Original Article

# The Impact of COVID-19 Severity on TNF-α and IFN-γ in T2DM Patients' PBMC Monocytes

**Dyahati Wahyurini,* Dicky L. Tahapary,** Heri Wibowo*****

**Master's Programme in Biomedical Sciences Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia*
***Division of Endocrinology, Metabolism, and Diabetes, Department of Internal Medicine, Dr. Cipto Mangunkusumo National General Hospital, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia*
***/***Metabolic, Cardiovascular, and Aging Cluster, The Indonesian Medical Education and Research Institute, Faculty of Medicine Universitas Indonesia,*
****Department of Parasitology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia*

***Abstract***

***Introduction:*** *Monocytes are very sensitive to changes in the metabolic environment, including hyperglycemia, such as type 2 diabetes mellitus (T2DM). T2DM is characterized by chronic low-grade inflammation and becomes one COVID-19 comorbid. TNF-α and IFN-γ are cytokines often linked to inflammation, the severity of COVID-19, and long COVID-19. This study aims to analyze the relationship between COVID-19 severity and TNF-α and IFN-γ in T2DM one month post-infection.*
***Methods:*** *This research is an experimental study at the Integrated Laboratory Faculty of Medicine, Universitas Indonesia, for four months. The total samples are 44 cryotubes of PBMC (18 T2DM and 26 Non-T2DM) from the CARAMEL study (COVID-19, Aging, and Cardiometabolic Risk Factors). PBMCs were stimulated with inactivated whole virions SARS-CoV-2 and incubated for 24 hours. Monocyte subsets and intracellular cytokines are detected by flow cytometry.*
***Results:*** *Research showed that IFN-γ in the T2DM group was higher in all subsets. There was no significant difference between the T2DM and non-T2DM groups. Based on the history of the severity of COVID-19, MedFI IFN-γ classic and intermediate monocytes differed significantly between the COVID-19 severity groups ($p = 0.049$ and $p = 0.022$). Further research is needed to analyze the risk factors involved.*
***Conclusion:*** *MedFI of classic and intermediate monocyte IFN-γ differed significantly between the severity of COVID-19. There were no significant differences between the T2DM and non-T2DM groups. The history of the severity of COVID-19 is significantly related to long COVID-19.*

***Keywords:*** *Type 2 Diabetes Mellitus, IFN-γ, Monocytes, SARS-CoV-2, TNF-α.*

**Korespondensi:** **Heri Wibowo**
E-mail: *bowoheri04@gmail.com*

# *Pengaruh Riwayat Keparahan COVID-19 terhadap TNF-α dan IFN-γ Monosit Pasien Diabetes Melitus Tipe 2*

*Dyahati Wahyurini,* Dicky L. Tahapary,** Heri Wibowo****

**Program Magister Ilmu Biomedik, Fakultas Kedokteran, Universitas Indonesia, Jakarta, Indonesia*
***Divisi Endokrin, Metabolik dan Diabetes, Departemen Ilmu Penyakit Dalam, Rumah Sakit Umum Dr. Cipto Mangunkusumo, Fakultas Kedokteran, Universitas Indonesia, Jakarta, Indonesia*
***/***Kluster Metabolic, Cardiovascular, and Aging, IMERI, Fakultas Kedokteran, Universitas Indonesia*
**** Departemen Parasitologi, Fakultas Kedokteran, Universitas Indonesia, Jakarta, Indonesia*

***Abstrak***

***Pendahuluan:*** *Monosit sangat peka terhadap perubahan lingkungan metabolik, termasuk hiperglikemia seperti pada Diabetes Melitus tipe 2 (DMT2). DMT2 ditandai inflamasi kronis derajat rendah sehingga menjadi komorbid dari COVID-19. TNF-α dan IFN-γ merupakan sitokin yang sering dikaitkan dengan inflamasi, keparahan COVID-19 dan long COVID-19. Tujuan penelitian ini adalah untuk melihat hubungan antara riwayat keparahan COVID-19 terhadap TNF-α dan IFN-γ monosit pada DMT2 pasca 1 bulan infeksi.*
***Metode:*** *Penelitian merupakan penelitian eksperimental dengan total 44 cryotube PBMC (18 DMT2 dan 26 Non-DMT2) dari penelitian CARAMEL (COVID-19, Aging, and Cardiometabolic Risk Factors). Penelitian dilakukan di Laboratorium Terpadu Fakultas Kedokteran Universitas Indonesia selama 4 bulan. PBMC diberikan stimulan (inactivated whole virion SARS-CoV-2) dan diinkubasi selama 24 jam. Analisis TNF-α dan IFN-γ dilakukan dengan flow cytometry.*
***Hasil:*** *Hasil Penelitian menunjukkan bahwa IFN-γ kelompok DMT2 lebih tinggi pada semua subset. Tidak ada perbedaan signifikan antara kelompok DMT2 dan Non-DMT2 Berdasarkan riwayat derajat COVID-19, MedFI IFN-γ monosit klasik dan intermediate berbeda signifikan diantara kelompok keparahan COVID-19 (p=0.049 dan p=0.022). Perlu dilakukan penelitian lebih lanjut untuk menganalisis faktor risiko yang terlibat.*
***Kesimpulan:*** *MedFI IFN-γ monosit klasik dan intermediate berbeda signifikan diantara riwayat keparahan COVID-19. Tidak ada perbedaan signifikan antara kelompok DMT2 dan non-DMT2. Riwayat derajat keparahan COVID-19 berhubungan signifikan dengan long COVID-19.*

***Kata kunci:*** *Diabetes Melitus tipe 2, IFN-γ, Monosit, SARS-CoV-2, TNF-α.*

## Introduction

Diabetes Mellitus type 2 (T2DM) is a metabolic disease that is of concern to Indonesia and the world. Indonesia is ranked 7th, with a total of 10.7 million sufferers, and the number of diabetes patients continues to increase.[1] In 2018, the prevalence of diabetes mellitus reached more than 10% of the adult population.[2] One of the risk factors for T2DM is obesity. Obesity conditions will trigger increased pro-inflammatory cytokines such as TNF-α and IL-6, resulting in chronic inflammation associated with hyperglycemia and insulin resistance in T2DM.[3] Hyperglycemia increases ROS[4] and activates pro-inflammatory cytokines.[5] Changes in metabolic conditions, increased oxidative stress, and inflammatory processes occur continuously and trigger chronic low-grade inflammation called metaflammation/ meta-inflammation.[4,6] The inflammation that occurs will disrupt metabolic processes and affect the immune system, resolution of inflammation, and insulin resistance.[7-9]

