

## Pulmonary Fibrosis with Increasing of Interleukin-6 in Sprague Dawley Rats With Metabolic Syndrome

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### ABSTRACT

**Background:** Pulmonary fibrosis is the result of chronic inflammation causing decreased lung function. Severe lung damage and worsening respiratory symptoms can be caused by metabolic syndrome. In metabolic syndrome, obesity, dyslipidemia, hypertension, and insulin resistance occurs, also chronic inflammation and increased interleukin-6. Research on rats with high-fat high-fructose diet caused metabolic syndrome that triggered chronic inflammation formed pulmonary fibrosis, which decreased lung function. The study result aims to increase awareness of pulmonary fibrosis due to chronic inflammation in metabolic syndrome.

**Methods:** The study design was true experimental in vivo test, pre and post test control group, with male Sprague-Dawley rats aged 37 weeks. Comparing each of 10 in control and metabolic syndrome groups. Metabolic syndrome expressed by increase of weight, triglycerides, blood sugar, and low HDL. Pulmonary tissue fibrosis assessed with modified Ashcroft score. Examination of plasma and tissue Interleukin-6 by ELISA, and the degree of tissue fibrosis by HE staining under a microscope. Different test with independent T-test and Mann-Whitney methods.

**Results:** There were significant differences in plasma and tissue Interleukin-6 and degree of lung tissue fibrosis between control and metabolic syndrome groups with each  $p < 0.05$ . There is an increase of plasma Interleukin-6, tissue Interleukin-6, and also degree of lung tissue fibrosis on metabolic syndrome group than control group.

**Conclusion:** There is an increase in the degree of pulmonary tissue fibrosis accompanied by an increase in plasma and tissue IL-6 in rats with metabolic syndrome.

**KEYWORDS:** IL-6, lung fibrosis, metabolic syndrome

### ARTICLE DETAILS

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### INTRODUCTION

Metabolic syndrome (METS/SM) is a group of various component risk factors consisting of central obesity, dyslipidemia, hypertension, and hyperglycemia. Metabolic syndrome is also a health problem throughout the world with prevalence increasing every year and is a risk for cardiovascular disease.<sup>1</sup> The prevalence of SM based on NCEP-ATP III criteria worldwide is around 15-30%, with a prevalence in Asian populations of around 10-15%.<sup>2</sup> SM can

cause disorders in various organs, namely adipocytes, liver, kidneys, lungs, eyes and heart.

Metabolic syndrome is a major and growing health problem worldwide affecting approximately 25% of adults worldwide. Metabolic syndrome increases the risk of developing type 2 diabetes (5-fold), stroke (2- to 4-fold), myocardial infarction (3- to 4-fold) and the risk of death (2-fold) regardless of previous cardiovascular history. Metabolic syndrome is defined by multiple pathophysiological disorders consisting of central obesity, insulin resistance, high blood

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pressure, and dyslipidemia. Although there are no universally accepted diagnostic criteria, most expert groups agree that this syndrome is defined by endothelial dysfunction, impaired insulin sensitivity and hyperglycemia, dyslipidemia, central obesity, and hypertension.<sup>3,4</sup> Several large studies published in the last ten years have shown an association between metabolic syndrome and impaired lung function in adults and children. Evidence linking metabolic syndrome and pulmonary disorders or lung diseases continues to grow and several mechanisms have been proposed to explain this association including the complex effects of insulin and insulin receptors in the lungs and airways, whose interactions begin early in life and contribute to normal lung development.<sup>5</sup>

Complications of metabolic syndrome occur due to chronic inflammation that occurs at the same time. Therefore, inhibiting the inflammatory process is important in preventing complications from metabolic syndrome. Metabolic syndrome triggers extensive tissue damage such as pulmonary fibrosis.<sup>6</sup>

The association between SM and lung disease has been proven by several studies. SM is a risk factor for worsening respiratory symptoms, severe lung damage, pulmonary hypertension and asthma. There is a positive relationship between lung impairment and various cardiovascular risks, such as hypertension, diabetes mellitus (DM), low density lipoprotein (LDL) cholesterol levels, and obesity.<sup>7</sup> Impaired lung function, assessed by a decrease in forced expiratory volume measured in the first second of exhalation (FEV1) and forced vital capacity (FVC), contributes significantly to several major health problems, such as chronic obstructive pulmonary disease (COPD) and death from cardiovascular disease.<sup>8</sup>

