UNIVERSITÀ DEGLI STUDI DI PADOVA

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DIPARTIMENTO DI SCIENZE DEL FARMACO

SCUOLA DI DOTTORATO DI RICERCA IN SCIENZE FARMACOLOGICHE

FARMACOLOGIA, TOSSICOLOGIA E TERAPIA

CICLO XXVIII

The role of ω-3 PUFA-enriched diet and DHA metabolites derived from aspirin-acetylated COX-2 on tumor growth and angiogenesis.

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A Martin.

... Al fin y al cabo, somos lo que hacemos para cambiar lo que somos.
La identidad no es una pieza de museo quietecita, sino la siempre asombrosa síntesis de las contradicciones de cada día.

... Alla fine, siamo ciò che facciamo per cambiare ciò che siamo.
L'identità non è un pezzo da museo, ma è sempre la sorprendente sintesi delle contraddizioni di ogni giorno.
(El Libro de los Abrazos-. Eduardo Galeano. 1989)

RIASSUNTO

Background: Il tumore alla mammella (TM) è il tumore più frequente che si verifica nelle donne. Dati epidemiologici hanno collegato l'assunzione degli acidi grassi polinsaturi omega-3 (ω-3 PUFA) con la minore incidenza di TM e diversi studi sperimentali hanno dimostrato gli effetti anti-proliferativi di ω-3 PUFA in diversi modelli di TM. L'olio di chia è ricco di acido α-linolenico (ALA, 18:3 ω-3), mentre l'olio di mais è ricco di acido linoleico (LA, 18:2 ω-6). L'angiogenesi è un processo strettamente regolato che coinvolge la proliferazione, la migrazione e la formazione di capillari da parte delle cellule endoteliali (CE). L'acido docosaesaenoico (DHA, 22:6 ω-3), un metabolita a valle dell'ALA, è stato dimostrato essere in grado di regolare l'angiogenesi correlatea al tumore, mentre l'acido arachidonico (AA, 20:4 ω-6) promuove l'angiogenesi. Inoltre, l'aspirina (ASA) ha effetti antineoplastici che sono mediati, almeno in parte, da metaboliti derivati dall'acetilazione della COX-2. Lo scopo dello studio è stato quello di determinare il ruolo degli ω-3 PUFA sulla crescita del un tumore alla mammella e, in particolare, di valutare l'effetto di metaboliti derivati dalla COX-2 acetilata in presenza di DHA nell'angiogenesi.

Metodi: 40 topi BALB/c sono stati nutriti con 1) una dieta ricca di ω-3 PUFA contenente il 10% di olio di chia (ChO); o 2) una dieta ricca d ω-6 PUFA contenente il 10% d'olio mais (CO). In seguito, i topi sono stati inoculati con una linea di cellule tumorali (LM3) per indurre il tumore primario ed i parametri di crescita tumorale sono stati registrati dopo 45 giorni. Nelle sezioni di tessuto tumorale sono state valutate le cellule mitotiche ed apoptotiche e l'angiogenesi mediante immunoistochimica. Gli esperimenti *in vitro* sono stati condotti con cellule endoteliali umane (HUVECs). Le HUVECs sono state trattate con DHA o AA (1-100 µM) per diversi tempi in presenza o assenza di ASA (50 µM). Inoltre, alcuni esperimenti sono stati effettuati con 17R-HDHA (100 nM - 3 µM), un metabolita derivato dalla COX-2 acetilata in presenza di DHA. La produzione

endogena di 17R-HDHA da parte delle HUVECs è stata analizzata mediante spettrometria di massa. La citotossicitá dei composti è stata valutata con il test MTT. La capacità angiogenica delle HUVECs è stata valutata utilizzando a) il saggio della chiusura della ferita, b) la chemiotassi in camera di di Boyden e c) la formazione dei capillari con il saggio di matrigel.

Risultati: Dopo 45 giorni l'incidenza tumorale era più alta nei topi sottoposti a dieta con olio di mais (CO) rispetto a quelli alimentati con la dieta chia (ChO) (100 vs 85%, $p < 0.05$). Il peso tumorale ed il volume del tumore, così come il numero delle metastasi erano più bassi nei topi che avevano ricevuto la dieta ChO, mentre il tempo di latenza del tumore è risultato più lungo nei topi alimentati con la dieta ChO. Inoltre, nel gruppo di topi sottoposti a dieta ChO sono state osservate un maggior numero di cellule apoptotiche, un minor numero di cellule mitotiche cosí come un minor numero di vasi rispetto al gruppo di controllo (CO). Alla luce di questi risultati *in vivo*, abbiamo studiato se ω-3 PUFA avevano attivitá anti-angiogenica diretta sulle CE umane. Non abbiamo osservato variazioni nella vitalità delle cellule endoteliali trattate con DHA fino a 50 µM o AA, in presenza o assenza di ASA per 24h, cosí come nelle cellule trattate con 17R-HDHA. La migrazione delle CE valutata mediante il saggio di chiusura della ferita, è significativamente diminuita rispetto al controllo nelle cellule pretrattate per 24 ore con 30 µM DHA, ma non in quelle pretrattate con 10 µM DHA. Tuttavia, è interessante sottolineare che 10 µM DHA in presenza di 50 µM ASA ha inibito significativamente la migrazione delle CE (-36%±5.4%, $p<0.001$) già dopo 24 ore. Per contro, AA in presenza o assenza di ASA non ha influenzato la migrazione cellulare a nessuna delle concentrazioni valutate (1-30 µM). In linea con questi risultati, le HUVECs trattate con DHA in presenza d'ASA ha prodotto una maggiore quantità di 17R-HDHA rispetto alle cellule trattate con solo DHA. Inoltre, il pretrattamento per 24h con 17R-HDHA (1-3 µM) ha diminuito la migrazione delle cellule endoteliali valutata con il test di chiusura della ferita (-

12%±4.1% and -28%±1.9%, $p<0.05$ e $p<0.01$, rispettivamente). Inoltre, 17R-HDHA (300 nM, 1-3 µM per 6 ore) ha ridotto la migrazione cellulare valutata in una camera di chemiotassi (-20%±1.4%, -30%±4.0% and -65%±3.5%, $p<0.01$ e $p<0.001$, rispettivamente), mentre DHA da solo non ha mostrato alcun effetto. Inoltre, 17R-HDHA ha diminuito la formazione di strutture capillaro-simili dopo 6 ore. In particolare, valutando diversi parametri, si è visto che i nodi, le maglie e la zona delimitata dalle maglie erano significativamente più bassi nelle cellule trattate con 10 µM DHA in presenza di 50 µM ASA, così come in quelle cellule trattate con 17R-HDHA (300 nM - 3 µM).

Conclusione: i risultati *in vivo* in un modello murino di tumore al seno, mettono in luce un ruolo degli ω-3 PUFA nella crescita tumorale. I risultati *in vitro* dimostrano che l'acetilazione della COX-2 da parte dell'aspirina porta alla produzione di metaboliti che potenziano gli effetti anti-angiogenici di DHA e possono avere un ruolo nel tumore.

ABSTRACT

Background: Breast cancer (BC) is the most frequent cancer occurring in women. Epidemiological data have linked ω-3 polyunsaturated fatty acids (PUFAs) consumption to lower incidence of BC and several experimental studies showed the anti-proliferative effects of ω-3 PUFAs in different BC models. Chia oil is rich in α-linolenic acid (ALA, 18:3 ω-3), while corn oil is rich in linoleic acid (LA, 18:2 ω-6). Angiogenesis is a tightly regulated process involving endothelial cells (ECs) proliferation, migration and tube formation. Docosahexaenoic acid (DHA, 22:6 ω-3) a downstream metabolite from ALA, has been demonstrated to regulate cancer-related angiogenesis, while arachidonic acid (AA, 20:4 ω-6) promotes angiogenesis. In addition, aspirin (ASA) has antineoplastic effects that are mediated at least in part by metabolites derived from acetylated COX-2. The study was aimed to determine a possible role of dietary ω-3 PUFAs on BC growth and, in particular, the effect of DHA metabolites from ASA-acetylated COX-2 in the angiogenic process.

Methods: 40 BALB/c mice were fed 1) a Chia Oil (ChO)-rich (ω-3), or 2) a Corn Oil (CO)-rich diet (ω-6). Afterwards, mice were inoculated with mouse BC cells (LM3) and tumor growth parameters were recorded after 45 days. Mitotic and apoptotic figures, as well angiogenesis in tumor sections were assessed by immunohistochemistry. Human endothelial cells (ECs) were treated with DHA or AA (1-100 μM) for different times in the presence or absence of ASA (50 μM). Selected experiments were performed with 17R-HDHA (100 nM - 3μM), a DHA metabolite derived from acetylated COX-2. Endogenous production of 17R-HDHA by HUVECs was analyzed by Liquid chromatography-tandem mass spectrometry. EC viability was analyzed with the MTT assay. The angiogenetic capability was evaluated using a) the wound healing assay, b) a chemotaxis chamber, and c) matrigel.

Results: After 45 days tumour incidence was higher in CO-fed compared with ChO-fed mice (100 vs 85%, $p<0.05$). Tumor weight and volume as well as metastasis number were lower, whereas tumor latency time was longer in ChO-fed mice. Compared to the control group, a higher number of apoptotic bodies, a lower number of mitosis and vessels were consistently observed in the ChO-fed mice group. Given these in vivo findings, we next studied whether ω-3 PUFAs had direct anti-angiogenic actions on human ECs. Cells were viable when treated with up to 50 µM DHA or AA in the presence or absence of ASA for 24h. Challenging the cells with 17R-HDHA for 24h did not affect cell viability. EC migration, as evaluated by wound healing assay, was significantly decreased in cells pretreated for 24h with 30 µM, but not 10 µM DHA compared to control. Interestingly, 10 µM DHA in the presence of 50 µM ASA significantly inhibited ECs migration (-36%±5.4%, $p<0.001$) already after 24h. By contrast, AA did not affect EC migration at any concentration tested (1-30 µM) in the presence or absence of ASA. Consistently, ECs treated with DHA+ASA produced higher amounts of 17R-HDHA with respect to cells treated with DHA alone. Pretreatment with 17R-HDHA (1-3 µM, 24h) decreased EC migration (-12%±4.1% and -28%±1.9%, $p<0.05$ and $p<0.01$, respectively) evaluated by the wound healing assay. Additionally, 17R-HDHA (300 nM, 1 or 3 µM, 6h) reduced EC migration as evaluated in a micro chemotaxis chamber (-20%±1.4%, -30%±4.0% and -65%±3.5%, $p<0.01$ and $p<0.001$, respectively), while DHA did not show any effect. Furthermore, 17R-HDHA inhibited EC tube formation after a 6h treatment. In particular, nodes, meshes and mesh area were significantly lower in 10 µM DHA-treated cells in the presence of 50 µM ASA, as well as in 17R-HDHA (300 nM-3 µM)-treated cells.

Conclusion: *In vivo* and *ex vivo* data point to a central role of ω-3 PUFAs in BC growth. *In vitro* data demonstrate that aspirin-acetylated COX-2 enhances the anti-angiogenic effects of DHA and may have a role in the setting of tumors.

CONTENTS

INTRODUCTION

1. INTRODUCTION

1.1. Cancer

Cancer arises through the accumulation of molecular changes that together allow cells to grow in an uncontrolled manner. Many factors contribute to this process, including hereditary genetic mutations and environmental hazards (Weinberg, 2013). The most common human cancers arise from epithelia and are called carcinomas. These tumors are responsible for more than 80% of the cancer-related deaths in the Western world. Included among the carcinomas are tumors arising from the epithelial cell layers of the gastrointestinal tract -which includes mouth, esophagus, stomach, and small and large intestines- as well as the skin, mammary gland, pancreas, lung, liver, ovary, uterus, prostate, gallbladder, and urinary bladder (Weinberg, 2013).

Cancer is defined as a polygenic and multifactorial disease where an interplay of factors may prevent or promote the disease. Mammalian cells have multiple safeguards to protect them against the potentially lethal effects of cancer gene mutations, and only when several genes are defective does an invasive cancer develop. Thus it is best to think of mutated cancer genes as contributing to, rather than causing, cancer. Cancer development involves multiple mutations within several key genes, including mutations in proto-oncogenes, tumor suppressor genes, and DNA repair genes. The process of accumulating mutations in several genes takes many years, and this is why cancer is more frequently seen in older individuals (Vogelstein and Kinzler 2004). Besides genetic influence, most adult cancers are caused mainly by environmental factors. This means that most cancers are at least in theory preventable. Among important causes of cancer are food and nutrition, physical activity and body composition (Rosato et al, 2013).

1.1.1. Cancer epidemiology: prevalence, incidence and mortality trends.

Cancer is the leading cause of mortality worldwide. According to WHO estimates for 2011 cancer causes more deaths than all coronary heart disease or all strokes. Indeed, 14.1 million new cancer cases (incidence), 8.2 million cancer deaths occurred in 2012 worldwide and 32.6 million men and women still alive in 2012, up to five years after their diagnosis (Ferlay et al, 2014). In Europe, more than 3.4 million new cases of cancer (excluding non-melanoma skin cancer) were diagnosed in 2012. The commonest cancers were those of breast, large bowel, prostate and lung, all of which represented more than 1.7 million cases annually.

The cancer with the largest number of incident and prevalent cases was breast cancer, while by far the largest number of deaths was due to lung cancer (Steliarova-Foucher, 2015).

1.1.2. Breast cancer epidemiology: prevalence, incidence and mortality trends.

Breast cancer (BC), the most frequently occurring cancer in women, is a major public health problem, with 1384155 estimated new cases worldwide with nearly 459000 related deaths. It is alarming to note that GLOBOCAN statistics for 2012 showed that approximately 1.7 million women were diagnosed with breast cancer in that year, with 522000 related deaths suggesting an increase in breast cancer incidence and related mortality by nearly 18% from 2008 (Tao et al, 2014). The incidence and mortality rates of breast cancer vary considerably across countries and regions, with a four to five-fold variation in incidence between different geographical areas. Rates are highest in Europe and North America and lowest in Asia (Iwasaki and Tsugane 2011).

1.1.3. Breast cancer risk factors

Besides genetic predisposition, scientific evidence suggests that age, hormone status, certain reproductive choices as well as life style factors such as diet, obesity and lack of physical activity, can modify the risk of breast cancer (Tumas et al, 2014).

Genetics: Mutations that predispose to breast cancer have been identified. These include mutations in the genes BRCA1 (breast cancer 1), BRCA2 (breast cancer 2), p53 (p53 tumor suppressor), PTEN (phosphatase and tensin homolog), and ATM (ataxia-telangiectasia mutated). Mutations in BRCA1 and BRCA2 can cause a high risk of breast cancer. Most studies on familial risk of breast cancer have found about two-fold relative risks for first-degree relatives (mothers, sisters, daughters) of affected patients. With affected second-degree relatives (grandmothers, aunts, grand-daughters), there is a lesser increase in risk (Key et al, 2001).

Age: The incidence of breast cancer increases rapidly with age during the reproductive years and then increases at a slower rate after about age 50 years, the average age at menopause (Key et al, 2001).

Hormone status: Hormones play an important role in breast cancer progression because they modulate the structure and growth of epithelial tumor cells. The epidemiologic evidence has consistently supported a positive

relationship between circulating *estrogen levels and postmenopausal breast cancer risk*, with a magnitude of association ranging from 1.4 (95% CI: 0.9–2.4) to 6.4 (95% CI: 2.8–14.9). All studies conducted to date have reported positive relationships, with seven of the nine individual studies and the initial pooled analysis reporting borderline or statistically significant positive associations that are stronger among the subset of women with estrogen receptor positive (ER+) tumors, which further establishes the role of endogenous estrogens in the etiology of postmenopausal breast cancer. Compared to postmenopausal women, data evaluating the relationship between endogenous *estrogens and premenopausal breast cancer risk* are more limited and less consistent. Prior studies have been limited by small case numbers and complicated by the large variations in estrogens and progesterone that occur during the menstrual cycle. Although the association for the doubling of estradiol levels ranged from 0.6 (95% CI: 0.2–1.4) to 1.8 (95% CI: 97 0.9–3.7), most studies have observed a positive relationship, which is qualitatively similar but weaker in magnitude when compared to the relationship in postmenopausal women. Furthermore, in a recent pooled analysis of seven prospective studies that evaluated sex hormones and risk of premenopausal breast cancer specifically, circulating estradiol levels were positively associated with breast cancer (OR for doubling in concentration of estradiol, 1.2, 95% CI: 1.1–1.4) (Brown and Hankinson, 2015; Key et al, 2002). In premenopausal women (before age 50) with breast cancer diagnosis, besides estradiol, estrone, androstenedione, DHEAS (didehydroepiandrosterone) and testosterone circulating concentrations were positively associated with breast cancer risk, whereas luteal phase progesterone and SHBG (sex hormone binding globulin) were not associated with risk (Key et al, 2013). Similarly to what observed in premenopausal women, a number of different sex hormones such as testosterone, DHEAS, and SHBG measured up to 16-20 years before breast cancer diagnosis were significantly associated with risk of postmenopausal breast cancer among women not using postmenopausal hormone therapy (Zhang et al, 2013).

Further, reproductive and hormonal risk factors such as *early age at menarche*, *late age at menopause*, *postmenopausal hormone therapy*, *oral contraceptives use*, *high postmenopausal body-mass index*, *late childbearing* and *no-breastfeeding practice* all of which contribute to a greater lifetime exposure to estrogen, have been linked to an increased risk of most of gynecological cancers such as breast cancer.

Menarche and the menstrual cycle: The older a woman is when she begins menstruating, the lower her risk of breast cancer. For each 1-year delay in menarche, the risk decreases by around 5%. There is also evidence that, although age at menarche is related to breast cancer risk at all ages, the effect may be stronger in younger (premenopausal) women (Key et al, 2001).

Menopause: Women who experience menopause at a late age are at a higher risk of breast cancer than those who cease menstruating earlier, with risk increasing by about 3% for each year older at menopause. The magnitude of this effect is similar whether menopause occurs naturally or as a result of bilateral oophorectomy. The protective effect of menopause can be seen in the slowing in the rate of increase in breast-cancer incidence with age that occurs at around age 50. Thus, premenopausal women are at higher risk of breast cancer than postmenopausal women of the same age, and peri-menopausal women are at intermediate risk (Key et al, 2001).

Postmenopausal body mass-index: In postmenopausal women, obesity increases the risk of breast cancer; risk is about 50% higher in obese women (body mass index >30 kg/m^2) than in lean women (body mass index 20 kg/m^2). This association is not observed in premenopausal women, among whom some, but not all, studies have observed that risk is slightly lower in obese women than in women of normal weight (Key et al, 2001).

Exogenous hormones: The use of *oral contraceptives* increased the risk of breast cancer by around 25% in current users of combined oral contraceptives, but the excess risk falls after cessation of use, such that 10 or more years after use stops, no significant increase in risk is evident. Risk does not vary significantly with duration of use, nor does the effect of combined oral contraceptives vary significantly according to other risk factors. The effect on risk of breast cancer does not vary with the type of estrogen or progestagen used. Although data on progestagen only oral contraceptives are limited, their effects seem to be broadly similar to those of combined preparations. The *postmenopausal hormone therapy* was also studied. Hormonal therapy use for the menopause occurs at a time when a woman is at high and increasing background risk of breast cancer. Current users of hormonal therapy for the menopause are at higher risk of breast cancer than women who have never used these preparations (Key et al, 2001).

Reproductive choices: Not bearing children and late (over 30) first pregnancy are recognized to increase the risk of breast cancer. Childbearing

seems to have a dual effect on breast cancer risk; it is increased in the period immediately after a birth, but this excess risk gradually diminishes and, in the longer term, the effect of a birth is to protect against the disease. Compared with nulliparous women, who have had at least one full-term pregnancy have around a 25% reduction in breast cancer risk. Furthermore, increasing protection is seen with increasing numbers of full-term pregnancies, such that women with five or more children have about half the risk of nulliparous women (Key et al, 2001).

Breastfeeding practice: Lactation is associated with increased differentiation of breast cells and with lower exposure to endogenous sex hormones during amenorrhea accompanying lactation. In addition, the strong exfoliation of breast tissue during lactation, and the massive epithelial apoptosis at the end of lactation, could decrease risk by elimination of cells with potential DNA damage. Women with breast cancer had, on average, fewer births than did controls (2.2 vs 2.6). Furthermore, fewer parous women with cancer than parous controls had ever breastfed (71% vs 79%), and their average lifetime duration of breastfeeding was shorter (9.8 vs 15.6 months). The relative risk of breast cancer decreased by 4.3% (95%, CI 2.9-5.8) after 12 months of breastfeeding. The size of the decline in the relative risk of breast cancer associated with breastfeeding did not differ significantly for women in developed and developing countries, and did not vary significantly by age, menopausal status, ethnic origin, the number of births a woman had, her age when her first child was born, or any of nine other personal characteristics examined (Collaborative Group on Hormonal Factors in Breast Cancer, 2002).

Lifestyle*:* Moderate physical activity is associated with a lower risk of breast cancer. The size of the effect of high physical activity has varied widely between studies, but a typical result is a reduction in risk of around 30% in association with a few hours per week of vigorous activity versus none (Key TJ et al, 2001). Several studies showed that between 80 and 90% of cancers are related to environmental factors and it is estimated that 35% are related to dietary factors (WCRF, 2007). The observation that breast cancer rates are much higher in countries with high-fat diets than in countries where fat intake is much lower, led to the hypothesis that high-fat diets increase breast cancer risk. More recent studies have looked at other possible dietary determinants of risk, such as consumption of meat, fiber, fruit and vegetables. There may be a moderate protective effect for a high consumption of vegetables, but results for meat, fiber, and fruit have been inconsistent, and breast cancer risk has not been shown to

be lower in vegetarians than in non-vegetarians in more developed countries (Key et al, 2001). In a large multicenter Italian case-control study, breast cancer risk was directly related to cereals, and hence available carbohydrates, and inversely related to vegetables and polyunsaturated fatty acids (Rosato et al, 2013).

1.1.4. Carcinogenesis: The six hallmark capabilities of cancer cells.

Normal cells evolve progressively to a neoplastic state (phenotype). Carcinogenesis is understood as a biological process occurring at multi-stages continuously. The hallmarks of cancer comprise specific capabilities acquired during the multistep development of human tumors. Indeed it is well known that, tumor cells develop *self-sufficiency in growth signaling*, *insensitivity to anti-growth signals*, *limitless replicative potential* and the capability to *evading apoptosis* that allow tumor growth. Moreover, the tumor-associated neovasculature, generated by the process of *angiogenesis,* as well as the activation of the processes of *invasion and metastasis,* allow carcinomas progress to higher pathological grades of malignancy (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011).

Self-sufficiency in growth signaling: It was the first tumor-specific capability to be clearly defined by cancer researchers. Normal cells require mitogenic growth signals before they can move from a quiescent state into an active proliferative state. These signals are transmitted into the cell by transmembrane receptors that bind distinctive classes of signaling molecules: diffusible growth factors, extracellular matrix components, and cell-to-cell adhesion/interaction molecules. Many of the oncogenes in cancer act by mimicking normal growth signaling in one way or another. Three common molecular strategies for achieving autonomy are evident, involving alteration of extracellular growth signals, transcellular transducers of those signals, or intracellular circuits that translate those signals into action. While most soluble mitogenic growth factors (GFs) are made by one cell type in order to stimulate proliferation of another -the process of heterotypic signaling- many cancer cells acquire the ability to synthesize GFs to which they are responsive, creating a positive feedback signaling loop often termed autocrine stimulation (Fedi et al, 1997). Clearly, the manufacture of a GF by a cancer cell obviates dependence on GFs from other cells within the tissue. The cell surface receptors that transduce growth stimulatory signals into the cell interior are themselves targets of

deregulation during tumor pathogenesis. Receptor overexpression may enable the cancer cells to become hyperesponsive to ambient levels of GFs that normally would not trigger proliferation (Hanahan and Weinberg, 2011).

Insensitivity to antigrowth signals: Within a normal tissue, multiple anti-proliferative signals operate to maintain cellular quiescence and tissue homeostasis; these signals include both, soluble growth inhibitors and immobilized inhibitors embedded in the extracellular matrix and on the surfaces of nearby cells. These growth inhibitory signals, like their positively acting counterparts, are received by transmembrane cell surface receptors coupled to intracellular signaling circuits. Antigrowth signals can block proliferation by two distinct mechanisms. Cells may be forced out of the active proliferative cycle into the quiescent (G0) state from which they may reemerge on some future occasion when extracellular signals permit. Alternatively, cells may be induced to permanently relinquish their proliferative potential by being induced to enter into post-mitotic states. Much of the circuitry that enables normal cells to respond to antigrowth signals is associated with the cell cycle clock. Incipient cancer cells must evade these anti-proliferative signals, which depend on the actions of tumor suppressor genes, if they are to prosper (Weinberg, 1995).

In breast cancer, the cell traits characterizing proliferation-related markers and insensitivity to growth factors inhibitor signals involve deregulated expression of cyclins, cyclin-dependent kinases (CDK) and CDK inhibitors (CDKIs) and oncogene products, such as ras, c-myc, steroid hormone receptors and growth factor receptors, e.g. members of the HER/erbB family (Daidone et al, 2014).

Limitless replicative potential: Many and perhaps all types of mammalian cells carry an intrinsic, cell-autonomous program that limits their multiplication. Cells in culture have a finite replicative potential. Once such cell populations have progresses through a certain number of doublings, they stop growing a process termed senescence. In cancer cells, this program must be disrupted in order for a clone of cells to expand to a size that constitutes a macroscopic. Most types of tumor cells that are propagated in culture appear to be immortalized, suggesting limitless replicative potential is a phenotype that was acquired *in vivo* during tumor progression and was essential for the development of their malignant growth state (Hanahan and Weinberg, 2011).

