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Study of new molecular markers with non-invasive methods in respiratory diseases, with particular regard to Idiopathic Pulmonary Fibrosis

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ABBREVATIONS

ADP	Aenosine diphosphate
AECs	Alveolar epithelial cells
AIP	Acute interstitial pneumonia
ALF	Airway lining fluid
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
BMI	Body Mass Index
COP	Cryptogenic organising pneumonia
DIP	Interstitial pneumonia
DLCO	Carbon monoxide diffusing capacity
DPLDs	Parenchymal lung diseases
EBC	Exhaled breath condensate
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ESTs	Expressed sequence tags
ET-1	Endothelin-1
ETC	Electron transport chain
FEV1	Forced expiratory volume in 1 s
FIP	Familial interstitial pneumonia
FVC	Forced vital capacity
GAP INDEX	Gender-Age-Physiology
GOR	Gastro-oesophageal reflux
GWAS	Genome wide association studies
H_2O_2	Hydrogen peroxide
HRCT	High resolution computed tomography
IIPs	Idiopathic interstitial pneumonias
IL-1α	Interleukin 1 alpha
IMM	Inner mitochondrial membrane
IPF	Idiopathic pulmonary fibrosis
KL-6	Krebs von den Lungen-6
KM curves	Kaplan-Meier curves

LIP	Lymphocytic interstitial pneumonia
LOH	Loss of heterozygosity
MAO	Mnoamine oxidases
MAs	Microsatellite alterations
MSI	Microsatellite instability
Mt/N	mitochondrial genome to nuclear genome ratio
MtDNA	Mitochondrial DNA
MUC5B	Mucin 5B
nDNA	Nuclear DNA
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NSIP	Non-specific interstitial pneumonia
OSAS	Obstructive Sleep Apnea Syndrome
$PaCO_2$	Partial Pressure of Carbon Dioxide
PaO_2	Partial Pressure of Arterial Oxygen
PDGF	Platelet-derived growth factor
RB-ILD	Respiratory bronchiolitis interstitial lung disease
RNS	Nitrogen species
ROS	Reactive oxygen species
SFTPA2	Surfactant proteins A2
SFTPC	Surfactant proteins C
STR	Short tandem repeats
TERC	Telomerase RNA component
TERT	Telomerase reverse transcriptase
TGF-β	Transforming growth factor β
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor α
TSGs	Tumor suppressor gene
UIP	Usual interstitial pneumonia
WB	Whole blood

Abstract

Background: Idiopathic pulmonary fibrosis (IPF) is a progressive fibrosing interstitial lung disease of unknown aetiology, which leads rapidly to death. As the diagnosis of IPF is complex, the search for non invasive biomarkers is of great relevance in consideration of the management of these patients.

The aim of this study was to investigate in exhaled breath condensate (EBC) of IPF patients the mitochondrial DNA (MtDNA) alterations, as oxidative stress marker, and the potential role of Periostin and genetic microsatellite alterations (MAs) in IPF pathogenesis.

Methods: 48 IPF patients were compared with 20 control subjects. Patients underwent EBC and blood collection. Content of mitochondrial DNA (MtDNA) and nuclear DNA (nDNA) was measured in EBC by Real Time PCR and the ratio between MtDNA/nDNA was calculated. Periostin was assessed by an enzyme-linked immunosorbent assay kit on EBC. Four microsatellite markers (THRA1, D17S579, D17S250 and D8S137) were used for the analysis of MAs. The EBC-DNA and WB-DNA were amplified by PCR; PCR products were analyzed using the ABI Prism 310 Genetic Analyzer.

Results: Exhaled MtDNA/nDNA was higher in IPF patients compared to healthy controls (16.59 ± 10.30 vs 7.94 ± 4.56; $p < 0.005$). The level of MtDNA/nDNA was negatively correlated with FVC% ($R = -0.4879$, $p = 0.006$) and FEV1% ($R = -0.4364$, $p = 0.018$). Moreover, we were able to detect higher Periostin levels in the EBC of patients with IPF compared control subjects (65,5 ± 23,5 pg/mL vs 33 ± 21,4 pg/mL, $p<0.05$), respectively. MAs were found in 58.82 % of EBC-DNA and 12.50 % of WB-DNA in patients with IPF ($p < 0.01$). None of the healthy subjects exhibited MAs in the studied

markers.

Conclusions: There is an increase of MtDNA/nDNA ratio in IPF subjects that led us to suggest that there is a presence of mitochondrial dysfunction that confirms an important role of the oxidative stress in IPF. We also found that Periostin is measurable in the airways and increased in patients with IPF. Unlike serum Periostin, which may be derived from several sources outside the lung, airways Periostin could be a useful marker to better understanding the pathogenesis of IPF. Furthermore, we reported that the genetic alterations, studied in EBC, may play an important role in the complex genetic basis of IPF.

Keywords: IPF, Exhaled Breath Condensate, Mitochondrial DNA, Oxidative stress, Periostin, Microsatellite alterations.

Riassunto

Introduzione: La fibrosi polmonare idiopatica (IPF) è una forma di polmonite interstiziale fibrosante ad eziologia sconosciuta, con andamento progressivo, che porta rapidamente alla morte. Poiché la diagnosi di IPF è complessa, la ricerca di nuovi biomarcatori, in particolare di quelli non invasivi è di grande importanza per la gestione di questi pazienti.

Lo scopo di questo studio è stato quello di valutare, nell'esalato condensato (EBC) dei pazienti IPF, le alterazioni del DNA mitocondriale (MtDNA), marcatore di stress ossidativo, ed il potenziale ruolo della Periostina e delle alterazioni genetiche dei microsatelliti (MAs) nella patogenesi dell'IPF.

Materiali e Metodi: 48 pazienti con diagnosi di IPF sono stati confrontati con 20 soggetti controllo. I pazienti sono stati sottoposti alla raccolta di EBC e di sangue. Il contenuto del DNA mitocondriale (MtDNA) e del DNA nucleare (nDNA) è stato amplificato e quantificato nell'EBC e nel sangue mediante Real Time PCR. Il rapporto tra MtDNA/nDNA è stato poi calcolato. La concentrazione della Periostina è stata valutata nell'EBC mediante test immunoenzimatico (ELISA). Quattro marcatori microsatellitari (THRA1, D17S579, D17S250 e D8S137) sono stati utilizzati per l'analisi delle MAs. Il DNA ottenuto dall'EBC e dal sangue è stato amplificato mediante PCR; i prodotti di PCR sono stati poi analizzati utilizzando un sequenziatore automatico (ABI Prism 310 Genetic Analyzer).

Risultati: Il rapporto MtDNA/nDNA nell'EBC dei pazienti IPF è risultato essere più alto rispetto al gruppo di controllo (16.59 ± 10.30 vs 7.94 ± 4.56; $p < 0.005$). I livelli di MtDNA/nDNA hanno mostrato una correlazione negativa con il FVC% ($R = -0.4879$, p

= 0.006) e con il FEV1% (R = -0.4364, p = 0.018). Inoltre, i livelli di Periostina misurati erano più alti nell'EBC dei pazienti IPF rispetto al gruppo di controllo (65,5 ± 23,5 pg/mL vs 33 ± 21,4 pg/mL, p <0.05), rispettivamente. Alterazioni dei microsatelliti sono state evidenziate nel 58,82% dei campioni di EBC analizzati e nel 12,50% dei corrispettivi campioni di sangue dei pazienti IPF (p <0,01). Nessuno dei soggetti controllo ha mostrato MAs nei marcatori studiati.

Conclusioni: Un aumento del rapporto MtDNA/nDNA nei pazienti IPF suggerisce la presenza di una disfunzione mitocondriale a conferma del ruolo dello stress ossidativo in questa patologia. Inoltre, abbiamo dimostrato la possibilità di dosare la Periostina nelle vie aeree e i livelli di quest'ultima sono risultati essere più alti nei pazienti IPF. A differenza della Periostina sierica, la Periostina delle vie aeree potrebbe rappresentare un utile marker per meglio comprendere la patogenesi dell'IPF. Infine, abbiamo riportato che le alterazioni genetiche, rilevate nell'EBC, potrebbero avere un ruolo importante nelle complesse basi genetiche dell'IPF.

Parole chiave: IPF, Esalato Condensato, DNA mitocondriale, Stress Ossidativo, Periostina, Microsatelliti.

1. INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a fibrotic lung disease of unknown origin. IPF has a dismal prognosis with a median survival between two and four years despite the current progress in our understanding of the disease pathogenesis and the therapeutic advances of the last years [Raghu G et al. 2018].

IPF is the most common form of the idiopathic interstitial pneumonias (IIPs) and has been estimated to account for between 17-37% of all interstitial lung disease cases [King TE Jr et al. 2011; Nalysnyk L et al. 2012].

The differentiation of IPF from the other IIPs is a challenge. In fact, a multidisciplinary approach with a combination of imaging, clinical and laboratory findings is required for diagnosing IPF [Raghu G et al. 2018].

The Fleischner Society recently published diagnostic criteria for IPF, highlighting as about 30% of patients with suspected IPF need invasive procedures such as a surgical lung biopsy to make a definite diagnosis because of a CT pattern indeterminate for usual interstitial pneumonia (UIP) [Lynch DA et al. 2018]. However, due to comorbidities, severe functional impairment and increased age this approach is not feasible in many patients, stressing the need for reliable biological markers which non-invasively can foster a confident diagnosis of IPF [Martinez FJ et al. 2017; Walsh SLF et al. 2017].

Biomarkers may have diagnostic and/or prognostic functions in disease states. They may help to discriminate between a particular disease and other disease conditions and to establish an early diagnosis, which is the prerequisite for better understanding of the pathogenesis of a disease. An ideal biomarker should be easily acquired through non-invasive means, have high validity and reliability, and be available for serial monitoring [Magnini D et al. 2017]. Taken into account that IPF is a disease affecting older individuals with many comorbidities and compromised respiratory reserve, the quest for

blood biomarkers or biomarkers in exhaled breath condensate seems more reasonable than the invasive measurement in the bronchoalveolar lavage (BAL) or in lung tissue [Rindlisbacher B et al. 2017; Tzouvelekis A et al. 2016; Guiot J et al. 2017; Guiot J et al. 2017; Yamada YI et al. 2017].

In particular, the exhaled breath condensate (EBC) is a fluid from airways that is collectable with complete non-invasiveness just breathing at tidal volume in the condenser device [Carpagnano GE et al. 2016]. The method allows to collected the airway lining fluid (ALF) to analyze the physiologic and pathologic processes in the lung [Effros RM. 2006].

Firstly, with this study we analyzed a new intriguing oxidative stress marker that is represented by the mitochondrial DNA. Mitochondria are independent organelles present in the eukaryotic cells [Wojtczak L et al. 2008], highly susceptible to be attacked by reactive oxygen species (ROS), due to lack of protective histones, introns and their limited DNA repair capacity [Pieters N et al. 2013]. Several lines of evidence demonstrate that ROS play a role in pulmonary fibrotic disease: oxidized lipids and proteins have been identified from the exhaled air, BAL fluid, and lung tissue of patients with fibrotic lung disease [Faner R et al. 2012; Kliment CR et al. 2010]; bleomycin-induced pulmonary fibrosis (the most common animal fibrosis model) is associated with increased levels of ROS, oxidized proteins, DNA, and lipids [Cheresh P et al. 2013; Gazdhar A et al. 2014]; increased oxidative DNA damage is seen in IPF, silicosis, and asbestosis patients, as well in experimental animal models of silicosis or asbestos-induced lung fibrosis [Liu G et al. 2013]; antioxidants and iron chelators can attenuate fibrosis induced by bleomycin or asbestos in rodent models [Cheresh P et al. 2013; Oury TD et al. 2001]. Our hypothesis is that in conditions of oxidative stress, the transcriptional and replication machinery of mitochondrial biogenesis will be up-

regulated resulting in increased mitochondrial biogenesis via replication of the mitochondrial genome; therefore in its presence they undergo an adaptive response that causes an increase of MtDNA copy numbers. This change could be detected in body fluids and airway of IPF patients.

In the second part of this study, we analyzed the Periostin concentrations in the airways of patients with IPF. Periostin is an extracellular matrix (ECM) protein belonging to the fasciclin family that is highly expressed at sites of injury or inflammation and promotes mesenchymal cell proliferation and fibrosis [Izuhara K et al. 2016; Conway SJ et al. 2014]. Periostin acts on fibroblasts together with inflammatory cytokines such as TNF α or IL-1 α activating NF- κ B, followed by production of various inflammatory cytokines and chemokines, leading to generation of fibrosis in the lungs [Uchida M et al. 2014; Taniguchi K et al. 2014].

Thus, Periostin is a key player in the pathogenesis of pulmonary fibrosis. Various authors found that serum Periostin was significantly up-regulated in IPF patients [Okamoto M et al. 2011; Naik PK et al. 2012; Tajiri M et al. 2015].

Serum Periostin was associated with decline of %FVC and %DLCO [Okamoto M et al. 2011], clinical progression [Tajiri M et al. 2015], and overall survival and time-to-event [Tajiri M et al. 2015]. Taken together, these results suggest the potential of Periostin as a prognostic biomarker in IPF.

In the last preliminary part of this study, we investigate the genetic alterations in EBC and blood of IPF patients, using four highly polymorphic microsatellite markers, located on several chromosomal arms, where there are some TSGs largely recognized in lung cancerogenesis, that could be part of the complex genetic basis of the IPF disease and explain the association of this disease and lung cancer. In fact, in patients with IPF, the incidence of lung cancer is much higher than that in the general population and with this

disease, it shares several characteristics such as short survival, epigenetic and genetic alterations, cellular and molecular aberrances and the activation of similar signaling pathways [Vancheri C et al. 2010; Vancheri C. 2013].

