UNIVERSITY OF NAPLES

FEDERICO II

FACULTY OF PHARMACY

Ph.D. STUDIES IN PHARMACEUTICAL SCIENCE

ALIAmides: EXPERIMENTAL STUDIES ON THE CONTROL OF ANGIOGENESIS

Ph. D. Student: MARIATERESA CIPRIANO

SCHOOL COORDINATOR: CH.MO PROF. MARIA VALERIA D'AURIA

ADVISOR: CH.MO PROF. TERESA IUVONE

CYCLE XXIV

Per le volte in cui abbiamo litigato
e per quelle in cui abbiamo fatto pace.

Per i momenti in cui stavi per arrenderti
e per quelli in cui non lo hai fatto.

Per avermi insegnato cos'è il coraggio.

Per questo, e per tanto altro ancora.

Ho perso un po' la vista, molto l'udito.
Alle conferenze non vedo le proiezioni
e non sento bene.
Ma penso più adesso di quando avevo vent'anni.
Il corpo faccia quello che vuole.
Io non sono il corpo: io sono la mente.

-- Rita Levi Montalcini

Index

Summary

Generally, pathological conditions or altered physiological events are the consequences of different mechanisms altering the normal homeostasis.
Between these events, *angiogenesis* that is the process that brings to the formation of new blood vessels from pre-existing ones, plays a very important role.
The angiogenic process begins with capillary sprouting and culminates in formation of a new microcirculatory bed composed of arterioles, capillaries and venules. The initiation of angiogenesis consists of at least three processes: 1) breakdown of the basement membrane of the existing vessels, 2) migration of endothelial cells from the existing vessels towards an angiogenic *stimulus*, and 3) proliferation of endothelial cells (Klagsbrun & D'Amore, 1991).

Physiological angiogenesis occurs during wound healing, organ regeneration, and in the female reproductive system during ovulation, menstruation, and the formation of the placenta; however, angiogenesis also occurs in pathological processes such as tumour growth, rheumatoid arthritis, diabetic retinopathy, and psoriasis.

A switch to the angiogenic phenotype depends on a local change in the balance between angiogenic stimulators and inhibitors (Post et al., 2008), since this series of events is subject to a tight control played by the "angiogenic balance", i.e. a physiological balance between the stimulatory and inhibitory signals for blood vessel growth, tightly controls angiogenesis (Folkman, 1995).

Growing evidences suggest that angiogenesis occurs in the development of human pathologies affecting the Central Nervous System (CNS) too, although its role is controversial. In fact, new vascularisation should be protective in post- ischemic brain (Beck et al., 2009), in cerebral stroke (Arai

et al., 2009), and in spinal cord injury (Han et al., 2010), where oxygen replacement is beneficial for neuronal cell survival in a hypoxic region. On the other hand, angiogenesis can be detrimental where inflammatory *foci* exist within the CNS, as in some neurodegenerative disorders (Candelario et al., 2009), since new blood supply may promote the increase of pro-oxidant, pro-inflammatory, and also pro- angiogenic mediators into the brain.

Among these neurodegenerative conditions, a negative role of angiogenesis has been recently described during Alzheimer Disease (Fioravanzo et al., 2010). In fact, it seems to be clear that, during hyperactivation of astroglial cells, where a scenario of "reactive gliosis" is already in progress, the formation of new vessels gets the damaged area worse, in relation to the increase of pro-oxidant, pro-inflammatory and pro- angiogenic mediators and despite the assumption that angiogenesis brings oxygen and nutrients to the hypoxic place (Chrystov et al., 2006).

In the periphery, a different cell type, mast cell, plays similarly to astroglia during neurodegeneration in sustaining angiogenic process.

Actually, several evidences have shown the important role of mast cells in supporting angiogenesis (Iuvone et al., 1999), since their critical presence near sites of new capillary sprouting is a fundamental event for the new vessel formation. In fact, during specific pathological conditions, such as inflammatory or allergic diseases, mast cells become activated and allowed to degranulate, in releasing a series of pro- angiogenic mediators (VEGF, MMPs, TNF- α).

In parallel, a new class of compounds, *ALIA*mides, (*Autacoid Local Injury Antagonism* Amides) possessing *in vitro* and *in vivo* anti- angiogenic activities, has been discovered (Aloe et al., 1993). Between these, recently it has been reported that Palmitoylethanolamide, *ALIA*mides' ancestor, is able to inhibit both astroglial and mast cell activation (Scuderi et al., 2011; De Filippis et al., 2010).

These evidences are the scientific starting point of my Ph.D. thesis, focused on studying the modulation of angiogenesis by *ALIA*mides through the control of astroglial and mast cell behaviour and leading, and, as a consequence, a modulation of both CNS and peripheral pathological inflammatory conditions.

Abbreviations

Aβ	*β-Amyloid*
AD	*Alzheimer's Disease*
ALIA	*Autacoid Local Injury Antagonism*
aFGF	*acidic Fibroblast Growth Factor*
Ang	*Angiopoietin*
bFGF (FGF-2)	*basic Fibroblast Growth Factor*
BM	*Basement Membrane*
BrdU	*BromodeoxyUridine*
CAD	*Contact Allergic Dermatitis*
CBs	*Cannabinoids*
CNS	*Central Nervous System*
COX-2	*CycloOXygenase-2*
DMEM	*Dulbecco's Modified Eagle Medium*
DNFB	*2,4-Dinitrofluorobenzene*
EBM	*Endotheial Basal Medium*
ECM	*ExtraCellular Matrix*
EC	*Endothelial Cell*
eCBs	*Endocannabinoids*
ECS	*Endocannabinoid System*
EGF	*Epidermal Growth Factor*
EGFR	*Epidermal Growth Factor Receptor*
ELISA	*Enzyme-Linked ImmunoSorbent Assay*
eNOS	*endothelial Nitric Oxide Sinthase*
EPCs	*Endothelial Progenitor Cells*
FBS	*Foetal Bovine Serum*
HSPG	*Heparin Sulphate ProteoGlycans*
HUVECs	*Human Umbelical Vein Endothelial Cells*

ICAM-1	*InterCellular Adhesion Molecule-1*
IFN	*Interferon*
ILs	*Interleukins*
iNOS	*inducible Nitric Oxide*
λ-carr	*λ-carragenin*
MC	*Mast Cell*
MITF	*Microphthalmia-Associated Transcription Factor*
MMPs	*MatrixMetalloProteases*
MTT	*3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazoliumbromide*
NFTs	*NeuroFibrillary Tangles*
NO	*Nitric Oxide*
PBS	*Phosphate Buffer Solution*
PDGF	*Platelet-Derived Growth Factor*
PEA	*Palmitoylethanolamide*
PI3K	*Phosphatidyl-Inositol 3 Kinase*
SP	*Senile Plaques*
RT-PCR	*Reverse Transcriptase- Polymerase Chain Reaction*
TGF-α	*Transforming Growth Factor-α*
TGF-β	*Transforming Growth Factor-β*
TNFR	*Tumor Necrosis Factor Receptor*
TNF-α	*Tumor Necrosis Factor-α*
VCAM-1	*Vascular Cell Adhesion Molecule-1*
VE-cadherins	*Vascular Endothelial-cadherins*
VEGF	*Vascular Endothelial Growth Factor*
VEGFR	*Vascular Endothelial Growth Factor Receptor*
vWf	*von Willebrand factor*

1. Introduction

1.1. Angiogenesis

1.1.1. Angiogenesis: an overview

The blood and the lymphatic vascular system penetrate every organ and tissue to supply cells with nutrients and oxygen, providing for the circulation of fluids and various signalling molecules. The formation of new vessels is a process divided in two different manners: **vasculogenesis** and **angiogenesis** (Ferrara, 2000).

Vasculogenesis, the development of the blood vascular system, is one of the first events in embryogenesis. During early embryonic development, mesodermal cells differentiate into hemangioblasts, progenitors of both hematopoietic and Endothelial Cells (ECs) giving rise to blood vessels. During differentiation, hemangioblasts produce angioblasts and their aggregation results in the formation of blood islands.

The fusion of blood islands results in the appearance of the primary blood vascular plexus consisting of fine capillaries formed by ECs. At this stage capillaries acquire an arterial or venous character. The stage of vasculogenesis is completed together with the formation of the primary vascular plexus and all further transformations of the vascular net proceed during angiogenesis (Karamysheva, 2008).

Angiogenesis is the outgrowth of new vessels from pre-existing vasculature. It is an essential process during embryogenesis, wound healing and the ovarian cycle but it also plays a major role in several pathologic processes such as tumor vascularisation, diabetic retinopathy, psoriasis and rheumatoid arthritis (Bouïs et al., 2006). In adults, formation and growth of new vessels are under strict control.

These processes are activated only under strictly defined conditions like wound healing. Strict regulation of this system and balanced functioning is very important for the organism because both excessive formation of blood vessels and their insufficient development lead to serious diseases

(Karamysheva, 2008). Scientists have studied the process of capillary growth since 1939, when Clark and Clark (1953) observed the process in real time using intravital microscopy of the microvascular network within rabbit ear chamber.

In the early 1970s Gimbrone and colleagues (1973) first achieved the establishment of long-term EC cultures. Subsequently the development of in vitro models of capillary network formation contributed to the understanding the angiogenic process (Folkman and Haudenschild, 1980).

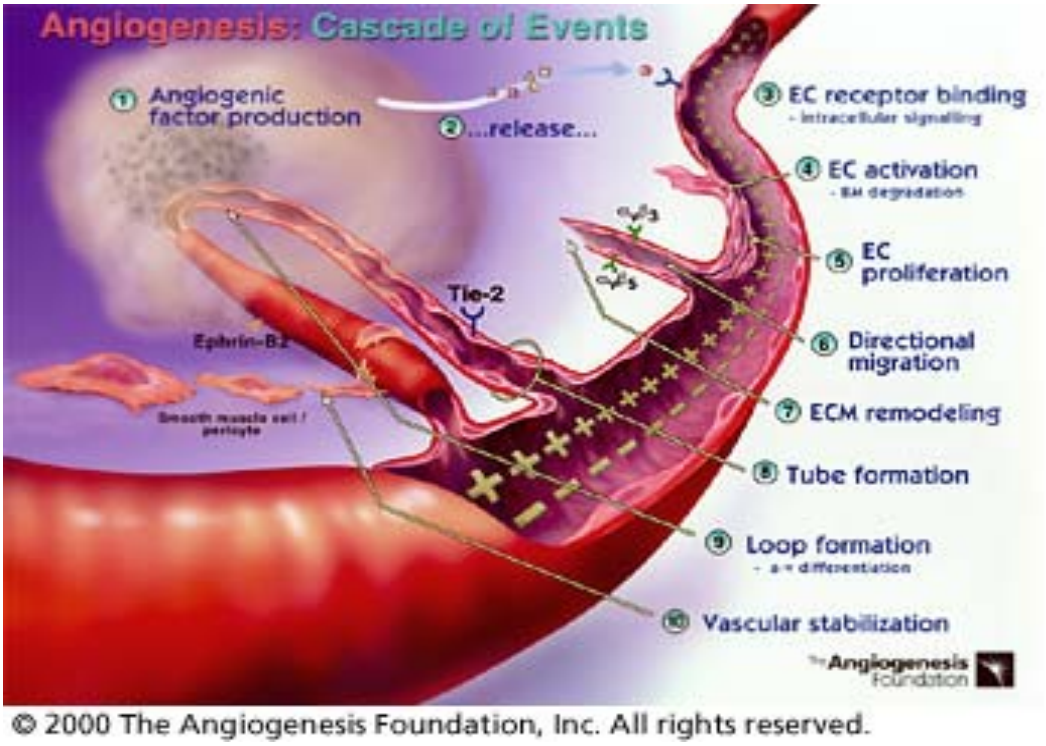


Figure 1.1.1.: ***Angiogenic process*** *(Folkman and Shing, 1992)*

1.1.2. Angiogenic Phases

The angiogenic process starts after different kinds of *stimuli*. *Stimuli* known to initiate angiogenesis include hypoxia, inflammation and mechanical factors such as stress and stretch. These *stimuli* either directly or indirectly activate ECs by initiating the autocrine or paracrine production and release of growth factors or cytokines. Angiogenic sprouting of blood vessels occurs in a series of events, which can roughly be divided into a destabilisation phase, a proliferation and migration phase and a maturation phase. All these steps

offer potential points for pro- or anti- angiogenic clinical intervention (Milkiewicz et al., 2006).

Vascular permeability, endothelial cell proliferation and migration

Angiogenesis initiates with vasodilation, a process involving nitric oxide (NO). Vascular permeability increases in response to VEGF, thereby allowin extravasation of plasma proteins that lay down a provisional scaffold for migrating endothelial cells. Ang1, a ligand of the endothelial Tie2 receptor, is a natural inhibitor of vascular permeability, tightening pre-existing vessels. Endothelial cells need to loosen interendothelial cell contacts and relieve periendothelial cell support to migrate from their resident site; so, mature vessels need to become destabilized. Ang2, an inhibitor of Tie2 signalling, may be involved in detaching smooth muscle cells from the extracellular matrix (Maisonpierre et al., 1997; Gale & Yancopoulos, 1999). Proteinases of the plasminogen activator, MMP, chymase or heparanase families influence angiogenesis by degrading basal membrane and extracellular matrix molecules as well as by activating or liberating growth factors (bFGF, VEGF and IGF-1) sequestered within the extracellular matrix (Coussens et al. 1999). MMP-3, MMP-7 and MMP-9 affect angiogenesis in neonatal bones (Vu et al., 1998) and tumours (Bajou et al., 1998), whereas TIMP-1, TIMP-3 or a naturally occurring fragment of MMP-2, by preventing binding of MMP-2 to $\alpha_v\alpha_3$ integrin, may limit the extent of migration and invasion of endothelial cells during tumour angiogenesis (Brooks et al., 1998).

Once the path has been cleared, proliferating endothelial cells can migrate to distant sites. Ang1 phosphorylates tyrosine in Tie2 and is chemotactic for endothelial cells, induces sprouting and potentiates VEGF, but fails to induce endothelial proliferation (Suri et al., 1998).

In contrast to VEGF, Ang1 itself does not initiate endothelial network organization, but stabilizes networks initiated by VEGF, presumably by stimulating the interaction between endothelial and periendothelial cells. This indicates that Ang1 may act at later stages than VEGF (Gale & Yancopoulos, 1999). Ang2, at least in the presence of VEGF, is also angiogenic. VEGF and its receptor VEGFR-2 affect physiological and pathological angiogenesis and

are therapeutic targets, although much remains to be learned about the involvement of the distinct VEGF isoforms or of the heterodimers of VEGF family members. Members of the fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) family are redundant during normal development; both affect angiogenesis probably by recruiting mesenchymal or inflammatory cells (Zhou et al., 1998; Lindahl et al., 1998).
TGF- α1 and tumour necrosis factor TNF-α can either stimulate or inhibit endothelial growth, and may be involved in tumour dormancy (Gohongi et al., 1999).

Nitric oxide, a downstream effector of VEGF, TGF α1 and other angiogenic factors, is not essential for embryonic vascular development, but affects pathological angiogenesis and improves the re-endothelialisation of naked vessels (Murohara et al., 1998).
A growing list of molecules is being discovered that are angiogenic after exogenous administration, but which molecules function as endogenous angiogenic factor remain undetermined (Carmeliet, 2000).

A balance of activators and inhibitors controls angiogenic sprouting (Fig. 1.1.2).
Angiogenesis inhibitors, suppressing the proliferation or migration of endothelial cells, include angiostatin (an internal fragment of plasminogen; O'Reilly et al., 1994), endostatin (a fragment of collagen XVIII; O'Reilly et al., 1997), antithrombin III, interferon- α (IFN-α), leukaemia inhibitory factor (LIF) and platelet factor 4 (PF4) (Carmeliet, 2000).

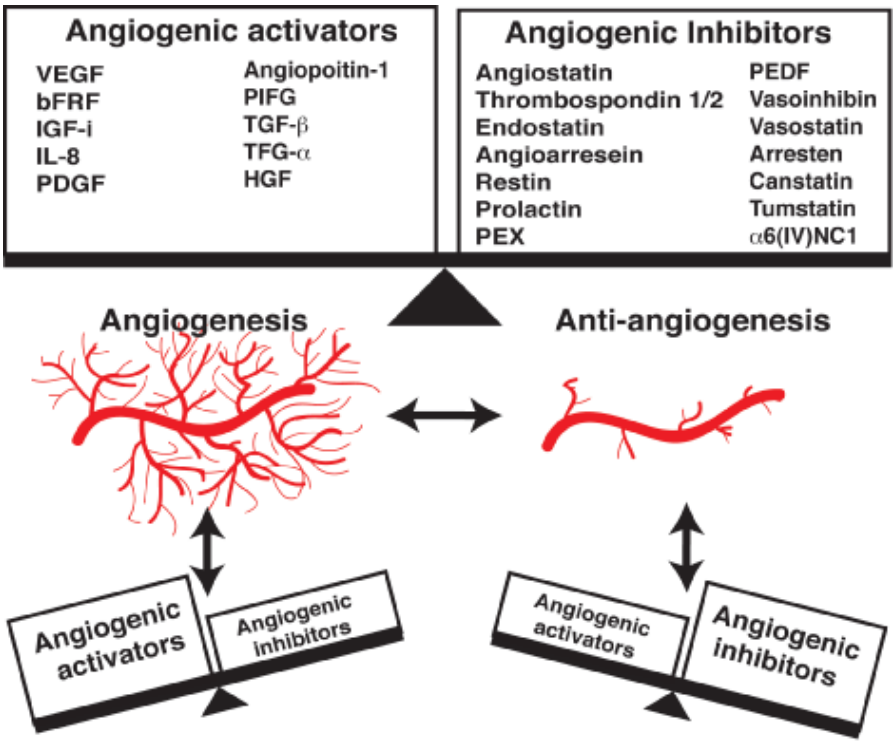


Figure 1.1.2.: ***Angiogenesis: the balance****. Illustration of "angiogenic balance".*

Lumen formation

Migrating endothelial cells often assemble as solid cords that subsequently acquire a lumen. Intercalation or thinning of endothelial cells and fusion of pre-existing vessels allow vessels to increase their diameter and length (Carmeliet, 2000). Specific VEGF isoforms play distinct roles in this moment, i.e. VEGF189 isoform decreases luminal diameter, whereas VEGF121, VEGF165 and their receptors increase lumen formation, in addition to increasing vessel length. Ang1 in combination with VEGF also increases luminal diameter (Suri et al., 1998). Other molecules affecting lumen formation are integrins ($\alpha_v\beta_3$ or α5) and the myocyte enhancer binding factor 2C (MEF2C) transcription factor. Excessive proteolysis may lead to cystic assembly of endothelial cells and prevent tube formation. Thrombospondin (TSP)-1 is an endogenous inhibitor of lumen formation (Carmeliet, 2000).

Endothelial survival and differentiation

Once assembled in new vessels, endothelial cells become quiescent and survive for years (Carmeliet et al., 1999). Endothelial apoptosis is a natural

mechanism of vessel regression in the retina and ovary after birth and a frequent (therapeutic) inhibitor of angiogenesis. Endothelial apoptosis is induced through deprivation of nutrients or survival signals when the lumen is obstructed by spasms, thrombi or the shedding of dead endothelial cells, or when a change in the angiogenic gene profile occurs (Jain et al., 1998; Gerber et al., 1999). The survival function of VEGF depends on an interaction between VEGFR2, α-catenin and vascular endothelial (VE)-cadherin (Carmeliet et al., 1999). Ang1 also promotes, whereas Ang2 suppresses, endothelial survival, at least in the absence of angiogenic *stimuli* (Gale & Yancopoulos, 1999; Holash et al., 1999).

Haemodynamic forces are essential for vascular maintenance, as physiological shear stress reduces endothelial turnover and abrogates TNF-α mediated endothelial apoptosis.

Endothelial apoptosis can be also induced by NO, ROS, angiostatin, TSP-1, the metallospondin METH-1, IFN-α, tissue factor pathway inhibitor (TFPI) and vascular endothelial growth inhibitor (VEGI) (Carmeliet, 2000).

To accommodate local physiological requirements, endothelial cells acquire specialized characteristics that are determined in part by the host tissue (Risau, 1998). For example, an interaction of astroglial cells expressing glial fibrillary acidic protein, pericytes and normal angiotensinogen levels is essential for development of the blood-brain barrier (Lindahl et al., 1998). In contrast, endothelial cells in endocrine glands, involved in the exchange of particles, become discontinuous and fenestrated; this is possibly mediated by interactions between VEGF and the extracellular matrix.

Remodelling, vessel maturation and stabilisation

The remodelling of the endothelial network involves the pruning of capillary-ike vessels with uniform size, and irregular organization into a structured network of branching vessels. Intussusceptions, resulting in replacement of vessels by extracellular matrix, underlie pruning and branching (Fig. 1.1.3.).

Gene inactivation studies indicate a morphogenetic function for VEGF and VEGFR- 3 (Carmeliet et al., 1996; Ferrara et al., 1996; Dumont et al., 1998), the endothelial receptor Tie1 (Patan, 1998), integrin, fibronectin and others (Carmeliet, 2000).

The maturation of the neovascular bed is characterised by a recruitment of mural cells. Endothelial cells from large vessels recruit smooth muscle cells whereas endothelial cells in microvessels recruit pericytes. The mural cells stabilise nascent vessels by inhibiting endothelial proliferation and migration, and by stimulating production of a new basal membrane and extracellular matrix.

They thereby provide haemostatic control and protect new endothelium-lined vessels against rupture or regression. Indeed, vessels regress more easily as long as they are not covered by smooth muscle cells (Benjamin et al., 1998). PDGF-B secreted by the endothelial cells recruits pericytes and smooth muscle cells (Lindahl et al., 1998). VEGF also promotes mural cell accumulation, presumably through the release of DGFB or binding to VEGF receptors (Benjamin et al., 1998).

Ang1, Tie2 and Ephrin- 2 affect growth and maintenance of blood vessels by stabilizing the interaction of mural cells with the nascent endothelial channel, and by inducing branching and remodelling (Suri et al., 1996; Maisonpierre et al., 1997; Gale & Yancopoulos, 1999).

TGF- α1, TGF- αR2 (Transforming Growth Factor- α Receptor II), endoglin (an endothelial TGF-α binding protein) and Smad5 (a downstream TGF-α signal) are involved in vessel maturation in a pleiotropic manner: they inhibit endothelial cell proliferation and migration, induce smooth muscle differentiation and stimulate extracellular matrix production (Dickson et al., 1995; Li et al., 1999).

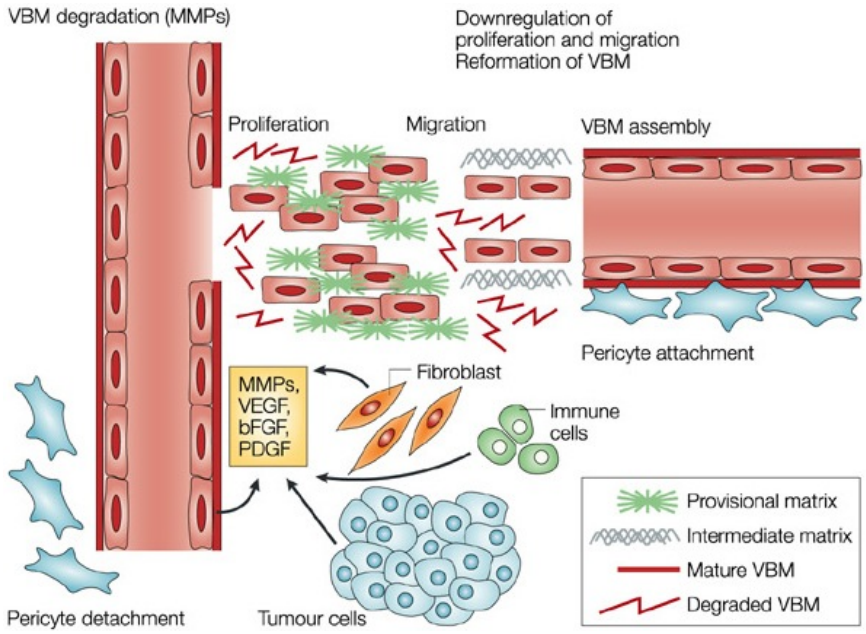


Figure 1.1.3.: ***Schematic angiogenic process****: the degradation of the ECM and vascular basal membrane (VBM) of existing blood vessel, proliferation and migration of endothelial cells towards an angiogenic stimuli, and formation and maturation of the new blood vessel sprout with the recruitment of pericytes are shown.*

1.1.3. Cell types involved in angiogenesis

Many cell types are involved in angiogenesis:

- *ECs;*
- *Endothelial Progenitor Cells (EPCs);*
- *Pericytes and smooth muscle cells;*
- *Inflammatory cells.*

ECs

ECs are the most quiescent and genetically stable cells of the body. Their turnover time is usually hundreds of days in contrast to bone-marrow cells which maintain an average turnover time of 5 days. ECs are bound to capillary BM which is composed of type IV collagen, laminin, heparin-sulphate proteoglycans, perlecans, nidogen/entactin, SPARC/BM-40/osteopontin, type XV collagen, type XVIII collagen and other molecules. This indicates that the primary signals originating from capillary BM inhibit proliferation and promote an environment that facilitates appropriate cell-cell adhesion (Kalluri, 2003).

During angiogenesis to prevent the disintegration of the vascular network under angiogenic *stimulus* and the blood supply to tissue in this region, just some ECs inside the capillary initiate angiogenesis. These cells are called “tip-cells” and occupy the leading position while new vessels grow: they react to the VEGF-A gradient that specifies the direction of their migration and which induces the capillary to grow towards the gradient (Karamysheva, 2008) sprouting phyllopodii towards the VEGF-A gradient (Gerhardt et al., 2003). This effect is caused by the interaction of VEGF-A with the VEGF receptor (VEGFR2), the concentration of which is high in tip-cells. Once tip cells are selected to move forward, formation of new capillaries should begin because of the proliferation and migration of other ECs (Karamysheva, 2008).

EPCs

The first EPCs were described as CD34-enriched mononuclear cells that acquired endothelial surface marker expression in culture (Asahara et al., 1997). Subsequent studies showed that a subpopulation of circulating CD34+ cells expressing CD34+ CD133+ VEGFR2+ could form endothelial colonies in vitro (Peichev et al., 2000; Gill et al., 2001). Other studies have shown that CD11b+ or CD14+ mononuclear cells give rise to endothelial cell-like colonies both in vitro and in vivo. Thus it appears that endothelial- like cells can arise from within bone marrow-derived haematopoietic progenitor cell populations or monocyte populations (Garmy-Susini and Varner, 2005). EPCs cultivated from different sources showed a marked expression of growth factors such as VEGF and Hepatocyte Growth Factor (HGF). The release of growth factors in turn may influence the classical process of angiogenesis, the proliferation and migration as well as survival of the mature cell (Fig. 1.1.4.). The capacity of EPCs to physically contribute to vessel-like structures may contribute to their potent capacity to improve neovascularisation (Urbich and Dimmeler, 2004).

Pericytes and smooth muscle cells

The maturation of primordial vascular tubes is accompanied by the emergence of peri-endothelial cells that participate in matrix formation and

that eventually become **pericytes** (cells locate in the same BM as the ECs) or vascular smooth muscle cells (cells locate outside the vascular BM and equipped with specific actin-myosin filaments). Recently the transmembrane molecule endoglin has been identified as an essential regulator of pericytes and vascular **smooth muscle cells** differentiation that modulate the interaction of Transforming Growth Factor β (TGFβ) superfamily members and their receptors and even is expressed in vascular smooth muscle cells (Kurz, 2000).

Inflammatory cells

Most components of the immune system are involved in angiogenic processes (Fig. 1.1.4.). A disruption of the microenvironment homeostasis leads to activation of resident cells as mast cells, resident macrophages and dendritic cells (Albini and Sporn, 2007). In particular macrophages are known to produce a series of angiogenic factors including VEGF.

Exposure of macrophages to hypoxia enhances expression of molecules related to angiogenesis, as MMP9, MMP12, Tumor Necrosis Factor-α (TNF-α) and Interleukin-10 (IL-10). In addition to being a source of VEGF macrophages are also influenced by this angiogenic factor, modulating their biological activity in response to VEGFR1 ligands.

In the last few years it has become apparent that neutrophils, like macrophages, are sensitive to the microenvironmental clues and adjust their behaviour in response to the local and distant signals (Noonan et al., 2008). They have been shown to play an important role in physiological and pathological angiogenesis (Heryanto et al., 2004). Neutrophil dependent angiogenesis in vivo has been shown to require VEGF-A release by neutrophils upon recruitment and stimulation. In response to stimulation the degranulate and release proteases, activate phagocytosis and oxidate burst generation before dying. In addition to VEGF-A neutrophils have been shown to make a wide range of angiogenesis molecules and an equally wide range of anti-tumour and anti- angiogenic molecules (Benelli et al., 2003). The key appears to lie within the response of these cells to the signal from the microenvironment (Noonan et al., 2008).

