Master Thesis:
Macrophage homeostasis in Idiopathic Pulmonary Fibrosis (IPF): a Bronchoalveolar Lavage Fluid Study

Graduate Program "The Molecular Basis of Human Disease"
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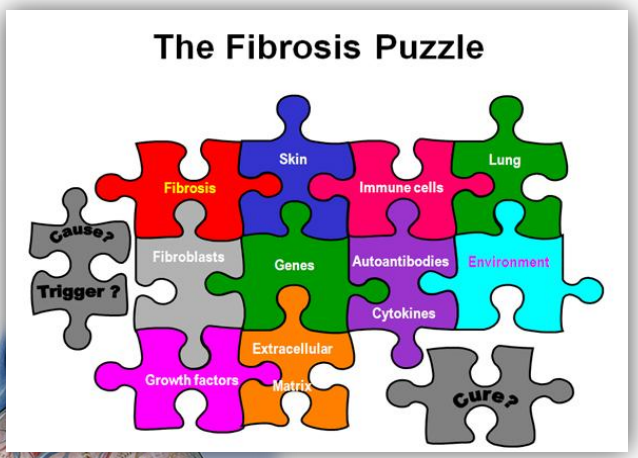

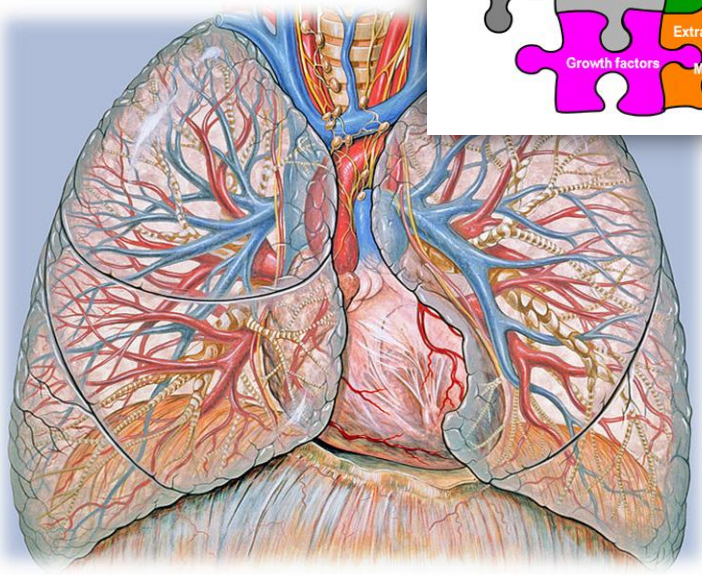

Τίτλος

Ομοιόσταση των μακροφάγων στην Ιδιοπαθή Πνευμονική Ίνωση (ΙΠΙ):
Μελέτη στο Βρογχοκυψελιδικό Έκπλυμα Ασθενών

Περίληψη

Η Ιδιοπαθής Πνευμονική Ίνωση (ΙΠΙ) είναι μία χρόνια και μη αναστρέψιμη διάμεση πνευμονοπάθεια με φτωχή πρόγνωση και περιορισμένες θεραπευτικές επιλογές. Την τελευταία δεκαετία έχουν γίνει σημαντικές ερευνητικές προσπάθειες για την ανακάλυψη των παθογενετικών μηχανισμών που συμβάλλουν στην ανάπτυξη αυτής της αινιγματικής πνευμονικής νόσου. Η αυτοφαγία αποτελεί ένα μηχανισμό για την ανακύκλωση των συστατικών και των οργανιδίων του κυττάρου και συνεισφέρει σημαντικά στην κυτταρική ομοιόσταση. Η σημασία των μιτοχονδρίων και ο καίριος ρόλος τους στη ρύθμιση του μεταβολισμού της ενέργειας του κυττάρου είναι ευρέως γνωστά. Παρόλα αυτά, παράγοντες όπως το αυξημένο οξειδωτικό στρες επηρεάζουν τη σωστή λειτουργία και δομή των μιτοχονδρίων. Είναι ζωτικής σημασίας για το κύτταρο να αποικοδομήσει αυτά τα δυσλειτουργικά μιτοχόνδρια κυρίως μέσω μίας επιλεκτικής μορφής της αυτοφαγίας που ονομάζεται μιτοφαγία. Πρόσφατες έρευνες σε επιθηλιακά κύτταρα και ινοβλάστες του πνεύμονα έχουν δείξει ότι η αυτοφαγία και τα δυσλειτουργικά μιτοχόνδρια πιθανόν να παίζουν κάποιο ρόλο στην ανάπτυξη της ΙΠΙ. Σε αυτή τη μελέτη επικεντρωθήκαμε στην αυτοφαγία, με ιδιαίτερη έμφαση στη μιτοφαγία και την οξειδωτική κατάσταση των μιτοχονδρίων σε ασθενείς με ΙΠΙ. Χρησιμοποιήσαμε τα κύτταρα από το βρογχοκυψελιδικό έκπλυμα ασθενών, που περιλαμβάνουν κυρίως μακροφάγα, και τα συγκρίναμε με υγιή άτομα αλλά και με ασθενείς με ρευματοειδή αρθρίτιδα (ΡΑ) οι οποίοι είχαν και πνευμονική νόσο (διάμεση πνευμονοπάθεια από ΡΑ). Τα αποτελέσματά μας έδειξαν αυξημένα πρωτεϊνικά επίπεδα της LC3 παράλληλα με μειωμένα επίπεδα της πρωτεΐνης p62 σε ασθενείς με ΙΠΙ, ενώ δεν φάνηκε κάποια διαφορά στους ασθενείς σε σχέση με υγιείς μάρτυρες, στα επίπεδα έκφρασης γονιδίων που εμπλέκονται στην αυτοφαγία και στη μιτοφαγία - όπως Beclin1, ULK1, S100A9, p62, BNIP3, AKT3. Ιδιαίτερα ενδιαφέρον αποτελεί το γεγονός πως βρήκαμε σημαντική πτώση της έκφρασης του γονιδίου PINK1 στους ασθενείς με ΙΠΙ, αλλά και με διάμεση πνευμονοπάθεια από ΡΑ, συγκρίνοντάς τα με τους υγιείς. Όσο αφορά τους ασθενείς με ΡΑ, παρατηρήσαμε ένα διαφορετικό πρότυπο γονιδιακής έκφρασης με σημαντικά αυξημένα επίπεδα Beclin1 και AKT3. Σχετικά με το οξειδωτικό στάτους των μιτοχονδρίων των ασθενών με ΙΠΙ, στόχος μας ήταν να εκτιμήσουμε την οξείδωση των μιτοχονδρίων με χρώση MitoSOX και ανάλυση κυτταρομετρίας ροής σε ζωντανά κύτταρα από ασθενείς που υποβλήθηκαν σε βρογχοσκόπηση κατά το χρονικό διάστημα που διήρκησε η μελέτη μας. Τα πρώτα αποτελέσματα, σύμφωνα με τις έως τώρα διαγνώσεις, δείχνουν μία σημαντική μετατόπιση του κυτταρικού πληθυσμού μετά από χρώση και ανάλυση της κυτταρομετρίας ροής, υποδηλώνοντας πως οι ασθενείς με ΙΠΙ είναι θετικοί για MitoSOX, παρά το γεγονός ότι το ποσοστό των έντονα θετικών κυττάρων για χρώση MitoSOX δεν έχει στατιστικά σημαντική αύξηση σε σχέση με τους υγιείς. Τέλος, αξιολογήσαμε την επίδραση του κύριου μεσολαβητή της ΙΠΙ, TGFβ1, σε κυτταρική σειρά μονοκυττάρων έπειτα από δράση ενός παράγοντα διαφοροποίησης προς μακροφάγα. Αποτελεί αρκετά ενδιαφέρον εύρημα ότι ο TGFβ1 φάνηκε να μειώνει τα επίπεδα των οξειδωμένων μιτοχονδρίων και την έκφραση του γονιδίου του PINK1 στα μακροφάγα.

Abstract

Idiopathic Pulmonary Fibrosis (IPF) is a chronic and irreversible interstitial lung disease with poor prognosis and limited therapeutic options. The last decade much research has been done to reveal the pathogenetic mechanisms involved in this enigmatic lung disease. Autophagy is a process for the turnover of subcellular components and organelles and represents a major cellular homeostatic mechanism. The significance of mitochondria and their main role in energy regulation are well known. However, upon oxidative stress, mitochondria become dysmorphic and dysfunctional and need to be removed via selective autophagy, called mitophagy. Recent evidence suggests a role of autophagy and mitochondrial dysfunction in the development of IPF focusing on lung fibroblasts or epithelial cells. In this study, we focused on the evaluation of autophagy with an emphasis in mitophagy and oxidation status in macrophages from patients with IPF, using the Bronchoalveolar Lavage Fluid (BALF) derived cells in comparison with control subjects and patients with Rheumatoid Arthritis (RA) and lung involvement (ILD-interstitial lung disease). Our results indicated an increase in LC3 protein levels, accompanied by a decrease in p62 protein levels in IPF patients, with no difference in mRNA expression levels in genes involved in autophagy and mitophagy - such as Beclin1, ULK1, S100A9, p62, BNIP3, or AKT3- in IPF patients when compared to controls. Interestingly, we demonstrated a significant downregulation of PINK1 mRNA levels in IPF and RA-ILD patients versus controls. However, RA-ILD patients seem to display a distinct expression pattern with upregulation of Beclin1 and AKT3. Regarding the oxidation levels of mitochondria in IPF patients, we evaluated MitoSOX staining in fresh BALF cells from newly recruited patients. Our preliminary data showed a distinct population shift of the cells following flow cytometry analysis, indicating MitoSOX positivity in IPF patients, although the percentage of strongly MitoSOX positive cells was not statistically significant different when compared to controls. Finally, we assessed the effect of the master mediator in IPF pathogenesis, TGFβ1, in THP1 cells treated with PMA. We interestingly observed that TGFβ1 decreased MitoSOX levels as well as PINK1 expression.

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1. Introduction

1.1. Idiopathic Pulmonary Fibrosis

Idiopathic Pulmonary Fibrosis (IPF) is a chronic and progressive fibrotic lung disease of unknown etiology (Figure 1) (1). It is characterized by the histopathologic pattern of usual interstitial pneumonia (UIP) and is the most common of the idiopathic interstitial pneumonias. Unfortunately it has a poor prognosis with median survival 2 to 6 years after diagnosis. Despite recent advances in understanding the pathogenic mechanisms, there are limited therapeutic options, whereas lung transplantation is actually the only effective therapy (2). It is currently believed that IPF is caused by repeated injuries in the alveolar epithelium, such as viral infection, gastroesophageal reflux and smoking, in a background of genetic predisposition (Figure 2) (3-6). After alveolar epithelium injury there is increased activity and proliferation of type II alveolar epithelial cells so as to repair the damage. Deregulation of this repair process leads to aberrant wound healing, differentiation of fibroblasts to myofibroblasts and excess accumulation of extracellular matrix (ECM) components, resulting in progressive lung remodeling and fibrosis (5). Endoplasmic reticulum stress of epithelial cells, epithelial to mesenchymal transition, high levels of transforming growth factor β (TGFβ1), fibrocyte recruitment, and involvement of immune cells along with increase in cytokines production, are among the mechanisms involved in the disease pathogenesis (Figure 3) (9).

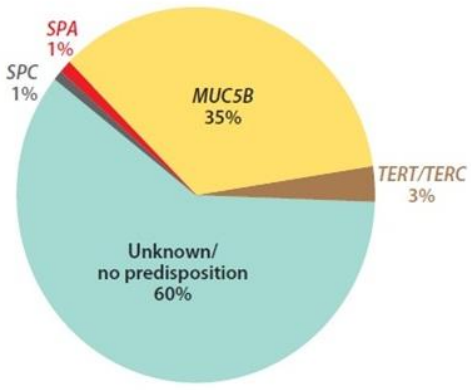


Figure 1. Estimated prevalence of genetic mutations predisposing to sporadic IPF (1).

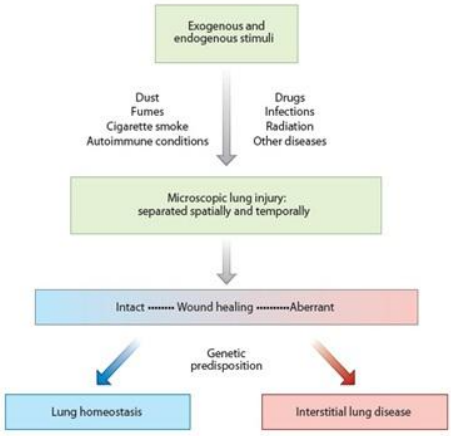


Figure 2. The pathogenetic stages for the development of IPF (2).

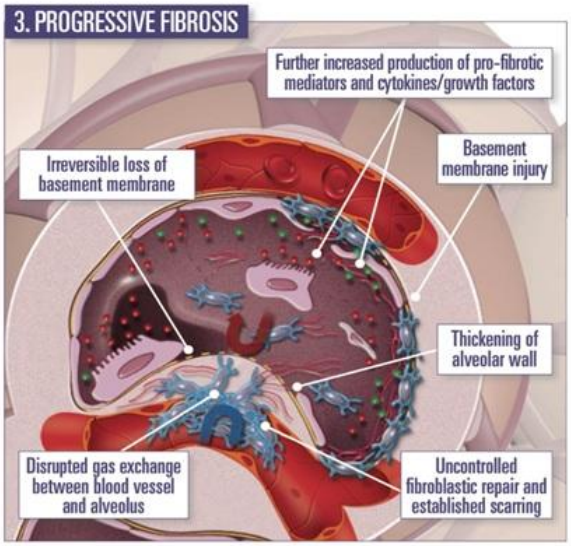


Figure 3. Epithelial damage, inflammation, EMT, myofibroblast activation, and repetitive cycles of tissue injury are important mediators of fibrosis (9).