The metainflammatory conditions in T2DM increase the risk of morbidity and mortality from infections, including COVID-19. The latest data shows mortality

rate for COVID-19 patients with comorbid T2DM is 21.4%.[10] T2DM increases the risk of COVID-19 mortality and tends to have more severe clinical symptoms.[10] Hyperglycemia and high glucose levels are also associated with the severity of COVID-19.[11] This is caused by dysfunction of the innate and adaptive immune response in T2DM conditions.[6] Monocytes play a role in fighting pathogens, assisting in the efficient resolution of inflammation, antigen recognition by T cells, and activating memory cells and antibodies.[12,13] Monocytes are immune cells most easily affected by metabolic changes, including T2DM. Dysregulation of monocytes in T2DM affects the cytokines produced.[12]

Dysfunction of monocytes and innate cells can interfere with the inflammatory process and the resolution of inflammation. Thereby, the degree of clinical symptoms.[12] Monocytes will increase during inflammatory processes, including chronic inflammation in T2DM.[14,15] Increasing monocytes in circulation was followed by an increase in TNF-α and IFN-γ and the risk of inflammation.[16] Research proves that in the T2DM group, there was an increase in levels of the cytokine TNF-α.[17] However, several studies have found a decrease in IFN-γ production, which disrupts the T cell response in fighting pathogens and contributes to immunity against infection.[18]

Disrupted interactions between monocytes and T cells and cytokines contribute to the severity of COVID-19 and the long-COVID-19 phenomenon. Increased cytokines occur in COVID-19 patients with comorbidities and severe symptoms.[19] Cytokine imbalance is also thought to be related to the occurrence of the long COVID-19 and the severity of reinfection. Long COVID-19 is a condition where residual symptoms are still felt for 1-3 months post-COVID-19. The frequency and intensity of long-COVID-19 varies. The symptoms caused are associated with increased monocytes and cytokines, especially IL-6, TNF-α, and IFN-γ.[20] Various studies suggest a bidirectional relationship between T2DM and COVID-19. T2DM affects the severity of COVID-19, and recent research shows that COVID-19 can trigger T2DM.[21] Rizvi *et al*.[22] explain the relationship between inflammation, DM, and post-COVID-19 syndrome. More significant inflammation will result in worse severity and prognosis of COVID-19 in T2DM patients and poor metabolic control. COVID-19 infection will result in changes in transcription pathways, resulting in increased levels of the cytokines IL-1, IL-6, IL-12, IFN-γ, and TNF-α[23] Each cytokine produced will carry out its respective function and have an impact on surrounding cells and body homeostasis which then becomes clinical manifestations.[24] The severity of COVID-19 will affect monocyte activity and the cytokines TNF-α and IFN-γ. This then has an impact on long-COVID-19. Therefore, this study aimed to analyze the relationship between the history of the severity of COVID-19,TNF-α and IFN-γ from monocytes of T2DM and non-T2DM patients.

## Method

This research used PBMC from COVID-19 with T2DM and COVID-19 without non-T2DM recruited in the CARAMEL study (COVID-19, Aging, and Cardiometabolic Risk Factors) between December 2020 and March 2022. The CARAMEL study was longitudinal research, but this research used only PBMC from the first month after negative PCR COVID-19. Inclusion criteria were set to find eligible subjects. The requirements were as follows: 1) Patients (>18 years old) were controlled after positive COVID-19 based on PCR one month ago. 2) Patients agreed with the informed consent. 3) Not yet vaccinated. 4) For the T2DM group, patients must have fasting blood glucose > 126 mg/dl, have been diagnosed with T2DM before, or be in antidiabetic treatment. Subjects were not included when they had one or more of the following criteria: 1) pregnant; 2) patients with autoimmune diseases, HIV, cancer, or acute infections. 3) Individuals were receiving treatment with immunosuppressants. Based on inclusion and exclusion criteria, the total samples were 44 PBMC isolation cryotubes (18 T2DM and 26 non-T2DM).

The research was conducted in May–October 2023 at the MVA IMERI cluster and the Integrated Laboratory of the Faculty of Medicine, University of Indonesia. Cryo-PBMC was thawed by warming it in a 37°C water bath for 1-2 minutes, then mixing it into the warm complete RPMI medium. Then, centrifuge at 400 g for 10 minutes. After that, cells are counted, seeded in a 48-well culture plate, and incubated overnight. After that, 10µL of the inactivated whole virion SARS-CoV-2 vaccine from PT. Biotis Pharmaceutical as a stimulant was given in the well and incubated for 24 hours. Then, monocytes were harvested and profiled using flow cytometry. Monocytes were grouped into three subsets based on CD14 and CD16: Classical (CD14+CD16-), Intermediate (CD14+CD16+), and non-classical (CD14loCD16+). The expression was detected with FITC anti-human CD14 antibody,[25] and human CD16 APC monoclonal antibody B73.1.[26] In-

tracellular cytokines TNF-α and IFN-γ were detected with the PE/Cyanine 7 anti-human TNF-α antibody[27] and APC-Cyanine 7 Mouse Anti-Human IFN-γ.[28] Analysis used FlowJo and IBM SPSS statistics 24. We analyzed clinical data such as age, gender, severity of COVID-19, and long COVID-19, then correlated it with the percentage of monocytes and cytokines produced. Mann-Whitney was used to analyze the differences between immunological parameters and diabetes status (T2DM and non-T2DM). Kruskal-Wallis was used to analyze the differences between immunological parameters and the severity of COVID-19 (asymptomatic, mild, moderate, severe). FlowJo was used to gate and visualized the percentage of monocytes and MedFI TNF-α and IFN-γ.

## Result

### Subject Characteristics

The research has been completed. Analysis of descriptions showed that 40.9% of PBMCs were from T2DM post-COVID-19 subjects, and 59.1% were from non-T2DM post-COVID-19 subjects. Isolation PBMC was more prevalent among males (65.9%) than females (34.1%). In COVID-19, the symptoms and degree of severity differ for each individual. In this study, the degree of severity has been grouped into 4 degrees: asymptomatic or without any complaints, the mild group with mild cough and fever, the moderate group with findings of clinical symptoms accompanied by X-ray results (pneumonia), and the severe group with severe clinical symptoms such as shortness of breath and requires NRM. All samples from the T2DM group had a history of mild, moderate, and severe degrees of COVID-19. Severe COVID-19 (33%) is the most common group in T2DM. In the non-T2DM group, moderate COVID-19 is the most cases (38.5%). This condition can also have an impact on the risk of long-COVID-19. All samples from the T2DM group experienced long-COVID-19; in the non-T2DM group, 76,028% experienced long-COVID-19. Based on body mass index (BMI), 75% of the sample was obese, and in the T2DM group, 88.9% was obese. Subject characteristics are shown in Table 1.

