in SSc, supporting their mobilisation from bone marrow. Furthermore, the subset of patients with digital vascular lesions and high severity score displayed low EPC counts, suggesting increased homing at this stage (57). Inflammatory infiltrates and an accumulation of extracellular matrix proteins, vascular changes are a hallmark in the pathogenesis of systemic sclerosis (SSc). Consistent with the ongoing endothelial cell apoptosis, several markers of EC damage are up-regulated in the serum of SSc patients. Vascular endothelial growth factor (VEGF), a very potent angiogenic molecule, is overexpressed in SSc patients despite the insufficient angiogenesis. VEGF can protect patients from fingertip ulcers, but a prolonged overexpression of VEGF might have paradoxical effects leading to the formation of irregular vessels similar to those observed in SSc. Besides defective angiogenesis, recent studies suggest that vasculogenesis is also impaired in SSc patients with reduced numbers and functional defects of endothelial progenitor cells.

In ANCA vasculitis, the CECs were found to be increased with a positive correlation of the disease activity (58).

Endothelial cell migration and cell proliferation assays have been used in the past for the study of promising therapeutic targets in autoimmune diseases. Albeit, it still remains challenging in the study of SLE.

In order to evaluate the above mechanisms, screening tools are needed. In our study we sought to establish a human umbilical vein endothelial cells line as well as primary endothelial arterial cells line. Our target is to apply classical cell biology and cutting edge genomic approach in order to study the mechanisms leading in vascular damage in SLE and the possible atheroprotective role of rHDL-apoE3.

2.1 Atherosclerosis

Atherosclerosis is a multifactorial, progressive, chronic inflammatory disease that primarily affects large and medium-sized arteries. It is characterized by the formation and development of atherosclerotic plaque, which consists of a well-circumscribed structure of lipids, necrotic cores, calcified areas, inflammatory SMCs and ECs, immune cells and foam cells. Consequently atherosclerosis is associated with cardiovascular disease (59). Today, atherosclerosis is considered an inflammatory disease that occurs as a result of inappropriate and uncoordinated immune activation. Furthermore fulfills the Witebsky and Rose criteria for classification as an autoimmune disease which is (a) the presence of specific autoantigens which in the case of atherosclerosis is the group of heat stress proteins HSP60/65, oxidized low density lipoprotein (oxLDL) and the B2-glycoprotein-1 (B2GP-1), (b) active immunization in experimental animal models induces atherosclerosis - immunization with HSP60, oxLDL, and B2GP1 induces the production of specific antibodies, (c) the evidence for the pathogenic role of autoantibodies - autoantibodies against HSP60, B2GP-1 enhance the development of atherosclerosis, and autoantibodies against oxLDL exhibit protective function, (d) passive transfer of the disease with specific T-lymphocytes - it has been shown that the administration to mice of specific anti- HSP60 and anti-B2GP1 T lymphocytes induces the initiation and progression of atherosclerosis and (e) the immunomodulatory therapy reduces the occurrence and intensity of experimental atherosclerosis in mice (60). It is however important to note that defining

atherosclerosis simply as an immune inflammatory disease, could oversimplify other stimuli such as metabolic and hemodynamic factors which are known to have an important role in the development of atherosclerotic plaque (61).

A crucial step in atherosclerosis initiation is endothelial dysfunction, with a gradual transition from the physiological role of the endothelium in the pathophysiological and finally pathological. Besides the key role in the initiation of atherosclerosis, endothelial dysfunction also contributes to the progression of atherosclerosis (62), confirming that endothelial dysfunction is an important factor throughout the process of vascular injury.

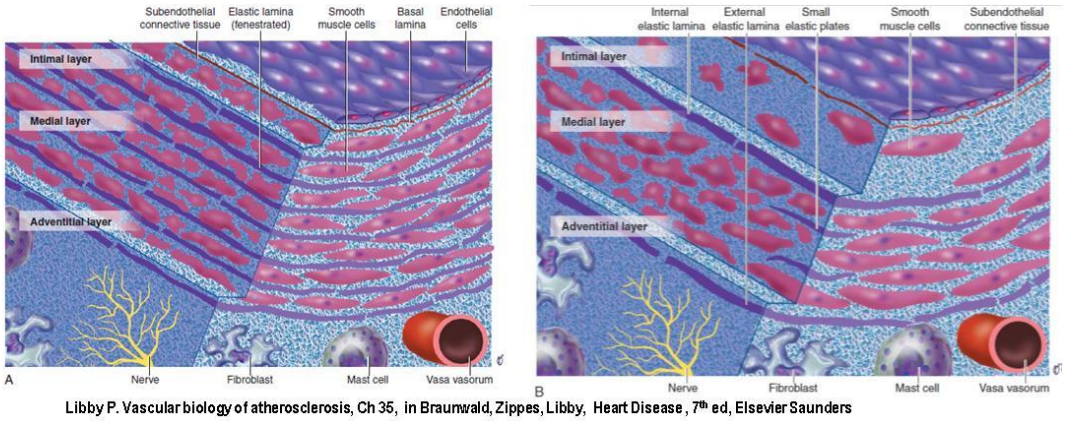
Epidemiological studies during the last 60 years have shown multiple risk factors for atherosclerosis and are shown in the table below.

Table 1. Genetic and Enviromental factors associated with atherosclerosis and Coronary Artery Disease.

Genetic and Enviromental factors associated with atherosclerosis and Coronary Artery Disease.
Factors with strong genetic background
High levels of LDL/VLDL
Low levels of HDL
High levels of Lp(a)
High blood pressure
Elevated levels of homocysteine
Family history
Diabetes mellitus and obesity
Elevated levels of coagulation factors
Depression and mood disorders
Male sex
Systemic inflammation
Metabolic syndrom
Enviromental factors
High fat diet
Tobacco use
Low anti-oxidant levels
Lack of physical activity
Inflammation factors

2.2 Anatomy of a normal artery.

The wall of a normal artery consists of three distinct layers. These layers are present in both elastic and muscular arteries but in various extent as seen in the picture below.



Libby P. Vascular biology of atherosclerosis, Ch 35, in Braunwald, Zippes, Libby, Heart Disease, 7th ed, Elsevier Saunders

Figure 2. Cross section of an elastic and a muscular artery wall

The **tunica intima** consists of a monolayer of endothelial cells, which line the lumen of blood vessels and are in direct contact with blood. The monolayer is located on a basal membrane composed mainly of type IV collagen, laminin, fibronectin and other extracellular matrix. Below the basal membrane are found smooth muscle cells within a layer of proteoglycans. The internal elastic membrane separates the tunica intima from the tunica media. Normally the tunica intima is very delicate area but with advancing age it's thickness increases, a state characterized as diffuse thickening of the intima and is present in many arteries in adults, but it is not necessarily accompanied by an increased atherogenic lipid load. In a healthy adult, endothelial cells have high half-life, have autocrine mechanisms of protection against various noxious stimuli, alter their shape to meet the needs of blood flow through oxygen sensors and hypoxia. The naive endothelial cells interconnect via ligand molecules such as VE-Cadherin and are surrounded by pericytes, which inhibit the proliferation of ECs and provide signals for survival.

The **tunica media** is found just below the inner elastic membrane. In elastic arteries such as the aorta, a well-developed concentric layers of smooth muscle cells can be found separated by layers of extracellular matrix rich in elastin. This structure serves as the storage of the kinetic energy derived from the contraction of the left ventricle while preserving the structural integrity of the arteries. In the smaller muscular arteries, smooth muscle cells have a rather non concentric laminar arrangement and are surrounded by matrix. Normally smooth muscle cells rarely divide and rates of cell division and death are extremely low. The same applies to the extracellular matrix, which is in equilibrium between synthesis and degradation. The tunica media is surrounded externally by the outer elastic membrane that separates it from the adventitia of the vessel.

The **adventitia** received special attraction in recent years due to its role in the vascular homeostasis and pathology. It consists mainly of collagen fibers in rather looser fashion than the tunica intima. Various nerve endings are found as well as vasa vassorum, whose role is important during the pathologic angiogenesis that characterizes the advanced atherosclerotic plaques. The cell populations consist mainly of fibroblasts and mast cells.

2.3 Steps in the evolution of atherosclerosis

The first step seems to be the extracellular accumulation of small lipoprotein particles (mainly LDL) in the intima of the vessel via passive diffusion through the connections of ECs in the arterial wall. These particles are rich in esterified cholesterol, LDL and triglycerides all encapsuled by a layer of phospholipids, free cholesterol and apolipoprotein-B100 (Apo-B100). Chylomicrons and Lipoprotein (a) are also present. Lp(a) has been identified as a major atherogenic factor implicated to fibrinolysis and the proliferation of SMCs (63). All the above particles become entraped via the proteoglycanes and are vulnerable to oxidization. Oxidized LDL is the crucial triggering in the evolution of atherosclerosis.

Much information are provided by animal models in several studies. Deficient mice in apolipoprotein E (ApoE-/ -) or mice deficient in the receptor of lipoprotein LDL (LDLR-/ -) develop atherosclerosis and marked changes in response to a high fat

diet (64). Following the inflammation that has been triggered, the migration and accumulation of leykocytes takes palce. Monocytes and lymphocytes penetrate the through the lining of the EC's and enter the vascular wall. Monocytes within the intima (stimulated by the cytokine M-CSF produced by activated Ecs) differentiate into macrophages, a process necessary for the development of atherosclerosis. Free oxygen radicals are produced by endothelial cells and differentiated macrophages. Along with the action of various other enzymes such as myeloperoxidase, sphingomyelinase and secretory phospholipase the inflammatoty atherosclerotic plaque is established. The extensively modified forms of LDL are uptaken rapidly by macrophages, leading to the formation of "foam cells", a phenomenon which is mediated by a group receptors expressed in macrophages and recognize a broad spectrum of ligands. These are scavenger receptors (65), a family of proteins that includes CD36, CD68, CXCL16, the receptor type lectin for oxidized LDL (LOX1) and scavenger receptor A and B1 (SR-A and SR- B1). These receptors are receptors that recognize molecular patterns (PRRs-Pattern-Recognition Receptors) and mediate uptake and degradation of lysosomal modified lipoproteins, lipopolysaccharides and apoptotic remnants. The signaling of these receptors does not lead directly to inflammation but leads to MHC-II-dependent antigen presentation thereby connecting the natural and acquired immunity which characterizes the further development of atherosclerosis (66).

These initial lesions called fatty streaks are not clinically significant but are precursors of more extensive lesions. Found even in infants and young children are present in the aorta and the first decade of life, the coronary arteries during the second decade and cerebral arteries in the third and fourth decades and sometimes can understeer.

2.4 The role of Apolipoprotein E (apoE) in atherosclerosis

Apolipoprotein E (apoE) was first described by Shore and Shore in 1973 as a part of the VLDL particles. It is now kown as a polypeptide composed of 229 amino acids which binds to various apolipoproteins (apart from VLDL) such as the chylomicrons and the HDL cholesterol. Almost one third of its molecule, the N-terminal region, enables binding to the LDL receptor (the region between amino acids

130 and 150). ApoE is a ligand for both the LDLr and the chylomicrons remnants' receptor.

The apoE gene is located in the long arm of chromosome 19. Unlike other apolipoproteins, apoE is synthesized not only in the liver, but also in the brain (astrocytes), spleen, lungs, kidneys, smooth muscle cells and ovaries, but not in the intestinal epithilium (67). Several apoE isoforms are known to date with the three major isoforms (E2,E3,E4) encoded by three alleles (epsilon 2, epsilon 3, epsilon 4 respectively).

ApoE has a major effect on the degradation of lipoproteins and thus on plasma levels. ApoE has a higher affinity for the LDLr than apoB itself. ApoE is also a ligand for the chylomicron remnants' receptor (LPR) and thus it binds chylomicron remnants rich in triglycerides which they enter the circulation mainly by the alimentary route.

All three apoE isoforms have different effects on the metabolism of lipoproteins. The predominant isoform in the population is allele E3, the most common genotype E3/3. Allele E2 is associated with lower LDL levels, whereas allele E4 with higher LDL levels, whern compared with allele E3. The occurrence of apoE2 is often associated with type III hyperlipoproteinemia. The low binding apoE2 activity to receptors decelerates the catabolic change of chylomicrons, VLDL and of remnant particles thus increasing their content in the plasma. Moreover the enhanced activity of hepatic LDL receptors lowers LDL-cholesterol concentrations and increases HDL-cholesterol concentration in the plasma. The presence of allele E2 even in the homozygous isoform does not necessary imply type III hyperlipoproteinemia and vice versa. Type III hyperlipoproteinemia may occur in patients with allele E3 or E4, but such an association is substantially lower.

On the other hand the presence of allele E2 is considered to be a protective factor against premature atherosclerosis symptoms, compared with the presence of allele E3 and particulary E4. The risk of ischemic heart disease depends on the occurrence of allele E4 which is connected with an inceased risk of ischemic heart disease, whereas the allele E2 has a contrary effect (68). ApoE4 isoform has also been linked with a higher incidence of Alzheimer's Disease.

Recently, it has been shown that apoE participates in a novel pathway of biogenesis of apoE-containing HDL particles through the action of ABCA1 lipid transporter and LCAT (69).

An important factor in atheroprotection is HDL cholesterol. It is well established today that HDL has anti-oxidant, anti-inflammatory, anti-thrombotic and atheroprotective role. Binding of HDL to its receptor (SR-BI) offers a dependent release of NO and protection of endothelial integrity. Previous studies have shown the important role of HDL on endothelial cell proliferation and migration. HDLnot only promoted the proliferation of HUVEC via mechanisms that increased intracellular Ca^{2+} and upregulated the production of prostacyclin (70). but also promoted endothelial cell migration that was promoted by signalling cascades mediated by interaction of S1P with S1P1 and S1P3 receptors that led to the activation of PI3 kinase, p38MAP kinase and Rho kinases (71). HDL can also activate the MAPK pathway either through processes that involve protein kinase C (PKC), Raf-1, MEK and ERK1/2 or PKC independent pathways. Another beneficial effect of HDL is its capacity to promote capillary tube formation in vitro. This function requires p44/42MAP kinase, which is downstream of Ras (72).

Atherosclerosis remains a major health issue and can be present in diseases affecting their progress. Systemic lupus Erythematosous and other autoimmune diseases are characterized by premature and progressive atherosclerosis leading in vascular events early in patient's life. On the other hand coronary artery disease is on a rise affecting today even young patients.

We sought to investigate the endothelial cell migration and proliferation combining classical biology methods and cutting edge genomic approach. We used human umbilical vein endothelial cells and human arterial endothelial cells to investigate cell migration and proliferation as promising therapeutic targets in the future.

3.1 HUVEC's Extraction from Umbilical Cords

The umbilical cords were obtained from the OBG Department of the University Hospital of Crete. Appropriate informed consent was obtained from both the parents of the newborns and are available on demand. Data are anonymous. Inclusion and exclusion criteria existed. Preterm newborns from unhealthy parents with APGAR score below 7 were excluded. Also, umbilical cords were available within 15 minutes in our lab after the labour. Umbilical cords with a size less than 15cm were excluded due to the expected low yield of HUVECs.