Particularly, upon epithelial injury, a wound healing process, which consists of four stages that include a coagulation phase, an inflammatory phase, a fibroblast migration and proliferation phase, and a final remodeling phase where normal tissue architecture is restored (Figure 4) (10). In the earliest stages after tissue damage, epithelial and endothelial cells release inflammatory mediators that initiate an antifibrinolytic-coagulation cascade that promotes blood clot formation and development of a temporary ECM. Then, destruction of the alveolar-capillary basement membrane and increased permeability allow efficient recruitment of inflammatory cells (neutrophils, macrophages, lymphocytes, and eosinophils) to the site of the injury. Neutrophils are the most abundant cells in the earliest stages of wound healing, but are quickly replaced by macrophages. During this initial leukocyte migration phase, activated macrophages and neutrophils remove dead cells and eliminate any invading organisms. Irritants that injure lung epithelial cells can be detected by the Nalp3 inflammasome in macrophages. These irritants stimulate the production of ROS, chemokines, and cytokines, enhancing further the recruitment and activation of leukocytes (Figure 5) (10). The recruited leukocytes, by secreting a variety of chemokines and profibrotic cytokines, trigger the inflammatory response and increase fibroblast proliferation. Moreover, epithelial cells release cytokines, such as IL-33, which can enhance the development of profibrotic Th2 responses. Th2 cells release IL-4 and IL-13, which promote the development of a profibrotic macrophage subpopulation that secretes TGFβ1 among other mediators. IL-13 can directly activate fibroblasts independently of TGFβ1. Thus, fibrocytes from the bone marrow and resident fibroblasts proliferate and differentiate into myofibroblasts, which release ECM components. It is suggested that fibroblasts and myofibroblasts may also be derived from epithelial cells undergoing epithelial to mesenchymal transition (EMT) (3, 7). In the final

remodeling and resolution phase, activated myofibroblasts can promote wound repair, leading to wound contraction and restoration of blood vessels. However, fibrosis often develops if any stage in the tissue repair program is deregulated or when the lung-damaging stimulus persists (10).

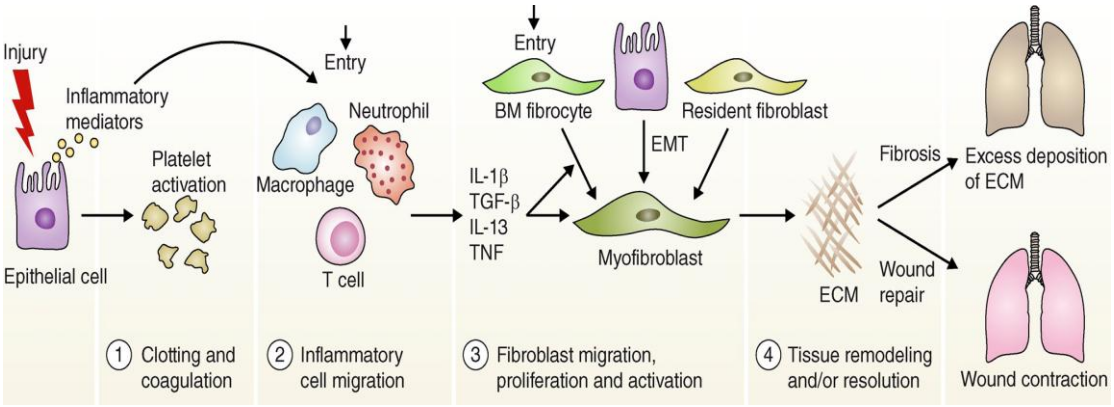


Figure 4. Alterations in normal wound healing contribute to the development of pulmonary fibrosis. Wound healing has four distinct stages: 1) a clotting/coagulation phase, 2) an inflammatory cell migration phase, 3) a fibroblast migration/proliferation/activation phase, and 4) a tissue remodeling and resolution phase (10).

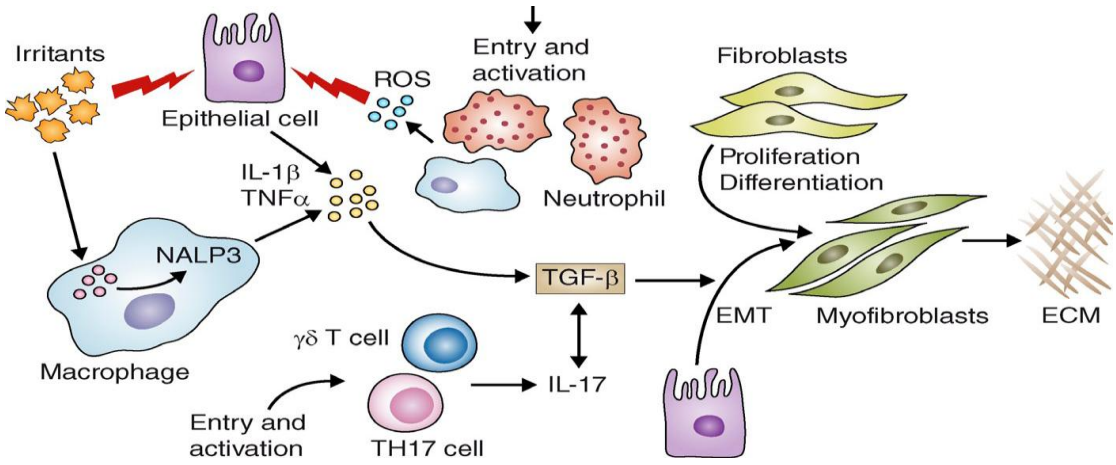


Figure 5. Proinflammatory and profibrotic mediators in the initiation and maintenance of fibrosis (10).

Alveolar Macrophages

Concerning macrophages, it is established that they represent very important cells in lung composition, since they play a central role in inflammation, host defense, and tissue homeostasis. They participate into all stages of the fibrotic process, since they serve as key regulators of fibroblast recruitment, proliferation, and activation. Interestingly, although some macrophages clearly promote tissue fibrogenesis, other macrophage subpopulations facilitate the resolution and/or reversal of fibrosis (11, 12).

Macrophages play a key role in IPF since they participate in the fibrotic mechanisms in the lung. It is currently believed that macrophages within the lung orchestrate the downstream progression and maintenance of fibrosis (13). It has been shown that the depletion of macrophages in the lung results in a reduction in lung collagen deposition, with a corresponding attenuation in BAL inflammation, in TGFβ1 transgenic mouse model. There is a vicious circle between fibroblasts and macrophages in IPF amplifying fibrosis (14, 15). Furthermore, there is evidence that shows a potent role of macrophages in epithelial to mesenchymal transition of A549 epithelial cell line (16, 17). Thus, the crosstalk between macrophages, fibroblasts and epithelial cells is important for the pathogenetic mechanisms in the lung environment.

Alveolar macrophages are unique amongst tissue-resident macrophages in both phenotype and function; e.g. their phenotype is similar to DCs, high levels of CD11c and CD205 expression. Alveolar macrophages also differ from the pulmonary interstitial macrophages in origin and lifespan. Pulmonary interstitial macrophages originate from bone marrow-derived monocytes and have a shorter lifespan. On the other hand, alveolar macrophages populate the lung during embryogenesis and renew from tissue resident populations, with little contribution from the bone marrow. It is widely believed that blood-borne precursors undergo an obligate intermediate differentiation step within the parenchyma transitioning to macrophages that subsequently migrate into the alveolar space (18).

Bronchoalveolar lavage (BAL) is a mildly invasive method, which allows the recovery of soluble and cellular components lining the alveolar epithelium. Alveolar macrophages constitute more than 70% of the cellular component in IPF which makes BALF ideal for their study (19, 20).

1.2. The Autophagic Pathway

Autophagy is a membrane-dependent mechanism for the turnover of subcellular components and represents a major cellular homeostatic mechanism. It serves as a source of metabolic fuel, it removes dysfunctional mitochondria or aggregated proteins, and determinates cell fate (21). During starvation, autophagy prolongs cell survival by recycling metabolic precursors from intracellular macromolecules (22). In addition to its basic role in the turnover of proteins and organelles, autophagy has multiple physiological and pathophysiological functions, including roles in cell differentiation and immune response (23). Autophagy has been proposed to be widely implicated in human health and disease (Figure 6), playing crucial role in cancer, metabolic, neurodegenerative, cardiovascular diseases, as well as pulmonary disorders (Figure 7) (24-26).

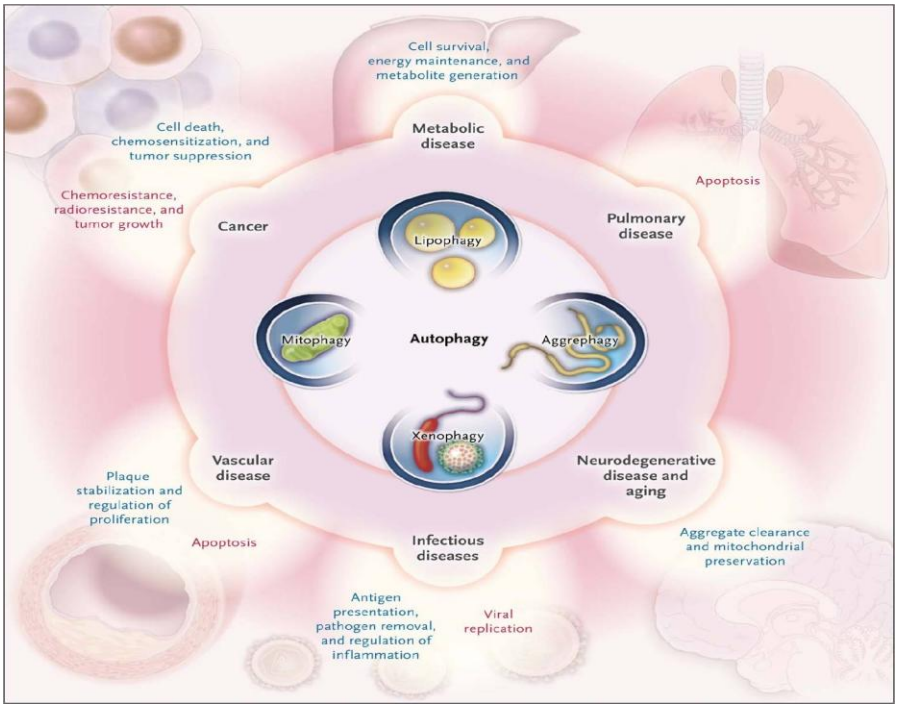


Figure 6. Autophagy in human health and disease (22).

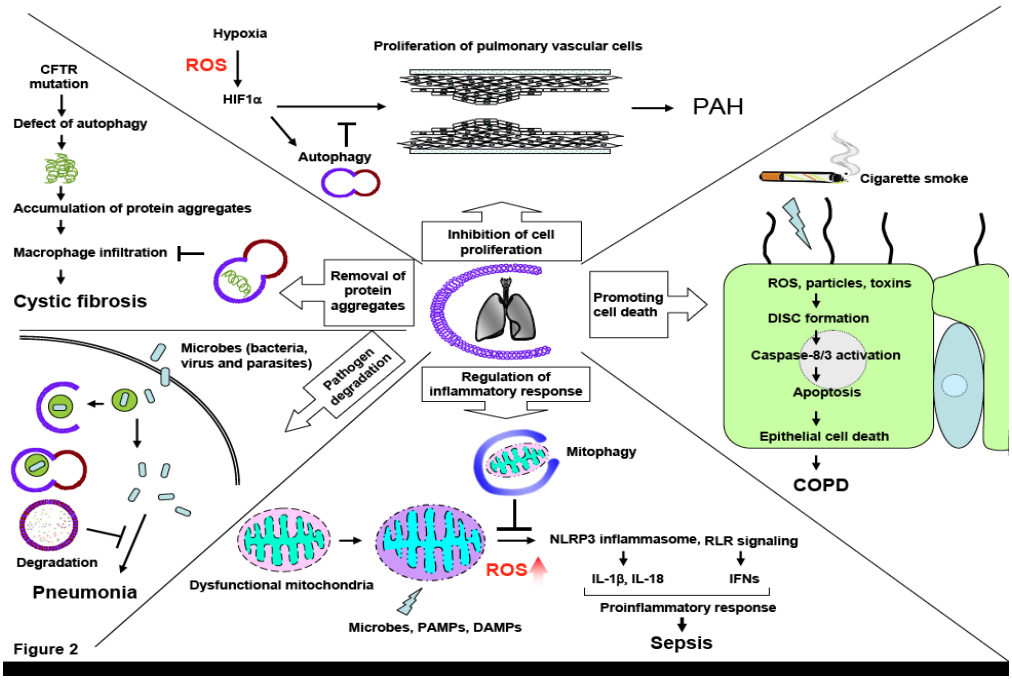


Figure 7. Autophagy in lung diseases (24).

There are several types of autophagy based on the selective component that is going to be degraded; macroautophagy, mitophagy, microautophagy (direct endocytosis of cytosolic material by lysosomes), aggrephagy (degradation of polyubiquitinated protein aggregates or aggresomes), xenophagy (digestion of pathogens), chaperone-mediated autophagy (molecular chaperones facilitate the transfer of proteins to the lysosomes), lipophagy (autophagy

regulates lipid metabolism) (Figure 8) (24, 27). Macroautophagy is the best studied mechanism and is often referred to as simply autophagy. It is characterized by the sequestration of cytoplasmic components in double-membrane vesicles, referred to as autophagic vacuoles or autophagosomes. Autophagosomes ultimately fuse with lysosomes, generating single membrane autophagosomes and degrading their components via enzymatic digestion (24).