### Intracellular Cytokine Analysis

TNF-α and IFN-γ from each monocyte were performed and yielded MedFI values. Detection was performed using Flow Cytometry with optimization of working steps and the amount of TNF-α and IFN-γ antibodies.[29,28,27,30] Classical, intermediate, and non-classical monocytes have different MedFI TNF-α and

**Table 1. The Characteristics of Research Subjects in the Non-T2DM and T2DM Groups**

| Characteristics | Total (n=44) | Groups | | *p-value* |
|---|---|---|---|---|
| | | Non-T2DM (n=26) | T2DM (n=18) | |
| Gender (n (%)) | | | | 0.042* |
| Male | 29 (65.9) | 14 (53.8) | 15 (83.3) | |
| Female | 15 (34.1) | 12 (46.2) | 3 (16.7) | |
| Age (median; IQR) | 47.5 (7.5) | 43 (23) | 51 (10) | 0.036* |
| Body mass index (n (%)) | | | | 0.086 |
| Underweight | 0 | 0 | 0 | |
| Normal | 5 (11.4) | 4 (15.4) | 1 (5.6) | |
| Overweight | 6 (13.6) | 5 (19.2) | 1 (5.6) | |
| Obese | 33 (75) | 17 (65.4) | 16 (88.9) | |
| COVID-19 Severity (n (%)) | | | | 0.054 |
| Asymptomatic | 6 (13.6) | 6 (23.1) | 0 | |
| Mild | 8 (18.2) | 5 (19.2) | 3 (16.7) | |
| Moderate | 19 (43.2) | 10 (38.5) | 9 (50) | |
| Severe | 11 (25) | 5 (19.2) | 6 (33.3) | |
| Long-COVID-19 | | | | 0.033* |
| No | 6 (13.63) | 6 (23.08) | 0 | |
| Yes | 38 (86.36) | 20 (76.92) | 18 (100) | |

*Notes: Data on patient characteristics was compared between T2DM and non-T2DM groups using the Mann–Whitney test, *p<0.05. Abbreviations T2DM, type 2 diabetes mellitus; IQR: interquartile range. Detail of body mass index: underweight (< 18.5kg), normal (18.5- 22.9 kg), overweight (23 – 24.9 kg), obese (>25kg).*

IFN-γ patterns. Statistical analysis showed higher MedFI IFN-γ in almost all monocyte subsets in the T2DM group, however, the statistical analysis showed no significant difference in the MedFI of IFN-γ and TNF-α between the T2DM groups and Non-T2DM. The analysis test continued by analyzing the relationship between the COVID-19 severity and IFN-γ and TNF-α post one month of COVID-19. The results showed that the mild severity had a higher MedFI IFN-γ than the asymptomatic group. The One-Way ANOVA analysis results prove a significant difference in classic and intermediate monocyte IFN-γ MedFI between COVID-19 severity groups (p=0.049 and p=0.022) (Table 2). The MedFI of TNF-α did not differ significantly between degrees of severity. The analysis continued by looking at the differences between degrees of severity and based on diabetes status. Based on the degree of severity, MedFI IFN-γ differed significantly between asymptomatic and moderate degrees (p=0.044). Intermediate Monocytes differ significantly between asymptomatic and mild (p=0.0274), asymptomatic to moderate (p=0.0278), and symptomatic with weight (p=0.0419).

Based on diabetes status, the pattern of monocyte percentage, MedFI IFN, and TNF did not differ significantly between the T2DM and non-T2DM groups. In non-T2DM, there was a significant difference in MedFI IFN-γ intermediate monocytes between the severity of COVID-19 (p=0.033), especially between asymptomatic and severe (p=0.039). In T2DM, there was a significant difference in MedFI IFN-γ non-classical monocytes between the severity of COVID-19 (p=0.040), especially between mild and severe (p=0.038) (Figure 1).

## Long COVID-19

History of the COVID-19 severity is related to long COVID-19. The statistical analysis results show a significant difference in the history of the severity of COVID-19 versus long COVID-19 (p=0.000). Based on T2DM status, the distribution of IFN-γ and TNF-α MedFI is shown in Figure 1. All samples that did not experience long COVID-19 were T2DM subjects with an asymptomatic history and showed low IFN-γ MedFI in all monocyte subsets (Figure 2a-2c ). Increasing COVID-19 severity was followed by an increase in MedFI IFN-γ. The Long COVID-19 group showed higher MedFI IFN-γ and TNF-α. MedFI IFN-γ and TNF-α distribution in the T2DM and Non-T2DM groups in the long COVID-19 group was almost the same, although it tended to be dominated by the T2DM group (Figure 2).

**Table 2. Analyses Univariate Result of Research Subject Characteristics**

| Cytokines – Monocyte subset | COVID-19 Severity | | | | *p-value* |
|---|---|---|---|---|---|
| | Asymptomatic | Mild | Moderate | Severe | |
| **IFN-γ (Median; min-max)** | | | | | |
| Total | 36.8 (32.5-46.10) | 71.25 (31.9-168) | 65.7(32.1-211) | 84.9 (42.8-147) | 0.053 |
| Classical ($CD14^+CD16^-$) | 17.65 (0.00-63.2) | 65.67 (29.3-310) | 93.3 (0.00-617) | 47.2 (0.00-371) | 0.049* |
| Intermediate ($CD14^+CD16^+$) | 32.96 (17.7) | 123.17 (67.4) | 111.03 (64.5) | 112.69 (46.46) | 0.022* |
| Non-classical ($CD14^{lo}CD16^+$) | 35.65 (31.6-46.7) | 36.5 (31.9-108) | 43 (0.00-173) | 43 (37.3-132) | 0.248 |
| **TNF-α (Median; min-max)** | | | | | |
| Total | 5.2 (3.06-61.30) | 3.99 (2.91-11.10) | 8.5 (2.51-32.4) | 6.55 (1.95-11.40) | 0.602 |
| Classical ($CD14^+CD16^-$) | 3.67 (2.1-44.5) | 7.45 (2.02-13.6) | 4.8 (0.00-137) | 3.26 (0.00-6.29) | 0.258 |
| Intermediate ($CD14^+CD16^+$) | 7.5 (2.88-71.7) | 9.96 (5.73-14.9) | 11.4 (0.00-18.5) | 10.4 (7.47-33.3) | 0.540 |
| Non-classical ($CD14^{lo}CD16^+$) | 14.55 (3.46-61.3) | 3.13 (2.88-8.8) | 6.48 (2.59-18.4) | 3.4 (0.00-28.8) | 0.092 |

