From MEDICAL BIOCHEMISTRY AND BIOPHYSICS
DEPARTMENT
Karolinska Institutet, Stockholm, Sweden

# EICOSANOIDS AND EXOSOMES: A LINK BETWEEN MACROPHAGES AND LUNG CANCER

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Stockholm 2017

# Eicosanoids and exosomes: a link between macrophages and lung cancer

## THESIS FOR DOCTORAL DEGREE (Ph.D.)

Public defense at Karolinska Institutet, Samuelssonssalen, Tomtebodavägen 6, Solna.

**Thursday November 23rd 2017, at 13:00.**

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# ABSTRACT

Chronic inflammation increases the risk of lung cancer. Macrophages (MO) are important players in inflammation, with regulatory and executive functions. Eicosanoids and exosomes can be both triggers and mediators of these functions. Cysteinyl leukotrienes (CysLTs) are the most potent mediators of broncho-constriction in the lungs, a function exerted via CysLT1 receptor. Their function in asthma is well described, but little is known about CysLTs and lung cancer.

In the first study we investigated how the interaction between pulmonary epithelium and leukocytes affects CysLTs formation. Monocytic cells and eosinophils formed $LTC_4$, which was exported and promptly converted to $LTD_4$ by pulmonary epithelial cells in a transcellular manner. The lung cancer cell line A549 expressing γ-glutamyl transpeptidase 1 (GGT-1) showed a high activity. Exosomes released by A549 cells also contained GGT-1 and efficiently converted $LTC_4$ to $LTD_4$. On the other hand, healthy bronchial epithelial cells (PBEC) expressing GGT-5 formed $LTD_4$ 12 times more slowly. The results highlight an active role for epithelial cells and their exosomes in biosynthesis of $LTD_4$, which may be of particular relevance in the lung, given that $LTD_4$ is the most potent agonist of CysLT1.

MOs can be differentiated from blood monocytes with GM-CSF and M-CSF, resulting in cells primed toward the inflammatory M1- and resolving M2-states. A comprehensive analysis of eicosanoid formation in these two *in vitro* models is missing and our second study focused on this gap. By LC-MS analysis, we observed that both MO phenotypes released pro-resolving lipid mediators (PGE metabolite, $LXA_4$) in resting conditions. When the same cells were incubated (30 min) with bacterial stimuli, there was a shift to pro-inflammatory eicosanoids: M-CSF MOs produced high amounts of $LTC_4$, relevant for M2 functions in asthma. GM-CSF cells expressed the highest levels of $cPLA_2$, 5-LO and FLAP; and in ionophore incubations these cells also produced the highest levels of 5-HETE. However, M-CSF MO formed more products apparently due to a better response to bacterial stimuli, demonstrated by enhanced mobilization and activation of $cPLA_2$ and 5-LO. In conclusion, GM-CSF and M-CSF can regulate specific pathways in MOs, and it appears that eicosanoid biosynthesis primarily reflect the cellular response and activation mechanisms, rather than the protein expression profile.

In colon cancer a pro-tumorigenic effect of $LTD_4$ but not $LTC_4$ has been demonstrated. A pro-tumorigenic effect has been shown also for exosomes. To extend the findings of our first study, we used pleura exudates from lung cancer patients to isolate primary cancer cells and exosomes. Both cells and exosomes metabolized $LTC_4$ to $LTD_4$, and we also found that exosomes stimulated CysLTs formation in the cancer cells. Cancer cells from all patients expressed CysLT1, and exosomes promoted their migration and survival in a CysLT1 dependent manner, as demonstrated by the inhibition by montelukast (MK) treatment, a CysLT1 antagonist used to treat asthma.

In cancer, interactions between the transformed cancer cells and other recruited cell types in the tumor are important. Tumor associated macrophages (TAMs) provide cancer cells with a suitable low-grade inflammation milieu including growth promoting factors. Taken together, the results in this thesis suggest a novel pro-tumorigenic mechanism based on this theme, driven by the exosomes/CysLT1 cascade: TAMs provide $LTC_4$ that lung cancer cells and their exosomes convert to $LTD_4$. Via CysLT1 receptor this promotes survival and migration of the cancer cells. A protective effect in lung cancer has been previously described for MK and our results suggest a possible mechanism for this, driven by the exosomes/ CysLT1 cascade, further encouraging the use of this drug in lung cancer treatment.

# LIST OF SCIENTIFIC PAPERS

I.   **Lukic, A**., Ji, J., Idborg, H., Samuelsson, B., Palmberg, L., Gabrielsson, S., & Rådmark, O. (2016). *Pulmonary epithelial cancer cells and their exosomes metabolize myeloid cell-derived leukotriene C4 to leukotriene D4.* Journal of lipid research, 57(9), 1659-1669

II.  **Lukic, A**., Larssen, P., Fauland, A., Samuelsson, B., Wheelock, C. E., Gabrielsson, S., & Radmark, O. (2017). *GM-CSF–and M-CSF–primed macrophages present similar resolving but distinct inflammatory lipid mediator signatures.* The FASEB Journal, 31(10), 4370-4381.

III. **Lukic, A**., Wahlund, C., Gomez, C., Brodin, D., Samuelsson, B., Wheelock, C. E., Gabrielsson, S., & Radmark, O. *Exosomes and malignant cells from lung cancer pleura exudates form LTD4, promoting cell migration and survival in a CysLT1 dependent mechanism.* Manuscript

**Publications not included in this thesis:**

I.   Torregrosa Paredes, P., Esser, J., Admyre, C., Nord, M., Rahman, Q. K., **Lukic, A.**, ... & Scheynius, A. (2012). *Bronchoalveolar lavage fluid exosomes contribute to cytokine and leukotriene production in allergic asthma.* Allergy, 67(7), 911-919.

II.  Basavarajappa, D., Wan, M., **Lukic, A**., Steinhilber, D., Samuelsson, B., & Rådmark, O. (2014). *Roles of coactosin-like protein (CLP) and 5-lipoxygenase-activating protein (FLAP) in cellular leukotriene biosynthesis.* Proceedings of the National Academy of Sciences, 111(31), 11371-11376.

III. Martinez-Bravo, M. J., Wahlund, C. J., Qazi, K. R., Moulder, R., **Lukic, A.**, Rådmark, O., ... & Gabrielsson, S. (2017). *Pulmonary sarcoidosis is associated with exosomal vitamin D–binding protein and inflammatory molecules.* Journal of Allergy and Clinical Immunology, 139(4), 1186-1194.

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AA | Arachidonic acid |
| AERD | Aspiring exacerbated respiratory disease |
| BAL | Bronco-alveolar lavage |
| BEC | Bronchial epithelial cells |
| BLT | Leukotriene $B_4$ receptor |
| CD | Cluster of differentiation |
| COPD | Chronic obstructive pulmonary disease |
| COX | Cyclooxygenase |
| $cPLA_2$ | Cytosolic phospholipase $A_2$ |
| CYP | Cytochrome P450 |
| CysLT | Cysteinyl leukotriene |
| DHA | Docosahexaenois acid |
| DiHET | Dihydroxyeicosatrienoic acids |
| DiHOME | Dihydroxyoctadecenoic acid |
| EDP | Epoxydocosapentaenoic acid |
| EET | Epoxyeicosatrienoic acid |
| EGFR | Epithelial growth factor receptor |
| EMT | Epithelial to mesothelial transition |
| EP | Prostaglandin $E_2$ receptor |
| EPA | Epoxydocosapentaenoic acid |
| EpOME | Epoxyoctadecenoic acid |
| ESCRT | Endosomal complex required for transport |
| FLAP | Five lipoxygenase activating protein |
| fMLP | N-formylmethionyl-leucyl-phenylalanine |
| GGT | Gamma-glutamyl transpeptidase |
| GM-CSF | Granulocyte macrophage-colony stimulating factor |
| M-CSF | Macrophage-colony stimulating factor |
| GSH | Glutathione |
| GTP | Guanosine Triphosphate |
| HDoHE | Hydroxydocosahexaenoic acid |
| HETE | Hydroxyeicosatetraenoic acid |
| HODE | Hydroxyoctadecadienoic acid |
| HpETE | Hydroperoxy eicosatetraenoic acid |
| HPLC | High-performance liquid chromatography |
| IL | Interleukin |
| IFN-$\gamma$ | Interferon $\gamma$ |
| LA | Linoleic acid |
| LC-MS | Liquid chromatography-mass spectrometry |
| LO | Lipoxygenase |
| LPS | Lipopolysaccharide |

| | |
|---|---|
| LT | Leukotriene |
| LTC4s | LTC$_4$ synthase |
| LTB4h | LTB$_4$ hydrolase |
| LX | Lipoxin |
| MDSC | Myeloid derived suppressor cells |
| MHC | Major histocompatibility complex |
| miRNA | Micro RNA |
| MK | Montelukast |
| MM6 | Mono Mac 6 |
| mPGES | Microsomal prostaglandin E$_2$ synthase |
| MSC | Mesenchymal stem cells |
| MVB | Multivesicular bodies |
| NSCLC | Non-small cell lung cancer |
| PBEC | Primary BEC |
| PBMC | Peripheral blood mononuclear cells |
| PE | Pleural exudate |
| PG | Prostaglandin |
| PGN | Peptidoglycan |
| PMN | Polymorphonuclear neutrophils |
| PTGIS | PGI$_2$ synthase |
| PUFA | Polyunsaturated fatty acids |
| RvE/RvD | Resolvins E/D series |
| TLR | Toll like receptor |
| SBC | Serine Borate Complex |
| sEH | soluble Epoxide Hydrolase |
| SPM | Specialized proresolving mediators |
| TAM | Tumor associated macrophages |
| Th | T helper cell |
| TME | Tumor micro-environment |
| TNFα | Tumor necrosis factor alpha |
| Treg | Regulatory T cell |
| TXAS | Thromboxane A synthase 1 |
| VEGF | Vascular endothelial growth factor |

# CONTENT

# INTRODUCTION

<u>A pinch of evolution</u>

In 1859, Darwin published *On the Origin of Species*. He postulated that all existing life forms originate from the same ancestor and that species evolve by natural selection. Although science and technology allow humans to challenge the action of evolution, our 'starting pack' is the result of millions of years of selection and whenever our physiological condition is perturbed, a cascade of defensive mechanisms is activated to restore homeostasis. Noxious stimuli will trigger inflammation, a refined arm of both innate and adaptive immunity that involves vascular leakage followed by leukocyte recruitment and activation at the site of damage, and finally "reconstructive" tissue healing. The mechanisms behind all these effects differ depending on the tissue, the type of trigger, and the effector cells [1, 2].

Evolution shapes life in term of cost and benefits. Although inflammation may compromise the physiological function of tissue, the benefit of clearing the harmful stimuli is superior. This biological response is affected by surrounding environmental factors: in the past century the development of antibiotics, and extensive changes in diet, stress and exercise have put a new selective force on our immune system, without providing time for evolution to readjust the inflammatory mechanisms. The inflammatory response can easily be dysregulated and loose its transitory properties in favor of a chronic condition [3]. Chronic inflammation is well connected with several diseases, including cancer, diabetes, obesity and cardiovascular diseases, which are the 'plague' of modern age. According to the last update by the World Health Organization (WHO), the second leading cause of death in 2015 across the world was lung related pathologies: cancer and inflammatory diseases, such as Chronic Obstructive Pulmonary Disease (COPD), and respiratory infection. The respiratory system is in fact particularly susceptible to inflammation, also in light of its 'open' anatomy: lungs are constantly exposed to viruses, bacteria, allergens, and the particles and smoke in air pollution. The pulmonary epithelium, assisted by resident leukocytes such as macrophages, represents the checkpoint where these external antigens may or may not be recognized as harmful and thus trigger the immune response [4]. An efficient immune response will fulfil the pathophysiological role in lung defense, clear the harmful stimuli, and restore the physiology of the tissue. To accomplish this task cells must communicate and eicosanoids and exosomes represent two fundamental categories of messengers in the lung.

In the following studies, we investigated eicosanoid biosynthesis (in particular one step in formation of leukotriene $D_4$) in connection to exosomes, to further understand how

macrophages and lung cancer cells may contribute to inflammatory conditions in the lung microenvironment.

## Chapter 1. Arachidonic acid derived eicosanoids

Eicosanoids are lipid mediators mostly derived from arachidonic acid (AA) that exert a pivotal role in inflammation. They have several biological effects, both in homeostasis and in pathological conditions, in the inflammatory phase as well as in the resolution of inflammation [5]. AA is the common name of 5, 8, 1, 14-cis-eicosatetraenoic acid, an ω-6 polyunsaturated fatty acid (PUFA) found in algae, plants and animals. AA can be metabolized via lipoxygenases (LO), cyclooxygenases (COX) and cytochrome P450 (CYP) .