The day before isolation of HUVECs Petri dishes 35-mm-diameter were coated with one drop of fibronectin solution per plate. Seven dishes were prepared per umbilical cord. The dishes were dried under the hood, with lid open. A sterile container was used, with 50 ml of buffer for conservation and transport, to the maternity hospital. One container was needed for each umbilical cord of 16–30 cm; the container was be kept at 4 C until cord collection and again immediately afterwards.

The day of HUVECs isolation and culture another container was filled up with 500 ml of sterile 0.15 M NaCl (''physiologic serum'' or 0.09% saline) and was maintain at 37 C in a water-bath. Under the hood, (for one cord) two syringes of 50 ml were prepared, one syringe of 30 ml and one syringe of 10 ml, one surgical clamping clip, two cannulae, suture, sterile compresses, sterile scalpels and sterile gloves. One aluminum foil was spread onto the working area, under the hood, for the subsequent cord manipulation. The cords were check whether are suitable for use and then each cord was treated one after another as follows. (Hematic or damaged cords were discarded and also those from infected parturient women such as HBV+, HIV+,

HCV+). Both ends of the cord were tidily cut with a scalpel and a cannula was introduced at each extremity of the vein and was tightly maintained with a suture (we use non absorbable wax coated, black braided silk surgical suture-Medipac). The umbilical vein then was washed with 1x PBS using the 50 ml syringe until the effluent buffer is transparent or slightly pink (all red blood cells were be removed). The 0.1% collagenase solution (Sigma IA C2654, stock is at 1% in M199; stores at-20oC) was injected at one extremity of the vein using the 30 ml syringe; when it leaked out of the other extremity, it was tightly clamped it with the surgical clamp. The syringe was maintained and protected both cord extremities with a clean aluminum foil. The cord was then incubated in the water-bath for 12 min. Under the hood, a sterile 50 ml tube was fill up with 10 ml of ‘‘complete M199 medium’’ and the cells were collected in this tube by washing the vein with 40 ml of 5% FCS/PBS.

The closed tube was then centrifuged at 1.700rpm for 5 min without temperature control. The supernatant was carefully discarded and the pellet of cells was suspended in a 14 ml of ‘‘complete M199 medium’’. The cells were dissociated by gentle aspiration and repulsing (three times) with the 30 ml syringe equipped with a needle (0.7 mm x 30 mm).

The volume was then splitted in two Petri dishes already covert with collagen. For our studies a pool of HUVECs of at least 5 umbilical cords was used and no possible rejection due to HLA-DR antigens was noticed.

The ECs cultures were then incubated at 37 1C in a 95% air/5% CO2 atmosphere saturated with H2O.

The following day (after less than 24 h), non-adherent cells were removed by changing the culture medium. The result was examined under phase-contrast microscopy; if some remaining blood clots were to contaminate the ECs, the culture medium was changed again. At this stage we also counted the adherent EC islets to determine a yield of adhesion.

The culture medium was change every 2 days (replacing with 1.5 ml medium each time). Usually, cell confluency was achieved in 7–8 days, although it is

dependent on the number of cells seeded. When confluent ECs showed contact inhibition, the EC cultures presented a ‘‘cobblestone appearance’’ (tightly packed polygonal ECs) in phase-contrast microscopy. Often we could wait 2 days more before treating the cells, because real confluency (with no mitosis) is achieved later than can be seen via microscope. Typically, in a 35 mm Petri dish, the constituted endothelium (organotypical culture) contains about 1 million cells.

Below are shown the mixing volumes of each solution used.

600mls MEDIUM FOR HUVEC

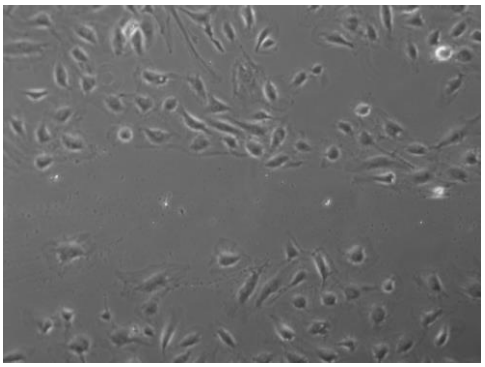
- GIBCO M_{199} 31150
- FCS GIBCO 10270 (final 20%)
- $3x10^3$ units Heparin
- 30 mg of commercial ECGS
- 1% Penicillin/Streptomycin

  The mix was stored in aliquots, covered with silver foil (light sensitive) and store at 4C

200mls COLLAGEN

- 200mls H_2O, tissue-culture tested, sterile
- 240ul acetic acid
- 10mg commercial rat tail collagen BD Bioscience 354236

  The mix was filtered and kept at 4C



Huvec’s at day 6

3.2 HUVEC's Proliferation and Migration under SLE sera stimulation

Blood samples from SLE patients were obtained from the Dpt of Rheumatology and Clinical Immunology, University Hospital of Crete. Control samples were obtained from healthy volunteers bank of our University. Appropriate informed consent was obtained and are available on demand. Data are anonymous.

Blood samples were centrifuged at 3.000rpm 4^0C for 15min. The RBC's pellet was discharged and the supernatant was harvested. Half of the sera undergone heating inactivation at 56^0C for 30min.

The final serum concentration in each row of our 96-well plate was scaled as being 1%, 5%, 10%, 20%.

1. HUVEC cells were seeded in a 96-well plate at a density of 104-105 cells/well in 100 μl of culture medium with or without compounds to be tested. The cells were cultured in a CO2 incubator at 37°C for 24-48 hours.

2. 10 μl of the reconstituted WST-1 mixture was added to each well using a repeating pipettor.

3. The plate was mixed gently for one minute on an orbital shaker.

4. The cells were incubated for two hours (adherent culture) to four hours (suspension culture) at 37°C in a CO2 incubator.

5. Before reading the plate, was mixed gently on an orbital shaker for one minute to ensure homogeneous distribution of color.

5. The absorbance of each sample was measured using a microplate reader at a wavelength of 450 nm.

Table 2. Setting of the 96well plate. Cells on grey background are not HUVEC containing.

		SLE Serum (active) #1 (OR CTRL)		SLE Serum (active) #2 (OR CTRL)		SLE Serum (inactive) #3 (OR CTRL)		EGM-2		EGM-2			
		1	2	3	4	5	6	7	8	9	10	11	12
A	2%	98µl EBM-2 + 2µl SLE SERUM	98µl EBM-2 + 2µl SLE SERUM	98µl EBM-2 + 2µl SLE SERUM	98µl EBM-2 + 2µl SLE SERUM	98µl EBM-2 + 2µl SLE SERUM	98µl EBM-2 + 2µl SLE SERUM	100µl EGM-2	100µl EGM-2	100µl EGM-2	100µl EGM-2	100µl EGM-2	100µl EGM-2
B	5%	95µl EBM-2 + 5µl SLE SERUM	95µl EBM-2 + 5µl SLE SERUM	95µl EBM-2 + 5µl SLE SERUM	95µl EBM-2 + 5µl SLE SERUM	95µl EBM-2 + 5µl SLE SERUM	95µl EBM-2 + 5µl SLE SERUM	100µl EGM-2	100µl EGM-2	100µl EGM-2	100µl EGM-2	100µl EGM-2	100µl EGM-2
C	10%	90µl EBM-2 + 10µl SLE SERUM	90µl EBM-2 + 10µl SLE SERUM	90µl EBM-2 + 10µl SLE SERUM	90µl EBM-2 + 10µl SLE SERUM	90µl EBM-2 + 10µl SLE SERUM	90µl EBM-2 + 10µl SLE SERUM	100µl EGM-2	100µl EGM-2	100µl EGM-2	100µl EGM-2	100µl EGM-2	100µl EGM-2
D	20%	80µl EBM-2 + 20µl SLE SERUM	80µl EBM-2 + 20µl SLE SERUM	80µl EBM-2 + 20µl SLE SERUM	80µl EBM-2 + 20µl SLE SERUM	80µl EBM-2 + 20µl SLE SERUM	80µl EBM-2 + 20µl SLE SERUM	100µl EGM-2	100µl EGM-2	100µl EGM-2	100µl EGM-2	100µl EGM-2	100µl EGM-2
E	2%	98µl EBM-2 + 2µl CONTROL SERUM	98µl EBM-2 + 2µl CONTROL SERUM	98µl EBM-2 + 2µl CONTROL SERUM	98µl EBM-2 + 2µl CONTROL SERUM	98µl EBM-2 + 2µl CONTROL SERUM	98µl EBM-2 + 2µl CONTROL SERUM	100µl EGM-2	100µl EGM-2	100µl EGM-2	100µl EGM-2	100µl EGM-2	100µl EGM-2
F	5%	95µl EBM-2 + 5µl CONTROL SERUM	95µl EBM-2 + 5µl CONTROL SERUM	95µl EBM-2 + 5µl CONTROL SERUM	95µl EBM-2 + 5µl CONTROL SERUM	95µl EBM-2 + 5µl CONTROL SERUM	95µl EBM-2 + 5µl CONTROL SERUM	100µl EGM-2	100µl EGM-2	100µl EGM-2	100µl EGM-2	100µl EGM-2	100µl EGM-2
G	10%	90µl EBM-2 + 10µl CONTROL SERUM	90µl EBM-2 + 10µl CONTROL SERUM	90µl EBM-2 + 10µl CONTROL SERUM	90µl EBM-2 + 10µl CONTROL SERUM	90µl EBM-2 + 10µl CONTROL SERUM	90µl EBM-2 + 10µl CONTROL SERUM	100µl EGM-2	100µl EGM-2	100µl EGM-2	100µl EGM-2	100µl EGM-2	100µl EGM-2
H	20%	80µl EBM-2 + 20µl CONTROL SERUM	80µl EBM-2 + 20µl CONTROL SERUM	80µl EBM-2 + 20µl CONTROL SERUM	80µl EBM-2 + 20µl CONTROL SERUM	80µl EBM-2 + 20µl CONTROL SERUM	80µl EBM-2 + 20µl CONTROL SERUM	100µl EGM-2	100µl EGM-2	100µl EGM-2	100µl EGM-2	100µl EGM-2	100µl EGM-2

For the Migration studying an in vitro wound-healing assay was performed. Confluent HUVECs plated in a 6-well plate, 16-20 h after treatment of SLE sera in 10% concentration were scratched with a pipette tip to create wounds. Randomly chosen fields (n=16) were used for imaging and the images were taken at identical locations at time 0 and overnight. The wound widths were measured by Photoshop (Adobe) software.

3.3 Production of reconstituted-HDL-apoE3 particles Global Gene Expression measurements (performed in Prof. V. Zannis laboratory)

Reconstituted-HDL-apoE3 particles were prepared in Prof. V. Zannis laboratory (Boston University) as previously described (73). rHDL-apoE3 contains apoE3, cholesterol and POPC (reconstituted discoidal particles consisting of apoE, 1-palmitoyl-2-oleoyl-l-phospatidylcholine (POPC).

Human Arterial Endothelial Cells were cultured under carefully selected conditions and incubated with rHDL prepared with apoE3 and phospholipids (ratio 1:100, designated rHDL-apoE3). Multiple cultures were prepared for each set of conditions. Total RNA was extracted from each cell culture (including cultures in the presence of saline or rHDL) by the standard Trizol method. The high quality total RNA was used for microarray analysis on the Human Gene 1.0 ST whole genome Arrays by Affymetrix.

Bioinformatic analyses performed in collaboration with Drs. M. Lenburg and D. Sanoudou, showed that the rHDL-apoE3 treatments caused differential expression (over 2-fold change and ≤ 0.05 FDR) in 198 genes.

3.4 Bioinformatical analysis and data mining for microarray gene expression data on rHDL-apoE3 treated HAECs

The 198 statistically significantly changed genes following HAEC exposure to rHDL-apoE3, were analyzed with the Ingenuity Pathways Analysis (Ingenuity Systems®, www.ingenuity.com). Ingenuity Pathways Analysis (IPA) enables the translation of large scale expression measurements to biologically meaningful information, by integrating data from a variety of databases and providing insight into the molecular and chemical

interactions, the biological processes, the molecular functions, and the predicted upstream regulators of the observed regulated processes.

The Core Analysis in IPA, allows the evaluation of biological processes, pathways and molecular networks. For the purposes of this project, the core analysis was performed on the 198 genes that were significantly altered (upregulated or downregulated) after rHDL-apoE3 treatment. Specific parameters were selected for the "data import" part of the analysis as shown in the series of Figures below. Initially, the list of 198 genes was uploaded in .xls as a flexible format (IPA requirements). The information contained in each column was indicated: the column header, was noted and the gene information was counted starting from the second row. The Gene symbol was chosen as an identifier type of the genes from the list and it was indicated that the array platform of Human Gene 1.0 ST Array was used. In the raw data 199 inserted elements were identifies (198, plus the header) and in the data summary 198 genes were successfully identified. As shown in the Figure 1 the first column was selected as the ID column, the description column was ignored in this phase and the third column was set as Observation 1/Fold change. The analysis was then saved and created.

Once the data were imported, a series of criteria/thresholds were selected for prior to running the data analysis. Specifically, in the section of the General Settings, the reference set of Human Gene 1.0 ST Array was inserted, since the population of genes to consider for p-value calculation was carried out with this array type. Direct and indirect relationships were preferred so that a more complete picture of the molecular networks and upstream regulators' analysis could be achieved. In the section of networks interaction endogenous chemicals were included and 35 molecules per network and 25 networks per analysis were chosen. All data sources used by the IPA software were included for the core analysis. These include the Ingenuity expert information, the Ingenuity supported third party information, the protein-protein interactions as well as additional sources (such as ClinicalTrials.gov, Gene Ontology, GVK Biosciences, Human Cyc, miRBase, Mouse genome database and obesity gene map database). To achieve a high level of confidence of the core analysis, only experimentally observed data were included, thus excluding the

predicted ones. In the section of species selection, we focused on human and mouse species, excluding the uncategorized ones. All tissues and all cell lines were included in the core analysis, since major molecular pathways of vascular endothelial cells are present in more than one tissue types and diseases (i.e. prostate cancer cell lines, kidney cancer cell lines). Finally, all mutations were selected including the ones having a translation impact or a functional effect, leading to a better understanding of the molecular pathways regulation under study.