2. IDIOPATHIC PULMUNARY FIBROSIS

2.1 Background

Idiopathic pulmonary fibrosis is one of the idiopathic interstitial pneumonias (IIPs), a group of diffuse parenchymal lung diseases (DPLDs), also known as the interstitial lung diseases [Raghu G et al. 2018]. Other types of IIP include desquamative interstitial pneumonia (DIP), respiratory bronchiolitis interstitial lung disease (RB-ILD), non-specific interstitial pneumonia (NSIP), acute interstitial pneumonia (AIP), cryptogenic organising pneumonia (COP) and lymphocytic interstitial pneumonia (LIP).

The IIPs are characterised by different patterns of inflammation and fibrosis. Further, they have a different prognosis and response to therapy, therefore the distinction of IPF from other forms of IIPs is highly important. In 2002, to provide a distinction, the ATS/ERS issued a statement in which are described the clinical, radiological and pathological manifestations of IPF and the other interstitial pneumonias. Figure 1 outlines the current classification and its chronological development.

Currently the diagnosis of IPF is linked to the definition of the ATS/ERS/JRS/ALAT: a chronic progressive fibrosing interstitial pneumonia of unknown cause, occurring primarily in adults, limited to the lungs and associated with the histological and/ or radiological usual interstitial pneumonia (UIP) pattern.

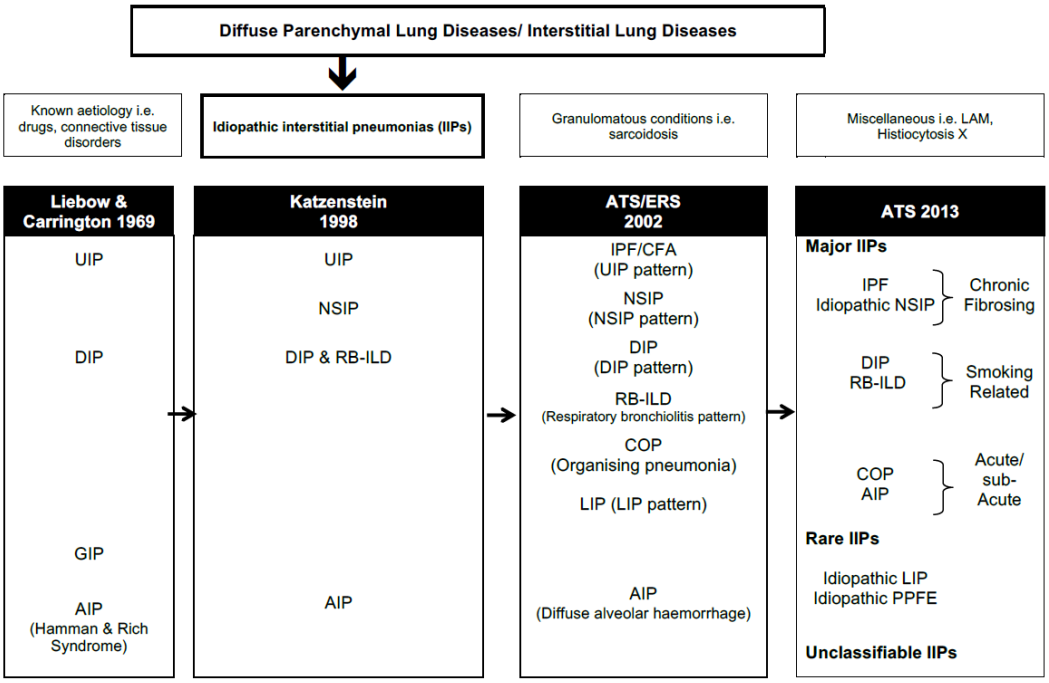


Figure 1 – Classification of Diffuse Parenchymal Lung Diseases and chronological development of the sub-classification of the Idiopathic Interstitial Pneumonias. Adapted from the ATS/ERS Guidelines (Agusti 2002; Travis WD et al. 2013).

2.2 Epidemiology

Reported prevalence and incidence data for idiopathic pulmonary fibrosis (IPF) vary and depend on ascertainment and reporting methods and also the age and geographic location of the population. IPF prevalence and incidence increase with advancing age, with presentation commonly occurring in the sixth and seventh decades; rarely is IPF seen in patients aged less than 50 years [Raghu G et al. 2018]. More men have been reported with IPF than women [Esposito DB et al. 2015]. IPF is a progressive condition which is ultimately fatal with a median survival of less than 4 years. Cause of death in most patients occurs from progression of lung fibrosis and respiratory failure rather than from commonly occurring co-morbid conditions as illustrated in Figure 2 [Ley B et al. 2011]. The incidence of IPF in the United States has most recently been estimated from 7 to 6

per 100,000 person-years in the USA [Raghu G et al. 2014], and in United Kingdom, the IPF incidence in 2008 was 7.4 per 100,000 person-years [Navaratnam V et al. 2011]. In Europe, IPF prevalence ranged from 1.25 to 23.4 cases per 100,000 population, and the annual incidence ranged between 0.22 and 7.4 per 100,000 population [Nalysnyk L et al. 2012]. Overall, European and Asian prevalence and incidence estimates appear to be lower relative to those reported in the US populations, although it remains unclear whether these are true differences or as a result of different methodologies [Caminati A et al. 2015]. However, a recent systematic review has demonstrated a global increase in IPF incidence, with the rates in different countries starting to come together [Hutchinson J et al. 2015].

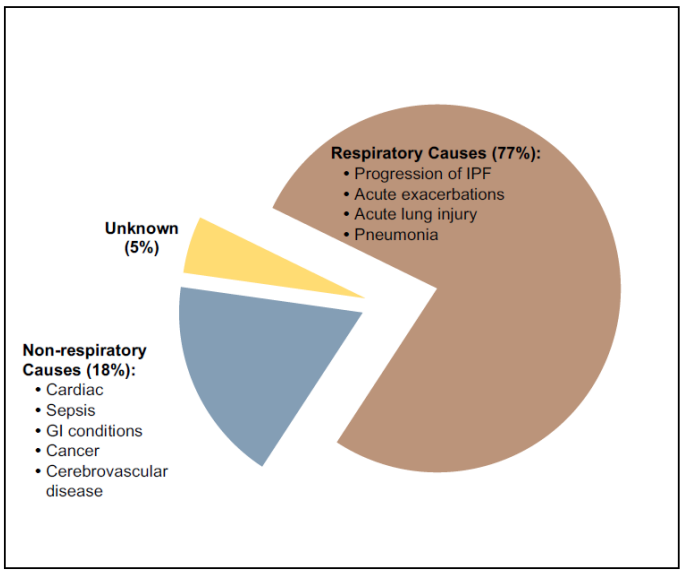


Figure 2 – Causes of death in patients with IPF (Ley B et al. 2011)

2.3 Pathogenesis

The pathogenesis basis of IPF has changed over time. Idiopathic pulmonary fibrosis is associated with the histological finding of UIP pattern. Usual interstitial pneumonia is

characterized by pathological proliferation of mesenchymal cells and uncoordinated collagen deposition [Selman M et al. 2011]. Subepithelial fibroblast foci, which are clusters of fibroblasts in the extracellular matrix, are the hallmark of UIP and are found throughout the lung in patients with IPF [Martinez FJ et al. 2017]. The number of fibroblast foci correlates with prognosis and survival [King TE et al. 2011].

The initial theory of IPF pathogenesis suggested that IPF was the result of a chronic inflammatory response to an unidentified trigger that caused lung injury and progressive fibrosis [Noble PW et al. 2005]. The assumption was that interrupting the inflammatory cascade, using anti-inflammatory therapy in the treatment of IPF, would prevent fibrosis.

However, it has been shown that inflammation is not the predominant histological feature in the vast majority of patients with IPF. In line with these findings, anti-inflammatory and immunosuppressive agents, in particular corticoids, not only failed to control the course of IPF, but seem to be even harmful [Raghu G et al. 2018; Martinez FJ et al. 2017; Selman M et al. 2001].

The ‘epithelial’ hypothesis has been proposed as an alternative theory of IPF pathogenesis. It is currently believed that the fibrotic process in IPF is a result of repeated micro-injury to the alveolar epithelium and a failure of normal wound healing [Gauldie J et al. 2001; Selman M et al. 2001].

In the normal lung, the alveolar epithelium is comprised of alveolar epithelial cells (AECs), predominantly of type I AECs and with a smaller number of type II AECs. Following alveolar epithelium injury, type II AECs proliferate and differentiate into type I AECs, thus repairing the alveolar epithelium [Crapo JD et al. 1982].

In IPF, the damage and apoptosis of alveolar epithelial type II cells causes an inadequate repletion of type I cells and altered epithelial cell phenotypes, resulting in failure to re-

establish a normal epithelium [Selman M et al. 2001; Barbas-Filho JV et al. 2001]. The cause of this atypical response of AECs is not fully understood.

The epithelial injury and consequent damage to the alveolar-capillary basement membrane is a critical step in the irreversible fibrotic process. The disruption of the basement membrane leads to the aberrant activation of alveolar epithelial cells, consequently leading to the enhanced migration of mesenchymal cells and to the intrusion of inflammatory cells to the alveolar space [Selman M et al. 2014].

The epithelial cells are the target of the injurious processes, but they also have a fibrogenic role. Following injury, they secrete factors which stimulate the activation, migration and proliferation of fibroblasts. These pro-fibrotic mediators including transforming growth factor β (TGF-β), platelet-derived growth factor (PDGF), tumour necrosis factor α (TNF-α), endothelin-1 (ET-1), connective tissue growth factor and osteopontin [Antoniades HN et al. 1990; Nash JR et al. 1993; Pardo A et al. 2005; Selman M et al. 2006]. TGF-β is the major factor involved in the pathogenesis of IPF. It promotes apoptosis of epithelial cells, epithelial-mesenchymal transition, collagen synthesis, fibroblast proliferation, fibroblast to myofibroblast trans-differentiation, angiogenesis and matrix metalloprotease expression [Tatler AL et al. 2012]. In IPF, the epithelial injury also activates the coagulation cascade which has several pro-fibrotic effect [Borensztajn K et al. 2008].

2.4 Aetiology

Idiopathic pulmonary fibrosis by definition is a condition of unknown aetiology. However, a number of potential risk factors have been described. These include environmental exposures, microbial agents, genetic factors and gastroesophageal reflux.

2.4.1 Environmental exposures

Several epidemiological studies have demonstrated that environmental exposures are involved in the pathogenesis of IPF. Different findings suggest that there is a strongly associated between cigarette smoking and metal dust with the risk of IPF, even for the familial form of pulmonary fibrosis [Taskar VS et al. 2006]. Hubbard et al. were first to report a statistically significant association between cigarette smoking and the development of IPF [Hubbard R et al. 1996]. Even after smoking cessation, smoke remains a risk factor by inducing a self-sustaining lung injury. Moreover, IPF patients with cigarette smoking history have a poorer survival compared to non-smokers [Spira A et al. 2004].

As well as cigarette smoking, further significant correlations have been established among IPF and agriculture and farming, livestock, wood dust and stone, sand and silica [Taskar V et al. 2008].

2.4.2 Microbial agents

Recent studies have characterised the 'microbiome' of IPF. Bacteria, virus and fungi play a potential role in the pathogenesis of IPF [Chioma OS et al. 2017]. An imbalance in bacterial community composition has been observed in patients with interstitial lung disease, when compared with healthy lungs. Studies suggested that the analysis of IPF lung microbiome composition may provide an explanation for disease pathogenesis and may be useful as a prognostic biomarker [Han MK et al. 2014]. Moreover, Huang and co-workers analysed the patients enrolled in the COMET-IPF study and demonstrated a relationship between peripheral blood immune gene expression and bronchoalveolar lavage (BAL) microbiome features in IPF [Huang Y et al. 2017]. Other risk factors

associated with IPF include viral infections, such as Epstein-Bar-virus, cytomegalovirus, hepatitis C virus, and human herpesvirus-8 [Molyneaux PL et al. 2013].

2.4.2 Genetic factors

Evidence suggests that genetic features characterized by a combination of gene variants and transcriptional changes, resulting in the loss of epithelial integrity, predispose to the development of IPF. Familial interstitial pneumonia (FIP) is identified when two or more member of the same biological family are affected [Schwartz DA 2016]. The mode of genetic transmission of FIP is autosomal dominant with variable penetrance and accounts from 2% to 20% of the overall cases of idiopathic interstitial pneumonias [Kropski JA et al. 2015]. Mutations in telomerase reverse transcriptase (TERT) and telomerase RNA component (TERC), which are genes involved in telomere length maintenance have been studied, with up to 15% of patients with familial pulmonary fibrosis and 3% of IPF patients having a mutation present [Armanios MY et al. 2007; Tsakiri KD et al. 2007 ; Cronkhite JT et al. 2008]. Several gene mutations have also been studied in patients with familial pulmonary fibrosis and IPF including surfactant proteins C and A2 (SFTPC and SFTPA2, respectively) [Nogee LM et al. 2001; Lawson WE et al. 2004; Lawson WE et al. 2006; Wang Y et al. 2009]. Two large genome wide association studies (GWAS) have identified common genetic variants as risk factors of IPF [Fingerlin T et al. 2013; Noth I et al. 2013]. Both studies established the role of the promoter of MUC5B gene as a risk factor of disease and characterized other common variants associated with IPF, e.g. TOLLIP and Toll-like receptor (TLR) 3. The polymorphism in the promoter of the gene encoding a mucin (MUC5B) has been confirmed as the strongest risk factor for development of both familial interstitial pneumonia and sporadic IPF [Hunninghake GM et al. 2013; Seibold MA et al. 2011; Roy MG et al. 2013]. The prevalence of this

polymorphism was found to be high in familial (34%) and idiopathic (38%) pulmonary fibrosis with IPF patients significantly more likely to have at least one copy of the polymorphism compared with controls, and MUC5B expression in the lung was 14 times higher in these patients [Seibold MA et al. 2011]. However, despite the association of this polymorphism with increased susceptibility, different studies have identified that its presence is also associated with a more favourable IPF phenotype [Yang IV et al. 2013], an improved survival [Peljto AL et al. 2013], and a trend to a slower decline in FVC [Stock CJ et al. 2013].

