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**CELLULAR AND MOLECULAR IMMUNITY IN TYPE 2
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CELLULAR AND MOLECULAR IMMUNITY IN TYPE 2 DIABETIC PATIENTS
AFTER COVID-19 RECOVERY

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November 2022

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Abu Bakr Abdulmajeed Kadhum AL-TAMEEMI

ABSTRACT

CELLULAR AND MOLECULAR IMMUNITY IN TYPE 2 DIABETIC PATIENTS AFTER COVID-19 RECOVERY

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Master of Science in Biology

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People with type 2 diabetes and Covid-19 infection had altered and distinct immune characteristics from patients without Covid-19 infection, and they are at a higher risk of developing health complications or being treated in a critical care unit than people without diabetes are. On the other hand, clinical data so far suggests that the Covid-19 may result in metabolic dysregulation and impaired glucose homeostasis. In addition, emerging data on new onset of diabetes mellitus in previously infected with Covid-19 patients reinforces the hypothesis of a direct effect of Covid-19 on glucose metabolism. The aim of this study is to investigate the role of Covid-19 infection in patients live with type 2 diabetes mellitus during the recovery state on some cellular immune response and its affection on the expression of interleukin-1 β as an important proinflammatory cytokine. The study included 75 participants. The median age in total was 50 years old (ranged from 23 to 65 years) who visited Al-Wafaa Center for Diabetes and Endocrine Disease-Ibn-sina Hospital-Mosul/Iraq and enrolled in the study from 30 August 2021 to 10 November 2021. The 25 participants with type 2 diabetes mellitus who had recovered from Covid-19 infection (8-10 weeks after symptoms onset) made up 33.3% of the total participants and had a mean age of 43.5. The second is that 25 participants (33.3%, mean age: 42.7) were type 2 diabetes mellitus without Covid-19 infection and with no other comorbidities. The last group consisted of 25 (33.3% mean age; 27.5) healthy control individuals. Both groups of type 2 diabetic patients were using insulin injections regularly as the only medication (insulin-dependent) over 3 years. The study found that the glucose level was higher in the type 2 diabetes mellitus recovered group compared with non-infected type

2 diabetic patients (mean 12.08 ± 5.18), (mean 10.12 ± 4.84), respectively. In addition, alterations in cellular immune response (including WBC count, neutrophil, phagocytic activity of neutrophil, monocyte, and eosinophil) have seen compared with other study groups. While hemoglobin was lower in recovered patients (130.12 ± 16.46) than type 2 diabetic mellitus and control (138.64 ± 12.67) and (148.68 ± 13.71), respectively. Furthermore, the results showed that interleukin-1 β expression in blood samples of the type 2 diabetes mellitus recovered group (28.1 ± 0.6) was higher in comparison with type 2 diabetic patients without Covid-19 infection (23.8 ± 0.8), and control (25.9 ± 2.8).

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Keywords: Type 2 diabetes, SARS-CoV-2, Covid-19, Interleukin-1 β , Cytokine storm, Innate immunity

