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The Effect of Wild Blueberry Bioactives on Endothelial Cell Migration and Angiogenesis: an in vitro Mechanistic, Genomic and Proteomic Approach

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THE EFFECT OF WILD BLUEBERRY BIOACTIVES ON ENDOTHELIAL CELL MIGRATION AND ANGIOGENESIS: AN *IN VITRO* MECHANISTIC, GENOMIC AND PROTEOMIC APPROACH

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An Abstract of the Dissertation Presented
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The goal of this study is to investigate the effects of wild blueberry fractions (anthocyanins and phenolic acids) on vascular function and physiology. More specifically the potential effects of the above fractions and their combination in physiological concentrations on endothelial cell migration, angiogenesis, gene expression and proteins synthesis of markers related to the above processes. The objectives are to study whether anthocyanins, phenolic acids and their combinations (ACNs:PAs) affect: a) cell proliferation, b) speed of endothelial cell migration, c) angiogenesis, d) gene expression of genes critical for cell migration and angiogenesis such as RAC1, RHOA, AKT1, eNOS and VEGF and finally e) synthesis of proteins that are critical for cell migration and angiogenesis such as RAC1, RHOA, AKT1, eNOS and VEGF.

This project utilized as an experimental model the human umbilical vein endothelial cells (HUV-EC-C [HUVEC] (ATCC® CRL-1730™)). Anthocyanins (ACNs) and phenolic acids (PAs) were extracted from the wild blueberry (WB) powder and a range of concentrations was

used (0.002 μg/mL, 8 μg/mL, 15 μg/mL, 60 μg/mL and 120 μg/mL). Cell cytotoxicity experiments were conducted to determine the appropriate concentrations for the following experiments; endothelial cell migration, angiogenesis, gene expression and Western Blot.

To determine possible cytotoxicity of the wild blueberry fractions, a broad range of concentrations were used (0.001 μg/mL - 1000 μg/mL for ACNs and 0.001 μg/mL – 500 μg/mL for the PAs) at different time points (30 min, 1 h, 3 h, 6 h, 12 h, 24 h, 48 h and 72 h). None of the concecrations were cytotoxic to the cells except the 1000 μg/mL of ACNs. Cell migration experiments documented an inhibitory effect on the speed of endothelial cells when ACNs at 60 μg/mL were used. However, PAs had the opposite effect on HUVECs speed. Exposure of the endothelial cells at 0.002 μg/mL, 60 μg/mL and 120 μg/mL significantly increased the speed of endothelial cell migration compared to control. Additionally, combination of both ACNs and PAs (ACNs:PAs) at 8μg/mL:8μg/mL and 60μg/mL:60μg/mL respectively, significantly increased endothelial cell migration speed compared to control. Angiogenesis experiments also revealed similar trends; ACNs inhibited the formation of the endothelial network while PAs and ACNs:PAs promoted a more stable endothelial network. Expression of genes related to the above cellular functions as well as protein analysis support the findings from cell migration and angiogenesis experiments.

In conclusion, ACNs, PAs and ACNs:PAs extracted from wild blueberries had a significant effect on endothelial cell function based on type of fraction and concentration not only at the mechanistic but also at the genomic and proteomic level. This may have clinical applications for degenerative diseases such as cancer, diabetic wounds and cardiovascular disease.

DEDICATION

To my mother Eleni, my grandparents Anastasios and Anna

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‘’τὸ ἔλεός σου καταδιώξει με πάσας τὰς ἡμέρας τῆς ζωῆς μου’’ (Ψαλμος κβ`)

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LIST OF ABBREVIATIONS

ACNs, Anthocyanins

ACNs:PAs, Combination of anthocyanins and phenolic acids

ACSs, Acute conditions of coronary syndromes

ASCVD, Atherosclerotic cardiovascular disease

BE, Bilberry anthocyanin-rich extract

bFGF, Basic fibroblast growth factor

C3G, Cyanidin-3-O-b-glucoside

CAD, Coronary artery disease

CaM, Calmodulin

CDC42, Cell division control protein 4

CHD, Coronary heart disease

CVAD, Cardiovascular atherosclerotic disease

CVCs, Calcified vascular cells

CVD, Cardiovascular disease

Cy, Cyanidin

Cy3glc, Cyanidin

DNA-PK, DNA-dependent protein kinase

Dp, Delphinidin

Dp3glc, Delphinidin

EC, Endothelial cell

ECGS, Endothelial cell growth supplement

ECM, Extracellular matrixes

eNOS, Endothelial nitric oxide synthase

EPCs, Endothelial progenitor cells

F-12K medium, Kaighn's modification of Ham's F-12 medium

FAD, Flavin adenine dinucleotide

FAK, Focal adhesion kinase

FBS, Fetal bovine serum

Flk-1, Fatal liver kinase-1 (Flk-1)

FMN, Flavin mononucleotide

HaCaT, Immortalized human keratinocyte cell line

HASMCs, Aortic smooth muscle cells

HIF, Hypoxia-inducible factor

HRMECs Human retinal microvascular endothelial cells

HUVECs Human umbilical vein endothelial cells

ICAM1, Intercellular adhesion molecule 1

IP, Immunoprecipitation

IRS, Insulin receptor substrate

LDL, Low density lipoprotein

LSD, Least significant difference

MCP1, Monocyte chemoattractant protein 1

MI, Myocardial infraction

mTOR, Mammalian target of rapamycin

Mv3glc, Malvidin

NADPH, Nicotinamide adenine dinucleotide phosphate

NO, Nitric oxide

P3G, Pelargonidin-3-glucoside

PAs, Phenolic acids

PASMCs, Pulmonary aortic smooth muscle cells

PCs, Pericytes (PCs)

PDGF, Platelet derived growth factor

PDK1, Dependent protein kinase 1

Pg3glc, Pelargonidin

PGE_2, Prostaglandin E_2

PI3K, Phosphoinositide-3-kinase

PIP_3, Phosphatidylinositol (3,4,5)-tris- phosphate

PMSF, Phenylmethylsulfonyl fluoride

Pn3glc, Peonidin

PRE, Purple rice extract

Pt, Petunidin

Pt3glc, Petunidin

RH, Relative humidity

SMCs, Smooth muscle cells

VCAM1, Vascular cell adhesion protein 1

VEGF, Vascular endothelial growth factor

VEGFR, Vascular endothelial growth factor receptor

VSMCs, Vascular smooth muscle cells

WB, Wild blueberries

CHAPTER 1

INTRODUCTION

Cardiovascular atherosclerotic disease (CVAD) is a major public health problem and is responsible for most deaths in developed societies, in developing countries and worldwide (1, 2). Atherosclerosis is a chronic inflammatory disease of the large arteries, and can cause heart disease and stroke (3). Macrophage accumulation in the intima is the hallmark of the atherosclerosis. High lipid concentration and storage in the intima increases the inflammatory response and subsequently leads to pathological conditions such as hemorrhage, thrombosis and blood vessel injury (4, 5). Atherosclerosis is a progressive disease involving an inflammatory disorder profile that leads to cardiovascular disease (CVD) and stroke (2, 4).

Early stages of atherosclerosis involve hypoxia, which leads to angiogenesis mediated by endothelial cells forming new vessels (6). It has been documented by evaluating early human lesions that angiogenesis is a phenomenon that occurs in early atherogenesis (6). The process of angiogenesis involves vascular cells and their extracellular matrix. In the presence of angiogenic factors such as vascular endothelial growth factor (VEGF), endothelial cells are recruited and under specific interactions are induced to proliferate, migrate and differentiate to form new capillaries from pre-existing vessels (angiogenesis) (7).

One fundamental process common to cell morphogenesis, immune function, physiology, development, regeneration and disease is cell migration (8, 9). The wound healing process involves hemostasis, inflammation, cell differentiation, proliferation and cell migration which promotes angiogenesis and finally tissue remodeling (10). Since wound healing is a cellular

response to injury, many cell types, including endothelial cells, are stimulated and coordinated in this complex biological phenomenon to perform a balanced wound healing process (10, 11). Several factors can affect wound healing and can be classified as local or systemic (10). Some of the systemic factors are: age, gender, stress, alcohol, smoking, malnutrition, obesity and diseases (10). Chronic diseases such as diabetes mellitus lead to impaired wound healing which is a result of unbalanced angiogenesis (11-13). Unbalanced wound healing is a typical problem in patients with diabetes mellitus (14). Moreover, predisposition of these patients to atherosclerosis and other implications such as increased chance of infection can make impaired wound healing a very serious threat for these patients (14). Reduced blood flow to the extremities, decrease in endothelial cell proliferation and angiogenesis result in improper response of diabetic patients to injury (11).

Blood vessels are responsible for carrying oxygen to all organs of the human body (15). The growth of blood vessels from pre-existing ones is known as the process of angiogenesis (15, 16). In cardiovascular biology there are three different types of blood vessel formation (angiogenesis, arteriogenesis and vasculogenesis) (17). In adults angiogenesis is initiated mainly from hypoxia-inducible factor (HIF)-1α expression (17). Arteriogenesis is the *de novo* formation of blood vessels (18, 19). Finally, vasculogenesis is the *in situ* formation of blood vessels from vascular progenitor cells and circulating endothelial progenitor cells (EPCs) (20, 21). All three processes are subcategories of neovascularization that can occur in adults (17). Control of angiogenesis can act as a therapeutic tool. Nearly four decades ago it was hypothesized that inhibition of angiogenesis would be a way to treat human cancer effectively (22). Clinical trials have documented promising results that angiogenesis can be an important target for cancer and other diseases (22). Therapeutic angiogenesis can play a significant role in diseases such as

ischemic disorders. However, a better understanding of molecular pathways involved in angiogenesis is critical (22).

Three major mechanisms, chemotaxis, haptotaxis and mechanotaxis (23), are involved in endothelial cell migration during angiogenesis. Haptotaxis is associated with response of endothelial cells to integrins binding to ECM components (23).

Small GTPases of the large RHO family are involved in the endothelial cell migration process in response to activation by VEGFR-2. Moreover, the production of nitric oxide (NO) from eNOS which is activated by AKT/PKB shows a significant role in the endothelial cell migration (23).

Early studies prove the importance of small G proteins in cell motility (24-27). RHOA, RAC and CDC42 are known members of the Rho family and they play a key role in the actin cytoskeleton (24). During cell migration RAC is involved in the formation of lamellipodia at the leading edge of migrating cells. RHOA is a regulator of actin stress fibers and required during focal adhesions. CDC42 is not directly involved in cell migration/movement but is essential for cell polarity that will control the direction of the cell movement (24).

During cell proliferation and other important cellular functions such as cell migration, cell growth and metabolism AKT kinase plays a key role (28, 29). It is a member of the AGC kinases, and there are three known isoforms of AKT that are critical in the cardiovascular system (29). Upstream regulators of AKT in the cardiovascular system are platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), epithelial growth factor (EGF) and basic fibroblast growth factor (bFGF) (29-31). During angiogenesis, AKT regulates the secretion of VEGF and during endothelial cell migration which is a critical function of angiogenesis AKT

regulates its function through the AKT-PI3K pathway (32-34). Moreover, AKT can directly phosphorylate eNOS at S1197. Activated eNOS then plays a major role in angiogenesis and vascular permeability (35).

The lowbush blueberry (*Vaccinium angustifolium)* has been ranked as one of the richest in flavonoids among other fruits and berries (36). Even though wild blueberries are low in antioxidant vitamins and minerals they are rich sources of bioactive compounds (polyphenols) such as anthocyanins (ACNs) and phenolic acids (PAs) as well as flavonoids that are generally found in fruits and vegetables (37). The antioxidant activity of wild blueberries is a result of anthocyanins, procyanidins, chlorogenic acid, and other flavonoid compounds (38). Anthocyanins from wild blueberries are primarily composed of delphinidin, malvidin, petunidin, cyanidin and peonidin (39, 40). The growing practices, location and harvesting methods are known to contribute to the phenolic content of wild blueberries.

Numerous *in vivo* and *in vitro* studies have documented the beneficial effects of wild blueberry (*Vaccinium angustifolium*) consumption on inflammation and cardiovascular disease (CVD) as well as many other chronic diseases (36-39, 41-44). Only a few studies have documented the effect(s) of single ACNs, PAs (nasunin, delphinidin, pelargonidin, ellagic acid and epigallocatechin-3 gallate) and fractions from different berries on cell migration, angiogenesis and wound healing (45-48).

Single ACNs such as nasunin ((Delph inidin-3-(p-coumaroylrutinoside)-5-glucoside) were found to inhibit HUVEC proliferation rate at 200 μM, 100 μM and 50 μM (16) and delphinidin (Dp) to increase vascular endothelial growth factor (VEGF)-induced tube formation of HUVECs (46). Diet-delivered polyphenols (apigenin, delphinidin, ellagic acid and

epigallocatechin-3 gallate) inhibited endothelial cell migration, proliferation and tubulogenesis through the JAK/STAT3 and MAPK signaling pathways (45). The inhibitory effect of ACNs (pelargonidin and its glucoside-conjugated form, pelargonidin-3-glucoside (P3G)) on cell proliferation and smooth muscle cell migration was documented by analyzing its effect on with focal adhesion kinase (FAK), a significant molecular target of ACNs (48). ACNs extracted from black soybean seeds coats were documented to stimulate wound healing in chronic wounds by reducing the inflammatory state of the wound (49).

Evidence documents the importance of small G proteins in cell motility (24-27). *In vitro* (human microvascular endothelial cells and HUVECs) and *in vivo* (C57BL/6 mice) studies have documented the effects of berry extracts and PAs (gallic acid from red raspberries) on cell migration and angiogenesis promoting expression of RHO GTPases, receptors (VEGFR2/NRP1) and other molecules (AKT, ERK 1/2, VEGFA and p38) involved in molecular pathways controlling those functions (47, 50, 51).

Individual anthocyanins as a class of polyphenols were evaluated *in vitro* using human umbilical vein endothelial cells after cells were treated with vascular endothelial growth factor (VEGF) (52). Inhibitory effect in the proliferation rate of endothelial cells was documented when cells were treated with anthocyanins at 1 μg/ml and 2 μg/ml (52). Moreover, in vitro tube formation assay experiment documented that anthocyanins inhibited the endothelial tube length in a dose depended manner (52). Finally, cell migration experiments revealed similar results about the inhibitory effect of anthocyanins (52).

Since there is a paucity of research on the effects of anthocyanin and phenolic acids on endothelial cell migration, angiogenesis and mechanisms thereof, the **goal** of this study is to

investigate the effect of ACN and PA fractions and their combination (ACNs:PAs) from wild blueberry powder *(Vaccinum angustifolium)* to determine whether and how they operate to alter cell migration and angiogenesis.

The **objectives** are to study whether ACNs, PAs and their combinations (ACNs:PAs) affect:

1. Proliferation rate of the endothelial cells by assessing cell cytotoxicity.

2. Speed of endothelial cell migration after acute exposure to different concentrations of the above fractions.

3. Angiogenesis (*in vitro*) endothelial network assay; such as: a) number of nodes, b) number of meshes c) area of meshes d) master segments length and e) number of master junctions.

4. Expression of genes critical for migration process such as RAC1 and RHOA and their synthesis.

5. Expression of genes critical during formation of endothelial network such as AKT, eNOS and VEGF.

6. Synthesis of proteins critical for the proper function of cell migration such as RAC1 and RHOA after acute exposure and

7. Synthesis of proteins critical for the proper development of angiogenesis such as AKT, eNOS and VEGF.

CHAPTER 2

LITERATURE REVIEW

2.1 Cell migration.

In nature there are different mechanisms of cell movement. Some of these mechanisms are haptotaxis, mechanotaxis, chemotaxis, durotaxis, electrotaxis and plithotaxis (53-55). In greater detail, haptotaxis (from the Greek άπτω or άπτομαι (hapto, ‘’touch’’) and τάξις (taxis ‘’order’’)) is the direct migration of a cell type e.g. endothelial cell (EC) from a surface of lower adhesion level towards a higher (56, 57). Mechanotaxis (from the Greek μηχανικό (mechano, ‘’mechanical’’) and τάξις (taxis ‘’order’’)) refers to cell migration that is directed by a mechanical force e.g. endothelial cell migration during angiogenesis (56, 57). Chemotaxis (from the Greek χημεικό (chemo, ‘’chemical’’) and τάξις (taxis ‘’order’’)), which is the most well studied in biology, is the ability of cells to reply with a directional movement to chemoattractant gradients (53, 58). Durotaxis (from the Greek συμπαγές (duro, ‘’stiff’’) and τάξις (taxis ‘’order’’)), which is similar to chemotaxis (only difference, chemotaxis usually refers to fluid chemoattractant gradients), is the process in which cell migrate towards an area of greater matrix stiffness (59-61). Electrotaxis (from the Greek ηλεκτρικό (electro, ‘’electrical’’) and τάξις (taxis ‘’order’’)) is defined as a directional mechanism of cell migration due to a direct-current electrical field (55, 60). Electrotaxis has been studied in epithelial cells for would healing closure experiments (62). Lastly, plithotaxis (from the Greek πλήθος (plitho, ‘’crowd’’) and τάξις (taxis ‘’order’’)), is described as the migration of cells that form a monolayer and thus have intact cell-cell junctions and tend to migrate towards the side that normal stress is greatest and sheer stress least (54). Plithotaxis refers only to a group of cells having tight cell-cell junctions forming a

monolayer such as epithelial and endothelial cells and not individual cells (53, 54). Moreover, this mechanism involves forces acting locally at the cell-cell junctions (53). In conclusion, there is a plethora of mechanisms of arrangements (‘’taxis’’) depending on the external stimuli, such as thermotaxis, phototaxis, geotaxis, magnetotaxis, thigmotaxis etc. Chemotaxis, haptotaxis and mechanotaxis are three major mechanisms of migration (23). During angiogenesis endothelial cells undergoing these three types of migration (23).

2.2 Angiogenesis.

Neovascularization is the mechanism that the human body triggers when it is in a stage of ischemia (63). Angiogenesis is one of the three processes that occur in neovascularization, the other two are arteriogenesis and vasculogenesis. Angiogenesis is the formation of new capillary blood vessels from existing ones (63-66). Angiogenesis is a normal biological process that starts in early stages of development (16). The first blood vessels ascend from endothelial precursors that share a common link with hematopoietic progenitors (16). These progenitors in early stages of development are forming a primitive vascular network of very small capillaries; the process is known as vasculogenesis (16). Even at this early stage capillaries are already programmed at the cellular level to an arterial or venous fate. During development in the angiogenic phase pericytes (PCs) and smooth muscle cells (SMCs) provide strength in endothelial cells allowing vessel perfusion regulation; a process known as arteriogenesis (16). Moreover, the lymphatic system is developed via transdifferentiation from veins (16). Normally angiogenesis is active after birth but during adulthood angiogenesis is occurring during the cycling ovary and pregnancy (16). Despite all that, ECs are capable of retaining their proliferative ability and as such angiogenesis can occur during wound healing and repair (16, 67).

2.3 The role of endothelial cells in angiogenesis.

Atherosclerosis is a chronic inflammatory disease (68). The vascular endothelial cells (ECs) form a monolayer that forms the tunica intima (69). In late stages of atherosclerosis when the endothelium is injured, the wound healing process begins with EC migration at the edge of the wound followed by EC proliferation (70). That sequence of events is crucial to the repair of the endothelium. In *in vivo* condition, the wound healing process takes a long time and requires the ECs to form tight junctions in a monolayer and adapt to the new laminar flow conditions (70). However, ECs do not migrate with the same speed from both edges of the wound. The ECs located upstream of the laminar flow migrate faster than the ones located downstream of the flow (70). Laminar flow has been documented to enhance the wound healing process. However, disrupted blood flow affects the migration speed of the cells towards the center of the wound. Researchers until recently (2014) have not been able to answer the question of how the ECs are able to coordinate the signaling events present in situations of different blood flows and then adjust their motility, migration and junction permeability (70). It is documented that vascular endothelial growth factor (VEGF) plays a key role in vasculogenesis (71). VEGF-A is the most well studied member of the VEGF family (71). Main receptors of the VEGF-A are the VEGFR1 and VEGFR2 and neuropilin-1 and 2 as coreceptors. Moreover, data from *in vivo* and *in vitro* studies suggests that VEGF-A regulates the survival and propagation of the endothelial cells rather than their differentiation (71). VEGF-A is responsible for stimulating endothelial cell proliferation through fetal liver kinase-1 (Flk-1). Furthermore, many studies have included tube formation assays to study angiogenesis. Tube formation of endothelial cells can be described as the formation of capillary-like structures. The first study on tube formation from primary human endothelial cells was described in 1988 (72). Recently, studies have documented that endothelial

cells are induced to form capillary tube-like structures when cultured on a matrix of basement membrane extract (Matrigel). The tubes from endothelial cells can form quickly (2 h - 24 h) depending on the cell line (73, 74).

2.4 The role of vascular smooth muscles cells in atherosclerosis.

Vascular smooth muscle cells (VSMCs) are located in the intima layer in humans and tunica media layer (69, 75). Smooth muscle cells are involved in the control of vascular tone and diameter through the mechanism of contraction (75). In the early stages of atherosclerosis, endothelial cells once activated will increase the production of nitric oxide (NO) and prostaglandin E_2 (PGE_2), Afterwards, the initiation of the atherosclerotic response to VSMCs will occur including vasodilation and excretion of anti-inflammatory factors (75). From that point on a cascade of signaling pathways is initiated. One of the final steps in the signaling cascade is the activation of RHOA/ROCK1 that will stimulate the VSMC migration (75). In conclusion, it is known that physiologically the VSMCs are switching phenotypically to trigger their proliferation and motility in case of vessel damage to induce repair. However, in this chronic inflammatory disease, atherosclerosis, VSMCs will change their phenotype into calcified vascular cells (CVCs) because of oxidative stress and pro-inflammatory stage conditions. Researchers have suggested that blocking the pro-inflammatory phenotype change in VSMCs might be of clinical importance (75).

2.5 Small GTPases RHOA and RAC1.

Small GTPases (monomeric, low-molecular-weight) are a super-family of hydrolase enzymes accounting for more than 100 members which can be categorized in five groups or subfamilies: Ras, RHO/RAC/CDC42, ARF/SAR1, RAB, and RAN (26). Ras GTPase was the first one linked to cardiac remodeling and the RHO/RAC1/CDC42 subfamily followed (26). The

RHO GTPase family has been studied in myocardial cells and there are 20 Rho gene products. RHOA and RAC1 are the most studied in myocardial cell signaling. The RHOA and RAC1 have a key role in the cytoskeletal organization such as formation of focal adhesions, actin stress fibers, lamellipodia, migration, smooth muscle contraction, neurite retraction, and cytokinesis (Figure 1) (26). There are three homologous proteins sharing 88% of the same amino acids in humans, RHOA, RHOB and RHOC (Figure 2) (26, 76). In a recent study, scientists have documented that NO can up-regulate RHOA, RAC1 and CDC42 expression, not only at the mRNA level, but also at the active and total protein levels (77). The activation of the small GTPases of the Rho family is involved in endothelial cell migration in response to activation of VEGFR-2 (23). Findings show that RHOA contributes to phosphorylation of VEGFR-2 which is very important in the endothelial cell migration process (23).

Figure 1: Two-dimensional HUVECs *in vitro* cell migration.

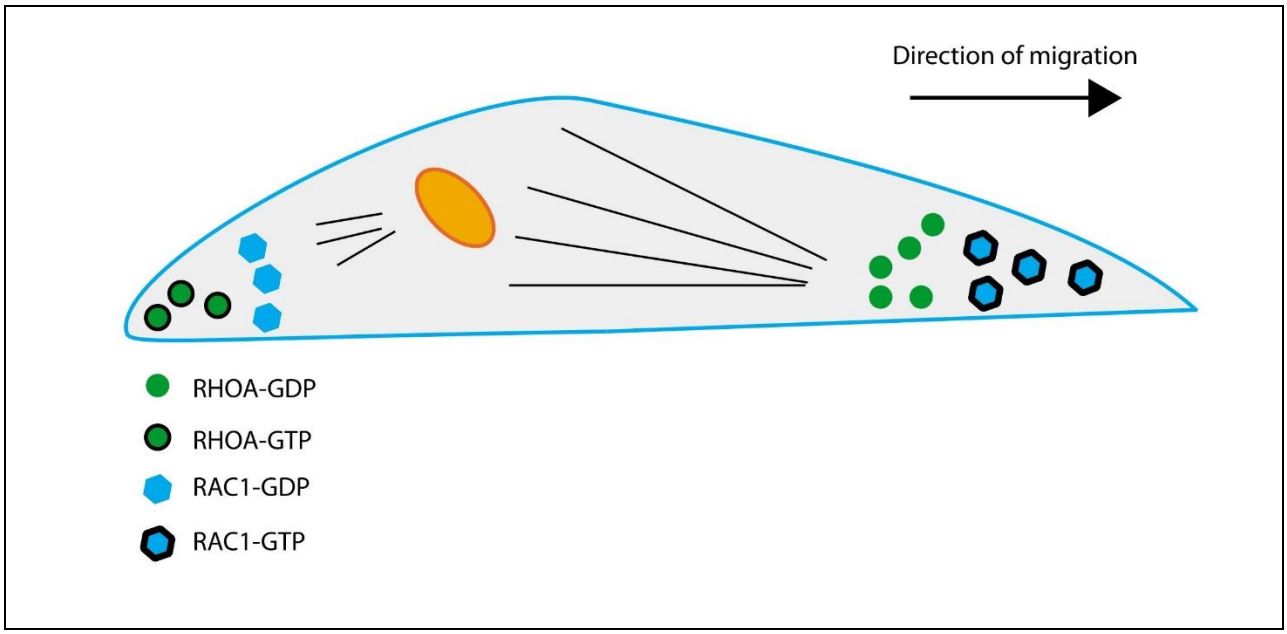
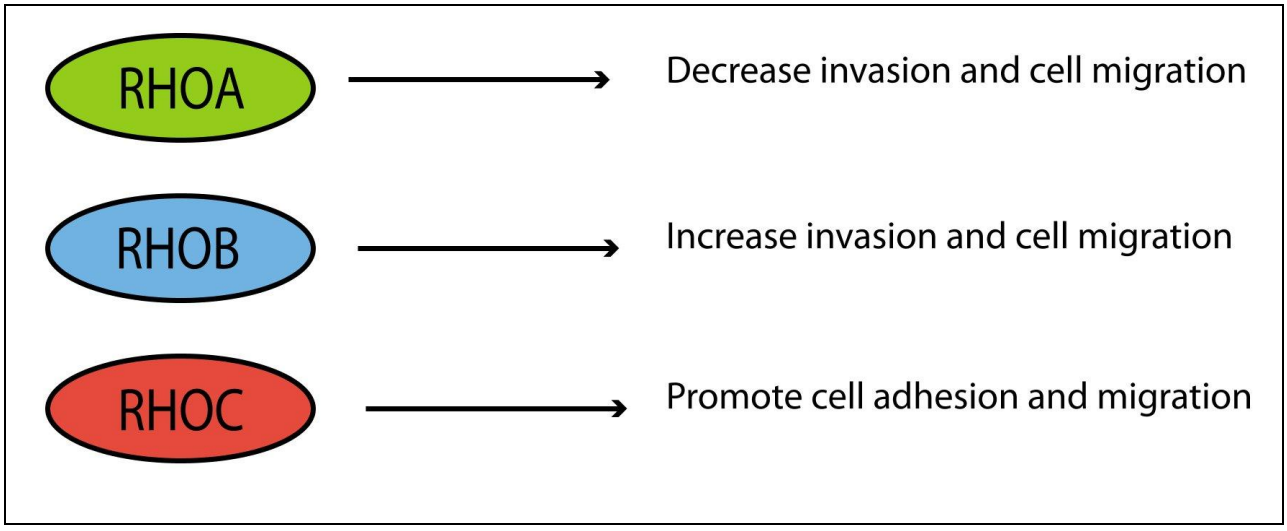


Figure 2: Functions of RHO family.