Inflammation will increase IL-6 levels in patients. So in metabolic syndrome it is suspected that there will be an increase in IL-6 levels in blood plasma. In metabolic syndrome there will also be chronic systemic inflammation. This is also related to the increase in body weight that occurs in obesity, which will cause a respiratory disease, namely obesity asthma. Furthermore, it will be proven that lung damage occurs in the tissue, namely tissue fibrosis occurs as a result of metabolic syndrome.<sup>9</sup>

Previous research conducted by Rohman et al. (2021) showed that treating rats with metabolic syndrome caused a lot of inflammation and fibrosis to occur in several cardiovascular tissues. Therefore, this research hopes to be a pioneer in finding the incidence of inflammation and fibrosis in lung tissue based on assessing IL-6 biomarker levels in blood serum and lung tissue, as well as looking at fibrosis that occurs in lung tissue through histological examination.

### METHODS

#### *Research Design*

This study used a true experimental in vivo test, post test only control group design using 20 male Sprague Dawley rats

(*Rattus norvegicus*) which were divided into 2 groups, namely the control group on a normal chow diet and the metabolic syndrome group given a high-fat, high-sucrose diet (High Fat High Sucrose / HFHS: powder pellets, sucrose, methionine, salt, MSG, egg yolk, and white butter) and Streptozotocin injection (STZ: 30 mg/kgBB). This HFHS diet intervention was given to rats aged 10-12 weeks for 37 weeks, STZ injection was given to rats weighing more than 500 grams (targeted to be achieved at week 18) for 10 weeks.

The research was conducted from June 2022 to March 2023. Care of experimental animals, treatment processes and organ harvesting were carried out in the Biomolecular Experimental Animal Laboratory, Faculty of Mathematics and Natural Sciences, Brawijaya University. Histology preparations were made at the Biomolecular Laboratory, Brawijaya University. Examination of tissue fibrosis using microscopic histo-pathological examination at the UB Anatomical Pathology Laboratory and examination of serum IL-6 in blood and tissue using ELISA at the UB Biomedical Laboratory.

Rats were divided into 10 control groups and 10 metabolic syndrome groups. Daily care and feeding is carried out every day. Body weight and fasting blood sugar measurements are carried out once a week, triglyceride and HDL measurements are carried out once a month. Rats were considered to have metabolic syndrome after measuring Blood Glucose > 126 mg/dL, triglyceride > 175 mg/dL, Bodyweight > 500 mg, and HDL < 45 mg/dL. The characteristics of the experimental animals were examined enzymatically as usual. After 37 weeks of treatment and both groups of rats reached a normal and metabolically stable condition for 4 weeks, the rats were sacrificed and blood was taken for ELISA examination and lung tissue was taken for ELISA and microscopic examination. The ELISA examination used is serum Interleukin-6 levels in blood plasma and lung tissue.

#### *Measurement of Plasma and Tissue IL-6 and Microscopic Examination of Lung Tissue*

The experimental animals were sacrificed after 37 weeks of treatment and in the group metabolic syndrome was achieved. Two parts were taken from each mouse in the group, namely blood plasma to check for IL-6 and lung tissue to check for IL-6 and HE smears for microscopic examination. IL-6 examination uses plasma and lung tissue by ELISA. For measuring IL-6 levels using the ELISA method using blood plasma material and lung tissue supernatant, expressed in ng/mL units and assessing lung tissue fibrosis using HE staining and expressed using the Modified Ashcroft scale<sup>11</sup> in the no category scale fibrosis formed (value < 5) and fibrosis formed (value ≥ 5).

#### *Data Analysis*

The data that was obtained was then tested for normality using the Shapiro-Wilk test. If the data is normally distributed, it is continued with the T-Test or ANOVA test with a significance level of 95%, and data that is not normally

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distributed is carried out using the Mann-Whitney test, with a p-value <0.05.