*Note: *p<0.05. Data are presented as median (minimun-maximum). The Kruskal Wallis test evaluated differences between MedFI IFN-γ and TNF-α between groups. Abbreviations: CD: cluster of differentiation; TNF-α; tumor necrosis factor-α; IFN-γ; interferon-γ.*

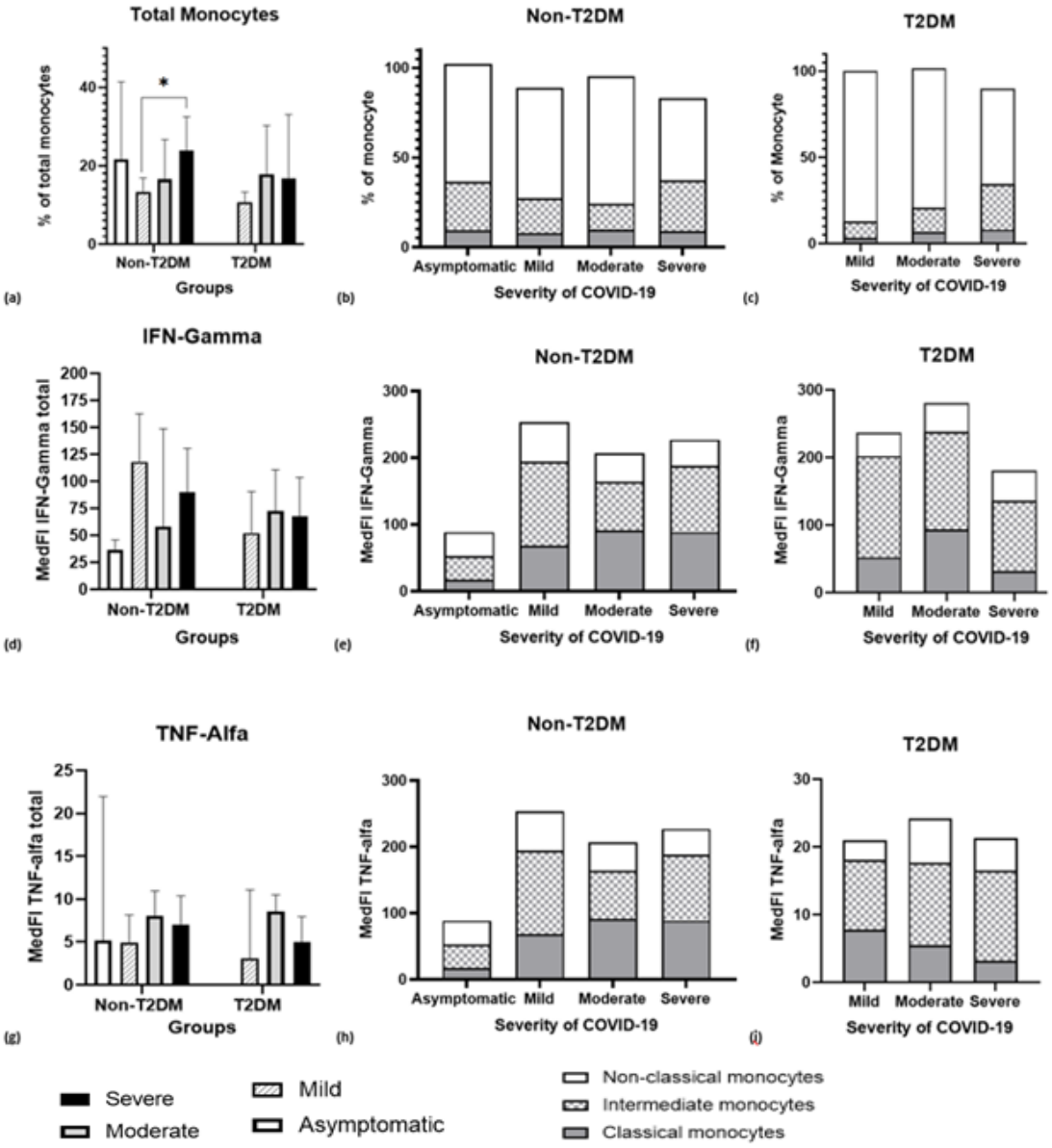


***Figure 1:*** *Percentage of subset monocytes, MedFI IFN-γ and TNF-α between COVID-19 severity groups in non-T2DM and T2DM patients. (a) proportion percentage of total monocyte in two groups; (b) proportion percentage of monocyte subset in non-T2DM group; (c) proportion percentage of monocyte subset in T2DM group; (d) proportion total MedFI IFN-γ in two groups; (e) proportion MedFI IFN-γ from monocyte subsets in non-T2DM; (f) proportion MedFI IFN-γ from monocyte subsets in T2DM; (g) proportion MedFI TNF-α in two groups; (h) proportion MedFI TNF-α in non-T2DM; (i) proportion MedFI TNF-α in T2DM. Note: *p<0.05. Kruskal Wallis was used for multiple group comparisons.*

## Discussion

Age, gender, and body weight are risk factors for T2DM. The body weight of most subjects was in the obesity category (75%). Obesity is associated with the development of T2DM and is a comorbid disease. Obesity and diabetes are risk factors for increasing the severity of COVID-19.[31,32] In T2DM patients, obesity is associated with poor early prognosis in COVID-19 patients. Albahrani et al.[33] prove that obesity was identified as an independent risk factor for the risk of decreased oxygen saturation in COVID-19 patients. The COVID-19 severity is associated with cytokines such as IFN-γ and TNF-α. IFN-γ is a type II, produced by monocytes, T, and NK cells. IFN-γ contributes to the control of inflammation and activates innate and acquired immunity.[34] IFN-γ participates in various innate immunological functions and adaptive and inflammatory processes.[35] The study found that people with T2DM had higher MedFI IFN-γ than non-T2DM. In T2DM, hyperglycemia will increase the formation of AGEs, sorbitol production, and activation of protein kinase C, thereby increasing ROS in the endothelium and triggering chronic inflamma-

