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Evaluation of Oxidative Stress Alveolar Dysfunction Biomarker and micro-RNA Signatures in Idiopathic Pulmonary Fibrosis: Case Control Study

A Dissertation

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

﴿إِنِّي جَزَيْتُهُمُ الْيَوْمَ بِمَا صَبَرُوا أَنَّهُمْ هُمُ الْفَائِزُونَ﴾

صَدَقَ اللَّهُ الْعَظِيمُ

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To the Prophet Muhammad and his family (peace be upon them)

My parents, the source of love and security

To my husband, my support and partner

To my children (**Muhammad, Rawan, and Ruqayya**), my efforts were
for a future worthy of you

To my husband's family, thank you for your support. My sincere
gratitude and appreciation to you all

**To all of you, I dedicate this effort, which would not have been
complete without you**

Asseel

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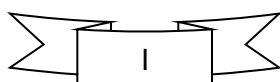
SUMMARY

Summary

Idiopathic pulmonary fibrosis (IPF) is a poorly understood pathogenesis. Alveolar epithelial cell dysfunction and oxidative stress are major contributing factors, yet comprehensive physiological and molecular studies remain limited. Therefore, the present study was designed to investigate the relationship between oxidative stress and alveolar dysfunction in IPF and to evaluate the diagnostic potential of certain physiological and molecular biomarkers.

The study was conducted in the Department of Biology /College of Science /Al-Qadisiyah University in cooperation with under the supervision specialist doctors at Imam Al-Sadiq Teaching Hospital in Babylon Governorate and the Respiratory Diseases Center in Diwaniyah City, during the period from December , 2024 until the end of November 2025 , the total number of samples was 120 including 60 IPF patients (39 males and 21 females ;mean age 58 ± 11.9 years), and 60 age/ sex-matched healthy control groups who had no history or clinical evidence of PF or asthma. Patients and control group information were collected via a questionnaire that included name, age, sex, and smoking status. Pulmonary function (FVC% (Forced Vital Capacity), FEV1/FVC%1) was assessed. Blood samples (6 ml) were drawn from all individuals ,then analyzed to assess changes in hematological parameters, in addition to alveolar dysfunction markers , including: integrin $\alpha\beta6$, cleaved cytokeratin-18 (cCK-18), and type COL1A1 collagen, as well as oxidative stress markers: growth factor differentiation factor 15 (GDF15), superoxide dismutase (SOD), and antioxidant response regulator protein 2 (Nrf2), using an ELISA assay. Expression of miRNA-374 and miRNA-153 was quantified by qRT-PCR.

The study results showed that no significant difference ($P > 0.05$) was observed between the groups of IPF patients with respect to mean age ,sex and BMI



However, the variation among the patient samples themselves, with the difference in the number of males to females. The study also indicated that smoking was present in only a limited percentage (15%) of older male patients. Clinical and hematological findings showed that the majority of patients (70%) had severe disease (forced vital capacity ratio = $36.11 \pm 6.9\%$). Patients with idiopathic pulmonary fibrosis exhibited a marked increase in white blood cell count (8.89 ± 1.8 vs. $7.82 \pm 1.3 \times 10^3/\mu\text{L}$, $p=0.015$), an increase in neutrophil count ($75.11 \pm 10.1\%$, $p=0.002$), and a decrease in lymphocyte count (1.66 ± 0.41 vs. 2.19 ± 0.32 , $p=0.001$), indicating a marked innate immune shift. Patients with idiopathic pulmonary fibrosis showed significantly elevated levels of the integrins $\alpha\beta6$ (71.47 ± 12.77 vs. 28.36 ± 4.71 ng/mL, $p<0.001$), cCK-18 (51.14 ± 11.3 vs. 38.07 ± 4.54 ng/mL, $p<0.001$), and COL1A1 (44.08 ± 9.81 ng/mL, $p<0.001$). ROC curve analysis demonstrated excellent diagnostic accuracy for both $\alpha\beta6$ (Area under the curve = 0.983, sensitivity 96.7%) and COL1A1 (Area under the curve = 0.982, sensitivity 96.7%).

Oxidative stress tests showed significant increases in serum GDF15 levels (554.02 ± 47.4 vs. 428.27 ± 18.99 pg./mL, $p<0.001$), SOD3 (10.07 ± 1.73 vs. 8.08 ± 1.42 ng/mL, $p=0.021$), and Nrf2 (53.52 ± 11.01 vs. 32.42 ± 3.25 ng/mL, $p<0.001$). These results demonstrated exceptional diagnostic power (Area under the curve = 0.983, sensitivity 96.7%). Significant negative correlations were also found between GDF15 ($r=-0.275$, $p=0.033$), Nrf2 ($r=-0.279$, $p=0.031$), and forced vital capacity (FVC%), directly linking oxidative stress to functional decline.

Regarding the molecular findings, patients with idiopathic pulmonary fibrosis showed elevated levels of miRNA-374 (2.51 ± 0.58 vs. 0.41 ± 0.1 , $p<0.001$) and decreased levels of miRNA-153 (0.25 ± 0.06 vs. 2.66 ± 0.91 , $p<0.001$). Both miRNAs demonstrated excellent diagnostic accuracy (Area under the curve = 1.000, sensitivity/specificity 100%). miRNA-374 was positively correlated with neutrophil count ($r=0.500$, $p=0.001$) and COL1A1 ($r=0.312$, $p=0.015$). Notably, miRNA-153

showed a strong inverse correlation with both the percentage of forced vital capacity ($r = -0.613$, $p = 0.001$) and the number of lymphocytes ($r = -0.493$, $p = 0.001$), suggesting a protective role, the loss of which leads to both fibrosis and immune system dysfunction. Logistic regression analysis revealed an inverse correlation between miRNA-374 and miRNA-153.

This study provides strong evidence that IPF is characterized by a vicious cycle of oxidative stress by GDF15/Nrf2 dysregulation and alveolar epithelial injury by $\alpha\text{v}\beta 6$ /cCK-18/COL1A1 dysregulation. The antagonistic regulatory profiles of miRNA-374 (promoting fibrosis) and miRNA-153 (anti-fibrosis) represent a novel molecular signature. The excellent diagnostic of $\alpha\text{v}\beta 6$, COL1A1, and Nrf2, and especially miRNA-374/153, qualifies these as potent non-invasive biomarkers for IPF. These findings open new avenues for early diagnosis and potential therapeutic targeting of the oxidative stress-miRNA-alveolar dysfunction axis

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List of Abbreviations

Abbreviation	Full Name
AECs	Alveolar epithelial cells
ANOVA	Analysis of Variance
$\alpha\text{v}\beta 6$	alpha-v beta-6 integrin
BAL	Bronchoalveolar Lavage
BLM	Murine Bleomycin
CBC	Complete Blood Count
cCK-18	Cleaved cytokeratin-18
COL1A1	Collagen 1A1
CT	Computed Tomography
DLCO	Diffusing Capacity of the Lung for Carbon Monoxide
ELISA	Enzyme- Linked Immunosorbent Assay
EBV	Epstein–Barr Virus
ECM	Extracellular Matrix
EDTA	Ethylene Diamine Tetra Acetic Acid
EMT	Epithelial-to-Mesenchymal Transition
FVC	Forced Vital Capacity
FEV1	Forced Expiratory Volume in 1 Second
GDF15	Growth factor differentiation factor 15
GRA	Granulocytes
HCT	Hematocrit
HGB	Hemoglobin
HRCT	High-Resolution Chest Scan
HRP	Horse Radish Peroxidase
ILD	Interstitial Lung Disease
IPF	Idiopathic Pulmonary Fibrosis
IL-1β	Interleukin-1 β
LYM	Lymphocytes
MONO	Monocytes

MUC5B	Mucin 5B
M1	Macrophage Type 1
M2	Macrophage Type 2
MDD	Multidisciplinary Discussion
MAPK	Mitogen-Activated Protein Kinase
MDTF	Myofibroblast-Derived Transforming Factor
MNT	MAX Network Transcriptional Repressor
NEU	Neutrophils
Nrf2	Nuclear factor erythroid 2
PLT	Platelets
PI3K	Phosphoinositide 3-Kinase
qPCR	quantitative analysis of RT-PCR
ROC	Receiver Operator Characteristic
ROS	Reactive Oxygen Species
SD	Standard Deviation
SNP	Single Nucleotide Polymorphism
SP-B	Surfactant Proteins-B
SPSS	Statistical Package for Social Sciences
SOD/EC-SOD3	extracellular superoxide dismutase
TBLB	Transbronchial Lung Biopsy
TBB	Transbronchial Biopsy
TERC	Telomerase RNA Component
TERT	Telomerase Reverse Transcriptase
TGF-β	Transforming Growth Factor- β
TLD	Terminal Lung Disease
TNF-α	tumor necrosis factor-alpha
UIP	Usual Interstitial Pneumonia
WBC	White Blood Cell

INTRODUCTION

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic and serious respiratory disease characterized by progressive fibrosis of the lung interstitial tissue, leading to progressive impairment of lung function, shortness of breath, and a reduced quality of life (Raghu et al., 2022).

Alveolar Epithelial cells exhibit metabolic changes and dysfunction during pulmonary fibrosis, leading to dysfunction in key regulatory pathways of fibrosis in the lung, including transforming growth factor beta (TGF- β). The integrin $\alpha\text{v}\beta 6$ is an important *in vivo* activator of TGF- β in the lung. $\alpha\text{v}\beta 6$ enters into a closed interaction with TGF- β , where each increases the activity of the other, and this causes fibrosis (Brzozowska & Deshmukh, 2022). This activation triggers a cascade of events that include the stimulation and differentiation of fibroblasts into myofibroblasts, and increased of fibroblast proteins such as COL1A1 (Madala *et al.*, 2025). The most abundant COL1A1, constitutes the matrix of lung tissue and plays an important role in tissue repair and remodeling (Devos *et al.*, 2023).

In addition to (cCK-18), which is part of a lung cell protein, it is produced during the apoptosis of alveolar epithelial cells (AECs) (Bai *et al.*, 2025). Studies have indicated that IPF patients exhibit significantly higher serum levels of cCK-18 compared to healthy controls (Ley *et al.*, 2014).

Recent evidence suggests that oxidative stress plays a fundamental role in the development of IPF by causing chronic damage to AECs (Fois *et al.*, 2018). These changes are linked to several physiological and molecular indicators, such as GDF15, the expression of this factor stimulated in response to cellular stress. (Takenouchi *et al.*, 2020) The results show that GDF15 is associated with cellular responses to oxidative stress and inflammation. (Cao *et al.*, 2022)

Studies indicate that superoxide dismutase (ECSOD/SOD3) plays a pivotal role in protecting tissues from damage caused by oxidative stress by removing the superoxide anion. which prevents the formation of other more potent oxidants such as peroxy nitrite and hydroxyl radical which represents the primary line of defense against free radicals in the lung(Kinnula *et al.*, 2006). Also, (Nrf2) is a central regulator of cellular antioxidative responses, inflammation, and restoration of redox balance It is a key factor in the development of IPF (Yu *et al.*, 2022).

Molecularly, some microRNAs, such as miRNA-374 and miRNA-153, have demonstrated a pivotal regulatory role in gene expression associated with endothelial cell damage and fibrosis.(Alonso *et al.*, 2023).Studies have shown that miRNA-374 may contribute to the regulation of gene expression related to the integrity of the pulmonary epithelial barrier, inflammatory processes, and the cell's response to oxidative stress (Bu *et al.*, 2017; Kim *et al.*, 2019).

Decreased expression of miRNA-374 may be associated with increased permeability in epithelial cells, contributing to the leakage of pro-inflammatory factors and fibrosis(Kim *et al.*, 2019). While miRNA-153 is associated with regulating the response to oxidative stress and cellular stress in many tissues, including the lung (Zhu *et al.*, 2018) .It is also associated with regulating the expression of genes which control apoptosis and cell survival, making it a vital player in the balance between survival and damage in epithelial cells affected by IPF(Liang *et al.*, 2015).

Studying the expression of miRNA-374 and miRNA-153 in IPF patients and relationship between these markers and oxidative stress on the one hand, and alveolar dysfunction in IPF patients on the other, is a fundamental step toward understanding disease mechanisms and developing accurate diagnostic tools that contribute to improving therapeutic intervention strategies.

1.1. Aims of the study

The main aim of this study was to elucidate the molecular interplay between oxidative stress and alveolar epithelial dysfunction in Idiopathic Pulmonary Fibrosis (IPF), focusing on the regulatory roles and epigenetic expression profiles of miRNA-374 and miRNA-153 to establish them as novel, non-invasive diagnostic biomarkers linked to disease progression and severity. This overarching aim was achieved through the following specific objectives:

- Study the demographic and clinical characteristics of patients diagnosed with IPF, including age, sex, BMI, smoking status, and disease staging.
- Assess the serum levels of selected biomarkers related to alveolar epithelial dysfunction including: integrin $\alpha\beta6$, cCK-18, COL1A1 and Oxidative Stress which include: GDF15, ECSOD or SOD3, Nrf2 in both IPF patients and healthy controls, to identify potential associations between these biomarkers.
- Investigate the expression levels of microRNA-374 and microRNA-153 in blood samples using molecular techniques, and examine their association with oxidative stress and alveolar epithelial injury in IPF patients.
- Also aims to determine the potential use of these biomarkers as diagnostic tools.

LITERATURE REVIEW

2. Literature Review

2.1. Definition & Epidemiology of IPF

IPF is a progressive, non-neoplastic, often fatal lung disease characterized by scarring of the lungs, leading to thickening of the lining of the lungs and causing irreversible loss of the tissues' ability to transport oxygen. (Spagnolo *et al.*, 2021) the mechanisms of fibrosis in IPF poorly understood, favored concepts of disease pathogenesis involve recurrent subclinical injuries to a genetically predisposed alveolar epithelium, followed by failure of alveolar re-epithelialization and repair .(Meltzer & Noble, 2008) . The incidence and prevalence of IPF are difficult to determine because uniform diagnostic criteria have only recently been defined. IPF is a rare disease which affects approximately 5 million persons worldwide.(Katzenstein & Myers, 1998), determining the incidence and prevalence of IPF has been challenging, as standardized diagnostic criteria have only been established in recent years. Global data indicate that IPF does not show a preference for any specific race, ethnic group, or socioeconomic background.(Golchin *et al.*, 2025) It is estimated that IPF affects at least 5 million people worldwide, and evidence suggests that its incidence has been increasing over the past decade (Directors & Committie, 2000)

IPF affects men more than women, the higher incidence of IPF in men is not fully understood, but research suggests a combination of genetic, hormonal, and environmental factors may be involved.(Gribbin *et al.*, 2006; Tondo *et al.*, 2025) In addition, the incidence of IPF increases with age. IPF most commonly appears between the fifth and seventh decades of life, with two-thirds of all cases arising in patients over 60 years of age (Meltzer & Noble, 2008).The average age at IPF diagnosis is 66 years, with the disease rarely occurring in individuals under 40 and almost never in children. A large U.S. population-based study also showed a clear increase in prevalence with age (Directors & Committie, 2000) .

2.2. Potential risk factors for developing IPF

The factors that trigger the plethora of profibrotic pathways involved in IPF pathogenesis remain unknown, but advancing age, cigarette smoking, viral infection, chronic aspiration of gastric content and genetic factors are all associated with an increased risk of IPF, probably by causing an alveolar epithelial barrier injury.(Spagnolo *et al.*, 2021)

2.2.1. Cigarette Smoking

Case-control studies have identified cigarette smoking as a potential risk factor for the development of IPF, with reported odds ratios (ORs) ranging from 1.6 to 2.9 across different regions worldwide. A strong association between smoking and IPF has been observed,(Gribbin *et al.*, 2006) with one study showing that individuals with a smoking history of 20–40 pack-years had an increased risk of developing IPF, reflected by an odds ratio of 2.3 (95% confidence interval: 1.3 to 3.8) (Gustafson *et al.*, 2007).

2.2.2. Aging

Population studies have demonstrated an increased incidence and prevalence of IPF with older age, with the majority of cases diagnosed in the fifth through seventh decades of life(Nho, 2017) Indeed, premature presentation with pulmonary fibrosis in patients with dyskeratosis congenita, a disease of accelerated aging, led to the sequencing of the telomerase genes that have been associated with IPF(Zaman & Lee, 2018)However, shortened telomeres have been observed in both peripheral blood leukocytes and lung tissue of IPF patients relative to controls have been reported even in those IPF patients without mutations in the telomerase genes.(Stuart *et al.*, 2014; Snetselaar *et al.*, 2017) Beyond abnormal telomere shortening, the molecular basis underlying the susceptibility of the ageing lung to a dysregulated

response to lung microinjury includes a senescent phenotype in IPF lung fibroblasts that alters the ratio of proliferation to apoptosis (Bailey *et al.*, 2013; Nho, 2017)

2.2.3. Sex

Across the world, IPF is more prevalent in men, who account for approximately 70% of all cases globally, based on data derived from national registries and international trials of IPF (Jo *et al.*, 2017). Initially, the increased risk among men was attributed to inhaled exposures, such as tobacco smoking or occupational exposures, which historically have been more common among men. However, sex-stratified analyses have demonstrated that specifically men with inhalational exposures are more likely to develop IPF than women (Gustafson *et al.*, 2007)

In the study of Carlsson *et al.* (1995) indicated that male sex hormones in mouse models were associated with accelerated fibrosis, while female sex hormones might be protective against pulmonary fibrosis. It has also been suggested that sex hormones are one possible cause of the differences between the sexes, particularly in the mouse model of bleomycin-induced pulmonary fibrosis, indicating an interaction between sex and exposure (Walters & Kleeberger, 2008).

The effect of sex hormones varies by organ and species, so it is necessary to study sex hormones in human samples to determine their role in IPF (Zaman & Lee, 2018).

2.2.4. Genetic Predisposition to IPF

Hereditary factors may play a role in IPF development, with the strongest evidence coming from familial cases. Familial IPF (FPF) is diagnosed when at least two first-degree relatives have histologically confirmed IPF. It is likely inherited in an autosomal dominant pattern with variable penetrance and affects both genders equally (Ley *et al.*, 2014). While no specific genetic markers have been confirmed, Some evidence suggests that genetic variations in the $\alpha 1$ -antitrypsin gene (which protects the lungs from protein degradation) may increase susceptibility to IPF, but

this is uncertain(Barnes, 2022). In contrast, there is no clear role for HLA(Human Leukocyte Antigen)genes in the disease, which distinguishes IPF from other autoimmune lung diseases and supports the idea that its primary mechanism stems from epithelial damage rather than an autoimmune dysfunction. (Zaman & Lee, 2018).

Clinical features of familial IPF are indistinguishable from those of the sporadic form, excepting for an earlier age of onset(Garcia, 2004). Familial IPF is defined as two or more confirmed cases within a single immediate family and accounts for 0.5–2% of all IPF cases. Lung inflammation has also been observed in unaffected family members.(Alonso *et al.*, 2023; Meltzer & Noble, 2008).

2.3. Clinical description of IPF

IPF patients experience breathlessness upon exertion. They are often bothered by a dry cough which interferes with daily activities. The onset of symptoms is slow, but symptoms become progressively worse over time. The initial presentation of breathlessness is commonly attributed to aging, cardiac disease, or emphysema which results in typical delays of diagnosis. Retrospective analysis of IPF patients suggests that symptoms precede diagnosis by a period of 6 months to 2 years(Dong *et al.*, 2006).

Symptoms such as weight loss, fever, and arthralgias are unusual in IPF and should prompt an investigation for secondary causes of pulmonary fibrosis.(Spagnolo *et al.*, 2021) Auscultation of the lungs reveals early inspiratory crackles, predominantly located in the lower posterior lung zones upon physical exam. These rales have a fine acoustic character reminiscent of the sound made by Velcro. Clubbing is found in approximately 50% of patients with IPF. There are no other physical manifestations, unless cor-pulmonale has developed in association

with end-stage disease. In that case, classic signs of right heart failure may be present.(Meltzer & Noble, 2008)

The examination of patients with IPF should attempt to identify those signs suggesting an alternative diagnosis such as systemic sclerosis or polymyositis that can be associated with secondary pulmonary fibrosis. To that end, the examiner should look for sclerodactyly, scleroderma, proximal muscle weakness and telangiectasias. The history should exclude Raynaud's phenomenon.(Herzog *et al.*, 2014)

2.4. Diagnosis of IPF

The diagnosis of IPF is often delayed up to several years. The objective of this study was to assess the impact of the diagnostic delay on progression- free survival, quality of life and hospitalization rates.(Hoyer *et al.*, 2022)

2.4.1. History and Physical

A common clinical presentation for IPF is dyspnea, which is usually progressive, debilitating, and persistent for >6 months in an older adult. Dry cough, unrelieved with antitussives is also common. On physical exam crackles are detected on chest auscultation in more than 80% of patients. These are often “dry,” occur at end-inspiration, and have a “velcro” quality.(Raghu *et al.*, 2006; Strykowski & Adegunsoye, 2023), Rales can also be heard, particularly with disease progression. Clubbing is present in up to half of all patients. Evidence of right sided heart failure including, an accentuated second pulmonic sound, right ventricular heave, and peripheral edema may be seen in the late stage of the disease and associated with cor pulmonale(Marinescu *et al.*, 2022)

2.4.2. Laboratory and Serological Tests

There are no laboratory tests to make the diagnosis of IPF, so the role of laboratory testing in patients with newly discovered ILD is to identify or exclude an etiology for the disease.(Raghu *et al.*, 2006) These laboratory tests are usually looking for subclinical rheumatologic disease or hypersensitivity pneumonitis. They should be interpreted cautiously as an antinuclear antibody $\geq 1:40$ is present in 17-25% of patients with IPF and a positive rheumatoid factor is present in up to 18% of patients with IPF(Moua *et al.*, 2014).

2.4.3. Chest Imaging

Most patients with IPF have an abnormal chest radiograph at the time of presentation. Peripheral reticular opacities, most profuse at the lung bases are typical findings on chest radiograph. The opacities are usually bilateral, often asymmetric, and associated with volume loss.(Gandhi *et al.*, 2023; Moua *et al.*, 2014).

High-resolution computed tomography (HRCT) is the preferred imaging method for diagnosing IPF, significantly improving the ability to detect the disease at earlier stages (Strykowski & Adegunsoye, 2023).

2.4.4. Pulmonary Function Testing

Comprehensive pulmonary function testing (PFT), including spirometry, lung volumes, and diffusing capacity for carbon monoxide (DLCO), is performed in all patients suspected of having IPF. These tests are typically done alongside measurements of oxygen saturation at rest and during walking. PFTs are conducted at baseline and repeated regularly to evaluate lung function and monitor disease progression. IPF usually shows a restrictive pattern characterized by reduced total lung capacity (TLC), reduced forced vital capacity (FVC), and decreased DLCO. Over time, patients may also show oxygen desaturation or reduced distance during a 6-minute walk test(Hasan, 2023; Raghu *et al.*, 2022; Strykowski & Adegunsoye, 2023).

2.4.5. Lung Biopsy

In the evaluation of suspected IPF, tissue biopsy can support diagnosis when imaging and clinical data are inconclusive. Transbronchial lung biopsy (TBLB) uses forceps to obtain small lung samples, but its diagnostic yield is limited—only about one-third of cases lead to a clear diagnosis, with most requiring further testing.(Raghu *et al.*, 2022)

Transbronchial cry biopsy (TBCB) offers a promising alternative, producing larger and higher-quality samples than TBLB, and may provide similar diagnostic value to surgical lung biopsy (SLB) when interpreted within a multidisciplinary discussion (MDD). However, its role in the diagnostic algorithm is still under investigation.(Hetzel *et al.*, 2020; Maldonado *et al.*, 2020).

Surgical lung biopsy remains the gold standard for histopathological confirmation of IPF and is recommended when the diagnosis is uncertain and the potential benefits outweigh the risks. It should be performed as part of an MDD and avoided in patients with advanced hypoxia or significant comorbidities. If (HRCT) clearly shows a usual interstitial pneumonia (UIP) pattern, biopsy is often unnecessary, as it is unlikely to change the diagnosis or management.(Raghu *et al.*, 2022; Strykowski & Adegunsoye, 2023; Zhao *et al.*, 2025).

2.5. Pathogenesis of IPF

While pathogenetic mechanisms are incompletely understood, the currently accepted paradigm proposes that injury to the alveolar epithelium is followed by a burst of pro-inflammatory and fibroproliferative mediators that invoke responses associated with normal tissue repair. For unclear reasons, these repair processes never resolve and progressive fibrosis ensues.(Meltzer & Noble, 2008) One leading theory suggests that IPF develops due to abnormal function of fibroblasts and epithelial cells, as well as disrupted epithelial-mesenchymal interactions, with

minimal or no involvement of inflammation. This is supported by histological evidence showing fibroblastic foci beneath damaged epithelial areas, without significant inflammatory cell presence.(Hecker & Thannickal, 2011) The onset and progression of fibrosis may be influenced by genetic predisposition, environmental exposures, oxidative stress, and imbalances in certain cytokines.(Strykowski & Adegunsoye, 2023).

Myofibroblasts and related mesenchymal cells are widely recognized as the key drivers of lung tissue distortion and fibrosis in IPF. The proliferation of fibroblasts and their transformation into myofibroblasts play a central role in disease development. However, the exact stage at which these cells acquire their pathological characteristics, as well as the mechanisms behind their abnormal differentiation and the influence of signals from epithelial cells, immune cells, and the extracellular matrix, remain unclear and require further investigation(Conroy *et al.*, 2016).

The lung's extracellular matrix (ECM) is made up of collagens, elastin, glycoproteins, and proteoglycans, providing structural support, mechanical stability, and elasticity for proper lung function. During normal wound healing, myofibroblasts produce ECM proteins and are then eliminated through apoptosis once repair begins. The initial scar tissue is usually replaced by stable, functional tissue. In IPF, however, myofibroblasts are resistant to apoptosis, leading to their prolonged presence and excessive ECM production. This results in persistent scarring. A defining feature of IPF is the presence of fibroblastic foci clusters of myofibroblasts—and widespread ECM buildup in the lung interstitium, which disrupts the normal alveolar structure (Bellaye & Kolb, 2015; Strykowski & Adegunsoye, 2023).