Detection of AA and the eicosanoid forming enzymes across the life domains indicate that the derived signaling molecules exert positive functions on survival that have been preserved over millions of years by natural selection [6, 7]. The CYP pathway is most likely the oldest, given that this superfamily of proteins is extensively present across all  domains of life [8]. The exact functions of AA-derived mediators and eicosanoid-like molecules in primordial organisms are yet to be described, but in plants and animals more is known. Interestingly, eicosanoids show mainly regulatory functions in plants: peaks in LO activities are detected during growth, while  prostaglandin-like molecules  participate in flowering [9, 10].

In animals, important functions of eicosanoids are exerted during the immune response. Both plants and animals possess an innate immune system, but inflammation is observed only in the animal kingdom, and eicosanoids seem evolutionary optimized in regulating the different phases of the inflammatory process. For example, leukotrienes (LTs) are the most studied LO products and their functional cascade, including enzymes and receptors, are found only in vertebrates [6]. 15-LO is an enzyme involved in formation of lipid mediators that can resolve inflammation. Recently it was demonstrated that the most evolved primates, including humans and chimpanzees, possess a 15-LO with higher capacity for the biosynthesis of resolving lipid mediators, in comparison to the 15-LO of other animals, including mice, rats and pigs [11]. The selection for an enhanced ability to form lipoxins in primates may have furnished an evolutionary advantage in resolving inflammation more efficiently, thus supporting a more balanced immune response and better survival. Moreover, it was proposed that development of a refined brain may compete with the development of the innate immune system [12], and the capacity to resolve inflammation more quickly may have been one of the factors promoting brain development in primates.

Different types of stimuli, such as pathogens, antigens, and cytokines, can lead to activation of phospholipase $A_2$ enzymes ($PLA_2$). $PLA_2$ is a superfamily of proteins and the most studied members are secreted $PLA_2$ ($sPLA_2$) and cytosolic $PLA_2$ ($cPLA_2$), both involved in AA-release, and calcium independent PLA2 ($iPLA_2$). $cPLA_2$ is the main enzyme to hydrolyze AA from the membrane. When activated by $Ca^{2+}$ binding and phosphorylation, $cPLA_2$ migrates to the nuclear membrane and releases AA from phospholipids [13]. Once AA is free, it undergoes one of the three metabolic routes – LO, COX, CYP- depending on the proximity, expression and activation state of each enzymatic route (Figure 1) [5].
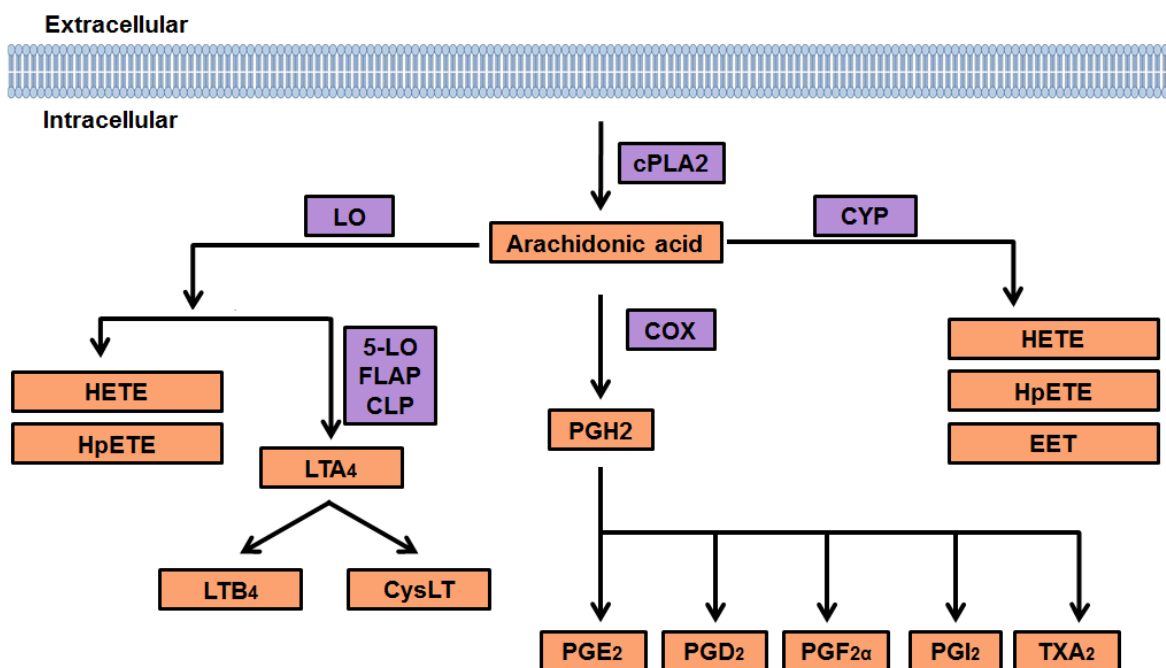


**Figure 1**. Summary of the AA-metabolism.

Chapter 1.1.1 5-LO pathway: the inflammatory side

AA may become substrate to 5-lipoxygenase (5-LO). Similarly to $cPLA_2$, 5-LO activity is tightly regulated. $Ca^{2+}$ signaling and ATP activate 5-LO by direct binding. Phosphorylation at serine-271 and serine-663 results in increased 5-LO activity, while the opposite effect is obtained when phosphorylation occurs at serine-523. Subcellular localization can also affect 5-LO activity: proximity to $cPLA_2$ and cell membranes results in higher activity [14]. The activation and the translocation of 5-LO is supported by two scaffold proteins, 5-LO activating protein (FLAP) and coactosin-like protein (CLP) [15]. Once activated, 5-LO

catalyzes the two steps necessary to convert AA to LTA$_4$, an unstable epoxide. LTA$_4$, can be metabolized by the cytosolic LTA$_4$ hydrolase to LTB$_4$ or it can be conjugated to glutathione (GSH) by nuclear membrane-associated LTC$_4$ synthase to form LTC$_4$. LTC$_4$ may be further converted to LTD$_4$ and LTE$_4$ by sequential peptide cleavages of the GSH moiety (Figure 2).
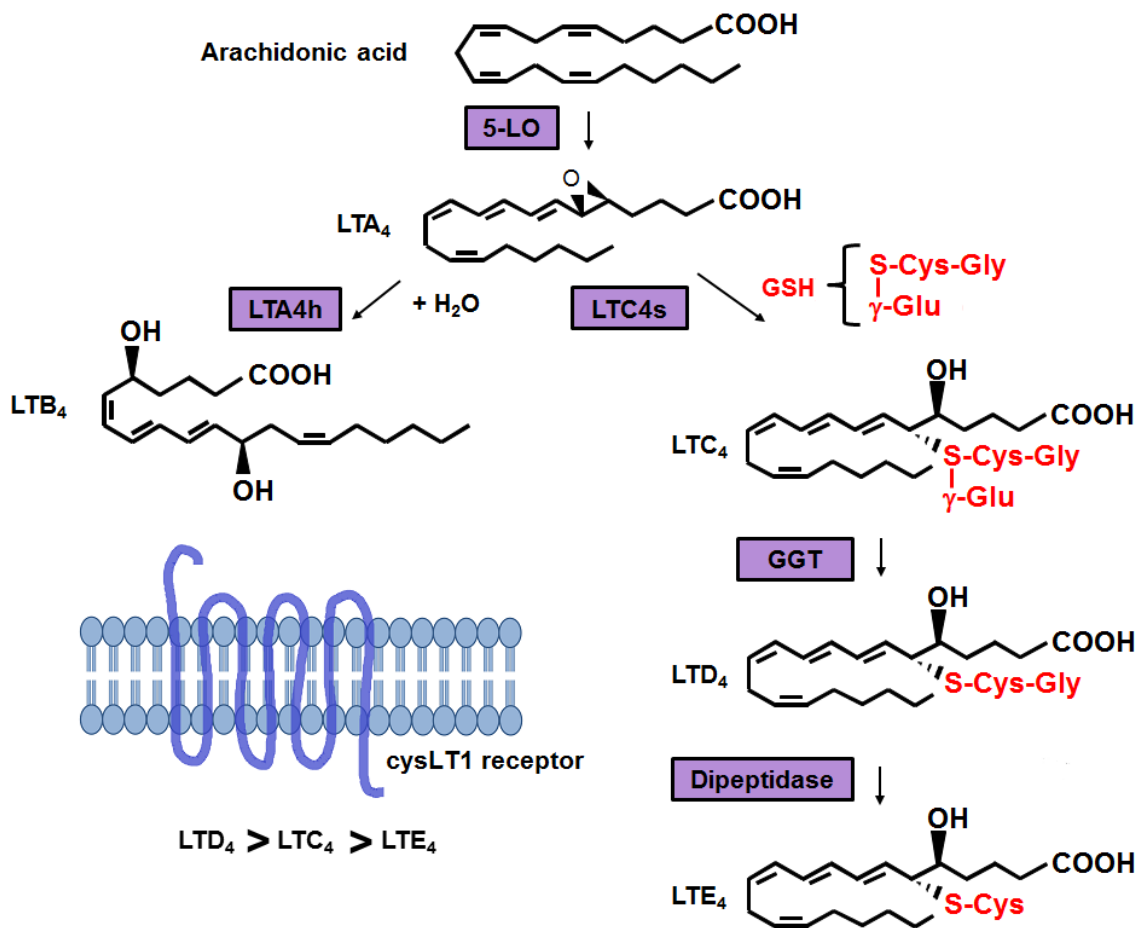


**Figure 2**. Overview of the leukotriene biosynthesis.

LTs are potent pro-inflammatory mediators which bind to receptors; LTB$_4$ exerts its role via BLT1/2, while CysLTs bind CysLT1/2 [16].In the last decade novel receptors for LTE$_4$ have been described, such as P2Y$_{12}$ and GPR99 [17, 18]. Depending on the receptor expression and distribution on different cell types, LTs mediate different effects in the inflammatory response. They are potent chemotactic agents; they increase cellular adhesion and motility, facilitating the recruitment and accumulation of leukocytes in the inflammatory microenvironment. LTB$_4$ is one of the most potent chemoattractants to neutrophils, while LTD$_4$ recruits dendritic cells and eosinophils. Both LTB$_4$ and LTD$_4$ can promote migration ot

Th17 cells [19] and $LTB_4$ also promotes the recruitment of activated regulatory T cells (Tregs) in lungs after acute injury [20]. $LTB_4$ contributes to host defense also by enhancing phagocytosis. CysLTs are fundamental players in respiratory inflammation: they regulate bronchoconstriction, vascular permeability, mucus secretion and airway remodeling [21, 22].

$LTD_4$, the first metabolite of $LTC_4$, is one of the most potent broncho-constricting molecules in humans, at least 1000 times more potent than histamine; it exerts a rapid effect, and in comparison to histamine it induces a prolonged bronchoconstriction [23]. $LTD_4$ has 10- to 100-fold higher affinity to CysLT1, the most expressed CysLT receptor in lung tissue [24] and this makes $LTD_4$ one of the most important mediators in lung inflammation. However, both $LTC_4$ and $LTD_4$ induce a similar broncho-constructive response [25], which probably results from the further metabolism of $LTC_4$ to $LTD_4$. Finally, dipeptidase enzymes catalyze metabolism of $LTD_4$ to the weakest CysLT1 agonist $LTE_4$ [26]. Two different strategies can prevent the action of LTs: inhibition of the biosynthetic enzymes or receptor antagonists. Today several drugs targeting the LTs pathway are commercially available, such as zileuton, a 5-LO inhibitor, or montelukast (MK) and zafirolukast, CysLT1 antagonists. The use of Cys-LT1 antagonists in treatment of asthma and allergic rhinitis is well established. Moreover, the discovery in the last two decades of the 5-LO role in other diseases than allergy, such as atherosclerosis and cancer, is driving the research toward new pharmacological applications [27].