ÖZET

COVID-19 İYİLŞEŞTİRME SONRASI TİP 2 DİYABETİK HASTALARDA HÜCRESEL VE MOLEKÜLER BAĞIŞIKLIK

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Tip 2 diyabetli ve Covid-19 enfeksiyonu olan kişiler, Covid-19 enfeksiyonu olmayan hastalardan belirgin ve farklı bağışıklık özelliklerine sahiptir, ve sağlık komplikasyonları geliştirme veya yoğun bakım ünitesinde tedavi görme riskleri, diyabeti olmayan kişilere göre daha yüksektir. Öte yandan, şimdiye kadarki klinik veriler, Covid-19'un metabolik düzensizlik ve bozulmuş glikoz homeostazına neden olabileceğini düşündürmektedir. Ek olarak, daha önce Covid-19 ile enfekte olmuş hastalarda yeni diyabet başlangıcına ilişkin ortaya çıkan veriler, Covid-19'un glikoz metabolizması üzerinde doğrudan bir etkisi olduğu hipotezini güçlendiriyor. Bu çalışmanın amacı, tip 2 diabetes mellitus ile yaşayan hastalarda iyileşme durumundaki Covid-19 enfeksiyonunun bazı hücresel immün yanıt üzerindeki rolünü ve bunun önemli bir proinflamatuvar sitokin olan interlökin-1 β ekspresyonu üzerindeki etkisini araştırmaktır. Çalışma 75 katılımcıyı içeriyordu. Irak Musul'da Al-Wafaa Diyabet Merkezi ve Ibn-sina Endokrin Hastalıklar bölümünü ziyaret eden ve 30 Ağustos 2021 ve 10 Kasım 2021 tarihlerinde çalışmaya alınan hastaların ortalama yaşı 50'dir (23 ve 65 yaş arası). Covid-19 enfeksiyonu (semptomların başlamasından 8-10 hafta sonra) iyileşen tip 2 diabetes mellituslu 25 katılımcı, toplam katılımcıların %33,3'ünü oluşturdu ve ortalama yaş 43,5 idi. İkincisi, 25 katılımcı (%33,3, ortalama yaş: 42,7) Covid-19 enfeksiyonu olmayan ve başka bir komorbiditesi olmayan tip 2 diabetes mellituslu kişilerdir. Son grup ise 25 (%33,3 yaş ortalaması; 27,5) sağlıklı kontrolden oluşuyordu. Her iki tip 2 diyabetli hasta grubu da 3 yıldır tek ilaç olarak (insüline bağımlı) insülin enjeksiyonlarını düzenli olarak kullanmaktaydı. Bu çalışmada, enfekte olmayan tip 2 diyabetli hastalarla (ortalama $10,12 \pm 4,84$) karşılaştırıldığında, tip

2 diabetes mellitus iyileşen (ortalama $12,08 \pm 5,18$) grupta glikoz seviyesinin daha yüksek olduğunu buldu. Ayrıca, diğer çalışma gruplarıyla karşılaştırıldığında hücresel immün yanıtta (WBC sayısı, nötrofil, nötrofilleri fagositik aktivitesi, monosit ve eozinofil) değişiklikler görülmüştür. İyileşen hastalarda hemoglobin ($130,12 \pm 16,46$) tip 2 diyabetli hastalardan ($138,64 \pm 12,67$) ve kontrolden ($148,68 \pm 13,71$) daha düşüktü. Ayrıca, sonuçlar, tip 2 diabetes mellitus iyileşen grubun ($28,1 \pm 0,6$) kan örneklerinde interlökin-1 β ekspresyonunun, Covid-19 enfeksiyonu olmayan tip 2 diyabetli hastalara ($23,8 \pm 0,8$) ve kontrole ($25,9 \pm 2,8$) kıyasla daha yüksek olduğunu gösterdi.

2022, 101 sayfa

Anahtar Kelimeler: Tip 2 diyabet, SARS-CoV-2, Covid-19, İnterlökin-1 β , Sitokin fırtınası, Doğuştan gelen bağışıklık

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CONTENTS

ABSTRACT	i
ÖZET.....	iii
PREFACE AND ACKNOWLEDGEMENTS	v
CONTENTS.....	vi
LIST OF SYMBOLS	viii
LIST OF ABBREVIATIONS	ix
LIST OF FIGURES	xi
LIST OF TABLES	xii
1. INTRODUCTION	1
2. LITERATURE REVIEW	4
2.1 SARS-CoV-2 (Covid-19).....	4
2.1.1 Response of innate immunity to infection with SARS-CoV-2 virus	6
2.2 Diabetes Mellitus	8
2.2.1 Type one diabetes mellitus	9
2.2.2 Type two diabetes mellitus	11
2.2.3 Gestational diabetes.....	16
2.3 SARS-CoV-2 and T2DM	17
2.3.1 Association between ACE2 receptors and infection by covid-19 in patients with diabetes	19
2.3.2 Role of diabetes in severity and susceptibility to infection with covid-19 and other viruses	22
2.3.3 Role of hyperglycemia in patients with DM during covid-19 infection	22
2.3.4 Role of obesity in DM patients during covid-19 infection.....	24
2.3.5 Innate immunity in T2DM patients with covid-19 infection	25
2.3.6 Adaptive immunity in T2DM patients with covid-19 infection.....	26
2.4 White Blood Cells (WBC) Overview	28
2.4.1 Myeloid cells	28
2.4.2 Lymphoid cells	34
2.5 Phagocytosis.....	38
2.5.1 The mechanism of phagocytosis	39