Vascular muscularization driven by repeated small injury is the main pathogenesis of pulmonary hypertension PH , leading to the sustained fibrotic

process of intima. Different cellular processes have been described during pulmonary artery remodeling of pulmonary hypertensin associated IPF, including endothelial dysfunction , Endothelial-Mesenchymal Transition as a source of myofibroblasts and pulmonary artery smooth muscle proliferation (Hashimoto *et al.*, 2010)

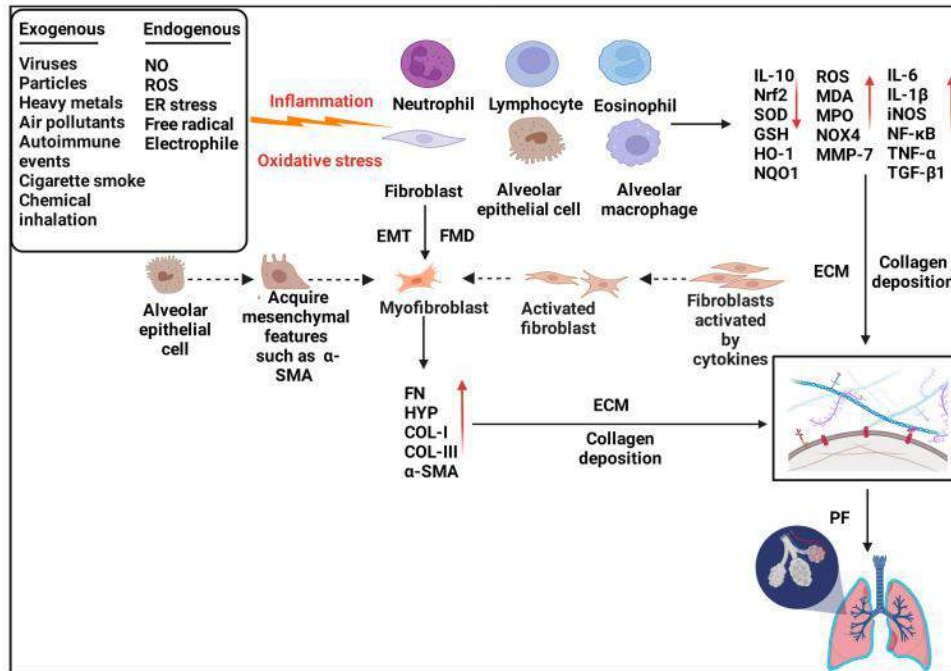
Monocytes and macrophages play a central role in tissue repair and fibrosis, and several molecular pathways involved in their function are being explored as potential therapeutic targets for IPF. Macrophages contribute to fibrosis by promoting fibroblast proliferation,(Song et al., 2000) regulating extracellular matrix (ECM) components, and releasing pro-fibrotic cytokines and growth factors.(Atabai *et al.*, 2009) However, they can also have antifibrotic effects. These contrasting roles are thought to be due to different macrophage subtypes: classically activated (M1) macrophages, which are generally antifibrotic, and alternatively activated (M2) macrophages, which are associated with profibrotic or regulatory functions(Tylah, 2022)

Adaptive immune responses play a role in the pathogenesis of IPF. Activated CD4+ T-cells are often found in the lungs of IPF patients even before symptoms appear. Additionally, mature dendritic cells infiltrate the lung tissue. Regulatory T-cells (FoxP3+), which help control immune responses, are reduced in both the blood and bronchoalveolar lavage fluid (BALF) of IPF patients (Koichi *et al.*, 2015)

2.6. Alveolar Dysfunction Biomarkers in IPF

Various exogenous and endogenous factors, can lead to damage to the alveolar epithelial cells (AECs), aberrant activation of immune cells and fibroblasts. These lung cells secrete multiple pro-inflammatory and pro-fibrotic factors which can accelerate the EMT process, induce the transition of AECs to lung fibroblasts, trigger the activation of quiescent fibroblasts, and promote the differentiation of fibroblasts

to myfibroblasts (Kulkarni *et al.*, 2016). Myofibroblasts can further release a large amount of collagen and ECM, resulting in hyperproliferation of fibroblasts and accelerates the progression of pulmonary fibrosis (figure 2-1) (Yu *et al.*, 2022).



Figure(2-1): Pathogenesis of pulmonary fibrosis (Yu *et al.*, 2022).

2.6.1. alpha-v beta-6 (α v β 6) integrin:

2.6.1.1. Definition and Localization:

Integrins are $\alpha\beta$ glycoproteins that bridge the cell membrane and connect the outside and inside. Integrins bind to certain extracellular proteins in the extracellular matrix or to other cells and then transduce signals intracellularly, influencing cell function.(Schnapp *et al.*, 2021) α v β integrin an integrin complex found mostly on the surface of airway epithelial cells. Its levels are quite low in healthy lungs, but they increase dramatically when there is chronic lung injury or inflammation, and subsequently become more prominent in fibrosis(Decaris *et al.*, 2021).

The ITGB6 gene in humans encodes integrin beta-6. This is the $\beta 6$ subunit of the integrin $\alpha v\beta 6$.(Fabregat et al., 2016) Integrin $\alpha v\beta 6$ is found only on epithelial cells. Most resting normal cells produce little ITGB6, although the largest quantities are found in the stomach, gall bladder, and lungs(Lian *et al.*, 2023).

2.6.1.2. Physiological effects of $\alpha v\beta 6$ integrin in IPF:

Idiopathic pulmonary fibrosis is characterized by elevated TGF- β signaling, which promotes fibroblast-to-myofibroblast transition, collagen gene expression, and scar tissue deposition that affects pulmonary function.(Kulkarni et al., 2016)

The $\alpha v\beta 6$ binds the RGD region of the inactive TGF- $\beta 1$ complex, which is related with ,latency-associated peptide) LAP.(Conroy et al., 2016) The cell's mechanical forces trigger the release of TGF- $\beta 1$ in a specific area of its surface. This activation triggers the (Smad2/3) pathway within neighboring cells, (figure2-2) causing fibroblasts to expand into myofibroblasts and, as a result, enhanced collagen deposition.(Golovina *et al.*, 2025; Margadant & Sonnenberg, 2010).

Integrin $\alpha v\beta 6$ -mediated TGF- $\beta 1$ activation is non-inflammatory as it inhibits cytokine diffusion to surrounding tissue, maintaining immunological homeostasis and promoting localized fibrosis. However, chronic overexpression—as seen in IPF—leads to excess ECM deposition, permanent alveolar remodeling, and eventually lung function decrease.(Margadant & Sonnenberg, 2010).

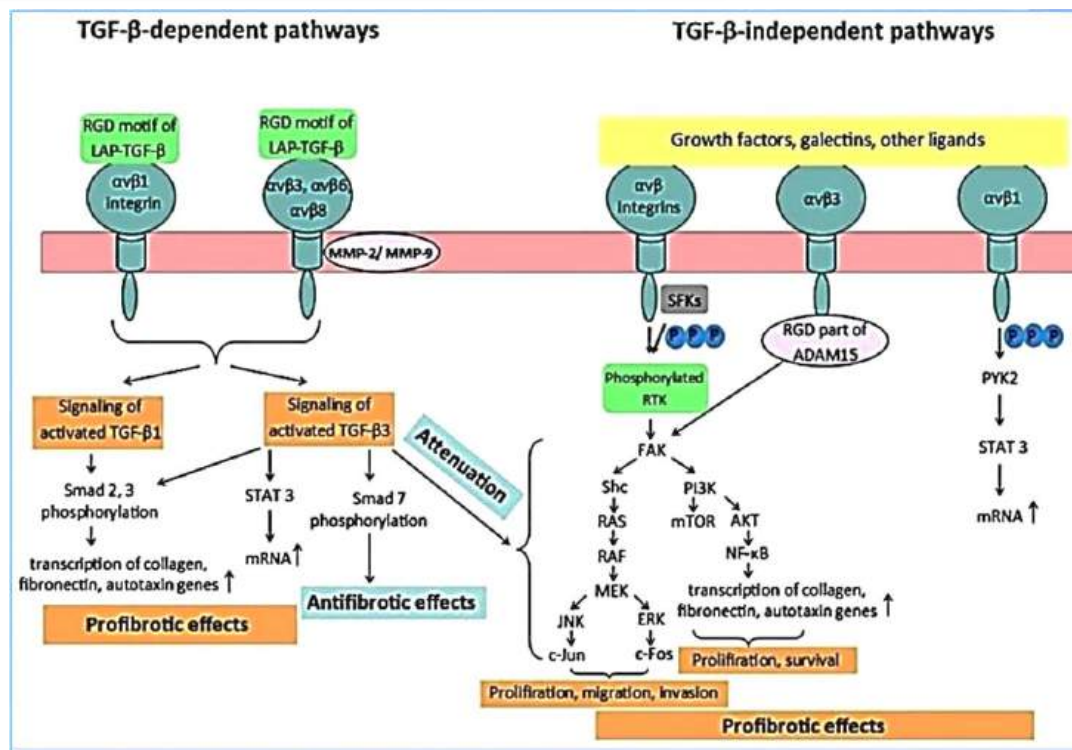


Figure2-2 : Integrin-associated fibrosis pathways. Direct and indirect such as. transforming growth factor beta TGF-β; MMP-2 - matrix metalloproteinase 2; MMP-9 - matrix metalloproteinase 9; P - phosphoric acid residues; SFKs - SRC family kinases; RTK - receptor tyrosine kinase; FAK - focal adhesion kinase; ERK - extracellular signaling regulator kinase; PI3K - phosphatidylinositol kinase 3; AKT - protein B kinase; mTOR - mammalian target of rapamycin; PYK2 - proline-rich tyrosine kinase 2; STAT - transcription activator; RGD - arginyl-glycyl-aspartic acid; ADAM15 - metalloproteinase 15.(Golovina et al., 2025)

2.6.2. CCK-18 and epithelial cell death

2.6.2.1. Definition and Localization:

Cleaved cytokeratin 18 (cCK-18), is a fragment of a lung cell protein, released when lung epithelial cells (AECs) die via apoptosis. It has been proposed that cCK-18 is produced during AEC apoptosis(Zamfir et al., 2023). and is detectable with M30 antibodies (Cha et al., 2012). It is a unique marker for alveolar epithelial cells because it is not released during the lysis of other cells (Conroy et al., 2016) A recent study investigated its diagnostic and prognostic role and suggested that IPF patients

present significantly increased serum cCK18 levels compared to healthy controls, while its prognostic role in IPF was not confirmed (Cha et al., 2012).

2.6.2.2. Physiological effects of (cCK-18) in IPF

In IPF patients, serum cCK-18 levels are significantly elevated compared to other ILDs. Furthermore, cCK 18 is activated early during unfolded protein response activation, before complete apoptosis. Thus, cCK 18 is a mechanistically informative biomarker of epithelial damage and ER stress in IPF, but it does not independently predict disease progression. (Cha et al., 2012; Zamfir et al., 2023).

2.6.3. Collagen type I alpha 1 Chain (COL1A1GENE)

2.6.3.1. Definition and Localization

The collagen family comprises 28 proteins whose function is to strengthen and support many body tissues, including cartilage, bones, tendons, skin, and the whites of the eyes. These proteins are primarily expressed in the extracellular matrix (ECM) and are characterized by a triple helical structure. (Tsitoura et al., 2021) They are associated with several diseases, most notably fibrosis and bone disorders. Collagen type I alpha 1 (COL α 1(I)) (COL1A1) is the most abundant extracellular matrix protein and is the one most strongly associated with fibrotic diseases. COL1A1 is regulated at the alveolar level by tumor growth factor beta (TGF β) (Devos et al., 2023) COL α 1(I) also interacts with a variety of cell receptors, including beta integrins. Therefore, it is highly expressed in fibrotic lung tissue. (Decaris et al., 2021)

2.6.3.2. Physiological effects of COL1A1 Gene

Fibrosis is a complex disease characterized by fibroblast activation, leading to matrix remodeling culminating in a stiff, type I collagen-rich fibrotic matrix. Which imparts abnormal tissue stiffness and impairs the lung's mechanical

properties.(Spagnolo et al., 2021) Alveolar epithelial cell (AEC) apoptosis is also a major feature of fibrogenesis, and AEC apoptosis is sufficient to initiate a robust lung fibrotic response. TGF- β (transforming growth factor- β) is a major driver of fibrosis and can induce both AEC apoptosis and fibroblast activation. that changes in extracellular matrix stiffness and composition can regulate the cellular response to TGF- β .

Progressive fibrosis is characterized by an accumulation of activated myofibroblasts, which produce a collagen-rich scar Thus, much of the prior fibrosis research has focused on the activation and function of myofibroblasts most studies focused on the role of myofibroblasts in collagen production (Agarwal *et al.*, 2020). recent research has shown novel role for monocyte-derived macrophages during tissue damage and wound healing, is the expression of collagens. Macrophages within the lung play regulate role the progression and maintenance of fibrosis.(Tsitoura *et al.*, 2021) As injury progresses into a more chronic phase, the provisional matrix is degraded and replaced by a fibrotic matrix primarily made up of fibrillar (COL1A1),Although collagen I is often regarded as an end product of fibrosis, it is also a key signaling molecule with a number of cell surface receptors such as integrins, that can influence cell behavior like adhesion, migration, and proliferation (Decaris *et al.*, 2021).The role of collagen I signaling during fibrosis has not been well studied. Early collagen deposition may therefore have an important role in further collagen production and may help determine the extent of fibrosis.(Agarwal *et al.*, 2020)

2.7. Oxidative Stress Biomarkers in IPF

Oxidative stress is defined as an imbalance in the production of reactive oxygen species (ROS) that exceeds the ability of cells/tissues to detoxify or scavenge them. Such oxidative stress can affect the structure/function of cellular macromolecules, resulting in tissue/organ malfunction (Zitka *et al.*, 2012). There is a significant and rising body of evidence demonstrating that oxidative stress is not only a pathogenic hallmark of IPF, but it likely plays a crucial role in the development of fibrosis in numerous organ systems.(Cui *et al.*, 2011; Ding *et al.*, 2011).

Lung tissue from IPF patients shows "signatures" of chronic oxidative stress. Although ROS creation plays a role in a variety of basic physiological functions, excessive ROS production can produce oxidative stress, altering cellular phenotypes and causing tissue damage over time.(Walters *et al.*, 2008) Oxidative stress can alter cellular phenotypes and fates, such as senescence and death, thereby promoting a tissue microenvironment that favors fibrosis over regeneration.(Cui *et al.*, 2011).

End-stage fibrosis may result from widespread effects on the epithelium, myofibroblasts, growth factors (e.g., TGF- β), inflammatory cells and the extracellular matrix (ECM) are also shown in.

2.7.1. Growth differentiation factor 15 (GDF15)

2.7.1.1. Definition and Localization

Growth differentiation factor 15 (GDF15)"also named macrophage inhibitory cytokine-1 (MIC-1) is a member of the transforming growth factor beta family, is produced by a variety of cells, including adipocytes and muscle cells, in response to cellular ischemia, mechanical stress, and oxidative stress(Dietzel *et al.*, 2026), is part of the senescence-associated secretory phenotype (SASP) protein. Also the studies indicated to the role of GDF15 in various physiological and pathological conditions,

including inflammation, aging, smoking, cancer, and hypoxia.(Cao *et al.*, 2022) Regarding it's in lung diseases , GDF15 has been associated with disease severity, exacerbation, prognosis, and decline of lung function in chronic obstructive pulmonary disease (COPD). serum GDF15 levels are correlate with disease activity, ILD, and declining lung function ,Similary, in IPF, an aggressive ILD associated with aging, GDF15 expression has been linked to disease severity, exacerbation, and prognosis(Dionet *et al.*, 2024).

2.7.2.2. Physiological effects of (GDF15)

Growth differentiation factor 15 is part of the stress response pathway and is activated in response to hyperoxia. Its Loss lead to decreased cell viability and increased oxidative stress under hyperoxia conditions (Dong *et al.*, 2018). GDF15 expression is low under normal physiological conditions except during pregnancy, due to its immunosuppressive effects. However, it increases during pathological conditions, including inflammation, aging, smoking, cancer, oxidative stress, and hypoxia.(Al-mudares *et al.*, 2020; Yingze *et al.*, 2019). Studies have recorded high expression levels in epithelial and phagocytic cells, as phagocytic cells play an important role in normal and pathological immune and inflammatory responses, due to their ability to release a variety of fibrosis-inducing chemicals such as phagocytic cell inhibitory cytokine 1 (MIC-1), which encodes transforming growth factor beta (TGF- β). (Takenouchi *et al.*, 2020)

GDF15 mRNA expression is increased in monocytes by activation stimuli such as interleukin-1 beta (IL-1 β), tumor necrosis factor-alpha (TNF- α), interleukin-2 (IL-2), phagocytic cell colony-stimulating factor, and transforming growth factor-beta (TGF- β) Increased MIC-1 expression leads to proteolytic cleavage of the progenitor peptide and the secretion of a cysteine-rich diprotein. Pure recombinant MIC-1 inhibits TNF- α production in phagocytic cells, suggesting an auto-regulatory role (Berini *et al.*, 2022; Dionet *et al.*, 2024). This protein is produced in response to

inflammatory cytokines and transforming growth factor-beta (TGF- β), and may subsequently limit phagocytic cell activation. This suggests multiple, even antagonistic, roles within the same disease state, where its effects can be dose- and time-dependent, and vary depending on the target tissue and the pathways subsequently activated. In IPF pathway, GDF15 tends to promote fibrosis as the disease progresses, indicating a regulatory dysfunction in its mechanism of action.(Al-mudares *et al.*, 2020) These findings suggest the potential use of GDF15 as a diagnostic and therapeutic option for IPF, a life-threatening condition associated with cellular responses to oxidative stress and inflammation (Takenouchi *et al.*, 2020).

2.7.2. Extracellular superoxide dismutase. (ECSOD) or SOD3

2.7.2.1. Definition and Localization

superoxide dismutase are a group of enzymes that catalyse the dismutation of superoxide into hydrogen peroxide and dioxygen. There are three isoforms of SODs : SOD1 (cytosolic), SOD2 (mitochondrial), and SOD3 (extracellular)(Wert *et al.*, 2018).EC-SOD is expressed in the bronchial and alveolar epithelium, macrophages, vessels, and interstitium in the human lung.(Fattman *et al.*, 2003; Kinnula *et al.*, 2006) EC-SOD expression has been found to be low/absent in the fibrotic areas and fibroblastic foci in UIP lungs, compared to less damaged nonfibrotic areas in the same lung, further suggesting that there is increased oxidative stress in the fibrotic areas of the lung (Koval *et al.*, 2010). It has been proposed that the cellular redox state and the balance of oxidants/antioxidants play a significant role in the progression of pulmonary fibrosis in animal models and many studies suggest this is also true for human IPF as well EC-SOD can significantly reduce the oxidative stress in the lung parenchyma both in experimental models of lung fibrosis and in human lung (Louis & Salières, 2025).

2.7.2.2. Physiological effects of SOD. (ECSOD)

Highly localized low-level ROS/RNS generation is required for proper physiological processes in the lung, such as smooth muscle relaxation in airways, blood vessels, and immunological responses. However, alteration of the lung's oxidant/antioxidant equilibrium is regarded to be a critical stage in the development of pulmonary diseases. As a result, antioxidant modulation, such as EC-SOD, may play a significant role in modifying or avoiding the causes of numerous lung illnesses (figure2-3)(Fei *et al.*, 2014)Studies have shown that 72 hours of exposure to 100% oxygen resulted in a considerable drop in EC-SOD levels in mice's lungs and bronchoalveolar lavage fluid. (Oury *et al.*, 2002)

studies have revealed that extracellular oxidative stress contributes to the development of acute lung injury, that normal EC-SOD levels give some protection, and that loss of EC-SOD from lung tissue may contribute to the disease progression(Fei *et al.*, 2014), In general, the human lung is characterized by a highly localized EC-SOD enzyme, which is disrupted in the lungs of patients with idiopathic pulmonary fibrosis/typical interstitial pulmonary fibrosis (UIP/IPF) (Kinnula *et al.*, 2006).

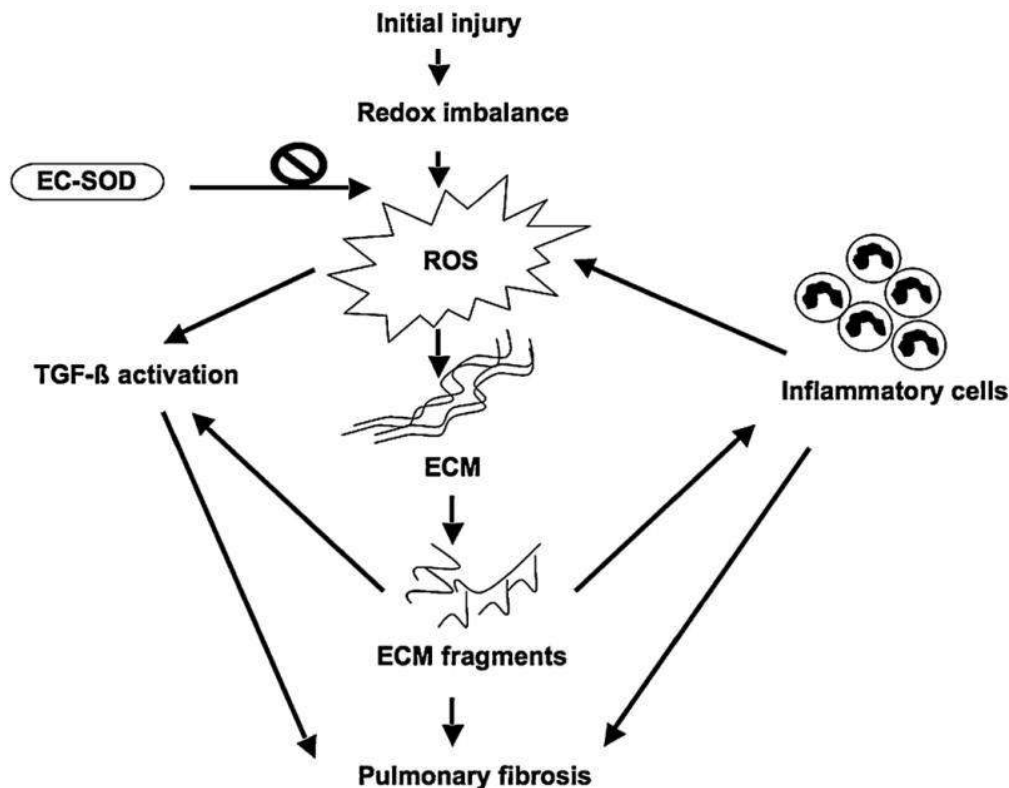


Figure2-3: The diagram shows that when the EC-SOD enzyme is inhibited, reactive oxygen species (ROS) levels rise dramatically, triggering three major damaging pathways: activation of transforming growth factor-beta (TGF- β), direct damage to the extracellular matrix (ECM), and structural breakdown, ultimately leading to pulmonary fibrosis(Liu & Desai, 2015).

2.7.3. Nuclear Factor Erythroid-Derived 2-like2 Protein (Nrf2)

2.7.3.1. Definition and Localization

One of the most significant regulators of oxidative stress in the human body is the transcription factor Nrf2. It is essential for the prevention of many diseases, including age-related, because it controls the expression of numerous cytoprotective genes (Fabiane *et al.*, 2019)

A proper balance of intracellular and extracellular oxidants and antioxidants is required for good pulmonary homeostasis. Protective antioxidants and antioxidant

enzymes protect the lungs from oxidants (Zeng *et al.*, 2022). The majority of these enzymes, which are located in bronchial and alveolar epithelial cells, alveolar macrophages, and the extracellular milieu, are regulated by the nuclear factor erythroid-derived 2-like2 protein (Nrf2). This protein induces the expression of numerous antioxidant pulmonary proteins. (Carlson *et al.*, 2020; Fois *et al.*, 2018).

The importance of Nrf2 in IPF has been demonstrated by experiments in mice where deficiency of this transcription factor significantly enhanced bleomycin-induced pulmonary fibrosis. (Fois *et al.*, 2018)

2.7.3.2. Physiological effects of (Nrf2)

The nuclear factor erythroid 2-related factor 2 (Nrf2) is a central regulator of cellular antioxidative responses, inflammation, and restoration of redox balance. Accumulating reports reveal that Nrf2 activators exhibit potent antifibrosis effects and significantly attenuate pulmonary fibrosis in vivo and in vitro. (Lee *et al.*, 2021; Yu *et al.*, 2022)

Activation of Nrf2 enhanced antioxidant defenses and reduced the expression of myofibroblast phenotypes, while in vitro cultures of primary lung fibroblasts from controls or IPF patients demonstrated that decreased Nrf2 expression is linked to increased expression of myofibroblast phenotypes (type I collagen and α -SMA) (Artaud *et al.*, 2013; Zeng *et al.*, 2022) indicate the Nrf2 might be a promising target for treatment of oxidative stress-induced fibrotic disorders, including IPF.

According to their functions, the downstream genes of Nrf2 can currently be categorized into three groups, phase II detoxifying enzymes; transporters and cellular antioxidants. Gene expression products can effectively regulate intracellular reactive oxygen species (ROS) levels, protect macromolecules from oxidative and chemical damage, and reduce the toxicity of exogenous drugs. Therefore, Nrf2 is essential for regulating oxidative stress and reducing inflammation. For this reason, researchers have focused on Nrf2 and sought to understand its complex role in IPF. (Artaud *et al.*, 2013; Lee *et al.*, 2021).

According to all information about of studied biomarkers of oxidative stress ,their roles are summarized in figure (2-4)

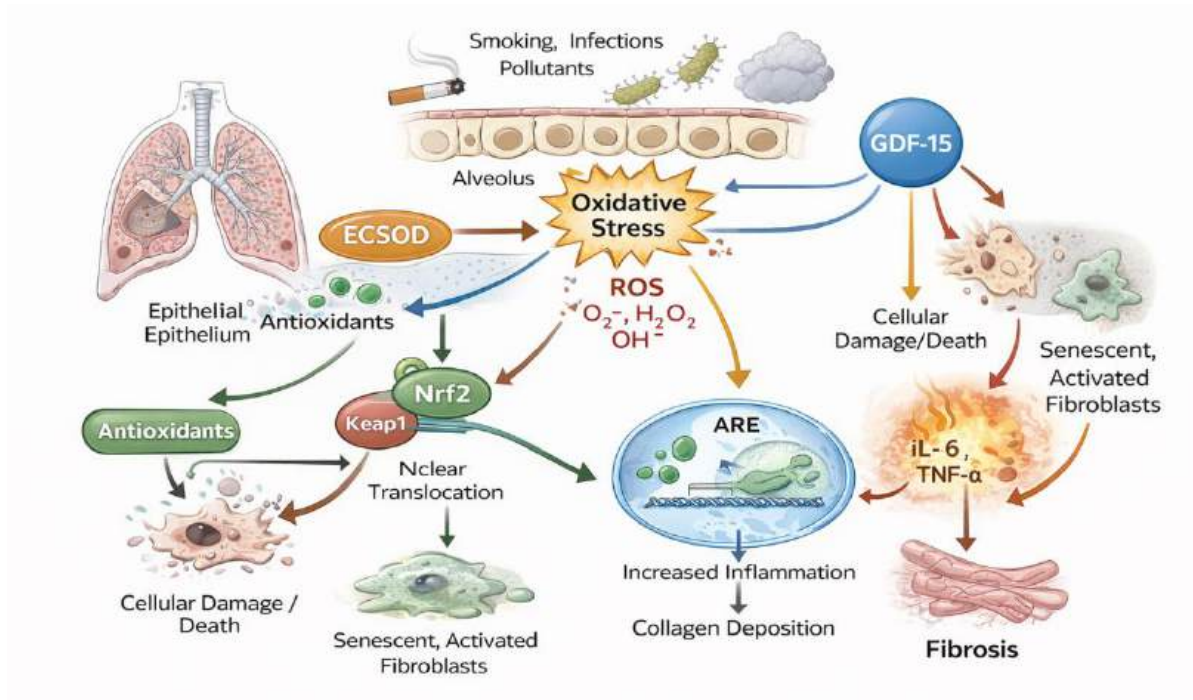


Figure 2-4: designed a diagram summarizing the role of oxidative stress factor in development of IPF. Environmental factors lead to increased production of reactive oxygen species (ROS), causing damage to alveolar epithelial cells. The figure illustrates the dual role of GDF-15 as a pro-inflammatory and fibrotic factor, and Nrf2 and ECSOD as antioxidant defense mechanisms that attempt to limit oxidative damage. When this balance between oxidative and antioxidant factors is disrupted, fibroblast activation and collagen deposition occur, leading to fibrosis. The diagram was created using : <https://www.biorender.com>.