Another possible way to target $LTD_4$ action is by acting on γ-glutamyl transpeptidase (GGT, formerly called γ-glutamyl transferase), the enzyme that converts $LTC_4$ to $LTD_4$. This enzyme is located on the extracellular side of the plasma membrane, mostly on epithelial cells [28] and it is fundamental in the metabolism of GSH, where it catalyzes the hydrolysis and transfer of γ-glutamyl moieties [29]. $LTC_4$, which is formed by conjugation of $LTA_4$ with a GSH molecule, can be further metabolized by GGT, by cleavage of the γ-glutamyl, to produce $LTD_4$. The GGT family includes different proteins, but the functional roles are known only for few of these. GGT-1 and GGT-5 are the two family members known to convert $LTC_4$ to $LTD_4$. Data from mouse and human models are different in regard of $LTD_4$ biosynthesis: in mice 90% of $LTD_4$ is formed by GGT-5, while in humans both GGT-1 and GGT-5 metabolize $LTC_4$, although GGT-1 is much faster [30]. GGT-1 is used as a diagnostic marker in several diseases characterized by high GSH levels, including cancer, but research failed to develop effective drugs to target this enzyme. GGT inhibitors tested in clinical trials include glutamine analogues, which proved to be toxic at concentrations required to inhibit the enzymatic activity. Only in recent years novel noncompetitive and non-cytotoxic

inhibitors have been developed, reviving once again the research for GGT-1 targeting drugs to test in clinical trials [31].

Chapter 1.1.2 5-LO pathway: anti-inflammatory and resolving products

The 5-LO metabolites derived from AA do not only have potent pro-inflammatory properties. This enzyme is involved in the biosynthesis of other molecules fundamental in the resolution phase of the immune response. $LTA_4$, the unstable epoxide, can be transported outside of the cells and further metabolized in a transcellular manner. Neutrophils release $LTA_4$, donating this substrate to cells that do not possess 5-LO, such as platelets [32]. These cells possess LTC4s and can produce $LTC_4$. They also possess 12-lipoxygenase, which can catalyze the conversion to lipoxin $A_4$ and lipoxin $B_4$ (LXs). These lipid mediators can be formed by the same enzymes with a reversed order: epithelium/endothelium possessing 15-LO can produce and export 15(S)-HpETE, which in presence of leukocytes may be converted via 5-LO to $LXA_4$/$LXB_4$ [33]. The biological action of LXs covers a range of effects in the resolution of lung inflammation: they reduce bronchoconstriction; they stimulate the phagocytosis of apoptotic PMN by macrophages; they block eosinophil trafficking by inhibition of eotaxin secretion [34]. The receptors that mediate these effects are still investigated; $LXA_4$ binds to ALX/FPR2, mediating the reduction of pro-inflammatory cytokine release and clearance of apoptotic PMN, but a $LXB_4$ receptor has not yet been clearly identified [22]. The lack of the complete description of the LXs signaling cascade did not stop the development of LXs analogs, currently investigated as potential drugs to stimulate resolution in inflammatory diseases.

Chapter 1.2 COX pathway

After the release from the nuclear membrane phospholipids, AA can be metabolized by COX-1 and COX-2, generating $PGH_2$, which is further metabolized by tissue-specific enzymes to generate the large family of prostanoids, which includes prostaglandins and thromboxane (Figure 1). COX-1 and COX-2 are homodimeric hemeproteins, bound to the nuclear membrane and to the adjacent endoplasmic reticulum. COX activity is not regulated, and when AA is available PGs are produced. Thus, in many cell types the regulation of $cPLA_2$ activity is important for regulation of PG biosynthesis. As for the LTs, the effects of prostanoids are mediated by G-protein coupled 7-TM receptors [35]. Prostanoid biosynthesis

is increased in inflammation, but the exact role exerted by these messengers is cell specific, in part due to the variance in the expression of the receptors. For instance, $PGE_2$, one of the most potent and studied inflammatory mediators, is involved in the regulation of four of the five signs of inflammation: rubor, tumor (swelling/edema), inflammatory pain, and fever. Yet, depending on the stage of the immune response, $PGE_2$ can act as an anti-inflammatory and immunosuppressive agent, for example by increasing infiltration and activity of Treg cells and MDSC cells [36]. $PGE_2$ can also stimulate production of LXs, in the so called "eicosanoid class-switching" [37]. The complementary ability to induce or reduce inflammation is explained by the presence of 4 receptors, expressed heterogeneously in human tissue, and characterized by different affinity to $PGE_2$, high for EP3 and EP4, low for EP1 and EP2 [36]. In lungs $PGE_2$ has a unique protective role, mediated by EP4, the most expressed $PGE_2$ receptor in this tissue. EP4 mediates the bronchodilator effect of $PGE_2$, the inhibition of eosinophil and neutrophil trafficking, as well as the decrease in cytokines production by human alveolar macrophages [38]. Other notable COX-products include $PGD_2$ and Thromboxane (TX) $A_2$, which most important functions are linked to airway inflammation and platelet activation, respectively [39, 40]. Inflammatory conditions can be treated with Nonsteroidal Anti-Inflammatory Drugs (NSAIDs), such as aspirin, ibuprofen and paracetamol. These drugs exert their action also by inhibiting COX activity and PGs biosynthesis. [41]. Because PGs can exert both inflammatory and anti-inflammatory properties, treatment with NSAIDs may cause negative side effects. An example: aspirin inhibits COX-derived $PGE_2$, which in turn can inhibit CysLTs biosynthesis in lung, as part of the anti-inflammatory and broncho-protective process [42]. A recent study showed that $PGE_2$ acts through EP2 to inhibit CysLTs biosynthesis by mast cells, resulting in less potent bronchoconstriction after challenge [43]. Thus, disruptions of $PGE_2$ production cause a significant percentage of patients with lung inflammatory disease to develop aspirin-exacerbated respiratory disease (AERD), characterized by severe eosinophilic inflammation driven by CysLTs, whose negative regulation is impaired by the lack of $PGE_2$ [44].

Chapter 1.3 CYP pathway

This is the third metabolic pathway that free AA can enter; it includes 57 known proteins and almost half of these enzymes can utilize AA as substrate [45]. In fact, several members can oxygenate AA on C16-C20 resulting in mono-HETEs. One of these is 20-HETE, a potent vascular modulator that execute some of its functions via EGFR [46] and that can modulate miR-133 and miR-143, known to affect the phenotype of smooth muscles cells [47]. The

unique mediators formed via this pathway are epoxyeicosatrienoic acids (EETs) and three major human CYP are involved in this metabolism: CYP2J2, CYP2C8 and CYP2C9; notably, the last CYP is the most expressed in the lungs [48]. The function of EETs is mainly studied in cardiovascular diseases, due to their ability to induce vasodilatation and thus act as protective agents [49]. Although EETs appear to have anti-inflammatory properties, they also have angiogenic and apparently pro-tumorigenic effects:  in mouse models of different types of cancer it was observed that the endothelial derived EETs play a crucial role in promoting tumor growth and metastasis [50]. An important mechanism to regulate EET functions is further metabolism by the soluble epoxide hydrolase (sEH), which results in the less potent dihydroxyeicosatrienoic acids (DHETs). As a consequence, targeting sEH may be a potential therapy where EETs function is crucial to pathogenesis. Last, but not least, CYP enzymes also contribute to PGs formation: PTGIS and TXAS, two of the downstream enzymes of the $PGH_2$ metabolism, belong to the CYP family and form $PGI_2$ and $TXA_2$ respectively.

Chapter 1.4 Eicosanoids in pulmonary disease

As described, AA metabolites are fundamental mediators of the immune response, with both pro- and anti-inflammatory actions. Several steps of the AA cascade are tightly regulated. Dysregulation of this metabolic network may lead to prolonged inflammation with aberrant tissue remodeling, a common feature of many lung associated diseases, such as asthma, COPD, and lung cancer [51, 52]. For example, asthma patients can have high levels of CysLT in body fluids, such as blood, BAL, urine, and sputum [53]. High levels of CysLTs in pulmonary tissue can lead to airway obstruction, due to increased broncho-constriction, mucus production, and vascular permeability. CysLTs also promote lung inflammation by enhancing the migration of eosinophils  in synergy with IL-33 [54] and later their trafficking toward lymph nodes [55]. Although higher $LTB_4$ levels have been reported as well, the role of $LTB_4$ in asthma is unclear: treatment with BLT1 antagonists did not inhibit the asthmatic response to allergen challenge in patients with allergic asthma [56], but these results do not exclude a primary role in non-allergic asthma. Alveolar macrophages isolated from broncho alveolar lavage (BAL) of patients with sarcoidosis release more $LTB_4$ compared to cells from healthy donors [57]. COX-derived metabolites display heterogeneous effects. Mast cell-derived $PGD_2$ triggers the asthmatic response [58], while $PGE_2$ exerts a protective effect, the disruption of which may lead to AERD. Patients with COPD have high levels of $LTB_4$ and $PGE_2$ in exhaled breath condensate (EBC) compared to healthy subjects [59]. A possible explanation for high $PGE_2$ in COPD may be its anti-inflammatory properties in the lung [38,

60]. $TXA_2$ is also significantly increased in urine of patients with COPD: increased production of $TXA_2$ may contribute to vascular complications such as pulmonary hypertension [61].

Dysregulation of the eicosanoid cascade has also been linked to cancer. Increased production of LTs and PGs has been observed for malignant tumors. $PGE_2$ and in some case also $LTB_4$ contribute to a favorable milieu for cancer cells, promoting their proliferation, migration, invasiveness, induction of angiogenesis and suppression of the immune response [62]. Inhibition of 5-LO can reduce lung tumorigenesis in mice [63] and proliferation in human lung cancer cell lines [64]. Interference with $LTB_4$ signaling also results in better survival, as demonstrated in a BLT1-/- knock out mouse model of lung cancer [65]. Evidence on the function of $LTB_4$ in tumorigenesis abounds in comparison to CysLTs, far less investigated. So far, two publications have presented different results on $LTC_4$ function in lung cancer. In a Lewis lung cancer mouse model, knock out of LTC4s did not affect primary tumor growth nor liver metastasis [66]. In the same study, depletion of 5-LO resulted in enhanced tumor growth caused by reduced cytotoxic CD8+ T cell recruitment [66]. This should reflect a function of LTs to stimulate the immune system to attack and kill cancer cells. However, in another study, 5-LO knock out reduced lung metastasis in a mouse model with breast cancer, in a $LTC_4$/CysLT2 and $LTB_4$/BLT2 manner [67]. This may reflect different functions of LTs in tumor progression, depending on the stage. For example, it is possible that LTs promote a low-grade inflammation tumor microenvironment, stimulating e.g. metastasis formation. Epidemiological studies support the hypotheses that CysLTs may contribute to lung cancer: recently, a Taiwanese 20 year retrospective study showed that asthma patients treated with MK have lower risk to develop lung cancer [68], suggesting that CysLT receptor antagonists may be beneficial to lung cancer patients. The same group showed that MK indeed induces apoptosis in A549 cells, a commonly investigated human lung cancer cell line, and reduces tumor growth in a lung cancer mouse model [69]. However, early clinical trials showed that patients with advanced non-small-cell lung cancer (NSCLC) did not respond to zileuton treatment or $LTB_4$ antagonists [70, 71]. Moreover, 5-LO expression did not correlate with prognosis, in contrast COX-2 expression did: in fact, patients with high COX-2 expression responded positively to celecoxib therapy [70]. A valid argument on why 5-LO inhibitors may fail as treatment against cancer depends on the nature of AA metabolism: blocking AA from entering 5-LO may shift this substrate toward COX, as observed in a co-culture model with lung epithelial cells and alveolar macrophages [72]. This will further enhance the production of PGs, as Poczobutt et al have observed in their lung cancer mouse model depleted of 5-LO [66]. $PGE_2$ has several pro-tumorigenic effects, including inhibition of

cytotoxic T cells response, which results in an immunosuppressed tumor microenvironment (TME) [36]. Another important way for PGE$_2$ to promote tumorigenesis is via Tregs: these cells are fundamental to maintain immunosuppression, and lung cancer cells can directly modulate Treg via COX-2/PGE$_2$ cascade [73]. In agreement with this, recently it was observed that urinary PGE$_2$ correlates positively with intratumor infiltration of Tregs in patients with lung cancer [74]. Moreover, PGE$_2$ action is not limited to the modulation of TME alone: A549 cells secrete this prostaglandin to self-promote proliferation in a EGFR dependent mechanism [75]. Furthermore, xenograft tumors derived from mPGES1-depleted A549 cells, display a slower growth and tumor progression, compared to WT cells [76]. The fact that both LO and COX pathways synthesize pro-tumorigenic metabolites is the driving force behind the development of dual 5-LO and COX inhibitors in the last years [77].