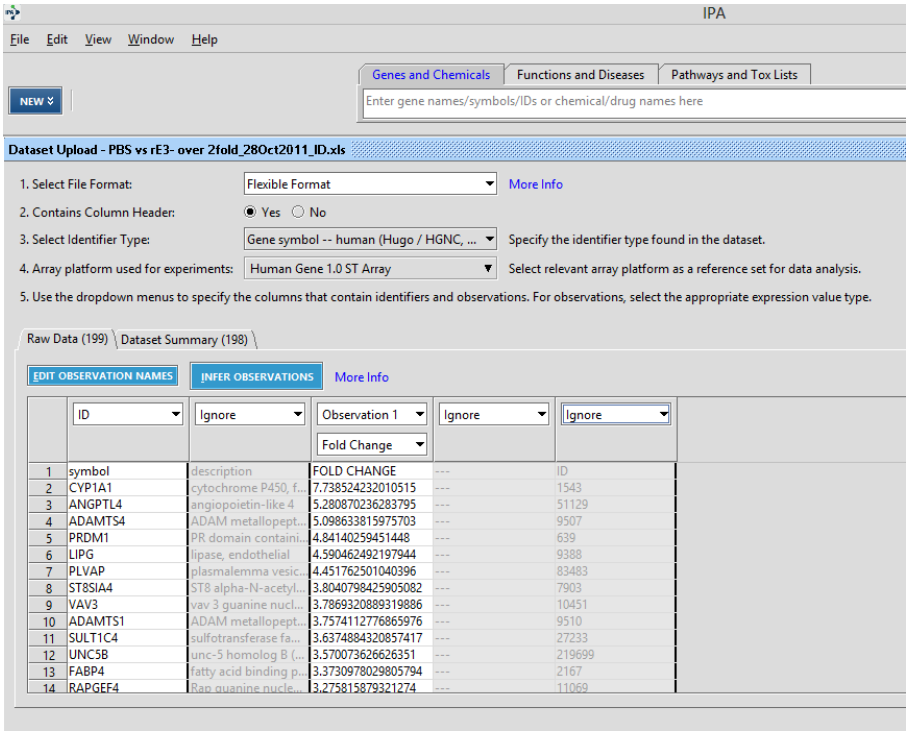


Figure 3. Settings selected for the 198 gene table import into the Ingenuity program.

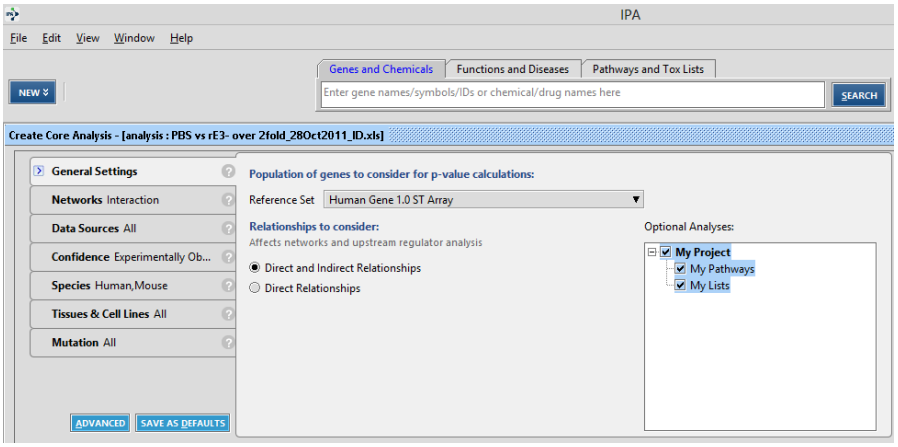


Figure 4. In the General settings both direct and indirect relationships were considered.

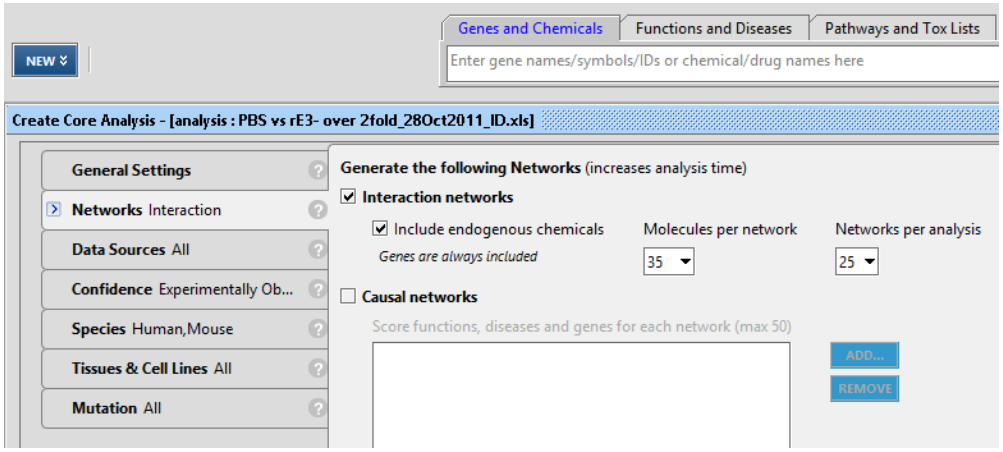


Figure 5. For the Interaction networks analysis a maximum of 35 molecules per network and 25 networks per analysis were selected. Endogenous chemicals were included.

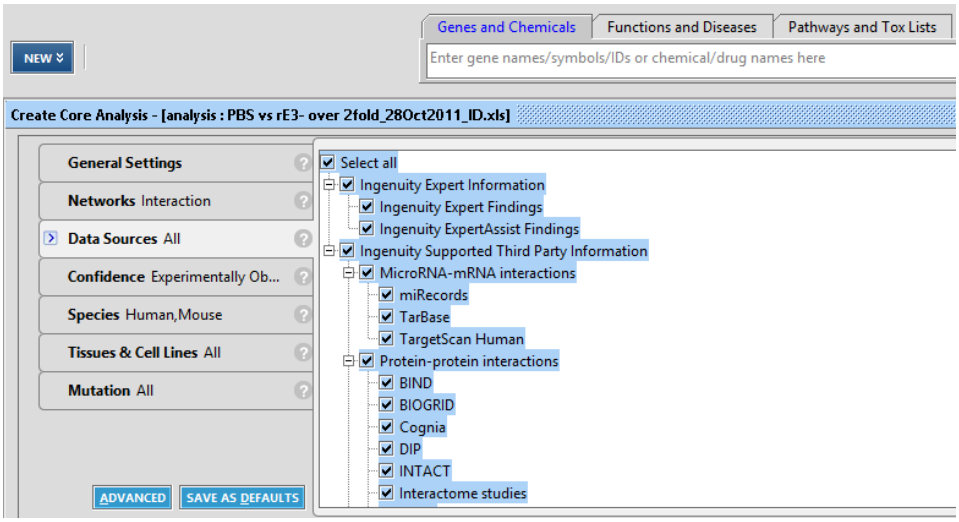


Figure 6. All data sources provided by the IPA software were included in the core analysis in order to achieve a high level of confidence in our results.

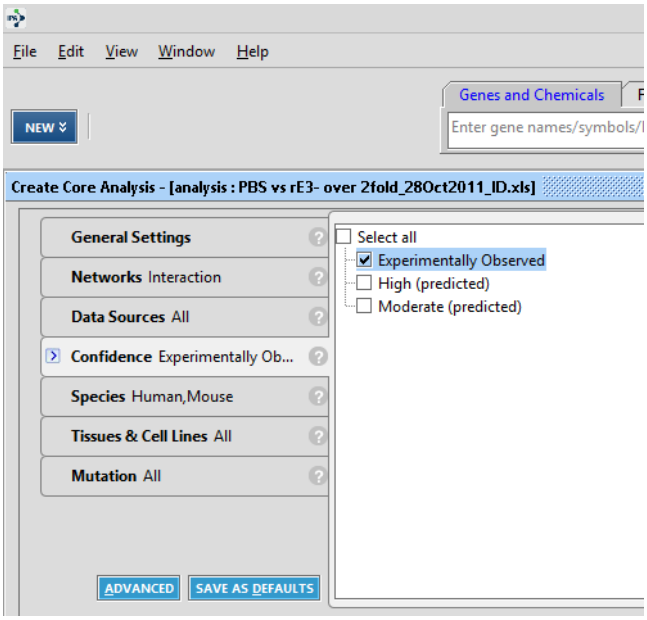


Figure 7. To strengthen the level of confidence only experimentally observed data were included in the 198-gene analysis.

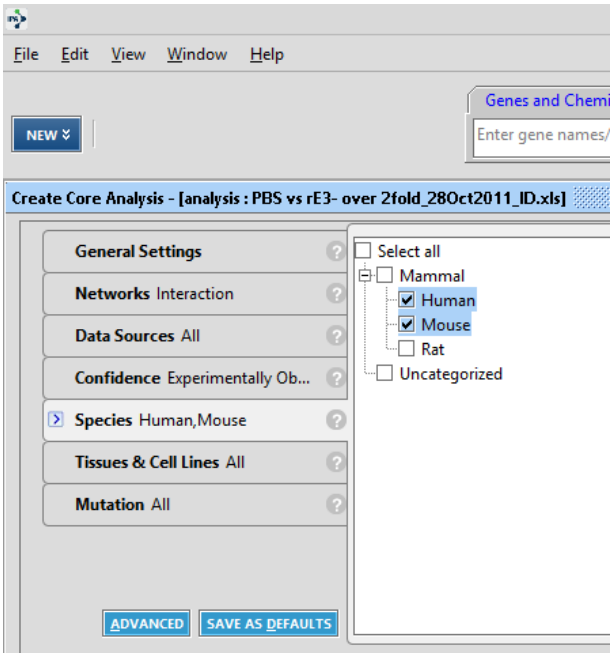


Figure 8. The species selected for this analysis were limited to human and mouse species.

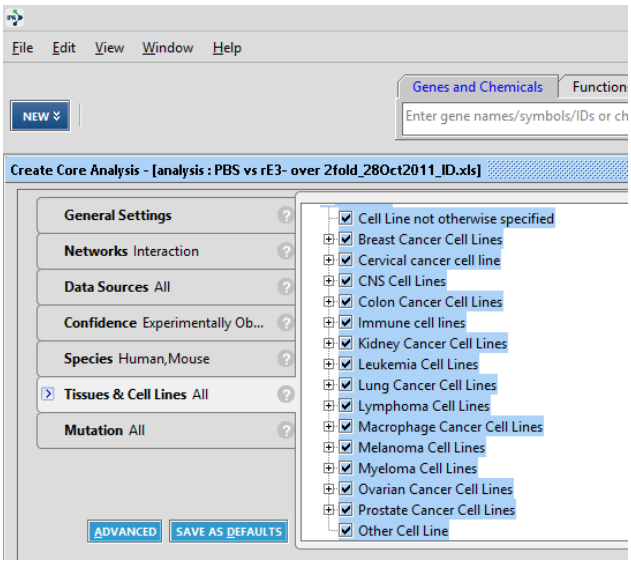


Figure 9. To obtain a comprehensive view of the molecular pathways affected by rHDL-apoE3 treatment of the HAECs, all tissues and cell lines were included in the core analysis.

Core analysis for the 198 genes revealed that 80 genes are implicated in "cellular growth and proliferation", a set of biological process with central role in atherosclerosis. Among these 80 genes, 16 genes emerged as especially interesting because of their direct role in *endothelial cell growth and proliferation*. Consequently, a second core analysis was performed, targeted to the selected 16 endothelial cell growth and proliferation genes.

A thorough literature research was performed using PubMed (http://www.ncbi.nlm.nih.gov/pubmed), and GeneCards databases (http://genecards.org/). The search terms used for PubMed were the gene name followed by the term "endothelial cell proliferation". The limitations for each category are shown in the table below.

Table 3. Limitations for the PubMed search

Search filters' categories	**Search filters**
Article types	Clinical trial, review, original articles
Text availability	Abstract available
Publication dates	10 years
Species	Human, mouse
Languages	English
Journal categories	Core clinical journals, MEDLINE

4.1 HUVEC's Proliferation and Migration under stimulation from SLE patient's sera

Below are shown the Elisa readings and the statistical analysis from the 96 well plate for each sample as described previously.

Table 4. Elisa Readings of the 96-well plate

	2%			M-NON	5%			M-NON	10%			M-NON	20%			M-NON				
	1	2	MEAN	HUVEC	3	4	MEAN	HUVEC	5	6	MEAN	HUVEC	7	8	MEAN	HUVEC	9	10	11	12
A	1.221	2.07	1.6455	1.5825	2.104	1.803	1.9535	1.8785	1.902	2.247	2.0745	1.9605	2.435	2.351	2.393	2.237	2.227	2.066	1.61	2.26
B	1.305	2.094	1.6995	1.6305	1.858	2.476	2.167	2.086	2.109	2.804	2.4565	2.3395	2.566		2.566	2.401	2.185	1.938	0.005	0.005
C	0.984	1.732	1.358	1.233	1.354	1.909	1.6315	1.4485	1.334	1.16	1.247	0.979	2.563	1.636	2.0995	1.7165	0.024	0.006	0.005	0.007
D	1.029	1.221	1.125	1.043	1.247	1.908	1.5775	1.4805	1.243	0.729	0.986	0.864	1.567	2.219	1.893	1.745	0.008	0.006	0.006	0.005
E	0.89	1.757	1.3235	1.2605	1.459	2.155	1.807	1.732	1.688	1.948	1.818	1.704	1.905	1.721	1.813	1.657	0.063	0.075	0.114	0.156
F	1.139	1.494	1.3165	1.2475	1.515	1.86	1.6875	1.6065	1.304	2.156	1.73	1.613	1.646	0.167	0.9065	0.7415	0.069	0.081	0.117	0.165
G	0.759	1.629	1.194	1.069	1.702	1.801	1.7515	1.5685	1.784	2.298	2.041	1.773	1.632	0.368	1	0.617	0.125	0.183	0.268	0.383
H	1.041	1.381	1.211	1.129	1.388	1.587	1.4875	1.3905	1.23	2.042	1.636	1.514	1.899	0.141	1.02	0.872	0.082	0.097	0.122	0.148
																	2%	5%	10%	20%

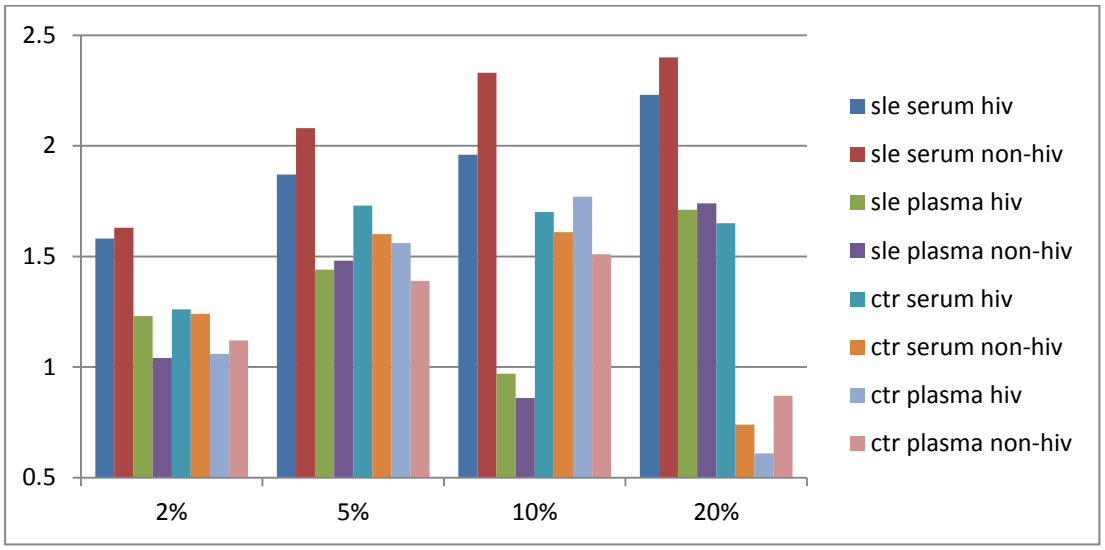
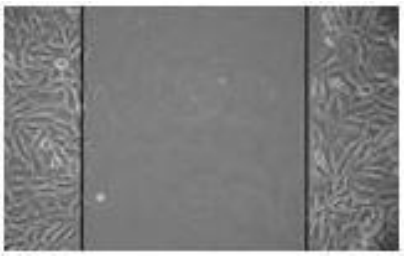


Figure 10. Statistical analysis of the Elisa Readings 96-well plate

Regarding the Migration assay the images obtained at time 0 and time 18hrs are shown below.