The high levels of VEGF may recruit leukocytes and natural killer cells. ECs

exposed to VEGF up-regulate InterCellular Adhesion Molecule-1 (ICAM-1) expression which play a key role for CD18 mediated neutrophil adhesion and recruitment.

VEGF also induce Vascular Cell Adhesion Molcecule-1 (VCAM-1) expression on ECs, which together with ICAM-1 recruit activated natural killer cells. Natural killer cells are known as negative regulators of angiogenesis but they can also induce angiogenesis by secretion of pro- angiogenic cytokines like VEGF, Platelet Growth Factor (PlGF) and Interleukin-8 (IL-8) (Hanna et al., 2006).

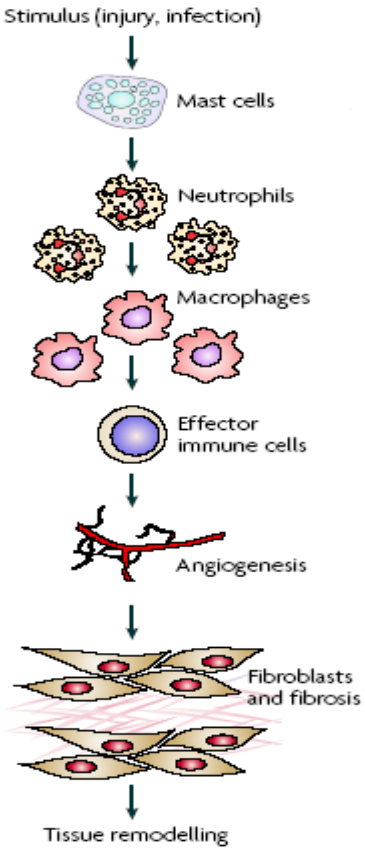


Figure 1.1.4.: ***The sequence of events in acute inflammation and tissue repair****. The process of inflammation initiates a series of catabolic and anabolic processes that occur in a defined order. Firstly the activation of resident cells (mast cells, resident macrophages and dendritic cells) and rapid entry of granulocytes in response to injury. Further recruitment of macrophages and infiltration of effector immune cells (lymphocytes) to enable ECs and fibroblast to form new blood vessels and a collagenous matrix. The last step is tissue remodelling (Albini and Sporn, 2007).*

1.1.4. Pro- angiogenic factors

The most known classification of pro- angiogenic factors is about their activation. They can be divided in two categories:

- direct factors, when they stimulate EC proliferation or migration directly;
- indirect factors, when EC proliferation and/or migration which is observed in vitro must have been induced by some other factors or cells, perhaps indirectly mobilized by the original angiogenic molecule (Folkman and Shing, 1992).

Numerous molecules perform critical functions within the complex angiogenic cascade. Production and activation of these growth factors, EC growth factor receptors and intracellular signalling mediators and transcription factors trigger the phases that comprise the abluminal sprouting form of angiogenesis (Milkiewicz et al., 2006). Some of the soluble growth factors, membrane-bound molecules and mechanical forces that mediate angiogenic process are summarized in Table 1.

Factors	Biological actions
Soluble mediators	
VEGF	increases EC permeability stimulates EC uPA/PAI-1 production stimulate EC proliferation inhibits EC apoptosis enhances EC migration stimulation *in vivo* angiogenesis
Ang-1	stimulates *in vitro* EC sprout formation increases girth and stability of endothelium
Ang-2	antagonizes Ang1 signalling/destabilizes endothelium
aFGF bFGF	stimulates EC proliferation enhances EC migration stimulates EC PA/collagenase production stimulates EC tube formation stimulates *in vivo* angiogenesis
PDGF	stimulates DNA synthesis in ECs stimulates ECs to form chord *in vitro* stimulates proliferation of smooth muscle cells and pericytes induces vWF, VEGF and VEGFR2 expression in cardiac ECs increases capillary wall stability
TGF-β	supports anchorage-independent growth of fibroblast inhibits proliferation and migration of ECs stimulates/inhibits formation of EC tubes *in vitro* produces net antiproteolytic activity via modulation of uPA/PAI-1 inhibits production of other proteases/stimulates production of pro stimulates VSMA production by pericytes chemotactic for monocytes and fibroblasts stimulates *in vivo* angiogenesis in presence of inflammatory respo increases vessel wall stability
TNF-α	stimulates angiogenesis *in vivo* stimulates formation of EC tubes *in vitro* inhibits EC proliferation
EGF, TGF-α	stimulate EC proliferation stimulate angiogenesis *in vivo*
G-CSF, GM-CSF	stimulate EC proliferation and migration
Angiogenin	stimulates angiogenesis *in vivo* supports EC binding and spreading
Angiotropin	stimulates random capillary EC migration stimulates EC tube formation stimulates *in vivo* angiogenesis
Tissue factor	contributes to development of yolk sac vasculature
Factor V	contributes to development of yolk sac vasculature
Prostaglandin	stimulates *in vivo* angiogenesis

Nicotinamide	stimulates *in vivo* angiogenesis
Monobutyrin	stimulates *in vivo* angiogenesis stimulates EC migration *in vitro*
Membrane bound-proteins	
$\alpha_v\beta_3$-integrin	highly expressed on activated ECs mediated EC attachment, spreading and migration present on angiogenic capillary spouts required for bFGF-stimulated angiogenesis *in vivo* localizes MMP-2 to capillary sprouts suppresses EC apoptosis
$\alpha_v\beta_5$-integrin	required for VEGF-stimulated angiogenesis *in vivo*
$\alpha_5\beta_1$-integrin	required for non-VEGF growth factor-stimulated angiogenesis *in vivo*
VE-cadherin	may mediate permeability of endothelium required for *in vivo* angiogenesis prevents EC apoptosis
Eph-4B/Ephrin-B2	colocalize at venous/arterial interfaces of developing embryo required for angiogenesis of head and yolk sac and for myocardial trabeculation
Ephrin-A1	required for *in vivo* angiogenesis induced by TNF-α chemotactic for ECs in vitro
Eph-2A	required for EC tube formation *in vitro*
Biochemical forces	
Blood flow/shear stress	Increases endothelial stress fiber formation (if laminar) promotes EC division (if turbulent) stimulates transcription of bFGF and TGF-β genes

Table 1.: ***Factors regulating normal angiogenesis****. VEGF; uPA, urokinase Plasminogen Acti- vator; PAI-1, Plasminogen Activator Inhibitor-1; Ang-1, Angiopoietin-1; Ang-2, Angiopoietin-2; vWf, von Willebrand factor; aFGF, Fibroblast Growth Factor a; bFGF, Fibroblast Growth Fac- tor b; PDGF, Platelet Derived Growth Factor; VEGFR2, Vascular Endothelial Growth Factor Receptor type 2; TGF-β, Transforming Growth Factor β; VSMA, Vascular Smooth Muscle Ac- tin; TNF-α, Tumor Necrosis Factor-α; EGF, Epidermal Growth Factor; TGF-α, Transforming Growth Factor-α; G-CSF, Granulocyte-Colony Stimulating Factor; GM-CSF, Granulo- cyte/Macrophage-Colony Stimulating Factor (Papetti and Herman, 2002).*

VEGF family and receptors

The VEGF family and its receptors have been known for long to play a central and specific role in angiogenesis as they mediate vascular permeability, EC proliferation, migration and survival (Bouïs et al., 2006 Ferrara, 2000). VEGF was originally described as a homodimeric 34-42kDa protein that increased vascular permeability in the skin (Fig. 1.1.5.). It was identified by a partial purification from the ascites fluid and cell culture supernatants of a guinea-pig hepatocarcinoma cell line and termed Vascular

Permeability Factor (VPF). In 1989 Ferrara and Henzel identified a growth factor for ECs in conditioned *medium* from bovine follicular pituitary cells and called it VEGF. This was subsequently sequenced and found to be identical to VPF (Leung et al., 1989; Keck et al., 1989).

The VEGF gene family consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and PlGF. These glycoproteins belong to a structural superfamily of 13 growth factors that includes PDGF. VEGF-A is mainly involved in angiogenesis while VEGF-C and VEGF-D are involved in lymphangiogenesis (Byrne et al., 2005).

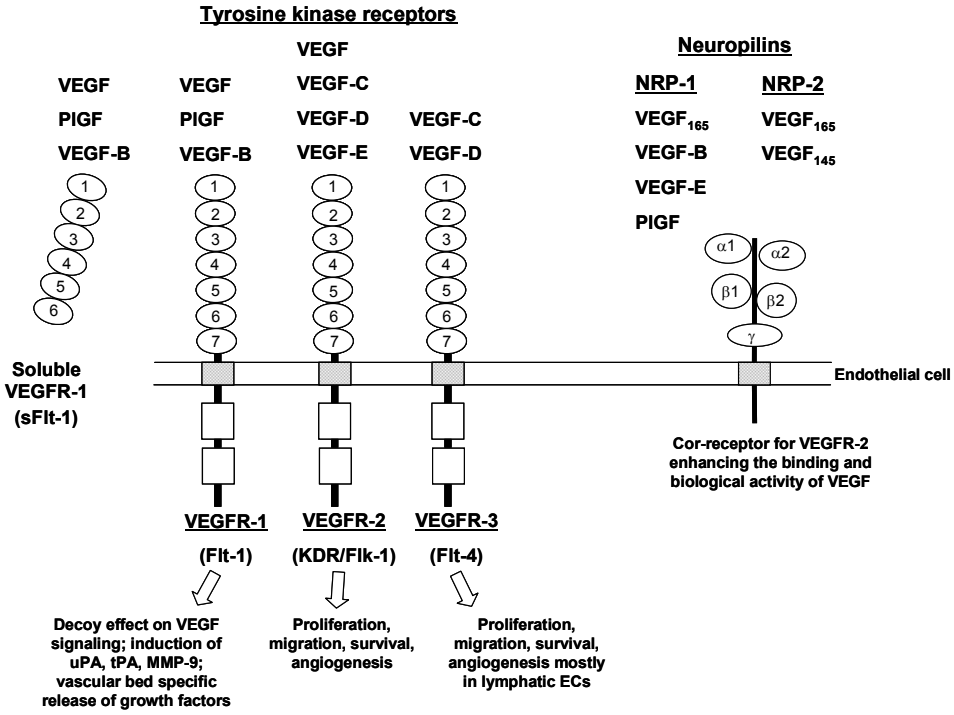


Figure 1.1.5.: ***The endothelial cell surface receptor for members of VEGF family and their biological activities.*** *VEGF tyrosine kinase receptors consist of seven extracellular Ig-like domains (numbered), a transmembrane region and an intracellular tyrosine kinase domain interrupted by a kinase-insert sequence. The soluble VEGFR-1 contains only the first six Ig-like domains. The neuropilins are isoforms-specific receptors for certain VEGF family members. The α1-α2 region has homology to components of the complement system; β1-β2 shares homology with coagulation factors V and VIII, whereas γ domain contain a MAM domain (Meprin/A5-neuropilin/Mu), a protein sequence also found in the metalloprotease meprin and receptor phosphatase μ (Robinson & Stringer, 2001).*

Growth factors of VEGF family exert their biological effect via interaction with receptors located on EC membranes. Three receptors have been identified: Flt-1 (VEGFR1), KDR/Flk-1 (VEGFR2) and Flt-4 (VEGFR3) (Papetti and Herman, 2002). The VEGF receptors are transmembrane proteins with a single transmembrane domain. The extracellular region of VEGF receptors is formed by seven immunoglobulin-like domains (IG I-VII), whereas the intracellular part exhibits tyrosine kinase activity and the tyrosine kinase domain in these receptors is separated into two fragments (TK-1 and TK-2) by an intekinase insert. All VEGF receptors are highly homologous (Ferrara 2000; Karamysheva 2008).

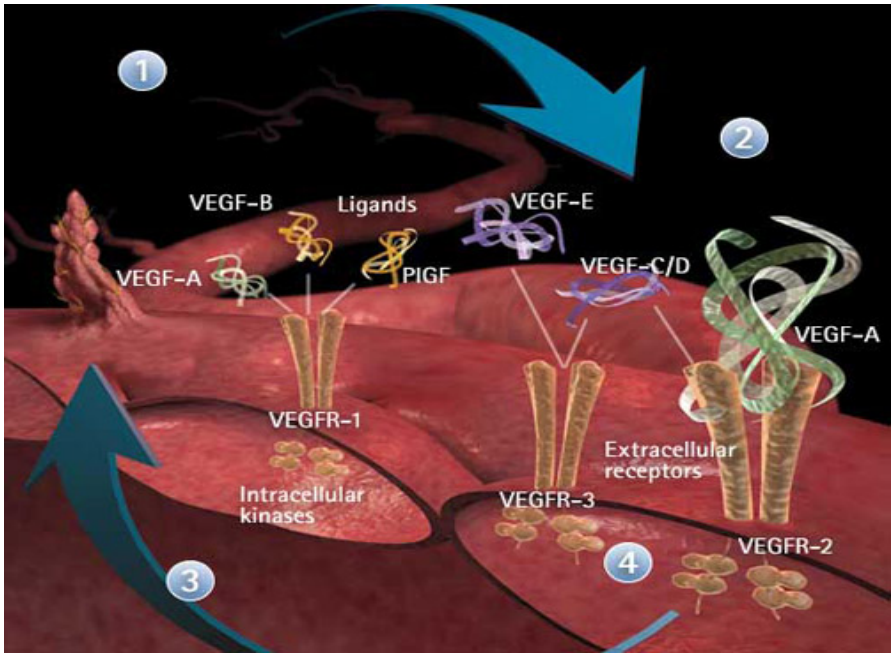


Figure 1.1.6.*: Image of VEGF family and VEGF receptors.*

VEGF-A is a secreted glycoprotein that assembles into a disulphide-linked homodimer. There are at least eight known human isoforms that are the result of alternative RNA splicing and termed VEGF121, VEGF145, VEGF162, VEGF165, VEGF165B, VEGF183, VEGF189 and VEGF206 according to their length in amino acid (Bouïs et al., 2006). Functional VEGF receptors were originally characterized as EC specific but they have recently been found on other normal cell types including vascular smooth muscle cells and monocytes/macrophages (Papetti and Herman, 2002).

VEGF-A transcription is stimulated by hypoxia as a result of Hypoxia Inducible Factor (HIF-1) binding to a Hypoxia Response Element (HRE) within the VEGF-A promoter.
Inflammatory mediators (IL-1α and IL-1β, TGF-β, prostaglandin E2) or activation of CycloOXygenase-2 (COX-2) as well, also increase VEGF-A production by mechanical forces of shear stress and cell stretch. VEGF promotes EC survival through activation of the Phosphatidyl-Inositol 3 Kinase (PI3K)/Akt pathway and through association with αvβ3 integrin and activation of focal adhesion kinase (Milkiewicz et al., 2006). It may increase EC permeability by enhancing the activity of vescicular-vacuolar organelles, clustered vesicles in EC lining small vessels that facilitate transport of metabolites between luminal and abluminal plasma membranes.
Alternatively VEGF may enhance permeability by loosening adherens junctions between ECs in a monolayer via rearrangement of cadherin/catenin com- plexes. Increased vascular permeability may allow for the extravasation of plasma proteins and formation of ECM favourable to ECs and stromal cell migration. Moreover VEGF stimulates EC production of Plasminogen Activator Inhibitor-1 (PAI-1) and interstitial collagenase. Therefore VEGF induces a balanced system of proteolysis that can remodel ECM components necessary for angiogenesis (Papetti and Herman, 2002).

VEGF-B is a growth factor that binds and activates VEGFR1 as well as NRP-1 (Papetti and Herman, 2002) inducing expression and increased activity of PAI-1 suggesting a role in ECM degradation and EC migration (Bouïs et al., 2006). VEGF-B is mitogenic for ECs and similarly to PlGF may cooperate with VEGF through its ability to form heterodimers with VEGF (Papetti and Herman, 2002) but it does not appear necessary for angiogenesis as demonstrated in mice deficient in VEGF-B that exhibit only minor cardiac defects (Aase et al., 2001).
VEGF-C and **VEGF-D** (also known as c-Fos Induced Growth Factor, FIGF) form a subgroup within the VEGF family as they consist of a central VEGF homology domain with N- and C-terminal extensions that are cleaved during protein maturation and are not seen in the other VEGFs or PlGF (Bouïs et al.,

2006). Both VEGF-C and VEGF-D stimulate angiogenesis *in vitro* and *in vivo* but their physiological roles are still undefined (Papetti and Herman, 2002).
VEGF-E refers to a group of VEGF-related proteins encoded by the *orf* virus, a parapoxvirus that infects sheep, goats and occasionally humans These viral proteins have retained VEGF function because they signal through VEGFR-2 ad stimulate angiogenesis *in vitro* and *in vivo* (Papetti and Hermann, 2002; Byrne et al., 2005).

Angiopoietins (Ang)
Four Ang have been identified and form a family of secreted proteins, Ang-1, Ang-2, Ang-3 and Ang-4 (Fielder and Augustin, 2006), that all bind to endothelium-specific receptor tyrosine kinases, the Tyrosine kinase with immunoglobulin and epidermal growth factor homology domains 1 and 2 receptor (Tie1 and Tie2) (Bach et al., 2007). The Tie receptors are almost exclusively expressed by ECs and hematopoietic stem cells. Ang and Tie receptors play a key role in angiogenesis (Otrock et al., 2007).
The best characterized are Ang-1 and Ang-2 which are 60% identical.

Ang-1 binds specifically to Tie2 thus activating it by inducing phosphorilation. This interaction does not promote the growth of cultured ECs however it induces migration, tube formation, spouting and survival (Bach et al., 2007). Many different cell types constitutively express ang-1: pericytes, smooth muscle cells, fibroblast and some tumor cells (Fielder and Augustin, 2006). In adults the Ang-1/Tie2 signalling system is essential for the maturation of vessels by maximizing the interaction between ECs and their surrounding support cells and matrix and stabilizing vessels (Post et al., 2008). Ang-1 is anti-inflammatory, protects against cardiac allograft arteriosclerosis and radiation-induced EC damage, promotes wound healing, inhibits VEGF-induced blood vessel formation and adhesion molecule expression. On the contrary its overexpression induces blood vessel and lymphatic angiogenesis (Fiedler and Augustin, 2006).

Ang-2 is the natural antagonist of Ang-1 by the inhibition of Tie2 signalling (Post et al., 2008). It is mainly produced by ECs and pericytes (Bach et al., 2007). Ang-2 mRNA is induced dramatically at the site of

vascular remodelling, promoting vessel destabilization (Bach et al., 2007), loosening of cell-matrix and cell-cell interaction. This antagonistic effect is thought to be a requirement for the sensitivity of ECs to other angiogenic factors such as VEGF (Post et al., 2008). Ang-2 up-regulation in the absence of other exogenous *stimuli* might result in vascular destabilization and subsequent vessels regression. Diversely to when present for a prolonged period or at high concentration, Ang-2 alone induces Tie2 phosphorylation in ECs and enhances their angiogenicity. Ang-2 expression is induced by various cytokines, including VEGF and bFGF and by microenvironmental factors (Fielder and Augustin, 2006). The temporal-spatial imbalance between the angiopoietins is crucial to the angiogenic switch. The presence of a higher level of Ang-2 compared to Ang-1 leads to increased angiogenesis and a more aggressive, larger, heavier tumour with a higher rate of proliferation that can be highly metastatic. A lower ratio of Ang-2:Ang-1 gives a slower rate of angiogenesis (Bach et al., 2007).

Ang-3 and **Ang-4** were described as ligands for Tie2 receptor in 1999 by homology cloning. Ang3 appears to be the mouse equivalent of the human protein Ang4. Ang4 has been shown to increase Tie2 phosphorylation and induce survival and migration unlike Ang3 that does not produce significant changes (Bach et al., 2006).

Fibroblast Growth Factor family (FGFs)

FGFs are major growth and differentiation factors in embryonic development as well as in the adult playing a role in neuronal signalling, inflammatory processes, hematopoiesis, angiogenesis, tumor growth and invasion. FGFs strongly bind to components of the ECM as HSPG from which they can be released by heparin or during ECM breakdown.

The FGFs binds the receptors FGFR. The two most extensively studied members of FGFs are acidic FGF (aFGF or FGF1) and basic FGF (bFGF or FGF2). Both aFGF and bFGF induce processes in ECs in vitro that are critical to angiogenesis (Papetti and Herman, 2002). bFGF has four known alternative splice forms all inducing proliferation, chemotaxis and urokinase type plasminogen activator activity, VEGF and VEGFR2 up-regulation in ECs, whereas only one isoform is known for aFGF (Bouïs et al., 2006).

Platelet-derived growth factor family (PDGF)

Besides VEGF and FGFs, **PDGF** has been shown to be one of the most potent angiogenesis inducers. Different cell types secrete it: platelets, macrophages, ECs, fibroblasts, keratinocytes (Bouïs et al., 2006), myoblasts, astrocytes and epithelial cells (Papetti and Herman, 2002). PDGF receptors (PDGFR) are made up of complexes between α- and β- subtypes: PDGFRα and PDGFRβ.

PDGFR are transmembrane proteins whose intracellular region contains the tyrosine kinase domain separated into two fragments by an interkinase insert but the extracellular region of PDGFR is formed by five immunoglobulin-like domains (Karamysheva, 2008). The effects of PDGF on vascular cells *in vitro* and *in vivo* suggest a role for this growth factor angiogenesis. Capillary ECs express PDGFRβ and are stimulated by PDGF-BB not only to increase DNA synthesis but also to form angiogenic chords and sprouts *in vitro*. PDGF also stimulates the proliferation and recruitment of cultured smooth muscle cells and pericytes both of which have been shown to express PDGFRβ (Papetti and Herman, 2002).

Transforming Growth Factor-β family (TGF-β)

TGF-β superfamily consists of close to 30 members including TGF-βs (TGF- β1, TGF-β2 and TGF-β3), bone morphogenetic proteins, activins and inhibins (Holderfield and Huges, 2008).

TGF-β is expressed by a wide variety of normal and transformed cells. It is found in ECM of many tissues. In the microvasculature both ECs and pericytes produce TGF-β and possess TGF-β receptors. It can stimulate or inhibit cell proliferation, control cell adhesion by regulating production of ECM, protease inhibitors and integrins and it can induce cellular differentiation. Moreover it regulates expression of a number of genes in ECs, particularly those involved in the establishment of, and interaction with, the BM (Papetti and Herman, 2002).

Tumor Necrosis Factor-α (TNF-α)

TNF is secreted by activated macrophages and activate T lymphocytes and it has pleiotropic proprieties between which is the ability to cause

apoptosis of tumor- associated ECs that can result in the complete destruction of the tumor vasculature.

TNF is a transmembrane protein, which upon cleavage by the metalloproteinase TACE produces a soluble trimer of 157 amino acid. Both isoforms of TNF (membrane and soluble) bind two distinct receptors that are ubiquitous, p55 or TNF- R1 and p75 or TNF-R2 (Lejeune et al., 2006).

Although TNF-α is primarily involved in inflammation and immunity, it shares many proprieties with TGF-β, stimulating angiogenesis *in vivo* and inhibiting EC growth (Papetti and Herman, 2002).

Epidermal Growth Factor (EGF)

EGF is secreted by platelets, macrophages and monocytes and in salivary, lacrimal and duodenal glands as well as in the kidney (Bouïs et al., 2006). EGF binds to the EGF receptor (EGFR) (also known as Human Epidermal growth factor Receptor, HER1) or other members of the HER family, HER2-4. HER2 signalling induces VEGF production via increases in HIFα protein synthesis (Milkiewicz et al., 2006). It has no direct effects on vascular endothelium but it is nevertheless involved in tumor proliferation, metastasis, apoptosis, angiogenesis and wound healing (Bouïs et al., 2006).

Others soluble growth factors

Many other soluble factors have been implicated in playing an important role in angiogenesis, but their effects on the vasculature are not as widespread as the previously discussed factors (Otrock et al., 2007).

Transforming growth factor-α (**TGF-α**) binds EGFR. It is a 5 to 6kDa protein that is mitogenic for ECs *in vitro* and induces angiogenesis *in vivo*.

Granulocyte Colony Stimulating Factor (**G-CSF**) and granulocyte/macrophage colony-stimulating factor (GM-CSF), proteins required for growth and differentiation of hematopoietic precursors, induce migration and proliferation of ECs to a limited extend (Papetti and Herman, 2002).

Several others soluble substances have been shown to affect angiogenesis.

Angiogenin induces angiogenesis in the chorioallantoic membrane and rabbit cornea assays (Otrock et al., 2007). It binds to extracellular

components and can support the adhesion and spreading of ECs *in vitro*. It is synthesized in small amount in the developing *fetus* and in large amount in the adult (Papetti and Herman, 2002).

Angiotropin is a polyribonucleopeptide that induces capillary EC migration. It is important in triggering the proliferative reactions in wound healing by activating microvascular ECs (Otrock et al., 2007).

Integrins

Integrins are a family of glycosylated, heterodimeric transmembrane adhesion receptors involved in cell-cell and cell-ECM interactions (D'Andrea et al., 2006). The attachment of ECs to the BM, ECM and each other usually occurs in specialised focal contacts where the cytoskeletal actin filaments are linked to **integrin** bound to the ECM through specialised cytoplasmatic proteins (Bouïs et al., 2006). They consist of non- covalently associated α- and β-subunits. There are 18 α- and 8 β-subunits known that can combine in more those 20 different heterodimeric receptors.

The role of αvβ3 integrin in mediating angiogenesis is evident in the binding of ECM components and matrix MMP-2 (Otrock et al., 2007). It is important for EC functions in neovascularization and is abundantly expressed on angiogenic blood vessels, in granulation tissue but not on vessels from normal skin. αvβ3 integrin mediates EC functions *in vitro* and plays an important role in angiogenesis *in vivo*. Its ligation also suppresses apoptosis in ECs, mediating EC survival by promoting proliferation and activation (Papetti and Herman, 2002).

Cadherins

Cadherins comprise a large family of Ca^{2+}-binding transmembrane molecules that promote homotypic cell-cell interactions.

Numerous studies highlighted the importance of VE-cadherin in neovascularization. VE-cadherin plays a role in regulating the passage of molecules across endothelium (Otrock et al., 2007). Cadherins not only establish EC junctional stability in the vessel wall but also enhance EC survival by promoting transmission of VEGF antiapoptotic signal to the

nucleus, a crucial result necessary for remodelling and maturation of vessels in angiogenesis (Papetti and Herman, 2002).

Eph-B4/Ephrin-B2

A group of RTKs and their ligands, playing a major role in angiogenesis are the ephrin (Eph) receptors and their ligands the **ephrins**.
Eph receptors belong to the largest known family of receptor tyrosine kinases consisting of at least 14 membrane-bound proteins (Papetti and Herman, 2002): A receptors (EphA1-A8) and B receptors (EphB1-B6) (Bouïs et al., 2006). Expression of ephrins can be induced by TNF-α and VEGF. They promote migration, repulsion, adhesion and attachment to the ECM via integrins (Bouïs et al., 2006). Moreover in the nervous system they appear to assist axon guidance through repulsive signals and establish borders between neuronal compartments (Papetti and Herman, 2002).

Biomechanical forces

The mechanical forces mediated by blood flow have profound effects on vessel growth (Papetti and Herman, 2002). A flowing fluid through a tube exerts a force tangential to the tube, called **shear stress** and another that is perpendicular to the tube wall and is caused by the pressure in the vessel, called **circumferential stretch**. Mature vessels can react to both these forces. Shear stress from blood flow has been shown to cause changes in morphology, cytoskeleton organization, ion channel activation and gene expression within ECs *in vitro*. In these mature systems chronic elevations in shear stress lead to increased vessel diameter, whereas chronic increases in pressure lead to decreased vessel diameters. This observation has lead to a pressure-shear hypothesis, whereby vessels adapt to shear stress as a function of local pressure.
The presence of **laminar flow**, which is a type of fluid flow where the stream-lines of the fluid motion are parallel, has been found to have atheroprotective effects on mature blood vessels. **Laminar shear stress** reduces apoptosis and induces antiapoptotic genes, such as Bcl-XL. Moreover it keeps cells from proliferating by inhibiting DNA synthesis. The signals that are created by flow are sensed through integrins and the cytoskeleton (Jones et al., 2006).

Thus microvascular blood vessels are remodelled in angiogenesis through several diverse mechanisms. Growth factors secreted from distant cells, transmembrane proteins binding to ECM components or to receptors on other cells and hemodynamic forces all act in concert to regulate normal angiogenesis. In a physiological setting, these factors exert both positive and negative influences on blood vessel growth to ensure that angiogenesis is confined to metabolic demands of growing and healing tissues (Papetti and Herman, 2002).

1.1.5. Endogenous inhibitors of angiogenesis

Much of the intracellular signalling associated with the angiogenic cascade appears to provide positive feedback for the continued activation of angiogenesis through their receptors (Bouïs et al., 2006). Some of the endogenous inhibitors are reported in Table 2.