Evading apoptosis: In principle, the resulting deregulated proliferation program should suffice to enable the generation of the vast cell populations that constitute macroscopic tumors. However, the ability of tumor cell populations to

expand in number is determined not only by the rate of cell proliferation but also by the rate of cell attrition. Programmed cell death -apoptosis- represents a major source of this attrition. The evidence is mounting, principally from studies in mouse models and cultured cells, as well as from descriptive analyses of biopsied stages in human carcinogenesis, that acquired resistance toward apoptosis is a hallmark of most and perhaps all types of cancers including breast cancer. The apoptotic machinery can be divided into two classes of components, sensors and effectors. The sensors are responsible for monitoring the extracellular and intracellular environment for conditions of normality or abnormality that influence whether a cell should live or die. These signals regulate the second class of components, which function as effectors of apoptotic death (Wyllie, 1980). Resistance to apoptosis can be acquired by cancer cells through a variety of strategies. The most common is the loss of p53 tumor suppressor function, which eliminates this critical damage sensor from the apoptosis-inducing circuitry. Alternatively, tumors may achieve similar ends by increasing expression of anti-apoptotic regulators (Bcl-2, Bcl-xL) or of survival signals (Igf1/2), by downregulating pro-apoptotic factors (Bax, Bim, Puma), or by short circuiting the extrinsic ligand-induced death pathway. The multiplicity of apoptosis avoiding mechanisms presumably reflects the diversity of apoptosis inducing signals that cancer cell populations encounter during their evolution to the malignant state (Hanahan and Weinberg, 2011). Apoptosis related proteins, such as bcl-2 (anti-apoptotic) and bax (pro-apoptotic), have been extensively studied in breast cancer as prognostic and predictive markers (Daidone et al, 2014).

Invasion and metastasis: Sooner or later during the development of most types of human cancer, primary tumor masses spawn pioneer cells that move out, invade adjacent tissues, and then travel to distant sites where they may succeed in founding new colonies. These distant settlements of tumor cells metastases are the cause of 90% of human cancer death. The capability for invasion and metastasis enables cancer cells to escape the primary tumor mass and colonize new terrain in the body where at least initially, nutrients and space are not limiting. The multistep process of invasion and metastasis has been schematized as a sequence of discrete steps, often termed the invasion metastasis cascade. This depiction envisions a succession of cell-biologic changes, beginning with local invasion, then intravasation by cancer cells into nearby blood and lymphatic vessels, transit of cancer cells through the lymphatic

and hematogenous systems, followed by escape of cancer cells from the lumina of such vessels into the parenchyma of distant tissues (extravasation), the formation of small nodules of cancer cells (micrometastases), and finally the growth of micrometastatic lesions into macroscopic tumors, this last step being termed colonization (Talmadge et al, 2010). Invasion and metastasis are exceedingly complex processes, and their genetic and biochemical determinants remain incompletely understood. At the mechanistic level, they are a closely allied process, which justifies their association with one another as one general capability of cancer cells. Both utilize similar operational strategies, involving changes in the physical coupling of cells to their microenvironment and activation of extracellular proteases. The activation of extracellular proteases and the altered binding specificities of cadherins, CAMs, and integrins are clearly central to the acquisition of invasiveness and metastatic ability (Hanahan and Weinberg, 2011).

Angiogenesis: The oxygen and nutrients supplied by the vasculature are crucial for cell function and survival, obligating virtually all cells in a tissue to reside within 100µm of a capillary blood vessel. During organogenesis, this closeness is ensured by coordinated growth of vessels and parenchyma. Once a tissue is formed, the growth of new blood vessels -the process of angiogenesis- is transitory and carefully regulated. Because of this dependence on nearby capillaries, it would seem plausible that proliferating cells within a tissue would have an intrinsic ability to encourage blood vessel growth. During tumor progression, an ‘‘angiogenic switch’’ is almost always activated and remains on causing normally quiescent vasculature to continually sprout new vessels that help sustain the expansion of neoplastic growths (Hanahan and Folkman, 1996). A compelling body of evidence indicates that the angiogenic switch is governed by countervailing factors that either induce or oppose angiogenesis (Baeriswyl and Christofari, 2009). This process will be described in details in the next section.

As in the case of other solid tumors, invasion and metastasis of breast cancer depends on activation of neoangiogenesis and on modifications of the extracellular matrix. In recent years many papers have been published on these topics, reporting suggestive data for a significant association between overexpression of markers related to or indicative of these functions and disease progression. In particular, hypervascularisation and surrogate markers related to the formation of new blood vessels, such as CD31 and vascular endothelial

growth factor (VEGF), have been proved to be associated with new disease manifestation (Daidone et al, 2014).

Overall, the hallmarks of cancer comprise six biological capabilities acquired during the multistep development of human tumors, which have provided a useful conceptual framework for understanding the complex biology of cancer and will increasingly affect the development of new means to treat human cancer.

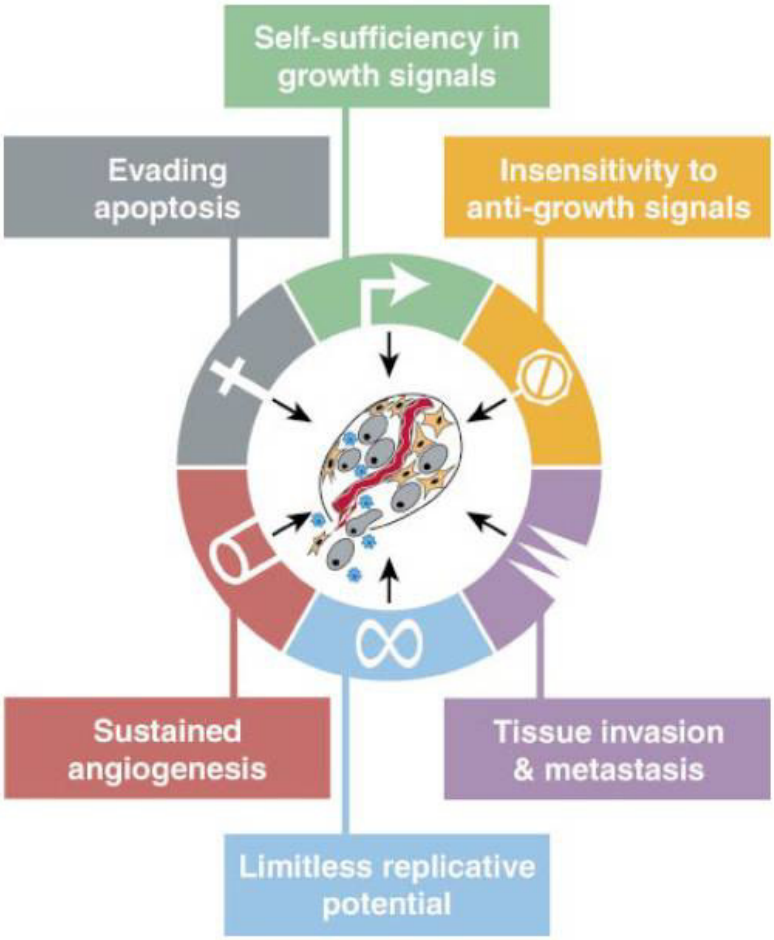

Figure 1.1: Hallmarks of cancers represented by the acquired capabilities of cancer cells (Hanahan and Weinberg et al, 2000).

1.2. Angiogenesis

Blood vessels supply oxygen and nutrients and provide gateways for immune surveillance. Endothelial cells (ECs) line the inner surface of vessels to support tissue growth and repair. As this network nourishes all tissues, it is not surprising that structural or functional vessel abnormalities contribute to many diseases.

Angiogenesis is both a physiological and pathological process, which leads to the formation of new blood vessels. Physiologically the process of angiogenesis begins at implantation, and during placental and embryonic development. In later life the process is prominent in the healing of wounds, osteogenesis, as well as in ovulatory-related and non-ovulatory-related reproductive processes. Furthermore, pathological angiogenesis is related to inflammatory disorders, pulmonary hypertension, blinding eye disease and the development of both benign and malignant tumors (Potente et al, 2011). In 1971 Folkman hypothesized that tumor growth is dependent on angiogenesis and subsequently experimental work demonstrated that for a tumor to grow beyond a size of 1-2 mm^3 a substantial new blood supply must develop to support the increasing metabolic requirements. In addition, in the setting of the tumor, vessels are also used as routes for tumor cells to metastasize (Folkman, 1971).

1.2.1. Factors affecting angiogenesis

Angiogenesis is subject to a complex control system with the involvement of both pro-angiogenic and anti-angiogenic factors. Under physiological conditions, angiogenesis is tightly controlled by an angiogenic balance, i.e., a physiological balance between stimulatory and inhibitory signals for blood vessel growth. A switch to the angiogenic phenotype depends on a local change in the balance between angiogenic stimulators and inhibitors, as a consequence of variation in tissue oxygen tension or endothelial damage (Arnal et al, 2010).

The stimuli that trigger angiogenesis such as hypoxia, inflammation, mechanical forces (shear stress/strech) and exercise initiate the production of growth factors (Milkiewicz et al, 2005). Besides growth factors, hormones such as estrogens, prolactin, insulin and melatonin also influence angiogenesis. In addition, neurotransmitters such as nicotine (nicotinic receptor) and catecholamine have been shown to modulate various aspects of angiogenesis acting on specific receptors (Mousa et al, 2013). Dietary factors also may act as an important component in regulating the angiogenic switch as they have an

important impact on cellular physiology and homeostasis, and hence could influence the equilibrium between anti- and pro-angiogenic factors (Bhat and Singh, 2008). Growth factors have both stimulatory and inhibitory roles within sprouting angiogenesis, the most investigated compound being vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), which are known to be potent stimulators of angiogenesis. Many others molecules such as platelet derived growth factor (PDGF), epidermal growth factor (EGF), angiopoietins (Ang), and TIE-receptors, Eph receptors and ephrins, hepatocyte growth factor (HGF), thymidine phosphorylase (TP), neuropeptide Y (NPY), platelet factor 4 (PF4) are considered the main mediators involved in angiogenesis (Bouïs et al, 2004). In contrast, there are a number of angiogenesis inhibitor molecules such as angiostatin, endostatin, tissue inhibitors of metalloproteinases (TIMPs), which serve to downregulate the angiogenesis response giving a negative feedback in vessel sprouting (Milkiewicz et al, 2005; Boosani and Sudhakar, 2011).

Endocrine hormones -steroids and peptides small molecules- are able to regulate angiogenesis. Most of these hormones appear to exert their effect on blood vessels only in specific tissues, but the angiogenic properties of these substances have not been systematically studied in tissues other than those that are classical targets (Mousa et al, 2013).

Estrogen and progesterone contribute to uterine angiogenesis in the menstrual cycle (Matsubara and Matsubara, 2012). Besides its role in menstrual cycle, estrogens have also been described to promote re-endothelization after arterial injury. The mechanisms involved in the pro-angiogenic effects of estrogens are probably multifactorial. Importantly, estrogen induces three of the most important pro-angiogenic factors: FGF-2, VEGF, and nitric oxide (NO). All these molecules could contribute to the neuro and cardioprotective effects of estrogens observed in models of ischemia/reperfusion injury (Arnal et al, 2010). In contrast, the endogenous metabolite of estrogen, 2-methoxyestradiol, has been described as a potent inhibitor of endothelial cell proliferation, migration and angiogenesis *in vitro* (Albini et al, 2010). Estrogen plays an important role in the development and growth of the mammary gland during puberty, pregnancy and lactation. In the setting of estrogen-sensitive tumors, estrogens modulate cell proliferation, adhesion, migration, and invasion. In addition, estrogens promote the formation of new blood vessels within the tumor mass, hence suggesting that

these steroids elicit a stimulatory role not only in cancer cells but also in components of the surrounding stroma (de Francesco et al, 2014).

Besides estrogen, prolactin, the lactation stimulating hormone that almost exclusively acts on the mammary gland is angiogenic in certain tissues (Reuwer et al, 2012). Prolactin can be proteolytically cleaved giving rise to angiostatic peptides with a strong anti-angiogenic activity, mediated by the inhibition of MAP-kinases (Albini et al, 2010). It has been suggested that chronic hyperinsulinemia associated with insulin resistance plays a role in breast cancer etiology and progression (Rostoker et al, 2015). In line with this, insulin promotes angiogenesis, at least in part via local VEGF production and stimulation of endothelial cell proliferation (Cubbon et al, 2012). In addition to female hormones and insulin, melatonin the main secretory product of the pineal gland, has been described to have a role in breast cancer angiogenesis. In particular, melatonin inhibits VEGF production in human breast cancer cells (Alvarez-Garcia et al, 2013).

Other factors besides hormones might affect tumor angiogenesis. In the past years, food and dietary components have been identified as modulators of angiogenesis. This is the case of genistein, contained in soy, epigallocatechin 3-gallate, abundant in green tea, resveratrol, contained in wine, kahweol, present in unfiltered coffee, oleocanthal and hydroxytyrosol, two constituents of virgin extra olive oil, carnosol and carnosic acid, two major components of rosemary extracts (Medina and Quesada, 2014). A particular attention has been devoted to the role of fatty acids (FAs), particularly to polyunsaturated fatty acids (PUFAs) in the setting of tumor angiogenesis.

It is well established that the metabolites of ω-6 and ω-3 PUFAs, which must be obtained through the diet have differential effects on cellular processes. ω-6 PUFAs-derived metabolites promote angiogenesis by increasing growth factor expression whereas ω-3 PUFAs have anti-angiogenic and anti-tumor properties (Kang and Liu, 2013). ω-3 PUFAs, principally from fish oil, modulate angiogenesis by suppressing downstream metabolites of arachidonic acid (AA), an ω-6 PUFAs (Kang and Liu, 2013). In particular, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) two ω-3 PUFAs, have potent anti-angiogenic effects inhibiting production of many important angiogenic mediators such as VEGF, PDGF, cyclo-oxygenase 2 (COX-2)-derived prostaglandin-E_2 (PGE_2) (Spencer et al, 2009). The role of fatty acids in angiogenesis will be further described (see on page 39).

1.2.2. Phases of angiogenesis

The formation of new vessels, i.e. angiogenesis, starts from abluminal sprouting from preexisting vessel. The angiogenesis sprouting steps can roughly be divided into a permeability phase, a proliferation, proteolysis and migration phase and a stabilization maturation phase (lumen formation phase) (Figure 1.2). This process is initiated by activation of endothelial cells by growth factors, mechanical and/or inflammatory stimuli.

First of all*, permeability* (1) across the endothelial cell layer increases, and this is followed by the *proliferation* of endothelial cells (2). Subsequently, *proteolysis* (3) of basement membrane components (controlled mainly by MMPs) enables sprouting of the endothelial cell into the interstitial space. After that, a continued coordination of cell adhesion and cytoskeletal remodeling components provide directional *migration* of the sprouting (4) endothelial cells. Eventually, the new sprout forms a *lumen* (5) by the process of intracellular vacuolar fusion or by stabilization of several cells around a central lumen. A new lateral branch will be formed when the sprout anastomosis with a pre-existing capillary. Alternatively, circulating endothelial progenitor cells (EPCs) (A) may contribute to the sprout process, adhering (B) to the activated endothelial cell, extravasating (C) through the endothelial cell layer, and clustering (D) within the intersitium. Some of these EPCs will integrate (E) into the sprout and comprise a portion of the newly formed capillary while others may remain as perivascular cells (Milkiewicz et al, 2005).

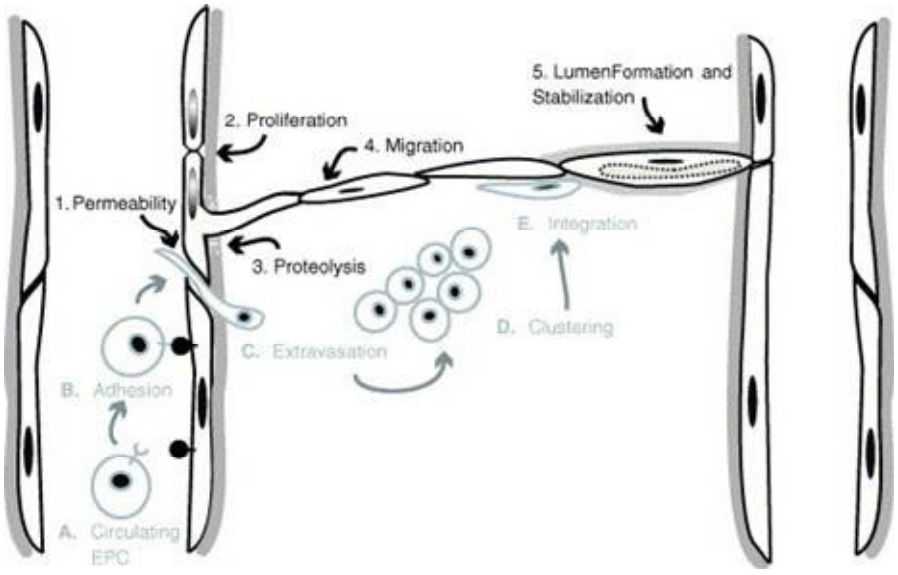

Figure 1.2: Phases of sprouting angiogenesis (Milkiewicz, 2005)**.**

The regulation of the process of abluminal sprouting involves specific molecules, which finely tune the angiogenesis sprouting steps.

Permeability: Angiogenic stimuli cause increased endothelial cell permeability through dissolution of adherens junctions responsible for cell–cell contacts. Release of endothelial cell junctions also promotes a pro-migratory phenotype. Endothelial permeability is regulated by adherens junctions, which are formed primarily through extracellular homophilic interactions between vascular endothelial (VE)-cadherins, with intracellular stabilization to the actin cytoskeleton via interactions with β-catenin and α-catenin (Corada et al, 1999; Dejana, 2004). EGF and HGF receptor tyrosine kinases both induce tyrosine phosphorylation of β-catenin and this correlates with decreased cell–cell adhesion and increased cell migration (Hoschuetzky et al, 1994).

Proliferation: Endothelial cell proliferation occurs early in angiogenesis, and continues as the new capillary sprout elongates. Activation of P13K/Akt promotes endothelial cell survival and proliferation through modulation of numerous cell cycle regulators, including cyclinD1, p27 and Bcl-X2 (Pages et al, 2000). Interestingly, β-catenin may dissociate from the adherens junction complexes, and translocate to the nucleus where it binds to T-Cell Factor, through which it acts as a transcriptional co-activator of numerous gene targets including proliferation mediators such as cyclinD1 and c-myc (Ben Ze'ev et al, 2000). Nuclear accumulation of β-catenin occurs with VEGF stimulation, and thus it may serve to couple initial events in growth factor triggered permeability with subsequent activation of cellular proliferation and proteolysis.

Proteolysis: The critical role that basement membrane degradation plays in the process of abluminal sprouting has been recognized for many years. Proteolysis is necessary to promote endothelial cell invasion into the surrounding interstitial matrix. Matrix metalloproteinases (MMPs) are zinc dependent proteases capable of degrading numerous matrix components. Several different MMPs are produced by endothelial cells and are implicated strongly in the process of capillary sprouting (Haas et al, 2005). Animal studies demonstrate that MMP inhibition can suppress both physiological (exercise-induced) angiogenesis and capillary growth within tumors, suggesting that regulation of MMP activity is a therapeutic target (Guedez et al, 2003). In addition to MMP, Plasmin is an active tryptic protease derived from proteolysis of plasminogen by tissue or urokinase plasminogen activators (tPA or uPA). This activation is facilitated by interaction of plasminogen and PA with the cell surface-localized receptor uPAR. uPA and

uPAR are upregulated by bFGF and VEGF stimulation and by hypoxic conditions (Pepper, 2001).

Migration: Cellular attachments to matrix molecules and the intracellular modulation of cytoskeletal components both determine the migratory properties of endothelial cells. Integrins play a prominent role in cell-matrix adhesion. Expression of integrins αvβ3 and αvβ5 is increased in proliferating endothelial cells, and by bFGF and VEGF stimulation (Nisato et al, 2003). Antagonists that disrupt endogenous matrix-integrin interactions can inhibit angiogenesis in various model systems (Brooks et al, 1994; Hammes et al, 1996; Nisato et al, 2003). Endothelial cell migration during angiogenesis is the integrated resultant of these 3 mechanisms. Typically, *chemotaxis* of endothelial cells is driven by growth factors such as VEGF and basic fibroblast growth factor (bFGF), whereas *haptotaxis* is associated with increased endothelial cell migration activated in response to integrins binding to ECM component. Because of their location at the inner face of blood vessels, endothelial cells are constantly in contact with shear stress, which contributes to activate migratory pathways. In fact, there is now accumulating evidence that fluid shear stress initiates *mechanotaxis* and modulates the various steps of migration (Lamalice et al, 2007).

Lumen formation and stabilization: The lumen of a blood vessel is essential for providing blood to any given tissues. In the vascular system, lumen formation involves a complex molecular mechanism composed of endothelial cell repulsion at the cell-cell contacts within the endothelial cell cords, junctional rearrangement, and endothelial cell shape change (Ribatti et al, 2012). It occurs as the sprout forms a multicellular structure because capillary sprouts generally are composed of one or two cells. These sprouts may form a lumen by intracellular canalization, which occurs through fusion of cytoplasmic vesicles, or by the alternative process of intercellular canalization, in which a lumen is created by the membrane apposition of two different cells (Egginton and Gerritsen, 2003). Stabilization of the nascent capillaries is a significant step in completion of the angiogenic process. The new capillary channel forms an anastomosis with a pre-existing capillary, creating a new patent capillary. The final stage requires stabilization of the capillary through the construction of basement membrane, adherens junctions and cessation of endothelial cell activation. This in part is accomplished through the production of factors that promote a differentiated and stable capillary morphology. Angiopoietin-1 (Ang-1) is produced constitutively in most adult tissues, and it binds and activates the endothelial cell specific tyrosine

kinase receptor (Tie-2). Tie-2 activation promotes endothelial quiescence through the MAPK and PI3K/Akt pathways that induce cell survival, but not endothelial cell proliferation (Peters et al, 2004). Placental derived growth factor (PDGF) may assist in the stabilization process. PDGF has a high affinity for VEGFR-1, and through activation of VEGFR-1 on pericytes or smooth muscle cells, stimulates increased coverage and stabilization of capillaries, and the formation of larger diameter collaterals (Autiero et al, 2003).

The knowledge of the specific steps and molecules involved in the angiogenic process is of paramount importance for the future development of novel therapeutic strategies targeting angiogenesis.

1.2.3. Antiangiogenic drugs in tumors

Antiangiogenic therapy has become a mainstay of cancer therapeutics, but clinical responses are generally active for short-term owing to the development of secondary resistance. A summary of the main drugs and pharmacological targets of angiogenesis is presented in Table 1.1 (Quintieri et al, 2014).

Currently, most of the FDA approved drugs, as well as those in phase III clinical trials target a single pro-angiogenic protein (Presta et al, 2009). In particular, the identification of VEGF as a key driver of the angiogenic process has converted the VEGF/VEGF receptor (VEGFR) signaling pathway into an attractive therapeutic target. VEGF blockers belong to two broad categories including: (I) VEGF ligand-blocking drugs and (II) drugs blocking VEGFR signaling. The use of neutralizing antibodies and other inhibitors has demonstrated that blockade of VEGF alone can substantially suppress tumor growth and angiogenesis in several experimental models. This has led to the development of the neutralizing anti-VEGF antibody bevacizumab, the first FDA-approved antiangiogenic molecule (Quintieri et al, 2014). Bevacizumab is currently used in a variety of therapeutic protocols (some including 5-fluorouracil) for solid tumors, particularly those for tumors of gastrointestinal and pulmonary origin (see table 1.1). Besides blockade of VEGF, also multi-targeted tyrosine kinase inhibitors, such as sorafenib and sunitinib, which block several tyrosine kinase receptor-mediated pathways, including VEGFR, represent a novel approach to angio-suppression (Presta et al, 2009).

Not surprisingly, given the redundancy of factors involved in the regulation of the angiogenic process, also other angiogenic factors and signaling pathways -

such as FGFs and angiopoietins- have been considered as potential therapeutic targets. In particular, FGFRs and associated ligands are a family of well-validated targets for therapeutic interventions in cancer diseases in relation to their prominent roles in cell growth, survival, differentiation and angiogenesis. Modulators of FGF or FGFR expression used to block completely or partially the activities of the FGF-FGFR complexes result in clinical drug candidates (Herbert et al, 2014). Overall, the current anti-angiogenic treatments when used alone have provided only a modest survival benefit (Mosele et al, 2014). Thus, there is increasing interest in combining antiangiogenic drugs with existing therapeutic modalities. As anti-angiogenics are generally cytostatic rather than cytoreductive, combinations involving conventional cytotoxic chemotherapies may be useful for maximizing therapeutic activity (Ma and Waxman, 2008).

Preclinical studies have confirmed that angiogenesis plays a central role also in **breast cancer** and is associated with metastatic potential of breast tumors. The microvessel density of breast cancers is known to be predictive of micrometastases, tumor recurrence and overall survival, establishing angiogenesis as a potential therapeutic target for breast cancer (Mackey et al, 2012). The anti-angiogenic drugs used in the setting of breast cancer comprise the monoclonal antibody bevacizumab, and multitargeted tyrosine kinase receptor antagonists such as sunitinib. Bevacizumab, for example, is highly recommended for triple negative phenotype (i.e. tumors not expressing estrogen, progesterone or Her2/neu receptor genes), which are highly proliferative tumors with high levels of VEGF Bevacizumab has been also approved for the use in metastatic breast cancer (Longatto et al, 2010; Miller et al, 2007).

Although the recognized beneficial effects of anti-angiogenic drugs in different tumors (see table 1), the currently used anti-angiogenic therapies has been also associated with a number of adverse events, including vascular complications (Daher and Yeh, 2008). The mechanisms of such toxicity are thought to involve the induction of endothelial dysfunction due to inhibition of vascular relaxation and impairment of VEGF functions. In addition, it is important to underline that the clinical benefit of anti-angiogenic drugs is restricted because of intrinsic and acquired limitations: (I) decrease the delivery of cytotoxic drugs to tumors, and hence reduce their efficacy, (II) intrinsic resistance characterized by tumor indifference to anti-angiogenic therapy, and (III) increase tumor aggressiveness in some cancer types due to pro-invasive mechanisms and increased dissemination and metastasis (Mosele et al, 2014).