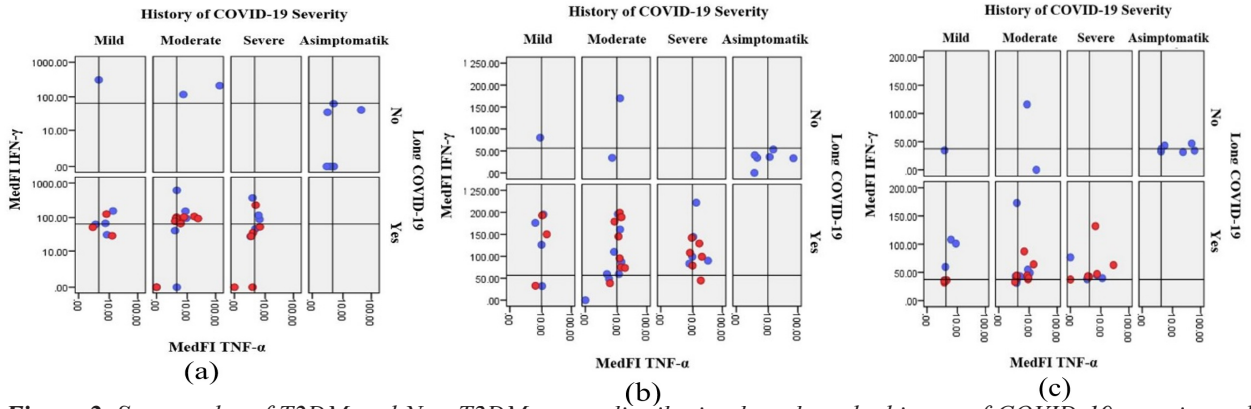


***Figure 2.*** *Scatter plot of T2DM and Non-T2DM group distribution based on the history of COVID-19 severity and long-COVID-19. (a) Classical monocytes (b) Intermediate monocytes (c) Non-classical monocytes. Red is T2DM, and Blue is Non-T2DM.*

tion.[4] Hyperglycemia increases the activation of the NF-κB transcription pathway, producing more pro-inflammatory cytokines, including IFN-γ. Rarani et al.[34] stated that IFN-γ could work by activating the JAK1, JAK2, and STAT signaling pathways. In the comparison group, non-T2DM, was not healthy but had a history of COVID-19. Other studies report that IFN-γ production tends to be lower in the COVID-19 and lower in the severe than in the moderate group.[34]

MedFI TNF-α intermediate monocytes in the T2DM group were higher than in the non-T2DM group. Coztella Ruiz *et al.*[35] concluded that TNF-α is a pro-inflammatory cytokine produced by monocytes, macrophages, T cells, and others. TNF-α is mediated by IL-1β and IL-6. TNF-α is involved in the regulation of inflammatory processes. Serum TNF-α levels increase in severe COVID-19 patients. TNF-α is one of the cytokines whose overproduction is associated with poor prognosis in SARS-CoV and MERS patients.[36] However, TNF-α did not show significant differences between the T2DM and non-T2DM groups in all monocyte subsets. People who have had COVID-19 in the past may have higher levels of cytokines, especially if they have long COVID-19. Even if a swab is negative for Sars-CoV-2, they can still have some symptoms of COVID-19, which are known as long COVID-19. Complaints can be felt for 1-3 months after COVID-19, and the symptoms caused are associated with increased monocyte frequency, increased IL-6 and TNF-α, and decreased HLA-DR.[20] The COVID-19 severity also affects the cytokines one month post-infection. Cytokine levels showed a sustained increase in COVID-19 patients with comorbidities and severe symptoms.[19]

Based on monocyte surface marker expression, circulating monocytes are grouped into three groups: classical (CD14+ CD16−), non-classic (CD14dim, CD16+), and intermediates (CD14+ CD16+)[36] Classical monocytes are pivotal in phagocytosis, innate sensing, and the migration process.[37] Classical monocytes can differentiate into many subsets of macrophages and monocyte derivatives. Intermediate monocytes (CD14+ CD16+) function to present antigens, secrete cytokines, regulate apoptosis, differentiate, and are responsible for the proliferation and stimulation of T cells. Intermediate monocytes are responsible for T-cell proliferation, stimulation, ROS, and angiogenesis.[14] Non-classical monocytes (CD14it CD16+) are concerned with complement, adhesion, Fc-γ-mediated phagocytosis, and linking innate and adaptive immune responses.[37] Non-classical monocytes are pro-inflammatory, anti-inflammatory, anti-metastatic, and atheroprotective,[36] maintain vascular integrity, react to inflammatory signals, and clear cellular debris.[37] Classical monocytes function in phagocytosis. Intermediate monocytes act as pro-inflammatories, releasing high levels of IL-1β and TNF-α. Non-classical monocytes play a role in patrolling the endothelium and releasing IL-1β and TNFα.[38] In healthy conditions, classical monocytes are the main population in blood circulation.[14] However, the percentage of the monocyte subset can change due to changes in conditions such as acute or chronic inflammatory conditions. In this study, PBMC samples were obtained from individuals with a history of COVID-19 with varying degrees of severity. In this study, non-classical and intermediate monocytes were pro-inflammatory monocytes with a high percentage. The changes in monocytes will be related to the IFN-γ and TNF-α produced. This study showed that intermediate monocytes have the highest MedFI IFN-γ and

TNF-α values among other subsets. At moderate grade, TNF-α from classic monocytes was lower than at mild grade, while TNF-α from intermediate monocytes was higher, although not statistically different. This proves that the increase in severity is in line with increased inflammation, characterized by higher levels of TNF-α in intermediate monocytes due to its role in pro-inflammation.

In Figure 1, when comparing the T2DM and non-T2DM groups, differences in classical and intermediate monocyte TNF-α patterns can be seen to a moderate degree. In the T2DM group, intermediate monocyte TNF-α was higher than classic monocytes, in contrast to the non-T2DM group with a history of moderate degrees of COVID-19, intermediate monocyte TNF-α was slightly lower than intermediate monocytes. In T2DM conditions, chronic inflammation occurs, and more pro-inflammatory monocyte subsets (intermediate and non-classical) are found. Chronic inflammation in T2DM causes the monocyte subset to shift to intermediate and non-classical, so TNF-α is highest in intermediate monocytes, as in this study. Hyperglycemia affects cytokine production in PBMC. Hyperglycemia also increases viral load, ACE2, and cytokine expression (IL-1b, TNF-a, IL-6, and IFN α, β, γ) in SARS-CoV-2-infected monocytes.[39] However, the pattern between the T2DM and non-T2DM groups was not statistically significantly different. Inflammation in the non-T2DM group is thought to originate from a history of COVID-19 one month ago. Persistent SARS-CoV-2 antigen in monocytes plays a role in the increased monocyte response in the T2DM and non-T2DM groups.