HUVEC coated plate at time 0.

HUVEC coated plate at time 18h

4.2 HAEC's Proliferation following rHDL-apoE3 incubation

The initial core analysis on the Functions of the 198 genes that exhibit statistically significant changes after HAEC's incubation with rHDL-apoE3 retrieved important information regarding their biological role. Fischer's exact test was used to calculate a –log (p-value) for each biological function (as shown in the y axis), determining the probability that each biological function assigned to that bar is due to random chance. The smaller the p-value is, the less likely is that the association is random. A p-values cutoff of 0.05 (shown as the threshold yellow line) was selected as an indication of statistical significance (i.e. non-random association). The biological functions that emerge as altered, at least at the gene expression level, are displayed along the x-axis. That is, taller bars are more significant than shorter bars. Only molecular and cellular functions are shown in the table below, excluding disorders and diseases and other physiological system development function categories.

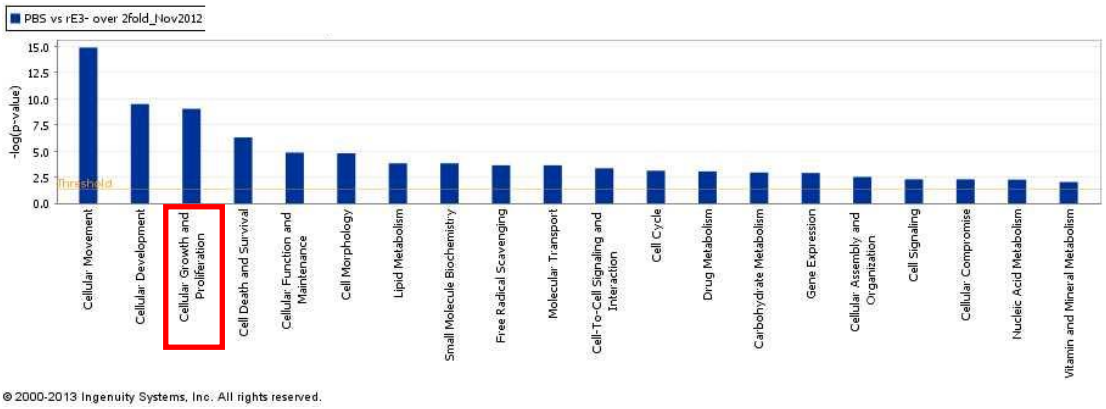


Figure 11. Bar chart showing statistically significant changed function categories in response to HAEC's incubation with rHDL-apoE3

Following the initial core analysis of the 198 genes that exhibit statistically significant changes after HAEC's incubation with rHDL-apoE3, we focused on genes that are especially implicated in atherosclerosis through mechanisms of cellular proliferation. Therefore a new core analysis was carried out for the 16 endothelial cell proliferation genes (shown in the table below). The 16-gene core analysis was performed using exactly the same criteria as in the 198-gene core analysis, to ensure consistency (see Materials and Methods section).

Table 5. Genes identified as implicated in endothelial cell proliferation, following the 198-gene core analysis for Functions.

Category	Genes
proliferation of endothelial cells	ADAMTS1,ANGPTL4,COL4A1, DKK1,DLL4, EDNRB,EFNB2,FLT1, IL8,NR4A1,PTGIR,PTGS2,STAT1
proliferation of vascular endothelial cells	DKK1,EFNB2,IL8,PTGIR
proliferation of microvascular endothelial cells	DKK1,EFNB2,IL8
proliferation of endothelial cell lines	CDK1,IL8,NTN4,PTX3

Table 6. Full name and fold change of the 16 endothelial cell proliferation genes.

symbol	description	FOLD CHANGE
ANGPTL4	angiopoietin-like 4	5.28
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	3.76
EFNB2	ephrin-B2	3.03
FLT1	fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor recep	2.77
PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	2.44
COL4A1	collagen, type IV, alpha 1	2.14
DLL4	delta-like 4 (Drosophila)	2.05
NR4A1	nuclear receptor subfamily 4, group A, member 1	2.05
NTN4	netrin 4	-2.05
EDNRB	endothelin receptor type B	-2.06
STAT1	signal transducer and activator of transcription 1, 91kDa	-2.08
CDK1	cyclin-dependent kinase 1	-2.10
PTGIR	prostaglandin I2 (prostacyclin) receptor (IP)	-2.21
DKK1	dickkopf homolog 1 (Xenopus laevis)	-3.65
IL8	interleukin 8	-4.41
PTX3	pentraxin 3, long	-7.86

Focusing on the targeted core analysis for the16 endothelial cell proliferation genes, a heat-map was generated for all the endothelial cell proliferation sub-categoreis, namely vascular endothelial cells proliferation, microvascular endothelial cells proliferation, and the endothelial cell line proliferation (Figures 12). The size of each box is associated with the –log(p-value) of the genes (i.e. the highest the –log(p-value) is for a certain category, the larger the box is for this category) and the color of each box depicts the z-score ranging from -1.238 (blue color) to 1.963 (red color). The z-Score is a statistical measurement of a category's relationship to the mean in a group of categories. A z-score of 0 means the score is the same as the mean's score.

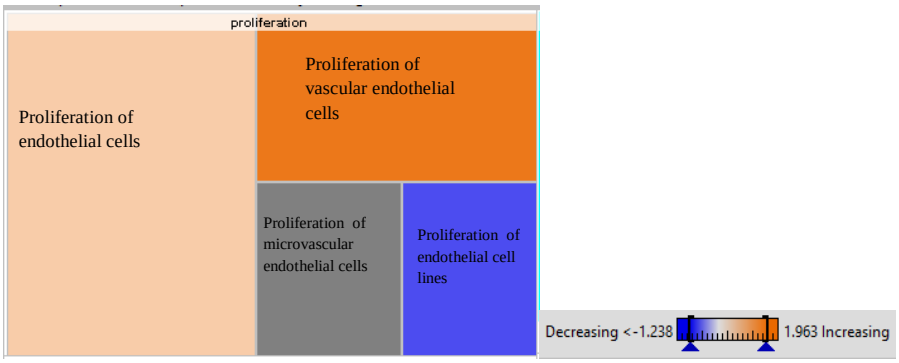


Figure 12. Heat map of the 16 genes altered categories in response to HAEC's incubation with rHDL-apoE3

Next, the specific molecular pathways in which the 16 endothelial cell proliferation genes participate were explored. Among the multiple options provided by the Ingenuity software, the canonical pathways of the cellular growth and proliferation were chosen, as shown in the Figure below. Again the threshold value is set to –log (p-value)=0.05.

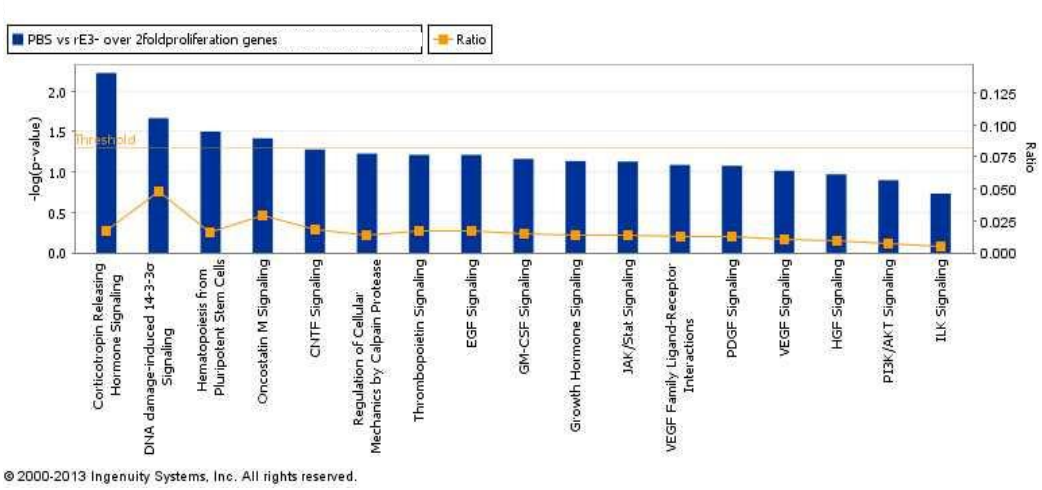


Figure 13. Bar chart showing statistically significant changed canonical pathways (x-axis) regarding cellular growth and proliferation. The y-axis displays the significance (i.e. –log(p-value) for each canonical pathway). The ratio is calculated as follows: number of genes in a given pathway that meet the cutoff criteria, divided by total number of genes that make up that pathway. The highest the ratio is found in a pathway, the highest the number of genes from our list participates in that molecular pathway.

Having identified the proliferation related biological functions and molecular pathways that are regulated in HAECs following rHDL-apoE3, we proceeded with the Network Analysis. The Network analysis mines the scientific literature that is publically available, in order to identify any kind of association between any two of the selected 16 genes. This resulted in three groups of inter-gene associations, namely networks.

Table 7. The 16 endothelial cell proliferation genes were subjected to network analysis. Three networks emerged. The statistically significant changed genes are indicated in bold, while the red arrows indicate upregulation and green arrows indicate downregulation.

ID	Molecules in Network	Score	Focus Molecul	Top Functions
1	↑**ADAMTS1,** Akt, Alpha catenin, CD3, ↓**CDK1,** Cg, ↑**COL4A1,** ↓**DKK1,** ↑**DLL4,** ↓**EDNRB,** ↑**EFNB2,** ERK, ERK1/2, ↑**FLT1,** Focal adhesion kinase, FSH, Hdac, Histone h3, Histone h4, ↓**IL8,** Jnk, KSR2, Lh, MYO1E, NFkB (complex), ↑**NR4A1,** P38 MAPK, PDGF BB, PI3K (complex), ↑**PTGS2,** ↓**PTX3,** RNA polymerase II, ↓**STAT1,** TCR, Vegf	33	13	Cardiovascular System Development and Function, Cellular Development, Cellular Growth and Proliferation
2	CCL13, Cyclin A, Dynamin, IFNG, IL6, IL1B, miR-208a-3p (and other miRNAs w/seed UAAGACG), ↓**PTGIR,** Ren2, SREBF1, TBXA2R	2	1	Cardiac Fibrosis, Cardiovascular Disease, Organismal Injury and Abnormalities
3	↑**ANGPTL4,** CITED2, CLOCK, DICER1, EPAS1, ERBB2, HDL-cholesterol, Hedgehog, HIF1A, HSD11B1, LEP, LEPR, LIPE, LPL, mir-373, mir-515, NR3C1, PCYT2, PGF, PLIN1, PPARA, PPARD, PPARG, proprotein convertase, PTGER1, SCD, Smad, SND1, TGFB1, TGFBR2, VLDL-cholesterol	1	1	Energy Production, Lipid Metabolism, Small Molecule Biochemistry

In order to further analyze the molecular pathways into which the genes under study are implicated, the Ingenuity database offers an up-to-date access to molecular pathways generated from our core analysis.

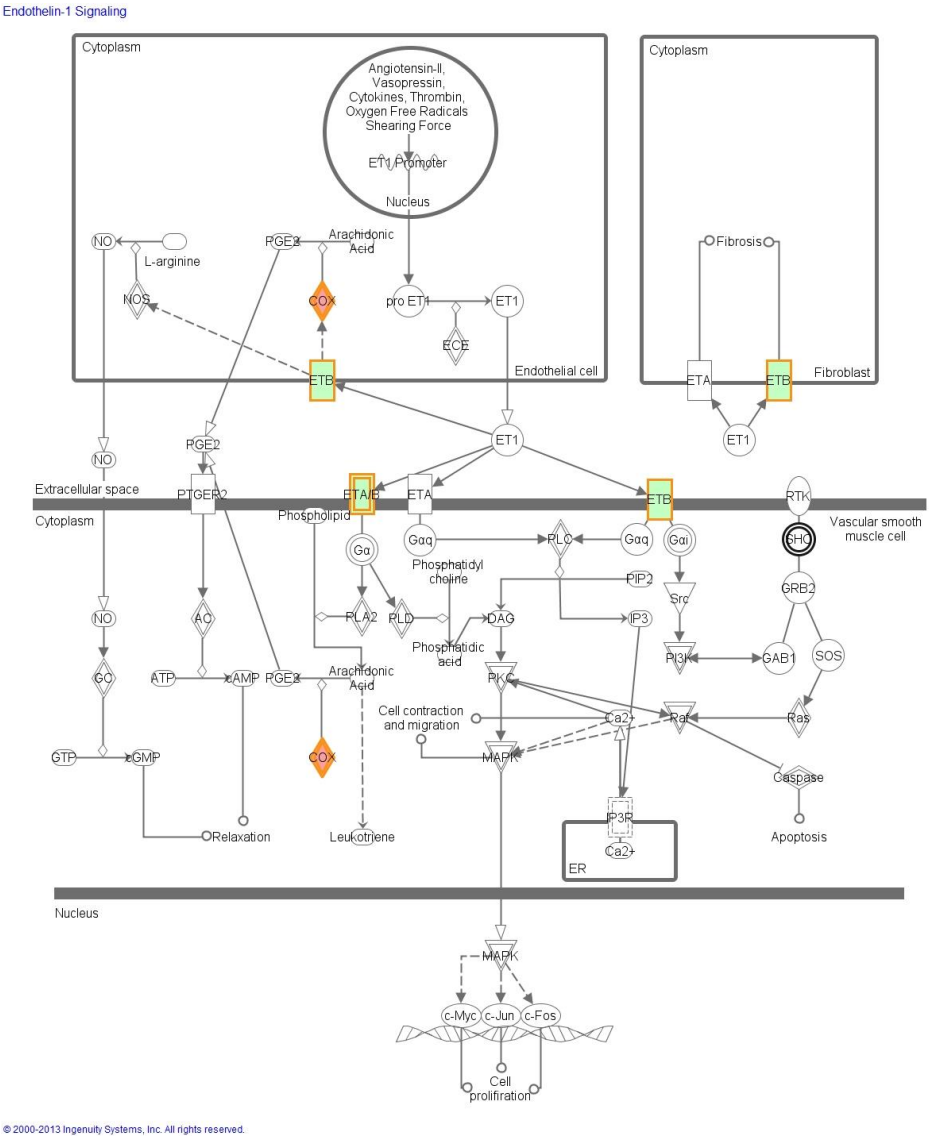


Figure 14. Molecular networks for the Cyclooxygenase and Endothelin receptor leading in endothelial cells proliferation through the PI3K and MAPK pathways activation