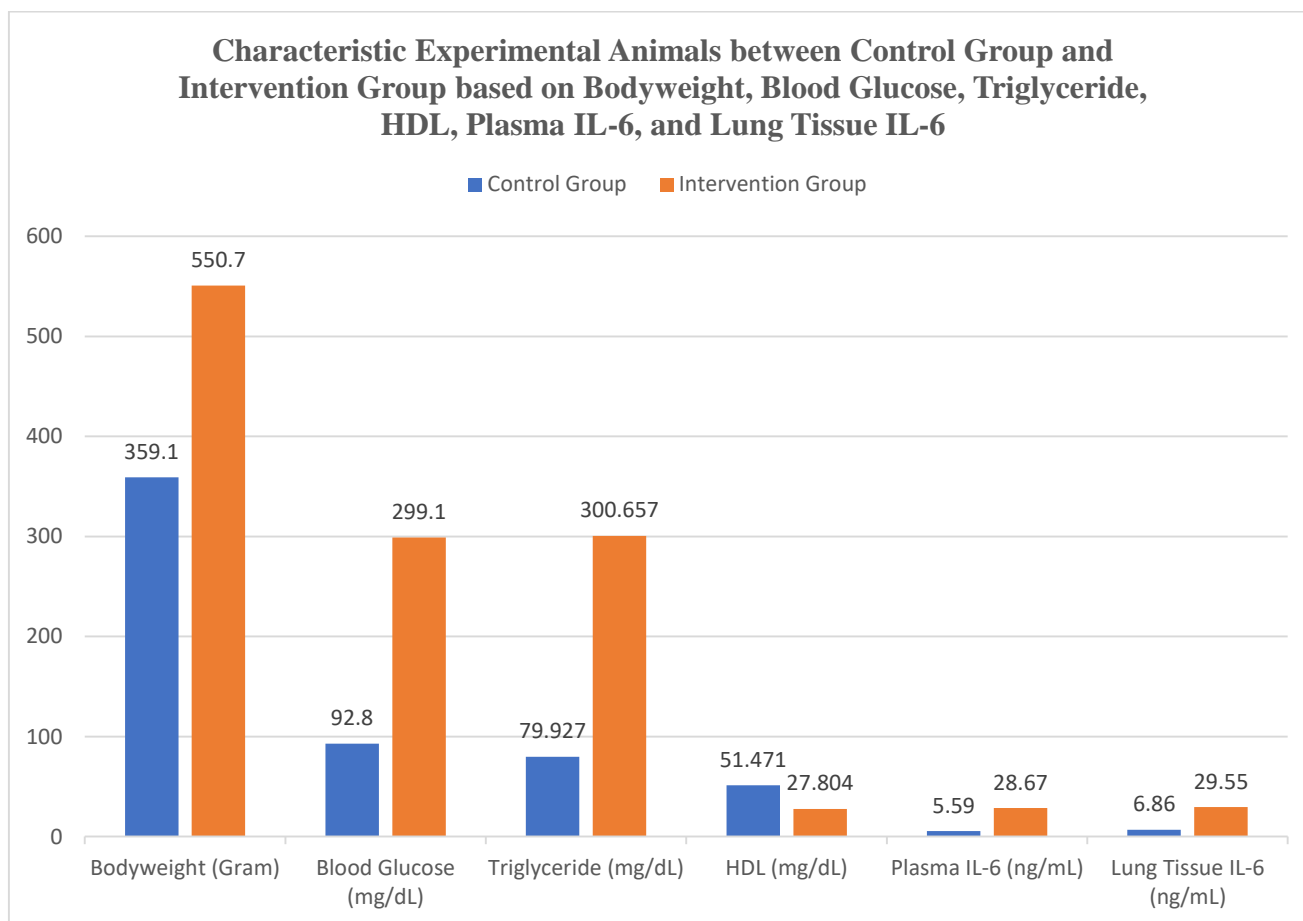
### RESULTS

A comparison of characteristics based on bodyweight, blood glucose, triglyceride, High-Density Lipoprotein (HDL), Plasma Interleukin-6 (IL-6), and Lung Tissue IL-6 in normal rats and rats with metabolic syndrome can be presented in Table 1 and Graphic 1 below.