Based on Tabel 1 and Figure 1, intermediate monocytes in the T2DM group with a history of severe disease had higher levels of IFN-γ and TNF-α than those with moderate degrees and then manifested long-term COVID-19. Turner et al. suggested that various interrelated factors influence long COVID-19.[40] The number and type of symptoms during initial infection are strong predictors of long COVID-19.[40] Patients who experience more than five symptoms during the first week of infection are at greater risk of experiencing long COVID-19, regardless of age or gender, according to research conducted by Menni and Sudre.[41,42] In this study, we found that long COVID-19 correlates with factors like BMI and history of the COVID-19 severity. These factors affected IFN-γ and TNF-α. However, when it comes to the connection with T2DM, more investigation is required to analyze it. The post-acute sequelae of COVID-19 can persist for 1-3 months after COVID-19.[20] Recent research states a relationship between COVID-19 infection and an increase in GDP and HbA1c. The relationship between T2DM and COVID-19 is two-way. T2DM affects the severity of COVID-19, and recent research shows that COVID-19 can trigger T2DM.[21] T2DM after COVID-19 is also considered a manifestation of COVID-19, known as New *Onset* DM (NODM) post-COVID-19. Based on research conducted by Vigili *et al.*[43] SARS-CoV-2 infection continuously damages pancreatic beta cells and causes insulin resistance; on the other hand, T2DM also supports long-term COVID-19. Poor metabolic health (such as T2DM and obesity) increases the risk of SARS-CoV-2 infection and COVID-19 complications through hyperglycemia, hyperinsulinemia, and insulin resistance.[43] Estiri *et al.*[44] found that T2DM was a risk factor for long-term COVID-19 among non-hospitalized COVID-19 patients. In his research on 9,025 subjects with positive test results, there were 33 indicative phenotypes of COVID-19.[44] The incidence and risk of hyperglycemia and T2DM increase after COVID-19 infection, especially in the group of men with an initial COVID-19 infection. Li Jet *et al,*[45] suspect that post-COVID-19 hyperglycemia is a phenomenon long after COVID-19 and is temporary so that blood glucose levels will return to normal.

The history of the severity of COVID-19 also plays a role in its occurrence *long* COVID-19. The research results describe all sufferers with COVID-19 who have a history of symptomatic COVID-19 and are predominantly moderate. All individuals with a history of severe degrees of severity manifested COVID-19 after one-month post-infection. Chenet *et al.*[46] *suggested* severe COVID-19 was associated with higher blood glucose and HbA1c than mild COVID-19 patients. The history of COVID-19 severity is related to GDP and HbA1c. Rizvi *et al.*[22] explain the relationship between inflammation, DM, and long-term COVID-19. Greater inflammation will result in worse severity and prognosis of COVID-19 in DM patients and poor metabolic control.[22] Prattichizzo *et al,*[47] explained that HbA1c is associated with higher-grade chronic inflammation and dysregulation of immune cells. This causes a linear relationship between HbA1c and the prognosis of COVID-19 and an increase in the death rate related to COVID-19 in DM patients with poor glycemic control before infection. COVID-19 causes hyperinflammatory and hypercoagulable syndromes with

the same severity in DM and non-DM patients. In T2DM, there is an increase in HbA1c and GDP up to 180 days after COVID-19.[48]

Several theories of post-COVID-19 hyperglycemia include the pancreas being one of the targets of SARS-CoV2, resulting in focal enlargement of the pancreas or dilatation of the pancreatic duct, resulting in pancreatic injury.[49,46] ACE 2 is thought to be involved in the relationship between COVID-19, hyperglycemia, and pancreatic injury.[50] SARS-CoV-2 binds to the ACE2 receptor on the surface of pancreatic islets.[46,51] Another hypothesis is that a stress response encourages the increased release of catecholamines and glucocorticoids. There is an increase in inflammatory activity that affects increasing insulin resistance.[51,52] Ren Researchers *et al.*[53] revealed that triglyceride, glucose, and insulin resistance indices were closely related to the severity of COVID-19. Insulin resistance is considered a cause of hyperglycemia in COVID-19 patients.[46]

Keerthi *et al.*[21] stated that hyperglycemia, which triggers NODM after COVID-19, can occur due to several risk factors such as family history, high BMI, and use of steroids, which can trigger NODM after COVID-19.[21] Kim SH et *al.*[54] concluded that the etiology of post-COVID-19 hyperglycemia is multifactorial, originating from the patient himself or the impact of the pandemic, such as psychosocial stress or limitations in carrying out controlled health checks. COVID-19 can also directly or indirectly affect pancreatic β-cell function and insulin sensitivity. This is related to acute COVID-19 infection and the treatment administered; persistent viral residency in multiple organs, including adipose tissue; endothelial dysfunction; and hyperinflammatory states.[51,54] T2DM is associated with chronic inflammation, resulting in dysfunction and immune cell dysregulation. This will result in a decrease in the immune system. When infected with COVID-19, dysregulation of the immune system will affect the clinical manifestations and severity of COVID-19.

## Limitation

This research has succeeded in answering the research objectives, trying to analyze the relationship between T2DM and COVID-19 in terms of monocytes and cytokines. Samples using all PBMCs can describe immune cell interactions as a whole. However, there are several limitations to this research. Samples in the form of cryo-PBMC have been stored for 1.5 years, which affects cell quality, and monocyte isolation cannot be carried out at an early stage. This research only used CD14 and CD16 as cell surface markers, which are still less specific because CD16 can be found in NK cells. It is best to use surface markers to eliminate other cells. In clinical application, many risk factors for T2DM were not analyzed in this study, such as lipid profile, smoking history, comorbidities, medication history, etc. All samples came from individuals with a history of COVID-19; this study did not compare with a healthy group without COVID-19.

## Conclusions

In Summary, the COVID-19 severity affects monocyte IFN-γ and TNF-α. IFN-γ from classical and intermediate monocytes differed significantly in the severity of COVID-19. Based on diabetes status, the pattern of monocyte percentage, MedFI IFN, and TNF did not differ significantly between the T2DM and non-T2DM groups. In non-T2DM, there was a significant difference in MedFI IFN-γ intermediate monocytes. In T2DM, there was a significant difference in MedFI IFN-γ non-classical monocytes between the COVID-19 severity. The COVID-19 severity is significantly different in long COVID-19.

## Conflicts of Interest

We declare no conflict of interest in this study. We declare no conflict of interest in this study. We declare no conflict of interest in this study. This research has received approval from the Health Research Ethics Committee, Faculty of Medicine, University of Indonesia (FK-UI), Dr. Cipto Mangunkusumo National General Hospital with No. KET-1112/UN2. F1/ETIK/2020.