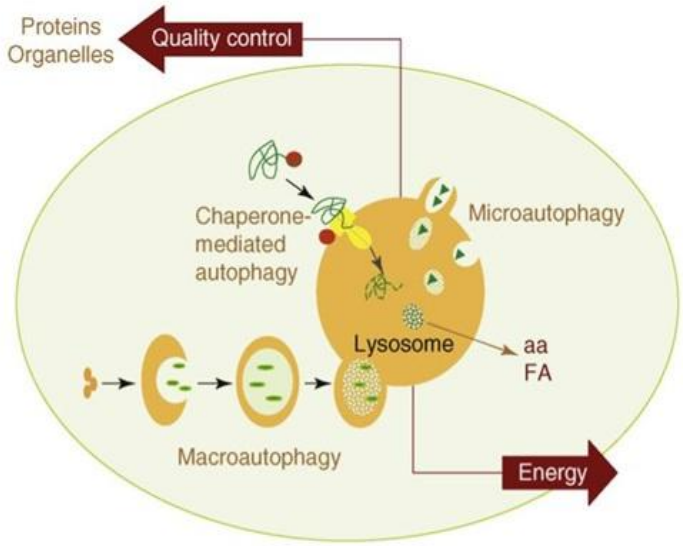


Figure 8. Autophagy represents a quality control system for the cell and consists of several types (21).

The macroautophagic pathway proceeds through several phases of membrane restructuring and translocation events (Figure 9). An initiation phase involves the activation and assembly of signaling components that trigger the process in response to environmental cues. In the nucleation phase, there is the formation of a pre-autophagosomal structure from subcellular membranes, and further evolves into an isolation membrane, named phagophore. During vesicle elongation, the isolation membrane expands to surround and engulf a cytosolic material targeted for degradation to form complete autophagic vacuoles or autophagosomes with a double-membraned structure. After autophagosome maturation and cargo sequestration, ultimately comes the fusion of autophagosomes with lysosomes (formation of autolysosomes). In this final stage, autophagosomal contents are degraded by lysosomal degradative enzymes (e.g., cathepsins and other acid hydrolases) and the contents of the autolysosome are released for metabolic recycling (22).

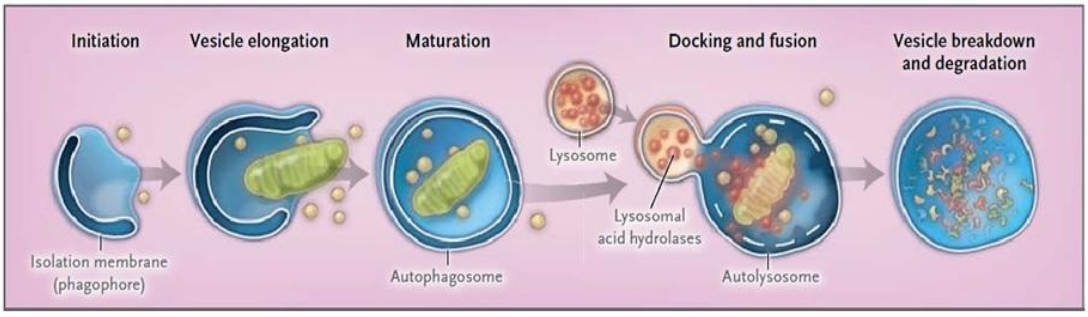


Figure 9.The stages of the autophagic process (22).

The autophagic pathway is a specific process responding to input from environmental cues. It represents a tightly regulated mechanism by several macromolecular signaling complexes (Figure 10) (22, 25). A number of autophagy-related genes (Atgs) have been identified from studies of lower organisms and homologs of many of these Atgs have been also identified in mammals. Atg gene products interact with cellular signaling pathways in a complex regulatory network that governs the autophagic program. The major negative regulator of autophagy is the mammalian target of rapamycin (mTOR). During nutrient-rich conditions - insulin and other growth factor signaling- class I phosphatidylinositol-3-kinase (PI3K)–AKT activates mTOR. The mTOR protein resides in a multiprotein complex, mTOR complex 1 (mTORC1), which represents the target of cascades responsive to nutrient availability, and regulates the mTOR substrate complex, consisting of the mammalian uncoordinated-51–like protein kinase 1 (ULK1), ATG13, ATG101, and FIP200.ULK1 kinase (Atg1 in yeast) is a major regulator of the initiation of starvation-induced autophagy. This interaction between mTORC1 and ULK1 suppresses ULK1 activity, blocking the activation of autophagy. On the contrary, through the mTOR pathway, starvation or stimulation with the antibiotic rapamycin acts as a potent inducer of autophagy by inhibiting mTOR serine/threonine kinase activity resulting in global dephosphorylation and activation of ULK1 kinase activity, and the consequently ULK1-dependent phosphorylation of FIP200 and Atg13. AMP-activated protein kinase (AMPK), which is regulated by AMP levels, negatively regulates mTOR and directly phosphorylates ULK1, thereby acting as a positive regulator of autophagy in response to energy depletion.

The nucleation step of autophagosome formation requires an additional regulatory complex involving a class III PI3K complex (PIK3C3, or VPS34), in association with p150, Atg14L, and the Bcl-2-interacting protein Beclin 1 (Atg6 in yeast). Beclin 1 is a major contributor in the regulation of autophagy. The activation of ULK1 leads to the recruitment of the class III PI3K complex to autophagosomes. The UV radiation resistance–associated tumor suppressor gene protein (UVRAG) forms an alternate Beclin 1–Vps34L complex, which also enhances autophagy. AKT negatively regulates autophagy by phosphorylating Beclin 1. Rubicon also

negatively regulates autophagy by binding to the class III PI3K complex. Several additional factors, including Ambra-1, Bif-1, and the antiapoptotic proteins Bcl-2 and Bcl-XL, interface with the class III PI3K complex and affect its activity (24).

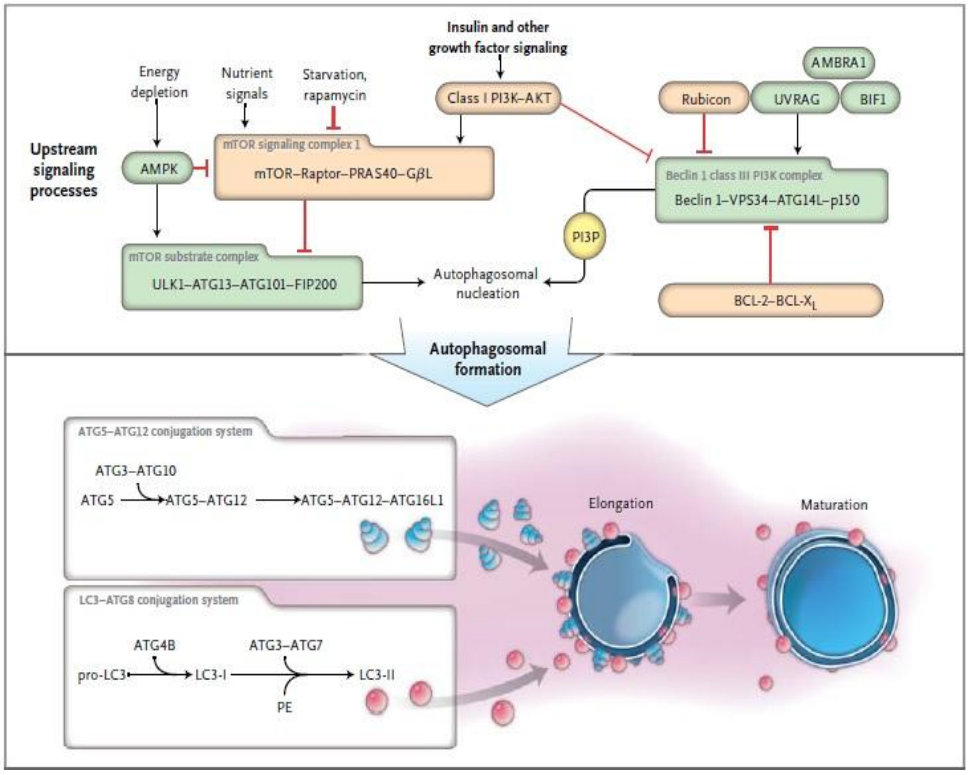


Figure 10. The regulation of the autophagic pathway (22).

The next step, autophagosomal elongation, requires two ubiquitin-like conjugation systems. In the first system, the ubiquitin-like protein ATG12 is conjugated to ATG5 by ATG7 and ATG10 enzymes. The resulting ATG5–ATG12 forms a complex with ATG16L1, which participates in the elongation phase. These factors dissociate from the autophagosome during maturation. The second conjugation system requires the ubiquitin-like protein microtubule–associated protein 1 light chain 3 (LC3, ATG8). LC3 is modified with the cellular lipid phosphatidylethanolamine (PE). The precursor form of LC3 is cleaved by the protease ATG4B to generate the LC3-I form, with an exposed lipid conjugation site at the C-terminal glycine residue. Conjugation of PE with LC3-I occurs from the sequential action of ATG7 and ATG3 enzymes. In mammals, the conversion of LC3-I (free form) to LC3-II (PE-conjugated form) is a key regulatory step in autophagosome formation. The cytosolic redistribution of LC3-II, as evidenced by a shift in the staining pattern of LC3 from diffuse to punctate staining, is a crucial indicator of autophagosome formation. The recruitment of LC3-II to the autophagosome is mediated by the ATG5-ATG12-ATG16L complex, which also facilitates LC3 conjugation. As a final step, when the autophagosome fuses with the

lysosome, LC3 becomes delipidated at the membrane surface by ATG4B or is degraded within the autolysosome by proteolytic activity. Lastly, very important role play specific adaptor molecules, including p62, that have been shown to bind to proteins or organelles promoting their selective degradation. The adaptor molecules are not required for autophagosomal formation, but represent specific substrates and recognition molecules for autophagy (22, 24, 25).

1.3. Mitochondria and Mitophagy

Mitochondria are essential organelles found in almost all eukaryotic cells, participating in many functions in the cell. Their main role is that of energy control and is achieved through oxidative respiratory phosphorylation. Reactive oxygen species (ROS) and reactive nitrogen species (RNS)such as O_2^- and H_2O_2can be released as byproducts by the mitochondrial electron-transport chain (mETC), where continuous electron leakage to O_2 occurs during aerobic respiration (27). Mitochondria are both generators and targets of oxygen/ nitrogen reactive species (28). ROS/RNS formation is part of normal cellular physiology however abnormal ROS/RNS production and accumulation result in oxidative stress, which is linked to mitochondrial dysfunction. Mitochondrial proteins themselves are primarily subjected to oxidative stress, whereas mitochondrial proteome imbalance causes further exacerbation of oxidative stress. Oxidative modifications cause reversible or irreversible damage to proteins. Reversible modifications occur in the sulfur-containing amino acids, cysteine and methionine. Mitochondria have a limited ability to repair some of the oxidized proteins using the antioxidant systems (thioredoxin/ thioredoxin reductase, glutaredoxin/ glutathione/ glutathione reductase and methionine sulfoxide reductase). Oxidatively damaged proteins may lose their proper tertiary structure and potentially form toxic aggregates within the organelle, leading to mitochondrial dysfunction. Cellular function and survival depends on the efficient clearance of the dysfunctional mitochondria, which can trigger cell death pathways (Figure 11) (29). Thus, the constant turnover and replacement of mitochondria is of great necessity, and the balance between mitochondrial biogenesis and degradation is critical for the elimination of oxidative damage and for the cellular homeostasis(30). Proliferator-activated receptor γcoactivator-1 (PGC-1a) is considered as the master regulator of bioenergetic functions and mitochondrial biogenesis (28). Akt3 also represents a key factor required for mitochondrial biogenesis, maybe through the regulation of the subcellular distribution of PGC-1a, and it has been proposed that Akt3 might inhibit autophagy in endothelial cells (31). Mitophagy is an individual form of autophagy and is defined as the selective digestion of mitochondria (32). Mitophagy is the major mechanism for the removal of the dysfunctional mitochondria featuring its important role in cellular homeostasis (28)(29). The impact of mitophagy is especially relevant to degenerative diseases in which the

occurrence of oxidative stress and the dysfunction in autophagy and mitophagy is well documented (26)(29)(33)(34). Autophagy plays an important role in both sensing oxidative stress and removing oxidatively damaged proteins and organelles, as well as the cellular machineries responsible for excessive ROS/RNS production. The crosstalk between autophagy, redox signaling and mitochondrial dysfunction is not well defined yet (27)(35). The accumulation of toxic proteins and the decrease in mitochondrial function may lead to further oxidative stress when the autophagic process is disrupted. Deregulated redox signaling or mitochondrial dysfunction can also influence autophagic activities (36).

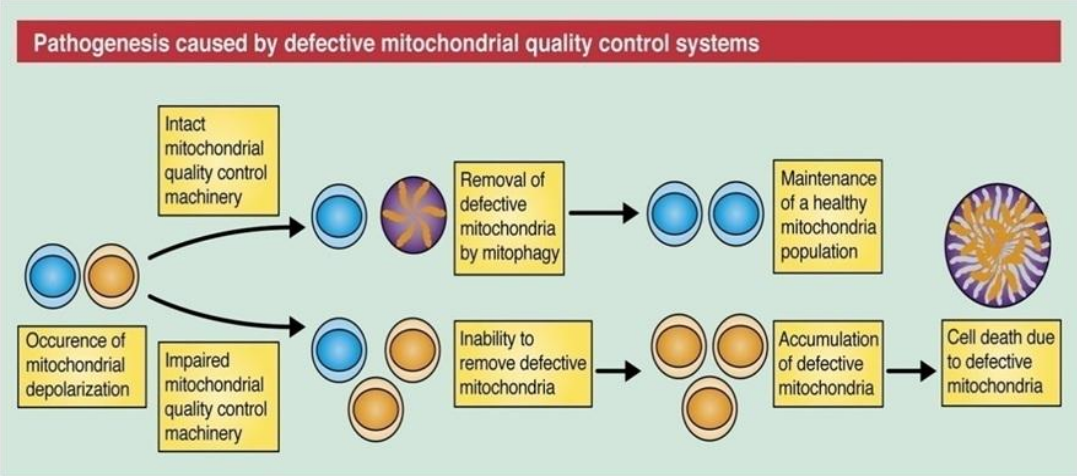


Figure 11. Defective mitophagy leads to cell damage (29).