2.8. epigenetics of IPF

Idiopathic pulmonary fibrosis (IPF) is a complex lung disease of unknown etiology. Development of IPF is influenced by both genetic and environmental factors. Recent studies have identified strong genetic predisposition factors for the development of pulmonary fibrosis while cigarette smoke remains the most strongly associated environmental exposure risk factor. Gene expression profiling studies of

IPF lung have taught us quite a bit about the biology of this fatal disease and those in peripheral blood have provided important biomarkers. However, epigenetic marks may be the missing link that connects the environmental exposure in genetically predisposed individuals to transcriptional changes associated with disease development. Moreover, epigenetic marks represent a promising therapeutic target for IPF(Yang & Schwartz, 2016)

The exact cause of IPF remains unknown, but increasing evidence supports the central role of epigenetic changes, particularly microRNA (miRNA), in IPF. Approximately 10% of miRNAs in IPF lung tissue exhibit differential expression compared to normal lung tissue. Diverse miRNA phenotypes exert either a pro-fibrotic or anti-fibrotic influence on the progression of IPF. In the context of IPF, epigenetic factors such as DNA methylation and long non-coding RNAs (lncRNAs) regulate differentially expressed miRNAs, which in turn modulate various signaling pathways implicated in this process, including transforming growth factor- β 1 (TGF- β 1)/Smad, mitogen-activated protein kinase (MAPK), and phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) pathways(Zhou et al., 2024).

2.9. microRNAs as Biomarkers of epigenetics

MicroRNAs (miRNAs) are short single-stranded RNA molecules of 19–25nucleotides in length that mediate posttranscriptional gene silencing of target genes, miRNAs regulate the onset and progress of a variety of diseases, such as cancer, infections, atherosclerosis. In lung diseases, many miRNAs contribute to the pathogenesis (Koichi & Munekazu, 2015).

In a study conducted on bronchoalveolar lavage fluid (BALF) from patients with idiopathic pulmonary fibrosis, the expression patterns of(miRNAs) were evaluated High-throughput quantitative detection of miR expression using a microarray indicated that miR-125b, miR-128, miR-21, miR-100, miR-140-3p and

miR-374b were upregulated in patients with IPF, while let-7d, miR-103, miR-26 and miR-30a-5p were downregulated. The expression level of miR-30a-5p was further examined, and its potential target genes were predicted using target gene prediction analysis software.(Bao et al., 2018) Analysis of the MDTE database and scientific literature revealed 12 specific IPF-associated miRNAs that originated from transposons. Eight of these 12 microRNAs (miR-153, miR-326, miR-340, miR-374, miR-487b, miR-493, miR-630, miR-1343) are unique, since the change in their expression is specific for IPF and has not been described with other diseases of the bronchopulmonary system(Mustafin, 2022)

2.9.1. miR-374 in Pulmonary Fibrosis

miR-374 a member of miR-374 family, which is considered a part of the broader miR-17/92 paralog cluster families, miR-374 has been less extensively studied in the context of pulmonary fibrosis Compared with miR-153. However, initial findings showed that miR-374 may also play a regulatory role in fibrotic signaling pathways. miR-374 has been indicate to modulate TGF- β signaling in other cell types and disease models, which may suggested a similar function in lung fibroblasts(Bu *et al.*, 2017)

recent studies showed that miR-374 might regulate epithelial-mesenchymal transition (EMT), a most important event in the development of fibrosis. Also, in study (Bao *et al.*, 2018) Quantitative analysis of miR-374 expression profiles revealed that miR-374 were upregulated by more than 2-fold in patients with IPF compared with healthy participants(Bao *et al.*, 2018). In 2015, Yang et al. identified significant changes in the levels of 47 different miRNAs in the blood plasma of IPF patients compared with healthy controls. These 47 microRNAs are involved in the signaling pathways of TGF- β , actin cytoskeleton regulation (Guanghai *et al.*, 2015),

Notably, miR-374 is among these dysregulated miRNAs and is functionally linked to TGF- β -associated fibrotic signaling pathways(Kang, 2017).

There some reports about the relationship between miR-374 and cancer. Bian et al., (2019) showed that the expression of miR-374b--5p was up-regulated and conducive to gastric cancer cell invasion and metastasis via inhibiting the expression of RECK (In a study focus at analyzing the expression of microRNA 374a in lung adenocarcinoma, miR-374a expression levels were measured in 111 samples using quantitative reverse transcription polymerase chain reaction (RT-PCR). Survival analysis revealed that patients with high miR-374a expression had significantly shorter survival times compared to patients with low miR-374a expression. The study concluded that miR-374a expression may be a potential predictive biomarker for predicting recurrence of early-stage lung adenocarcinoma after curative surgery (Kim *et al.*, 2019).

various possibilities for the diagnosis and treatment of the miR-374 family members in diseases view of the relationship between the expression of these members and multiple diseases, their expressions can be measured as a novel biomarker, in serum and/or tissue from patients to assess the likely hood of illness and the prognosis of disease, especially in cancer.(Hernandez *et al.*, 2023)

2.9.2. miR-153 in Pulmonary Fibrosis

miR-153 has recently become well-known mainly as a tumor suppressor miRNA molecule, regulating genes responsible for cancer cell proliferation, and epithelial-mesenchymal transformation. It has also recently been found to play an important role in the pathogenesis of (IPF) by regulating key fibrosis pathways. The expression of miR-153 is downregulated in affected lung tissues. This downregulation is primarily due to the inhibitory effects of TGF- β 1, a cytokines central to fibrogenesis.

Conversely, increasing miR-153 levels can attenuate the profibrotic activities of TGF- β 1. (figure2-6)(Liang *et al.*, 2015).

The dysregulation of miR-153 in IPF underscores its potential as a therapeutic target. Restoring miR-153 expression could disrupt the TGF- β signaling cascade, offering a novel approach to modulate fibrotic processes in the lungs. Further research is necessary to explore miR-153-based therapies and their efficacy in clinical settings(Guanghai *et al.*, 2015).miR-153 is a microRNA molecule located within genes that encode a protein associated with the islets of Langerhans. It has been implicated in several diseases, including Parkinson's and Alzheimer's (Tsuyama *et al.*, 2015) Furthermore, it has been reported to inhibit the proliferation and spread of various malignant cell types, such as lung cancer cells(Niu *et al.*, 2015).Therefore, it is considered a tumor suppressor. A recent study demonstrated miR-153 dysregulation in mice with experimental lung damage, specifically showing reduced miR-153 levels in the lungs of a mouse model of bleomycin-induced pulmonary fibrosis(Xie *et al.*, 2011) miR-153 has been shown to inhibit the fibrotic activity of TGF- β 1, which stimulates fibroblasts to differentiate into myofibroblasts. These myofibroblasts are characterized by enhanced fibrotic, contractile, and migration capacities, which are important factors in the pathogenesis of idiopathic pulmonary fibrosis (Hardie *et al.*, 2009). Furthermore, miR-153 has been shown to play a role in weakening the contractility of pulmonary fibroblasts and to significantly inhibit the migration of lung fibroblasts, supporting the role of miR-153 as an inhibitor during the progression of pulmonary fibrosis. Furthermore, it has been shown that TGF- β 1 reduces miR-153 levels and that it operates within an amplification circuit to enhance TGF- β 1 signal transduction in human fibroblasts. Data suggest that miR-153 is an inhibitor of pulmonary fibrosis; therefore, its reduced levels promote fibrosis. miR-153 targets the TGF- β receptor type II (TGFB2), a critical component in the TGF- β signaling pathway . By binding to TGFB2, miR-153 inhibits its

expression, leading to reduced phosphorylation of downstream signaling molecules SMAD2/3, thereby reduction fibroblast activation and collagen deposition (Liang *et al.*, 2015). Also overexpression of miR-153 affects TGF- β 1-induced SMAD2/3 phosphorylation, two of the most important TGF- β signaling pathways from the cell membrane to the nucleus. SMAD2/3 phosphorylation was significantly reduced with increased miR-153 expression in MRC-5 cells, while total SMAD2/3 levels remained unchanged. Conversely, miR-153 inhibition, by introducing a miR-153 inhibitor into the cells, increased SMAD2/3 phosphorylation, while total SMAD2/3 levels were unaffected. These data suggest that miR-153 may be involved in the process of pulmonary fibrosis by interfering with TGF- β signaling and thus fibrogenesis of fibroblasts (Margadant & Sonnenberg, 2010).

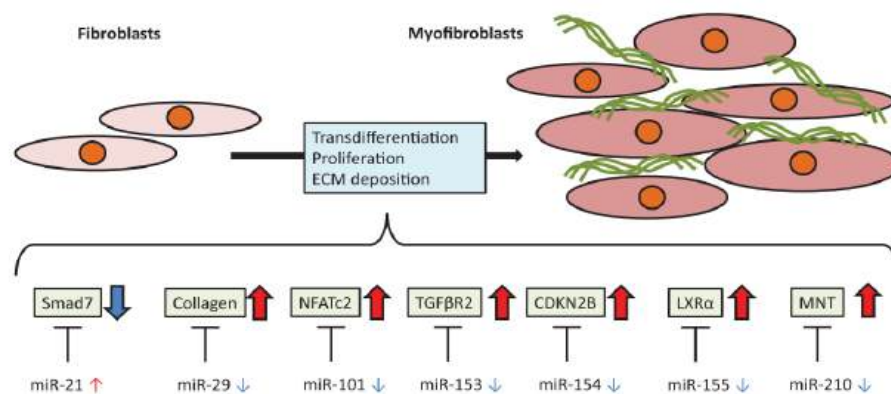


Figure2-6: role of miR-153 in the pathway of (TGFB2)(Liang *et al.*, 2015)

MATERIALS

AND

METHODS

3.1. Materials

3.1.1. Instruments and Equipments:

All Equipment and instruments that used in this study are listed in table (3-1)

Table (3-1): Equipment and instruments that are used in this study

No.	Equipment& instruments	company	Country
1	Digital camera	Samsung	china
	EDTA Tube	Afcovac	Jordan
	ELISA Reader	Paramedical	Italia
2	Eppendorf tubes	Sigma	England
3	Exispin vortex centrifuge	Bioneer	Korea
4	Gel electrophoresis	Bioneer	Korea
	Gel Tube	Fcovaca	Jordan
5	High Speed Cold centrifuge	Eppendorf	Germany
6	Incubator	Memmert	Germany
7	Microcentrifuge tubes	Biobasic	Canada
8	Micropipettes (different volumes),	CYAN	Belgium
9	MiniOpticon Real Time PCR	Bio-Rad	USA
10	Refrigerator	Concord	Lebanon
11	Spectrophotometer	Shimadzu	Japan
12	Thermocycler PCR	BioRad	USA
13	Vortex	CYAN	Belgium
14	Water Bath	Memmert	Germany

3.1.2. Chemicals

The chemical and biological materials used in this work are listed in Table (3-2) below:

Table (3-2): Chemical materials with their remarks

No.	Chemicals	Company	Country
1	Absolute Ethanol	Labort	India
2	Chloroform	Labort	India
3	DEPC water	Bioneer	Korea
4	Isopropanol	Labort	India
5	Nuclease free water	Bioneer	Korea

3.1.3. Kits

3.1.3.1. Kits of ELISA assay

The Kit used in this study with their companies and countries of origin is listed in Table (3-3)

Table (3-3): Kits of ELISA assay in this study

No.	Names of ELISA kits	Company	Country
1	Collagen Type I Alpha 1	Elabscience	China
2	Cytokeratin 18	Elabscience	China
3	Growth Differentiation Factor 15	BT LAB	China
4	Integrin Alpha-V	BT LAB	China
5	Nuclear Factor Erythroid 2	BT LAB	China
6	Superoxide Dismutase 3	BT LAB	China

3.1.3.2. Kits of Molecular study

The kits used in this study with their companies and countries of origin are shown in Table (3-4):

Table (3-4) Kits of the molecular assay in this study

No.	Kit	Company	Country
1	AccuZol Total RNA Extraction Kit	Bioneer	Korea
	Trizol reagent 100ml		
2	DNase I enzyme kit	Promega	USA
	DNase I enzyme		
	10x buffer		
	Free nuclease water		
	Stop reaction		
3	M-MLV Reverse Transcriptase kit	Bioneer	Korea
	M-MLV Reverse Transcriptase (10,000U)		
	5X M-MLV RTase reaction buffer		
	dNTP		
	100mM DTT		
	RNase Inhibitor		
4	GoTaq® qPCR Master Mix	Promega	USA
	2X SYBER green dye		
	Taq DNA polymerase		
	dNTPs (dATP, dCTP, dGTP, dTTP)		
	10X buffer		

3.1.4. Primers

The qPCR Primers for mature hsa-miR-374 ([MIMAT0000727](#)) and miR-153 ([MIMAT0000439](#)) were design in this study by using (The Sanger Center miRNA database Registry) to selected miRNA sequence and using miRNA Primer Design Tool. Whereas, qPCR Housekeeping gene (GAPDH) (NM_001256799.3) were design in this study by using NCBI-Database and Primer3 plus design online. These primers were provided by (Macrogen company, Korea) as following table(3-5):

Table (3-5): primer sequences of miR-374 and miR-153 for real time-qPCR

PRIMER		SEQUENCE (5'-3')
miRNA universal RT primers		GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGAT ACGACTTTT TTTT TTTVN
miR-374 qPCR primer	F	AAGCGACCTTATAATACAACCTGAT
	R	GTCGTATCCAGTGCAGGGT
miR-153 qPCR primer	F	AACAAGTTGCATAGTCACAAAAGTG
	R	GTCGTATCCAGTGCAGGGT
GAPDH qPCR primer	F	AAAATCAAGTGGGGCGATGC
	R	TTCTCCATGGTGGTGAAGACG

3.2. Methods

3.2.1. Study Design and Blood Sample Collection

The current study included a case-control study, total number (120) participants including 60 patients diagnosed with IPF. (clinically and radiographically) by a specialist physician, as well as 60 healthy individuals as a control group, who had no medical history or clinical evidence of pulmonary fibrosis or asthma. Samples of IPF Patients were from visitor of Imam Al-Sadiq Hospital in Babylon Governorate and the Specialized Allergy and Asthma Center in Diwaniyah from December 2024 to November 2025. All patient and control group data were recorded in a questionnaire distributed during personal interviews. The questionnaire included information on name, age, gender, and smoking status , weight, height , family history, degree of IPF (Appendix 1).

Lung function, including forced expiratory volume in first second (FEV1) and forced vital capacity (FVC), was assessed by clinicians using spirometry. These measurements help clinicians differentiate between obstructive lung diseases such as asthma/COPD (low FEV1/FVC) and restrictive lung diseases such as pulmonary

fibrosis (low FVC, normal FEV1/FVC). The data were recorded with each patient's information.

To ensure the homogeneity of the study population and isolate the specific characteristics of IPF, strict exclusion criteria were applied. Patients with a known history or clinical evidence of other confounding respiratory diseases including bronchial asthma, Chronic Obstructive Pulmonary Disease (COPD), or any other chronic or acute pulmonary disorders were systematically or any confounding factors excluded from the study.

Approximately 6 ml of blood was collected venous blood from each participant. Each blood sample was then divided into several portions for physiological and molecular analysis as follow:

- 1- One milliliter of blood was placed in a tube containing an anticoagulant (EDTA) to perform a complete blood count (CBC) test.
- 2- Three milliliters were placed in anticoagulant-free tubes to obtain the serum. The samples were left to stand for 20–30 minutes to allow coagulation, and then the serum was separated by centrifugation at 2500 rpm for 10 minutes. The serum was then collected in sterile Eppendorf tubes and stored at -20°C until measurements of physiological biomarkers were performed using enzyme-linked immunosorbent assay (ELISA).
- 3- Two milliliters of the blood sample were transferred directly into Eppendorf tubes containing TRIzol reagent then stored at -20°C until RNA extraction for molecular study using Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR). as in study design figure (3-1) .

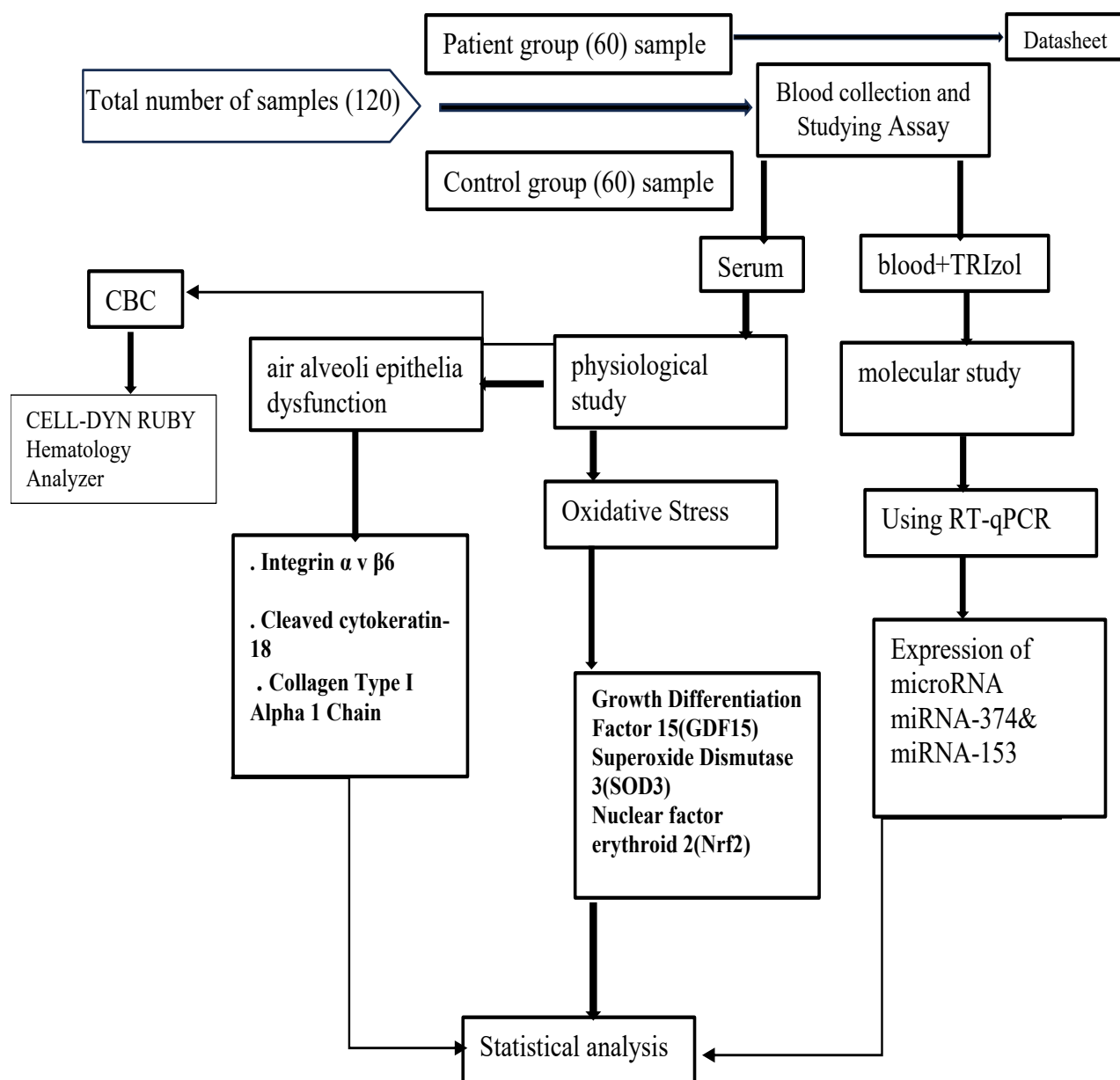


Figure (3-1) Study design

3.2.2. physiological study

3.2.2.1. Hematological Assay (Complete Blood Count (CBC))

A complete blood count was performed on each blood sample using the CELL-DYN RUBY Hematology Analyzer (Abbott Diagnostics, USA). We followed the manufacturer's instructions. The samples were gently mixed before analysis to ensure homogeneity of the blood components. The tubes were then placed in a special holder inside the analyzer, which automatically drew the sample and analyzed it. Within a few minutes, the device provides a range of many hematological indicators, but the counting of lymphocytes and neutrophils and percentage of neutrophils is considered specific to IPF, therefore, the results of these indicators were relied upon regardless of other in the CBC test.

3.2.2.2. Serological assay (Enzyme-Linked Immunosorbent Assay (ELISA))

3.2.2.2.A. Quantification of alveolar dysfunction biomarker

① $\alpha\text{v}\beta 6$ integrin (ng/mL)

$\alpha\text{v}\beta 6$ integrin was quantified using Human Integrin Alpha-V, ITGAV ELISA Kit according to the manufacturer's instructions (BT LAB Bioassay technology laboratory, China). Components of $\alpha\text{v}\beta 6$ Integrin ELISA Kit as follow:

No.	Components	Quantity
1	Standard Solution (2400 ng/L)	0.5ml x1
2	Pre-coated ELISA Plate	12 * 8 well strips x1
3	Standard Diluent	3ml x1
4	Streptavidin-HRP	6ml x1
5	Stop Solution	6ml x1
6	Substrate Solution A	A 6ml x1
7	Substrate Solution B	6ml x1
8	Wash Buffer Concentrate (25x)	20ml x1
9	Biotinylated Human ITGAV Antibody	1ml x1

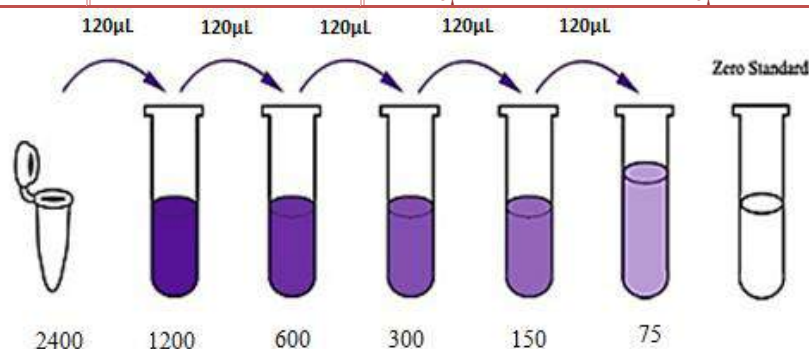
A: Assay Principle

This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with Human ITGAV antibody. ITGAV present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Human ITGAV Antibody is added and binds to ITGAV in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated ITGAV antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of Human ITGAV. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450nm.

B. standard preparation

The stock standard solution (2400 ng/L) was diluted to obtain the following concentrations in the table:

1200ng/L	Standard No.5	120μL Original standard + 120μL Standard diluent
600ng/L	Standard No.4	120μL Standard No.5 + 120μL Standard diluent
300ng/L	Standard No.3	120μL Standard No.4 + 120μL Standard diluent
150ng/L	Standard No.2	120μL Standard No.3 + 120μL Standard diluent
75ng/L	Standard No.1	120μL Standard No.2 + 120μL Standard diluent



C: Assay procedure

1. All reagents, standard solutions and samples were prepared according to kit instruction and bring at room temperature before use.

2. Standard addition: 50 μ L serial dilution was added into to standard well. Without added antibody to standard well because the standard solution contains biotinylated antibody.
3. sample addition 40 μ l sample was added to sample wells and then add 10 μ l Human ITGAV antibody to sample wells, then 50 μ l streptavidin-HRP added in to sample wells and standard wells (Not blank control well).
4. incubation : 50 μ l of streptavidin-HRP was added to sample wells and standard wells (Not blank control well), then mix well, and the plate is covered with a sealer and it was incubated for 60 minutes at 37°C.
5. washing : the sealer was removed and the plate was washed 5 times with washing buffer. Wells were soaked with at least 300ul of wash buffer for 30 seconds to 1 minute per wash. The plate is dried on paper towels.
6. stop solution addition : 50 μ l of stop solution was added to each well, and the blue color changed to yellow immediately.
7. assay : optical density (OD value) of each well was determined immediately with a microplate reader set to 450 nm within 10 min after addition of the stop solution.

D. Calculation of results:

The ELISA results were calculated depend on the optical density reading for each standard and samples. Then the standard curve was plotted by the mean OD value for each standard on the Y-axis against the concentration on the X-axis and draw a best fit curve through the points on the graph. The concentration of samples α v β 6 integrin in ng/ml was calculated by using the standard curve (Fig 3-2).

