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Clinical and Experimental Pathology

Supervisor: PD Dr. Torsten Goldmann

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Abstract

Annexin A8 (AnxA8) is the eighth member of the Ca^{2+}/phospholipid (PL) binding annexin family. Annexins are known to be ubiquitous and abundant membrane-binding proteins. As membrane-binding proteins, annexins function as membrane organizers and targeting proteins of various processes, thereby they participate in the assembly of enzyme and signalling complexes, vesicle trafficking and repair. Less is known about AnxA8; it specifically binds to phosphatidylinositol-4,5-bisphosphate (PIP_2), F-Actin and associates with late endosomes (LE), which suggests its role in the regulation of epidermal growth factor receptor (EGFR) and/or endocytic transport. In contrary to other annexins, AnxA8 is a less ubiquitous and generally low expressed protein, reflecting its tissue-specific function. AnxA8 was found to be upregulated in different cancer types, like pancreatic cancer or breast cancer. Since most cancer types indicate a deficient EGFR, a correlation between AnxA8 and EGFR is speculated. This study focused on the specific role of AnxA8 in lung, which represents one of the AnxA8-positive localizations. In fact, the expression and cellular localization of AnxA8 was investigated in normal lung, lung cancer (adenocarcinoma and squamous-cell carcinoma), (lipopolysachharides (LPS), lipopeptide (PAM3) and the glucocorticoid analogue dexamethasone (DEXA)) stimulated lung, and pathogen-infected lung tissues. In addition, the expression was examined in bronchoalveolar lavage cells (BALs) of chronic obstructive pulmonary disease patients and LPS/PAM3 stimulated A549 cells. Simultanously, all samples were compared to EGFR using immunohistochemistry, RT-PCR and western blot techniques. IHC results show a membrane-bound localization of both AnxA8 and EGFR, although the expression of AnxA8 appeared lower. AnxA8 was predominantly found in alveolar macrophages and epithelial cells type II (possibly in alveolar epithelial cells type I as well). Stimulation by DEXA affected the expression of AnxA8 in lung, indicated by increased signals in alveolar macrophages. An increased expression in inflammatory cells could also been detected in BAL cells of COPD patients.

Abbreviations

AnxA	annexin group A
A549	modified tumor alveolar epithelial cell line
AEC I, II	alveolar epithelial cell type 1, 2
Akt	protein kinase B
AM	alveolar macrophage
BAL	bronchoalveolar lavage
CC	cholangiocarcinoma
COPD	chronic obstructive pulmonary disease
DEXA	dexamethasone glucocorticoid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
FOXO4	forkhead box O4
GAPDH	glyceraldehde-3-phosphate-dehydrogenase
HOPE	hepes-glutamic acid buffer mediated organic solvent Protection Effect
IHC	immunohistochemistry
JNK	c-Jun N-terminal kinases
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
NSCLC	non-small cell lung cancer
PI3K	phosphatidylinositide 3- kinases
PA	phosphatidic acid
PAM3	lipopeptide Pam3-Cys-Ala-Gly
PE	phosphatidylethanolamine
PG	phosphatidyglycered
PI	phosphatidylinositol
PL	phospholipid
PS	phosphatidylserine
PCR	polymerase chain reaction
PIP_2	phosphatdylinositol-4,5- bisphosphate
PKC	protein kinase c
RPMI	roswell park memorial institute medium
RT	reverse transcription
RT-PCR	reverse transcription polymerase chain reaction
STST	Short-Term Stimulation of Tissues
TK	tyrosine kinase
TMA	tissue microarray
VAC-β	vascular anticoagulant β

1. Introduction

1.1. Overview of the Annexin Superfamily

Thirty-six years ago the first Annexin was discovered by Carl Creutz et al. and named „Synexin“ (Annexin A7) (1). Synexin was isolated from the adrenal medulla and identified to cause the accumulation of isolated chromaffin granules by reversible binding to Ca^{2+} (at concentrations more than 6 μM). Discovery of annexins started at the end of 1970 when they were differently named by their biochemical characteristics and ultimately agreed on the final name “annexin”, based on phospholipid binding properties together with a numeric system (2,3). The name “annexin” originates from the latin word *annexus* meaning “attachment/connection”. In 1980s and 1990s, annexin sequence data increased, representing their diversity and expression in all organisms except of few unicellular organisms. Nowadays, annexins are known to form an evolutionary conserved multigene family, which bind to acidic phospholipids in a reversible and Ca^{2+} dependent manner (biochemical hallmark) (4). They are classified in five groups (A-E) (5,6). Human annexins and related orthologs represent group A, consisting of 12 annexin members (A1-A11 and A13). **(Table 1)**

Name	**Synonyms/Former name(s)**	**Human gene symbol**
Annexin A1	Lipocortin 1, annexin I	ANXA1
Annexin A2	Calpactin 1, annexin II	ANXA2
Annexin A3	Annexin III	ANXA3
Annexin A4	Annexin IV	ANXA4
Annexin A5	Annexin V	ANXA5
Annexin A6	Annexin VI	ANXA6
Annexin A7	Synexin, Annexin VII	ANXA7
Annexin A8	Annexin VIII	ANXA8
Annexin A9	Annexin XXXI	ANXA9
Annexin A10		ANXA10
Annexin A11	Annexin XI	ANXA11

Annexin A12	unassigned	
Annexin A13	Annexin XIII	ANXA13

Table 1 *Annexins are classified in five groups (A-E). Group A: Human and vertebrate orthologues, 12 human subfamilies with 100 orthologues in chordate metazoa; B: Non-vertebrate metazoans; C: Mycetozoa and fungi; D: Plants and E: Alpha giardins (groups B-E are now shown)* (5,6)

Numerous studies on annexins have discovered their different intra- and extracellular functions, like interacting with diverse partners, being membrane-membrane or membrane-cytoskeleton linkers, stabilizing specific domains of organelle membranes and the plasma membrane, participating in Ca^{2+}-regulated exocytosis, endocytosis, cell signalling, regulating inflammation and coagulation, and possessing calcium channel activity. Annexin members were found to have different tissue-specific expression patterns and subcellular localizations. However, their biochemical properties allow them to interact with specific partners and to be targets for phosphorylation, thereby enabling them to accomplish their functions by translocating, migrating to the nucleus or being secreted. The absence of annexins in yeast plus their activity, which depends on cellular circumstances, complicate their precise functional investigation by gene knock-out models in mice and cell lines. However, it gives them a pivotal role. Less is known about annexin A8 or previously named vascular anticoagulant (VAC-β), a Ca^{2+}-/PL binding protein that inhibits coagulation and phospholipase A_2 activity, which Hauptmann et al. discovered as a new annexin member late in 1989 (7).

1.2. Annexin A8

Annexins are ubiquitous and *"any single cell type appears to express a range of annexins, or an "annexin fingerprint," but no single annexin is expressed in all cells, implying that regulation of annexin gene expression is tightly controlled"* (5). In contrast, the expression and localization of AnxA8 in different tissues is less ubiquitous and less abundant. In humans, the expression of AnxA8 was found in skin, placenta, lung, lung lamellar bodies and the cornea, whereas the highest expression was found in lung amongst these tissues (8,9). The gene is also found to be

specifically overexpressed in acute myelocytic leukemia. A specialized function of AnxA8 is postulated. Here we focus on its role in the lung.

1.2.1. Expression of Annexin A8

AnxA8 was firstly identified as a blood anticoagulant (VAC-β) based on in vitro studies. A role in proliferation and/or differentiation of leukemic cells was postulated, since AnxA8 is overexpressed in acute promyelocytic leukemia (APL) and undetected in acute myeloid leukemia (AML) subtypes other than APL. A translocation within the retinoic acid receptor-α (RARAα) gene is the cause of APL, affecting the expression of many genes regulated by RARA (10). The specific expression of AnxA8 in APL, its phospholipase inhibiting property and the lack of a secretory sequence suggests its role in the signal transduction in APL cells and in normal hematopoietic cell differentiation (11,12). Developmental regulation of AnxA8 in APL (maturation inducible) cell line NB4 was shown by induction of the differentiation activator all-trans retinoic acid (ATRA). AnxA8 is downregulated by ATRA induction. These results support a role of AnxA8 in signal transduction and modulation of protein kinase C (PKC) and provide a link between AnxA8 expression and cell growth, which has already been reported for AnxA1 (11,13). The synthetic glucocorticoid dexamethasone inhibits proliferation of A549 cells (human lung adenocarcinoma line) and leads to the appearance, or a further increase (3-fold) in AnxA1 expression on the cell surface.

Various studies have started to focus on the role of the less known member AnxA8. The identification of AnxA8 as a 36 kDa calcium/phospholipid binding protein in placenta was confirmed by Sarkar et al., who also found a normal expression of AnxA8 in placenta and lung (11). A predominant expression of AnxA8 in the plasma membrane indicates that AnxA8 is localized in the cytoplasm or the cell membrane (12,13). In addition, reports show that murine AnxA8 mRNA and AnxA8 protein are specifically found in the suprabasal layers of the stratified epithelia of the oesophagus, the forestomach, in the tongue epithelium and in all layers of the cornea epithelium and in the cornea endothelium of the eye. Detection of AnxA8 and RARA in specific cell layers in the stratified epithelia of the skin and tongue suggests a specific function of AnxA8 in keratinocyte differentiation. Hence, AnxA8 might play

a role in the terminal differentiation of epithelial cells in these tissues (14). Recent reports demonstrate an upregulated expression of AnxA8 in osteoclasts by bone (15). Upregulated expression of AnxA8 was identified to regulate the formation of actin ring and cytoskeletal reorganization late in osteoclast differentiation. Again, it supports its role in cell differentiation. Expression of AnxA8 in HeLa cells (with epithelial-like morphology) was detected as well (8).

1.2.2. AnxA8 in lung

The lung is an essential respiration organ. Its main function is the exchange of O_2 and CO_2 with blood and respiratory air. Two types of cells are found in the epithelium of alveoli, which are linked to annexins. (**Fig. 2**) These are long, flat pneumocytes type I (AECI), and small, fat, surfactant producing pneumocytes type II (AECII). In fact, AECI has a flat nucleus surrounded by some cell organelles, pinocytotic vesicles in the cytoplasm and microfilaments. AECII have a round-shaped nucleus and contain lamellar bodies and keratins found in the cytoplasm. Furthermore, alveolar macrophages (AM) are also found, they arise from monocytes and ingest bacteria and particles. In addition, the connective tissue found in alveolar septum contains fibroblasts, which has actin filaments and possibly a contractile function as myofibroblasts.

Basal cells, which transform into cancer cells by basal cell hyperplasia or dysplasia in the respiratory epithelium of bronchi, are the main cause of squamous-cell carcinoma (SC) (16). Together with adenocarcinoma (AC) they are the most frequent types of non-small-cell lung carcinoma (NSCLC) of epithelial lung cancer, consisting of different histological patterns. Histologically, SC arises centrally in larger bronchi and it can develop cavitation by necrosis. Intercellular bridges and keratosis are histological hallmarks. On the other hand, AC shows a frequency of at least 25% in lung cancer. In fact, it reaches a frequency of 45% in malignant lung tumors. Primary AC is localized in the periphery, whereas fibrosis occurs centrally. Histologically, AC presents a glandular and mucous pattern. Furthermore, chronic obstructive pulmonary disease (COPD) defines a group of lung diseases (chronic bronchitis or emphysema), which can be triggered by bacterial infections (e.g.

Haemophilus influenzae). It shows a specific inflammatory response including the release of early cytokines and the recruitment of macrophages, neutrophils and dendritic cells, resulting in epithelial cell hyperplasia (17). Annexins are associated with inflammatory processes, since they interact with phospholipase A_2 (PLA_2) (18).

AnxA8 is significantly lower expressed (70-100 times) in lung compared to AnxA5, which is expressed ubiquitously and abundantly (8). IHC results revealed an exclusive localization of AnxA8 in the endothelia. Both HeLA cells and lung endothelial cells have low proliferative rate and express AnxA8. **(Fig. 3)** Thus, the general low expression of AnxA8 can occur in un/differentiated and non-/proliferating cells. AnxA8 presents a tissue and cell-type specific protein with a highly specialised function in lung.

The characteristic expression and intracellular distribution of AnxA1, A2 and A4 in different tissues support their role in differentiation and/or tissue-specific functions (19). **Fig. 2** represents the expression of AnxA1, A2 and A4 in the respiratory tract.

Tissue	Annexin		
	I	II	IV
Respiratory tract			
Nasal mucosa			
Epithelium	++	+++	++
Ciliae	+++	+++	+++
Endothelium of swell bodies	+	+++	+
Paranasal sinuses			
Epithelium	++	+++	++
Ciliae	+++	+++	+++
Endothelium of capillaries	+	+++	+
Larynx epithelium			
Stratified epithelium			
Basal epithelium	+	–	++
Intermediate layer	++	–	+
Superficial layer	+++	+	–
Respiratory type epithelium			
Epithelium	++	+++	+++
Ciliae	+++	+++	+++
Cartilage	–	–	–
Trachea			
Epithelium	++	++	+++
Ciliae	+++	+++	+++
Cartilage	–	–	–
Bronchi			
Epithelium	++	–	+++
Ciliae	+++	+++	+++
Clara cells	–	–	–
Bronchioles			
Epithelium	++	–	+++
Ciliae	+++	+++	+++
Clara cells	–	–	–
Respiratory bronchioles			
Epithelium	++	–	+++
Alveoli			
Type I cells	–	+++	+
Type II cells	–	+++	–
Pleura			
Mesothelial cells	+++	+++	++

Figure 1 *Overview of the expression of AnxA1, A2 and A4 in the respiratory tract. (- negative, + weak positivity, ++ moderate positivity and +++ strong positivity)* (19)

These results show that AnxA2 was strongly expressed on the luminal membrane of both alveolar epithelial type I (AECI) and type II (AECII) cells, but lacked in the bronchi, the bronchioles and the respiratory bronchioles. AnxA4 was

weakly found in AECI. The expression of all three annexins was the same in non-keratinised stratified squamous epithelium throughout the body. However, an increased expression dependent on the differentiation status has been shown. All three annexins were absent in clara cells of bronchi.

1.2.3. Expression level

Annexins are found as cytosolic and extracellular proteins, which points out their intra- and extracellular functionality. Several studies extend and confirm their intra- and extracellular functions by showing a nuclear localization of AnxA2 and AnxA7. In contrast, AnxA8 has no secretory sequence peptide and is not shown to be an extracellular protein (20–22). Promoter regulatory studies were used to investigate the gene expression and the control of it. **Fig. 2** represents the expression levels of all human annexin members examined in millions of expressed sequence tags (ESTs) originating from human and mouse tissue cDNA libraries, thereby underlining promoter strength and mRNA stability. These results show that AnxA8 is expressed moderately in comparison to the highly expressed AnxA1 and AnxA2. Different expression levels are providing information about their different gene expression regulation and their functional variance (9).

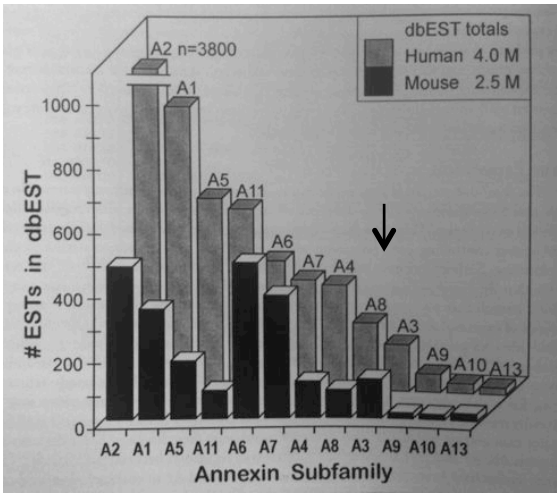

Figure 2 *Annexin expression level profiles in human and mouse derived from millios of expressed sequence tags (ESTs) from human and mouse tissue cDNA libraries. (The ratio of the gene expression level in human and mouse is approx. 100:60 ESTs.) AnxA8 is expressed at low levels* (9).

1.2.4. Classification of AnxA8

Functional differences of annexins and the hypothesis of a tissue-specific role of AnxA8 require molecular genetic changes throughout the evolution gained by gene duplications and homologous recombinations. Based on cladistic studies, human annexins are classified into 3 groups, which might represent their functional similarities: The first group includes AnxA7, A11, A13, second group includes AnxA4, A5, A8, and third group includes AnxA1-3 plus AnxA9 and A10 as distant members (5). These three groups might have established functional variety in order to match the needs of evolving tissues and organisms. In fact, Anx8 and AnxA4 are proposed to be sequential duplication products from AnxA11. AnxA8 evolved about 383 and 323 million of years ago in this order: ANX11 → ANX4 → ANX8. (**Fig. 4)** Furthermore, AnxA8 together with AnxA1, A3-5 and A13 indicates a fast evolution and/or tissue-specific expression via limited functionality or adaptation to new species requirements, or it acts in counter-function (23).

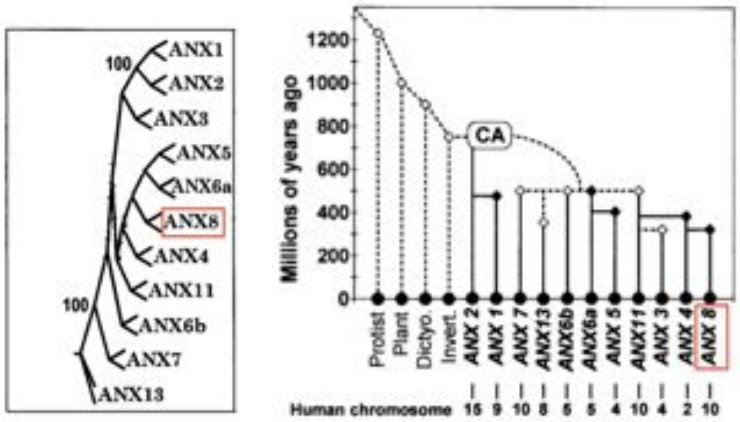

Figure 3 *Clastidic analysis of annexins (left) and a divergence time chart in comparison to their chromosomal location (right)* (23).

1.3. Structural characteristics of the annexins shed light on their function

Annexin A5 was the first structurally studied member of the annexin family, the so-called prototype for other annexins (24). Annexins consist of two domains: the highly conserved C-terminal core domain (a 34 kDa C-terminal region) and a unique N-terminal domain, coherent with post-translational modifications and protein-ligand binding sites. The core domain contains a related and specific tetrad structure of

homologous repeats (annexin repeats), generally consisting of four repeats filled into an α-helical disk (25). Ca^{2+} and PL binding sites are harboured in the core domain, thus enabling membrane binding. Interestingly, annexins have a low intrinsic affinity for Ca^{2+} (K_d 25 - 1000 μm), and they can bind 10 to 12 Ca^{2+} ions. However, annexins bound to acidic PL can increase their Ca^{2+} affinity up to 100-fold. Concentration of free Ca^{2+} necessary for membrane translocation differs between annexin members (26).