**Table 1. Data on Characteristic Variables in Experimental Animals**

Characteristics	Control Group (N=10)	Intervention Group (N=10)	p value (T-test p < 0.05)
Bodyweight (Gram)	359.10±27.97	550.70±13.22	< 0.01*
Blood Glucose (mg/dL)	92.80±14.39	299.10±70.36	< 0.01*
Triglyceride (mg/dL)	79.93±9.60	300.66±73.25	< 0.01*
HDL (mg/dL)	51.47±6.92	27.80±4.73	< 0.01*
Plasma IL-6 (ng/mL)	5.59±3.21	28.67±9.08	< 0.01*
Lung Tissue IL-6 (ng/mL)	6.86±4.05	29.55±13.82	< 0.01*

\*Significant



**Graphic 1. Data on Characteristic Variables in Experimental Animals**

From the results of the comparison test, the average Bodyweight, Blood Glucose, Triglyceride, HDL, Plasma IL-6, and Lung Tissue IL-6 between control group and intervention group using the T-test were obtained. The result of p value is <0.01 (p <0.05), so it can be concluded that there is a significant difference in the comparison of Bodyweight, Blood Glucose, Triglyceride, HDL, Plasma IL-6, and Lung Tissue IL-6 between control group and intervention group, where the average levels of Bodyweight, Blood Glucose,

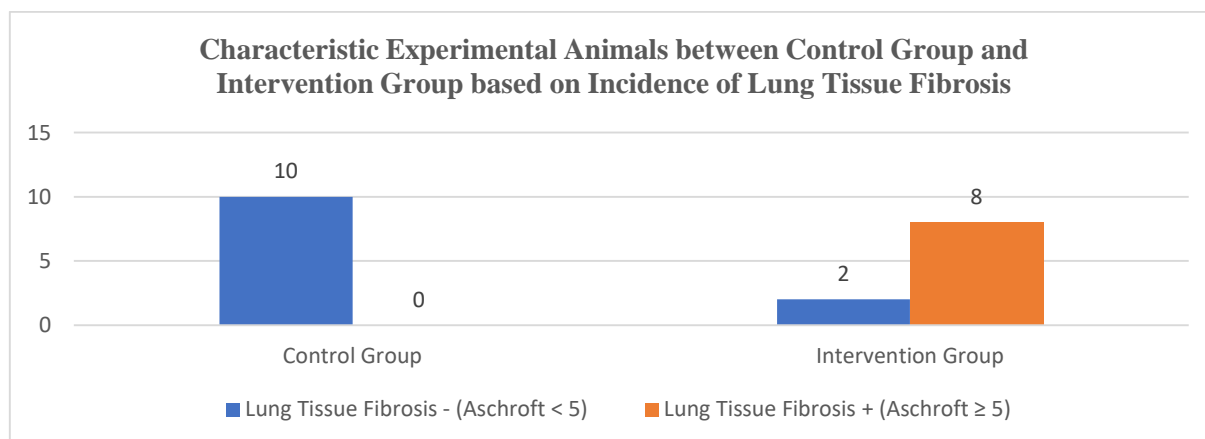
Triglyceride, Plasma IL-6, and Lung Tissue IL-6 of animals trials in the control group were higher than result levels in intervention group, but inversely proportional to HDL which was higher in control group and lower in intervention group. Based on the categories of histopathological variables of animals trials in the control group and intervention group, a cross table (Table 2), Graphic 2, and Figure 1 (a, b, c, d) can be obtained as follows.

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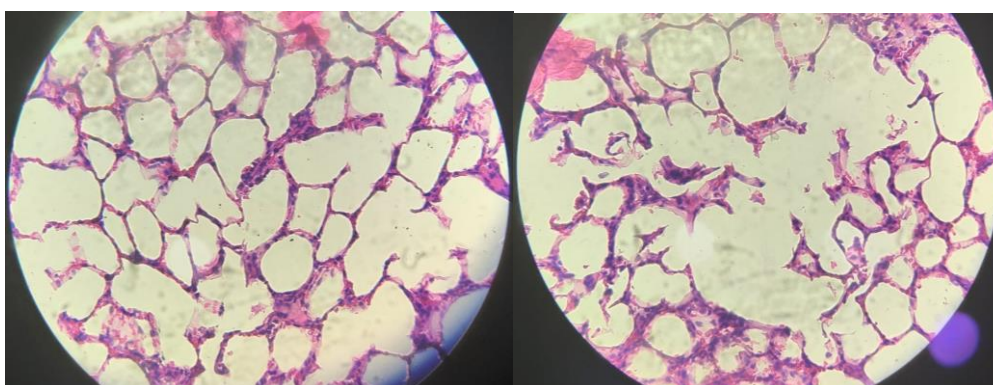
**Table 2. Data Variabel on the Incidence of Lung Tissue Fibrosis in Experimental Animals**