2.6 AKT.

In angiogenesis AKT has a key role by stimulating the secretion of the vascular endothelial growth factor (VEGF) (29). During endothelial cell migration which precedes angiogenesis; VEGF is induced through AKT pathway (29, 33, 78).

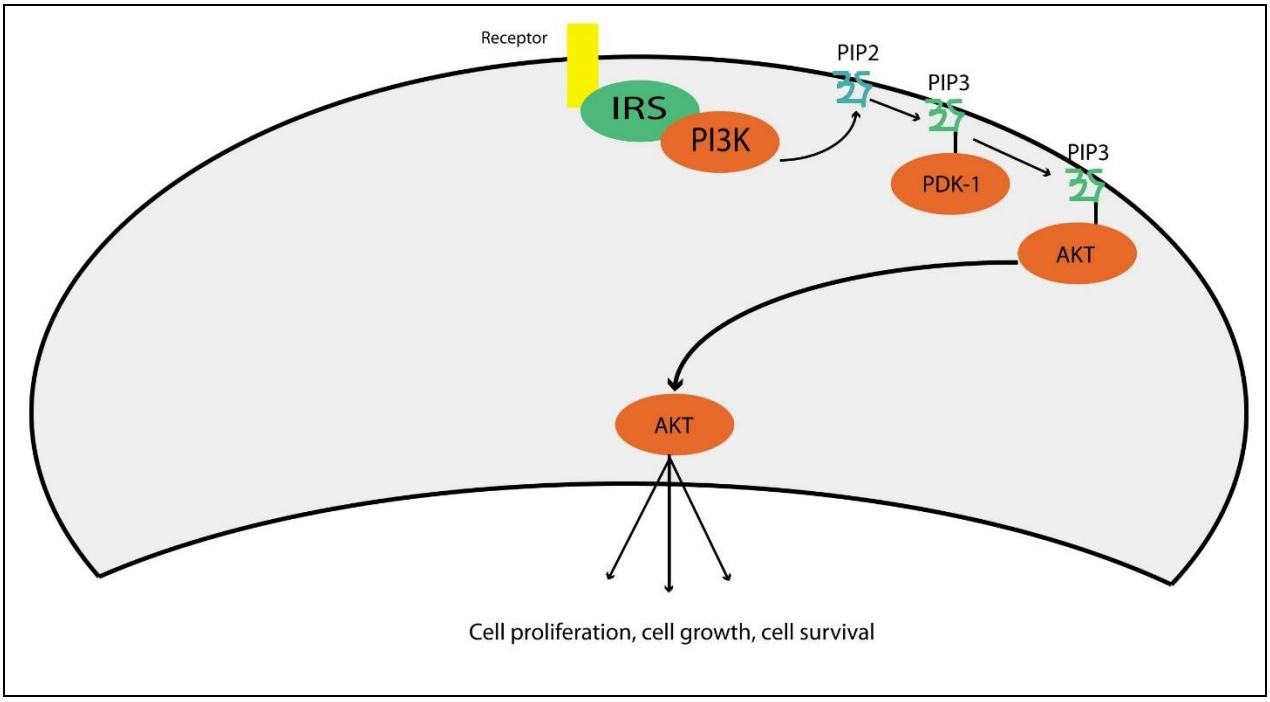
In general, protein kinases are enzymes that play a key role in different functions of the cell by attaching a phosphate group on a Ser, Thr or Tyr of a substrate (79). The AKT family kinases are serine/threonine kinases that are part of the bigger general class of AGC kinases which has 518 members in humans (29, 80). It is also known that 60 out of the 518 kinases of the AGC group are highly conserved in eukaryotic cells (28). Moreover, 42 of the 60 kinases are capable of having some other function other than the catalytic (kinase) domain (28). Finally, alternative splicing and different isoforms add more complexity to the AGC family (79). The AGC kinases are most related to cAMP-dependent protein kinase 1 (PKA; also known as PKAC), cGMP-dependent protein kinase (PKG; also known as CGK1α) and protein kinase C (PKC) base on the sequence alignment of their catalytic domain (79).

Activation of AGC kinases depends on phosphorylation (79). There are two segments where the activation can occur, one located in the catalytic domain and the other one in a non-catalytic domain known as hydrophobic motif. However, there are several AGC kinases that have another significant phosphorylation site, important for their integrity, the turn motif (79).

The tyrosine kinase receptors are a family of receptors found on the cell surface and are responsible for many critical cellular processes such as cell proliferation, cell migration, cell survival, metabolism and general cell-cycle regulation (80). Scientists have discovered 58 tyrosine kinase receptors which can be subcategorized into 20 subfamilies (80).

The AKT pathway is highly conserved and activation of this molecular pathway is tightly controlled with a multistep system (Figure 3) (81).

Figure 3: Steps of activation of AKT molecular pathway.



A tyrosine kinase receptor after activation will stimulate the phosphoinositide-3-kinase (PI3K) via an insulin receptor substrate (IRS). This event will convert the catalytic domain to be converted from phosphatidylinositol (3,4)-bis- phosphate (PIP_2) lipids to phosphatidylinositol (3,4,5)-tris- phosphate (PIP_3). AKT will bind to PIP_3 while in the plasma membrane and 3-phosphoinositide-dependent protein kinase 1 (PDK1) will partially activate AKT through phosphorylation (81). In order to fully activate AKT, either mammalian target of rapamycin (mTOR) or DNA-dependent protein kinase (DNA-PK) need to phosphorylate AKT at S473 in the carboxyl end (81). The fully activated form of AKT is implicated in many cellular functions

such as cell proliferation, angiogenesis, cell growth, cell metabolism, cell survival and apoptosis (81).

2.7 Endothelial Nitric Oxide Synthase (eNOS).

Nitric oxide (NO) is a major regulator of endothelial cell migration during angiogenesis (23). NO is quickly produced by endothelial nitric oxide synthase after activation of AKT (23, 82).

Endothelial nitric oxide synthase (eNOS) is the primary source of nitric oxide in the cardiovascular system (83). Primary role of the endothelial nitric oxide synthase is in vasodilation (83); however, there is not a lot of information on the microvascular permeability role (83). The exact signaling mechanisms underlining the regulation of vascular permeability from eNOS are not been discovered yet (83). There are different stimuli that can trigger eNOS to regulate vascular permeability (84-86). The phosphorylation of eNOS and location of that event is critical and determines the activity of the molecule; functions such as shear stress, molecules such as hormones and other molecules can activate eNOS by phosphorylation/dephosphorylation mechanisms (83, 87-90). Nitric oxide has numerous molecular targets and can regulate functions such as neurotransmission, vascular tone, regulation of gene transcription and mRNA translation (91). All NOS are dimers of identical subunits that make up the functional enzyme as a homodimer (91). There are two steps of NO synthesis. During the first step the NOS hydroxylates L-arginine to N^{ω}-hydroxy-L-arginine and the second step includes the oxidation of N^{ω}-hydroxy-L-arginine to L-citrulline and NO (92, 93). Endothelial NOS is targeted to the caveolin and binding to caveolin-1 (cav-1) inhibits its activity (90, 94, 95). However, calcium-calmodulin and intracellular calcium can release eNOS from cav-1 (94, 96). In addition to the above mechanisms also important is the location of the molecule in the cytosol; so localization of

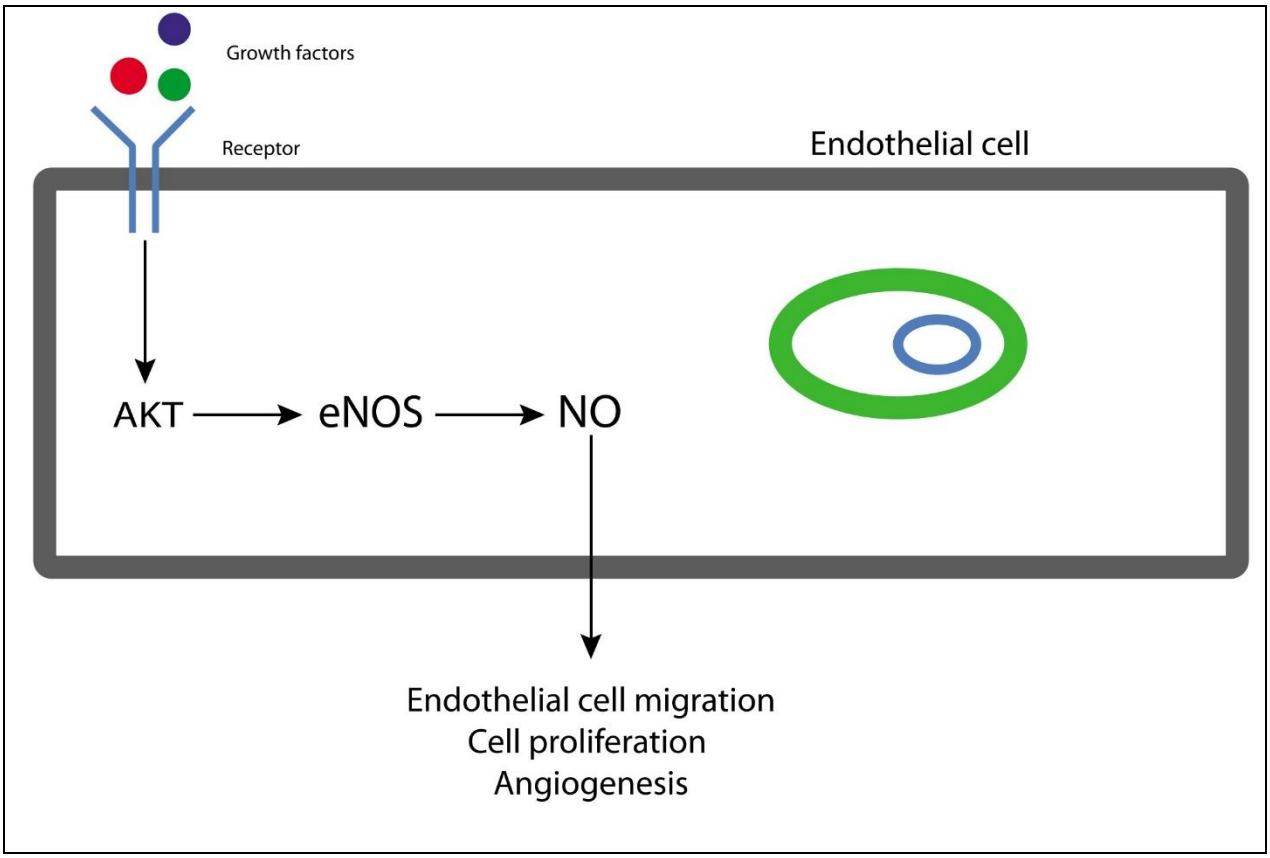
the molecule can potentially play a key role in the activity of eNOS (97-100). Nitric oxide synthase is a multi-domain enzyme (101). The oxygenase domain in the N-terminal (amino acids 1-491) has binding sites for heme, L-arginine (Glu361) and (6R-)5,6,7,8-Tetrahydrobiopterin (BH_4) and a reductase domain (amino acids 492-1205) that has binding sites for flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), nicotinamide adenine dinucleotide phosphate (NADPH) and calmodulin (CaM) (101, 102). Thus, cofactors of the enzyme can be considered FAD, FMN, BH_4 and heme.

The catalytic activity of eNOS is shown here:

2 L-arginine + 3 NADPH + 4 O_2 = 2 L-citrulline + 2 nitric oxide + 3 $NADP^+$ + 4 H_2O

According to Uniprot protein database there are three isoforms of this enzyme. However, only one containing 1205 amino acids is considered canonical and functional. Endothelial NOS is a homodimer and BH_4 may help stabilize the dimerization. The two identical subunits of the enzyme are myristoylated and palmitoylated. The monomers are inactive and unable to bind either L-arginine or BH_4 (101). The molecular pathway of AKT and eNOS are connected and a general scheme is shown in Figure 4.

Figure 4: Interaction between AKT and eNOS.



2.8 Vascular endothelial growth factor (VEGF).

Precursors of endothelial cells, angioblasts, when under the influence of VEGF will start to migrate and start begin forming new angiogenic networks (23).

One of the most important molecular pathways activated in endothelial cells is the vascular endothelial growth factor (VEGF) (103). This signaling cascade can activate plethora of molecules involved in angiogenesis, cell migration, cell proliferation, vascular permeability and remodeling (103, 104). The VEGF family members can stimulate vasculogenesis during development and angiogenesis in adulthood (105). Vascular endothelial growth factor is part of the cysteine knot growth factor superfamily and it consists of five structurally homologous

similar secreted mammalian glycoproteins members (VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF)) and one parapoxvirus-encoded member (103, 106). The different isoforms of VEGF have different solubilities, bioavailability and they act like ligands in an overlapping pattern to the three different tyrosine kinases VEGF-receptors (103, 106). The best representative of the VEGF family could be considered VEGF-A. The VEGF-A gene is located on chromosome 6 (6p21.1) (103). Through alternative mRNA splicing there are six VEGF-A isoforms (VEGF 121, VEGF 145, VEGF 165, VEGF 183, VEGF 189 and VEGF 206) (103). All isoforms are products of a single pre-mRNA and the final active ~14kb mRNA contains eight exons (106). The difference between the VEGF-A isoforms is the ability to bind to heparin sulfate and the extracellular matrix (ECM) (106). VEGF-A is the predominant human isoform, which can also be secreted into systemic circulation. Moreover, during hypoxia conditions, VEGF-A expression is increased making it a valuable marker of angiogenesis that is implicated in many diseases such as cancer (106). There are three tyrosine kinase VEGF-receptors (VEGFR1, VEGFR2 and VEGFR3) (103). These receptors share the same structure; a) an extracellular binding domain, b) a transmembrane helix and c) the cytoplasmic kinase domain (Figure 5) (103). When a ligand binds the extracellular side of these receptors, the intracellular domain to autophosphorylates and forms a dimer with another receptor (105). VEGFR1 is found on the vascular endothelium as well as in other non-endothelial cells such as macrophages and monocytes; it has a 180kDa molecular weight and it's ligands are VEGF-A, VEGF-B and PlGF (Figure 6) with higher affinity to VEGF-A (K_d 10 pM) (103, 106, 107). VEGFR2 is a 200-230kDa receptors that binds VEGFR-A, VEGF-C, VEGF-D, and VEGF-E (Figure 6) (103, 106). Even though VEGF-A binds with lower affinity (K_d 75-125 pM) to VEGFR2 the receptors tyrosine kinase activity is stronger than VEGFR1 (103). VEGFR2 can be found on the lymphatic

and vascular endothelium. This receptor is known to be associated with activation of cell migration by activating focal adhesion kinase (FAK) and RHOA/ROCK pathways. Moreover, it is involved in cell proliferation and regulation of the actin cytoskeleton (103). Lastly, VEGFR3 has a molecular weight of 195kDa. VEGFR3 binds VEGF-C and VEGF-D (Figure 6) and it is highly expressed in the lymphatic endothelium (103, 106). VEGFR3 like VEGFR2 is also responsible for cell migration and cell survivability via AKT and p42/p44MAPK pathway (108). Studies suggest that VEGFR3 acts as a negative switch of VEGFR2 (109). Recently VEGFR3 has been implicated with the Notch signaling pathway which suggests an association in sprouting angiogenesis and an interaction between tip and stalk cell (110).

In summary, VEGFR2 is critical regulator on numerous processes in endothelial cells such as cell survival, proliferation, migration, vascular permeability and invasion; thus, a crucial regulator of angiogenesis. Finally, all the above signal transduction molecules; GTPases, AKT, eNOS and VEGF are interrelated in many cellular functions Figure 7.

Figure 5: General structure of VEGF receptor.

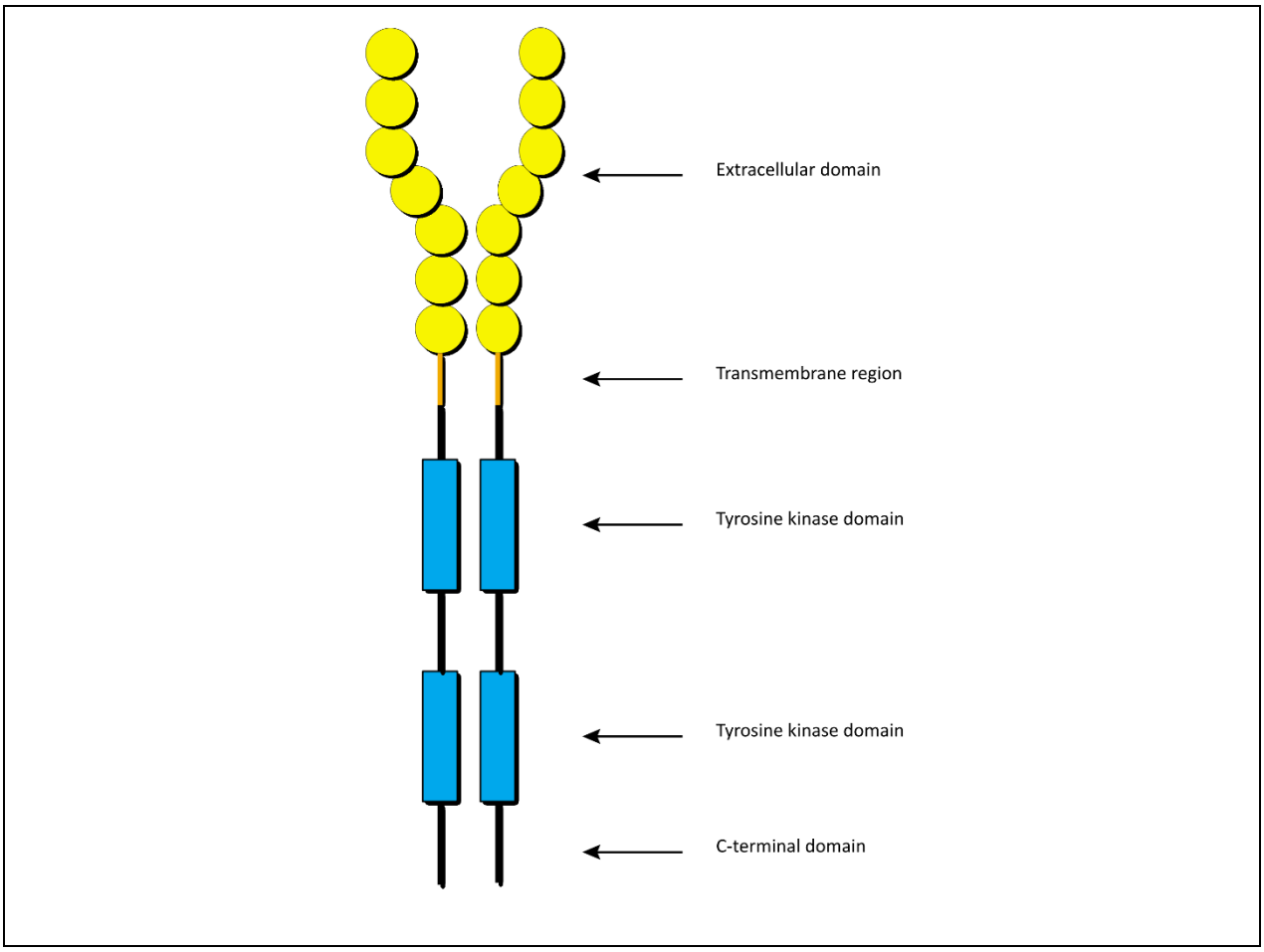


Figure 6: The three different VEGF receptors and their ligands.

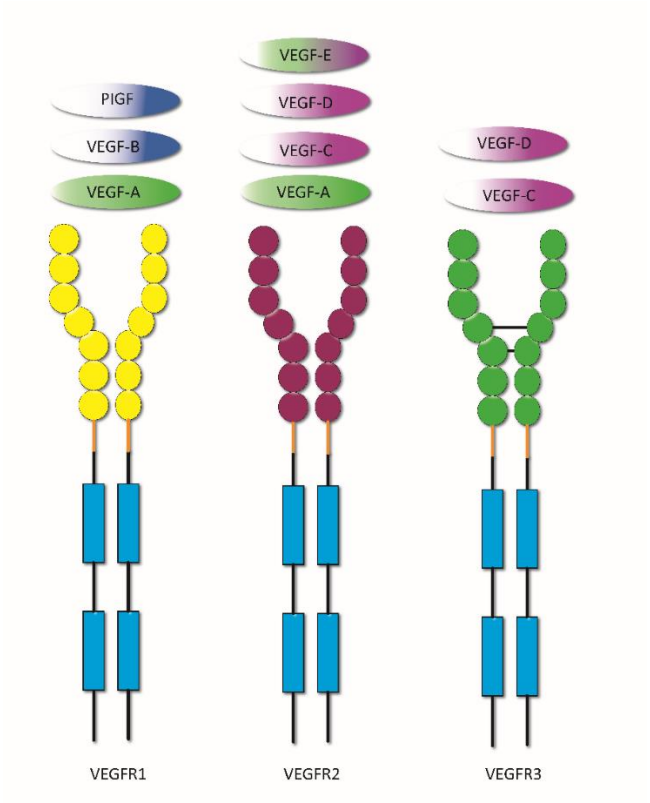
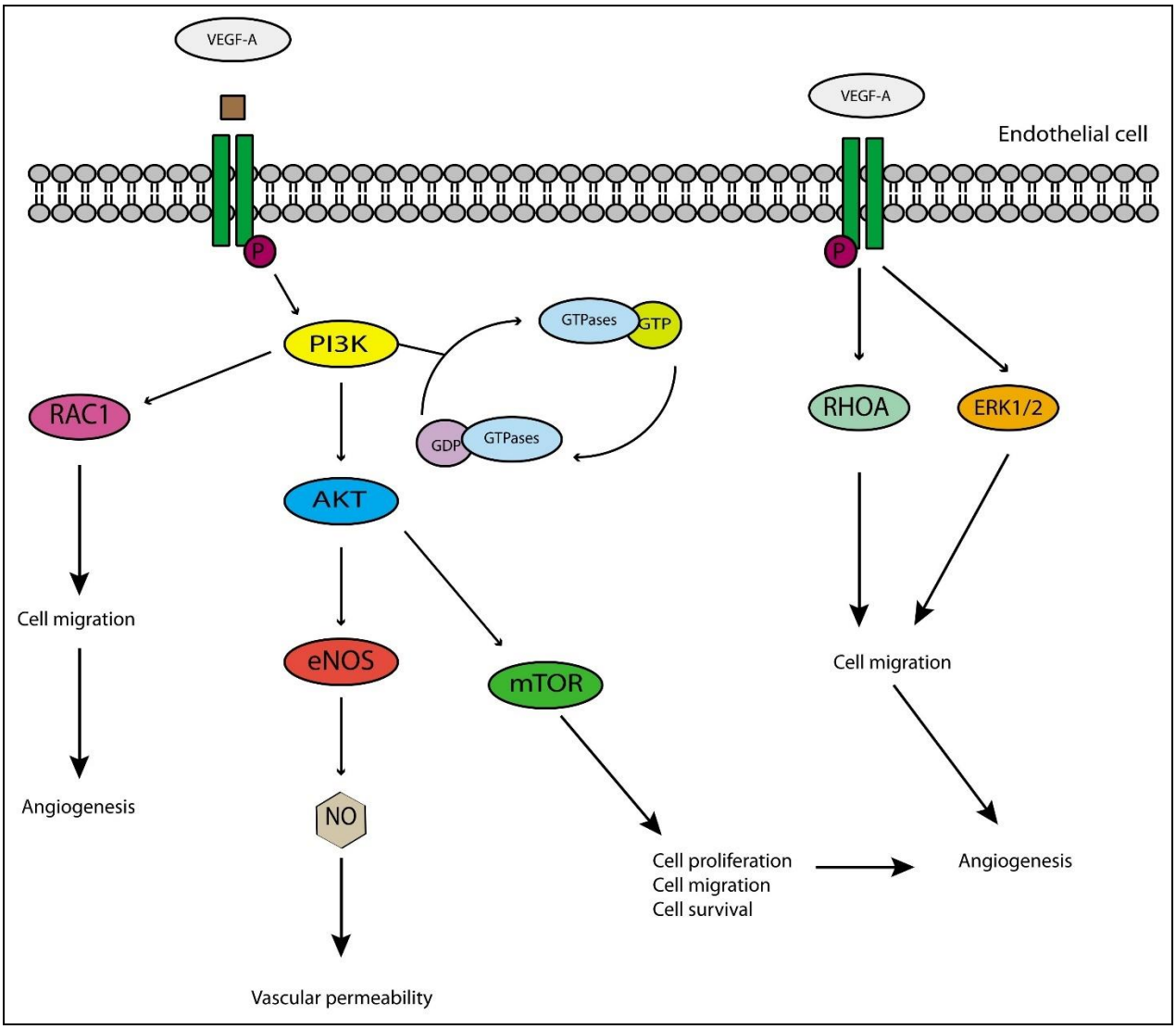


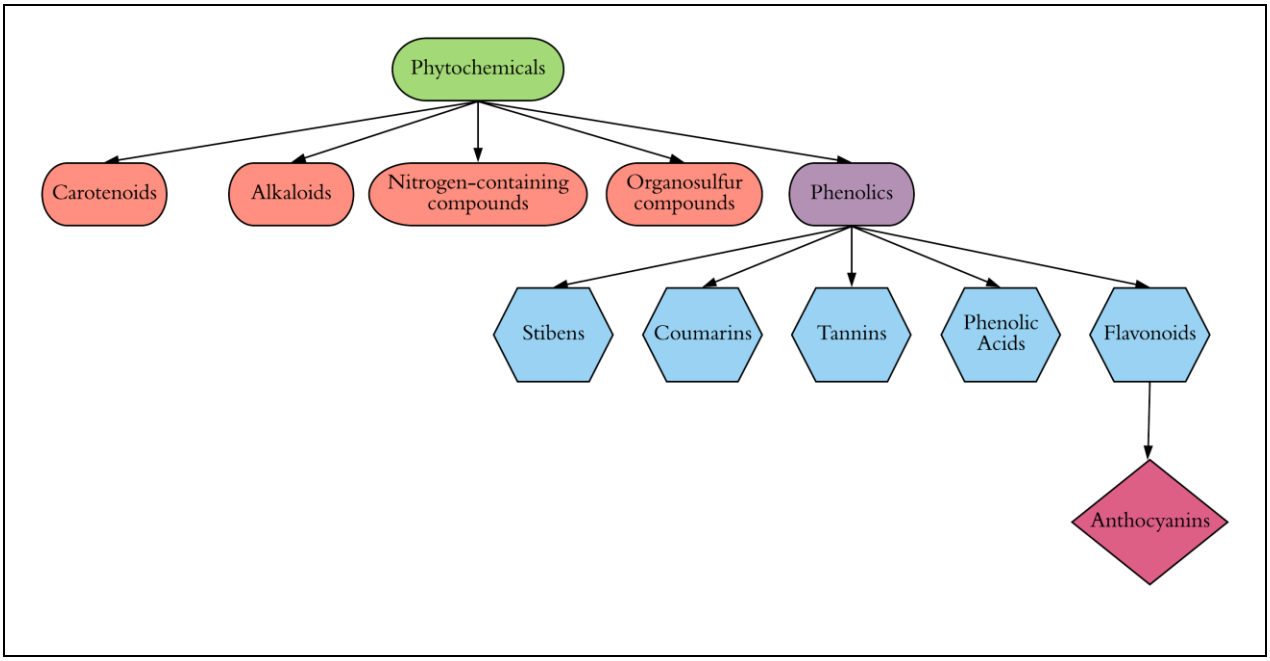
Figure 7: Critical signaling pathways involved in cell migration and angiogenesis in endothelial cells.