1.3.1. Annexin Core – a conserved domain provides Ca^{2+}/phospholipid binding sites

The core domain is about 70 amino acids (aa) long and contains four similar repeats (endonexin fold), each is folded into five α-helices (A-E). However, AnxA6 has exceptionally eight repeats. Annexin repeats are highly conserved homologous domains, which provide Ca^{2+}/phospholipid binding sites by their consensus endonexin sequence *GXGT-[38 residues]-DE* (a specific 'type II' motif) (4). The four annexin repeats are located around a central hydrophilic hole, which has a voltage-dependent Ca^{2+}-channel activity (27). **(Fig. 5)** Each domain has five α-helices, which are found on the rounded surface of the protein. Structurally, short loops are holding these five helices together, thus allowing Ca^{2+}-binding. Specific loops exist; amongst them is the "AB" loop (also Type II; loop between helices A and B) and the "DE" loop (loop between helices D and E), representing Ca^{2+}-binding sites together with the "B" site. Most proteins have two to four type II high-affinity Ca^{2+}-binding sites, they alone are sufficient for membrane binding. Some annexins have two additional type III ("B" or "DE" sites) Ca^{2+}-binding sites with lower affinity, which increases the affinity of binding. Ca^{2+} is coordinated by three carbonyl oxygens located in a short interhelical loop (between helices A and B) and two carboxyl oxygens of an acidic residue, located at the end of helix D ('cap') and water molecules. Joining of "AB" and "DE" helices is accomplished more precisely by binding of Ca^{2+} within the loop (27–30). Annexins have different domain preferences, which interact with phospholipid bilayers. Binding to membranes depends on the net charge of the polar head of phospholipids, annexins prefer binding to phosphatidylglycerol (PG), phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylethanolamine (PE) or

phosphatidylinositol (PI). Interestingly, annexins A8 and A2 preferably bind to phosphatidylinositol-4,5-bisphosphate (PIP_2) (31–33). Thus, phospholipid-binding properties of AnxA8 explain its anticoagulant and anti-phospholipase activity and specific PL-binding supports its specialised functionality.

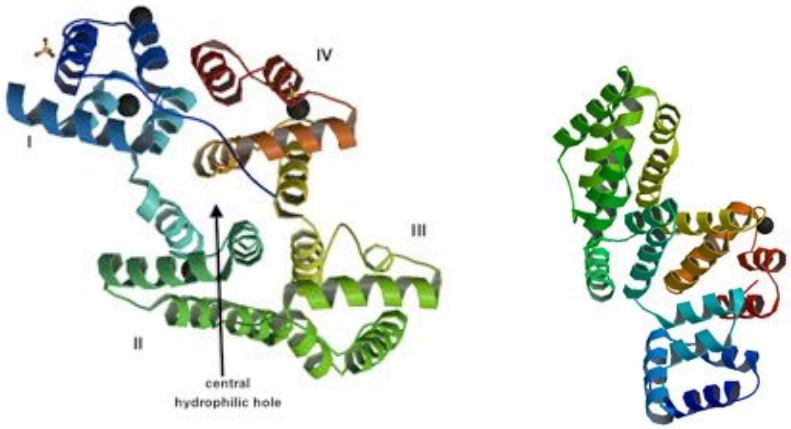

***Figure** 4 Crystal and molecular structure of human annexin A5 after refinement (PDB 1AVR)* (34,35). *Annexin A5 is shown from the concave side. The core contains four domains (I-IV), which consist of five a-helices (A-E). The central hydrophilic hole is displayed (arrow), suggested to have voltage-dependent Ca^{2+}-channel activity when annexin binds to Ca^{2+}. AB, DE and B sites (not shown) are responsible for Ca^{2+}-binding. (Left) The 2.1 angstrom resolution structure of annexin A8* (36) *(right).*

1.3.2. The N-terminal interaction domain

In contrast to the conserved annexin core, the smaller N-terminal domain is unique for each protein. It has neither a conserved sequence nor length or structure homology. However, the N-terminal domain is responsible for the variety of protein functions, it is considered as the key element in regulating structure and function (5). Protein–protein interactions (e.g. with the EF-hand family protein S100 and actin) and post-translational modifications (e.g. phosphorylation) of the N-terminus affect the structure of the core and contribute to structural stability. The length of the N-terminal domain varies between few to 200 aa residues, that led to a size-specific arrangement into three groups of this domain: short (less than 20 aa), intermediate (22-55 aa) and long (+56 aa) N-terminus.

The first annexin member with defined full-length crystal structure together with its complete N-terminal domain (in absence of Ca^{2+} ions) was AnxA1 (37).

Proteins with intermediate and long N-terminal domains were shown being active in membrane aggregations (Annexin A1, A2, A7 and A13b), thus suggesting a role of N-terminus in membrane aggregations and fusion events (38). The N-terminal domain of AnxA1 is composed of 41 aa residues. Two consecutive amphipathic α-helices (NA: 2-16 and NB:18-26) are found in the N-terminal domain, which are connected to the core domain via linker (residues 27-41). Precisely, the N-terminal domain is located across from the Ca^{2+}-binding site of the core domain, on the concave side of the core. Residue at position 17 is displaying a bend between both helices. The most N-terminal helix (NA: 2-16), which points towards the convex side of the protein, is inserted into the core domain III (helix D). (**Fig. 6**) Subsequently, helix D is displaced and unwound forming an extended loop (D-flap). The second helix (NB: 18-26) interacts with the surface of the core domain IV. NB helix, together with the linker region, is in contact with core repeats I and IV. Presence of Ca^{2+} ions lead to conformational changes involving the movement of the N-terminus out of the core domain III through linker while the unwound D-flap folds into a helical conformation forming a Ca^{2+}-binding site (37,39). Thus, Ca^{2+} ions induce a conformational change including a N-terminal domain presentation and binding of annexin to a membrane. Consistent of these facts, a new term for the N-terminus is proposed: "N-terminal interaction domain" (30).

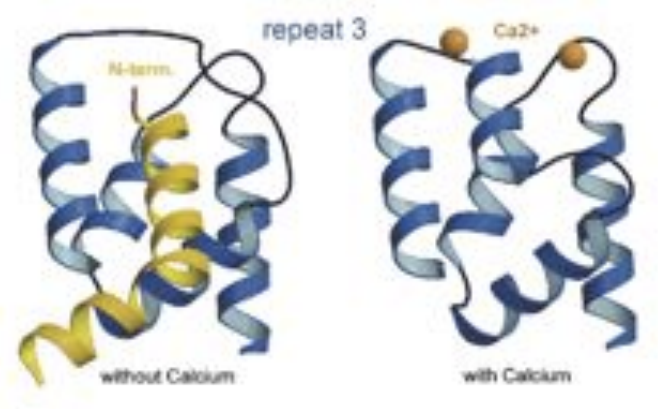

Figure 5 *Core domain III of the full-length Annexin A1 is displayed in absence (left) and presence (right) of Ca^{2+} ions. N-terminus consists of two consecutive amphipathic α-helices (NA and NB). NA helix is inserted into helix D, resulting in unwinding of helix D into an enxtended loop. NB helix is interacting with the surface of core domain IV (not showns). In presence of Ca^{2+}, a conformational rearrangement is induced, which leads to folding of helix D and formation of a Ca^{2+} -binding site. N-terminal domain is shown being excluded from the hydrophobic pocket of the core domain.* (39)

There is a limited report on the interacting partners and modifications of AnxA8. The crystal structure of AnxA8 is similar to that of AnxA3 (40). (**Fig. 5**) Both are considered as medium-length N-terminal proteins consisting of three Trp residues and several Ser and Thr residues that can be phosphorylated. Previous findings have shown that AnxA8 lacks the tyrosine residue that is phosphorylated by EGFR or other kinases. In fact, Thr14 might be a PKC phosphorylation site that allows membrane binding, as seen in other annexins (7). Moreover, AnxA8 cannot interact with S100 proteins due to different composition of the N-Terminus and lacks the formation of an amphipathic helix. Structural similarity between AnxA8 and AnxA3 suggests a functional relationship in some cells undergoing differentiation, since both proteins are found in promyelocytic cells during their differentiation.

Figure 6 *The structure and molecular weight of AnxA8 (AnxA1 and AnxA2 have the same size; AnxA6: 68 kDa)* (41).

Furthermore, AnxA8 has a potential proteolytic cleavage site. In the course of Ca^{2+}-binding, it is lacking the first 16 residues of the N-terminus, which might regulate its activity. In addition, the core domain III might play an important role in the function of annexins and the flexibility, as even low levels of Ca^{2+} induce a conformational change in domain III of AnxA8. These findings are supported by recent results of structural related AnxA3. They show that AnxA3 has a greater flexibility due to a different position of domain III relative to the other domains in comparison to AnxA5 and A1 (42).

1.3.3. Post-translational N-terminal modification

N-terminal domains enable post-translational modifications. Reversible phosphorylation is controlled by the activity of protein kinases (Ser/Thr kinase, Tyr kinase or Ser/Thr/Tyr kinase), thereby changing the activity and function of proteins. Tyrosine kinase is an important enzyme that catalyses essential cellular signalling events: growth, differentiataion, proliferation and transformation. As mentioned before, AnxA8 has the same phosphorylation site as other annexins, which is the threonine at position “14”. Thus, AnxA8 is a substrate for protein kinase C (PKC) (7). Accompanied by conformational changes in the core domain, modification of AnxA8

results in its functional regulation. Indeed, variance in the N-terminal sequences might allow specific modifications, thus explaining functional differences of annexins.

Certain annexins are cellular targets for Ser/Thr protein kinases like protein kinase C (PKC) and cAMP-dependent protein kinase A (PKA), which affects their functions (vesicle aggregation and lipid binding characteristics) (43). Phosphorylation has been shown to affect (positively and negatively) the "bivalent" activity of certain annexins (AnxA1, A2, A4, A6 and A7), leading to membrane aggregation. Phosphorylation was catalysed by the epidermal growth factor (EGF) receptor kinase and Src-family tyrosine kinases (TK) in AnxA1 and A2. Hence, a link between EGFR and annexins has been made. Further results support this link by showing that AnxA1 is phosphorylated in the multivesicular body (MVB) during the processing of the EGFR. A role of some annexins in EGFR sorting and recycling upon phosphorylation by an active EGFR kinase is postulated (44). Furthermore, phosphorylation can also affect the Ca^{2+} sensitivity of annexins, resulting in the release of their N-terminal domain and allowing their proteolysis. Additionally, phosphorylation affects the location of AnxA2, which can be located both in cytoplasm and nucleus (45). It enters the nucleus upon PKC-phosphorylation, while it is exported by the nuclear export signal via its N-terminal domain, which is a substrate for the leptomycin-sensitive nuclear transport machinery.

1.3.4. N-terminal interacting partners

The regulatory NH2-terminal domains harbour binding sites for EF-hand proteins in the first 10-14 aa. Annexins complex with several EF hand proteins (*S100A6: AnxA11, S100A10: AnxA2, S100A11: AnxA1, Sorcin: AnxA7, S100A1 and S100B: AnxA6*) and gain functional activity and/or help to translocate EF-hand proteins to membranes (5,46). Binding to EF-hand proteins can be applied to other proteins. It is accomplished by a conformational change and induced by Ca^{2+} ions. Thus, Ca^{2+} regulates the exposure of membrane or protein binding sites (ligand binding and membrane aggregation) and the access for modifications (e.g. phosphorylations by PKC), leading to specific annexin activities and affecting specific localizations of annexins (39).

AnxA8 shows a unique F-actin and PL binding property. AnxA8 is recruited to F-actin-associated PIP_2-rich membrane domains formed in HeLa cells upon infection with non-invading enteropathogenic E. coli. (32). The interaction with F-actin and actin-associated membrane domains, as well as the association with late endosomes, highlights the link between AnxA8 and EGFR in context of EGFR trafficking, signalling and consequently cancer.

1.4. Overview of Annexin Functions

The exact role of AnxA8 and individual annexins is still unknown. However, all members have a common conserved core domain and a variable N-terminal domain that determines particular functional roles and possible additional features. In regard to the naming "*annexus*", annexins bind to acidic phospholipid membranes, whereas AnxA8 binds specifically to PIP_2, activated by intracellular Ca^{2+}, which itself is induced by diverse stimuli. In contrast, AnxA8 cannot bind to phosphatidiyserine (PS), which excludes its role in modulation of cell removal by phagocytosis as described for AnxA3-A5 and AnxA13 (47). It is essential to keep in mind that annexins use their Ca^{2+} sensitivity and specific PL compositions, or pH-sensitivity to regulate their function, and they can gain additional activity by modification of their unique N-terminus. In fact, specificity is preserved in the annexin core. The activities include Ca^{2+}-dependent and Ca^{2+}-independent binding to PL membranes especially to cytosolic leaflets of the PM and certain organelle membranes. Thereby, annexins (AnxA1, A2, A4 and A7) can act as membrane scaffolds via its "bivalent" activity and enable membrane vesicle aggregation, which is regulated by phosphorylation (30). **(Fig. 8)** Furthermore, Ca^{2+}-independent activity is associated with pH level (48). AnxA2 and AnxA6 are shown to bind to endosomal membranes at neutral pH.

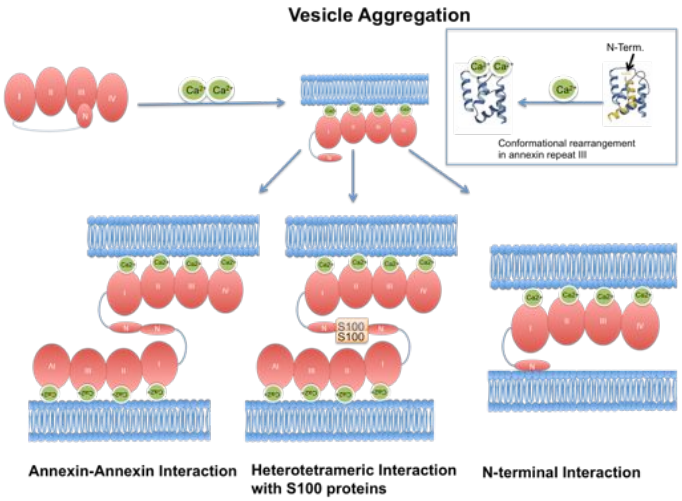

Figure 7 *Vesicle aggregation is an important quality of annexins. Linking of two membranes is dependent on the PL-composition and the* Ca^{2+} *level and can be further regulated by modifications and partners they interact with. (modified image:* (33)*)*

In response to increased $[Ca^{2+}]_{cyt}$, annexins move around the cell, where they can bind to membranes, receptors or other proteins and affect e.g. EGFR signalling and trafficking (49)**.** In addition to their structural features, they don´t have a signal peptide for cellular export; they can be secreted (e.g. AnxA1 by ATP-binding cassette (ABC) transporter system); and they act extracelullarly on coagulation (50,51). Thus, annexins are described to be involved in following intra- and extracellular processes (5,52,53):

- Exocytosis
- Endocytosis
- Regulation of membrane: trafficking, structure, dynamics, membrane-cytoskeleton interactions
- Calcium-channel activity
- Signalling (mediation of $[Ca^{2+}]_{cyt}$ sensing and signalling, linking $[Ca^{2+}]_{cyt}$ dynamics to cytoskeletal responses and mediating immune or stress responses)
- Inhibition of phospholipase activity
- EGFR modulation
- Apoptosis?
- Proliferation and movement of cancerous cells

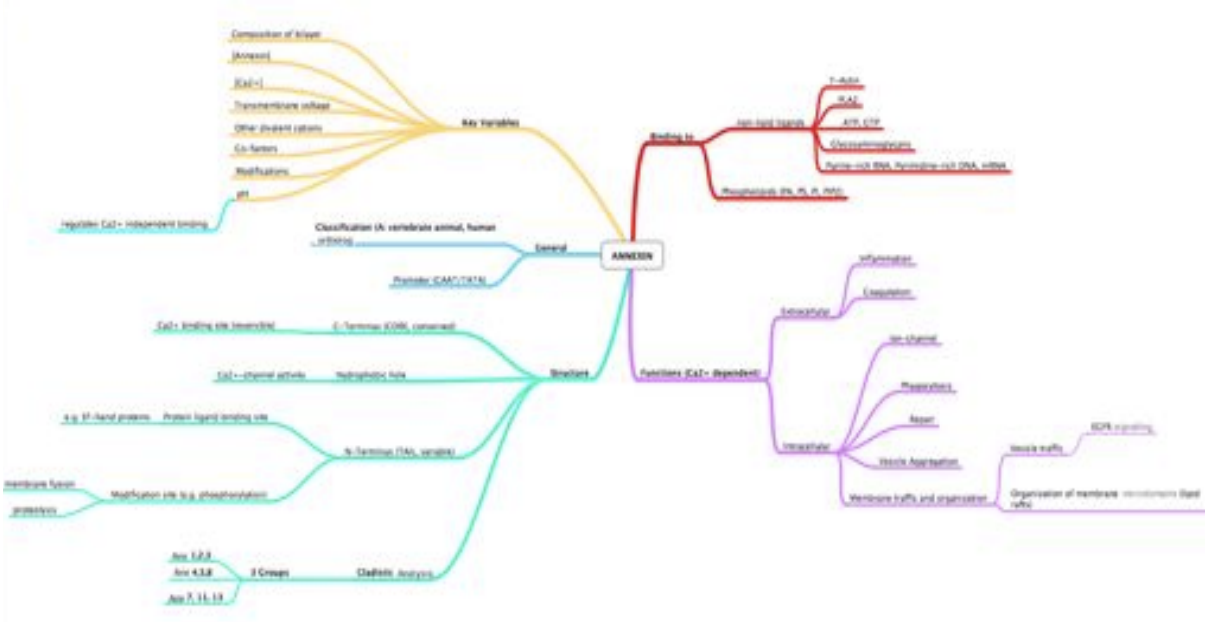

Figure 8 *Overview of the structure and functions of annexins*

1.4.1. Exocytosis

Fusion of the secretory-vesicle membrane with the PM is necessary for the secretory pathway and can be explained by the "bivalent" activity (to link to membranes) of annexins. AnxA2 is expressed in AECII, where it plays a role in lung surfactant secretion from AECII by binding to S100A10 (54). Recent studies show another family member (AnxA7) to associate with AECII (55). In fact, AnxA7 plays a role in the fusion between lamellar bodies and the PM during surfactant secretion in AECII. Alveolar type II cells produce lung surfactant (mainly comprised of phospholipids, combined with carbohydrate and protein, released by exocytosis), store it in lamellar bodies and secrete it into the alveolar hypophase by fusion between lamellar body with the PM, where it prevents surface tension and optimises conditions for gas exchange. AECI establishes the surface for gas exchange (56). Interestingly, annexins are associated with the formation of lipid rafts (49,57). These specialized microdomains can be also found in the PM of AECII, where they contain some proteins (e.g. SNARE) linked to the surfactant secretion process. Additionally, stimulation of AECII with surfactant secretagogues has shown to increase the surface AnxA7, which interacts with lamellar bodies and the PM. Furthermore, binding to PM was increased by AnxA7 phosphorylation in vitro. In fact, post-translational

modifications and protein interactions might enable annexins to insert into membranes.