2.4.2 Gastro-oesophageal reflux (GOR)

Gastro-oesophageal reflux (GOR) and micro-aspiration has been linked with a number of respiratory conditions and several studies have identified GOR as a possible risk factor for the development of IPF [El-Serag HB et al. 1997]. Patients with IPF have been found to have a high prevalence of GOR and hiatus hernia, and are often asymptomatic [Raghu G et al. 2006a; Sweet MP et al. 2007; Noth I et al. 2012]. Despite evidence of an association between GOR and IPF, a causal relationship has yet to be established [Hershcovici T et al. 2011].

2.5 Diagnosis

A diagnosis of IPF is difficult, because the clinical manifestations are similar to other conditions (e.g., sarcoidosis) and is in part a diagnosis of exclusion [du Bois RM et al. 2012]. Other conditions that resemble IPF are interstitial lung diseases related to connective tissue disorders such as rheumatoid arthritis, systemic lupus erythematosus and progressive systemic sclerosis, and conditions triggered by environmental exposures, such as chronic hypersensitivity pneumonitis [Castelino FV et al. 2010; Selman M et al.

2006]. An accurate diagnosis of IPF is important considering the differences in prognosis, IPF shows only a ~50% 3-year survival rate. More importantly, no therapies have been proven to reverse, halt or delay the progression of disease in IPF in large, well-conducted, double-blinded, placebo-controlled, prospective clinical trials [Raghu G. et al. 2011]. Current guidelines recommend a systematic, multi-disciplinary approach to the investigation of patients with suspected IPF [Raghu G et al. 2018].

Diagnosing IPF requires three main criteria: the exclusion of other known sources of interstitial lung diseases (including occupational, environmental, and drug toxicities obtained through a thorough patient history); a pattern of usual interstitial pneumonia on high resolution computed tomography (HRCT) (if a surgical lung biopsy is not available/safe to obtain); specific combinations of patterns from HRCT and surgical lung biopsy when both tests are available. and histopathology patterns in patients subjected to lung tissue sampling (Figure 3).

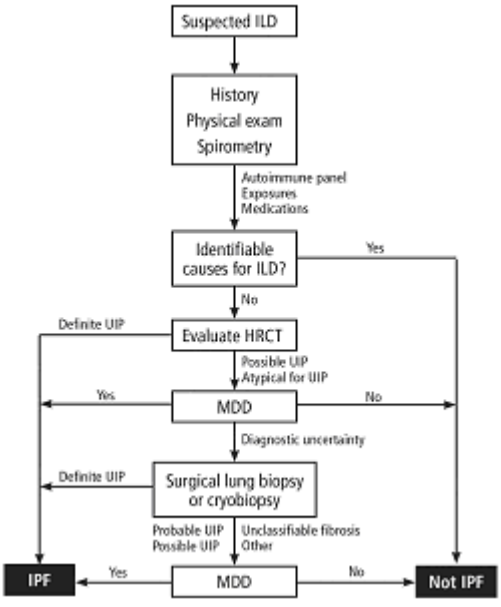


Figure 3 – Diagnostic algorithm for patients with suspected IPF. Adapted from (Raghu G et al. 2011)

2.6 Prognosis

Despite new therapeutic strategies the prognosis for the majority of patients with IPF remains poor with a median survival of less than 4 years according to Fernandez Perez ER et al. [Fernandez Perez ER et al. 2010]. The rate of decline varies between individual patients and clinically three different forms of progression can be described: slowly progressive IPF, rapidly progressive IPF and periods with relative stability interposed by periods of rapid acceleration [Ley B et al. 2011]. The slowly progressive form of IPF is characterized by gradual decline in lung function and worsening of dyspnea without any periods of acceleration. Patients with rapidly progressive IPF were characterized by Selman et al. A subset of patients has a short duration of symptoms before first presentation, with rapid progression to death, in contrast to patients who experienced symptoms for a few years before presentation.

2.7 Treatment

IPF remains a major cause of illness and mortality and thus reflects a largely unmet medical need [Spagnolo et. al, 2015]. Because inflammation was long thought to be associated with the development of IPF, glucocorticoids or immunosuppressive agents represented the conventional approach to the treatment of patients, especially those with milder cases of the disease. However, the ATS/ERS/JRS/ALAT guidance recommended that IPF not be treated with corticosteroid monotherapy, colchicine, cyclosporine A, or the combination of corticosteroid with immunosuppressant (azathioprine or cyclophosphamide).

Since October 2014, mild-to-moderate IPF is now treated with either of two recently FDA-approved oral antifibrotics: pirfenidone and nintedanib.

Pirfenidone may decrease the rate of decline in VC and may increase the progression free survival time over one year [Taniguchi H et al. 2009.].

Nintedanib, a tyrosine kinase inhibitor first used to treat IPF. It has been shown to have a broad inhibitory activity on the downstream signaling cascades of fibroblasts and myofibroblasts [Wollin L et al. 2015]. Nintedanib was found to reduce the decline in FVC while also delaying the onset of acute exacerbation [Richeldi L et al. 2014].

IPF is the second most frequent disease for which lung transplantation is performed and within the interstitial lung diseases the most common among referrals for lung transplantation [Orens JB et al. 2006]. IPF patients have the highest waiting list mortality compared to other diseases [Thabut G et al. 2003; ten KL et al. 2012]. This pleads for early referral to a transplantation centre [Steinman TI et al. 2001].

3. OXIDATIVE STRESS AND IPF

Oxidative stress is define as an imbalance between oxidant production and antioxidant defence in favour of oxidants, that leads to cellular dysfunction and tissue damage.

Within a cell there are two main cellular source of ROS. The mitochondria are the largest source of ROS, since the reactions that occur during the oxidative phosphorylation processes frequently lose electrons during their transfer between the electron transport chain complexes [Adam-Vizi V et al. 2006; Halliwell B et al. 2007; Turrens J F. 2003]. Other sources are many enzymes that generate ROS for diverse purposes, in particular the phagocytic NADPH oxidase, NOX2 (gp91), which uses NADPH to reduce molecular oxygen, thus producing superoxide [Lambeth JD. 2004; Brown DI et al. 2009].

THE REDOX SYSTEM is essential in maintaining cellular homeostasis. Under physiologic conditions, cells maintain redox balance through generation and elimination of reactive oxygen/nitrogen species (ROS/RNS). This delicate balance between ROS generation and elimination is maintained by many complex mechanisms, and a dysfunction of any of these mechanisms could lead to alterations in cellular redox status.

The shift in the balance between oxidants and antioxidants with an increase in ROS production or a decrease in ROS-scavenging capacity due to exogenous stimuli or endogenous metabolic alterations can disrupt redox homeostasis, leading to an overall increase of intracellular ROS levels, termed *oxidative stress*.

Oxidative stress contributes to many pathological conditions, including cancer, neurological disorders, idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease, asthma [Jenner P. 2003; Sayre LM et al. 2001; Asami S. et al, 1997; Comhair SA et al. 2005; Fitzpatrick AM et al. 2009].

ROS are highly reactive molecules and can damage cell structures such as carbohydrates,

nucleic acids, lipids, and proteins and alter their functions.

Lipids are most susceptible to oxidative modification. Lipid peroxidation generates lipid radicals, which can further attack the subsequent lipid molecules and propagate as a chain reaction. Oxidative modifications to proteins can be deleterious, irreversible (excluding cysteine and methionine) and ultimately leading to protein inactivation and degradation; the damaged protein becomes the target of various endogenous proteases, such as cathepsin c, calpain, trypsin and especially of the proteasome. Compared with lipids and proteins, nuclear DNA seem less susceptible to oxidative modifications because of its double-helix structure and the protective shield from histone and other coating proteins.

In extreme cases, the oxidative damage by ROS leads to alteration and impairment of all structures and cellular macromolecules resulting in cell death.

3.1 Mitochondria as Ros Targets

Despite mitochondria are a major source of ROS, but they are also extremely susceptible to their attack, because mitochondrial membranes contain polyunsaturated fatty acids targets of lipid peroxidation, of which ROS are powerful initiators.

In particular, the mitochondrial phospholipid bilayer, where the respiratory chain is located, contains fatty acids and, therefore, the lipoperoxidation can inhibit energy transduction and modify mitochondrial membrane potential.

Secondly, the inner membrane contains numerous oxidizable enzymes and cofactors, such as NADH-CoQ reductase and ATP synthase. In addition, the mitochondrial DNA is more susceptible to oxidative damage, because of its close proximity to the inner membrane, the major site of ROS generation [Inoue M et al. 2003].

3.2 Oxidative Stress can induces MtDNA damage

In most eukaryotic cells, mitochondria are dynamic organelles with the ability to fuse and divide (fission), forming constantly changing tubular networks, rather than solitary organelles according to old vision [Bereiter-Hahn J et al. 2008].

Within cell, the mitochondrial mass is controlled through either the production (biogenesis) or the degradation (mitochondrial quality control), however, an abnormal signal might also initiate a retrograde response, enabling cell adaptation through increased mitochondrial biogenesis [Michel S et al. 2012].

Mitochondrial morphology is complex, dynamic and changes constantly due to fusion and fission events in response to specific cellular needs.

Damaged mitochondria are fissioned, and then the whole organelle is degraded by mitochondrial autophagy or mitophagy [Kim EH et al., 2007]. But extreme conditions of oxidative stress might alter this balance, allowing an accumulation in the cell of damaged mitochondria [Scherz-Shouval R et al. 2007]. In these damaged mitochondria the electron transport chain might be blocked, resulting in accumulation of ROS [Giacco F et al. 2010]. As MtDNA is located close to the source of ROS production, the mitochondrial DNA could be damaged resulting in accumulation of deletions and mutations [Indo HP et al. 2007]. Accumulation of damaged MtDNA in the cell could cause a chronic innate inflammatory response in the cells. There is growing evidence that oxidative stress plays a significant role in IPF [Kinnula VL et al. 2005; Kliment CR et al. 2010]. Due to its exposure to relatively higher oxygen tensions than other tissues, the lung is particularly sensitive to oxidative stress. Exogenous oxidants and pollutants further increase oxidant production and activate inflammatory cells to generate free radicals. It has been long believed that pulmonary fibrosis begins with alveolar inflammation and that chronic inflammation modulates fibrogenesis [Keogh BA et al. 1982], but the role of the

inflammatory process in the pathogenesis of IPF is however much-discussed. However, Balestro et al. recently highlighted the importance of inflammation among the factors that contribute to determining the rate of disease progression in patients with IPF [Balestro E et al. 2016]. As a direct consequence of inflammation, redox imbalance in the lower respiratory tract has also been proposed to play a role in the lung injury of IPF [MacNee W et al. 1995]. Our hypothesis is that the presence of oxidative stress can induce in IPF patients an alteration of the transcriptional and replication machinery of mitochondrial biogenesis which would be up-regulated resulting in an increased mitochondrial biogenesis by replication of the mitochondrial genome; therefore in its presence they undergo an adaptive response that causes an increase of MtDNA copy numbers. This change could be detected in body fluids (Figure 5).

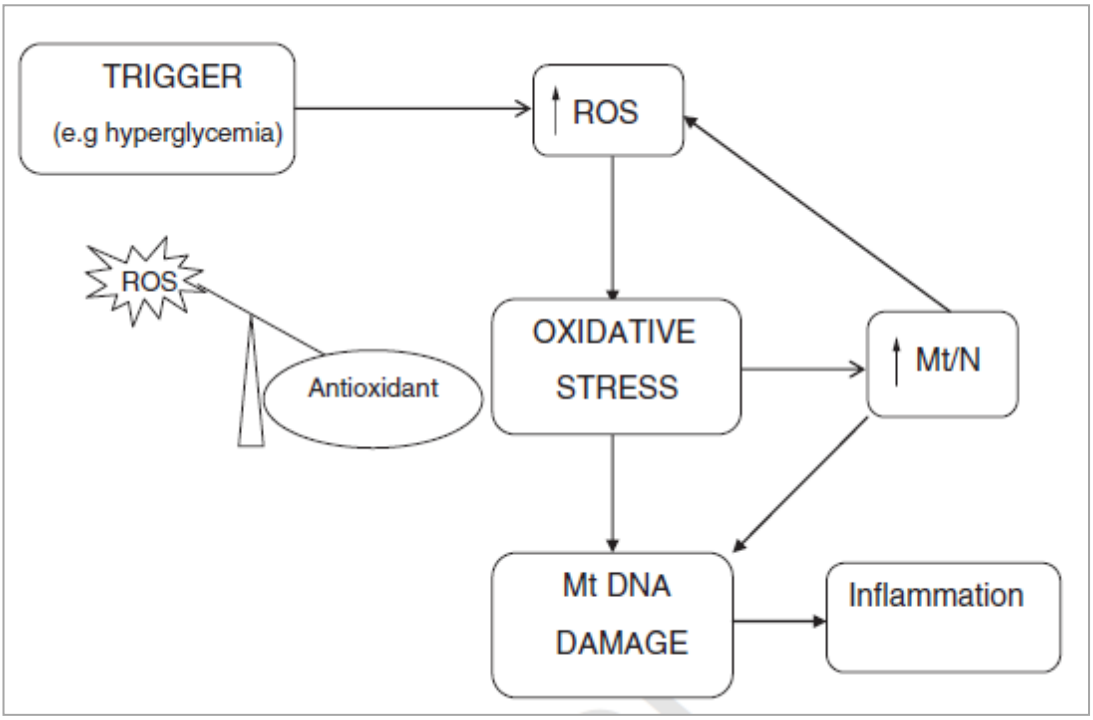


Figure 5 - The Mt/N hypothesis: mitochondrial DNA content measured as Mt/N (mitochondrial to nuclear genome ratio) is a biomarker of mitochondrial dysfunction [Malik AN et al. 2013].