2.9 Phytochemicals.

Plant chemicals are considered as one term phytochemicals. The world derives from the Greek word ‘’phyto’’ (φυτό) which means plant (111). There are five major subcategories of phytochemicals: carotenoids, phenolics, alkaloids, nitrogen-containing compounds and organosulfur compounds (Figure 8). Phenolics are divided into five subcategories: phenolic acids, stilbenes, coumarins, tannins and flavonoids. Flavonoids also contains additional classes of active compounds and one major class is anthocyanins. There are more than 5000 individual phytochemicals that have been discovered by scientists in fruits and vegetables (111, 112).

Figure 8: Classification of phytochemicals.



2.10 Anthocyanins (ACNs).

In nature there are many phytonutrients such as ACNs and PAs (113, 114). These bioactive compounds can be found in the skin of fruits and vegetables as well as in cell vacuoles (115). The most important bioactive compounds are the ACNs and more than 500 have been described, and six of them pelargonidin, cyanidin, delphinidin, petunidin, peonidin, and malvidin are generally found in fruits and vegetables (115). Lowbush blueberries contain all of the above anthocyanins with the exception of pelargonidin (114, 116). In their majority anthocyanins (Figure 9) from wild blueberries are composed from delphinidin, malvidin, petunidin, cyanidin and peonidin (Table 1) (115, 117). Scientists have documented that a person on average can consume 180-215 mg/day of ACNs (118, 119). However, the human body doesn't have the ability to absorb ACNs very well. It has been documented that less than 1 % can been found in the plasma and the concentration can be from 10-50 nmol/L (120). Additionally, most of the ACNs are broken down and metabolized further (115). Anthocyanins are polar molecules and that makes them water soluble as well as capable to dissolve in other solvents such as methanol, ethanol and acetone (114). It is important to note that anthocyanidins are a different form of anthocyanins. Anthocyanidins are the anthocyanins with no aglycons. They are oxygenated derivatives of flavylium (2-phenylchromenylium) salts (121).

Absorption of anthocyanins and other flavonoids takes place rapidly in the stomach and the small intestine (113, 122). Bilitranslocase is an enzyme involved in this process (122). Bilitranslocase acts as a flavonoid membrane transporter (123). Anthocyanins after entering the circulatory system intact in the blood, pass through the liver before reaching any further organs (113). Moreover, anthocyanins that are not absorbed within 15 min - 2 h and subsequently are exposed to the microbiota mainly in the colon (113).

As mentioned earlier anthocyanins are the glycosylated version of flavylium (2-phenylchromenylium) more specifically the 3-OH position is found more frequently than any other (124). The most abundant anthocyanins are six: pelargonidin (Pg3glc), cyanidin (Cy3glc), peonidin (Pn3glc), delphinidin (Dp3glc), petunidin (Pt3glc) and malvidin (Mv3glc) (113, 117, 124). However, scientists have reported 539 different anthocyanins isolated from plants and vegetables (124). Moreover, anthocyanins since they are highly reactive molecules and are very sensitive to many factors such as light, temperature, oxygen, enzymes and pH (124).

Figure 9: General chemical structure of anthocyanins.

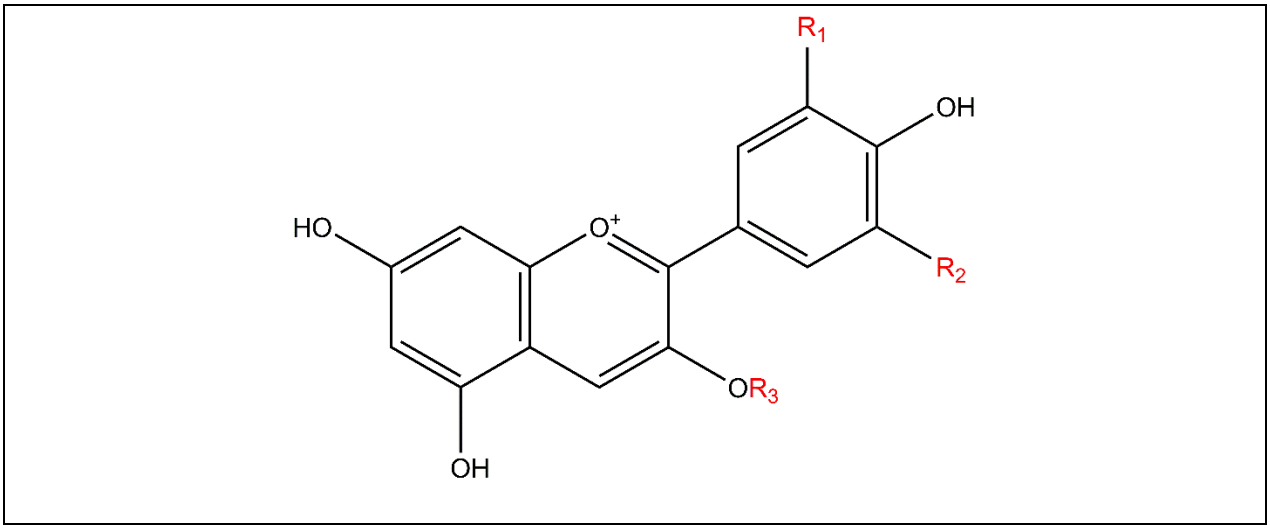


Table 1: The six most abundant anthocyanins in nature in their flavylium form.

Anthocyanins	Pg3glc	Pn3glc	Cy3glc	Mv3glc	Pt3glc	Dp3glc
R_1	H	OCH_3	OH	OCH_3	OCH_3	OH
R_2	H	H	H	OCH_3	OH	OH
R_3	Sugar moiety					

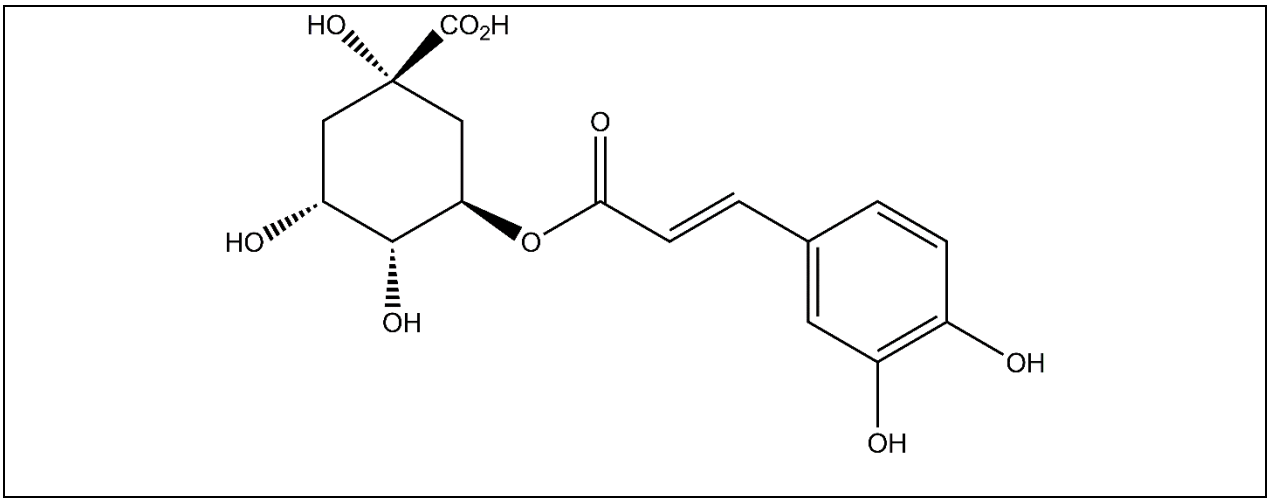
2.11 Phenolics and Phenolic acids (PAs).

Phenolics are compounds that have one or more aromatic rings in their structure and one or more hydroxyl groups (111). Phenolics are useful as a line of defense for plants as they can act against pathogens, parasites and predators They are also important for the color of plants (111). Phenolic acids are a subcategory of phenolics and they can also be divided in two major groups, hydroxybenzoic acids and hydroxycinnamic acids. The first group is mainly found in complex structures such as lignin while the second group usually can be found in cell wall structure components (111). In addition, food processing techniques can increase the release of bound phenolic acids.

2.11.1 Chlorogenic acid.

Caffeic acid is a major phenolic acid that is abundant in the plant world (111). Chlorogenic acid (Figure 10) is a product of enzymatic oxidation and is derived as an ester from caffeic acid (111). Chlorogenic acid can be found in many dietary sources such as apples, pears and berries (125, 126). Chlorogenic acid can enter human blood circulation and it can have biological effects; it is an antioxidant along with caffeic acid and it can protect DNA structures *in vitro* (125, 127, 128).

Figure 10: Chemical structure of chlorogenic acid.



2.12 The Wild Blueberry (WB) (*V. angustifolium*).

Numerous studies have documented in the past the beneficial effects of wild blueberry (*Vaccinium angustifolium*) consumption on inflammation and cardiovascular disease (CVD) as well as many other chronic diseases (113, 115, 129-133). The lowbush *Vaccinium angustifolium* has been ranked as one of the richest in flavonoids among other fruits and berries (129). Wild blueberries are rich source of bioactive compounds (polyphenols) such as anthocyanins (ACNs) and phenolic acids (PAs) as well as flavonoids (117, 130). It has been documented that both the high and lowbush blueberries have three times higher content of total ACNs compared to strawberries and raspberries (114). Studies demonstrated that a wild blueberry diet can have a positive effect on the remodeling of the endothelium structure on Sprague-Dawley rats and in adult spontaneously hypertensive rats (SHR) after eight (8) weeks (134, 135). Moreover, according to Bushway and co-workers in 1983, wild blueberries are low in antioxidant vitamins and minerals however, they have high concentration of phenolic compounds, especially anthocyanins (117, 129).

2.13 Effect of Anthocyanins and Phenolics on endothelial cell migration and angiogenesis.

The effects of various anthocyanins and phenolic acids from different types of berries are summarized in Table 2. Previous studies have evaluated cell migration and angiogenesis in conditions such as cancer while others in healthy conditions. Moreover, investigators used many *in vitro* and *in vivo* models used to evaluate the effects of ACNs and PAs derived from different sources.

Table 2: Anthocyanins and phenolic acids and their effect on cell migration and angiogenesis.

Author (date)	Journal	Cell line	Fraction	Source	Concentrations	Results
Matsubara, K. et al. (2005)	Journal of Agricultural and Food Chemistry	HUVECs (Osaka, Japan)	Nasunin (delphinidin-3-5 glucoside)	Eggplant peels	0-200 µM	No effect on tube formation and chemotaxis
Chen, P. et al. (2006)	Cancer Letters	A549 (ATCC)	Anthocyanins (cyanidin 3-rutinoside or cyanidin 3-glucoside)	Mulberry	0, 25, 50, 75, and 100 µM	Inhibition of cell invasion
Lamy, S. et al. (2006)	Carcinogenesis	HUVECs (Clonetics)	Anthocyanidins	Commercially obtained	5, 10, 15, 20 and 25 µM	Inhibition on cell motility and tube formation
Lamy, S. et al. (2008)	Journal of Cellular Biochemistry	U-87 (ATCC)	Anthocyanidins	Commercially obtained	5, 10, 15, 20, 25 and 50 mM Or 10, 25, 50, or 100 mM	Inhibition on cell motility and migration
Huang, H. et al. (2008)	Journal of Agricultural and Food Chemistry	B16-F1 (BCRC, Hsin-Chu, Taiwan)	Anthocyanins	Mulberry	1, 2 and 3 mg/mL	Inhibition on cell motility and migration
Nizamutdinova et al. (2009)	Food and chemical toxicology	HUVECs (Clonetics)	Anthocyanins	Black Soybean	50 µg/mL and 100 µg/mL	Increased cell migration

Table 2 Continued

Author (date)	Journal	Cell line	Fraction	Source	Concentrations	Results
Matsunaga, N et al. (2010)	Evidence-Based Complementary and Alternative Medicine	HUVECs (Kurabo) human dermal fibroblast (Cascade) and HaCaT	Anthocyanins	Bilberry	0.3, 3 and 30 μg/ml	Inhibition on cell motility and tube formation
Mauray, A. et al. (2011)	Nutrition, metabolism, and cardiovascular diseases	apoE$^{-/-}$ mouse model	Anthocyanin-rich extract	Bilberry	Diet supplemented with 0.02 % of Bilberry extract	Changes on gene expression underling beneficial properties of bilberries
Lamy. S, et al. (2012)	Experimental Cell Research	HUVECs (Clonetics)	Ellagic Acid	Commercially obtained	25 μM	No significant differences
Kausar, H. et al. (2012)	Cancer Letters	Beas2b, H1299 and A549	Anthocyanidins	Commercially obtained	25, 50, 75 and 100 μM	Inhibition on cell motility and migration
Tanaka, J. et al. (2012)	Phytotherapy Research	HUVECs (Kurabo)	Anthocyanins	Commercially obtained	1, 3 and 10 μM for tube formation 3, 10 and 30 μM for cell migration	Inhibition on cell motility and tube formation

Table 2 Continued

Author (date)	Journal	Cell line	Fraction	Source	Concentrations	Results
Wang, N. et al. (2012)	Breast Cancer Res Treat	HUVECs (ATCC)	Ellagic Acid	Commercially obtained	2.5 – 10 µM	Inhibition of angiogenesis at nontoxic dosages
Zhang, Y. et al. (2013)	Journal of Nutrition	Endothelial progenitor cell from apoE2/2 mice	Anthocyanin cyanidin-3-O-b-glucoside (C3G)	Commercially obtained	0.2 % wt:wt for six weeks	Normalization of results
Son, J. et al. (2014)	Biochemical Pharmacology	HASMCs and HUVECs (Lonza)	Pelargonidin and pelargonidin-3-glucoside (P3G)	Commercially obtained	10, 20 and 40 µM	Inhibition of wound healing, migration and aortic sprouting with pelargonidin on HASMCs (No effect on HUVECs)
Son, J. E. et al. (2014)	Cardiovascular Research	Human aortic smooth muscle cells (HASMCs) (Lonza	Anthocyanidins	Commercially obtained	5, 10, and 20 µM	Inhibition on cell motility and migration

Table 2 Continued

Author (date)	Journal	Cell line	Fraction	Source	Concentrations	Results
Li et al. (2014)	Scientific Reports	RAW264.7 (ATCC)	Anthocyanin-rich fractions	Mulberries Red Raspberries Black raspberries	100 – 200 µg/mL	Inhibition of proinflammatory genes (iNOS, COX-2 and IL-6)
Park, J. et al. (2015)	Cellular Oncology	HUVECs (ATCC)	Chlorogenic acid (CGA)	Commercially obtained	2 µM or10 µM	Inhibition of angiogenesis through HIF-1α and AKT
Del Bo et al. (2016)	Molecular Nutrition and Food Research	HUVECs Unspecified THP-1 (ATCC)	Anthocyanins and Phenolic acids	Wild Blueberry fractionation	0.01 to 10 µg/mL	ACNs and PAs can decrease adhesion of monocytes to HUVECs

2.13.1 Effect of Anthocyanins on cell migration and angiogenesis.

An *in vitro* study by Matsubara et al. documented that nasusin which is an anthocyanin antioxidant was able to inhibit *ex vivo* angiogenesis using a rat aortic ring model. Moreover, HUVEC cell proliferation assay documented an inhibitory effect of nasusin in high concentrations (50-200 μM) compared to the control (136). Interestingly, *in vitro* tube formation showed no significant differences in any of the tested concentrations. Cell migration in a chemotaxis *in vitro* setup did not exhibit any significant differences either (136).

In 2006 Chen et al. tested anthocyanins cyanidin 3-rutinoside and cyanidin 3-glucoside from mulberries on A549, a human lung cancer cell line (137). In all tested concentrations (0-100 μM) of cyanidin 3-rutinoside or cyanidin 3-glucoside, nosignificant effect of cell cytotoxicity was observed. In addition, cell motility, cell invasion and cell adhesion were evaluated after cells were treated with anthocyanins for 24 h with concentrations ranging from 0, 25, 50, 75, or 100 μM. Cell adhesion was inhibited compared to the control under all concentrations of cyanidin 3-glucoside except 75 μM, cell motility was unaffected and cell invasion was affected with an inhibitory manner with 100 μM. Cyanidin 3-rutinoside had no effect on cell adhesion while cell motility and cell invasion decreased with treatment of 100 μM (137).

In 2007 and 2008 Lamy and his team studied the effects of anthocyanins on glioblastoma cells in the first study and HUVECs and pulmonary aortic smooth muscle cells (PASMCs) in the second study (138, 139). In the first study anthocyanins commercially available were used on glioblastoma cells (U-87) to assess the effect on cell migration and invasion. Using Transwell chambers they documented that cyanidin (Cy), delphinidin (Dp), and petunidin (Pt) in concentrations ranging from 5 to 50 μM inhibited cell migration. These studies concluded that

anthocyanins structures such as the ortho-dihydroxyphenyl structure on the B-ring of anthocyanidins and the presence of a free hydroxyl group at position 3 might be responsible for the documented results (138). Moreover delphinidin (Dp) was documented to inhibit cell invasion as well in a concentration dependent manner in a range of 5 – 50 μM (138). For the second study the OptiBerry BX-600 anthocyanin-rich combination berry extract was used to evaluate the effect on tube formation and cell migration (139). Lamy et al suggested that delphinidin (5 – 15 μM) inhibited cell migration for smooth muscle cells while cell proliferation was not affected (139). Cocultured cells (SMCs and HUVECs) treated with delphinidin (25, 50 and 75 μM) showed reduce tube length in angiogenesis experiment after six days of treatment (139).

One other study evaluated the effects of anthocyanins extracted from mulberries (140). The B16-F1, a murine melanoma cell line, was used to test the effect of anthocyanins on cell viability, wound healing and cell migration. MTT assay documented that mulberry ACNs in various concentrations (0 – 5 mg/mL) were significantly cytotoxic to the cells after three days of treatment. Scratch assays were used to evaluate cell motility after cells were treated with ACNs from 0 – 3 μg/mL for up to three days and a significant inhibitory effect was documented in cell motility. Cell migration experiments also documented the inhibitory effects of ACNs at a concentration of 3 mg/mL (140).

Black soybeans have also been used in research to evaluate the effects of anthocyanins on cell migration. In 2009 Nizamutdinova et al. used HUVECs, human dermal fibroblast and immortalized human keratinocyte cell line, HaCaT as models to test anthocyanins extracted from black soybeans (49). Wound healing assays documented that for both cell lines cell migration

was induced after 48 h with 50 and 100 μg/mL of ACNs. After 24 h fibroblasts showed an increased with 100 μg/mL and keratinocytes with 50 and 100 μg/mL of ACNs (49).

An *in vivo* study in 2010 documented that dietary supplementation with a bilberry anthocyanin-rich extract (BE) attenuated atherosclerotic lesion development in apolipoprotein E-deficient ($apoE^{-/-}$) mice (141). The aim of this study was to investigate the *in vivo* mechanisms of the action of bilberry extract, administered by supplementation at a nutritional level, in the aorta of $apoE^{-/-}$ mice using a global transcriptomic approach. For the best assessment of the BE action this study focused on the early stages of atherosclerosis. Two animal groups were used, one on control diet and one on the same control diet supplemented with 0.02% BE. Animals were fed for two weeks. After the two-week period, plasma lipid, antioxidant capacity and global genomic analysis were evaluated using pangenomic microarrays. Results showed significantly improved hypercholesterolemia on the BE diet group compared to the control diet group. Moreover, nutrigenomic analysis identified 1261 genes which expression was modulated by BE in the aorta samples isolated from the animals. Bioinformatic analysis revealed that these genes are implicated in different cellular processes such as oxidative stress, inflammation, transendothelial migration and angiogenesis, processes associated with atherosclerosis development/protection (141).

Another *in vitro* study in 2012 documented the effects of berry anthocyanins on cell migration, invasion and motility. Kaiser et al used tumorigenic H1299 cells as a model and commercially available anthocyanins (chloride forms of Cy, Mv, Pe, Pt and Dp) were used (142). Cell migration and invasion was significantly decreased compared to the control. It was obvious that Pe was the most effective anthocyanins and a concentration of 6.25 μM was enough to show an effect. Combination of all individual anthocyanins was also able to inhibit cell invasion with

3.12, 6.25 and 12.5 μM (142). However, cell motility inhibition was documented only with the anthocyanins mixture in a dose dependent manner (142).

An *in vitro* study in 2012 documented the protective effects of anthocyanins from purple rice (51). In a previous study three anthocyanins from purple rice (PRE) (cyanidin 3-O-glucoside, peonidin 3-O-glucoside and cyanidin 3-O gentiobioside) were isolated (143). Along with other evidence, it was hypothesized that these anthocyanins could potentially inhibit VEGF induced angiogenesis. HUVECs and human retinal microvascular endothelial cells (HRMECs) were used as an *in vitro* model (51). PRE inhibited VEGF induced angiogenesis in HUVECs co-cultured with fibroblasts. Parameters of tube formation such as area, length, joints and paths were significantly suppressed with PRE 3 – 30 μg/mL. Moreover, cell migration in the form of a wound healing assay showed that HRMECs migration was decrease with 10 – 30 μg/mL of PRE (51).

In 2013, an *in vitro* study evaluated the effects of the anthocyanin cyanidin-3-O-b-glucoside (C3G) on cell adhesion, cell migration and tube formation. Zhang et al isolated endothelial progenitor cells (EPCs) from blood and bone marrow of both nondiabetic and diabetic apoE$^{-/-}$ mice (144). The diabetic mice were fed the AIN-93 diet or an AIN-93 diet supplemented with C3G (0.2 % wt:wt) for six (6) weeks. C3G improved cell adhesion and cell migration indicating beneficial effects of anthocyanins. Moreover, tube formation was normalized compared to the diabetic mice without the C3G supplementation (144).

One more *in vitro* study showed the anti-inflammatory activities, biochemical functions and improved immune response from seven different berry varieties tested in macrophages (145). In this study seven berry varieties were evaluated. Researchers conducted experiments

using three different concentrations of anthocyanins (100, 150 and 200 mg/ml) to measure the cell viability, nitric oxide level, NF-κB and their gene expression. Results from the cell viability assay showed no cell toxicity. Evaluation of the production of nitric oxide showed a 20.70% to 25.47% NO inhibition when the cells were treated with 150 and 200 mg/ml of anthocyanin extracts. It can be concluded from this study that anthocyanins from berries can significantly reduce the state of inflammation by suppressing the expression of pro-inflammatory genes such as iNOS (145).

An *in vitro* study in 2014 evaluated the effects of an individual anthocyanin; pelargonidin-3-glucoside (P3G) (48). Son et al used pelargonidin and glucoside-conjugated form, pelargonidin-3-glucoside (P3G) commercially available and HPLC-purified to evaluate the effect on cell cytotoxicity, cell proliferation, cell migration, wound healing and aortic sprouting. As a model aortic smooth muscle cells (HASMCs) and HUVECs were used. Concentrations of up to 40 μM of P3G did not documented any cytotoxic effect. After HASMCs were induced with platelet-derived growth factor (PDGF)-BB pelargonidin but not its glucoside conjugated form exhibited strong inhibitory effects were exhibited (48). Wound healing as a migration assay was used and after cells were treated with PDGF, pelargonidin inhibited cell migration in a dose depended manner. Additional migration assay; Boyden chamber also documented similar results. Further investigation with an aortic ring-sprouting assay documented that pelargonidin significantly reduced PDGF-BB-induced aortic sprouting (48). The same assays and *in vitro* model was used to evaluate the effects of six anthocyanins (Petunidin, delphinidin, cyanidin, pelargonidin, malvidin, and peonidin) (146). However, petunidin resulted in the strongest inhibitory effect on HASMCs during cell migration (146).

We conclude from previous research that the beneficial anti-inflammatory, anti-metastatic effects of ACNs are well documented. However, there are some contradicting results when the evaluation comes to cell migration especially with different types of anthocyanins and the use of ACN concentrations that are usually higher than the ones normally found in the blood stream.

2.13.2 Effect of Phenolic acids on cell migration and angiogenesis.

One *in vitro* study evaluated the effects of ellagic acid on HUVECs during cell migration, cell proliferation and angiogenesis (45). Ellagic acid (25 μM) was able to significantly inhibit HUVECs proliferation rate in the presence of IL-6. Cell migration evaluation with Transwell documented no significant inhibition of migratory cells with ellagic acid at 25 μM (45). Tube formation assay to evaluate the effects of ellagic acid on HUVECs documented no significant results (45).

A 2012 *in vitro* study outlined the beneficial effects of phenolic acid such as ellagic acid. Wang et al used HUVECs as an experimental model and ellagic acid (0 – 10 μM) commercially obtained (147). The effect of ellagic acid on would healing, angiogenesis and cell invasion was evaluated. Before conducting these assays, researchers evaluated the proliferation rate of HUVECs when induced with VEGF. When cells were treated with ellagic acid proliferation rate was significantly reduced in a time (12 – 48 h) dependent manner (147). Transwell and wound healing assays were both used for cell migration evaluation. Inhibitory effect of ellagic acid was documented with both assays. Finally tube formation assay of HUVECs on Matrigel documented distraction of the endothelial tube network when cells were treated with ellagic acid (147).

In 2015, another *in vitro* study documented the beneficial effects of chlorogenic acid (CGA). Park et al evaluated the effect of CGA (10 μM) on cell migration, cell invasion and tube formation using HUVECs as a model (148). The researchers used tube formation assay to assess angiogenesis and used hypoxic conditions along with CGA treatment to HUVECs for up to 24 h. CGA significantly inhibit hypoxia induced network formation by forcing endothelial cells to form shorter and broken tubes (148). Cell motility tested with a wound healing assay documented that hypoxia induced mobility was significantly reduced by CGA. Finally, Transwell assay was used to evaluate the endothelial cell invasion. CGA was able to significantly inhibit cell invasion due to hypoxic conditions (148).

Other *in vitro* studies demonstrated that anthocyanins and phenolic rich fractions from wild blueberries can reduce lipid accumulation in macrophages derived from monocytes (37). The goal of this study was to evaluate the anti-atherosclerotic role of anthocyanins and phenolic compounds from blueberries. The concentrations of polyphenols used for these experiments were 0.05, 0.1, 0.3, 0.6, 1.25, 2.5, 5, and 10 μg/mL (range of concentration: minimum 0.09 μM and maximum 64.9 μM depending of the compound). The cell cytotoxicity assay showed no significant differences between the control and the treatments. Additionally, the cell viability was above 90% even when the cells were exposed to the highest concentration of polyphenols for 24 h (37).