## Acknowledgment

The Authors express gratitude to the Master's Programme in Biomedical Sciences Faculty of Medicine, the Ministry of Research, Technology and Higher Education Indonesia, and PT. Biotis Pharmaceutical. This research was funded by the Ministry of Research, Technology and Higher Education Indonesia grant.

## References

1. Kemenkes. Infodatin tetap produktif, cegah, dan atasi Diabetes Melitus 2020 [Internet]. Pusat Data dan Informasi Ke-

mentrian Kesehatan RI. 2020. hal. 1–10. Tersedia pada: https://pusdatin.kemkes.go.id/resources/download/pusdatin/infodatin/Infodatin-2020-Diabetes-Melitus.pdf
2. Tahapary DL, Soewondo P. Burden of metabolic diseases in Indonesia: An even more critical issue during COVID-19 pandemic. Med J Indones. 2020;29(4):347–9.
3. Larasati RA, Harbuwono DS, Wibowo H, Rahajeng E, Pradipta S, Nuraeni HS, et al. The role of butyrate on monocyte migration and inflammation response in patient with type 2 diabetes mellitus. Biomedicines. 2019;7(4):1–13.
4. Aravindhan V, Madhumitha H. Metainflammation in Diabetic Coronary Artery Disease: Emerging Role of Innate and Adaptive Immune Responses. J Diabetes Res. 2016;2016(1).
5. Mouton AJ, Li X, Hall ME, Hall JE. Obesity, hypertension, and cardiac dysfunction novel roles of immunometabolism in macrophage activation and inflammation. Circ Res. 2020;789–806.
6. Susantiningsih T, Mustofa S. |Ekspresi IL-6 dan TNF-α Pada Obesitas JK Unila | Volume 2| Nomor 2 | Juli. 2018.
7. Russo L, Lumeng CN. Properties and functions of adipose tissue macrophages in obesity. Immunology. 2018;155(4):407–17.
8. Chylikova J, Dvorackova J, Tauber Z, Kamarad V. M1/M2 macrophage polarization in human obese adipose tissue. Biomed Pap. 2018;162(2):79–82.
9. Teixeira FC, Pereira FEF, Pereira AF, Ribeiro BG. Metabolic syndrome's risk factors and its association with nutritional status in schoolchildren. Prev Med Reports [Internet]. 2017;6:27–32. Tersedia pada: http://dx.doi.org/10.1016/j.pmedr.2017.02.002
10. Harbuwonoa DS, Handayani DOTL, Wahyuningsih ES, Novita Supraptowati A, Kurniawan F, Tahapary DL, et al. Impact of diabetes mellitus on COVID-19 clinical symptoms and mortality: Jakarta's COVID-19 epidemiological registry. Prim Care Dibetes. 2022;16(January):65–8.
11. Lazarus G, Audrey J, Wangsaputra VK, Tamara A, Tahapary DL. High admission blood glucose independently predicts poor prognosis in COVID-19 patients: A systematic review and dose-response meta-analysis. Diabetes Res Clin Pract [Internet]. 2021;171:108561. Tersedia pada: https://doi.org/10.1016/j.diabres.2020.108561
12. Alzaid F, Julla J, Diedisheim M, Potier C, Potier L, Velho G, et al. Monocytopenia, monocyte morphological anomalies and hyperinflammation characterise severe COVID -19 in type 2 diabetes. EMBO Mol Med. 2020;12(10):1–12.
13. Knoll R, Schultze JL, Schulte-Schrepping J. Monocytes and Macrophages in COVID-19. Front Immunol. 2021;12(July):1–12.
14. Orozco SL, Canny SP, Hamerman JA. Signals governing monocyte differentiation during inflammation. Curr Opin Immunol [Internet]. 2021;73:16–24. Tersedia pada: https://doi.org/10.1016/j.coi.2021.07.007
15. Wu H, Ballantyne CM. Metabolic Inflammation and Insulin Resistance in Obesity. Circ Res. 2020;1549–64.
16. Flynn MC, Pernes G, Lee MKS, Nagareddy PR, Murphy AJ. Monocytes, macrophages, and metabolic disease in atherosclerosis. Front Pharmacol. 2019;10(JUN):1–13.
17. Wibowo H, Harbuwono DS, Tahapary DL, Kartika R, Pradipta S, Larasati RA. Impact of Sodium Butyrate Treatment in LPS-Stimulated Peripheral Blood Mononuclear Cells of Poorly Controlled Type 2 DM. Front Endocrinol (Lausanne). 2021;12(July):1–8.
18. Kartika R, Purnamasari D, Pradipta S, Larasati RA, Wibowo H. Impact of low interferon-γ and il-10 levels on tnf-α and il-6 production by pha-induced PBMCS in type 2 diabetes mellitus. J Inflamm Res. 2020;13:187–93.
19. Qin S, Jiang Y, Wei X, Liu X, Guan J, Chen Y, et al. Dynamic changes in monocytes subsets in COVID-19 patients. Hum Immunol. 2021;82(3):170–6.
20. Pal R, Banerjee M. Are People with Uncontrolled Diabetes Mellitus at High Risk of Reinfections with COVID-19? 2020;(January).
21. Keerthi BY, Sushmita G, Khan EA, Thomas V, Cheryala V, Shah C, et al. New onset diabetes mellitus in post-COVID-19 patients. 2022;
22. Rizvi AA, Kathuria A, Mahmee W Al, Al-Rasadi K, Al-Alawi A, Banach M, et al. Post-COVID syndrome, Inflammation, and Diabetes. J Diabetes Complications. 2022;36(October).
23. Olajide OA, Iwuanyanwu VU, Lepiarz-Raba I, Al-Hindawi AA. Induction of Exaggerated Cytokine Production in Human Peripheral Blood Mononuclear Cells by a Recombinant SARS-CoV-2 Spike Glycoprotein S1 and Its Inhibition by Dexamethasone. Inflammation. 2021;44(5):1865–77.
24. Dhar SK, K V, Damodar S, Gujar S, Das M. IL-6 and IL-10 as predictors of disease