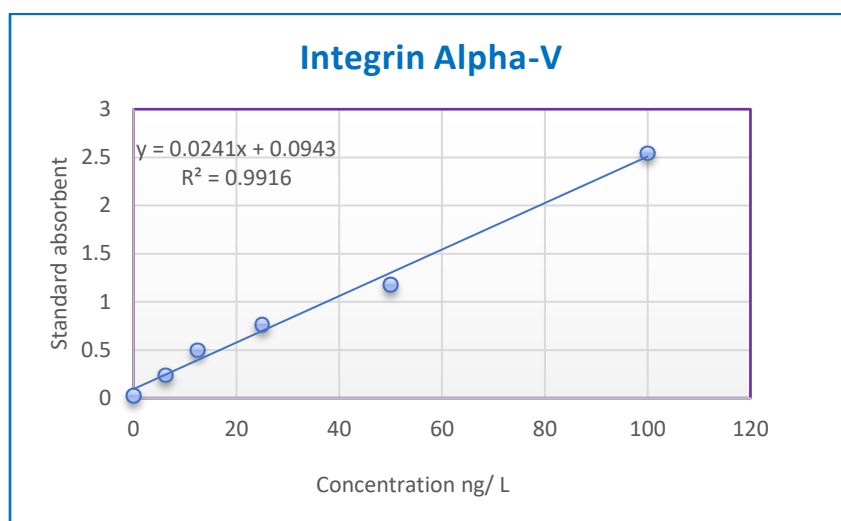


Figure (3-2): Standard curve for Human Integrin Alpha-V by ELISA

x: concentration of samples , y: optic density of samples

② Cytokeratin 18 (mIU/ mL)

Cytokeratin 18 was quantified using Human CK-18/KRT18(Cytokeratin 18) ELISA Kit according to the manufacturer's instructions (Elabscience Biotechnology Co.Ltd, China). Components of CCK-18 ELISA Kit as follow:

No.	components	Quantity
1	Reference Standard (400 mIU/ mL)	2 vials
2	Pre-coated ELISA Plate Micro ELISA Plate (Dismountable)	12 * 8 well strips ×12
3	Concentrated Biotinylated Human CK-18 antibody (100×)	1×120 μL
4	Concentrated HRP Conjugate (100×)	1 ×120 μL
5	Reference Standard & Sample Diluent	1 × 20 mL
6	Biotinylated Human CK-18 antibody Detection Diluent	1 × 14 mL
7	HRP Conjugate Diluent	1 × 14 mL
8	Concentrated Wash Buffer(25×)	1 × 30 mL
9	Substrate Reagent	1 × 10 mL
10	Stop Solution	1 × 10 mL

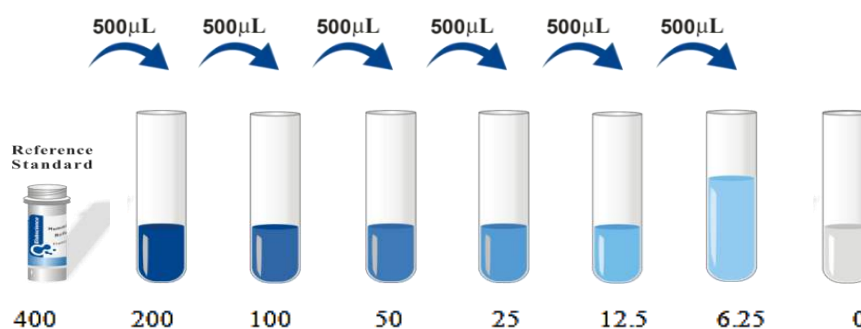
A. Assay Principle

This ELISA kit uses the Sandwich-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to Human CK-18. Samples (or Standards) are added to the micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Human CK-18 and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Human CK-18, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 ± 2 nm. The OD value is proportional to the concentration of Human CK-18. You can calculate the concentration of Human CK-18 in the samples by comparing the OD of the samples to the standard curve.

B. standard preparation

The stock standard solution (400 mIU/mL) was diluted to obtain the following concentrations **in** the table:

200 mIU/ mL	Standard No.6	500µL Original standard + 500µL Standard diluent
100 mIU/ mL	Standard No.5	500µL Standard No.6 + 500µL Standard diluent
50 mIU/ mL	Standard No.4	500µL Standard No.5 + 500µL Standard diluent
25 mIU/ mL	Standard No.3	500µL Standard No.4 + 500µL Standard diluent
12.5 mIU/ mL	Standard No.2	500µL Standard No.3 + 500µL Standard diluent
6.25 mIU/ mL	Standard No.1	500µL Standard No.2 + 500µL Standard diluent



C: Assay procedure

1. Diluted standard, blank and sample addition : 100 μL each dilution of standard, blank and sample were added into the appropriate wells ,then, the plate was covered with the sealer and Incubated for 90 min at 37°C.
2. biotinylated detection addition: the liquid decanted from each well, then 100 μL of biotinylated Human CK-18 anti-body solution was added to each well. Then , the plate covered with a new sealer and Incubate for 1 hour at 37°C.
3. washing : the sealer was removed and the solution decanted from each well the plate was washed 3 times with washing buffer. Wells were soaked with at least 350 μL of wash buffer for 1 minute per wash. The plate is dried on paper towels or other absorbent material.
4. HRP Conjugate addition : 100 μL of HRP Conjugate working solution was added to each well. Then the plate was covered with a new sealer and Incubate for 30 min at 37°C.
5. Washing : the solution decanted from each well, the plate was washed 5 times as conducted in step 3.
6. Substrate Reagent addition: 90 μL of Substrate Reagent was added to each well. the plate was Covered with a new sealer, and Incubate for about 15 min at 37°C.
7. Stop Solution addition :50 μL of Stop Solution was added to each well, and

the blue color changed to yellow immediately.

8. assay: optical density (OD value) of each well was determined at once with a micro-plate reader set to 450 nm within 10 min after addition of the stop solution.

D. Calculation of results:

The ELISA results were calculated depend on the optical density reading for each standard and samples optical density. Then the standard curve was plotted by the mean OD value for each standard on the X-axis against the concentration on the Y-axis and draw a best fit curve through the points on the graph. The concentration of samples Human CK-18 in 400 mIU/mL was calculated by using the standard curve (Fig.3.3).

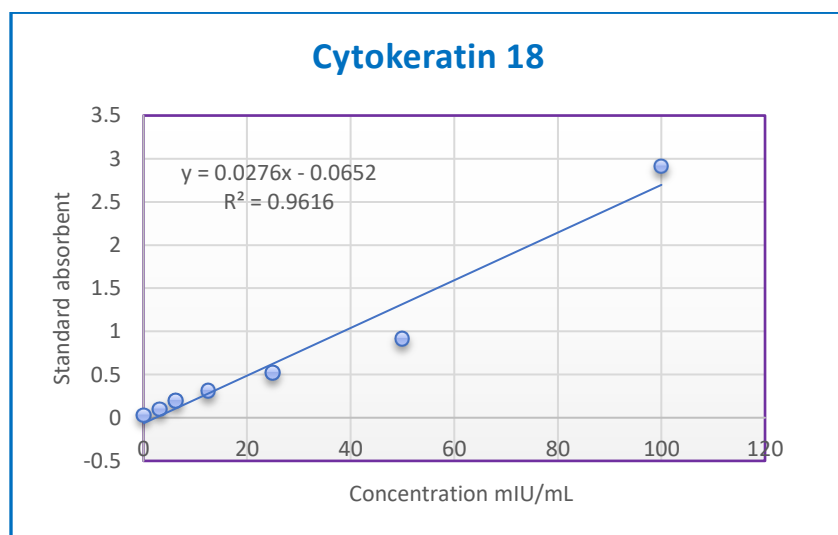


Figure (3-3): Standard curve for Human Cytokeratin 18 by ELISA

x: concentration of samples, y: optic density of samples

③Collagen Type 1 Alpha (COL1α1)

Collagen Type 1 Alpha was quantified using Human COL1α1(Collagen Type Alpha 1) ELISA Kit according to the manufacturer's instructions (Elabscience Biotechnology Co.Ltd, China). Components of Human COL1α1ELISA Kit as follow:

No.	components	Quantity
1	Reference Standard (20 ng/L)	2 vials
2	Pre-coated ELISA Plate Micro ELISA Plate (Dismountable)	12 * 8 well strips×12
3	Concentrated Biotinylated Human COL1 α 1 antibody (100×)	1×120 μ L
4	Concentrated HRP Conjugate (100×)	1 ×120 μ L
5	Reference Standard & Sample Diluent	2 × 20 mL
6	Biotinylated Human COL1 α 1 antibody Detection Diluent	1 × 14 mL
7	HRP Conjugate Diluent	1 × 14 mL
8	Concentrated Wash Buffer(25×)	1 × 30 mL
9	Substrate Reagent	1 × 10 mL
10	Stop Solution	1 × 10 mL

A. Assay Principle

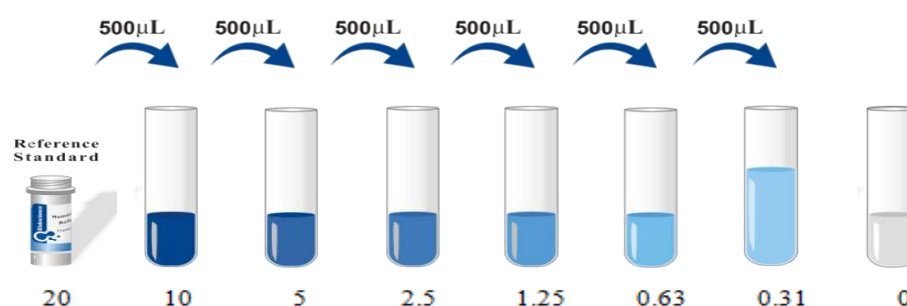
This ELISA kit uses the Sandwich-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to Human COL1 α 1. Samples (or Standards) are added to the micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Human COL1 α 1 and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Human COL1 α 1, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 ± 2 nm. The OD value is proportional to the concentration of Human COL1 α 1. You can calculate the concentration of

Human COL1 α 1 in the samples by comparing the OD of the samples to the standard curve.

B. standard preparation

The stock standard solution (20 ng/L) was diluted to obtain the following concentrations in the table:

10ng/L	Standard No.6	500 μ L Original standard + 500 μ L Standard diluent
5ng/L	Standard No.5	500 μ L Standard No.6 + 500 μ L Standard diluent
2.5ng/L	Standard No.4	500 μ L Standard No.5 + 500 μ L Standard diluent
1.25ng/L	Standard No.3	500 μ L Standard No.4 + 500 μ L Standard diluent
0.63ng/L	Standard No.2	500 μ L Standard No.3 + 500 μ L Standard diluent
0.31ng/L	Standard No.1	500 μ L Standard No.2 + 500 μ L Standard diluent



C: Assay procedure

All Subsequent steps of assay procedure were performed as described in steps 1,2 ,3,4,5,6,7and 8 for CK-18 assay.

D. Calculation of results:

The ELISA results were calculated depend on the optical density reading for each standard and samples optical density. Then the standard curve was plotted by the mean OD value for each standard on the X-axis against the concentration on the Y-axis and draw a best fit curve through the points on the graph. The concentration of samples Human COL1 α 1 in 20 ng/L was calculated by using the standard curve (Fig.3.4).

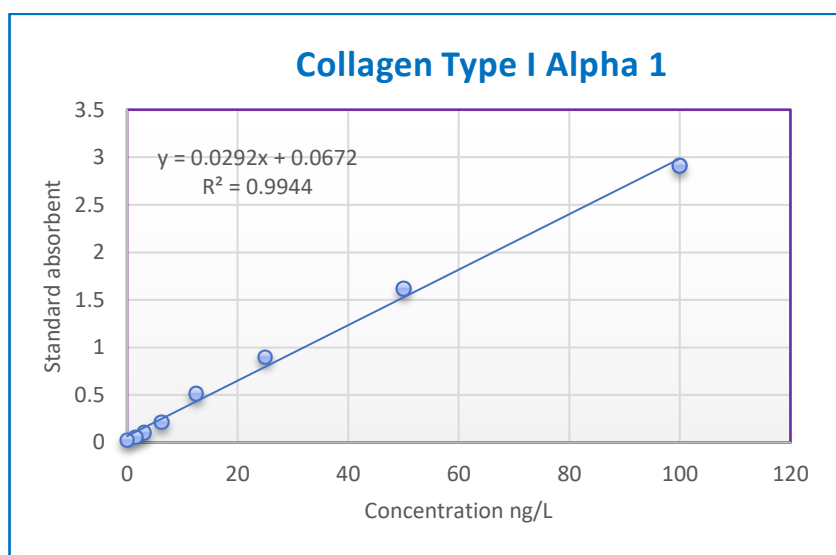


Figure (3-4): Standard curve for Human COL1α1 by ELISA

x: concentration of samples, y: optic density of samples

3.2.2.2.B. Quantification of oxidative stress biomarkers

① Growth Differentiation Factor 15 (GDF15) (ng/L)

Growth Differentiation Factor 15 was quantified using Human Growth Differentiation Factor 15, GDF15 ELISA Kit according to the manufacturer's instructions (BT LAB Bioassay technology laboratory, China). Components of Human GDF15 ELISA Kit as follow:

No.	Components	Quantity
1	Standard Solution (3200 ng/ml)	0.5ml x1
2	Pre-coated ELISA Plate	12 * 8 well strips x1
3	Standard Diluent	3ml x1
4	Streptavidin-HRP	6ml x1
5	Stop Solution	6ml x1
6	Substrate Solution A	6ml x1
7	Substrate Solution B	6ml x1
8	Wash Buffer Concentrate (25x)	20ml x1
9	Biotinylated Human GDF15 Antibody	1ml x1

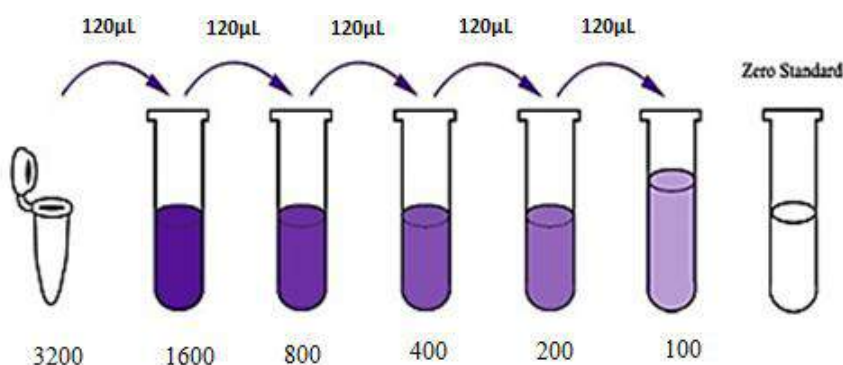
This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with Human GDF15 antibody. GDF15 present in the sample is

added and binds to antibodies coated on the wells. And then biotinylated Human GDF15 Antibody is added and binds to GDF15 in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated GDF15 antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of Human GDF15. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm

B. standard preparation

The stock standard solution (3200 ng/L) was diluted to obtain the following concentrations in the table:

1600ng/L	Standard No.5	120μL Original standard + 120μL Standard diluent
800ng/L	Standard No.4	120μL Standard No.5 + 120μL Standard diluent
400ng/L	Standard No.3	120μL Standard No.4 + 120μL Standard diluent
200ng/L	Standard No.2	120μL Standard No.3 + 120μL Standard diluent
100ng/L	Standard No.1	120μL Standard No.2 + 120μL Standard diluent



C: Assay procedure

All Subsequent steps of assay procedure were performed as described in steps 1,2 ,3,4,5,6,7and 8 for Integrin $\alpha v \beta 6$ assay.

D. Calculation of results:

The ELISA results were calculated depend on the optical density reading for each standard and samples optical density. Then the standard curve was plotted by the mean OD value for each standard on the X-axis against the concentration on the Y-axis and draw a best fit curve through the points on the graph. The concentration of samples Human GDF15 in ng/L was calculated by using the standard curve (Figure 3.5).

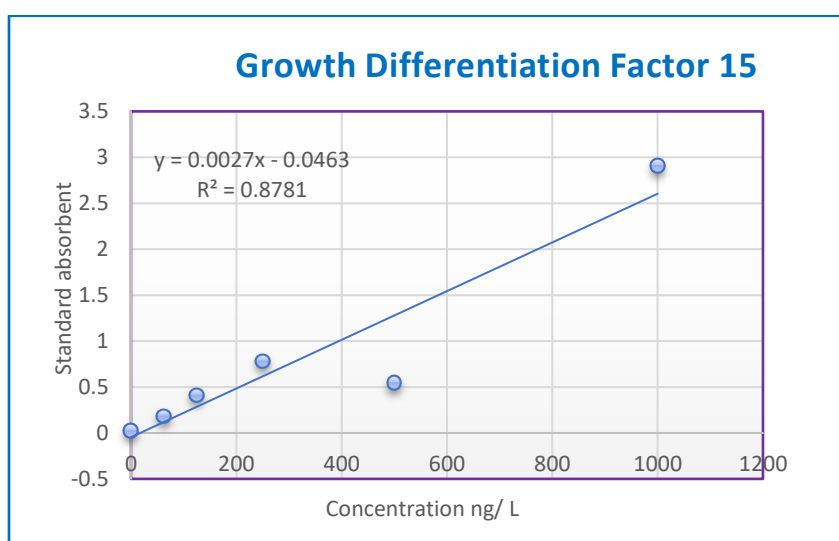


Figure (3-5): Standard curve for GDF15 by ELISA

x: concentration of samples , y: optic density of samples

② Extracellular Superoxide Dismutase (Cu-Zn), SOD3

Superoxide Dismutase was quantified using Human Extracellular Superoxide Dismutase (Cu-Zn), SOD3 ELISA Kit according to the manufacturer's instructions (BT LAB Bioassay technology laboratory, China) . Components of SOD3 ELISA Kit as follow:

No.	Components	Quantity
1	Standard Solution (960 ng/L)	0.5ml x1
2	Pre-coated ELISA Plate	12 wells * 8 detachable strips
3	Standard Diluent	3ml x1
4	Streptavidin-HRP	6ml x1
5	Stop Solution	6ml x1
6	Substrate Solution A	A 6ml x1
7	Substrate Solution B	6ml x1
8	Wash Buffer Concentrate (25x)	20ml x1
9	Biotinylated Human SOD3 Antibody	1ml x1

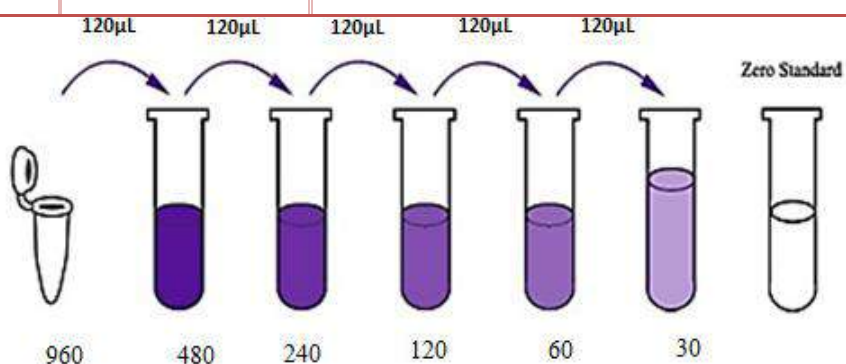
A. Assay Principle

This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with Human SOD3 antibody. SOD3 present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Human SOD3 Antibody is added and binds to SOD3 in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated SOD3 antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of Human SOD3. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

B. standard preparation

The stock standard solution (960 ng/L) was diluted to obtain the following concentrations in the table:

480ng/L	Standard No.5	120μL Original standard + 120μL Standard diluent
240ng/L	Standard No.4	120μL Standard No.5 + 120μL Standard diluent
120ng/L	Standard No.3	120μL Standard No.4 + 120μL Standard diluent
60ng/L	Standard No.2	120μL Standard No.3 + 120μL Standard diluent
30ng/L	Standard No.1	120μL Standard No.2 + 120μL Standard diluent



C. Assay procedure

All Subsequent steps of assay procedure were performed as described in steps 1,2,3,4,5,6,7and 8 for $\alpha\text{v}\beta 6$ Integrin assay.

D. Calculation of results:

The ELISA results were calculated depend on the optical density reading for each standard and samples optical density. Then the standard curve was plotted by the mean OD value for each standard on the X-axis against the concentration on the Y-axis and draw a best fit curve through the points on the graph. The concentration of samples Human SOD3 in ng/L was calculated by using the standard curve (Figure 3.6).

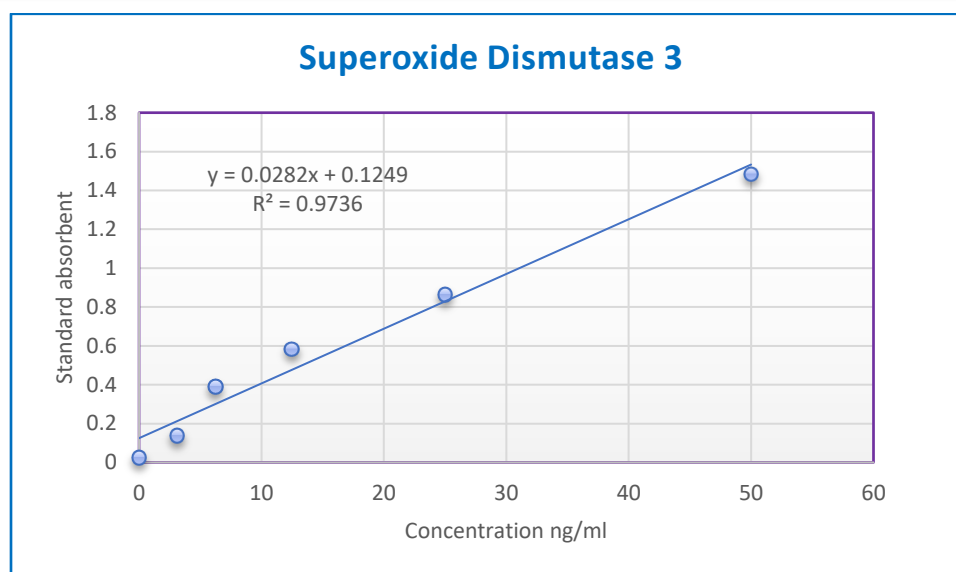


Figure (3-6): Standard curve for SOD3 by ELISA

x: concentration of samples , y: optic density of samples

③ Human Nuclear Factor Erythroid 2-related Factor 2, NRF2

Nuclear Factor Erythroid 2-related Factor 2 was quantified using Human NRF2 ELISA Kit according to the manufacturer's instructions (BT LAB Bioassay technology laboratory, China) . Components of NRF2 ELISA Kit as follow:

No.	Components	Quantity
1	Standard Solution (80 ng/ml)	0.5ml x1
2	Pre-coated ELISA Plate	12 wells * 8 detachable strips
3	Standard Diluent	3ml x1
4	Streptavidin-HRP	6ml x1
5	Stop Solution	6ml x1
6	Substrate Solution A	A 6ml x1
7	Substrate Solution B	6ml x1
8	Wash Buffer Concentrate (25x)	20ml x1
9	Biotinylated Human NRF2 Antibody	1ml x1

A. Assay Principle

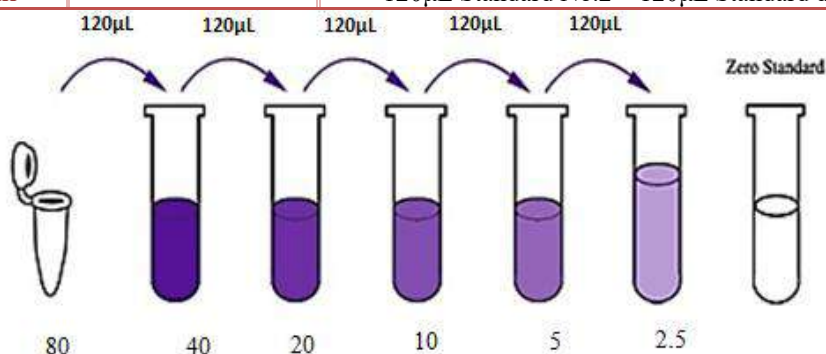
This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with Human NFE2L2 antibody. NFE2L2 present in the sample is

added and binds to antibodies coated on the wells. And then biotinylated Human NFE2L2 Antibody is added and binds to NFE2L2 in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated NFE2L2 antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of Human NFE2L2. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

B. standard preparation

The stock standard solution (80 ng/ml) was diluted to obtain the following concentrations in the table:

40ng/ml	Standard No.5	120μL Original standard + 120μL Standard diluent
20ng/ml	Standard No.4	120μL Standard No.5 + 120μL Standard diluent
10ng/ ml	Standard No.3	120μL Standard No.4 + 120μL Standard diluent
5ng/ ml	Standard No.2	120μL Standard No.3 + 120μL Standard diluent
2.5ng/ ml	Standard No.1	120μL Standard No.2 + 120μL Standard diluent



C. Assay procedure

All Subsequent steps of assay procedure were performed as described in steps 1,2,3,4,5,6,7and 8 for $\alpha\text{v}\beta 6$ Integrin assay.

D. Calculation of results:

The ELISA results were calculated depend on the optical density reading for each standard and samples optical density. Then the standard curve was plotted by the mean OD value for each standard on the X-axis against the concentration on the Y-axis and draw a best fit curve through the points on the graph. The

concentration of samples Human , NRF2 in ng/ml was calculated by using the standard curve (Figure 3.7).

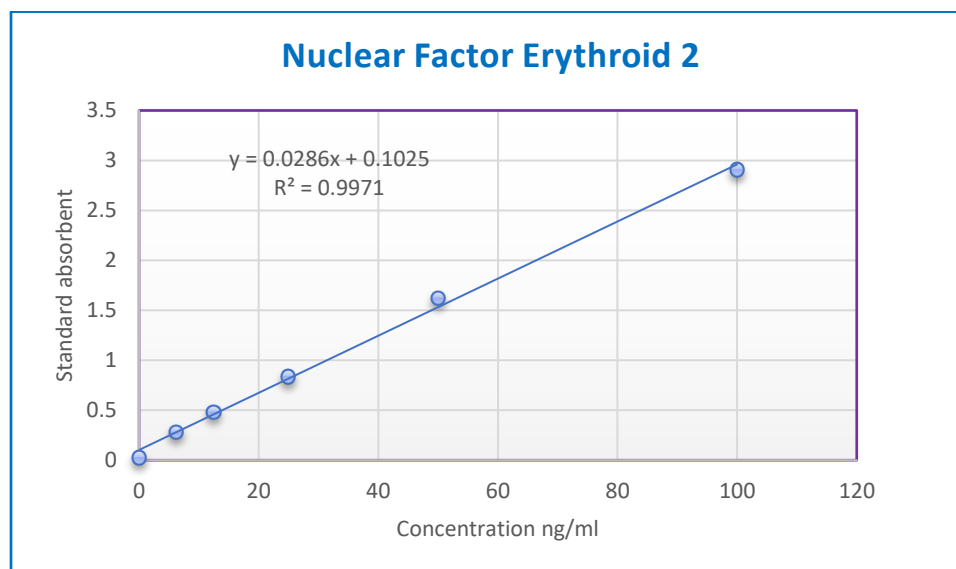


Figure (3-7): Standard curve for NRF2 by ELISA

x: concentration of samples , y: optic density of samples

3.2.3. Molecular study:STEM-LOOP RT-qPCR

The stem loop RT-qPCR was used in expression analysis quantification of mature hsa-miR-374 and miR-135 as well as housekeeping gene (GAPDH) in blood patients and normal samples by using Real-Time PCR technique and this method was carried out according to method described by (Varkonyi-Gasic and Hellens 2011) and included the following steps:

① Total RNA extraction

Total RNA was extracted from blood samples by using (TRIzol® reagent kit) and done according to company instructions as following steps:

- 1- 250µl blood samples or blood samples were placed in 1.5 microcentrifuge tube then 750µl TRIzol® reagent was added to each tubes.
- 2- Then, 200µl chloroform was added to each tube and shaken vigorously for 60 seconds.