Angiostatin	
Anti-angiogenic anti-thrombin III	
Canstain	
Endostatin (collagen XIII fragment)	
Fibronectin fragment	
Heparinases	Interferon-α (IFN-α)
	Interferon–β (IFN-β)
	Interferon–χ (IFN-χ)
Interleukin-4 (IL-4)	
Interlukin-12 (IL-12)	
Interleukin-18 (IL-18)	
Plasminogen activator inhibitor	
Pigment epithelium derived factor (PEDF)	
Prolactin 16KDa fragment	
Thrombospondin-1 (TSP-1)	
Retinoids	

Table 2.: ***Endogenous inhibitors of angiogenesis***

Angiostatin

Angiostatin is a fragment of plasminogen and was first determined to have anti- angiogenic proprieties because it inhibited neovascularization in a mouse tumor model. Angiostatin also inhibit EC proliferation, resulting in tumor dormancy through decreased cell replication and increases in

apoptosis (Milkiewicz et al., 2006). Several MMPs as well as tumor-cell-derived plasmin thiolreductase have been shown to cleave plasmin to angiostatin (Bouis et al., 2006).

Endostatin

Endostatin is a carboxy-terminal fragment of collagen XVIII that was shown to specifically inhibit EC proliferation and tumor growth. Endostatin inhibits VEGF-induced migration in Human Umbilical Vein ECs (HUVECs). Endostatin interferes with VEGF-stimulated VEGFR2 signalling, which may occur through direct interaction of endostatin with VEGFR2 (Milkiewicz et al., 2006). Angiostatin and endostatin has been shown to act synergistically to inhibit tumor angiogenesis (Bouis et al., 2006).

Other anti- angiogenic factors

Anti-thrombin, a plasma proteinase inhibitor of the serpin superfamily, is a heparin-binding protein and the major plasma inhibitor of coagulating proteases, primarily thrombin and factor Xa. Anti- angiogenic and antitumor growth proprieties have been attributes to both the cleaved and the uncleaved latent forms of human plasma-derived anti-thrombin (Cao et al., 2002).

Interferons (IFNs) have shown a significant anti-tumor activity in pre-clinical models and are the most commonly used cytokines in patients. IFNs expression precedes the majority of the innate response cytokines and it has been proposed to be the first cytokine secreted by antigen-presenting cells after antigen stimulation (Minuzzo et al., 2007).

Vasohibin was described as a gene induced in VEGF-stimulated ECs. The gene encodes for a secreted protein that is an inhibitor of tumor angiogenesis. The precise mechanism by which vasohibin blocks angiogenesis is not yet known, although direct antagonism of growth factor receptors has been excluded probably as a negative feedback regulator of angiogenesis (Milkiewicz et al., 2006).

Arrestin, canstatin, restin as well as **fragments of prolactin** and other small fragments could inhibit angiogenesis (Bouïs et al., 2006)

1.2. Angiogenesis in specific pathological conditions

1.2.1. Angiogenesis during Neurodegeneration

Great emphasis has been addressed to the role of angiogenesis during neurodegeneration, a process characterized by neuronal loss in specific areas of the central nervous system (Vagnucci and Li, 2003).

Neurodegenerative diseases are commonly divided into two main groups according to phenotypic features: conditions causing problems with movements or conditions affecting memory and related to dementia (Kim et al., 2009).

Recent data established that angiogenesis plays a crucial role in the evolution of neurodegenerative disorders since it has been demonstrated that vascular remodeling occurs during the evolution and the progression of these disorders (Fioravanzo et al., 2010).

The most studied neurodegenerative disorder is Alzheimer's disease (AD), characterized by senile plaques (SPs) of β-amyloid (Aβ) and by neurofibrillary tangles (NFTs) of hyperphosphorylated tau protein (Esposito et al., 2006).

A plentiful inflammatory reaction is a common hallmark of several neurodegenerative diseases, and it has recently been considered as a pivotal mechanism responsible for the progression of the neurodegenerative process (Skaper, 2007).

Microglia and astrocytes are the resident innate immune cells in the brain and produce a plethora of factors, as IL-1, TNF-a, NO, prostaglandins, superoxide, which result toxic for neurons (Heneka et al., 2007; Hoozemans et al. 2002; for a review, see De Filippis et al., 2009). Behind pro-inflammatory and pro-oxidant mediators, activated glial cells are able to release the whole machinery necessary for the development of the angiogenetic process, such as the pro-angiogencic factors VEGF, TNF-α together with MMPs, the enzymes degrading extracellular matrix (Tarkowski et al., 2002; Thirumangalakudi et al., 2006; Fioravanzo et al., 2010; Fig. 1.2.1.).

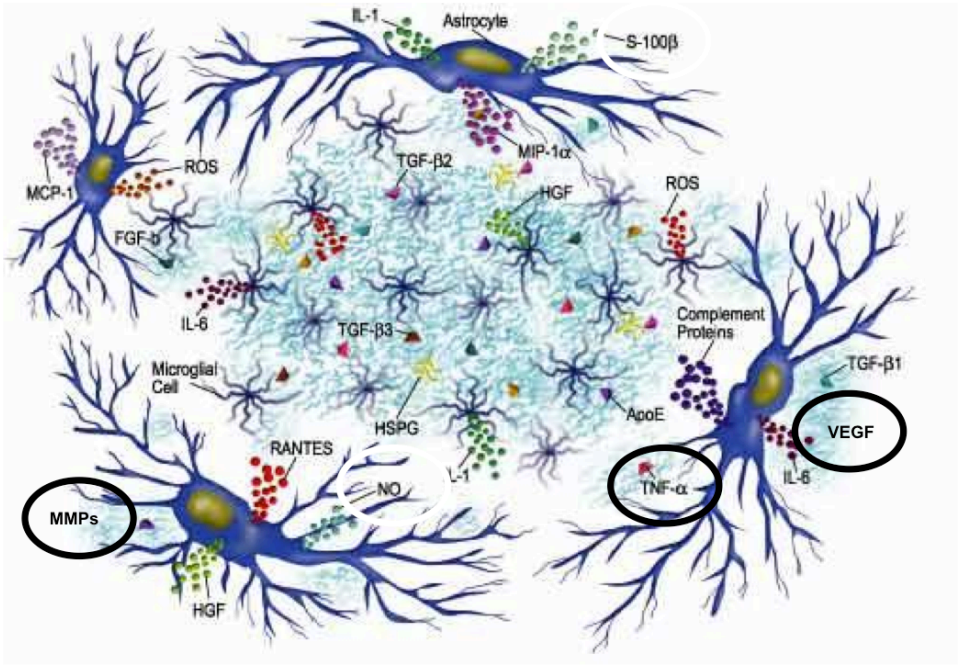


Figure 1.2.1.: ***Image of reactive gliosis.*** *Pro-inflammatory, pro- angiogenic and neurotoxic mediators released during reactive gliosis.*

Even if during neuroinflammation the formation of new vessels brings oxygen and nutrients to the injured tissue, several evidences show that this process gets worse the reactive gliosis present in the damaged area (Chrystov et al., 2006).

Many evidences show the presence of new vessels formation during AD (Cantara et al., 2004; Yang et al. 2004). In the intratecal lumen of post mortem brain of patients affected by AD, i. e., a significant increase of the pro- angiogenic VEGF and TGF-β expression was found (Chiappelli et al., 2006). Moreover, recent data obtained by histological analysis from the hippocampus, midfrontal cortex, *substantia nigra pars compacta* (SNpc), and *locus ceruleus* of AD patients, reported an increased number and diameter of blood vessels, thus confirming the occurrence of angiogenesis in AD. In the same study, furthermore, it has been recognized that angiogenic vessels are different from patent vessels, not only in diameter, but also in structure, since they present numerous *fenestrae*, widened inter-endothelial junctions, abnormal endothelial cell shapes, and abnormal basement membranes (Carmeliet et al., 2000).

Another confirmation that angiogenesis occurs during neurodegeneration in AD brains is that new vessels formation have been found into the Aβ senile plaques, too. Besides this, vascular Aβ deposition, also known as cerebral amyloid angiopathy, is associated with vascular dysfunction in animal and human studies. Finally, AD is related with morphological changes in capillary networks, and soluble Aβ produces abnormal vascular responses to physiological and pharmacological *stimuli*. This last evidence is emphasized by the role of hypoxic transcription factor-1a (HIF-1a), regulator of hypoxia in AD, that seems to increase the expression of beta-secretase (BACE), bringing about the proteolytic cleavage of the Aβ precursor protein (APP) in Aβ peptide (Zhang and Xu, 2007). Moreover, the same HIF-1a, that is up-regulated in a hypoxic cell, is able to activate the transcription of VEGF; subsequently, VEGF binds VEGFR on the capillary surface and leads to a key- series of events: the change of vessel permeability, the activation of endothelial cells which start to proliferate and to release mediators able to break down the basement membrane and, finally, the activation of MMPs, that cleave the surrounding extracellular matrix, allowing the endothelial cells to migrate leading to tube formation (Qutub et al., 2009).

Furthermore, a recent post-mortem study indicated that brain regions affected by AD pathology (i.e., *hippocampus, midfrontal cortex, substantia nigra, and locus ceruleus*) are sites of active angiogenesis. In particular, in the hippocampus, the on-going angiogenesis resulted in increased vascular density, that directly correlated with Aβ load, suggesting that angiogenesis in AD and may be related to tissue injury (Desai et al., 2009; for a review, see De Filippis et al., 2010).

These results are confirmed not only by several experimental studies indicating that the injection of pro- angiogenic mediators in normal brain induces AD-related symptom and signs, but also by other evidences on animal models of AD, which are habitually accompanied with important vasculature changes (Esiri, 2000).

In view of all these evidences, to underline the correlation between new vessel formation and AD and its possible pharmacological modulation still remains very important for the progression of this disease.

1.2.2. Angiogenesis during Inflammation

The signals controlling angiogenesis, while directed at the endothelial cells, come from cells in the nearby tissues (Folkman and Brem, 1992). As previously described, different cells are able to produce angiogenic factors when the environment becomes hypoxic or inflammatory, including tumor cells, keratinocytes, monocytes, macrophage and mast cells. Of all these cells, the inflammatory monocyte- macrophage-mast cell type can be found at most sites where angiogenesis is occurring in an abnormal environment, including wound healing and disease (De Filippis et al., 2008). Activated monocytes and/or macrophages alone are sufficient to induce angiogenesis in the avascular cornea (Koch et al. 1996). In fact, almost every growth factor and cytokine known to regulate angiogenesis can be produced by macrophages (Sunderkotter et al., 1994).

Factor capable of inducing angiogenesis are categorized as either indirect, those that act via intermediary mechanisms, often being found to induce angiogenesis only in vivo, or direct, those also able to induce proliferation, migration, and/or differentiation of endothelial cells directly in vitro.

Obviously, as well explicated in the first paragraph, VEGF appears to be the most endothelial cell proliferation and migration; in vivo, it is potently angiogenic and causes vascular permeability. Together with VEGF, after adamage, other factors released by inflammatory cells have direct or indirect effects on endothelial cells, including TGF-α, TGF-β PDGF (Sunderkotter et al., 1994). During chronic inflammation, i.e, several pro- angiogenic mediators are implicated, since angiogenesis occurs for the maintenance of tissue perfusion, in allowing the increase in cellular trafficking necessary for chronicity (De Filippis et al. 2008).

In granulomatous tissue, a chronic inflammation, for example, nitric oxide produced by the endothelium and macrophages by inducible NO synthase is an important mediator of tissue destruction and oedema in inflammation (Iuvone et al., 1994; Appleton et al., 1996). Granuloma is a specific well-characterized experimental procedure during which there is a strong implication of pro- angiogenic and pro-inflammatory factors (Iuvone et al., 1994; Iuvone et al., 1999).

In fact, it is characterized by the formation of a specific tissue that includes an intense angiogenesis and infiltration of inflammatory cells (Lage and Andrade, 2000). Granulomatous tissue is histologically characterized by the presence of infiltrating macrophages, epithelioid and giant cells, activated mast cells (Forehand and Johnston, 1995)

Among the cells participating in granuloma formation, connective MCs, especially present in the skin, importantly contribute to the development of chronic inflammation. MCs, shown to be in a large part near sites of new capillary sprouting, are implicated in angiogenesis; in fact they produce, store and release several mediators, both vasoactive amines (Metcalfe et al., 1997) and several enzymes degrading the connective tissue matrix, in providing new space for neovascular sprouts

The implication of these cells and of their releasing factors give to the granulomatous chronic inflammation an important explicative role to characterize angiogenic process.

1.2.3. Angiogenesis during Dermatitis

Several evidences show that there is a strong implication between angiogenesis and dermatitis. Dermatitis is most commonly defined as an inflammation of the skin. Symptoms of dermatitis include red, swollen, itchy and/or blistered skin. There are two basic types of dermatitis: endogenous ("from within") dermatitis is an often-chronic allergic skin condition that has no apparent external cause; exogenous dermatitis, also known as contact dermatitis has recognisable external causes.

The evidence of a presence of new vessel formation during dermatitis, is well reported in particular during contact allergic and atopic dermatitis, since the participation of a specific immunity cell, in particular mast cells, able to release different kinds of pro- angiogenic factors, is testified in literature (Groneberg at al., 2005).

Between these types of dermatitis, contact allergic dermatitis (CAD) is a specific one defined as a delayed hypersensitivity reaction that occurs after repeated exposure to an allergen.

Numerous irritants and/or allergens (nickel, rubber, plants, medications) can induce CAD, and the corresponding rash develops within 48–72 hs of re-exposure to the same antigen. Additional symptoms are itching, redness, swelling and the formation of small skin blisters. CAD develops in two steps: (1) contact of an allergen with the skin and its binding to Langerhans cells, which then travel to lymphnodes, where the allergen is exposed to T-lymphocytes; the allergen is also taken up by resident and newly recruited dendritic cells, which migrate to lymph nodes and prime the T-lymphocytes; and (2) re-exposure to the allergen, which causes the activation of primed T-lymphocytes, with subsequent release of inflammatory mediators that are responsible for the features of inflammation and keratinocyte injury (Karsak et al., 2007; Imada et al., 2002).

In a mice model of 21 days- DNFB induced CAD, explained by Karsak et al., i.e, it has been well-characterized the presence of activated keratinocytes during the early phase of the disease (Petrosino et. al., 2011), while a strong involvement of another different cell type, mast cells, it has been shown during the final phase, as it normally happens during other similar types of dermatitis. Mast cells, once activated, are able to release different types of mediators, between which pro- angiogenic mediators.

In parallel, during the last phase of CAD, it has been described the presence of small calibre vessels, as a proof of on-going angiogenesis.

Therefore, the modulation of mast cell activation results a momentous approach to regulate mast cell sustained angiogenesis during CAD.

1.3. Mast Cells

1.3.1. Mast cells during angiogenesis and inflammation

In 1879 Paul Ehrlich firstly described mast cells.

Initially, in rodents, MCs were divided into two subtypes depending on the variable content of the neutral serine proteases, tryptase and chymase. Thus, MCs containing only tryptase represent the mucosal MCs, preferentially localised in the mucosa of the airways and gastrointestinal tract. MCs containing chymase, tryptase, carbossipeptidase and chatepsin G are defined as connective-tissue MCs, which are typically found in the skin, synovium, peritoneum and peri-vascular tissues. However, in other species, including humans, another subtype has been identified containing chymase and carbossipeptidase and possessing a different tissue localisation (Bradding et al., 1995).

Their strategic localisation that directly interfaces with the external environment makes MCs acting as surveillance antennae against different types of injury and, whenever necessary, they can activate and regulate both innate and adaptive immune mechanisms (Bienenstock et al., 1986). Moreover, MCs possess important physiological roles that support homeostatic control, such as in tissue remodelling, wound healing, and the neuroimmune response to stress (for a review, see Bradding et al., 1995) and are located in close proximity to blood and lymphatic vessels (Sacchi et al., 2003) and nerves (Newson et al., 1983) in order to perform this function.

MCs possess several biological mediators that are released from cytoplasmic granules primarily due to *stimulus*-induced degranulation; including vasoactive amines such as histamine, proteoglycans (mainly heparin and chondroitin sulphate), neutral serine proteases, such as tryptase and chymase, cytokines and growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor, NGF, transforming growth factor (TGF)-β and tumour necrosis factor alpha (TNF-α) (Bradding et al, 1995). However, activated MCs can also induce de novo synthesis of several inducible enzymes, such as inducible nitric oxide synthase (NOS) or cyclo-oxygenase-2 (and their products nitric oxide, prostaglandins and leukotrienes), together with a plethora of chemokines and cytokines, TNF-a,

IL-1a, IL-1b, IL-6, IL-9, IL-12, IL-18 and TGF-b, which function to amplify the inflammatory / immune reaction. Hence, MCs have long been known to participate in the inflammatory process and this knowledge was based on the evidence that MCs are present and recruited to all inflammatory sites.

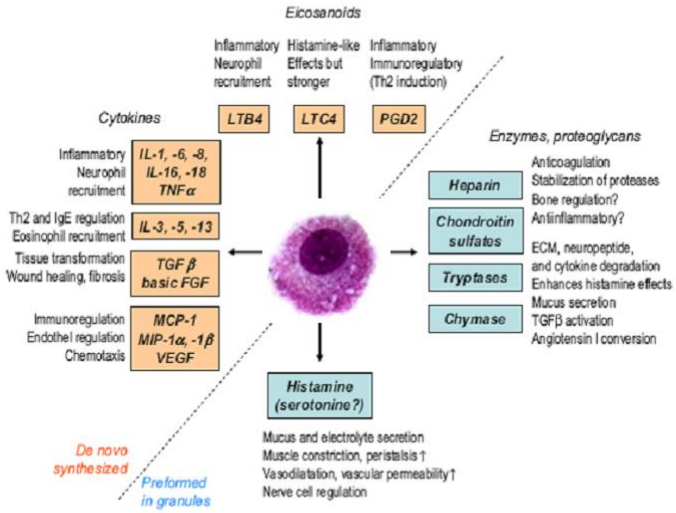


Figure 1.3.1.: ***Mast cells activation.*** *Realising of preformed and/or new synthesis mediators*

Recently, it became clear that MCs play an important role in orchestrating the whole inflammatory process from initiation events to chronic activation. MCs are the first immune cells to considerably stimulate the inflammatory process due to a rapid release of pro-inflammatory and vasoactive mediators. In particular, histamine, cytokines and proteases facilitate a pro-inflammatory microvascular environment due to increased vasodilatation, increased vascular permeability and local oedema formation, thus increasing the tissue delivery of other pro-inflammatory cells (Fig. 1.3.1.)

Moreover, histamine and TNF-a, released by MCs, increase inflammatory cell recruitment due to an increase in (i) endothelial adhesion molecules expression (selectin and integrins), and thus the stimulation of leucocyte endothelial adhesion and rolling, and (ii) MC-derived leukotrienes and IL-8, which act as chemotactic signals for eosinophils, neutrophils and basophils (Ribeiro et al., 1997). Therefore, even if the main goal of MC activation is to

initiate the inflammatory response to maintain internal homeostasis, excessive MC activation could lead to unwanted chronic inflammation.
Previous studies demonstrate the role of MCs in the chronic inflammatory process, showing that granuloma formation in rats was accompanied by massive mast cell degranulation, whereas the depletion of MC granule contents reduced granulomatous tissue formation by more than 50% (Russo et al., 2005; Fig. 1.3.2.).

The important role of MCs in chronic inflammation is also linked to their fundamental role in angiogenesis. In fact, they are considered dues ex machine during new vessel formation, since they are localised in tight contact with blood vessels, where they release the main vasoactive compound, such as VEGF, TNF-α, together with important extracellular matrix degrading enzymes, as metalloproteinases (MMPs) and chymase.

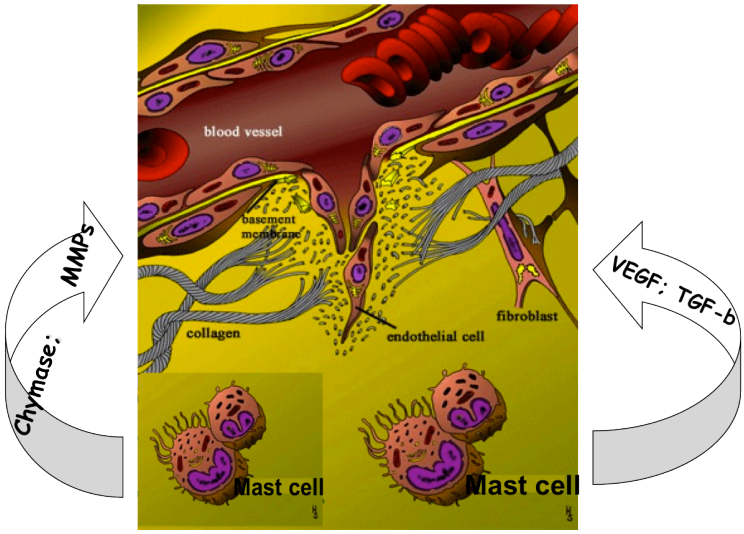


Figure 1.3.2.: ***Mast cell sustaining angiogenesis.*** *Mast cells are able to regulate angiogenesis through the release of pro- angiogenic mediators*

Chymase is a peptidase with chymotrypsin-like activity, almost selectively expressed by MCs. Together with fibronectin and collagen, chymase directly contributes to extracellular matrix degradation, and, indirectly, it plays also an important role in the activation of MMPs.
Thanks to chymase, MCs possess the complete angiogenesis “package”: they secrete MMPs, and they can activate and inhibit them, in promoting the matrix degradation and, at the same time, in realising several factors which

activate endothelial cells. Within chymases family, *rat mast cell protease-5* (rMCP-5) is the most studied and the most known.

1.3.2. Rat Mast Cell Protease-5 and Microphthalmia-associated Transcription Factor

Rat mast cell protease-5 belongs to a family of serine proteases classified as chymases. The chymase family can be further subdivided into two groups: *α*- and *β*-chymases (Sanker et al., 1997). Among rodent α-chymases, rMCP-5 is predominantly expressed in connective tissue type mast cells (MCs) and also early in MC development (McNeil et al., 1991).

Like other MC chymases, rMCP-5 is packed in the MC secretory granules intimately bound to proteoglycans and it is released, together with several other stored mediators, upon activation (Forsberg et al., 1999). Several evidences show that rMCP-5 plays a crucial role in the inflammatory/immune process.

My research group has previously demonstrated that rMCP-5 exhibits pro-inflammatory and pro- angiogenic effects in rat λ-carrageenin-induced granuloma, i.e., a model of chronic inflammation actively sustained by mast cell activation (Russo et al., 2005).

It is also known that MC chymase plays an important role in the allergen-induced biphasic skin reaction (Tomimori et al., 2002) and in eliciting/maintaining cutaneous inflammation in atopic dermatitis (Badertscher et al., 2005).

Moreover, MC chymase has been proposed to increase vascular permeability both in skin disease and brain edema during intracerebral hemorrhage (He and Walls, 1998; Strbian et al., 2009).

At a cellular level, MC chymase has been shown to induce the release of neutrophil chemoattractants by eosinophils and to mediate interaction between MCs and eosinophils in allergic diseases (Terakawa et al., 2006;).

An appealing pharmacological strategy for controlling rMCP-5 is offered by the modulation of its transcriptional pathway. In fact, it has been demonstrated that several mouse MCPs (mMCP) are under the transcriptional control of Microphthalmia-associated Transcription Factor

(MITF) and that, more particularly, mMCP-5 gene expression appears to be regulated by a MITF-mediated mechanism (Levy et al., 2006)

MITF belongs to Myc supergene family of basic helix-loop-helix leucine zipper (bHLH-Zip) DNA-binding protein; these proteins are dimeric transcription factors and important regulators of cell development and survival (Hershey and Fisher, 2004). MITF protein is predominantly expressed in MCs, melanocytes, heart and skeletal muscles. MITF regulates the transcription of a spectrum of genes in MCs, many of which are crucial for their differentiation and survival, including several MCPs, protease inhibitors, adhesion molecules, metabolic enzyme and growth factor receptors (Kitamura et al., 2006).

Phosphorylation/dephosphorylation of proteins is a key-regulatory step in protein-protein interactions (Sonnenblick et al., 2004.). MITF takes part to the coordinated expression of its target genes through its association with different proteins, as a result of protein kinase-dependent phosphorylation (Shibahara et al., 2001). Three serine sites (Ser73, Ser298 and Ser409) for MITF phosphorylation have been reported (Wu et al., 2000); the phosphorylation of these serine sites positively correlates with the up-regulation of MITF transcriptional activity and degradation of MITF (Fig. 1.3.3.). In particular the phosphorylation of Ser73 is due to the activation of ERK pathway (Hamesath et al., 2008)

Thus, the phosphorylation/dephosphorylation of MITF plays an important role in regulating MCs proliferation and the relative expression of their mediators. Hence, these evidences suggest a strong correlation between MITF, MCs and their stored mediators (chymase *in primis)* and inflammation.

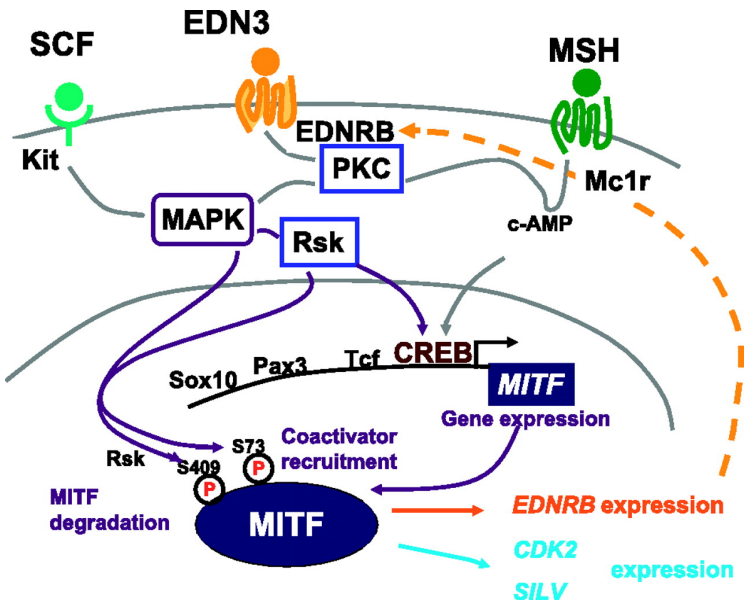


Figure 1.3.3.: ***MITF.*** *Mechanism of activation/ de-activation of MITF.*

2. *ALIA*mides: Palmitoylethanolamide & Congeners

2.1. *ALIA* hypothesis

The acronym *ALIA* (*Autacoid Local Inflammation Antagonism*) was formulated when Aloe et al. (1993), when he indicated that some endogenous N-acylethanolamines exerted a local antagonism on inflammation (Fig. 2.1.1.).

Rita Levi-Montalcini and co-workers (1996) modified the acronym into *Autacoid Local Injury Antagonism*, in order to explain the mechanism of action of Palmitoylethanolamide (PEA) and related fatty acid amides. This change was made following the observation that the pharmacological effects of PEA appear to reflect the consequences of supplying the tissue with a sufficient quantity of its physiological regulators of cellular homeostasis (Levi-Montalcini et al., 1996). The effect was first attributed to mast-cell activity control. Concomitantly, Mazzari et al. (1996) demonstrated that the in vivo anti-inflammatory effects of PEA were due to down-regulation of mast-cell degranulation.

Similar results have recently been obtained in dogs and cats. In fact, densitometric analysis performed on skin biopsies from cats with eosinophilic granuloma complex and treated with PEA, revealed an increase in the granular density of cutaneous mast-cells, thereby suggesting a decrease in mast-cell degranulation (Scarampella et al., 2001).

Using similar techniques, an inhibitory control over mast-cell degranulation has been demonstrated in dogs locally exposed to PEA analogues.

Together with PEA, other compounds acting as *ALIA*mide has been described; however, starting from these evidences, Palmitoylethanolamide is considered as the early *ALIA*mide.