Additionally, mitochondrial remodeling through fission, fusion or mitophagy is important for mitochondrial homoeostasis (32). Mitochondria dynamically undergo a fission-fusion cycle with their morphology to vary accordingly. Mitofusin 1 and 2 (Mfn-1,-2), and Optic atrophy 1 (Opa1) seem to be major regulators of mitochondrial fusion, whereas the dynamin related protein 1(Drp1) and Mitochondrial Fission protein 1 (Fis1) play an important role in fission. Maintenance of the balance between fission and fusion is important for mitochondrial function (28).

Mitophagy is controlled either under mechanisms of general macroautophagy or selectively through specific mitophagy genes. The selectivity of mitophagy is regulated by a variety of proteins, including PINK1 [PTEN (phosphatase and tensin homologue deleted on chromosome 10)-induced kinase 1], Parkin (cytosolic E3 ubiquitin ligase) and BNIP3 (Bcl-2/adenovirus E18 19-kDa-interacting protein), as well as the processes of mitochondrial fission and fusion (37). It is proposed that PINK1 functions as a mitochondrial stress sensor via its import and degradation, which is dependent on the mitochondrial membrane potential. In healthy mitochondria, PINK1 is imported into mitochondria and rapidly undergoes

degradation at the inner mitochondrial membrane. However, in response to mitochondrial damage and mitochondrial membrane depolarization, PINK1 becomes stabilized on the outer mitochondrial membrane and induces Parkin translocation to mitochondria. Following recruitment, Parkin ubiquitylates several outer mitochondrial membrane proteins, such as voltage-dependent anion channel proteins (VDAC) resulting in the recruitment of the autophagy receptor p62 and finally the degradation by the autophagic machinery (Figure 12) (26). Furthermore, BNIP3 is localized to the outer mitochondrial membrane and acts as receptor for targeting autophagosomes to mitochondria.BNIP3 induces the translocation of Drp1 protein to mitochondria resulting in mitochondrial fission, which then activates Parkin-dependent mitophagy (38).

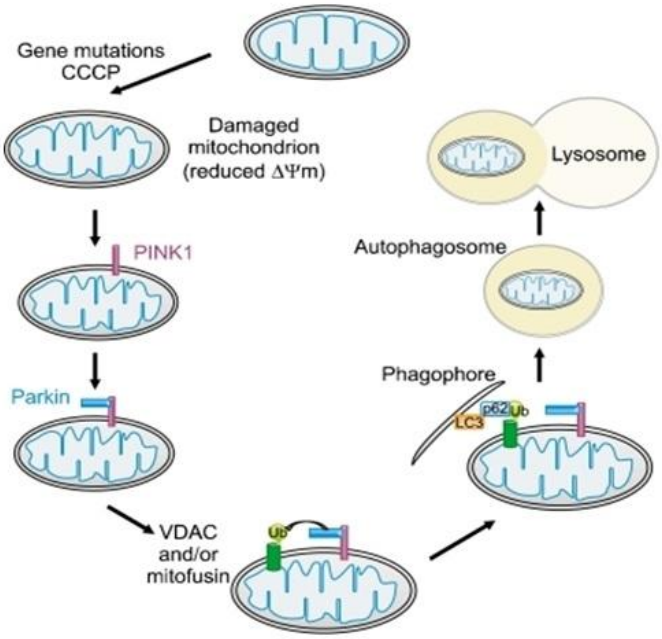


Figure 12. PINK1-parkin pathway upon mitochondrial damage (26).

1.4. Implications of Autophagy and Mitophagy in Lung Diseases

There is evidence implying that autophagy and dysfunction of mitochondria may be involved in the pathogenesis of IPF. Oxidative stress, endoplasmic reticulum stress and hypoxia are not only mechanisms implicated in the pathogenesis of IPF, but they are also inducers of autophagy(39). A study by Patel and colleagues examined markers of autophagic activity (LC3 and p62) in human IPF lungs and the number of autophagosomes and it was detected that autophagy is not induced in human IPF lungs. The suggested mechanism is the TGFβ1 induced activation of PI3K and mTOR, which inhibits autophagy (40). However, it is more likely that autophagy deregulation in IPF is cell specific. Another study claimed that there is

insufficient autophagy in alveolar epithelial cells in IPF lungs, which may lead to epithelial cell senescence, whereas in fibroblasts insufficient autophagy may induce differentiation to myofibroblasts. However, this study proposed that TGFβ1 enhances autophagy in lung fibroblasts (41). Bueno et al recently proposed that there may be an induction of the autophagy process in alveolar epithelial cells (AECs) in IPF, but finally the autophagy flux is impaired. They also detected a decreased expression of the major regulatory molecule of mitophagy, PINK1, in AECs from IPF patients, but interestingly there was no difference in fibroblasts (42).

Dysfunction of mitochondria has been already proposed in lung diseases. Recent studies showed altered mitochondrial structure (elongation) and function in alveolar epithelial cells (43), and in primary bronchial epithelial cells (44) upon cigarette smoke exposure (CSE).In contrast, Hara et al demonstrated mitochondrial fragmentation in primary human bronchial epithelial cells treated with cigarette smoke extract for 48 hours (45).Increased mitochondrial fragmentation and greater mitochondrial fission in airway smooth muscle cells isolated from patients with clinically diagnosed moderate asthma relative to cells from non-asthmatic and nonsmoker patients after CSE has been also reported(46). It has been suggested that while mitochondria initially adapt to cigarette smoke and oxidative stress by altering their structure, this may be not beneficial in the long term due to decrease in mitophagy (43). Recently, Ahmad et al proposed smoke-induced cellular senescence due to impaired mitophagy (47). In alveolar macrophages from COPD patients there seems to be defective autophagy and blockage of clearance of damaged and dysfunctional mitochondria (48).

A recent study indicated the importance of mitochondria in IPF. It was shown that LYCAT (lysocardiolipin acyltransferase, a cardiolipin-remodeling enzyme) expression is significantly altered in peripheral blood mononuclear cells and lung tissues from patients with IPF. LYCAT expression in peripheral blood mononuclear cells directly and significantly correlated with pulmonary function outcomes and overall survival. Using animal models of IPF, they showed that LYCAT overexpression reduced several indices of lung fibrosis; conversely, down-regulation of LYCAT expression enhanced fibrogenesis. It was suggested that this LYCAT- mediated lung protection may involve suppression of mitochondrial ROS generation and apoptosis of alveolar epithelial cells (49). Furthermore, Bueno and colleagues claimed that reduction in PINK1 expression leads to mitochondrial damage and enhances lung fibrosis (42). A more recent study by Patel et al indicated damaged mitochondria in IPF lungs and mitochondrial depolarization in TGFβ1 treated lung epithelial cells (50).

IPF is now considered a disease of premature aging (51-53). It is proposed that changes related with aging, such as oxidative stress, mitochondrial dysfunction, modifications of ECM among others, may be responsible for the fibrotic phenotype (54, 55). Extensive oxidative damage has been linked with premature aging and development of age-related diseases (56,

57). During aging, proteins and cellular debris are ineffectively cleared and accumulate in the cell. Accelerated accumulation of damage under conditions of environmental and genetic stress has been associated with short lifespan and incidence of age-related phenotypes. Recent data showed that PINK1 deficiency related with aging may lead to defective mitophagy in alveolar epithelial cells and fibrosis in the aging lung (42). Mitochondrial turnover is dependent on autophagy, which declines with age, due to insufficient formation of autophagosomes or to deficient elimination after fusion with lysosomes (56)(58). Such a defect in cellular housekeeping mechanisms leads to senescence or apoptosis. On the other hand, insufficient autophagy in lung fibroblasts may results in their differentiation into myofibroblasts without any effect on their senescence, and to increased production of extracellular matrix which are critical steps in the fibrogenetic process (41).

1.5. Rational and aim of the study

Although the current hypothesis on IPF pathogenesis emphasizes the role of alveolar epithelial cells, recent studies re-establish the significant role of immune cells such as monocytes and macrophages in IPF (13, 15). Macrophages are the major cell component of the alveolar space and are key cells for the removal of pathogens and debris.

Autophagy is a vital process for the cellular homeostasis and regulates the intracellular turnover of proteins and organelles. It is a cell-specific process , thus it is important to evaluate this mechanism in each cell subtype. Regarding the autophagy process in macrophages in fibrosis little is known. Interestingly, a study by Drakopanagiotakis and colleagues identified that alveolar macrophages of patients with IPF have a decreased apoptotic rate, which may enhance the progression of IPF (59). In addition, it has been shown that there is an autophagy defect in alveolar macrophages of smokers resulting in mitochondrial dysfunction and damage (48). Mitochondrial dysfunction seems to be associated with the induction of fibrosis and recent evidence suggests that defective mitophagy is related with smoking and hypoxia (43-45, 60).

The purpose of our study is therefore to evaluate the autophagy with an emphasis in mitophagy and oxidationstatus in macrophagesfrom patients with IPFusing the Bronchoalveolar Lavage Fluid (BALF) derived cells in comparison with control subjects and patients with Rheumatoid Arthritis (RA) and lung involvement (ILD-interstitial lung disease) since RA-ILD and IPF present some rather intriguing similarities at the clinical and molecular level (61-63).

2. Materials and Methods

2.1. Human Subjects

IPF patients: The diagnosis of IPF was based on open or video-assisted thoracoscopic biopsy, with all biopsies reviewed by the same two histopathologists, or using ATS/ERS clinical and HRCT criteria (64). In accordance with the aforementioned criteria any patient presenting any known cause of pulmonary fibrosis, such as a systemic connective tissue disorder, was excluded from this study using both immunologic screening and rheumatologic clinical evaluation. All IPF patients were newly diagnosed and had not received previous treatment.

RA-ILD patients: Criteria for the diagnosis of CTD included the American College of Rheumatology (ACR) 1987 revised criteria for the classification of rheumatoid arthritis (RA) (65); Patients with RA-ILD had HRCT findings indicative of definite interstitial lung disease.

Control patients: The control subjects were patients undergoing bronchoscopy for the investigation of haemoptysis, without any overt pulmonary comorbidities and with normal bronchoscopic findings and cytology results.

2.2. BALF cell isolation

Bronchoalveolar Lavage Fluid (BALF) was obtained from all patients at room temperature. Briefly, a flexible bronchoscope was wedged into a sub-segmental bronchus of a predetermined region of interest based on radiographical findings. A BAL technique was performed by instilling a total of 180 ml of normal saline in 60-mL aliquots, each retrieved by low suction.

The BALF samples were subsequently kept on ice and were processed within 2 hours of collection. Samples were filtered through sterile 70nm cell strainers (BD) and centrifuged at 1500rpm for 5 minutes at 4^{o}C. Cell pellets were washed and re-suspended with cold PBS. Total cell count and cell viability were subsequently assessed using Trypan blue (ICN).

1-1.5 million cells were centrifuged and cell pellets were homogenised in TriReagent^{TM} (MBL) for total RNA, or RIPA buffer (Invitrogen) containing protease and phosphatase inhibitors, Pierce) for SDS-PAGE/Western blot analysis, followed by storage at -80^{o}C. 0.5 million cells were kept in RPMI-1640supplemeted with 2% FCS for subsequent MitoSOX staining and Flow Cytometry analysis.

Differential cell population count was analysed following May-Grunewald-Giemsa staining.

2.3. BALF cell culture and treatments

0.5 million cells were cultured O/N in RPMI1640 (Biosera) supplemented with 2% FCS (Biosera) and 10% penicillin-streptomycin in a humidified incubator at 37^{o}C containing 5% CO_2.

2.4. RNA extraction and mRNA expression

Total RNA was isolated using the mirVana^{TM} miRNA isolation kit (Ambion) with minor modifications. 350μl of TriReagent was used for cell lysis and storage of samples, followed by addition of 350μl of mirVana cell lysis solution and 35μl of mirVana miRNA additive. All further steps for the isolation of total RNA were performed as recommended by the manufacturer. Quality and quantity of isolated RNA was assessed by agarose gel electrophoresis and spectrophotometry (Nanodrop) respectively. For gene expression analyses, 500ng of total RNA were first treated with DNAfree (Ambion) in order to remove genomic DNA contamination, followed by 1st strand cDNA synthesis using Maxima RT ^{TM} (Fermentas) and real time qPCR analysis using Maxima SYBR Green pPCRmix (Fermentas) on a Mx3005P qPCR system (Agilent Technologies).

Probe and primer sequences are summarized in Table 1. GAPDH levels were used as endogenous control for the normalization of mRNA expression levels in BALF samples.

Table 1. The sequences of primers used.

Gene Name	**Primer Sequences**
GAPDH	F: agccacatcgctcagaca
	R: ccaatacgaccaaatccgtt
Beclin1	F: tcaccatccaggaactcaca
	R: tggctcctctcctgagttagtc
p62	F: agctgccttgtacccacatc
	R: cagagaagcccatggacag
Akt3	F: ttgctttcagggctcttgat
	R: cataatttcttttgcatcatctgg
PINK1	F: ggagtatggagcagtcacttacag
	R: ggcagcacatcagggtagtc
ULK1	F: gccctcgtacccaagctc
	R: gaggccagggtcttctgc
BNIP3	F: tgctgctctctcatttgctg
	R:gactccagttcttcatcaaaaggt

Parkin	F: cgaccctcaacttggctact
	R:tctttaatcaaggagttgggaca
S100A9	F: gtgcgaaaagatctgcaaaa
	R: tcagctgcttgtctgcattt

2.5. Western blot analysis

Total protein lysates (20ng) of BALF samples were separated in 12% SDS-PAGE, transferred to 0.45nm nitrocellulose membrane (Biorad), followed by detection of p62 with an anti-p62 mouse monoclonal antibody (MBL), LC3A using an anti-LC3Amouse monoclonal antibody (Abgent) and b-actin using an anti-b-actin mouse monoclonal antibody (Sigma).Appropriate anti-mouse HRP conjugated secondary antibody (Chemicon) was used and immunodetection was performed with enhanced chemiluminescence reagent Luminata™ (Millipore). Bands were visualised with the ChemiDoc XRS+ system (Biorad) and densitometry analyses were performed using Image Lab ™ software (Biorad).