Last, CYP products have also been studied in relation to cancer. EETs have positive effect on cancer migration and invasion, as observed in both mammary cancer cell line and lung cancer cell line, including A549 cells; similarly, overexpression of CYP2J2 promotes both tumor growth and lung cancer formation in a mouse model [78]. In a more recent study, it was reported that tumor growth and number of metastasis in the lungs is increased in sEH-depleted mice, further supporting a pro-tumorigenic effect for EETs [50]. One of the mechanisms behind EETs pro-tumorigenic effects relies on neutrophil recruitment; in fact ablation of these cells blocks the pro-metastatic effect of 14,15-EET treatment [79].

## Chapter 2. Other substrates for the LO-COX-CYP cascades

Archaeological and evolutionary studies showed that pathologies characterized by chronic inflammation were not that common 10.000 years ago as today; one of the major contributors to these differences seems to be the change in our diet: the ω-6/ω-3 fatty acids ratio in the past was 1 while the typical modern western diet has a ratio of 15/1 [80]. Today it is generally believed that a diet enriched in ω-3 fatty acids results in a more balanced inflammatory response , and possibly better resolution of inflammatory conditions [81, 82]. The ω-6 family includes AA and linoleic acid (LA), a PUFA with 18-carbon chain length. In the ω-3 group, docosahexaenoic acid (DHA) and epoxydocosapentaenoic acid (EPA) are the most studied members, with 22-carbon long and 20-carbon long chains respectively. In the past decades, science has unveiled fundamental anti-inflammatory and pro-resolving function of DHA and EPA derived mediators, which strengthens the hypothesis that an ω-3 enriched diet is beneficial. One of the most exiting aspects of the eicosanoid metabolism is that many of the

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enzymes metabolizing AA are active with other PUFA substrates, including LA, DHA and EPA (Figure 3).
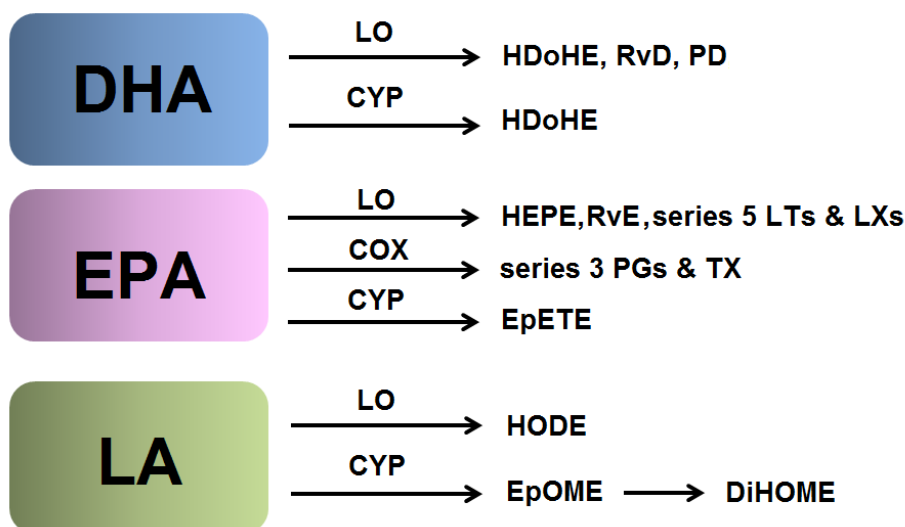


**Figure 3**. Other PUFAs metabolism via LO-COX-CYP pathways.

LA is the second most abundant PUFA (after AA) in PBMC [83] and it's metabolite 13-HODE, formed via 15-LO, contributes to bronchial injury in asthmatic patients [84]. The pathogenesis of COPD also correlates with LA metabolites: a recent lipidomic study on BALF from female, but not male, patients showed increased concentration of the CYP derived LA metabolites EpOMEs and DiHOMEs, suggesting a gender specific lipid mediator signature [85]. In cancer research, LA metabolites have shown contrasting results, with some models supporting an anticancer effect, while others demonstrate a pro-tumorigenic one [86]. EDPs are class of active lipid mediators derived from DHA, via CYP: they can counteract VEGF effect on HUVEC and inhibit metastasis in a Lewis lung metastasis model [87].

The most studied DHA and EPA metabolites have potent resolving functions: they have anti-inflammatory properties and promote resolution of inflammation, and return to homeostasis [37]. Specialized pro-resolving mediators (SPM) include EPA-derived E-series resolvins (RvE), and DHA-derived D-series resolvins (RvD), protectins (PD) and maresins: all are formed via several enzymatic steps including, in different order, 5-LO, 12/15-LO, CYP and COX-2 [88]. The LXs was the first subgroup of SPM to be described and their functions are the best characterized so far [89]. Of interest, aspirin not only induces the formation of some of the LXs, but also of some resolvins: aspirin-triggered RvD1 promotes resolution of the

inflammatory response in a sterile pneumonia mouse model, by enhancing bacterial clearance by macrophage phagocytosis among other factors [90]. The RvD1 can also counteract EMT transition in A549 cells: EMT stands for epithelial-to-mesenchymal transition, a fundamental event in cancer dissemination and metastasis formation. [91]. The D and E series of resolvins often have congruent effects: for example, both RvE1 and RvD1 downregulated TNFα + IL-6 induced overexpression of COX-2 and VEGF in human pulmonary arteries [92]. Protectin D1 (PD1) inhibits replication of influenza virus in infected A549 cells [93]. In the same study, other known AA, DHA and EPA metabolites, including 15-HETE and 17-HDoHe (a precursor of D-resolvins), showed similar inhibiting properties. However, neither RvE1 nor RvD1 affected virus replication, suggesting that the inhibitory effect is not retained from 17-HDoHe [93].  Maresins are the latest addition to the SPM family; they are DHA derived metabolites, formed via 12/15-LO and macrophages are a primary source [94]. Recently it was observed that macrophages exploit the LTC4s-GGT enzymatic cascade to further metabolize maresins, forming conjugated forms [95]. Stable analogs of SPM are currently under investigation as potential therapy to stimulate or reduce aberrant inflammatory responses [96].

## Chapter 3. Exosomes

Intercellular communication is a fundamental aspect of the immune system and it occurs in different ways. Many cells secrete small molecules that can target themselves in an autocrine loop, or other cells, in the neighborhood or in distant sites. Eicosanoids can target cells in the close proximity, in a paracrine fashion. Another channel of intercellular communication is by extra cellular vesicles, membrane vesicles defined primarily by size and origin. Microvesicles, sized 100-1000 nm, are shed from the plasma membrane while exosomes, sized 40-100 nm, are of endocytic origin [97].  In 1987 Johnstone et al described the vesicles released by reticulocytes during the maturation stage and called these exosomes. They concluded that other cell types present in blood did not release exosomes, and that the primary function of these vesicles was to remove unnecessary functional proteins during erythropoiesis [98]. After 30 years of research scientists proved that all cell types investigated so far produce exosomes, containing proteins and other classes of biomolecules. One of the most interesting properties of exosomes is their ability to transfer functional mRNA and miRNA between cells [99]. From an evolutionary point of view, exchange of genetic material in form of DNA and RNA represents a type of horizontal transfer, one of the oldest and most important ways to exchange genetic information prior to the development of sexual

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reproduction [100]. After infection of cells, viruses can exploit the exosome machinery to spread viral miRNA [101]. Other pathogens, including parasites, fungi and bacteria, can exploit exosomes as a shuttle to disseminate infection [102]. This raises the question on how ancient and evolutionary selected the exosomal apparatus is. Except mammalian cells, plants also appear to form exosomes-like vesicles [103] but a clear evolutionary perspective has yet to be explored. The general nomenclature of extracellular vesicles is not clearly defined but it is generally recognized that exosomes share a specific set of molecules and a typical biogenesis [104].

## Chapter 3.1 Biogenesis

Multivesicular bodies (MVBs) originate from the late endosome, a cellular compartment containing molecules from the plasma membrane, the cytosol and the Golgi. Here the protein sorting of the future exosomal cargo is performed by a multi-protein complex called Endosomal Complex Required for Transport (ESCRT). Exosomes are released when MVBs do not fuse with lysosomes for degradation, but with the plasma membrane, thus releasing the nano-vesicles formed by inward budding of the MVB membrane (Figure 4) [105].
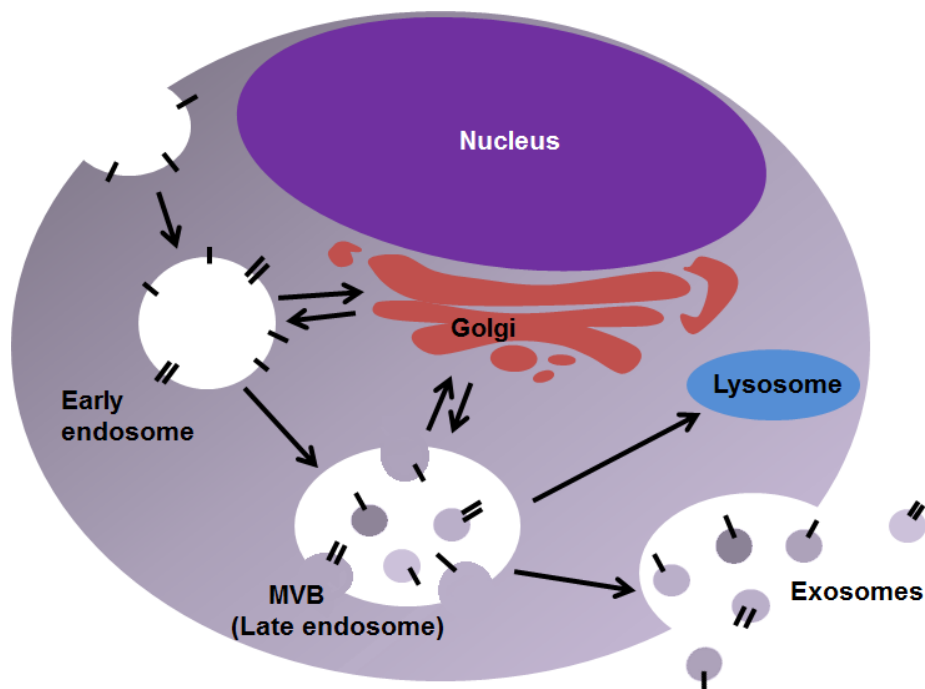


**Figure 4.** Formation and release of exosomes.

Exosomes constitute a heterogeneous population of vesicles and the ESCRT molecular machinery may be responsible for the genesis of many subtypes of exosomes. However, in recent years also ESCRT-independent exosome release has been demonstrated in different cell types [97]. The biogenesis of exosomes explains why these vesicles are enriched in endosomal markers such as tetraspanins (CD9, CD63, and CD81). Their content of cytosolic and plasma membrane proteins, lipids and RNA usually reflect the parent cells. However, the cargo is specifically selected and not just a sample of the cell content.

Chapter 3.2 Function of exosomes

Investigations of the exosomal load unveiled their role as mediators of intercellular communication: exosomes deliver mRNAs, which may be translated to functional proteins following the uptake by recipient cells. Also miRNAs are packed within the vesicles, and higher expression of distinct miRNA sequences can be detected in exosomes compared to parental cells [99]. Exosomes released by immune-competent cells may carry the parental cell function during the immune response [106]. For example, dendritic cell derived exosomes carry different surface molecules with a specific function, such as adhesion (CD54), co-stimulation (CD80, CD86) or antigen presentation (MHC-I/II). Dendritic cell exosomes can activate T cells, more efficiently when derived from mature dendritic cells compared to immature dendritic cells and also independently of MHC molecules [107, 108]. Dendritic cell exosomes can also act indirectly, via other mediators formed by B cells or other dendritic cells [109, 110]. Intriguing results have been published regarding exosomes and lung diseases. Admyre et al first reported that BAL fluid contains exosomes, enriched in co-stimulatory molecules such as MHC-I/II and CD54, similarly to dendritic cell derived exosomes [111]. BALF exosomes from sarcoidosis patients are also enriched in pro-inflammatory molecules and increase cytokine release from PBMC, indicating that these exosomes may participate in disease progression [112]. The first report of exosome secretion by eosinophils showed that more vesicles are released by eosinophils from asthmatic patients compared to healthy donors [113].