Emerging concepts suggest that, in addition to growth factors, vascular metabolism also regulates angiogenesis and is a viable target for manipulating the microvasculature. It is well known that endothelial cells rely on glycolysis for ATP production, and recently has been shown that the key glycolytic protein 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3) regulates angiogenesis. Thus, pharmacological blockade of PFKFB3 causes a transient, partial reduction in glycolysis, and reduces pathological angiogenesis with minimal systemic harm (Stapor et al, 2014).

This quest for angiogenesis inhibitors is not confined to conventional chemo-preventative compounds but extends to substances found in the diet, such as PUFAs, which have long been associated with lower rates of cancer (Spencer et al, 2009).

Drug	Target	Structure	Indications
Bevacizumab	All VEGF isoforms	mAb	mCRC, mRCC, NSCLC, rGBM, metastatic breast cancer (EMA approval), advanced ovarian cancer (EMA approval)
Sunitinib	VEGFR 1-3, c-Kit, PDGFR-α and -β, RET, CSF-1R, and FLT3	TKI	mRCC, GIST, PNET
Sorafenib	VEGFR 2-3, PDGFR-β, FLT3, c-Kit, CRAF, BRAF, and P38-alpha	TKI	mRCC, advanced HCC
Pazopanib	VEGFR 1-3, c-Kit, PDGFR-α and -β, FGFR-1 and-3, c-Kit, and c-Fms	TKI	mRCC, advanced ovarian cancer, soft tissue sarcoma
Cediranib	VEGFR 1-3 and c-kit	TKI	mCRC, recurrent ovarian cancer
Aflibercept	PIGF, VEGF-A, and VEGF-B	Fusion protein	mCRC
Regorafenib	VEGFR 1-3, c-Kit, PDGFR-α and -β, FGFR 1-2, TIE2, RET, DDR2,RAF-1, and BRAF	TKI	mCRC, GIST
Vandetanib	EGFR, VEGFR, RET, TIE2, and Src	TKI	Advanced medullary thyroid cancer
Trebananib [AMG 386]	Ang-1/2	Peptibody	Recurrent ovarian cancer
Brivanib	FGFR-1 and VEGFR-2	TKI	Advanced HCC

Table 1.1: Overview of anti-angiogenic drugs approved for clinical use in cancer (Quintieri et al, 2014). *c-Fms: transmembrane glycoprotein receptor tyrosine kinase, c-kit: stem cell factor receptor, CSF-1R: colony stimulating factor-1 receptor, EGFR: epidermal growth factor receptor, FGFR: fibroblast growth factor receptor, FLT3: fetal liver tyrosine kinase receptor-3, GIST: gastro-intestinal stromal tumor, HCC: hepatocellular carcinoma, mCRC: metastatic colorectal cancer, mRCC: metastatic renal cell carcinoma, NSCLC: Non-small cell lung cancer, PDGFR: platelet derived growth factor receptor, PIGF: placental growth factor, PNET: Pancreatic Neuro-ectodermal tumor, RAF: murine leukemia viral oncogene homolog, RET: rearranged during transfection, rGBM: recurrent glioblastoma, Src: v-src sarcoma viral oncogene homolog, TIE2: tyrosine kinase with immunoglobulin like and epidermal growth factor like domains 2, VEGF: vascular endothelial growth factor, VEGFR: vascular endothelial growth factor receptor, mAb: monoclonal antibody, TKI: small molecule tyrosine kinase inhibitor.*

1.3. Fatty acids

In the body, fatty acids (FAs) deriving from the dietary fats or synthesized endogenously from acetyl CoA (Comba et al, 2011) are incorporated in blood lipids, fat deposits or structural lipids in biological membranes. Chemically, a FA is a carboxylic acid with an aliphatic tail. The predominant fatty acids are straight chain, can be saturated or contain carbon double bonds with an even number of carbon atoms and are termed 'unsaturated fatty acids'. When the FA contains one double bond, it is called 'monounsaturated' (MUFAs; e.i. palmitoleic acid, 16:1). If it contains more than one double bond, it is called 'polyunsaturated' (PUFAs, e.i. arachidonic acid, 20:4) (Ratnayake et al, 2009).

There are at least four families of PUFAs, depending on the parent FAs from which they are synthesized. The ω-9 and the ω-7 series are synthesized in our body starting from oleic acid (OA, 18:1 ω-9) and palmitoleic acid (PA, 16:1 ω-7), respectively (Calder, 2013). In addition, there are 18-carbon PUFAs, ω-3 and ω-6 series of PUFAs derived from α-linolenic acid (ALA 18:3, ω-3) and linoleic acid (LA, 18:2 ω-6), respectively. Since humans cannot synthesize ALA and LA, they are considered essential fatty acids (EFAs) (Azrad et al, 2013). LA and ALA are synthesized in large quantities in plants, while they are not produced in humans and other mammals because of lack of Δ12- desaturase and Δ15-desaturase, the enzyme that allow synthesize of EFAs. Although animals cannot synthesize ALA or LA they can metabolize them by further desaturation and elongation (Calder, 2013). EFAs metabolism is shown in Figure 1.3.

Among the main dietary sources of LA are vegetable oils such as sunflower, safflower and corn oil (Das, 2011), while chia and rapeseed oils are rich in ALA (Espada et al, 2007; Das, 2011). LA is the predominant PUFA in the Western diet, and it is converted to arachidonic acid (AA, 20:4 ω-6), while ALA is converted via desaturases and elongases to yield the eicosapentaenoic acid (EPA, 20:5 ω-3), which can be further elongated to docosahexaenoic acid (DHA, 22:6 ω-3) (Azrad et al, 2013).

Plant (ALA) and marine (EPA and DHA) ω-3 FAs used to be abundant in traditional Mediterranean diets. These ω-3 PUFAs are considered as the major mediators of the protective effect provided by this diet. On the other hand, Western diet is rich in ω-6 compared to ω-3 FAs and this PUFA profile has been associated with higher breast cancer risk (Das, 2011; de Lorgeril and Salen, 2012).

1.3.1. Essential Fatty Acids

Starting from ALA and LA which are considered respectively, ω-3 and ω-6 essential fatty acids (EFAs) incorporated through the diet different PUFAs are synthesized (Figure 1.3). The term omega (ω or n) is a structural descriptor for a family of PUFAs. “ω” signifies the position of the double bond that is closest to the methyl terminus of the acyl chain of the fatty acid. For example, all ω-3 fatty acids have this double bond on carbon 3, counting the methyl carbon as carbon one (Calder, 2013). The structure of ω-3 and ω-6 EFAs is shown in Figure 1.4.

As mentioned above, mammalian cells metabolize EFAs by successive elongation and desaturation reactions. Enzymes endowed with elongase and desaturase activities, such as Δ5- and Δ6, metabolize different PUFAs families. In eukaryotic cells, these enzymes are localized on the cytosolic face of the endoplasmic reticulum (Grammatikos et al, 1994). As shown in Figure 1.3, ALA can be converted to stearidonic acid (SDA, 18:4 ω-3), which can be further elongated to eicosatetraenoic acid (ETA, 20:4 ω-3) by Δ-6 desaturase. ETA is then desaturated by Δ-5 desaturase to yield EPA. It is important to note that the conversion of ALA to EPA (ω-3 family) is in competition with the conversion of LA to AA (ω-6 family), since the same enzymes are used (Figure 1.3). In particular, PUFA precursors compete with each other for the same enzymes, with hierarchical preference given for ω-3 over ω-6 FA (Das, 2011).

Several factors are known to influence the metabolism of EFAs. For example, saturated fats, cholesterol, trans-fatty acids, alcohol, adrenaline, and glucocorticoids inhibit Δ6 and Δ5-desaturases. Pyridoxine, zinc, nicotinic acid, and magnesium are co-factors for normal Δ6-desaturase activity. Moreover, the activities of Δ5 and Δ6-desaturases are slow in humans (Δ5>Δ6). As a result, the conversion of LA and ALA to their respective metabolites may be inadequate under certain circumstances. In such an instance, it is necessary to supplement EPA and DHA (to bypass Δ5 and Δ6-desaturases). Generally, supplementation of AA is not necessary since; it can be obtained from the diet (Das, 2011).

Following dietary intake, ω-3 PUFAs are incorporated into cell membranes in all tissues of the body (Surette, 2008) where they play important roles in membrane structure, potentially influencing membrane functions and transmembrane and intracellular protein activity via a number of mechanisms (Lundbæk et al, 2010). Feeding laboratory animals a diet containing fish oil, which provides EPA and DHA, results in a higher content of these fatty acids in the cell membrane of lymphocytes, macrophages, Kupffer cells and neutrophils;

typically, an enrichment in marine ω-3 PUFAs is accompanied by a decrease in AA content (Calder, 2013). Several studies showed that ω-3 PUFAs incorporation differs between individuals due to dissimilar rates of metabolism, enzymatic activity, background diet, age, and sex (Arterburn et al, 2006). For example, DHA and EPA plasma concentrations measured from day 7 to day 28 were significantly higher than at baseline in healthy subjects receiving a 28-day oral treatment with 3 g/day of DHA/EPA (Rusca et al, 2009). Moreover, plasma EPA concentrations increase in a linear manner in response to dietary EPA, whereas dietary DHA causes a dose-dependent, saturable increase in plasma phospholipid DHA concentrations with doses up to 2 g/day. Both DHA and EPA similarly reduce AA concentrations in plasma. Overall, tissue content of long-chain ω-3 fatty acids increases in response to dietary DHA or EPA supplementation (Arterburn et al, 2006).

Once incorporated into cell membranes, esterified PUFAs are released by the action of several different phospholipase A2 (PLA_2s) (Calder 2013). In particular, it has been demonstrated that DHA release from astrocytes is provided by Ca^{2+}-independent PLA_2 ($iPLA_2$), whereas AA is mainly released by another isoform, the Ca^{2+}-dependent PLA_2 ($cPLA_2$) (Strokin et al, 2004). In the case of endothelial cells, bovine aortic endothelial cells (BAECs) incorporate EPA as well as DHA when these fatty acid are present in the culture medium (Hadjiagapiou et al, 1986; Hadjiagapiou and Spector, 1987). PUFAs either released from cell membranes or from dietary intake are substrate for cyclooxygenases (COXs), lipoxygenases (LOXs) and cytochrome P450 (CYP450), three families of enzymes that generate bioactive lipid mediators (Shimizu et al, 2009).

AA acting as a substrate for COX enzymes leads to prostaglandins and thromboxanes (Samuelsson et al, 1978). In addition, complex families of prostaglandin isomers called isoprostanes derive as free radical products of oxidative metabolism (Hamberg et al, 1975). LOX enzymes metabolize AA to leukotrienes and lipoxins, and CYP450 enzymes to hydroxyeicosatetraenoic and epoxyeicosatrienoic acids. These lipid mediators play critical roles in diverse physiological and pathological processes, such as cancer (Greene et al, 2011).

Besides AA, also ω-3 PUFAs can be metabolized by COXs, LOXs and CYP450. A widely accepted theory explain that the health-promoting effects of ω-3 PUFAs is that they suppress the metabolism of AA or serve as alternative substrates to generate ω-3 lipid mediators with beneficial actions (Rose and Connolly, 1999). In particular, it is believed that the protective effects of EPA

(20:5 ω-3) are based upon a) preventing conversion of AA to pro-inflammatory eicosanoids (prostaglandins and leukotrienes), b) serving as an alternate substrate producing 5-series leukotriens that are less potent; and/or c) conversion by cyclooxygenase (COX) to 3-series prostanoids (such as PGI_3). These explanations have not been generally accepted because of the lack of molecular evidence *in vivo* and the high concentrations of ω-3 PUFAs required to achieve putative beneficial actions *in vitro* (Serhan et al 2000). Other potential mechanisms associated to the protective role of PUFAs will be discussed in more details later (see on page 38).

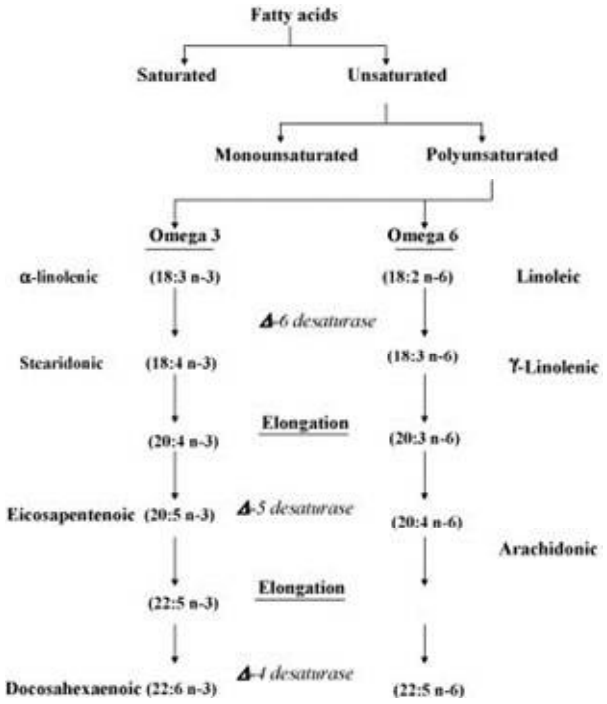

Figure 1.3: Conversion of essential ω-6 and ω-3 PUFAs to their longer chain, more unsaturated derivatives (Siddiqui et al, 2008).

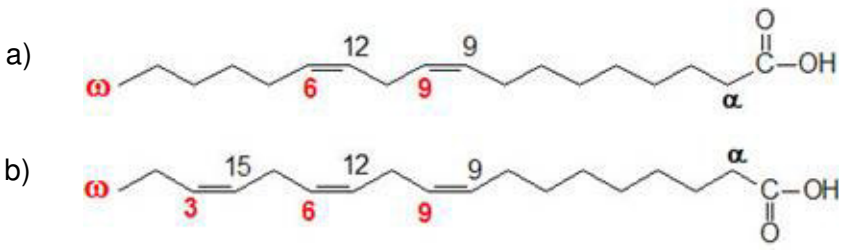

Figure 1.4: Structural representation of 18-carbon fatty acids.
PUFAs with 2 and 3 double bonds. a) LA (18:2 ω-6) and b) ALA (18:3 ω-3). The red numbers represents the carbon atoms counting from the methyl end of the chain (ω).

1.3.2. The Role of PUFAs in tumorigenesis

Fatty acids may promote or inhibit cancer. In the current literature there are inconsistent data about the potential role or implication of PUFAs in carcinogenesis. Several studies have found that, MUFAs and ω-6 PUFAs promote tumor growth, while ω-3 PUFAs may exert inhibitory action (Vara-Messler et al, 2015; Azrad et al, 2013).

ω-3 PUFAs, mainly EPA and DHA, have been shown to have multiple antitumor actions that affect all of the alterations that dictate malignant growth (Stephenson et al, 2011) reported above (see on page 35). We will report some examples of this large body of evidence from laboratory-based studies that support a beneficial effect of ω-3 PUFAs on the hallmarks of cancer (Stephenson et al, 2011).

ω-3 PUFAs profoundly affect *growth factor signal transduction*, with numerous putative pathways identified in the *in vitro* and *in vivo* setting. For example, it has been demonstrated that EPA and DHA have beneficial effects on breast cancer cells growth through a marked decrease of epidermal growth factor receptor (EGFR) expression in lipid rafts, leading to alteration in EGFR signaling (Schley et al, 2007). Indeed, it has been shown that ω-3 PUFA exerts anti-cancer effects by reducing the expression of some growth factors including human epidermal growth factor receptor-2 (HER-2), epidermal growth factor receptor (EGFR) and insulin-like growth factor receptor 1 (IGF-1R), inhibiting cell proliferation by either activating PPARγ or decreasing levels of fatty acid synthase (FAS) protein and/or promoting cell apoptosis via blocking PI3K/Akt pathways, downregulating phosphorylated Akt and lowering Bcl-2/Bax ratio (Liu et al, 2004). In addition, *in vivo* studies demonstrated that the levels of COX-2 and its metabolite PGE2 are reduced by supplementation of ω-3 PUFAs. In particular, it has been shown that reduced levels of COX-2 and PGE_2 were related to a reduction in tumor growth in a prostate cancer cell xenograft in mice (Rao et al, 2001). Moreover, in a rodent model of breast cancer, DHA induced a reduction in mammary tumor accompanied by an upregulation of BRCA 1 (breast cancer 1) tumor suppressor protein, thus suggesting that ω-3 PUFAs affect *tumor sensitivity to growth-inhibitory signals* (Jourdan et al, 2007). *In vitro*, EPA derived metabolites of COX and LOX have been shown to decrease growth of human breast cancer cell lines (Rose and Connolly, 1990), while in xenografted rats carrying a neuroblastoma tumor supplementation with a DHA-enriched diet prior to tumor cell injection delayed tumor formation. In the same study, it was

investigated the effect of DHA as a therapeutic agent in rats who had established tumors. Tumors in animals receiving high dose DHA showed response better response in terms of disease progression compared to animals receiving low dose DHA or controls (Gleissman et al, 2011). Another study performed in a pancreatic tumor xenograft in nude mice showed tumor inhibition by DHA (Swamy et al, 2008).

ω-3 PUFAs affect also *tumor evasion of programmed cell death*, which is a complex process controlled by an intricate milieu of intra-cellular signal transduction pathways. Evidence suggests that DHA and EPA have beneficial effects on many of these pathways. For example, an increased induction of apoptosis with ω-3 PUFAs has been demonstrated on HT-29 colon cell line (Chen and Istfan, 2000; Clarke et al, 2000). Other studies have shown that DHA induces a dose-dependent effect on cancer cell apoptosis (Gleissman et al, 2011; Lindskog et al, 2006). EPA and DHA have also been shown to modulate the expression of the Bcl-2 family proteins. In particular, they downregulate anti-apoptotic proteins Bcl-2 and Bcl-xL and increase the levels of the pro-apoptotic proteins Bak and Bcl-xS (Gleissman et al, 2010). In a mouse model of colon carcinogenesis the initiation of tumor growth was restricted by increased apoptosis related to ω-3 PUFAs supplementation (Hong et al, 2000).

There are also evidence for effects of ω-3 PUFAs on *tumor invasion and metastasis*, which may be decreased by EPA and DHA supplementation. EPA and DHA have been demonstrated to affect tumor cell migration through the suppression of tumor derived nitric oxide (NO) production (Ohata et al, 1997). Inhibition of metastasis by ω-3 PUFAs enriched diets has been demonstrated in both mouse and rat models of colorectal cancer (Iwamoto et al, 1998; Kontogiannea et al, 2000). EPA and DHA have also been shown to suppress development of lung metastases (Rose and Connolly, 1996).

In addition, ω-3 PUFAs have also been shown to have a profound effect on angiogenesis. Experiments in which breast carcinomas were implanted into nude mice fed with diets high in EPA or DHA indicated that both tumor microvessel density counts and levels of VEGF measured in the resected tumors were significantly lower in animals receiving these ω-3 PUFAs (Rose and Connolly, 1999). Consistently, ω-3 PUFAs inhibits primary mammary tumor growth through modulation of selected determinants of vascularization in a murine model of breast cancer (Mukutmoni-Norris et al, 2000). Besides PUFAs, also lipid mediators from ω-3 PUFAs may play a role in angiogenesis. This issue

will be discussed further (see on page 48). Overall, the *in vitro* studies on different cell culture models as well as *in vivo* studies in animals widely suggest that ω-3 PUFAs have promising anticancer effects. Conversely, the data in humans are controversial. In respect to epidemiological evidence, several prospective cohort studies investigated the association between PUFAs (either specific fatty acids or mixtures) and risk for cancer (Azrad et al, 2013). In particular, on the epidemiological studies on breast cancer produced inconsistent findings. In the Multiethnic Cohort Study, no association between PUFAs and risk for breast cancer was observed. In contrast, the Vitamins and Lifestyle (VITAL) study showed a trend toward a protective association for both EPA and DHA dietary intake and breast cancer risk. Additionally, when EPA and DHA from diet and also supplementation with fish oil were considered, both EPA and DHA were significantly associated with reduced risk for breast cancer. Besides, in the French E3N study population the intake of ω-3 PUFAs *per se* showed no association with breast cancer risk, except in subjects also consuming higher amounts of ω-6 PUFAs. Finally, in the Shanghai Women's Health Study, no association between PUFAs and breast cancer risk was found. Taken together, the studies indicated that the ratio of ω-3 to ω-6 rather than ω-3 *per se* is important to reduce breast cancer risk. The discrepancy observed between studies may reflect the difficulties in collecting accurate dietary data; however, it also may reflect genetic variation in PUFAs metabolism which has been shown to modify physiological levels of PUFAs and cancer risk (Witte and Hardman, 2015).

An interventional study in breast cancer patients where DHA was combined with FEC 75 chemotherapy [intravenous infusion of cyclophosphamide (500 mg m^{-2}) and fluorouracil (500 mg m^{-2}), followed by epirubicin (75 mg m^{-2}) showed delayed time to tumor progression and longer overall survival with respect to FEC alone. However, these findings were only observed when patients were stratified into 2 groups of either high or low incorporation of DHA into plasma and erythrocytes. Patients who had higher incorporation of DHA into plasma and erythrocytes benefited compared to those who had lower DHA incorporation (Bougnoux et al, 2009).

1.4. Cyclooxigenases and their metabolites

Cyclooxygenases (COXs), also known as prostaglandin endoperoxide synthase (PGHS), are the enzymes responsible for the conversion of AA or other fatty acids into the various prostanoids, a family of lipid mediators that have widespread and diverse biological function. AA represents the main substrate for COXs which are the enzymes responsible for the transformation of AA in the unstable intermediate known as prostaglandin H_2 (PGH_2). PGH_2 can be further metabolized into the 2-series prostanoids by tissue specific isomerases such as prostacyclin synthase (PGI_2s) and prostaglandin E_2 synthase (PGE_2s) to originate prostacyclin (PGI_2) and prostaglandin E_2 (PGE_2), respectively (Smyth et al, 2009).

Also, EPA has been deeply investigated for its metabolism by COXs since it is an analogue of AA. Through the COX pathway, EPA is converted into prostaglandin H_3 (PGH_3) and after that to PGs and TXs of the 3-series. These prostanoids exert a number of specific functions, sometimes opposite to those exerted by the 2-series prostanoids (Lagarde et al, 2013). Besides AA and EPA, also DHA is highly susceptible to oxidation by COXs. For example, COX-2 converts DHA to 13-hydroxy-DHA (13-HDHA) in human microvascular endothelial cells (Christie, 2011). It is widely accepted that DHA-derived metabolites switch the classical AA derived pro-inflammatory toward anti-inflammatory mediators. In addition, DHA when metabolized by LOX can act as a precursor of metabolites promoting tissue repair (Figure 1.5) (Serhan et al, 2002).

More recently, it has been demonstrated that aspirin-acetylated COX-2, in the presence of AA as a substrate endogenously produces a number of novel lipid mediators called AT-Lipoxins, which exerts protective effects both *in vitro* and *in vivo*. In the presence of substrates different from AA such as DHA and EPA, aspirin-acetylated COX originates other metabolites endowed with anti-inflammatory activity (Figure 1.5) (Serhan et al, 2002). Overall these metabolites including lipoxins (from AA), E-resolvins (from EPA) and D-resolvins (from DHA), are called aspirin-triggered metabolites (Figure 1.5). The combined use of DHA and aspirin became an attractive therapeutic avenue for the treatment of inflammatory related conditions (Makriyannis and Nikas, 2011). We will further discuss specific roles of these metabolites (see on page 51). COXs exist in two main isoforms: COX-1 constitutively expressed under basal conditions in most cells including vascular endothelial cells, and COX-2 which is primarily an inducible enzyme, but it is now known to be constitutively expressed in specific cells including endothelial cells. COX-2 has been mainly associated with a variety

of clinical inflammatory states and increased cell replication or differentiation; for instance COX-2 is upregulated by growth factors, tumor promoters, as well as by cytokines (Smyth et al, 2009).

1.4.1. COX-2 in the setting of tumors

In respect to the role of COXs in cancer development, COX-1 expression remains unaltered in most of cancers, although some findings indicate that COX-1 might play a role in intestinal polyposis and ovarian cancer. COX-1 might also be responsible for prostanoids synthesis at early stages in pre-cancerous lesions (Salvado et al, 2012). In contrast, COX-2 is overexpressed in a series of human cancers such as lung, breast, stomach, prostate, ovarian, head and neck, pancreatic, brain, gliomas, melanomas and colorectal adenocarcinomas. While there are several data on the involvement of COX-2 in tumor, the molecular mechanisms through which COX-2 contributes to tumorigenesis have not been yet completely identified (Rizzo et al, 2011). In particular, it has been demonstrated that COX-2 contributes to neovascularization, which is essential for tumor progression. Among the various prostanoids, PGE_2 is the best studied in the context of tumor progression. Both endothelial and tumor cells produce PGE_2. The production of prostanoids in the vasculature is due to the presence of both, COX-1 and COX-2 isoforms. COX-2 is inducible in cultured endothelial cells and human vessels following stimulation by pro-inflammatory factors, including IL-1β, TNF-α which are also abundant in the tumor microenvironment (Gately, 2000). Co-culturing endothelial and tumor cells has been reported to promote COX-2-dependent endothelial cells motility and assembly into capillary-like structures, an effect attributed to the release of angiogenic peptides, nitric oxide, and prostaglandins such as PGE_2 from the tumor cells. An alternative interpretation is that prostanoids synthesized by endothelial COX-2 might contribute to this effect, and recent findings support the idea that COX-2 expression in the tumor endothelium might promote tumor growth and neovascularization by producing bioactive prostanoids with paracrine actions on nearby cancer cells. This suggests that the landscaping tumor promoter action of endothelial COX-2 would arise both from its ability to synthesize prostanoids intracellularly and via transcellular metabolism through crosstalk with neighboring tumor cells. Indeed, transcellular metabolism could be a common mechanism for producing PGE_2 with crosstalk occurring between endothelial, stromal, tumor, and other cells present in the tumor microenvironment (Salvado et al, 2009; Salvado et al, 2012).