1.4.2. Endocytosis

Annexins are subcellular differently distributed and switch between cytoplasm and PM upon Ca^{2+} level. Their role in membrane trafficking is supported by their choice of membrane, which varies within the family members. Mostly they bind to membranes of the biosynthetic and endocytic pathway. AnxA2, which was previously linked to the secretory pathway, was identified on early endosomes, associated in a Ca^{2+} independent manner (5,48). Furthermore, another annexin member (AnxA1) is associated with multivesicular endosomes (MVE), in fact it is mediating the inward vesiculation (58). AnxA8 was also found associated with the endocytic pathway and will be discussed in the next chapter. Localization and function of endosomes is dependent on actin filaments. Binding to F-acting has already been described for different annexins, amongst them AnxA8 and AnxA2. In fact, AnxA2 was detected together with actin filaments in human lung adenocarcinoma cells (A549), where it binds Ca^{2+} in/dependently (59).

1.4.3. Phagocytosis

Amongst other phospholipids, annexins also bind to phosphatidylserine (PS). PS signalises phagocytosis of apoptotic cells and they are found on the surface of apoptotic cells. It has been reported that AnxA8 cannot bind to PS and therefore it is not involved in phagocytosis by modulating cell removal (60). Interaction of annexins require negatively charged amino acids found in the type II binding motif of AnxA3, A4, A5 and A13. These are replaced by positively charged amino acids in AnxA8 and prevent its binding to PS at physiological conditions.

1.4.4. Inflammation

The functional variety of annexins has extended due to their detection to inhibit phospholipase A2 (PLA_2), which is crucial in inflammatory processes (18). Inflammation is an initial response to pathogens or other harmful stimuli, which is

stopped by anti-inflammatory mediators possibly in a time- and space-specific manner so that it ensures fixing of tissue structure and homeostasis. Glucocorticoids are anti-inflammatory mediators and function in two ways: genomically (transactivation or transrepression of gene transcription) or non-geneomically (rapid and independent of de novo protein synthesis) (61). AnxA1 was described to have inflammatory activity due to its extracellular detection upon inflammation (4,61). Glucocorticoids (endogenous and exogenous) were found to regulate the synthesis and function of AnxA1 and they can mediate their anti-inflammatory effects via AnxA1. In fact, studies with the glucocorticoid analogue dexamethasone (DEXA) on the sensitivity of the promoter (CAAT and TATA boxes are minimal required for promoter activity) revealed AnxA1 not to be a glucocorticoid primary response gene (62). However, results are controversial and may depend on the use of different cell lines or time of induction. Studies on the cytokine responsiveness of the AnxA1 promoter showed the gene to be induced by interleukin-6 (IL-6), suggesting an AnxA1 role in acute inflammatory response. In another study, stimulation of RAW 264.7 macrophages (MAK) with lipopolysaccharide (LPS) lead to a downregulation of AnxA1, while its expression was significantly increased with DEXA or DEXA and LPS (63). The incubation of MAKs results in the release of cytokines and induces the inflammatory response.

1.4.5. Cancer

Annexins can be phosphorylated by EGFR TK, which suggests their role in EGFR signalling and consequently cancer. This postulation is supported by following findings: AnxA1 is expressed in prostate cancer, leukemia, cervical cancer and lung squamous cell carcinoma (SC) where it might be involved in genesis, progression, recurrence and metastasis (64). This implicates that multiple functions of AnxA1 can be assumed for other proteins as well. Furthermore, the expression levels of AnxA2 mRNA and protein in human non-small cell lung cancer (NSCLC) tissues were significantly higher than in normal tissues, indicating another link between annexin and cancer (65). There is limited information of AnxA8 in cancer. It was firstly described in APL, where it was overexpressed in APL but not in other leukemia. A link with EGFR-signalling was supported by the findings of AnxA8 in

cholangiocarcinoma (CC) and pancreatic cancer; AnxA8 is associated with breast cancer as well (52,66,67).

1.5. AnxA8 – controlling EGFR signalling and trafficking

Activation of the EGF receptor upon EGF-ligand binding triggers different signalling events, containing the janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, ERK (RAS/extracellular signal regulated kinase) pathway and phosphatidylinositol 3-kinase/AKT (PI3 kinase/AKT) pathway.

At cell surface, EGF binds to its receptor and mediates an EGFR dimerization that stimulates its intrinsic tyrosine kinase (TK) activity. This results in autophosphorylation of some tyrosine residues that allows proteins to bind via their Src homology 2 (SH2) domains, proceeding in the activation of downstream signalling cascades. These pathways regulate important cellular processes like cell differentiation, proliferation, apoptosis and motality. EGFR signalling does not only occur on the cell surface, activated receptor enhances signalling upon endocytic transport by lateral movements and the formation of specific signalling protein complexes. AnxA8 and other individual annexins are found to be involved in trafficking and compartment-specific microenvironment for EGFR signalling. Thereby, annexins interact with different proteins and membranes, organize membrane microdomains and form/stabilize compartment-specific signalling platforms. Ca^{2+}, cholesterol, pH levels and the lipid composition affect the target specificity of each annexin.

AnxA2 and AnxA6 are associated with the formation of lipid rafts (clolesterol-rich PM domains), which are rich in protein receptors and possibly acting as membrane signalling platforms (57). As already mentioned, they are found in the plasma membrane of AECII and allow its surfactant secretion. Their exact role in EGFR signalling is controversial. They might act as inhibitors of the EGFR signalling or only of certain pathways, which require modulators/scaffolds as annexins could be. Nevertheless, lipid rafts seem to be important to EGFR by providing a platform for ligand binding and signalling.

1.5.1. AnxA8 affects EGFR by interacting with late endosomes and F-actin

Internalization and lysosomal targeting are important steps of the signalling termination that requires downregulation of the activated EGFR via endocytosis. Inactivation of EGFR occurs at the same time as its activation. It is a regulatory step that ensures the optimal strength and duration of signalling, whereas hyperstimulation can lead to cancer. As mentioned before, lipid rafts are involved in EGFR signalling. They are also involved in the internalization of EGFR, which occurs via clathrin-coated-pits (CCP) and via caveolae/lipid rafts in [EGFR]-dependent manner (68). At low doses of EGFR, internalization occurs via CCPs, and EGFR is recycled (signalling is maintained). At high doses of EGFR, internalization can occur via both ways, whereas internalization via lipid rafts results in receptor degradation.

Furthermore, AnxA8 and AnxA2 are proposed to play a role in multivesicular body (MVB) biogenesis (57). Specific sorting to internal vesicles of multivesicular endosomes (MVE) enables the final degradation of EGFR in late endosomes (LE)/lysosomes. Indeed, proper movement of endosomes ensures their proper functions. Mobility of LE requires cytoskeletal elements such as actin filaments to correctly deliver transported EGFR. They are also associated with perinuclear aggregation, morphology, fusion and trafficking of the LE, thereby controlling the endocytosis and degradation of EGFR, and affecting EGFR signalling.

Interestingly, AnxA8, which is a F-actin and membrane binding protein, was reported to regulate the association and function of LE by linking LE to actin cytoskeleton either directly or by the organization of membrane/actin attachment sites. These results are supported by the identification of AnxA8 being specifically linked with late endosomes (32,69). In addition, depletion of AnxA8 decreases the association of LE with actin and leads to their relocation to the cell periphery. Deficient movement of LE results in a delayed transport and degradation of EGFR. Additionally, delayed transport affects the signal transduction by prolonging the activation of ERK1/2 (MAP kinase signalling), since EGFR can still signal from the early endosomes. Recently it has been shown that the depletion of AnxA2 also delays endocytic trafficking of EGFR via cofilin activation (an actin binding protein) and enhances EGFR signalling (JNK and Akt, but not Erk) pathways (70).

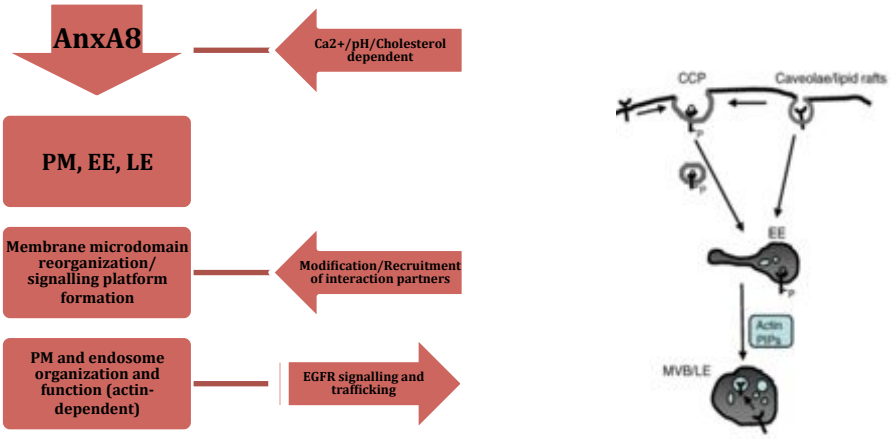

Figure 9 *AnxA8 can modulate the microdomain formation (lipid rafts). It associates with late endosomes and F-actin and enables the activity of EGFR (signalling and trafficking) in a* Ca^{2+}*, pH and cholesterol dependent manner* (57).

The early detection of AnxA1 to be phosphorylated in the multivesicular body (MVB) during processing of EGFR provided first insights into the role of annexins in EGFR sorting, which goes along with a phosphorylation by an active EGFR kinase. So far, detection of other annexins (AnxA2, A6 and A8) in endocytic compartments supports their role in modulating trafficking and EGFR/Ras signalling (69,71).

AnxA8 has further similarities to AnxA2; it might organize or stabilize actin-associated membrane domains (via raft clustering) by specific binding to F-acting and PIP_2 (via lateral interactions). Binding to PIP_2 is strictly Ca^{2+}-dependent and binding to F-actin occurs substoichiometrically (32,53).

These results, together with the moderate expression level solely found in lung, skin, liver and kidney, may explain the tissue-specific and modulating role of AnxA8 in the signalling and trafficking of EGFR. This includes the lateral movement of EGFR, kinetics of EGFR endocytosis and the direct interaction with EGFR and regulators/effectors of the EGFR/Ras signalling (e.g. Src tyrosine kinase, Pyk2, PKC, SH2-domain-containing tyrosine phosphatase 2 (SHP2), Rho, Cdc42, Ras and GTPase activating protein p120GAP (57). The ability to interact with both F-actin as well as the endosomes defines a specific mechanism by which annexins can mediate EGFR endocytosis.

1.5.2. AnxA8 – role in ADAM-mediated ectodomain shedding

A specific function of AnxA8 in keratinocyte differentiation was reported (14). Its expression was found in specific cell layers in the stratified epithelia of the skin and tongue. Recently it has been reported that AnxA8 interacts with ADAM17 in keratinocytes (72). ADAM17 is a preotease involved in ectodomain shedding. ADAM17 is also found in the endothelial cells, same as AnxA8. However, interaction of AnxA8 with ADAM17 occurred extracellular, which provides a new localization and function of AnxA8. Ectodomain shedding is a cell surface remodelling process, which is an important step in the activation of the Notch signalling (endothelial differentiation or tumor angiogenesis) or the Tie-1 signalling (blood-vessel formation). It has also been reported that keratinocyte migration requires ectodomain shedding of EGFR ligand precursors in order to activate EGFR and it's signalling. Knockdown of AnxA8 has shown to affect the cell motility, which was reduced, indicating its role in keratinocyte migration. Regulation of ectodomain shedding remains important for cellular functions; incorrect shedding leads to abnormal development, such as cancer. These results agree with the previously mentioned EGFR-modulating activity of AnxA8.

1.6. AnxA8 and Cancer

The effect on EGFR goes beyond its modulation during trafficking and signalling. Previous results show that depletion of AnxA2 not only delays EGFR trafficking and enhances EGFR signalling, it also enhances the metastasis formation in breast cancer cell lines (70). In neoplasia, annexins have a tumour type-specific pattern of expression, due to their different expression levels and/or specific subcellular localizations (52). For instance, IHC analyses in lung cancer show a diffuse expression of AnxA1 in neoplastic cells in lung tumor tissues and a high expression of AnxA2 at the cell surface (73). A deficient EGFR-TK, resulting in a hyperstimulation of EGFR, is found in many human cancers.

In cholangiocarcinoma (CC), AnxA8 is highly expressed but is downregulated upon tumor progression and tumor metastasis by EGF-mediated FOXO4 phosphorylation within the EGFR/PIP3/AKT signalling pathway (66). **(Fig. 9)**

Triggered by EGF, the morphologic changes of the epithelial-to-mesenchymal transition (EMT) in CC cells enable spreading of tumor via migration. Moreover, ectopic AnxA8 reverses the morphology of cells, it is accompanied with the deregulation of focal adhesion kinase (FAK) and F-Actin dynamics, and thereby it influences the morphology of EMT. FAK plays a role in cellular adhesion and spreading processes, therefore it is required for invasion and metastasis. FAK activity is inhibited upon EGF-induced EMT and is restored later on. In CC, both proteins AnxA8 and FOXO4 are simultaneously expressed in the cytoplasm of malignant biliary epithelial (BE) cells. On the other hand, expression of AnxA8, FAK and EGFR was reduced during tumor progression. These results suggest a role of AnxA8 in cell spreading and tumor metastasis in cholangiocarcinoma (CC) by EGF-FOXO4 signalling, whereas AnxA8 inhibits the cell migratory and metastatic properties of CC cells.

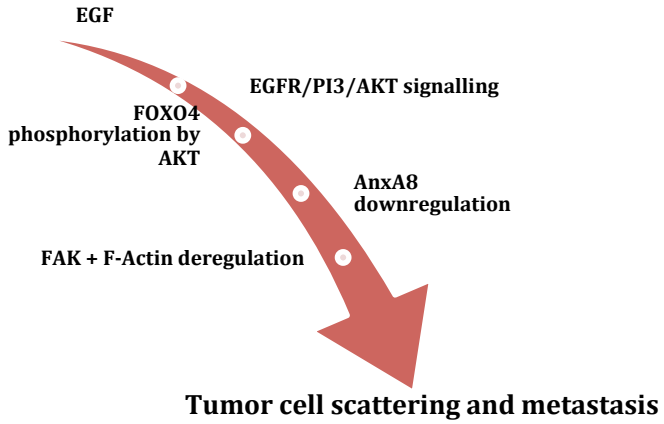

Figure 10 *Downregulation of AnxA8 upon EGF-induced EGFR/FOXO4 signalling during tumor progression.*

Linking of AnxA8 with cancer is further supported by its upregulated expression found in pancreatic cancer, APL, cholangiocarcinoma (CC) and basal-cell like subtypes in breast cancer (52,66,67). Further, upregulation of AnxA8 expression was documented in murine mammary glands upon induced mammary gland involution indicating a link between AnxA8 expression and breast cancer prognosis.

Annexin A8 was preferentially expressed in cultured basal cells but predominant luminal expression was found in normal human breast tissue in vivo (67).

AnxA8 might be used as a marker of pancreatic cancer, since a high expression in infiltrating adenocarcinomas was reported. The strong and diffuse immunohistochemical signal was neclear and cytoplasmic (74). The finding of highly expressed AnxA1, A2, A4 and A5 in pancreatic cancer supports these results (75).

Further results show that AnxA8 is upregulated in squamous cell carcinoma (SC) of the uterine cervix (76). These results strengthen the role of annexins in the tumorigenesis. **(Fig. 12)**

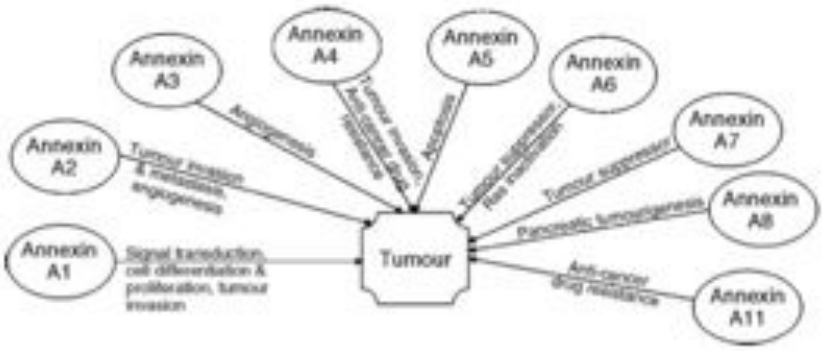

Figure 11 *The roles of annexins in tumourigenesis. A tumour type-specific pattern of expression is linked with tumor development and progression. AnxA8 is involved in pancreatic tumourigenesis* (52).

1.7. Aim of the study

AnxA8, which is a poorly understood annexin member, might have a specialized function according to a restricted tissue and cellular distribution, and a general low expression level. Since the highest amount of AnxA8 was found in the lung, their distribution in lung will be further explored. Thus, this study is investigating the expression and cellular localization of AnxA8 together with EGFR in human lung and in lung cancer tissues (SC and AC), adenocarcinomic human alveolar basal epithelial cells (A549), as well as in bronchoalveolar lavage cells (BALs) obtained from chronic human lung disease (COPD) patients. It might give rise of a specific distribution of AnxA8 in lung cancer, which is simultaneously compared with the expression of EGFR. Additionally, it can provide a link to EGFR. On the other hand, COPD BALs allow investigating the localization and the role of AnxA8 in inflammation. Pathogenic infection studies on AnxA8 were not described yet. Hence, the influence of different pathogen infections (Haemophilus influenzae (HI), Streptococcus pneumonia (SP) and Chlamydia pneumonia (CWS)) on AnxA8 might also give insights into the cellular role of AnxA8. In addition, short-term stimulation of tissues (STST) is implemented to examine the response to inflammatory agents LPS and the bacterial lipopeptide Pam3, and the glucocorticoid analogue dexamethasone (DEXA). Following three techniques were applied: Immunohistochemistry (IHC), conventional Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) and Western blot.