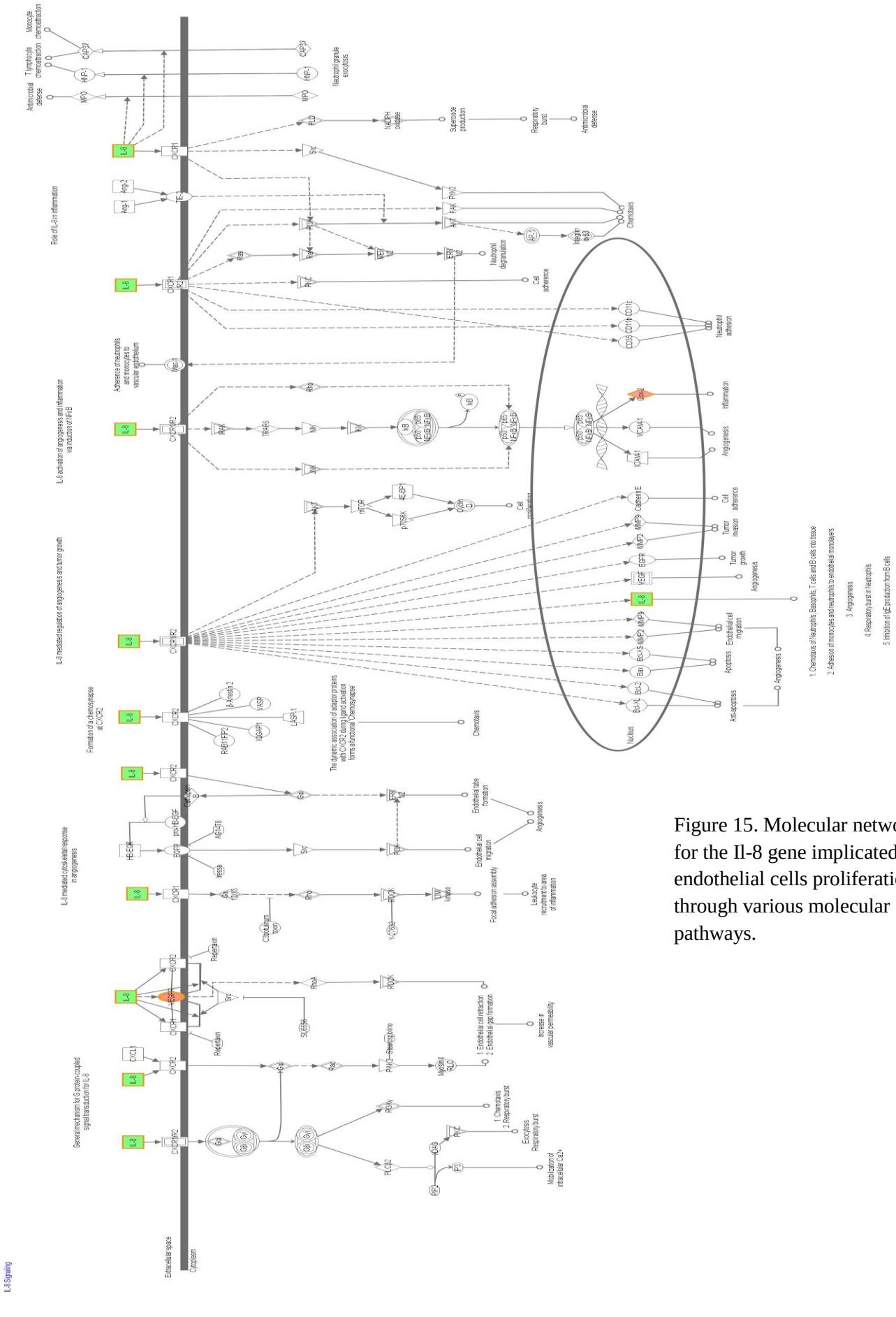
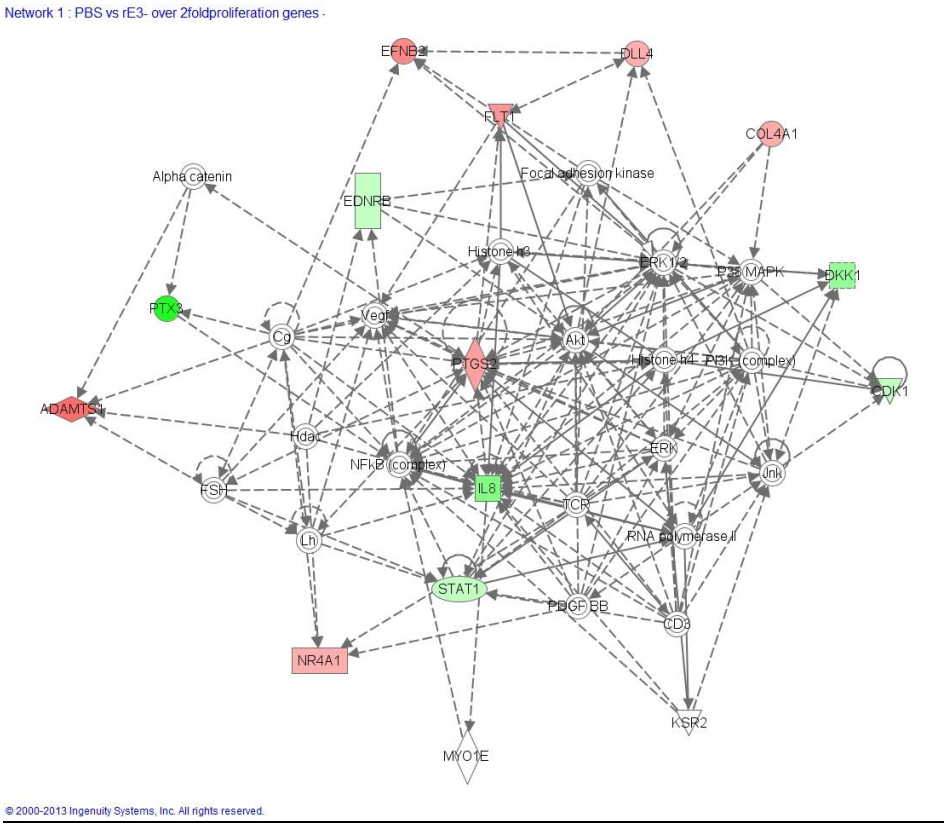


Figure 15. Molecular networks for the Il-8 gene implicated in endothelial cells proliferation through various molecular pathways.

Importantly, 13 of the 16 genes of interest were included in the same network. As shown in Figure 16-17 there are numerous direct and indirect interactions between these genes, and extensive experimental / scientific literature data to support these.



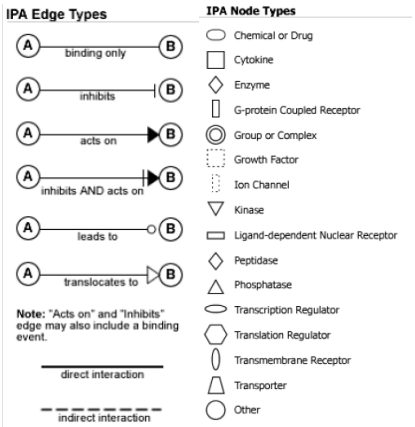


Figure 16-17. Molecular networks for the genes implicated in endothelial cells proliferation
Legends for the edge and node types of the networks

The other two networks contain only one significantly changed gene each, and are therefore for very limited value for the purposes of this study.

To investigate the upstream regulators potentially orchestrating the expression changes of the 16 endothelial cell proliferation related genes, the IPA Upstream Analysis was employed. In total 136 transcription factors were found to be associated with the 16 genes as shown in the Figure 18 below. Notably the most important transcription factors in terms of number of controlled genes seem to be NFKB1, JUN and TP53 each one associated with 6/16 genes, and PPARD associated with 5/16 genes.

Upstream Regulator	Molecule Type	Activation z-score	p-value of overlap	Target molecules in dataset
AHR	ligand-dependent nuclear receptor		1.08E-03	↓CDK1, ↑COL4A1, ↑PTGS2
Ap1	complex		4.33E-02	↓IL8
AR	ligand-dependent nuclear receptor		1.55E-02	↓EDNRB, ↓STAT1
Atf	group		1.88E-03	↑PTGS2
ATF1	transcription regulator		1.68E-02	↑PTGS2
ATF4	transcription regulator		2.38E-03	↑PTGS2, ↓PTX3
BCL10	transcription regulator		1.03E-02	↓IL8
BCL3	transcription regulator		1.31E-02	↓IL8
C/ebp	group		3.75E-03	↑PTGS2
C1QBP	transcription regulator		2.82E-03	↓IL8
CBL	transcription regulator		1.22E-02	↓STAT1
Cbp/p300	group		4.69E-03	↑PTGS2
CBX3	transcription regulator		6.56E-03	↓CDK1
CCNT1	transcription regulator		3.75E-03	↓IL8
Cebp	complex		5.63E-03	↓IL8
CEBPA	transcription regulator		4.69E-05	↑EFNB2, ↓IL8, ↑PTGS2, ↓PTX3
CEBPB	transcription regulator		1.07E-03	↑EFNB2, ↓IL8, ↑PTGS2
CEBPD	transcription regulator		4.15E-02	↓IL8
CITED2	transcription regulator		1.40E-02	↑ANGPTL4
CREB1	transcription regulator		1.14E-04	↑FLT1, ↑NR4A1, ↑PTGS2
CREBBP	transcription regulator		8.12E-05	↓IL8, ↑NR4A1, ↑PTGS2
CTNNB1	transcription regulator		4.33E-02	↑COL4A1, ↑PTGS2
DEK	transcription regulator		7.49E-03	↓IL8
E2F1	transcription regulator		1.45E-02	↓CDK1, ↑FLT1
E2F2	transcription regulator		1.68E-02	↓CDK1
E2F3	transcription regulator		3.15E-02	↓CDK1
EHF	transcription regulator		6.56E-03	↓CDK1
ELF4	transcription regulator		2.05E-02	↓IL8
ELK4	transcription regulator		8.43E-03	↑NR4A1
EPAS1	transcription regulator		2.63E-03	↑ANGPTL4, ↑FLT1
ESR1	ligand-dependent nuclear receptor		1.29E-03	↓IL8, ↑PTGS2, ↓PTX3
estrogen receptor	group		1.27E-02	↑COL4A1, ↓IL8
EZH2	transcription regulator		9.80E-05	↓DKK1, ↓IL8, ↑PTGS2
FOS	transcription regulator		2.67E-02	↓IL8, ↑PTGS2
FOSL1	transcription regulator		1.49E-02	↓IL8
FOSL2	transcription regulator		1.40E-02	↓IL8
FOXC1	transcription regulator		9.36E-03	↑EFNB2
FOXC2	transcription regulator		2.87E-04	↑EFNB2, ↑PTGS2
FOXL2	transcription regulator		4.78E-02	↑PTGS2
FOXM1	transcription regulator		8.00E-04	↓CDK1, ↑FLT1
GFI1	transcription regulator		1.98E-03	↓IL8, ↓STAT1
HAND1	transcription regulator		5.43E-05	↑EFNB2, ↑FLT1
HBP1	transcription regulator		1.88E-03	↑PTGS2
Hdac	group	0.000	2.83E-06	↑ADAMTS1, ↓EDNRB, ↓IL8, ↑NR4A1
HDAC1	transcription regulator		8.12E-05	↓CDK1, ↓IL8, ↑PTGS2
HDAC2	transcription regulator		5.41E-04	↓DKK1, ↑PTGS2

HDAC3	transcription regulator		4.56E-04	↓IL8, ↑PTGS2
HHEX	transcription regulator		2.82E-03	↑FLT1
HIF1A	transcription regulator	1.135	2.17E-05	↑ANGPTL4, ↑FLT1, ↓IL8, ↑PTGS2
Hmga1	transcription regulator		1.12E-02	↑PTGS2
HMGB1	transcription regulator		2.05E-02	↓IL8
HOXA10	transcription regulator		8.39E-05	↓DKK1, ↑FLT1, ↑NR4A1
HOXB9	transcription regulator		6.56E-03	↓IL8
HTT	transcription regulator		5.08E-03	↑COL4A1, ↓EDNRB, ↑NR4A1
Ikb	group		1.88E-03	↑NR4A1
IRF1	transcription regulator		2.32E-03	↓IL8, ↑PTGS2
IRF2	transcription regulator		3.06E-02	↑PTGS2
IRF3	transcription regulator		3.97E-02	↓IL8
IRF8	transcription regulator		4.78E-02	↓STAT1
IRF9	transcription regulator		1.68E-02	↓STAT1
JUN	transcription regulator	-0.068	2.56E-09	↓CDK1, ↓DKK1, ↓IL8, ↑PTGS2, ↓PTX3, ↓STAT1
KAT2B	transcription regulator		9.36E-03	↑PTGS2
KAT5	transcription regulator		2.42E-02	↓IL8
KLF2	transcription regulator		1.86E-02	↑PTGS2
LMX1B	transcription regulator		8.43E-03	↓PTX3
MEF2A	transcription regulator		9.36E-03	↑NR4A1
Meg3	transcription regulator		3.75E-03	↑FLT1
MITF	transcription regulator		4.60E-02	↓IL8
MLL2	transcription regulator		2.14E-02	↓DKK1
MSX2	transcription regulator		2.14E-02	↓DKK1
Mucin	group		9.40E-04	↑PTGS2
MYBL2	transcription regulator		2.14E-02	↓CDK1
MYC	transcription regulator		2.96E-02	↓CDK1, ↑COL4A1
N-cor	group		3.24E-02	↑PTGS2
NCOA2	transcription regulator		2.23E-02	↑PTGS2
NCOA3	transcription regulator		3.24E-02	↑PTGS2
NCOR1	transcription regulator		1.68E-02	↑PTGS2
NCOR2	transcription regulator		1.89E-04	↓IL8, ↑PTGS2
NEUROG3	transcription regulator		1.22E-02	↑DLL4
Nfat (family)	group		9.36E-03	↑PTGS2
NFATC1	transcription regulator		1.59E-02	↑PTGS2
NFATC2	transcription regulator		3.37E-03	↑PTGS2, ↓STAT1
NFATC3	transcription regulator		1.77E-02	↑PTGS2
NFE2L2	transcription regulator		2.76E-02	↓IL8, ↑PTGS2
NFIX	transcription regulator		1.12E-02	↓IL8
NFkB (complex)	complex		1.05E-03	↓EDNRB, ↓IL8, ↑PTGS2
NFKB1	transcription regulator	0.871	6.30E-10	↑DLL4, ↓IL8, ↑NR4A1, ↑PTGS2, ↓PTX3, ↓STAT1
NFKB2	transcription regulator		2.78E-02	↑PTGS2
NFKBIA	transcription regulator		1.24E-03	↓IL8, ↑PTGS2
NFYB	transcription regulator		1.59E-02	↓CDK1
NKRF	transcription regulator		2.82E-03	↓IL8
NOTCH1	transcription regulator		2.21E-03	↑EFNB2, ↑FLT1
NOTCH3	transcription regulator		1.59E-02	↓PTX3
NOTCH4	transcription regulator		2.97E-05	↑DLL4, ↑EFNB2
NR1D1	ligand-dependent nuclear receptor		1.12E-02	↓STAT1
Nr1h	group		3.79E-04	↓IL8, ↑PTGS2
NR1H2	ligand-dependent nuclear receptor		4.33E-02	↑PTGS2
NR3C1	ligand-dependent nuclear receptor		1.03E-02	↑ANGPTL4, ↓IL8, ↑PTGS2
NR4A2	ligand-dependent nuclear receptor		3.06E-02	↓PTX3
NRIP1	transcription regulator		8.36E-04	↑PTGS2, ↓PTX3

Figure 18. Transcription factors predicted to serve as upstream regulators of the 16 endothelial cell proliferation related genes.