3.3 Quantification of mitochondrial Copy Number

Mitochondria contain their own extra-chromosomal genome, so a common method for measuring MtDNA content is to quantify a mitochondrial encoded gene relative to a nuclear encoded gene to determine the mitochondrial genome to nuclear genome ratio (Mt/N) [Malik AN et al. 2009; Malik AN et al. 2011]. This approach is interesting because the methodology to quantify nucleic acids is more advanced and widely available than the methods to measure the entire mitochondrial organelle or the components of the OXPHOS system, using imaging, cell biology, or protein quantification techniques.

Previous studies measured the Mt/N ratio by nucleic acid hybridization [Veltri KL et al. 1990], e.g. slot blot or Southern blotting, but in the last decade real time qPCR became the main method used [Malik AN et al. 2011].

The method requires the purification of genomic DNA from cells or tissue, the use of qPCR to quantify a mitochondrial encoded gene and a nuclear encoded gene, and then calculate the MtDNA/nDNA ratio [Malik AN et al. 2011; Andreu AL et al. 2009; Gourlain K et al. 2003]. Real-Time qPCR requires less sample and it is a fast technique and widely available. MtDNA is effective as a putative biomarker for the study of mitochondrial dysfunction.

4. MITOCHONDRION

4.1 Mitochondrion definition

The mitochondrion (plural mitochondria) is a membrane-bound organelle found in the cytoplasm of eukaryotic cells. Mitochondria are typically round to oval in shape and range in size from 0.5 to 10 μm. The number of mitochondria per cell varies widely; for example, in humans, erythrocytes (red blood cells) do not contain any mitochondria, whereas liver cells and muscle cells may contain hundreds or even thousands.

It is the power house of the cell; in fact, the primary function of which is to generate large quantities of energy in the form of adenosine triphosphate (ATP). In addition to producing energy, mitochondria store calcium for cell signaling activities, generate heat, and mediate cell growth and death.

The mitochondrion is surrounded by a double-membrane system, consisting of inner and outer mitochondrial membranes that separate it from the cytosol and the rest of the cell components. The membranes are lipid bilayers with proteins embedded within the layers. The inner membrane is folded to form cristae; this increases the surface area of the membrane and maximizes cellular respiration output. The region between the two membranes is the intermembrane space. Inside the inner membrane is the mitochondrial matrix (Figure 6). The outer mitochondrial membrane is freely permeable to small molecules and contains special channels capable of transporting large molecules. In contrast, the inner membrane is far less permeable, allowing only very small molecules to cross into the gel-like matrix that makes up the organelle's central mass.

The matrix contains several copies of the mitochondrial DNA genome, special mitochondrial ribosomes, tRNA a highly concentrated mixture of hundreds of enzymes, including the enzymes of the Krebs cycle, which metabolizes nutrients into by-products

the mitochondrion can use for energy production. The processes that convert these by-products into energy occur primarily on the inner membrane, which is bent into folds known as cristae that house the protein components of the main energy-generating system of cells, the electron transport chain (ETC). The ETC uses a series of oxidation-reduction reactions to move electrons from one protein component to the next, ultimately producing free energy that is harnessed to drive the phosphorylation of ADP (adenosine diphosphate) to ATP. This process powers nearly all cellular activities, including those that generate muscle movement and fuel brain functions.

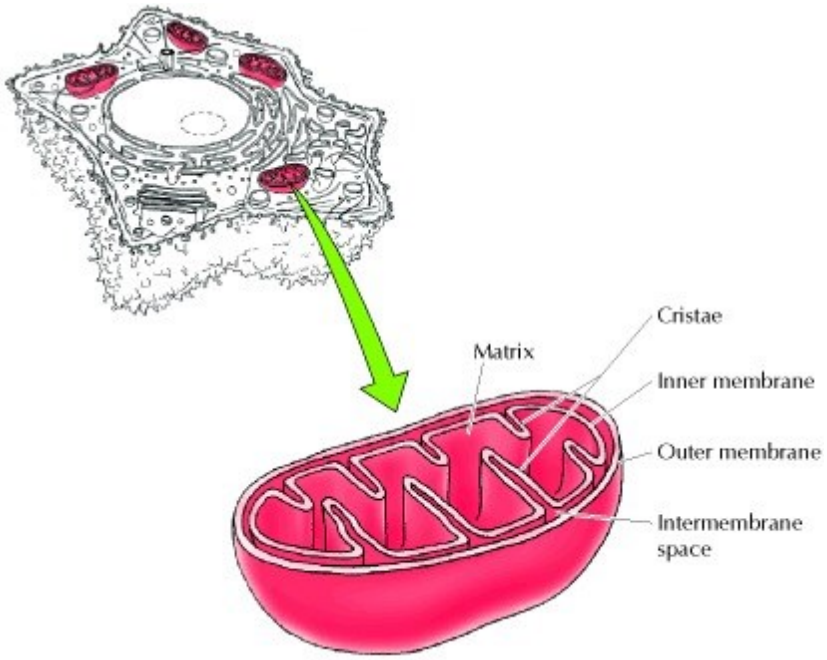


Figure 6 - Structure of a mitochondrion (The Cell, 2nd edition A Molecular Approach Geoffrey M Cooper).

4.2 Mitochondrion function

The mitochondrion is able to perform many functions, but the most prominent roles of mitochondria are to produce the energy currency of the cell through respiration and to regulate cellular metabolism.

However, the mitochondrion are involved in many other functions:

- apoptosis-programmed cell death;
- regulation of cellular cycle;
- regulation of the membrane potential;
- heme synthesis reactions;
- cholesterol synthesis;
- heat production;
- storage of calcium ions and calcium signaling.

Moreover, mitochondria are also the main source of intracellular reactive oxygen species (ROS), this is a consequence of electron transport along the respiratory chain. The inhibition of the mitochondrial respiratory chain promotes the formation of ROS.

Another source of ROS are monoamine oxidases (MAO), enzymes anchored to the outer mitochondrial membrane, which catalyze the oxidation of biogenic amines and during this process they generate hydrogen peroxide (H_2O_2).

The reactive oxygen species, in particular the hydroxyl radical, are harmful because they are able to interact and to oxidize all biological macromolecules (lipids, proteins, nucleic acids and carbohydrates).

Because they are involved in several cellular processes, mitochondrial dysfunction is the focus of numerous oxidative stress diseases: diabetes and obesity, tumors, HIV, OSAS, fertility and growth. Numerous studies have shown how these disorders are related to a condition of oxidative stress.

4.3 Mitochondrial DNA

Mitochondria contain their own genetic system, mitochondrial DNA (MtDNA), which is separate and distinct from the nuclear genome (nDNA) of the cell.

An individual mitochondrion can contain more than one mitochondrial genome, the number has been estimated to be between 0 and 11 copies with a mean of 2.0 [L. Cavelier et al. 2000]. The mitochondria genome is an unmethylated circular double-stranded DNA molecule composed of 16,569 nucleotide bases and contained 37 genes. It encodes 13 polypeptides involved in electron transport chain (ETC) and oxidative phosphorylation, 22 transfer RNAs, and two ribosomal RNAs located in the inner mitochondrial membrane (IMM) matrix (Figure 7). Unlike nuclear DNA, which is passed on from both parents, the mitochondrial genome is inherited maternally. This is because the mother's egg cell donates the majority of cytoplasm to the embryo, and mitochondria inherited from the father's sperm are usually destroyed. Like the DNA of nuclear genomes, mitochondrial DNA can be altered by mutations. The lack of introns, protective histones, and the close proximity to the electron transport chain result in mithocondrial DNA (MtDNA) being more susceptible to oxidative damage than nuclear DNA (nDNA). In addition to this, mitochondria have limited DNA repair capacity [Lee HC et al. 1998]. Therefore an increase of oxidative stress could be very harmful for it.

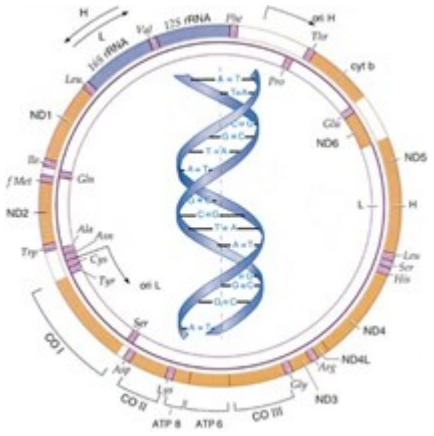


Figure 7 - The human mitochondrial genome.

5. PERIOSTIN

Periostin is a member of the matricellular family of proteins. Matricellular proteins are defined by the ability to bind both to ECM and to cell surface receptors. Other matricellular protein family members implicated in lung fibrosis include osteopontin [White ES et al. 2016], tenascin C [Bhattacharyya S et al. 2016] and secreted protein acidic and rich in cysteine (SPARC) [Chang W et al. 2010]. Periostin was originally identified as osteoblast-specific factor 2 in a mouse osteoblast cell line [Horiuchi K et al. 1999] and is expressed in the periosteum and in the periodontal ligament.

The expression of matricellular proteins can be induced by various cytokines including transforming growth factor (TGF-β), interleukin-4 (IL-4), and IL-13 [Takayama G et al. 2006; Ashley SL et al. 2017].

Periostin is composed of a cysteine-rich domain in its N-terminal portion, four tandemly lined fasciclin I domains in the middle, and an alternative splicing domain in its C-terminal portion [Oka T et al. 2007; Ruan K et al 2009]. Interestingly, up to nine splice variants have been identified, but the full-length transcript encodes an approximately 90 kDa secreted protein that includes all exons [Morra L et al. 2012].

The functional significance of the splice variants is not well understood. Periostin is known to bind type I collagen and fibronectin and has been shown to be involved in collagen fibrillogenesis [Kudo A et al. 2011]. Cells can bind Periostin through cellular integrin receptors and stimulation of cells by Periostin can influence cell adhesion, proliferation, migration and angiogenesis [Horiuchi K et al. 1999].

Not surprisingly, Periostin has been implicated in invasion and metastasis of various tumors [Ruan K et al. 2009].

5.1 Periostin and IPF

Periostin is highly expressed in the lungs and detected in the circulation of patients with IPF [Okamoto M et al. 2011; Naik PK et al. 2012]. It is highly expressed at sites of injury or inflammation and promotes mesenchymal cell proliferation and fibrosis, and plasma levels of Periostin at baseline predict clinical progression in patients with IPF [Naik PK et al. 2012].

Preclinical evidence supporting a role for Periostin in lung fibrosis comes from studies using a mouse model in which Periostin expression has been shown to coincide with the appearance of fibrosis [Uchida M et al. 2012; Naik PK et al. 2012]. Serum Periostin levels in IPF patients were found significantly higher than those in control subjects or patients with other IIPs and are inversely correlated with pulmonary function in patients with IPF; these findings suggest Periostin is a component of fibrosis in IIP and could potentially be used as a biomarker to distinguish histopathologic types of IIP [Okamoto M et al. 2011].

Other studies have also shown that increased serum levels of Periostin are associated with IPF progression [M. Tajiri et al. 2015; Naik PK et al. 2012; Ohta S et al. 2017].

Change in Periostin serum levels were also found to be associated with increasing extent of honeycombing on HRCT; moreover, in the same study population Periostin concentrations demonstrated to be an independent predictor of survival, while a more established biomarker such as KL-6 was not, suggesting that Periostin is a promising ECM biomarker of disease progression and long term outcome in IPF [Tajiri M et al. 2015].

Periostin is produced from bronchial epithelial cells in response to IL-13, a fibrogenic cytokines which are highly expressed in IPF lung tissue and which was found to be involved in the development/progression of IPF [Passalacqua G et al. 2017].

6. MICROSATELLITES

The genomes of higher organisms contain three types of multiple copies of simple repetitive DNA sequences (satellite DNAs, minisatellites and microsatellites) arranged in arrays of vastly differing size [Armour JAL et al. 1999; Hancock JM 1999].

Microsatellites or short tandem repeats (STR), are polymorphic DNA loci containing repeated nucleotide sequences, typically from 2 to 7 nucleotides per unit. The main types of microsatellite are mono-, di-, tri- and tetranucleotide repeats, but also repeats of five (penta-) or six (hexa-) nucleotides are usually classified as microsatellites as well.

Microsatellites are highly polymorphic, abundant and fairly evenly distributed throughout the euchromatic part of the genomes. For these properties, microsatellites result one of the most popular genetic markers for mapping, paternity testing and population genetics [Goldstein DB et al. 1999].

STR are scattered through the genome, mostly they constitute a large fraction of non-coding DNA. However, recently several reports have shown that a large number of STR are also located in transcribed regions of genomes, including protein coding genes and expressed sequence tags (ESTs), although, repeat number of SSRs in these regions are comparatively low [Morgante M et al. 2002; Li YC et al. 2002]. STR variations within these regions can cause frame-shift, alteration in gene expression, inactivation of gene, change of function and eventually phenotypic changes and can cause disease [Li YC et al. 2002].