- 3- The mixture was incubated on ice for 5 minutes. Then centrifuged at 12000 rpm, 4C°, for 15 minutes.
- 4- Supernatant was transferred into a new eppendorf tube, and 500µl isopropanol was added. Then, mixture mixed by inverting the tube 4-5 times and incubated at 4C° for 10 minutes. Then, centrifuged at 12,000 rpm , 4C° for 10 minutes.
- 5- Supernatant was discarded, and 1ml 80% Ethanol was added and mixed by vortex again. Then, centrifuge at 12000 rpm, 4C° for 5 minutes.
- 6- The supernatant was discarded and the RNA pellet was left to air to dry.
- 7- 50µl DEPC water was added to each sample to dissolve the RNA pellet, Then, the extracted RNA sample was kept at (-20).

② Estimation RNA yield and quality

The extracted genomic RNA was checked by using Nanodrop spectrophotometer (THERMO. USA) that check RNA concentration and estimation of RNA purity through reading the absorbance in at (260 /280 nm) as following steps:

1. After opening up the Nanodrop software, chosen the appropriate application (Nucleic acid, RNA).
2. A dry wipe was taken and cleaned the measurement pedestals several times. Then carefully pipet 2µl of ddH₂O onto the surface of the lower measurement pedestal.
3. The sampling arm was lowered and clicking OK to blank the Nanodrop, then cleaning off the pedestals.
4. After that, the pedestals are cleaned and pipet 1µl of RNA sample for measurement.

③DNase I Treatment

The extracted RNA were treated with DNase I enzyme to remove the trace amounts of genomic DNA from the eluted total RNA by using samples (DNase I enzyme kit) and done according to method described by Promega company, USA instructions as follow:

Mix	volume
Total RNA 100ng/ul	10ul
DNase I enzyme	1ul
10X buffer	4ul
DEPC water	5ul
Total	20ul

After that, The mixture was incubated at 37C° for 30 minutes. Then, 1µl stop reaction was added and incubated at 65C° for 10 minutes for inactivation of DNase enzyme .

④cDNA synthesis

A. cDNA synthesis for target miRNAs (miRNA-374a and miRNA-153)

DNase-I treated RNA samples were used in miRNA cDNA synthesis step for target miRNA by using M-MLV Reverse Transcriptase kit and done according to company instructions as following tables:

Step 1:

RT master mix	volume
Total RNA 100ng/ul	8ul
Target miR RT primer	1ul
DEPC water	1ul
Total	10ul

Than RNA and primer was denatured for 10 min at 65 °C, after that immediately cool on ice.

Step 2:

RT master mix	volume
Step 1 RT master mix	10ul
M-MLV RTase (200u)	1ul
5X M-MLV RTase reaction buffer	4ul
100mM DTT	2ul
dNTP	2ul
RNase inhibitor	1ul
Total	20ul

Then the tubes were placed in vortex and briefly spinning down. The RNA converted into cDNA in thermocycler under the following thermocycler conditions:

Step	Temperature	Time
cDNA synthesis (RT step)	42 °C	1 hour
Heat inactivation	95 °C	5 minutes

B. cDNA synthesis for GAPDH gene

DNase-I treated RNA samples were also used in cDNA synthesis step for GAPDH gene by using M-MLV Reverse Transcriptase kit and done according to company instructions as following tables:

Step 1

RT master mix	volume
Total RNA 100ng/ul	8ul
Random Hexamer primer	1ul
DEPC water	1ul
Total	10ul

Then RNA and primer was denatured for 10 min at 65 °C, after that immediately cool on ice.

Step 2

RT master mix	volume
Step 1 RT master mix	10ul
M-MLV RTase (200u)	1ul
5X M-MLV RTase reaction buffer	4ul
100mM DTT	2ul
dNTP	2ul
RNase inhibitor	1ul
Total	20ul

Then the tubes were placed in vortex and briefly spinning down. The RNA converted into cDNA in thermocycler under the following thermocycler conditions:

Step	Temperature	Time
cDNA synthesis (RT step)	42 °C	1 hour
Heat inactivation	95 °C	5 minutes

⑤ Quantitative Real-Time PCR (qPCR) for target miRNAs and GAPH

The quantitative Real-Time PCR used in quantification of target miRNAs (miRNA-374 and miRNA-153) and GAPDH gene expression analysis as following steps:

A. qPCR master mix preparation

qPCR master mix was prepared by using GoTaq® qPCR Master Mix kit that dependent on SYBER Green dye detection of gene amplification in Real-Time PCR system and include the follow:

Standard protocol:

qPCR master mix for target miRNA	volume
miRNA cDNA template (20ng)	5μL
Forward primer(10pmol)	1μL
Reverse primer (10pmol)	1μL
qPCR master mix	10 μL
Nucelaes free water	3μL
Total	20 μL

qPCR master mix for GAPH	volume
cDNA template (20ng)	5 μ L
Forward primer(10pmol)	1 μ L
Reverse primer (10pmol)	1 μ L
qPCR master mix	10 μ L
DEPC water	3 μ L
Total	20 μ L

After that, this qPCR master mix component that mentioned above placed in qPCR strip standard plate tubes and mixed by Exispin vortex centrifuge for 3 minutes, then placed in Miniopicon Real-Time PCR system.

B. miRNA qPCR Thermocycler conditions

After that, the qPCR plate was loaded and the following thermocycler protocol in the following table:

qPCR step	Temperature	Time	Repeat cycle	Time
Initial Denaturation	95 °C	5min	1	1 hour
Denaturation	95 °C	20 sec	45	5 minutes
Annealing\Extention Detection(scan)	60 °C	30 sec		

The data results of q RT-PCR for target and housekeeping gene were analyzed by the relative quantification gene expression levels (fold change) (The Δ CT Method Using a reference gene) that described by (Livak and Schmittgen, 2001). as following equation:

$$\Delta\text{CT (Test)} = \text{CT (target gene, test)} - \text{CT (HKG gene, test)}$$

$$\Delta\text{CT (Control)} = \text{CT (target gene, control)} - \text{CT (HKG gene, control)}$$

$$\Delta\Delta\text{CT} = \Delta\text{CT (Test)} - \Delta\text{CT (Control)}$$

$$\text{Fold change (target / HKG)} = 2^{-\Delta\Delta\text{CT}}$$

3.3. Statistical analysis

Data were summarized, presented and analyzed using statistical package for social science (SPSS version 23) and Microsoft office Excel 2016. Numeric data were presented as mean, standard deviation, range, median and interquartile range (IQR) after performance of Kolmogorov-Smirnov normality test and making decision about normally and non-normally distributed variable. An Independent sample t-test was used to study the difference in mean between any two groups provided that the variable is normally distributed. A one-way ANOVA test was used to study the difference in mean among more than two groups provided that the variable is normally distributed. The Chi-square test was used to study the association between any two categorical variables. The odds ratio and 95% confidence interval were estimated to measure risk. To detect the cutoff value that predicts a positive finding, receiver operator characteristic (ROC) curve analysis was used with its, . the level of significance was considered at P-value of 0.05 or less and highly significant level at 0.01 or less (Kwak, 2023)

RESULTS AND DISCUSSION

4. Results and Discussion

4.1. Demographic characteristics of patients and control subjects enrolled in the current study

The current study included a total of (120) participants, comprising (60) patients diagnosed with IPF and (60) apparently healthy controls matched for age and sex. The sample size was determined based on availability of confirmed cases during the study period and in accordance with inclusion and exclusion criteria to ensure homogeneity and reduce confounding variables.

Table (4-1) shows the demographic characteristics of patients and healthy individuals, The mean age of patients with idiopathic pulmonary fibrosis was 58.23 ± 11.9 years, while the mean age of healthy individuals was 53.95 ± 11.6 years. No statistically significant difference was observed between the two groups in mean age ($P = 0.113$). Similarly, no statistically significant difference was observed in the frequency distribution of patients and healthy individuals according to age group ($P = 0.651$). Moreover, most of the patients with IPF were within age (≥ 60 years) .

The patient group included 39 males (65.0%) and 21 females (35.0%), while the healthy group included 45 males (75.0%) and 15 females (25.0%). No statistically significant difference was observed in the frequency distribution of patients and healthy individuals according to sex ($P = 0.232$). the absence of a statistically significant difference in the distribution of individuals in the two groups according to age and sex is a prerequisite for avoiding bias in this case-control study. While the most of IPF patients in this study were male (65%).

No statistically significant difference was observed in the frequency distribution of the two groups according to smoking status ($P = 0.068$). This study included 42 patients (70.0%) with a normal weight ($BMI \geq 18.5-24.9 \text{ kg/m}^2$), 14 patients (23.3%) with an overweight weight ($BMI \geq 25.0-29.9 \text{ kg/m}^2$), and only 4

patients (6.7%) with obesity ($\text{BMI} \geq 30 \text{ kg/m}^2$). There was no statistically significant difference in the frequency distribution of the two groups according to the obesity classification ($P = 0.893$). While there was statistically significant difference in the frequency distribution of the IPF according to the obesity classification ($P = 0.001^*$), in which the higher percentage of IPF patients were normal weight (70%). Also, no statistically significant difference was observed in the frequency distribution of the two groups according to BMI ($P = 0.808$).

The present finding, that is the lack of significant difference in distribution of individuals in both groups according to age and gender are a pre-requisite in order to avoid bias in such case control study.

(4-1): Demographic characteristics of patients with IPF and healthy control subjects

Characteristic	IPF Patients n = 60	Healthy control n = 60	P
Age (years)			
Mean ±SD	58.23 ± 11.9	53.95 ± 11.6	0.113 † NS
< 40 years, n (%)	9 (15.0)	12 (20.0%)	0.651 ¥ NS
40-49 years, n (%)	12 (20.0%)	15 (25.0%)	
50-59 years, n (%)	12 (20.0%)	8 (13.3%)	
≥ 60 years, n (%)	27 (45.0%)	25 (41.7%)	
P value	0.004**	0.015**	
Gender			
Male, n (%)	39 (65.0 %)	45 (75.0 %)	0.232 ¥ NS
Female, n (%)	21 (35.0%)	15 (25.0%)	
M:F ratio	1.86:1	3:1	
P value	0.020**	0.001**	

Smoking			
Smoking, <i>n</i> (%)	9 (15.0%)	2 (3.3%)	0.068 ¥ NS
Non-smoking, <i>n</i> (%)	51 (85.0%)	58 (96.7%)	
P value	0.001**	0.001**	
Body mass index (BMI) kg/m2			
Mean ±SD	23.1 ± 3.96	22.90 ± 3.69	0.808 † NS
Range	18.70–31.80	18.90– 30.80	
Obesity Classification			
Normal weight, <i>n</i> (%)	42 (70.0%)	44 (73.3%)	0.893 ¥ NS
Overweight, <i>n</i> (%)	14 (23.3%)	13 (21.7%)	
Obese, <i>n</i> (%)	4 (6.7%)	3 (5.0%)	
P value	0.001**	0.001**	

n: number of cases; **SD**: standard deviation; †: independent samples t-test; ¥: Chi-square test; NS: not significant at $P > 0.05$

The results of this study showed that the comparative design between patients with IPF and the control group was well-balanced in terms of age, sex, and body mass index, with no statistically significant differences between the two groups in these variables. This similarity is a fundamental methodological requirement in case-control studies to minimize the influence of confounders and enhance the reliability of causal inferences. This methodology aligns with the guidelines of the American Thoracic Society and the European Respiratory Society (Graham *et al.*, 2019) which recommend careful control of demographic variables when studying factors influencing IPF, given the disease's association with advanced age and male predominance (Raghu *et al.*, 2022)

The results of this study are consistent with several previous studies that have indicated a strong relationship between age, sex, and the occurrence of idiopathic pulmonary fibrosis. Age is a factor associated with worsening health conditions and

decreased survival rates in patients with idiopathic pulmonary fibrosis due to degenerative changes affecting alveolar epithelial cells, reduced efficiency of tissue repair mechanisms, and increased levels of oxidative stress associated with aging (Koo *et al.*, 2016). The likelihood of developing the disease increases with age (over 60 years), particularly in men, while it rarely affects individuals under 40 and is almost nonexistent in children. This is what our study indicated, and this finding is consistent with the results of several previous studies (Golchin *et al.*, 2025; Koo *et al.*, 2016; Redente *et al.*, 2011). Genetic variations associated with an increased risk of idiopathic pulmonary fibrosis have recently been identified, but they may also increase the likelihood of developing other age-related lung diseases (Meltzer & Noble, 2008).

Our results are consistent with literature indicating sex-related differences in disease prevalence. Most studies show a clear male predominance in disease incidence compared to females, with a ratio reaching 3:1 in some studies. Males also exhibit higher incidence and mortality rates than females. However, information remains limited regarding potential differences in disease symptoms and comorbidities between the sexes (Golchin *et al.*, 2025). This association may be attributed to several possible factors, including increased male exposure to environmental and occupational factors such as smoking and industrial dust, as well as the potential protective role of female hormones, which may contribute to reducing the severity of the inflammatory response and oxidative stress, potentially playing a role in modulating the inflammatory response and fibrosis pathways (Zaman *et al.*, 2020).

Studies indicate that the effect of smoking is still unclear. Is it due to direct exposure to and ingestion of the fine particles contained in cigarette smoke, which may lead to apoptosis, or to the reactive oxygen and nitrogen species emitted from cigarette smoke that stimulate cell activation and death (Casal *et al.*, 2024; Oh *et al.*,

2012).Recent studies suggest that smoking may have a detrimental effect on the survival of patients with (IPF). Reference to a study by Kulkarni *et al* (2016) reported that lifelong non-smokers have better outcomes than former smokers. Studies also indicate that exposure to cigarette smoke may increase susceptibility to bleomycin-induced IPF.(Oh *et al.*, 2012) Smoking is a risk factor that increases the likelihood of developing IPF because it induces a specific phagocytic cell phenotype.(Gandhi *et al.*, 2023) Therefore, the results of this study do not negate the role of smoking, but rather indicate that it is a risk factor and may be a contributing or aggravating factor in the disease, rather than an independent direct cause. This agreement with the current view that IPF results from a complex interaction between environmental and biological factors (Raghu *et al.*, 2022).

Body mass index (BMI) is a common indicator for assessing nutritional status and is calculated using the formula: $BMI = \text{weight (kg)} / \text{height (m}^2\text{)}$. This index is used to classify individuals into categories such as underweight, normal weight, overweight, and obesity. According to the Asia Pacific region's obesity criteria, BMI is classified as: underweight ($<18.5 \text{ kg/m}^2$), normal weight ($18.5\text{--}22.9 \text{ kg/m}^2$), overweight ($23\text{--}24.9 \text{ kg/m}^2$), and obesity ($\geq 25.0 \text{ kg/m}^2$). The World Health Organization has adopted this formula as an international standard for assessing body composition.(Okawa & Mitsuhashi, 2025)

In the results of the current study, no statistically significant difference was observed in the frequency distribution between patients and the healthy control group according to body mass index (BMI) ($P = 0.808$), indicating that obesity was not a distinguishing factor in this sample. This finding is consistent with studies suggesting that IPF is not a metabolic disease, but rather a degenerative fibrosis associated with impaired lung tissue repair after repeated injuries(King *et al.*, 2014; Zinellu *et al.*, 2023) . A review has shown that “there is no clear evidence of a direct association between BMI and the risk of developing IPF”(Zinellu *et al.*, 2023).

Many studies have focused on the predictive role of body mass index (BMI) in disease progression, a decrease in BMI or weight loss has been found to be associated with an increased risk of death and worsening clinical condition in IPF patients (Xing *et al.*, 2024). Analytical studies have also shown that a higher BMI may be associated with a relatively lower risk of death, a phenomenon known as the (obesity paradox) It is worth noting that malnutrition in advanced stages can result from anorexia and chronic shortness of breath.(Pan *et al.*, 2024; Zinellu *et al.*, 2023). In light of our study, which showed that the average BMI of the patients was within normal limits, with no significant difference compared to the control group, this can be explained by the fact that the patients in our sample did not reach the advanced stages of clinical deterioration accompanied by weight loss or wasting, which is often observed in the later stages of the disease.

4.2. Family history.

The presence of family history is an important contributory factor in IPF. The present results show that 9 (15.0%) of IPF patients have positive family history and 51 (85.0%) of patients have negative family history, figure (4-1).

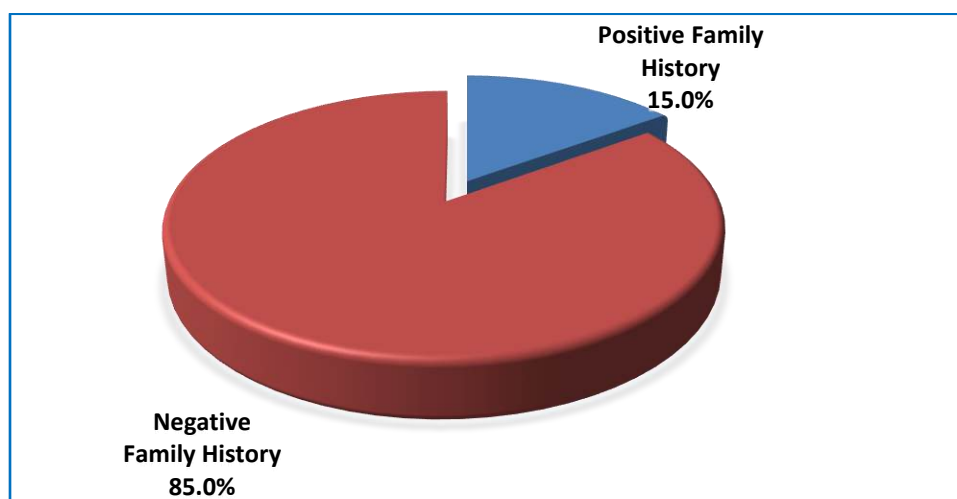


Figure (4-1): Distribution of patients according to family history.

This result is consistent with epidemiological studies indicating that the familial form of pulmonary fibrosis accounts for a small proportion (approximately 0.5–2%) of all cases, with most cases lacking a clear family history (Bailey *et al.*, 2013; Marshall *et al.*, 2000). However, 15% of IPF patients were positive in this study, which may reflect an increased detection of familial cases due to improved awareness and diagnosis. Having a family history does not necessarily mean that the disease is transmitted directly, but rather reflects a genetic predisposition that interacts with environmental factors such as smoking, occupational exposure, and oxidative stress (Meltzer & Noble, 2008; Zaman & Lee, 2018). This supports the idea that IPF is a multifactorial disease resulting from a complex interaction between genes and the environment (Fabiane *et al.*, 2019). Accordingly, the results of our study support the idea that IPF is mostly a non-familial disease, although there is a limited percentage of cases that may be related to a genetic predisposition. This highlights the importance of studies related to the early detection of this category of patients and a deeper understanding of the mechanisms of the disease.

4.3. clinical pathological characteristics of IPF patient and control.

4.3.1. The frequency distribution of IPF patients according to severity

The frequency distribution of IPF patients according to severity was shown in figure (4-2), as the followings: 42 (70.0%) of IPF patients was severe cases and 18 (30.0 %) of IPF patients was moderate cases.

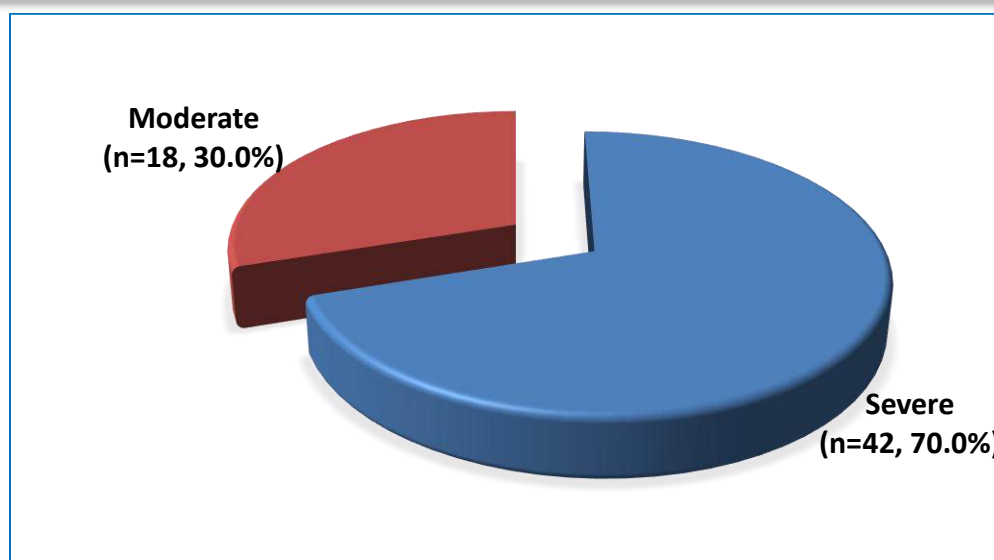


Figure (4-2): Distribution of patients according to severity

This finding reflects a common pattern in IPF of diagnosis at relatively advanced stages. This finding is consistent with the scientific literature, which describes idiopathic pulmonary fibrosis as a progressive disease characterized by a continuous decline in lung function, often discovered only after significant tissue damage has occurred due to delayed diagnosis. Studies have shown a clear time lag between the onset of symptoms and diagnosis, averaging more than two years (Wang *et al.*, 2025). Because of the nonspecific nature of early symptoms such as shortness of breath and dry cough, which are often attributed to old age or other respiratory diseases, diagnosis may be delayed for several years in some cases (Goel *et al.*, 2012).

IPF is a progressive disease by nature, so a delay in diagnosis directly leads to clinical deterioration, as the progression to advanced stages accelerates upon diagnosis, lung function deteriorates, and quality of life declines (Hoyer & Bendstrup, 2022). IPF is characterized by a gradual and irreversible decrease in lung vital capacity due to increased fibrous tissue deposition over time, leading to progression from moderate to severe stages if early intervention does not occur (Strykowski & Adegunsoye, 2023). Therefore, initiating treatment at advanced

stages reduces its effectiveness in slowing disease progression, underscoring the importance of early diagnosis(Herberts *et al.*, 2023). The high percentage of severe cases in the current study may reflect limited early detection or delays in referring patients to specialized centers, a finding supported by numerous international studies(Herberts *et al.*, 2023; Raghu *et al.*, 2022). The results of the current study are consistent with the scientific direction that confirms that IPF is often diagnosed at late stages, due to the nature of the disease's gradual symptoms, which are similar to other less severe lung diseases, leading to a delay in its clinical recognition(Wang *et al.*, 2025). Therefore, there is an urgent need to strengthen early diagnosis strategies to improve the prognosis and reduce the rate of disease progression.

4.3.2. Results of Lung function test (FVC% and FEV1/FVC%1) in patients and healthy controls.

The comparison of Lung function test (FVC% and FEV1/FVC%1) in IPF patients and healthy controls has been carried out and the results were demonstrated in table (4-2). Mean levels of Forced Vital Capacity (FVC%) were 41.81 ± 6.11 and 97.00 ± 4.36 , in IPF patients and healthy control group respectively; the mean levels was lower in IPF patient in compared to healthy control and the difference was significant ($P < 0.001$). But the mean ratio of the forced expiratory volume in one second (FEV1) to the forced vital capacity (FVC) (FEV1/FVC%1) were 91.64 ± 10.1 and 80.45 ± 1.41 , in IPF patients and healthy control group respectively; the mean levels was higher in IPF patient in compared to healthy control and the difference was significant ($P < 0.001$). The Logistic regression model show that the correlation lung function test such as FVC% in which have indirectly correlate with FEV1/FVC%1 among IFP patients as in figure (4-3) this result might be refer to that IPF condition enhances production of FVC% in suppression the expression of FEV1/FVC%1.The comparison of Lung function test (FVC% and FEV1/FVC%1) according to disease severity has been carried out and the results were demonstrated

in table (4-3) In all Lung function test (FVC% and FEV1/FVC%1), the present results show there was significant difference between patients with moderate severity and patients with severe severity according to each of FVC% and FEV1/FVC%1 ($p < 0.05$).

Table (4-2) :Comparison of Lung function test (FVC% and FEV1/FVC%1) in patients and healthy controls

Groups		Forced Vital Capacity (FVC%) level	FEV1/FVC%1
IPF patients	Mean $\pm$ SD	41.81 $\pm$ 6.11	91.64 $\pm$ 10.1
Control	Mean $\pm$ SD	97.00 $\pm$ 4.36	80.45 $\pm$ 1.41
p-value		0.001**	0.017**

SD: standard deviation; †: Independent T test; **: significant at $P < 0.05$

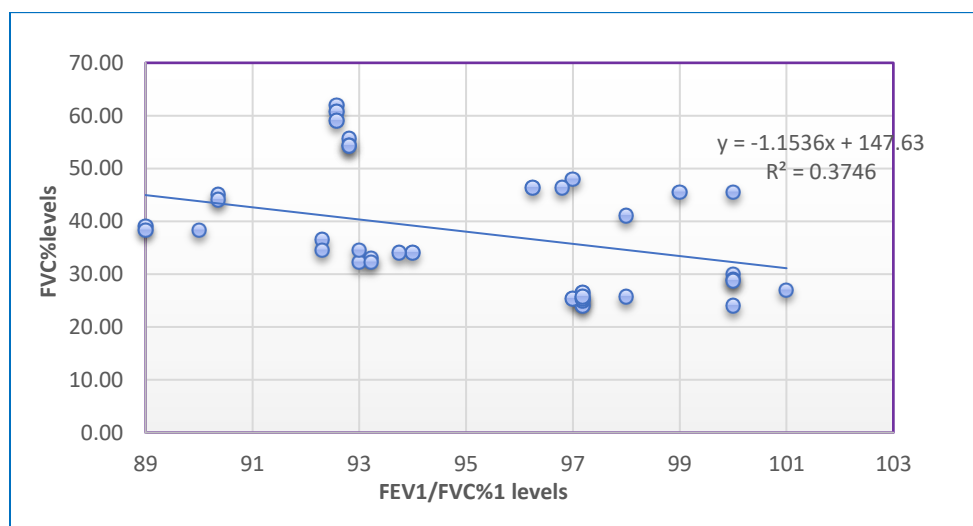


Figure (4-3): The Linear Regression of FVC% and FEV1/FVC%1 levels among patient

Table (4-3): Frequency distribution of Lung function test (FVC% and FEV1/FVC%1) according to disease severity

Characteristic	FVC%	FEV1/FVC%1
Disease severity		
Moderate	55.14 ± 4.44	87.63 ± 4.57
Severe	36.11 ± 6.9	93.23 ± 4.71
p-value	0.001** †	0.001** †

†: Independent T test; **: significant at $P < 0.05$

One of the most important signs of fibrosis in IPF is collagen deposition and thickening of the alveolar septum, leading to increased stiffness of the lung tissue(Alonso *et al.*, 2023). This limits the lung's ability to expand during inspiration and results in a significant decrease in total exhaled volume (FVC)(Raghu *et al.*, 2022). Forced expiratory volume in the first second (FEV1) decreases, but to a lesser extent than FVC. This is due to the increased elastic rebound forces of the lungs in restrictive diseases, which cause air to be expelled more rapidly at the beginning of exhalation.(Graham *et al.*, 2019). Consequently, the ratio between FEV1 and FVC remains normal or increases, despite the overall decrease in lung volumes(Fu *et al.*, 2024). This confirms that the decline in lung function is directly related to the progression of fibrosis, reflecting the gradual expansion of fibrotic areas and a decrease in the effective air space for gas exchange(Strykowski & Adegunsoye, 2023)This finding supports clinical studies indicating that assessing disease severity based on forced vital capacity (FVC) is a cornerstone of disease classification and management(Kulkarni *et al.*, 2016).