2.6. MitoSOX treatment – Flow Cytometric Analysis

Mitochondrial ROS was measured by MitoSOXRed (Invitrogen) staining. Cells were stained with MitoSOX Red (5 μM) for 10 minutes at 37^{o}C. To evaluate cell viability, we used Propidium Iodide (PI) Red 1μl/ml, 1minutebefore performing the flow cytometry, for the detection of necrotic/apoptotic cells. Data were acquired with Beckman Coulter flow cytometer and analyzed with FlowJo 8.7 (Treestar, Ashland, OR).

2.7. THP-1 cell culture and treatments

THP-1 cells were cultured in RPMI1640 (Biosera) supplemented with 10%FCS (Biosera) and 1% penicillin-streptomycin in a humidified incubator at 37^{o}C containing 5% CO_2. Prior to experiments, 2 millions cells were treated with phorbol-12-myristate-13-acetate (PMA) for six hours at a final concentration of 100ng/ml, followed by addition of TGFβ11 at final concentration of 1-5-10 ng/ml, LPS 1μg/ml or 0.1μg/ml for 24 hours and ATP at final concentration of 5μM for 60 minutes. The cells were detached using PBS-10mM EDTA and either stained with MitoSOX, or lysed in TriReagent™ (MBL) for total RNA extraction.

2.8. Immunofluorescence

0.2 million cells overlaid on glass slides using Cytospin were fixed with 4% formaldehyde for 5 minutes followed by washing with PBS. Permeabilisation buffer (0.5%FBS, 0.2%Triton in TBS) was added for 10 minutes followed by blocking buffer (0.5%FBS, 0.1%Triton, 2mg/ml BSA in TBS) for 10 minutes at room temperature. Primary antibody incubations were carried

out for 60 minutes at room temperature followed by washing with TBS. Secondary antibody incubations were performed for 30 minutes followed by washing with TBS. Topro was added for nuclear staining. Finally, TrueBlack (Biotium) was added for 30 seconds so as to eliminate autofluorescence signal due to intracellular lipofuscin granules. Images were taken by Leica confocal microscope.

2.9. Statistical analysis

Gene expression analysis was performed using Prism 5 software following incorporation of relative expression values in average (duplicates) normalized by GAPDH or 18s. Relative expression values for the patient cohort were calculated by the equation:

Relative Gene Expression=

Effgoi^(`Calibrator Ctgoi – SampleCtgoi) / Effref^(Calibrator Ctref – SampleCtref)

(Eff: efficiency, goi: gene of interest, ref: reference gene)

Gene expression analysis for THP1 treatments was calculated by the equation:

Relative Gene Expression=

Effgoi^(NTCtgoi – TreatedCtgoi) / Effref^(NTCtref – TreatedCtref)

(NT: non treated cells)

Group comparisons were made by *t* test, Mann-Whitney test, Wilcoxon test, or One-way ANOVA or Kruskal-Wallis test, as appropriate. A *P* value less than 0.05 was considered statistically significant.

3.Results

3.1. Human Samples

Bronchoalveolar Lavage Fluid (BALF) cells from a cohort of ninety-eight subjects, that underwent bronchoscopy was analysed retrospectively, consisting of previously characterised patients with IPF (n=55), RA-ILD (n=20), and healthy control subjects (n=23).

Patients were classified as current smokers, former smokers (defined as having smoked a minimum of one cigarette a day for a minimum of 1 year, stopping at least 6 months before presentation) or non-smokers.

Some differences regarding the smoking status of the three groups were observed. The IPF group contained less current and former smokers relative to the control group (Table 2). Additionally, RA-ILD group included more current smokers when compared with IPF (Table 2).

Table 2. Patients' characteristics. Fisher's exact test. p1: controls versus IPF, p2: controls versus RA-ILD, p3: IPF versus RA-ILD.

Characteristics	Controls	IPF	RA-ILD	p value
Number	23	55	20	
Gender (male/female)	18/5	47/8	13/7	p1.p2,p3 ns
Non smokers	1	16	3	p1,p2,p3 ns
Former smokers	3	29	4	p1<0.05, p2 ns, p3<0.05
Current smokers	11	9	10	p1<0.001, p2 ns , p3<0.01

Analysis of BALF cellular composition showed no significant differences in the populations of macrophages, lymphocytes and neutrophils among the three groups (Table 3). In contrast the percentage of eosinophils was significantly higher in the IPF group relative to controls (p: 0.002) and RA-ILD (p: 0.039).

Table 3. BALF cell population analysis. Mann Whitney test. Values represented as means±SD. p1: controls versus IPF, p2: controls versus RA-ILD, p3: IPF versus RA-ILD.

BALF cell populations	Controls	IPF	RA-ILD	p value
Macrophages	80,41 ±19,37	77,34 ± 15,65	80,26 ±12,69	p1, p2, p3 ns
Lymphocytes	13,94 ±18,99	11,43 ±16,74	12,71 ±12,92	p1, p2, p3 ns
Neutrophils	3,656 ±4,225	6,693 ±6,884	3,520 ±2,651	p1, p2, p3 ns
Eosinophils	0,5508 ± 1,089	3,279 ± 3,519	1,211 ± 1,201	p1=0.0002 p2= 0.06 p3 = 0.039

3.2. Autophagy status in IPF macrophages

Recent evidence suggests a significant role of autophagy in lung diseases. Particularly in IPF it has been proposed that an inhibition of autophagy in epithelial cells may be a trigger of increased apoptosis. To the best of our knowledge, analysis of autophagy in IPF macrophages has not been reported. Therefore it is of great importance to evaluate the status of macrophages, since a deregulation of autophagy could affect their function and may participate in the disease pathogenesis. For the assessment of autophagy in BALF cells of IPF

patients we measured the protein levels of LC3 and the adaptor protein p62 in a subset of samples (10 controls, 15 IPF, 8 RA-ILD). Commonly, increased levels of LC3 correlate with increased number of autophagic vesicles, whereas increased levels of p62 are associated with a defect in autophagy mechanism, due to the accumulation of aggregated proteins or damaged organelles that interact with p62.

We observed a statistically significant increase of LC3A protein levels in RA-ILD patients in comparison to the controls (p: 0.0176) (Figure 13A). In addition in IPF patients there was a trend of higher levels of LC3 versus controls, although this was not statistically significant (Figure 13 A, C). P62 in IPF tended to be lower than in controls (p: 0.0782) while in RA-ILD patients p62 levels were higher relative to IPF (p: 0.0426) (Figure 13 B, C). Thus, our results indicate an important difference in the levels of key autophagy protein markers in RA-ILD and IPF patients' BALF cells relative to controls. Although further evaluation of LC3B, LC3 isoforms I and II and autophagic vesicle quantification are required for an assessment of autophagy levels, our results may indicate a different state of autophagy activation in BALF cells from IPF and RA-ILD patients.

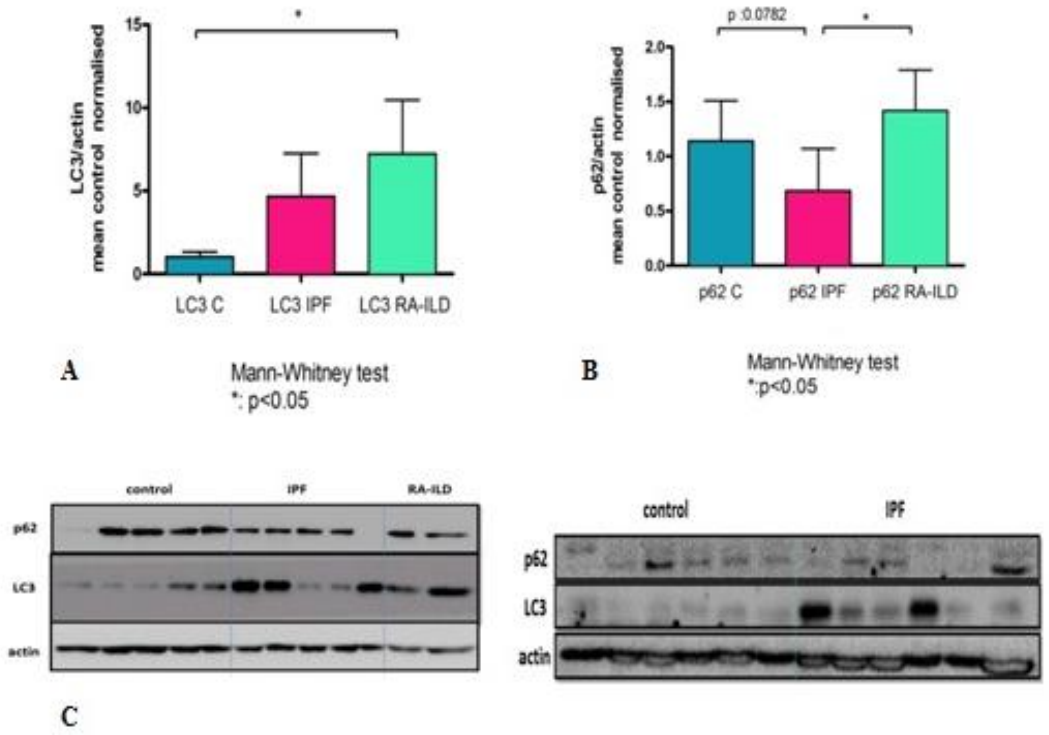


Figure 13. A. Densitometry. Quantification of LC3 protein levels in controls, IPF and RA-ILD patients. Protein levels were normalised to b-actin. **B.** Densitometry. Quantification of p62 protein levels in BALF cells. **C**. Representative immunoblots of LC3 and p62 in protein lysates of BALF cells.

Furthermore, we examined the mRNA levels of genes that encode proteins involved in the regulation of autophagy. Expression of ULK1 and Beclin1, key molecules for the initiation of autophagy showed no difference between IPF and controls (Figure 14 A, B). Interestingly, Beclin1 mRNA levels were significantly upregulated in the RA-ILD group compared to the IPF group ($p<0.05$) (Figure 14 B). P62 mRNA in contrast to protein levels was not differentially expressed among the three groups (Figure 14 C). We also examined S100A9, a calcium-binding protein previously described as a potent biomarker in BALF that may help differentiate IPF from other idiopathic interstitial pneumonias and maybe involved in the induction of autophagy (35, 66, 67). However, we did not detect differences in S100A9 mRNA levels between the three groups (Figure 14 D).

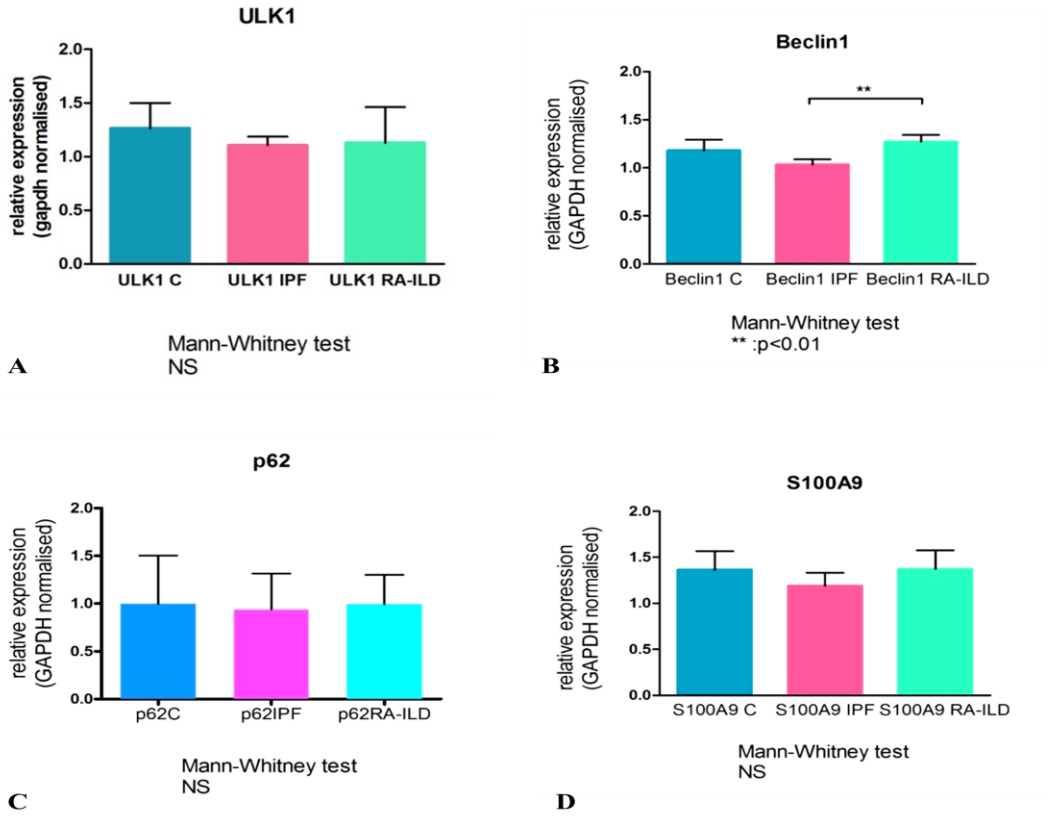


Figure 14. RT-PCR results; m RNA expression levels of A. ULK1, B. Beclin1, C. p62 and D. S100A9, normalised to gapdh, in controls, IPF and RA-ILD patients.