severity in COVID-19 patients: results from meta-analysis and regression. Heliyon [Internet]. 2021;7(2):e06155. Tersedia pada: https://doi.org/10.1016/j.heliyon.2021.e06155
25. BD Pharmingen. Technical Data Sheet FITC Mouse Anti-Rat Granulocytes. 2017;
26. BioLegend Inc. APC anti-human CD16 Antibody. 2014;2. Tersedia pada: https://www.biolegend.com/en-gb/products/apc-cy7-anti-human-cd16-antibody-1904
27. BioLegend. PE / Cyanine7 anti-human TNF-α Antibody. 2013;6–8.
28. BioLegend. APC / Cyanine7 anti-human IFN-γ Antibody. 2019;6–8.
29. BioLegend. Intracellular Flow Cytometry Staining Protocol. BioLegend [Internet]. 2021;(420801):9–11. Tersedia pada: https://www.biolegend.com/en-us/protocols/intracellular-flow-cytometry-staining-protocol
30. BD. Kit Manual "Cytoperm, B D Cytofix Kit, Fixation Permeabilization." (554714).
31. Ohno M, Dzúrová D. Body Mass Index and Risk for COVID-19-Related Hospitalization in Adults Aged 50 and Older in Europe. Nutrients. 2022;14(19).
32. Alberti A, Freiberger V, Ventura L, Grigollo L, Dias P, Comim CM, et al. Obesity in people with diabetes in COVID-19 times: Important considerations and precautions to be taken. World J Clin Cases. 2021;9(20):5358–71.
33. AlBahrani S, Al-Maqati TN, Al Naam YA, Alqahtani JS, Alqahtani AS, AlRabeeah S, et al. The Association of Body Mass Index with COVID-19 Complications and Survival Rate at a Tertiary Hospital. Life. 2023;13(7).
34. Rarani FZ, Rashidi B, Jafari Najaf Abadi MH, Hamblin MR, Reza Hashemian SM, Mirzaei H. Cytokines and microRNAs in SARS-CoV-2: What do we know? Mol Ther - Nucleic Acids [Internet]. 2022;29(September):219–42. Tersedia pada: https://doi.org/10.1016/j.omtn.2022.06.017
35. Costela-ruiz VJ, Illescas-montes R, Puerta-puerta JM, Ruiz C, Melguizo-rodríguez L. SARS-CoV-2 infection: The role of cytokines in COVID-19 disease. Cytokine Growth Factor Rev. 2020;54(June):62–75.
36. Ożańska A, Szymczak D, Rybka J. Pattern of human monocyte subpopulations in health and disease. Scand J Immunol. 2020;92(1):1–13.
37. Tahir S, Steffens S. Nonclassical monocytes in cardiovascular physiology and disease. Am J Physiol - Cell Physiol. 2021;320(5):C761–70.
38. Yang J, Zhang L, Yu C, Yang XF, Wang H. Monocyte and macrophage differentiation: Circulation inflammatory monocyte as biomarker for inflammatory diseases. Biomark Res. 2014;2(1):1–9.
39. Zhou Z, Wang H, Tan S, Zhang H, Zhu Y. The alterations of innate immunity and enhanced severity of infections in diabetes mellitus. Immunology. 2023;(July):1–11.
40. Turner S, Khan MA, Putrino D, Woodcock A, Kell DB, Pretorius E. Long COVID: pathophysiological factors and abnormalities of coagulation. Trends Endocrinol Metab [Internet]. 2023;34(6):321–44. Tersedia pada: https://doi.org/10.1016/j.tem.2023.03.002
41. Menni C, Valdes AM, Freidin MB, Sudre CH, Nguyen LH, Drew DA, et al. Real-time tracking of self-reported symptoms to predict potential COVID-19. Nat Med [Internet]. 2020;26(7):1037–40. Tersedia pada: http://dx.doi.org/10.1038/s41591-020-0916-2
42. Sudre CH, Murray B, Varsavsky T, Graham MS, Penfold RS, Bowyer RC, et al. Attributes and predictors of long COVID. Nat Med. 2021;27(4):626–31.
43. Vigili de Kreutzenberg S. Long COVID-19 and diabetes mellitus: a short review. Metab Target Organ Damage. 2023;3(1).
44. Estiri H, Strasser ZH, Brat GA, Semenov YR, Consortium T, Ehr C-, et al. Evolving phenotypes of non-hospitalized patients that indicate long COVID. 2021;1–10.
45. Li J, Li Y, Wang Z, Liu N. Increased risk of new-onset diabetes in patients with COVID- : a systematic review and meta-analysis. 2022;(November).
46. Chen J, Wu C, Wang X, Yu J, Sun Z. The Impact of COVID-19 on Blood Glucose: A Systematic Review and Meta-Analysis. Front Endocrinol (Lausanne). 2020;11(October):1–8.
47. Prattichizzo F, de Candia P, Nicolucci A, Ceriello A. Elevated HbA1c levels in pre-Covid-19 infection increases the risk of mortality: A systematic review and meta-analysis. Diabetes Metab Res Rev. 2022;38(1):1–8.
48. Alberca RW, Álefe Y, Ramos L, Pereira NZ, Beserra DR, Cláudia A, et al. Long-term effects of COVID-19 in diabetic and non-diabetic patients. 2019;19(5):2–6.
49. Furong Liu,*,‡ Xin Long,*,‡ Bixiang Zhang,*,‡ Wanguang Zhang,*,‡ Xiaoping Chen,* ‡ and Zhanguo Zhang. ACE2 Expression in Pancreas May Cause Pancreatic Damage. Clin Gastroenterol Hepatol.

2020;18(August):2128–30.
50. Xuan X, Gao F, Ma X, Huang C, Wang Y, Deng H, et al. Activation of ACE2/ angiotensin (1-7) attenuates pancreatic β cell dedifferentiation in a high-fat-diet mouse model. Metabolism [Internet]. 2017;81:83–96. Tersedia pada: http://dx.doi.org/10.1016/j.metabol.2017.12.003
51. Pantea A, Bica SI, Salmen T, Al W, Khalid M, Maciej KA, et al. New-Onset Diabetes Mellitus in COVID-19 : A Scoping Review. Diabetes Ther [Internet]. 2023; Tersedia pada: https://doi.org/10.1007/s13300-023-01465-7
52. Gupta R Das, Atri A, Mondal S, Bhattacharjee A. Characterizing progressive beta-cell recovery after new-onset DKA in COVID-19 provoked A-β+ KPD (ketosis-prone diabetes): A prospective study from Eastern India. 2020;(January).
53. Ren H, Yang Y, Wang F, Yan Y, Shi X, Dong K, et al. Association of the insulin resistance marker TyG index with the severity and mortality of COVID-19. Cardiovasc Diabetol [Internet]. 2020;1–8. Tersedia pada: https://doi.org/10.1186/s12933-020-01035-2
54. Kim SH, Arora I, Hsia DS, Knowler WC, Leblanc E, Mylonakis E, et al. New-Onset Diabetes After COVID-19. J Clin Endocrinol Metab [Internet]. 2023;108(11):1164–74. Tersedia pada: https://doi.org/10.1210/clinem/dgad284