2.6 Interleukin-1 Family	40
2.6.1 Expression of IL-1 in tissues and target cells	42
2.6.2 IL-1 β gene and its expression	43
2.6.3 IL-1 β and its role in SARS-CoV-2 and T2DM.....	46
3. MATERIALS AND METHODS.....	48
3.1 Samples Collection	48
3.2 Blood Glucose Test.....	48
3.3 White Blood Cell Count (WBCc) Test	49
3.4 Determination of Phagocytic Activity of Neutrophil	50
3.4.1 Nitro-blue tetrazolium (NBT) test.....	50
3.4.2 Peripheral blood smear preparation with NBT	50
3.4.3 Microscopic examination and counting.....	51
3.5 Molecular Study	51
3.5.1 Measuring the expression of IL-1 β	51
3.6 Statistical Analysis	56
4. RESULTS AND DISCUSSION.....	57
4.1 Molecular Study	57
4.1.1 Investigating of IL-1 β expression	57
4.2 Statistical Analysis	59
4.2.1 Glucose level	59
4.2.2 WBC count	66
4.2.3 Monocytes.....	68
4.2.4 Neutrophil and their phagocytic activity.....	70
4.2.5 Lymphocytes	73
4.2.6 Eosinophils	74
4.2.7 Hemoglobin	75
5. CONCLUSIONS AND RECOMMENDATION.....	77
5.1 Conclusions	77
5.2 Recommendations	77
REFERENCES.....	79
CURRICULUM VITAE.....	101

LIST OF SYMBOLS

%	Percentage
~	Approximately, equal (weak approximation)
>	Strict inequality (greater than)
±	Plus-minus (both plus and minus operations)
≤	Inequality (less than or equal to)
°C	Celsius degree
μL	Microliter
μm	Micrometer
dL	Deciliter
Kbs	Kilobase
kDa	Kilodalton.
Kg	Kilogram
m	Meter
mg	Milligram
mmol	Millimole
ng/mL	Nano gram/milliliter
nm	Nanometer
rpm	Round per minute
UV	Ultraviolet
α	Alpha
β	Beta
γ	Gamma
δ	Delta
Δct	Delta Ct, Ct (cycle threshold)

LIST OF ABBREVIATIONS

ACE2	Angiotensin-converting enzyme 2
ADE	Antibody-dependent enhancement
ALT	Alanine transaminase
APCs	Antigen-presenting cells
ARDS	Acute respiratory distress syndrome
ASC	Antibody-secreting cell
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BG	Blood glucose
BMI	Body mass index
CCL	Chemokine C-C motif ligand
CCXL	C-X-C motif chemokine ligand
CCXL-10	C-X-C motif chemokine ligand 10
CF	Complement factor
CK	Creatinine kinase
Covid-19	Coronavirus disease 2019
CRP	C-reactive protein
CRS	Cytokine release syndrome
CVD	Cardiovascular disease
DAMPs	Damage-associated molecular patterns
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
DPP4	Dipeptidyl peptidase-4
ERS	Early recovery stage
ESR	Erythrocyte sedimentation rate
FPG	Fasting plasma glucose
G-CSF	Granulocyte-colony stimulating factor
GDM	Gestational diabetes mellitus
GI	Glucose intolerance
GTT	Impaired glucose tolerance test
HDPP4	Human DPP4
HSC	Hematopoietic stem cell
ICU	Intensive care unit
IFN	Interferon
IFN-I	Type I interferon
IgG	Immunoglobulin G
IL	Interleukin
ILCS	Innate lymphocytes or innate lymphoid cells
IR	Insulin resistance