The 4 most important transcription factors and their relationship with the significantly changed endothelial cell proliferation genes are depicted in the Figure 19 below.

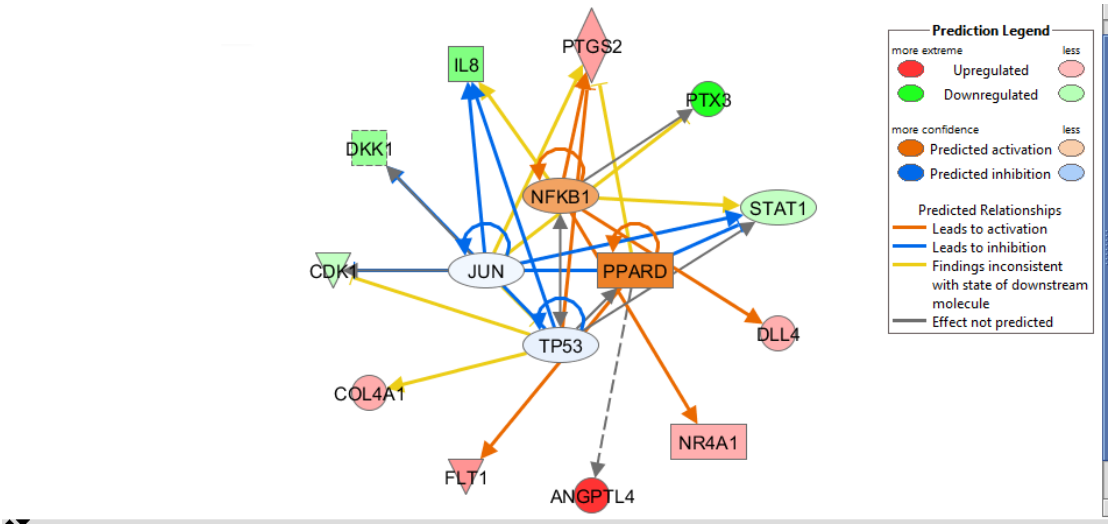


Figure 19. Transcription factors as upstream regulators with the highest impact on the gene's expression

Investigation of the migration and proliferation mechanisms of endothelial cells

HUVECs represent a valuable model for endothelial cell studies, As an example of the interest for the scientific community, more than 100,000 publications have cited ECs in general, and at least 10,000 HUVECs in particular. HUVECs model is useful for any research on general properties of human ECs, but other sources of ECs could be better models for studies on specific pathological areas, for example, atherosclerosis development or metastasis dissemination in particular microvascular areas. Nevertheless, HUVECs are the most simple and available human EC type, accurate for the preparation of large quantities of cells.

We established in our laboratory an easy method for isolation and culture of HUVECs to supply our future experiments. The confluent primary cultures of HUVECs can be obtained in 8 days, although you have to wait for an additional 48 h before starting a special treatment, such as the addition of a drug to study a particular effect. As for many other cell types, particularly for adherent cell lines, HUVECs can be used in subcultures, obtained with or without trypsin treatment, for amplification of the number of cells, but most often the primary culture is preferred because of the decrease in expression of many proteins at each passage due to a mechanism of accelerated senescence, with spontaneous apoptosis. Moreover, no additional growth factor is required for this protocol, as many growth factors and related products can modify protein synthesis and intracellular trafficking.

As shown at the table there seems to be a strain for increased viability of HUVEC cells after incubation with sera from lupus patients, either heat inactivated or not vs. the controls (most evident at 4 hours in a 20% concentration).

Regarding the Migration assay there seems to be a time-dependent increase in HUVEC's migrating ability, when incubated with SLE sera. These findings are consistent with the current literature and show that the inflammatory environment is playing a crucial role in the vascular remodeling.

However, HUVECs present with a number of limitations, compared to arterial cells. For example HUVECs are venous endothelial cells lacking the well-organized wall of an artery where important processes take place during atherosclerosis.

Effect of rHDL-apoE3 on endothelial cells proliferation

In addition to cell migration, cell proliferation is also a critical aspect of atherosclerosis. In order to investigate the potential beneficial effects of rHDL-apoE3 on endothelial cell function, and specifically on proliferation, we utilized a global gene expression approach. The gene expression analysis of HAECs exposed to rHDL-apoE3, revealed 198 altered genes post treatment (Prof. V. Zannis and Dr D. Sanoudou labs). Among them, proliferation was among the most significantly changed mechanisms (Figure 11). Further analysis indicated that 16 genes were directly involved in endothelial cells proliferation. Twelve of these genes were tested and validated in Prof. V. Zannis lab, by high throughput RT-qPCR, namely: ADAMTS1, ANGPTL4, COL4A1, DKK1, IL8, PTX3, EFNB2, FLT1, CDK1, PTGIR and PTGS2.These genes are associated with a number of different molecular pathways that are consequently predicted to be up- or down-regulated. Details on the role of each one of these genes and their respective pathways are presented in detail below.

The 16 genes have been categorized regarding the molecular pathways they are implicated in (P38 MAPK/ PAK/ ROCK kinase, PI3K/ Akt/ eNOS, ERK1/2, WNT and the Notch pathway).

The P38 MAPK/ PAK/ ROCK kinase pathway

The FLT1 (fms-related tyrosine kinase 1, vascular endothelial growth factor/vascular permeability factor receptor, Entrez Gene ID: 2321, also known as VEGFR1) showed upregulation with a fold change of 2.768. This gene encodes a member of the vascular endothelial growth factor receptor (VEGFR) family. VEGFR family members are receptor tyrosine kinases (RTKs) which contain an extracellular ligand-binding region with seven immunoglobulin (Ig)-like domains, a transmembrane segment, and a tyrosine kinase (TK) domain within the cytoplasmic domain. This protein binds to VEGFR-A, VEGFR-B and placental growth factor and plays an important role in angiogenesis and vasculogenesis (74). Isoform sFlt1 may

have an inhibitory role in angiogenesis. VEGF-A executes various physiological functions largely by binding to two tyrosine kinase receptor domains, Flt1 (Fms-like tyrosine kinase or VEGFR1), and Flk1 (Fetal liver kinase 1 or VEGFR2). Flk1 binds VEGF- A, albeit with lower affinity relative to Flt1. Flt1 undergoes weak tyrosine autophosphorylation in response to VEGF-A. Therefore, Flt1 is a ‘‘decoy’’ receptor, which functions as a negative regulator of VEGF-A signaling on vascular endothelial cells. Consistent with these reports, loss of Flt1 causes embryonic lethality because of vascular overgrowth and disorganization (75,76). PTK7 (protein tyrosine kinase 7) promotes vascular endothelial growth factor-A (VEGF-A)-induced endothelial cell migration in vitro and angiogenesis in vivo (77). PTK7 joins a receptor complex with Flt-1, but not with KDR/Flk-1 (VEGFR2) or with Flt-4 (VEGFR3). PTK7 serves an essential role in Flt-1 phosphorylation and activation of downstream signals of Akt, and focal adhesion kinase (FAK). Flt-1 regulates the VEGF-mediated cell migration of vascular endothelial cells via activation of RACK1/PI3K/Akt and Rac1 pathways (78). RACK1, a receptor for activated protein kinase C1), binds to Flt1 in vitro and plays a crucial regulatory role in promoting VEGF-induced PI3K/Akt-Rac1 activation. Flt-1 regulates VEGF-mediated cell migration in human umbilical vein endothelial cells (HUVECs) through modulating actin reorganization, which is essential for cell motility (79,80). Additionally it has been sown that VEGF/Flt-1 signaling plays a significant role in vascular inflammation and neointima formation by regulating OPN expression in adventitial fibroblasts and provides insight into Flt-1 as a potential therapeutic target for vascular diseases (81,82). The Flti1 upregulation could potentially indicate an activation of the VEGF through the Ras/MEK/ERK and the Akt/eNOS pathways, possibly ultimetly leading to increased HAEC’s proliferation and migration (83,84).

Another important gene, the EFNB2 (ephrin-B2, Entrez Gene ID: 1948) was upregulated with a fold change of 3.03. This gene encodes a member of the ephrin (EPH) family. The ephrins and EPH-related receptors comprise the largest subfamily of receptor protein-tyrosine kinases and have been implicated in mediating developmental events. Based on their structures and sequence relationships, ephrins are divided into the ephrin-A (EFNA) class, which are anchored to the membrane by a

glycosylphosphatidylinositol linkage, and the ephrin-B (EFNB) class, which are transmembrane proteins. This gene encodes an EFNB class ephrin which binds to the EPHB4 and EPHA3 receptors. Ephrin-B2 (EFNB2), a transmembrane protein which binds to the receptor tyrosine kinases, such as EPHA4, EPHB4 and EPHA3 is upregulated (f=3.03). It has been reported that Ephrin B2/EphB4 receptor signaling regulates endothelial cell migration through phosphoinositide (PI) 3-kinase-mediated activation of Rac1 (85,86). Also as shown later EFNB2 is implicated in the ERK1/2 pathway (87,88). Upregulation of EFNB2 could lead to HAECs proliferation through these mechanisms.

An important gene, PTX3 (pentraxin 3, long / Entrez Gene ID: 1948, also known as TNF-inducible gene 14 protein (TSG-14)) was downregulated in our experiments with a fold change of -7.86. Pentraxin 3 is a member of the pentraxin superfamily. This super family characterized by cyclic multimeric structure. PTX3 is rapidly produced and released by several cell types, including endothelial cells, mononuclear phagocytes, and fibroblasts, in response to primary inflammatory signals. Recent observations have shown that PTX3 binds Fibroblast growth factor-2 (FGF2) with high affinity and specificity (91,92). FGF2 is a major heparin-binding angiogenic growth factor that induces cell proliferation, chemotaxis and protease production in cultured endothelial cells. In vivo, FGF2 shows angiogenic activity in different experimental models and modulates neovascularization during wound healing, inflammation, atherosclerosis and tumor growth, thus representing a possible target for anti-angiogenic therapies (89,90). Preclinical studies demonstrate that FGF2 antagonists inhibit tumor growth and vascularization. FGF2 exerts its activity on endothelial cells by interacting with high affinity tyrosine-kinase FGF receptors (FGFRs). The biological activity of PTX3 is related to its ability to interact with different ligands via its N-terminal or C-terminal domain as a consequence of the modular structure of the protein. Accordingly, PTX3 inhibits FGF2-dependent endothelial cell proliferation in vitro and angiogenesis in vivo (93). Also, PTX3 inhibits FGF2-dependent smooth muscle cell activation and intimal thickening after arterial injury (94). The downregulation of PTX3 in our experiments would relieve the inhibitory effect of PTX3 on the FGFR-PI3K and ERK1/2 pathways, therefore

allowing their contribution to HAEC's proliferation and migration, with a potential beneficial effect in the setting of atherosclerosis.

At the level of transcription factors, STAT1 (signal transducer and activator of transcription 1, Entrez Gene ID: 6772) showed a downregulation in our experiments with a fold change of -2.08. The protein encoded by this gene is a member of the STAT protein family. In response to cytokines and growth factors, STAT family members are phosphorylated by the receptor associated kinases, and then form homo- or heterodimers that translocate to the cell nucleus where they act as transcription activators. This protein can be activated by various ligands including interferon-alpha, interferon-gamma, EGF, PDGF and IL6 and mediates the expression of a variety of genes, which is thought to be important for cell viability in response to different cell stimuli and pathogens. A reverse correlation has been reported between VEGF and STAT1-regulated genes expression (95). Also an anti-angiogenic role of STAT1 has been reported. The anti-angiogenic role of STAT1 activation that acts in mutant Tie2-R849W-expressing ECs impairs VEGF-A-mediated STAT3 signaling, bFGF production, and smooth muscle cell recruitment (96,97,98). A balancing activity of STAT1 and STAT3 may be important for the overall vascular homeostasis (99). Downregulation of STAT1 in our experiments could potentially contribute to the VEGFA-PI3K pathway activation leading in HAEC's proliferation and migration.

NTN4 (Netrin 4, Entrez Gene ID: 59277) was downregulated in our experiments with a fold change of -2.05. NTN4 belongs to a family of proteins related to laminins (105) initially identified as axonal guidance molecules, which have been shown to play roles in angiogenesis, lymphangiogenesis and tumor metastasis (103,104). It has been reported that Netrin-4 binds endothelial α6β1 integrin and that netrin-4-induced lymphatic endothelial cell (EC) migration, adhesion, focal adhesion contact and phosphorylation of Src Family kinase (a key element of both integrin signaling and cell migration), are suppressed by inhibition of α6 or β1 (100,101,106). Downregulation of NTN4 in our experiments has an impact to the Integrins-PI3K

pathway as well as to the PI3K/Akt/eNOS pathway showing its role in HAEC's proliferation and migration.

The above are summarized in the table 8 and the following figure 20 below.