3.3. Mitochondrial homeostasis in IPF macrophages

Mitochondria are major organelles in cell metabolism, and their homeostasis, which is defined by the balance of biogenesis and turnover, is essential for the cell and therefore highly regulated. The role of mitochondrial damage and dysfunction has been recently described in lung diseases. In IPF it has been reported that mitochondrial damage in epithelial cells may be

implicated in the development of the disease. Therefore, we focused on the evaluation of mitochondrial homeostasis in BALF cells in IPF patients. Initially, we assessed the mRNA levels of several genes involved in mitochondrial homeostasis including PINK1, Parkin, BNIP3 and AKT3.

PINK1 is the major molecule that is associated with mitochondrial damage and promotes mitophagy. We found a statistically significant downregulation of PINK1 mRNA in IPF and in RA-ILD patients relative to controls (Figure 15A). Additionally, we identified strong correlations of the expression of PINK1 with the expression of ULK1, Beclin1 and p62 in IPF patients (Table 4) (Figure 16). Interestingly, there was no correlation of PINK1 with Beclin1 in RA-ILD patients, in accordance with our previous finding of the upregulation of Beclin1protein levels in these patients (Table 4).

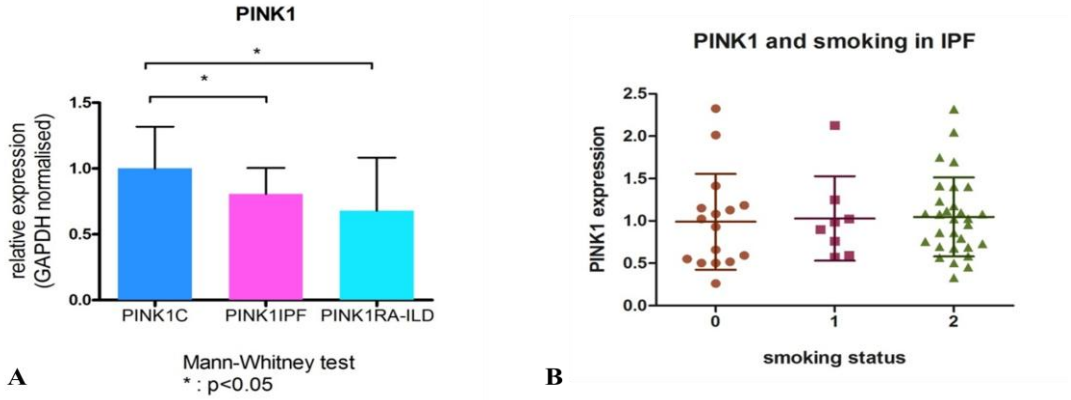


Figure 15. A. mRNA expression levels of PINK1 in patients. **B.** Correlation analysis of PINK1 expression levels with smoking status in IPF patients. 0: non smokers, 1: current smokers, 2: ex-smokers.

Table 4. Correlations of PINK1 with Beclin1, ULK1 and p62 m RNA levels in IPF and RA-ILD patients.

Correlations of PINK1 expression				
	IPF		RA-ILD	
genes	spearman r	p value	spearman r	p value
Beclin1	0,86	<0,0001	0,22	<0,001
ULK1	0,65	<0,0001	0,71	<0,001
p62	0,75	<0,0001	0,31	0,18

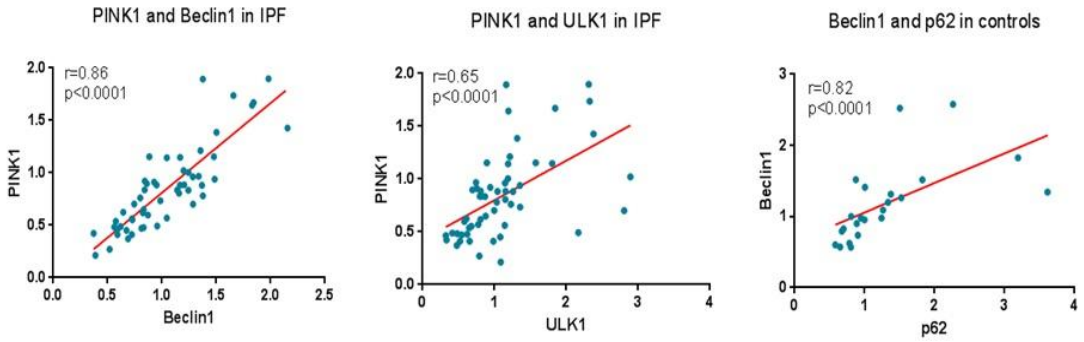


Figure 16. Correlations of PINK1 with Beclin1, ULK1 and p62 mRNA levels in IPF patients.

Parkin and BNIP3 were not significantly altered among the three groups (Figured 17 A, B); however we detected strong correlation between parkin and ULK1 and PINK1expression (Table 5).

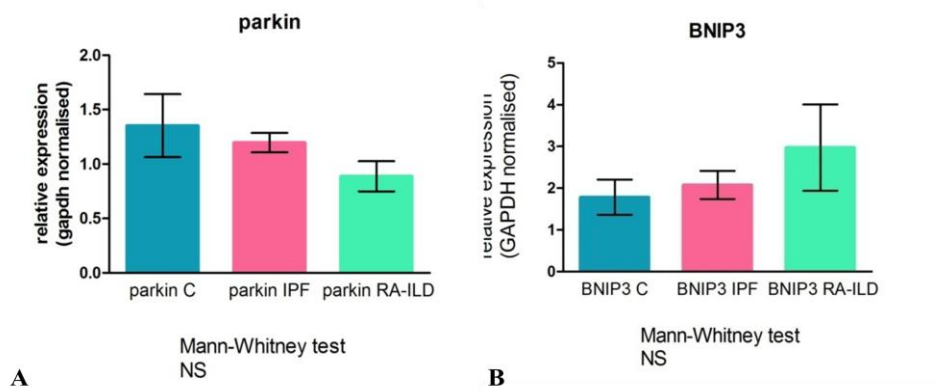


Figure 17. A. Parkin expression levels by RT-PCR. **B.** BNIP3 expression levels by RT-PCR.

Table 5. Correlations of parkin expression levels and PINK1, Beclin1 and ULK1 in IPF and RA-ILD patients.

Correlations of parkin expression				
	IPF		RA-ILD	
genes	spearman r	p value	spearman r	p value
PINK1	0,50	<0,0001	0,82	<0,0001
Beclin1	0,55	<0,0001	0,14	0,55
ULK1	0,66	<0,001	0,68	<0,001

Next we examined AKT3 levels, since this kinase participates in the de novo mitochondrial biogenesis, and we found a significant upregulation in the RA-ILD group compared with IPF

(Figure 18A). AKT3expression was strongly positively correlated withPINK1 in IPF patients, but not in RA-ILD (Figure 18B).

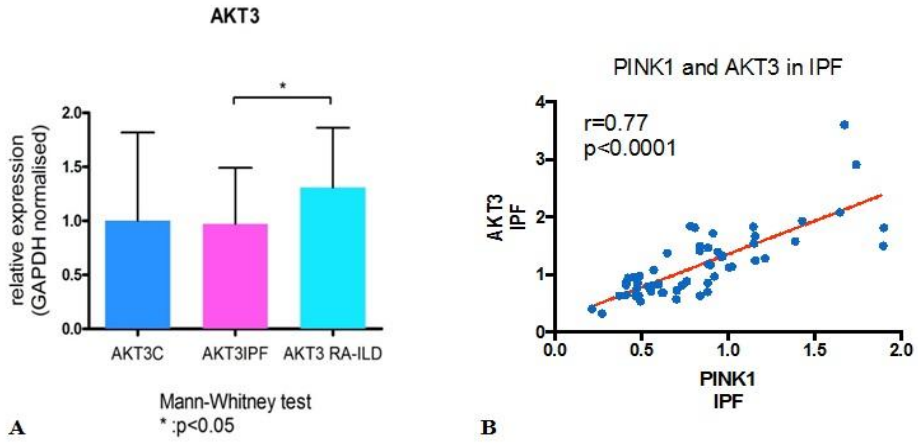


Figure 18. A. RT-PCR results of AKT3 mRNA levels. **B.** Correlation of PINK1 and AKT3 expression levels in IPF patients.

Noticeably, although smoking has been involved in mitochondrial dysfunction and mitophagy impairment, we found no correlation with smoking status in the above gene expression analysis (Figure 15B).

Overall, we observed a significant downregulation of PINK1 in IPF and RA-ILD patients relative to control, and an upregulation of AKT3 only in RA-ILD suggesting again differential regulation of the pathways of mitochondria biogenesis and turnover in the two ILD groups and relative to controls.

3.4. Oxidation status of mitochondria

3.4.1. Evaluation of MitoSOX Red staining under oxidizing conditions in A549 epithelial cells and THP1 monocytes.

Next we sought to evaluate the oxidation status of mitochondria. To this end we used MitoSOX Red, a mitochondrial superoxide indicator which is a live-cell permeant fluorogenic dye highly selective for the detection of oxidized mitochondria in live cells (68, 69).

Initially, we assessed the staining of oxidized mitochondria with MitoSOX following treatment with hydrogen peroxide (H_2O_2). We treated A549 a non-small cell lung cancer (NSCLC)-derived human alveolar epithelial cell line with 1000μM H_2O_2.Using confocal microscopy the characteristic mitochondrial network/web staining in the cytoplasm was observed which was enhanced upon H_20_2 treatment (Figure 19).

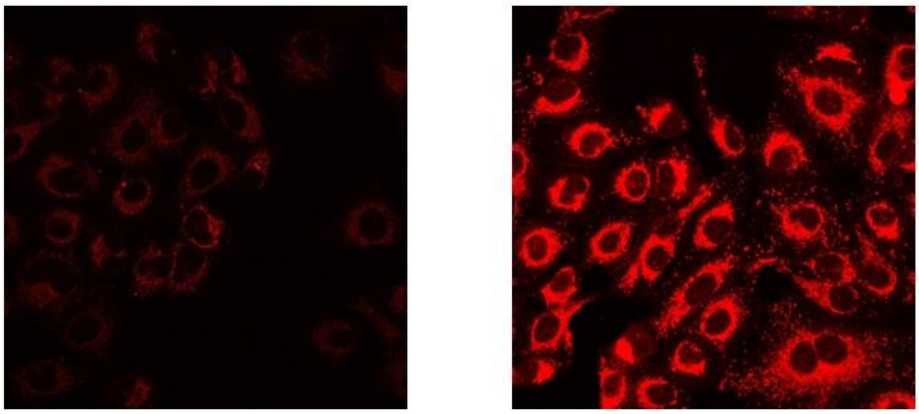


Figure 19. A. MitoSOX Red staining of non treated A549 epithelial cells. **B.** MitoSOX Red staining of A549 cells treated with 1000μ M H_20_2.

Next, we evaluated MitoSOX Red staining of THP1 human monocytes by flow cytometry analysis. We tested two MitoSOX Red concentrations; 0.5μM and 5μM, and a strong fluorescent signal was observed at concentration of 5μM which was used hereafter (Figure 20A). In addition, similarly to the A549 cells, we measured the levels of MitoSOX Red staining in THP1 cells following treatment with 1000μM H_2O_2. In parallel, since necrotic and apoptotic cells are expected to contain oxidized mitochondria whileH_2O_2treatment is known to induce apoptosis, we have stained the cells with Propidium Iodide (PI) (Figure 20B) which allowed for the exclusion of the MitoSOX Red positive necrotic/apoptotic population from the analysis of the alive MitoSOX Red positive cells (Figure 20C). A small percentage of PI positive cells was still among the analysed population and it was decided to subtract this percentage of PI positive cells from the MitoSOX Red positive population for the quantification of MitoSOX Red percentage for the rest of our analyses. Hence focusing on the cells that were PI negative we observed a high proportion of MitoSOX positive cells upon H_2O_2treatment (Figure 20D).

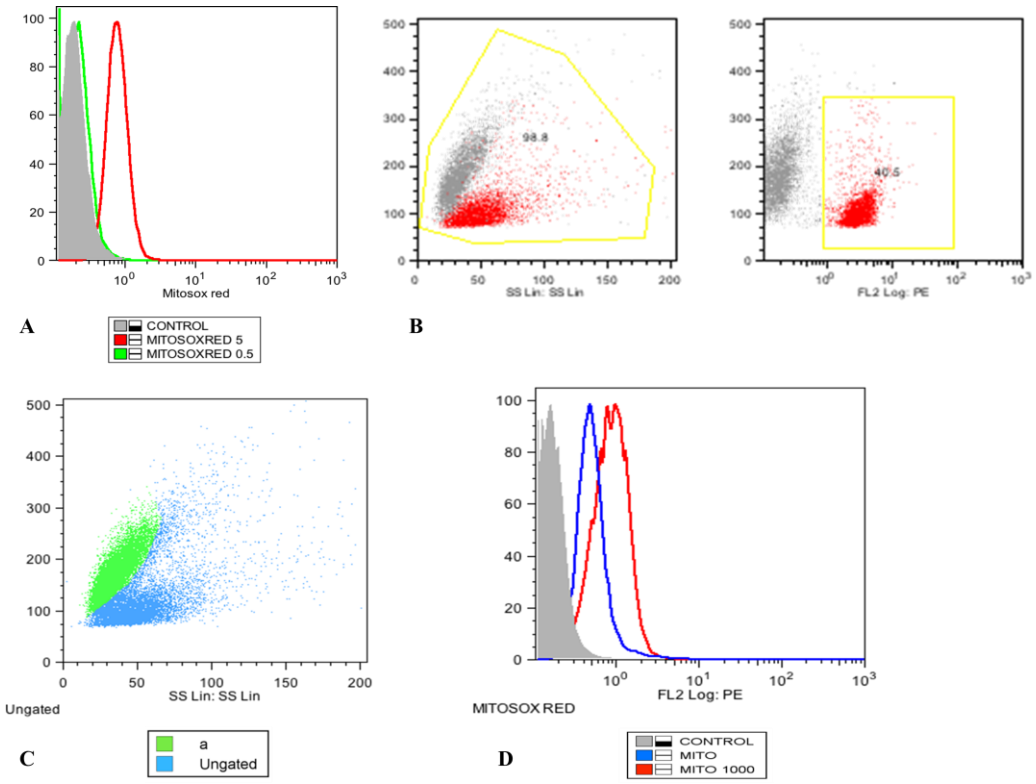


Figure 20. A. Histogram of flow cytometry analysis. THP1 cells stained with MitoSOX Red at final concentrations of 0.5μ M and 5μ M. **Figure B**. THP1 cells treated with 1000 μ M H_2O_2. PI staining detecting the necrotic/apoptotic cell population. **C.** MitoSOX staining (5μ M) in THP1 cells treated with 1000 μ M H_2O_2 . Analysis in the gated population of the PI negative cells. **D.** MitoSOX staining (5 μM) results of THP1 cells treated with 1000μ M H_20_2 in the PI negative population. Histogram of the gate a of Figure C.