IRFS	Interferon regulatory factors
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MAS	Macrophage activation syndrome
MCP-1	Monocyte chemoattractant protein-1
MERS	Middle east respiratory syndrome
MHC	Major histocompatibility complex
MHV	Mouse hepatitis virus
MIg	Monoclonal immunoglobulin
MLR	Monocyt-lymphocyte ratio
MRNA	Messenger ribonucleic acid
NBT	Nitro-blue tetrazolium
NETs	Neutrophil extracellular traps
NF-KB	Nuclear factor kappa B
NK	Natural killer
NLR	Neutrophil-lymphocyte ratio
NLRP	Nod-like receptor pyrin
PAMPS	Pathogen-associated molecular patterns
PRR	Pattern recognition receptor
PYD	N-terminal pyrin domain
qRT-PCR	Quantitative reverse transcription PCR
RBD	Receptor-binding domain
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RPG	Random plasma glucose
SARS-CoV	Severe acute respiratory syndrome coronavirus
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
T1DM	Type 1 diabetes mellitus
T2D	Type2 diabetes
T2DM	Type 2 diabetes mellitus
TC	T-cytotoxic
TCR	T-cell receptor
TH CELL	T helper cell
TH	T-helper
TH1	T-helper 1
TLR	Toll-like receptor
TMPRSS2	Transmembrane protease serine 2
TNF	Tumor necrosis factor
TNF- α	Tumor necrosis factor- α
TREG CELL	Regulatory T cell
WBC	White blood cells
WHO	World health organization

LIST OF FIGURES

Figure 2.1	Structure of human coronavirus causing acute respiratory syndrome (SARS-CoV-2) (Rando <i>et al.</i> 2021).....	4
Figure 2.2	The mechanism of SARS-CoV-2 transmission (Harrison <i>et al.</i> 2020).....	5
Figure 2.3	Coronavirus recognition and immune response (Brodin 2021)	8
Figure 2.4	Impairment in immune response mechanisms during hyperglycemia (Ley <i>et al.</i> 2007).....	16
Figure 2.5	Significance role of inflammation in the development of T2DM and its relationship with other metabolic disorders (Tsalamandris <i>et al.</i> 2019).	16
Figure 2.6	Potential pathogenic mechanisms in patients with T2DM and Covid-19 (Lim <i>et al.</i> 2021).....	18
Figure 2.7	Structure of ACE2 as SARS-CoV-2 cell entry receptor (Yang <i>et al.</i> 2020)	20
Figure 2.8	Neutrophil and NETosis formation (Rai 2019).....	30
Figure 2.9	Stages of phagocytosis	39
Figure 2.10	The inflammasome; how the pro-IL-1 β converted into active IL-1 β by the NLRP3 inflammasome. Through signaling, different PAMPs or DAMPs cause pro-IL-1 to prduction (Leung <i>et al.</i> 2010).....	45
Figure 3.1	Spectrophotometer/EMC-11D-V/ used in measuring blood glucose of samples	49
Figure 3.2	Auto hematology analyzer	49
Figure 3.3	(A) qRT-PCR, (B) kit for PCR.....	53
Figure 3.4	Implen Gmbh-Nano drop	53
Figure 3.5	qPCR software MxPro3005P (Ecological Research Software)	55
Figure 4.1	Difference in IL-1 β gene expression in monocyte between the diabetic patients groups. The 0 line refer to control group, DM refer to T2DM, and DM.R refer to T2DM.R.....	57
Figure 4.2	The components of amplification curve, this figure represent the increase of fluorescence with the number of cycles for different samples during amplification process.....	59
Figure 4.3	Differences in mean glucose levels between the three study groups	60
Figure 4.4	Correlation between glucose level and other parameters in T2DM.R group.....	63
Figure 4.5	Correlation between glucose level and other parameters in T2DM group	64
Figure 4.6	Phagocytic activity of neutrophil for different sample of the study (A, B and C), neutrophil in phagocytosis state with the presence of formazan crystals deposits on neutrophils, compare with healthy control cells in D, E, and F	70