Hystopathological Characteristic (Aschroft Modification)	Control Group		Intervention Group		p value (Mann-Whitney test p <0.05)
	N (10)	%	N (10)	%	
Hystopathology					< 0.01*
Lung Tissue Fibrosis - (Aschroft < 5)	10	100%	2	20%	
Lung Tissue Fibrosis + (Aschroft ≥ 5)	0	0%	8	80%	

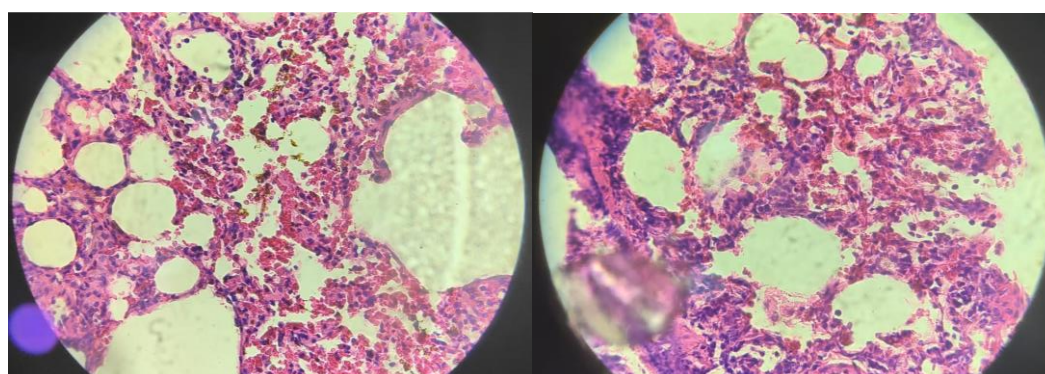
\*Significant



**Graphic 2. Data on the Incidence of Lung Tissue Fibrosis in Experimental Animals**



**Figure 1 (a-b). Control Group Lung Tissue.** In image a, it shows group C (grade 2), the alveolar septum appears clearly fibrotic with enlarged alveolar structures but no visible fibrosis. Image b shows group D (score 3) with an alveolar septum with walls starting to become fibrotic and some of the alveoli are enlarged even though the fibrosis is not yet very visible. (Aschroft modification<sup>11</sup>; image resource are from the original research of this study).



**Figure 1 (c-d). Intervention Group Lung Tissue.** In image c shows group F (grade 5) the alveolar septum appears varied with more fibrosis and widespread damage to the alveolar structure. Image d shows group G (score 6) with varying alveolar septum and most of it has been deformed with fibrotic masses in large alveoli with very extensive structural damage. (Aschroft modification<sup>11</sup>; image resource are from the original research of this study).

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For hystopathological (Ascroft modification) in the control group and intervention group (Figure 1.a-b, Figure 1.c-d) each has a non-normal distribution, so the test can use non-parametric statistics (Mann-Whitney test, or chi square). Based on the table above, it can be seen that of the 10 experimental animals in the control group, all of them had hystopathology results (Ascroft) which were classified as no fibrosis ( $< 5$ ). Meanwhile, of the 10 experimental animals in the intervention group, 20% had hystopathology (Ascroft) results that were classified as no fibrosis ( $< 5$ ), and the other 80% had hystopathology (Ascroft) results that were classified as fibrosis ( $\geq 5$ ). So it can be concluded that there is a significant difference in the comparison of hystopathology (Ascroft) results between the control group and intervention group, where 80% of fibrosis occurred in the intervention group, while in the control group no fibrosis occurred (100%).