6.1 Polymerase Slippage

The predominant mechanism of microsatellite length variation is polymerase slippage during DNA replication or repair [Stephan W et al. 1998].

Slippage is a mistake made by the proteins involved in DNA replication (DNA Polymerase), due to the misalignment of the DNA after denaturation, which leading to base-pairing errors and continued lengthening of the new strand and an increase in the number of repeats (i.e. additions). In this case the error occurs on the daughter strand on the contrary, when the error occurs on the parent strand causing a decreased number of repeats (i.e. deletions) [Goldstein DB 1999; Ellegren H 2004].

Despite the tendency to microsatellite instability, occurring in all dividing cells, microsatellites remain stable in length, due to the efficiency of the mismatch repair system [Ben Yehuda A et al. 2000]. But in some cases these control systems could fail and these errors become mutations. It is usually assumed that replication slippage is the main source of mutation in STR.

6.2 Recombination

Another possible cause of mutation is recombination. It may change STR length by unequal crossing over or by gene conversion. This could happen both during meiosis or mithosis. Depending on the motif, unequal exchange may generate unidirectional (either contraction or expansion) or bidirectional changes (both contraction and expansion). In heteroduplex DNA formations (e.g. in the Halliday structure in recombinating homologous chromosomes), slippage and recombination may interact, affecting STR stability. Mutation rates may vary respect to repeat types, base composition of repeats, microsatellite type (perfect, compound or interrupted), length of the region, chromosome position and nature of the flanking sequences. Differences are also found among taxonomic groups [Balloux F et al. 2002].

6.3 Microsatellites and IPF

Alterations of microsatellite markers (MAs) are mainly due to the deletion or insertion, respectively with loss of heterozygosity (LOH) and microsatellite instability (MSI). MSI has been correlated with a high mutational rate and DNA repair processes [Borrill ZL et al. 2008; Kharitonov SA et al2001a]. LOH and MSI are have been previously reported in malignancies of various origin, including lung carcinoma [Ionov Y et al. 1993; Field JK et al. 1995; Froudarakis MEG et al. 1998]. MAs have also been previously studied in DNA samples from IPF patients [Dimitris A et al. 2000]. There is abundant epidemiologic evidence suggesting that patients with IPF exhibit a greater risk for lung cancer development [Vancheri C et al. 2010; Vancheri C 2013; Archontogeorgis K et al. 2012; Bouros D et al. 2002].

This evidence has increased the interest in the identification of common pathways between the two disorders. The basis of this relationship is not yet known. On the molecular level, previous studies have shown that p53 and p21 are expressed especially in hyperplastic bronchial and alveolar epithelial cells of lung tissues from all patients with IPF [Kuwano K et al. 1996]. It is suggested that p53 and p21 are upregulated in association with chronic DNA damage. Tumorigenesis in IPF could be the result of a *p53* mutation due to chronic DNA damage and repair leading to *p53* upregulation [Kuwano K et al. 1996]. Information on the molecular pathway of cancer development is provided by the identification of novel tumor-suppressor genes (TSGs). The inactivation of TSGs plays a critical role in multistage carcinogenesis. At present, the most common methodology used for localizing sites in the genome with high probability for the presence of candidate TSGs is the detection of loss of heterozygosity (LOH), using highly polymorphic microsatellite markers [Sourvinos G et al. 1997].

7. AIMS

Our hypothesis is that the presence of oxidative stress can induce in IPF patients an alteration of the transcriptional and replication machinery of mitochondrial biogenesis which would be up-regulated resulting in an increased mitochondrial biogenesis by replication of the mitochondrial genome, and this change could be detected in body fluids.

To test this hypothesis we used real time qPCR to measure mitochondrial to nuclear genome ratio (Mt/N) in accordance with the early theory which suggests that Mt/N is a biomarker of mitochondrial dysfunction [Malik AN et al. 2013].

The aim of this study is therefore to investigate the oxidative stress, through the measurement of MtDNA/nDNA in the EBC of patients affected by IPF, to give a further contribution to determine if the oxidative stress is really a protagonist in the development of IPF. To better analyze what happens to mithocondria locally and systemically we compared MtDNA/nDNA in blood and EBC of paired patients.

Furthermore, in our study was analyzed the Periostin concentrations in the airways of patients with IPF, evaluating its role as a biomarker to assess specific subtype of ILDs.

Moreover, a small preliminary study was carried out, with the aim to investigate the MAs, in EBC and in the paired whole blood (WB) of IPF patients, using 4 highly polymorphic microsatellite markers, located on several chromosomal arms, where there are some tumor-suppressor genes (TSGs) largely recognized in lung cancerogenesis, that could be part of the complex genetic basis of the IPF and explain the association of this disease and lung cancer. With this preliminary study, we hope to contribute to clarifying the genetic basis of the IPF disease and its relationship with lung cancerogenesis.

8. MATERIALS AND METHODS

8.1 Population

Patients were consecutively recruited for the study from the outpatient facility of the Institute of Respiratory Diseases of the University of Foggia, Italy, between October 2015 and December 2017.

We enrolled 48 IPF patients and 20 ages matched normal subjects. IPF was diagnosed according to the criteria of ATS/ ERS/JRS/ALAT statement for IPF after evaluation of all clinical, laboratory, functional, imaging and histological data [Raghu G et al. 2018]. All patients were treatment naive. This study was conducted in accordance with the amended Declaration of Helsinki. Written informed consent was obtained from all subjects, and the study was approved by our Institutional Ethics Committee of Foggia.

All patients underwent collection of EBC and peripheral blood (WB) sample at enrollment.

8.2 Pulmonary function test

Pulmonary function tests were performed. Forced expiratory volume in 1 s (FEV1), forced vital capacity (FVC) and Carbon Monoxide Diffusing Capacity (DLCO) were measured using a spirometer (Sensormedics, USA). The best value of three maneuvers was expressed as a percentage of the predicted normal value.

8.3 EBC and Blood collection and DNA extraction

1 mL of EBC was collected in one setting from each patient at the time of diagnosis, by using a condenser, which allowed for the non-invasive collection of non-gaseous components of the expiratory air (EcoScreen Jaeger, Wurzburg, Germany). The

condensate was collected on ice at -20°C, transferred to 1,5 ml polypropylene tubes, and immediately stored at -70°C for subsequent analysis. We used the condensate within one month from the storing. The EBC was concentrated with Microcon-30kDa Centrifugal Filter Unit with Ultracel-30 membrane (Merk Millipore, Billerica, MA, USA) and then analyzed.

Blood samples were collected in the morning. A total of 3 ml peripheral blood sample was collected in EDTA tubes and then was stored at -80° C. Whole blood DNA was extracted with QIAamp DNA MiniKit according to the manufacturer's protocol (Qiagen, Hilden, Germany). The concentration of extracted DNA was measured at 260 nm with NanoDrop Spectrophotometer (Thermo Scientific NanoDrop, USA) and was adjusted to 10 ng/µl. Extracted DNA was stored at -20°C until further use.

8.4 Quantitative Real-Time PCR

Mitochondrial DNA copy number was measured by qPCR method using an Applied Biosystems 7300 real-time PCR System (PE Applied Biosystems). MtDNA was measured by quantification of a unique mitochondrial fragment relative to a single copy region of beta-2-microglobulin nuclear gene (β2M) [Malik AN et al. 2011]. Primers, probes (IDT, Integrated DNA Technologies, USA) and gene accession numbers are listed in Table 1. Mitochondrial DNA and β2M probes were labelled at 5' end with 6 FAM and MAX fluorescent dyes respectively and both probes contained BHQ- 1 as a quencher dye at 3' end. The PCR mix was: 1x TaqMan® Universal PCR Master Mix (PE Applied Biosystems), 200 nM of each primer, 125 nM of TaqMan Probe, 50 ng of total DNA extract in a 20 µl PCR reaction. Quantitative real-time PCR conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s of denaturation at 95°C and 60 s of annealing/extension at 60°C. The data presented are the means of 3 measurements.

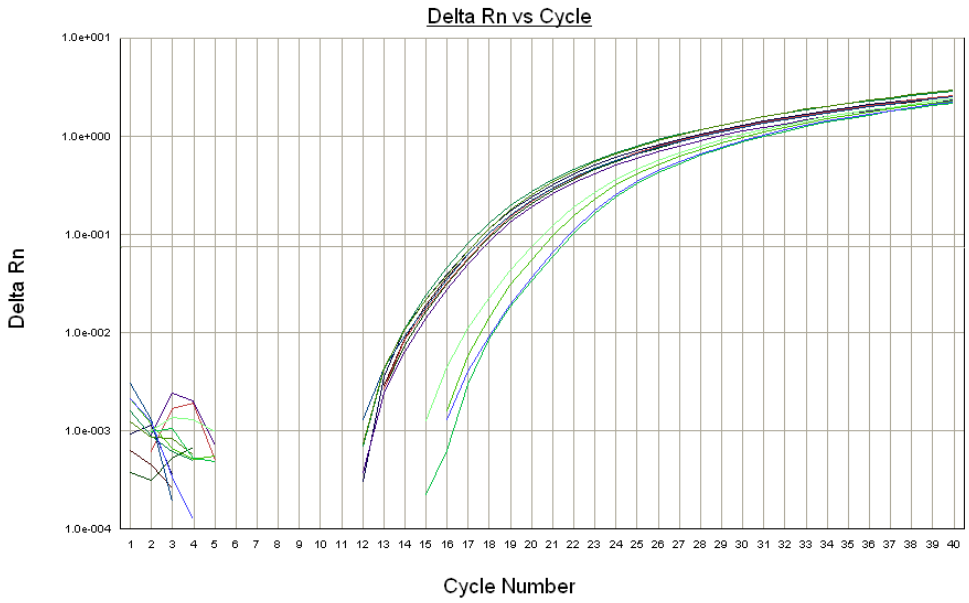
The positive controls (extracted from normal healthy persons) and negative control (DDW + master mix) were added for every PCR run. Standard curves obtained from serial dilutions of PCR-amplified target sequences were used for the quantification of MtDNA and nuclear genome (nDNA), and then the ratio of MtDNA/nDNA was calculated (Figure 8).

Table 1 - Primers/probes used in the study.

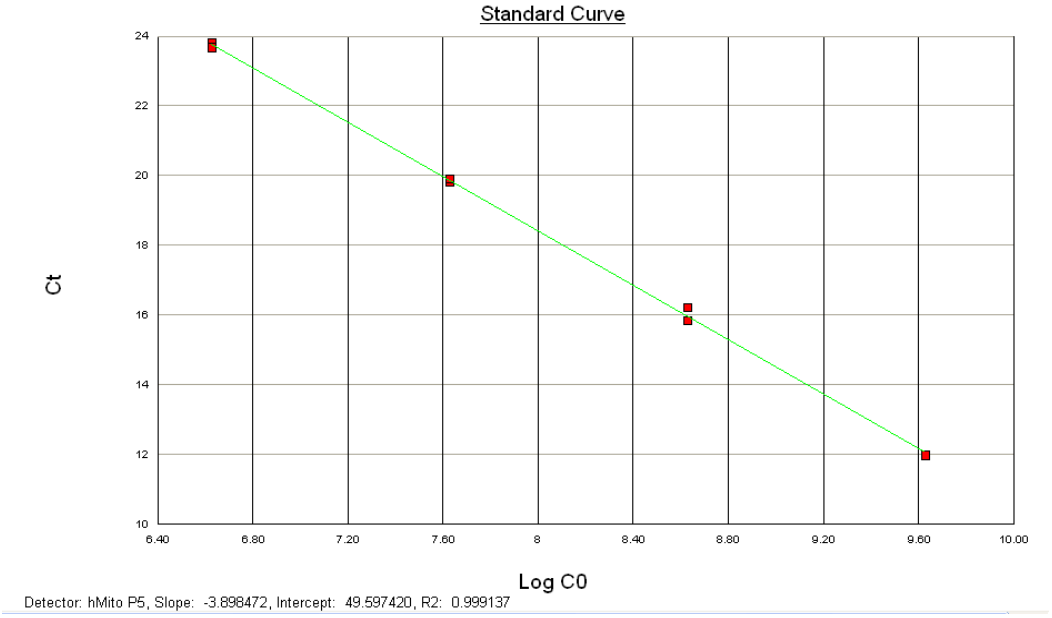
Gene accession number	Primer/probe	Sequence	Product size (bp)
Human mithocondrial genome NC_012920	Mito F	TTAAACACATCTCTGCCAAACC	150
	Mito R	AGATTAGTAGTATGGGAGTGGGA	
	Mito P	AA CCC TAA CAC CAG CCT AAC CAG A	
Human β2M accession number M17987	β2M F	CTTTCTGGCTGGATTGGTATCT	100
	β2M R	CAGAATAGGCTGCTGTTCCTAC	
	β2M P	AG TAG GAA GGG CTT GTT CCT GCT G	

Figure 8 - (A) Kinetic curves of MtDNA obtained by q-Real Time PCR. The reported signal (Rn) is calculated by dividing the amount of fluorescence emitted by the reporter by the amount of fluorescence emitted by a passive report (log ΔRn). Fluorescence is plotted vs cycle number. (B) MtDNA standard curve.

A



B



8.5 Periostin analysis

Initially, a proprietary sandwich enzyme-linked immunosorbent assay (Human Periostin ELISA Kit; Thermo-Fisher Scientific) was used to determine Periostin levels in undiluted samples of EBC. The intra-assay variability was < 10% and the interassay variability was < 12%, with a detection limit of 0.08 ng/mL. Because we achieved unexpected results, below the detection limit, we chose to measure Periostin levels in undiluted samples of EBC using a specific enzyme immunoassay kit (DuoSet ELISA; R&D Systems) according to the manufacturer's recommendations because this is a more sensitive assay. Prior to the test, the EBC was concentrated with Microcon-30kDa Centrifugal Filter Unit with Ultracel-30 membrane (Merk Millipore) and then analyzed together with non-concentrated EBC samples. We carried out several experiments with both EBC samples (concentrated and not), and then we compared the results. Our results showed no significant difference in the concentrations of Periostin; therefore, we decided to use the unprocessed EBC samples for the next test.