We conclude from the above studies that there is lack of research on the effects of PAs on cell migration and angiogenesis. The above studies document contradictions and fail to give a clear understanding of the impact these compounds have on the above processes.

CHAPTER 3

MATERIALS AND METHODS

3.1 Cell culture.

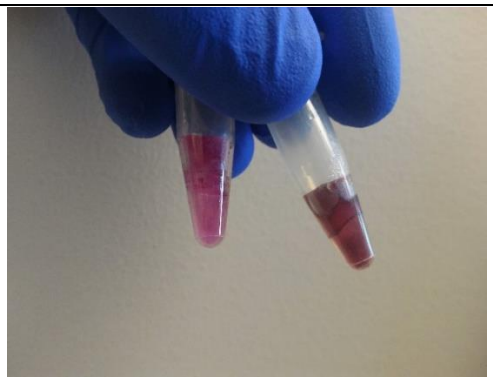
Human umbilical vein endothelial cells (HUV-EC-C [HUVEC] (ATCC® CRL-1730™)) were purchased from the American Type Culture Collection (ATCC®) Manassas, VA, USA. Human umbilical vein endothelial cells were maintained in F-12K medium (Kaighn's modification of Ham's F-12 medium) (ATCC® 30-2004™) with 10 % fetal bovine serum (FBS) (ATCC® 30-2020™) and 1 % penicillin-streptomycin solution (ATCC® 30-2300™), heparin 0.1 mg/mL (Sigma, H3149) and endothelial cell growth supplement (ECGS) 0.03 mg/mL (Sigma E2759). The culture vessel for the growth of the cells were the Corning® T-75 flask (catalog #3276). The culture conditions for the cell line were air 95 %, carbon dioxide (CO_2) 5 %, 90 % of relative humidity (RH) and temperature of 37 ^{o}C.

3.2 Extraction of ACNs and PA fractions from WB powder.

Wild blueberries (WB) were provided as a composite by Wyman's (Cherryfield, Maine, USA) and processed following standard procedures to obtain a freeze-dried powder (FutureCeuticals, Momence, Ill., USA) (44). Vacuum-packed plastic bags with the wild blueberry powder were stored at −20 °C until use. The wild blueberry powder had a total content of 1.5 % w/w of anthocyanins, with malvidin-3-galactoside and peonidin-3-glucoside being the most abundant forms, as previously reported (149). From the freeze-dried wild blueberry powder three fractions were isolated: **1.** Phenolic-rich fraction (ethyl acetate soluble, containing mainly chlorogenic acid) **2.** Anthocyanin-rich fraction (methanol soluble fraction, containing mainly anthocyanins) and **3.** Water soluble fraction.

Extraction of ACNs and PAs from the WB powder was performed according to the method previously published with the use of STRATA cartridges (Phenomenex, California) (37). The protocol followed for the extraction of the bioactive compounds (Figure 11) is described in detail by Del Bo' (37). Determination of total phenolic concentration was conducted by the Folin-Ciocalteu method (150-152). For the determination of the anthocyanin fraction concentration, a pH differential method was used (153).

Figure 11: ACNs (left) and PAs (right) after wild blueberry powder fractionation method.



3.3 Analysis of ACNs and PA fractions.

Wild blueberry ACN and PA fraction concentration was determined by HPLC. The system consisted of an Alliance mod. 2695 (Water, Milford, MA) equipped with a mod. 2998 photodiode array detector (Waters) as previously reported (37).

3.4 Growth curve of HUVECs.

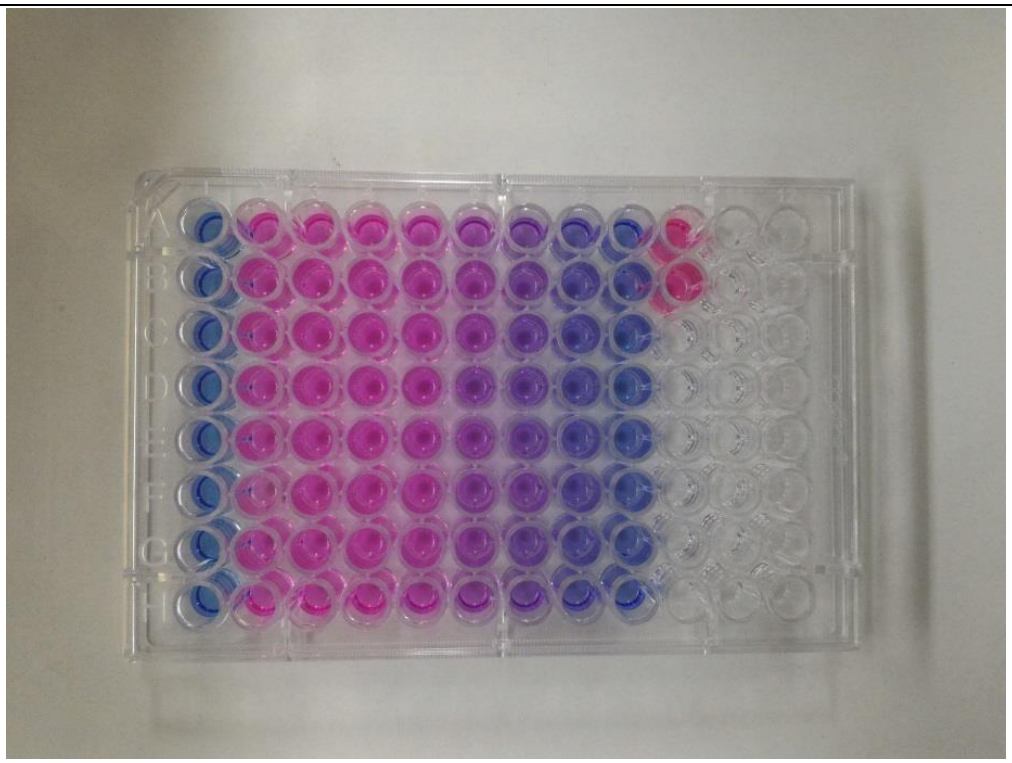
Calculating a growth curve for the HUVECs provides useful information for the growth characteristics of this cell line. For this experiment the multiwell protocol was used (154). Six 6-

well plates (Corning, Product #3335) were used. From each six-well plate, two wells were plated with $1x10^4$ cells/mL, two with $3x10^4$ cells/mL and two with $1x10^5$ cells/mL. The six-well plates were placed in a humid incubator (humidity level 90 %) with 5 % CO_2 level and 37 ºC temperature. After 24 h, cells from one well from each cell density ($1x10^4$ cells/mL, $3x10^4$ cells/mL and $1x10^5$ cells/mL) were aspirated with 500 µL of TrypLE™ Express Enzyme (1X), containing no phenol red (LifeTechnologies 12604-013). Erythrosine B stain solution (ATCC® 30-2404™) was used to stain dead cells, and a hemocytometer was used to count the live and dead cells from each well. The remaining wells from the six-well plate were returned to the incubator. Every 24 hours the same procedure was executed up to the twelfth day. After plotting the cell concentration (y axis) versus days after subculture (x axis) the lag phase, log phase and plateau phase were calculated (154).

3.5 Cell proliferation and cytotoxicity assay.

The cell growth curve experiment provided information on the doubling time of the HUVECs. The ACN and PA cytotoxicity assay was conducted so that the optimum concentration and exposure time of the active compounds was used for the rest of the experiments. Different concentrations (0.001 µg/mL - 1000 µg/mL for ACNs and 0.001 µg/mL – 500 µg/mL for the PAs) were tested. Moreover, different exposure times were tested (30 min, 1 h, 3 h, 6 h, 12 h, 24 h, 48 h and 72 h). Cell proliferation and cytotoxicity were conducted by using the alamarBlue assay (Life Technologies, DAL1025). Before conducting the cell proliferation and cytotoxicity experiments, a standard curve of alamarBlue was generated (Figure 12). Cells for both cell proliferation and cytotoxicity assay were measured by using Synergy 2 multiwell plate reader (Bio-Tek Instruments Inc., Winooski, VT) with excitation/emission (530 nm-560 nm/590 nm) as previously described (155).

Figure 12: AlamarBlue standard curve on a 96-well plate.



Prior to deciding the concentrations used for the cell migration experiment a cell proliferation assay was performed. During that assay dose response curve was generated for ACNs and PAs with concentrations ranging from 0.001 μg/mL - 1000 μg/mL for ACNs and 0.001 μg/mL – 500 μg/mL for the PAs. Based on results from the cell proliferation assay using ACNs, PAs and ACNs:PAs specific concentrations of the above were chosen for the cell migration experiments. The aim of this study was to delineate the effects of ACNs, PAs and ACNs:PAs from low concentrations reachable in the blood stream from normal consumption of wild blueberries to higher concentrations found in the reposted literature (37, 45, 48, 146, 147).

3.6 Wound healing assay.

3.6.1 *In vitro* wound healing assay.

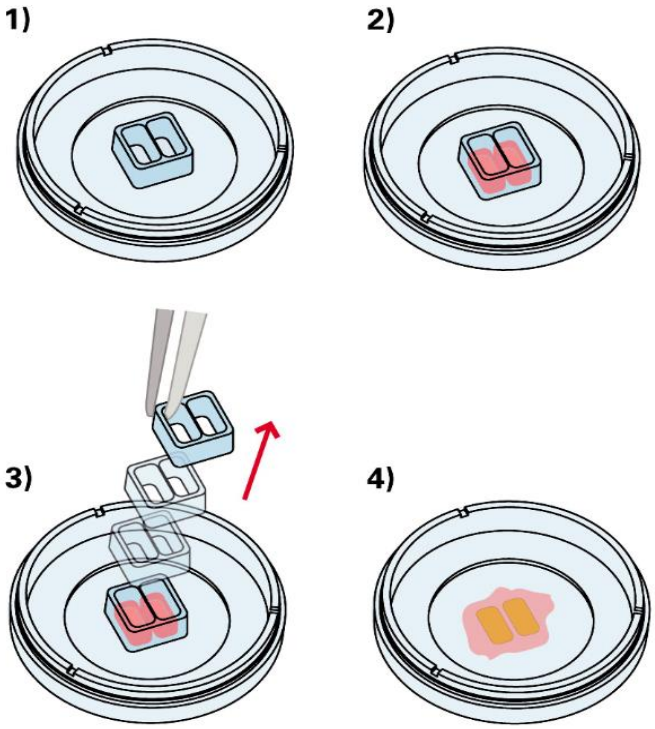
To analyze cellular migration a confluent monolayer population of cells were scratched to induce an artificial wound. The cells in the wound edge of the monolayer will start moving towards the newly available area until the opening is fully covered with new cells (156, 157). This *in vitro* scratch assay is very straightforward and economical (156). The main advantage of this assay is that it can mimic to some extent migration process *in vivo*; for example if endothelial cells are removed from a blood vessel, migration process will be initiated in order to heal the wound and cover the empty area (156, 158). Another advantage of the scratch assay is that it can be used to study interactions of cells with extracellular matrixes (ECM) (156). Despite all the advantages there are some limitations; the time required for the formation of the monolayer it can be too long so it is not recommended for primary cell lines. Also, it cannot compared and substituted for the well establish chemotaxis assays (156). However, time of preparation of the assay has been significantly decreased with the newly used μ-dish used during this project.

3.6.2 Endothelial cell migration assay.

The effect of ACN, PAs and the combination of both fractions on cell migration was evaluated by the IBIDI Culture–Insert (Figure 11) (Ibidi, Munich, Germany). The culture inserts were seeded with $5x10^5$ cells/mL and the final volume in each insert chamber was 70 μl (Figure 13). The IBIDI Culture–Insert was placed in the incubator for 24 hours. After cells reached ≥90% confluence they were treated with the PA and ACN fractions and their combinations at concentrations determined by the results of the cytotoxicity experiment (0.002 μg/mL, 8 μg/mL, 15 μg/mL, 60 μg/mL and 120 μg/mL). Cells in the treated (ACN and PA fractions and

combinations, n=7 replicates) and untreated (control, n=10 replicates) wells were observed under an inverted phase-contrast optical microscope with 10x objective (Nikon TS100) for up to 24 h. At the end of each experiment the speed of closure was calculated as the cell migration rate ($v_{migration}$ in μm/hr). Analysis was conducted with the TScratch software as described in detail by Geback and Jonkman (159, 160).

Figure 13: Steps of cell migration assay.



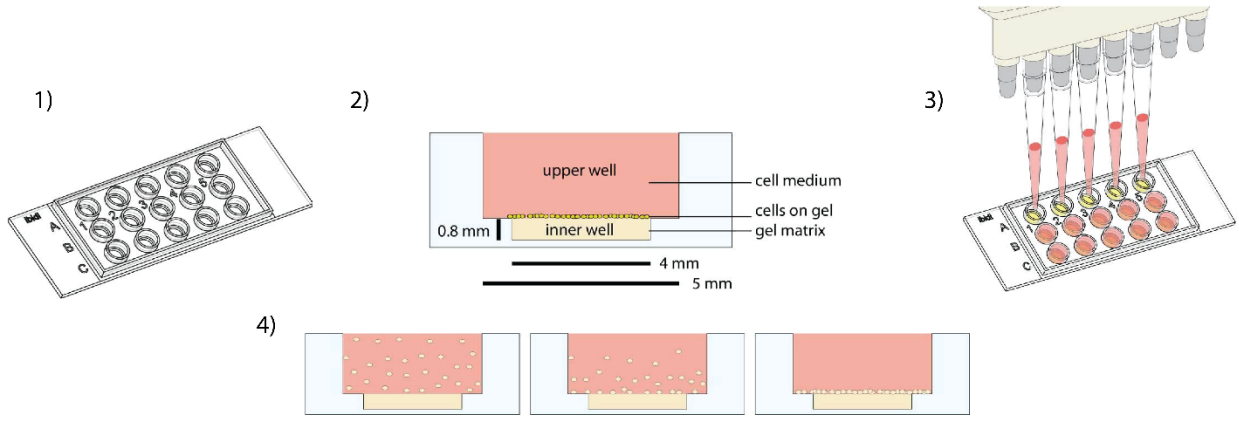
IBIDI dish comes with the culture insert already pre-installed (1), HUVECs were seeded with $5x10^5$ cells/mL and the final volume in each insert chamber was 70 μl (2). After 24 hours the culture insert was removed (3) and the rest of the dish was being filled with F-12K medium (4) (picture source: www.ibidi.com).

3.7 Angiogenesis assay.

Angiogenesis is a critical process during tissue development and wound healing (74). *In vitro* angiogenesis is a fast, reliable and quantifiable technique to evaluate angiogenesis. Since angiogenesis is also linked to various pathological conditions such as cancer, psoriasis, diabetic retinopathy, arthritis, asthma, autoimmune disorders, infectious diseases, and atherosclerosis it is critical to have the right tools to access this complex biological phenomenon (74, 161-163). The most critical limitation in this *in vitro* angiogenesis assay is that endothelial cells are not interacting with smooth muscle cells or pericytes that are normally surrounding the endothelial cells throughout the human body.

Based on previous published results on endothelial cell migration critical concentrations of ACNs, PAs and ACNs:PAs used in this study were (60 μg/mL for ACNs, 0.002 μg/mL, 60 μg/mL and 120 μg/mL for PAs and 8 μg/mL and 60 μg/mL for ACNs:PAs). Endothelial cells ($1x10^4$ cells/well) were plated and cultured on Matrigel (BD Biosciences) that will be applied on an IBIDI μ-slide Angiogenesis plate (Ibidi, Martinsried, Germany) and incubated at 37 °C and 30 min for gel construction (Figure 14). With this method, cells will be induced to form capillary-like tubes. After exposing the cells to a 4 h treatment of anthocyanin, phenolics and combinations of both fractions, including a control (untreated cells), the effect on the tube formation was photographed by using an inverted phase-contrast optical microscope with 4x objective (Nikon, TS100) after 4 h based on the treatment. The **a.** number of meshes **b.** total meshes area **c.** number of nodes **d.** number of muster junctions and **e.** total master segment length was measured and analyzed with the computer program Image J with the Angiogenesis Analyzer plugin (74, 164).

Figure 14: Steps of angiogenesis assay.



µ-Slide Angiogenesis (1), cross section of one well (2), seeding HUVECs (1x10^4 cells/well) (3) and adhesion process of cells (4) (picture source: www.ibidi.com).

Figure 15: Parameters of Angiogenesis measured.

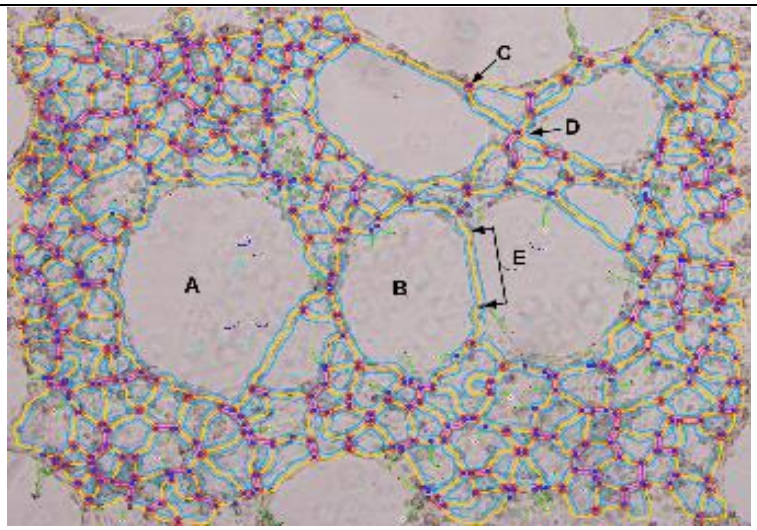


Image J with the Angiogenesis Analyzer plugin and five parameters were evaluated for the tube network integrity (right). **a.** number of meshes **b.** total meshes area **c.** number of nodes **d.** number of master junctions and **e.** total master segment length (Figure 15).

3.8 Gene expression, real-time RT-PCR analysis.

Endothelial cells were cultured and maintained as described previously. HUVECs were treated with ACNs, PAs and combination of both bioactive compounds for 2 h and 6 h. mRNA was isolated using the RNeasy Kit (Qiagen) and DNase Digestion (Qiagen) was also used for RNA purification. QuantiTect Reverse Transcription Kit (Qiagen) was used for cDNA synthesis and removal of genomic DNA. A two-step RT-PCR was followed using a CFX96 (BioRad) PCR system. A 20 µl PCR reaction volume was performed using TaqMan gene expression master mix (Invitrogen) and TaqMan primers (Invitrogen): RHOA (Hs00357608_m1), RAC1 (Hs01902432_s1), VEGFA (Hs00900055_m1), NOS3 (Hs01574665_m1), AKT1 (Hs00178289_m1) and GAPDH (Hs99999905_m1).

3.9 Immunoprecipitation (IP) and immunoblot analysis.

For immunoprecipitation studies, cells were lysed using the Cell Signaling active Rho detection kit (8820) and active Rac1 detection kit (8815) following manufacturer's instructions. To ensure the immunoprecipitation procedures were working properly, the samples were treated with GTPγS (positive control) and GDP (negative control). Moreover, in the 1X Lysis/Binding/Wash Buffer, phenylmethylsulfonyl fluoride (PMSF) (Cell Signaling, 8553) was used at a concentration of 1 mM. For the detection of Akt1, VEGF, eNOS and β-tubulin, total protein was extracted from the cells in RIPA lysis and extraction buffer (Thermo Fisher, 89901) supplemented with protease/phosphatase inhibitor cocktail (Cell Signaling, 5872). The concentration of total protein in these samples was measured using a BCA assay (Thermo Fisher,

23225). Immunoprecipitated materials and total protein samples were resolved by 4 - 20 % mini-protean TGX stain-free protein gel electrophoresis (BioRab, 4568094). After transfer using Trans-Blot Turbo System (BioRad, 1704150), LF-PVDF membranes (BioRad, 1704274) were blotted with anti-Rac1 (1:1000, Cell Signaling, 8631), anti-RhoA (1:667, Cell Signaling, 8789), anti-Akt1 (1:1000, Cell Signaling, 2938), anti-Phospho-Akt1 (1:1000, Cell Signaling, 9018), anti-Phospho-Akt1 (1:5000, Abcam, ab81283), anti-VEGF (1 μg/mL, Thermo Fisher, PA5-16754), anti-VEGF (5 μg/mL, Abcam, ab1316), anti-VEGF (1 μg/mL, Abcam, ab46154), anti-eNOS (1:1000, Cell Signaling, 9572), anti-eNOS (1 μg/mL, BD Biosciences, 612392), anti-Phospho-eNOS (1:1000, Cell Signaling, 9571), anti-Phospho-eNOS (1:500, Abcam, ab184154) and anti-β-Tubulin (1:1000, Cell Signaling, 2128). Proteins were detected with antibodies specific for either mouse or rabbit IRDye® 800CW Goat anti-Mouse IgG, (1:15000, Li-COR, 925-32310) and IRDye® 800CW Goat anti-Rabbit IgG, (1:15000, Li-COR, 925-32211) using the Li-COR Odyssey imaging system (Li-COR Biosciences). In other cases, either anti-Mouse IgG, HRP linked antibody (1:1000, Cell Signaling, 7076) or anti-Mouse IgG, HRP linked antibody (1:1000, Cell Signaling, 7074) was used along with the Syngene, G:Box imaging system (Syngene: Cambridge, UK). Moreover, for western blot analysis VEGFA positive control was used (Abcam, ab55566 and R&D Systems, 293-VE-010). All assays were repeated at least four times in independent experiments.

3.10 Statistical analysis.

Statistical analysis was performed using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA). For the cell migration experiments, the data were analyzed by one-way ANOVA. For post-hoc comparisons Fisher's least significant difference (LSD) test was

used. For the cell migration experiments, the control included 10 replicates (n=10) and each treatment group (ACNs, PAs and ACNs:PAs) included 7 replicates (n=7). For the tube formation/angiogenesis experiments, the data were analyzed by one-way ANOVA. For post-hoc comparisons Fisher's least significant difference (LSD) test was used. Each experiment was repeated four times (n=4) and each independent sample per group was tested four times (n=4). For the gene expression and western blot experiments a two-tailed Mann-Whitney U-test was performed to compare the control to ACNs while one-way ANOVA was used for the PAs and combination (ACNs:PAs) groups. For post-hoc comparisons Fisher's least significant difference (LSD) test was used. For gene expression experiments the control included 10 replicates (n=10) and each treatment group (ACNs, PAs and ACNs:PAs) included 10 replicates (n=10). For the IP experiments the control included 4 replicates (n=4) and each treatment group (ACNs, PAs and ACNs:PAs) included 4 replicates (n=4). All data in the graphs are expressed as mean ± SEM. A p-value of <0.05 was considered significant.

CHAPTER 4

RESULTS

4.1 Wild Blueberry profile analysis.

HPLC profile analysis of the ACN fraction is reported in Table 3 and Figure 15. The total ACN concentration was 45.11 ± 0.35 mg/mL with 15 different ACNs detected. Malvidin glucosides were higher in concentration (26.5 %) followed by malvidin galactoside (14.8 %) while delfinidin glucoside (8.9 %), petunidin glucoside (8.2 %) and cyanidin glucoside (7.4 %) followed. The PA fraction contained mainly chlorogenic acid (10.23 ± 1.8 mg/mL) with traces of ferulic and caffeic acids (Figure 16).

Table 3: Characterization of the ACN fraction extracted from the wild blueberry powder (*V. angustifolium*).

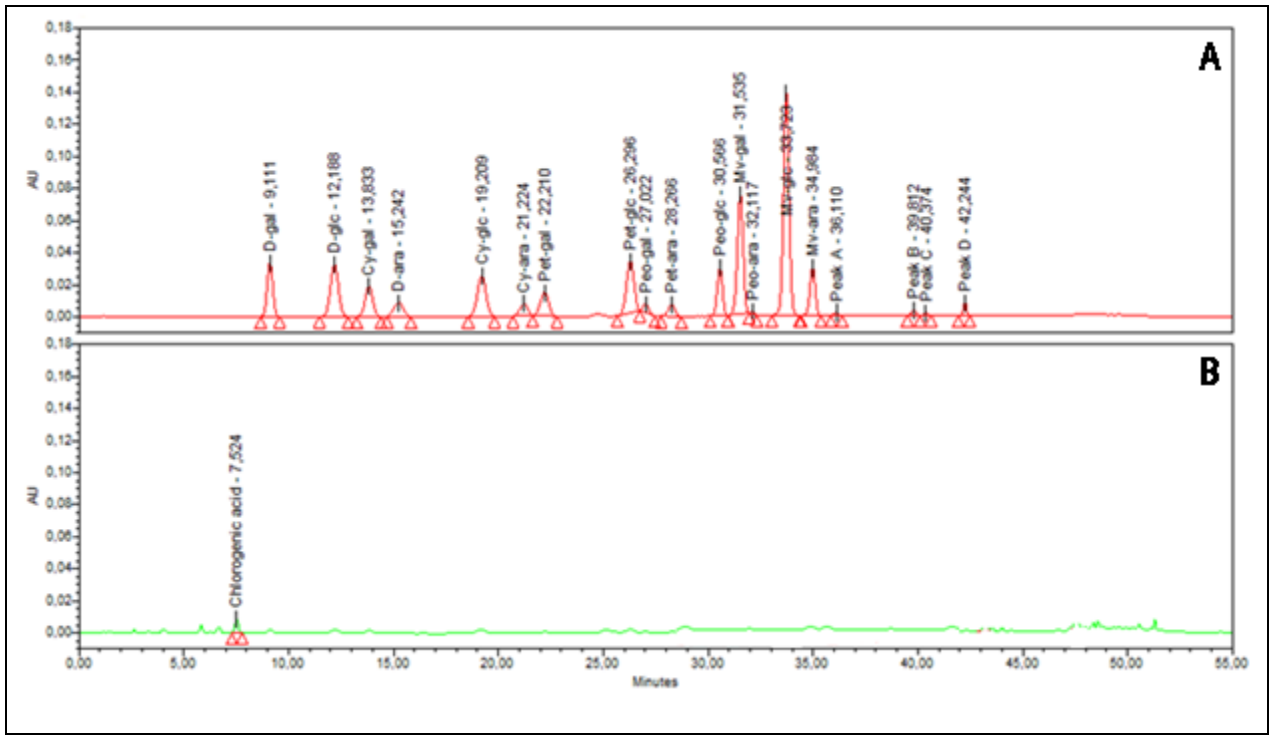
Peak	Name	Mean* (µg/mL)	SD
1	D-gal	7.1	0.14
2	D-glc	10.005	0.13
3	Cy-gal	5.3	0.14
4	D-ara	2.885	0.02
5	Cy-glc	8.415	0.02
6	Pet-gal	2	0.14
7	Cy-ara	4.6	0.00
8	Pet-glc	9.365	0.09
9	Peo-gal	1.1	0.00
10	Pat-ara	1.75	0.07
11	Peo-glc	5.845	0.36
12	Mv-Gal	16.85	0.07
13	Peo-ara	0.09	0.01

Table 3 Continued

14	Mv-glc	30.05	0.07
15	Mv-ara	6	0.00
Peak A	Dp-glc-ac	0.19	0.01
Peak B	Cy-glc-ac	0.38	0.03
Peak C	Pt-glc-ac	0.1	0.00
Peak D	Mv-gal-ac	0.75	0.07
	Total	45.11	0.35

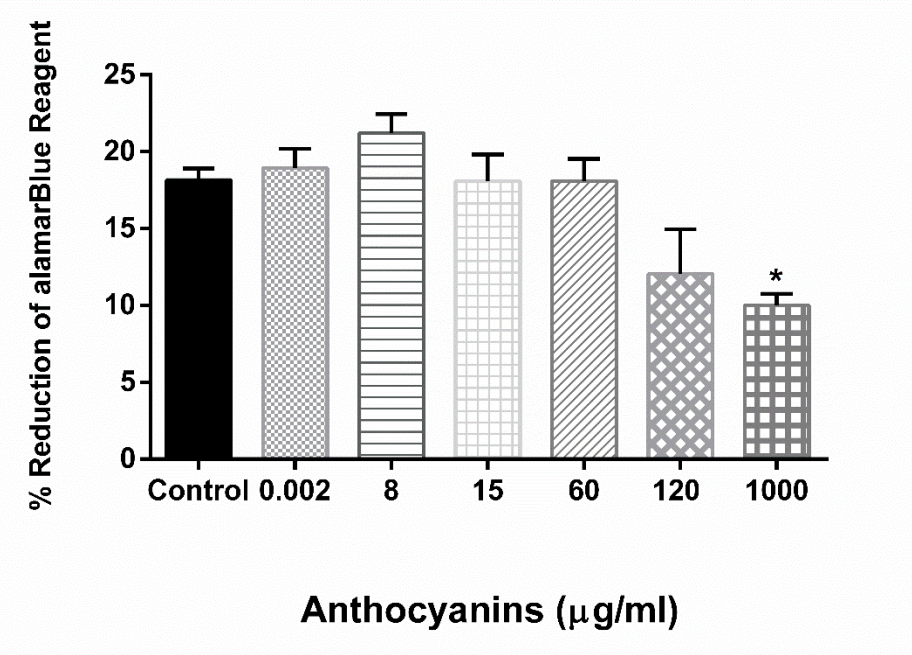
* Mean ± standard deviation of *ara* arabinoside, *gal* galactoside and *glc* glucoside.