### DISCUSSION

In this study, there was a strong significant difference in body weight in the control group with an average body weight of 359.10 gr and the METs group was 550.7 gr with a correlation test coefficient of 0.977 ( $p < 0.05$ ). This is in accordance with previous research conducted by Rohman, 2021. As in previous research, central obesity, lipid metabolism disorders, and changes in energy homeostasis are also associated with the nature of MetS. In patients with MetS, the process of lipolysis in adipose tissue is accelerated, resulting in increased release of FFA into the portal circulation. In addition, macrophage infiltration in adipose tissue, changes in the immune response, and imbalance in the synthesis of pro- and anti-inflammatory cytokines affect insulin activity in the liver and muscle. Macrophages make up approximately 40% of the total cells of obese adipose tissue, compared with 10% in lean adipose tissue. They can generally be categorized as classically activated macrophages (M1) and alternatively activated macrophages (M2). The M1 phenotype is characterized by increased expression of inflammatory proteins, such as TNF- $\alpha$ , IL-6, and IL-12 as well as inducible nitric oxide synthase, and M2 by increased expression of anti-inflammatory proteins such as arginase. There is an increased ratio of M1 to M2 in obese adipose tissue, while M1 is also correlated with inflammation and insulin resistance. Adipose tissue is the most metabolically active endocrine organ in the body and a source of hormones such as adiponectin, IL-6, and TNF- $\alpha$ .<sup>4</sup>

In this study too, there was a strong significant difference in Blood Glucose in the control group with an average of 92.80 mg/dL and the intervention group was 299.10 mg/dL with a correlation test coefficient of 0.906 ( $p < 0.05$ ). In conditions of increased blood sugar levels, according to the existing consensus, there will be an increase in risk factors for metabolic syndrome in patients. The core component of MetS is insulin resistance (IR), hence the alternative name "insulin resistance syndrome". IR is a

reduction in the ability of target organs, such as liver, skeletal muscle, and adipose tissue, to respond to normal insulin levels. Glycogen synthesis in cells pathway is primarily responsible for mediating the various actions of insulin on metabolism by regulating the activity and expression of transcription factors, enzymes, and proteins responsible for cell proliferation and apoptosis.<sup>12</sup>

We found a strong significant difference in triglyceride in the control group with an average of 79.93 mg/dL and the intervention group was 300.66 mg/dL with a correlation test coefficient of 0.912 ( $p < 0.05$ ). And the study also showed a strong difference in the HDL results of the control group of 51.47 mg/dL and the METs group of 27.80 mg/dL with a negative correlation test coefficient of -0.903 ( $p < 0.05$ ). In some study before, it was found that patients with triglycerides were the main lipid component in food. Triglyceride levels in the blood are influenced by high intake of sugar and fat. High triglycerides and low HDL (high-density lipoprotein) cholesterol are features of metabolic syndrome.<sup>13</sup>

There was a strong significant difference in plasma IL-6 in the control group with an average plasma IL-6 of 5.59 mg/dL and the intervention group was 28.67 mg/dL with a correlation test coefficient of 0.873 ( $p < 0.05$ ). This can also be seen showing a strong significant difference in tissue IL-6 in the control group with an average tissue IL-6 of 6.86 mg/dL and the intervention group was 29.55 mg/dL with a correlation test coefficient of 0.761 ( $p < 0, 05$ ). According to Rubini (2013), Interleukin-6 is a multifunctional cytokine that plays an active role in the pathogenesis of respiratory system diseases and respiratory mechanisms. Inflammation is the response of an organism to pathogens and mechanical alterations in tissue, in the form of a series of reactions that occur at the site of tissue injury. Like many other cytokines, Interleukin-6 has dual properties, both pro-inflammatory and anti-inflammatory. Interleukin-6 has a causative role in determining increased airway resistance. Interleukin-6 determines a significant impact on the resistance of the respiratory system to increased mechanical work of breathing during inspiration. Systemic inflammatory reactions are triggered by the release of pro-inflammatory cytokines which are dominant in patients with impaired lung function, namely TNF- $\alpha$  (Tumor Necrosis Factor- $\alpha$ ) and IL-6 (Interleukin-6) in the respiratory and extremity muscles.<sup>14</sup>