Table 8. Genes implicated in the p38 MAPK pathway and their fold change

Molecular Pathway	Gene Symbol	Gene Name	Fold change
P38 MAPK/ PAK/ ROCK kinase	FLT1	fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)	2.768
	EFNB2	ephrin-B2	3.032
	PTX3	pentraxin 3, long	-7.861
	STAT1	signal transducer and activator of transcription 1	-2.084
	NTN4	Netrin 4	-2.052

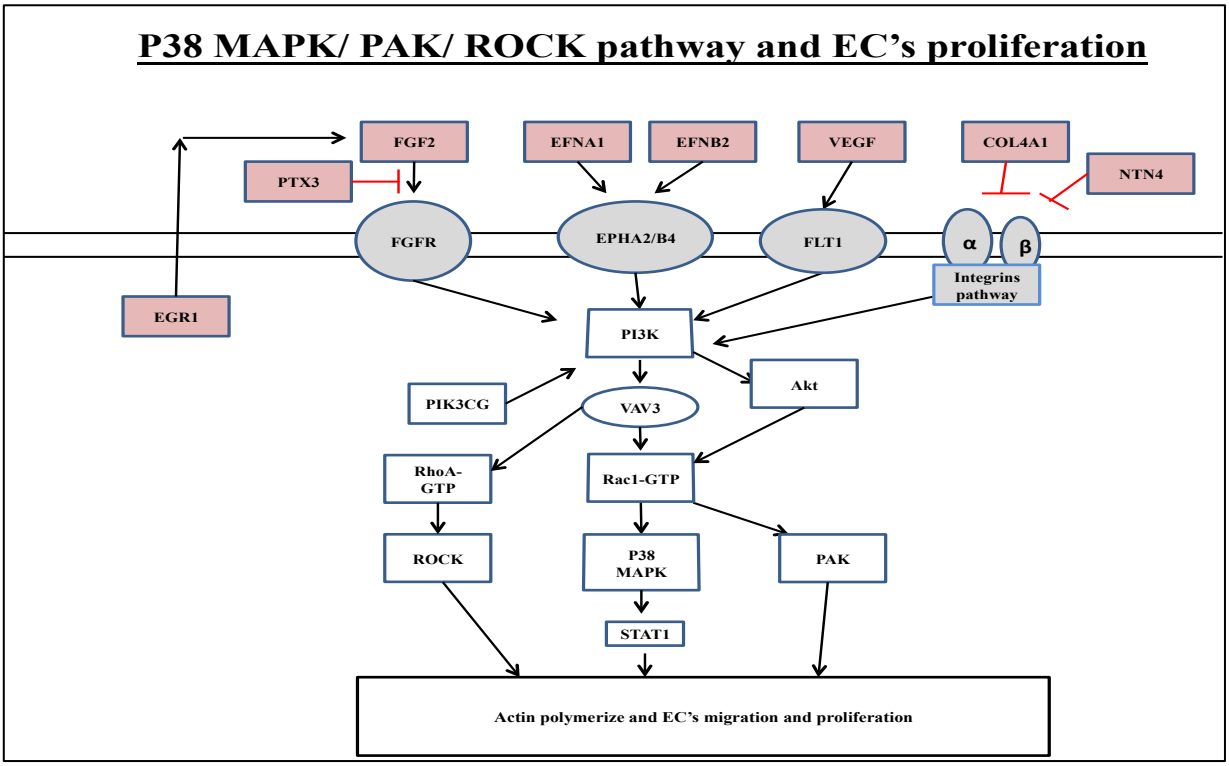


Figure 20. Altered gene's expression impact on the p38 MAPK/PAK/ROCK pathway

The PI3K/Akt/eNOS pathway

Another important gene that interacts with the VEGFR, ADAMTS1 (a disintegrin and metalloproteinase with thrombospondin motif, Entrez Gene ID: 9510) showed upregulation in our experiments with a fold change of 3.75. This gene encodes a member of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motif) protein family. Members of the family share several distinct protein modules, including a propeptide region, a metalloproteinase domain, a disintegrin-like domain, and a thrombospondin type 1 (TS) motif (107,108). ADAMTS1 has been demonstrated to inhibit angiogenesis in vivo and to suppress endothelial cell proliferation in vitro (110). Also ADAMTS1 is a potent inhibitor of angiogenesis: it inhibits endothelial cell proliferation in vitro, suppresses fibroblast growth factor-2–induced vascularization in the cornea pocket assay, and inhibits VEGF-induced angiogenesis in the chorioallantoic membrane. In addition, it has been shown that ADAMTS1 directly binds VEGF and blocks VEGFR2 phosphorylation, with consequent suppression of endothelial cell proliferation (112,113). In vivo the ADAMTS1 protein was also significantly increased in ECs during flow-induced expansion of the basilar artery in a recent study (109). In the same study ADAMTS1 was able to drastically decrease VEGFR2 phosphorylation by a mechanism that involved direct binding and sequestration of VEGF._Upregulation of ADAMTS1 in our experiments contributes to the blockage of the VEGFR pathway as shown in the Figure 21.

COL4A1**(**collagen, type IV, alpha 1, Entrez Gene ID: 1282) was upregulated in our experiments with a fold change of 2.139. This gene encodes the major type IV alpha collagen chain of basement membranes. This gene is organized in a head-to-head conformation with another type IV collagen gene so that each gene pair shares a common promoter. It has been shown that inhibits angiogenesis and tumor formation (114). The C-terminal half is found to possess the anti-angiogenic activity. Specifically inhibits endothelial cell proliferation, migration and tube formation (115,116). It also inhibits expression of hypoxia-inducible factor 1alpha and ERK1/2

and p38 MAPK activation. Ligand for alpha1/beta1 integrin. Upregulation of COL4A1 in our experiments shows its important role to the integrins pathway activation as shown in the Figure 22.

EDNRB (endothelin receptor type B, Entrez Gene ID: 1910 also known as ETB) was downregulated in our experiments with a fold change of -2.063. The protein encoded by this gene is a G protein-coupled receptor, which activates a phosphatidylinositol-calcium second messenger system. Its ligand, endothelin, consists of a family of three potent vasoactive peptides: ET1, ET2, and ET3. EDNRB is located primarily in the vascular endothelial cells where they play a role in vasoconstriction, vasodilation, bronchoconstriction and cell proliferation. It has been shown that EDNRB blockage restores NO-mediated endothelial function and inhibits atherosclerosis in apolipoprotein E-deficient mice (119). Also EDNRB has been shown to enhance oxidative stress in HUVEC's (118). Downregulation of EDNRB in our experiments has an important impact to the PI3K/Akt/eNOS pathway contributing to HAEC's proliferation and migration.

Table 9. Genes implicated in the PI3K/Akt pathway and their fold change

Molecular Pathway	Gene Symbol	Gene Name	Fold change
PI3K/ Akt/ eNOS	EFNB2	ephrin-B2	3.032
	ADAMTS1	a disintegrin and metalloproteinase with thrombospondin motif	3.757
	COL4A1	collagen, type IV, alpha	2.139
	EDNRB	endothelin receptor type B	-2,063
	NTN4	Netrin 4	-2.052

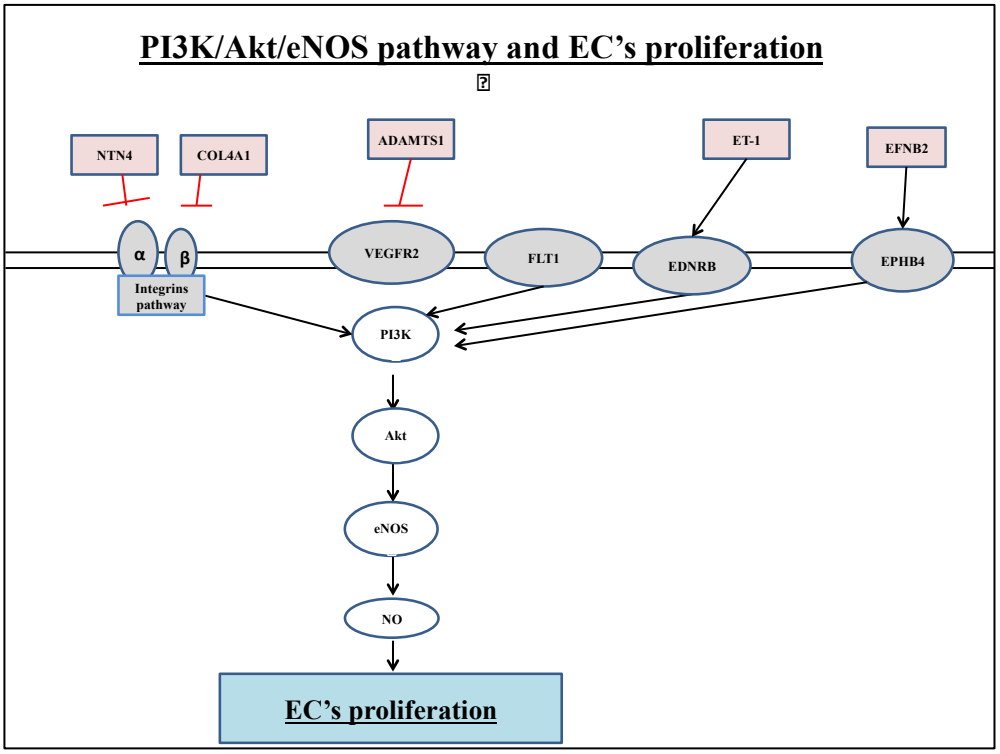


Figure 21. Altered gene's expression impact on the PI3K/Akt/eNOS pathway

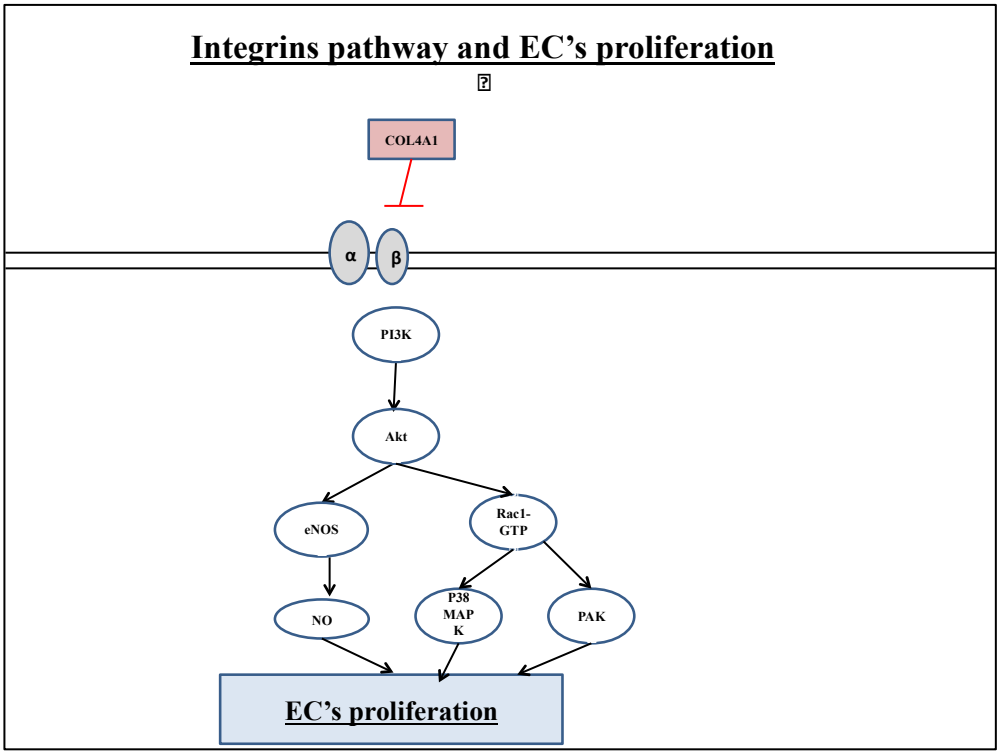


Figure 22. COL4A1 impact in the integrins molecular pathway

The ERK1/2 pathway

ANGPTL4 (angiopoietin-like 4, Entrez Gene ID: 51129), showed upregulation in our experiments with a fold change of 5.28. This gene is a member of the angiopoietin/angiopoietin-like gene family and encodes a glycosylated, secreted protein with a fibrinogen C-terminal domain (124). This gene is induced under hypoxic conditions in endothelial cells and is the target of peroxisome proliferation activators. The encoded protein is a serum hormone directly involved in regulating glucose homeostasis, lipid metabolism, and insulin sensitivity and also acts as an apoptosis survival factor for vascular endothelial cells (125). It is a protein with hypoxia-induced expression in endothelial cells. It may act as a regulator of angiogenesis and modulate tumorigenesis (125,126). It inhibits proliferation, migration, and tubule formation of endothelial cells and reduces vascular leakage and may exert a protective function on endothelial cells through an endocrine action. In response to hypoxia, the unprocessed form of the protein accumulates in the

subendothelial extracellular matrix (ECM). The matrix-associated and immobilized unprocessed form limits the formation of actin stress fibers and focal contacts in the adhering endothelial cells and inhibits their adhesion. It also decreases motility of endothelial cells and inhibits the sprouting and tube formation. In a recent study it has been shown that the carboxyl terminus of Angptl4 alone is sufficient to suppress angiogenesis, possibly acting through Tie and the integrins receptor inhibiting the Raf/MEK/ERK1/2 MAP kinase pathway in endothelial cells (128). ANGPTL4 is upregulated in our experiments, ultimetly leading to increased HAEC's proliferation and migration

Il-8(Interleukin-8, Entrez Gene ID: 3576) was downregulated in our experiments with a fold change of -4.410. The protein encoded by this gene is a member of the CXC chemokine family. This chemokine is one of the major mediators of the inflammatory response. This chemokine is secreted by several cell types. It functions as a chemoattractant, and is also a potent angiogenic factor. The interaction of Il-8 with the vascular endothelial growth factor (VEGF), and its receptor (CXCR1) has been shown to increase endothelial cell proliferation and capillary tube formation (130). PTGIR (Prostaglandin I2 receptor, Entrez Gene ID: 5739) was downregulated in our experiments with a fold change of -2.21. The protein encoded by this gene is a member of the G-protein coupled receptor family 1 and has been shown to be a receptor for prostacyclin. Prostacyclin, the major product of cyclooxygenase in macrovascular endothelium, elicits a potent vasodilation and inhibition of platelet aggregation through binding to this receptor. The important athero-protective role of prostacyclin is becoming increasingly evident as recent studies have revealed adverse cardiovascular effects in mice lacking the prostacyclin receptor, in patients taking selective COX-2 inhibitors, and in patients in the presence of a dysfunctional prostacyclin receptor genetic variant (135,136). Kasza et al. (137) reported a novel PKA-, Akt-1- and ERK1/2-dependent prostacyclin-induced prostacyclin release that appears to play an important role in propagation of the quiescent, differentiated phenotype through adjacent arterial smooth muscle cells in the vascular media (138). Both Il-8 and PTGIR are downregulation in our experiments. This may seem contradictory to the inflammatory environment that accompanies atherosclerosis but

we have to keep in mind that our experiments were not in vivo experiments and were based on an RNA level and that these genes are implicated in more than one molecular pathways.

NR4A1 (nuclear receptor subfamily 4, group A, member 1, Entrez Gene ID: 3164) showed upregulation in our experiments with a fold change of 2.047. This gene encodes a member of the steroid-thyroid hormone-retinoid receptor superfamily. The encoded protein acts as a nuclear transcription factor and the translocation of the protein from the nucleus to mitochondria induces apoptosis (136). Members of this subfamily have been characterized as early response genes regulating essential biological processes including inflammation and proliferation.It has been recently shown that Overexpression of NOR1 in human endothelial cells increased the expression of VCAM-1 and ICAM-1 while NOR1 deficiency altered adhesion molecule expression in response to inflammatory stimuli (134). NR4A1 is upregulated in our experiments, which would be anticipated to promote ECs proliferation.