8.6 Microsatellite alterations analysis

The analysis of microsatellite alterations was performed using four polymorphic microsatellite markers, located on chromosomal arms 8p and 17q. These markers are D8S137, D17S579, D17S250, THRA1. Nucleotide sequences of primers for microsatellite analysis are available through the Genome database (http://www.ncbi.nlm.nih.gov/genemap-99) (Table 2). One of each paired primer was fluorescent-labeled with FAM and HEX.

Table 2. Primer sequences

Locus	Primer
D8S137	GCTAATCAGGGAATCACCCAA
D17S579	AGTCCTGTAGACAAAACCTG
D17S250	GGAAGAATCAAATAGACAAT
THRA1	CTGCGCTTTGCACTATTGGG

Both EBC-DNA and WB-DNA were amplified by fluorescent PCR.

PCR amplification was performed in 25 µl final volume, and performed on a GeneAmp 9700 thermal cycler (Applied Biosystems) by combining the template with 1 U Taq Master Mix-Hot Start PCR Master Mix (Euroclone) in PCR buffer and 200 nM of each primer. The PCR protocol consisted: initial denaturation at 94°C for 10 minutes followed by 35 cycles of denaturation at 94°C for 50 seconds, annealing at 52°C for 1 minute, extension at 72°C for 2 minutes and a final extension at 60°C for 30 minutes. A negative control (buffer and enzyme without DNA template) was included in every PCR series.

PCR products were separated by electrophoresis on agarose gel to 1.5% and display the transilluminator to UV light at 302 nm. PCR products for each clinical specimen were analyzed by laser fluorescence using an ABI Prism DNA sequencer 310 equipped with GeneScan 2.1 software (Applied Biosystems). This technique allowed to perform a sensitive and quantitative estimation of allelic portions by measuring the height peak of both alleles. These assays allowed us to identify a possible alteration in allelic portion of DNA. Loss of heterozygosity (LOH, reduction of at least 30% in allele intensity) and the presence of allele shifts, indicating genomic instability (MI), were recorded in the various samples of EBC and compared with the profile obtained in the DNA from blood cells.

MI was defined as the appearance of a clear novel band that was absent in the lane from

the healthy control DNA. In our study, each result of amplification was confirmed by at least two independent analyses (Figure 9).

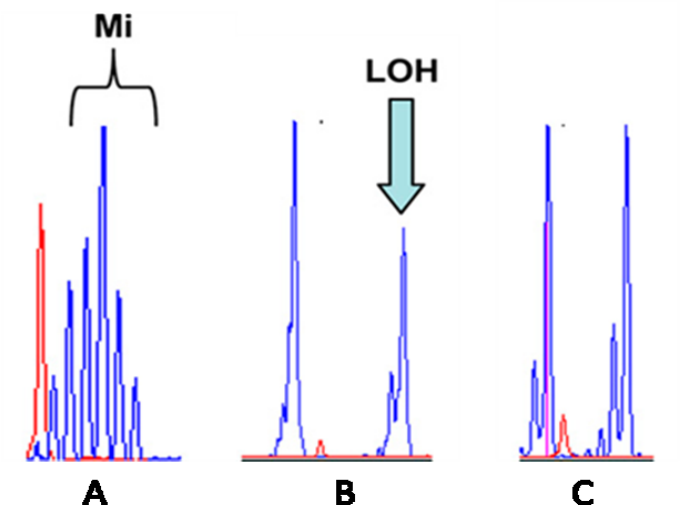


Figure 9 - Analysis of microsatellite DNA. (A) example of microsatellite instability (MI); (B) example of loss of heterozygosity (LOH); (C) example of heterozygosity of the two alleles.

8.7 Statistical analysis

Descriptive statistics (i.e., means, standard deviations, percentages) were applied to summarize the continuous and categorical variables. The relationship between two continuous variables was determined by measuring the Pearsons correlation coefficient. All variables analyzed were normally distributed so Student's T-test was used to compare the mean values. Multiple regression analysis was also used to evaluate the influence of different factors on MtDNA level. The Kaplan-Meier method was used to estimate the probability of survival as a function of time, and survival differences were analyzed using the log rank test.

All P value < 0,05 was considered significant. Statistical Software (Statistica version 8.0, StatSoft, Inc. 2007, USA) was used to analyze the data.

9. RESULTS

Demographic, clinical and functional data of patients affected by IPF and healthy controls enrolled in the study are reported in Table 3.

MtDNA/nDNA was detectable in all EBC samples. IPF patients showed a higher level of MtDNA/nDNA ratio (16.59 ± 10.30 vs 7.94 ± 4.56; $p < 0.005$) than the control group (Figure 10). The level of MtDNA/nDNA was negatively correlated with FVC% in all subjects ($R = -0.4879$, $p = 0.006$), and also we found a negative correlation between MtDNA/nDNA and FEV1% ($R = -0.4364$, $p = 0.018$) (Figure 11 and Figure 12). Others clinical parameters such as age, BMI were not associated with MtDNA directly.

IPF patients showed an increase of MtDNA/nDNA in blood. MtDNA/nDNA in blood of healthy subjects was lower compared to group of IPF patients (170.2 ± 116.06 vs 65.97 ± 20.56; $p < 0.005$) that we analysed (Figure 13). The level of MtDNA/nDNA in blood was significantly correlated with the stage of the disease (Figure 14 – Table 4). Levels of exhaled MtDNA/nDNA were correlated with blood MtDNA/nDNA ($R = -0.4717$, $p = 0.010$) (Figure 15).

Moreover, Periostin was measurable in the EBC of 30 patients of IPF and 10 healthy controls enrolled. We found a significantly higher exhaled Periostin in IPF patients respectively compared to healthy controls (65,5 ± 23,5 pg/mL vs 33 ± 21,4 pg/mL, $p<0.05$). There was no significant correlation between Periostin levels and sex or pulmonary function tests (Figure 16).

To investigate the prognostic effect of blood and EBC mtDNA content in IPF patients, we dichotomized the patients into high- or low-mtDNA copy number group by the median value (141.28 and 19.69 respectively) of mtDNA copy number (Figure 17). We then assessed the prognostic significance of EBC Periostin level, we divided the patients into two subgroups by the median value (60.75) of EBC Periostin content and estimated

the association between this marker and the overall survival of IPF patients (Figure 17). Patients' survival was calculated as the interval between the date of diagnosis and date of death or date of the last follow-up visit and accounted for the most important clinical endpoint. The results of this analysis indicate that both blood and EBC MtDNA/nDNA ratio, and Periostin levels in the EBC were not significantly associated with unfavourable prognostic value (Table 5).

Table 3 - General characteristics of patients with idiopathic pulmonary fibrosis and controls.

	Patients (N=48)	Controls (N=20)	
	Mean ± DS	*Mean ± DS*	*p*
General characteristics			
Males	41	6	0.001
Age (years)	68.6 ± 5.82	66.18 ± 3.72	0.08
BMI (Kg/m^2)	29.8 ± 2.59	27.80 ± 4.64	ns
pH	7.40 ± 0.02	7.40 ± 0.01	0.60
PaO_2 (mm Hg)	72,52 ± 12,06	98.90 ± 18.0	ns
$PaCO_2$ (mmHg)	39.11 ± 4.41	40.42 ± 5.51	ns
FVC (%)	72.86 ± 5.76	103.25 ± 14.05	< 0.0001
FEV_1 (%)	76.79 ± 15.26	90.97±6.63	0.0002
% DLCO (% predicted)	49.73 ± 13.05	-	-
GAP INDEX	3.85 ± 1.33	-	-
Biological Data			
MtDNA/nDNA Blood	170.20 ± 116.0	65.97 ± 20.56	0.0011
Log MtDNA Blood	2.13 ± 0.30	1.80 ± 0.16	0.0011
MtDNA/nDNA EBC	16.59 ± 10.30	7.94 ± 4.56	0.0017
Log MtDNA EBC	1.10 ± 0.35	0.77 ± 0.31	0.0017
Periostin (pg/mL)	65.5 ± 23.5	33 ± 21.4	< 0.05

Abbreviations: BMI: Body Mass Index; FVC: Forced Vital Capacity; DLCO: Carbon Monoxide Diffusing Capacity; FEV1: Forced Expiratory Volume in 1 Second; PaO_2: Partial Pressure of Arterial Oxygen; $PaCO_2$: Partial Pressure of Carbon Dioxide; GAP INDEX: Gender-Age-Physiology.

Figure 10 – Differences between ratio Mitochondrial/Nuclear DNA in EBC of IPF subjects and in control group.

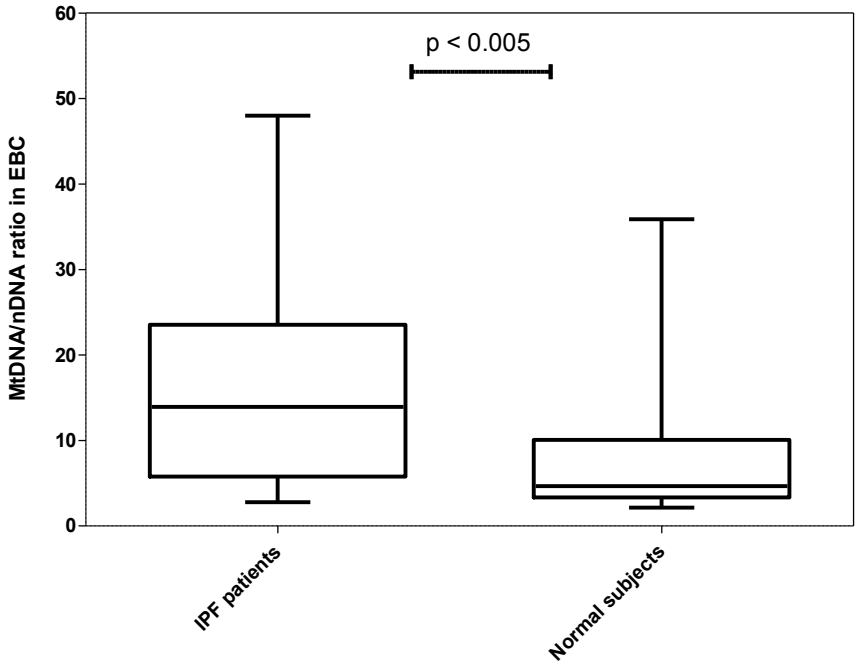


Figure 11 – MtDNA/nDNA ratio correlates negatively with FVC% in EBC of IPF patients: R = -0,4879, p < 0.005.

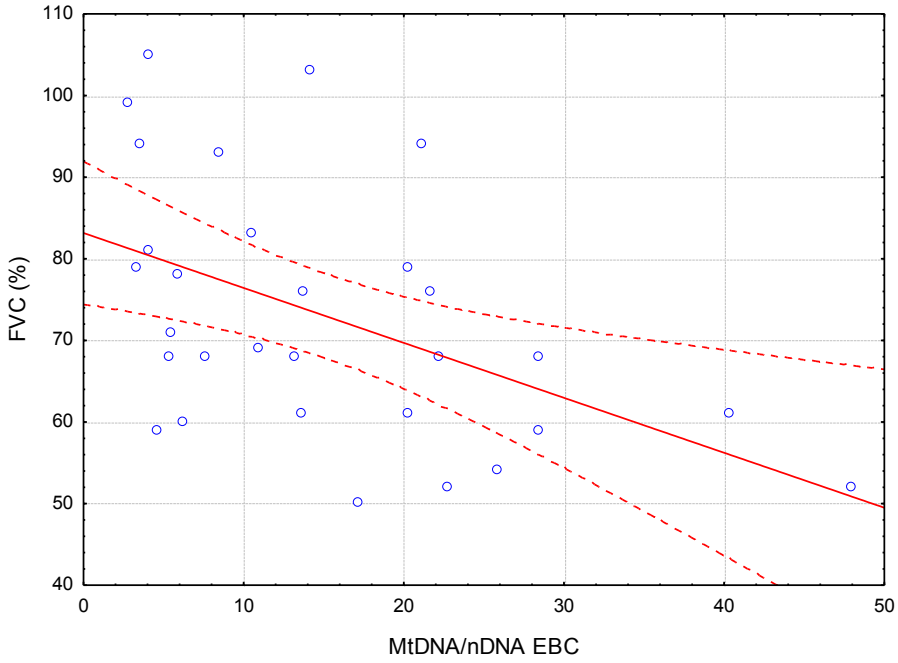


Figure 12 – MtDNA/nDNA ratio correlates negatively with FEV1% in EBC of IPF patients: R = -0,4364, p = 0.018.