Tumor derived exosomes participate in maintaining the tumorigenic milieu through several routes. For example, due to their immunomodulatory properties, tumor exosomes support immunosuppression, by impairing dendritic cells maturation and promoting Treg activation; they also modulate the TME directly, by enhancing angiogenesis and promoting the formation of favorable metastatic niches, all to support the tumor growth [114]. The 'seed

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and soil' theory postulates that metastatic spread of cancer cells is not random but tightly regulated, and requires compatibility between disseminated malignant cells and microenvironment so that new metastatic loci can form [115]. It is now recognized that exosomes are among the factors that prepare a favorable TME, the soil, thus promoting metastasis formation initiated by circulating cancer cells, the seed [116]. In a lung cancer model, it was recently demonstrated that tumor derived exosomes promote metastasis by directly activating TLR-3 in alveolar epithelium, resulting in neutrophil recruitment [117]. Also TLR-8 is involved in lung tumorigenesis: lung cancer exosomes contain miRNAs that activate macrophages via TLR-8 to secrete pro-inflammatory and tumorigenic cytokines, such as TNFα and IL-6 [118]. Recruitment of macrophages is a fundamental step in establishing the TME: lung cancer exosomes induce mesenchymal stem cells (MSC) with an inflammatory phenotype which secrete cytokines such as MCP-1, the most potent macrophage chemoattractant, resulting in their higher infiltration and larger tumors in comparison to unstimulated MSC [119]. Lung cancer exosomes, especially when released in hypoxic conditions, can promote vascularization and at the same time vascular permeability, which is a crucial step to allow infiltration of tumor supporting cells [120]. Exosomes also promote EMT: notably, serum derived exosomes from lung cancer patients induce expression of vimentin as well as migration of healthy bronchial epithelial cells [121]. Since exosomal cargo (miRNA and proteins) mirrors parental cells, these vesicles have been suggested as diagnostic biomarker candidates in disease. For example, the concentration of plasma exosomes and their total miRNA amount in patients with lung adenocarcinoma is higher compared to control groups, and elevated levels of miRNAs associated to lung tumor tissue were also detected in the exosomes of patients, strengthening the use of exosomes as diagnostic tools in lung cancer [122]. A more recent study further support the use of exosomal miRNA as diagnostic marker in lung carcinogenesis [123].


Chapter 3.3 Eicosanoids & exosomes

Eicosanoids and exosomes represent two different types of messengers. Eicosanoids are hormone-like compounds, acting via G-protein coupled receptors on target cells, which are normally neighboring cells, since these lipid mediators have a short half-life. On the other hand, exosomes are large vesicles containing a selected cargo, which could exert a multitude of effects and functions, as such or after uptake into cells. Some organ-derived exosomes retain their specificity: intestine derived exosomes are taken up mainly by the intestine itself within 24 hours, and only a prolonged circulation will lead to clearance by other organs,

mainly liver [124]. Malignant exosomes isolated from cancer cell lines appear to be taken up mainly by liver and spleen [125] but tumor derived exosomes can also travel to specific niches, notably lung and liver, to promote metastasis formation, a phenomenon dictated by the expression of specific integrins [126]. Moreover, if not cleared immediately, exosomes are quite stable in body fluids, and they protect their content. For example, free miRNA in circulation is more vulnerable to ribonucleases in comparison to exosomes-carried miRNA, suggesting the higher validity as biomarker resource of the latter type [127]. This is true also for compounds of different nature: exosome-bound curcumin, a potent $cPLA_2/COX/5$-LO inhibitor [128] displayed longer bioavailability *in vivo* and enhanced anti-inflammatory properties in comparison to free curcumin [129], suggesting that exosomes may be exploited as specific and efficient drug delivery systems [130].

Rather than being two parallel ways for cells to communicate, exosomes and eicosanoids often converge, with the first behaving as a tool box for the second. For example, exosomes are enriched in phospholipids, ceramides and cholesterol but they also carry PUFAs, mainly AA and LA [131]. Furthermore, depending on the parental cells, exosomes may carry enzymes involved in the different steps of AA. $PLA_2$ members including $iPLA_2$, $sPLA_2$ and $cPLA_2$, responsible for the cleavage of PUFA from membrane phospholipids, have been detected in mast cells derived exosomes; surprisingly, these enzymes showed functional activity via a GTP-dependent mechanism, an unknown pathway in the cellular system [131]. Other active enzymes involved in the AA metabolism have been detected in the same exosomes, such as COX-1 and COX-2 [131]. Transcellular biosynthesis of AA metabolites is a well-described process, and exosomes can function as substrate supplier in the local or distant microenvironment. Due to their ability to transport active enzymes, exosomes may also participate in the transcellular biosynthesis as 'nanocells'. For example, macrophages and dendritic cells express enzymes involved in the biosynthesis of LTs and also secrete exosomes loaded with LTC4s and LTA4h, but apparently less 5-LO. Moreover, exosomal LTC4s and LTCA4h metabolize $LTA_4$ more efficiently compared to the parental cells, and their contribution to LT biosynthesis may explain the ability of the exosomes to induce PMNL chemotaxis [132]. Majumdar et al demonstrated that also exosomes released by PMNL carry 5-LO, FLAP and LTA4h. After fMLP stimulation these vesicles are directly loaded with $LTB_4$ and can efficiently promote PMNL migration in a $LTB_4$-BLT1 manner [133]. LTA4h is also packed in exosomes isolated from BAL fluid of sarcoidosis patients, and so are 5-LO and FLAP [134]. Exosomes from BAL fluid of asthma patients have higher LTA4h activity compared to LTC4s activity; in comparison to exosomes from healthy

donors, exosomes from patients also induce increased LTs formation and IL-8 release in bronchial epithelial cells [135].

Remarkably, exosomes have also been reported to carry bioactive lipid molecules directly, such as prostaglandins. Regarding tumor progression, breast cancer derived exosomes are enriched for $PGE_2$ and when injected in mice they induce accumulation of myeloid derived suppressor cells (MDSC), and release of pro-inflammatory cytokines such as IL-6 and VEGF, thus promoting tumor growth [136]. In the tumor micro-environment MDSC are capable of differentiating in macrophages, further enlarging the population of tumor associated macrophages (TAM), fundamental contributors to tumorigenesis [137]. Exosomes pretreated with anti-$PGE_2$ antibodies displayed a partial decrease in MDSC induction and cytokines release, suggesting that exosomal $PGE_2$ may favor the creation or maintenance of the tumorigenic milieu [136]. Intestinal exosome-like particles also carry $PGE_2$, which mediate, in part, the anergic state of natural killer T cells in the liver, where these vesicles migrate and where they may play a pivotal role in the liver immune system [124].

## Chapter 4. Macrophages, polarization and cancer

Phagocytosis evolved as a mechanism for nutrition in primitive unicellular organisms, but in multicellular organisms this process turned into a system for protection [138]. Today, macrophages represent the first defense line of phagocytes in vertebrates and play a crucial role as sentinels of the immune system in response to pathological events that may trigger inflammation in tissue [139]. The origin of macrophages has been vastly investigated: tissue resident macrophages originate from the yolk sac and the fetal liver; during inflammation, peripheral blood monocytes derived from bone marrow will be recruited and differentiated to macrophages in the inflamed tissue [140]. Macrophages can recognize harmful stimuli, destroy them and communicate with the surrounding environment to restore physiological conditions. In the year 2000 Mills proposed for the first time the M1/M2 polarization concept in relation to Th1/Th2 response [141], providing a simple model to better understand the role of macrophages. Their function is far more heterogeneous compared to T cells: it depends on the broad range of stimuli that can activate them and shift their phenotype within a broad spectrum, delimited by pro-inflammatory and healing/resolving functions (Figure 5) [142, 143].
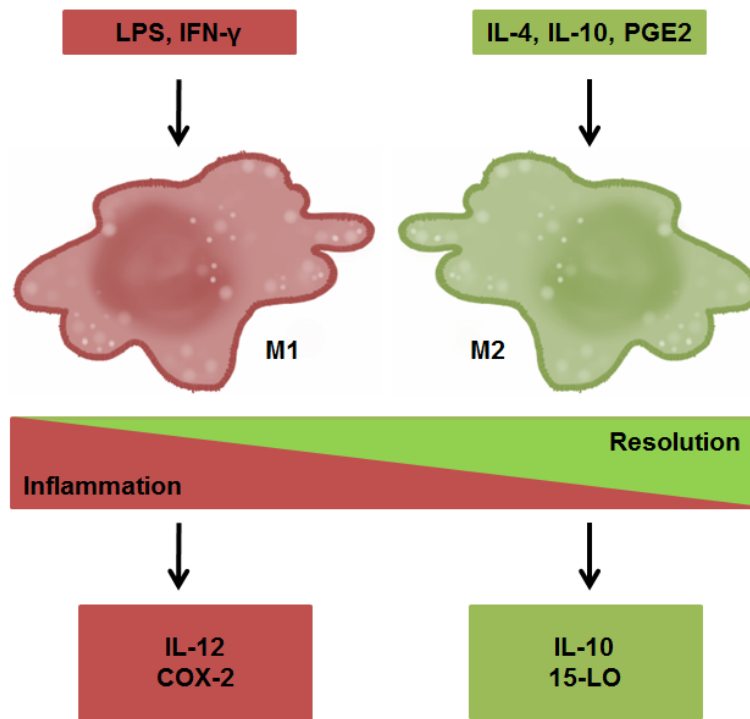
**Figure 5**. Schematic representation of the most common stimulating factor, type of function, and markers that characterize M1/M2 macrophages

One of the most important discoveries that pushed research on M1/M2 polarization arouse when M2 macrophages were shown to promote cancer [144]. TAMs are formed mainly by recruited monocytes [145], as demonstrated also in a mouse model for lung cancer [146]. TAMs are fundamental supporters of tumorigenesis and the majority of them exhibit typical markers of M2 polarized cells [147, 148]. In 1998, a few years before the concept of M1 and M2 polarization would storm into the macrophages research, Huang et al demonstrated that macrophages grown in presence of supernatant from lung cancer cells increased IL-10 production and decreased IL-12 production [149]. Today these two cytokines are considered specific M2 and M1 markers [150], and is well known that cancer cells can turn macrophages toward M2 [151]. In the same paper, Huang et al demonstrated that the factor released by cancer cells that shifted the cytokine profile from M1 to M2 was $PGE_2$ [149]. $PGE_2$ has indeed a tight connection with cancer and macrophages. For example, $PGE_2$ can promote tumorigenesis also by enhancing TAM's expression of PD-L1, thus polarizing them toward an immunosuppressive M2 phenotype [152]. Doxorubicin resistant breast cancer cells also release $PGE_2$ to expand an M2 polarized population of MDSC [153]. This is in agreement with a celecoxib (COX-2 inhibitor) treatment in a mouse model with lung cancer, where blocking of $PGE_2$ signaling inhibited tumor growth by increasing M1 and decreasing M2,

thus changing the M2/M1 ratio [154]. The overall tissue M2/M1 ratio observed in tumor tissue of NSLCC lung cancer patient is close to 70:30; however, higher M1 density in tumor islets positively correlated with patient survival time [155]. Similar results were obtained in another study: infiltrating TAMs in tumor specimens from patients with lung adenocarcinoma show a M2 phenotype, and higher % of M2 correlated with poor survival [156]. So far, targeting M2 or reprogramming these cells toward M1 appears as a possible therapy against cancer. For example, in a melanoma model, antibody therapy against the receptor MARCO expressed by M2-like but not M1-like macrophages resulted in reduced tumor growth and a shift toward M1 populations. This was one of the first studies to demonstrate successful immunotherapy based on TAM reprogramming as tool to treat cancer [157]. Ablation of dicer, the miRNA processing enzyme, is another approach that results in inhibition tumor growth, caused by enrichment of M1 macrophages whereas M2 macrophages are shut down by dicer inactivation [158]. Zanganeh et al also reported that activation of M1 macrophages by iron oxide nanoparticles could inhibit primary tumor growth, and lung and liver metastasis in a mammary tumor mouse model [159]. All these studies support M1 reprogramming as a therapy against malignant cancer. However, the tumor heterogeneity is a crucial factor to consider [160] and it is important to point out that tumor infiltrating macrophages are never made by M2 macrophages only, as mentioned earlier [155, 156]: this raises the question whether the M1 infiltrating cells are the last defense line of the immune system, still fighting against the tumor itself. However, there may be the possibility that a small but significant percentage of M1 actually may contribute to tumorigenesis, given their higher ability to form pro-tumorigenic molecules such as IL-6 [150], which in turn can promote M2 polarization [161]. M1 macrophages also release larger amounts of $PGE_2$ [162], which can effectively promote M2 polarization as mentioned above.