Our results showed a significant decrease in forced vital capacity (FVC%) in patients with IPF compared to the control group , while the FEV1/FVC% ratio was significantly higher in these patients, these results reflect the known functional pattern of IPF classified as a restrictive lung disease(King *et al.*, 2014; Raghu *et al.*,

2022). The decrease in FVC% is consistent with findings in clinical studies, where it is considered a key indicator of fibrosis severity. This is due to increased collagen deposition and loss of lung tissue elasticity, leading to reduced lung expansion during inhalation (Tsitoura *et al.*, 2021). International guidelines indicate that a decrease in FVC is used as a primary indicator for assessing disease severity and monitoring its progression, and it is directly associated with increased mortality in IPF patients (Marinescu *et al.*, 2022). The markedly elevated FEV1/FVC% ratio in patients, which is a hallmark of restrictive disorders, is what distinguishes IPF from obstructive diseases such as asthma and chronic obstructive pulmonary disease (COPD), which are characterized by a lower ratio (Zamfir *et al.*, 2023)

4.4. physiological biomarker

4.4.1. Evaluation Complete Blood Count of Studied Groups

The comparison of some blood parameters ((WBC, neutrophils, lymphocyte and neutrophils%) in IPF patients and healthy controls has been carried out and the results were demonstrated in table (4-4). Mean levels of white blood cells (WBC) count were 8.89 ± 1.8 and 7.82 ± 1.3 , in IPF patients and healthy control group respectively; the mean levels was higher in IPF patients in compared to healthy control, and the difference was significant ($P > 0.05$). Also the Neutrophils count and Neutrophils % were significantly higher in IPF patients in compared to healthy control, ($P < 0.05$). But the present results show significant decrease of Lymphocyte count in IPF patients compared to healthy control, ($P < 0.05$). The comparison of blood parameters (WBC, neutrophils, lymphocyte and neutrophils%) according to disease severity has been carried out and the results were demonstrated in table (4-5). In all blood parameters (WBC, neutrophils, lymphocyte and neutrophils%), the present results show there was significant difference between patients with moderate severity and patients with severe severity according to each of neutrophils, lymphocyte and Neutrophil% ($p < 0.05$). But there was non-significant difference

between patients with moderate severity and patients with severe severity according to WBC, ($p>0.05$). The correlations between blood parameters (WBC, neutrophils, lymphocyte and neutrophils%) and lung function test parameters in IPF patients were shown in tables (4-6). There are significant positive correlation between WBC and Neutrophils counts ($r=0.527$ and $p=0.001$), WBC and Lymphocyte counts ($r=0.302$ and $p=0.019$), neutrophils and neutrophils% ($r=0.499$ and $p=0.001$), in IPF patients. Furthermore, there are significant negative correlation between Lymphocyte and neutrophils% ($r=-0.813$ and $p=0.001$), in IPF patients. But there are were non-significant correlation between all other parameters.

Table (4-4): Comparison of some blood parameters (WBC, neutrophils, lymphocyte and neutrophils%) in IPF patients and healthy controls.

Characteristic	IPF patients n=60	Healthy control n=60	P
White blood cells (WBC) count ($\times 10^9/L$)			
Mean $\pm$ SD	8.89 $\pm$ 1.8	7.82 $\pm$ 1.3	0.015 † S
Neutrophils count ($\times 10^9/L$)			
Mean $\pm$ SD	6.70 $\pm$ 1.2	5.23 $\pm$ 1.1	0.001 † S
Lymphocyte count ($\times 10^9/L$)			
Mean $\pm$ SD	1.66 $\pm$ 0.41	2.19 $\pm$ 0.32	0.001 † S
Neutrophils%			
Mean $\pm$ SD	75.11 $\pm$ 10.1	63.81 $\pm$ 4.46	0.002 † S

SD: standard deviation ; †: Independent T test; S: significant at $P < 0.05$

Table (4-5): Frequency distribution of blood parameters (WBC, neutrophils, lymphocyte and neutrophils%) according to disease severity

Characteristic	WBC	Neutrophil	Lymphocyte	Neutrophil%
Disease severity				
moderate	8.43 ± 1.61	5.90 ± 1.20	1.93 ± 0.61	68.91 ± 5.61
sever	9.08 ± 1.94	7.03 ± 1.69	1.54 ± 0.41	77.78 ± 8.6
p-value	0.220 †	0.032** †	0.029** †	0.001** †

†: Independent T test; **: significant at $P < 0.05$

Table (4-6): Correlation between blood parameters (WBC, neutrophils, lymphocyte and neutrophils%) and lung function test parameters in IPF patients

Characteristic	blood parameters							
	WBC		Neutrophils		Lymphocyte		neutrophils%	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
WBC	1							
Neutrophils	0.527	0.001*	1					
Lymphocyte	0.302	0.019*	- 0.11 1	0.401	1			
neutrophils%	- 0.074	0.576	0.499	0.001*	-0.813	0.001*	1	
FVC%	- 0.201	0.124	-0.188	0.151	0.198	0.129	-0.177	0.177
FEV1/FVC%1	0.064	0.627	0.130	0.322	-0.153	0.244	0.199	0.127

r: correlation coefficient.

The results of the current study indicate a significant increase in the number and percentage of white blood cells (WBCs) and neutrophils, along with a marked decrease in lymphocytes, leading to an elevated neutrophil-to-lymphocyte ratio (NLR), which is used as an important inflammatory marker.(Zinellu *et al.*, 2020) Studies have shown that an elevated NLR is associated with increased disease severity and decreased lung function (Bergantini *et al.*, 2020; Zhang *et al.*, 2024)

These results reflect an immune imbalance characterized by the dominance of innate immunity and a diminished role of adaptive immunity, consistent with the modern understanding of the mechanism of pulmonary fibrosis(Alonso *et al.*, 2023). The findings of the current study can be further interpreted by comparing them with the study by Tsitoura *et al.* (2021), which highlighted the pivotal role of Macrophages In fibrosis, type M2 is overactive, secreting substances such as TGF- β and IL-13 that stimulate collagen production. Studies have shown that phagocytic cells in the lungs of fibrosis patients express collagen 1a1 abnormally, meaning that they not only function as immune cells but also become direct contributors to the fibrosis process(Fu *et al.*, 2024; Malaviya *et al.*, 2023).

The transformation of M2 macrophages is involved in tissue repair and, when dysregulated, can drive fibrosis. is linked to the secretion of inflammatory mediators and chemokines that attract neutrophils to the site of inflammation, These cells secrete enzymes such as elastase and extracellular neutrophil networks, which accelerate fibrosis (Bellaye & Kolb, 2015; Song *et al.*, 2000; Zhao *et al.*, 2025). One study indicated that higher concentrations of these cells in the lungs are associated with a poor prognosis(Bellaye & Kolb, 2015). This supports the idea that neutrophils are not merely an inflammatory marker, but an active component in the progression of fibrosis(Zhao *et al.*, 2025). Therefore, the elevated neutrophil counts observed in our study can be explained in light of these findings.

In normal lung tissue, Th1 cells secrete cytokines such as IFN- γ , which act as antifibrotic agents by inhibiting the activation of Th2 cells which stimulate fibrosis this process reduces fibroblast proliferation and collagen production It modulates macrophage activity shifting it from the fibrotic M2 to the inflammatory M1 subtype (Malaviya *et al.*, 2023; Tylah, 2022). Tsitoura stated that the inflammatory environment in IPF shifts from an adaptive immune response to a fibrotic response driven by phagocytic cells. Activated phagocytic cells secrete TGF, IL-10, TNF- α and β DGF (platelet-derived growth factor)(Malaviya *et al.*, 2023; Tsitoura *et al.*, 2021). These factors promote fibrosis and suppress the lymphatic response. TNF- α binds to its receptors on T-cells, activating caspase-8 and apoptosis. TGF- β also inhibits T-cell proliferation and induces cell death (Wynn, 2011; Li *et al.*, 2006) Therefore, the reduced lymphocyte count in blood in our results reflects the suppression of adaptive immunity in favor of the fibrosis pathway.

4.4.2. Levels of air alveoli epithelia dysfunction parameters

The results of this study, presented in Tables (4-7),the studied group showed a significant increase in the levels of biomarkers of alveolar epithelial dysfunction, specifically alpha-v beta-6 ($\alpha\text{v}\beta 6$) integrin, with an average level of 71.47 ± 12.77 ng/L in patients with IPF and 28.36 ± 4.71 ng/L in the healthy control group. Elevated levels of cleaved cytokeratin 18(cCK-18) and COL1 α 1 were also observed in patients compared to healthy individuals. These differences were statistically significant ($P < 0.001$).

The comparison of air alveoli epithelia dysfunction parameters according to severity of disease has been carried out and the results were demonstrated in table (4-8). In all parameters, the present results show there was non-significant difference between patients with moderate severity and patients with severe severity according to each of $\alpha\text{v}\beta 6$ integrin, cCK-18 and COL1A1 ($p > 0.05$).

4.4.2.A. Diagnostic accuracy of air alveoli epithelia dysfunction parameters.

Receiver operating characteristic (ROC) analysis was performed to reveal the prognostic accuracy of using $\alpha\text{v}\beta 6$ integrin concentrations to distinguish IPF patients from healthy control subjects. Table (4-9) An optimal $\alpha\text{v}\beta 6$ integrin cut-off value more than of 34.82 resulted in an AUC value of 0.983 (95% confidence interval [CI], 0.951- 1.000, $P= 0.001$), sensitivity of 96.7%, specificity of 98.3 %, PPV of 98.3 %, and NPV of 96.7%. The present results indicate $\alpha\text{v}\beta 6$ integrin is considered as excellent prognostic marker to distinguish IPF patients from healthy control. Also, the present results indicate COL1 $\alpha 1$ is considered as excellent prognostic marker to distinguish IPF patient from healthy control.

In addition, an optimal cCK-18 cut-off value of more than 40.37 could be used to distinguish IPF patient from healthy control subjects with a sensitivity of 80.0%, specificity of 81.7%, PPV of 81.4%, and NPV of 80.3% and 0.808 (0.720- 0.896). The present results indicates cCK-18 is considered as a good prognostic marker to distinguish IPF patient from healthy control (Figure 4-4).

4.4.2.B. Regression correlations between air alveoli epithelia dysfunction parameters.

The Logistic regression model show that the correlation air alveoli epithelia dysfunction parameters such as $\alpha\text{v}\beta 6$ integrin in which have directly correlate with cCK-18 and COL1 $\alpha 1$ among IPF patients as in figure (4-5) and (4-6) this result might be refer to that IPF condition enhances production of $\alpha\text{v}\beta 6$ integrin in relation the expression both of cCK-18 and COL1 $\alpha 1$. Also cCK-18 in which have directly correlate with COL1 $\alpha 1$ among IPF patients as in figure (4-7).

Table (4-7): Comparison of air alveoli epithelia dysfunction parameters ($\alpha\beta6$ integrin, cCK-18 and COL1 α) in patients and healthy controls

Groups		$\alpha\beta6$ integrin (ng/L)	cCK-18 (mIU/mL)	COL1 α 1(ng/L)
IPF patients	Mean $\pm$ SD	71.47 $\pm$ 12.77	51.14 $\pm$ 11.3	44.08 $\pm$ 9.81
Control	Mean $\pm$ SD	28.36 $\pm$ 4.71	38.07 $\pm$ 4.54	22.68 $\pm$ 5.95
p-value		0.001**	0.001**	0.001**

SD: standard deviation; †: Independent T test; **: significant at $P < 0.05$

Table (4-8): Frequency distribution of air alveoli epithelia dysfunction parameters according to Severity of disease

Groups	$\alpha\beta6$ integrin(ng/L)	cCK-18(mIU/mL)	COL1 α 1(ng/L)
Severity of disease			
Moderate	70.92 $\pm$ 11.3	52.41 $\pm$ 11.4	44.82 $\pm$ 8.4
Severe	73.67 $\pm$ 9.98	51.23 $\pm$ 13.7	42.33 $\pm$ 9.54
P value	0.644 †	0.832 †	0.536 †

†: independent samples t-test; **: significant at $P < 0.05$

Table (4-9): air alveoli epithelia dysfunction parameters

Characteristic	$\alpha\beta6$ integrin (ng/L)	cCK-18 (mIU/mL)	COL1A1(ng/L)
Cutoff value	> 34.82	> 40.37	> 29.69
P value	0.001	0.001	0.001
Sensitivity %	96.7 %	80.0 %	96.7 %
Specificity %	98.3 %	81.7 %	95.0 %
PPV %	98.3 %	81.4 %	95.1 %
NPV %	96.7 %	80.3 %	96.6 %
AUC (95% CI)	0.983 (0.951- 1.000)	0.808 (0.720- 0.896)	0.982 (0.960- 1.000)

CI: Confidence interval, AUC: Area under curve.

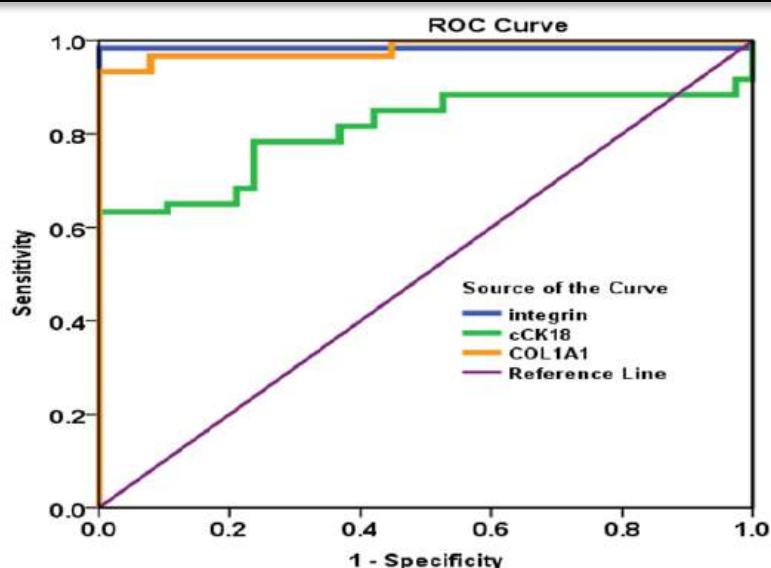


Figure (4-4): (Blue curve) Receiver operating characteristic curve for $\alpha\text{v}\beta 6$ integrin levels to distinguish IPF patients from healthy control subjects. (Green curve) Receiver operating characteristic curve for cCK-18 levels to distinguish IPF patients from healthy control subjects. (Orange curve) Receiver operating characteristic curve for COL1A1 levels to distinguish IPF patients from healthy control subjects.

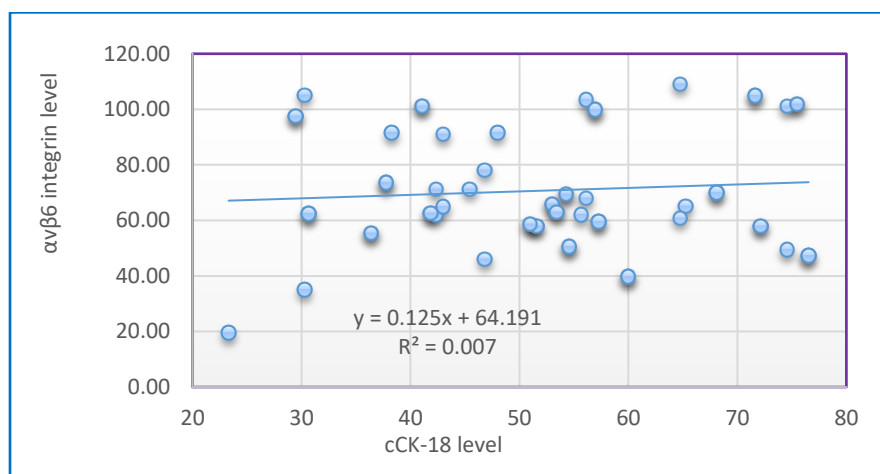


Figure (4-5): The Linear Regression of $\alpha\text{v}\beta 6$ integrin and cCK-18 levels among patients.

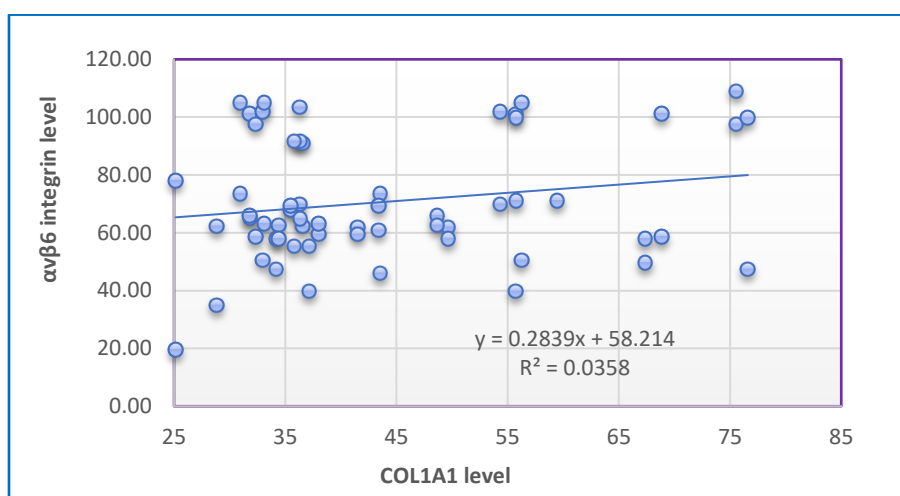


Figure (4-6): The Linear Regression of αvβ6 integrin and COL1A1 levels among patients.

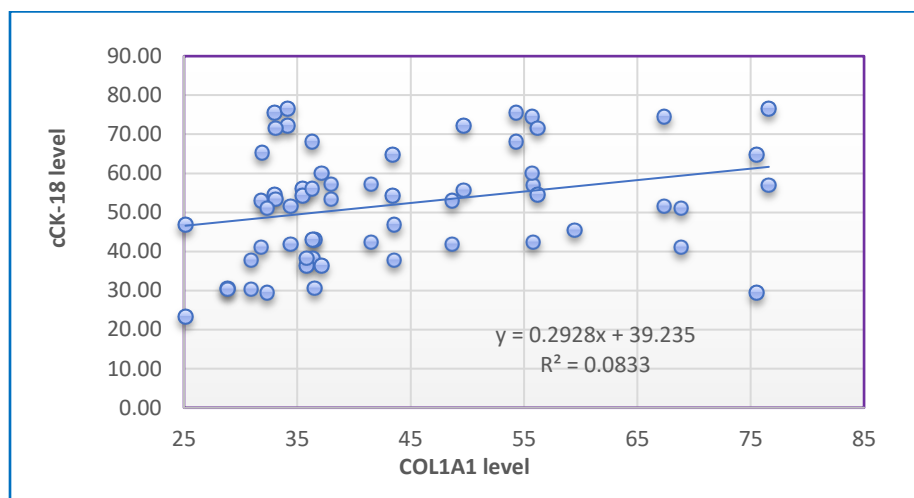


Figure (4-7): The Linear Regression of cCk-18 and COL1A1 levels among patients.

4.4.2.C. Correlation between air alveoli epithelia dysfunction parameters and other parameters in patients with IPF.

The correlation between air alveoli epithelia dysfunction parameters and other parameters in patients with IPF were shown in tables (4-10). There are significant positive correlation between αvβ6 integrin and Neutrophils counts ($r=0.316$ and $p=0.016$), αvβ6 integrin and neutrophils% ($r=0.329$ and $p=0.011$), in IPF patients. But there are were non-significant correlation between all other parameters.

Table (4-10): Correlation between air alveoli epithelia dysfunction parameters and other parameters in patients with IPF

Parameters	Air alveoli epithelia dysfunction parameters					
	$\alpha\beta6$ integrin		cCK-18		COL1 α 1	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
WBC	0.045	0.733	0.031	0.813	0.133	0.810
Neutrophils	0.310	0.016*	0.126	0.375	0.069	0.600
Lymphocyte	-0.210	0.107	-0.086	0.570	-0.022	0.806
neutrophils%	0.329	0.011*	0.083	0.531	0.079	0.547
FVC%	-0.065	0.621	-0.011	0.933	-0.121	0.359
FEV1/FVC%1	0.019	0.886	0.016	0.905	0.081	0.537

r: correlation coefficient.

significant increase in $\alpha\beta6$ integrin levels in patients with idiopathic pulmonary fibrosis compared to the control group showed in the results of this study, can be explained by the fact that this protein is a key receptor in damaged alveolar epithelial cells. It plays a crucial role in activating (TGF- β), the molecular pathway most strongly associated with pulmonary fibrosis. These results are consistent with what other studies have indicated, which find that $\alpha\beta6$ integrin is one of the most important biomarkers associated with the development of pulmonary fibrosis, and could serve as potential therapeutic target (Brzozowska & Deshmukh, 2022; Madala *et al.*, 2025).

This contributes to stimulating the transformation of fibroblasts into myofibroblasts and increasing extracellular matrix production, ultimately leading to collagen accumulation and the development of pulmonary fibrosis. (Lian *et al.*, 2023)

TGF- β itself has been found to stimulate the expression of the integrin $\alpha\beta6$ through an Smad3-dependent regulatory mechanism, creating a positive feedback loop that promotes fibrosis (Decaris *et al.*, 2021). Recent studies have shown that

dual inhibition of $\alpha\text{v}\beta 6$ and $\alpha\text{v}\beta 1$ proteins significantly reduces COL1A1 gene expression in lung sections from patients with idiopathic pulmonary fibrosis, confirming a causal relationship between the activity of this integrin and collagen synthesis, which is consistent with our findings. Furthermore, clinical trials have demonstrated that the $\alpha\text{v}\beta 6/\alpha\text{v}\beta 1$ inhibitor (Bexotegast) reduces the deposition of active type I collagen in the lungs of patients with idiopathic pulmonary fibrosis (Golovina *et al.*, 2025).

The current study also revealed an obvious increase in COL1A1 levels in patients with IPF compared to healthy individuals. COL1A1 consider a major component of the extracellular matrix in fibrotic tissues, and its accumulation leads to loss of lung elasticity and reduced gas s exchange efficiency(Devos *et al.*, 2023). Furthermore, COL1A1 gene expression is closely linked to the activation of fibroblasts and their transformation into myofibroblasts via the TGF- β pathway, as demonstrated by numerous studies. These studies also indicate that increased production of COL1 α 1 is a key feature of pulmonary fibrosis (Tsitoura *et al.*, 2021)

COL1A1 is a gene that encodes the alpha-1 chain of type I collagen, the primary component of the ECM in fibrotic tissue(Tsitoura *et al.*, 2021). Multiple studies have shown that COL1A1 is a hub gene in IPF, and its high expression is associated with poor prognosis and reduced survival rates(Sun *et al.*, 2025). The direct correlation shown by ROC curve analysis in our results between $\alpha\text{v}\beta 6$ integrin and COL1A1 can be readily explained by the well-established role of $\alpha\text{v}\beta 6$ in activating TGF- β , the primary driver of type I collagen synthesis(Madala *et al.*, 2025). When activated, TGF- β stimulates myofibroblasts to produce large quantities of type I collagen, encoded by the COL1A1 gene(Devos *et al.*, 2023). Therefore, our findings suggest the potential use of integrins and the COL1A1 gene as diagnostic tools for early disease diagnosis. ROC curve analysis showed that $\alpha\text{v}\beta 6$ and COL1A1 possess high diagnostic power in distinguishing between patients with

idiopathic pulmonary fibrosis and healthy individuals, consistent with previous findings(Agarwal *et al.*, 2020; Tsitoura *et al.*, 2021).

As for cCK-18, it is demonstrated good diagnostic accuracy but relatively lower compared to the other two markers, suggesting that it may be more closely associated with epithelial cell death rather than fibrosis itself.

The results of the current study showed a significant increase in serum cCK-18 levels in patients with IPF. This increase is consistent with the scientific literature indicating that cCK-18 can be a biomarker for apoptosis of alveolar epithelial cells (AECs), a pivotal process in the pathogenesis of IPF cCK-18 is considered a direct result of the cleavage of cytokeratin-18 protein by caspases during apoptosis (Wu *et al.*, 2024). Therefore, the high level recorded in our results is laboratory evidence of active programmed cell death in lung tissue, consistent with the findings reported by Cha *et al.*, (2012)who found that cCK-18 levels were significantly elevated in IPF patients compared to healthy individuals, and suggested its use as a potential diagnostic tool . It is important to note that this elevation may not necessarily reflect disease severity, as some studies have not shown a strong correlation between cCK-18 levels and pulmonary function indicators such as forced vital capacity (FVC) or (FEV1/FVC%1) (Cha *et al.*, 2012)

However, its value lies in its being an indicate While the prevailing model focuses on cCK-18 as a marker of cell death, other evidence points to a possible role for cytokeratin-18 in triggering an autoimmune response. Dobashi *et al.* (2000) found autoantibodies against cK-18 and CK-18-containing immune complexes in the serum of IPF patients (Dobashi *et al.*, 2000). This finding opens the door to a complementary interpretation of our results: it is possible that the elevated cCK-18 resulting from cell death stimulates the immune system, generating a sustained autoimmune response that exacerbates epithelial cell damage(Stainer *et al.*, 2021).

In our case, determining whether there is an association with autoantibodies requires further analysis.

The direct correlation between cCK-18 and COL1 α 1 demonstrated in our results represents an important link between epithelial cell death (reflected by cCK-18) and excessive collagen production (reflected by COL1 α 1). This correlation may be explained by the fact that TGF- β activation by α v β 6 leads to epithelial cell apoptosis, thereby simultaneously increasing cCK-18, myofibroblast activation, and COL1 α 1 (Cha *et al.*, 2012;Decaris *et al.*, 2021).