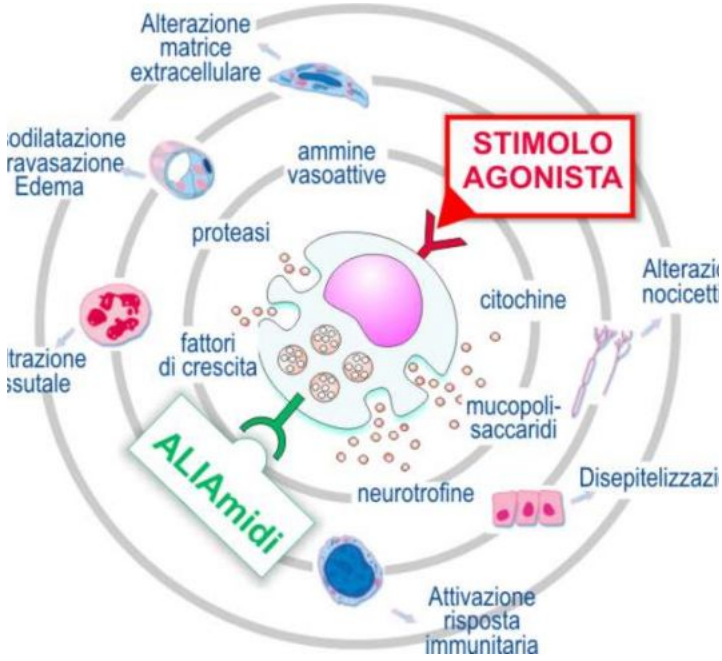


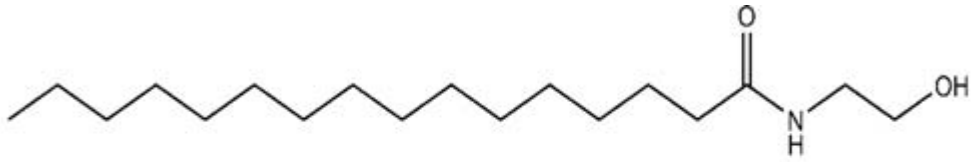
Figure 2.1.1.: ***ALIA mechanism***

2.2. Palmitoylethanolamide

The endogenous fatty acid amide palmitoylethanolamide (PEA), chemical structure N-(2-hydroxyethyl) esadecanamide (Fig. 2.2.1.) is naturally present both in animal and in vegetable tissues and is able to enhance both the cannabinoid and vanilloid signalling was initially considered as an autacoid, acting mainly as an anti-inflammatory agent through the down-regulation of mediator release from mast-cells, monocytes and macrophages (Aloe et al., 1993, Facci et al., 1995; Mazzari et al., 1996; Ross et al., 2000; Scarampella et al., 2001).

In addition to the hypothesis that PEA has potent immunoregulatory properties, recent data have demonstrated that PEA may also play a key role in the regulation of complex systems involved in the inflammatory response, pruritus, neurogenic and neuropathic pain (Di Marzo et al., 2001).

In general, PEA is thought to be involved in endogenous protective mechanisms that are activated in the body as a result of different types of tissue damage or stimulation of inflammatory responses and nociceptive fibres.



Palmitoylethanolamide
(PEA)

Figure 2.2.1.: ***Palmitoylethanolamide structure.*** *N-(2-hydroxyethyl) esadecanamide*

PEA and related fatty acids amides are classified on the basis of their mode of action as endocannabinoids and cannabimimetics (Petrosino et al., 2010). There is a great deal of evidence showing that PEA may act both as an anti-inflammatory and anti-nociceptive agent (De Filippis et al., 2011; Lambert et al., 2002). However, the real mechanism of action of this endogenous compound that mediates its anti-inflammatory, analgesic and anti-pruritic effects is still debated. There are at least three hypotheses, supported by experimental evidence concerning PEA pharmacodynamics, all seemingly different, but for several aspects they may be considered complementary and synergistic.

2.2.1. Biosynthesis and inactivation.

Unlike classical neurotransmitters and hormones which are stored in and relased from intracellular secretory vesicles, the production of FAE occurs through on-demand synthesis within the lipid bilayer (Cadas et al., 1996; Schmid et al., 1990). In mammalian tissues, two concerted and independent biochemical reactions are responsible (Fig. 2.2.2.).

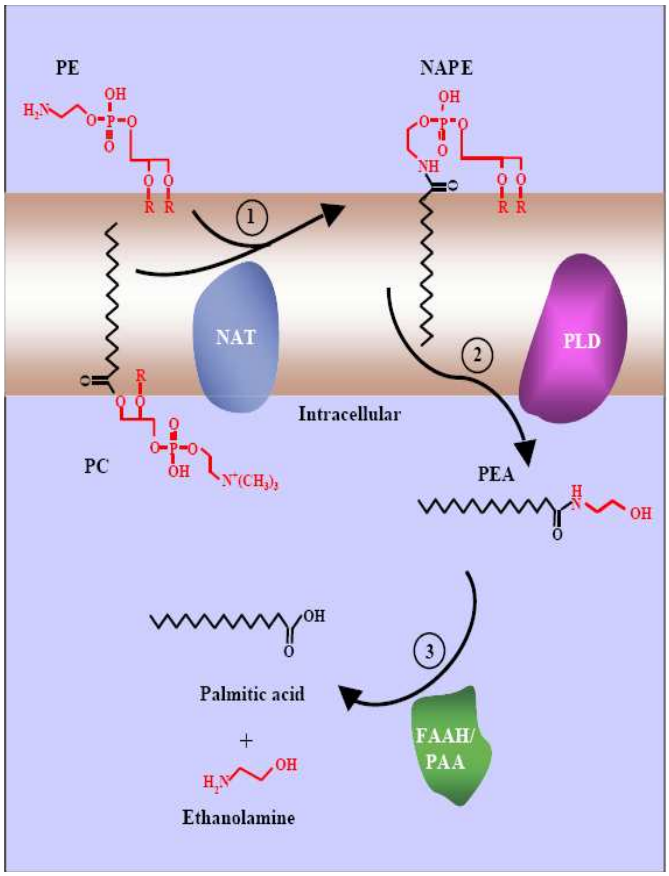


Figure 2.2.2.: ***PEA biosynthesis and inactivation***

The first is the transfer of a fatty acid from membrane-bound phospholipids to phosphatidylethanolamine (PE), catalyzed by a calcium ion and cyclic-AMP regulated N-acyltransferase (NAT), to form the FAE precursor N-acyl phosphatidylethanolamine (NAPE).

Different FAE precursors are generated according to which fatty acid is initially transferred to PE (i.e., the initial transfer of palmitic acid will yield a PEA precursor, while that of arachidonic acid will yield an anandamide precursor). The second step in FAE synthesis is the cleavage of membrane-bound NAPE to release free PEA, which is mediated by a NAPE-specific phospholipase D (PLD). This lipid hydrolase shares little sequence homology to other members of the PLD family and recognizes multiple NAPE species, producing PEA along with other FAEs (Okamoto et al., 2004).

Alternatively, a separate mechanism of synthesis has been proposed involving a similar two-step reaction: (1) the hydrolysis of NAPE to N-palmitoyl-lysoPE (lyso-NAPE) by soluble phospholipase A2 (sPLA2) and (2) the subsequent cleavage of lyso-NAPE by a lysophospholipase D (lyso-PLD) (Natarajan et al., 1984). The activities of these two enzymes are highest in the stomach, brain, and testis (Sun et al., 2004). The relative contribution of each of these synthetic pathways is unknown at present.

PEA inactivation primarily consists of its intracellular hydrolysis by lipid hydrolases (Fig. 2.2.2.) (Schmid et al.,1985). One of these enzymes, called fatty acid amide hydrolase (FAAH), has been molecularly cloned (Cravatt et al., 1996) and extensively characterized (Bracey et al., 2002), and selective inhibitors that block its activity in vivo have been developed (Kathuria et al., 2003).

A second enzyme, referred to as PEA-preferring acid amidase (PAA), has also been identified (Ueda et al., 2001). FAAH, a membrane-bound intracellular serine hydrolase, for which PEA is an excellent substrate (Désarnaud et al., 1995; Hillard et al., 1995; Ueda et al., 1995; Fowler et al., 2001), is present in all mammalian tissues, but is particularly abundant in brain and liver (Cravatt and Lichtman, 2002).

In fact, mice lacking the faah gene have dramatically reduced PEA hydrolysis and increased PEA levels in brain and liver tissues (Cravatt et al., 2004; Lichtman et al., 2002). In contrast to FAAH, PAA activity is most abundant in the rodent intestine, spleen, and lung (Ueda et al., 2001). PAA recognizes all FAEs, suggesting that it may play a broad role in the deactivation of these compounds by intact cells; however, in the presence of detergent, this activity displays a marked preference for PEA as a substrate (Ueda et al., 2001).

2.2.2. Hypothesis of action

The molecular mechanism of action of PEA is still controversial and several hypotheses have been put forward to explain its anti-inflammatory and analgesic effects. These hypotheses include the following:

i. an *Autacoid Local Injury Antagonism* through which PEA acts by down- regulating mast-cell degranulation (Aloe et al., 1993);

ii. the direct stimulation of the cannabinoid CB2 receptor or of an as-yet uncharacterized CB2-like receptor, as suggested by results obtained in different in vivo studies using the CB2 antagonist SR144528;

iii. an 'entourage effect' (Smart et al., 2002) through which PEA acts by enhancing the anti-inflammatory and anti-nociceptive effects exerted by another fatty acid ethanolamide, anandamide (AEA), which is often produced together with PEA. AEA acts by activating the cannabinoid CB1 and CB2 or the transient receptor potential vanilloid receptor type 1 (TRPV1) channel, and PEA might potentiate these actions either via inhibition of the expression of FAAH (LoVerme et al., 2005) for which AEA is also a substrate, or through allosteric stimulation of TRPV1 receptors, or both. Activation of TRPV1 receptors is then immediately followed by their desensitization and refractoriness to s*ubsequent* stimulation by inflammatory or nociceptive *stimuli*;

iv. a binding to the orphan G-protein coupled receptor (GPR55), that is not yet well-clarified;

v. last, a specific molecular target has been found for PEA, the nuclear peroxisome proliferator- activated receptor-a (PPAR-a), which clearly mediates several anti-inflammatory effects of this compound (D'Agostino et al., 2007; Scuderi et al., 2011)

2.3. PEA Congeners

2.3.1. Adelmidrol and Fumidrol

Recent studies have shown the existence of other molecules, classified as *ALIA*mide, able to acts as anti-inflammatory compounds, following the most considered exemple of PEA: Adelmidrol and Fumidrol. Adelmidrol is a synthetic derivate of azelaic acid, a naturally occurring saturated dicarboxylic acid, that is found in some whole grains and intrace amounts in the human body (Bonner et al., 1999), its plasma levels normally ranging from 20 to 80 ng/mL, while Fumidrol is a synthetic derivate of fumaric acid.

Between Adelmidrol and Fumidrol, the most studied, according to their pharmacological activity, is Adelmidrol.

Adelmidrol is an amphiphilic or amphipathic compound, possessing both hydrophilic and hydrophobic properties, that favour its solubility both in aqueous and organic *media.* The effect of Adelmidrol has been shown to depend, at least in part, on the control of MCs granular density, in suggesting a decrease in their degranulation (De Filippis et al., 2009).

In particular, the topical application of a gel formulation containing the fatty acid amide Adelmidrol produced a significant increase in the densitometric measurement for cutaneous mast-cells located in the lips of dogs with experimentally induced wounds. In the dog, the percentage surface re-epithelialisation associated with the increase in mast-cell densitometric measurement was significantly higher in the lesions exposed to aldemidrol than in those treated with vehicle.

Finally, De Filippis et al. (2009), have shown strong anti-inflammatory and anti- angiogenic activities of Adelmidrol during granuloma, a specific chronic inflammation sustained by mast cell activation in rats, as a proof of its potent action in controlling mast cells behaviour.

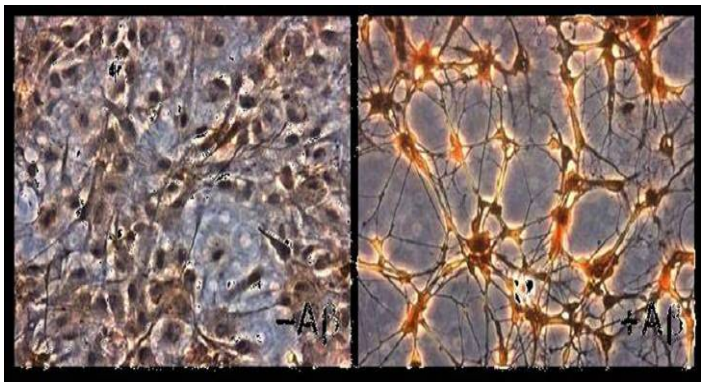
3. Material and Methods

3.1. Cell Cultures

3.1.1. C6 Rat Glioma Cell Cultures

C6 rat glioma cells (ATCC CCL-107) were cultured in 10 cm i.d. Petri dishes ($3x10^3$/dish) and in 24 multiwells plates ($6.25x10^4/cm^2$), in Dulbecco's modified Eagle's *medium* supplemented with 5% FBS (*fetal bovine serum)*, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in 5% CO_2, 95% air.

Confluent cells were treated with 1 µg/ml Aβ 1-42 (Tocris, Ellisville, MO) in the presence or absence of the following substances: PEA 10^{-8}-10^{-6} M (Epitech Group, Italy) and the selective PPAR-α antagonist GW6471 (2,5-10µM) (Tocris, Ellisville, MO). After 48 hours incubation time, cells in Petri dishes were subjected to the different treatments, as described below. *Media* of cells in 24 multiwells were collected and used to stimulate HUVEC cells.



(A) **(B)**

Figure 3.1.1.: ***Images of C6 cells.*** *C6 cells in* ***(A)*** *normal conditions and* ***(B)*** *after Aβ-insult.*

3.1.2. Human Umbilical Vein Endothelial Cells

Clonetics® HUVEC Cells (Lonza, Basel, CH) were cultured in Clonetics® EGM® Endothelial Growth *Medium* (Lonza Basel, CH) at 37°C, 5% (v/v)

CO_2. Confluent cells were seeded in 96 multiwells plates ($3.125x10^4/cm^2$) and incubated for 24 hours. After this period of incubation, they were treated with supernatant-*media* of C6 pre-treated cells, and incubated for 48 hours. 16 hours before the end of incubation time, BrdU was added to evaluate cell proliferation.

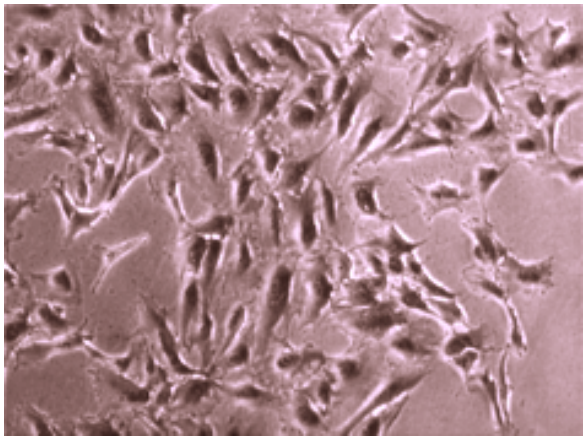


Figure 3.1.2.: ***Image of HUVEC cells.***

3.1.3. Cell Functionality Assay

To determinate cells functionality, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-Formazan Assay (MTT assay) was performed. Cells were plated at the density of $1×10^5$ cells/well, left to adhere for 2 h at 37 °C, and then treated as described previously. After 48-h incubation time, 25µl of 5g/L 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium solution were added to the cells. Cells were incubated for an additional 3 h at 37 °C before being lysed, and then dark blue crystals were solubilized with 125 µl of a 50% N,N-dimethyl formamide/20% sodium dodecyl sulfate solution with an adjusted pH of 4.5. Optical density (OD) was measured with a spectrophotometer (Titertek Multiskan MCC/340; Titertek Instruments, Huntsville, AL) equipped with a 620-nm filter. Cell viability was calculated as OD treated/OD control ratio.

3.1.4. Cell Proliferation Assay

To determine the effect of treatment of C6 cells on HUVEC cellular proliferation, Cell Proliferation ELISA BrdU assay (Roche Applied Science,

Monza, Italy) was used. The assay is based on the measurement of BrdU incorporation during DNA synthesis. Cells were seeded ($3.125x10^4$ cells/cm^2) in 96 wells plates and cultured for 24 hours. The *medium* was removed and C6 *media* in absence or in presence of Aβ 1-42 1 μg/mL (Tocris, Ellisville, MO), PEA (10^{-8}; 10^{-7}; 10^{-6} M) and the selective PPAR-α antagonist GW6471 (2,5μM; 5μM; 10μM), were added for 48 hours. Two controls were performed:

1. blank: has to be performed in each experimental setup. The blank provides information about the unspecific binding of BrdU and anti-BrdU- POD conjugate to the plate. The absorbance value obtained in this control has to be subtracted from all other values.

2. control of spontaneous proliferation: to determine the proliferation degree of non-treated cells. 18 hours before the end of the incubation BrdU was added to the cells.

During this labelling period, the pyrimidine analogue BrdU was incorporated in place of thymidine into the DNA of proliferating cells. After removing the culture *medium* the cells were fixed and the DNA was denaturated by adding FixDenat for 30 minutes at room temperature. This step was essential to improve the accessibility of the incorporated BrdU for detection by the antibody. The anti-BrdU-POD was added to cells for 90 minutes at room temperature. It bound to the BrdU incorporated in newly synthesized endothelial cellular DNA. The immune complexes were detected by the subsequent substrate (TMB) reaction. The reaction product was quantified by measuring the absorbance at the respective wavelength using Microplate Autoreader at 405nm, reference wavelength 490nm.

3.2. Animal Models

3.2.1. Sponge implantation for granuloma induction in rats

Male Wistar rats (Harlan, Italy), weighing 200–220 g, were used in all experiments. Animals were provided with food and water ad libitum. The light cycle was automatically controlled (on 07 hrs 00 min.; off 19 hrs 00 min.) and the room temperature thermostatically regulated to 22 ± 1°C with 60 ± 5% humidity. Prior to the experiments, animals were housed in these conditions for 3–4 days to become acclimatized. Sponges were implanted as previously described by Iuvone et al., 1999. Briefly, two polyether sponges (0.5; 1.5; 2.0 cm) weighing 0.035 ± 0.002 g were implanted subcutaneously on the back of rats (n = 12–18 for each group) under general anaesthesia with ketamin/xylazin 5 mg/mL. Sponges and surgery tools were sterilized by autoclaving for 20 min at 120°C. λ-carrageenin (1% w/v) (Sigma) was dissolved in pyrogen-free saline and injected into each sponge in presence or absence of 100 µL of PEA solution (200, 400, 800 µg/mL) in final volume of 0.5 ml/sponge. Ninety-six hours after sponge implant, rats were sacrificed in atmosphere of CO_2. The granulomatous tissue around the sponge was dissected by using a surgical blade (Fig. 3.2.1.), weighted, quickly frozen in liquid nitrogen, and stored at -80°C. Animal care as well as all experiments were in accordance with European Community Council Directive 86/609/EEC and efforts were made to minimize animal suffering and to reduce the number of animals used.

A) ***B)***

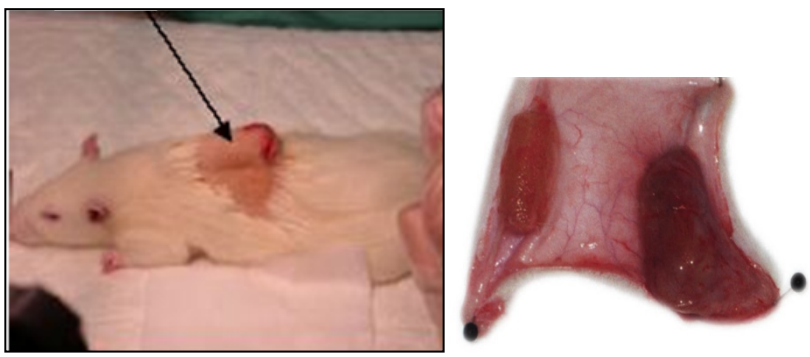


Figure 3.2.1.: *Evaluation of **(A)** granuloma formation 96 hours after sponge implant and **(B)** after explant.*

3.2.2. Induction of Contact Allergic Dermatitis in mice

Eight- to ten-week-old female C57BL/6J mice (Fig. 3.1.4.) were obtained from Harlan. Animal experiments were approved by the University of Naples, Federico II. The institutional and national guidelines for the care and use of laboratory animals were followed.

2,4-Dinitrofluorobenzene (DNFB) was diluted in acetone/olive oil (4:1) immediately before use. Mice were sensitized by painting 50 μL of 0.2% DNFB on the shaved abdomen on two consecutive days. Controls were treated with 50μL acetone/ olive oil. Ears of mice were painted with 10 μL of 0.3% DNFB on day 5. Ear thickness was measured 24, 48 and 72 h after challenge using an engineers micrometer, and ear swelling was calculated in each mouse as the difference in ear thickness between the unchallenged and the challenged ear. Statistical significances were evaluated with the Wilcoxon– Mann–Whitney two-samples test. After the third challenge and sacrifice, the ears were removed and immediately immersed into liquid nitrogen, to be stored at -80° until extraction and purification of PEA. N = 9 mice per group were used for these experiments. These mice were the same that had been used previously for AEA and 2-AG level determination (38).

Figure 3.2.2.: *C57BL/6J mouse.*

3.3. Biochemical Evaluations

3.3.1. ELISA Assay

For the quantitative determination of rat Vascular Endothelial Growth Factor (VEGF) concentrations in C6 stimulated cells *media*, Quantikine® Rat VEGF Immunoassay (R&D Systems, Minneapolis, MN) was used. This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody pre-coated onto the microplate react with standard, control and samples pipetted into the wells and any VEGF present is bound to the immobilized antibody. After washing away the unbound substances an enzyme-linked polyclonal antibody is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop solution is added. The intensity of the colour measured is in proportion to the amount of VEGF bound in the initial step. The samples values are then read off the standard curve.

C6 cells were seeded ($6.25x10^4/cm^2$), in 24-well plates for 24 hours in DMEM *medium* supplemented with 5% FBS (PromoCell, Heidelberg, Germany). The *medium* was then replaced with DMEM 2.5% FBS (PromoCell, Heidelberg, Germany) in absence or in presence of Aβ1-42 1 µg/mL (Tocris, Ellisville, MO), PEA (10^{-8}; 10^{-7}; 10^{-6} M; Epitech Group, Italy) and the selective PPAR-α antagonist GW6471 (2,5µM; 5µM; 10µM) (Tocris, Ellisville, MO) for 48 hours. At the end of incubation *media* were collected into polypropylene tubes. Samples were stored at lower -20°C temperature until use. After adding 50µl of Assay Diluent RD1-41, 50µl of standards (composed of dilution of rat VEGF standard from 2000pg/ml to 31.2pg/ml), control (given with kit) and samples were added to the pre-coated plate.

The plate was covered and incubated 2 hours at room temperature on a horizontal microplate shaker. Five washings were performed with 400µl Wash Buffer to ensure the removal of any unbound substances. 100µl of Rat VEGF Conjugate were added to each well and the plate was incubated for 1 hour at room temperature on the shaker. Five washings were performed and 100µl of Substrate Solution were added to each well. After 30 minutes of incubation at room temperature on the benchtop protected from light, 100µl

of Stop solution were added to each well, gently tapping the plate to ensure thorough mixing. The optical density of each well was determine within 30 minutes, using the Microplate Autoreader ELISA (BioRad) set to 450 nm, reference wavelength 570nm. Standard curve and quantitative analysis were obtained using the specific Microplate Autoreader Software.

3.3.2. Nitrite Determination

NO was measured as nitrite (NO^-_2, 10^6nmol) accumulated in the incubation *medium* after 48 hours on C6 cells and on HUVEC cells in C6 conditioned *media.* A spectrophotometric assay based on the Griess reaction was used (D'Acquisto et al., 2001). Briefly, Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine in phosphoric acid) was added to an equal volume of cell culture supernatant and the absorbance at 550 nm was measured after 10 min using the Microplate Autoreader (BioRad). The nitrite concentration was determined by reference to a standard curve of sodium nitrite.

3.3.3. Evaluation of Haemoglobin content in granulomas

The granulomatous tissue, i.e. the new formation tissue encapsulating the sponge, was collected and measured with a balance weighting min 0.02 to max 300 g (KERN EG300-EM) always by the same person who was blinded for the treatments. In some experiments, the granulomatous tissue was homogenized on ice with the Polytron PT300 tissue homogenizer in 1 ml PBS (4 ml each g of wet weight).

Briefly, after centrifugation at 2500 g for 20 min at 4°C, the supernatants were further centrifuged at 5000 g for 30 min and haemoglobin concentration in the supernatant was determined spectrophotometrically at 450 nm performed with the haemoglobin assay kit (Sigma Diagnostic). The haemoglobin content was expressed as mg haemoglobin/g of wet weight.

3.3.4. Reverse Transcriptase-Protein Chain Reaction (RT-PCR)

Cytosolic and nuclear extracts from granulomatous tissues were performed as previously described.

The Bio-Rad protein assay kit determined protein concentration. The mRNA level of rMCP-5 in granulomatous tissue was determined using the semi-quantitative RT-PCR method. The PCR-primers were selected according to the rat rMCP-5 cDNA sequence (forward primer 5'-TCCTGCAAACACTTCACCAG-3', reverse primer 5'-CGAGATCCAGAGTTAATTCT- 3'); and rat β-actin cDNA (forward primer 5'-GGCACCACACCTTCTACA-3' nucleotide positions 330-348, and reverse primer 5'-CAGGAGGAGCAATGATCT -3'). 15ml aliquots of PCR products were electrophoretically fractionated through 1% agarose gel containing the fluorescent Vistra green dye (Amersham Pharmacia Biotech, GE Healthcare; Switzerland). Labeling intensity of the PCR product, which is linear to the amount of DNA, was quantified using the Molecular Imager FX and Quantity One software (Biorad, Milan, Italy).

3.3.5. Western Blot Analysis

Western blot analysis was performed on C6 cells, on rat granulomatous tissue and on mice ears extracts, treated as described previously.
Depending upon the experiments, cells or tissues were mechanically lysed.

Briefly, 24 hours after treatment, C6 harvested cells (1×10^6cells) were washed twice with ice-cold PBS, and then it was centrifuged at 180g for 10 min at 4°C. The pellet was re-suspended in 50µl of ice-cold hypotonic lysis buffer and incubated on ice for additional 15 min. The total protein extract was obtained by centrifugation at 13,000g for 15 min at 4°C.
Rat granulomatous tissue and mice ears, previously mechanically homogenised, were lysed and the total protein extract obtained as described for cells.
Samples were subjected to SDS-polyacrylamide gel electrophoresis, and proteins were transferred onto nitrocellulose membrane and incubated with one of the following antibodies: mouse anti-VEGF (1:1000 v/v; Neomarkers, Fremont CA), mouse anti-S100B (1:250 v/v; Abcam, Cambridge, UK), mouse anti-iNOS (1:1000 v/v; Transduction, Cambridge, UK), mouse anti-TNF-α (1:250 v/v; Neomarkers, Fremont CA), mouse anti- MMP9 (1:100 v/v; Neomarkers, Fremont CA), mouse anti-MITF (1:1000 v/v; Lifespan

Biosciences, Seattle, WA), mouse anti-p-ERK (1:2000 v/v; CellSignalling, Beverly, MA), mouse anti-β actin (1:1000 v/v; Santacruz, CA) and mouse anti-tubulin (1:1000 v/v; Santacruz, CA). Appropriate peroxidase-conjugated secondary antibodies (1:1000 v/v; Santacruz, CA) were used, and proteins were visualized using an enhanced chemiluminescence kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Protein expression was quantified by densitometric analysis of the acquired images by ImageQuant (GE Healthcare), with a GS 700 imaging densitometer (Bio-Rad, Hercules, CA) and a computer program (Molecular Analyst; IBM, White Plains, NY)

3.3.6. Electrophoretic Mobility Shift Assay (EMSA)

Double stranded oligonucleotides containing the MITF recognition sequence (5'-CCT AGA CAG ACA AAA CCT AGA CAA TCA CGT GGC TGG-3') were endlabelled with 32P-γ-ATP. Nuclear extracts containing 5μg protein were incubated for 15 min with radiolabeled oligonucleotides (2.5-5.0 x 10^4 cpm) in 20μl reaction buffer containing 2 μg poly dI-dC, 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol. The specificity of the DNA/protein binding was determined by competition reaction in which a 50-fold molar excess of unlabeled wild 80 type, mutant or Sp-1 oligonucleotide was added to the binding reaction 15 min before addition of radiolabeled probe. In supershift assay, antibodies reactive to MITF proteins were added to the reaction mixture 15 min before the addition of radiolabeled probe. Nuclear protein-oligonucleotide complexes were resolved by electrophoresis on a 6% non-denaturing polyacrylamide gel in 1x TBE buffer at 150 V for 2 h at 4 °C. The gel was dried and auto-radiographed with intensifying screen at –80 °C for 20 h. Subsequently, the relative bands were quantified by densitometric scanning of the X-ray films with a GS-700 Imaging Densitometer (Bio-Rad, Milan, Italy) and a computer program (Molecular Analyst; IBM).were endlabelled with ^{32}P-g-ATP (Amersham, Milan, Italy).