In particular, in human breast cancer the role of COX-2 as a critical component of tumor progression has been thoroughly investigated. COX-2 is upregulated in 40% of breast cancers, with up to 84% increases in some studies. Moreover, COX-2 plays a crucial role as a prognostic factor for malignancy and has been associated with carcinogenesis, particularly neoangiogenesis and tumor progression. As for the role of COXs in breast cancer related angiogenesis, it was demonstrated that COX-2 induces the expression of angiogenic factors, such as VEGF, basic fibroblast growth factor (bFGF), transforming growth factor 1 (TGF-1), platelet-derived growth factor (PDGF) and endothelin. In particular COX-2 derived PGE_2 binds at its receptor thus enhancing VEGF expression (Hoellen et al, 2011).

1.4.2. Pro-resolving aspirin-triggered metabolites

Aspirin, the first chemically produced medication was synthesized in the 1850s and continues to be widely used for its analgesic and anti-inflammatory properties. However, aspirin has also served as an invaluable pharmacological probe for unraveling several aspects of the inflammatory syndrome. During the 1970s, John Vane demonstrated that aspirin attenuates the biosynthesis of prostaglandins by acting as an inhibitor of COX-1. This discovery served as a basis for the development of a number of the widely used non-steroidal anti-inflammatory drugs (NSAIDs) (Makriyannis and Nikas, 2011). To date, aspirin is well appreciated for its ability to irreversibly inhibit COX-1 and inactivate this enzyme, blocking both PGs and TXs production in cells that possess these biosynthetic pathways. The mechanism of aspirin's action involves acetylation of COX within the enzyme's catalytic region. This prevents alignment of the substrate AA for oxygenation within the catalytic center that produces the prostaglandin endoperoxide intermediate (PGG_2).

Aspirin's action within cells that possess COX-2 is different. The catalytic region of COX-2 is larger than that of COX-1, and when it is acetylated by aspirin, the biosynthesis of endoperoxide is blocked. Yet, unlike COX-1, acetylated COX-2 remains active producing a number of metabolites in the presence of different substrates. In particular, aspirin-acetylated COX-2 generates 15R-HETE (15-hydroxy-eicosatetraenoic acid) from AA, 18R-HEPE (18-hydroxy-eicosapentaenoic acid) from EPA and 17R-HDHA (17-hydroxy-docosahexaenoic acid) from DHA (Figure 1.5). The first aspirin-acetylated COX-2 metabolite characterized was derived from AA, and was termed 15R-HETE. 15R-HETE,

18R-HEPE and 17R-HDHA can be further transformed by 5-LOXs expressed in human neutrophils and macrophages to aspirin-triggered lipoxins (AT-L) or aspirin-triggered resolvins (AT-Rvs). This process of cell-cell cooperation is termed transcellular metabolism or transcellular biosynthesis, and could represent an important contribution to the profile of lipid mediator produced in physiological as well as in pathologic circumstances by a particular organ or cell. The resulting metabolites have potent anti-inflammatory effects inhibiting human neutrophil migration and enhancing macrophage clean up overall improving the resolution of inflammation (Serhan et al, 2011).

Endothelial cells are able to incorporate different PUFAs into cell membrane phospholipids and to convert them into specific metabolites. In bovine aortic endothelial cells, it has been demonstrated that EPA as well as DHA are incorporated in the cell membrane phospholipids. Moreover, when DHA or EPA were available, less AA was incorporated into membrane phospholipids, and in turn converted to prostacyclin (PGI_2) (Hadjiagapiou and Spector, 1987). Conversely, in BAECs chronically supplemented with EPA or DHA PGI_3 production increased indicating that when challenged with EPA or DHA endothelial cells easily convert EPA to PGI_3 (Hishinuma et al, 1999). When treated with aspirin, EPA enriched human vein endothelial cells (HUVECs) or microvascular cells generate 18R-HEPE, that is released and further converted to AT-RvE_1 by activated human neutrophils through 5-LOX (Serhan et al, 2000). On the other hand, in presence of DHA, aspirin-treated endothelial cells generate 17R-HDHA, which can be further metabolized by 5-LOX to AT-RvD_1 (Serhan et al, 2002).

Specialized pro-resolving mediators (SPM) including those derived from COX-2 such as AT-Rvs have been shown to directly promote the resolution of inflammation (Serhan et al, 2011). The majority of the evidence comes from animal models. For example, the role of aspirin-triggered resolvin D_1 (AT-RvD_1) in immune complex-induced lung injury has been determined in mice. Lung vascular permeability was significantly reduced in the AT-RvD_1-treated mice when compared to control mice. Furthermore, administration of AT-RvD_1 caused a significant decrease in the broncho-alveolar lavage fluid content of neutrophils, inflammatory cytokines, and chemokines. Of interest, it has been demonstrated that IgG immune complex-induced activation of NFκB was significantly inhibited by AT-RvD_1 (Tang et al, 2014). Additionally, intra venous (iv) administration of AT-RvD_1 1h after LPS challenge protected mice from kidney injury associated

with tubular damage by decreasing neutrophil infiltration and by suppressing LPS-induced ICAM-1 and VCAM-1 expression in the kidney (Chen et al, 2014). The *in vivo* protective effects of this family of compounds have been demonstrated also in other disease models. RvE_1 treatment significantly increased post-traumatic sleep in brain-injured mice while AT-RvD_1 treatment mitigated motor and cognitive deficits suggesting that AT-RvD_1 may impart functional benefit through mechanisms other than resolution of inflammation alone (Harrison et al, 2015). Moreover, in a model of endometriosis in female Sprague-Dawley rats both RvD_1 and AT-RvD_1 significantly decreased vascular permeability in ectopic endometrial tissue and AT-RvD_1 alleviated the severity of vaginal hyperalgesia, suggesting that these compounds can be considered for further investigation of their therapeutic potential in endometriosis (Dmitrieva et al, 2014). Finally, in the setting of pathological angiogenesis, AT-Lipoxin (a downstream metabolite of COX-2 acetylated in presence of AA) (Figure 1.5) as well as RvE_1 and RvD_1 reduce inflammatory corneal angiogenesis (Jin et al, 2009).

AT-L leads to the resolution of inflammation, for example, AT-L blocks the generation of reactive oxygen species in endothelial cells, and it is a potent anti-inflammatory, inhibiting cell chemotaxis of polymorphonuclear (PMN) cells, leukocyte-endothelial interaction and NFkB activation as well as tumor necrosis factor-alpha (TNF-α) secretion in activated T cells. Accordingly, AT-L shows promise for treating a number of inflammatory diseases, as it possesses potent protective actions in a variety of experimental animal models of disease, including peritonitis, colitis, dermal inflammation, ischemia-reperfusion injury, and periodontitis. Also, AT-L reduces the PMN-endothelial cell adhesion initiated by preeclamptic plasma when human PMN were incubated with AT-L prior to addition to endothelial monolayers (Romano et al, 2015). Besides AT-metabolites from AA, also AT-EPA metabolites were characterized. *In vitro*, HUVECs treated with ASA convert EPA to 18R-HEPE. *In vivo*, 18R-HEPE blocked PMN infiltration and trans-endothelial migration and inflammation in a murine air pouch (Serhan et al, 2000). In addition, the biological activity of 18R-HEPE was examined using a murine model of zymosan-induced peritonitis. 18R-HEPE (2.5 μg) or vehicle alone was administered intraperitoneally at the time of zymosan-A injection. 18R-HEPE significantly reduced neutrophil infiltration as compared to vehicle alone (Krishnamurthy et al, 2011). Consistently, AT-RvE_3 (a downstream metabolite of 18R- or S- HEPE) has been proved to be a potent inhibitor of PMN chemotaxis *in*

vitro and also significantly reduced PMN numbers in zymosan-induced peritonitis model *in vivo*. Intravenous administration of 18R- and 18S-RvE_3 (10 ng/mouse) gave 30-45% inhibition of PMN infiltration that was maintained at the 100ng doses (Isorbe et al, 2012).

Recently, healthy volunteers were supplemented with ω-3 PUFAs (2.4 g/day) for 7 days with random assignment to take aspirin (300 mg/day) or placebo. Blood was collected at baseline and after 7 days. Plasma 18R/S-HEPE and 17R/S-HDHA as well as derivated E and D series resolvins were measured. Supplementation with ω-3 PUFAs significantly increased RvE_1, 18R/S-HEPE and 17R/S-HDHA, thus demonstrating that these metabolites are formed *in vivo* in humans. However, the addition of aspirin after ω-3 PUFAs supplementation did not affect AT-metabolite generation (Barden et al, 2014). Also, the study of Oh et al showed that in blood samples from volunteers receiving aspirin (81 mg at 12 and 24h) prior to a single dose (1 g) of fish oil the concentrations of the 18R-HEPE isomer in human plasma were 3-folds higher than the 18(S)-HEPE (Oh et al, 2011).

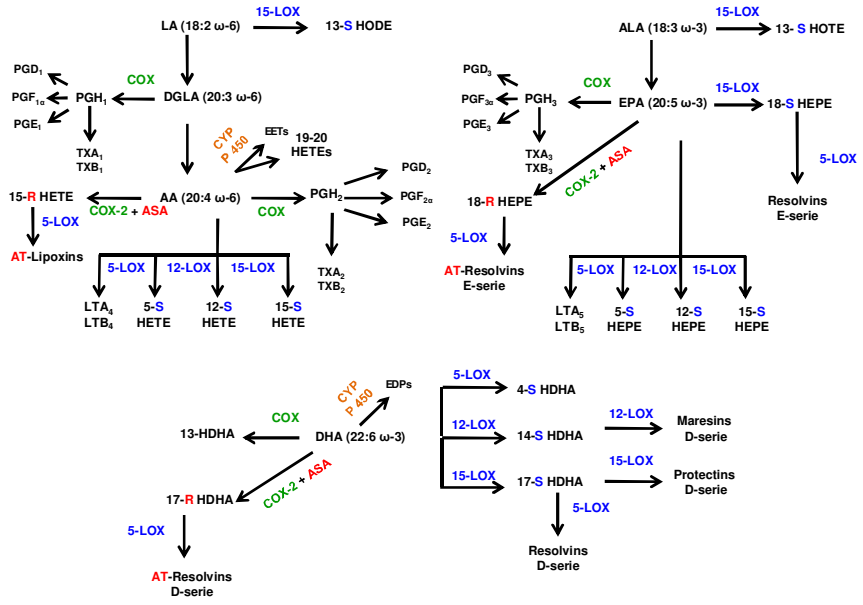

Figure 1.5: Outline of the pathways of PUFAs metabolism (Vara-Messler et al, 2015). *LA (linoleic acid); ALA (α- linoleic acid); AA (arachidonic acid); DGLA (dihomo-gamma-linolenic acid); EPA (eicosapentaenoic acid) or DHA (docosahexaenoic acid); ASA (aspirin); COX (cyclooxygenase); LOX (lipoxygenase); CYTP450 (cytochrome P450); HODE (hydroxyoctadecadienoate); HOTE (hydroxyoctadecatrienoate); PGs (prostaglandin); TXs (thromboxane); LTs (leukotriene); HETEs (hydroxyeicosatetraenoic acid); HEPE (hydroxy-eicosapentaenoate); EETs (epoxyeicosatrienoic acid); EDPs (epoxydocosapentaenoic acid); HDHA (hydroxy-docosahexaenoic acid); AT (aspirin-triggered).*

In this thesis, we will focus on the role of metabolites generated in DHA-treated cells from aspirin acetylated COX-2, namely 17R-HDHA, an upstream metabolite of AT-RvsD, which is generated by endothelial cells (Figure 1.5). Until now, the role of 17R-HDHA and the downstream metabolites were extensively characterized *in vitro* and *in vivo* models by Serhan (Serhan et al, 2002). In particular, these metabolites have been studied in the setting of inflammation. For instance, di- and tri-hydroxy products derived from 17R-HDHA inhibited microglial cell cytokine expression in an *in vivo* model of dermal inflammation and peritonitis by reducing 40-80% leukocytic exudates (Serhan et al, 2002). In addition, it has been demonstrated that a systemic treatment with 17R-HDHA (as well as its downstream metabolites, AT-RvD_1, RvD_2) in a nanogram range greatly improved disease activity index, body weight loss, colonic damage, and polymorphonuclear infiltration in two models of experimental colitis. Also, these treatments reduced colonic cytokine levels, as well as mRNA expression of NFkB and adhesion molecules (Bento et al, 2011). Additionally, decreased colitis activity was demonstrated in another murine model. The analysis of lipid mediators showed a sustained formation of DHA-derived 17R-HDHA after treatment with ASA and DHA. Overall, the results showed a protective effect of ASA and DHA in a murine colitis model and could give a rationale for a careful reassessment of ASA therapy in patients with inflammatory bowel disease and particularly ulcerative colitis, possibly combined with DHA supplementation (Köhnke et al, 2013). The effects of 17R-HDHA have been also evaluated in adjuvant-induced arthritis in rats treated with mechanical and thermal stimuli. Under these experimental conditions, 17R-HDHA, given systemically, inhibited the development and the maintenance of mechanical hyperalgesia (Lima-Garcia et al, 2011).

Of interest, at the end of ’90, the Gruppo Italiano per lo Studio della Sopravvivenza nell’Infarto Miocardico (GISSI-Prevenzione) reported data on the protective effect of ω-3 PUFAs in aspirin treated patients. Patients (n=11323) surviving a very recent myocardial infarction (MI) were randomized to 1.0 g daily of fish oil ω-3 PUFAs (PUFAs, n=2835), vitamin E (300 mg daily, n=2830), both (n=2830), or no treatment (n=2828) (GISSI, 1999). This study provided evidence that ω-3 PUFAs supplementation decrease cardiovascular death of about 45%. However, the impact of ASA alone was not evaluated (Serhan et al, 2002).

Overall, these results indicate that at sites of inflammation-resolution ω-3 PUFAs in the presence of aspirin generate novel oxygenated bioactive products. Given their potent actions, the production of AT-metabolites may, in part, provide

a molecular rationale underlying the beneficial actions of ω-3 PUFAs in neoplasia, chronic immune and cardiovascular diseases and serve for new therapeutic development.

1.5. Role of PUFA-derived metabolites in angiogenesis

Angiogenesis, one of the necessary steps in tumor growth and metastasis, is influenced by ω-6 and ω-3 PUFAs, as well as their downstream metabolites produced by either COX, LOX or CYT P450 (Figure 1.5). It is generally believed that ω-6 PUFAs and their metabolites promote tumor angiogenesis through a variety of signaling pathways, encouraging cell proliferation and migration, and decreasing tumor apoptosis. Conversely, ω-3 PUFAs and their metabolites are endowed with anti-angiogenic properties. Given that ω-6 and ω-3 PUFAs have opposing effects and compete for the same enzymes, lowering ratio ω-6:ω3 PUFA ratio shows great potential for controlling pathological angiogenesis and reducing cancer development (Kang and Liu, 2013).

1.5.1. Arachidonic acid metabolites and angiogenesis

The pro-angiogenic effects of COX-2 are mediated primarily by products of AA metabolism such as prostaglandin E_2 (PGE_2) and thromboxane A_2 (TXA_2). A number of tumor overexpressed COX-2 and COX-2 derived PGE_2 induces tumor-associated angiogenesis through an increased expression of VEGF. Indeed, in a preclinical breast cancer model, COX-2 inhibition by celecoxib suppresses angiogenesis and tumor growth, prevents metastases, and increases overall survival (Xin et al, 2012). COX-2 has been found overexpressed also in pulmonary, colonic, and mammary tumors as compared to negligible levels of COX-2 in non-neoplastic epithelium. Furthermore, patients with metastases have higher COX-2 expression, PGE_2 levels, and microvessel densities than cancer patients without metastases (Kang et al, 2013). Under hypoxic conditions, COX-2/PGE_2 enhances HIF-1 transcriptional activity, which in turn increases VEGF levels, angiogenesis and tumor cell survival in this hostile environment (Greenhough et al, 2009). Besides, it has been shown that PGE_2-mediated elevation of intracellular cAMP level leads to an increase in eNOS activation and endothelial NO and cGMP production by PKA-dependent PI3K/Akt activation and then promotes angiogenesis *in vitro* and *ex vivo* (Namkoong et al, 2005). Recently, it has been established a role also for COX-1 in angiogenesis. Indeed,

it has been demonstrated that the PGE_2 biosynthesis was dependent on COX-1, rather than COX-2 since neither the pharmacological inhibition nor COX-2 gene silencing affected PGE_2 production and angiogenesis evaluated by the formation of capillary like structures *in vitro* (Salvado et al, 2013). Moreover, it was demonstrated that other COX metabolites including PGJ_2 and its derivative PGD_2 dose-dependently inhibit tube formation in endothelial cells *in vitro* while PGE_1 (from DGLA, 20:3 ω-6) and PGE_2, had no significant effects. Indeed, 15d-PGJ_2 was the most potent inhibitor of HUVEC tube formation (Xin et al, 1999; Mousa et al, 2013). In line with these results, Kim et al. (2006) demonstrated that in endothelial cells treated with 15 deoxy-PGJ_2 VEGFR-1 and 2 mRNA levels were reduced and this in turn was associated with its anti-angiogenetic activity (Kim et al, 2006).

In addition, others COX metabolites have been demonstrated to have a role in angiogenesis. The inhibition of endogenous TXA_2 biosynthesis with a thromboxane synthase inhibitor, CI (20 μM) reduced VEGF or bFGF stimulated endothelial cell migration *in vitro* and bFGF-induced angiogenesis *in vivo* (Nie et al, 2000). Consistently, the TXA_2 receptor (TP) agonist U46619 stimulated while a TXA_2 receptor antagonist, inhibited endothelial cell migration in response to VEGF or bFGF. In the context of cardiovascular disease where revascularization and angiogenesis play a protective role, it has been reported that HUVECs express both TPα and TPβ. The signaling downstream TPβ but not TPα stimulation abrogates the pro-angiogenic and chemotactic properties of VEGF on endothelial cells and that the mechanism of inhibition involves the suppression of NO and FAK/Src activation. Overall these data suggest a different role for the 2 TP receptor subtypes in mediating angiogenesis: Stimulation of TPα receptors produced no inhibition of VEGF-induced angiogenesis, while TPβ expression is necessary and sufficient to reduce the angiogenic potential of VEGF-treated ECs in the presence of TP ligands (Ashton et al, 2004).

Beside COX metabolites, also metabolites derived from LOX have been described to modulate the angiogenic process. In particular, metabolites derived from 5-LOX have been demonstrated to increase angiogenesis. Indeed, 5-LOX inhibitors decreased the incidence of colonic adenoma formation and this was associated to reduced angiogenesis, decreased activity of the metalloprotease 2 (MMP-2) as well as decreased VEGF protein expression in the colons of tumor bearing animals (Ye et al, 2004). In line with this result, in a rat mammary tumor model, the overexpression of 5-LOX has been accompanied by the increase in 5-

HETE and LTB_4 production as well as the increase of key angiogenic factors such as MMP-2 and VEGF (Chatterjee et al, 2013). Beside 5-LOX, also 12-LOX through its metabolite 12(S)-HETE has been demonstrated to have a pronounced pro-angiogenic effect. Prostate tumors bearing mice overexpressing 12-LOX showed a remarkable increased angiogenesis with respect to healthy mice evidenced by a high microvascular density. Moreover, 12(S)-HETE has been shown to stimulate endothelial cell migration and to up-regulate the surface expression of integrin αvβ3 (an integrin predominantly associated with angiogenic blood vessels) in tumors. The potential of 12(S)-HETE as a significant stimulator of pathological angiogenesis may lie in its ability to induce the VEGF gene and protein expression (Pidgeon et al, 2007). In regards to 15-LOX metabolites, there are conflicting results and both pro- and anti-angiogenic effects have been demonstrated. In particular, lipoxin A_4 pretreatment of endothelial cells has been shown to be associated with anti-angiogenetic effects against various pro-angiogenesis stimulus such as bFGF or VEGF (Mousa et al, 2013), while 15(S)-HETE have been demonstrated to have pro-angiogenetic effect (Soumya et al, 2012).

It is well recognized that arachidonic acid can be metabolized also by CYT P450 epoxygenases giving rise to epoxygenated fatty acids (EpFAs) such as epoxyeicosatrienoic acids (EETs) (Spector et al, 2015). These lipid mediators are known to regulate various biological processes including angiogenesis. The pro-angiogenic effect of EETs was firstly demonstrated in astrocytes co-cultured with endothelial cells where EETs released from astrocytes stimulated endothelial cell proliferation and tube formation. Further in vitro studies showed that treatment with synthetic EETs stimulated angiogenesis in endothelial cells. In particular, 11,12- and 14,15-EETs have been shown to increase endothelial cell proliferation, migration and invasion. Animal studies were followed up to support the pro-angiogenic effects of EETs. Consistently, treatment with 11,12- or 14,15-EET stimulated neovascularization in a Matrigel plug assay and a chicken chorioallantoic membrane assay. Moreover, 5,6- and 8,9-EET stimulated angiogenesis in a subcutaneous sponge assay in mice (Zhang et al, 2014). Additionally, it has also been demonstrated that EETs elicit endothelial cell proliferation and angiogenesis increasing COX-2 gene and protein expression (Michaelis and Fleming, 2006) or up regulating VEGF-A expression (Panigrahy et al, 2011). Overall, evidence largely supports a pro-angiogenic role for AA and its

metabolites. The exception is represented by the PGD_2 metabolite 15 deoxy-PGJ_2.

To summarize, two lines of evidence support the pro-angiogenic effects of AA metabolites; *first*, high eicosanoid concentration due to high activity of COX-2, 12-LOX, 5-LOX and CYP 450 upregulate pro-angiogenetic factors through a variety of mechanisms. *Second*, inhibition of COX-2, 12-LOX and 5-LOX attenuates angiogenesis through downregulating pro-angiogenetic factor (Kang and Liu, 2013).

1.5.2. EPA and DHA metabolites and angiogenesis

ω-3 PUFAs can exert their anti-angiogenic properties mainly by two main mechanisms: 1) by inhibiting the production of pro-angiogenic metabolites derived from AA or 2) by producing anti-angiogenic metabolites from COXs, LOXs or cytochrome P450 (CYP) enzymes.

As we previously explained, ω-3 PUFAs work as "natural inhibitors" of ω-6 eicosanoids. Moreover, ω-3 PUFAs inhibited the production of many important angiogenic mediators such as VEGF, PDGF, COX-2, PGE_2, nitric oxide, and matrix metalloproteinases (Spencer et al, 2009).

For example*, in vitro* exposure of endothelial cells to DHA under conditions that efficiently incorporate DHA into membrane phospholipids decreased stimulated COX-2 mRNA transcription and COX-2 protein expression as well as the production of AA-derived metabolites with a role in angiogenesis (Massaro et al, 2007; Pola et al, 2004). Moreover, it has been demonstrated that ω-3 PUFAs are able to inhibit VEGF expression in colon cancer cells involving the negative regulation of the COX-2/PGE_2 pathway (Dommels et al, 2003). Several *in vitro* and *in vivo* studies on animal models show that ω-3 PUFAs have inhibitory effects on COX-2 and PGE_2 production by tumor cells (Spencer et al, 2009).

Besides suppressing the formation of pro-angiogenic eicosanoids from AA (20:4, ω-6) by endothelial and cancer cells (Spencer et al, 2009; Salvado et al, 2009; Salvado et al, 2012), ω-3 PUFAs are capable to biosynthesize specific anti-angiogenetic lipid mediators. In particular, the roles of COXs metabolites in the presence of ω-3 PUFAs have been studied *in vitro.* It has been demonstrated that PGE_3, that is biosynthesized from EPA by COX metabolism, did not stimulate the pro-angiogenic mediator angiopoietin-2 (Ang-2) differently from AA-derived PGE_2 (Szymczak et al, 2008). These findings are consistent with the

suggestion that PUFAs undergo biotransformation by COX-2 to generate lipid mediators that modulate tumor angiogenesis. Regarding the specific role of EPA in angiogenesis, it has also been demonstrated that human umbilical vein endothelial cells (HUVECs) treated with EPA decreased VEGF-stimulated tube formation and angiogenesis. In addition, VEGF-stimulated migration of HUVECs was suppressed and certain matrix metalloproteinases (MMPs) associated with endothelial cell migration was diminished in HUVECs treated with EPA (Tsuzuki et al, 2007). Moreover, a study on the effect of EPA on VEGF-induced endothelial cell proliferation showed that bovine carotid artery endothelial cells treated with EPA displayed a dose-dependent suppression of VEGF-induced endothelial cell proliferation (Yang et al, 1998). *In vivo*, rats with fibrosarcoma were assigned to diets supplemented with corn oil, normal saline or EPA. After resection of the tumor, rats on EPA diet had significantly decreased levels of VEGF-α mRNA, demonstrating that EPA supplementation inhibits tumor growth, potentially through alterations in the expression of the pro-angiogenic VEGF-α (Tevar et al, 2002). Few data are available on the role of DHA metabolites on angiogenesis. Recently, a key role for 4-HDHA (a metabolite from DHA by 5-LOX metabolism) has been described. 4-HDHA has potent effects in inhibiting endothelial cell sprouting (Sapieha et al, 2011). Moreover, it has been demonstrated *in vivo* that increasing ω-3 PUFAs tissue levels by dietary or genetic modification decreased the avascular area of the retina by increasing vessel regrowth after injury, thereby reducing the hypoxic stimulus for neovascularization. More interesting, the bioactive ω-3 PUFAs derived mediators NPD_1, RvD_1 and RvE_1 also potently protected against neovascularization (Connor et al, 2007). Besides the role on COXs- and LOXs-derived metabolites, ω-3 PUFAs are also substrates of cytochrome P450 (CYP) epoxygenases. This enzyme, for example, synthetizes epoxydocosapentaenoic acids (EDPs) from DHA. It has been recently reported that EDPs suppressed endothelial cell migration and protease production via a VEGF receptor 2-dependent mechanism. Also, EDPs inhibited VEGF- and FGF 2-induced angiogenesis *in vivo*. Moreover, when EDPs are co-administered with a low-dose soluble epoxide hydrolase inhibitor, EDPs are stabilized in circulation, causing ~70% inhibition of primary tumor growth and Overall, EDPs mediates the angiogenic switch to decrease tumor growth (Zhang et al, 2013). Thus, ω-3 PUFAs, through COX, LOX or cytochrome P450 (CYP) enzymes are able to synthetize metabolites which are able to directly affect angiogenesis as well as to counteracting the pro-angiogenic actions of ω-6 PUFAs (Kang and Liu, 2013).