In other words, these markers may represent e sequential stages in the mechanism of severe pulmonary fibrosis, beginning with epithelial cell damages, followed by cell death, then fibroblast activation, and finally collagen deposition.

4.4.3. Oxidative stress parameters (GDF15, SOD3 and Nrf2) in studied group

The comparison of oxidative stress parameters (GDF15, SOD3 and Nrf2) in IPF patients and healthy controls has been carried out and the results were demonstrated in table (4-11). Mean levels of Growth Differentiation Factor 15 were 554.02 ± 47.4 ng/L and 428.27 ± 18.99 ng/L, in IPF patients and healthy control group respectively; the mean levels was higher in IPF patient in compared to healthy control and the difference was significant ($P < 0.001$). Also the mean of SOD3 level were 10.07 ± 1.73 ng/L and 8.08 ± 1.42 ng/L ,in IPF patients and healthy control group respectively; the mean levels was higher in IPF patient in compared to healthy control and the difference was significant ($P < 0.001$). Furthermore, the mean levels of Nrf2 was higher in IPF patient in compared to healthy control and the difference was significant ($P < 0.001$).

The comparison of oxidative stress parameters according to severity of disease has been carried out and the results were demonstrated in table (4-12). In all

parameters, the present results show there was non-significant difference in the levels of GDF15, SOD3 and Nrf2 ($p>0.05$) between IPF patients with moderate severity and IPF patients with severe severity .

4.4.3.A. Diagnostic accuracy of oxidative stress parameters.

Receiver operating characteristic (ROC) analysis was performed to reveal the prognostic accuracy of using GDF15 concentrations to distinguish IPF patients from healthy control subjects. Table (4-13) An optimal GDF15 cut-off value more than of 453.92 resulted in an AUC value of 0.911 (95% confidence interval [CI], 0.849-0.974, $P= 0.001$), sensitivity of 90.0%, specificity of 91.7 %, PPV of 91.5 %, and NPV of 90.2%. The present results indicates GDF15 is considered as excellent prognostic marker to distinguish IPF patients from healthy control. Also the present results indicates Nrf2 is considered as excellent prognostic marker to distinguish IPF patient from healthy control.

In addition, an optimal SOD3 cut-off value of more than 9.28 could be used to distinguish IPF patient from healthy control subjects with a sensitivity of 76.7%, specificity of 75.0%, PPV of 75.4%, and NPV of 76.3% and 0.768 (0.699- 0.809). The present results indicates SOD3 is considered as acceptable prognostic marker to distinguish IPF patient from healthy control. Figure (4-8).

4.4.2.B. Regression correlations between oxidative stress parameters.

The Logistics regression model show that the correlation oxidative stress parameters such as GDF15 in which have directly correlate with SOD3 and Nrf2 among IPF patients as in figure (4-9) and (4-10) this result might be refer to that IPF condition enhances production of GDF15 in relation the expression both of SOD3 and Nrf2. Also SOD3 in which have directly correlate with Nrf2 among IPF patients as in figure (4-11).

Table (4-11) :Comparison of oxidative stress parameters (GDF15, SOD3 and Nrf2) in patients and healthy controls

Groups		GDF15 (ng/L)	SOD3 (ng/L)	Nrf2 (ng/ml)
IPF patients	Mean $\pm$ SD	554.02 $\pm$ 47.4	10.07 $\pm$ 1.73	53.52 $\pm$ 11.01
	Range	381.78-795.83	6.55-13.06	32.35-77.61
Control	Mean $\pm$ SD	428.27 $\pm$ 18.99	8.08 $\pm$ 1.42	32.42 $\pm$ 3.25
	Range	393.05-464.06	5.91-10.67	30.00-35.73
p-value		0.001**	0.021**	0.001**

SD: standard deviation; †: Independent T test; **: significant at $P < 0.05$

Table (4-12): Frequency distribution of oxidative stress parameters according to severity of disease

Groups	GDF15 (ng/L)	SOD3 (ng/L)	Nrf2 (ng/ml)
Severity of disease			
Moderate	561.91 $\pm$ 52.3	10.06 $\pm$ 1.54	52.03 $\pm$ 11.1
Severe	535.61 $\pm$ 44.7	10.11 $\pm$ 2.16	54.07 $\pm$ 11.3
P value	0.342 †	0.932 †	0.107 †

†: independent samples t-test; **: significant at $P < 0.05$

Table (4-13): Roc curve of oxidative stress parameters (GDF15, SOD3 and Nrf2)

Characteristic	GDF15 (ng/L)	SOD3 (ng/L)	Nrf2 (ng/ml)
Cutoff value	> 453.92	> 9.28	> 34.75
P value	0.001	0.001	0.001
Sensitivity %	90.0 %	76.7 %	96.7 %
Specificity %	91.7 %	75.0 %	98.3 %
PPV %	91.5 %	75.4 %	98.3 %
NPV %	90.2 %	76.3 %	96.7 %
AUC (95% CI)	0.911 (0.849-0.974)	0.768 (0.699-0.809)	0.983 (0.962-1.000)

CI: Confidence interval, AUC: Area under curve.

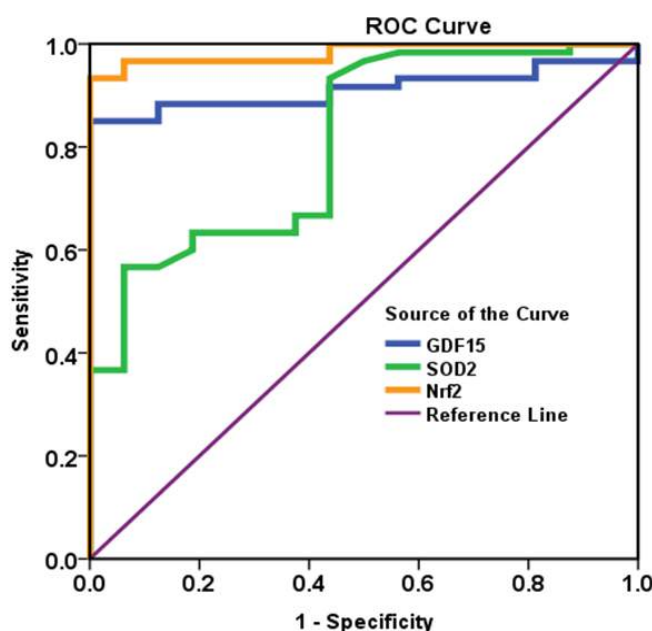


Figure (4-8): (Blue curve) Receiver operating characteristic curve for GDF15 levels to distinguish IPF patients from healthy control subjects. (Green curve) Receiver operating characteristic curve for SOD3 levels to distinguish IPF patients from healthy control subjects. (Orange curve) Receiver operating characteristic curve for Nrf2 levels to distinguish IPF patients from healthy control subjects.

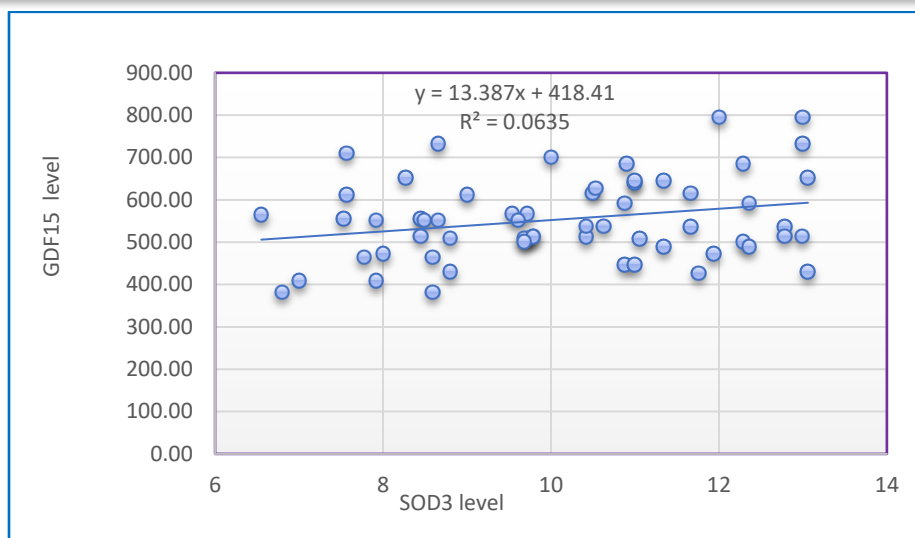


Figure (4-9): The Linear Regression of GDF15 and SOD3 levels among patients.

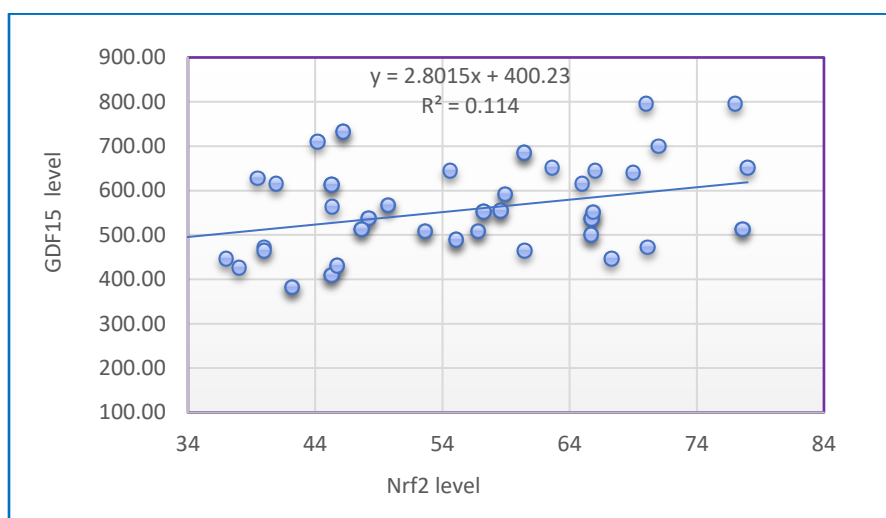


Figure (4-10): The Linear Regression of GDF15 and Nrf2 levels among patients.

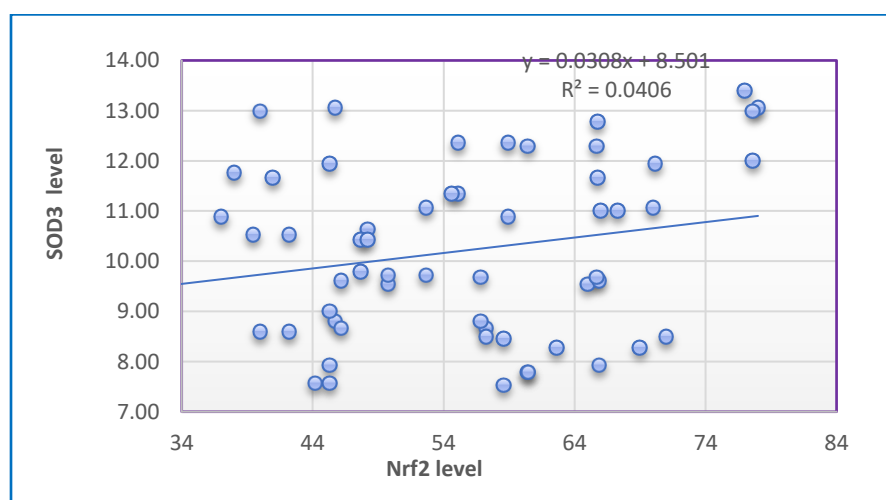


Figure (4-11): The Linear Regression of SOD3 and Nrf2 levels among patients.

4.4.3.C. Correlation between oxidative stress parameters and other parameters in patients with IPF.

The correlation between oxidative stress parameters and other parameters in patients with IPF were shown in tables (4-14). There are significant positive correlation between Nrf2 and Cck-18 ($r=0.296$ and $p=0.022$ in IPF patients. Furthermore, there are significant negative correlation between GDF15 and FVC% ($r=-0.275$ and $p=0.033$), and Nrf2 and FVC% ($r=-0.279$ and $p=0.031$), in IPF patients. But there are were non-significant correlation between all other parameters.

Table (4-14): Correlation between oxidative stress parameters and other parameters in patients with IPF

Parameters	Oxidative stress parameters					
	GDF15 (ng/L)		SOD3 (ng/L)		Nrf2 (ng/ml)	
	<i>r</i>	<i>P</i>	<i>R</i>	<i>P</i>	<i>r</i>	<i>P</i>
WBC	0.162	0.217	0.081	0.539	0.152	0.245
Neutrophils	0.103	0.437	0.062	0.639	0.228	0.079
Lymphocyte	-0.085	0.519	-0.049	0.712	-0.016	0.905
neutrophils%	0.094	0.475	0.026	0.844	0.101	0.443
FVC%	-0.275	0.033*	-0.133	0.322	-0.279	0.031*
FEV1/FVC%1	0.189	0.148	0.039	0.768	0.062	0.637
$\alpha\text{v}\beta 6$ integrin	0.096	0.465	0.081	0.537	0.039	0.766
cCK-18	0.225	0.084	0.088	0.503	0.296	0.022*
COL1A1	0.013	0.921	0.086	0.511	0.110	0.403

r: correlation coefficient.

The results presented in the tables above indicate a significant imbalance in oxidative stress markers in IPF patients compared to healthy individuals, along with excellent diagnostic potential for some of these markers. An imbalance between free radical and antioxidant production is one of the factors leading to alveolar epithelial damage and, consequently, the activation of fibrosis pathways (Zhao *et al.*, 2025).

GDF15 messenger RNA (mRNA) is expressed in aged alveolar lung epithelial cells and can be detected in peripheral blood and bronchial lavage in response to inflammation, oxidative stress, and cell damage (Abhinav *et al.*, 2017). It is also expressed after bleomycin injection in mice. In patients with idiopathic pulmonary fibrosis, GDF15 mRNA expression in lung tissue is markedly increased, acting as a fibrotic risk factor by activating macrophages and fibroblasts (Michele *et al.*, 2021). Single-cell RNA sequencing of human lungs identifies epithelial cells as the primary source of GDF15, and circulating GDF15 concentrations are markedly elevated, correlated with survival rates in several independent cohorts (Yingze *et al.*, 2019). This agrees with our result in the current study, which indicate a considerable rising in GDF15 levels in patients compared to the healthy individual.

SOD3 is a key antioxidant found in mitochondria, where it scavenges superoxide radicals and represents the first line of defense against oxidative stress (Wert *et al.*, 2018). data indicate that SOD3 is differentially localized in the cell to prevent oxidative damage to the adjacent tissue. studies shows that dysregulation of SOD3 interactions and activity may be critical in the pathophysiology o oxidative stress-related dresses. (Kinnula *et al.*, 2006). Recent evidence suggests that SOD3 activity is inhibited in IPF via genetic mechanisms (Koval *et al.*, 2010). Studies have shown that IPF tissues exhibit elevated levels of the enzyme DNMT3A, leading to hypermethylation of the SOD3 gene and consequently, its reduced expression. This inhibition results in impaired mitochondrial function and increased production of reactive oxygen species (ROS), promoting fibroblast proliferation and fibrosis. (Xian *et al.*, 2025). The decreased levels of EC-SOD in IPF tissues can be explained by several mechanisms, including inhibition of the enzyme by growth factors, or degradation of the heparin/cellular matrix specific to EC-SOD in affected lung regions, leading to its diffusion from the extracellular matrix and a marked increase in its levels (Behr, 2003).

Inghilleri *et al.* (2011) observed decreased SOD levels in foci of pulmonary fibrosis, while its levels were elevated in body fluids and blood. This is similar to the findings of the current study, where SOD levels in the blood sample were also elevated. Another interesting finding is that the increased expression of EC-SOD in interstitial mast cells of the lungs of patients with idiopathic interstitial pulmonary fibrosis (UIP) may reflect the marked increase in serum enzyme levels (Gao *et al.*, 2014), this leads us to suggest that elevated SOD3 levels in our patients' blood may not necessarily reflect intracellular functional activity. Rather, inhibition of its production within lung cells may lead to increased oxidative stress, which could overwhelm defense mechanisms due to ongoing tissue damage. This could reflect the need for systemic compensatory action (Wert *et al.*, 2018). or the leakage of the protein from within the mitochondria of fibrotic cells into the extracellular matrix may increase its circulating levels (Behr, 2003; Gao *et al.*, 2014). Ultimately, the decreased intracellular and increased levels in body fluids indicate a general imbalance in the body's homeostasis (Ołdakowska *et al.*, 2020)

The role of EC-SOD in mast cells of damaged lungs remains unclear, but these cells may serve as a source of replacement for this enzyme, similar to inflammatory cells in animal models of fibrosis. In general, the human lung is characterized by a high concentration of EC-SOD, which is disrupted in the lungs of patients with idiopathic/usual interstitial pulmonary fibrosis (UIP/IPF) (Kinnula *et al.*, 2006).

The transcription factor Nrf2 is one of the most important cellular regulators of the antioxidant response. Previous studies have established that it controls the expression of several genes responsible for fighting free radicals in cells. In other words, it protects lung epithelial cells from oxidative damage under normal conditions (Lee *et al.*, 2021). The transcription factor Nrf2 is one of the most important regulators of the antioxidant response at the cellular level. Previous studies have shown that it controls the expression of several genes responsible for fighting

free radicals in cells. In other words, Nrf2 protects lung epithelial cells from oxidative damage under normal conditions (Lee *et al.*, 2021). Under normal conditions, Nrf2 is consistently expressed, but at low levels in the cytoplasm, where it binds to Keap1 and is targeted for proteolysis. In cases of chronic oxidative stress resulting from ongoing cell damage, this pathway may be disrupted. Oxidative stress leads to cysteine-sensitive modification of Keap1, causing a structural change that prevents it from targeting Nrf2 for degradation (Gao *et al.*, 2025; Izumi *et al.*, 2015).

Vomund *et al.* (2017) indicated that oxidative stress leads to increased levels of Nrf2 protein without an increase in the expression of its mRNA, resulting in an inadequate compensatory antioxidant response despite elevated levels of some of its components, as demonstrated in this study. This response cannot keep pace with the accumulation of free radicals caused by the disease (Lin *et al.*, 2023). This supports our hypothesis that increased oxidative stress may stimulate a as a compensatory defense response via the Nrf2 pathway (Izumi *et al.*, 2015; Vomund *et al.*, 2017). Despite this compensatory increase continued cell damage ultimately leads to increased epithelial cell death, reflected in elevated cCK-18 levels a finding consistent with previous studies. This aligns with our results, which demonstrated a strong positive correlation between Nrf2 and cCK-18, a marker of epithelial cell death. (Cha *et al.*, 2012).

From our findings, we conclude that oxidative stress plays a significant role in the development of IPF. Reviewing the ROC curves of the GDF15 and Nrf2 markers, we find they possess significant diagnostic potential, leading us to conclude that they have a distinct diagnostic value. Meanwhile, we found that SOD3 showed acceptable diagnostic value.

Our results showed a significant negative correlation between serum GDF15 levels and forced vital capacity (FVC%) in IPF patients ($r = -0.275$, $p = 0.033$). This correlation is a quantitative reflection of the well-established clinical relationship between disease progression and deteriorating lung function. A decrease in FVC% reflects progressive fibrosis and impaired lung elasticity(Raghu *et al.*, 2022), while an increase in GDF15 indicates an active cellular stress response(Abhinav *et al.*, 2017). Recent research has shown that GDF15 expression in the lung tissue of IPF patients is significantly elevated and correlates with lung function, and its circulating blood levels are directly related to disease severity and survival rates(Dionet *et al.*, 2024). Therefore, this moderate negative correlation supports the hypothesis that GDF15 can be a biomarker of epithelial stress in IPF, with higher levels indicating deteriorating lung function.

Similarly, the results revealed a statistically significant negative correlation between total serum Nrf2 levels and FVC% ($r = -0.279$, $p = 0.031$). This discrepancy may seem paradoxical, given that Nrf2 is a key transcription factor responsible for regulating the expression of antioxidant genes(Yu *et al.*, 2022). However, this correlation can be explained in the context of IPF by several subtle mechanisms. First, basic research demonstrates that the Nrf2 pathway is cell-specific in fibrotic lung, where it is highly expressed in hyperplastic alveolar epithelium and inflammatory cells, while being absent from the fibroblastic foci that characterize IPF(Macari *et al.*, 2013;Tylah, 2022).This discrete spatial distribution suggests that measurable elevations in serum Nrf2 may not reflect sufficient functional activity in the primary target cells of fibrosis, but rather may be a marker of widespread epithelial injury that releases this factor into the bloodstream although Nrf2 activation stimulates antioxidant protective mechanisms(Zhang *et al.*, 2023).

Studies suggest that expression of the Nrf2 pathway in the human lung affected by IPF is further evidence that the pathogenesis is mediated by oxidative

factors and originates in the alveolar epithelium (Fois *et al.*, 2018). Therefore, elevated total Nrf2 levels may reflect the severity of the response to oxidative stress, rather than the effectiveness of antioxidant mechanisms, thus explaining its association with reduced lung function.

Another important finding was a weak but statistically significant positive correlation between Nrf2 and cCK-18 in patients with idiopathic pulmonary fibrosis ($r = 0.296$, $p = 0.022$). This correlation provides statistical evidence linking two key pathways in the mechanism of idiopathic pulmonary fibrosis: oxidative stress (represented by Nrf2) and apoptosis (represented by cCK-18). Notably, recent studies in other medical fields, such as Hashimoto's disease, have already demonstrated a strong correlation between Nrf2 and CK18, with researchers suggesting that elevated levels of both may be a consequence or cause of pathophysiological pathways in those conditions (Yetim *et al.*, 2023) Therefore, their positive correlation likely reflects a synchronized pathological relationship, as oxidative stress (which Nrf2 attempts to counteract) is a major driver of epithelial cell apoptosis (resulting in cCK-18). This suggests that oxidative imbalance and apoptosis are not separate events, but rather two sides of the same coin in the development of IPF (Artaud *et al.*, 2013).

4.5 .Molecular biomarkers

4.5.1. Efficiency of the amplification analysis of miRNA-374 and miRNA-153

The melting curve examination of the amplified products from miRNA-374 and miRNA-153 and their internal reference genes are shown in Figure (4.12) which explained a high specific amplification without non-specific reactivity or primer dimer. Also, the amplification curve of the studied samples represents of miRNA-374 and miRNA-153 along with their internal reference genes amplification. This also points to that RNA extraction and cDNA synthesis was successful as in figure (4-13).

4.5.2. Analysis of the qRT-PCR expression data of miRNA-374 and miRNA-153

In this study, a quantitative analysis of RT-PCR analyzed *Expression of miRNA-374* and *miRNA-153* comparison of their expression between, apparently control group and IPF group. The change in gene expression was calculated using a relative quantitative measurement (Livak and Schmittgen, 2008)

The comparison of miRNA-374 and miRNA-153 genes expression between IPF patients and healthy control subjects has been carried out and the results were demonstrated in table (4- 15). Mean of miRNA-374 gene expression were 2.51 ± 0.58 and 0.41 ± 0.1 in IPF patients and healthy control respectively; the mean levels was higher in IPF patients in compared to healthy control and the difference was highly significant ($P < 0.001$). While the mean of miRNA-153 expression were 0.25 ± 0.06 and 2.66 ± 0.91 in IPF patients and healthy control respectively; the mean levels was lower in IPF patients in compared to healthy control and the difference was highly significant ($P < 0.001$).

These results indicate that IPF was associated with the highest number copies of *miRNA374* and lowest of number copies of *miRNA153*, which reflect of up-regulation of *miRNA374* and down-regulation of *miRNA153*.

The comparison of miRNA-374 and miRNA-153 according to Severity of disease has been carried out and the results were demonstrated in table (4-16). The present results show non-significant difference of miRNA-374 and miRNA-153 according to Severity of disease ($p < 0.05$).

4.5.2.A. Diagnostic accuracy of miRNA-374 and miRNA-153.

To assess the diagnostic accuracy of miRNA-374 and miRNA-153 in differentiating between IPF patients and healthy individuals, receiver operating characteristic (ROC) curve analysis was performed (table 4-17 and figure 4-14). An optimal cutoff value for miRNA-374, more than of 0.91 resulted in an AUC value of 0.998 (95% confidence interval [CI], 0.995- 1.000, P= 0.001), sensitivity of 98.3.0%, specificity of 95.0%, PPV of 95.2%, and NPV of 98.3%. In addition, an optimal borderline value for miRNA-153, less than of 0.775 resulted in an AUC value of 0.980 (95% confidence interval [CI], 0.961- 0.999, P= 0.001), sensitivity of 93.3%, specificity of 95.0%, PPV of 94.9%, and NPV of 93.4%.. These results indicate that miRNA-374 and miRNA-153 is an excellent diagnostic marker for differentiating between IPF patients and healthy individuals.

4.5.2.B. Regression correlations between miRNA-374 and miRNA-153.

Logistic regression analysis suggests an inverse correlation between miRNA-374 and miRNA-153 in IPF patients as shown in figure (4-15). The results indicate that elevated miRNA-374 levels are associated with decreased miRNA-153 levels, suggesting a possible antagonistic regulation of these two microRNAs during disease progression.