3.3.7. Immunoprecipitation Assay

For immunoprecipitation assay, 1 mg of protein extracts was incubated with 30 μl of protein A/G agarose beads coated with 5 μg of antibody against

MITF at 4 °C for 12 h. The beads were washed and boiled in the SDS sample buffer. The eluted proteins were loaded on 12% SDS-PAGE. The membranes were blocked in PBS, 0.2% Tween and 5% dry milk for 2 h, and then challenged with anti-phosphoserine (1:1000 v/v; Pierce, Rockford, Illinois). The proteins were visualized chemiluminescence detection reagents (Amersham, GE Healthcare; Switzerland) according to the manufacturer's instructions in Image quant 800 apparatus. The protein bands were analysed by densitometric analysis with a GS-800 imaging densitometer.

3.4. Histological Investigations

3.4.1. Mast cell Counting

After excision of the implant, the rat granulomatous tissue around the sponge and the excised ears of the mice were put in 10% formalin. Thin (0.5µm) paraffin sections were prepared and stained with toluidine blue according to Iuvone *et al*. (1999) and then processed for light microscopy examination. Mast cells were counted in five randomly selected sections using a x100 objective lens, differentiating between deep blue (un-degranulated) and light blue (degranulated) MCs.

3.4.2. Blood Vessels Evaluation

The granulomatous tissue around the sponges on the back of rats was collected and measured with a balance weighting min 0.02 - max 300 gr (KERN EG300-EM) always by the same person who was blinded for the treatments. In some experiments, the granulomatous tissue was fixed in formol-methanol (9:1, v/v) solution at 4°C for 24h. After dehydration in an ethanol series and infiltration with xylol paraffin-wax sections were cut at 4-6 µm and stained with haematoxylin and eosin. A vessel counting was performed on five randomly selected sections using a 100X objective lens.

3.4.3. Small Calibre Vessels Evaluation

Excised ears of the mice were put in 10% formalin. Thin (0.5µm) paraffin sections were prepared and stained with a specific trichrome staining.

The final blood vessels staining is due to the combination of three different staining from different solutions:

- Solution A, also called plasma stain, containing acid fuchsin, Xylidine Ponceau, glacial acetic acid, and distilled water.
- Solution B contains phosphomolybdic acid in distilled water.
- Solution C, also called fibre stain, contains Light Green SF yellowish, or alternatively Fast Green FCF.

3.5. Statistical Analysis

Results are expressed as the means ± S.E.M. of *n* experiments. Statistical significance was calculated by one-way analysis of variance (ANOVA) and Bonferroni-corrected *P*-value for multiple comparison tests. The level of statistically significant difference was defined as $P<0.05$.

Linear associations between variables were assessed by the use of standard-least-square linear regression.

Correlation coefficient (r) was presented as measure of linear association for regression relationship.

4. Results

4.1. Effect of PEA on angiogenesis during neurodegeneration

4.1.1. Effect of PEA on reactive gliosis in C6 cells stimulated with Aβ (1-42) for 48 hoursù

After *Aβ* stimulation glial cells undergo to reactive gliosis characterized by an increase of cell functionality and the release of several pro-inflammatory mediators. Therefore, in order to evaluate reactive gliosis, we measured those parameters. First of all, PEA effect on functionality of C6 stimulated cells has been evaluated through MTT assay.

As shown in Fig. 4.1.1., C6 cell functionality was significantly (P<0.001) increased in cells treated with A*β* (1-42) (1µg/mL) in confront to control (un-treated) cells. The functionality of A*β* (1-42) stimulated C6 cells was significantly decreased after the administration of PEA (10^{-8}-10^{-6}M), in a concentration-dependent manner.

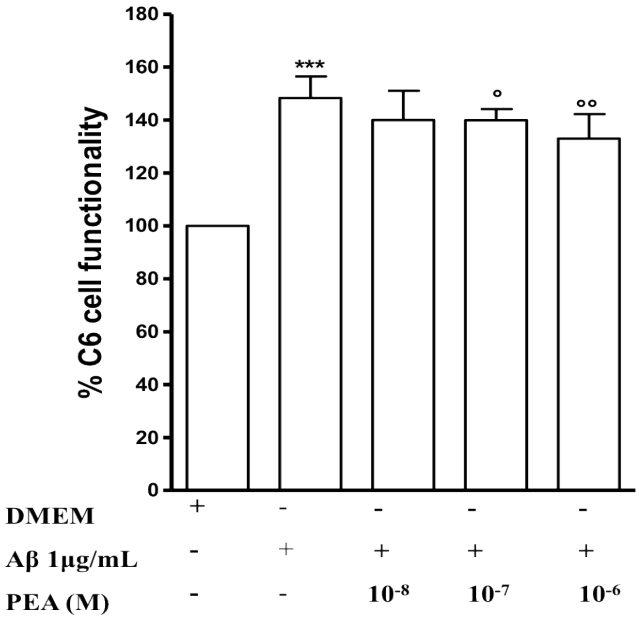


Figure 4.1.1.: ***Effect of PEA on functionality of Aβ-stimulated C6 cells.*** *Results are expressed as mean ± SEM of 3 experiments. ***p<0.001 vs. Control; °p < 0.05, °°p < 0.01 vs. Aβ (1-42) 1µg/mL.*

Besides, cell stimulation with A*β* (1-42) (1µg/mL) for 48 hours, caused a significant (P<0.001) increase of nitrite levels in confront to the control.
Interestingly, the treatment with PEA (10^{-8}; 10^{-7}; 10^{-6}M) significantly and in a concentration dependent manner reduced the nitrite production (Fig 4.1.2.).

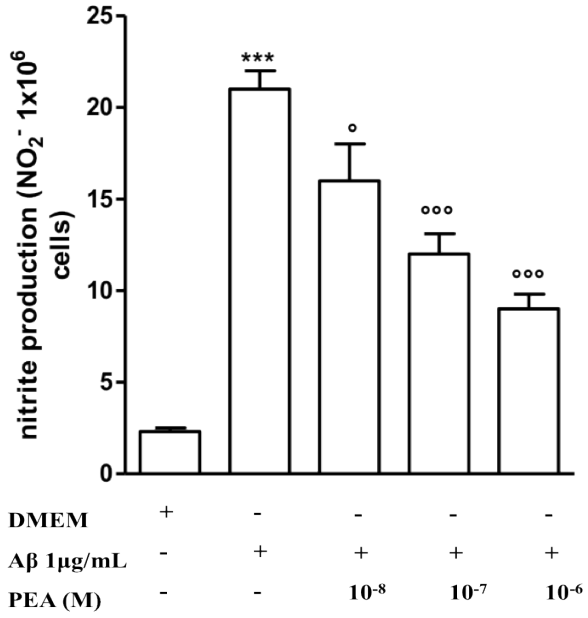


Figure 4.1.2.: ***Effect of PEA on nitrite production in Aβ-stimulated C6 cells.*** *Nitrite production has been evaluated through Griess reaction. Results are expressed as mean ± SEM of 3 experiments. ***P<0.001 vs. Control; ° p <0.05, °°°p < 0.001 vs. Aβ (1-42) 1µg/mL.*

Since several data suggest that the effects of PEA in astroglial cells are mainly mediated by a PPAR-α receptor signalling, we used GW6471, the selective antagonist of PPAR-α.
As shown in Fig. 4.1.3., GW6471 (2,5 µM, 5 µM and 10 µM), significantly (P<0.001) and in a concentration-dependent manner, reverted the effect of PEA (higher concentration of 10^{-6}M) on nitrite production from A*β*-stimulated C6 cells, thus confirming PEA agonistic activity on PPAR-α.

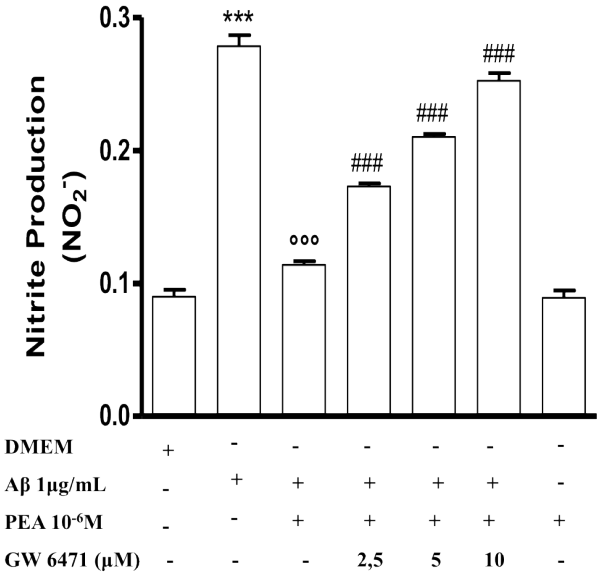


Figure 4.1.3.: ***Effect of GW6471 in reverting PEA effect on nitrite production from Aβ-stimulated C6 cells.*** *Nitrite production has been evaluated through Griess reaction. Results are expressed as mean ± SEM of 3 experiments. ***P<0.001 vs. Control; °°°p < 0.001 vs. Aβ (1-42) 1µg/mL; ###p < 0.001 vs. PEA 10-6M.*

Other pro-inflammatory markers, as S100β and iNOS, were evaluated in C6 cells in order to estimate their expression consequent to A*β* insult.

Western blot analysis showed that the expression of both S100β and iNOS protein were considerably increased in C6 cells stimulated with A*β* (1-42) (1µg/mL) for 48 hours. PEA (10^{-8}-10^{-7}-10^{-6}M) treatment significantly (P<0.5; P<0.01; P<0.001,) and in a concentration dependent manner reduced the expressions of both S100β (Fig. 4.1.4. ***A***) and iNOS (Fig. 4.1.4. ***B***) in C6 cells.

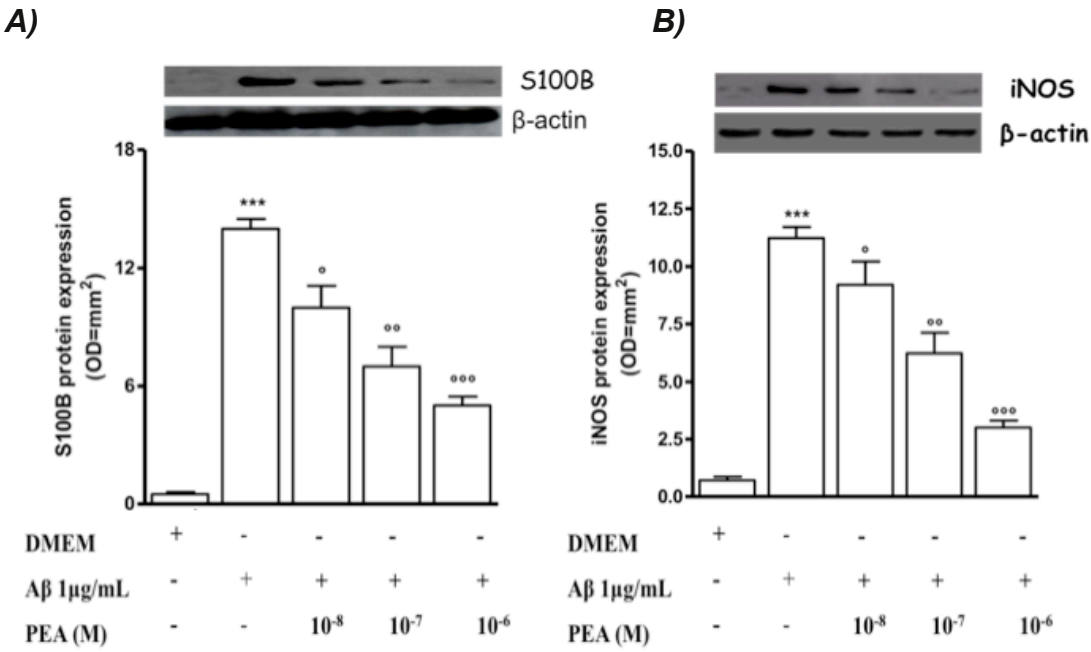


Figure 4.1.4.: ***Effect of PEA on S100β (A) and iNOS (B) protein expressions in Aβ-stimulated C6 cells.*** *S100β* ***(A)*** *and iNOS* ***(B)*** *protein expressions have been evaluated through Western Blot analysis. β-actin protein expression is shown as control. Results are expressed as mean ± SEM of 3 experiments. ***P<0.001 vs. Control; ° p<0.05, °°p<0.01, °°°p<0.001 vs. Aβ 1μg/mL.*

4.1.2. Effect of PEA on pro- angiogenic factors in C6 cells stimulated with Aβ (1-42) for 48 hours

Next we were aimed to demonstrate that C6 cells, stimulated with A*β* (1-42) (1μg/mL) for 48 h, were also able to produce and release pro- angiogenic factors.

Hence, Western Blot analysis and ELISA assay for VEGF, MMP-9 and TNF-*α* have been performed on A*β* stimulated cells.

As shown in Fig. 4.1.5., the expressions of VEGF ***(A)***, MMP-9 ***(B)*** and TNF-*α* ***(C)*** were significantly (P<0.001) increased by A*β* treatment in confront to control (un-treated) cells.

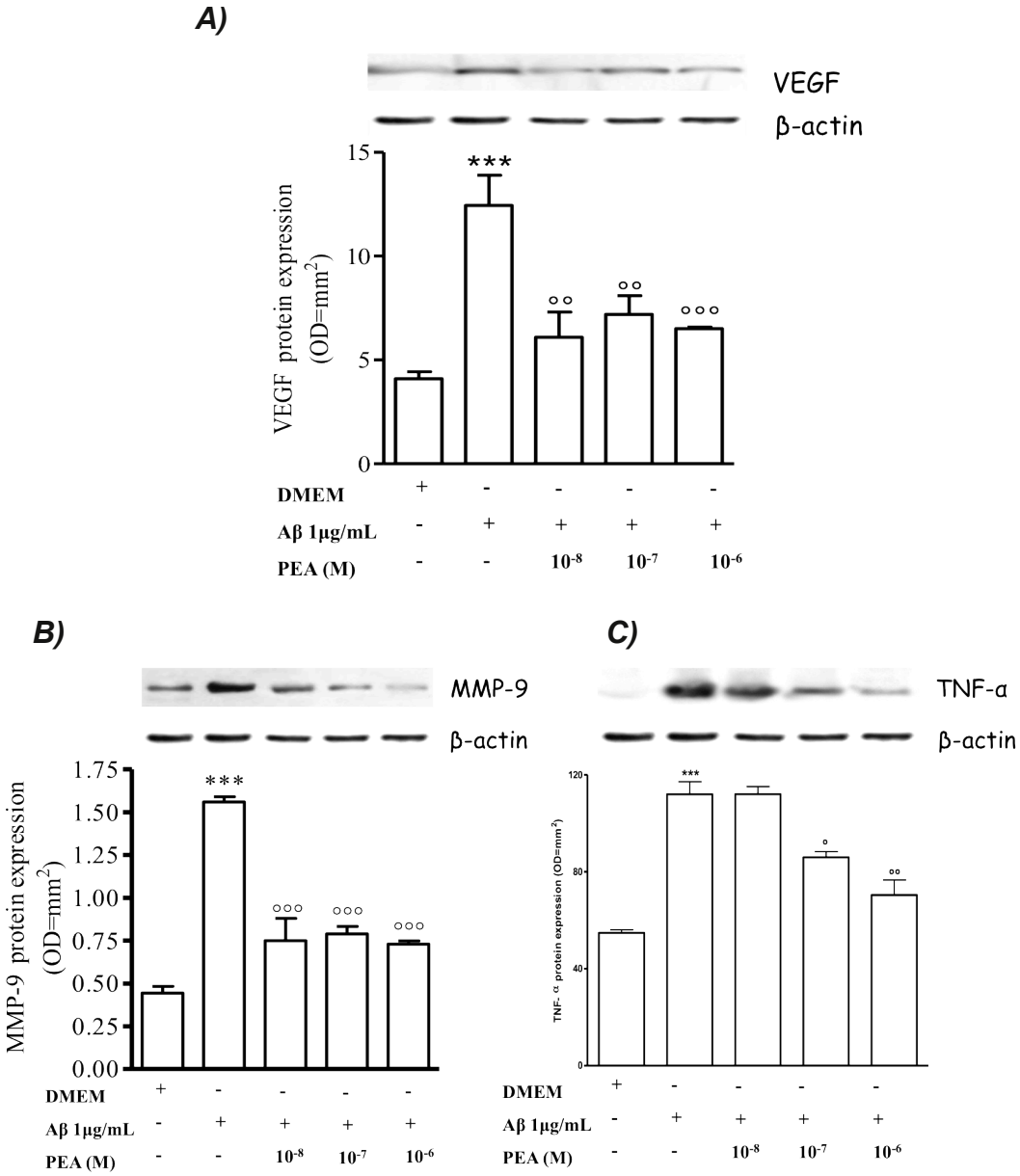


Figure 4.1.5.: ***Effect of PEA on pro- angiogenic factors protein expressions in Aβ-stimulated C6 cells.*** *PEA effect on pro- angiogenic* ***(A)*** *VEGF,* ***(B)*** *MMP-9 and* ***(C)*** *TNF-α protein expressions has been evaluated through Western Blot analysis. β-actin protein expression is shown as control. Results are expressed as mean ± SEM of 3 experiments. ***P<0.001 vs. Control; ° p<0.05, °°p<0.01, °°°p<0.001 vs. Aβ 1µg/mL.*

Treatment with PEA (10^{-8}; 10^{-7}; 10^{-6}M) of A*β* stimulated C6 cells strongly reduced the expression of VEGF, as well as MMP-9 and TNF-α, even if a good concentration-effect correlation was not always observed (Fig. 4.1.5. ***A***; ***B***; ***C***).

In order to clarify whether PEA was able to reduce not only the expression but also the release of pro- angiogenic mediators from A*β* stimulated C6 cells, ELISA assay of VEGF release, the most important pro- angiogenic factor, has been performed on supernatants of A*β* stimulated cells.

VEGF release from C6 cells was considerably increased in A*β* (1µg/mL) stimulated cells in respect to the un-treated cells, and significantly decreased by PEA (10^{-8}; 10^{-7}; 10^{-6}M) treatment in a concentration dependent manner (Fig. 4.1.6.).

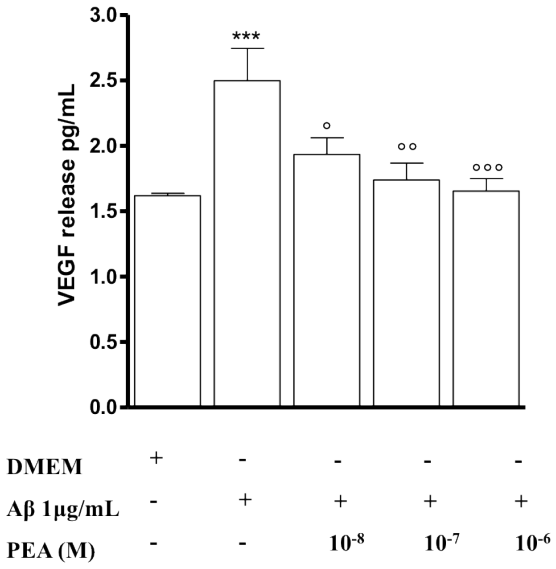


Figure 4.1.6.: ***Effect of PEA on VEGF release in Aβ-stimulated C6 cells.*** *The evaluation of PEA effect on VEGF release has been performed through ELISA assay. Results are expressed as mean ± SEM of 3 experiments. ***P<0.001 vs. Control; ° p<0.05, °°p<0.01, °°°p<0.001 vs. Aβ 1µg/mL.*

In order to clarify whether the effect of PEA on pro- angiogenic mediator release was also related to a PPAR-α-mediated mechanism, we used the selective antagonist of PPAR-α, GW6471.

As shown in Fig. 4.1.7., GW6471 (2,5 µM, 5 µM and 10 µM) was able to revert, in a concentration dependent manner, PEA (10^{-6}M) effect in reducing VEGF release from Aβ-stimulated C6 cells. The treatment with GW6471 alone had no effect on VEGF release.

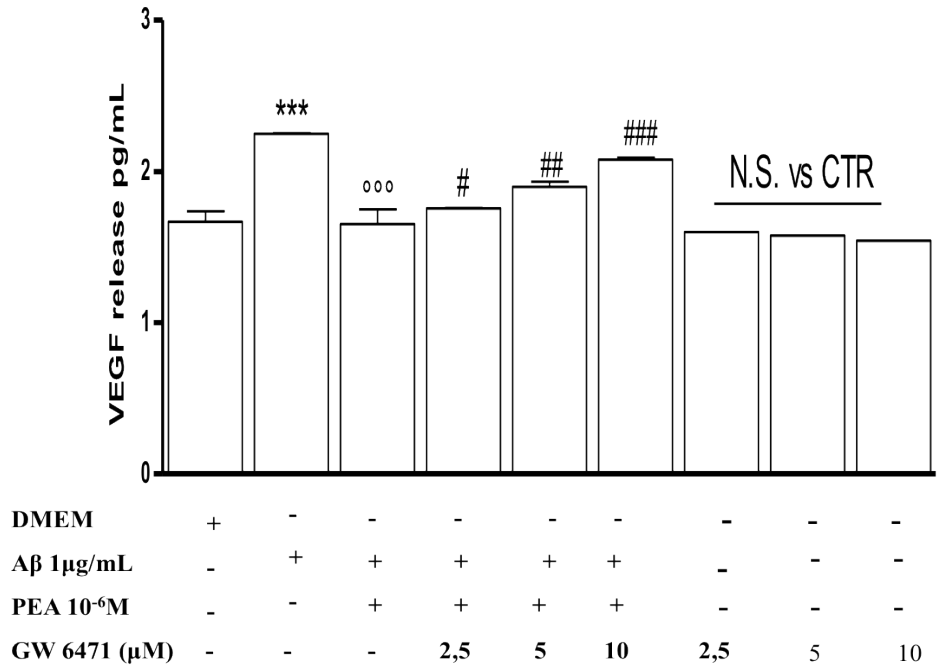


Figure 4.1.7.: ***Effect of GW6471 in reverting PEA effect on VEGF release from Aβ-stimulated C6 cells.*** *The evaluation of GW6471 on VEGF release has been made through ELISA assay. Results are expressed as mean ± SEM of 3 experiments. ***P<0.001 vs. Control; °°°p < 0.001 vs. Aβ (1-42) 1µg/mL; ###p < 0.001 vs. PEA 10-6M.*

4.1.3. Effect of PEA on proliferation of HUVEC, incubated with C6-conditioned medium

The obtained results, explaining the capability of astroglial cells in expressing and releasing pro- angiogenic factors, have been an encouraging starting point for the subsequent aim: to study the effects of pro- angiogenic factors released by C6 cells, stimulated with A*β* 1µg/mL and treated with different concentrations of PEA (10^{-8}-10^{-6}M), on endothelial cells, which are responsible of tube formation, after the binding of VEGF to their membrane receptor, VEGFR-2.

To assess the effect of Aβ (1-42) 1µg/mL and PEA (10^{-8}-10^{-6}M) on endothelial proliferation, BrdU experiments were performed on HUVEC cells, previously incubated for 24 hours with C6 conditioned *media*.

Interestingly, proliferation was significantly (P<0.001) increased in HUVEC cells treated with Aβ (1-42) C6-conditioned *medium*, while PEA was able to contrast this effect in a concentration dependent manner, with having no effect on HUVEC that were treated with PEA alone (Fig. 4.1.8.)

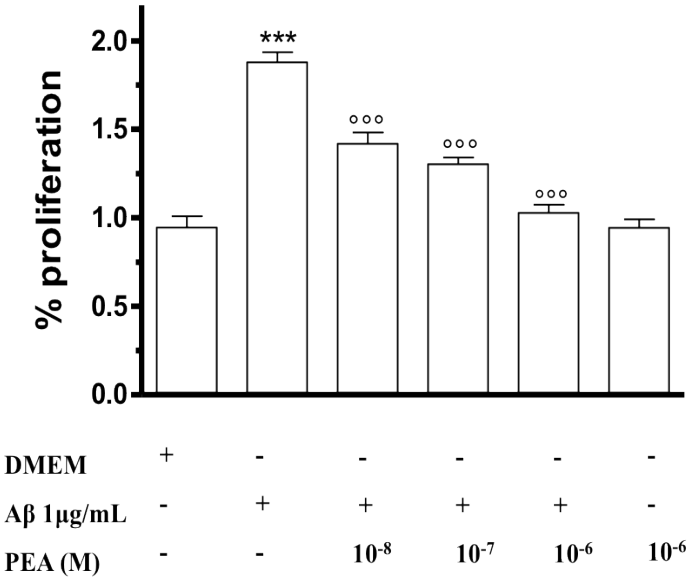


Figure 4.1.8.: ***Effect of PEA on proliferation of HUVEC, incubated with C6-conditioned medium.*** *The evaluation of PEA effect on proliferation of HUVEC incubated with C6-conditioned media has been made through BrdU assay. Results are expressed as mean ± SEM of 3 experiments. ***P<0.001 vs. Control; °°°p < 0.001 vs. Aβ (1-42) 1µg/mL.*

Finally, in order to confirm the agonistic effect of PEA on PPAR-α, BrdU experiments were newly performed on HUVEC cells, previously incubated for 24 hours with C6-conditioned *media* containing also GW6471 treatment.
GW6471 at the concentrations of 2,5 µM, 5 µM and 10 µM was able to revert, in a concentration dependent manner, PEA 10^{-6}M activity, that significantly had reduced HUVEC proliferation consequent to the incubation with Aβ-stimulated C6 cells *media.* The treatment with GW6471 alone did not influence HUVEC proliferation (Fig. 4.1.9.).

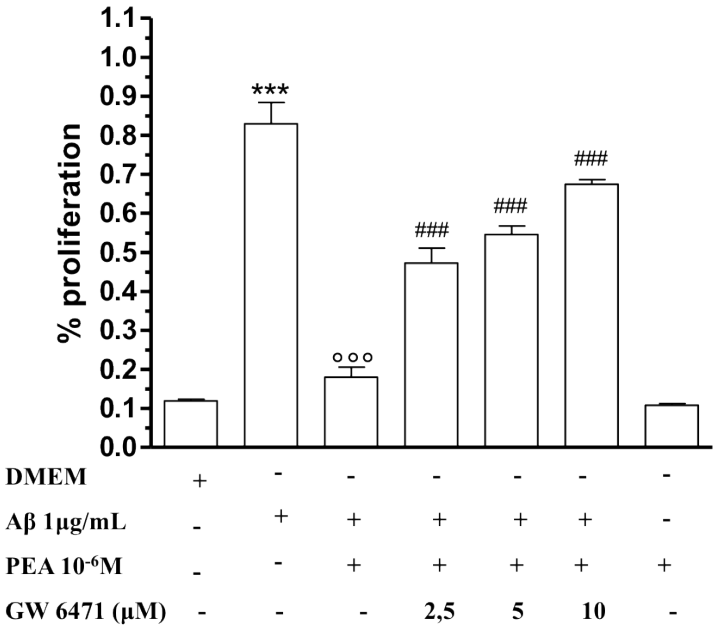


Figure 4.1.9.: ***Effect of GW6471 in reverting PEA effect on proliferation of HUVEC, incubated with C6-conditioned medium cells.*** *The evaluation of GW6471 on HUVEC proliferation has been performed through BrdU assay. Results are expressed as mean ± SEM of 3 experiments. ***P<0.001 vs. DMEM; °°°p < 0.001 vs. Aβ (1-42) 1µg/mL; ###p < 0.001 vs. PEA 10-6M.*

4.2. Effect of PEA on angiogenesis during granuloma

4.2.1. Effect of PEA on wet weight of granulomatous tissue and on the Haemoglobin content

In order to evaluate the effect of PEA on angiogenesis in chronic inflammation, it has been used a model of granuloma, that is a chronic inflammatory disease actively sustained by mast cell involvement (Iuvone et al. 1999): MCs are activated and their degranulation produces the release of different mediators, among which pro- angiogenic factors.

The first evaluation that has been performed is the effect of PEA on granulomatous tissue formation, evaluated as wet weight of tissues and on the Haemoglobin content.

The implant of sponges soaked with λ−carrageenin 1μg/mL on the back of rats, for 96 hours, caused a substantial increase ($P<0.001$) of granulomatous tissue in confront to the un-treated animals, that was significantly reduced by PEA 200, 400, 800 μg/mL, administered at the moment of implantation (Fig. 4.2.1. ***A***).

Moreover, PEA (200, 400, 800 μg/mL) significantly ($P<0.001$) and concentration-dependently reduced also Haemoglobin content in the tissue, in comparison to λ-carrageenin alone (37.4±1.72 mg Hb/g tissue; Fig. 4.2.1. ***B***).