1.5.3. Aspirin-triggered metabolites and angiogenesis

Aspirin-acetylated COX-2 metabolite from AA (20:4, ω-6) are named aspirin-triggered lipoxins (AT-L), while those from EPA (20:5, ω-3) or from DHA (22:6, ω-3) are named aspirin-triggered resolvins (AT-Rvs) from E or D series, respectively. The impact of AT-L and their analogues on angiogenic pathways, such as endothelial proliferation, VEGF and VEGF receptor expression has been well characterized (Romano et al, 2015), while few data are available on AT-Rvs. It has been reported that 15-epi-16-(parafluoro)-phenoxy-lipoxin A4 (AT-L1), the stable analogue of AT-L, inhibits angiogenesis by inhibiting VEGF-stimulated endothelial migration in a concentration-dependent manner. Moreover, AT-L1 inhibited endothelial cell proliferation in cells stimulated with VEGF. *In vivo*, in a granuloma model of inflammatory angiogenesis, AT-L1 treatment (10 g/mouse) reduced by approximately 50% angiogenic parameters (Fierro et al, 2002). Moreover, ATL-1 inhibited EC adhesion to fibronectin via interaction with its specific receptor. Furthermore, VEGF-induced MMP-9 activity and expression were reduced by pretreatment with AT-L1. AT-L1 inhibited NFκB nuclear translocation and strongly decreased VEGF-dependent phosphorylation of phosphainositide 3-kinase (PI3-K) as well as extracellular signal-regulated kinase-2 (ERK-2), two signaling kinases involved in EC proliferation (Cezar-de-Mello 2008). Furthermore, AT-L1 modulates essential components of the motile process, by impairing actin polymerization and focal adhesion assembly. Pretreatment of EC with AT-L1 caused a reduction in VEGF-induced stress fibers and therefore reduced the intracellular content of filamentous actin. A concomitant impairment in stress-activated protein kinase (SAPK2/p38) phosphorylation suggests that ATL-1 inhibition of VEGF-stimulated actin polymerization involves the SAPK2/p38 pathway. Moreover, ATL-1 treatment inhibited focal adhesion clustering due to inhibition of focal adhesion kinase (FAK) phosphorylation and the subsequent association of FAK with the actin cytoskeleton. Together these results provide evidence that AT-L1 inhibits EC migration via the concerted inhibition of actin polymerization and proper assembly of focal adhesions, supporting a role for these novel lipid mediators as angiogenesis modulators (Cezar-de-Mello 2006). To date, few studies have addressed the role of resolvins series E and D, derived from the metabolism of EPA and DHA, respectively. It has been demonstrated that besides AT-L, RvE1 and RvD1, reduced inflammatory corneal hematoangiogenesis. Mice treated with these lipid mediators have had reduced mRNA expression of VEGF (A, C and

R2), TNF-α, IL-1 alpha, IL-1 beta, and suppressed hemoangiogenesis, but not lymphangiogenesis. In addition, AT-L directly inhibits VEGFA-mediated corneal neovascularization and is the most potent inhibitor of angiogenesis among these lipid mediators (Jin et al, 2009).

At our knowledge, there is no data on aspirin-acetylated COX-2 derived DHA metabolites in the setting of tumor-angiogenesis.

AIM OF THE STUDY

2. AIM OF THE STUDY

The general objective of the present study was to determine the role of dietary ω-3 polyunsaturated fatty acids (PUFAs) from chia oil on a syngeneic mouse model of breast cancer (BC). The major specific aim of this thesis work was to investigate the effects of docosahexaenoic acid (DHA 22:6, ω-3) metabolites from aspirin-acetylated COX-2 in angiogenesis. The present work began in Argentina, where mice were fed a chia oil-rich diet containing α-linolenic acid (ALA 18:3, ω-3). *Ex vivo* and *in vitro* angiogenesis experiments have been carried out in Italy.

Cancer is the leading cause of mortality worldwide, and the tumor with the largest number of incident and prevalent cases is BC. Beside the highest prevalence and incidence, BC is also the most frequently occurring tumor in women (Ferlay et al, 2014). Angiogenesis is a tightly regulated process that contributes to tumor growth, which is controlled by a balance of pro- and anti-angiogenic molecules. The angiogenic process represents an important target to suppress tumor growth and metastasis; however, patients become resistant to anti-angiogenic therapy over time. Moreover, currently used drugs have been associated with several side effects including bleeding and defects in wound healing (Moserle et al, 2014).

Epidemiological and preclinical data have linked ω-3 PUFAs consumption to a lower incidence of BC. Furthermore, experiments performed in breast carcinoma-implanted mice fed diets rich in ω-3 PUFAs showed that tumor microvessel density in resected tumors were significantly lower with respect to control diet-fed animals. Consistently, a fish oil-based diet rich in ω-3 PUFA, inhibits primary mammary tumor growth through modulation of vascularization in this murine model of BC (Mukutmoni-Norris et al, 2000). Moreover, it has been shown that dietary intake of ALA from flaxseed suppresses breast tumor growth in ovariectomized athymic BALB/c mice (Chen et al, 2007). Previously published data from the Argentinian lab demonstrated that ALA-rich diets based on mystol or perilla oils reduced mammary tumor growth (Muñoz et al, 1995; Nakamaya et al, 1993). In addition, preliminary data demonstrated that the ALA-rich oil from chia (*Salvia hispanica L.*) seeds, used by pre-Hispanic civilization living in South America, significantly reduces growth of a transplantable mammary adenocarinoma in mice. At present, the role of an ALA-enriched diet in tumor angiogenesis has not been addressed. Conversely, several *in vitro* and *in vivo*

studies have shown that docosahexaenoic acid (DHA 22:6, ω-3), a downstream metabolite from ALA, as well as DHA epoxymetabolites, decrease angiogenesis, while arachidonic acid (AA 20:4, ω-6) promotes angiogenesis (Kang and Liu, 2013; Zhang et al, 2013). Several COX-1 and COX-2 AA-derived eicosanoids have shown to have a pro-angiogenic effect (Salvado et al, 2013; Nie et al, 2000; Pola et al, 2004). In particular, AA-derived PGE_2 has been largely studied in the context of tumor progression, including the angiogenic process.

In addition, whereas COX-1 acetylated by aspirin (ASA) is permanently inactivated, acetylated COX-2 is still capable to metabolize PUFAs to a novel series of specialized lipid mediators with pro-resolving activity. In particular, acetylated COX-2 in presence of either AA or ω-3 PUFAs generates several metabolites including 18R-HEPE from eicosapentaenoic acid (EPA 20:5, ω-3) and 17R-HDHA from DHA (Serhan, 2014). Interestingly, metabolites produced *in vivo* by ω-3 PUFAs have potent anti-inflammatory effects and considering that inflammation can stimulate angiogenesis, it is possible to hypothesize that reduced inflammation modulates angiogenesis *in vivo*. However, although the ability of metabolites from ASA-acetylated COX-2 to promote the resolution of inflammation is well recognized, their direct role in angiogenesis is almost unexplored (Connor et al, 2007).

The present study was aimed at evaluating the role of metabolites generated by ASA-acetylated COX-2 in the presence of either DHA or AA in several *in vitro* models of angiogenesis. The study of metabolites required for antiangiogenic and, more generally, anti-tumor effects of ω-3 PUFA is relevant considering the high degree of inter-individual variability in metabolizing fatty acids. This may explain, at least in part, the inconsistent results from clinical trials of ω -3 PUFAs in human cancer (Wang et al, 2014).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Materials

ω-3 PUFAs source, chia seed oil, was purchased from Nutraceutica Sturla S.R.L. (Argentina), while ω-6 PUFAs source, corn seed oil was purchased from Arcor S.A. (Argentina). LM3 cells, a line of murine mammary cancer cells, were kindly provided by Dr. Bal de Kier-Joffe (Instituto Oncologico Angel Roffo, Argentina). Fatty acids, as well as 17R-HDHA were obtained from Cayman Chemical (Ann Arbor, MI, USA) and fatty acids-methyl esters standard came from Nu-Chek Prep Inc (Minneapolis, MN, USA).

MIB-5 mAb (for Ki-67), as well as secondary Ab HRP labelled anti-rabbit and 3,3'-diaminobenzidine (DAB) was purchased from DAKO Corp (Carpinteria, CA, USA). The Fluorometric kit TUNEL System was purchased from Roche Applied Sciences (Mannhein, Germany) and VEGF Receptor 2 mAb from Cell Signaling Technology, Inc. (Danvers, MA, USA). CD31 anti-human Ab was purchased from BD Biosciencies Pharmingen (San Diego, CA, USA).

Matrigel® was purchased from Becton Dickinson (Waltham, MA, USA) and DiffQuick staining kit from Thermo Fisher Scientific Inc (Waltham, MA, USA). Hematoxylin and eosin, trypsin 0.25%-EDTA, Minimal Essential Medium (MEM) and Medium 199 (M199), fetal bovine serum (FBS), endothelial cell growth factor (ECGF), antibiotics solution (10.000 U/mL penicillin/10 mg/mL streptomycin), as well as other analytic grade chemical agents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.2. Study design for *in vivo* and *in vitro* studies

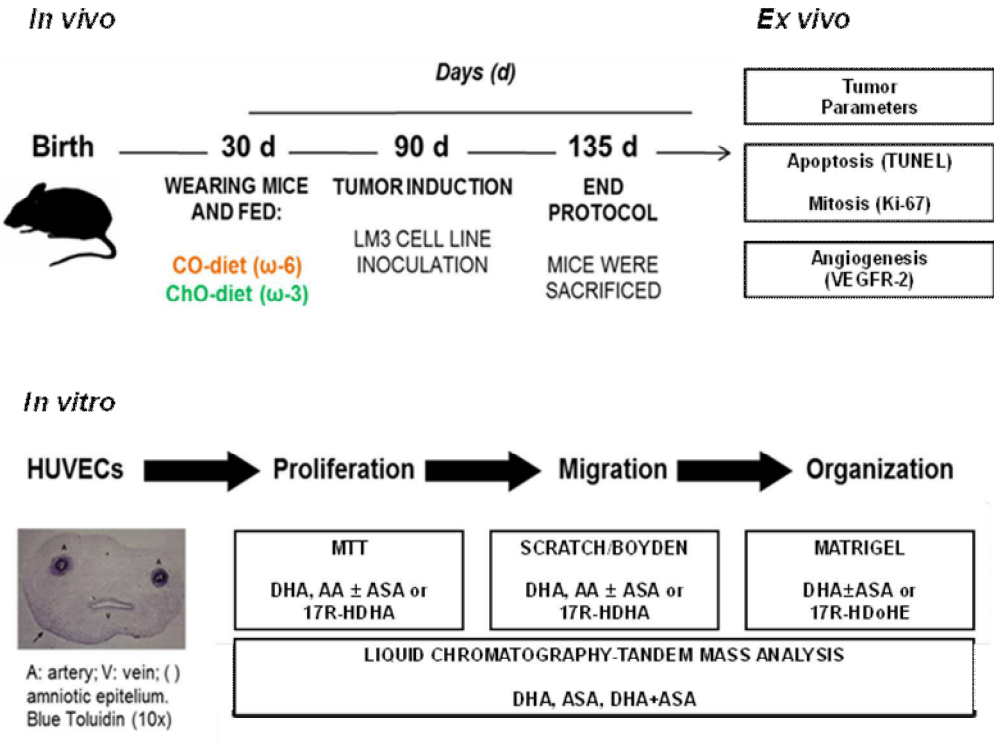

Figure 3.1: A schematic line drawing the experimental approach.

3.3. *In vivo* experiments

3.3.1. Mice and diets

After weaning, 40 BALB/c mice (male and female) were randomized and housed in polycarbonate cages in groups of four. Animals were kept in a 12h light and 12h darkness cycle at a constant temperature of 23°C. Animal studies were conducted in accordance with the Guidelines set by the National Institutes of Health (NIH) - Guide for the Care and Use of Laboratory Animals. All the procedures were approved by the Institutional Committee for the Care and Use of Laboratory Animals at the Facultad de Ciencias Médicas (Universidad Nacional de Cordoba, Argentina). Animals were fed with two isocaloric diets (3.86 Kcal/g) differing in their fatty acid composition. The final composition of both diets consisted of (%): casein (16.0), sucrose (34.0), corn starch (39.0), fiber (2.0), salt mixture (3.5) and vitamin mixture (0.5). The chia oil diet (ChO) contained chia oil (10.0) and the control diet contained corn oil (CO) (10.0). Food and water were provided ad libitum. As previously published (Espada et al, 2007), the fatty acid composition of chia oil used for dietary oil supplementation showed approximately a 60% ALA (18:3, ω-3) and 20% LA (18:2, ω-6), whereas corn oil

contains around 50% of LA and trace amounts of ALA (Table 3.1). Values for the oils agree with those published by the American Oil Chemists Society (Adlof, 2003). Animals were inspected daily; food consumption and weight were recorded weekly.

Major fatty acids (%)					
	Saturated FAs		MUFAs	PUFAs	
			ω-9	ω-6	ω-3
	14:0	16:0	18:1	18:2	18:3
CORN OIL	1.04	30.50	13.43	53.01	2.02
CHIA OIL	6.40	4.42	2.29	23.65	63.24

Table 3.1: Fatty acid composition (%) of corn and chia oil added to the diet.
Values represent mean of each fatty acid. Common names: 14:0 *Myristic acid*, 16:0 *Palmitic acid*, 18:0 *Stearic acid*, 18:2 *Linoleic acid*, 18:3 *α-Linolenic acid*. PUFAs: *polyunsaturated fatty acids*; MUFA: *monounsaturated fatty acids*; FA: *fatty acids.*

3.3.2. Tumor induction and cancer cell culture

LM3 cells were isolated from mouse mammary gland tumors that exhibited moderate metastatic behaviour (Bal de Kier Joffre et al, 1983). LM3 cells were maintained in MEM, supplemented with 10% (v/v) fetal bovine serum, 1% antibiotics solution (10000 U/mL penicillin/10 mg/mL streptomycin) and incubated at 37°C under 5% CO2. Mice have been fed for three months with either the ChO or the CO diet, and after that tumor was induced with LM3 murine cancer cell line (106 cells in 200 µl fresh MEM) injected subcutaneously into the left flanks of the mice. in order to obtain a syngeneic breast cancer murine model. After tumor induction mice were fed with their respective diets. Meanwhile, the latency time (days) for palpable tumors was recorded. Animals were sacrificed at 45 days after inoculation.

3.4. *Ex vivo* experiments

3.4.1. Tumor parameters

During necropsy, tumor weight (g), volume (mm3) and the number of grossly macroscopic metastasis were recorded in all organs of the host fed

animals with the aid of magnifying lens, as published in previous studies (Espada et al, 2007). Samples from tumor tissues were fixed in 4% (w/v) paraformaldehyde and, after that tissues were dehydrated and embedded in paraffin for histological analyzes. Histological analyses were performed on sections from 3 animals for each dietary condition.

3.4.2. Apoptosis and mitosis determination

Apoptotic and mitotic figures were recognized by characteristic morphological changes in fixed tumor tissues stained with hematoxylin and eosin (HE). Apoptotic figures were recognized by shrinking, condensing and fragmenting of nuclei, and mitotic figures as cells on metaphases (Figure 3.2).

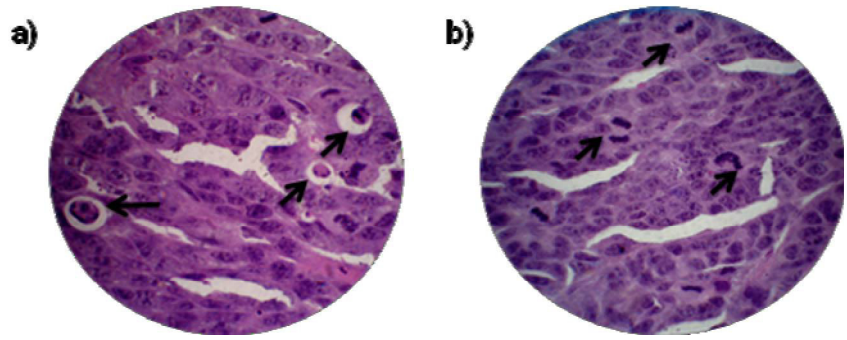

Figure 3.2: Apoptotic and mitotic figures from tumors.
Apoptotic (a) and mitotic (b) figures from neoplastic tumor tissue fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin and stained with hematoxylin and eosin (100x magnification).

Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay has been used to detect apoptotic cells that undergo extensive DNA degradation during the late stages of apoptosis (Kyrylkova et al, 2012). An in situ cell death detection kit Fluorometric TUNEL System assay was conducted in the paraffin-embedded tumors. Tumor sections were dewaxed and rehydrated. After that, slides were immersed in 0.85% (w/v) NaCl for 5 minutes and samples were permeabilizated with 0.25% (v/v) Triton X-100 in PBS buffer (TBS). Tumor sections were labeled adding 50 µl of TdT reaction mix and slides were incubated for 1h at 37°C in a humidified chamber avoiding exposure to light. The reaction was stopped by washing for 15 minutes and slides were counterstained with 4',6-diamino-2-fenilindol (DAPI) to visualize all nuclei.

Ki-67 staining was performed in order to detect mitotic cells. Ki-67 nuclear antigen is expressed in cell cycle S, G1, G2, and M phases, but is non-existing in G0 phase (Inwald et al, 2013). Tumor sections (8 µm thickness) were dewaxed and rehydrated. Following, antigen retrieval was performed bringing the slides to boil in 1mM Na-citrate buffer pH: 6.0 during 45 minutes at 95ºC. Sections were washed and endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 minutes. Non-specific binding sites were blocked with 3% albumin in Tris-buffered saline buffer, 0.1% Tween-20 (TBST) for 1h. The staining with MIB-5 mAb (1:400) was performed overnight at 4ºC. After incubation, slides were washed three times and sections were covered with a secondary Ab (HRP labelled polymer) for 1h. After that, sections were developed with 3,3'-diaminobenzidine (DAB) for 10 minutes at room temperature and counterstained with hematoxylin.

TUNEL and Ki-67 positive cells in ten high-power fields were counted in a blinded manner from three animals for each dietary condition using a light microscope equipped with a digital camera (40x magnification) Olympus BH2. Results were expressed as a mean ± SEM of TUNEL or Ki-67 positive cells/mm2.

3.4.3. Angiogenesis determination

It has been shown that downregulation of VEGF-Receptor 2 (VEGFR-2) resulted in a significant reduction of the sprouting capability and hence in decreasing angiogenesis. Angiogenesis was determined by immunohistochemistry (IHC) in embedded paraffin tumor sections (4 µm thickness). Sections were dewaxed, rehydrated and antigen retrieval was performed bringing the slides to boil in 1 mM ethylenediaminetetraacetic acid (EDTA) pH: 8.0 during 15 minutes at 95ºC. After that, sections were washed and endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 minutes. Non-specific binding sites were blocked with 5% serum in Tris-buffered saline buffer 0.1% Tween-20 (TBST) for 1h. The staining with VEGF-R2 Rabbit mAb (1:250) was performed overnight at 4ºC. After incubation, slides were washed three times in TBST and sections were covered with a secondary Ab (HRP labelled polymer anti-rabbit) for 1h. Subsequently, sections were developed with 3,3'-diaminobenzidine (DAB) for 10 minutes at room temperature and counterstained with hematoxylin. Vessels were counted in ten high-power fields in a blinded manner from three animals for each dietary condition using a Nikon

Eclipse Ti equipped with a digital camera (40x magnification). Results were expressed as a mean ± SEM of vessel density/mm2.

3.5. *In vitro* experiments

3.5.1. Endothelial cell isolation and culture

Human umbilical vein-endothelial cells (HUVECs) were obtained from human umbilical cords. Cords were washed with 0.9% (w/v) NaCl and enzyme digestion [0.1% (w/v) collagenase] was performed at 37°C for 15 minutes. HUVECs were eluted with a phosphate saline buffered and collected by centrifugation at 1100 rpm for 5 minutes. Pelleted HUVECs were suspended in fresh M199 supplemented with 15% (v/v) FBS, 1% (v/v) gentamicin, 0.1 mg/mL (w/v) endothelial cell growth factor (ECGF), and 100 g/mL (w/v) heparin and placed in T-25 flask kept in a humidified atmosphere of 95% air and 5% CO2 at 37°C. To determine the culture purity, HUVECs (5 x 104 cells) were incubated with an anti-CD31 antibody or with an isotype control IgG1 antibody at room temperature. An unstained sample was included and cells were analysed by flow-cytometry. The background staining observed in isotype controls was subtracted from the signals obtained in CD31+ cells. Flow cytometry analysis of CD31 revealed that purity of isolated HUVECs was 99.6% (corrected by background subtraction) (Figure 3.3). We have randomly performed a similar experiment in order to control cell purity.

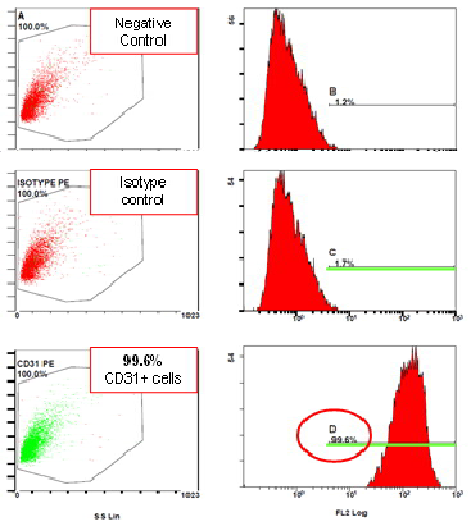

Figure 3.3: Representative image of CD31-positive cells.
Percentage of endothelial cells defined by CD31-positive cells.

3.5.2. Endothelial cell treatments

HUVECs were used at 2nd to 6th passage. Cells were treated from 24 to 72h with DHA (1-50 µM) or AA (1-50 µM) in the presence or absence of ASA (50 µM). Selected experiments were performed with 17R-HDHA (100 nM - 3 µM), a DHA metabolite derived from COX-2 acetylated by ASA.

3.5.3. MTT assay for cell viability determination

The MTT reduction assay was performed to determine cell viability. The yellow 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) salt is converted to purple formazan crystals by the succinatetetrazolium reductase system of the mitochondrial respiratory chain, which is active in viable cells only. HUVECs (at the density of 2 x 103, 1.5 x 103 or 104 cells/well) were seeded in 96-well plates in 100 µl medium/well. Cells were incubated overnight to allow adhesion. Cells were treated for 24 to 72h with different concentrations of DHA (1-50 µM) or AA (1-50 µM) in the presence or absence of ASA (50 µM) and 17R-HDHA (100 nM - 3 µM); four hours before the end of protocol, 10 µl of MTT [5 mg/mL (w/v)] was added to each well. Finally, the incubation medium was removed and the formazan crystals were dissolved in 100 µl/well dimethyl sulfoxide (DMSO). MTT reduction was quantified by measuring the light absorbance at 570 nm−630 nm using a multilabel plate counter (VICTOR–Wallac) (Stratford et al, 1989). Background absorbance from control wells (cell-free media) was subtracted and the cell viability was expressed as a percentage of the control.

3.5.4. Wound healing assay

In order to determine haptotaxis, wound healing assay were conducted. HUVECs were seeded in a 12 well plate at the density of 105, 1.5 x 105 or 2 x 105 cells/well depending on the duration of the experiment. After 24h of adhesion cells were treated with DHA (1-50 µM) or AA (1-50 µM) in the presence or absence of ASA (50 µM) and 17R-HDHA (100 nM-3 µM) for 24 to 72h. After that, the cell monolayer was scraped using a sterile p1000-pipet tip and the debris were removed carefully by washing (Liang et al, 2007). The treatment was replaced and pictures were taken at time 0h (T0) and after 16h (T16) using a phase contrast inverted microscope Nikon Eclipse Ti equipped with a digital camera (4x magnification, 3 unit fields/well).

Quantitative analysis of cell migration was performed using an average wound space from those fields and the percentage of change in the wound space was calculated using the following formula:

$$\% \text{ change} = \frac{(\text{average space at T0}) - (\text{average space at T16})}{(\text{average space at T0})*100.}$$

Relative cell migration was calculated by dividing the percentage change in the wound space of treated cells by that of the control cells in each experiment. Results were expressed as a percentage of cell migration.

3.5.5. Boyden chamber assay

Chemotaxis describes cell migration based on chemicals in a cell's surrounding environment. In order to evaluate the chemo-attractive effect of 17R-HDHA, a chemotaxis assay was performed using a modified 48-well Boyden chamber with a polycarbonate 8-µm pore size membrane previously coated collagen [10mg/mL (w/v)].. HUVEC suspension (1.6 x 105 cells/mL) was seeded into the upper chamber to migrate toward a chemoattractant in the lower chamber (Boyden, 1962). In the lower chamber was placed either a medium supplemented with 15% (v/v) FBS (positive-control), a medium without FBS (negative-control) or a medium supplemented with 17R-HDHA (300 nM -3 µM). Following 6h incubation, HUVECs in the upper side of the membrane were removed and the membrane was immediately transferred to a dish with metanol and placed for 10 minutes at -20ºC in order to fix migrated cells. Thereafter, the membrane was stained with Diff-Quick® kit according to the manufacturer's instructions. Pictures were taken with a microscope Nikon Eclipse Ti (20x magnification, 5 unit fields/well). Cells were counted using a Cell Counter plugging developed by Image-J (http://imagej.nih.gov/ij/ U.S. National Institutes of Health. Bethesda, Maryland, USA). Results were expressed as a percentage of migrated cells respect to the positive-control.