Figure 16: HPLC profile analysis of the ACN (panel A) and PA (panel B) fractions of wild blueberry (*V. angustifolium*) powder.



The HUVEC proliferation rate was calculated to seed the appropriate number of cells in the 96-well plate for the cell cytotoxicity assay. Quadruplicate samples were used for each different concentration of ACNs and PAs. Results after twenty-four hours of treatment with ACNs and PAs are presented in Figure 17.

Figure 17: AlamarBlue cytotoxicity assay.



Percent reduction of alamarBlue reagent after twenty-four hours of treatment with different concentrations of the ACN fraction. Treatment with ACNs at 1000 μg/mL inhibited endothelial cell proliferation rate compared to the control (*: $p \leq 0.05$)

After twenty-four hours of treatment with ACNs, no significant difference in proliferation rates were detected for the HUVECs at the concentrations tested compared to the control (Figure 4). However, inhibition of the proliferation rate of endothelial cells treated with the highest

concentration of ACNs (1000 μg/mL) was documented to be significantly different from the control (Figure 17).

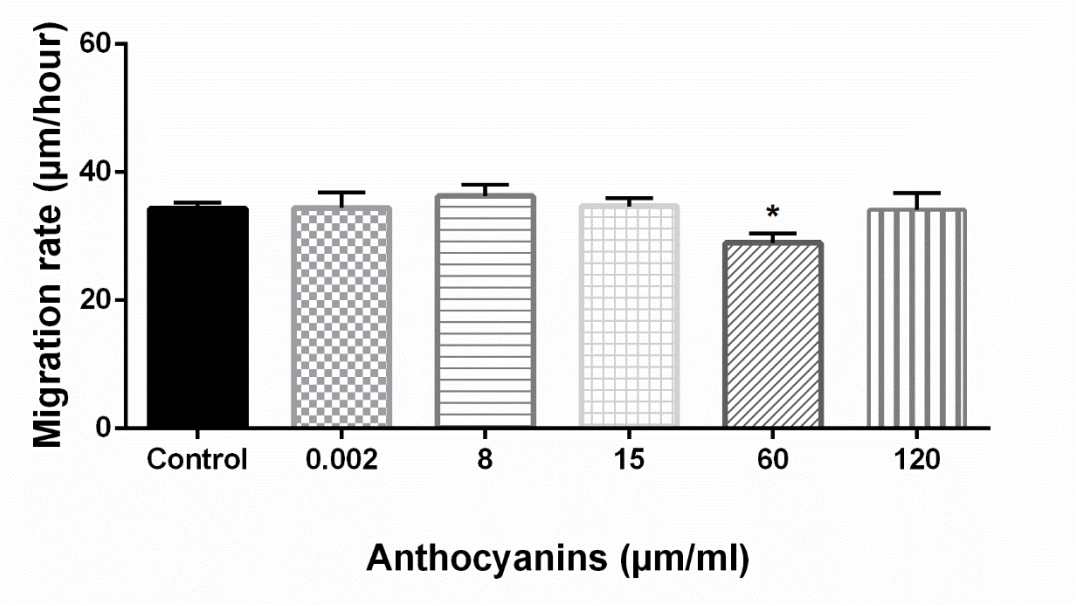
Similarly, exposure of HUVECs for twenty-four hours to the PA fraction did not show any significant differences in HUVEC proliferation rate at the concentrations tested (data not shown).

Exposure of HUVECs to the above ACN and PA fractions concentrations for seventy-two hours documented no statistically significant difference on cell proliferation (data not shown).

4.2 Cell migration assay.

After determining the appropriate concentrations of ACN and PA fractions that were not cytotoxic to the HUVECs, the cell migration assay was performed to evaluate the effect of five (0.002 μg/mL, 8 μg/mL, 15 μg/mL, 60 μg/mL and 120 μg/mL) concentrations of ACNs, PAs and their combination (ACNs:PAs). After treatment with ACNs (Figure 18), endothelial cell migration speed was reduced at the 60 μg/mL compared to control ($p \leq 0.05$). No statistically significant differences in cell migration were detected at ACN concentrations of 0.002 μg/mL, 8 μg/mL, 15 μg/mL and 60 μg/mL.

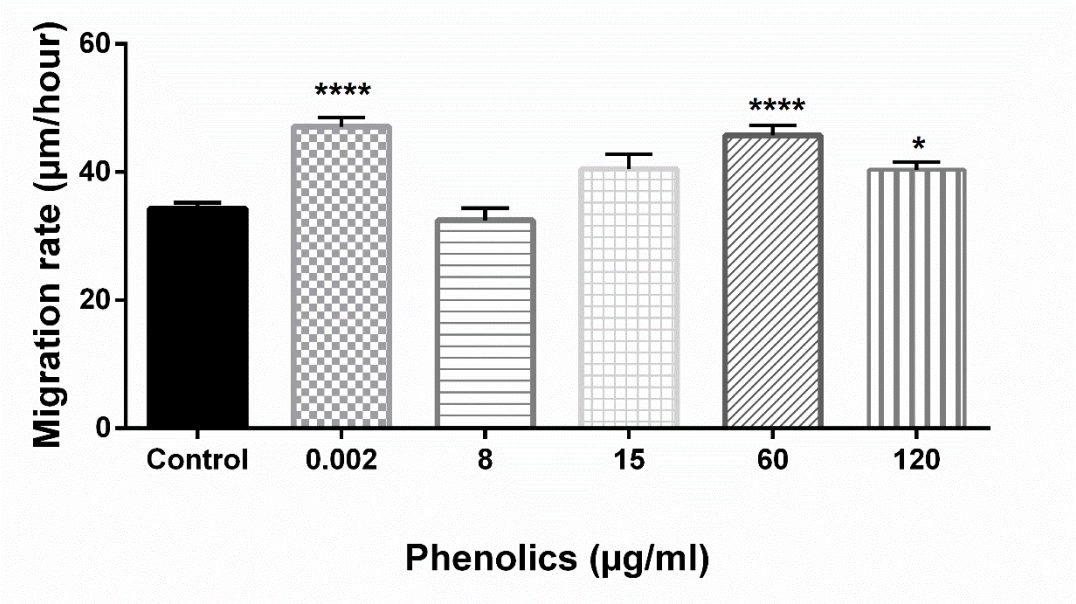
Figure 18: Migration rate of endothelial cells after acute exposure to ACNs.



Endothelial cells were treated with ACNs. Treatment with ACNs at 60 µg/mL inhibited endothelial cell migration rate compared to the control (*: $p \leq 0.05$)

Endothelial cell migration was promoted after treatment with PAs (Figure 19). Exposure of the endothelial cells at 0.002 µg/mL, 60 µg/mL ($p \leq 0.0001$) and 120 µg/mL ($p \leq 0.01$) significantly increased the speed of endothelial cell migration compared to control.

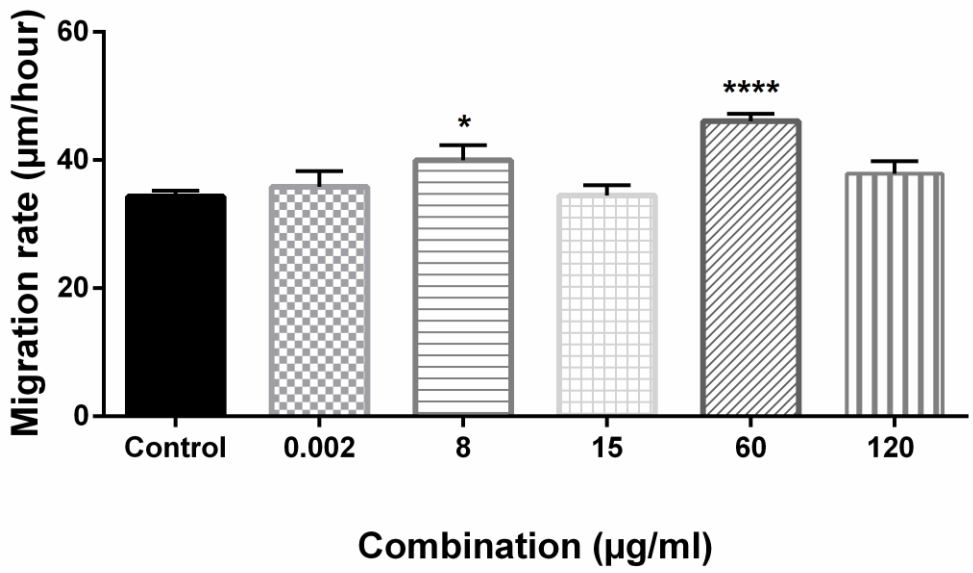
Figure 19: Migration rate of endothelial cells after acute exposure to PAs.



Endothelial cells were treated with PAs. Treatment with PAs at 0.002 µg/mL, 60 µg/mL (****: $p \leq 0.0001$) and 120 µg/mL (**: $p \leq 0.01$) increased endothelial cell migration rate compared to control.

Additionally, combination of both ACNs and PAs (ACNs:PAs) (Figure 20) at 8µg/mL:8µg/mL and 60µg/mL:60µg/mL respectively, significantly increased endothelial cell migration rate compared to control. Statistical differences among groups were not detected at any of the other tested ACN:PA combinations.

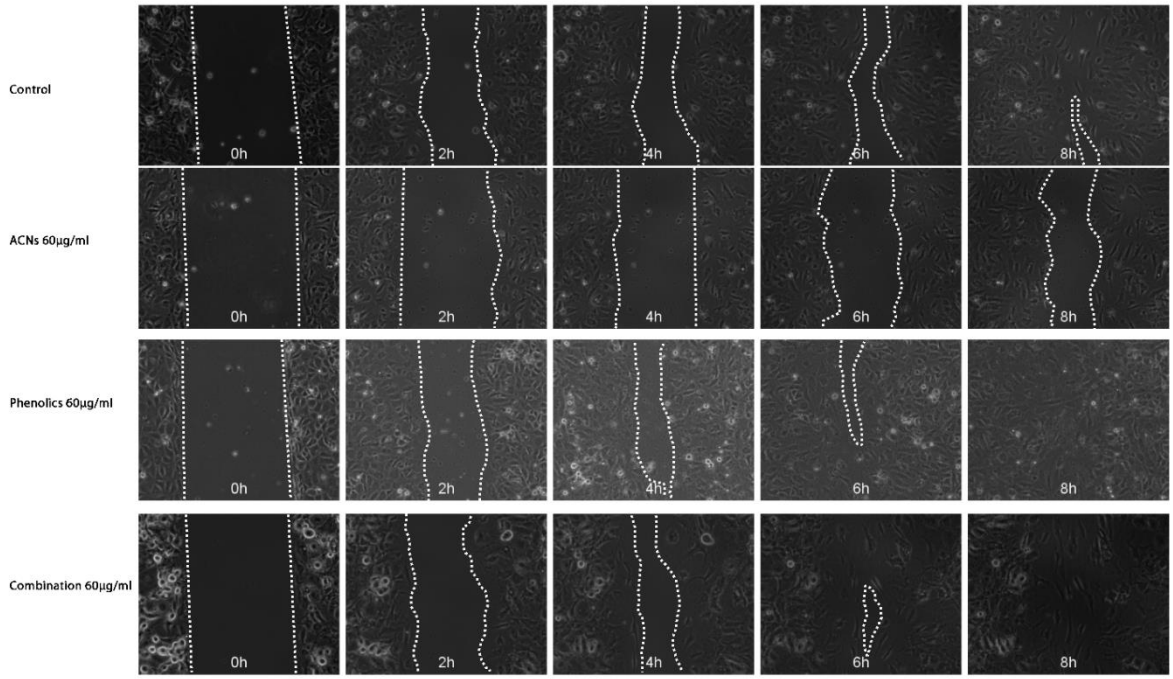
Figure 20: Migration rate of endothelial cells after acute exposure to ACNs:PAs.



Endothelial cells were treated with combination of both ACNs and PAs. Treatment with ACNs:PAs 8 µg/mL:8 µg/mL (*: $p \leq 0.05$) and ACNs:PAs 60 µg/mL:60 µg/mL (***: $p \leq 0.001$) increased endothelial migration rate compared to control.

Visual depictions of cell migration from all treatments of ACNs, (60 µg/mL), PAs (60 µg/mL) and combination of both fractions (60 µg/mL:60 µg/mL) (Figure 21) were observed by in captured time-lapse video frames from exposure of HUVECs at different time points (0 h, 2 h, 4 h, 6 h and 8 h). In this montage, pictures were taken from the time-lapse video at the above-mentioned time-points and the cell migration border was marked with white dotted lines so that the progression of endothelial cell migration towards the empty area can be clearly observed.

Figure 21: Captured frames from time-lapse video from time-points at 0 h, 2 h, 4 h, 6 h and 8 h of HUVEC migration after acute exposure to ACNs, PAs and ACNs:PAs.

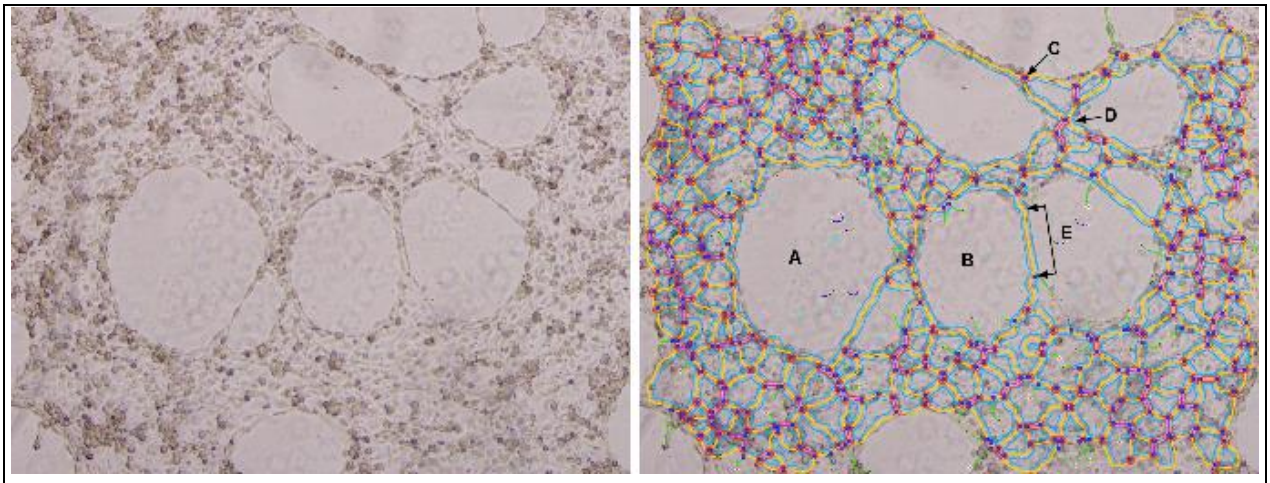


Summary of all treatments (ACNs 60 µg/mL and PAs 60 µg/mL) that altered endothelial cell migration rate (magnification is 10x).

4.3 Angiogenesis assay.

Tube formation *in vitro* assay was first standardized for the HUVEC cell line. After determining the appropriate cell density and incubation time experiments were performed in quadruplicates for each treatment. Analysis from the tube formation assay pictures are presented in Figure 22.

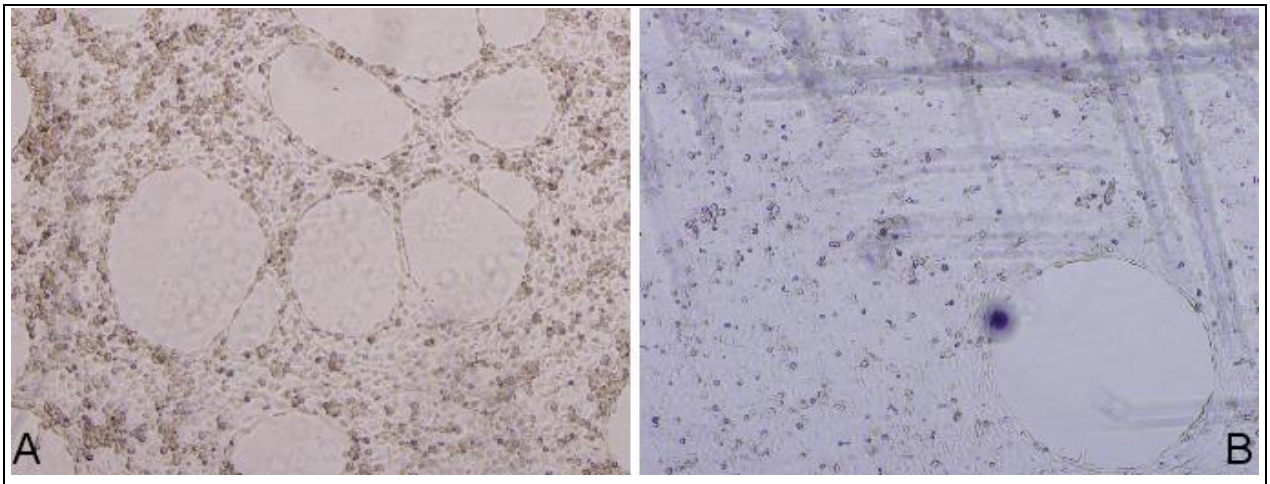
Figure 22: Untreated (control) HUVECs. Before (left) and after (right) analysis with Image J Angiogenesis Analyzer plugin.



Picture was captured (objective 4x) after four hours of cells plated in the well. The initial picture (left) was analyzed with Image J with the Angiogenesis Analyzer plugin and five parameters were evaluated for the tube network integrity (right). **a.** number of meshes **b.** total meshes area **c.** number of nodes **d.** number of master junctions and **e.** total master segment length (Figure 22).

Cells treated with ACNs at 60 μg/mL were not able to form a complete tube network after four hours of incubation time (Figure 23).

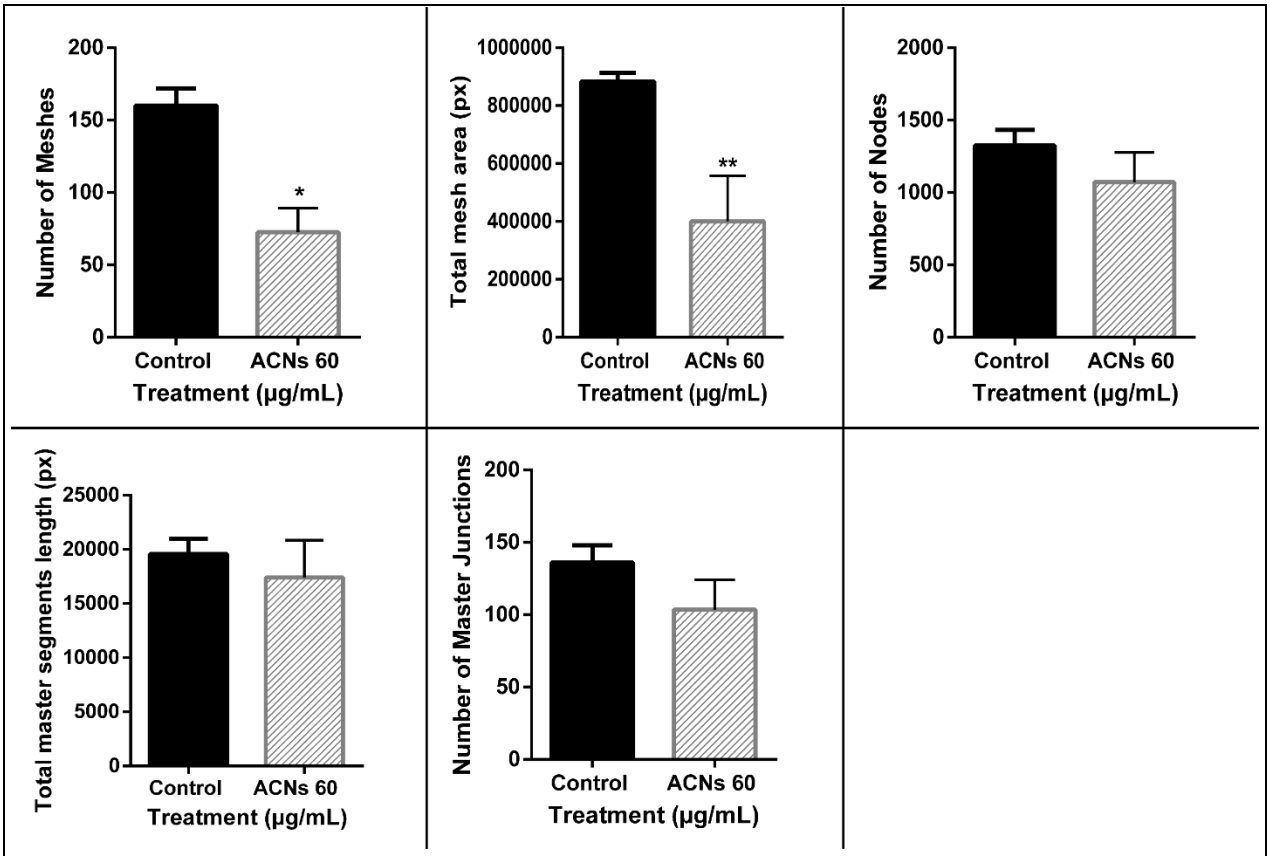
Figure 23: Untreated HUVECs (A) and HUVECs treated with 60 μg/mL of ACNs (B).



Objective 4x was used

Only two parameters (number of meshes and total mesh area) from the five tested documented decreased numbers compared to the control (Figure 24). However, the number of the meshes and the area of the mesh are critical and of high importance for the formation of a complete endothelial tube network.

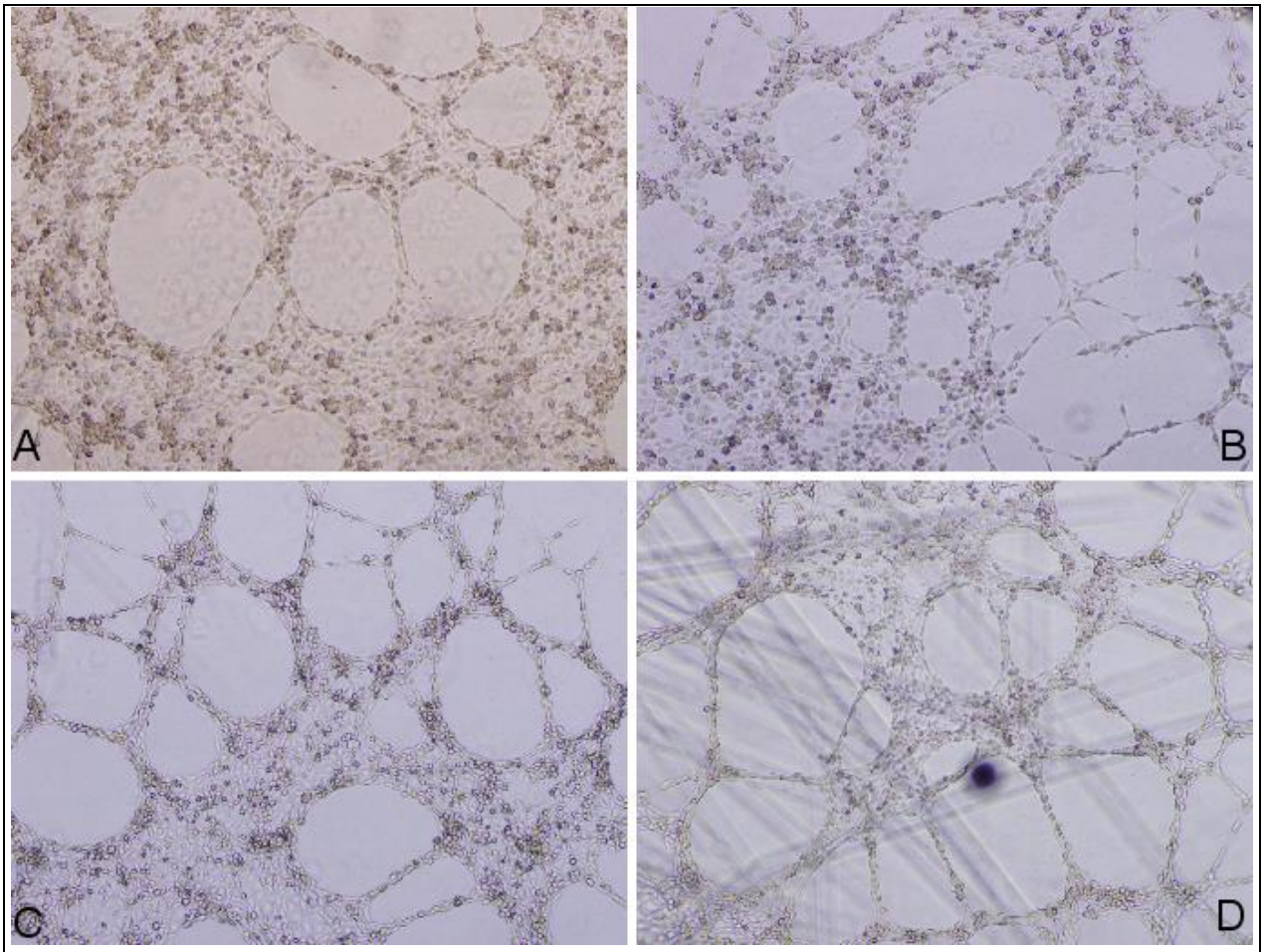
Figure 24: Parameters of endothelial tube formation integrity after cells were treated with ACNs at 60 µg/mL.