LIST OF TABLES

Table 3.1	Tools and devices used in the research	50
Table 3.2	Kits used in the research	52
Table 3.3	IL-1 β Gene primer that used in this study in human	56
Table 4.1	Independent comparisons between each pair of the study groups in glucose level, (* P $\leq$ 0.05 refer to significant differences)	60
Table 4.2	Mean values and standard deviation ($\pm$ Std.) for samples of each study group in glucose level.	60
Table 4.3	Correlation between glucose level and other parameters in T2DM.R group.....	62
Table 4.4	Correlation between glucose level and other parameters in T2DM.....	63
Table 4.5	Pairwise comparisons of WBC count between the three groups (*P $\leq$ 0.05)	67
Table 4.6	Mean values and standard deviation ($\pm$ Std.) for samples of each group in WBC count.....	67
Table 4.7	Pairwise comparisons of absolut number of monocyte between groups as pairs (* P $\leq$ 0.05=significant).....	69
Table 4.8	Mean values of absolute number of monocyte in the three study groups	69
Table 4.9	Pairwise comparisons of absolut number of neutrophil between groups (* P $\leq$ 0.05=significant)	71
Table 4.10	Pairwise comparisons of absolut number of phagocytic cell between groups (* P $\leq$ 0.05=significant).....	71
Table 4.11	Mean values of absolute number of neutrophil in groups	72
Table 4.12	Mean values of absolute number of phagocytic cell activity in groups ..	72
Table 4.13	Mean values of absolute number of lymphocyte in the three study groups	73
Table 4.14	Pairwise comparisons of absolute number of eosinophil between groups (*P $\leq$ 0.05 is significant)	74
Table 4.15	Mean values of absolute number of eosinophil in the three study groups	75
Table 4.16	Pairwise comparisons of hemoglobin between groups (*P $\leq$ 0.05 is significant).....	75
Table 4.17	Mean values of hemoglobin in the three study groups.....	76

1. INTRODUCTION

The very first incidence of the coronavirus infection-2019, which brought on by the SARS-CoV-2 (severe acute respiratory syndrome) virus, and reported in Wuhan, China, in the end of December 2019. SARS-CoV-2 is a phylogenetic relative of the coronavirus 2002 (Kong *et al.* 2020). According to an epidemiological research carried out in China, the majority of Covid-19 patients experience moderate or uncomplicated illness. However, 14% of patients experience severe disease that requires hospitalization and oxygen assistance, and 5% need to be admitted to an intensive care unit (Team 2020). To date, 10 May 2022, there have been 515.748.861 cases of Covid-19 confirmed, including 6.255.835 deaths, reported to WHO. A total of 11.593.897.033 vaccine doses administered (WHO 2021). For 80% of symptomatic patients, the condition presents as moderate flu-like symptoms (such as fever and cough) and upper respiratory tract virus infection. For the remaining 20% of individuals, however, SARS-CoV-2 may spread to the lower respiratory tract, causing serious diseases such as pneumonia and acute respiratory distress syndrome (ARDS) (Lauer *et al.* 2019). Several studies have revealed that advanced age and male sex, as well as the comorbidity of chronic conditions such as hypertension, diabetes (type 1 and 2), cardiovascular and respiratory disorders, might raise the risk of severity and death in such a way that these susceptibilities can be correlated with the pathogenesis of Covid-19 infection. Notably, chronic diseases have certain characteristics, including an inflammatory state, immune response issues, and an increased propensity to contracting contagious diseases (Bloomgarden 2020, Holman *et al.* 2020, Chávez-Reyes *et al.* 2021, Rahmani-Kukia and Abbasi 2021).

Patients with diabetes, however, also had distinct immunological characteristics from those without diabetes (Han *et al.* 2021). Diabetes was the second highest incidence rate among chronic Covid-19 comorbidities (7.4 % to 19.0%), following hypertension (15% to 30%) (Xu *et al.* 2020). Several pathophysiological pathways behind the relationship between diabetes mellitus and SARS-CoV-2 are yet to be investigated. Few early investigations have demonstrated that underlying cardiovascular disease and diabetes are high among Covid-19 hospitalized to intensive care units (Piva *et al.* 2020). Limited data

are available about the immunological and inflammatory response to Covid-19 in diabetic individuals. Diabetes patients with Covid-19 infection have been found to have increased vulnerability to develop an inflammation and severity of infection, which associated with a rise of critical care unit hospitalization. The cytokine storm usually accompanied with glucose metabolism dysregulation, resulting in metabolic failure (Van Wyngene *et al.* 2018).