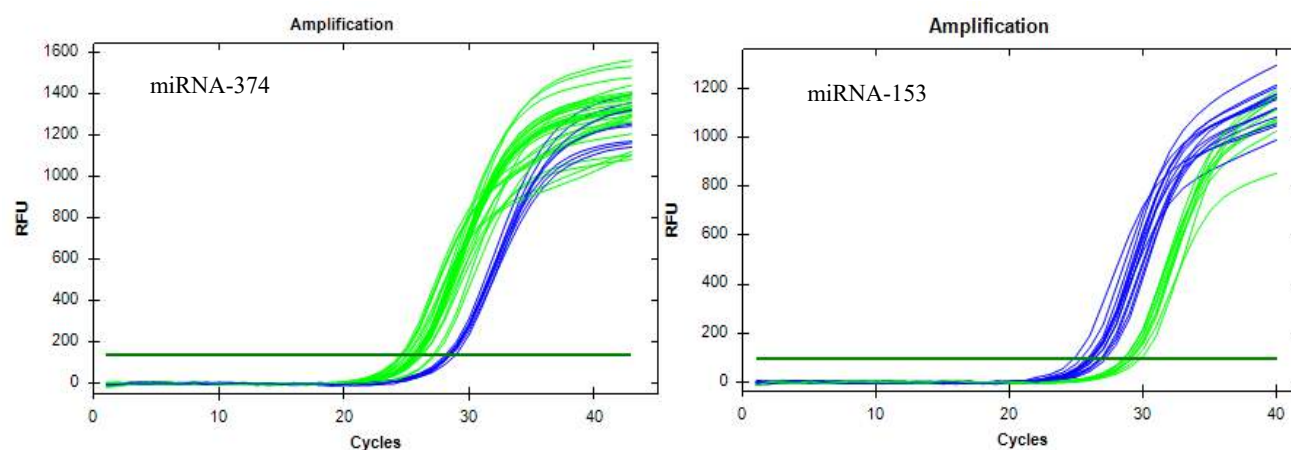


Figure (4-12): qPCR amplification plots of miRNA153in IPF patients (Green plots) and healthy control in (blue plots)

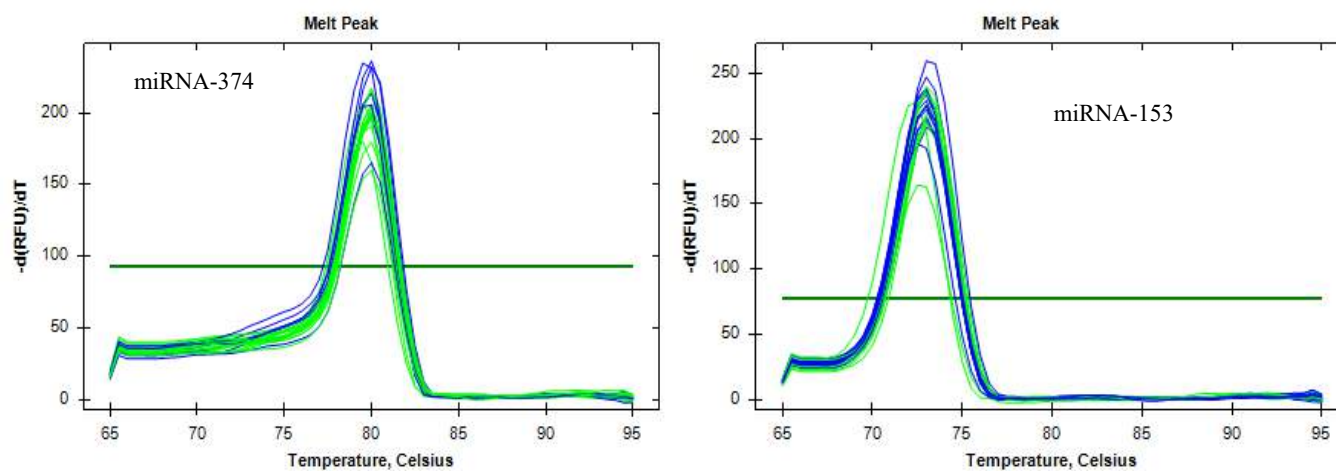


Figure (4-13): qPCR melting curves of miRNA-374 in IPF patients (Green plots) and healthy control in (blue plots). The specific amplification was show at 80C melting peaks

Table (4-15): Comparison of mean of *miRNA-374* and *miRNA-153* gene expression between patients and healthy controls

Groups	<i>miRNA-374</i>			<i>miRNA-153</i>		
	Mean	SD	SE	Mean	SD	SE
IPF patients	2.51	0.58	0.21	0.25	0.06	0.01
Control	0.41	0.10	0.02	2.66	0.91	0.31
p-value	0.001**			0.001**		

SD: standard deviation; SE: standard error; **: significant at $P < 0.05$ **Table (4-16): Frequency distribution of *miRNA-374* and *miRNA-153* according to severity**

Severity of disease	<i>miRNA-374</i> (fold change)	<i>miRNA-153</i> (fold change)
	Mean $\pm$ SD	Mean $\pm$ SD
Moderate	2.31 $\pm$ 0.36	0.22 $\pm$ 0.07
Severe	2.44 $\pm$ 0.64	0.27 $\pm$ 0.06
P value	0.566 † NS	0.104 † NS

n: number of cases; SD: standard deviation; †: independent samples t-test; NS: non-significant at $P > 0.05$ **Table (4-17): Roc curve of *miRNA-374* and *miRNA-153* gene expression**

Characteristic	<i>miRNA-374</i> gene	<i>miRNA-153</i> gene
Cutoff value	> 0.91	< 0.775
P value	0.001	0.001
Sensitivity %	98.3 %	93.3 %
Specificity %	95.0 %	95.0 %
PPV %	95.2 %	94.9 %
NPV %	98.2 %	93.4 %
AUC (95% CI)	0.998 (0.995- 1.000)	0.980 (0.961- 0.999)

CI: Confidence interval, AUC: Area under curve

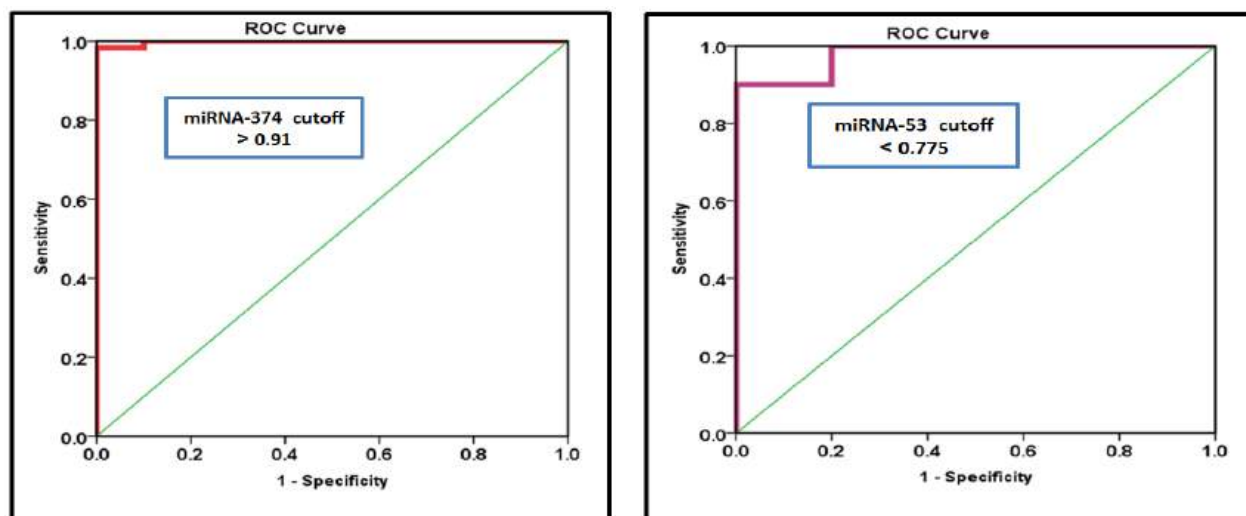


Figure (4-14): Receiver operating characteristic curve for *miRNA-374* and *miRNA-153* to distinguish IPF patients from healthy control subjects.

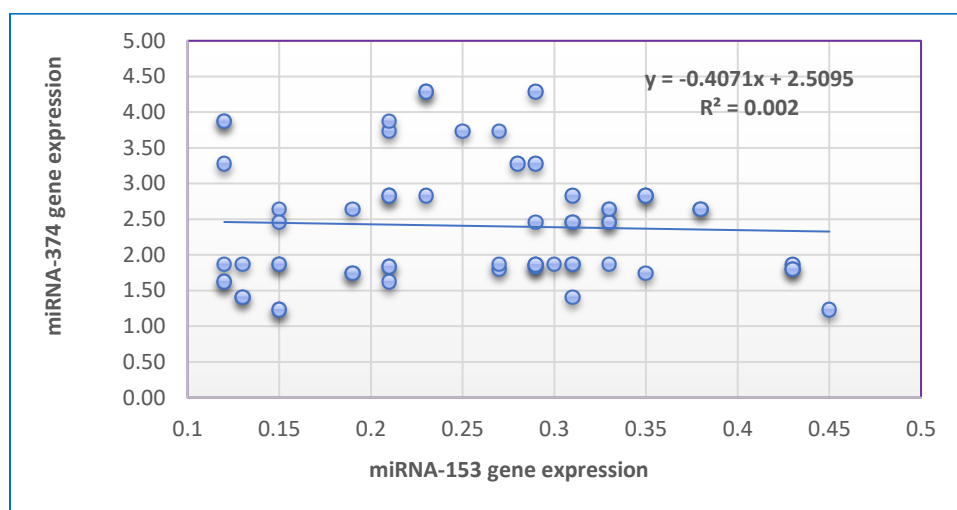


Figure (4- 15): The Logistic scatter *miRNA-374* and *miRNA-153* among patients.

4.5.2.C. Correlation between *gene expression parameters* and other parameters in patients with IPF.

The correlation between *miRNA-374* and *miRNA-153* expression and other parameters in patients with IPF were shown in tables (4-18). We observed expression of both *miRNA-374* and *miRNA-153* and certain blood parameters in these patients. A strong positive correlation between neutrophil ratio with expression of both *miRNA-374* and *miRNA-153*. In contrast, *miRNA-153* showed a strong, significant inverse correlation with lymphocyte count.

In this study, did not show a direct relationship between miRNA-374 and forced vital capacity (FVC%) ,while, the results of miRNA-153 recorded a strong and significant inverse correlation with (FVC%) ($r = -0.613$, $P = 0.001$), this suggests that decreased miRNA-153 levels may be associated with reduced lung function. On the other hand, there are significant positive correlation between miRNA-374 and COL1A1 ($r=0.312$ and $p=0.015^*$) in IPF patients. But there was non-significant correlation between all other parameters and miRNA-374, also between miRNA-153 and other parameters.

Table (4-18): Correlation between *gene expression* parameters and other parameters in patients with IPF

Parameters	<i>gene expression</i>			
	<i>miRNA-374</i>		<i>miRNA-153</i>	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
WBC	-0.055	0.678	0.080	0.543
Neutrophils	0.200	0.125	0.232	0.075
Lymphocyte	0.178	0.173	-0.493	0.001*
neutrophils%	0.500	0.001*	0.341	0.008*
FVC%	-0.012	0.925	-0.613	0.001*
FEV1/FVC%1	0.004	0.987	0.176	0.178
$\alpha\text{v}\beta 6$ integrin	0.252	0.053	-0.004	0.978
Cck-18	0.159	0.225	-0.028	0.839
COL1A1	0.312	0.015*	-0.231	0.075
GDF15	0.064	0.626	-0.195	0.135
SOD3	0.151	0.250	-0.011	0.934
Nrf2	0.163	0.213	-0.207	0.113

r: correlation coefficient.

The study results showed a significant increase in miRNA-374 expression (Bao) and decrease in miRNA-374 expression in patients with IPF compared to healthy individuals ($P < 0.001$). This suggests that these molecules may have a role associated with the disease. The increasing of miRNA-374 expression may have a stimulatory role or be positively associated with the disease. Research shows that miR-374 can influence and mediate crucial inflammatory pathways. For example, one study found that miR-374 can regulate the secretion of interleukin-6 (IL-6) and transforming growth factor-beta 1 (TGF- β 1) two key molecules in the fibrosis pathway (Yuan *et al.*, 2019), by affecting the oncogene Myc (a key transcription factor that regulates cell proliferation, growth, apoptosis, and metabolism, and controls approximately 15% of human genes. When its regulation is disrupted, the immune system is also disrupted (Dhanasekaran *et al.*, 2022). A strong positive correlation between neutrophil percentage and miRNA-374 in our results can be support the relation of miRNA-374 and inflammatory pathway. This is consistent with the known inflammatory nature of the disease.

On the other hand the lack of a statistically significant correlation between miRNA-374 and FVC% which demonstrated in this study, This may be because FVC reflects the terminal damage to lung tissue structure; therefore, miRNA-374 may be associated with the inflammatory phase preceding fibrosis (Jo *et al.*, 2017). This supports the researchers' hypothesis, this molecule is likely more involved in the early inflammatory phases preceding functional fibrosis, while FVC% reflects the final structural damage to the lung (Ruth & Arely, 2022).

It is worth noting that some previous studies have shown contradictory results. For example, a study on bronchial and alveolar lavage fluid (BALF) found that miR-374b was elevated in patients with IPF, which is consistent with the findings of the current study (Xianglong *et al.*, 2019)

Researchers found elevated levels of miR-374 in the blood serum of patients, and that increased levels exacerbated fibrosis and impaired cardiac function by

inhibiting the anti-inflammatory cytokine IL-10 and activating the NF- κ B inflammatory pathway (Li *et al.*, 2022). A 2024 study showed that increased levels of miR-374a-5p in psoriasis lesions activate the Wnt5a/NF- κ B pathway, leading to increased cell proliferation and the production of potent inflammatory cytokines such as IL-1 β , IL-6, IL-8, and TNF- α (Ma *et al.*, 2024)

In contrast, the study showed a significant decrease in miRNA-153 expression in patients with IPF ($P < 0.001$), suggesting that it may have a protective or inhibitory role in the disease. Our results strongly support the well-established scientific mechanism of miR-153. It has been shown that TGF- β 1, the central regulator of fibrosis, reduces miR-153 expression in lung fibroblasts (Koichi *et al.*, 2015) Studies have demonstrated that increased miR-153 weakens fibrosis, while its inhibition enhances the fibrotic activity of TGF- β 1. The molecular mechanism of this effect lies in miR-153's ability to directly target the TGFBR2 molecule, disrupting the TGF- β /Smad signaling pathway and thus inhibiting fibroblast activation (Guanghai *et al.*, 2015). These results are also consistent with research linking dysregulation of this molecule to the mechanisms of epithelial cell transformation into fibroblasts (EMT), a key process in fibrosis (Li *et al.*, 2025)

Our results showed a strong and statistically significant inverse correlation between miRNA-153 and FVC% , a very important clinical finding. This may indicate that a decrease in this protective molecule is associated with a severe decline in lung function. This inverse relationship with FVC% supports the hypothesis of its relative protective role, as its decline coincides with a decrease in forced vital capacity, the most important indicator for assessing IPF severity and prognosis (Raghu *et al.*, 2018). Similarly, the inverse association between miRNA-153 and lymphocytes suggests that its deficiency may contribute to the immune dysfunction associated with IPF, and this is consistent with (Ruth & Arely, 2022). This suggests that decreased miRNA-153 levels may be associated with worsening immune dysfunction and deteriorating lung function. (Liang *et al.*, 2015)

This may suggest that microRNA is involved in the inflammatory processes and functional changes associated with disease progression

Logistic regression analysis revealed an inverse relationship between the two molecules, suggesting opposing regulation of their metabolism during disease progression. This relationship makes sense from a pathophysiological perspective. While miR-153 is inhibited by TGF- β 1(Liang *et al.*, 2015), miR-374 appears to be involved in other inflammatory and fibrotic pathways (Yuan *et al.*, 2019). This complex interaction between the two molecules may be key to a deeper understanding of disease progression. Numerous studies support the general idea that these molecules possess promising diagnostic potential. miR-153 has been proposed as a diagnostic and prognostic biomarker in multiple cancers (Yousefnia, 2022).The miR-374 family has also been proposed as a promising diagnostic tool in several disease processes(Bian *et al.*, 2019).

The lack of a statistically significant relationship between either molecule and disease severity ($P > 0.05$) may indicate that these molecules reflect the presence of the disease itself, rather than necessarily its stage of progression. The positive correlation between miRNA-374 and COL1A1 ($r=0.312$, $P=0.015^*$) is consistent with the role of miRNA-374 in stimulating collagen-producing cells, a central process in fibrosis(Yuan *et al.*, 2019).On the other hand, the lack of correlation between miRNA-153 and collagen may reflect its inhibitory role in fibrosis.

These results confirm the dual and complementary roles of both miRNA-153 (as a protective inhibitor) and miRNA-374 (as an inflammation-associated factor) in IPF. The study provides strong evidence for the potential of these two molecules as promising diagnostic biomarkers and sheds light on the molecular mechanisms underlying disease progression. Further functional and clinical studies are recommended to confirm these findings and explore their potential as future therapeutic targets.

CONCLUSIONS

AND

RECOMMENDATIONS

Conclusions

1. Age and sex, are considered risk factors for IPF in this study. Most IPF patients were male, and the age groups most susceptible to the disease were elderly.
2. The significant increase in neutrophils and a decrease in lymphocytes considered a clear shift in the innate immune response in IPF patients, indicating an active inflammatory environment associated with the progression and development of IPF.
3. The sharp increase in integrin $\alpha\beta6$, and COL1A1 indices reflects structural deterioration in the alveoli. The results also showed that COL1A1 and integrin $\alpha\beta6$ possess exceptional diagnostic accuracy ($AUC > 0.98$), making them promising tools for clinically accurate assessment of tissue fibrosis.
4. The inverse relationship between (GDF15 and Nrf2) levels and the forced vital capacity ratio (FVC%). suggest a role for oxidative stress in the severity of IPF disease
5. A complex molecular relationship exists between studied microRNAs characterized by up-regulation of miR-374 acts as a promoter of fibrosis and inflammation (correlated with COL1A1 and neutrophils) and down-regulation miR-153 represents a loss of a bio-protective mechanism, provides a novel molecular indicator of dysfunction in the miR-374 and miR-153 regulatory axis ,which influence fibrosis pathways in IPF patients.

Recommendations

1. Future studies should be conducted on larger and more geographically diverse samples within Iraq to enhance statistical power and generalize the findings more broadly.
2. Development of targeted therapeutic pathways by Focusing on research that targets blocking of $\alpha\text{v}\beta 6$ integrin or improving Nrf2 pathway to reduce the progression of fibrosis.
3. The studied miRNAs, such as miR-374 and miR-135, should be considered as potential biomarkers for early diagnosis or monitoring disease progression, which could pave the way for developing new therapeutic targets.
4. Conduct longitudinal studies to monitor changes in miRNA gene expression as the disease progresses or responds to treatment.
5. A cohort study design to prospectively follow IPF patients, using Kaplan-Meier curves and the Cox proportional hazards model to compute survival rates and precisely determine the impact of risk factors on IPF progression.
6. Encourages collaboration between research centers and hospitals in Iraq, to establish a national database for (IPF) patients, include both genetic and clinical aspects.
7. Promoting the use of modern molecular techniques in local laboratories and training staff in their application for the diagnosis and management of chronic diseases such as IPF. .
8. Necessary to study the effects of sex hormones in human samples to look for their effectiveness in IPF

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APPENDIX

Appendix: Patient Questionnaire

Questionnaire

Name:-----

Age: -----

Gender: -----

History of diseases: -----

Type of sample:-----

Type of diseases: -----

Underwent treatment: -----

الخلاصة

الخلاصة.....

يُعدّ التليف الرئوي مجهول السبب (IPF) مرضًا غير مفهوم جيدًا من حيث آليته الأساسية. ويُعتبر خلل وظائف الحويصلات الهوائية والإجهاد التأكسدي من العوامل الرئيسية المساهمة فيه، إلا أن الدراسات الفيسيولوجية والجزيئية الشاملة لا تزال محدودة. لذلك، صُممت هذه الدراسة للتحقق من العلاقة بين الإجهاد التأكسدي وخلل وظائف الحويصلات الهوائية في التليف الرئوي مجهول السبب، ولتقييم الإمكانات التشخيصية لبعض المؤشرات الحيوية الفيسيولوجية والجزيئية.

أُجريت الدراسة في قسم الأحياء/كلية العلوم/جامعة القادسية بالتعاون مع أطباء متخصصين في مستشفى الإمام الصادق التعليمي بمحافظة بابل ومركز أمراض الجهاز التنفسي بمدينة الديوانية، خلال الفترة من ديسمبر 2024 إلى نهاية نوفمبر 2025. بلغ إجمالي عدد العينات 120 عينة، منها 60 مريضًا مصابًا بالتليف الرئوي مجهول السبب (39 ذكرًا و21 أنثى؛ متوسط العمر 58 ± 11.9 سنة)، و60 فردًا سليمًا من نفس العمر والجنس كمجموعة ضابطة، ليس لديهم تاريخ مرضي أو دليل سريري على الإصابة بالتليف الرئوي أو الربو. جُمعت معلومات المرضى والمجموعة الضابطة عبر استبيان تضمن الاسم والعمر والجنس وحالة التدخين. وقُيِّمت وظائف الرئة (النسبة المئوية للسعة الحيوية القسرية، ونسبة حجم الزفير القسري في الثانية الأولى إلى النسبة المئوية للسعة الحيوية القسرية). تم سحب عينات دم (6 مل) من جميع الأفراد، ثم تحليلها لتقييم التغيرات في المؤشرات الدموية، بالإضافة إلى مؤشرات خلل وظائف الحويصلات الهوائية، بما في ذلك: الإنتغرين $\alpha v \beta 6$ ، والسيتوكيراتين-18 المنشطر (cCK-18)، والكولاجين من النوع COL1A1، فضلاً عن مؤشرات الإجهاد التأكسدي: عامل التمايز والنمو 15 (GDF15)، وإنزيم ديسموتاز الفائق (SOD)، وبروتين منظم الاستجابة المضادة للأكسدة 2 (Nrf2)، باستخدام اختبار ELISA. وتم قياس التعبير عن miRNA-374 و miRNA-153 كميًا باستخدام تقنية qRT-PCR.

أظهرت نتائج الدراسة عدم وجود فرق ذي دلالة إحصائية ($P > 0.05$) بين مجموعات مرضى التليف الرئوي مجهول السبب فيما يتعلق بمتوسط العمر والجنس ومؤشر كتلة الجسم. ومع ذلك، لوحظ تباين بين عينات المرضى أنفسهم، مع اختلاف في نسبة الذكور إلى الإناث. كما أشارت الدراسة إلى أن التدخين كان موجودًا لدى نسبة محدودة فقط (15%) من المرضى الذكور الأكبر سنًا. وأظهرت النتائج السريرية والدموية أن غالبية المرضى (70%) يعانون من مرض شديد (نسبة السعة الحيوية القسرية $36.11 \pm 6.9\%$). أظهر المرضى المصابون بالتليف الرئوي مجهول السبب زيادة ملحوظة في عدد خلايا الدم البيضاء (1.8 ± 8.89 مقابل $7.82 \pm 1.3 \times 10^3$ ميكرو لتر، $p=0.015$)، وزيادة في عدد العدلات ($75.11 \pm 10.1\%$ ، $p=0.002$)، وانخفاضًا في عدد الخلايا الليمفاوية (0.41 ± 1.66 مقابل 0.32 ± 2.19 ، $p=0.001$)، مما

يشير إلى تحول ملحوظ في المناعة الفطرية. أظهر المرضى المصابون بالتليف الرئوي مجهول السبب ارتفاعاً ملحوظاً في مستويات الإنتغرينات $\alpha\text{v}\beta 6$ (12.77 ± 71.47 مقابل 4.71 ± 28.36 نانوغرام/مل، $p < 0.001$)، و cCK-18 (11.3 ± 51.14 مقابل 4.54 ± 38.07 نانوغرام/مل، $p < 0.001$)، و COL1A1 (9.81 ± 44.08 نانوغرام/مل، $p < 0.001$). وأظهر تحليل منحنى ROC دقة تشخيصية ممتازة لكل من $\alpha\text{v}\beta 6$ (المساحة تحت المنحنى = 0.983، الحساسية 96.7%) و COL1A1 (المساحة تحت المنحنى = 0.982، الحساسية 96.7%).

ظهرت اختبارات الإجهاد التأكسدي ارتفاعاً ملحوظاً في مستويات GDF15 في مصل الدم (47.4 ± 554.02 مقابل 18.99 ± 428.27 بيكوغرام/مل، $p < 0.001$)، و SOD3 (1.73 ± 10.07 مقابل 1.42 ± 8.08 نانوغرام/مل، $p = 0.021$)، و Nrf2 (11.01 ± 53.52 مقابل 3.25 ± 32.42 نانوغرام/مل، $p < 0.001$). وقد أثبتت هذه النتائج قدرة تشخيصية استثنائية (مساحة تحت المنحنى = 0.983، حساسية 96.7%). وُجدت أيضاً ارتباطات سلبية معنوية بين GDF15 ($r = -0.275$ ، $p = 0.033$)، و Nrf2 ($r = -0.279$ ، $p = 0.031$) والسعة الحيوية القسرية (%FVC)، مما يربط الإجهاد التأكسدي مباشرة بالتدهور الوظيفي.

أما فيما يتعلق بالنتائج الجزيئية، فقد أظهر المرضى المصابون بالتليف الرئوي مجهول السبب ارتفاعاً في مستويات miRNA-374 (0.58 ± 2.51 مقابل 0.1 ± 0.41 ، $p < 0.001$) وانخفاضاً في مستويات miRNA-153 (0.06 ± 0.25 مقابل 0.91 ± 2.66 ، $p < 0.001$). وأظهر كلا النوعين من miRNA دقة تشخيصية ممتازة (مساحة تحت المنحنى = 1.000، حساسية/نوعية 100%). ارتبط miRNA-374 ارتباطاً إيجابياً بعدد العدلات ($r = 0.500$ ، $p = 0.001$) و COL1A1 ($r = 0.312$ ، $p = 0.015$). ومن الجدير بالذكر أن miRNA-153 أظهر ارتباطاً عكسياً قوياً مع كل من النسبة المئوية للسعة الحيوية القسرية ($r = -0.613$ ، $p = 0.001$) وعدد الخلايا اللمفاوية ($r = -0.493$ ، $p = 0.001$)، مما يشير إلى دور وقائي، يؤدي فقده إلى كل من التليف واختلال وظائف الجهاز المناعي. وكشف تحليل الانحدار اللوجستي عن وجود ارتباط عكسي بين miRNA-153 و miRNA-374.

تقدم هذه الدراسة أدلة قوية على أن التليف الرئوي مجهول السبب يتميز بحلقة مفرغة من الإجهاد التأكسدي الناتج عن خلل في تنظيم GDF15/Nrf2 وإصابة الظهارة السنخية الناتجة عن خلل في تنظيم $\alpha\text{v}\beta 6$ /cCK-18/COL1A1. تمثل الأنماط التنظيمية المتضادة لـ miRNA-374 (المحفز للتليف) و miRNA-153 (المضاد للتليف) بصمة جزيئية جديدة. وتوهم القدرة التشخيصية الممتازة لـ $\alpha\text{v}\beta 6$ و COL1A1 و Nrf2، وخاصة miRNA-374/153، هذه المؤشرات الحيوية غير الجراحية الفعالة لمرض

التليف الرئوي مجهول السبب. وتفتح هذه النتائج آفاقًا جديدة للتشخيص المبكر والاستهداف العلاجي المحتمل لمحور الإجهاد التأكسدي-miRNA-خلل وظائف الحويصلات الهوائية.



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كلية العلوم / قسم علوم الحياة

تقييم الاجهاد التأكسدي والمؤشرات الحيوية لخلل الوظيفة السنخية وبصمات الحمض الريبي النووي الميكروي في التليف الرئوي مجهول السبب: دراسة حالات وشواهد

أطروحة

مقدمة إلى مجلس كلية العلوم / جامعة القادسية وهي جزء من متطلبات نيل

درجة دكتوراه فلسفة في علوم الحياة

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