Treatment with ACNs at 60 µg/mL documented decreased number of meshes (*: $p \leq 0.05$) and area of meshes (**: $p \leq 0.01$) compared to the control.

Cells treated with PAs at 0.002 μg/mL, 60 μg/mL and 120 μg/mL were able to form a complete tube network that was also quantified for further analysis (Figure 25).

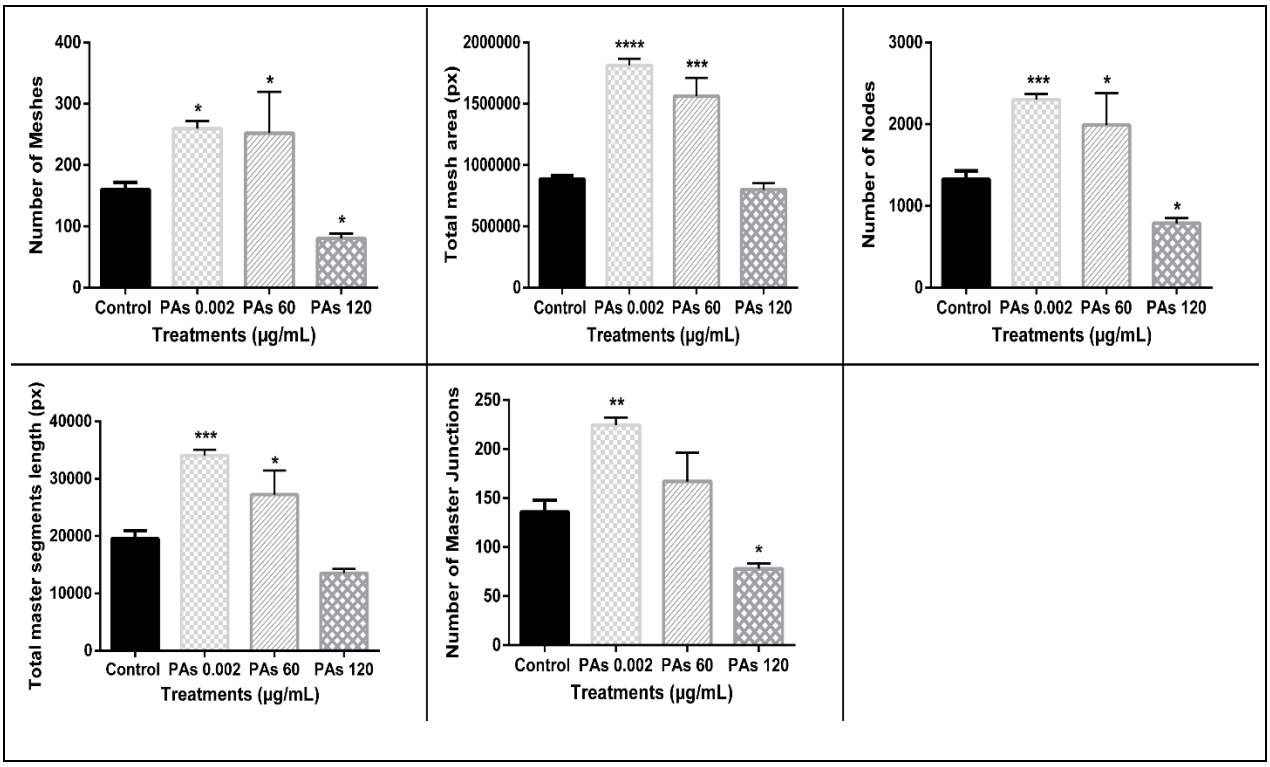
Figure 25: Untreated HUVECs (A), HUVECs treated with PAs at 0.002 μg/mL (B), 60 μg/mL (C) and 120 μg/mL (D).



Objective 4x was used

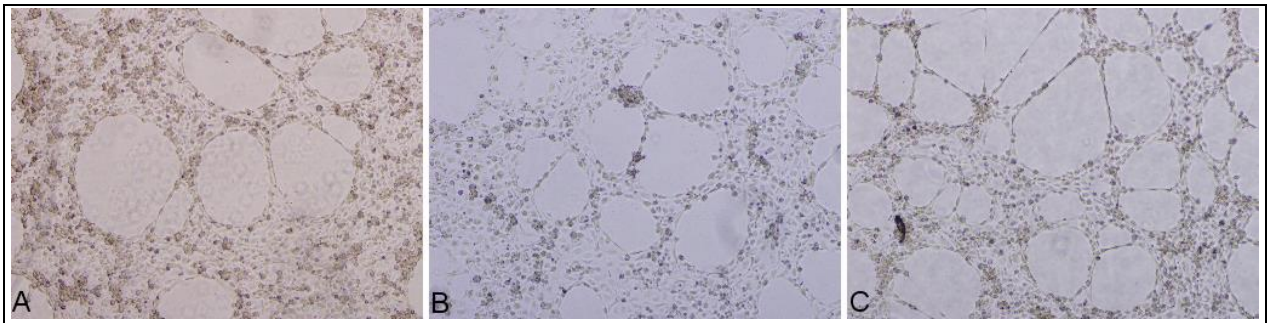
PAs at 0.002 μg/mL increased all the parameters measured for endothelial tube formation compared to the control. PAs at 60 μg/mL increased all the parameters but one (the number of the master junctions) compared to the control. Treatment with PAs at 120 μg/mL documented decreased number of meshes, nodes and number of master junctions compared to the control. PAs at the lowest concentration tested documented the highest effect on the endothelial tube network (Figure 26). Increased number of meshes (*: $p \leq 0.05$), area of the mesh (****: $p \leq 0.0001$), number of nodes (***: $p \leq 0.001$), total master segment length (***: $p \leq 0.001$) and number of master junctions (**: $p \leq 0.01$) compared to the control (Figure 25). Moreover, PAs at 60 μg/mL increased number of meshes (*: $p \leq 0.05$), area of the mesh (***: $p \leq 0.001$), number of nodes (*: $p \leq 0.05$) and number of master junctions (*: $p \leq 0.05$) compared to the control while no effect was documented on the number of the master junctions (Figure 26). However, PAs at 120 μg/mL had opposite results compared to the lower concentrations of PAs. PAs at 120 μg/mL decreased the number of the meshes (*: $p \leq 0.05$) and the number of nodes (*: $p \leq 0.05$) as well as the number of the master junctions (*: $p \leq 0.05$) compared to the control (Figure 26) while no effect was documented on the mesh area and total master segment length compared to the control (Figure 26).

Figure 26: Parameters of endothelial tube formation integrity after cells were treated with PAs at 0.002 µg/mL, 60 µg/mL and 120 µg/mL.



Finally, cells treated with combination of both ACNs and PAs managed to form a complete endothelial network (Figure 27).

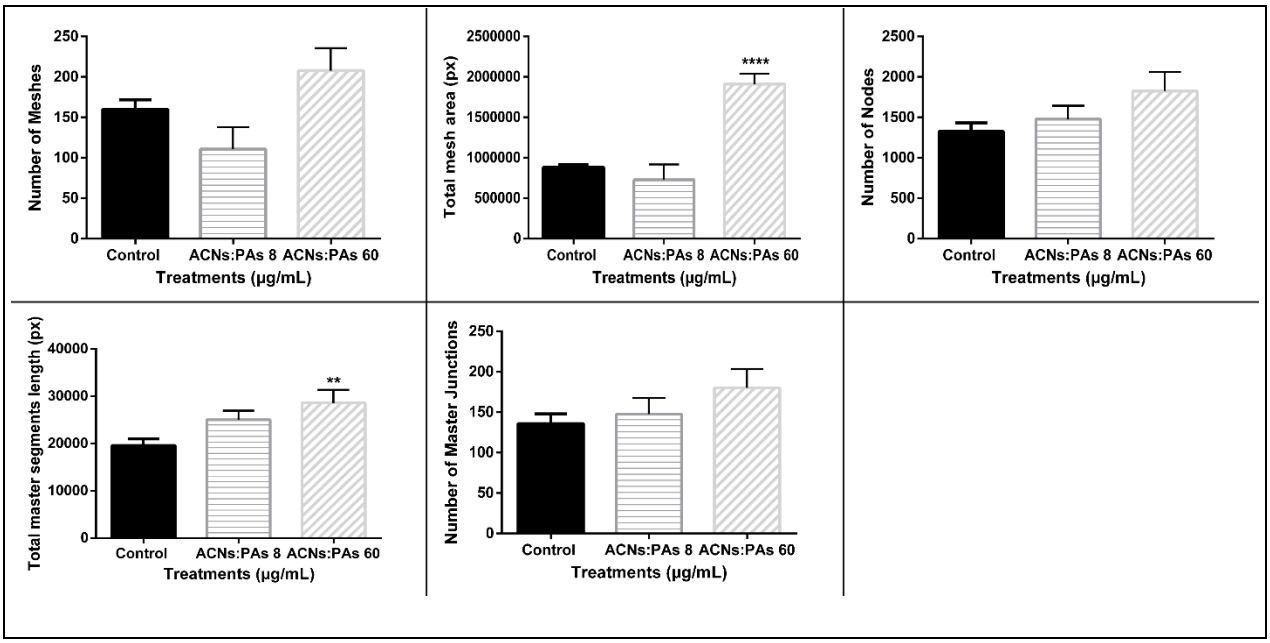
Figure 27: Untreated HUVECs (A), HUVECs treated with ACNs:PAs at 8 µg/mL (B) and 60 µg/mL (C).



Objective 4x was used

Parameters of endothelial tube formation integrity after cells treated with combination of both ACNs and PAs at 8 µg/mL and 60 µg/mL. ACNs:PAs at 60 µg/mL increased the total mesh area (****: p≤0.0001) and the total master segment length (**: p≤0.01) compared to the control (Figure 28).

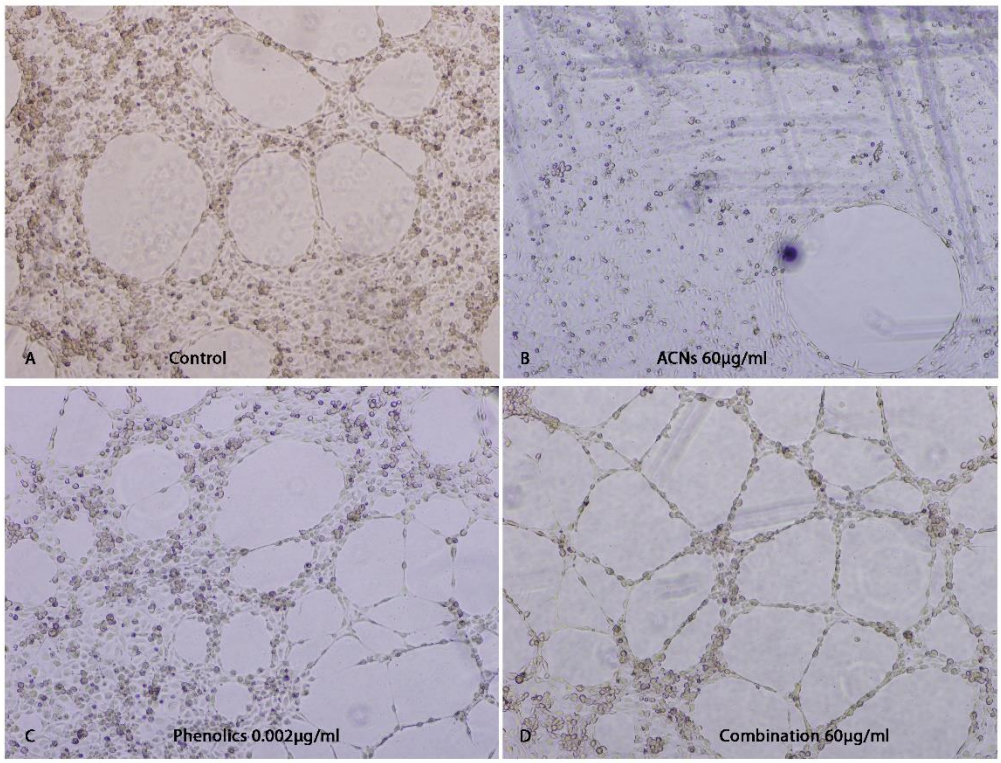
Figure 28: Parameters of endothelial tube formation integrity after cells were treated with combination of both ACNs and PAs at 8 µg/mL and 60 µg/mL. ACNs:PAs at 60 µg/mL.



Combination of both ACNs and PAs at 8 µg/mL documented no statistical significant differences compared to the control on any of the five parameters of endothelial tube network integrity. However, ACNs:PAs at 60 µg/mL increased the total mesh area (****: p≤0.0001) compared to the control without having an effect on the number of meshes. Moreover, the total master segment length (**: p≤0.01) was increased compared to the control.

A summary from tube formation assay can be observed from all treatments of ACNs, (60 µg/mL), PAs (60 µg/mL) and combination of both fractions (60 µg/mL:60 µg/mL) (Figure 29). Pictures were obtained after four hours of treatment.

Figure 29: Summary of the three different treatments at the same concentration (A: control, B: ACNs at 60 µg/ml, C: PAs at 0.002 µg/ml and D: ACNs:PAs at 60 µg/ml).



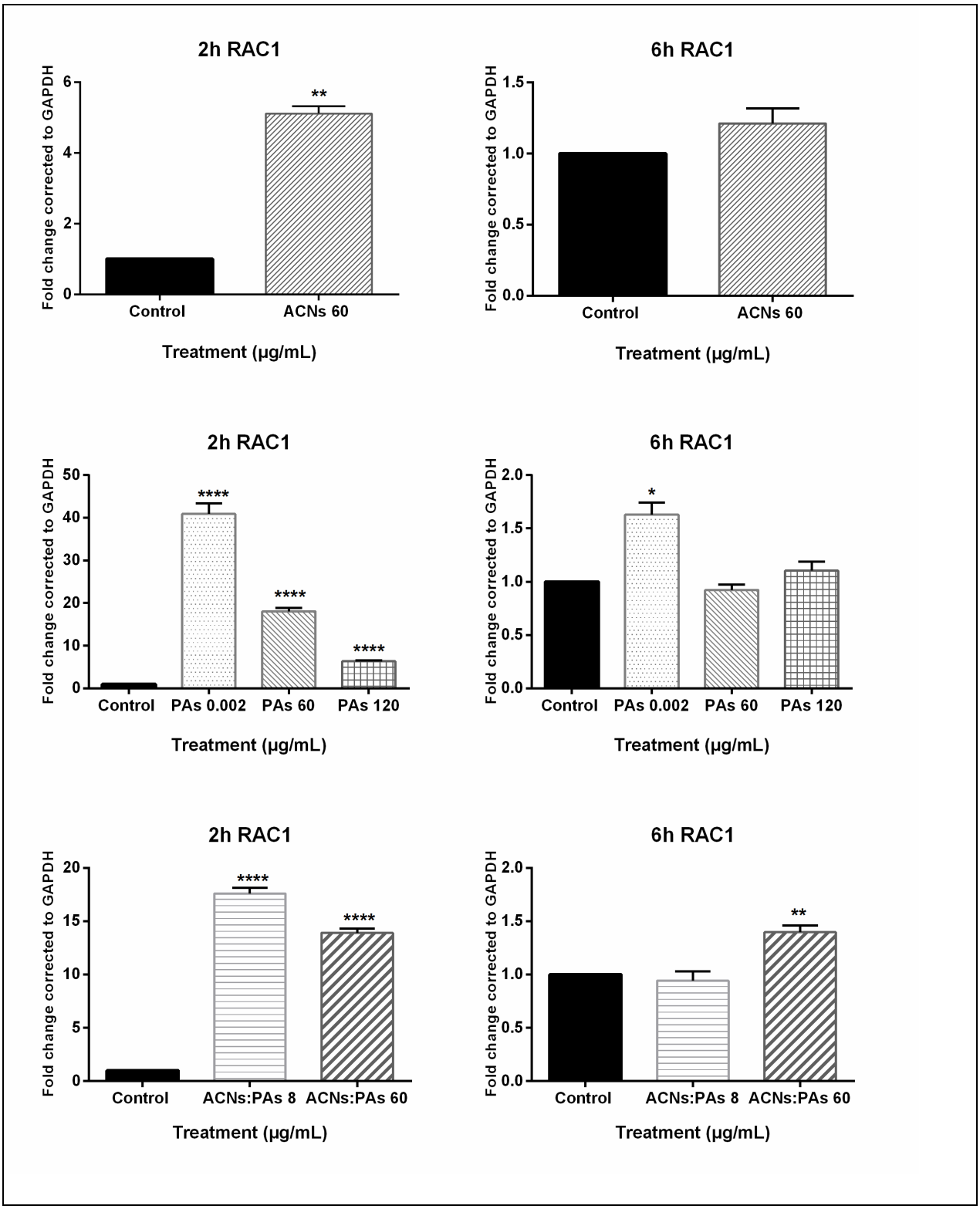
Objective 4x was used

4.4 Gene expression and IP for RAC1 and RHOA.

After determining the ACNs, PAs and ACNs:PAs concentrations that demonstrated a significant effect on endothelial cell migration, gene expression and IP experiments were pursued to determine whether there was a change in RAC1 and RHOA gene expression and

protein levels. Concentrations tested were ACNs at 60 μg/ml, PAs at 0.002 μg/ml, 60 μg/ml and 120 μg/ml and ACNs:PAs at 8 μg/ml and 60 μg/ml. Gene expression for RAC1 was tested at two and six hours after treatment (Figure 30). After 2 hours of HUVEC exposure to ACNs, gene expression of RAC1 increased almost fourfold compared to control but was not significant after 6 hours of exposure. Similarly, exposure to PAs for 2 hours significantly upregulated RAC1 expression compared to control, at all concentrations tested. Longer exposure time (6 hours) significantly increased RAC1 expression at 0.002 μg/ml only.

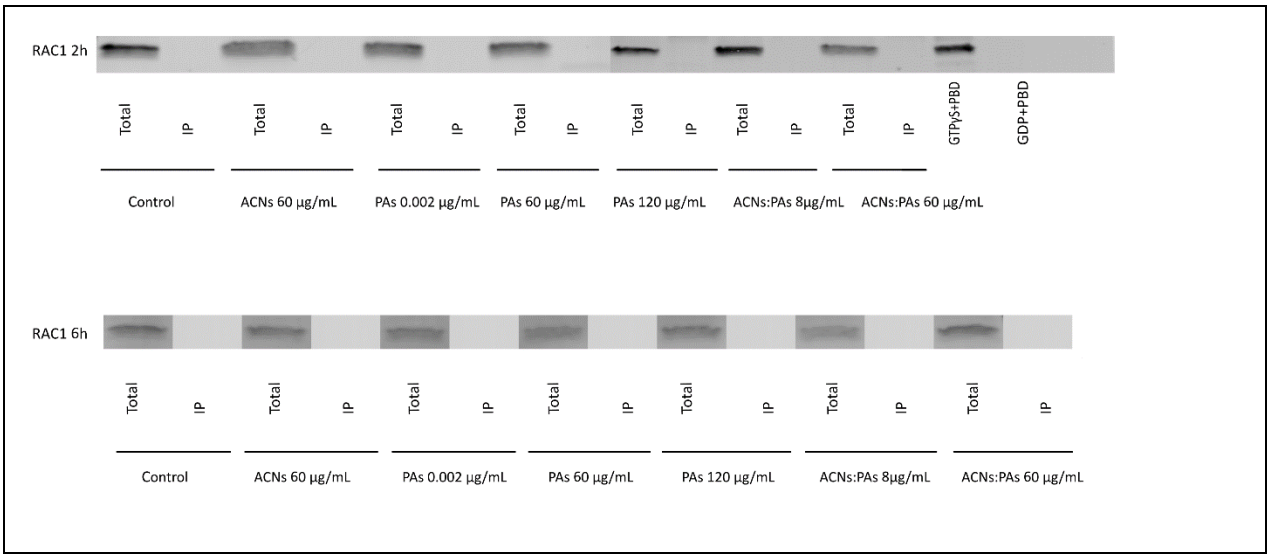
Figure 30: Gene expression of RAC1 after acute treatment (2 and 6 hours) of ACNs, PAs and ACNs:PAs.



Gene expression for RAC1 was increased compared to the control for all tested concentration only two hours after treatment, ACNs 60 μg/mL (**: p≤0.01), PAs at 0.002 μg/mL (****: p≤0.0001), 60 μg/mL (****: p≤0.0001) and 120 μg/mL (****: p≤0.0001) and ACNs:PAs 8μg/mL and 60 μg/mL (****: p≤0.0001) while six hours post treatment PAs at 0.002 μg/mL (*: p≤0.05) and ACNs:PAs at 60 μg/mL (**: p≤0.01) still documented an increased gene expression compared to the control.

Immunoprecipitation for RAC1 was conducted following the appropriate protocol according to the manufacturer and the positive and negative controls indicates that the procedure was performed accurately, the samples underwent the process of IP did not detecting any RAC1 (Figure 31).

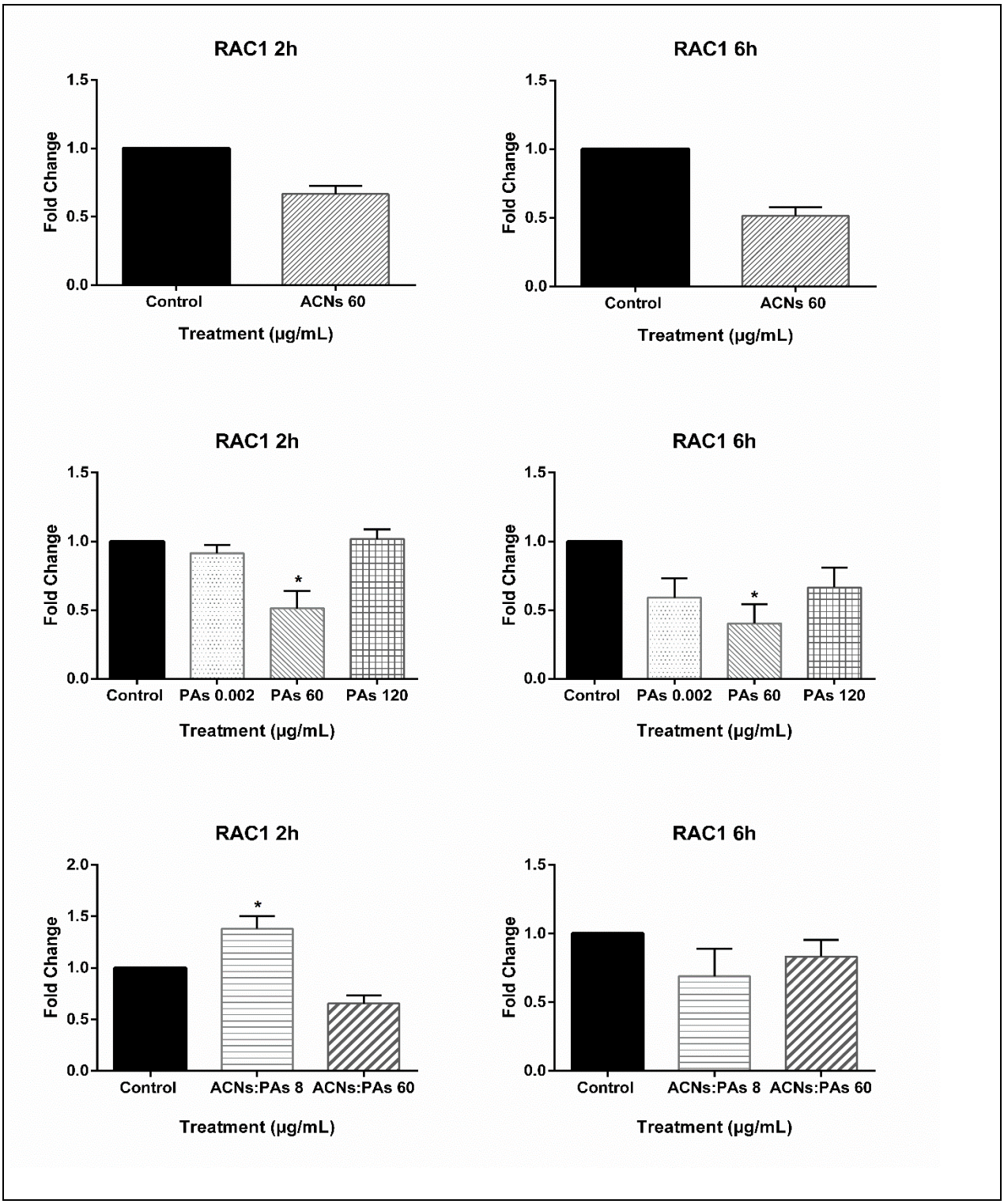
Figure 31: Total and active form of RAC1 after acute treatment (2 and 6 hours) of ACNs, PAs and ACNs:PAs.



Quantitative western blot analysis of total RAC1 documented inhibition of total RAC1 when cells were treated with PAs at 0.002 μg/ml after 2 h and 6 h of treatment. ACNs:PAs at 8

μg/ml documented an increased level of total RAC1 after 2 h without documenting the same effect after 6 h (Figure 32).

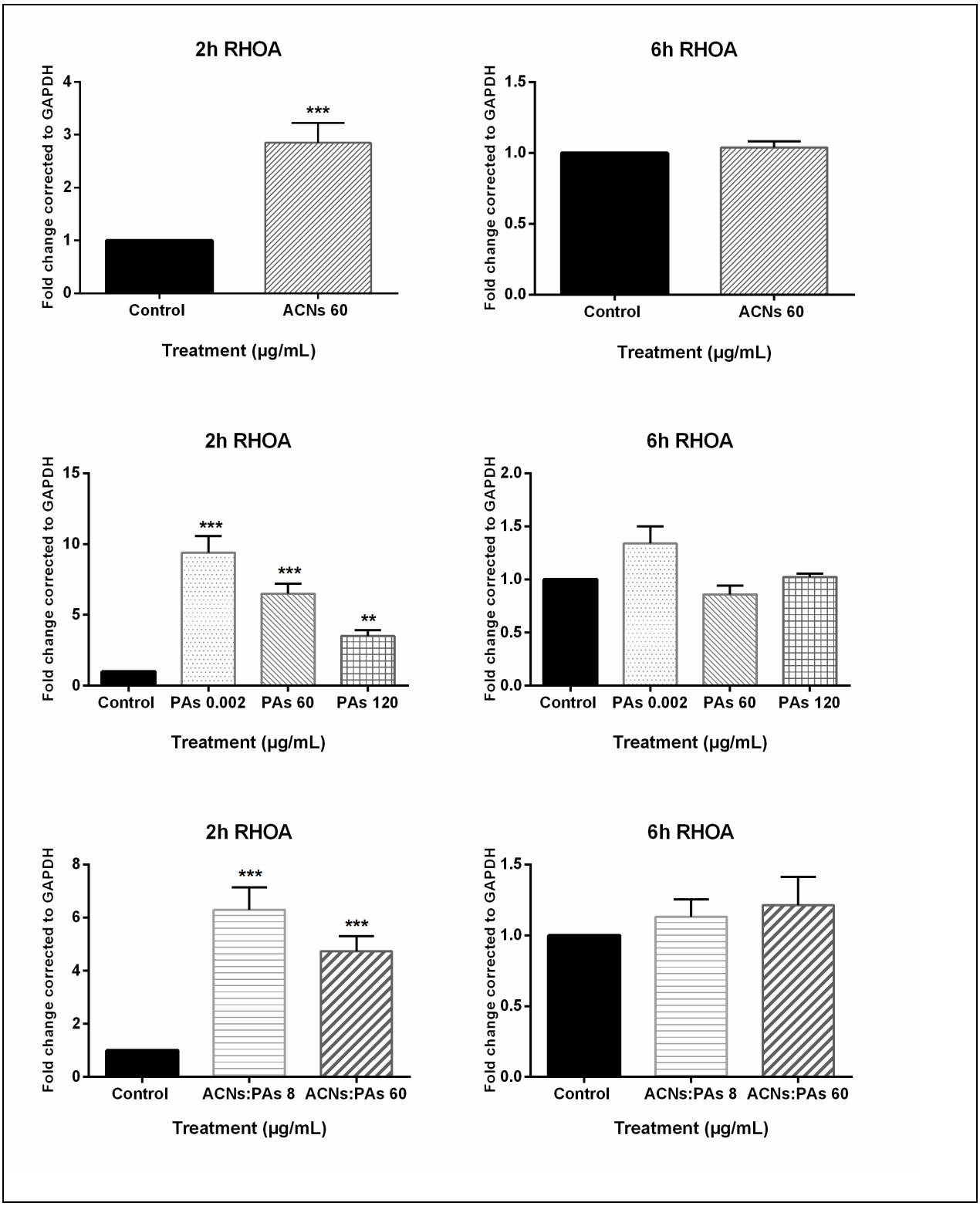
Figure 32: Quantitative analysis of total form of RAC1 after acute treatment (2 and 6 hours) of ACNs, PAs and ACNs:PAs.