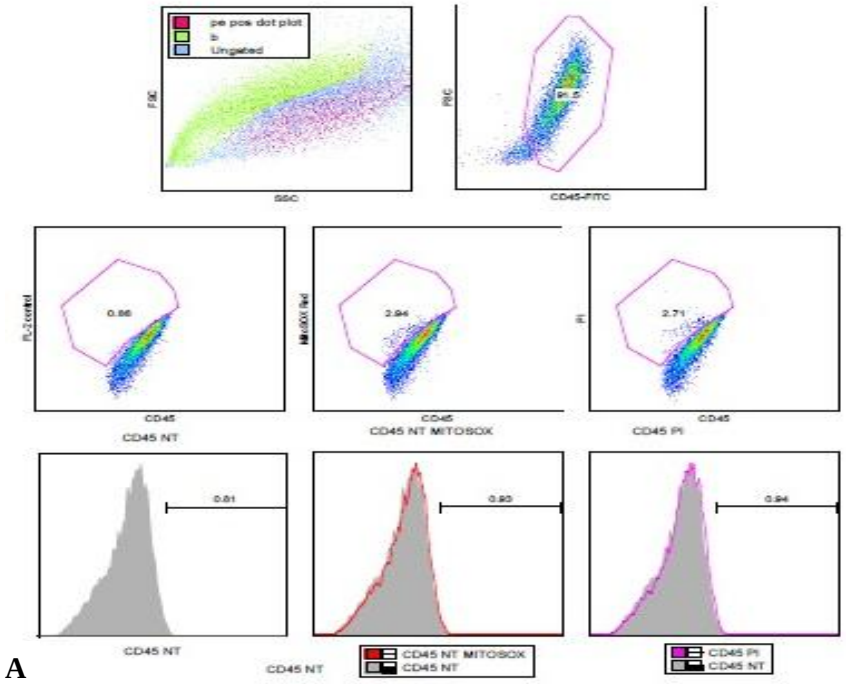
3.4.2. Oxidation level of mitochondria in BALF cells

Our next goal was to estimate the presence of oxidized mitochondria in live BALF cells from IPF and other ILDs relative to control samples. To this end, a second cohort of consecutively recruited patients for bronchoscopy and BAL was also included in our study. Provisional diagnoses for this group are summarised in Table 6.

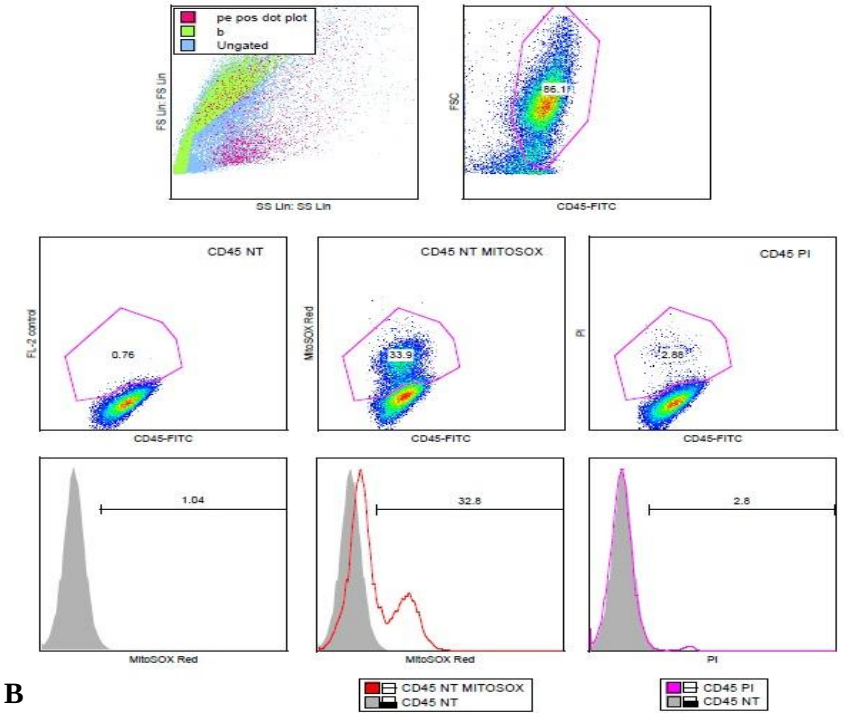
Table 6. The characteristics of patients recruited for fresh BALF cells analysis. Preliminary data with provisional diagnosis.

Characteristics	Controls	IPF	ILDs	lung Ca
Number	4	4	5	2
Gender (male/female)	4/0	4/0	2/3	2/0
Smoking status (non/ former/current)	0/2/2	1/2/1	3/0/2	0/0/1

Freshly isolated BALF cells were stained with CD45-FITC pan-leucocyte marker and MitoSOX Red or PI. Following exclusion of the majority of PI positive cells, the population of CD45 positive cells was evaluated for MitoSOX Red staining. Examples of positive and negative MitoSOX Red samples are shown in Figure 21. During the analysis of our flow cytometry results, we observed that two parameters could be evaluated, the MitoSOX red percentage and a small but distinct shift in the total CD45 positive population (Figure 21).



A



B

Figure 21. Representative images of flow cytometry analysis (histograms and dot plots). BALF cells were stained with CD45-FITC for the evaluation of MitoSOX Red and PI positive cells in CD45 positive cell population. **A.** Control sample with 91.5% CD45 positive cells, out of which there are 2.94% MitoSOX positive cells, and 2.71% PI positive. In histogram there is no shift in MitoSOX or PI stained cells. **B.** IPF patient with 86.1% CD45 positive cells, out of which there are 33.9% MitoSOX positive and 2.88% PI positive cells. It is also demonstrated a strong shift in MitoSOX stained cells compared with non treated or PI stained.

MitoSOX staining of the BALF cells was verified by confocal microscopy, where strongly positive cells lacking strong CD45 staining were identified as dead cells while double positive CD45 and MitoSOX Red cells were confirmed as the population of BAL cells containing oxidized mitochondria (Figure 22).

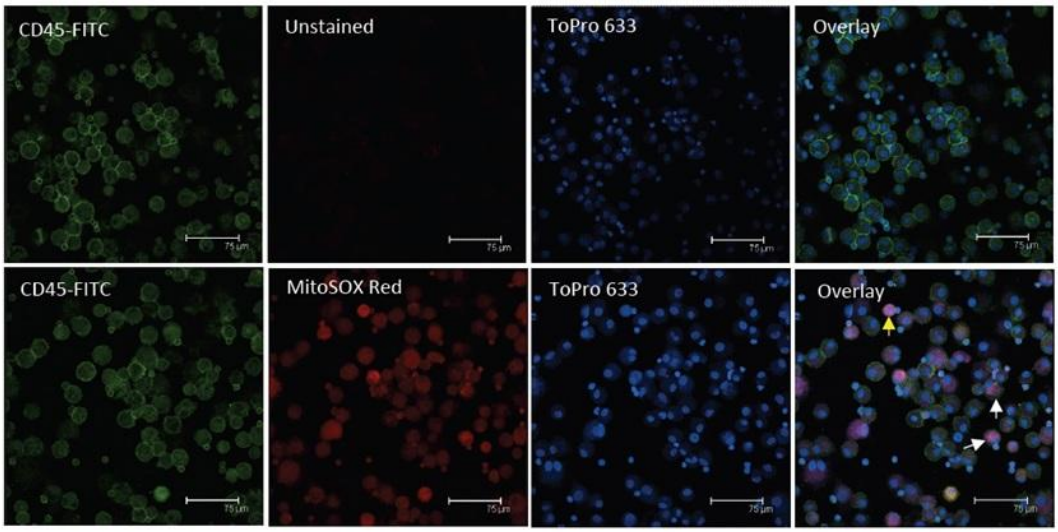


Figure 22. Confocal microscopy images. BALF cells of ILD patient stained with CD45-FITC and Topro 633, with or without MitoSOX Red. Yellow arrow shows a highly red stained cell lacking CD45 strong staining. White arrows show double positive cells for MitoSOX Red and CD45-FITC.

Table 7 summarizes our results from all analysed fresh BALF samples, regarding the percentage of MitoSOX positive cells and the presence of a shift in the cell population in histograms of flow cytometry analysis. Statistical analysis revealed that IPF patients did not display significantly higher percentage of MitoSOX positive cells (Figure 23). However, a clear trend in the presence of a shift in the CD45 positive / PI negative population towards a more pronounced MitoSOX Red staining was observed in all 4 IPF samples and only in 1 out of 4 controls.

Table 7. Flow Cytometry results of MitoSOX staining. Two parameters were evaluated during analysis; the percentage of MitoSOX positive cells and the shift in the total CD45 positive cell population.

Prov. Diagnosis	MitoSOX Shift	MitoSOX %
control	-	4,24
control	-	0
control	-	0
control	+	7,2
IPF	+	1,94
IPF	+	1,16
IPF	+	30,5
IPF	+	5,08
Lung Ca	-	0
Lung Ca	-	0
RA-ILD	-	13,2
RB-ILD	-	0,3
Sarcoidosis	-	0
CTD-ILD	+	1,89
HP	-	0

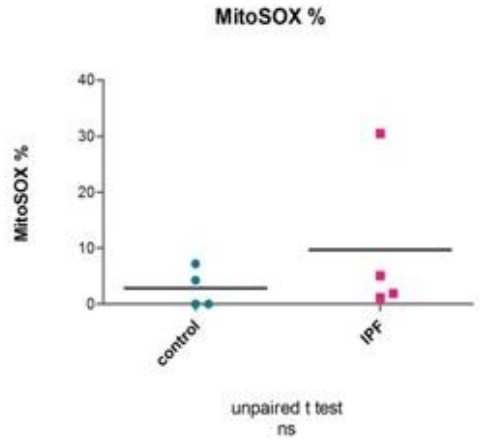


Figure 23. Statistical analysis of the percentage of MitoSOX in IPF patients and control subjects. Unpaired t test non significant (n=4).

3.5. TGFβ1 effect on THP1 cells

Since TGFβ1 is considered as the main mediator in IPF pathogenetic mechanisms, we investigated the response to TGFβ11 in THP1 monocyte cell line treated with phorbol-12-myristate-13-acetate (PMA), which promotes their differentiation into macrophages. We used THP1 cells treated with PMA for 6 hours and followed by treatment with three concentrations of TGFβ1; 1ng/ml, 5ng/ml and 10 ng/ml overnight. Following flow cytometry

analysis, we detected a decrease in MitoSOX levels upon treatment with 5 and 10 ng/ml TGFβ1 (Figure 24 A, C). Interestingly, in the PMA treated THP-1 ells we observed a second cellular population with high MitoSOX incorporation. Evaluation of the high MitoSOX population following TGFβ1 treatments revealed that it significantly decreased upon treatment with 5ngr/ml and10ngr/ml TGFβ1 (Figure 24 B, C). This result was intriguing since in bronchial epithelial cells TGFβ1 treatment results in increased MitoSOX staining (50).