Both diseases characterized by increased obesity accompanied by persistent systemic low-grade inflammation, which induces immunological dysregulation and increases susceptibility to infection. However, the impact of high glucose level or DM on innate and adaptive immune response in Covid-19 patients is yet unclear, and more research needed. An increased in lymphocytopenia, immune system dysfunction, and cytokine storm was found in earlier studies on Middle East respiratory syndrome (MERS) and SARS-CoV (Yin and Wunderink 2018). According to study comparing patients with diabetes with healthy individuals during the Covid-19 infection, diabetic individuals were more likely to experience high blood glucose and lymphocytopenia and coupled with increased level of myoglobin, ferritin, D-dimer, and urea nitrogen. Diabetes patients also exhibited obviously raised mortality and cytokine levels (Han *et al.* 2021). In accordance with, a study on Covid-19 patients, the infected individuals had significantly decreased T cell counts, and the remaining T cells, seeming functionally fatigued (Diao *et al.* 2020). Some research on these patients showed impaired glucose metabolism and homeostasis (Korytkowski *et al.* 2020, Müller *et al.* 2021). Alteration in white blood cell population, and other immunity members (Kwiecień *et al.* 2021), in addition to the secretion of cytokines including interleukin (IL)-6, IL-1 β and TNF- α (Viurcos-Sanabria and Escobedo 2021), leading to more susceptibility to infection, disease severity, and ultimately death. In addition, a research on convalescent patients with diabetes and other comorbidities indicated that Covid-19 patients had lower absolute counts and proportions of lymphocytes, monocytes, eosinophils, and basophils than convalescent patients in the study cohorts. Furthermore, changes were also observed in neutrophils activity (Kwiecień *et al.* 2021). According to another research of convalescents, patients at the early recovery stage (ERS) of Covid-19 patients had higher levels of monocytes; also, there was a larger

proportion of classical CD14⁺⁺ monocytes with high inflammatory gene expression as well as a higher number of CD14⁺⁺ IL1⁺ monocytes (Wen *et al.* 2020).

Concerning all of the above-mentioned data, it is essential to explore the role and percentage of cellular immunity, especially members of innate immunity, which are considered to be the first line of defense against Covid-19 infection and other viral infections. Using quantitative real-time polymerase chain reaction (qRT-PCR), the aim of this study was to examine the expression of proinflammatory cytokine (IL-1 β), as an inflammatory mediator during the course of inflammation, from monocyte gene in Type 2 diabetes mellitus (T2DM) recovered patients from SARS-CoV-2 infection in comparison to those without previous infection.

The characterization of the key cellular subsets and their states in Covid-19 is a crucial step in developing critical insights on the immune clearance mechanism, and understanding the pathophysiology of Covid-19 infection on the immunity and its association to T2DM, as well as the development of new Covid-19 treatments. Although, the mechanism by which immune cell subsets change during Covid-19 is still largely unknown.

2. LITERATURE REVIEW

2.1 SARS-CoV-2 (Covid-19)

Since the beginning of the 21st century, coronaviruses have triggered lethal pneumonia outbreaks in people. SARS-CoV emerged in 2002 and caused an epidemic with a 10% death rate that spread to five continents before being contained in 2003 (with additional cases reported in 2004). MERS has emerged in 2012, in the Arabian Gulf, and has produced repeated outbreaks with a death rate of 35%. SARS and MERS are zoonotic viruses that crossed the species barrier with the help of bats/palm civets and dromedary camels, respectively (Tortorici and Veessler 2019).

Coronaviruses are members of the Nidovirales family Coronaviridae. Corona displays crown-like spikes on the outer surface of the virus; hence, it was designated a coronavirus. The family of Coronaviruses have genetic material as a single-stranded RNA with a size 26 to 32 kbs and tiny in diameter (65-125 nm) (Figure 2.1). The subgroups of the family of coronaviruses are alpha (α), beta (β), gamma (γ), and delta (δ), as well as omicron coronavirus. The transmission rate of SARS-CoV-2 is higher than that of SARS-CoV, and a genetic modification at the spike (S) protein in the receptor-binding domain (RBD) region of Covid-19 virus may be responsible for this kind of spreading rate (Shereen *et al.* 2020).