Fold change western blot analysis of total form of RAC1 after acute treatment (two and six hours) of ACNs, PAs and ACNs:PAs. Inhibition of RAC1 levels documented with PAs at 0.002 μg/mL (*: $p \leq 0.05$) after 2 h and 6 h. Increased levels of RAC1 documented with ACNs:PAs at 8 μg/mL (*: $p \leq 0.05$) after 2 h.

Gene expression for RHOA also shows a similar pattern as RAC1, but in this case, no significant effect was observed six hours post treatment (Figure 33).

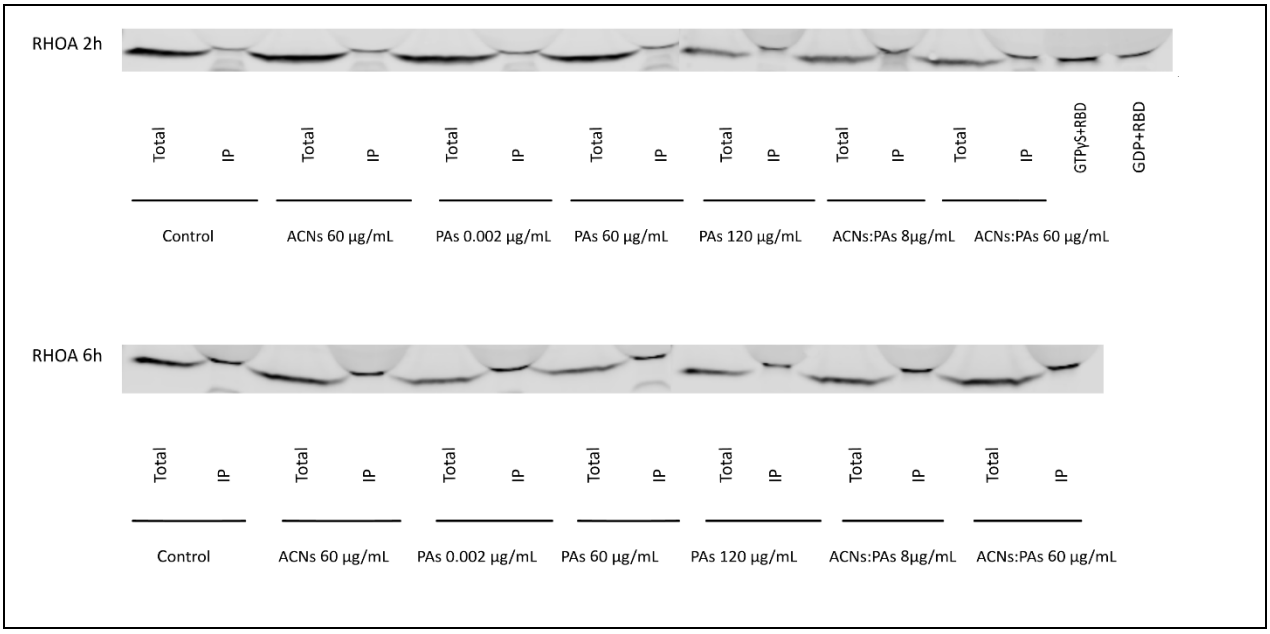
Figure 33: Gene expression of RHOA after acute treatment (2 and 6 hours) of ACNs, PAs and ACNs:PAs.



Gene expression for RHOA is increased compared to the control for all tested concentrations two hours after treatment, ACNs 60 μg/mL (***: p≤0.001), PAs at 0.002 μg/mL (***: p≤0.001), 60 μg/mL (***: p≤0.001) and 120 μg/mL (**: p≤0.01) and ACNs:PAs 8μg/mL and 60 μg/mL (***: p≤0.001).

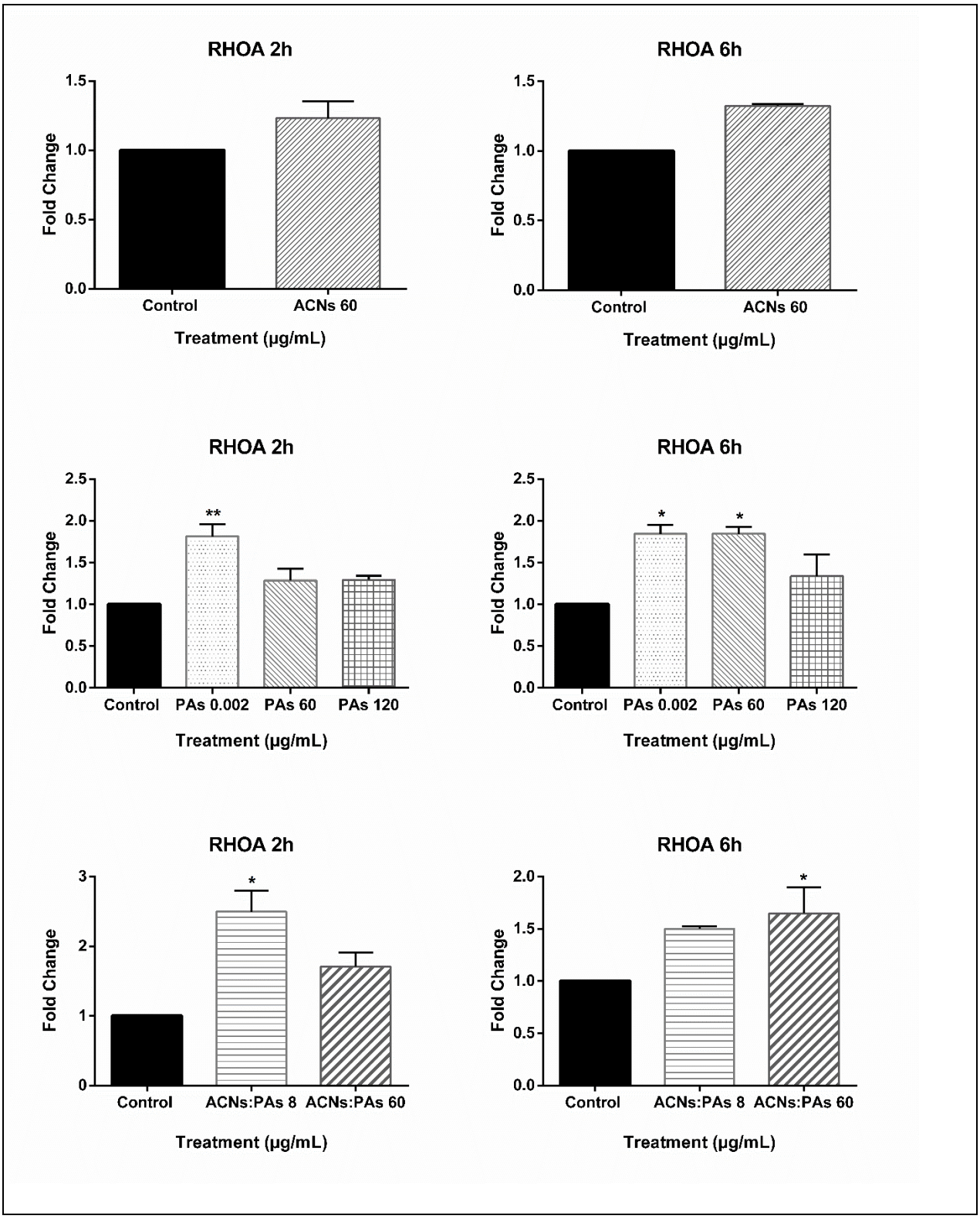
Immunoprecipitation for RHOA was conducted following the appropriate protocol according to the manufacturer and the positive and negative controls indicates that the procedure was performed accurately (Figure 34).

Figure 34: Total and active form of RHOA after acute treatment (2 and 6 hours) of ACNs, PAs and ACNs:PAs.



Quantitative western blot analysis of RHOA documented increased levels of active RHOA when cells were treated with PAs at 0.002 μg/mL after 2 h (**: p≤0.01) and 6 h (*: p≤0.05) and at 60 μg/mL after 6 h (*: p≤0.05). ACNs:PAs at 8 μg/mL (*: p≤0.05) documented an increased level of RHOA after 2 h while 6 h of treatment for ACNs:PAs at 60 μg/mL (*: p≤0.05) (Figure 35).

Figure 35: Quantitative analysis of active form of RHOA after acute treatment (2 and 6 hours) of ACNs, PAs and ACNs:PAs.



Fold change western blot analysis of RHOA after acute treatment (two and six hours) of ACNs, PAs and ACNs:PAs. Increased levels of RHOA with PAs at 0.002 μg/mL (**: $p \leq 0.01$) after 2h and after 6 h (*: $p \leq 0.05$), PAs at 60 μg/mL (*: $p \leq 0.05$), ACNs:PAs at 8 μg/mL (*: $p \leq 0.05$) after 2h and ACNs:PAs at 60 μg/mL (*: $p \leq 0.05$) after 6 h.

4.5 Gene expression for AKT1, eNOS and VEGF.

Gene expression was conducted for AKT1, eNOS and VEGF (Figure 36). Testing the gene expression of AKT1 for 2 and 6 hours post treatment with all tested concentrations resulted in having a significant effect. When cells treated with ACNs at 60 μg/mL resulted a reduced expression compared to the control. PAs at 0.002 μg/mL and 60 μg/mL increased the expression at 2 hours but at 6 hours the expression was reduced compared to the control. PAs at 120 μg/mL have no effect after 2 hours of treatment, however after 6 hours decreased the gene expression of AKT1 compared to the control. Combination of both fractions ACNs:PAs at 8 μg/mL resulted in a decreased gene expression for both time points while ACNs:PAs at 60 μg/mL had no effect at 2 hours however, we documented decreased gene expression at 6 hours compared to the control.

Similarly, ACNs at 60 μg/mL documented reduced gene expression for eNOS at 2 and 6 hours. No effect was documented with PAs for both time points while ACNs:PAs at 8 μg/mL and 60 μg/mL resulted decreased gene expression levels at 2 and 6 hours post treatment.

After treatment with ACNs 60 μg/mL, no effect was documented on VEGF gene expression levels for both time points compared to the control. Treatment with PAs at 0.002 μg/mL, 60 μg/mL and 120 μg/mL for 2 hours resulted in an increased gene expression level compared to the control but had no effect at 6 hours. Similarly to the PAs, combination of

ACNs:PAs 8 µg/mL and ACNs:PAs 60 µg/mL documented increased gene expression levels compared to the control after 2 hours but no effect was documented at 6 hours.

Figure 36: Gene expression of AKT1, eNOS and VEGF after acute treatment (2 and 6 hours) of ACNs, PAs and ACNs:PAs.

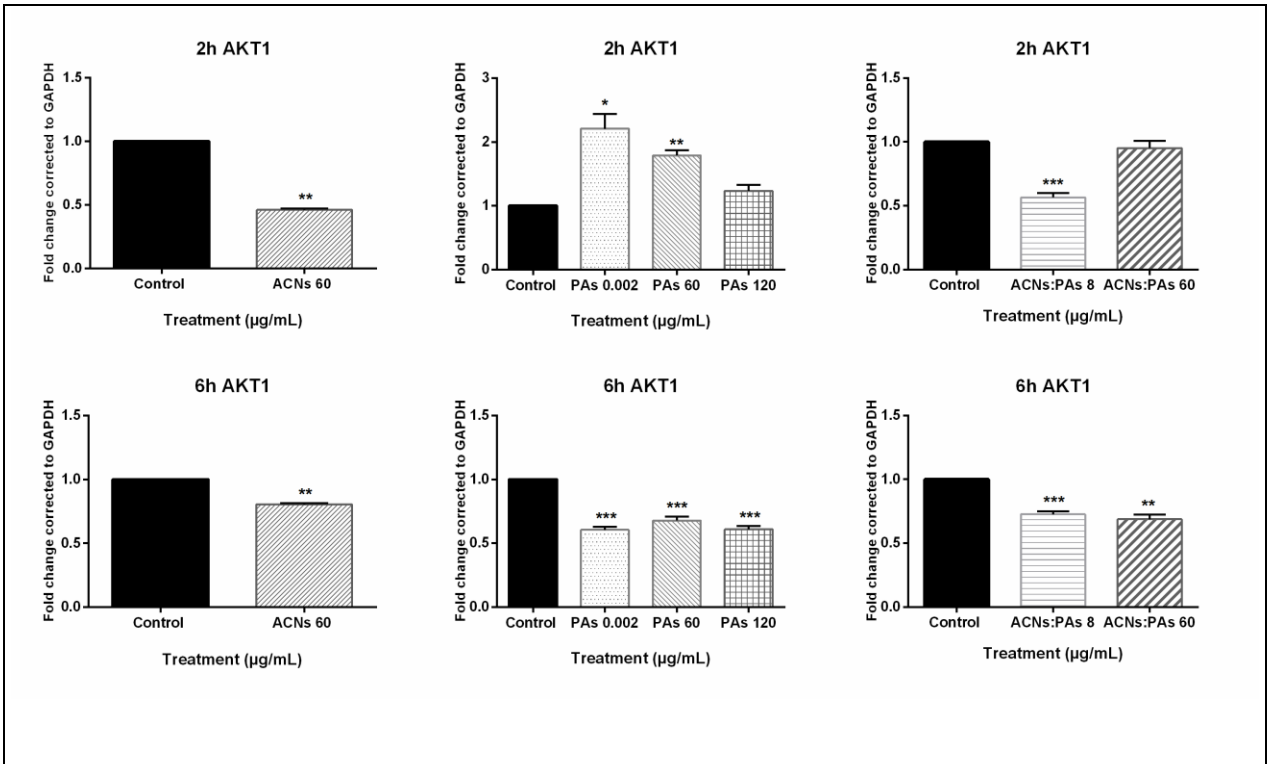
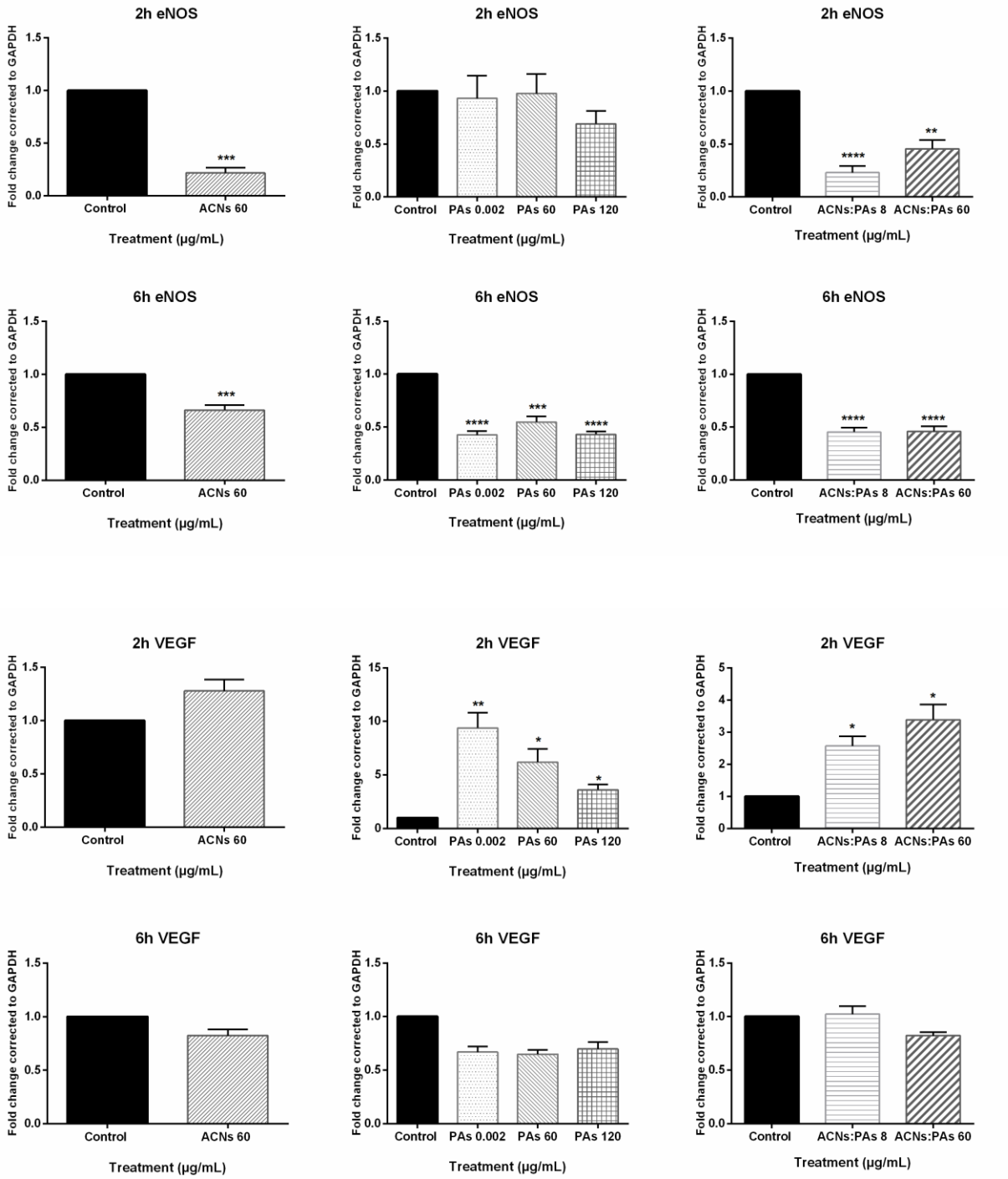


Figure 35 Continued



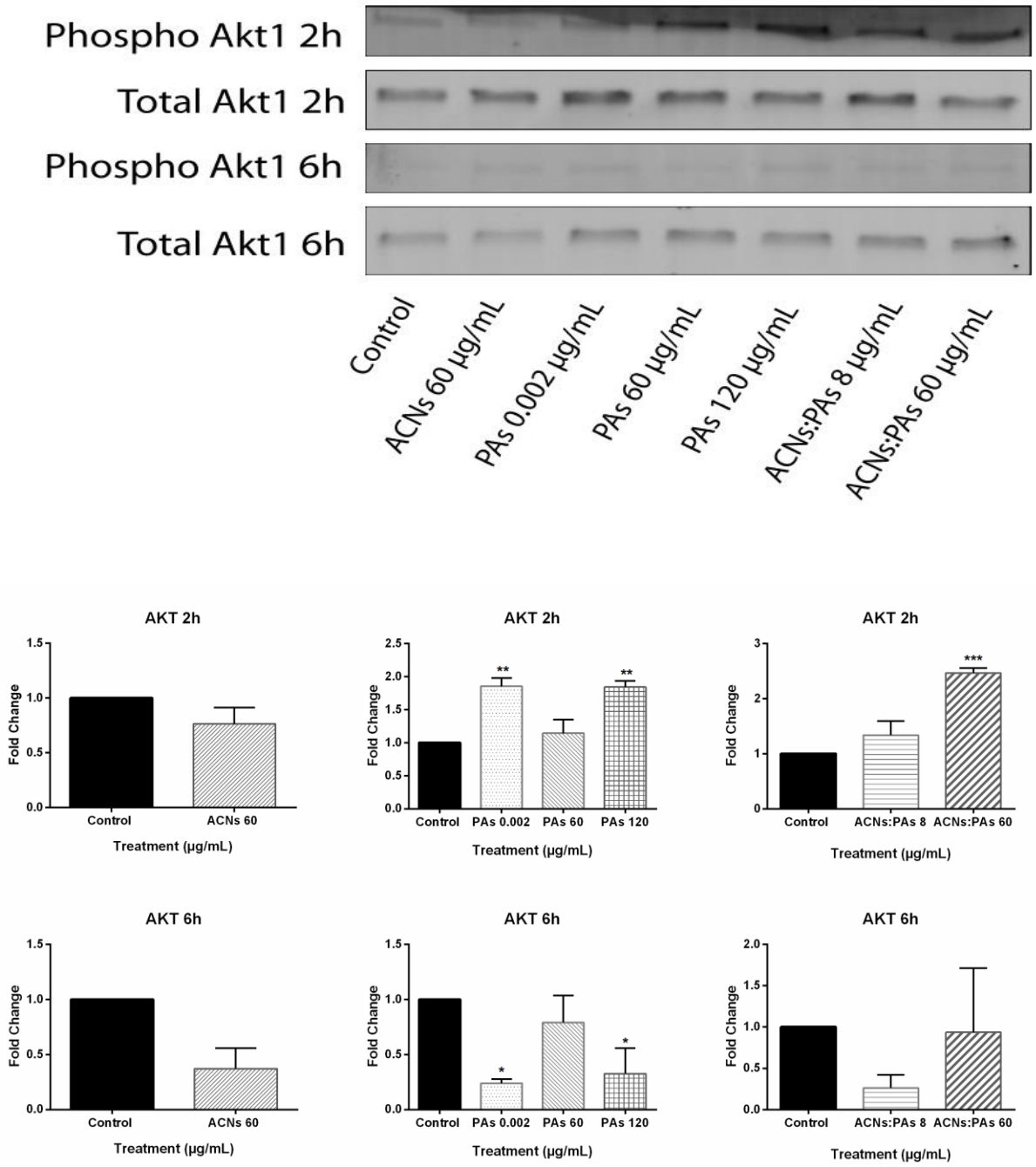
The values are reported as fold change corrected to GAPDH housekeeping gene.

(*: p≤0.05) (**: p≤0.01) (***: p≤0.001) (****: p≤0.0001)

4.6 Western Blot analysis of AKT1, eNOS and VEGF.

Western blot analysis for AKT1 (Figure 37) documented that there was a statistically significant difference compared to the control after 2 hours of treatment with PAs at 0.002 μg/mL (**: $p \leq 0.01$), 120 μg/mL (**: $p \leq 0.01$) and ACNs:PAs at 60 μg/mL (***: $p \leq 0.001$). However, 6 hours post treatment with PAs at 0.002 μg/mL (*: $p \leq 0.05$) and 120 μg/mL (*: $p \leq 0.05$) we documented decreased levels of AKT1 were observed.

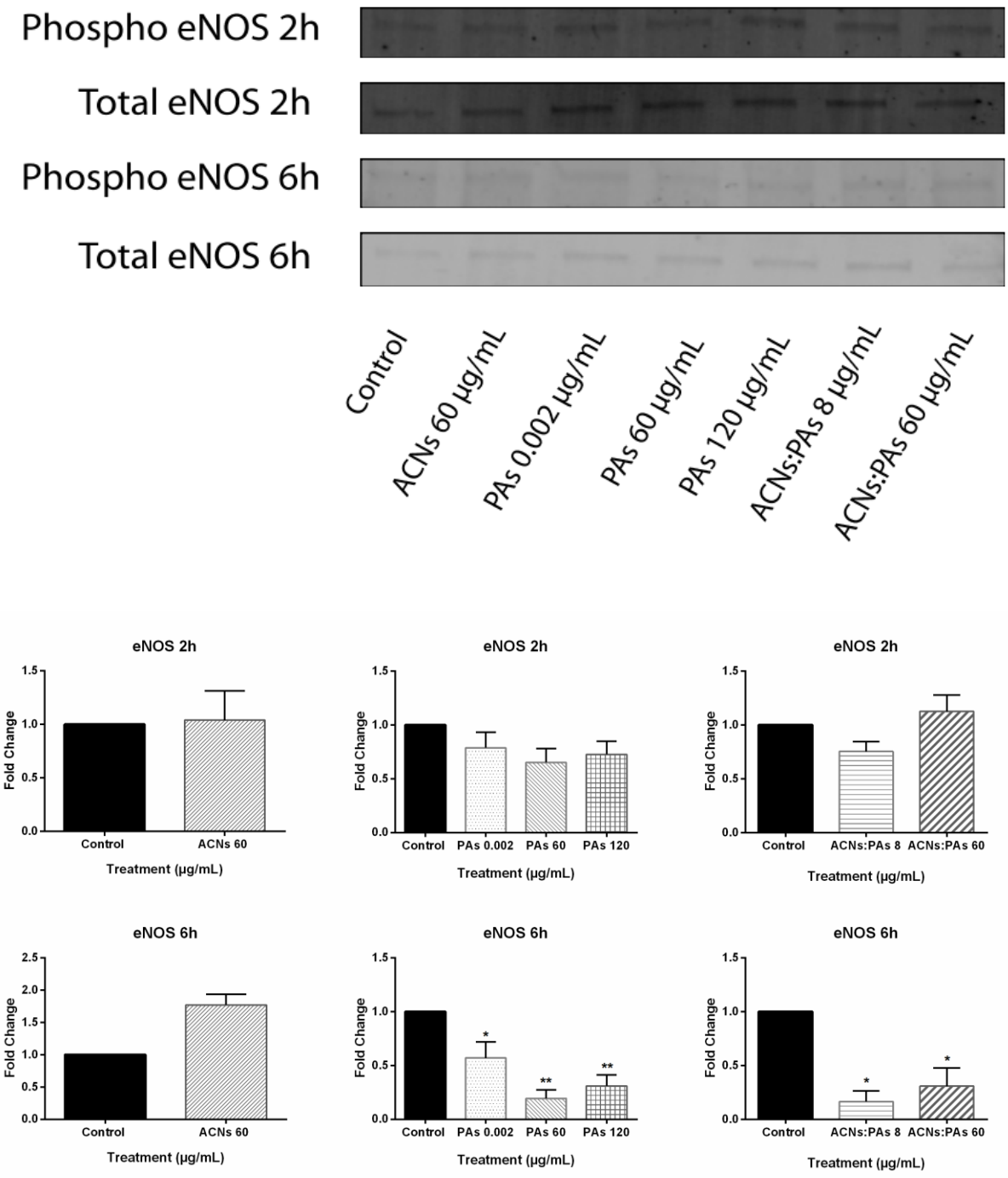
Figure 37: Qualitative and quantitative western blot analysis for AKT1 after acute treatment (2 and 6 hours) of ACNs, PAs and ACNs:PAs.