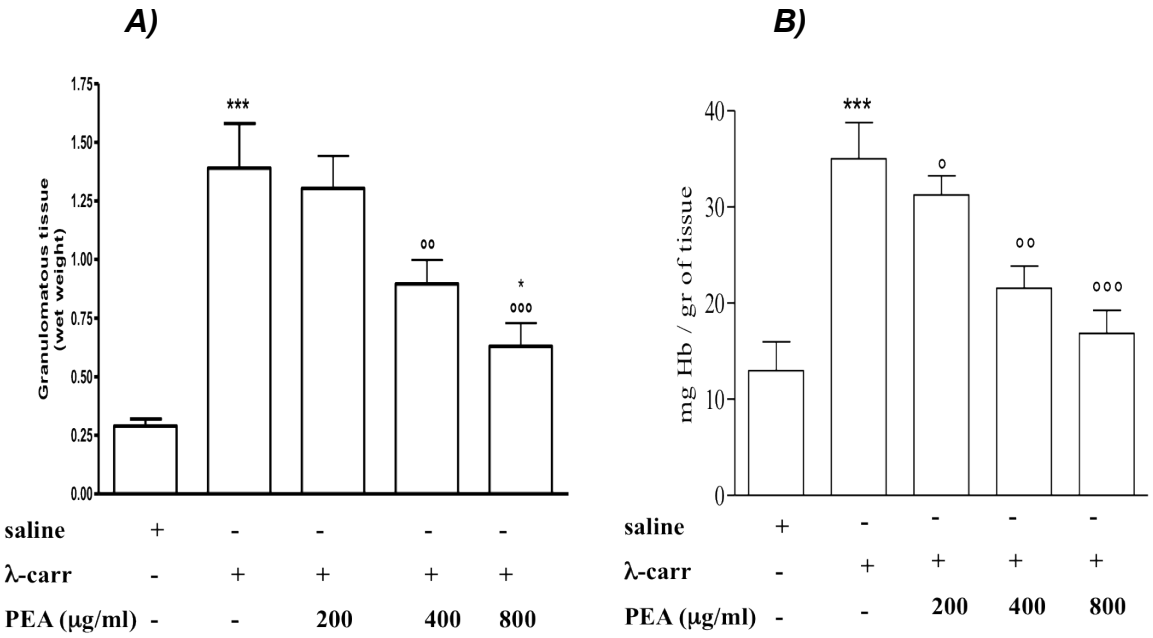


Figure 4.2.1.: ***Effect of PEA on angiogenesis evaluated as (A) wet weigh of granulomatous tissue and (B) Haemoglobin content of granulomas.*** *Results are expressed as mean ± SEM of 3 experiments. ***P<0.001 vs. Saline; ° p < 0.05, °°p < 0.01, °°°p < 0.001 vs. λ-carrageenin alone.*

4.2.2. Effect of PEA on the expression of pro- angiogenic mediators in granulomatous tissue

In order to evaluate if PEA action on granulomatous tissue was linked to its activity in modulating angiogenesis, protein expression assays have been performed.

More specifically, the expressions of VEGF, most important pro- angiogenic protein, TNF-α a pro- angiogenic and pro-inflammatory mediator have been evaluated through Western Blot Assay.

The expressions of VEGF and TNF-α in granuloma, both drastically increased by the treatment with λ-carrageenin in confront to the un-treated rats, were significantly (P<0.001) decreased after the administration of PEA 200, 400, 800 μg/mL, in a dose-responding manner (Fig. 4.2.2. ***A;B***).

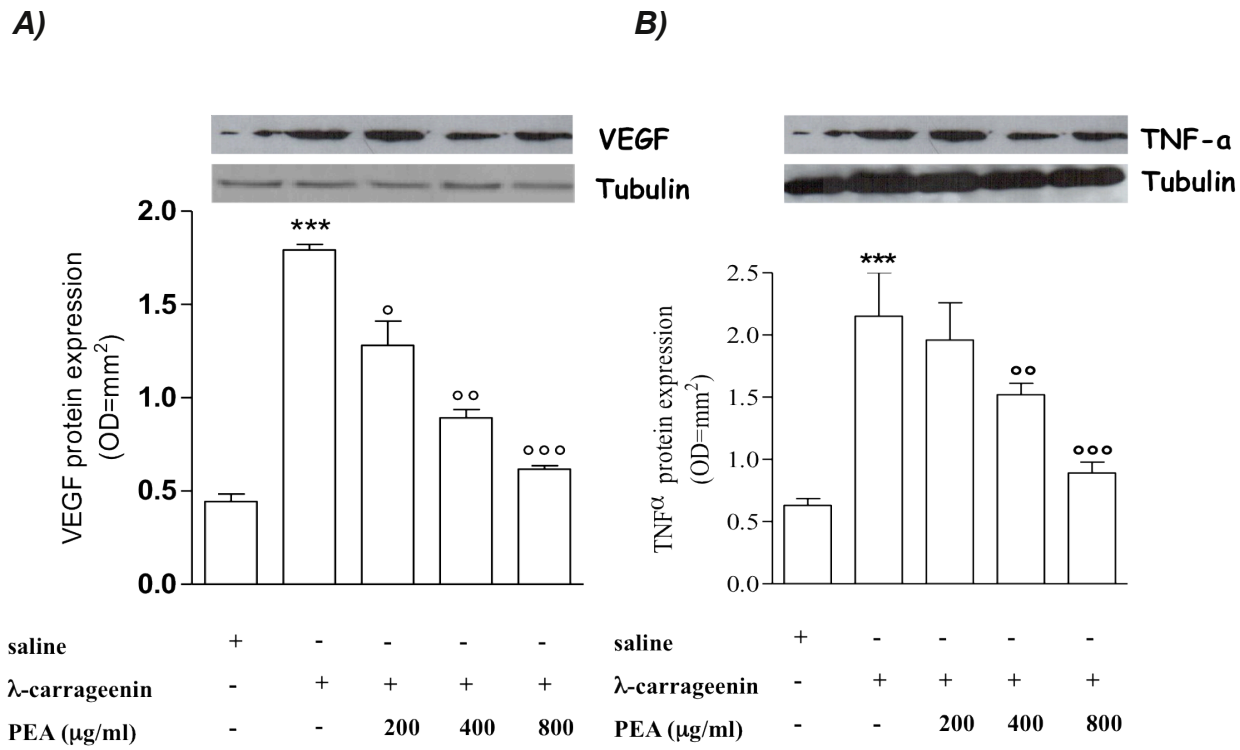


Figure 4.2.2.: ***Effect of PEA on expressions of (A) VEGF and (B) TNF-α pro- angiogenic factors.*** *PEA effect on pro- angiogenic* ***(A)*** *VEGF,* ***(B)*** *TNF-α protein expressions has been evaluated through Western Blot analysis. Tubulin expression is shown as control. Results are expressed as mean ± SEM of 3 experiments. ***P<0.001 vs. Saline; ° p<0.05, °°p<0.01, °°°p<0.001 vs. λ-carrageenin alone.*

Starting to the assumption that, after the release of VEGF, angiogenetic process prosecutes with its binding to the endothelial cells receptors, an evaluation of the expression of CD31, marker of endothelial cells, has been performed.

As expected, CD31 protein expression was significantly increased by the treatment with λ-carrageenin, in confront to the un-treated animals. PEA was able to drastically reduce the expression of CD31 (8.45±0.78 OD=mm^2; Fig. 4.2.3.).

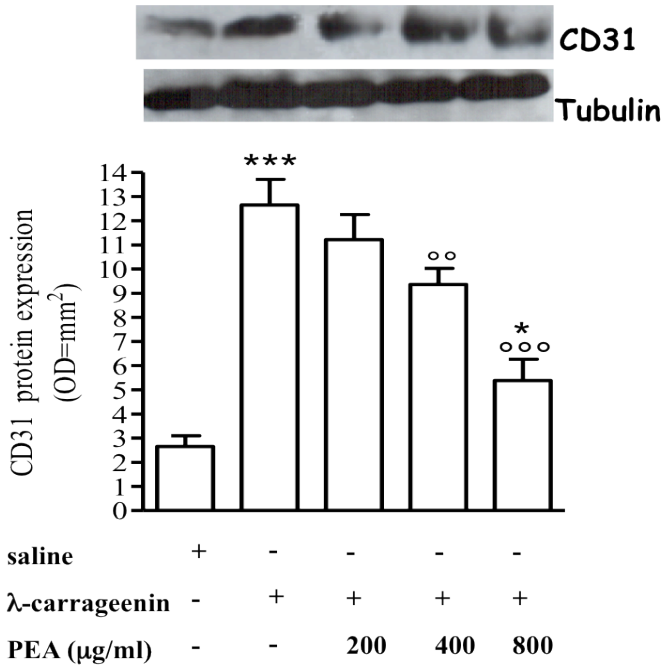


Figure 4.2.3.: ***Effect of PEA on CD31 protein expression.*** *PEA effect on CD31 protein expression has been evaluated through Western Blot analysis. Results are expressed as mean ± SEM of 3 experiments. Tubulin expression is shown as control. ***P<0.001 vs. Saline; ° p < 0.05, °°p < 0.01, °°°p < 0.001 vs. λ-carrageenin alone.*

4.2.3. Effect of PEA on new vessel formation in granulomatous tissue

As the treatment with of λ-carrageenin 1% was able to increase pro-angiogenic factors, granulomatous tissue wet weight and its content of haemoglobin, histological analysis to evaluate blood vessels have been performed.

Fig. 4.2.4. shows granulomatous tissues stained with haematoxylin and eosin: in granulomas from λ-carrageenin-treated rats, it is evident the elevated number of small calibre vessels, as an indication of on-going formation vessels. Vessels number was drastically reduced after the treatment with PEA at the higher dose (800 µg/mL), in confirming its effect in obstructing angiogenic process.

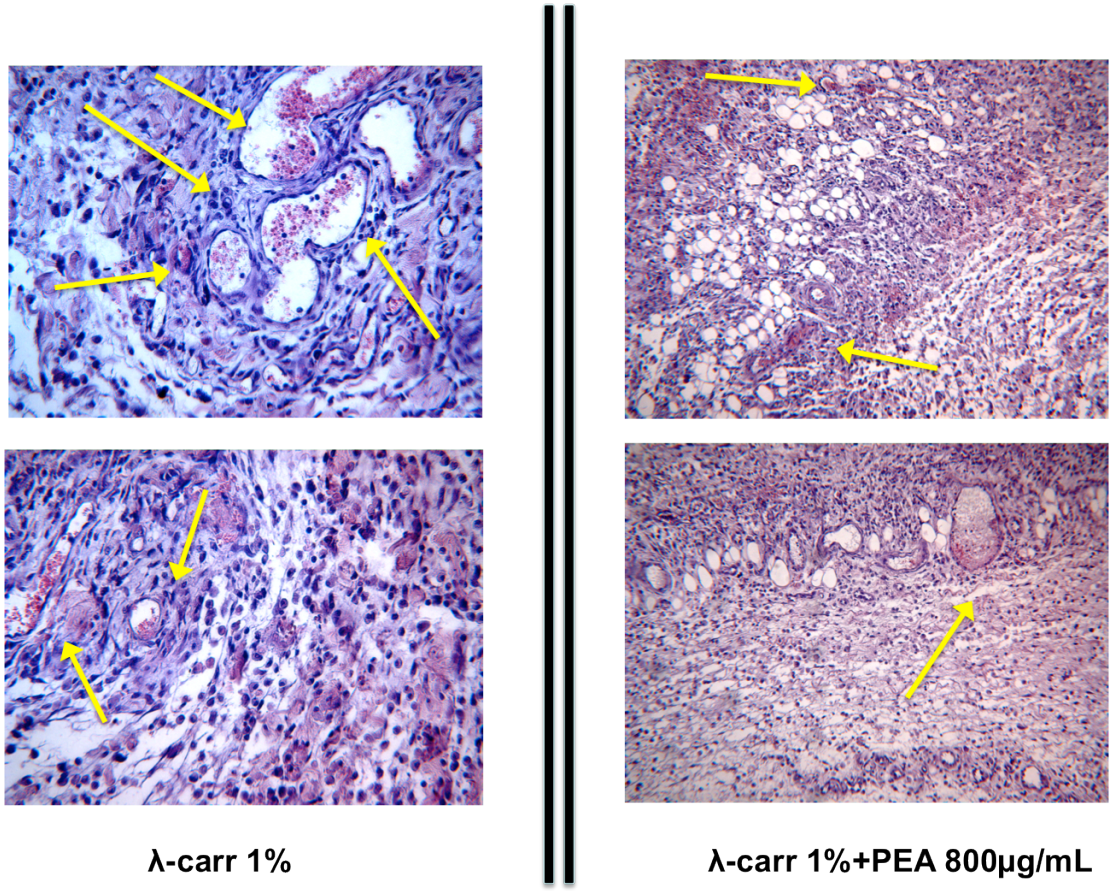


Figure 4.2.4.: ***Effect of PEA on small calibre vessels in granulomatous tissue.*** *Representative histological analysis of granulomatous tissues stained with haematoxylin and eosin.*

4.2.4. Effect of PEA on mast cell number and degranulation in granulomatous tissue

Previously, the role of mast cells in sustaining the process of granuloma formation has been perfectly clarified (Iuvone et al. 1999). In parallel, stimulation of MCs and their consequent degranulation produce the release of various mediators, i.e. pro- angiogenic factors. Therefore, in order to elucidate whether PEA modulation of pro- angiogenic factors expression in granulomatous tissue was due to an *ALIA* mechanism, the number and the degranulation of MCs have been evaluated.

Histological analysis of granulomas, stained with Toluidine Blue, revealed that PEA treatment (800 µg/mL) modulates MCs activity, in reducing their total number (deep blue, un-degranulated) and their degranulation (light blue,

degranulated) in respect to the tissues from rats treated with λ-carrageenin alone (Fig. 4.2.5.).

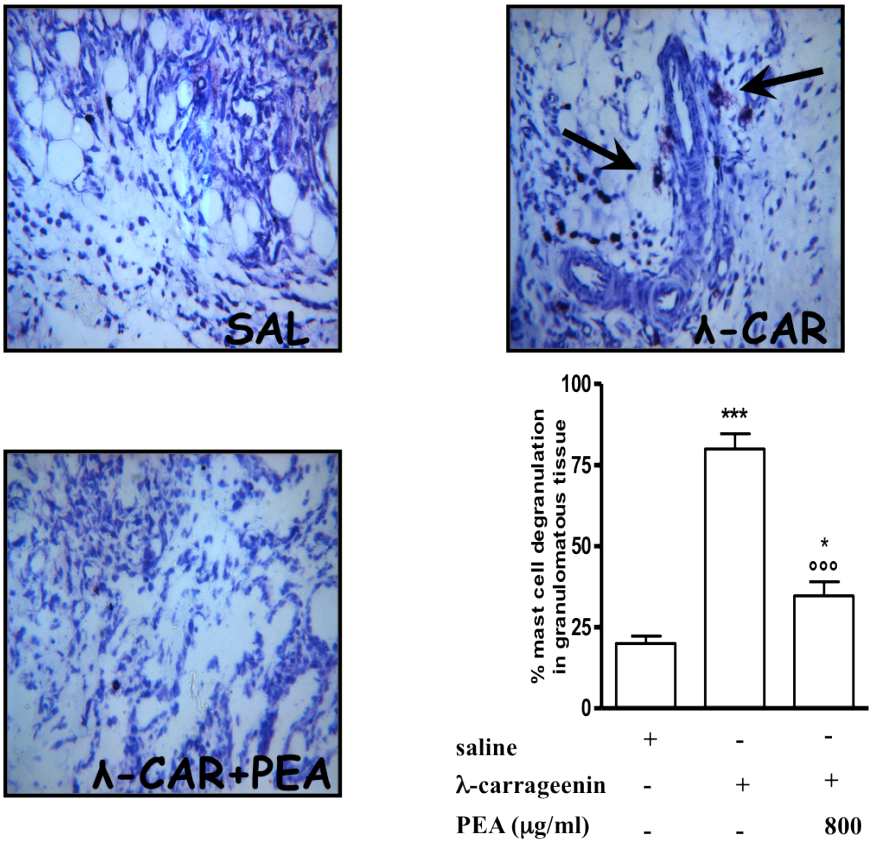


Figure 4.2.5.: ***Effect of PEA on λ-carrageenin activated mast cell.*** *Representative histological analysis of granulomatous tissues stained with toluidine blue. Results are expressed as mean ± SEM of 3 experiments. *p < 0.05, ***p<0.001 vs. saline; °°°p < 0.001 vs. λ-carrageenin alone.*

4.2.5. Effect of PEA on rMCP-5 transcription and expression in granulomatous tissue

In order to investigate whether PEA was able to influence rMCP-5 behaviour, a specific mast cell chymase possessing pro- angiogenic properties, different analysis of the amount of rMCP-5 in λ-carrageenin-induced granulomatous tissue in the presence or absence of PEA have been performed.

Co-injection of PEA (200, 400 and 800 µg/ml) caused a dose-dependent reduction of rMCP-5 mRNA levels in tissue homogenates compared to λ -carrageenin 1% (Fig. 4.2.6. ***A***).

The inhibition of rMCP-5 expression operated by PEA was also confirmed by Western Blot analysis, where chymase was dose-dependently reduced by PEA treatment compared to λ –carrageenin 1% (Fig. 4.2.6. ***B***).

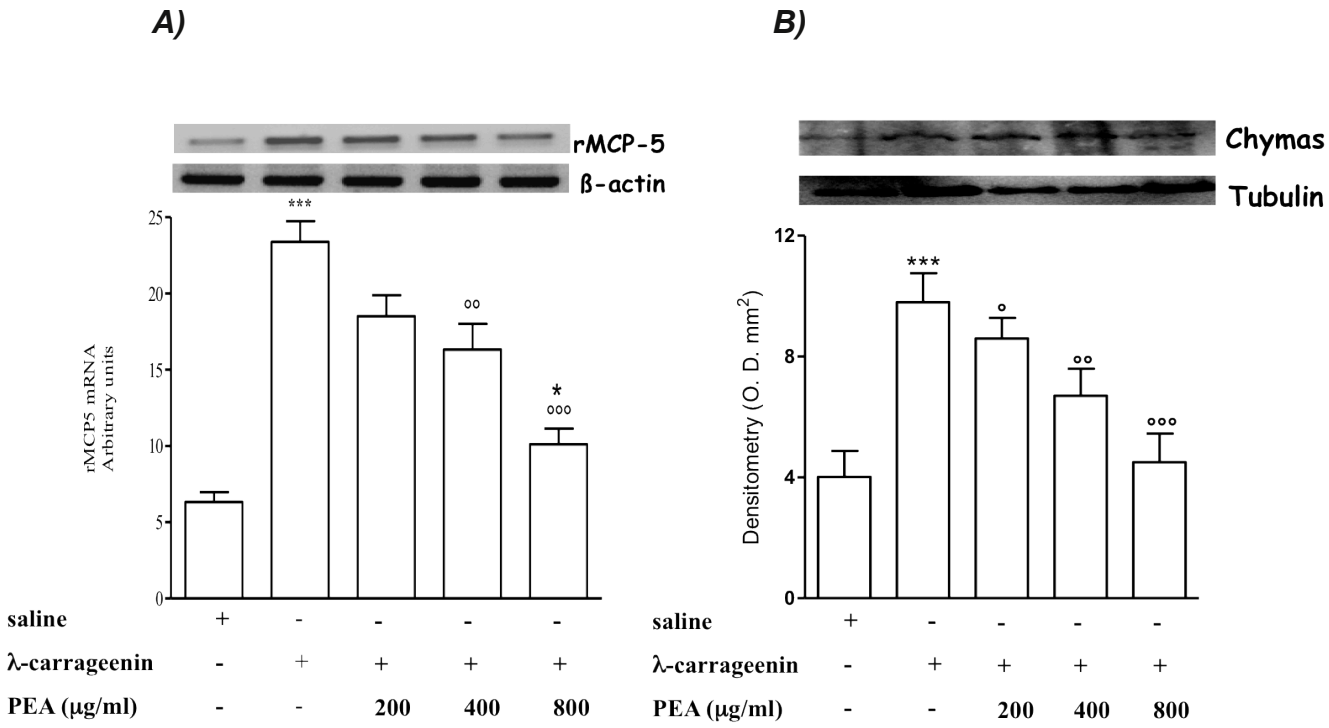


Figure 4.2.6.: ***Effect of PEA on rMCP5 transcription (A) and expression (B) induced by λ-carrageenin in granulomatous tissue.*** *Representative Vistra green-stained agarose gel of RT-PCR products (A), and Western Blot product (B). Tubulin expression is shown as control. Results are expressed as mean ± SEM of 3 experiments. ***P<0.001 vs. Saline.; °p < 0.05, °°p < 0.01, °°°p < 0.001 vs. λ-carrageenin alone.*

4.2.6. Effect of PEA on MITF protein expression in granulomatous tissue

The evaluation of MITF protein expression has been performed in considering its activation mechanism. Since MITF is activated by phosphorylation, that brings its translocation into the *nucleus*, a double analysis has been performed, according to its phosphorylation.

The total MITF protein expression was significantly decreased in λ-carrageenin-induced granulomatous tissues at 96 hours, if compared to the control tissues (Fig. 4.2.7. ***A***), as a proof of its activation by inflammatory

stimuli. PEA administration produced a significant restoring of MITF protein levels into the granulomatous tissue, as compared with λ -carrageenin alone.

In another set of experiments, MITF was specifically immune-precipitated from protein extracts of tissues. Figure 4.2.7.***B*** shows a significant (P<0.001) increase (2.6 fold) of the amount of phosphorylated MITF in λ -carrageenin-induced granulomatous tissue compared to the control tissues. On the contrary, PEA treatment caused a decrease in the levels of phosphorylated MITF, if compared to the effect of λ –carragenin treatment.

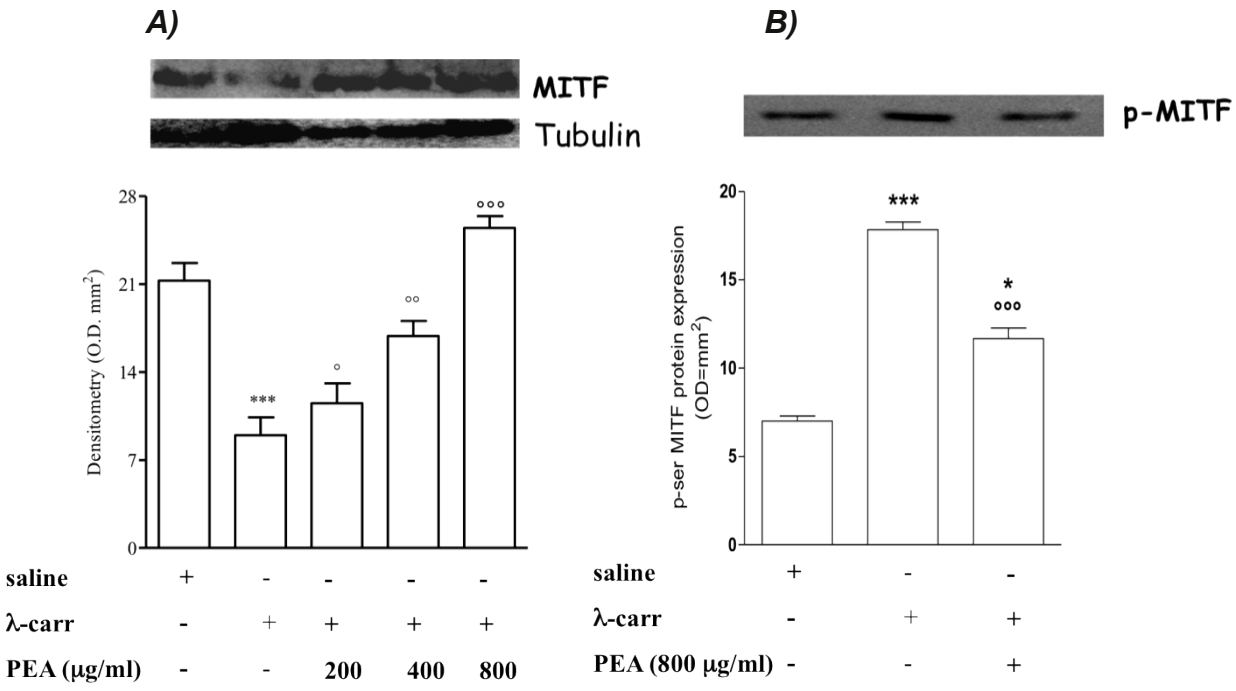


Figure 4.2.7.: *Effect of PEA on λ-carrageenin-induced total (A) and phosphorylated (B) MITF protein expression in granulomatous tissue*. *Representative Western blot analysis and relative densitometric analysis of MITF protein levels in sponges injected with saline, λ-carrageenin (1% w:v), or λ-carrageenin in the presence of increasing amount of PEA (200, 400, 800 mg/ml) . Tubulin expression is shown as control. Results are expressed as mean ± SEM of 3 experiments. ***P<0.001 vs. Saline; °p < 0.05, °°p < 0.01, °°°p < 0.001 vs. λ-carrageenin alone.*

4.2.7. Effect of PEA on p-ERK protein expression in granulomatous tissue

Starting from the assumption that the phosphorylation of MITF is a key-regulator event for its activation, concurrent to the ERK-*pathway* activation, Western Blot for p-ERK has been performed.

As a result, the expression of p-ERK, which was significantly (P<0.001) increased by the treatment of λ-carrageenin 1% in respect to the control, was drastically decreased after the administration of PEA (200, 400, 800 μg/mL), in a dose-responding manner (Fig. 4.2.8.).

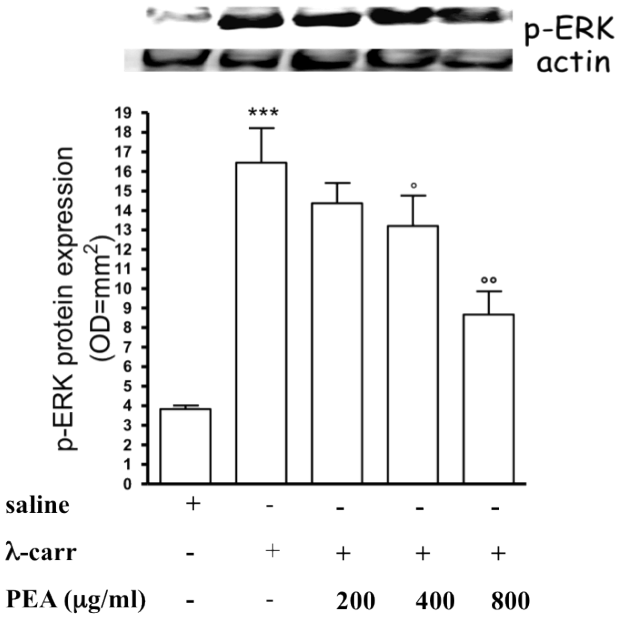


Figure 4.2.8.: ***Effect of PEA on λ-carrageenin-induced p-ERK expression in granulomatous tissue****. PEA effect on p-ERK protein expression has been evaluated through Western Blot analysis. Tubulin expression is shown as control. Results are expressed as mean ± SEM of 3 experiments. ***P<0.001 vs. Saline; °p < 0.05, °°p < 0.01 vs. λ-carrageenin alone.*

4.2.8. Effect of PEA on MITF/DNA binding activity in granulomatous tissue

To detect MITF/DNA binding activity, nuclear extracts from granulomatous tissues were analysed by EMSA. A basal level of MITF/DNA binding activity

was detected in nuclear extracts from tissues of saline-treated sponges evaluated 96 hours after implant. λ-Carrageenin treatment induced a marked increase of MITF/DNA binding activity that was significantly and dose-dependently reduced by the local administration of PEA (Fig 4.2.9. ***A***). The composition of MITF/DNA binding complexes was determined by competition experiments. In the reaction of competition, the specificity of MITF/DNA binding complexes was evidenced by the complete displacement of MITF/DNA binding in the presence of a 50-fold molar excess of unlabeled MITF probe. In contrast, a 50-fold molar excess of unlabeled mutated MITF probe or Sp-1 had no effect on DNA/binding activity (Fig 4.2.9. ***B***).