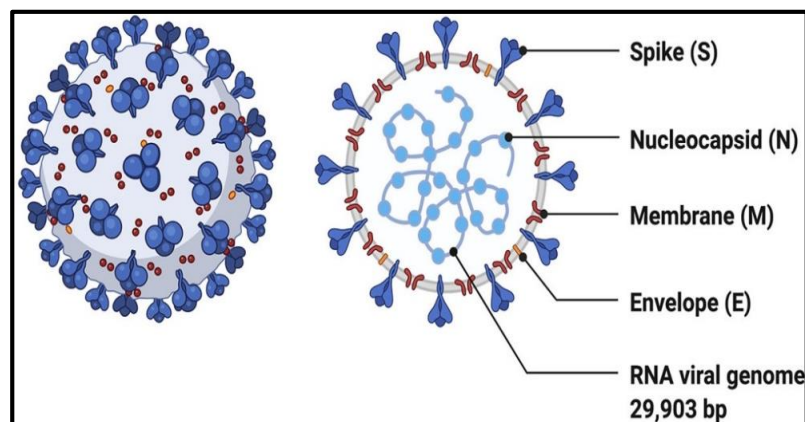


Figure 2.1 Structure of human coronavirus causing acute respiratory syndrome (SARS-CoV-2) (Rando *et al.* 2021)

The International Committee on the Taxonomy of Viruses (ICTV) named SARS-CoV-2 as the virus and Covid-19 as the disease (Cui *et al.* 2019). The WHO pronounced Covid-19 a global pandemic on March 11, 2020 (Zhu *et al.* 2020). The symptoms of Covid-19 virus are similar to those of SARS and MERS and include tiredness, fever, dry cough, and discomfort in the upper chest, occasionally shortness of breath, and diarrhea. While in acute cases were observed multi-organ failure, cytokine storm-driven sepsis, and secondary infections (Zhu *et al.* 2020). According to clinical chemistry studies, alanine transaminase (ALT), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), creatinine kinase (CK), erythrocyte sedimentation rate (ESR), C-reactive protein, D-dimer level white blood cell (WBC) count, procalcitonin, urea, and creatinine have increased. Covid-19 patients have been shown to have decreased levels of hemoglobin, lymphocytes, eosinophils, and serum albumin (Cascella *et al.* 2022).

SARS-CoV-2 can spread both directly (through droplets and human-to-human contact) and indirectly by (contaminated objects and airborne contagion). As previously stated, SARS-CoV-2 is believed to transmit mostly through respiratory droplets, such as when a patient cough, sneezes, or even talks or sings (Figure 2.2). Typically, droplets cannot travel more than six feet (almost two m) and have a limited period in the air. However, SARS-CoV-2 stays infectious and viable in droplets (less than five μm in diameter) for up to three hours. Therefore, airborne isolation, room ventilation, and the right use of disinfection may limit the virus' aerosol dissemination (Lotfi *et al.* 2020).

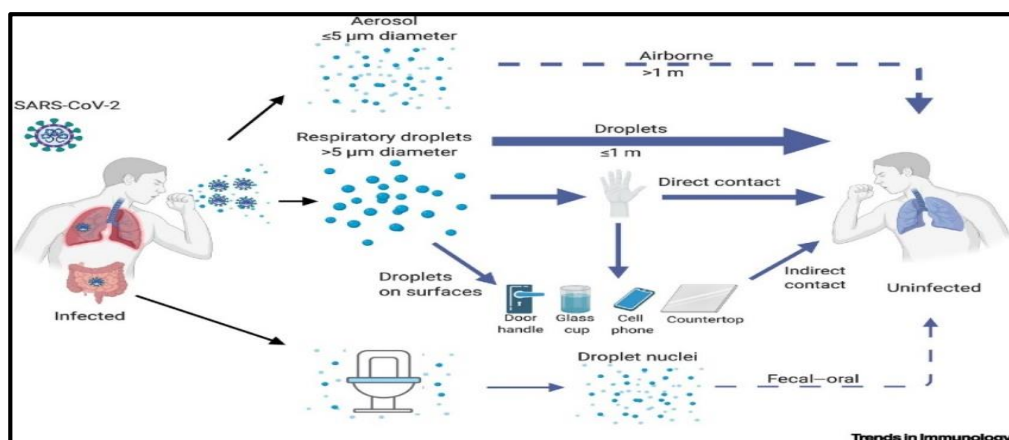


Figure 2.2 The mechanism of SARS-CoV-2 transmission (Harrison *et al.* 2020)