Another complex issue is the contrasting nature of the TME, immunosuppressive and yet inflammatory, tightly linked to TAMs. Smoke and chronic inflammation are well known contributors to lung cancer and COPD patients have a higher risk in developing lung cancer [163]. COPD patients present high and prolonged expression of NF-kB [164], resulting in immunosuppression supported by the presence of Tregs and M2 macrophages [165]. SPM are investigated as therapy for chronic inflammation, also in light of their potential to modulate macrophages polarization. For example, RvD1 treatment promotes M2 polarization resulting in a reduced neutrophilic lung inflammation in a mouse model of smoke-induced lung inflammation [166]. Similarly, other studies demonstrated that resolvins promote M2 polarization [167, 168]. It is reasonable that M2 macrophages sustain their phenotype in a positive autocrine loop, in light of their effective endogenous SPM biosynthesis,  in

comparison to M1 macrophages [162]. However, recently it was reported that SPM such as RvD1, RvD2 and Maresin 1 can also regulate adaptive immunity and induce immunosuppression by enhancing Treg and decreasing CD8 and CD4 T cells activity [169]. It is plausible that a constant attempt to resolve dysregulated inflammation actually initiate immunosuppression in the TME, thus creating a favorable tumor milieu. Consequently, a deeper understanding of the eicosanoid/macrophage networking is required in order to use macrophage polarization for effective therapies.

# MATERIALS AND METHODS

The following studies were performed according to the ethical guidelines at Karolinska Institute, with approved ethical permits for Paper I and Paper III.

Detailed information on material and methods can be found in the respective papers. Here is presented a brief list of the performed methods.

| | Paper I | Paper II | Paper III |
|---|---|---|---|
| Cell culture (MM6 cells, MDMs, MDDCs, Eosinophils, A549 cells, PBEC, primary lung cancer cells) | x | x | x |
| Handling of human pleural exudates | | | x |
| Exosome isolation from culture supernatants and pleural exudates | x | | x |
| Western blot analysis | x | x | x |
| Cell incubations and solid phase extraction of lipid mediators | x | x | x |
| High performance liquid chromatography (HPLC) | x | x | x |
| Mass spectrometry analysis | x | x | x |
| Flow cytometry analysis | x | x | x |
| Enzyme-linked immunosorbent assay (ELISA) | | x | x |
| Immunocytochemistry | x | x | |
| Viability assay | | | x |
| Scratch assay | | | x |
| Exosome size analysis by Nanosight | x | | x |
| Sucrose gradient fractionation | | | x |
| Electron microscopy | | | x |

# PAPERS

**Paper I: the co-culture project**

<u>Background and aim</u>

Asthma is an obstructive lung disease, characterized by chronic inflammation [51], and the global frequency has been increasing since the '70 [170]. AERD is considered a subtype of asthma that develops in response to NSAID treatment, which results in COX inhibition and a consequent shunt of AA toward other pathways, mainly LOs [171]. In fact, patients with AERD present higher expression of LTC4s [172] and overproduction of CysLTs, sustained by platelet-adherent leukocytes [173]. The increased formation of inflammatory CysLTs may not depend only on higher availability of AA but also on regulatory mechanisms. Inhibition of COX results in decreased $PGE_2$ formation, which can directly regulate LTs formation, via cAMP-PKA catalyzed phosphorylation of serine-523 on 5-LO [174]. Also, it was shown that prolonged exposure to zymosan, a TLR-2 agonist, results in decreased LTC4s activity in monocytes; this inhibitory phosphorylation depends on endogenous $PGE_2$ formation and can be counteracted by aspirin treatment [175].

*In vitro* experiments normally investigate a single cell type and this is a major limit compared to *in vivo* models. Co-culture systems represent an upgrade, allowing researchers to investigate biological events considering cells interactions [176, 177]. A549 is an alveolar type II epithelial cell line established from a lung cancer patient, widely used as *in vitro* model for lung epithelial cells. This cell line is also exploited as a valid substitute for lung epithelial cells in co-culture models that mimic the interaction between lung epithelium and macrophages [178-183]. Inspired by a previous study on $PGE_2$-LTC4s regulation within macrophages [175], we aimed to understand if also $PGE_2$ from the lung epithelium could regulate LTC4s activity in monocytes/macrophages.

<u>Results and discussion</u>

We selected Mono Mac 6 (MM6), a leukemia-derived macrophage-like cell line, to combine with A549 in a co-culturing system. This represents a valuable model for our study, given the possibility to regulate both 5-LO in MM6 and mPGES1 in A549 [184, 185]: in fact, MM6 cells were treated with TFG-β and Vitamin D3, to upregulate 5-LO expression and activity, while A549 cells were stimulated with IL-1β, in order to increase $PGE_2$ production. Finally, cells were co-cultured for 24 hours and further experiments were performed. LT biosynthesis

assays showed that monocultures of MM6 cells produced mainly $LTC_4$, while A549 cells could not produce LTs from AA (Figure 2, Paper I). Importantly, there was no detectable change in the formation of $LTC_4$ from exogenous $LTA_4$ or endogenous AA when MM6 were co-cultured with A549 for 24 hours. Although IL-1β clearly upregulated mPGES1 in A549 cells as observed by western blot, the concentration of $PGE_2$ in the co-culture medium was ~3-5 nM, as measured by LC-MS. Apparently, this was not sufficient to modulate LTC4s in MM6. However, the co-culture did show a significant change: formation of $LTD_4$, which was almost undetectable when both cells were grown in monocultures.

The formation of $LTD_4$ is relevant for several reasons: here we mimic the lung microenvironment, where $LTD_4$ exert important functions in asthma, as the most potent agonist of CysLT1. Moreover, A549 is a cancer cell line but in lung cancer a specific effect of $LTD_4$ has not been described. On the other hand, increasing evidence over the last two decades demonstrated the pro-tumorigenic effect of $LTD_4$ in colon cancer [186]. In detail, several colon cancer cell lines form CysLTs [187, 188], which may further promote survival of cancer cells via CysLT1 [187]. Moreover, higher expression of CysLT1 correlates with poor prognosis in colon cancer patients, similarly to COX-2 overexpression [189]. Interestingly, $LTD_4$ but not $LTC_4$ upregulates COX-2 expression in intestinal epithelium [190] and the positive effect of $LTD_4$ on COX-2 expression was shown also for malignant intestinal epithelial cells [188, 191].

Given the established functions of $LTD_4$ in the lung and in cancer, we further investigated $LTD_4$ formation in our model. $LTC_4$ must be exported to be converted to $LTD_4$: once exported $LTC_4$ can access GGT, a plasma membrane protein that convert GSH and $LTC_4$. Activity assays with different substrates and western blots (Figure 2-3, Paper I) indicated that MM6 cells produced and exported $LTC_4$ (Table 1, Paper I), which was further converted by A549 cells via GGT-1 in a transcellular manner. A549 cells formed $LTD_4$ quickly and more efficiently upon IL-1β stimulation (Figure 4, Paper I). It was previously shown that macrophages and dendritic cells exosomes carry active LTC4s and LTA4h [132] and we hypothesized that exosomes could also carry active GGT-1. Indeed, when MM6 cells were incubated with $LTA_4$ in presence of A549 derived exosomes, more $LTD_4$ was produced, due to GGT-1 present in exosomes (Figure 5, Paper I). We also treated the co-cultures with Serine Borate complex (SBC), an inhibitor of GGT-1 to confirm that $LTD_4$ is truly formed via this protein: SBC treatment blocked $LTD_4$ formation, and unexpectedly it also upregulated 5-LO activity in MM6 cells, allegedly by increasing the nuclear translocation of 5-LO.

Increased formation of $LTD_4$ was also observed in short time co-incubations of A549 cells with monocyte-derived dendritic cells and with eosinophils, while these cells alone formed $LTC_4$ and almost no $LTD_4$ (Figure 6, Paper I). A deeper investigation in literature confirmed that most leukocytes lack the capacity to form all CysLTs: in the majority of studies mainly $LTC_4$ had been detected, as we also observed with MM6. Not only monocytes/macrophages [192-194], but also eosinophils [195-197] and mast cells mainly form $LTC_4$ [198-200]. Interestingly, when MM6 were incubated with primary bronchial epithelial cells (PBEC), longer time was required to obtain a significant increase in $LTD_4$ formation. Further investigation showed that PBEC form $LTD_4$ via GGT-5, another member of the GGT family, which converts $LTC_4$ slowly compared to GGT-1 (Figure 7, Paper I). While the gene family for GGT includes several members [29], only two of these are $LTD_4$ producing enzymes, GGT-1 and GGT-5. In vitro data on purified human GGT-1 and GGT-5 showed that both enzymes have similar affinity for $LTC_4$ but the first converts $LTC_4$ faster, indicating a more active "leukotrienase" activity [30]. In co-incubations of MM6 with the normal PBEC (expressing GGT-5) a long incubation time (6 hours) was required for substantial conversion of LTC4 to $LTD_4$, while co-incubations of MM6 with A549 cells (expressing GGT-1) produced comparable amounts of $LTD_4$ within 30 min: this demonstrated that human GGT-1 has the highest $LTD_4$ forming activity both as purified protein and in whole cells.

In conclusion, this study elucidated the differences between GGT-1 and GGT-5 in $LTD_4$ formation on a cellular level. Our model indicates that myeloid derived cells produce and donate $LTC_4$ and that pulmonary epithelium, normal and malignant, can transform $LTC_4$ to $LTD_4$. These results emphasize an active role of epithelial cells and their exosomes in the biosynthesis of $LTD_4$, a fundamental eicosanoid in pulmonary inflammation.


Future plans

It is well known that CysLTs may be formed in a trans-cellular manner. For example. neutrophils form and export $LTA_4$, which can be converted to $LTC_4$ by platelets [201]. Alveolar macrophages and lung epithelium can exchange AA and enhance 5-LO or COX derived products [72]. Given the intricate nature of AA metabolism, and the knowledge that several enzymatic steps follow a transcellular route [202], it is vital to understand the function of each player. Administration of drugs, systemic or locally, lack the ability to target a specific cell type. Liposomes and exosomes are investigated as cell specific-delivery systems, which may become feasible in the next future [203]. Therefore, it is fundamental to resolve

all the metabolic steps that a complex cellular network may exploit to form eicosanoids. Co-culture models with multiple players, such as leukocytes-epithelial-endothelial cells, may provide more clear pictures of how single cell types contribute to eicosanoid biosynthesis, thus indicating the most suitable drug-target.

When MM6 cells were incubated for 30 min with $LTA_4$ in presence of exosomes, we detected $LTD_4$, formed via GGT-1 packed within the vesicles. However, when MM6 cells were incubated with $LTA_4$ after 24 hours treatment with exosomes, no $LTD_4$ was formed (data not shown); instead, MM6 showed a decreased 5-LO activity, with both endogenous and exogenous AA. Studies exploiting co-culture models can expand our knowledge on how exosomes regulate the eicosanoid biosynthesis and metabolism, given that this communication channel can easily be manipulated. MiRNA and proteins delivered by exosomes may retain their function in recipient cells. For example PTEN, a tumor-suppressant protein, can be transported between cancer cells via exosomes [204]. We know that cells take up exosomes and can further process/sort the exosomal content [205, 206]. Our data indicate that MM6 did not retain the exosomal GGT-1 activity. The other way around, in a pilot experiment we observed that MM6 derived exosomes could deliver 5-LO to THP-1, an immature-macrophage like cell line, but this transfer did not occur when the recipient cells were A549 cells. These preliminary results raise the question: which component of the eicosanoid metabolic cascade can be transferred between cells? Enzymes and receptors of the eicosanoid cascade are not ubiquitous: if exosomes contain these proteins, a spontaneous question is whether this packing is just a tool to shed unnecessary functions or if it might be a mechanism to increase and disseminate formation of eicosanoids.

**Paper II: the macrophage project**

Background and aim

Blood derived monocytes are the main resource to obtain differentiated macrophages in vitro, induced by GM-CSF or M-CSF and in previous projects in the group one or the other of these cytokines have been used [15, 132]. In a pilot experiment we compared macrophages differentiated with both cytokines and we observed a significant difference in the ability to form 5-LO products. GM-CSF and M-CSF prime macrophages toward the two end points of the broad spectrum of macrophage phenotypes, M1 and M2 respectively, but without fully reaching these [142, 207]. Specific transcripts differentiated between M1 and M2 macrophages include enzymes involved in eicosanoid metabolism [208] and the lipidomic profile of these two phenotypes indeed shows significant differences: for example, M1 macrophages release higher amounts of inflammatory PGs and LTs, while M2 macrophages release higher amounts of LXs [162]. These lipid signatures are in agreement with the commonly accepted concept that M1 are pro-inflammatory macrophages while M2 are resolving macrophages [142].