PTGS2 (prostaglandin-endoperoxide synthase 2, Entrez Gene ID: 5743) was upregulated in our experiments with a fold change of 2.436. Prostaglandin-endoperoxide synthase (PTGS), also known as cyclooxygenase, is the key enzyme in prostaglandin biosynthesis, and acts both as a dioxygenase and as a peroxidase. There are two isozymes of PTGS: a constitutive PTGS1 and an inducible PTGS2, which differ in their regulation of expression and tissue distribution. This gene encodes the inducible isozyme. It is regulated by specific stimulatory events, suggesting that it is responsible for the prostanoid biosynthesis involved in inflammation and mitogenesis. It has been already shown that it is selectively upregulated by laminar shear stress (149). It mediates the formation of prostaglandins from arachidonate and may have a role as a major mediator of inflammation and/or a role for prostanoid signaling in activity-dependent plasticity. Upregulation of PTGS2 in our experiments depicts the HAEC’s trend for enhanced proliferation and migration.

CDK1(cyclin-dependent kinase 1, Entrez Gene ID: 983) was downregulated in our experiments with a fold change of -2.010. The protein encoded by this gene is a member of the Ser/Thr protein kinase family. This protein is a catalytic subunit of the highly conserved protein kinase complex known as M-phase promoting factor (MPF), which is essential for G1/S and G2/M phase transitions of eukaryotic cell cycle. Mitotic cyclins stably associate with this protein and function as regulatory subunits. The kinase activity of this protein is controlled by cyclin accumulation and destruction through the cell cycle. It has been shown that cell proliferation and migration are modulated by Cdk1 (144). The phosphorylation and dephosphorylation of this protein also play important regulatory roles in cell cycle control (143,144) ultimately altering endothelial cells proliferation.

Table 10. Genes implicated in the ERK1/2 pathway and their fold change

Molecular Pathway	Gene Symbol	Gene Name	Fold change
ERK1/2	EFNB2	ephrin-B2	3.032
	ADAMTS1	a disintegrin and metalloproteinase with thrombospondin motif	3.757
	ANGPTL4	angiopoietin-like 4	5.280
	COL4A1	collagen, type IV, alpha	2.139
	DLL4	delta-like 4	2.047
	IL 8	Interleucin 8	-4.410
	NR4A1	nuclear receptor subfamily 4, group A, member 1	2.047
	PTGIR	prostaglandin I2 (prostacyclin) receptor (IP)	-2.212
	PTGS2	prostaglandin-endoperoxide synthase 2	2.436
	NTN4	Netrin 4	-2.052
	PTX3	pentraxin 3, long	-7.861
	CDK1	cyclin-dependent kinase 1	-2.010

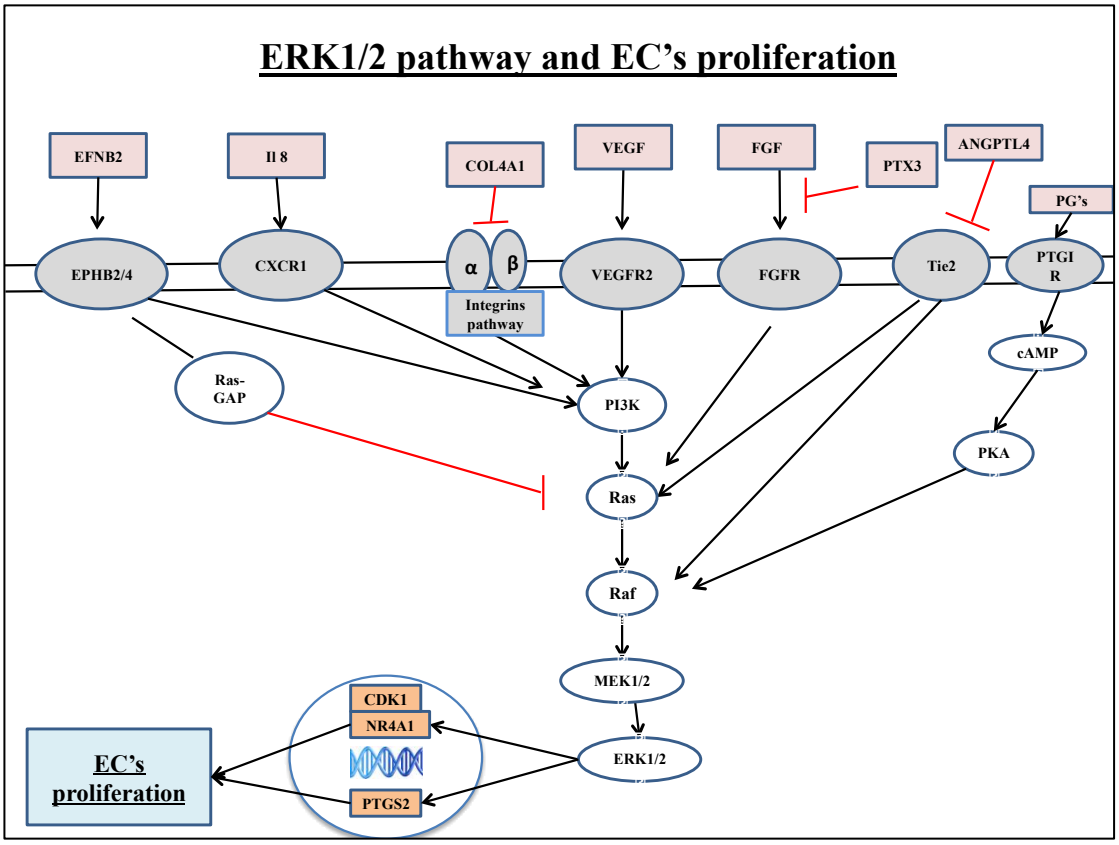


Figure 23. Altered gene's expression impact on the ERK1/2 molecular pathway

The WNT molecular pathway

DKK1 (dickkopf 1 homolog, Entrez Gene ID: 22943) was downregulated in our experiments with a fold change of -3.648. This gene encodes a protein that is a member of the dickkopf family. It is a secreted protein with two cysteine rich regions and is involved in embryonic development through its inhibition of the WNT signaling pathway. It has been found that carbonic anhydrase IX (CA9) modulates tumor-associated cell migration and invasion, and that dickkopf-1 (DKK-1) is a novel CA9-interacting protein (145). The N-terminal domain of CA9 is participated to interact with the Val60–Tyr168 site of DKK-1. Also DKK-1 inhibits endothelial cell angiogenesis of CA9 in tumorigenesis. Furthermore, induction of CA9-mediated mTOR phosphorylation and angiogenesis was significantly inhibited by over-expression of DKK-1.It has been reported that DKK1 increases endothelial progenitor cell angiogenic potential (147). The Wnt pathway is responsible for the activation of β-catenin and it's role for a cell-to-cell communication. DKK1 inhibition of the Wnt pathway, leads in β-catenin complex intracellular degradation (148,149). The DKK1 downregulation relieves the inhibitory effect on Wnt1, potentially leading to polymerization of the B-catenin complex and induction of proliferation.

Table 11. Gene implicated in the Wnt pathway and its fold change

Molecular Pathway	Gene Symbol	Gene Name	Fold change
WNT	DKK1	dickkopf 1 homolog	-3.648

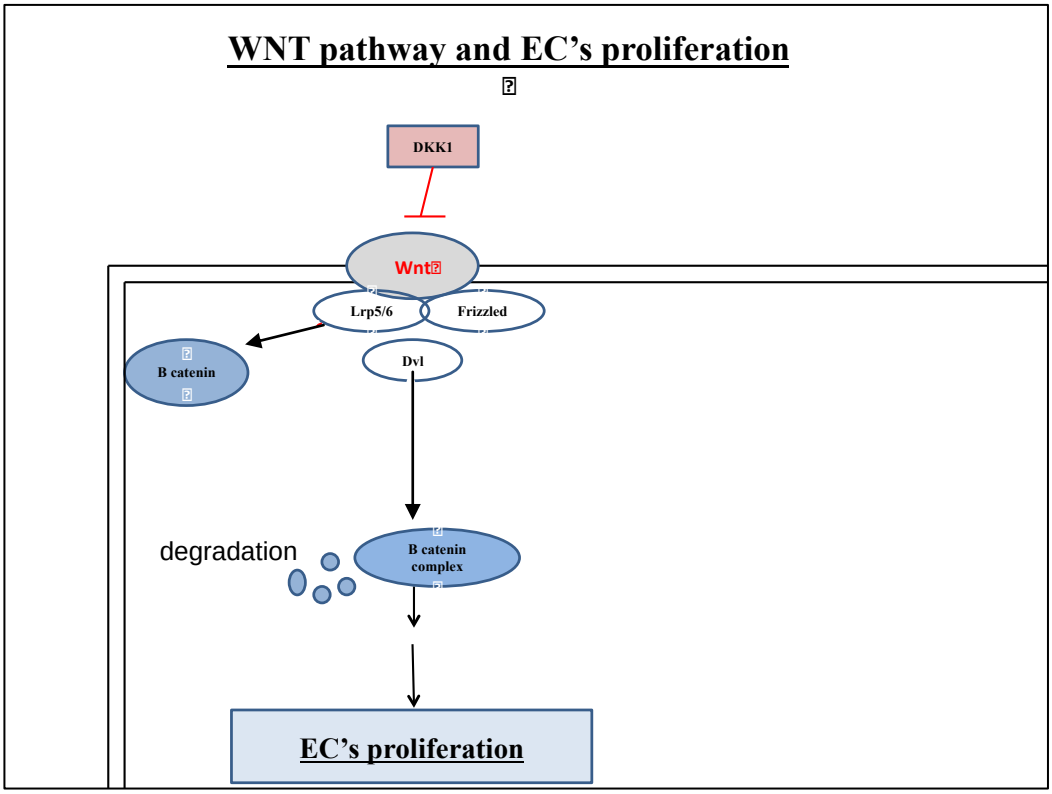


Figure 24. DKK1 gene's expression impact on the WNT molecular pathway

The Notch/DLLS molecular pathway

DLL4 (delta-like 4, Entrez Gene ID: 54567) was upregulated in our experiments with a fold change of 2.047. This gene is a homolog of the Drosophila delta gene. The delta gene family encodes Notch ligands that are characterized by a DSL domain, EGF repeats, and a transmembrane domain (150,151). Notch signaling induces lateral inhibition and gives rise to a nonuniform population of endothelial cells in the presence of VEGF-A stimulation (152). Below is a schematic illustration of the VEGF-A-Notch feedback loop controlling tip-stalk specification: stalk cells receive high Notch signal, which represses transcription of the VEGF receptors KDR, NRP1, and FlLT4, while stimulating expression of the decoy receptor(s)FLT1. Tip cells receive low Notch signal, allowing for high KDR, NRP1, and FLT4 expression, but low (s)FLT1 expression. Dll4 also acts in the ERK1/2 pathway as shown earlier. Dll4 seems to control the fate of a cell (stalk or tip) during angiogenesis (153,154). The DLL4 upregulation could potentially indicate the activation of the Notch

molecular pathway, possibly ultimetly leading to increased HAEC's proliferation and migration.

Table 12. Gene implicated in the Notch pathway and its fold change

Molecular Pathway	Gene Symbol	Gene Name	Fold change
Notch	DLL4	delta-like 4	2.047

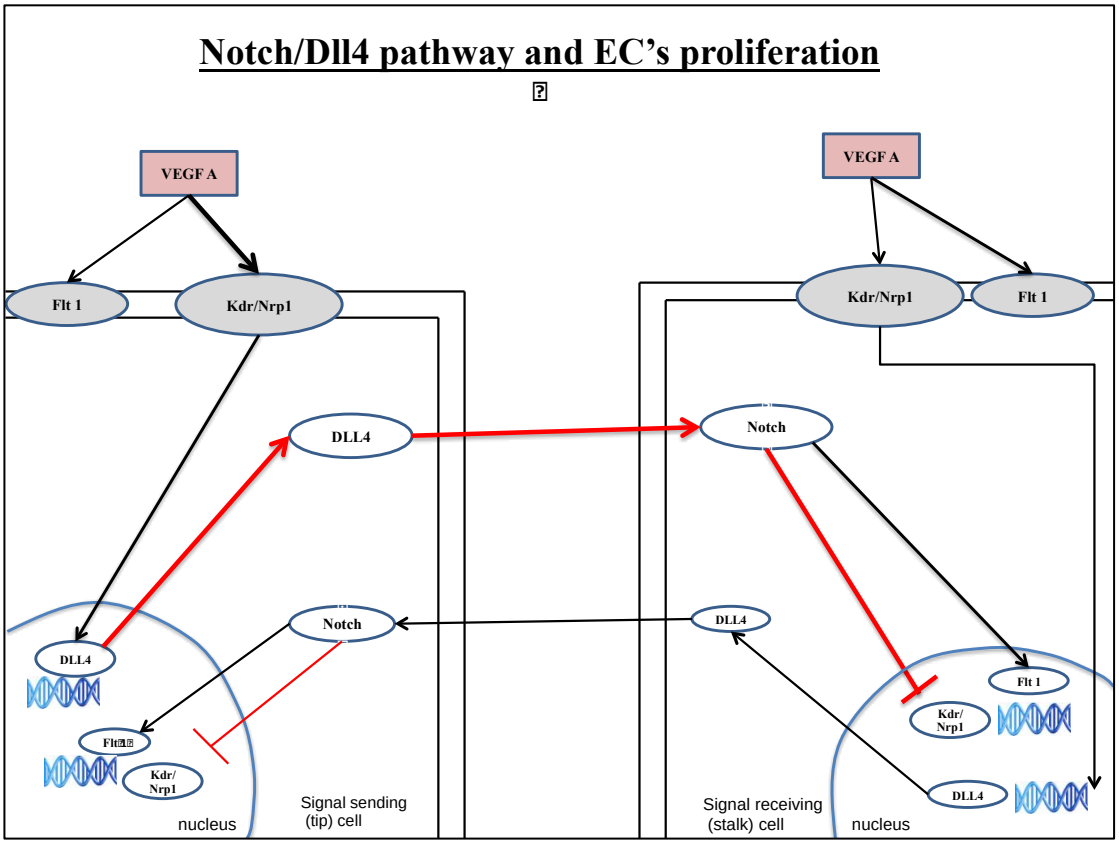


Figure 25. Altered DLL4 gene's expression impact on the Notch molecular pathway

HUVECs and HAECs, although a widely used and highly valuable in vitro approach for the study of endothelial cells, they inevitably present with a number of limitations, as the in vitro experiments lack all the metabolic capacities and the responses in physio- pathology and toxicity related to the different types of ECs found in an organism.

A series of new experiments is needed to elucidate the exact mechanisms that drive HAEC's proliferation and the role rHDL-apoE3 that will eventually lead in the discovery of promising therapeutic targets against atherosclerosis. Western Bolt analyses are needed to elucidate the molecular functions at the protein level. Cell functional assays for the migration and proliferation are needed for further validation along with miRNA studies focusing on the selected upregulated genes.

It is evident that various significantly changed genes, triggered by rHDL-apoE3 in HAECs are implicated in endothelial cell's proliferation through specific molecular mechanisms (P38 MAPK/ PAK/ ROCK kinase, PI3K/ Akt/ eNOS, ERK1/2, WNT and the Notch pathway). Considered as a whole, these molecular changes are predicted to induce proliferation in HAECs. In conclusion, the results from this study, suggest a promising role for rHDL-apoE3 in modulating endothelial cell proliferation, and ultimately in the atherosclerosis setting in vivo.

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