A significant increase in serum and lung tissue IL-6 levels was found in the intervention group compared to controls. Interleukin-6 (IL-6) plays an important role in the body's immune defense mechanisms. IL-6 functions as a pro-inflammatory and anti-inflammatory agent.<sup>15</sup> IL-6 is a cytokine released by macrophages and adipocytes. IL-6 also plays a role in the regulation of fat and glucose metabolism and contributes to insulin resistance through various mechanisms. This cytokine is increased in cases of insulin resistance and obesity as well as metabolic syndrome.<sup>16</sup>

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Metabolic syndrome is a collection of metabolic disorders such as insulin resistance, atherogenic dyslipidemia, central obesity, and hypertension. The development of metabolic syndrome involves a combination of genetic and acquired factors associated with insulin resistance and low levels of chronic inflammation.<sup>16</sup> The presence of insulin resistance causes the release of pro-inflammatory cytokines from adipose tissue, thereby increasing serum and tissue IL-6 levels.<sup>17</sup>

The results of this study are in line with previous research that the results showed that metabolic syndrome patients had higher IL-6 levels compared to controls.<sup>18</sup> Another study aimed to evaluate the prevalence of metabolic syndrome in Indian patients with COPD and determine the effectiveness of IL-6 and insulin resistance (measured by HOMA-IR) as screening markers for MetS in COPD. Results from the study showed that IL-6 had higher sensitivity than HOMA-IR as a screening marker for MetS in COPD.<sup>19</sup>

The association between metabolic syndrome (MetS) and pulmonary disease has been observed in several cross-sectional and longitudinal studies. This syndrome has been identified as an independent risk factor for worsening respiratory symptoms, greater lung function weight loss, pulmonary hypertension and asthma. Several potential mechanisms to explain this association, include dietary factors and the effects of adiposity and fat-induced inflammation on the lung, and the role of other morbidities that often co-occur with MetS, such as obstructive sleep apnea (OSA) and obesity. In contrast to the well-known association between asthma and obesity, recognition that MetS affects the lungs is relatively recent. However, there is uncertainty regarding the relative contribution of each metabolic factor to adverse effects on the respiratory system. Also, it is unclear how much of MetS and its pulmonary effects occur independently of obesity. Despite these epidemiological limitations, the proposed mechanistic pathways strongly suggest that this association is likely causal.<sup>6</sup>

This study shows that increasing body weight, triglycerides and decreasing HDL cause pulmonary fibrosis in mice. In patients with metabolic syndrome, lipid metabolism disorders occur. Alveolar macrophages in the lung tissue of fibrosis patients have been shown to increase due to lipid accumulation. These macrophages will secrete inflammatory mediators which cause the acceleration of the fibrosis process. Additionally, in fibroblasts, impaired lipid metabolism results in the accumulation of pathological myofibroblasts.<sup>20</sup>

Pulmonary fibrosis is the result of lung inflammation that is not immediately treated. So the main complication is a decrease in lung function. Metabolic syndrome can cause idiopathic pulmonary fibrosis (IPF). The process of pulmonary fibrosis is influenced by several protein signals. Dysregulation of lipid metabolism in the onset and progression of IPF. Serum adiponectin concentration may be

useful for predicting IPF prognosis because it is inversely correlated with body mass index.<sup>21</sup>

In summary, increased oxidative stress may be a major cause of diabetic pulmonary fibrosis, as can other diabetes-induced complications. Increases in reactive oxygen species and/or reactive nitrogen species that damage the lung occur directly or indirectly through the induction of pro-inflammatory and profibrotic cytokines, leading to accumulation and fibrosis of the interstitial extracellular matrix, and ultimately pulmonary dysfunction.<sup>22</sup>

### CONCLUSION

There was a significant increasing levels in characteristic of experimental animals of bodyweight, blood glucose, triglyceride, HDL, and IL-6 levels in plasma blood and lung tissue in rats with metabolic syndrome compared to the control group.

As much as 80% of the pulmonary tissue was formed in the metabolic syndrome (intervention) group of rats, meanwhile fibrosis was not formed in all the control group rats.

### CONFLICT of INTEREST

The authors have no conflict of interest.

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### ETHICAL STATEMENT

This study was approved by Ethical Medical Research Committee of Faculty of Medicine, Universitas Brawijaya, Registered number: 400/069/K.3/102.7/2023.

### AUTHOR CONTRIBUTION

All authors contributed equally to this study.

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