The M1/M2 concept applies mainly to *in vitro* models and it does not take in account the kinetics of the different pathways and mechanisms exploited by macrophages to orchestrate the inflammatory process, from the earliest onset to the resolution phase [209]. The time course of inflammation is normally simplified in few specific phases, delimited by initiation and resolution [1]. *In vitro* activation and polarization of macrophages normally requires 18-24 hours of stimulation, in order to induce transcriptional changes [150]. Considering the time required to activate macrophages, this means that *in vitro* generated M1 and M2 macrophages can be placed somewhere in the middle or late phase of an ongoing inflammatory processes. However, eicosanoids are products of pre-existing enzymes that can be activated within minutes; in fact, these mediators are produced during all phases of inflammation, from the earliest stage to the latest resolving phase. This means that studies on fully activated M1 and M2 macrophages fail to observe differences in eicosanoid metabolism that arise in the onset of inflammation, when bacterial alarm signaling triggers the immune response and the consequent phenotypic shifts. Lipidomic analysis has been successfully applied to study the modulation of AA metabolism, and several papers have been published for mouse derived macrophages [193, 194, 210, 211]. However, only one comprehensive report was published for human M1/M2 macrophages [162] and formation of lipid mediators in GM/M-CSF primed macrophages, the earliest inflammatory phase, had not been investigated. The study described in Paper II aimed to fill in this gap.

<u>Results and discussion</u>

We differentiated human monocytes with GM-CSF or M-CSF and after 7 days, we obtained primed but not fully polarized M1 and M2 cells (Figure 1, Paper II). We then determined by LC-MS/MS the eicosanoid and other lipid mediators released from resting cells, and produced upon short (30 min) bacterial stimulation. We chose LPS/fMLP and peptidoglycan (PGN), both physiological stimuli representative of Gram- and Gram+ infection. During the last 24 hours of differentiation, resting M-CSF macrophages released more anti-inflammatory $LXA_4$ (Figure 2, Paper II), similarly to M2 macrophages [162]. Basal formation of PGs was comparable between GM-CSF and M-CSF macrophages (Figure 2, Paper II), in contrast to published data, reporting that PGs release is higher in M1 in comparison to M2 [162, 212]. Resting GM-CSF cells released more CYP derived products: EETs metabolites are generally regarded as anti-inflammatory [213], however we detected mainly the inactivated DHETs form, derived from further metabolism by sEH.

We also characterized for the first time the eicosanoid pathway in human macrophages in response to short bacterial stimulation. M-CSF macrophages released larger amounts of 5-LO products, particularly following LPS/fMLP stimulation (Figure 3, Paper II). Specifically, M-CSF macrophages shunted the intermediate $LTA_4$ to LTC4s rather than to LTB4h. Asthma is generally considered a Th2 type disease [214], associated with M2 accumulation [215]. M-CSF macrophages display considerably higher $LTC_4$ production in line with priming to M2, associated to asthma where release of CysLTs exert crucial functions, particularly as modulators of bronchoconstriction and tissue remodeling [16]. Interestingly, conversion of AA to 15-HETE, catalyzed by 15-LO, was about 20 times lower compared to 5-HETE formation. The total amount of COX-derived metabolites showed minor differences between the two phenotypes (Figure 3, Paper II), in agreement with comparable levels of COX-1 expression between GM-CSF and M-CSF macrophages (Figure 4, Paper II) and both macrophages phenotypes released a negligible amount of CYP products. We also detected 17-HDoHE, precursor of resolvins, and other anti-inflammatory mediators (Figure S2, Paper II). These were all more abundant in M-CSF MOs, in agreement with the higher ability of M2 macrophages to release pro-resolving mediators [162].

Differentiation with GM-CSF or M-CSF was not sufficient to induce COX-2, which accounts for a large part of PGs biosynthesis in M1 macrophages, obtained by long-term stimulation with IFN-γ and/or LPS [5, 208]. However, GM-CSF and M-CSF differentiation was sufficient to modulate the protein expression of other eicosanoid forming enzymes. Expression of $cPLA_2$, 5-LO and FLAP was significantly higher in GM-CSF cells (Figure 4,

Paper II). Literature on 5-LO in M1 and M2 macrophages is contrasting: Lehmann et all reported similar results for 5-LO/FLAP expression in M1 and M2, in agreement with our findings for GM-CSF and M-CSF macrophages [216]. In contrast, others have shown that ALOX5, the gene encoding 5-LO, was downregulated more prominently in M1 compared to M2 [208, 212]. Transcriptome levels do not always compare to protein levels [217] and possibly transcriptional changes in ALOX5 require longer time to translate to changes in the expression of 5-LO protein. M-CSF macrophages expressed higher levels of 15-LO (Figure 3), in line with reports for M2 [208, 212].

The most prominent mismatch between protein expression and effective metabolic activity was observed for the 5-LO pathway: the protein expression of 5-LO was significantly higher in GM-CSF macrophages. Our findings suggest that M-CSF macrophages could overcome the lower 5-LO protein expression and release larger amounts of LTs by enhanced activation and mobilization of key enzymes, $cPLA_2$ and 5-LO, in response to bacterial stimulation (Figure 5). In line with several publications on CysLTs in monocytic cells (see Paper I), only 10% of $LTC_4$ was further metabolized to $LTD_4$, in both M-CSF and GM-CSF macrophages, via GGT-5 (Figure 4, Paper II). However, this was surprising since GM-CSF macrophages displayed a clear western blot band for GGT-1 (Figure 4, Paper II).

Tissue resident macrophages are among the first cells to sense alarm signals. They respond, and orchestrate the immune response by recruiting neutrophils and additional macrophages during progression of inflammation. M-CSF is constitutively expressed and its depletion leads to reduction of several tissue resident macrophages populations while GM-CSF is known to be increased during inflammation. In fact, macrophages differentiated with the M-CSF *in vitro* are close to normal homeostatic macrophages while macrophages differentiated with GM-CSF are considered inflammatory macrophages [218]. Resident macrophages need to respond quickly, including mobilization of endogenous substrate when bacterial stimulation triggers the immune response: here we did observe that M-CSF macrophages mobilized AA more efficiently compared to GM-CSF macrophages (Figure 5, Paper II), despite lower expression of $cPLA_2$ (Figure 4). This is in agreement with a recent report: bone marrow derived macrophages also release larger amounts of AA when polarized toward the M2 endpoint [219]. The M-CSF cells here investigated also released larger amounts of CysLTs, known to increase vascular permeability, a key event during early inflammation [220]. Similarly, tissue macrophages of murine origin showed preference for $LTC_4$ when the 5-LO cascade is triggered [193, 194, 219]. Moreover, resemblance of M-CSF macrophages to tissue macrophages agrees with an M-CSF priming toward the M2 state, since steady state

macrophages in homeostatic conditions should be closer to the reparative/resolving endpoint, than to the inflammatory end of the spectrum. On the other hand, GM-CSF release is augmented during inflammation. Other leukocytes recruited to the inflamed tissue, such as neutrophils and platelets, can deliver free AA [202]. When such exogenous substrate is provided, GM-CSF macrophages can exploit more efficiently the higher expression of the enzymatic machinery behind LTs productions, as we showed when cell were incubated with exogenous AA (Figure 5, Paper II) and thus contribute to amplify the ongoing inflammation.

In conclusion, we report for the first time a comprehensive eicosanoid analysis in human GM-CSF and M-CSF differentiated macrophages. GM-CSF and M-CSF alone can prime macrophages for specific lipid metabolism, demonstrating that polarization toward M1 and M2 has started. However, full activation is fundamental to exploit all components of the eicosanoid metabolism, notably COX-derived products. Moreover, the distinct eicosanoid profiles in response to bacterial stimulation do not reflect protein expression but rather the efficiency to activate eicosanoid-forming enzymes.

Future plans

Inflammatory conditions are promoted by several factors. Among these are Pathogen-Associated Molecular Patterns (PAMPs) [221] and alarm molecules of endogenous origin, called Damage-Associated molecular patterns (DAMPs) [222]. DAMPs, for example ATP and DNA, are often released by cancer cells and may contribute to inflammation in the tumor micro-environment [223]. Here we observed distinct lipid metabolism when macrophages were stimulated with LPS/fMLP or PGN, which can be identified as PAMPs. So far, there is no clear evidence on how DAMPs may affect eicosanoid metabolism in macrophages. A comprehensive lipidomic analysis of macrophages stimulated with tumor associated DAMPs may unveil how eicosanoid formation is modulated by the TME.

The immunity of lymphocytes, and their crosstalk with macrophages, notably via cytokines, inspired the M1/M2 notion; however, depletion of lymphocytes does not block M1 and M2 generation [141]. Recent studies [224-227] suggest that eicosanoids may also contribute to the phenotypic shift of macrophages during inflammation, given the early activation of this metabolic cascade and the ability of eicosanoids to modulate cytokines and other immune molecules [58, 228, 229]. Therefore, further research may clarify how eicosanoids contribute to the development of M1/M2 phenotypes. Intervention on the eicosanoid cascade during activation of macrophages may block the phenotypic shifts associated with diseases like M2

in asthma [215] and cancer [137] and M1 in obesity [215, 230], thus providing an additional therapeutic tool based on macrophage manipulation.

**Paper III: the pleura exudate project**

<u>Background and aim</u>

In Paper I we showed that myeloid cells and bronchial epithelial cells transform $LTC_4$ to $LTD_4$ slowly, via GGT-5. On the other side, the lung cancer cell line A549 formed $LTD_4$ quickly, via GGT-1. A rapid formation of $LTD_4$ by the malignant cells raises the question whether CysLTs have a function in lung cancer. The pro-tumorigenic effect of the $LTD_4$-CysLT1 cascade in colon cancer is well established [186]. However, recently it was demonstrated that $LTC_4$ execute anti-tumor effects via CysLT2, by upregulating 15-PGHD, the enzyme that promotes catabolism and inactivation of $PGE_2$ [231]. This suggests that a fast conversion of $LTC_4$ is crucial to promote tumorigenesis, highlighting the function of GGT-1 as the fast $LTD_4$ forming enzyme. GGT-1 is a well established marker for hepatic diseases, including liver cancer [232, 233]. It plays a crucial role in redox regulation and it has been linked to oxidative stress and chemoresistence in cancer [234, 235]. However, little is known about GGT-1 and lung related diseases. In the lungs, GGT-1 and GGT-5 are detected mainly in alveolar macrophages [28, 236]. An aberrant expression of GGT-1 is found in specimens from patients with lung cancer, mainly adenocarcinoma [237, 238]. A recent epidemiological study demonstrated that higher serum levels of GGT-1 correlate with specific types of cancers, including stomach, colon and lung [239] and GGT-1 activity might be exploited to visualize small tumor loci in the lungs [240]. In paper I we observed that GGT-1 is the fastest $LTD_4$ forming enzyme and we also showed exosomes from the NSCLC cell line A549 form $LTD_4$ via GGT-1. Several reports unveil the function of exosomes in lung tumorigenesis [114, 117-121] and exosomes of malignant (prostate) origin carry active GGT-1 [241]. In paper III we investigated the link between GGT-1, $LTD_4$, exosomes and lung cancer using biological specimens from patients with lung cancer.

<u>Results and Discussion</u>

In collaboration with the Department of Respiratory Medicine and Allergy, Karolinska Hospital Solna, we obtained pleural exudates (PE) from 14 patients with lung cancer. From these PEs we isolated exosomes and malignant cells for further studies (Figure 1-2, Paper III), as previously shown [242, 243]. Both PE-derived exosomes and malignant cells contained GGT-1 and rapidly formed $LTD_4$ (Figure 3, Paper III). In contrast to macrophages and dendritic cell derived exosomes, PE-exosomes did not form any $LTC_4$ or $LTB_4$ when incubated with $LTA_4$ (data not shown) but did form $LTD_4$ from $LTC_4$ similarly to A549

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exosomes, suggesting that epithelial/cancer cell exosomes are different from antigen presenting cell exosomes in terms of LT biosynthesis. Moreover, malignant cells from 9 out of 12 patients were positive for GGT-1, further suggesting that this protein may indeed be a valid marker for lung adenocarcinoma [239, 240].