3.5.6. Matrigel assay

To assess the capability of endothelial cells to form capillary-like structures, HUVECs were seeded on Matrigel®, a soluble basement membrane extract of the Engelbreth-Holm-Swarm tumor that gels at room temperature to form a reconstituted basement membrane. HUVECs (2 x 104 cells/well) were

plated onto a thin layer of the basement membrane Matrigel® in a 48-well plate and incubated for 30 minutes at 37°C in a 5% CO2 for 6h in complete cell culture medium with or without tested compounds in presence of ECGF [100 mg/mL (w/v)]. At the end of protocol, the capillary-structures were observed using a phase contrast inverted microscope Nikon Eclipse Ti equipped with a digital camera and pictures were taken (40x magnification, 3 unit fields/well). Images were analysed using Angiogenesis Analyzer, a plugging developed by Image-J software (http://imagej.nih.gov/ij/ U.S. National Institutes of Health. Bethesda, Maryland, USA). Data on dimensional parameters (total tubule length) and topological parameters (number of nodes, junctions and meshes and total mesh area) of the capillary-like network were analysed in control and treated wells (Gandin et al, 2015). Results were expressed as a percentage of change from control cells.

3.5.7. Liquid chromatography-tandem mass spectrometry analysis

In order to verify the endogenous production of 17R-HDHA by HUVECs release, HUVEC supernatants from cells treated with DHA or ASA or DHA+ASA were harvested and were analysed by Liquid chromatography-tandem mass spectrometry. Samples were centrifuged to remove any cellular material. 50 µl of internal standard was added to 700 µl of sample. The resulting supernatants were immediately applied to C18 SPE cartridges that had been preconditioned with 1 mL Methanol and 1 mL water. Finally, the hydroxyl fatty acids were eluted using 400 µl of a solution composed by acetonitrile:methanol (65%:35%). Samples were evaporated to dryness with a speed vacuum and recovered in 100 µl of a solution 70% (v/v) phase A (water, acetic acid 0.05%, pH: 5.7) and 30% (v/v) phase B (65% Acetonitrile and 35% methanol). 10 µl of each samples were injected in a liquid chromatography Agilent 1100 equipped with a reverse phase column (Kinetex 5u C18, 50 x 2.1 mm). The column was eluted with a linear gradient from 30 to 100% solvent B (methanol:acetonitrile, 65:35) over 9 minutes (Solvent A: 0.05% acetic acid pH: 5.7 with ammonia). The effluent from the high-performance liquid chromatography (HPLC) column was directly infused into an API4000 triple quadrupole operated in negative ion mode. Quantitation was performed using standard curves obtained with synthetic standard and recovery was calculated using deuterated internal standards.

3.6. Data analysis

Data are expressed as means ± standard error (SEM) of at least three independent experiments. Statistical analysis was performed using t-Test (2-tailed probability value) to compare two groups. Multiple comparisons were made by ANOVA followed by Dunnet multiple comparison tests using GraphPad Prism version 6.00 for Windows (San Diego, CA, US). Each of the $p \leq 0.05$ changes was considered statistically significant.

RESULTS

4. RESULTS

4.1. ALA-enriched diet inhibits BC growth and metastasis

40 BALB/c mice were fed for 90 days an α-linolenic (ALA)-rich diet (10% chia oil, ChO) or with a linoleic acid (LA)-rich diet (10% corn oil, CO) before tumor induction. At the end of the experimental protocol, 45 days after tumor induction, animals' general status was optimal. Diet and water consumption were similar for both groups. Tumor incidence, considered as the number of mice which developed tumors at sacrifice, was higher in CO-fed mice (100%) compared with ChO-fed mice (ChO) (85%). Moreover, tumor weight (1.0 ± 0.2 vs 2.2 ± 0.2 g, $p<0.05$) and volume (4.4 ± 0.4 vs 7.2 ± 1.0 mm^3, $p<0.05$) were lower in ChO- vs CO-fed mice, whereas the latency time (22 ± 1 vs 15 ± 2 d, $p<0.05$) was longer in ChO-fed mice. Likewise, ChO-fed mice had significantly fewer metastases when compared to CO-fed mice group (70 ± 0.8 vs 10 ± 0.1, $p<0.05$) (Figure 4.1). In particular, the highest percentage of metastases was located in the lung, retroperitoneum and liver.

a)

Tumor Parameters	CORN DIET	CHIA DIET
Tumor incidence (n)	20	17 *
Tumor weight (g)	2.2± 0.2	1.0± 0.2 *
Tumor volume (mm^3)	7.2± 1.0	4.4± 0.4 *
Metastasis number (n)	10± 1.0	7.0± 0.8 *
Latency time (days)	15± 2.0	22± 1.0 *

b)

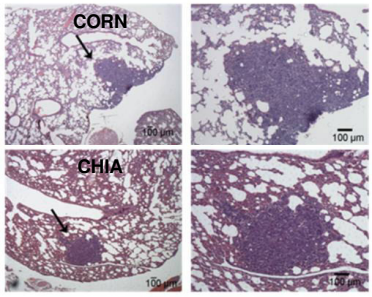

Figure 4.1: Tumor parameters comparison between corn- and chia oil-fed mice.
a) Tumor incidence was considered as the number of mice with tumors (n). Tumor weight was measured using a precision scale (g). Tumor volume was calculated using a digital caliper (mm^3). The number of macroscopic metastases was recorded in all organs (n). Latency was determined as the time between tumor induction and detection of palpable tumors (days). Values are means ± SEM.; n=17-20. Corn vs. chia. t-Test, *p ≤ 0.05. b) Representative images of lung metastasis of hematoxylin/eosin stained tissues from corn and chia oil-fed mice. (4x and 10x magnifications, scale bar 100 µm).

4.2. ALA-enriched diet enhances apoptosis while lowering mitosis

Given that an imbalance between cell proliferation and apoptosis may contribute to tumor progression (Liu et al, 2001), we evaluated apoptosis and mitosis in tumor tissues from ChO- and CO-fed mice stained with hematoxylin and eosin. We found that TUNEL positive cells (as an index of apoptosis)

significantly increased in tumor tissue from ChO-fed mice compared to the CO-group (31.49 ± 0.57 vs. 17.59 ± 2.60 cell/mm^2, $p<0.05$, n=3) (Figure 4.2, a). Consistently, we also found that Ki-67 positive cells (as an index of mitosis) were lower in ChO-fed mice with respect to CO-fed mice (89.88 ± 16.07 vs. 155.17 ± 20.34 cell/mm^2, $p<0.05$, n=3), indicating that cell proliferation was lower in tissues from ChO-fed mice (Figure 4.2, b). These findings are in accordance with the smaller tumor size found in mice receiving ChO-diet with respect to controls.

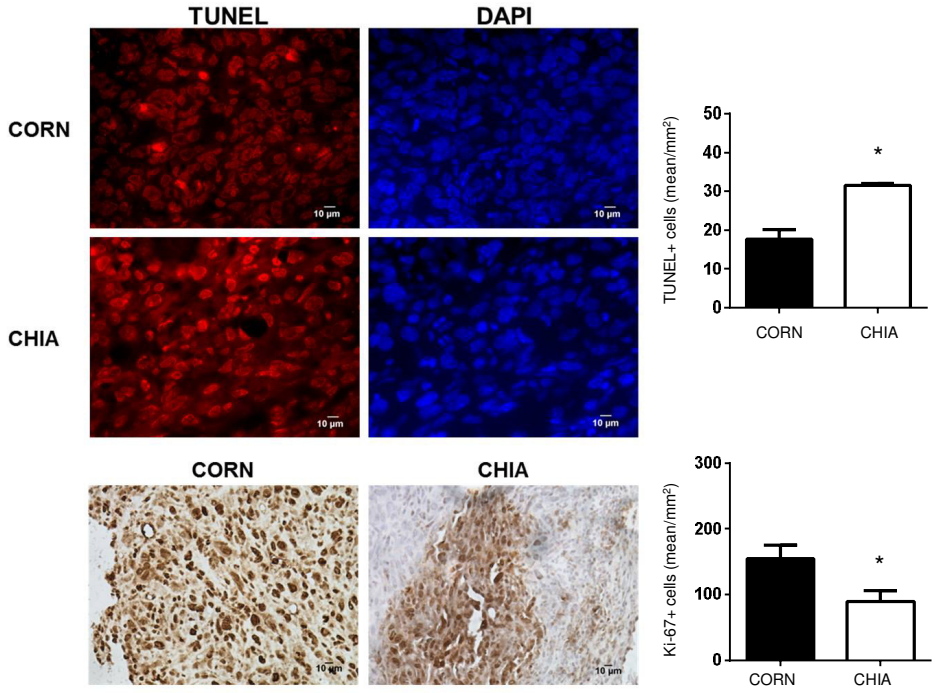

Figure 4.2: TUNEL and Ki-67 assay in tumor tissues of corn- and chia oil-fed mice. a) Apoptosis evaluation in primary tumor section tissues by TUNEL Assay. TUNEL-positive cells in the nuclei are stained in red and total nuclei are stained with DAPI in blue (40x magnification, scale bar 10 µm). Bars show TUNEL-positive cells expressed as mean ± SEM/mm^2. Corn vs. chia. t- Test. *p ≤ 0.05. Experiments were repeated in tumor tissue sections from 3 mice for each dietary condition. b) Mitosis evaluation in primary tumor section tissues by Ki-67 assay. Ki-67-positive cells are stained in brown (40x magnification, scale bar 10 µm). Bars show Ki-67-positive cells expressed as mean ± SEM/mm^2. Corn vs. chia. t-Test. *p ≤ 0.05 Experiments were repeated in tumor tissue sections from 3 mice for each dietary condition.

4.3. ALA-enriched diet decreases tumor-angiogenesis

Since vascular endothelial growth factor (VEGF) is a potent angiogenic factor with a major role in different steps of angiogenesis, we evaluated VEGFR-2 expression in tumor tissues from ChO- and CO-fed mice by immunohistochemistry. We found that the number of vessels was significantly lower in tumor tissues from animals fed ALA-rich diet with respect to controls

(48.9 ± 17.88 vs 146.4 ± 22.24 vessel/mm^2, p<0.05) (Figure 4.3). A large body of evidence indicates that the VEGF-VEGF receptor (R) complex, in particular VEGFR-2, is involved in the pathological angiogenesis promoting the malignancy of solid tumors. Therefore, it is possible to infer that reduced tumor size was influenced by a lower vascularization.

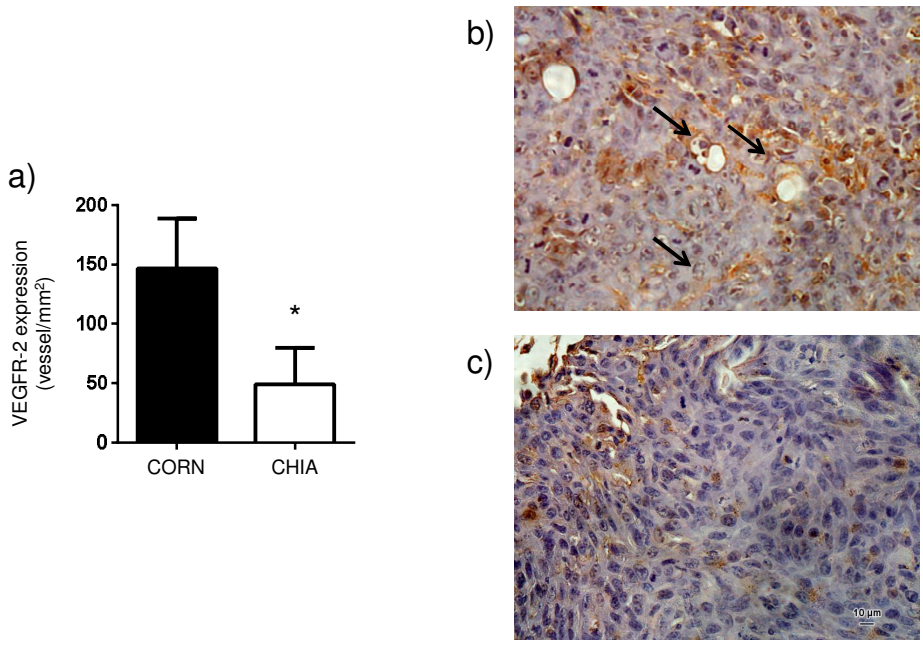

Figure 4.3: Effects of corn- and chia- oil diet on tumor angiogenesis.
Vessel formation was evaluated in tumors tissues from corn- or chia oil fed-mice. a) Bars show vessel density expressed as mean ± SEM/mm^2. Corn vs. chia. t- Test. *p ≤ 0.05. Experiments were repeated in tumor tissue sections from three mice for each dietary condition..b) and c): microphotographs of VEGFR-2 immunostained tumor sections from corn- and chia-fed mice, respectively (40x magnification, scale bar 10 µm).

Given these *in vivo* findings, we next studied whether ω-3 PUFAs have direct anti-angiogenic actions on endothelial cells. In particular we used docosahexaenoic acid (DHA 22:6, ω-3) a downstream metabolite from α-linolenic acid (ALA 18:3, ω-3).

4.4. DHA and AA did not decrease endothelial cell viability

In order to determine possible effects of PUFAs on endothelial cell viability, an MTT assay was performed. We found that HUVECs were maximally viable when treated with 1 to 50 µM DHA for 24h (Figure 4.4, a). Similar results were obtained in cells treated with 1 to 30 µM AA for 48h (Figure 4.4, d). Conversely, when cells were challenged with 50 µM DHA for 48h (65.3 ± 13.0% vs control, p<0.05) or 72h (42.7 ± 13.8% vs control, p<0.05) cell viability

significantly decreased (Figure 4.4, b and c). Overall, these data indicate that DHA concentrations up to 30 µM DHA were suitable to carry out further experiments since cell viability was not significantly affected.

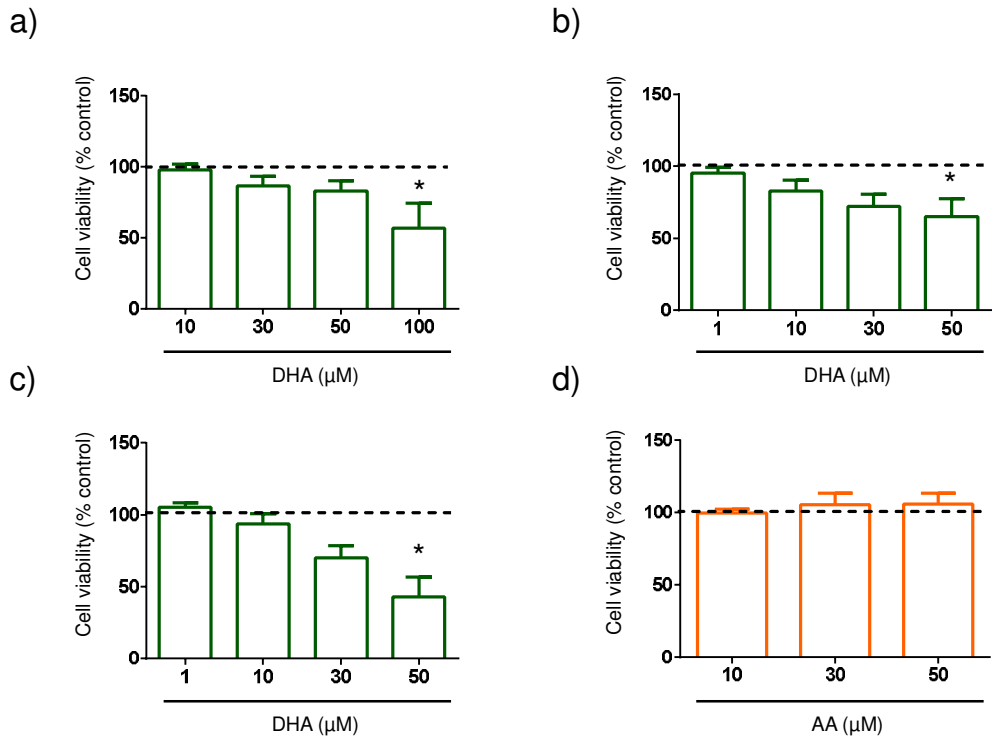

Figure 4.4: Effects of DHA (ω-3) and AA (ω-6) on HUVEC viability after 24, 48 or 72h. HUVECs were incubated for: a) 24, b) 48 or c) 72h with DHA (1-100 µM) or d) 48h with AA (1-50 µM) and cell viability was measured by MTT. Data are expressed as percent of untreated, control cells. Bars show the mean ± SEM of 3-4 independent experiments. Control vs. treatment. ANOVA, *post hoc*-test Dunnet. *p<0.05.

4.5. DHA decreases endothelial cell migration (haptotaxis) in a time- and concentration-dependent manner

In order to evaluate the effects of ω-3 and ω-6 PUFAs on integrin-mediated non-directional motility of endothelial cells (haptotaxis), we used a wound healing migration assay in cells pre-treated with 1-30 µM DHA for 24-72h. As shown in Figure 4.5, we demonstrated that treatment with 30 µM DHA for 24h significantly decreased EC migration (-25% ± 6.8%, p<0.01) (Figure 4.5, a) when compared to untreated control cells taken as 100%. After 48h, 10 or 30 µM DHA significantly reduced endothelial cell migration (-20% ± 1.54% and -44% ± 8.9%, p<0.001, respectively) (Figure 4.5, b). Finally, 72h pre-treatment with increasing (1-30 µM) DHA concentrations significantly reduced cell migration with respect to

controls (Figure 4.5, c). Overall, DHA decreases ECs migration in a time-concentration fashion.

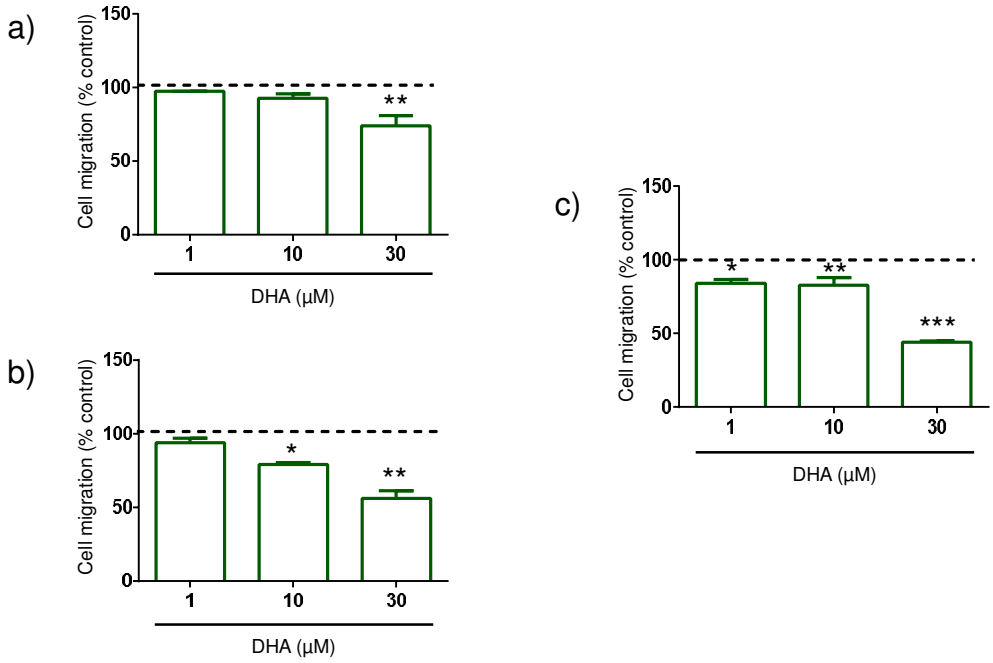

Figure 4.5: Effect of DHA on HUVEC migration (haptotaxis) after 24, 48 or 72h. HUVECs were pre-treated with 1-30 µM DHA for: a) 24, b) 48 or c) 72h. Thereafter, monolayers were wounded (t0), and treated as above for 16h (t16). For each experimental condition, 3 micrograph images were taken at t0 and t16; wound closure was calculated as described in Methods. Bars show the mean ± SEM of 3 independent experiments. Control vs. DHA. Control vs. treatment. ANOVA, *post hoc*-test Dunnet. *p<0.05; **p<0.01; ***p<0.001.

4.6. DHA in the presence of ASA decreases endothelial cell migration (haptotaxis) without affecting viability

We further analyzed the effect of COX-2 acetylated by aspirin in presence of DHA on EC migration by the wound healing assay. Differently from treatment with DHA alone, treatment with 10 µM DHA in presence of 50 µM aspirin (ASA) significantly inhibited EC migration (-36% ± 5.4%, p<0.001) already after 24h (Figure 4.6) while 10 µM AA in the presence of ASA did not induce any effect. Neither ASA nor AA alone affected EC migration as evaluated by the wound healing assay. In order to determine the cytotoxic effects of ASA alone or in presence of DHA, ECs were challenged with increasing concentrations of ASA (10-100 µM) in the presence or absence of 10 µM DHA and the viability test was performed. HUVECs were maximally viable when treated with these compounds for 24h (Figure 4.6, c).

These data suggest that aspirin-acetylated COX-2 metabolites decreased endothelial cell migration by enhancing the anti-angiogenic effects of ω-3 PUFAs without affecting cell viability.

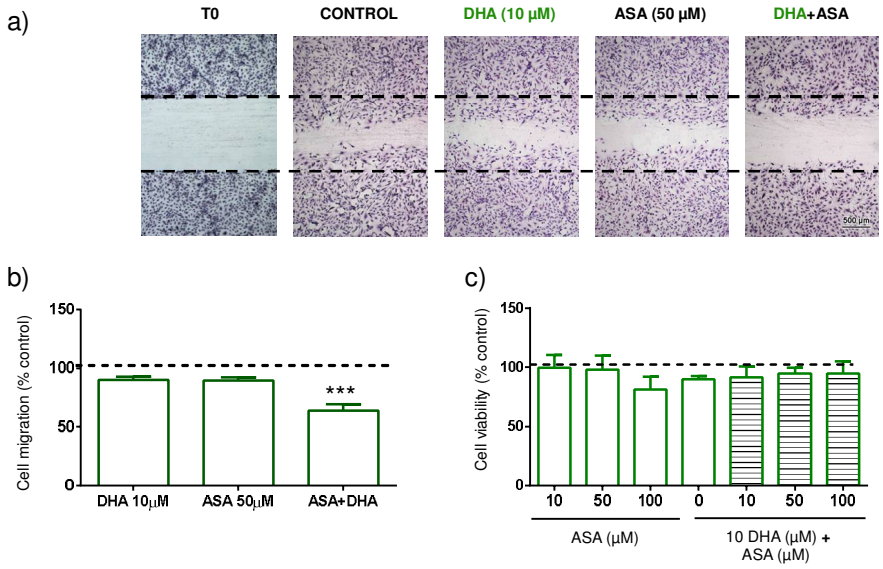

Figure 4.6: Effect of DHA±ASA on HUVEC migration and viability after 24h.
a) Representative images of a wound healing experiment in hematoxylin and eosin stained cells (4x magnification, scale bar 500 µm). b) HUVECs were pre-treated with 10 µM DHA, 50 µM ASA or 10 µM DHA + 50 µM ASA for 24h, wounded (t0), and treated as above for 16h (t16). For each experimental condition, 3 micrograph images were taken at t0 and t16; wound closure was calculated as described in Methods. Bars show the mean ± SEM of 4 independent experiments. Control vs. treatment. ANOVA, *post hoc*-test Dunnet, ***p<0.001. c) HUVECs were incubated for 24h with ASA (10-100 µM) or 10 µM DHA + ASA (10-100 µM) and cell viability was measured by MTT assay. Bars show the mean ± SEM of 3 independent experiments performed in quadruplicate. Data are expressed as % of control. Control vs. treatment. ANOVA, *post hoc*-test Dunnet.

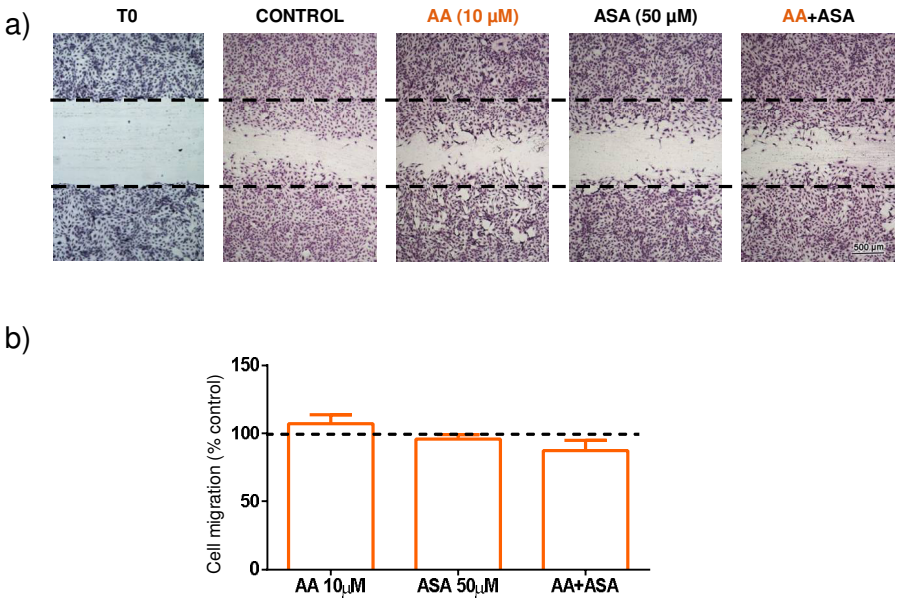

Figure 4.7: Effect of AA in presence or absence of ASA on HUVEC migration after 24h. a) Representative images of a wound healing experiment in hematoxylin and eosin stained cells (4x magnification, scale bar 500 µm). HUVECs were pre-treated with 10 µM AA, 50 µM ASA or 10 µM AA + 50 µM ASA for 24h, wounded (t0), and treated as above for 16 h (t16). For each experimental condition, 3 micrograph images were taken at t0 and t16; wound closure was calculated as described in Methods. Bars show the mean ± SEM of 3 independent experiments. Control vs. treatment. ANOVA, *post hoc*-test Dunnet.