2. Materials

Reagent	Description	Note
Primary Antibody	**AB-1** AnxA8 - rabbit polyclonal antibody	LS-B2459; [1mg/ml], size: 50μg, LifeSpan BioSciences
Primary Antibody	**AB-2** AnxA8 - rabbit polyclonal antibody	GTX119471; [1mg/ml], size: 25μg, GeneTex
Primary Antibody	**AB-3** AnxA8 – rabbit polyclonal antibody (Annexin A8-like 2)	GTX114249; [1mg/ml], GeneTex
Primary Antibody	EGFR - mouse monoclonal antibody	Ref MSK014-05 clone 21E1/ Lot A419-5
Buffer	Antibody Dilution	Order no. ZUC025, Zytomed Systems, Berlin, Germany
Deparaffinization Reagents	Ispropanol; Acetone	Merck, German; J.T. Baker
ZytoChem-Plus HRP Polymer-Kit	Blocking Solution, PostBlock, HRP-Polymer	REF/Cat.No. POLHRP-100 Zytomed Systems
Permanent AEC Kit	Reagent 1, 2, 3 (Chromogen), 4 (H_2O_2)	REF/Cat. No. ZUC054-200 Zytomed Systems
Buffer (10x)	T-TBS pH 7.6 with 0.05% Tween 20	Tween- Tris buffered saline, sigma-Aldrich, Germany
Dehydration Reagents	70%, 80%, 90%, 96%, absolute EtOH and Xylene	Merck, Germany
Counterstain Reagent	Meyers Hemalaum	Merck, Germany
Mounting media	Kayser´s glycerol gelatin	Merck, Germany
Peroxid-Block	3% H_2O_2	Merck, Germany
Rneasy Mini Kit	RTL buffer, RW 1 buffer and RPE buffer	Catalog No. 7416, Qiagen, Germany
Reducing Reagent	ß-Mercaptoethanol	Sigma, Germany

RT-PCR Reagents	Buffer	10x PCR buffer	Invitrogen, Germany
		10x DNAse buffer	
		5x First Strand buffer	
	Primer	Oligo dt ^{15} Primers	
		GAPDH Primers (each 20μMol)	
		AnxA8 Primers	
		dNTPs (each 2,5 mM)	
		Superscript II Reverse Transcriptase	
		DNase I	
		MgCl2 (50mM)	
		EDTA (25mM)	
		DTT	
		Taq DNA Polymerase(5U/μl)	Fermentas
Gel Electrophoresis Reagents		Loading dye xylene-cyanol	Sigma, Germany
		2% Agarose gel	Invitrogen
		Ethidium Bromid	Invitrogen
		DNA marker pBr322-Msp1	NewEnglandBiolabs
		Running Buffer 1x TAE (Tris-base 40mM; 0. 5 M EDTA pH 8; glacial acetic acid pH 7. 5	
Western Blot Reagents		Tris	Roth
		Glycine	Merck
		SDS	Roth
		Glycerol	Merck
		Ammoniumpersulfat (10%)	Biorad
		Temed	Roth
		Acrylamid	Roth

	Butanol	Merck
	Running buffer:	50mM Triss, 384 mM Glycine, 0,1 % SDS
	Buffer	1,5M Tris/HCl pH 8,8
	Buffer	0,5M Tris/HCl pH 6,8
	Reducing buffer	200mM Tris/HCl pH 8,8, 20mM EDTA, 25% Glycerine (v/v), 2% SDS (v/v), 1% DTT (w/v), Bromphenol blue
	DIG Wash and Block Buffer Set	Boehringer Mannheim Roche
	Washing buffer 1	TBS-T pH 7,4 1M Tris, 1M NaCl, 25mM $MgCl_{2*}6H_20$, 0,5% Tween
	Washing buffer 2:	0,5M Tris, 0,5M NaCl, 25mM $MgCl_{2*}6H_20$) pH 9,5
	Protein extraction buffer:	7M Urea, 2M thiourea, 100mM DTT, 4% CHAPS, 2% IGEPAL, 1% Triton X, 5mM PMSF, 0,5mM EDTA, 40mM Tris
	Coomassie Bradford Protein Assay Kit	Thermo Scientific Pierce 23200
Secondary Antibody	Alkaline Phosphatase-conjugated AffiniPure Goat Anti-rabbit IgG, Fc Fragment Specific	Dianova

3. Methods

3.1. Samples

Human lung and lung carcinoma (AC and SC) tissues were used as samples to investigate the expression of AnxA8. Furthermore, samples were stimulated with inflammatory agents (LPS, PAM3, DEXA) and used as single tissue section slices (STSS). Additionally, adenocarcinomic human alveolar basal epithelial cells (A549 cells) were investigated as stimulated (with inflammatory agents) and non-stimulated cells. Further, bronchoalveolar lavage method was performed to take out respiratory secretions and to examine (a)cellular components. These BAL samples originated from chronic obstructive pulmonary disease (COPD) patients and healthy, unaffected lung patients. Moreover, Chlamydia pneumonia (CWS), Haemophilus influenzae (HI) and Streptococcus pneumonia (SP) stimulated samples - generated as tissue microarray slides (TMAs) - were used for further investigations. Samples originated from patients of the Medical Clinic Borstel and the University of Lübeck obtained after lobectomies. All tissues were previously HOPE-fixed and paraffin embedded in the laboratories of the Research Center Borstel. (see Appendix Table 1 + Table 2) Applied formalin-free Hepes-glutamic acid buffer mediated Organic solvent Protection Effect (HOPE) fixation technique is comparable to formalin fixation, resulting in formalin-like morphology. Additionally, HOPE-fixation is avoiding the distinct nucleic acid degradation and methylene cross-linking problems emerging using Formalin-fixation (77,78).

Total amount of non-stimulated tissues: 130 samples

- Lung: 30 samples
- AC: 50 samples
- SC: 50 samples

Total amount of LPS, PAM3 and DEXA stimulated tissues: 10 samples

- Lung: 8 samples
- Tumor: 2 samples

Total amount of A549 cells: 13 samples

- Non-stimulated: 4 samples
- LPS-stimulated: 5 samples
- PAM3-stimulated: 4 samples

Total amount of BAL cells: 7 samples

- Normal lung: 2 samples
- COPD: 5 samples

3.1.1. Short-Term Stimulation of Tissues (STST)

STST (Short-Term Stimulation of Tissues) is an *ex vivo* short-term tissue culture model combined with the HOPE-fixation and paraffin embedding method (79). It gives insights into the cellular events taking place and the responsiveness upon stimulation with inflammatory agents (LPS, PAM3 and DEXA) investigated in human lung tissue samples and A549 cells. Lung tissue samples were obtained at least 5 cm away from tumor front. Thereby, samples of $1cm^3$ size were cultured in RPMI 1640 with 10% fetal calf serum (FCS), 2% 1M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonicacid, 1% of 10mM sodium pyruvate and 1% of Penicillin-Streptomycin at 37°C with 5% CO_2. Inflammatory agents (LPS (lipoglycan of gram-negative bacteria), PAM3 (represents the gram-positive compound peptidoglycan) and glucocorticoid (DEXA) of each 200ng/ml concentration were incubated for 24 hours and fixed with HOPE. Incubation was performed simultaneously with a non-stimulated medium control.

3.1.2. Hope-Fixation Method

Hepes-glutamic acid buffer mediated Organic solvent Protection Effect (HOPE) technique is a new fixation tissue method (80). HOPE provides FFPE-like (Formalin-fixed) morphology together with good preservation of nucleic and antigenic structures. In addition to that, HOPE technique is valued for single –cells preparations as well.

3.1.3. Tissue Microarray (TMAs)

For analysing the expression of genes simultaneously, tissue microarrays (TMAs) were performed in multiple individual tissue samples on one slide (81). Therefore 0.6 to 3.0 mm cores from tissue of interest from paraffin-embedded tissue blocks were produced by a microtome and further analysed in IHC. (**Fig. 13**)

Figure 12 *Tissue Microarray Process* (81)

3.2. Immunohistochemistry (IHC)

Protein expression in tissue sections can be localized by immunohistochemistry (IHC), where sections were exposed to antibodies. Application of antibodies was performed by a Horse Radish Peroxidase (HRP) Polymer method and visualized by the conversion of the colourless AEC (3-Amino-9-

ethylcarbazol) substrate into a red signal. This method is avoiding the usage of endogenous biotin, which produces augmented background signals. In contrast, immunocytochemistry (ICC) was performed for A549 cells and BAL cells and treated like described for IHC.

3.2.1. Standard protocol of IHC

HOPE-fixed paraffin-embedded lung samples were cut on a microtome (Leica SM 2000R), placed on slides and dried at 37°C overnight. TMAs were generated previously in the laboratory and were kept at 4 degrees before usage. Then, single tissue section slides and TMAs were deparaffinised according to the standard IHC protocol.

- Incubation for 10 minutes in isopropanol at 60°C
- Washing in fresh isopropanol for 2 minutes
- Drying at room temperature for 10 minutes
- Incubation in 70% acetone for 10 minutes at 4°C (rehydration)
- Washing with DEPC-treated water for 10 min at 4°C
- Tempering in dH_2O at room temperature for 5 minutes
- Blocking of endogenous peroxidases by 3% H_2O_2 for 10 minutes
- Washing with dH_2O
- Transfer into a new cuvette with T-TBS pH 7.6 with 0.05% Tween 20
- Washing with T-TBS pH 7.6 (3x á 2 minutes)
- Proteinblock for 5 minutes
- Immunodetection with primary antibody diluted in antibody diluent (for 1 hour at room temperature
- Washing with T-TBS pH 7.6 (3x á 5 minutes)
- PostBlock for 15 minutes (only for mouse monoclonal antibody (=EGFR))
- HRP-Polymer for 20 minutes
- Washing with T-TBS pH 7.6 (3x á 2 minutes)
- Colour reaction by permanent AEC Kit (incubation with 200μl AEC)
- Counterstaining with Mayer ´s hemalaun (cell nuclei staining)

- Dehydration by increasing series of Ethanol (10 sec. in 70% EtOH, 10 sec. in 80% EtOH, 10 sec. in 90% EtOH, 2x 10 sec. in 96% EtOH, 2x 10 sec. in absolute EtOH, 10 sec. in Xylene)
- Covering of slides with Kayser´s glycerol gelatin
- Analyses on microscope

3.2.2. Optimization of immunohistochemistry

In order to investigate the expression and localization of AnxA8 and EGFR, antibodies and their concentrations were experimentally optimized. Annexin A8 AB-2 (same as AB-1) was used for further IHC tests (exclusions are mentioned). Placenta tissue - (Placenta – 01.07.10 (Seppl)) was defined as a suitable positive control. (**Fig. 14**)

Optimal AnxA8 antibody dilution: 1/400
Optimal EGFR antibody dilution: 1/250

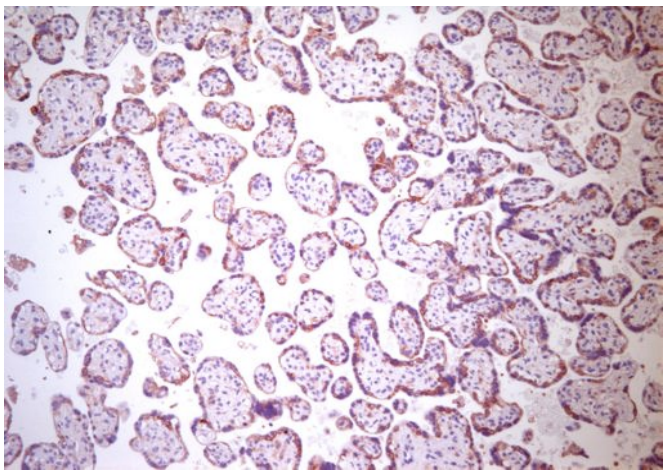

Figure 13 *– HOPE-fixed-paraffin-embedded placenta tissue stained for AnxA8 (AB2, 1/400 dilution) was selected as a positive control (magnification: 100x)*

3. 3. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR is a combination of a reverse transcription (RT) and a polymerase chain reaction (PCR) where RNA is reverse transcribed into complementary DNA (cDNA) by reverse transcriptase and further amplified by PCR. Tissue slices have been generated using microtome. RNA was purified from following samples:

Sample Number	**Description**
22032/08 (lung)	RPMI
Stimulated	+ 20 μl LPS
	+ 20 μl PAM3
	+ 20 μl DEXA
23418/08 (lung)	RPMI
Stimulated	+ 20 μl LPS
	+ 20 μl PAM3
	+ 20 μl DEXA

3.3.1. RNA-Extraction and measurement of RNA by optical density

RNA was extracted according to the manufacturer´s protocol (Rneasy –Kit). Total RNA dissolved in the DEPC-treated water was analysed by measuring the absorption of nucleic acids at 260 nm in a spectrophotometer (Gene Quant pro RNA/DNA Calculator, Amersham Pharmacia Biotech). For this purpose, RNA was diluted 1:100 (2µl RNA + 98µl DEPC-treated water). For assessing protein contamination, the ratio of 260/280 nm was determined. Samples were kept at -80 °C and used for further analyses.

Sample Number	**Description**	**Concentration (ng/μl)**
22032/08 (lung)	RPMI	1.500
Stimulated	+ 20 μl LPS	1.478
	+ 20 μl PAM3	1.429
	+ 20 μl DEXA	1.389
23418/08 (lung)	RPMI	1.605
Stimulated	+ 20 μl LPS	1.512
	+ 20 μl PAM3	1.482
	+ 20 μl DEXA	1.459

3.3.2. Polymerase Chain Reaction (PCR)

Remaining DNA was destroyed by DNase I treatment. Exctracted RNAs served as templates to synthesize complementary DNAs catalyzed by the enzyme reverse transcriptase. Therefore, RNA was reverse transcribed to cDNA using Superscript II and Taq polymerase (Superscript II, Invitrogen). Resulting cDNA was used to determine the presence of the AnxA8 gene by its corresponding primers performing PCR.

PCR is a method performed to amplify DNA fragments by using specific primers. Thus, AnxA8 primers (forward and reverse) have been designed (size: 191 bp). (**Table 2)** Further, Glycerinaldehyd-3-phosphat-Dehydrogenase (GAPDH) is a housekeeping gene, which is expressed at constant levels. Detection of GAPDH using GAPDH primers allows normalizing the results of AnxA8-PCR. **Table 3** is displaying the designed forward and reverse primer of GAPDH (size: 257 bp).

PRIMER FORWARD	PRIMER REVERSE
5´-CAG CAG CAT GAT CAT GGA AG-3´	5´-ATA GAA GAC CGA AAG GCT GG-3´

Table 2 *Forward and reverse primer of Annexin A8*

PRIMER FORWARD	PRIMER REVERSE
5´-AGA ACG GGA AGC TTG TCA TC-3	5´-TGC TGA TGA TCT TGA GGC TG-3

Table 3 *Forward and reverse primer of GAPDH*

According to the standard PCR protocol, a master mix containing d. water, PCR buffer, dNTPs, MgCl2 and Taq DNA Polymerase was prepared in a single tube and later aliquoted into individual PCR tubes. Forward and reverse primers and the template DNA were mixed together and solutions were added into PCR tubes containing the master mix. (**Table 4 and 5**)

Reagent	**Concentration**	**Quantity for 50µl of reaction mixture**
dH20	-	34,7 µl
10x PCR buffer	1x	5 µl
2,5mM dNTP mix	0,2 mM of each	1 µl
Primer reverse GAPDH	0,1µM	2 µl
Primer forward GAPDH	0,1µM	2 µl
Taq DNA Polymerase (Fermentas)	1u/50µl	0,3 µl
25mM MgCl2	1,25 mM	2 µl
Template DNA	1µg	3 µl

Table 4 *GAPDH - Reaction Mixture (total volume: 50µl)*

Reagent	**Concentration**	**Quantity for 50µl of reaction mixture**
dH20	-	32,7 µl
10x PCR buffer	1x	5 µl
2,5mM dNTP mix	0,2 mM of each	1 µl
Primer reverse AnxA8	0,1µM	2 µl
Primer forward AnxA8	0,1µM	2 µl
Taq DNA Polymerase (Fermentas)	1u/50µl	0,3 µl
25mM MgCl2	1,25 mM	2 µl
Template DNA	1µg	5 µl

Table 5 *AnxA8 - Reaction Mixture (total 50µl)*

Next, PCR tubes were placed into a 94°C preheated thermal cycler (Biometra TGradient). Details of the PCR program are shown in **Table 6**.

1	1:00` 94°C
2	1:00` at 56°C and 57°C (AnxA8) 1:00` 55°C (GAPDH)
3	1:30` 72°C
39 cycles	
4	15:00` 72°C
5	∞ 4°C

Table 6 *PCR program for AnxA8 and GAPDH*

3.3.3. Optimization of the PCR

AnxA8 PCR was initially performed with only one sample (22032/08 – RMPI), in order to examine optimal conditions for further studies. Thus, the optimal annealing temperature was identified to be at 57°C. (**Fig. 15)** Presence of AnxA8 in the selected sample (22032/08 – RMPI) was confirmed by visualizing the corresponding band of 191 base pairs size. Afterwards, AnxA8 PCR was performed with 8 stimulated lung samples and with a GAPDH-PCR as an intern control.

Figure 14 *AnxA8 PCR performed at annealing temperature of 56°C (left) and 57°C (right). The optimal annealing temperature was found to be 57°C.*

3.3.4. Gel Electrophoresis

Gel electrophoresis is performed using agarose and a tris buffering solution. Further details are shown in **Table 7.** PCR products and the DNA marker pBr322-Msp1 were loaded onto gel. Electrophoresis was performed at 90 volts for 30 minutes and visualized by a UV-transilluminator (Gel documentation system, INTAS, Germany).

2,0% Agarose	2,8 g Agarose
	140 ml TBE buffer
	10 μl EtBr
Loading scheme	1,5 μl loading dye xylene-cyanol

Table 7 *DNA agarose gel electrophoresis - PCR products were seperated on a 2% agarose gel*

3.4. Western Blot

Western blot was applied to detect and compare the amounts of AnxA8 in lung tissue samples that were stimulated with LPS, PAM3, DEXA and pathogenic microorganisms (CWL, SP and HI).