We performed LC-MS analysis on PEs, and on resting and stimulated cancer cells. Although PE-derived cancer cells express the enzymes to form LTs, only few pg of CysLTs were formed while the most abundant metabolites were PGs: we detected 37 $ng/10^6$ cells of $PGE_2$ in cell culture medium and over 53 $ng/10^6$ cells in presence of exogenous AA. On the other hand, these cells could form over 28 $ng/10^6$ cells of $LTD_4$ when exogenous $LTC_4$ was provided, in the same range of $PGE_2$ biosynthesis. We also detected 22 pg/ml of $PGE_2$ and 17 pg/ml of $LTD_4$ in the original PE used to establish the cancer cells (Table S2, Paper III). We believe that the similar amounts detected in the PEs, which can be considered very close to *in vivo*, suggest that $PGE_2$ and $LTD_4$ equally contribute to lung tumorigenesis. There may be several advantages for the cancer cell to release high amounts of both mediators. For example, $LTD_4$ can directly promote $PGE_2$ formation by increasing COX-2 expression in colon cancer cells [188] and both mediators promote tumor growth in a colon cancer model [244]. Moreover, $PGE_2$ and $LTD_4$ cooperate to promote vascular inflammation [245], which may promote formation of pleural effusions [246]

The drastically different capacities of lung cancer cells to form $LTC_4$ from AA, compared to the conversion of $LTC_4$ to $LTD_4$, raises the question which other cells may donate $LTC_4$ in the TME. We isolated macrophages from PE in parallel to malignant cells: when endogenous metabolism of AA was stimulated with A23187, PE-derived macrophages formed high amounts of LTs, and more $LTC_4$ than $LTB_4$, similarly to M-CSF macrophages, as determined in Study II. TAM are leukocytes that infiltrate the TME and promote tumorigenesis [148]. Their phenotype shifts between M1 and M2 endpoints, but a higher percentage of M2 is associated with poor prognosis in patients with lung cancer [156]. Given the similar LT formation of PE-derived macrophages as in M-CSF macrophages, which are primed toward the M2 phenotype, we believe that M2-polarized TAM represent an ideal resource of $LTC_4$ in the TME. Other effective candidates to form $LTC_4$ in the TME are exosomes of myeloid origin. As we mentioned before, neutrophils are enriched in the TME [247]. So far, neutrophils are considered the best donors of $LTA_4$, given their ability to export over 50% of it [32]. Exosomes released by macrophages and dendritic cells efficiently convert $LTA_4$ to $LTC_4$ [132]. Thus, we believe that TAM and myeloid-derived exosomes may contribute to

cancer progression by providing the $LTC_4$ that malignant exosomes and cells convert to $LTD_4$.

Since both exosomes and eicosanoids can promote a pro-tumorigenic environment, we investigated the effect of PE-derived exosomes on the eicosanoid metabolism in PE-cancer cells by LC-MS/MS. Malignant cells increased the formation of CysLTs after 24 hours treatment with PE-exosomes (Figure 4, Paper III). Similarly, BAL exosomes from asthmatic patients can enhance CysLTs formation by bronchial epithelial cells [135]. PE-exosomes induced changes in the formation of other mediators, including $PGD_2$, 20-HETE and $TXB_2$. These results clearly shows that PE-exosomes can influence the release of tumor-promoting lipid mediators by cancer cells. The same vesicles also induced migration of both A549 cell and malignant cells from patient 12 (Figure 6, Paper III). The CysLT1 antagonist MK, a drug for asthma treatment, abolished this effect. MK is normally administrated as a 10 mg pill, which results in ~ 1 μM in blood after ~3 hours [248], the concentration which abrogated the exosome induced migration.

Analysis of CysLT1 expression showed that PE-derived cancer cells from all 12 patients were positive for this receptor. In normal lungs CysLT1 is identified in smooth muscles and tissue macrophages, but not epithelium [24]. Inhibition of the $LTD_4$/CysLT1 signaling pathway leads to cell death in malignant cells overexpressing CysLT1, indicating that CysLTs promote survival of cancer cells [249, 250]. In our study MK treatment induced apoptosis of the malignant lung cancer cells isolated from PEs in a dose-dependent manner, with $IC_{50}$ ~6 μM (Figure 7, Paper III). Interestingly, PE-exosomes could partially counteract the cell death induced by MK, most likely by increasing the formation of CysLTs.

In conclusion, exosomes and malignant cells present in PEs effectively form $LTD_4$ and we speculate that $LTD_4$ promotes the formation of novel metastatic niches. In fact, PE-cancer cells modulate the release of specific eicosanoids in response to exosomes, including CysLTs and the same PE-exosomes promote migration and survival of primary lung cancer cells via CysLT1. This is the first report to unveil the role of the exosomes/$LTD_4$ machinery, in lung cancer, suggesting a novel way exosomes and LTs can contribute to tumor progression, in agreement with the 'soil and seed' theory.

<u>Future plans:</u>

It is crucial to clarify which player of the TME contributes to the biosynthesis and metabolism of specific eicosanoids that may promote tumorigenesis [62]. Exosomes can promote tumorigenesis and the polarization of macrophages toward the M2 phenotype [136, 251, 252]. Inspired by the effects on the PE-exosomes on PE-cancer cells, a next step can be to study the effect of PE-exosomes on macrophage polarization, with focus on the lipidomic profile, in order to understand how the phenotype of macrophages may affect the eicosanoid spectrum in the TME. Stimulation of macrophages with exosomes may also represent a model to mimic DAMP induced eicosanoid metabolism.

Moreover, the results here reported promote the concept that targeting CysLTs may be a valid therapeutic approach in treatment of lung cancer. In fact, there is evidence in both animal models and humans that targeting CysLTs signaling pathways promote anti-tumor effects. Ablation of 5-LO in mice results in decreased influx of neutrophils to sustain metastasis formation in lungs, an effect driven by $LTB_4$ and CysLTs [67]. MK treatment results in increased survival rate in mice with lung cancer [253] and a similar protective effect has been described for humans as well [68]. A 60 mg pill would produce a ~6 μM in plasma, the $IC_{50}$ required to induce apoptosis. This dosage does not give additional side effects, as demonstrated with patients taking 200 mg of MK for 5 months [254]. Moreover, we observed that MK could further enhance the cytotoxic effect of Doxorubicin and Cisplatin, common chemotherapeutic drugs. Thus, the mentioned studies and our results request further research to clarify the exact function of CysLT1 in lung cancer. Large cohorts of biological samples from patients with lung cancer may define the relationship between CysLT1 expression and prognosis, showed to be negative for patients with colon and breast cancer [255, 256]. Given that MK is an approved medication, we believe that there is potential for MK to be quickly included in clinical trials as therapy for patients with lung cancer.

## Conclusions

This thesis brings together two different players of the inflammatory response, eicosanoids and exosomes. While we focused on macrophages and lungs from beginning, the initial goal of the first study was related to asthma. As it is well known, science is unpredictable, and we ended up with lung cancer. The connection of the main findings of this thesis is summarized in Figure 6.
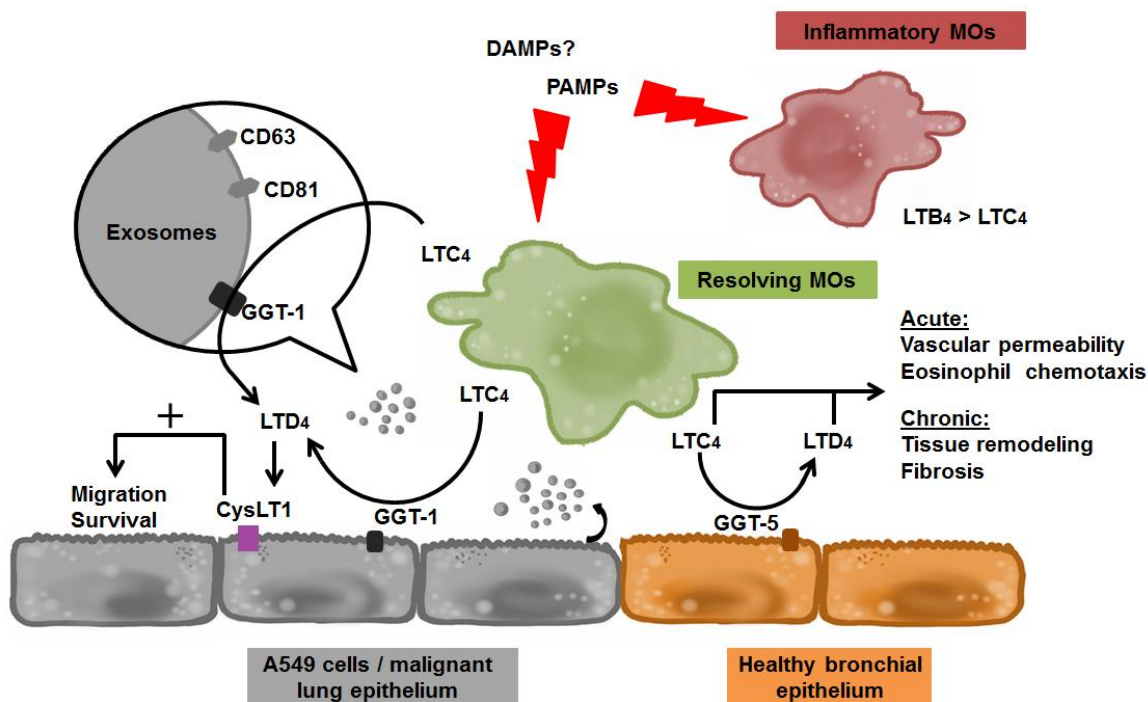


**Figure 6**. Three studies in one picture.

In lungs, epithelial cells directly contribute to $LTD_4$ biosynthesis: $LTC_4$ is metabolized quickly via GGT-1 and slowly via GGT-5. Tumor exosomes also account for efficient formation of $LTD_4$ via GGT-1. Myeloid cells are the principle source of $LTC_4$, Macrophages display specific eicosanoid metabolism, depending on the differentiating factor and the trigger. M-CSF macrophages, primed toward the resolving endpoint, forms $LTC_4$ effectively, due to efficient activation of LTs forming enzymes in response to PAMPs, and despite the lower expression of these enzymes. In the tumor microenvironment, macrophages donate $LTC_4$ that malignant cells and exosomes convert to $LTD_4$: $LTD_4$/CysLT1 promotes migration and survival of cancer cells, possibly contributing to metastasis formation, suggesting a novel target for treatment of lung cancer.

# ACKNOWLEDGEMENTS

lab meeting presentations! Special thanks to **Dolores,** and **Min**, who answered 1001 questions at beginning of my PhD and to **Victoria** and **Michaela**, for your help even when it was not your duty.

Thanks to the 'exosome-group', C**asper, Maria, Pia, Rosanne, Gözde, Jarred, Roberta, Maria-Jose, Stefi** and **Zek**. I always enjoyed going to the 'other side' of Karolinska: thank you for making me feel welcome and for all the scientific inputs. I have learned so much from all of you, especially hard-core immunology. And thanks **Zek** and **Stefi**, for teaching me everything when I joined the group.

A famous italian proverb says "Chi trova un amico, trova un tesoro". I'm lucky to have met you, **Ylva, Fra, Ele** and **Eva**. You made the tea-time sweeter and winters warmer. For sure, if I ever k#&% somebody, I know where to look for accomplices ;)

Sono quasi 8 anni qua nel freddo Nord. Tante cose sono cambiate e ma alcune sono rimaste. **Thilelli, Gian,** e **Diana**. Sono sempre felice di sentirtvi, e vedere come procede la vita, anche perche' sembra sempre che l'ultima volta che ci siamo visti era ieri. Ed e' cosi' bello sapere che tra 10-20 anni saremo ancora li, ad aggiornarci sugli ultimi sussulti di vita. Grazie!

**Lara, Daniela** e **Claudio**, grazie per avermi accolto in famiglia, anche se mi sono presa un ¼ di voi e me lo son portata 2100km a Nord!

Hvala teta **Lizi**, šta ima snagu da putuje svugdje, i šta uvijek me podsjeti koliko su Lukići velika familija. Cekam vrijeme kada ćemo svi biti zajedno.

Фала на **Баба, Мони, Пане, Борис** и. **Ивона.** Обозавам да патувам, али Стип ми е нај умилено место, фала на сите вас. Да бидете сите здрави и срекни, така да моземе да се видеме се по често.

Gazie na Sile i Joze. **Mamo** i **tato**, zemale ste odluki u zivoto sto sa mi dale prilike sta nema svako. Dali ste mi dobri geni i totale sloboda, ako deneska sam uspesna e' grazie a voi. Najvise vas volim.

**Marco**. Ne e' passata di acqua sotto i ponti negli ultimi dieci anni. Siamo passati da Zambo a R.A.M.A. ed infine A.M.M.A..... Per qualcuno il PhD e' un lavoro di coppia, e nel mio caso questo e' totalmente vero: non sarei mai arrivata alla fine di questo lungo viaggio senza di te. Grazie di tutto, questa tesi e' per te. ♥

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