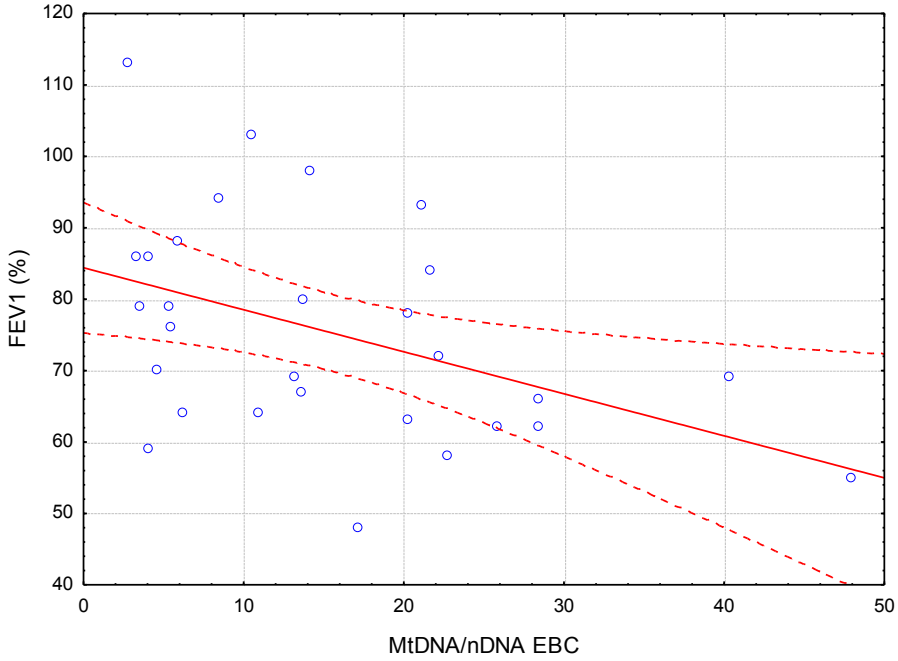


Figure 13 – Differences between ratio Mitochondrial/Nuclear DNA in Blood of IPF subjects and in control group.

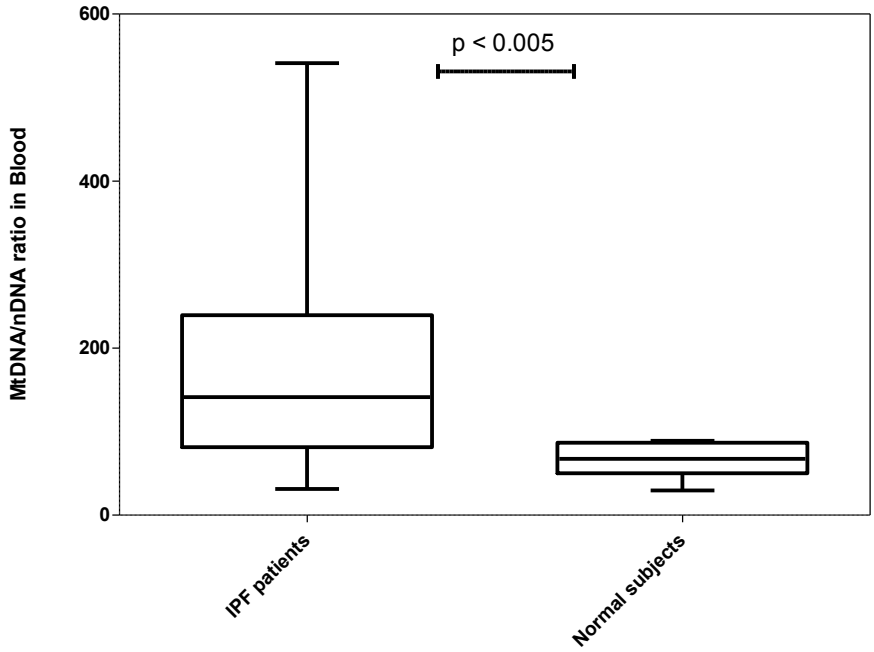


Table 4 - Biological data according to the stage of the disease

	Stage 1	Stage 2	Stage 3	p
Patients (N)	21	25	4	
MtDNA/nDNA Blood	174.09 ± 122.65	144.09 ± 71.73	393.65 ± 149.54	0.01
Log MtDNA Blood	2.14 ± 0.32	2.16 ± 0.26	2.58 ± 0.14	0.01
MtDNA/nDNA EBC	18.54 ± 11.11	13.60 ± 11.83	25.61 ± 4.00	ns
Log MtDNA EBC	1.18 ± 0.32	0.99 ± 0.36	1.41 ± 0.07	ns
Periostin (pg/mL)	73.03 ± 32.40	58.96 ± 20.76	57.75 ± 17.15	ns

Figure 14 – Blood MtDNA/nDNA ratio correlates with the stage: p < 0.05

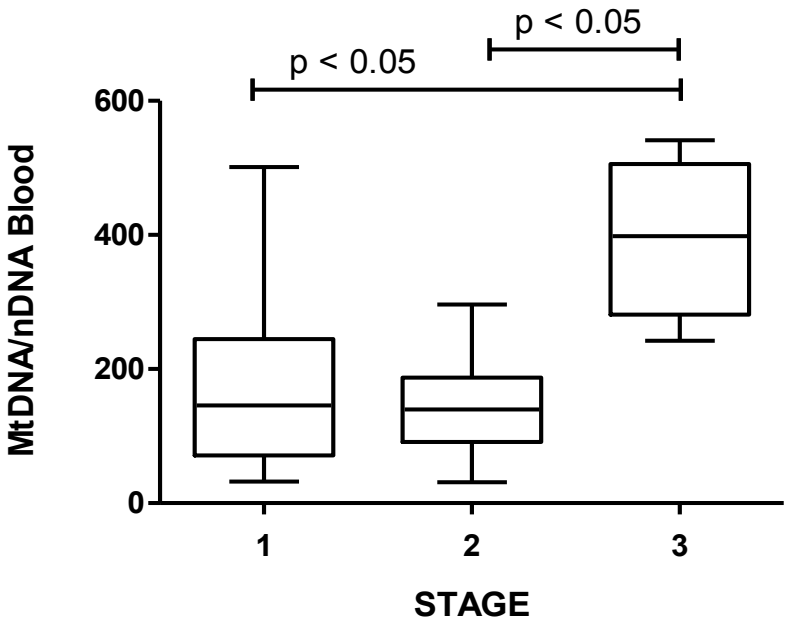


Figure 15 – Correlation between Mitochondrial/Nuclear DNA in EBC and Blood: R = 0.6, p < 0.005.

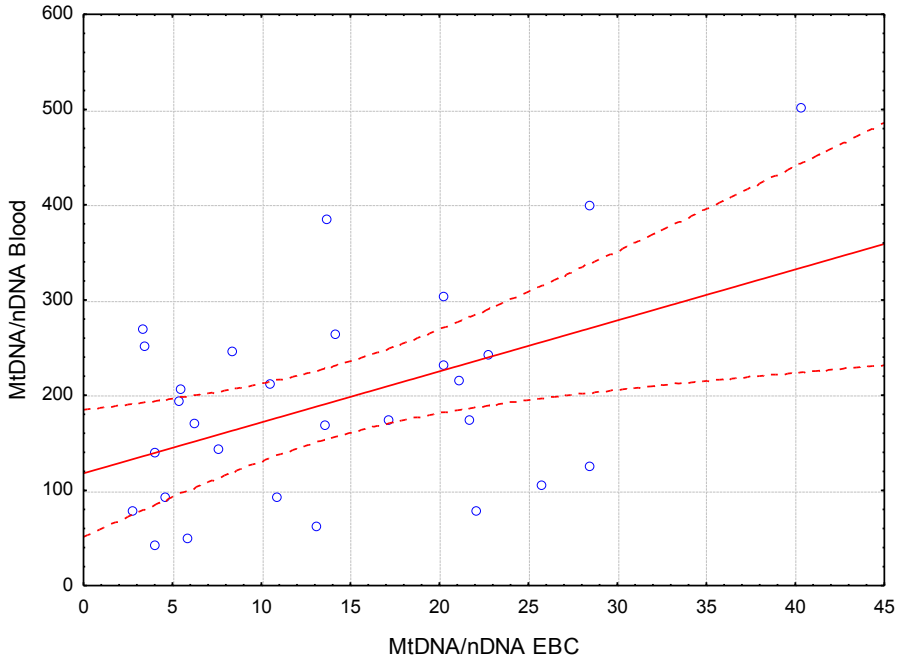


Figure 16 – Periostin levels in the EBC of IPF patients and healthy controls.

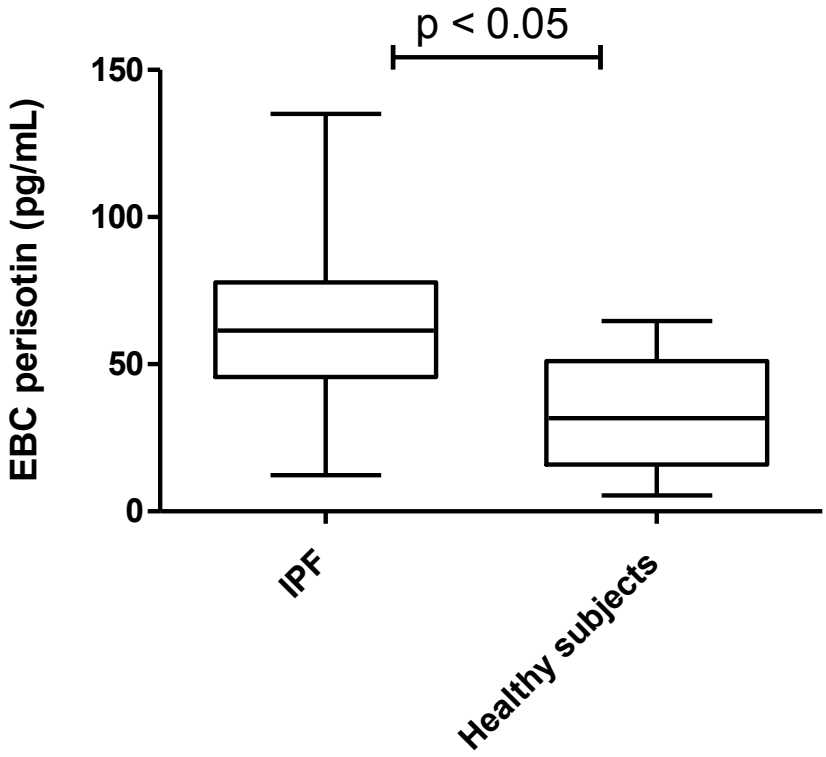


Figure 17 – KM curves of the overall survival analyses in IPF patients. (A) MtDNA/nDNA Blood; (B) MtDNA/nDNA EBC; (C) EBC Periostin levels.

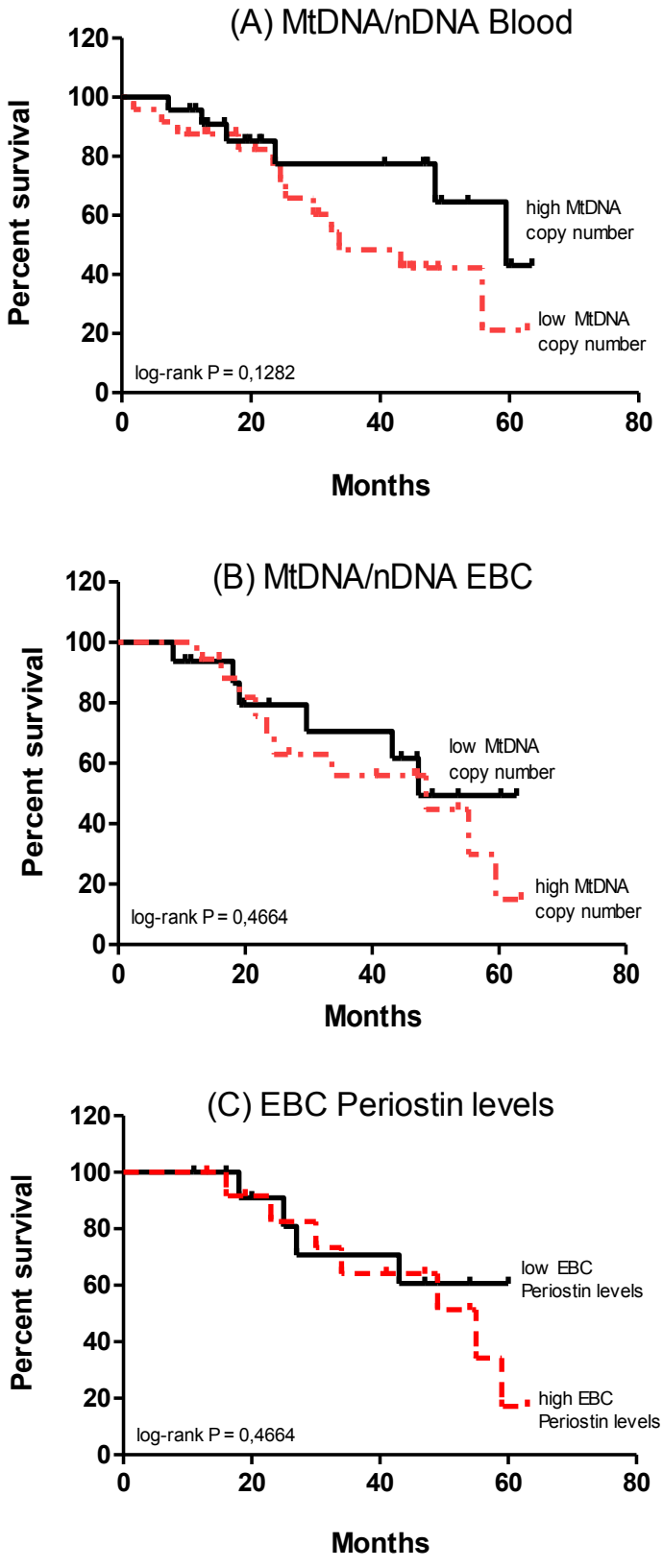


Table 5 – Survival analysis (X^2 and p value for log rank test) for blood and EBC MtDNA content and Periostin content.