3.4.1. Protocol

Tissue samples were selected, cut on a microtome and deparaffinizated before protein extraction. Bradford protein assay was applied to measure the protein concentration of the cell lysates. Thereby, 2 µL of each sample was added to 1 mL Bradford reagent, respectively. The lysates were then mixed with 4x Laemmli reducing sample buffer in ratio of 1:4 for a total protein concentration of 30 µg (sometimes 50 µg or 100 µg) and boiled at 95°C for 5 minutes. Proteins were separated by 12% SDS-PAGE polyacrylamide gels (at 50 V until samples reached the resolving gel, then 180 V for ca. 90 minutes total). 5 µL Page Ruler Prestained Protein was used as the marker, 2 µL nitrated ovalbumine (OVA) (0.82 µg/µL) was used as a positive control, and 2 µL OVA (0.82 µg/µL) was used as a negative control. They were blotted onto polyvinylidene fluoride (PVDF) membranes:

- Washing of the blot with TBS-0.1%T for 5 minutes.
- Blocking in blocking buffer (5% NDM/TBS-T) for 1 h at room temperature.
- (Alternative blocking with 5% Gelatine/TBS-T for 1 h at room temperature)
- Washing with TBS-0.1%T, 2x 5 minutes.
- Primary antibody was used: rabbit polyclonal antibody GTX119471 (1:100) in 1% NDM/TBS-0.1%T. Incubation overnight at 4°C.
- Washing with TBS-0.1%T 3 x 5 minutes.
- Secondary antibody was used: anti-rabbit HRP (1:5,000) in 1% NDM/TBS-0.1%T. Incubation for 1h at room temperature.
- Washing with TBS-0.1%T 3 x 10 minutes
- Substrate ECL chemiluminescence Western Blot kit 1:40 (1 mL directly on membrane). Analysis with ChemiDoc

4. Results

4.1. Immunohistochemistry

4.1.1. Expression and cellular localization of AnxA8 in lung and lung cancer (adenocarcinoma and squamous-cell carcinoma) tissues

130 samples (*of lung and lung-cancer tissues*) were used to investigate the expression and cellular localization of AnxA8 by immunohistochemistry. Results of positive cases show that signals of AnxA8 were detected in 19 of 30 lung samples. In adenocarcinoma (AC) and squamous-cell carcinoma (SC), signals of AnxA8 appeared almost at the same extent. A minimal decrease of AnxA8-positive cases was found in adenocarcinoma samples. Following table shows the amount of AnxA8-positive cases compared to the total amount of samples including the type of tissue examined:

AnxA8 (positive)	**91 of 129 samples (70,5%)**
Lung	19 of 30 (76,6%)
AC	34 of 50 (68,0%)
SC	38 of 49 (77,6%)

Although AnxA8 was detected in almost 80% of the lung samples, these results confirm the localization of AnxA8, which was in previous studies described to be the lung. Furthermore, AnxA8 was also detected in NSCLC (AC and SC) of epithelial lung cancer. Histologically, AnxA8-positive cases are showing a membrane-bound staining in both lung and lung-cancer tissues. These results support the initial identification of AnxA8 in the plasma membrane. With the exception of two samples, almost all AnxA8-positive lung tissues displayed signals in alveolar macrophages (AM) and alveolar epithelial cells type II (AECII). (**Fig. 16)** Furthermore, results show signals of AnxA8 in epithelial lining of alveoli. These signals were distributed along the alveolar epithelium and indicate the expression of AnxA8 in alveolar epithelial cells type I (AECI). These results support the previously documented expression of annexins in alveolar epithelial cells.

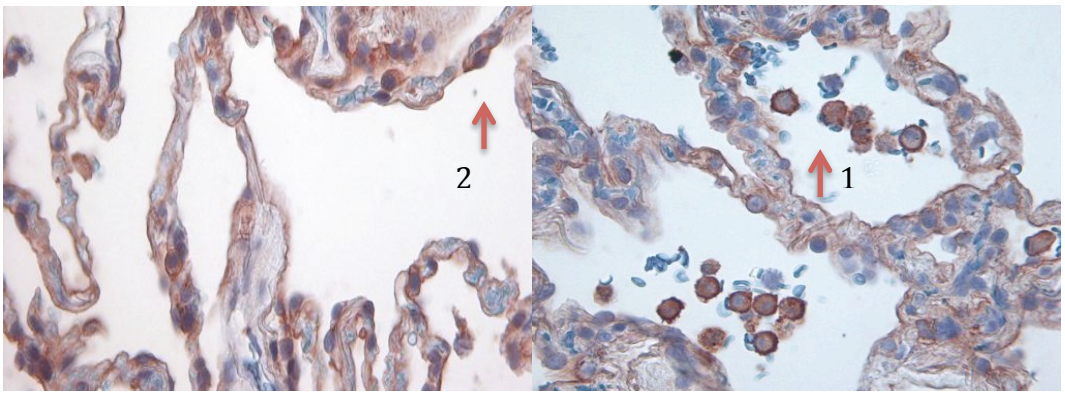

Figure 15 *Immunohistochemical detection of AnxA8 in lung – Positive AnxA8 signals were found in alveolar macrophages (AM) (1) and in alveolar epithelial cells type II (AECII) (2). A membrane-bound staining is displayed and might signalise the expression of AnxA8 in AECI. (magnification: left: 400x and right: 200x)*

Furthermore, epithelial cells of bronchi displayed signals of AnxA8. In fact, these signals can be referred to the basal cells of bronchus epithelium. (**Fig.17**.)

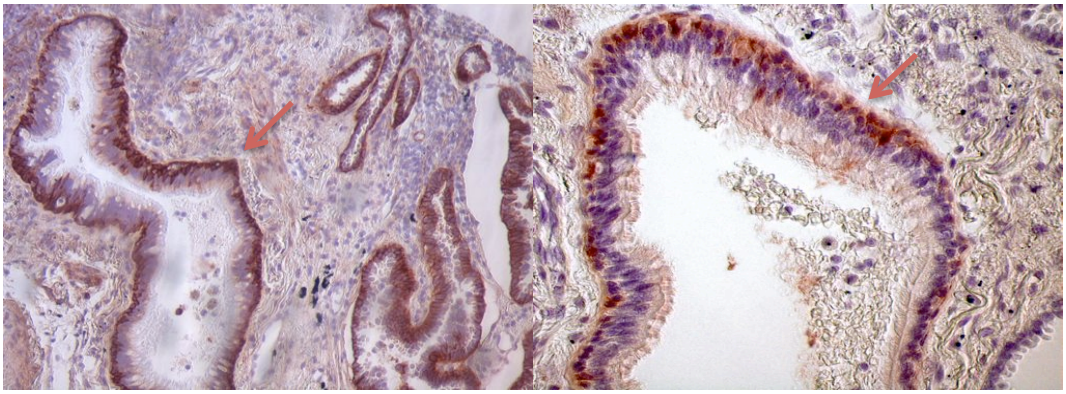

Figure 16 - *Immunohistochemical detection of AnxA8 in epithelial cells of bronchi. (magnification: left: 100x, right: 200x)*

Among lung cancer, signals of AnxA8 were of almost the same amount - slightly more (+10%) AnxA8-positive cases were found in squamous-cell carcinomas. The protein was not upregulated in comparison to lung tissues. Interestingly, AnxA8-positive squamous-cell carcinoma tissues displayed a recurring pattern ("brick-shaped"), which resembles the shape of a brick in addition to the membrane-bound staining. In fact, signals were not always homogen, a heterogen staining was observed as well. **(Fig. 18)** In contrast, AnxA8-positive adenocarcinoma tissues did not display a" brick-shaped" pattern, although it showed a membrane-bound staining. **(Fig. 19)** Signals were detected in alveolar macrophages in both AnxA8-positive lung cancer tissues.

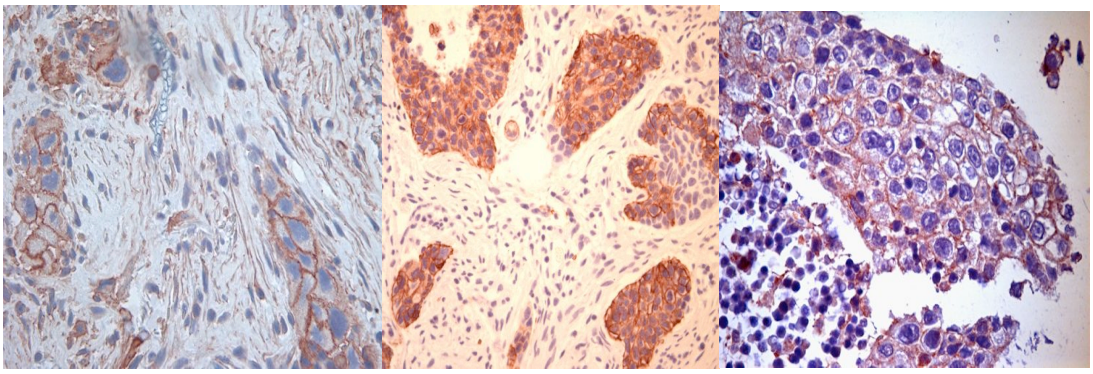

Figure 17 – *Immunohistochemical detection of AnxA8 in squamous-cell carcinomas – A "brick-shaped", membrane-bound and heterogenic staining is displayed in AnxA8-positive SC tissues. (magnification: left and right: 400x, middle: 200x)*

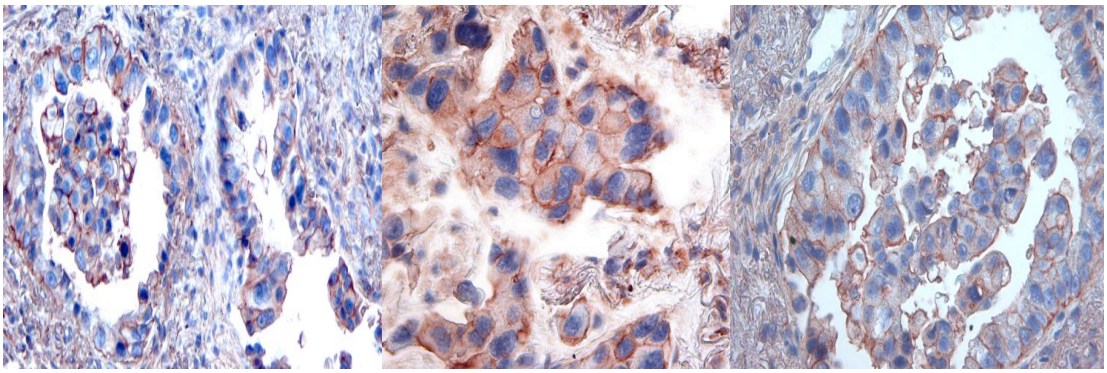

Figure 18 *Immunohistochemical detection of AnxA8 in adenocarcinomas – A membrane-bound staining is displayed in AnxA8-positive AC tissues. It is lacking the "brick-shaped" pattern observed in SC tissues. (magnification: left: 200x, middle and right: 400x)*

4.1.2. Expression and cellular localization of EGFR in lung cancer tissues – in comparison with AnxA8

70 samples of adenocarcinoma and squamous-cell carcinoma tissues were used to investigate the expression and cellular localization of EGFR by immunohistochemistry. This allows studying the correlation of AnxA8 with EGFR in context of their expression and localization.

Results of positives cases show that signals of EGFR were detected in 80% of all cases, same percentage of positive cases is found in both lung cancer types. EGFR-positive cases compared to total amount of samples in adenocarcinoma (AC) and squamous-cell carcinoma (SC) tissues are displayed in the following table:

EGFR (positive)	**56 of 70 samples (80%)**
AC	24 of 30 (80%)
SC	32 of 40 (80%)

These results show that EGFR has a slightly higher amount of positive cases in comparison to AnxA8. In fact, identical percentage of EGFR-positive cases was found in both cancer types, whereas AnxA8-positive cases were identified more (+10%) in squamous-cell carcinoma tissues. (**Fig. 20)**

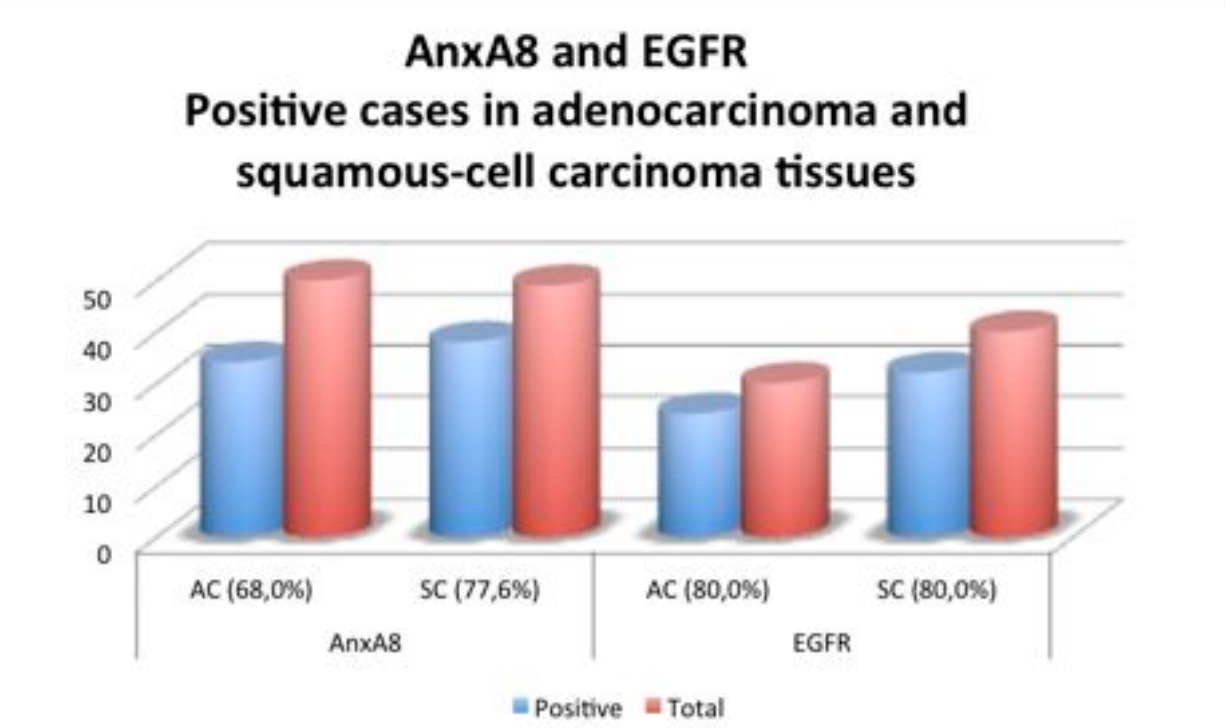

Figure 19 *AnxA8 and EGFR - positive cases in lung-cancer tissues (AC and SC). The amount of positive cases (blue) in comparison to total amount of cases (red) in lung cancer tissues is illustrated. Signals of both proteins appeared in the range from 68% to 80% in both lung cancer tissues.*

EGFR is a membrane-bound protein. As expected, signals of EGFR were membrane-bound in both lung cancer tissues and again it was a "brick-shape" pattern in squamous-cell carcinoma tissues identified. In contrast to the results of AnxA8, signals of EGFR appeared stronger. (**Fig. 21 and Fig. 22)**

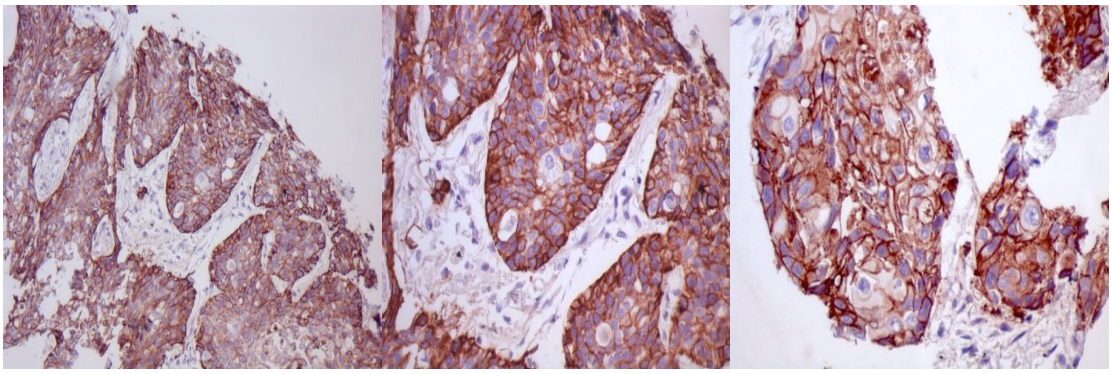

Figure 20 *- Immunohistochemical detection of EGFR in squamous-cell carcinomas – A membrane-bound staining with a "brick-shape" pattern is displayed in EGFR-positive SC tissues. Left: An heterogenic distribution of EGFR signals is displayed (magnification: left: 100x, middle and right: 200x)*

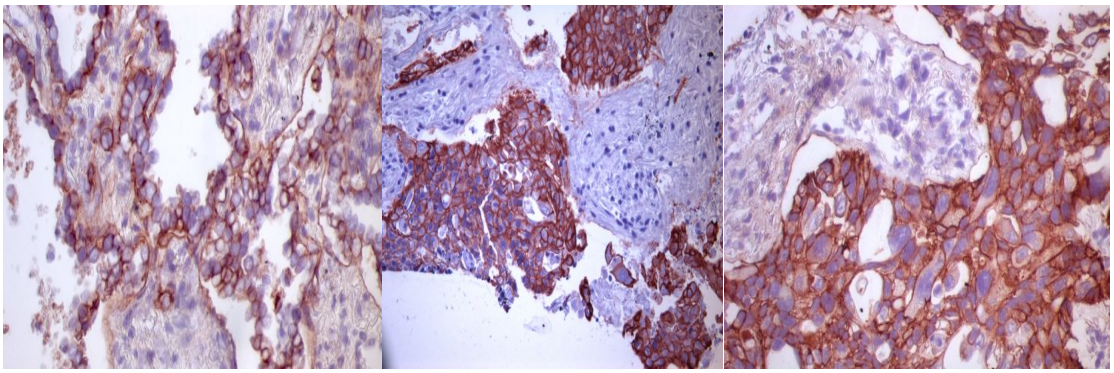

Figure 21 *- Immunohistochemical detection of EGFR in adenocarcinomas – A membrane-bound staining is displayed in EGFR-positive AC tissues. Left: A heterogenic distribution of EGFR signals is displayed (magnification: left and right: 200x, middle: 100x)*

4.1.3. Effects of LPS, PAM3 and DEXA on the expression of AnxA8 in lung and lung cancer tissues

The expression of AnxA8 was examined in 8 cases of short-term stimulated (STS) lung tissues and 2 cases of STS tumor tissues, and furthermore compared with EGFR. Tissues were stimulated for 24 hours with 200ng/ml of each stimuli (LPS, PAM3 and DEXA). Inflammatory stimuli are assumed to affect annexins with a time and dose-dependent manner.