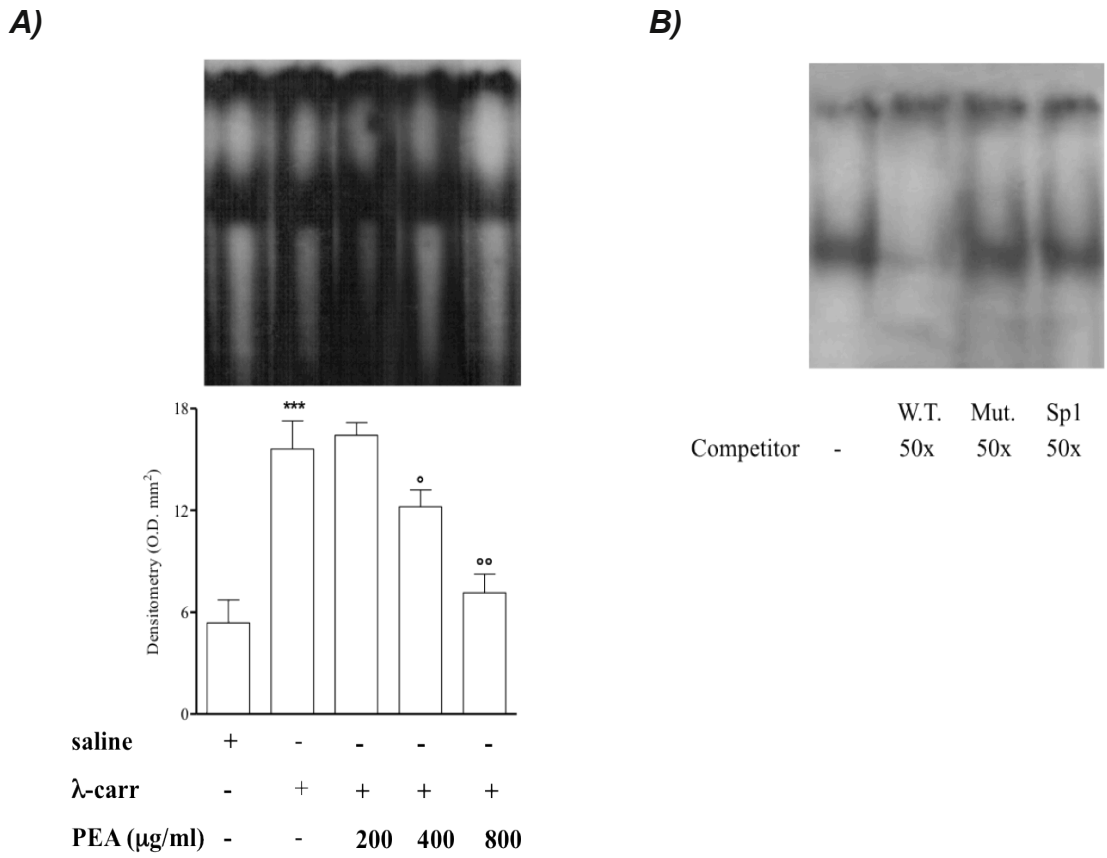


Figure 4.2.9.: ***Effect of PEA on DNA binding activity and characterization of MITF complex in λ-carrageenin-induced granuloma****. (**A**) EMSA showing the MITF binding activity in nuclear extracts from granulomatous tissues. (**B**) Characterization of the MITF/DNA complex. In competition reaction, nuclear extracts were incubated with radiolabeled MITF probe in absence or presence of: identical but unlabeled oligonucleotide (WT, 50x), mutated nonfunctional MITF probe (Mut. 50x) or unlabeled oligonucleotide containing the consensus sequence for Sp-1 (Sp-1, 50x). Data are from a single experiment representative of 3 separate assays. Densitometric data are expressed as mean±S.E.M. of 3 sponges from 3 rats. ***p<0.001 vs. saline; °p<0.05, °°p<0.001 vs. λ-carragenin.*

4.3. Effect of PEA on angiogenesis during Contact Allergic Dermatitis

4.3.1. Effect of PEA on mast cell number and degranulation in CAD-mice ears

The role of MCs in dermatitis has been well clarified, since several studies demonstrate their presence during the last phase of the disease.

In order to investigate MC presence in CAD, and PEA effect in this model, histological analysis on mice ears, stained with Toluidine Blue, were performed.

The staining reveals the effect of PEA treatment (5 mg/kg) in modulating MCs activity.

Interestingly, as shown in Fig. 4.3.1., PEA did not reduce total number of MCs (deep blue, un-degranulated,).

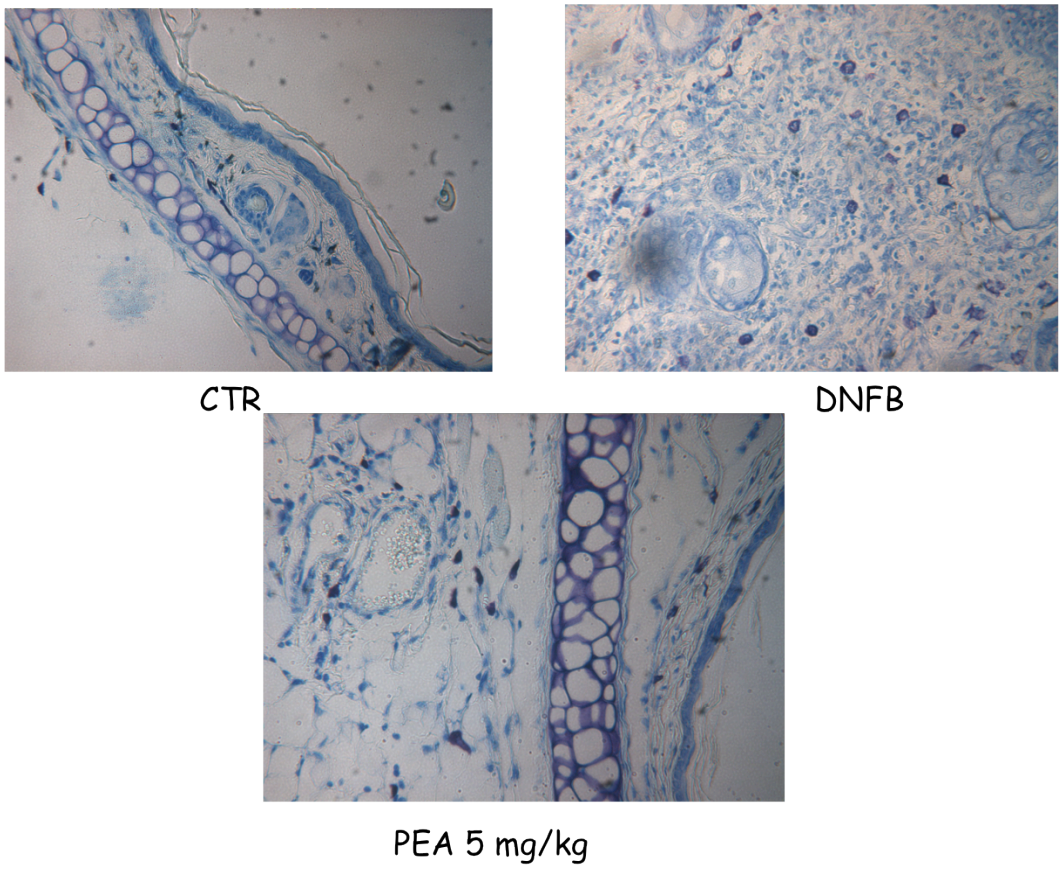


Figure 4.3.1.: ***Effect of PEA on number mast cells after DNFB treatment.*** *Representative histological analysis of CAD mice ears stained with toluidine blue.*

Fascinatingly, the effect of PEA was visible on MC activation (light blue, degranulated).

In fact, histological analysis of ears explanted from mice treated *i.p.* with PEA 5mg/kg, revealed its efficacy in reducing MC degranulation, in respect to the ears obtained from rats treated with DNFB alone (Fig. 4.3.2.)

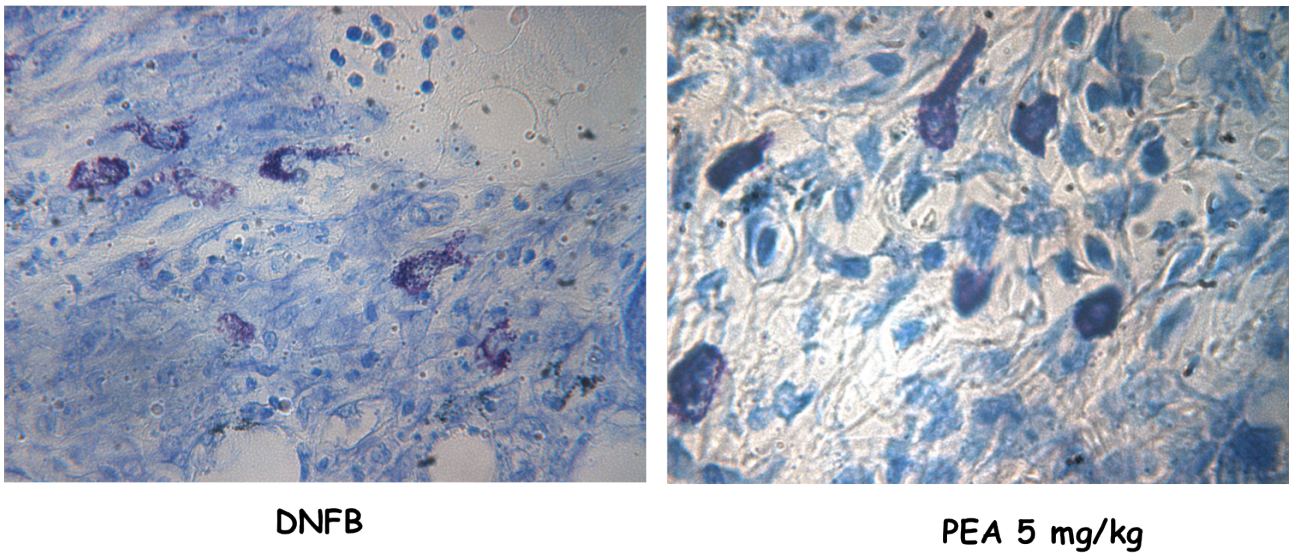


Figure 4.3.2.: ***Effect of PEA on degranulation of DNFB activated mast cells.*** *Representative histological analysis of CAD mice ears stained with toluidine blue.*

4.3.2. Effect of PEA on the expression of pro- angiogenic mediators in CAD- mice ears

Starting from the assumption that several studies in DAC show the development of angiogenesis during the last phase of insult, and, since MC presence has been evaluated during the same phase, in order to evaluate PEA effect on angiogenesis related to MC activation, protein expression of pro- angiogenic mediators through Western Blot analysis has been performed on mice ears extracts.

Definitely, VEGF and TNF- α expressions were measured, not only as they are important pro- angiogenic factors, but also since they are critical mediators that are released during MC degranulation.

As shown in Fig. 4.3.3.,TNF-α ***(A)*** and VEGF ***(B)*** protein expressions were significantly (P<0.001) increased in extracts of the ears obtained by DNFB 0,3%-injured mice, as compared to the control. Interestingly, PEA 5mg/kg administration determined a significant (P<0.01) decrease of both TNF-α and VEGF protein expressions.

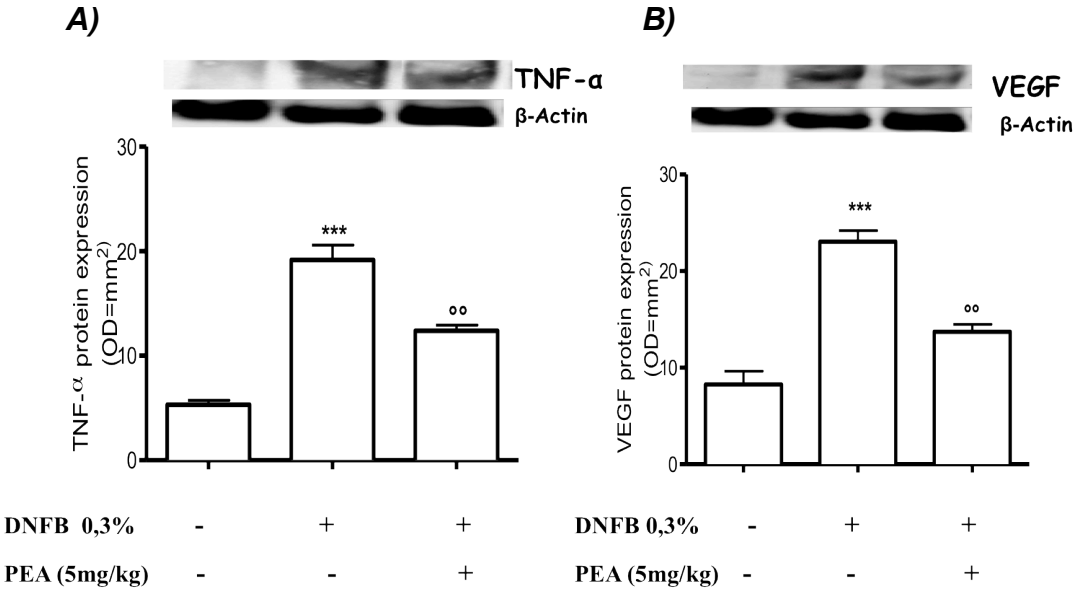


Figure 4.3.3.: ***Effect of PEA on expressions of (A) TNF-α and (B) VEGF pro-angiogenic factors.*** *β-actin expression is shown as control. Results are expressed as mean ± SEM of 3 experiments. ***P<0.001 vs. Control; °°p<0.01 vs. DNFB 0,3% alone.*

4.3.3. Effect of PEA on new vessel formation in CAD- mice ears

Last, in order to verify if the capability of PEA in modulating MCs degranulation and the consequent release of pro- angiogenic factors resulted also in a modulation of number of new vessel formation, histological analysis with a specific trichrome coloration has been performed.

As shown in Fig. 4.3.4., the treatment with DNFB 0,3% strongly increased the number of new vessels in mice ears, in respect to the ears of the un-treated mice. This evaluation has been done according to the vessels calibre: small

calibre vessels, presumably meaning of angiogenesis, were increased by DNFB treatment.

Moreover, the new vessel progression was located, basically, beside the external side of mice ears, where the DNFB injure had been executed.

Interestingly, PEA treatment at the dose of 5 mg/kg, drastically reduced the effect of DNFB, in significantly decreasing the number of small calibre vessels.

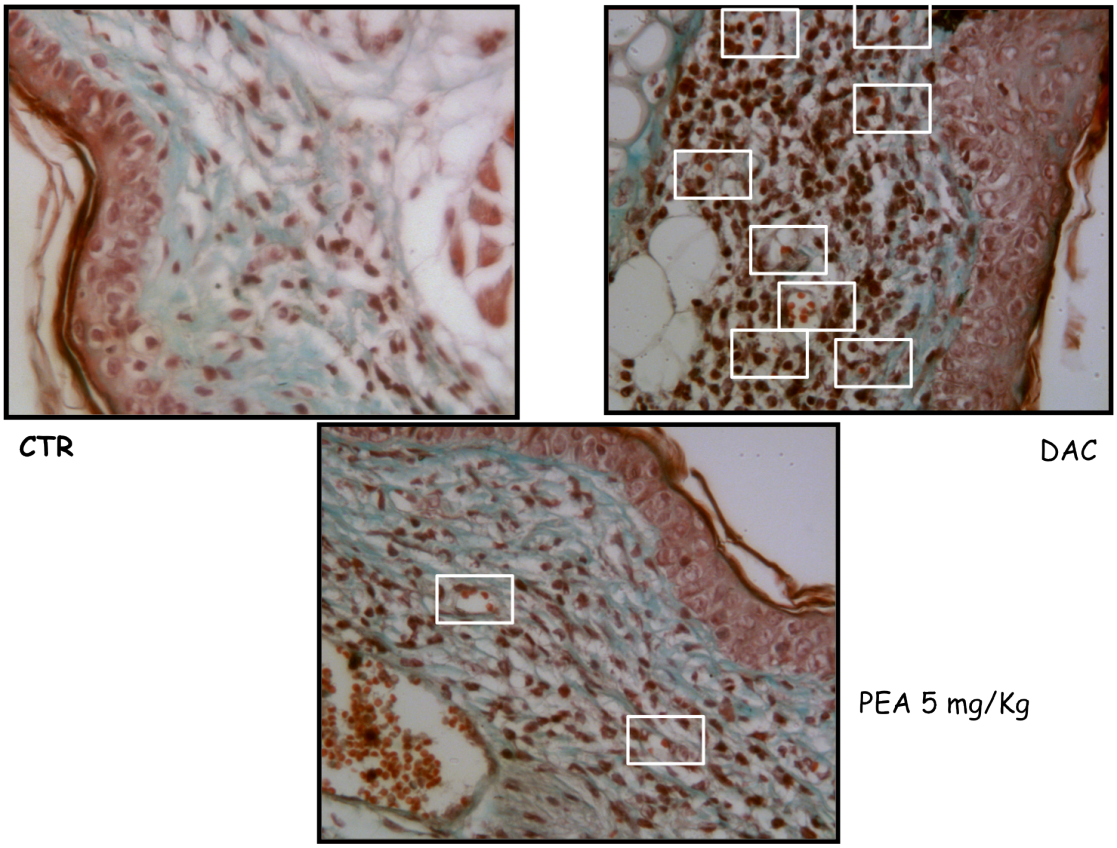


Figure 4.3.4.: ***Effect of PEA on small calibre vessels during CAD.*** *Representative histological analysis of mice ears tissues stained with tri-chromic staining.*

5. Discussion

The results of my Ph.D. study show, for the first time, the effect of *ALIA*mides in the control of pathological angiogenesis during inflammatory conditions, with main regard to Palmitoylethanolamide (PEA) which was able to reduce, both *in vitro* and *in vivo*, the angiogenic process occurring in astrogliosis, as well in granuloma formation and in contact allergic dermatitis.

In order to investigate the mechanism of the anti- angiogenic effect of PEA, we focused our attention on two different cell types, strictly responsible for the starting and the progression of angiogenesis occurring in CNS and in periphery: the astroglial cells and the mast cells. Both these two cell-types are endowed with the entire machinery necessary to the ignition and progression of angiogenesis. In fact, several evidences show that, the activated astroglial cells during neurodegeneration, as well as hyper-activated mast cells during inflammatory/allergic conditions, are able to release, together with pro-inflammatory mediators, a series of pro-angiogenic factors. Among them, mainly VEGF that, once released binds to its receptor, VEGFR-2, on the endothelial cells inducing their proliferation, and MMPs, which degrades extracellular matrix leading to new tube formation sprouting (Fioravanzo et al., 2010).
In our experiments, administration of Aβ for 48 hours to rat astroglial cells, produced an increase of pro-inflammatory mediators, nitric oxide and S100β, as well of pro- angiogenic VEGF, TNF-α, MMP-9 protein expression. The increase of these mediators was strongly and significantly reduced by PEA treatment, in part confirming that PEA is able to manage the reactive gliosis after Aβ insult (Scuderi et al., 2011). Moreover, our results showed that the treatment with Aβ induced also the release of VEGF in *medium* thus suggesting that this diffusible pro- angiogenic mediator could stimulate the near vasculature to proliferate through the binding to its receptors on endothelial cells. Since PEA treatment significantly reduced, not only the expression of pro- angiogenic mediators, but also VEGF release than,

consequently, could prevent the VEGF-induced EC proliferation, in order to confirm this, we evaluated the HUVEC endothelial cell proliferation exposed to the conditioned *medium* deriving from Aβ-stimulated astroglial cells. As expected, the proliferation of HUVEC was increased by the treatment with conditioned *medium* from Aβ-stimulated cells, while PEA, given to astroglial cells, reduced HUVEC proliferation in view of the above reported reduction of pro- angiogenic factors release. Thus, the results here obtained support our idea that PEA could modulate angiogenesis occurring during neurodegeneration, since the *in vitro* model we used, is a reproducible and validate model to study angiogenesis associated to neurodegenerative process (Fioravanzo et al., 2010).

As already stated, PEA mechanism of action still remains controversial, hence, in order to clarify its anti-proliferative effect on endothelial cells, we focused our attention on the reported effect of PEA on PPAR- α receptor.

In fact, recent data have shown that PEA effect in ameliorating reactive gliosis is played through an agonism on PPAR- α receptor on astroglial cells (Sasso et al., 2011; Scuderi et al., 2011); in line with these evidences, we tested the selective PPAR- α antagonist, GW6471, which strongly reverted PEA action in our model, confirming that PEA anti-proliferative effect on endothelial cells is played by a PPAR- α-dependent mechanism on astroglial cells.

In addition, part of my Ph.D. study was aimed to consolidate and expand previous evidences obtained by my group on the ability of PEA in controlling mast cell activation during chronic inflammation (De Filippis et al., 2011). Therefore, we evaluated the effect of PEA on angiogenesis occurring during in granuloma formation and allergic contact dermatitis (CAD), two experimental models where mast cells activation has been strongly validated (De Filippis et al., 2010; Petrosino et al., 2011), identifying in these cells the “orchestrator” of these pathologies. In particular, if the role of mast cell is important during all the phases of the granulomatous process, on the contrary during CAD, despite the strong involvement in the earlier phase of the disease of a different cell type, (keratinocyte), mast cell has been identified to contribute to the final phase (Iuvone al., 1999; Petrosino et al., 2011).

Histological analysis from granulomatous tissues of rats treated with of λ-carrageenin, as from the ears of the mice treated with DNFB, stained with Toluidine blue, evidenced an increasing of mast cell degranulation, in confront to the untreated animals. PEA administration, both in rats as in mice, significantly reduced MC degranulation, as showed by histological analysis. Moreover, PEA strongly reduced also the expression of pro- angiogenic mediators, VEGF, TNF-α, which were both increased in rats treated with λ-carrageenin and in mice treated with DNFB, in respect to the untreated animals, thus suggesting that PEA could reduce new vessel formation in inflamed tissues by controlling mediators release from mast cell release.

In order to confirm these, we performed a staining with haematoxylin/eosin of histologic sections of rats' granulomas and mice's ears to evidence the small vessel formation index of angiogenesis. Here, we evidenced a significant increase of the number of small calibre vessels in the rats' granulomas treated with λ-carrageenin, as in the mice's ears treated with DNFB, in respect to the untreated animals. In particular, during CAD the increase in the number of small calibre vessels has been reported beside the external side of mice ears, where the insult of DNFB has been executed. Treatment with PEA during CAD resulted in a significant decrease of small calibre vessels number, confirming PEA ability to control new vessel formation.

Furthermore, in order to clarify the molecular mechanism of action of PEA during chronic inflammation, we studied the involvement of the transcription factor MITF during granuloma, which regulates the transcription of several genes in mast cells, including that coding for rMCP-5 protein. We demonstrated that local administration of PEA was able to prevent MITF activation and in parallel to down-regulate rMCP-5 expression (mRNA and protein levels), in granulomatous tissue from λ -carrageenin-treated rats. These data are well in accordance to a previous study of my group, showing the key pro- angiogenic role played by rMCP-5 during granuloma formation in rats (Russo et al., 2005). In particular phosphorylation/de-phosphorylation is a key regulatory step in MITF activation, since three serine sites (Ser73, Ser298 and Ser409) for its phosphorylation have been reported (Wu et al., 2000); the phosphorylation of these sites positively correlates with the up-regulation of MITF transcriptional activity. To confirm the ability of PEA to

prevent MITF activation, we measured the phosphorylate state of MITF*:* interestingly, granulomatous tissue from λ-carrageenin exhibited a decrease of the total amount of MITF and an increase of phosphorylated MITF protein levels. PEA treatment reversed these effects, restoring total MITF protein levels to those one of granulomatous tissues from saline sponges. Thus, it is plausible that PEA is able to avoid the phosphorylation of MITF and, consequently, its phosphorylation-directed proteolytic degradation. Therefore, we investigated the main phosphorylation pathways leading to MITF activation, the MAPK cascade, studying ERK signalling. The phosphorylation of ERK is one of the key- events for the switch of the inactive state to the active form of several pro-inflammatory cascades. Our data evidenced that PEA reduced the amount of phosphorylated ERK, the activate form of this kinase. Interestingly, these data are in agreement with previous results demonstrating that PEA is able to control the activation fate of pro-inflammatory cascades ending to the activation of important transcription factors, as AP-1 and NF-κB (D'Agostino et al., 2009).

In conclusion, the present thesis show the strong effect of *ALIA*mide in controlling pathological angiogenesis which accompanies chronic inflammatory states both at central levels and in periphery through the modulation of two different cell types, astroglial cells and mast cells. Although these two cell types are completely different in function and in anatomic localisation, however are both of critical importances in the control of angiogenic process by the ability to undergo to activation and to release a plethora of both inflammatory and pro- angiogenic mediators that exacerbate the damage. The results here shown demonstrate, for the first time the capability of Palmitoylethanolamide in counteract the negative side of angiogenesis, acting as a pleiotropic molecule of a broad spectrum of activity.

For this reason, it is conceivable to hypothesize the usage of PEA to prevent the chronicity of diseases during which angiogenesis plays the strong role in getting them worse and, in particular, in all those inflammatory conditions linked to astroglial cell hyperactivation and mast cell activation.

References

- Aase K, von Euler G, Li X, Pontén A, Thorén P, Cao R, Cao Y, Olofsson B, Gebre-Medhin S, Pekny M, Alitalo K, Betsholtz C, Eriksson U, *Vascular endothelial growth factor-B-deficient mice display an atrial conduction defect*, Circulation. 2001;104(3):358-64
- Albini A, Sporn MB, *The tumour microenvironment as a target for chemoprevention*, Nat Rev Cancer. 2007;7(2):139-47, Review
- Aloe L, Leon A, Levi-Montalcini R. *A proposed autacoid mechanism controlling mastocyte behaviour*. Agents and Actions. 1993.39, C145-C147.
- Appleton I, Brown NJ, Willis D, Colville-Nash PR, Alam C, Brown JR, Willoughby DA. *The role of vascular endothelial growth factor in a murine chronic granulomatous tissue air pouch model of angiogenesis*. J Pathol. 1996;180(1):90-4.
- Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM, *Isolation of putative progenitor endothelial cells for angio- genesis*, Science. 1997;275(5302):964-7
- Bach F, Uddin FJ, Burke D, *Angiopoietins in malignancy*, Eur J Surg Oncol. 2007;33(1):7-15. Review
- Badertscher K, Brönnimann M, Karlen S, Braathen LR, Yawalkar N. *Mast cell chymase is increased in chronic atopic dermatitis but not in psoriasis*. Arch Dermatol Res. 2005;296:503-6.
- Benelli R, Albini A, Noonan D, *Neutrophils and angiogenesis: potential initiators of the angiogenic cascade*, Chem Immunol Allergy. 2003;83:167-81, Review
- Bienenstock J. *Mucosal immunological protection mechanisms in the airways.* Eur J Respir Dis Suppl. 1986;147:62-71. Review.
- Bouïs D, Kusumanto Y, Meijer C, Mulder NH, Hospers GA, *A review on pro- and anti-angiogenic factors as targets of clinical intervention*, Pharmacol Res. 2006;53(2):89-103, Review
- Bracey MH, Hanson MA, Masuda KR, Stevens RC, Cravatt BF. *Structural*

adaptations in a membrane enzyme that terminates endocannabinoid signaling. Science. 2002. 298 (5599), 1793-1796.

- Brinton RD, Wang JM. *Preclinical analyses of the therapeutic potential of allopregnanolone to promote neurogenesis in vitro and in vivo in transgenic mouse model of Alzheimer's disease.* Curr Alzheimer Res. 2006;3(1):11-7.
- Bradding P, Okayama Y, Howarth PH, Church MK, Holgate ST. *Heterogeneity of human mast cells based on cytokine content.* J Immunol. 1995;155(1):297-307.
- Byrne AM, Bouchier-Hayes DJ, Harmey JH, *Angiogenic and cell survival functions of vascular endothelial growth factor (VEGF)*, J Cell Mol Med. 2005;9(4):777-94, Review
- Cadas H, di Tomaso E, Piomelli D. *Occurrence and biosynthesis of endogenous cannabinoid precursor, N-arachidonoyl phosphatidylethanolamine, in rat brain.* J Neurosci. 1997. 17, 1226-1242.
- Carmeliet P, Jain RK. *Angiogenesis in cancer and other diseases*. Nature. 2000, 407(6801), 249-57. Review.
- Cantara S, Donnini S, Morbidelli L, Giachetti A, Schulz R, Memo M, Ziche M., *Physiological levels of amyloid peptides stimulate the angiogenic response through FGF-2*, FASEB J. 2004;18(15):1943-5
- Cao Y, Lundwall A, Gadaleanu V, Lilja H, Bjartell A, *Anti-thrombin is expressed in the benign prostatic epithelium and in prostate cancer and is capable of forming complexes with prostate-specific antigen and human glandular kallikrein 2*, Am J Pathol. 2002;161(6):2053-63
- Chiappelli M, Borroni B, Archetti S, Calabrese E, Corsi MM, Franceschi M, Padovani A, Licastro F, *VEGF gene and phenotype relation with Alzheimer's disease and mild cognitive impairment*, Rejuvenation Res. 2006;9(4):485-93
- Christov A, Ottman JT, Grammas P, *Vascular inflammatory, oxidative and protease- based processes: implications for neuronal cell death in Alzheimer's disease*, Neurol Res. 2004;26(5):540-6
- Clark ER, Clark EL, Growth and behavior of epidermis as observed microscopically in observation chambers inserted in the ears of rabbits, Am J Anat. 1953;93(2):171-219.
- Costa B, Comelli F, Bettoni I, Colleoni M, Giagnoni G. *The endogenous fatty acid amide, palmitoylethanolamide, has anti-allodynic and anti-hyperalgesic*

effects in a murine model of neuropathic pain: involvement of CB(1), TRPV1 and PPARgamma receptors and neurotrophic factors. Pain. 2008, 133-145-56.