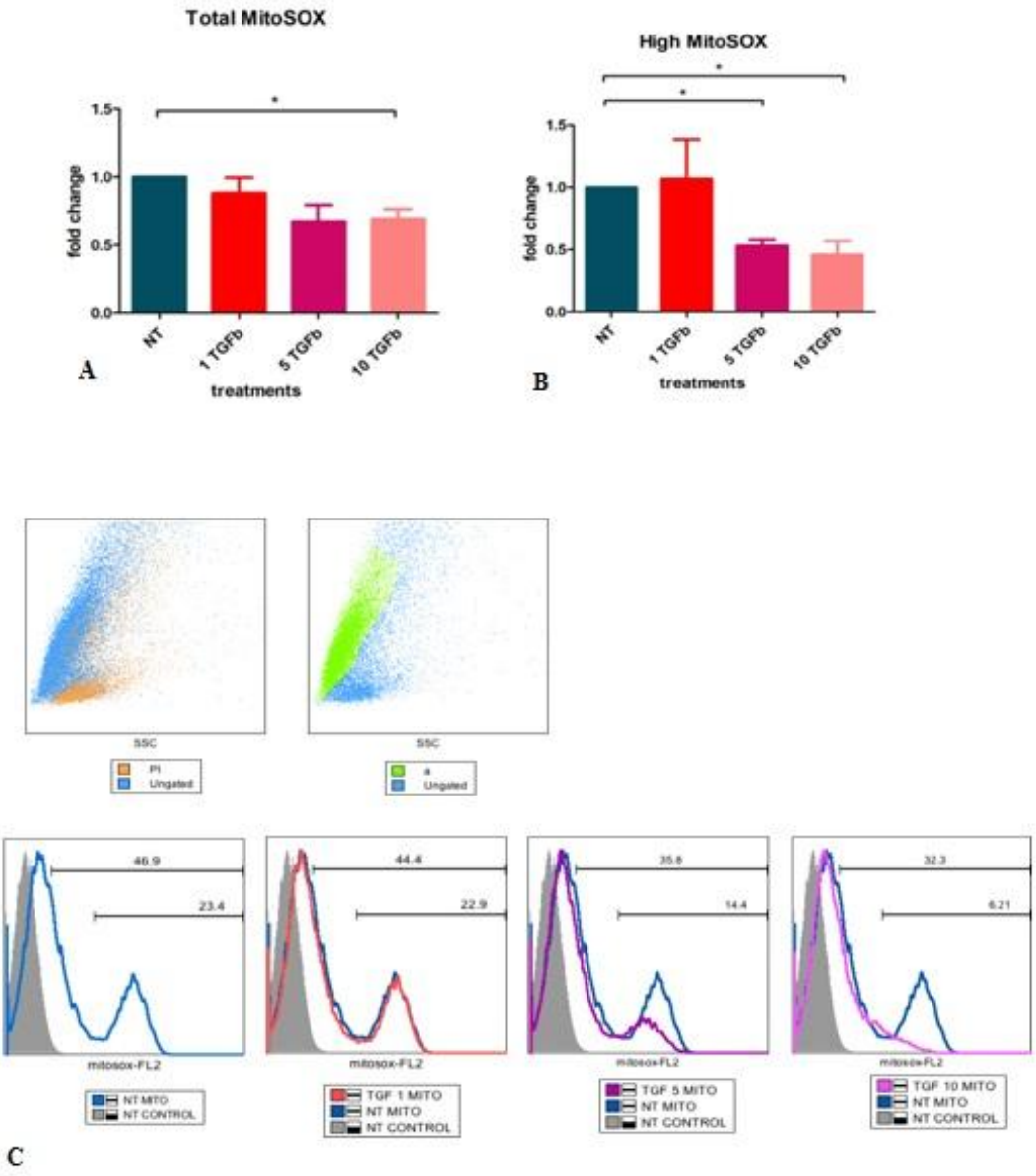


Figure 24. MitoSOX staining in THP1 cells treated with PMA followed by several TGFβ1 treatments. **A**. Statistical analysis of total MitoSOX percentage using one sample t test (n=4), *:p value<0.05 . **B.** Statistical analysis of the second population, that displays high peak in MitoSOX staining. One sample t test (n=4) *: p value< 0.05. **C**. Representative histograms of flow cytometry analysis, comparing non treated cells and TGFβ1 treated cells stained with MitoSOX. The percentage of each positive population (total and high peak) is shown.

Next, we investigated the expression of PINK1 levels in THP1 cells treated with PMA, after treatment with TGFβ1. It would be expected that decreased MitoSOX levels could be due to an increase in PINK1 expression. However, we found reduced expression of PINK1 expression, which was statistically significant between non treated and 10 ng/ml TGFβ1 treated cells (Figure 25).

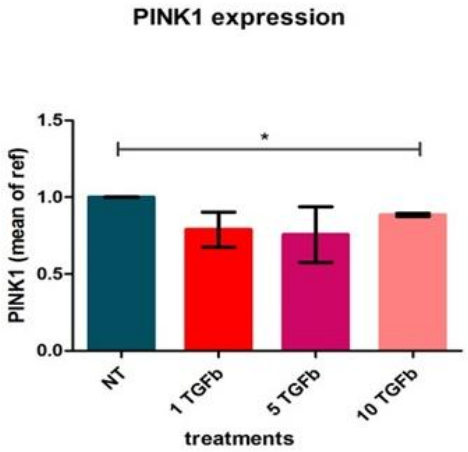


Figure 25. Fold change of PINK1 expression in THP1 cells treated with PMA after TGFβ1 treatments. PINK1 relative expression was normalised with the mean of two reference genes; GAPDH and 18s. One sample t test (n=3), *: $p<0.05$.

4. Discussion

IPF is a progressive fibrotic lung disease with poor prognosis and insufficient therapy. Thus, it is of great importance to identify the pathogenetic mechanisms that lead to fibrosis. It is currently believed that autophagy and mitophagy may play an important role in the development of IPF. This pathway seems to be deregulated in lung diseases and in several cell subtypes. We examined autophagy and mitochondria in BALF cells from IPF and RA-ILD patients as well as control subjects.

Initially we examined the protein levels of autophagy associated proteins in stored BALF samples. Our results may indicate an enhancement of autophagy in IPF BALF cells based on an observed trend for higher LC3 protein levels coupled with a decrease in p62 protein relative to controls. However, we also examined the expression of inducible genes involved in the autophagy process such as Beclin1, ULK1, p62 and S100A9 and we found no difference in their mRNA levels in IPF patients relative to controls. Hence the accumulation of LC3 protein could be also indicative of an inhibition of the autophagic flux and the turnover of the autophagosomes.

Despite the fact that inducers of autophagy are present in IPF pathogenesis, it has been reported that autophagy is not induced in whole lung tissue measuring the levels of LC3 and

p62 (40). Another study showed insufficient autophagy in epithelial cells in IPF lungs, leading to epithelial cell senescence, as well as in fibroblasts, maybe resulting in myofibroblast differentiation (41). Recently it has been reported that although there may be a potent induction of autophagy and high levels of LC3 in AECs in IPF, autophagy flux is impaired (42). Our findings do not suggest defect in autophagy concerning LC3 and p62 protein levels. However, the protein levels of other regulatory molecules, such as Beclin1, as well as the numbers of LC3 positive vesicles/autophagosomes should be assessed in order to claim that these results do not imply an inhibition of the autophagy flux.

Concerning RA-ILD patients, we observed an increase in both LC3 and in p62 protein levels. In parallel RA-ILD patients' BALF cells display higher expression levels of Beclin1 when compared with IPF patients. Taken together these results may suggest an activation of autophagy in RA-ILD, which could be expected following the increased inflammatory profile of this disease (23).

Mitochondria represent essential organelles in cell metabolism, but they can also cause cellular damage via the toxic byproducts of oxidative phosphorylation. Regarding mitochondrial homeostasis, our major finding is the significant down regulation of PINK1 expression levels in IPF and RA-ILD patients. PINK1 regulates mitochondrial turnover and thereby protects mitochondria from stress. It has been recently reported down regulation of PINK1 in AECs in IPF (42). The significance of PINK1 deficiency in AECs has been demonstrated; it leads to accumulation of dysfunctional mitochondria, it increases the release of profibrotic factors when exposed to ER stress, and influences susceptibility to lung fibrosis (42).

PINK1 expression is induced by the canonical PTEN (70). The downregulation of PINK1 could be therefore explained by a decrease in PTEN levels in the BALF cells. PTEN expression has been found decreased in fibroblasts and epithelial cells from IPF lungs in parallel with activation of AKT, which may lead to the apoptosis resistance phenotype of IPF fibroblasts (71-73). It has also been demonstrated that deletion of PTEN induces fibrosis (74). An increased pro-survival profile of IPF macrophages has also been previously reported (59), without yet existence of evidence regarding PTEN levels in IPF macropahges. Moreover, it is currently known that PINK1 expression is regulated by FOXO3a transcription factor (75). Conclusively, further analysis should be done to evaluate PTEN and FOXO3a m RNA levels in IPF BALF cells in order to examine a potent decrease in levels of genes that regulate PINK1 expression.

In addition, we demonstrated a strong co-regulation of PINK1 and the genes of autophagy regulation such as ULK1, Beclin1 and p62 in IPF patients. Interestingly, we did not find correlation between PINK1 expression and smoking status, despite evidence has been

proposed a potent smoking induced mitochondrial dysfunction (44, 46). However, in our study there is the limitation of the significant smaller number of smokers in IPF patients when compared with the other two groups.

Parkin and BNIP3, adaptor molecules for mitophagy, did not seem altered in the transcriptional level among control, IPF and RA-ILD patients. However, AKT3 that is involved in mitochondrial biogenesis is upregulated in RA-ILD versus IPF.

In parallel with our previous finding of upregulated expression levels of Beclin1 and a potentially increase in autophagy, the upregulation of AKT3 likely indicates that RA-ILD patients have a distinct pattern in cellular mechanisms. This fact would be expected as RA is a systemic inflammatory disease with different cause and pathogenesis than IPF (76)(77). However, we think the comparison with RA-ILD patients as a non-IPF ILD group is useful, since these two diseases share some similarities in the clinical level. The radiologic and histopathologic pattern of IPF is that of usual interstitial pneumonia (UIP). UIP, unlike the rest of the collagen tissue disorders (CTDs), is also the most frequent pattern when interstitial involvement accounts in patients with RA (61, 63).

Our next aim was the evaluation of mitochondrial oxidation levels in IPF patients. ROS are physiologically produced by all cells and mostly derived from leakage of the electron transport chain in mitochondria. Increased ROS generation is associated with mitochondrial dysfunction and this induces increased mitophagy in order for the cell to degrade the dysmorphic mitochondria and promote mitochondrial biogenesis (27, 29).

We therefore analysed fresh BALF samples from consecutive patients undergoing bronchoscopy for the last 9 months. Despite the small number of diagnosed patients, flow cytometry analysis showed increased MitoSOX positivity in IPF patients. In particular, we observed a shift in the CD45 positive BALF cell population of IPF patients upon staining with MitoSOX Red implying a higher level of oxidized mitochondria. Interestingly, we also observed a second peak upon MitoSOX staining representing a second cell population with high MitoSOX Red. This population was not PI positive, thus it was not necrotic/apoptotic cells. We measured the entire percentage of MitoSOX positive cells in BALF samples, always excluding the percentage of the PI positive cells, and performed statistical analysis of the MitoSOX positive percentage. We found no statistically significant difference between IPF and control patients. However, we should take into consideration that our sample size is very small yet, including only 4 IPF and 4 control diagnosed patients. To sum up, our finding regarding a clear shift upon MitoSOX staining in all IPF patients imply an increase in MitoSOX levels, thus we plan to include more patients that will be recruited for bronchoscopy, so as to a detect a potentially more significant difference in IPF patients.

Intriguingly, in accordance with our preliminary results, Patel et al recently found increased MitoSOX in alveolar epithelial cells in the absence of PINK1 (50).

TGFβ1 is the master molecule that mediates the pathogenetic mechanisms in IPF. However, the effect of TGFβ1 on autophagy in IPF is not well established yet. Patel et al suggested that TGFβ1 inhibits autophagy in human lung fibroblasts via activation of PI3K, Akt and mTORC1 (40). On the contrary, Araya et al proposed that TGFβ1 enhances autophagy in primary lung fibroblasts (41). These differences could be attributed in the different cell lines used or in differences in experimental conditions. In THP1 macropahges treated with PMA, we observed that TGFβ1 stimulation downregulates PINK1 expression. Interestingly, we also demonstrated a decrease in oxidation level of mitochondria upon TGFβ1 treatment. It has to be mentioned that during flow cytometry analysis upon MitoSOX staining, we observed a second cellular population with high peak, similar to our observation in BALF cells. TGFβ1decreases MitoSOX percentage whether this population is analysed separately, or not. This result is contradictory to a recent study by Patel et al that proposed higher PINK1 levels and increased MitoSOX staining upon TGFβ1 treatment (50). However, this study investigated bronchial epithelial cells.

Furthermore, it is interesting to notice a higher steady state of oxidized mitochondria by MitoSOX staining in tissue cultured cells, such as A549 epithelial cell line and THP1 monocytes. Moreover, PMA which is widely used for the growth inhibition of THP1 cells in order to differentiate them and represent tissue macrophages, induces itself ROS generation (78). On the contrast, freshly isolated BALF cells from patients do not display such a high oxidation level. Maybe this difference is attributed to the culture conditions, whereas lower oxygen concentrations better represent the physiologic conditions (79).

Conclusively, the impact of TGFβ1 on oxidation level of mitochondria and mitochondrial turnover remains unclear. Further research need to be done to explore the actions of this key mediator in IPF lungs and in different cell populations.

Demonstrating a new mechanism involved in IPF pathogenesis could help us develop a novel therapeutic strategy in the future, targeting mitochondria(80). This is an attractive prospect provided the extensive research on mitochondria targeting therapeutics for other diseases. Achieving a balance between mitochondria biogenesis and mitophagy is believed to have beneficial effects (28). Despite the fact that mitophagy is considered as a major protective mechanism in response to stress, it can also lead to cell death or cellular senescence upon overactivation. Mitochondrial stress has been closely associated with aging processes and age-related diseases (58). Thus, manipulating mitochondrial state may benefit aging cells and aging patients. Recent studies with mesenchymal stem cells suggest that mitochondrial

transfer from MSCs contributes to the reconstitution of alveolar bioenergetics and the protection against experimental model of acute lung injury (81).The role of mitochondrial biogenesis highlighted another recent study by Missios et al suggesting glucose administration at advanced age, which could be beneficial in mitochondrial and energy homeostasis in aging tissues (82). Moreover, the induction of autophagy is considered to protect against the development of lung fibrosis (39-41), suggesting a potential therapy targeting this mechanism. Although there may be an enhancement of autophagy process, there is evidence implying a defect in autophagy flux in IPF. Autophagy modulation has been already proposed in lung diseases (25, 39). In conclusion, manipulating autophagy and mitochondrial homeostasis is a really promising target for lung diseases. Therefore, future studies regarding alveolar macrophages and other cell types in lung composition, should be performed to provide a new hope for the diminution of IPF development or progression.

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