The results show that AnxA8 was expressed in all stimulated lung and lung cancer tissues as well as in the medium control (RPMI). Thus, all cases were AnxA8-positive. Furthermore, LPS and PAM3 induced neither significant upregulation nor downregulation compared to the medium control in lung and tumor tissues. (**Fig. 23**)

Interestingly, DEXA seems to induce the expression of AnxA8 in lung compared to the medium control. The glucocorticoid analogue dexamethasone appears to affect the expression of AnxA8, indicated by signals in alveolar macrophages. (**Fig. 24)**

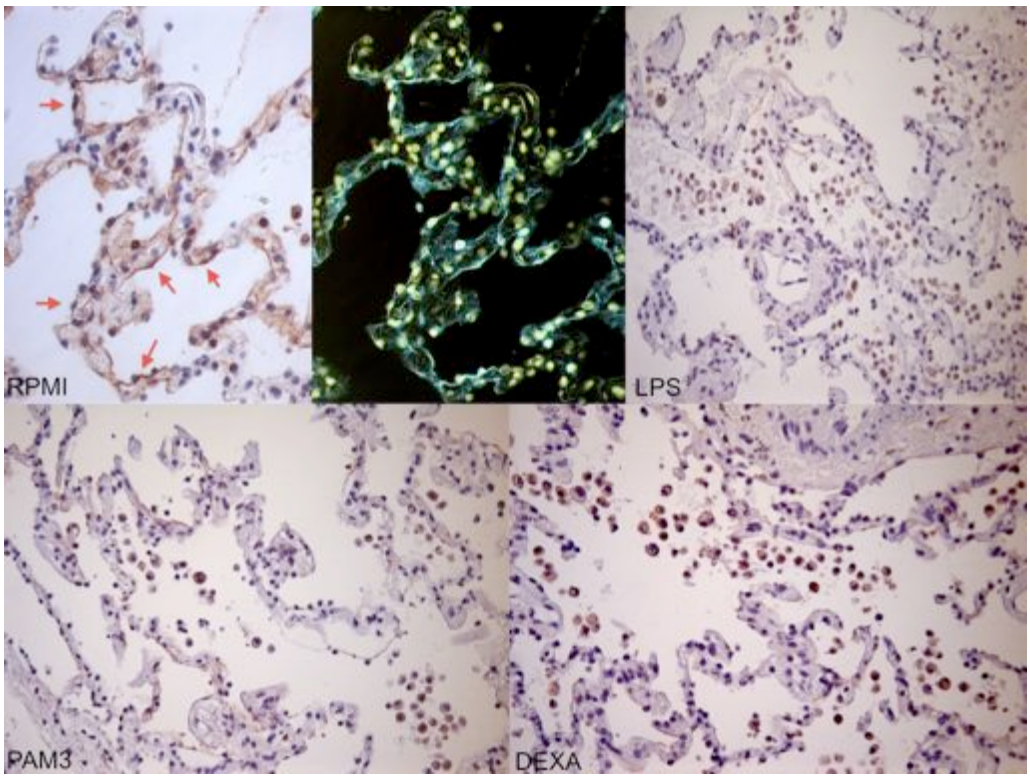

Figure 22 *- Immunohistochemical detection of AnxA8 in LPS, PAM3 and DEXA stimulated lung tissues – LPS and PAM3 did not stimulate AnxA8 expression compared to medium (RPMI). DEXA appears to induce the expression of AnxA8 detected in alveolar macrophages (AM). Signals were membrane-bound and found in AM and alveolar epithelial cells type I and II (arrow) (magnification: 200x)*

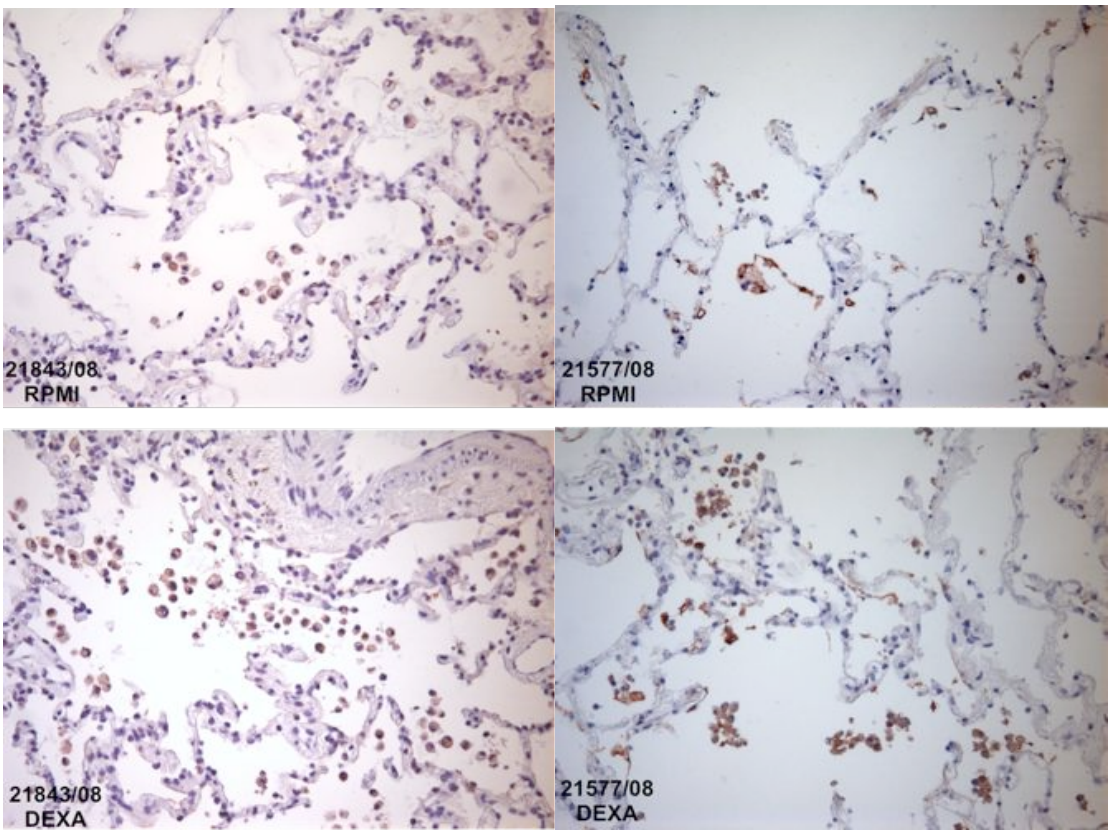

Figure 23 - *Immunohistochemical detection of AnxA8 in DEXA stimulated lung tissues - Expression of AnxA8 appears upregulated by DEXA indicated by increased signals in alveolar macrophages. (magnification: 100x)*

The signals were both cytoplasmic and membrane-bound. Histologically, expression of AnxA8 was detected in alveolar macrophages of all cases, again in basal cells of bronchi and very few in alveolar epithelial cells type II. In addition, signals were distributed along the alveolar epithelium and again they indicated the expression of AnxA8 in alveolar epithelial cells type I (AECI). These results support the expression of annexins in alveolar epithelial cells and epithelial cells (basal cells) of bronchi. **(Fig. 25)**

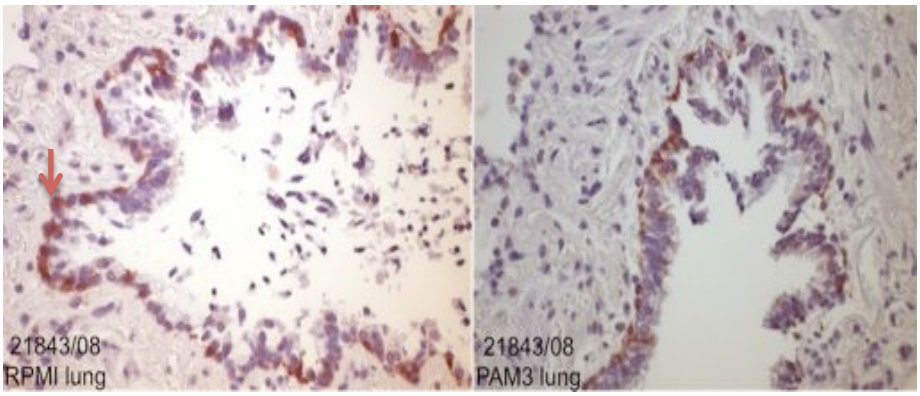

Figure 24 *- Immunohistochemical detection of AnxA8 in medium (RPMI) and PAM3 stimulated lung tissues – Signals were detected in basal cells (arrow) of all bronchi. Neither LPS or DEXA nor PAM3 (shown here) did stimulate the expression of AnxA8. (magnification: 200x)*

Furthermore, the expression of AnxA8 was examined in two cases of stimulated tumor lung tissues. Both cases were AnxA8-positive indicated by cytoplasmic and membrane-bound signals. Stimulation by LPS, PAM3 or DEXA did not affect the expression of AnxA8. (**Fig. 26)**

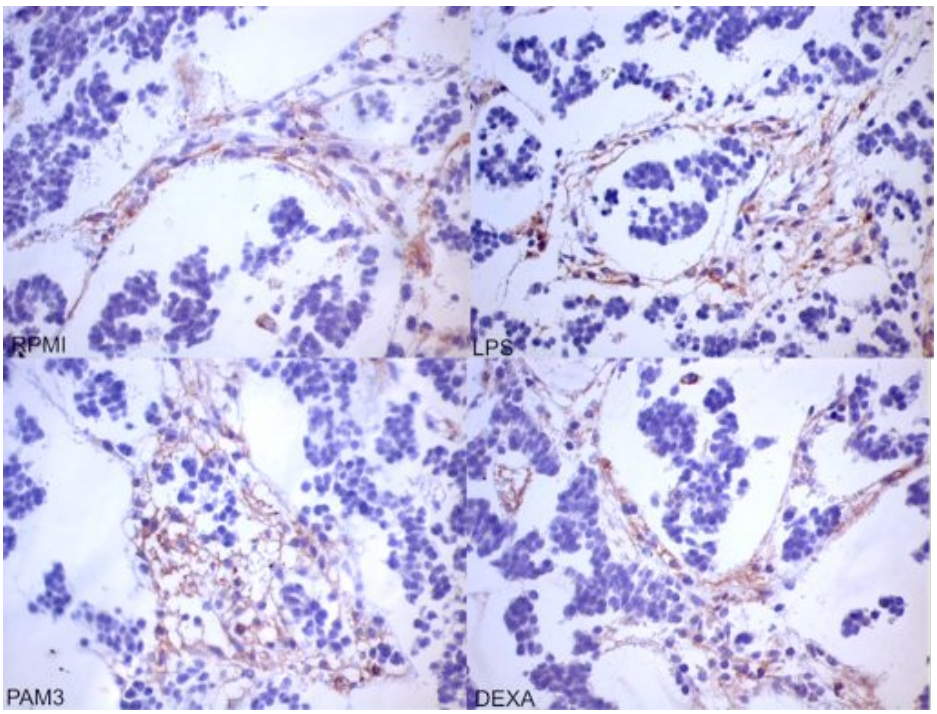

Figure 25 *- Immunohistochemical detection of AnxA8 in LPS, PAM3 and DEXA stimulated tumor lung tissues – Signals were cytoplasmic and membrane-bound. Neither LPS or PAM3 nor DEXA did stimulate the expression of AnxA8. (magnification: 200x)*

4.1.4. Effects of LPS, Pam3 and DEXA on the expression of EGFR in lung and lung cancer tissues – in comparison to AnxA8

In order to investigate the correlation between AnxA8 and EGFR, same cases of (LPS, PAM3 and DEXA) stimulated lung and tumor tissues were used to examine the expression of EGFR.

The results show that the expression of EGFR was detected in all of the tested lung tissues and in only one of the tumor tissues. In comparison to the medium (RPMI), there was no significant upregulation or downregulation by LPS, PAM3 or DEXA in lung or tumor tissues. In fact, positive signals were detected in alveolar macrophages, basal cells of bronchi, AECII and along the alveolar epithelium in all cases. The latter indicates the expected membrane-bound signals of EGFR. (**Fig. 27 and Fig. 28)**

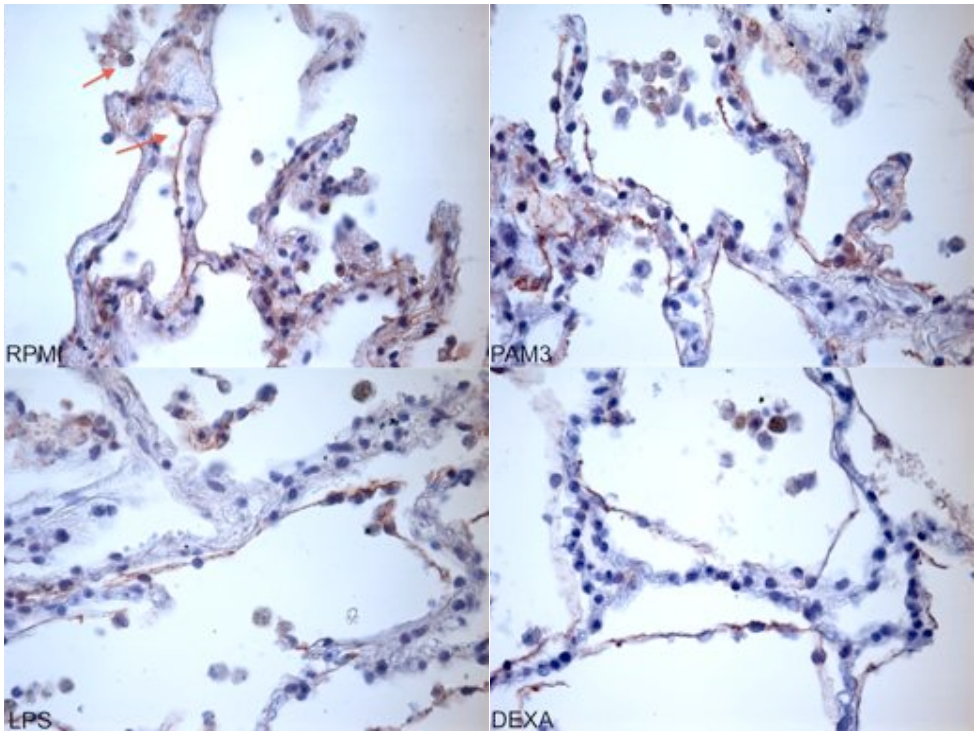

Figure 26 *- Immunohistochemical detection of EGFR in LPS, PAM3 and DEXA stimulated lung tissues – Signals were detected in alveolar macrophages and along the alveolar epithelium (arrow). Neither LPS or PAM3 nor DEXA did stimulate the expression of EGFR. (magnification: 200x)*

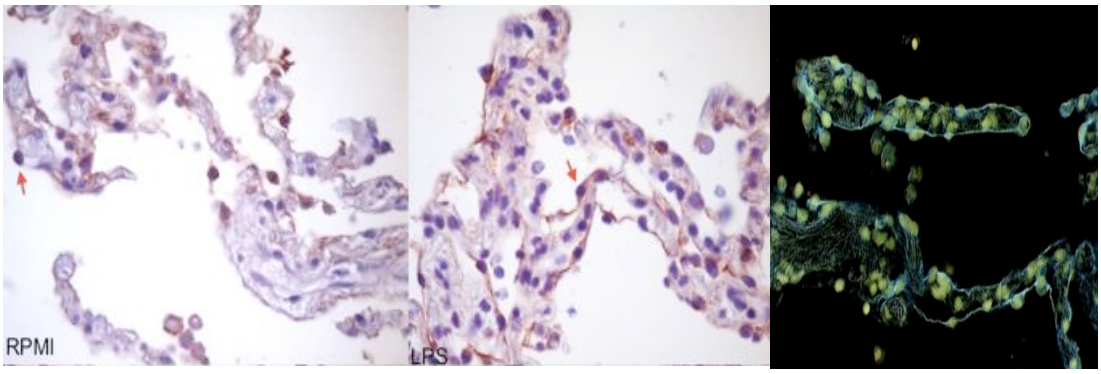

Figure 28 *- Immunohistochemical detection of EGFR in RPMI and LPS stimulated lung tissues – Signals (arrow) were detected in AM, AECII and along the alveolar epithelium (left). Neither LPS or PAM3 nor DEXA did stimulate the expression of EGFR. Right: Negative image of detected EGFR signals along the alveolar epithelium in LPS stimulated lung tissue. (magnification: 200x)*

Positive signals of EGFR were detected within the epithelium of bronchi in both medium control and stimulated lung tissues. Indeed, EGFR signals were found in the basal cells of bronchi, resembling the results of AnxA8. (**Fig. 29)**

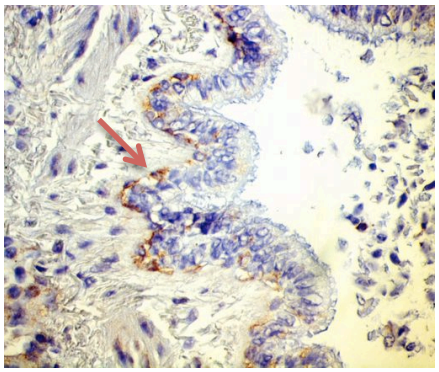

Figure 29 *- Immunohistochemical detection of EGFR in medium control (RPMI) in lung tissue – Signals (arrow) were detected in basal cells of bronchi. (magnification: 200x)*

In summary, there was no significant change (upregulation/downregulation) of EGFR and AnxA8 expression in LPS or PAM3 stimulated lung and lung tumor tissues detected. Neither did DEXA affect the expression of EGFR. In contrast, DEXA affected the expression of AnxA8 only in lung, indicated by increased signals in alveolar macrophages. In general, AnxA8 signals were detected in all bronchi, specifically in basal cells, alveolar epithelial cells II (possibly in AECI) and in alveolar macrophages, corresponding to signals of EGFR-positive tissues. In addition, AnxA8 signals were both cytoplasmic and membrane-bound, whereas EGFR signals were only membrane-bound.

4.1.5. Expression of AnxA8 and EGFR in A549 cells and bronchoalveolar lavage cells

4.1.5.1. Effects of LPS and PAM3 on the expression of AnxA8 in A549 cells

Adenocarcinomic human alveolar basal epithelial cells (A549 cells) were used to further analyse the expression of AnxA8 and to compare it with the expression of EGFR. Hence, 13 samples of A549 were investigated: five samples were stimulated with LPS (200ng/ml), four samples were stimulated with PAM3 (200ng/ml) and another four samples were medium control.

The results show that AnxA8 was expressed in all four PAM3 stimulated cells, 4/5 LPS-stimulated cells and in 3/4 medium control cells. There was no significant change in the expression of AnxA8 by LPS or PAM3 in comparison to the medium control.