	Log-rank test	
	X^2	*P*
MtDNA/nDNA Blood	2,314	0,1282
MtDNA/nDNA EBC	0,5305	0,4664
EBC Periostin levels	0,2710	0,6026

9.1 Prelimanary results

9.1.1 Microsatellite analysis in EBC sample from patients with IPF

We found that all IPF patients showed genetic alterations in their EBC sample, manifested as either MI or LOH, in at least one of the studied markers. Considering each microsatellite as informative when heterozygosity was evident, only three patients had informative results in all four considered loci; regarding the total number of analyzed loci (four for each of 11 patients), 77.27 % (34/44) of the analyses resulted in informative microsatellite. Table 5 shows the results of the microsatellite analysis in EBC-DNA for each locus and patient. LOH was found in 44.11 % (15/34) and MI in 14.7 % (5/34) of the informative loci studied. The most commonly affected microsatellite markers were D17S579 (in 80 % of the informative EBC samples for that locus), D17S250 (in 75 % of the informative EBC samples for that locus) and THRA1 (in 83 % of the informative EBC samples for that locus).

9.1.2 Microsatellite analysis in WB-DNA sample from patients with IPF

We found that three of 11 (27.27 %) IPF patients showed genetic alterations in their WB-DNA sample, manifested only LOH, in at least one of the studied markers. Considering each microsatellite as informative when heterozygosity was evident, only three patients had informative results in all four considered loci; regarding the total number of analyzed loci (four for each of 11 patients), 72.72 % (32/44) of the analyses resulted in informative microsatellite. Eight patients did not present MAs in any of the studied markers. LOH was found in 12.50 % (4/32) of the informative loci studied, and no patients presented MI (Table 7). The most frequently altered microsatellite in WB-DNA was D8S137 (in 20 % of the informative WB-DNA samples for that locus).

9.1.3 Comparison of microsatellite analysis in EBC-DNA and WB-DNA from patients with IPF

A significantly higher number of MAs were present in EBC-DNA with respect to WB-DNA (58.82 vs. 12.50 % of MAs, respectively; $p<0.01$). All MAs found in WB-DNA were also evident in EBC-DNA; conversely, the MAs shown in EBC-DNA were not found in WB-DNA.

Table 6 - Results of loss of heterozygosity and microsatellite instability in exhaled breath condensate sample of 11 patients with idiopathic pulmonary fibrosis.

Patient ID	D8S137 8p21.3-q11.1	D17S579 17q21	D17S250 17q11.2	THRA1 17q12
1	N	H	LOH	LOH
2	LOH	-	LOH	-
3	H	MSI	-	LOH
4	H	MSI	-	LOH
5	H	LOH	H	LOH
6	H	LOH	LOH	H
7	H	LOH	LOH	LOH
8	H	H	LOH	N
9	H	MSI	N	N
10	H	MSI	LOH	N
11	H	MSI	H	N

Abbreviations: LOH: loss of heterozygosity; MSI: microsatellite instability; H: heterozygosity; N: non-informative; – no data available.

Table 7 - Results of loss of heterozygosity and microsatellite instability in blood sample of 11 patients with idiopathic pulmonary fibrosis.

Patient ID	D8S137 8p21.3-q11.1	D17S579 17q21	D17S250 17q11.2	THRA1 17q12
2	H	-	H	-
3	H	H	-	H
4	H	H	-	H
5	H	H	H	H
6	H	H	H	H
7	LOH	H	H	H
8	H	LOH	LOH	-
9	H	N	N	-
10	H	H	H	-
11	LOH	H	H	-

Abbreviations: LOH: loss of heterozygosity; MSI: microsatellite instability; H: heterozygosity; N: non-informative; – no data available.

10. DISCUSSION

The main finging of this study, is that IPF patients have an alteration of MtDNA content and this seems to be related with the increase of oxidative stress levels.

The results lead us to suggest that a positive feedback may exist between pulmonary fibrosis and oxidative stress: increased oxidative stress accelerates pulmonary fibrosis following alveolar cell injury, and progression of pulmonary fibrosis may potentially increase oxidative stress over time.

Oxidative stress is the result of a disorder of the redox balance of the cell, resulting in excessive oxidation of intracellular proteins. Oxidation and reduction of proteins are a major signalling mechanism of intracellular control and are usually mediated via sulfhydril groups of cysteines in proteins and can affect almost all cellular processes including protein folding, protein activity, and numerous biochemical pathways [Hurd TR et al. 2005]. Therefore, an alteration of cells redox balance can have major implications on cell signaling, resulting in cell alterations that could impair the normal function and lead to disease [Lee J et al.2012].

It is known that chronic oxidative stress can cause damage to proteins, lipids and DNA molecules within the cell and it is considered to play a role in many common diseases such as diabetes and its complications, cancer [Dasgupta S et al. 2012], neurodegenerative disorders and IPF [Matsuzawa Y et al. 2015].

In the same way, oxidative stress induces mitochondrial dysfunctions even if its molecular mechanisms are not well understood [Indo HP et al. 2007].

Under conditions of oxidative stress there is an accumulation of excess free radicals, as mitochondrial DNA is located close to the source of ROS production, the DNA itself can become damaged resulting in accumulation of deletions and mutations [Indo HP et al. 2007]. Therefore, in condition of oxidative stress the transcriptional and replication

machinery of mitochondrial biogenesis via replication of the mitochondrial genome is potentiated and the increase in mitochondrial DNA quantity resulting detectable in body fluids [Malik AN et al. 2013; Yang Ai SS et al. 2013].

The human mitochondrial genome is particularly susceptible to oxidative stress because mithocondria have few repair mechanisms so, in its presence they undergo an adaptive response that causes an increase of content of MtDNA as a result of increased mitochondrial biogenesis [Malik AN et al. 2013].

To better explore the role of oxidative stress in IPF we studied a new effective oxidative stress marker that is the abundance of mitochondrial DNA.

Different methods to study MtDNA are available, however, several studies suggest that the measure of content of MtDNA estimated by the mitochondrial to nuclear genome ratio (Mt/N) is a simple way to evaluate the presence of mitochondrial dysfunctions [Malik AN et al. 2009].

Earlier studies measuring MtDNA/nDNA utilized hybridization [Veltri KL et al 1990], but in the last decade real-time quantitative PCR has become a straight forward detection method [Malik AN et al. 2013; D'Souza AD et al. 2007], because it requires less sample and it is a technique which is fast and widely available.

The number of mitochondria in a particular cell type can vary depending on many factors, including the stage of cell cycle, environment and redox balance of the cell, stage of differentiation, and a different cell signaling mechanisms [Michel S et al. 2012]. Individual mitochondria can contain several copies of the mitochondrial genome [Navratil M et al. 2007]. The presence of oxidative stress seems to be the main cause of change in MtDNA copy number. Mitochondria are the major site of reactive oxygen species (ROS) generation, produced during the ATP production by electron leakage that occurs in the mitochondrial electron transport chain [Bonner MR et al. 2009]. As well as

in energy production, mitochondria are also involved in the regulation of numerous other cellular functions including cell proliferation, apoptosis, and intracellular calcium homeostasis [Malik AN et al. 2013]. Several studies have shown how these disorders are related to a condition of oxidative stress [Malik AN et al. 2013]. Because mitochondria are involved in several fundamental cellular processes, their dysfunction can affect a range of important cellular functions and can lead to a variety of diseases [Michel S et al. 2012]. The role of mitochondrial dysfunction in numerous diseases is well documented [Malik AN et al. 2011].

The results of this part of the study indicate that an increased abundance of mitochondrial DNA is present in the EBC and blood of individuals with IPF suggesting an increased prevalence of oxidative stress and is in line with Daniil et al. who reported an increase of the oxidative stress level in IPF [Daniil ZD et al.2008] and Naik et al. who suggested that the oxidative stress, mediated by ROS and NADPH oxidase (NOX) activity, promotes pulmonary fibrosis [Naik E et al. 2011]. Also, Zhou et al., previously demonstrated that after an inflammatory stimulus, the accumulation of damaged mitochondria precipitates in an increase in mtROS production [Zhou R et al.2011].

In the second part of this study, we have shown that Periostin levels are detectable in EBC with significant differences between patients with IPF and ILDs and healthy control subjects. Periostin is highly expressed in the lungs and is found at increased levels in the circulation of patients with IPF [Naik PK et al. 2012; Okamoto M et al. 2011]. This same study have shown that elevated levels of Periostin in the serum o plasma can predict a decrease in lung function over 6 months or 48 weeks respectively [Naik PK et al. 2012; Okamoto M et al. 2011]. Importantly, Periostin may be a relevant biomarker for disease activity in these older patients as new research suggests that in normal subjects, Periostin levels are stable from age 32 past 70 [Walsh JS et al. 2017]. This elevation in Periostin

during IPF disease is not surprising when you consider that two well-known pro-fibrotic mediators, namely TGF-β [Khalil N et al.1991; Khalil N et al. 1991] and IL-13 [Passalacqua G et al. 2017] are also highly increase in Periostin seen in IPF. It is believed that Periostin may play an important role in helping to stiffen the lung ECM. For instance, cross-linking between collagen fibrils is catalyzed by lysyl oxidases. Periostin can activate bone morphogenetic protein-1 to cleave lysyl oxidase; this in turn activates lysyl oxidase while also localizing this active enzyme to the ECM [Maruhashi T et al. 2010]. Ultimately, this leads to crosslinking of the collagen fibers and stiffening of the ECM. This increase in the stiffness of the ECM is believed to promote ongoing fibroblast activation, which may perpetuate the progressive nature of IPF [Tschumperlin DJ et al. 2012].

To our knowledge, this is the only study to date where Periostin levels have been measured in the airways (EBC) of patients with IPF. Furthermore, EBC is a collection method from airways that is rapid, that is completely noninvasive, that can be used in patients with IPF, and that is safe. These characteristics may be important when the patients to be analyzed are those with IPF, frustrated for numerous diagnostic tests that they underwent for diagnostic and follow up purposes and moreover, they soon develop early respiratory failure becoming unable to perform also common tests required.

Our findings show that Periostin concentrations in EBC reach values that may be detected by a commercially available ELISA kit.

A limitation of this part of the study is instead measurement of Periostin in matched serum samples. However, now we focused on airways, but we are planning to evaluate this aspect in a future study. Anyway, these results support our working hypothesis that Periostin is dosable in the airways of patients with IPF. As the circulating one, airways

Periostin may be a potential biomarker to support IPF diagnosis and to monitor disease progression during follow-up.

In the last preliminary part of this study, we detected genetic alterations at the microsatellite level, located within or near TSGs implicated in lung cancer. The 11 patients with IPF demonstrated that these could be part of the complex genetic basis of this disease and in its frequent association with cancer. We found that all patients showed genetic alterations in their EBC-DNA, consisting of either MI or LOH. No healthy subjects exhibited these alterations. Nine (81.81 %) patients exhibited LOH and five (45.45 %) exhibited MI in at least one microsatellite marker. To reveal MI or LOH, we have selected three microsatellites at the 17q arm surrounding the BRCA1 region and one microsatellite at the 8p21.3–q11.1 arms, where interesting TSGs are located, due to the fact that deletions of this region occur frequently in lung tumors [Lerebours F et al. 1999]. Lung cancer has been largely associated with deletions at 17q near the BRCA1 region [Fong KM et al. 1995]. We have found a high incidence of MAs at the level of 17q11.2–q21 locus in IPF subjects enrolled, where important TSGs are located, that could explain their predisposition to lung cancer. We found, for the first time, an increased number of MAs in EBC of IPF patients, thus making this sample suitable for this type of analysis also in this fibrosing disease. Moreover, we need to remember that patients with IPF are particularly frustrated for the numerous diagnostic tests that they undergo; therefore, we believe that research markers in noninvasive samples as EBC could have an important advantage in the monitoring and follow-up of IPF patients. We believe that it would be very interesting for future studies, to monitor these IPF patients to see whether the presence of genetic alterations at the level of the studied loci may predispose them to the development of lung cancer. However, this is an observational preliminary study that we are planning to continue on a larger population that will be

followed prospectively over time. In the case this will be proven, we believe that it would be interesting to develop a new therapeutic approach, taking advantage of the vast knowledge that already exists on cancer biology or even “borrowing” drugs, as in the case of nintedanib, which has been developed or used for cancer and can be particularly useful in IPF associated with or at risk of lung cancer. Finally, the recognition of new common pathogenic mechanisms between IPF and cancer may also encourage new research studies on this topic and new IPF clinical trials directed toward personalized treatments as largely experimented in cancer.

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11. CONCLUSION

Our main results suggest that in IPF subjects the transcriptional and replication machinery of mitochondrial biogenesis will be up-regulated resulting in an increased mitochondrial biogenesis via replication of the mitochondrial genome and this change could be detected in body fluids and airway. The MtDNA/nDNA may be considered an easy and potential marker of the oxidative stress. This study highlights the fact that it makes sense to study oxidative stress in IPF as it plays a key role in the development and progression of the disease and might be a new target of biological therapeutic strategies. Furthermore, from our point of view, this research field could open interesting sceneries to better understand the pathophysiology of others pulmonary diseases and non-pulmonary diseases in which oxidative stress is always involved, because if we can control the oxidative stress pathway we may be able to create therapeutics for a wide range of chronic diseases.

With the results of the second part, we confirmed that it is possible to study Periostin in EBC. For the first time, we analyzed Periostin in airways of patients with IPF. Airways Periostin may be a potential biomarker to support IPF diagnosis and to monitor disease progression during follow-up.

In the last preliminary part of the study, we showed that it is possible to study MAs of 17q in the EBC of patients with IPF and that these could be implicated in the etiopathogenesis of the disease, often complicated by association with lung cancer. Further studies are needed to evaluate the clinical significance and the prognostic value of these genetic alterations in light of important clinical implications that they might have.

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