Moreover, when HUVECs were challenged with DHA+ASA for 48h (Figure 4.8, a and b) or 72h (Figure 4.9, a and b) the inhibitory effect on cell migration was accentuated (-44 ± 4.9% and -53 ± 1.2%, $p<0.001$, respectively). Viability of treated cells was not different with respect to control cells at any time or concentration tested (Figure 4.8, c and 4.9 c). Differently from what observed for DHA, pre-treatment of HUVECs with AA in presence of ASA for 48h did not affect cell migration (Figure 4.10, a). Cell viability was also similar with respect to control (Figure 4.10, b). Overall, DHA in presence of ASA enhances its antiangiogenic capabilities and this effect is unrelated to cell proliferation. These data suggest a possible role for metabolites from ASA-acetylated COX-2 in endothelial cell migration.

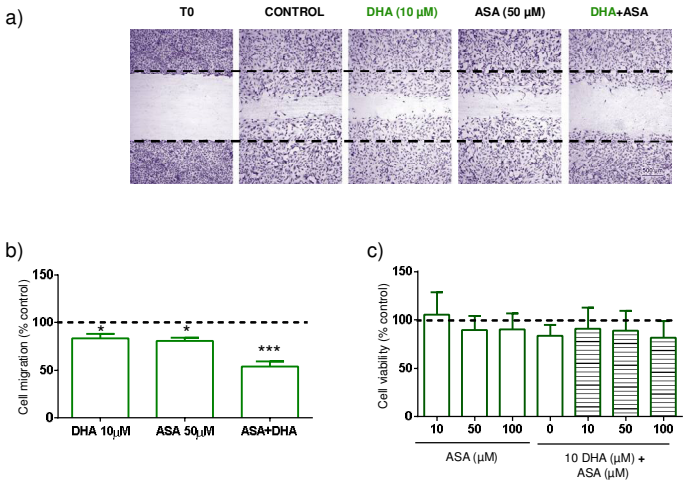

Figure 4.8: Effect of DHA±ASA on HUVEC migration and viability after 48h.
a) Representative images of a wound healing experiment in hematoxylin and eosin stained cells (4x magnification, scale bar 500 µm). b) HUVECs were pre-treated for 48h with 10 µM DHA, 50 µM ASA or 10 µM DHA + 50 µM ASA, wounded (t0), and treated as above for 16h (t16).. For each experimental condition, 3 micrograph images were taken at t0 and t16; wound closure was calculated as described in Methods. Bars show the mean ± SEM of 3 independent experiments. Control vs. treatment. ANOVA, *post hoc*-test Dunnet. *p<0.05; ***p<0.001. c) Cells were incubated for 24h with ASA (10-100 µM) or 10 µM DHA + ASA (10-100 µM) and cell viability was measured by MTT assay. Bars show the mean ± SEM of 3 independent experiments performed in quadruplicate. Data are expressed as % of control. Control vs. treatment. ANOVA, *post hoc*-test Dunnet.

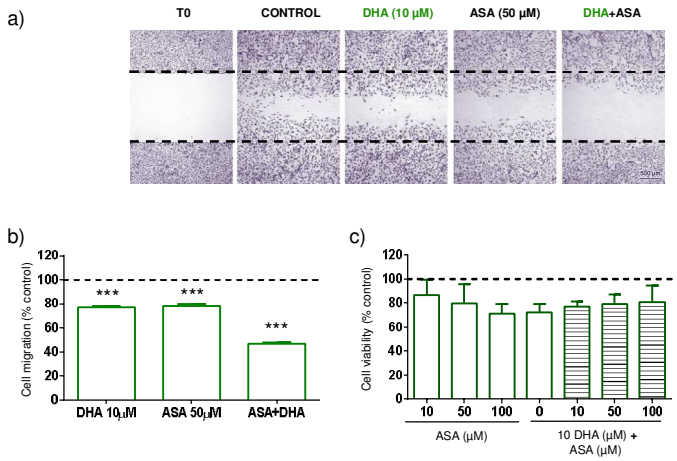

Figure 4.9: Effect of DHA±ASA on HUVEC migration and viability after 72h.
a) Representative images of a wound healing experiment in hematoxylin and eosin stained cells (4x magnification, scale bar 500 µm). b) HUVECs were incubated for 72h with 10 µM DHA, 50 µM ASA or 10 µM DHA + 50 µM ASA, wounded (t0), and treated as above for 16h (t16). For each experimental condition, 3 micrograph images were taken at t0 and t16; wound closure was calculated as described in Methods. Bars show the mean ± SEM of 3 independent experiments. Control vs. treatment. ANOVA, *post hoc*-test Dunnet. ***p<0.001. c) Cells were incubated for 24h with ASA (10-100 µM) or 10 µM DHA + ASA (10-100 µM) and cell viability was measured by MTT assay. Bars show the mean ± SEM of 3 independent experiments performed in quadruplicate. Data are expressed as % of control. Control vs. treatment. ANOVA, *post hoc*-test Dunnet.

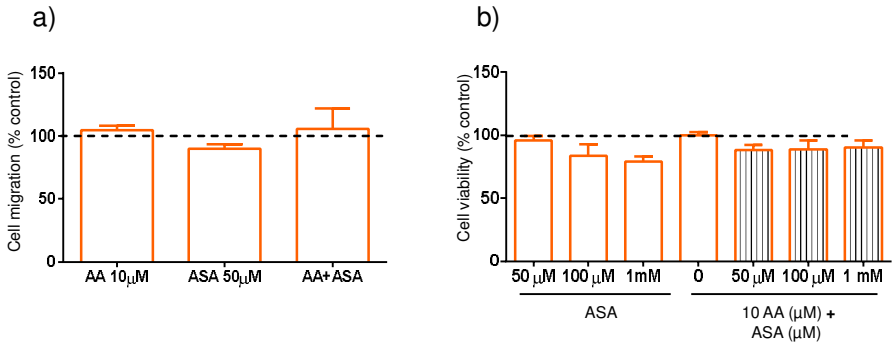

Figure 4.10: Effect of AA±ASA on HUVEC migration and viability after 48h.
a) HUVECs were pre-treated with 10 µM AA, 50 µM ASA or 10 µM AA + 50 µM ASA for 48h, wounded (t0), and treated as above for 16h (t16) with the same treatment. For each experimental condition, 3 micrograph images were taken at t0 and t16; wound closure was calculated as described in Methods. Bars show the mean ± SEM (%) of 3-4 independent experiments. Control vs. treatment. ANOVA, *post hoc*-test Dunnet. b) Cells were incubated for 48h with ASA (10-1000 µM) or 10 µM AA + ASA (10-1000 µM) and cell viability was measured by MTT assay. Bars show the mean ± SEM of 3 independent experiments performed in quadruplicate. Data are expressed as % of control. Control vs. treatment. ANOVA, *post hoc*-test Dunnet.

4.7. 17R-HDHA decreases endothelial cell migration (haptotaxis) without affecting viability

In order to assess the role of DHA metabolites from acetylated COX-2 in cell migration, we tested the anti-angiogenetic capability of 17R-HDHA. We found that after 24h pre-treatment, 1 and 3 µM 17R-HDHA decreased EC migration (-12% ± 4.1% and -28% ± 1.9%, $p<0.01$, respectively) (Figure 4.11, b). Moreover, this effect was higher after 48h (-22% ± 3.7% and -37% ± 7.4%, $p<0.01$, respectively) (Figure 4.11, a and c), without affecting EC viability at any time or concentration tested (Figure 4.11, d). These data support an effect of 17R-HDHA diminishing EC migration towards a gradient of cell adhesion sites.

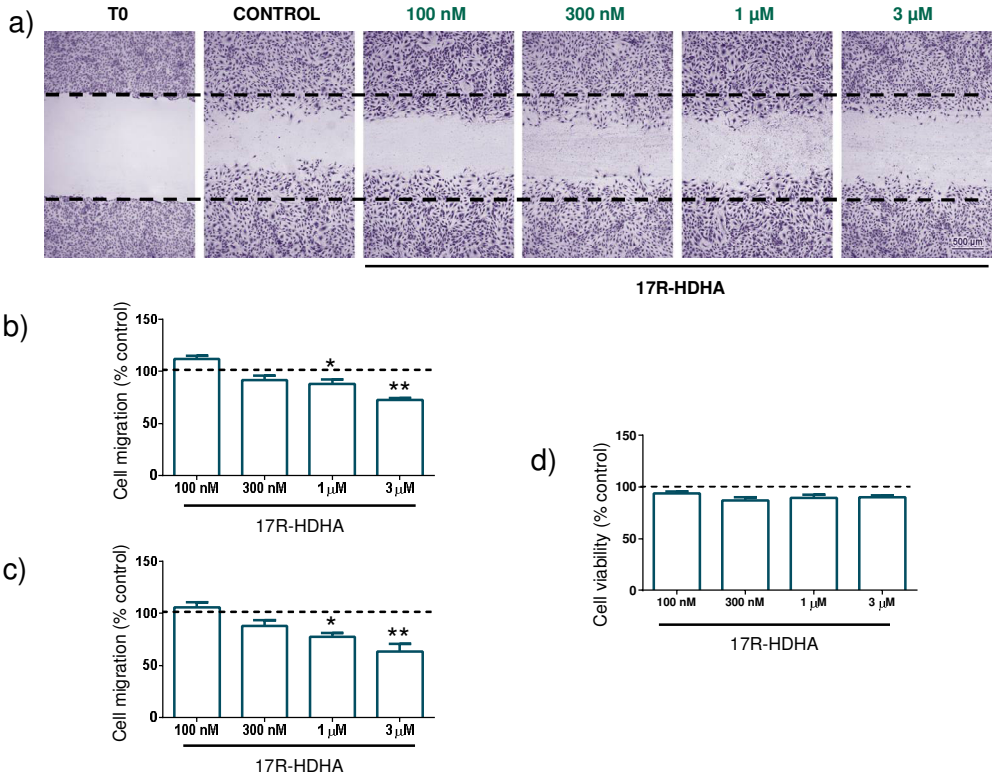

Figure 4.11: Effect of 17R-HDHA on HUVEC migration and viability.
a) Representative images of a wound healing experiment in hematoxylin and eosin stained cells after 48h treatment (4x magnification, scale bar 500 µm). HUVECs were pre-treated with 100 and 300 nM or 1 and 3 µM 17R-HDHA for a) 24h or b) 48h, wounded (t0), and treated as above for 16h (t16). For each experimental condition, 3 micrograph images were taken at t0 and t16; wound closure was calculated as described in Methods. Bars show the mean ± SEM of 3 independent experiments. Control vs. treatment. ANOVA, *post hoc*-test Dunnet. *p<0.05; **p<0.01. d) Cells were incubated for 48h with 100 nM-3 µM 17R-HDHA and cell viability was measured by MTT assay. Bars show the mean ± SEM of 3 independent experiments performed in quadruplicate. Data are expressed as % of control. Control vs. treatment. ANOVA, *post hoc*-test Dunnet.

4.8. 17R-HDHA decreases endothelial cell migration (chemotaxis) in a concentration-dependent manner

Chemotaxis assays are useful to measure HUVEC migration in response to an attractant gradient, an essential step in tumor angiogenesis. In order to investigate the role of 17R-HDHA on EC migration in response to a chemical stimulus at shorter time points, we used a microchemotaxis chamber. Already after 6h, 17R-HDHA (300 nM, 1-3 µM) reduced FBS-induced EC migration (-19.5% ± 1.4%, -30% ± 4.0% and -64% ± 3.5%, respectively; **p<0.01 and ***p<0.001,) (Figure 4.12, a and b), while DHA alone did not show any effect (data not shown).

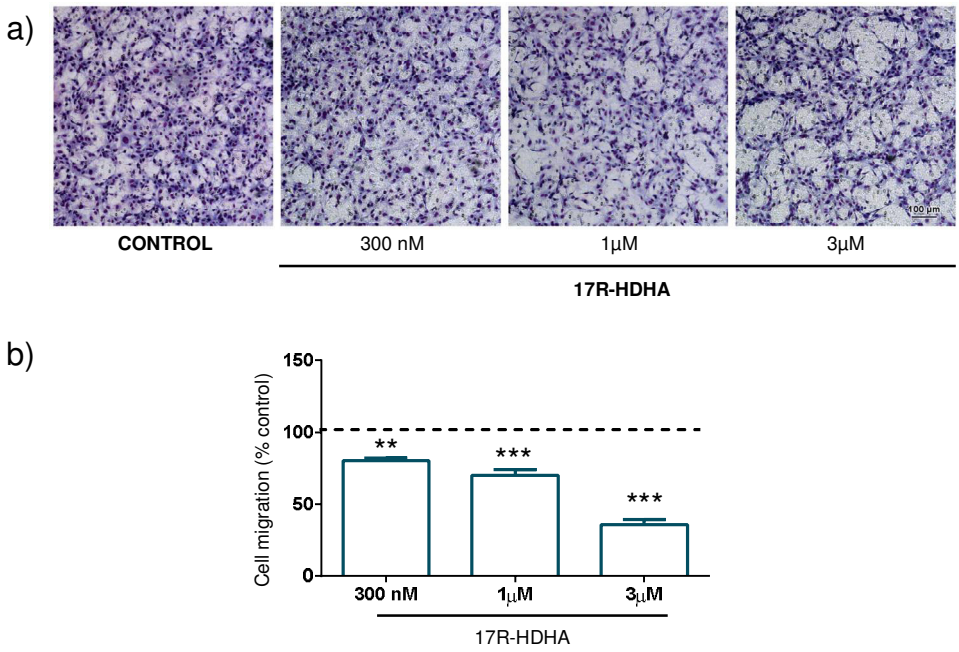

Figure 4.12: Effect of 17-R HDHA on HUVEC chemotaxis. HUVEC migration toward 15% FBS (control) was measured in a microchemotaxis chamber in the presence of increasing concentration of 17R-HDHA (300 nM-3 µM). a) Representative images of migrated cells on the bottom of the filter membrane as detailed in Methods. Migrated cells were counted under a light microscope (20x magnification, scale bar 100 µm). b) Bars show the mean ± SEM of 3 independent experiments performed in sextuplicate and expressed as % of control. Control vs. treatment. ANOVA, *post hoc*-test Dunnet. **p<0.01; ***p<0.001.

4.9. DHA in presence of ASA or 17R-HDHA decrease capillary tube-like formation

We further analyzed the effect of 17R-HDHA on tubularization, the process of organization of endothelial cells in capillary tube-like structures when cultured onto extracellular matrix proteins. To determine whether cells treated with 10 µM DHA in the presence of 50 µM ASA or 17R-HDHA at concentrations 300 nM or 3 µM affected the formation of capillary-like structures, we used a Matrigel® assay.

Qualitative analysis of imagines shows decreased formation of capillary-like structures in ECs treated with either 10 µM DHA in the presence of 50 µM ASA or 17R-HDHA for 6h (Figure 4.13).

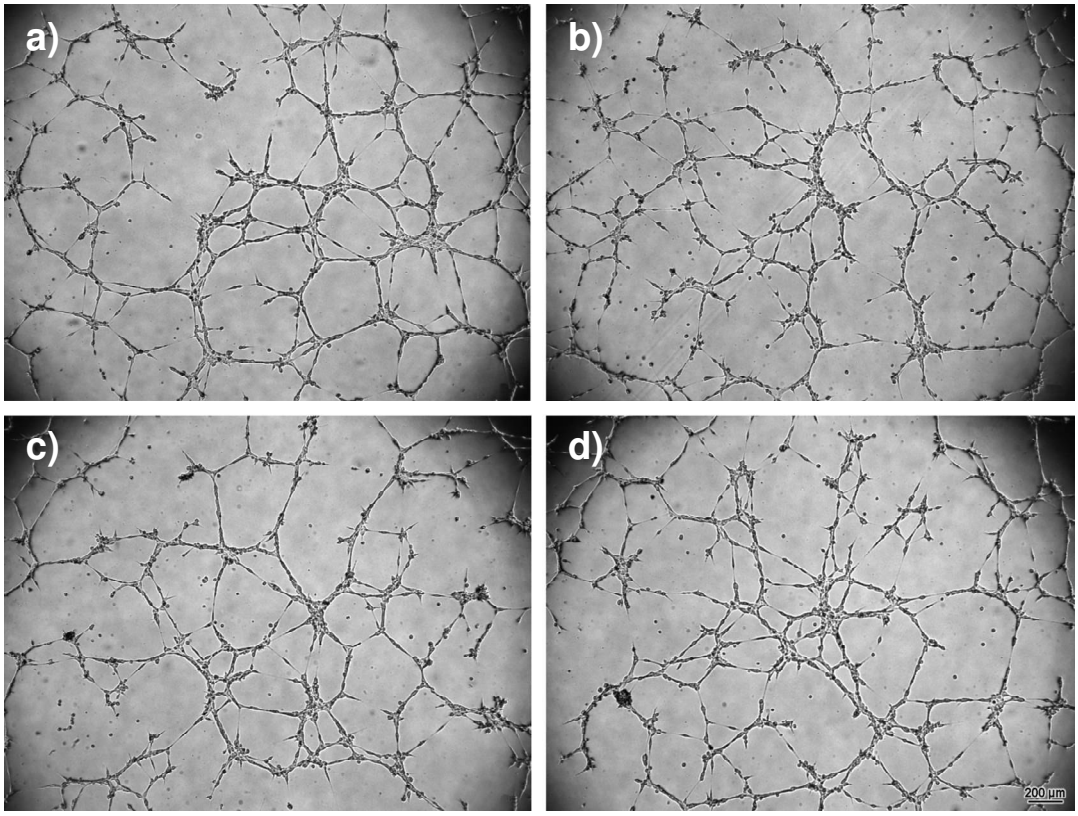

Figure 4.13: Effect of 10 µM DHA in the presence of 50 µM ASA or 17R-HDHA on HUVEC capillary tube-like formation. Representative phase contrast microphotographs showing the effect of: a) control b) 10 µM DHA in the presence of 50 µM ASA, c) 300 nM 17R-HDHA and d) 3 µM 17R-HDHA on HUVECs cultured onto Matrigel® for 4h (40x magnification, scale bar 200 µm).

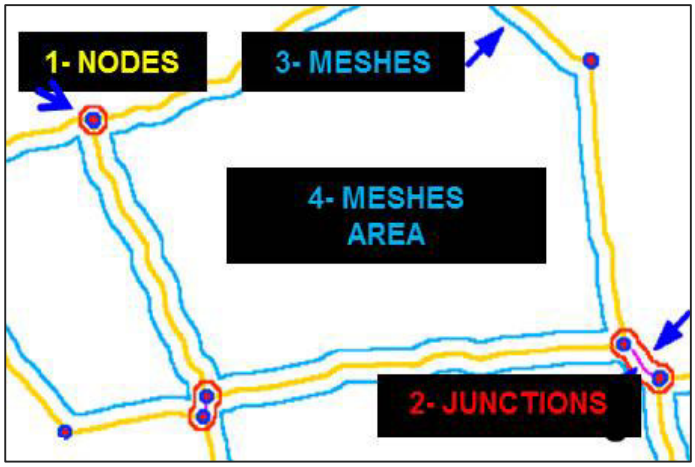

Figure 4.14: Schematic representation of parameters used for the quantitative analysis.

Moreover, quantitative analysis (Figure 4.15) of topological parameters (number of nodes, junctions, meshes and total mesh area, see figure 4.14) shows that 10 µM DHA in the presence of 50 µM ASA (-33 ± 11%, $p<0.05$) as well as 300 nM and 3 µM 17R-HDHA (-30% ± 9.6% and -32% ± 2.8%, $p<0.05$, respectively), decreased node formation (Figure 4.15, a). The number of junctions showed a tendency to decrease but the difference was not significant with respect to control cells (Figure 4.15, b). Meshes (Figure 4.15, c) as well as meshes area (Figure 4.15, d) significantly decreased in treated compared to control cells. As for *dimensional parameters* (total tubule length), no significant differences were observed in treated ECs with respect to controls (Figure 4.15, e). These data demonstrate that DHA in presence of ASA as well metabolites from acetylated COX-2 exerted an anti-angiogenic effect by decreasing capillary tube-like formation.

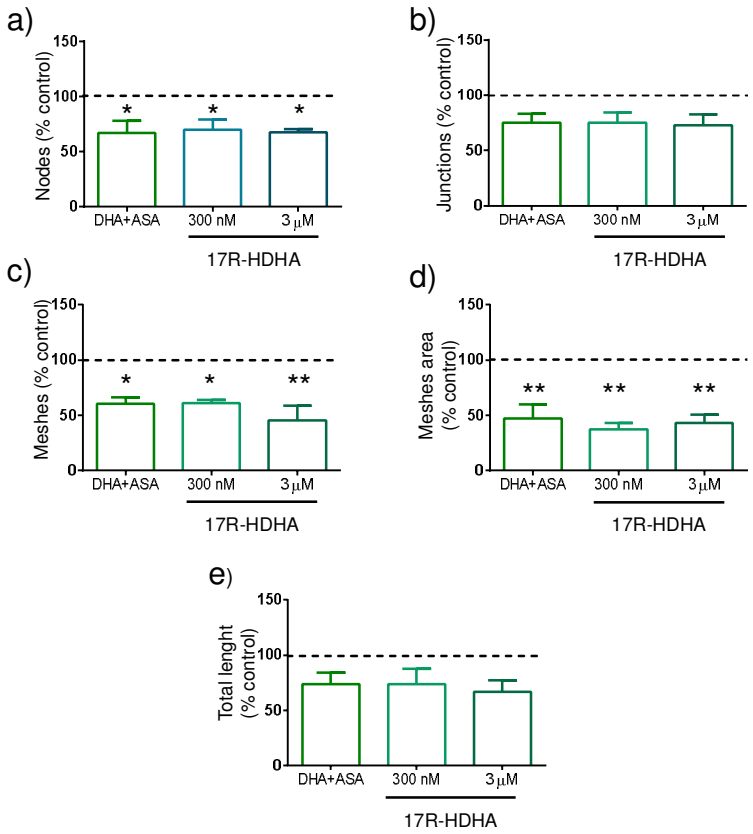

Figure 4.15: Effect of 10 µM DHA in the presence of 50 µM ASA and 17R-HDHA on HUVEC capillary tube-like formation. Quantitative analysis of the effect of 10 µM DHA in the presence of 50 µM ASA and 17R-HDHA on topological parameters: a) number of nodes, b) junctions, c) meshes and d) mesh area; and on dimensional parameters: e) total length. After 6h incubation, 3 micrograph images/each experimental condition were taken. Bars show the mean ± SEM of 3 independent experiments. Data are expressed as % of control. Control vs. treatment. ANOVA, *post hoc*-test Dunnet. *$p<0.05$; **$p<0.01$.

4.10. DHA in the presence of ASA increases endothelial 17R-HDHA production.

In order to demonstrate the ability of HUVEC to produce 17R-HDHA, we analyzed the supernatants of cells pre-treated with DHA (10 µM), ASA (50 µM) or DHA (10 µM) in presence of ASA (50 µM) for 24 hours by liquid chromatography-tandem mass spectrometry. We demonstrated that HUVECs treated with DHA in presence of ASA produced higher amounts of 17R-HDHA with respect to cells treated with DHA alone (Figure 4.16). These data suggest that ECs treated with aspirin in the presence of DHA are able to synthesize 17R-HDHA.

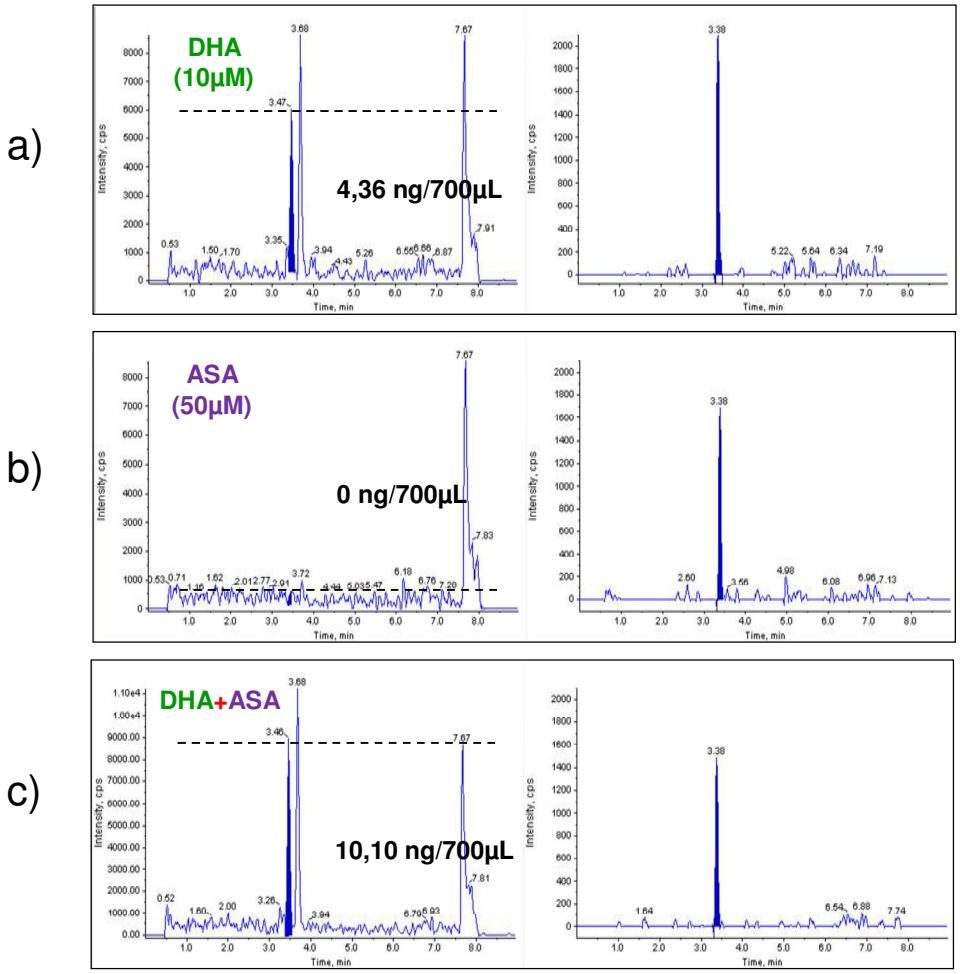

Figure 4.16: Synthesis of 17R-HDHA in HUVECs treated with DHA, ASA or DHA+ASA. Cell supernatants were analyzed using LC-MS/MS. Representative MS spectrum of 17R-HDHA from HUVECs incubated for 24h with a) 10 µM DHA, b) 50 µM ASA and c) 10 µM DHA in the presence of 50 µM ASA. In the right graph, added 17R-HDHA was used as a standard (retention time: 3.38 min).