- Cravatt BF, Lichtman AH. *The enzymatic inactivation of the fatty acid amide class of signaling lipids.* Chemistry and Physics of Lipids. 2002. 121 (1-2), 135-148.
- Cravatt BF, Giang DK, Mayfield SP, Boger DL, Lerner RA, Gilula NB. *Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides.* Nature. 1996. 384 (6604), 83-87.
- Cravatt BF, Saghatelian A, Hawkins EG, Clement AB, Bracey MH, Lichtman AH. *Functional disassociation of the central and peripheral fatty acid amide signaling systems.* Proceedings of the National Academy of Sciences of the United States of America. 2004. 101 (29), 10821-10826.
- D'Acquisto F, de Cristofaro F, Maiuri MC, Tajana G and Carnuccio R. *Protective role of nuclear factor kappa B against nitric oxide-induced apoptosis in J774 macrophages*. Cell Death Differ.2001 8:144-151.
- D'Agostino G, La Rana G, Russo R, Sasso O, Iacono A, Esposito E, Mattace Raso G, Cuzzocrea S, Loverme J, Piomelli D, Meli R, Calignano A. *Central administration of palmitoylethanolamide reduces hyperalgesia in mice via inhibition of NF-kappaB nuclear signalling in dorsal root ganglia*. Eur J Pharmacol. 2009 613:54
- D'Agostino G, La Rana G, Russo R, Sasso O, Iacono A, Esposito E, Raso GM, Cuzzocrea S, Lo Verme J, Piomelli D, Meli R, Calignano A. *Acute intracerebroventricular administration of palmitoylethanolamide, an endogenous peroxisome proliferator-activated receptor-alpha agonist, modulates carrageenan-induced paw edema in mice.* J Pharmacol Exp Ther, 2007. 322(3):1137-43.
- D'Andrea LD, Del Gatto A, Pedone C, Benedetti E, *Peptide-based molecules in angiogenesis*, Chem Biol Drug Des. 2006;67(2):115-26, Review
- De Filippis D, **Cipriano M**, Esposito G, Scuderi C, Steardo L, Iuvone T. *Are anti-angiogenic drugs useful in neurodegenerative disorders?* CNS Neurol Disord Drug Targets. 2010;9(6):807-12. Review.

- De Filippis D, D'Amico A, **Cipriano M**, Petrosino S, Orlando P, Di Marzo V, Iuvone T. *Levels of endocannabinoids and palmitoylethanolamide and their pharmacological manipulation in chronic granulomatous inflammation in rats*. Pharmacol Res. 2010;61:321-8.
- De Filippis D, Luongo L, **Cipriano M**, Palazzo E, Cinelli MP, de Novellis V, Maione S, Iuvone T. *Palmitoylethanolamide reduces granuloma-induced hyperalgesia by modulation of mast cell activation in rats.* Mol Pain. 2011;7:3
- De Filippis D, Steardo A, D'Amico A, Scuderi C, **Cipriano M**, Esposito G, Iuvone T. *Differential cannabinoid receptor expression during reactive gliosis: a possible implication for a nonpsychotropic neuroprotection.* ScientificWorldJournal. 2009;9:229-35. Review
- De Filippis D, D'Amico A, Cinelli MP, Esposito G, Di Marzo V, Iuvone T. *Adelmidrol, a palmitoylethanolamide analogue, reduces chronic inflammation in a carrageenin-granuloma model in rats*. J Cell Mol Med. 2009;13:1086-95.
- De Filippis D, D'Amico A, Iuvone T. *Cannabinomimetic control of mast cell mediator release: new perspective in chronic inflammation*. J Neuroendocrinol. 2008;20 Suppl 1:20-5.
- De Filippis D, Russo A, D'Amico A, Esposito G, Pietropaolo C, Cinelli M, Russo G, Iuvone T. *Cannabinoids reduce granuloma-associated angiogenesis in rats by controlling transcription and expression of mast cell protease-5.* Br J Pharmacol. 2008;154(8):1672-9.
- Desai BS, Schneider JA, Li JL, Carvey PM, Hendey B. *Evidence of angiogenic vessels in Alzheimer's disease. J Neural Transm*. 2009;116:587-97
- Désarnaud F, Cadas H, Piomelli D. Anandamide amidohydrolase activity in rat brain microsomes. *Identification and partial characterization*. Journal of Biological Chemistry. 1995. 270 (11), 6030-6035.
- Di Marzo V, Fontana A, Cadas H, Schinelli S, Cimino G, Schwartz JC, Piomelli D. *Formation an inactivation of endogenous cannabinoid anandamide in central neurons*. Nature. 372, 686-691.
- Di Marzo V, Melck D, Orlando P, Bisogno T, Zagoory O, Bifulco M, Vogel Z., De Petrocellis L., *Palmitoylethanolamide inhibits the expression of fatty acid amide hydrolase and enhances the antiproliferative effect of anandamide in human breast cancer cells*. Biochem J. 2001. 122 358, 249-255.

- Edelberg JM, Reed MJ, *Aging and angiogenesis*, Front Biosci. 2003;8:1199- 209, Review
- Esiri MM. *Which vascular lesions are of importance in vascular dementia?* Ann. N. Y. Acad. Sci. 2000, 903, 239-43. Review
- Esposito G, De Filippis D, Steardo L, Scuderi C, Savani C, Cuomo V, Iuvone T. *CB1 receptor selective activation inhibits beta-amyloid-induced iNOS protein expression in C6 cells and subsequently blunts tau protein hyperphosphorylation in co-cultured neurons.* Neurosci. Lett. 2006, 404(3), 342-6.
- Facci L, Dal Toso R, Romanello S, Buriani A, Skaper SD, Leon A. *Mast cells express a peripheral cannabinoid receptor with differential sensitivity to anandamide and palmitoylethanolamide.* Proc Natl Acad Sci USA **1995** 92:3376–3380
- Ferrara N. *Vascular endothelial growth factor and the regulation of angiogenesis.* Recent Prog Horm Res. 2000;55:15-35; discussion 35-6. Review.
- Fiedler U, Augustin HG, *Angiopoietins: a link between angiogenesis and inflammation*, Trends Immunol. 2006;27(12):552-8. Review
- Fioravanzo L, Venturini M, Liddo RD, Marchi F, Grandi C, Parnigotto PP, Folin M. *Involvement of rat hippocampal astrocytes in β-amyloid-induced angiogenesis and neuroinflammation.* Curr Alzheimer Res. 2010;7(7):591-601.
- Folkman J, Haudenschild C, *Angiogenesis in vitro*, Nature. 1980;288(5791):551-6,
- Folkman J, Shing Y, *Angiogenesis*, J Biol Chem. 1992;267(16):10931-4, Review
- Forehand JR, Johnston RB Jr. *Chronic granulomatous disease: newly defined molecular abnormalities explain disease variability and normal phagocyte physiology.* Curr Opin Pediatr. 1994;6(6):668-75. Review. Erratum in: Curr Opin Pediatr 1995;7(1):126.
- Forsberg E, Pejler G, Ringvall M, Lunderius C, Tomasini-Johansson B, Kusche-Gullberg M, Eriksson I, Ledin J, Hellman L, Kjellén L. *Abnormal mast cells in mice deficient in a heparin-synthesizing enzyme. Nature. 1999;400:773-6.*
- Fowler CJ, Jonsson KO, Tiger G. *Fatty acid amide hydrolase: biochemistry, pharmacology, and therapeutic possibilities for an enzyme hydrolyzing anandamide, 2- arachidonoylglycerol,*

palmitoylethanolamide, and oleamide. Biochem Pharmacol. 2001;62(5):517-26. Review.

- Gagnon E, Cattaruzzi P, Griffith M, Muzakare L, LeFlao K, Faure R, Béliveau R, Hussain SN, Koutsilieris M, Doillon CJ, *Human vascular endothelial cells with ex tended life spans: in vitro cell response, protein expression, and angiogenesis*, Angiogenesis. 2002;5(1-2):21-33
- Garmy-Susini B, Varner JA, *Circulating endothelial progenitor cells*, Br J Cancer. 2005;93(8):855-8, Review
- Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, Jeltsch M, Mitchell C, Alitalo K, Shima D, Betsholtz C, *VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia*, J Cell Biol. 2003;161(6):1163-77.
- Gill M, Dias S, Hattori K, Rivera ML, Hicklin D, Witte L, Girardi L, Yurt R, Himel H, Rafii S, *Vascular trauma induces rapid but transient mobilization of VEGFR2(+)AC133(+) endothelial precursor cells*, Circ Res. 2001;88(2):167- 74
- Gimbrone MA Jr, Cotran RS, Folkman J, *Endothelial regeneration: studies with human endothelial cells in culture*, Ser Haematol. 1973;6(4):453-5,
- Groneberg DA, Bester C, Grützkau A, Serowka F, Fischer A, Henz BM, Welker P. *Mast cells and vasculature in atopic dermatitis--potential stimulus of neoangiogenesis*. Allergy. 2005;60(1):90-7
- Hanna J, Goldman-Wohl D, Hamani Y, Avraham I, Greenfield C, Natanson-Yaron S, Prus D, Cohen-Daniel L, Arnon TI, Manaster I, Gazit R, Yutkin V, Benharroch D, Porgador A, Keshet E, Yagel S, Mandelboim O, *Decidual NK cells regulate key de- velopmental processes at the human fetal-maternal interface*, Nat Med. 2006;12(9):1065-74
- He S, Walls AF. *The induction of a prolonged increase in microvascular permeability by human mast cell chymase.* Eur J Pharmacol. 1998;352:91-8.
- Heneka MT, O'Banion MK, *Inflammatory processes in Alzheimer's disease*, J Neuroimmunol. 2007;184(1-2):69-91Review
- Hershey CL, Fisher DE *Mitf and Tfe3: members of a b-HLH-ZIP transcription factor family essential for osteoclast development and function*. Bone. 2004;34(4):689-96. Review.
- Heryanto B, Girling JE, Rogers PA, *Intravascular neutrophils partially mediate the endometrial endothelial cell proliferative response to oestrogen in ovariectomised mice*, Reproduction. 2004;127(5):613-20.

- Hillard CJ, Wilkison DM, Edgemond WS, Campbell WB. *Characterization of the kinetics and distribution of Narachidonylethanolamine (anandamide) hydrolysis by rat brain.* Biochimica et Biophysica Acta. 1995.1257 (3), 249-256.
- Holderfield MT, Hughes CC, *Crosstalk between vascular endothelial growth factor, notch, and transforming growth factor-beta in vascular morphogenesis*, Circ Res. 2008;102(6):637-52, Review
- Hoozemans JJ, Veerhuis R, Rozemuller AJ, Eikelenboom P, *The pathological cascade of Alzheimer's disease: the role of inflammation and its therapeutic implica tions*, Drugs Today (Barc). 2002;38(6):429-43, Review
- Imada T, Komorita N, Kobayashi F, Naito K, Yoshikawa T, Miyazaki M, Nakamura N, Kondo *Therapeutic potential of a specific chymase inhibitor in atopic dermatitis.* Jpn J Pharmacol. 2002;90:214-7.
- Iuvone T, Carnuccio R, Di Rosa M. *Modulation of granuloma formation by endogenous nitric oxide*. Eur J Pharmacol. 1994;265(1-2):89-92.
- Iuvone T, Den Bossche RV, D'Acquisto F, Carnuccio R, Herman AG. *Evidence that mast cell degranulation, histamine and tumour necrosis factor alpha release occur in LPS-induced plasma leakage in rat skin.* Br J Pharmacol. 1999 128:700-4
- Jones EA, le Noble F, Eichmann A, *What determines blood vessel structure? Genetic prespecification vs. hemodynamics*, Physiology (Bethesda). 2006;21:388-95, Review
- Kalluri R, *Basement membranes: structure, assembly and role in tumour angiogene sis*, Nat Rev Cancer. 2003;3(6):422-33, Review
- Karamysheva AF, *Mechanisms of angiogenesis*, Biochemistry (Mosc). 2008;73(7):751-62, Review
- Kathuria S, Gaetani S, Fegley D, Valino F, Duranti A, Tontini A, Mor M, Tarzia G, La Rana G, Calignano A, Giustino A, Tattoli M, Palmery M, Cuomo V, Piomelli D. *Modulation of anxiety through blockade of anandamide hydrolysis.* Nature Medicine. 2003. 9 (1), 76-81.
- Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J, Connolly DT, *Vascular permeability factor, an endothelial cell mitogen related to PDGF*, Science. 1989;246(4935):1309-12
- Karsak M, Gaffal E, Date R, Wang- Eckhardt L, Rehnelt J, Petrosino S,

Starowicz K, Steuder R, Schlicker E, Cravatt B, Mechoulam R, Buettner R, Werner S, Di Marzo V, Tüting T, Zimmer A. *Attenuation of allergic contact dermatitis through the endocannabinoid system.* Science. 2007;316(5830):1494-7.

- Kim WY, Lee HY. *Brain angiogenesis in developmental and pathological processes: mechanism and therapeutic intervention in brain tumors*. *FEBS J.* 2009, 276(17),4653-64.
- Kitamura Y, Oboki K, Ito A. *Molecular mechanisms of mast cell development.* Immunol Allergy Clin North Am. 2006;26:387-405.
- Klagsbrun M, D'Amore PA. *Regulators of angiogenesis.* Annu Rev Physiol. 1991;53:217-39.
- Kurz H. *Physiology of angiogenesis*, J Neurooncol. 2000;50(1-2):17-35, Review
- Lage AP, Andrade SP. *Assessment of angiogenesis and tumor growth in conscious mice by a fluorimetric method*. Microvasc Res. 2000;59(2):278-85.
- Lambert DM, Vandevoorde S, Jonsson KO, Fowler CJ. *The palmitoylethanolamide family: a new class of anti-inflammatory agents?* Curr Med Chem. 2002;9(6):663-74. Review.
- Lejeune FJ, Liénard D, Matter M, Rüegg C, *Efficiency of recombinant human TNF in human cancer therapy*, Cancer Immun. 2006;6:6, Review
- Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N, Vascular endothelial growth factor is a secreted angiogenic mitogen, Science. 1989;246(4935):1306-9,
- Levy C, Khaled M, Fisher DE. *MITF: master regulator of melanocyte development and melanoma oncogene.* Trends Mol Med. 2006;12:406-14.
- Levi-Montalcini R, Skaper SD, Dal Toso R, Petrelli L, Leon A. *Nerve growth factor: from neurotrophin to neurokine*. Trends in Neuroscience 1996, 19, 514–520.
- Lo Verme J, Fu J, Astarita G, La Rana G, Russo R, Calignano A, Piomelli D. *The nuclear receptor peroxisome proliferator-activated receptor-alpha mediates the anti-inflammatory actions of palmitoylethanolamide.* Mol Pharmacol, 2005. 67(1):15-9
- McNeil HP, Austen KF, Somerville LL, Gurish MF, Stevens RL. *Molecular cloning of the mouse mast cell protease-5 gene. A novel secretory granule protease expressed early in the differentiation of serosal mast cells*. J

Biol Chem. 1991;266:20316-22.

- Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN, Yancopoulos GD, *Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis*, Science. 1997;277(5322):55-60
- Mandriota SJ, Pepper MS, *Vascular endothelial growth factor-induced in vitro angiogenesis and plasminogen activator expression are dependent on endogenous basic fibroblast growth factor*, J Cell Sci. 1997;110 (Pt 18):2293-302
- Mazzari S, Canella R, Petrelli L, Marcolongo G, Leon A. *N-(2-hydroxyethyl)hexadecanamide is orally active in reducing edema formation and inflammatory hyperalgesia by down-modulating mast cell activation.* European Journal of Pharmacology. 1996. 300 (3), 227-236.
- Metcalfe DD, Baram D, Mekori YA. *Mast cells*. Physiol Rev. 1997;77(4):1033-79. Review.
- Milkiewicz M, Ispanovic E, Doyle JL, Haas TL, *Regulators of angiogenesis and strategies for their therapeutic manipulation*, Int J Biochem Cell Biol. 2006;38(3):333-57. Review
- Minuzzo S, Moserle L, Indraccolo S, Amadori A, *Angiogenesis meets immunology: cytokine gene therapy of cancer*, Mol Aspects Med. 2007;28(1):59-86. Review
- Natarajan V, Schmid PC, Reddy PV, Schmid HH. *Catabolism of N-acylethanolamine phospholipids by dog brain preparations*. Journal of Neurochemistry. 1984.42 (6), 1613-1619.
- Newson B, Dahlström A, Enerbäck L, Ahlman H. *Suggestive evidence for a direct innervation of mucosal mast cells.* Neuroscience. 1983;10(2):565-70.
- Noonan DM, De Lerma Barbaro A, Vannini N, Mortara L, Albini A, *Inflammation, inflammatory cells and angiogenesis: decisions and indecisions*, Cancer Metastasis Rev. 2008;27(1):31-40, Review
- Okamoto Y, Morishita J, Tsuboi K, Tonai T, Ueda N. *Molecular characterization of a phospholipase D generating anandamide and its congeners.* Journal of Biological Chemistry.2004. 279 (7), 5298-5305.
- Otrock ZK, Mahfouz RA, Makarem JA, Shamseddine AI, *Understanding the biology of angiogenesis: review of the most important molecular*

mechanisms, Blood Cells Mol Dis. 2007;39(2):212-20. Review.

- Papetti M, Herman IM, *Mechanisms of normal and tumor-derived angiogenesis*, Am J Physiol Cell Physiol. 2002;282(5):C947-70, Review
- Peichev M, Naiyer, Pereira D, Zhu Z, Lane WJ, Williams M, Oz MC, Hicklin DJ, Witte L, Moore MA, Rafii S, *Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors*, Blood. 2000;95(3):952-8
- Petrosino S, Iuvone T, Di Marzo V. *N-palmitoyl-ethanolamine: Biochemistry and new therapeutic opportunities.* Biochimie. 2010;92:724-7.
- Pogue AI, Lukiw WJ, *Angiogenic signaling in Alzheimer's disease*, Neuroreport. 2004;15(9):1507-10
- Post S, Peeters W, Busser E, Lamers D, Sluijter JP, Goumans MJ, de Weger RA, Moll FL, Doevendans PA, Pasterkamp G, Vink A, *Balance between angiopoietin-1 and angiopoietin-2 is in favor of angiopoietin-2 in atherosclerotic plaques with high microvessel density*, J Vasc Res. 2008;45(3):244-50.
- Qutub AA, Mac Gabhann F, Karagiannis ED, Vempati P, Popel AS. *Multiscale models of angiogenesis.* IEEE. Eng. Med. Biol. Mag. 2009, 28(2), 14-31. Review.
- Raso GM, Esposito E, Vitiello S, Iacono A, Santoro A, D'Agostino G, Sasso O, Russo R, Piazza PV, Calignano A, Meli R. *Palmitoylethanolamide stimulation induces allopregnanolone synthesis in C6 Cells and primary astrocytes: involvement of peroxisome-proliferator activated receptor-α.* J Neuroendocrinol. 2011;23(7):591-600
- Ribatti D, Gualandris A, Bastaki M, Vacca A, Iurlaro M, Roncali L, Presta M, *New model for the study of angiogenesis and antiangiogenesis in the chick embryo chorioallantoic membrane: the gelatin sponge/chorioallantoic membrane assay*, J Vasc Res. 1997;34(6):455-63
- Ribatti D, Nico B, Vacca A, Roncali L, Burri PH, Djonov V, *Chorioallantoic membrane capillary bed: a useful target for studying angiogenesis and anti-angiogenesis in vivo*, Anat Rec. 2001;264(4):317-24, Review
- Ribatti D, Urbinati C, Nico B, Rusnati M, Roncali L, Presta M, *Endogenous basic fibroblast growth factor is implicated in the vascularization of the chick embryo chorioallantoic membrane*, Dev Biol. 1995;170(1):39-49
- Ribeiro RA, Souza-Filho MV, Souza MH, Oliveira SH, Costa CH, Cunha FQ,

Ferreira HS. *Role of resident mast cells and macrophages in the neutrophil migration induced by LTB4, fMLP and C5a des arg*. Int Arch Allergy Immunol. 1997;112:27-35.

- Ross RA, Brockie HC, Pertwee RG, *Inhibition of nitric oxide production in RAW264.7 macrophages by cannabinoids and palmitoylethanolamide*. European Journal of Pharmacology. 2000. 401 (2), 121-130.
- Russo A, Russo G, Peticca M, Pietropaolo C, Di Rosa M, Iuvone T. *Inhibition of granuloma-associated angiogenesis by controlling mast cell mediator release: role of mast cell protease-5.* Br J Pharmacol. 2005 145:24-33.
- Sacchi G, Weber E, Aglianó M, Lorenzoni P, Rossi A, Caruso AM, Vernillo R, Gerli R, Lorenzi M. *Lymphatic vessels in colorectal cancer and their relation with inflammatory infiltrate.* Dis Colon Rectum. 2003;46(1):40-7.
- Sanker S, Chandrasekharan UM, Wilk D, Glynias MJ, Karnik SS, Husain A. *Distinct multisite synergistic interactions determine substrate specificities of human chymase and rat chymase-1 for angiotensin II formation and degradation*. J Biol Chem. 1997;272:2963-8.
- Sastre M, Klockgether T, Heneka MT, *Contribution of inflammatory processes to Alzheimer's disease: molecular mechanisms*, Int J Dev Neurosci. 2006;24(2-3):167-76. Review.
- Sastre M, Walter J, Gentleman SM, *Interactions between APP secretases and inflammatory mediators*, J Neuroinflammation. 2008;5:25, Review
- Scarampella F, Abramo F, Noli C. *Clinical and histological evaluation of an analogue of palmitoylethanolamide, PLR 120 (comicronized Palmidrol INN) in cats with eosinophilic granuloma and eosinophilic plaque: a pilot study*. Vet Dermatol. 2001;12(1):29-39.
- Schmid HH, Schmid PC, Natarajan V, *N-acylated glycerophospholipids and their derivatives.* Progress in Lipid Research. 1990. 29 (1), 1-43.
- Scuderi C, Esposito G, Blasio A, Valenza M, Arietti P, Steardo L Jr, Carnuccio R, De Filippis D, Petrosino S, Iuvone T, Di Marzo V, Steardo L. *Palmitoylethanolamide counteracts reactive astrogliosis induced by beta-amyloid peptide*. J Cell Mol Med. 2011
- Shibahara S, Takeda K, Yasumoto K, Udono T, Watanabe K, Saito H, Takahashi K. *Microphthalmia-associated transcription factor (MITF): multiplicity in structure, function, and regulation*. J Investig Dermatol Symp

Proc. 2001;6:99-10.

- Skaper SD. *The brain as a target for inflammatory processes and neuroprotective strategies*. Ann. N. Y. Acad. Sci. 2007, 1122, 23-34.
- Smart D, Jonsson KO, Vandevoorde S, Lambert DM, Fowler CJ. *'Entourage' effects of N-acyl ethanolamines at human vanilloid receptors. Comparison of effects upon anandamide-induced vanilloid receptor activation and upon anandamide metabolism.* Br J Pharmacol. 2002;136(3):452-8.
- Sonnenblick A, Levy C, E Razin E. *Interplay between MITF, PIAS3, and STAT3 in mast cells and melanocytes*. Mol Cell Biol.; 24;2004:10584-92.
- Strbian D, Kovanen PT, Karjalainen-Lindsberg ML, Tatlisumak T, Lindsberg PJ. *An emerging role of mast cells in cerebral ischemia and haemorrhage.* Ann Med. 2009;41:438-50 .
- Sun YX, Tsuboi K, Okamoto Y, Tonai T, Murakami M, Kudo I, Ueda N *Biosynthesis of anandamide and N-palmitoylethanolamine by sequential actions of phospholipase A2 and ysophospholipase D.* 2004. Biochemical Journal. 380 (Part 3), 749-756.
- Tarkowski E, Issa R, Sjögren M, Wallin A, Blennow K, Tarkowski A, Kumar P, *Increased intrathecal levels of the angiogenic factors VEGF and TGF-beta in Alzheimer's disease and vascular dementia*, Neurobiol Aging. 2002;23(2):237-43
- Terakawa M, Tomimori Y, Goto M, Fukuda Y. *Mast cell chymase induces expression of chemokines for neutrophils in eosinophilic EoL-1 cells and mouse peritonitis eosinophils*. Eur J Pharmacol. 2006;538:175-81
- Thirumangalakudi L, Samany PG, Owoso A, Wiskar B, Grammas P, *Angiogenic proteins are expressed by brain blood vessels in Alzheimer's disease*, J Alzheimers Dis. 2006;10(1):111-8, Review
- Tomimori Y, Tsuruoka N, Fukami H, Saito K, Horikawa C, Saito M, Muto T, Sugiura N, Yamashiro K, Sumida M, Kakutani S, Fukuda Y. *Role of mast cell chymase in allergen-induced biphasic skin reaction.* Biochem Pharmacol. 2002;64:1187-1193.
- Ueda N, Kurahashi Y, Yamamoto S, Tokunaga T. *Partial purification and characterization of the porcine brain enzyme hydrolyzing and synthesizing anandamide.* Journal of Biological Chemistry.1995. 270 (40), 23823-23827.
- Ueda N, Yamanaka K, Yamamoto S. *Purification and characterization of an*

acid amidase selective for N-palmitoylethanolamine, a putative endogenous anti-inflammatory substance. Journal of Biological Chemistry. 2001.276 (38), 35552-35557.

- Urbich C, Dimmeler S, *Endothelial progenitor cells: characterization and role in vascular biology*, Circ Res. 2004;95(4):343-53, Review
- Vagnucci AH Jr, Li WW, *Alzheimer's disease and angiogenesis*, Lancet. 2003;361(9357):605-8, Review
- Wong CK, Ng SS, Lun SW, Cao J, Lam CW. *Signalling mechanisms regulating the activation of human eosinophils by mast-cell-derived chymase: implications for mast cell-eosinophil interaction in allergic inflammation*. Immunology. 2009;126(4):579-87.
- Wu M, Hemesath TJ, Takemoto CM, Horstmann MA, Wells AG, Price ER, Fisher DZ, Fisher DE. *c-Kit triggers dual phosphorylations, which couple activation and degradation of the essential melanocyte factor Mi.* Genes Dev. 2000;14:301-1
- Yang SP, Bae DG, Kang HJ, Gwag BJ, Gho YS, Chae CB, *Co-accumulation of vas cular endothelial growth factor with beta-amyloid in the brain of patients with Alz- heimer's disease*, Neurobiol Aging. 2004;(3):283-90
- Zhang YW, Xu H. *Molecular and cellular mechanisms for Alzheimer's disease: understanding APP metabolism*. Curr. Mol. Med. 2007, 7(7), 687-96. Review.

Acknowledgements

First of all I would like to thank my Supervisor, Prof. Teresa Iuvone, for her presence and help, for the constant encouragements, for trusting me, and for all the opportunities she gave me during the years of Ph.D. studies, that allow me growing both by scientific and personal side.
Thanks to "Def", Dr. Daniele De Filippis, for his scientific and friendly support, for the "smiling lab moments" and for being, for the second time, my "moral" Assistant-Supervisor…
Now I realize that it is impossible to have a good research without a great group.

I would like to thank my Ph.D. Coordinator, Prof. Maria Valeria D'Auria, for the helpful and patient presence during the Ph.D. studies period.

Thanks to Epitech Group, and especially to Dr. Francesco della Valle & Collaborators, for the financial support of my Ph.D.

Thanks to Prof. Luca Steardo, Dr. Giuseppe Esposito and Dr. Caterina Scuderi, for the great and instructive permanence in Rome.

Thanks to Prof. Vincenzo Di Marzo and the Endocannabinoid Research Group, for constantly and productively collaborating with my group: thanks also to Dr. Maria Pia Cinelli, for the cooperation in our common studies.

A special thanks is for Prof. Christopher Fowler and the Department of Pharmacology ad Clinical Neuroscience of Umeå University: I felt at home from the first day of my permanence, working with nice people of a high level of scientific knowledge and research.

Thanks also to the moral and scientific support of Prof. Rosa Carnuccio and Prof. Armando Ialenti.

Thanks to my colleagues/friends, dr.ssa Alessandra D'Amico, dr.ssa Daniela De Stefano, dr.ssa Maria Vittoria Di Lauro, dr.ssa Marcella Maddaluno, dr.ssa Elisa Panza and dr.ssa Barbara Romano: for our "full" days of these full years… Simply, I will miss you.
Thanks also to the "masculine side of the research": dr. Antonio Di Pascale, dr. Gianluca Grassia, dr. Francesco Maione and dr. Antonio Parisi: strange, but I will miss you, too…
Thanks to Massimo, Luana, Maria, Irene, Carmela, Andrea, Elisabetta, Stefania, to have been so comprehensive in my "bad and nervous moments".

A special thanks is for you, which are looking for your name without finding: "I'll be your mirror"…

My closing acknowledgements are for my lovely family: my mother, Peppino & Karolina, Nicola, Roberto & Serena, to have sustained me every moment of my daily life.
And finally, thanks to Matteo: the emotions of all the degrees of the world are not comparable to being called "Ghia"…

"Be less curious about people and more curious about ideas"

Marie Curie