In contrast, the expression of EGFR was positive in all examined A549 cells. Again, no significant differences were detected in LPS and PAM3 stimulated tissues in comparison to medium control. However, expression of AnxA8 was weak within all positive cells, while EGFR-positive cells showed strong signals in all cells. However, LPS and PAM3 did neither upregulate nor downregulate the expression of AnxA8 or EGFR compared to medium. Furthermore, membrane-bound signals were most prominent in EGFR stained cells, whereas both membrane-bound and cytoplasmic signals were detected in AnxA8 stained cells. (**Fig. 30**)

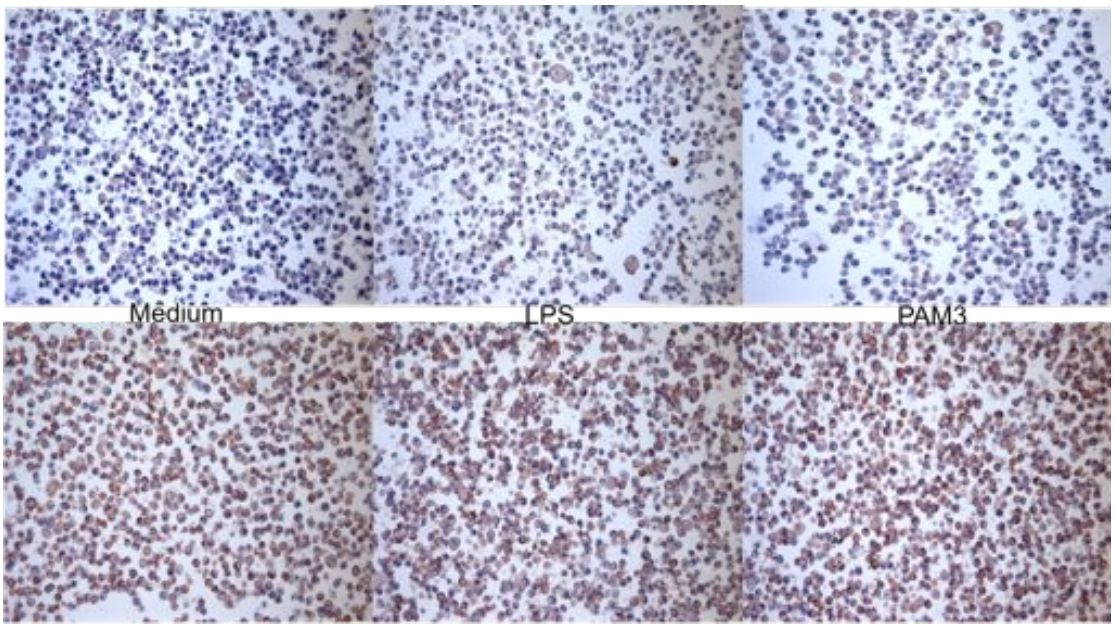

Figure 30 - *Immunocytochemical detection of AnxA8 (top) and EGFR (bottom) in medium control (RPMI), LPS and PAM3 stimulated A549 cells – Signals of AnxA8 were cytoplasmic and membrane-bound, whereas signals of EGFR were preferably membrane-bound and strong. (Magnification: 200x)*

4.1.5.2. Expression of AnxA8 in chronic disease

Bronchoalveolar lavage cells (BALs) were obtained from patients with chronic obstructive pulmonary disease (COPD) and used to analyse the expression of AnxA8 and EGFR in comparison to BAL cells of healthy patients. An increased number of inflammatory cells like macrophages are found in BAL cells of COPD patients.

Expression of AnxA8 was analysed in six samples of COPD BALs and seven samples of normal (healthy) BALs. Signals of AnxA8 were detected in all samples with the exception of one sample obtained from a healthy patient. Signals were cytoplasmic and less membrane-bound. (**Fig. 31**)

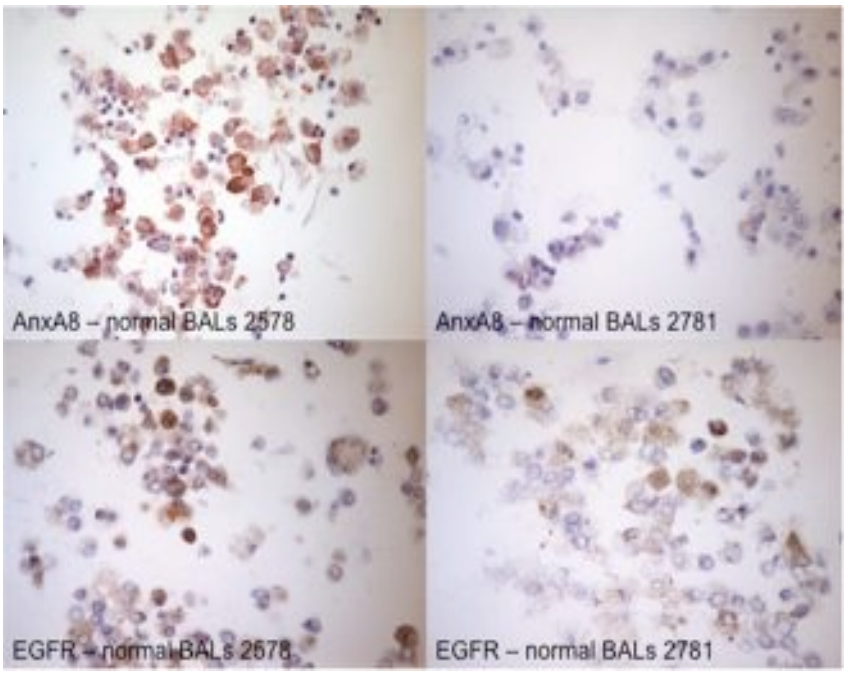

Figure 31 – *Immunocytochemical detection of AnxA8 and EGFR in bronchoalveolar lavage cells obtained from two healthy patients. (magnification: 400x)*

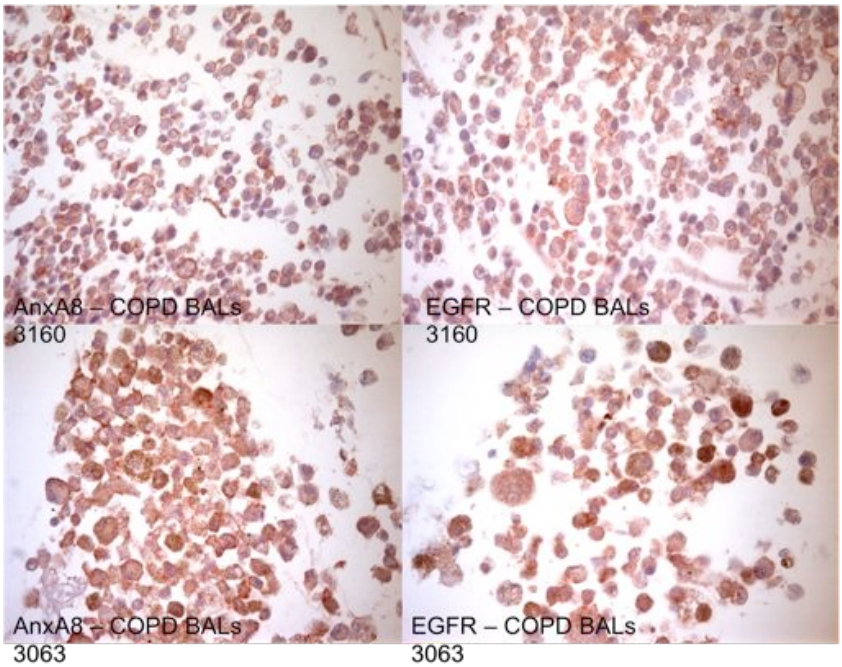

Figure 32 – *Immunocytochemical detection of AnxA8 and EGFR in bronchoalveolar lavage cells obtained from two COPD patients. Both proteins are abundantly expressed in inflammatory cells. (magnification: 400x)*

An increased expression of AnxA8 in COPD BALs when compared to normal BALs was detected, although the signals vary between weak and moderate in normal BALs. (**Fig. 32**) This shows an enlarged expression of AnxA8 in inflammatory cells. Expression of EGFR was examined in four samples of COPD BALs and three samples of normal (healthy) BALs. All examined samples were positive for EGFR expression and showed cytoplasmic signals. Signals of EGFR were detected being stronger in COPD BALs compared to normal BALs, indicating a strong expression in inflammatory cells. In summary, AnxA8 expression was previously demonstrated in AM and AECII. Even stronger expression was found in COPD, indicating its expression in inflammatory cells.

4.1.6. Expression of AnxA8 and EGFR in infected lung tissues (Haemophilus influenzae, Streptococcus pneumoniae and Chlamydia pneumonia)

The expression of AnxA8 was investigated in Haemophilus influenzae (HI), Chlamydia pneumonia (CWL) and Streptococcus pneumoniae (Strep) infected lung tissues.

Fig. 33 illustrates the expression of AnxA8 in three different infected lung tissues, which were stimulated for 24h and 72h in the case of streptococcus pneumonia. Results were showing no alterations of AnxA8 expression in all infected tissues in comparison to the medium control. The expression of AnxA8 was most prominent in MAKs and AECII, which correlates with previous results. (**Fig. 34**)

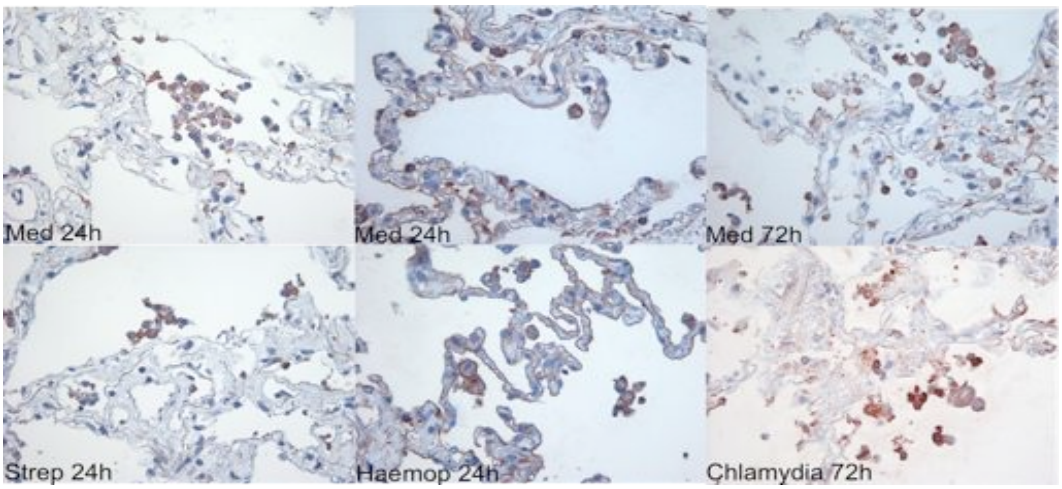

Figure 33 - *Immunohistochemical detection of AnxA8 in Streptococcus pneumonia (24h stimulation) (left), Haemophilus influenza (24h stimulation) (middle) and Chlamydia pneumonia (72h stimulation) (right) infected lung tissues. Expression of AnxA8 was unaffected upon infection, when compared to medium control (24h/72h stimulation). (magnification: 400x)*

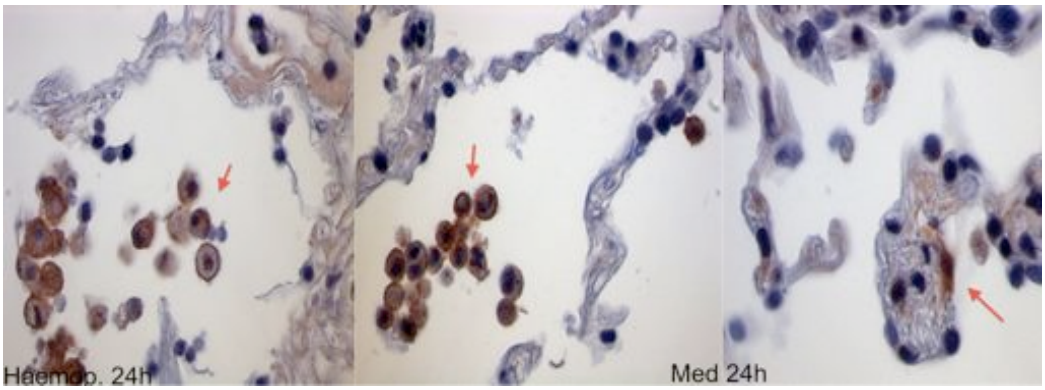

Figure 34 *- Immunohistochemical detection of AnxA8 in Haemophilus influenza (HI) infected lung and medium control tissues. Expression of AnxA8 was unaffected in HI tissue. Signals were detected in AM and AECII (arrow). Same as in Strep infected tissues, results were showing no differences between Med 24h and Haemop 24h. (Stained with AnxA8 AB-3 primary antibody) (magnification 400x)*

Additionally, expression of EGFR was examined only within Strep infected tissues and compared to the expression of AnxA8. (**Fig. 35**) There was neither upregulation nor downregulation observed when compared to the results of AnxA8 in Strep infected tissues. In summary, infections and different stimulation times had no influence on AnxA8. Signals were detected in MAKs and AECII, matching with previous results.

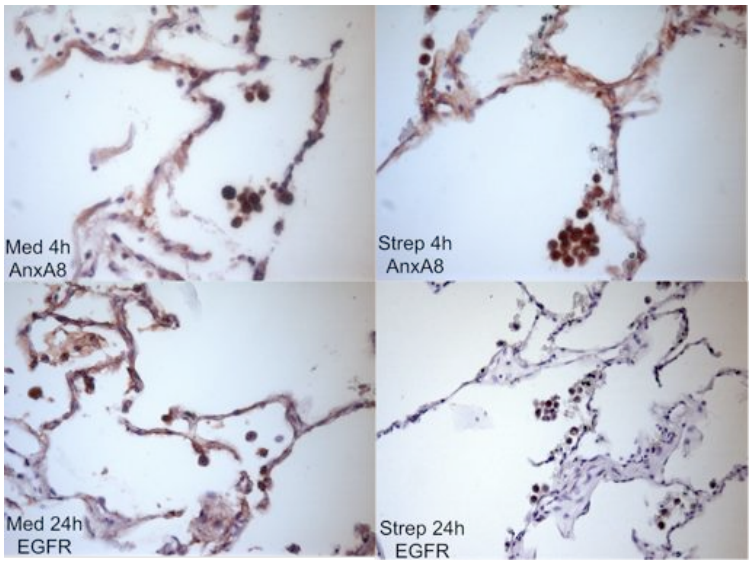

Figure 35 *– Immunohistochemical detection of AnxA8 and EGFR in Streptococcus pneumoniae infected lung tissues in comparison to medium control. No change in AnxA8 and EGFR expression was detected in Strep. Infected tissues, both stimulated for 4h and 24h. (magnification: 200x)*

4.2. Transcription of AnxA8 and transcriptional regulation to different stimuli

Transcripts of AnxA8 are further analysed by RT-PCR. The method was performed to confirm the transcription of AnxA8 in two selected cases: 22032/08 and 23418/08. Each analysed case included LPS, PAM3 and DEXA stimulated tissues and a medium control (RPMI). An intern control by the housekeeping gene GAPDH using GAPDH primers was performed for later normalizations, and all bands were showing a corresponding 250bp size. (**Fig. 36)**

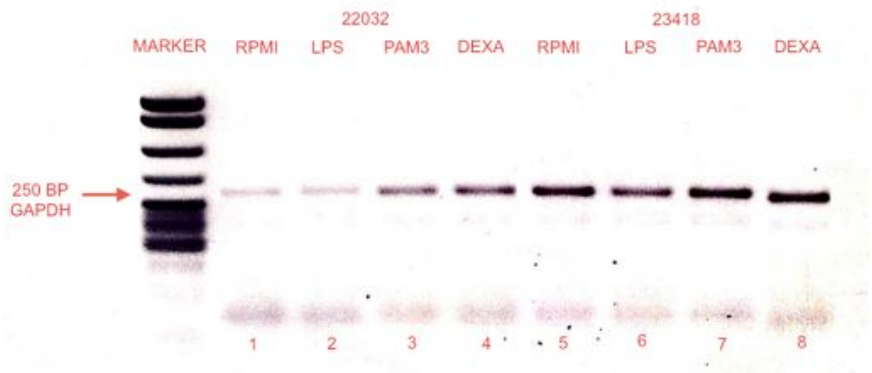

Figure 36 *- RT-PCR- GAPDH fragment of 250bp is shown in all samples (RPMI, LPS, PAM3 and DEXA stimulated lung samples: 22032 and 23418).*

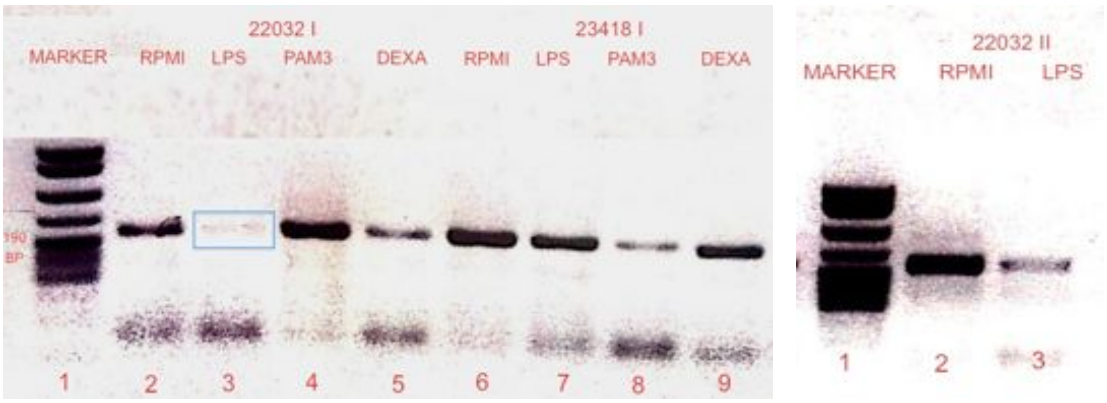

Figure 37 *- RT-PCR - The AnxA8 fragment of 190bp is shown in both stimulated cases of lung tissues. RT-PCR was repeated in case of 22032 LPS (blue) due to weak results and confirmed in second AnxA8-PCR (right). (Marker: pBr322-Msp1)*

Fig. 37 illustrates the results of the RT-PCR detecting AnxA8 transcripts in LPS, PAM3 and DEXA stimulated lung. Each case (22032 and 23418) was compared to the medium control (RPMI). This confirms the results of IHC and the transcription of AnxA8 in stimulated lung tissues.

Transcriptional levels of AnxA8 were compared with medium control (RPMI) and normalized to GAPDH transcript levels using a semi-quantitative method (band-leader). Results are shown in **Fig. 38.** AnxA8 transcript levels were distinct within LPS (+12% and -57%) and PAM3 (+40% and -16%) stimulated lung compared to medium in both samples. A decrease of -45% occurred in DEXA stimulated lung compared to medium in one sample, whereas no change occurred in another sample. These results are distinctive and cannot identify regulations in lung-stimulated tissues.