DISCUSSION

5. DISCUSSION

This research began in Argentina where mice were fed a diet based on chia (*Salvia hispanica L*) seeds oil (ChO diet) as a source of α-linolenic acid (ALA18:3, ω-3). The first specific aim was to determine the anti-growth and antiangiogenic effects of a ChO diet on a syngeneic mouse model of breast cancer (BC). The second specific aim was to investigate the role of metabolites from aspirin (ASA)-acetylated COX-2 in the presence of either docosahexaenoic acid (DHA 22:6, ω-3), a downstream product of ALA, or arachidonic acid (AA 20:4, ω-6), in several *in vitro* models of angiogenesis. *Ex vivo* and *in vitro* angiogenesis experiments have been carried out in Italy.

We showed reduced tumor growth parameters in mice fed the ALA-enriched diet. The direct route through which ω-3 PUFAs are hypothesized to act in tumor cells is via incorporation into cell membranes (Turk et al, 2013). Vegetable oils with more than 50% of ALA in their composition such as rosa canina, sacha inchi and chia oil are good sources of ω-3 PUFAs. Recent studies performed in rodents fed vegetable oils have shown that ALA can be converted to DHA in organs such as liver and brain (Valenzuela et al, 2014). Moreover, it has been demonstrated that an ALA-rich diet increases the level of ALA and its downstream metabolites, such as eicosapentaenoic acid (EPA 20:5, ω-3) and DHA, in tumor tissues (Mason et al, 2015; Espada et al, 2007). Thus, physical-chemical alterations of the lipid microenvironment may affect the structural and functional properties of membranes, thereby altering the growth characteristics of neoplastic cells (Corsetto et al, 2012). An indirect possible route through which ω-3 PUFAs are hypothesized to act is related to their metabolic conversion into bioactive metabolites. An important mechanism for the health-promoting effects of ω-3 PUFAs is the suppression of prostanoid generation derived from the

metabolism of linoleic acid (LA 18:2, ω-6) and AA, which in turn might be involved in cell growth, apoptosis, angiogenesis and metastasis.

In particular, it has been demonstrated that EPA and DHA inhibit the formation of ω-6 lipid mediators via multiple mechanisms, including reduced release of AA from membrane phospholipids, inhibition of the enzymatic activities of metabolizing enzymes and direct competition with AA for enzymatic conversions. AA, EPA and DHA also serve as alternative substrates of lipid metabolism enzymes, leading to increased formation of ω-3 lipid mediators (Wang et al, 2014). In particular, 13(S)-HODE (produced from LA metabolism by 15-LOX) is associated with rapid proliferation, aggressive grade and metastases in breast cancer (O'Flaherty et al, 2013).

We evaluated the effects exerted by a ChO-diet enriched in ALA on tumor cell proliferation and apoptosis. We found that Ki-67-positive cells were less abundant in mammary tumors from mice fed the ChO-diet compared to control mice (fed corn oil diet, enriched in LA), whereas the number of TUNEL-positive cells significantly increased. The effects on tumor cell growth observed in ChO-fed mice could be explained, at least in part, by changes in the lipid composition of membrane microdomains, which in turn may lead to the downregulation of growth factor receptors such as epidermal growth factor receptors (EGFR, HER-2) involved in BC growth (Corsetto et al, 2011). Our data are in line with previously published data obtained in animal fed different ω-3 enriched diets. In particular, dietary intake of ALA from flaxseed has been shown to suppress breast tumor growth in ovariectomized athymic BALB/c mice (Chen et al, 2007). In addition, previously published data from the Argentinian lab demonstrated that ALA-rich diets based on mystol or perilla oils reduced mammary tumor growth (Muñoz et al, 1995; Nakamaya et al, 1993). *In vitro* data support an anti-proliferative and pro-apoptotic role of ALA in several cell lines. In particular, ALA

upregulates Bax expression (pro-apoptotic) and downregulates Bcl-2 (anti-apoptotic) expression in a dose-dependent manner in an ER-positive breast cancer cell line (Kim et al, 2009). This finding suggests that ALA mediates growth-inhibitory and pro-apoptotic effects in estrogen-positive breast cancer cells.

Angiogenesis is a tightly regulated process that contributes to tumor growth and metastasis (Potente et al, 2011). A large body of evidence indicates that the vascular endothelial growth factor (VEGF)-VEGF-receptor (VEGFR) complex, in particular VEGFR-2, is involved in pathological angiogenesis, which promotes the malignancy of solid tumors. Moreover, VEGFR-2 targeted therapy was demonstrated to restore the abnormal vasculature in tumors, enhancing their susceptibility toward conventional therapy (Kampen, 2012). In order to determine the effect of ω-3 PUFAs in tumor angiogenesis, we evaluated VEGFR-2 expression in primary tumor sections from animals fed ChO- and control diets. In our model, the vessel number was significantly lower in tumor tissues from animals fed the ALA-rich diet with respect to controls. These data suggest that the reduced tumor size observed in ChO-fed mice could also be due to impaired vascularization. Although several *in vivo* studies showed that ω-3 PUFAs inhibit angiogenesis (Spencer et al, 2009), this is the first evidence for an antiangiogenic effect of a ChO diet as a source of ALA. In particular, a study carried out in a murine model of breast cancer showed that a ω-3-rich diet inhibited primary mammary tumor growth through the modulation of specific determinants of vascularization, such as CD31 and VEGF (Mukutmoni-Norris et al, 2000). It has also been demonstrated that fish oil-fed rats inoculated with a highly metastatic cell line display decreased microvessel density along with decreased metastasis, suggesting that angiogenesis contributes to the anti-metastatic effect of dietary ω-3 PUFAs (Mannini et al, 2009). In addition, in transgenic Fat-1 mice (able to

convert ω-6 to ω-3 PUFAs) inoculated with a lung cancer cell line, tumor growth and angiogenesis were significantly diminished compared to wild-type mice (Taguchi et al, 2014). Moreover, it has been shown that VEGF circulating blood levels are reduced in individuals with lower serum ω-6 PUFAs/ω-3 PUFAs ratio (due to higher ω-3 PUFA levels), with respect to individuals with higher ω-6 PUFAs/ω-3 PUFAs ratio (Ambring et al, 2006).

Starting from in vivo data by our group and others supporting a role for ω-3 PUFAs in decreasing tumor-angiogenesis, we next investigated whether ω-3 PUFAs have direct anti-angiogenic actions on human endothelial cells. In particular, we used DHA (22:6, ω-3), a downstream metabolite of ALA (18:3, ω-3). We compared the effects of DHA with those of AA (20:4, ω-6). Interestingly, in mammals DHA is found predominantly in neural tissue, where it may constitute up to 25-35% of the total membrane acyl chains (Strokin et al, 2004). Therefore, in order to test specific effects in endothelial cells, DHA has to be added exogenously.

Several *in vitro* and *in vivo* studies have shown that DHA decreases, while AA promotes angiogenesis (Kang and Liu, 2013; Zhang et al, 2013). However, compared to specific AA-derived metabolites, less data are available on the role of DHA metabolites (Wang et al, 2014). In particular, it has been recently demonstrated that acetylated COX-2 in presence of either AA or ω-3 PUFAs generates several metabolites from DHA including 17R-HDHA (Serhan, 2015). Interestingly, metabolites produced *in vivo* by ω-3 PUFAs have potent anti-inflammatory effects and, considering that inflammation can stimulate angiogenesis, it is conceivable that reduced inflammation modulates angiogenesis *in vivo*. However, although the ability of metabolites from ASA-acetylated COX-2 to promote the resolution of inflammation is well recognized, their direct role in angiogenesis is almost unexplored (Serhan, 2015).

First, we assessed the effects of PUFAs on endothelial cell viability. We found that human umbilical vein endothelial cells (HUVECs) were viable when treated with DHA at concentrations up to 30 µM for 24 to 72 hours. Similarly, in HUVECs challenged with AA, no effects on HUVEC viability were observed in the concentration range 10-50 µM after treatment for up to 48h. Thereafter, we tested cell viability on EC challenged with 10 µM DHA (24-72h) or 10 µM AA (48h) in presence of acetylsalicylic acid (ASA) or with ASA alone (10 µM – 1 mM); also in this case cell viability did not differ with respect to controls. Consistently, Szymczak et al. (2008) treated HUVECs with DHA or AA at concentrations up to 160 µM for 20h, and observed that cell viability decreased only when PUFA concentrations exceeded 40 µM. Similarly, in the human microvascular endothelial cell line HMEC-1 co-treated with VEGF (20 ng/mL) and 50 µM DHA for 48h, cell viability decreased with respect to control cells (Zhuang et al, 2013). In contrast, other authors showed no adverse effects on cell growth by 100 µM DHA in the presence or absence of VEGF (50 ng/mL) (Chao et al, 2014). In an *in vitro* study aimed to investigate whether ASA interferes with the proliferative stage of angiogenesis, the authors found that treating HUVECs with 0.01-0.10 mM ASA for 24h did not change cell proliferation. However, treatment with 1 mM ASA inhibited HUVEC proliferation at later time points (Pearce et al, 2003). Moreover, Borthwick et al. (2006) found that in a well-characterized microvascular cell line (HMEC-1) challenged with ASA (0.5-5 mM) no effects on cell proliferation were detectable with up to 0.5 mM ASA, whereas 2 and 5 mM ASA caused a significant decrease in cell viability after 48h. Overall, decreased proliferation and increased apoptosis have been reported as major effects of ω-3 PUFAs in cultured endothelial cells as well as in other cells. However, some of these effects may be due to lipid peroxidation promoted by high PUFA concentrations (100-300 µM) (Artwohl et al, 2004). The present study was

performed using PUFA concentrations (10-30 µM) that do not affect cell proliferation in order to avoid effects on the proliferative stage of angiogenesis. Interestingly, a recent paper demonstrated that incorporation of DHA in cell membranes of rat cardiomyocytes challenged with 25 µM DHA approximates the incorporation levels reached after treating rats with a diet corresponding to about 4 g total ω-3/day (Lamaziere et al, 2015). This dietary intake is likely to correspond to that provided by supplementation with recommended dose in humans (Shearer et al, 2012).

We further analyzed endothelial cell migration, which represents an important step of angiogenesis. Cell migration requires tight regulation of the cell contractile and non-contractile states as well as integration of signals elicited by haptotactic and chemotactic stimuli (Lamalice et al, 2007). We first performed wound healing assays in order to evaluate endothelial cell haptotaxis, a process of cell migration activated in response to integrins binding to extracellular matrix (ECM) components (Lamalice et al, 2007). We observed a significantly decreased cell migration in HUVECs pre-treated with 30 µM DHA for 24h. At later time points (48 or 72 h), endothelial cells migration was significantly and to a greater extent reduced in cells challenged with lower DHA concentrations (10 µM) with respect to controls. Overall, DHA decreased HUVEC migration in a time- and concentration-dependent fashion. A recent study demonstrated that in the HMEC-1 cell line treatment with 50-100 µM DHA for 24 h inhibits wound closure by up to 30% (Zhuang et al, 2013). As noted above, DHA decreases while AA promotes angiogenesis (Kang and Liu, 2013). Recently, Chao et al. (2014) demonstrated that 8h treatment with 100 µM DHA inhibits VEGF-induced cell migration via binding to G-protein coupled receptor 120 (GPR120), inhibition of extracellular-signal-regulated kinases (ERK1/2) and endothelial nitric oxide synthase (eNOS) phosphorylation, which is critical to cell migration. However, the

mechanisms underlying the inhibition of cell migration by DHA, which is critical for angiogenesis and wound repair, are not fully understood. The ω-3 PUFAs, act in part via formation of certain lipid metabolites (LMs) such as cyclooxygenase (COX)-derived prostaglandin E3 (PGE_3) (Szymczak et al, 2008), lipoxygenase (LOX)-derived 4-hydroxy-docosahexaenoic acid (4-HDHA) (Sapieha et al, 2011), cytochrome P450 (CYP)-derived epoxydocosapentaenoic acids (EDPs) (Zhang et al, 2013), as well as unique ω-3 lipid autacoids such as resolvins and protectins (Kang and Liu, 2013).

It has been shown that several COX-1 and COX-2 AA-derived eicosanoids have a pro-angiogenic effect (Salvado et al, 2013; Nie et al, 2000; Pola et al, 2004). In particular, AA-derived PGE_2 has been extensively studied in the context of tumor progression, including the angiogenic process. For example, AA-derived PGE_2 augmented, whereas EPA (20:5 ω-3, a precursor of DHA)-derived PGE_3 suppressed growth factor-induced angiopoietin 2 synthesis, suggesting a beneficial effect of ω-3 PUFAs in reducing angiogenesis (Szymczak et al, 2008). Moreover, pharmacological and gene silencing experiments have recently demonstrated that endothelial PGE_2 biosynthesis is dependent on COX-1, rather than COX-2, and plays a pivotal role in the formation of capillary-like structures (Salvado et al, 2013). Previously, other authors supported a role for PGE2 in angiogenesis (Greenhough et al, 2009; Namkoong et al, 2005). For example, a study demonstrated a role for TXA_2, another metabolite generated from AA, in angiogenesis (Nie et al, 2000; Ashton et al, 2004). Conversely, the direct role of DHA metabolites in the setting of angiogenesis has been less investigated. However, generation of AA metabolites decreases when ω-3 PUFAs are added to the culture medium of endothelial cells. In particular, enrichment of bovine endothelial cells with DHA and EPA reduces their capacity to produce PGI_2 (Hadjiagapiou et al, 1986; Hadjiagapiou et al, 1987), an AA

metabolite with angiogenic properties *in vivo* mediated through VEGF induction (Pola et al, 2004).

Recent research showed a high degree of inter-individual variability in metabolizing ω-3 PUFAs to generate LMs upon dietary intake of ω-3 PUFAs. Clearly, it is important to elucidate the specific lipid metabolizing enzymes and metabolites required for the anti-tumor and anti-angiogenic effects of ω-3 PUFAs. The identified enzymes and metabolites could serve, for example as biomarkers to screen the sub-populations which are most likely to respond to ω-3 PUFAs, or develop personalized doses for ω-3 supplementation. In addition, bioactive ω-3 LMs could serve as biotemplates to design more potent and safer therapeutic drugs. Compared with the ω-6 series LMs, the roles of ω-3 series LMs in angiogenesis and cancer have been less investigated (Wang et al, 2014).

Starting from this knowledge, we further investigated the role of DHA metabolites from aspirin-acetylated COX-2 in angiogenesis. We found that, when treating HUVECs with low DHA concentration (10 µM) in the presence of 50 µM ASA, differently from what observed with DHA alone, cell migration was significantly inhibited already after 24h. Moreover, in HUVECs challenged with low DHA concentration (10 µM) in the presence of 50 µM ASA for 48h or 72h the inhibitory effect on cell migration was accentuated. In contrast to what observed with DHA, when HUVECs were challenged with 10 µM AA in the presence of ASA, no effects on endothelial cell migration were observed after 24 or 48h. In addition, neither ASA nor AA alone affected EC migration as evaluated by the wound healing assay. These findings are partially in contrast with previously published data demonstrating that ASA-triggered lipoxins (AT-L, generated by acetylated COX-2 in the presence of AA) inhibit VEGF-induced EC migration in a concentration-dependent fashion (Fierro et al, 2002). In the latter study, however, the authors did not test the effects of AA in the presence of ASA on cell

migration, rather they directly evaluated the effect of AT-L. In our work, we did not observe any effect, probably because endogenous AT-L precursors are unstable. Future experiments with exogenous 15R-HETE could help address these discrepancies.

It is widely recognized that, while COX-1 acetylated by ASA is permanently inactivated, acetylated COX-2 is still capable to metabolize PUFAs to a novel series of R-containing precursors that are specialized lipid mediators with pro-resolving activity (Dovizio et al, 2013; Serhan et al, 2002). In particular, acetylated COX-2 in the presence of either AA or ω-3 PUFAs generates several metabolites including 18R-HEPE from EPA and 17R-HDHA from DHA. These metabolites, after oxygenation by 5-LOX generally expressed by inflammatory cells such as neutrophils, generate ASA-triggered lipoxins (AT-L, from AA) or resolvins (AT-Rv D, from DHA) (Serhan et al, 2002). There is little evidence for the expression of 5-LOX by endothelial cells (Walker et al, 2002), and we were unable to detect this enzyme in HUVECs (unpublished data). Overall, based on results in HUVECs treated with DHA and ASA, we hypothesize that aspirin-acetylated COX-2 metabolites decreased endothelial cell migration by enhancing the anti-angiogenic effects of ω-3 PUFAs.

We found that pre-treating HUVECs with 17R-HDHA (1 and 3 μM) for 24h decreased EC migration as evaluated by the wound healing assay, and this effect further increased after 48h treatment. We found no significant effect on cell viability using 17R-HDHA up to 3 μM for 48h. Next, we evaluated the effect of 17R-HDHA on EC migration in response to FBS (containing a mixture of growth factors). Already after 6h, 17R-HDHA (300 nM-3 μM) reduced HUVEC migration in a dose-dependent manner, while DHA alone did not show such short-term effect (data not shown). These data are compatible with the production of bioactive metabolites from DHA, and strongly support a role for DHA metabolites

generated by ASA-acetylated COX-2 in inhibiting endothelial cell migration. To further explore the role of 17R-HDHA, we tested whether treatment with 10 µM DHA in the presence of 50 µM ASA or with 17R-HDHA alone affected the formation of capillary-like structures by endothelial cells when cultured on extracellular matrix proteins. We found that 10 µM DHA in the presence of 50 µM ASA as well as exogenously added 17R-HDHA decreased the ability of endothelial cells to organize into capillary-like structures, strongly supporting a role for this metabolite in the above-described effects.

Finally, we analyzed the ability of HUVEC to produce 17R-HDHA endogenously. The supernatants of HUVECs pre-treated with DHA (10 µM) or ASA (50 µM) or DHA plus ASA for 24h were analyzed by liquid chromatography-tandem mass analysis. The data show that HUVECs treated with DHA in presence of ASA produced higher amounts of 17-HDHA with respect to cells treated with DHA alone. These results suggest that 17-HDHA mediates the antiangiogenic effects observed in cells treated with DHA in the presence of ASA. Unfortunately, we were not able to distinguish S- or R-containing isoforms, but since HUVECs do not express detectable mRNA levels of 15-LOX (Lee et al, 2001), the enzyme capable of converting DHA to 17S-HDHA, we inferred that the 17-HDHA detected was the isomer R. This is the first evidence demonstrating the endogenous production of 17-HDHA in non-stimulated human endothelial cells.

The *in vivo* formation of 17-HDHA has been already demonstrated in different cells and tissues. Serhan and co-authors (2002) demonstrated that brain of aspirin-treated mice produces endogenous 17R-HDHA as do human microglial cells. Similarly, the same authors also demonstrated that in hypoxic HUVECs stimulated with inflammatory cytokines, COX-2 converted DHA to 13-hydroxy-DHA that switched with ASA to 17R-HDHA. In addition, 17-HDHA has been

detected in a murine colitis model after treatment with ASA and DHA (Köhnke et al, 2013).

Overall, our data support a role for DHA metabolites, in particular 17-HDHA generated by ASA-acetylated COX-2, in inhibiting endothelial cell angiogenesis. We cannot exclude that high levels of ω-3 PUFAs incorporated into cell membranes reduce angiogenesis through decreased production of pro-angiogenic AA-derived eicosanoids. However, the basal production of prostanoids (except prostacyclin) in unstimulated endothelial cells is relatively low, and no detectable levels of other prostanoids have been found by liquid chromatography-tandem mass analysis (data not shown). Another mechanism that could, at least in part, explain the antiangiogenic effects of DHA in ASA-treated endothelial cells involves COX-2 expression (Bolego et al, 2009). Previously published studies indeed reported that DHA can reduce COX-2 expression in different cellular models (Song et al, 2014; Li et al, 2013). For example, it has been reported that in human saphenous vein endothelial cells (HSVECs) challenged with pro-inflammatory stimuli COX-2 expression and activity was reduced after 24h exposure to DHA, while COX-1 expression was not affected by DHA (Massaro et al, 2006). Moreover, DHA treatment of macrophages decreased LPS-induced PGE_2 synthesis, primarily through reducing COX-2 expression (Li et al, 2013). Overall, these data support a role for DHA in reducing COX-2 expression in cells treated with inflammatory stimuli and provide a new explanation for some of the anti-inflammatory effects of DHA. Since the present work has been carried in the absence of any inflammatory stimulus, the modulation of unstimulated COX-2 expression is probably less relevant.

To our knowledge, this is the first evidence for a role of DHA metabolites generated by ASA-acetylated COX-2, such as 17-HDHA as a precursor of AT-

resolvins, in the setting of angiogenesis. Other authors demonstrated a functional role of these novel metabolites in different *in vitro* and *in vivo* models. For example, 17R-HDHA was recently shown to have anti-inflammatory properties in animal models of experimental colitis and arthritis (Lima-Garcia et al, 2011; Bento et al, 2011), while *in vitro* experiments showed that 17(R/S)-HDHA suppresses LPS-triggered tumor necrosis factor-α formation in a murine macrophage cell line (Weylandt et al, 2011). In addition, in the fat-1 transgenic mouse model, which endogenously forms ω-3 PUFAs from ω-6 PUFAs, lipidomic analyses of lipid mediators revealed significantly increased levels of 17-HDHA in the livers of mice carrying tumors with respect to controls, suggesting a possible role of this ω-3 PUFA-derived lipid mediator in suppressing liver tumorigenesis (Weylandt et al, 2011).

In addition, few data document the functional role of DHA-derived metabolites other than resolvin precursors in the setting of angiogensis. Recently, it has been demonstrated that epoxydocosapentaenoic acids (EDPs), which are lipid mediators produced by cytochrome P450 epoxygenases from DHA, inhibit VEGF and fibroblast growth factor 2-induced angiogenesis *in vivo*, and suppress endothelial cell migration and protease production in vitro via a VEGF receptor 2-dependent mechanism (Zhang et al, 2013). Moreover, a key role for 4-HDHA (a metabolite from DHA by 5-LOX metabolism) has been described. 4-HDHA has potent effects in inhibiting endothelial cell proliferation and sprouting (Sapieha et al, 2011).

To conclude, we found that *in vivo* an ALA-rich diet generates tumor-microenvironmental changes resulting in an anti-growth effect in a murine breast cancer model. This effect is mediated, at least in part, by decreased tumor angiogenesis. This data supports a role for a chia oil diet, as an ALA dietary source, in the prevention and treatment of breast cancer. Moreover, we identified

a role for DHA metabolites derived from aspirin-acetylated COX-2 *in vitro* in inhibiting angiogenesis. Further experiments in animal models of tumor-associated angiogenesis will shed more light on the potential beneficial direct effects of aspirin-triggered metabolites on angiogenesis and tumor growth.

Limitations of the present work

The present work was conducted on endothelial cell (EC) that can mimic individual steps of the angiogenic cascade, such as cell migration, proliferation and capillary-like formation. However, different cell types, such as fibroblasts, pericytes and smooth muscle cells, play a crucial role during new vessel formation by releasing soluble factors and establishing homotypic and heterotypic interactions. In addition, the interaction between HUVECs and extracellular matrix protein represents another critical step in the angiogenic process (Conway et al, 2001). Although the molecular and cellular mechanisms of angiogenesis have been elucidated *in vivo*, the complexity of animal models often limits detailed mechanistic interpretation of experimental findings. A functional solution to bridge the gap between *in vitro* endothelial cultured cells and *in vivo* animal models is the use of *ex vivo* assays, in which vascular tissue explants are embedded in extracellular matrix gels and produce new vascular sprouts that differentiate in capillary-like structures such as rat and mouse aortic ring assays, frequently used to evaluate the efficacy of pro- and anti-angiogenic molecules (Seano et al, 2013). Adaptations of this *ex vivo* assay may provide insights into the mechanism exerted by ω-6 or ω-3 PUFAs and their metabolites in the angiogenic process.

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RINGRAZIAMENTI

Al termine di questi anni di dottorato desidero ringraziare tutte le persone che in vario modo mi hanno accompagnata in questo percorso.

Vorrei ringraziare la Prof.ssa Chiara Bolego per offrirmi il suo preziosissimo contributo teorico e metodologico durante tutte le fasi del mio lavoro di ricerca.

Voglio, inoltre, ringraziare tutti i Professori, cosí come colleghi e amici dottorandi con cui ho condiviso giornate in dipartimento, preoccupazioni e frustrazioni, oltre a idee e soddisfazioni. La vostra amicizia è stata un tesoro in questa avventura!

Ringrazio la mia famiglia per avermi condotta nel mio percorso di studi fino alla scelta di iniziare il dottorato di ricerca al estero, e per non avermi mai fatto mancare il sostegno. Un caro pensiero va inoltre a la mia nonna che mi protegge e mi guida.

Infine, ringrazio immensamente a Martin, per avermi sostenuta ed appoggiata dal primo giorno in cui ho deciso di impegnarmi in questa esperienza al estero! per aver condiviso le soddisfazioni e le difficoltà, per i saggi consigli nei momenti dificile di questo percorso.

A tutti, semplicemente Grazie!

Vuelvo Al Sur

Astor Piazzolla.

Letra: Fernando Pino Solanas (1988).

Vuelvo al Sur,

como se vuelve siempre al amor,

vuelvo a vos,

con mi deseo, con mi temor.

Llevo el Sur,

como un destino del corazón,

soy del Sur,

como los aires del bandoneón.

Sueño el Sur,

inmensa luna, cielo al revés,

busco el Sur,

el tiempo abierto, y su después.

Quiero al Sur,

su buena gente, su dignidad,

siento el Sur,

como tu cuerpo en la intimidad.

Vuelvo al Sur,

te quiero Sur...