The values are reported as fold change (*: $p \leq 0.05$) (**: $p \leq 0.01$) (***: $p \leq 0.001$)

Western blot analysis for eNOS (Figure 38) documented that there were statistical significant differences compared to the control after 6 hours of treatment with PAs at 0.002 μg/mL (*: $p \leq 0.05$), 60 μg/mL (**: $p \leq 0.01$), 120 μg/mL (**: $p \leq 0.01$) and ACNs at 8 μg/mL (*: $p \leq 0.05$) and 60 μg/mL (*: $p \leq 0.05$). No effect was documented at 2 hours for PAs and ACNs:PAs or ACNs at both time points.

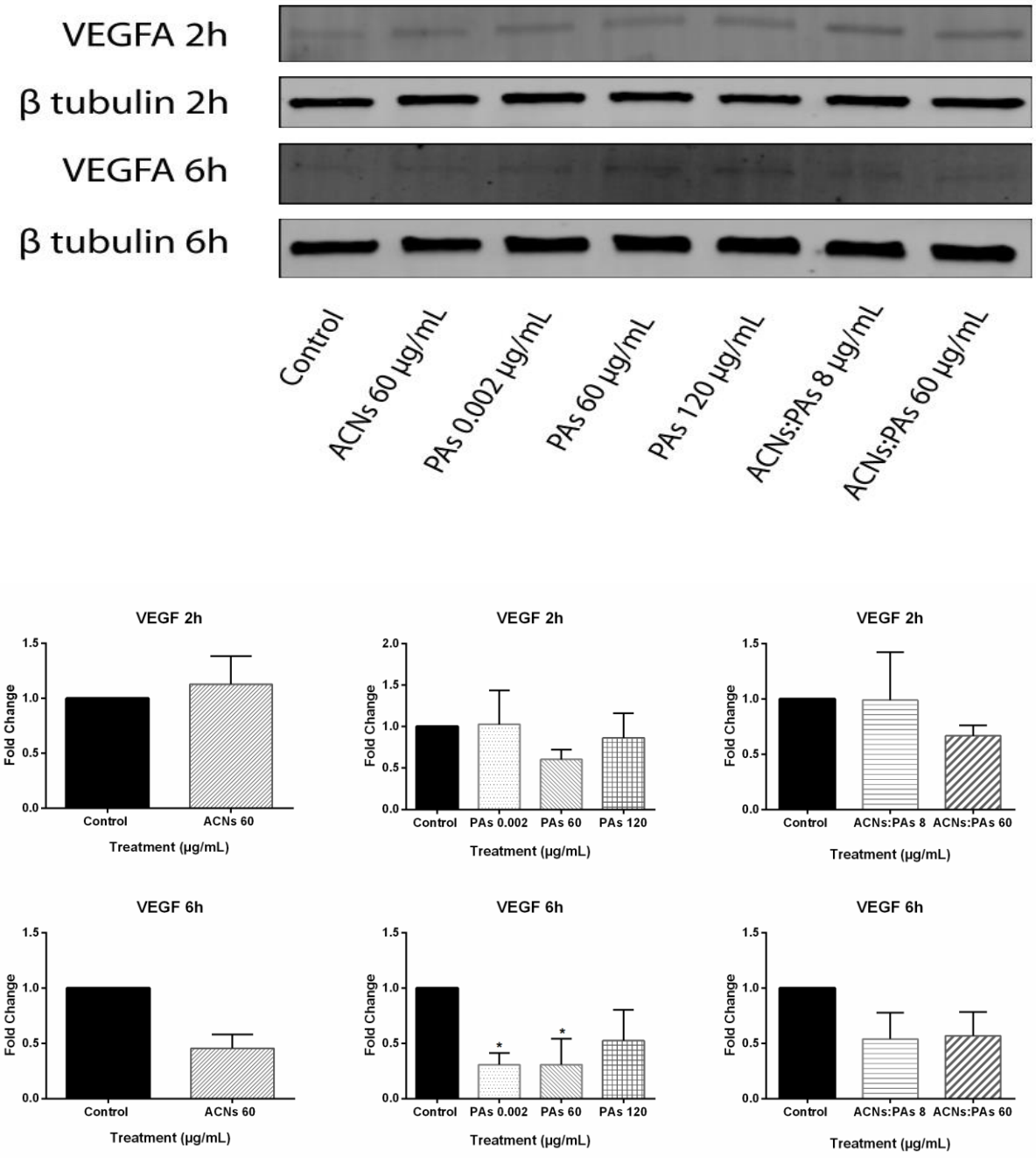
Figure 38: Qualitative and quantitative western blot analysis for eNOS after acute treatment (2 and 6 hours) of ACNs, PAs and ACNs:PAs.



The values are reported as fold change (*: $p \leq 0.05$) (**: $p \leq 0.01$)

Similarly, to eNOS western blot analysis for VEGF (Figure 39) documented decreased levels with PAs at 0.002 μg/mL (*: $p \leq 0.05$) and 60 μg/mL (*: $p \leq 0.05$) 6 hours post treatment. No differences documented for the remaining treatments at any of the two time points.

Figure 39: Qualitative and quantitative western blot analysis for VEGF after acute treatment (2 and 6 hours) of ACNs, PAs and ACNs:PAs.



The values are reported as fold change (*: $p \leq 0.05$)

CHAPTER 5

DISCUSSION

5.1 Effect of ACNs, PAs and combination on endothelial cell migration.

The present study investigated the effects of different concentrations of extracts from wild blueberries on endothelial cell migration speed, gene expression and protein synthesis of RAC1 and RHOA as well as their effect on the proliferation rate of endothelial cells. This is a novel study that documented that anthocyanin and phenolic acid fractions from wild blueberries differentially modulate endothelial cell migration and gene expression.

Endothelial cell migration is critical for normal wound healing processes, tissue formation and angiogenesis (55). Collective cell migration is a type of migration that plays a key role in wound healing (165). *In vitro* experiments with HUVECs, have recently unraveled possible mechanisms of collective cell migration and the role of cadherin as well as the role of RAC1, RHOA, CDC42 and F-actin in the above process (166).

In our experiments, we observed reduction of cell proliferation only at 1000 μg/mL of ACNs treatment compared to control, twelve hours after treatment as also documented in previous studies (38, 45, 137, 167, 168). Under our experimental conditions, we documented inhibition of cell migration when HUVECs were exposed to the anthocyanin wild blueberry extract at 60 μg/mL and increased expression of RAC1 and RHOA at 2 hours after exposure. (47). Additionally, past studies have shown that extracts from bilberries (25% anthocyanins) inhibit HUVEC cell proliferation and migration by directly inhibiting ERK 1/2 and AKT but not PLCγ pathways (47). The use of individual anthocyanins (petunidin, delphinidin, cyanidin,

pelargonidin, malvidin, and peonidin) were shown to inhibit smooth muscle cell migration at 20 μM by targeting focal adhesion kinase (FAK) (146) while pelargonidin at 40 μM exhibited an anti-proliferative and anti-migratory effect on smooth muscle cells (48). Even though there are adequate number of studies on the role of ACNs on cell migration, there is paucity of research on the role of PAs and specifically chlorogenic acid, the major component of the PA fraction used in this study.

While we observed inhibition of endothelial cell migration when HUVECs were exposed to 60 μg/mL wild blueberry anthocyanin fraction, we documented significantly greater speed of migration at phenolic acid concentrations of 0.002 μg/mL, 60 μg/mL and at 120 μg/mL. The major component of the phenolic acid fraction extracted from the wild blueberry powder was chlorogenic acid which is the most abundant polyphenol in the human diet (61). This phenolic acid has not been previously studied to assess its role on endothelial cell migration.

We also chose to test the combination of both fractions simultaneously since they are more relevant to whole berry consumption and have not been tested to date. The combination of fractions of ACNs:PAs at 8 μg/mL:8 μg/mL and 60 μg/mL:60 μg/mL significantly promoted endothelial cell migration similar to the phenolic acid fraction.

Past studies on cell migration using gallic acid at ranges of 2-3.5 mM, over a 24 h period, revealed inhibition of cell motility at 6 h, 12 h and 24 h, inhibition of NF-κB activity as well as downregulation of PI3K/AKT pathway (169). In contrast, our phenolic acid fraction containing primarily chlorogenic acid, increased endothelial cell motility when cells were treated at 0.002 μg/mL, 60 μg/mL and 120 μg/mL concentrations. The differences in endothelial cell migration speed observed in our study when cells were treated with the same concentration but different

fractions may be explained based on chemical and structural differences among them, stability of the compounds and availability to HUVEC cell receptors (170). Additionally, the level of oxidation, glycosylation, ability to form polymeric molecules and the existence of stereoisomers may explain the results documented in our experiments (170).

RHOA and RAC1 are critical players during cell migration; RHOA acting in the back of the cell while RAC1 in the front of the cell along with CDC42. RHOA plays a key role in actin cytoskeleton formation and is involved in mechano-transduction through RHOA/RHO-kinase signaling (44, 45). RAC1 also plays a significant role in endothelial function by controlling eNOS and the phosphorylation of proteins critical for cell to cell junction such as occludin, VE-cadherin, and b-catenin (171). In this study, gene expression of RAC1 and RHOA increased compared to the control after 2 h exposure with all treatments while western blot analysis of total RAC1 was significantly reduced after exposure to 60 μg /mL (for 2 and 6 h) of PAs and was significantly induced after exposure to the 8 μg /mL combination. Finally, western blot analysis of active RHOA was significantly increased after 2 h exposure to 0.002 μg/mL PAs and 8μg/mL combination. These are novel findings that to our knowledge have not been previously reported. Previous studies have reported that caffeic acid at 0 – 100 μmL on smooth muscle cells significantly decreased RAC1 protein synthesis after 24 hour exposure at 10 μmol/L and 100 μmol/L compared to untreated cells (172) and protocatechuic acid downregulated the RAS/AKT/NF-κB pathway by targeting RHOB (173).

Cell migration experiments proved that ACNs and PAs can differentially modulate endothelial cell speed which requires changes in the cytoskeleton that are in part regulated by RHO GTPases. However, since the RHO family is composed of many members there is a possibility that other members such as CDC42 may be responsible for the observations made.

Since the present study evaluated collective cell migration, cadherin finger formation has been proposed to also be important for cell to cell interaction between the leading and following cell and RHOA is more critical for the formation of cadherin fingers than RAC1, that is self-governing (8, 166). It has also been documented that a critical activator of RAC1 is RHOG (174). In addition to that, shear stress can also act as a RAC1 activator (175).

The present study examined for the first time the effects of wild blueberry fractions on HUVECs after acute exposure. We examined a range of low and high concentration of anthocyanins and phenolic acids on cell proliferation, endothelial cell migration and gene expression of RAC1 and RHOA. Some of the concentrations used in our experiments (anthocyanins and phenolic acids at 0.002 μg/mL and 8 μg/mL) were close to the physiologically reported single compound concentrations that have been observed in the blood stream (176). Anthocyanins can be found in the blood stream after consumption of food in the plasma at 274 nM (177). Research evaluating the fate of chlorogenic acids after coffee ingestion in healthy humans documented a concentration of 385 μmol isolated from ileal fluid (178, 179). The presence of four anthocyanins, delphinidin 3-O-β-rutinoside (D3R), cyanidin 3-O-β-rutinoside (C3R), delphinidin 3-O-β-glucoside (D3G), and cyanidin 3-O-β-glucoside (C3G), in the plasma of healthy humans after consumption of black currant was detected at concentrations for D3R, C3R, D3G and C3G of 73.7±35.0 nmol/L, 46.3±22.5 nmol/L, 22.7±12.4 nmol/L and 5.0±3.7 nmol/L respectively (180). A range of anthocyanin and phenolic acids from 0.05 to 10 μg/mL (37) was used for lipid accumulation in macrophages. In our experiments the concentrations of our extracts were 9 nmol/L to 579 nmol/L for anthocyanins and 5.6 nmol/L to 338.6 nmol/L for phenolic acids, comparable to the above studies.

Thus, we document for the first time that different fractions extracted from wild blueberries have a significant and differential effect on endothelial cell migration which plays a key role on many physiological phenomena such as angiogenesis and wound healing. These differential effects seem to be dose and compound dependent and be orchestrated by the induction of RAC1 and RHOA, two proteins involved in cell motility. These are novel findings that to our knowledge have not been previously reported.

5.2 Effect of ACNs, PAs and combination on angiogenesis.

The present study examined the effects of different fractions from the wild blueberries on endothelial *in vitro* tube formation assay and protein synthesis of AKT, eNOS and VEGF. This novel study documented that different fractions at different concentrations have a different impact on the integrity of the endothelial network. This particular assay is simple, rapid and reliable, can generate quantitative results and is overall better than any other *in vitro* assay to assess angiogenic regulators (73, 181, 182).

In the previous section we documented the effects of these fractions and their combination on the endothelial cell migration that is highly correlated with angiogenesis. We documented that ACNs are able to inhibit cell migration while PAs and combination of both fractions have the opposite results as inducing the speed of endothelial cell migration. Moreover, even low concentrations such as 0.002 μg/mL are efficient to change the endothelial migration speed.

In this study we documented the effect of ACNs on angiogenesis through the tube formation assay. When HUVECs were treated with ACNs at 60 μg/mL the number of meshes (*: $p \leq 0.05$) and the total area of the meshes (**: $p \leq 0.01$) were significantly reduced compared to the

control with number of nodes, total master segment length and number of master junctions remaining unaffected. The effects of ACNs on angiogenesis were documented through measurement of AKT, eNOS and VEGF gene expression and protein synthesis. Anthocyanins were found to inhibit AKT (**: $p \leq 0.01$) and eNOS (***: $p \leq 0.001$) gene expression for up to 6 h post treatment. Interestingly, ACNs had no significant effect on the VEGF gene expression. However, western blot analysis documented no significant results for all three markers. Changes in gene expression does not necessarily translate to increased protein levels since proteins can undergo posttranslational modification. Our results documented inhibition of angiogenesis with ACNs. This finding also agrees with most of the previous studies (46, 47, 51, 137, 140, 142) using commercially available anthocyanins that have been found to be in high concentrations in food/berry sources.

For phenolic acids we documented a different pattern from ACNs. From the tube formation experiments we documented that all parameters of angiogenesis (number of meshes, total area of the mesh, number of nodes, total master segment length and number of master junctions) were significantly increased compared to the control when HUVECs were treated with PAs at 0.002 μg/mL. The same results were documented at 60 μg/mL only without the significant effect on the number of the master junctions. Surprisingly, inhibition of number of the meshes, nodes and master junctions was documented with PAs at 120 μg/mL while total mesh area and length of master segments remaining unaffected. This may imply that higher concentrations of chlorogenic acid can also allow cells to form an endothelial network but with fewer number of meshes.

Following evaluation of PAs on angiogenesis through gene expression of AKT, eNOS and VEGF we documented that gene expression was increased with PAs at 0.002 μg/mL (*:

p≤0.05) and 60 μg/mL (**: p≤0.01) for only 2 h post treatment and that effect turns into inhibition at 6 h for all three tested concentrations (0.002 μg/mL, 60 μg/mL and 120 μg/mL). Protein levels of AKT match the gene expression profile. Gene expression for eNOS documented no effect at 2 h, however inhibition for all three concentrations tested was documented at 6 h (***: p≤0.001). Also, in this case, protein levels agreed with gene expression. VEGF gene expression significantly increased its expression only at 2 h (*: p≤0.05) which did not translate to the protein level. However, increased protein synthesis of VEGF was documented 6 h post treatment with PAs at 0.002 μg/mL and 60 μg/mL (*: p≤0.05). Previous studies have documented similar results with ours, however without testing concentrations close to the physiologically found in the blood stream and some other studies are in contrast with our results (41, 45, 147). In our PA fraction, the only phenolic acid found was chlorogenic acid which has been studied to a lesser extent than other phenolic acids such as caffeic acid and ellagic acid.

Angiogenesis assay results for combination of both fractions documented only increased total mesh area and total master segment length when cells were treated with 60 μg/mL:60 μg/mL. No significant differences were documented for 8 μg/mL:8 μg/mL Finally, combination of both fractions documented decreased gene expression for AKT and eNOS while increased expression was documented for VEGF at 2 h post treatment. Even though gene expression of AKT decreased compared to the control increased protein synthesis was documented at 2 h post treatment at 60 μg/mL:60 μg/mL. Protein synthesis of eNOS agrees with the gene expression results while no effect was documented for VEGF protein levels.

To our knowledge, combination of both fractions in angiogenesis experiments evaluating the effect on AKT, eNOS and VEGF has not been done before. These are also novel findings.

Most of the previous studies documented similar results with ours for the ACNs (46, 47, 51, 137, 140, 142). However, results are different for PAs since we documented increased angiogenesis and modulation of markers of angiogenesis such as AKT, eNOS and VEGF under specific time and concentration parameters. Moreover, this is a study evaluating chlorogenic acid more than general fraction of PAs since chlorogenic acid is predominant in our sample.

An *in vitro* study documented that HUVECs treated with 0.1 μg/mL, 1 μg/mL and 2 μg/mL of ACNs from black beans inhibited tube formation after cells were induced by VEGF (52). HUVECs with ACNs were not able to develop a complete endothelial network (52). Additionally, in the tube formation assay it was documented that the length of the tube network induced by VEGF was reduced when cells were treated with 1 μg/mL (52). Similarly, to the tube formation experiment wound healing assay was performed and shown the inhibitory effect of ACNs (52).

The effects of the anthocyanin cyanidin-3-O-b-glucoside (C3G) on endothelial progenitor cells (EPCs) isolated from blood and bone marrow of both nondiabetic and diabetic $apoE^{-/-}$ mice were evaluated (144). In this study, the EPCs were used in a tube formation assay and the results showed enhanced levels of angiogenesis (144). Moreover, in a complete setup of cell adhesion, migration and angiogenesis analysis EPC functions showed a significant impairment in diabetic $apoE^{-/-}$ mice compared with nondiabetic mice (144). Additionally, C3G supplementation significantly altered diabetes induced impairment in increased angiogenesis (144).

Among different anthocyanins (commercially available) tested (cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin) delphinidin had the strongest anti-angiogenic effect (46). HUVECs were pretreated for 18 hours with various concentrations (1, 5, 10, 15 or 25

mM) of delphinidin and the length of the tube network was quantified (46). Moreover, delphinidin inhibited VEGF-dependent tyrosine phosphorylation of VEGFR-2 in a dose- and time-dependent manner (46).

A recent *in vitro* study documented the pro-apoptotic effects of anthocyanin-rich dietary bilberry extract (Antho 50) on B CLL cells from 30 patients and on peripheral blood mononuclear cells (PBMCs) from healthy subjects (183). Results revealed a pro-apoptotic effect of Antho 50 on B CLL cells but no effect on PBMCs. In this study B CLL cells were treated with 75 μg/mL of Antho 50 which contains mainly delphinidin-3-*O*-glucoside and delphinidin-3-*O*-rutinoside. Cells were treated for up to 6 h and Antho 50 inhibited the phosphorylation of AKT (183).

Additionally, more *in vitro* studies investigated the inhibitory effects of polyphenols (45). In this study five polyphenols were evaluated, delphinidin (an anthocyanidin from berries), epigallocatechin gallate (a catechin from green tea), ellagic acid (a phenolic acid from raspberries), apigenin and luteolin (flavones from parsley and celery) (45). The aim of the study was to determine the effect of the above polyphenols on inflammatory cytokine (IL-6) induced angiogenesis (45). Apigenin and luteolin were the most effective angiogenic inhibitors on the inflammatory cytokine IL-6/STAT3 pathway (45).

An *in vitro* and *in vivo* study evaluated the effect of *Vaccinium myrtillus* (Bilberry) extracts (VME) (0.3–30 μg/mL) on angiogenesis (47). HUVECs were induced with VEGF-A and tube area, length, joints and paths of endothelial network were quantified. At 3 μg/mL or more, VME reduced all four parameters to the untreated control level (47). In the *in vivo* part VME inhibited the formation of neovascular tufts during oxygen-induced retinopathy in mice

(47). Moreover, in a protein level VME (30 μg/mL) was able to inhibit VEGF-A induced phosphorylation of extracellular signal-regulated kinase 1/2 (ERK 1/2) and AKT (47).

More *in vitro* studies confirmed the antiangiogenic properties and antioxidant activities of bilberry main anthocyanidins (delphinidin, cyanidin and malvidin) (184). Tube formation assay using HUVECs documented inhibition in the tube formation network when cells were treated with concentrations from 1 – 3 μM (184).

Furthermore, purple rice fractions were documented to inhibit ERK 1/2 and p38 pathways leading to inhibition of cell proliferation and migration with greater impact on VEGF-induced angiogenesis (51). Tube formation experiments evaluated by measuring the tube area, length, joint and path documented inhibitory effect with concentrations from 3-30 μg/ml (51).

Additionally, anthocyanin nasunin, isolated from eggplant peels documented to suppress micro vessel outgrowth (10 – 100 μM) in an *ex vivo* angiogenesis experiment using rat aortic rings (136). However, nasunin had no effect in tube formation, and chemotaxis experiments but was able to inhibit HUVEC proliferation rate (136).

The present study examined for the first time the effects of wild blueberry fractions on HUVECs after acute exposure. We examined a range of low and high concentration of anthocyanins and phenolic acids, as well as combination of both, on cell cytotoxicity, angiogenesis, gene expression and protein synthesis of AKT1, eNOS and VEGF. Some of the concentrations used in our experiments (anthocyanins, phenolic acids and combination at 0.002 μg/mL and 8 μg/mL) were close to the physiologically reported concentrations that have been observed in the blood stream (176). Scientists have documented that poly-phenolic aglycones in

plants can be found in concentrations in low μM to mM range (176). Anthocyanins can be found after consumption of food in the plasma at 274 nM (177).

In recent years there have been significant research on the effects of polyphenols on the angiogenesis (185). Polyphenols are capable of controlling/altering molecular pathways that are critical in the initial steps of angiogenesis (185). Effect of polyphenols on growth factors can easily affect cell proliferation and angiogenesis. For example, inhibition of epidermal growth factor receptor (EGFR) can lead to reduction of angiogenesis as a phenomenon. Moreover, VEGFR stimulation can also trigger the PI3K/AKT/mTOR pathway however, there are polyphenols such as quercetin and curcumin that can inhibit this pathway resulting reduction of angiogenesis (186, 187). On the contrary, there are polyphenols such as delphinidin, epigallocatechin gallate, ellagic acid, apigenin, and luteolin that can promote angiogenesis (185).

5.3 Summary of results

In this study we were able to document for the first time the different effects or wild blueberry bioactives on endothelial cells during cell migration and angiogenesis. The differential effects of ACNs, PAs and ACNs:PAs had a significant impact on genetic and proteomic levels.

Anthocyanins at 60 μg/mL inhibited endothelial cell migration and angiogenesis. Even though we documented increased levels of gene expression for RHOA and RAC1, protein levels were unaffected. Gene expression of AKT1 and eNOS was decreased after acute exposure which is expected since they are downstream of the signaling molecular pathway while VEGF was unchanged (Figure 7).

All three concentrations of PAs (0.002 μg/mL, 60 μg/mL and 120 μg/mL) increased speed of endothelial cell migration and resulted in a more developed endothelial network

compared to the control. Increased gene expression and protein levels of RAC1 and RHOA agrees with the cell migration assay. Moreover, increased gene expression of VEGF resulted in increased expression of AKT1 gene that resulted in decreased gene expression of eNOS downstream in the signaling pathway.

Combination of both fractions ACNs:PAs at 8 μg/mL and 60 μg/mL increased endothelial cell migration and angiogenesis compared to the control/untreated cells. Gene expression and protein levels of RAC1 and RHOA were increased which is expected since we observed promotion of endothelial cell migration speed. Even though, gene expression of VEGF increased; which targets AKT and eNOS, the expression of these genes decreased, with no differences in their protein levels 6 h post treatment. This may have allowed adequate time for the endothelial cells to form a well-developed network 4 h post treatment as documented during the angiogenesis assay.

5.4 Future steps

Future experiments should target additional molecules associated with cell migration, shear stress and angiogenesis to reveal the mechanisms by which the above fractions confer their differential action. Additionally, evaluation of single anthocyanins, phenolic acid compounds and their metabolites on endothelial cell migration and angiogenesis will provide useful information on their *in vivo* role on the vascular system. Moreover, additional molecules that are involved during endothelial cell migration and angiogenesis are important to be studied such as intercellular adhesion molecule 1 (ICAM1), vascular cell adhesion protein 1 (VCAM1), FAK, monocyte chemoattractant protein 1 (MCP1) and cell division control protein 42 (CDC42).

Finally, our results suggest that ACNs, PAs and combination of both have a different effect on different parameters on the endothelial tube network, gene expression and protein synthesis of important molecules such as AKT1, eNOS and VEGF. Interestingly, the treatments tested in this study were able to alter the gene expression level of critical genes (AKT1, eNOS and VEGF) and protein synthesis after acute exposure. Angiogenesis is a very complex phenomenon that plays a key role in health and disease. Unlocking the molecular pathway that these fractions act upon could potentially provide a useful tool on using angiogenesis as a mechanism of treatment.

5.5 Significance.

According to the world Health Organization CVDs are leading the cause of death worldwide accounting more than 17.3 million deaths per year (188). Despite the latest improvements in treatment of cardiovascular disease (CVD), it is still the leading cause of mortality and morbidity (189). According to the American Heart Association, CVD is the leading cause of death in the United States accounting 17 % of the overall national health expenditures (190). Based on the rising healthcare cost and subsequently on the economy it is critical to understand and outline the future of cardiovascular disease (190-192). Moreover, cell migration and angiogenesis are very important processes of diseases that are related to CVD and atherosclerosis such as stroke (2). Thus, finding economic and effective ways to control processes such as angiogenesis, sprouting and collective cell migration that are involved in plethora of pathological conditions are of high importance and priority. There are several studies that have documented the beneficial effects of berries and their role as anti-metastatic and anti-inflammatory agents. Angiogenesis being highly regulated in the human body and having cell

migration as a precursor of that process it is very critical to have the ability to possible control that process.

This study documented for the first time that ACNs and PAs from wild blueberries can differentially modulate endothelial collective migration and angiogenesis in human umbilical vein endothelial cells. For the first time we showed that even low (achievable concentrations possible found in human body) concentrations of ACNs and PAs can control endothelial cell migration and angiogenesis. Moreover, some of the concentrations of the ACNs and PAs used can alter expression of genes (RHOA, RAC1 and AKT) critical for the formation of new blood vessels and wound healing. Additionally, ACNs and PAs were able to affect protein synthesis of molecules (AKT, eNOS and VEGF) that are critical components of several molecular pathways that are not only involved in processes such as cell migration, angiogenesis and more such as cell survival, cell growth, cell proliferation, vasorelaxation and metabolism. Furthermore, with the findings of this project we were able to delineate functions of ACNs and PAs as well as their combination from wild blueberries as a possible tool of controlling fundamental processes that are playing a key role to several pathological conditions such as unbalanced wound healing and ischemia.

From an industry perspective our findings allowed our laboratory to apply for a provisional U.S. patent that may utilize ACNs and PAs combinations primary compounds in future pharmaceutical products against impaired wound healing and therapeutic angiogenesis treatment.

Lastly State of Maine is one of the largest producers of wild blueberries worldwide and the findings of this project can support the importance of investing towards their continuous high

production and sales. Thus, our findings also have a beneficial impact in the agricultural economy in the State of Maine.

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