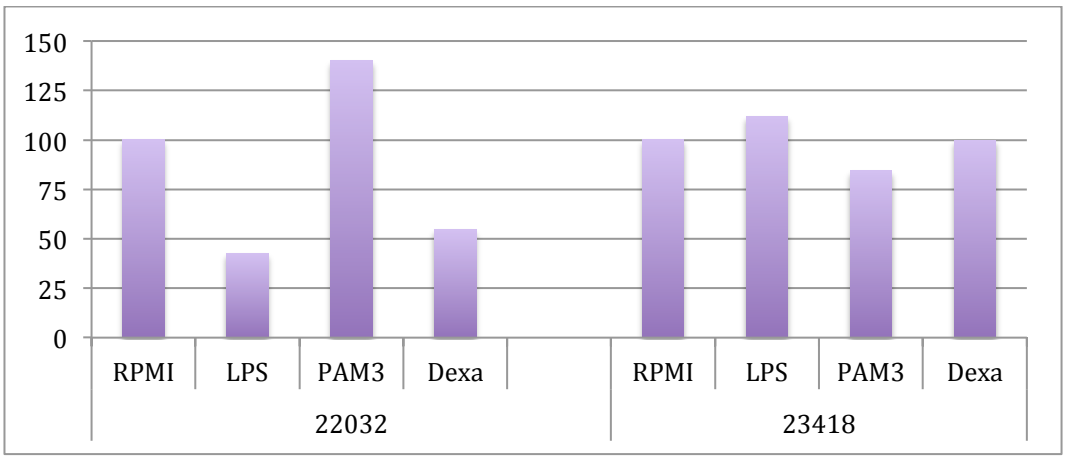

Figure 38 *AnxA8 transcript levels compared to medium control (RPMI= 100%) and referred to GAPDH levels. Measurement by Band-Leader (semi-quantitative).*

4.3. AnxA8 protein immunoblot (Western Blot)

Western blot was performed to detect AnxA8 protein in (pathogenic microorganisms and LPS/PAM3/DEXA) stimulated lung tissue samples. Western blot analyses turned out to be more difficult than expected. Different blocking solutions and non-/denaturating conditions did not lead to optimal western blot results. However, (weak) positive signals were obtained by using 5% gelatine as a blocking solution and by preparing samples under denaturating conditions. Thus, bands at 39 kDa signalized the detection of AnxA8 in HI, CWL and SP samples after 24h and 72h (in case of CWL) of stimulation. (**Fig. 39 and Fig. 40**)

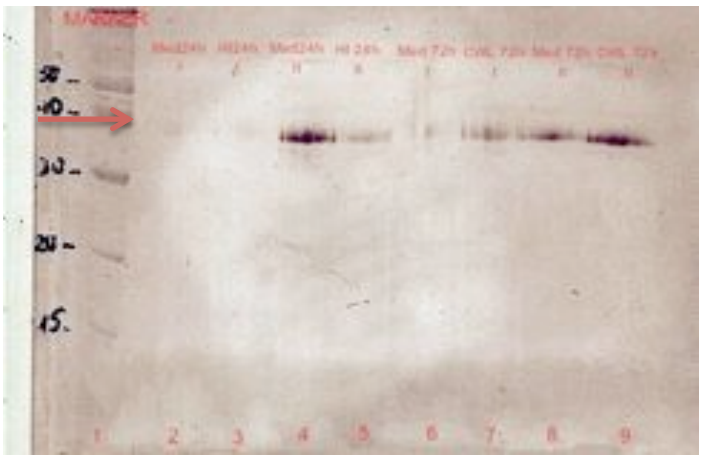

Figure 39 *Western Blot – AnxA8 protein of 39kDa (arrow) detected in Med, HI and CWL infected lung samples. (Med: medium, HI: Haemophilus, CWL: Chlamydia)*

Figure 40 *Western blot – Med and SP samples were labelled for AnxA8 protein. Bands were identified for AnxA8 protein of 39kDa (arrow) in Med and SP infected lung. (Med: medium, SP: Streptococcus)*

5. Discussion

Annexins are a family of Ca^{2+} and PL binding proteins (4). Although their structure is well defined, they are still in need of functional identification, even more the young member AnxA8. Their variable N-terminus and the ability to be modified (e.g. phosphorylated) are supposed to define their functional role. There is a strong assumption that detection of annexins in diverse organisms may be due to their functional variety, which include Ca^{2+}-in/dependent membrane binding, vesicular trafficking, modulation of EGFR (related to cell differentiation, migration and proliferation) and many more (5). Thereby, interaction with protein-ligands and binding to distinct membranes (plasma membranes or internal membranes) is regulated by Ca^{2+}, pH or cholesterol levels. Membrane-related activities (membrane dynamics, segregation, aggregation, remodelling of cytoskeleton) of Ca^{2+} sensitive annexins seem to be their key mode of action. Their ability to interact with phospholipase A_2 suggests their role as lipid messengers of lipid signalling via three possible mechanisms: first, formation of signalling platforms (lipid rafts) and lateral reorganizations of lipids in the cytosolic leaflet of the membrane into microdomains containing lipids and proteins, which establishes the environment of specific signalling proteins; second, interaction with phospholipase A_2 or other lipid kinases may recruit other proteins required in signalling events; and third, interaction with lipid mediators of the signalling pathway, for instance phosphatidylinositol (PI) or phosphatidylinositol 4,5 biphosphate (PIP_2) (47,49,57). The latter was identified to be specifically associated with AnxA2 and AnxA8 (31,32). PIP_2 was identified being associated with F-actin and AnxA2 at membranes, this might be important for membrane-associated actin assembly and bacterial adhesion during bacterial infection (47). In addition, it was also found in epithelium where it plays a role in formation of apical region of the plasma membrane required for cell polarity. Indeed, specific binding to PIP_2 and F-actin are demonstrated for AnxA8 as well. It is not far fetched to assume similar activity for AnxA8. Despite their binding property, AnxA8 are involved in following signalling-related activities: the control of EGFR signalling by functioning as a “shedding platform”, in order to determine the substrate selectivity of ADAM17; by affecting EGFR downregulation via association with late endosomes,

or by being involved in lipid rafts (signalling platform) formation (49,69,72). Thereby, activity is connected to membranes and includes the association with F-actin. Cellular processes are exerted by F-actin, including cell motility, cell division and cytokinesis, vesicle and organelle trafficking, cell signalling, and the maintenance of the cell shape. AnxA8 is linked to differentiation steps (osteoclasts and keratinocytes), which may be explained by the association with the activities of F-actin (14,15). Since AnxA8 was identified in the terminal step of osteoclast differentiation and keratinocyte differentiation, both processes require the stop of proliferation. This is supported by the association of AnxA8 with the degradation of EGFR (signal termination) via EGFR-endocytosis (69).

Association with signalling processes extends the functionality of annexins. Besides differentiation, annexins are also involved in cancer progression and inflammation processes. AnxA1 has been reported being involved in inflammatory processes (61). In fact, glucocorticoids (endogenous and exogenous) were found to regulate the synthesis and function of AnxA1 and they can mediate their anti-inflammatory effects via AnxA1. Indeed, DEXA seems to affect the expression of AnxA8 in lung tissues, detected by increased signals in macrophages. How does it relate to the present role of AnxA8? Exogenous glucocorticoids (GC), like DEXA, use many effector molecules to facilitate their numerous anti-inflammatory effects, in order to re-establish the tissue structure. Function of GCs is accomplished in a tissue, time and dosage dependent manner by two mechanisms: genomic mechanism (including transactivation or transrepression of gene transcription) and non-genomic mechanisms (that are rapid and independent of de novo protein synthesis). Their molecular targets can be detected by analysing their effects on immune cells. AnxA1, which is found in the cytoplasm of neutrophils, monocytes and macrophages in resting conditions, is induced by GC analogue dexamethasone, applying both mechanisms. Upon activation, AnxA1 is localized at the cell surface and cell-specifically secreted.

In this study, AnxA8 is demonstrated being also induced by DEXA in lung tissues, which were stimulated for 24h. Signals were cytoplasmic and membrane-bound, and highly detected in macrophages. No induction was observed in DEXA-stimulated tumor tissues, which might be due to tissue-specific effects by

dexamethasone. An increased expression of AnxA8 in chronic disease (COPD BALs) supports its role in inflammatory processes. Since membrane-related interactions are the key mode of action, expression of AnxA8 might also be mediated by DEXA via genomic mechanism. AnxA8 might help forming lipid rafts on plasma membranes, resulting in signalling platforms associated with signalling pathways like EGFR/PI3K/Akt, which is located and activated at lipid rafts and plays an important role in inflammatory processes.

Bacterial lipopolysaccharides (LPS) and the synthetic analogue lipopeptide PAM3, as well as pathogen infection affect the inflammation by induction of cytokine gene expression and secretion in macrophages (82,83). All stimuli have an individual pattern of cytokine induction, which can be the secretion of both cytokines interleukin-6 (IL-6) and IL-8 by LPS or IL-8 by PAM3. In this study, no effect was detected by LPS, PAM3 or pathogen infections. Again, these results may be explained by tissue, time and dosage dependent stimulations. A previous study supports these results, stimulation of RAW 264.7 macrophages (MAK) with LPS lead to a downregulation of AnxA1, while its expression was significantly increased with DEXA or DEXA and LPS (63).

In neoplasia, annexins have a tumour type-specific pattern of expression, due to their different expression levels and/or specific subcellular localizations (52). AnxA8 shows to be slightly more (+10%) expressed in squamous-cell carcinomas than adenocarcinomas, although expression appeared to be low and comparably the same as in normal lung tissues – indicated by weak membrane-bound signals. Comparison to EGFR revealed no significant changes, although signals appeared stronger in A549 cells, lung and lung cancer tissues. This could be explained by the constant low expression of AnxA8.

Interestingly, besides alveolar macrophages, the expression of AnxA8 was also recorded in AECII and possibly also in AECI. Signals of AnxA8 were detected in the epithelial lining of alveoli, which might be referred to AECI, since other annexins (AnxA1, A2, A4 and A7) are expressed in alveolar epithelial cells (19,55). Detection of AnxA8 in surfactant producing pneumocyte type II might indicate its role in surfactant secretion, which was previously reported for AnxA7. Lipid rafts can also

be detected on the plasma membrane of AECII, where they contain some proteins (e.g. SNARE) linked to the surfactant secretion process. Phosphorylation of AnxA7 has lead to its transfer to the plasma membrane, where it interacts with lamellar bodies and the membrane. Post-translational modifications and protein interactions might enable annexins to additionally insert into membranes. AnxA8 might also play a role in the surfactant secretion, which is again a membrane-related activity of annexins.

Furthermore, expression of AnxA8 has also been recorded in basal cells of bronchi, which are stem cells of the epidermis. Basal cells are made of basal keratinocyte cells and differentiate into keratinocytes, supporting the role of AnxA8 in keratinocyte differentiation.

Thus, AnxA8 remains a target protein for further research, since it seems to be involved in various processes, especially of the signalling pathway. Considering its tissue- and distribution-specificity, future experiments should focus on the detection of AnxA8 in different cell lines and knock-out animals. This might help to explain the role of AnxA8.

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„Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.“

7. Appendix

7.1. Samples

Table 1.

Single Tissue Section Slides – Non-stimulated			**Single Tissue Section Slides - Stimulated (K1T)**	
BALs	COPD		1	21843/08
1	2992			Lu + RPMI
2	3063			Lu + 20μl LPS
3	3160			Lu + 20μl Pam3
4	3159			Lu + 20μl Dexa
5	3098			Tu + RPMI
6	3084			Tu + 20μl LPS
BALs	Lung (normal)			Tu + 20μl Pam3
1	2640			Tu + 20μl Dexa
2	3072		2	21577/08
3	3170			Lu + RPMI
4	3952			Lu + 20μl LPS
5	2578			Lu + 20μl Pam3
6	2781			Lu + 20μl Dexa
7	2855			Tu + RPMI
	A549			Tu + 20μl LPS
1	Med			Tu + 20μl Pam3
2	Med			Tu + 20μl Dexa
3	Med - 27.9.11		3	22032/08
4	Med			Lu + RPMI
1	LPS			Lu + 20μl LPS
2	LPS			Lu + 20μl Pam3
3	LPS			Lu + 20μl Dexa
4	LPS		4	23438/08
5	LPS			Lu + RPMI
1	Pam3			Lu + 20μl LPS
2	Pam3			Lu + 20μl Pam3
3	Pam3			Lu + 20μl Dexa
4	Pam3		5	22369/08
	Tumor			Lu + RPMI
1	12740/06 AC			Lu + 20μl LPS
2	6215/06 AC			Lu + 20μl Pam3
3	3191/05 AC			Lu + 20μl Dexa

4	15768/06 SC	6	22521/08
5	18315/06 SC		Lu + RPMI
6	6021/04 SC		Lu + 20μl LPS
7	14305/06 SC		Lu + 20μl Pam3
8	5757/08 SC		Lu + 20μl Dexa
	Array	7	12554/08
1	755/02 AC		Lu + RPMI
2	6683/02 AC		Lu + 20μl LPS
3	8469/03 AC		Lu + 20μl Pam3
4	10214/05 SC		Lu + 20μl Dexa
5	11661/05 SC	8	10656/08
6	17363/02 SC		Lu + RPMI
7	19172/05 AC		Lu + 20μl LPS
8	20512/06 AC		Lu + 20μl Pam3
			Lu + 20μl Dexa

Table 2.

Sample	Array	Type	#
1	ZL1	Lung	4858/06
2			11174/06
3			8848/06
4			13263/06
5			6374/06
6			12331/06
7			3277/09
8			9265/06
9			13106/06
10			8365/06
11			12727/06
12			4346/06
13			9378/06
14			8578/06
15			12740/06
16	ZL2	Lung	16566/06
17			20512/06
18			17652/06
19			21833/06
20			17072/06
21			20837/06
22			14305/06
23			18315/06
24			21988/06

Sample	Array	Type	#
1	Daniel I	AC	1636/01
2			15532/02
3			3375/02
4			7263/03
5			17309/03
6			5571/02
7			8469/03
8			755/02
9			9986/04
10			6683/02
11			13049/02
12			13763/03
13			17556/04
14	Daniel II	AC	403/05
15			21527/06
16			10731/05
17			6212/06
18			18305/06
19			11227/05
20			6224/06
21			21987/06
22			375/07
23			3191/05
24			19301/06

25			17279/06
26			21527/06
27			15768/06
28			19301/06
29			17082/06
30			21094/06
31	ZL1	AC	3277/06
32			8848/06
33			13263/06
34			13106/06
35			8365/06
36			12727/06
37			4346/06
38			8578/06
39			12740/06
40			4858/06
41	ZL2	AC	17652/06
42			21833/06
43			17072/06
44			20837/06
45			21988/06
46			17279/06
47			21527/06
48			19301/06
49			17082/06
50			20512/06
51	ZL1	SC	6374/06
52			12331/06
53			9265/06
54			9378/06
55			11174/06
56	ZL2	SC	14305/06
57			18315/06
58			15768/06
59			21094/06
60			16566/06
61	STSS	AC	6215/06
62			3191/05
63		SC	6021/04
64			5757/08

25			2041/07
26			5775/05
27			20512/06
28			19172/05
29	Daniel I	SC	3140/02
30			3970/03
31			14751/03
32			15693/02
33			16733/02
34			1852/04
35			6427/02
36			17363/02
37			8192/03
38			2386/02
39			964/03
40			11813/03
41			16359/04
42			2821/02
43			1187/03
44			2835/02
45			15312/02
46			2829/03
47			14254/03
48			18610/04
49	Daniel II	SC	10214/05
50			352/06
51			9378/06
52			21833/06
53			18315/06
54			2537/05
55			11661/05
56			9265/06
57			18488/06
58			17836/05
59			6374/06
60			18168/05
61			6757/06
62			2805/07
63			9045/05
64			7947/06
65			21094/06
66			3487/07

7.3. Zusammenfassung

Annexin A8 (AnxA8) ist das achte Mitglied der Ca^{2+}- und Phospholipid-bindenden Annexin Familie, die ubiquitär, häufig auftretend und vor allem membranbindend sind. Als membranbindende Proteine sind sie Zielproteine verschiedener Prozesse und sind beteiligt an der Organisation von Membranen (z.B. Formierung einer Signalübertragungsebene), dem Vesikeltransport oder dem Reparaturmechanismus. Nur wenig ist bekannt über AnxA8, das spezifisch mit phosphatidylinositol-4,5-bisphosphat (PIP_2), F-Aktin und späten Endosomen assoziiert. Eine Rolle in der Regulation von EGFR (epidermal growth factor receptor) und/oder EGFR Endocytose wird vermutet. AnxA8 scheint eine gewebespezifische Funktion auszuüben, da es im Vergleich zu den anderen Annexinen nur gering ubiquitär und wenig exprimiert vorliegt. In verschiedenen Krebsarten (z.B. Bauchspeicheldrüsenkrebs oder Brustkrebs) ist AnxA8 sogar überexprimiert, dies verstärkt die Beziehung zu EGFR, welcher in den meisten Tumorarten defekt ist. In dieser Studie wird der Fokus auf die Rolle von AnxA8 in der Lunge gelegt, da es dort am Häufigsten aufgefunden wurde. Die Expression und zelluläre Lokalisation von AnxA8 wird genauer in der Lunge, zwei Lungenkrebsarten (Adenokarzinom und Plattenepithelkarzinom), stimulierten Lungengeweben (LPS/PAM3 und DEXA) sowie Pathogen-infizierten Lungengeweben untersucht. Des Weiteren wurde die Expression auch in krankhaften BALs (Bronchoalveoläre Lavage Zellen von chronisch obstruktiven Lungenerkrankten) und stimulierten A549 Zellen (LPS und PAM3) untersucht. Dabei wurden Immunohistochemie (IHC), RT-PCR und Western Blot Methoden angewendet und parallel mit den Ergebnissen von EGFR verglichen. Die Ergebnisse der IHC zeigen eine deutlich membrangebundene Lokalisation von AnxA8 und EGFR, die von der Intensität schwächer für AnxA8 ausgefallen ist. Hinzu wurde AnxA8 vermehrt in den Alveolarmakrophagen und Alveolarepithelzellen Typ II gefunden (möglich auch in Alveolarepithelzellen Typ I). Dexamethason zeigt Einfluss auf die Expression von AnxA8 zu haben; in der Lunge wurden erhöhte Signale in den Alveolarmakrophagen festgestellt. Ebenfalls wurden erhöhte Signale in krankhaften BALs entdeckt, die auf die Lokalisation von AnxA8 in Immunzellen schließt.

7.2. Curriculum Vitae

Personal Information

Name:	Lejla Ziga
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Day of Birth:	01.03.1985
Place of Birth:	Priboj
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Education

Since 10/2006	Molecular Biology (diploma degree); Focus: molecular medicine, immunology/microbiology and genetics, University of Vienna, Austria
03/2013 – 06/2013	Biology (bachelor degree), University of Vienna, Austria
1995-2005	Albert-Schweitzer-Gymnasium, (upper secondary school), Hamburg, Germany
1993-1994	Albert-Schweitzer-Schule, (elementary school), Hamburg, Germany
1991-1993	Alija-Nametak, (elementary school), Sarajevo, Bosnia and Herzegovina

Degrees

June 2013	Bachelor of Science in Biology (BSc),
June 2005	General qualification for university entrance (Abitur)

Training

2/2010 - 06/2010	Clinical and Experimental Pathology - Research Center Borstel
7/2009 – 09/2009	Department of Microbiology and Immunology - McGill University Health Center, Montreal
7/2008	Institute of Inorganic Chemistry – University of Vienna
05/2006 - 06/2006	Practical nursing internship – Alsterdorf Hospital, Hamburg
05/2001	Internship - „Sundance“ event agency, Hamburg
07/2000	Internship - Child and Youth Services „Kinder- und Jugendhilfe e.V.“, Hamburg

Qualifications and Skills

Languages:	Bosnian:	native language
	German:	native language
	English:	good skills, fluently
	Spanish:	good skills

Computer skills: MC Office: Access, Word, Excel and Powerpoint