The initial step in infection is the binding of a virus to its target receptor on a host cell. Earlier, literature on SARS-CoV2 revealed that this virus primarily targets airway epithelial cells, alveolar epithelial cells, vascular endothelial cells, and macrophages in the lung, which all express the angiotensin-converting enzyme 2 (ACE2). Multiple studies have demonstrated that excessive expression of ACE2 in the intestinal epithelium results in viral shedding via feces (Xu *et al.* 2020). Although ACE2 does not appear to be expressed by immune cells. SARS-CoV target receptor on the host as SARS-CoV-2 employs the same entrance receptor, these cell subgroups are possible targets of this virus (Tay *et al.* 2020).

2.1.1 Response of innate immunity to infection with SARS-CoV-2 virus

The earliest line of defense against viruses is the innate immunity reaction, and it is vital for protection from viruses' infection (Vabret *et al.* 2020). The humoral components of antiviral innate immunity include complement and coagulation-fibrinolysis system components, soluble proteins that detect glycan on cell surfaces (e.g. mannose binding lectin (MBL)), chemokines, interferons (IFN), and naturally occurring antibodies (mainly IgM but also IgA and IgG). It also contains a number of biological components, including natural killer (NK) cells, other innate lymphoid cells (ILCs), and γ , δ , and T cells, which limit the spread of viral infection by cytotoxic action on target cells, cytokine synthesis, and activation of an adaptive response (Boechat *et al.* 2021).

In the majority of cases, the immune response eliminates the infection and has no lasting repercussions; nevertheless, the immune system, in some individuals, becomes overstimulated resulting in a cytokine storm and severe lung injury (Hasan *et al.* 2021). One of the basic causes of Covid-19 pathophysiology is the cytokine storm, which results in severe inflammation, lung injury, ARDS, and other organ failure. In addition, elevated levels of IL-10, CXCL-10, IL-7, TNF- α , granulocyte colony stimulating factor (G-CSF), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1A (MIP-1A) levels, indicate the critical role of "cytokine releasing hormone" in pathogenesis of Covid-19 virus (Chen *et al.* 2020). The IFN response is an important early line of defense against viruses. IFN types I and III (the so-called "innate" IFNs) appear to

have a role in limiting infection for many respiratory viruses, including SARS-CoV-1, by developing a cellular state of viral resistance and initiating adaptive immune responses (Park and Iwasaki 2020).

SARS-CoV-2, like SARS-CoV and MERS, is an RNA virus with a single strand. Once entering a target cell, the virus is identified by pattern recognition receptors (PRRs) such as toll-like receptors (TLR), viral-infection sensors melanoma differentiation-associated gene 5 (MDAG5), and retinoic acid-inducible gene I (RIG-I), leads to the viral recognition induces the type I IFN response program and IFN-stimulated genes (Figure 2.3). The TLR3 response induces transcription of the NLR family pyrin domain containing 3 (NLRP3) gene, which, along with other cellular responses to viral infection, such as the formation of reactive oxidative species, calcium flux from cytoplasmic stores, protein aggregation, and the release of danger-associated patterns, helps to the activation of the NLRP3 inflammasome and possibly other inflammasome complexes (Brodin 2021). The NLRP3 inflammasome triggers caspase-1-dependent cleavage and release of major proinflammatory cytokines IL-1 β and IL-18, as well as pyroptotic cell death mediated by gasdermin-D. The degree of NLRP3 activation corresponds with the severity of Covid-19 illness (Rodrigues *et al.* 2021). As a consequence of pyroptotic cell death, LDH is secreted. Patients with Covid-19 have elevated LDH levels in their blood, and these enzyme levels associate with disease severity (Huang *et al.* 2020). These findings imply that inflammasome activation is an essential characteristic of Covid-19 (Han *et al.* 2020).

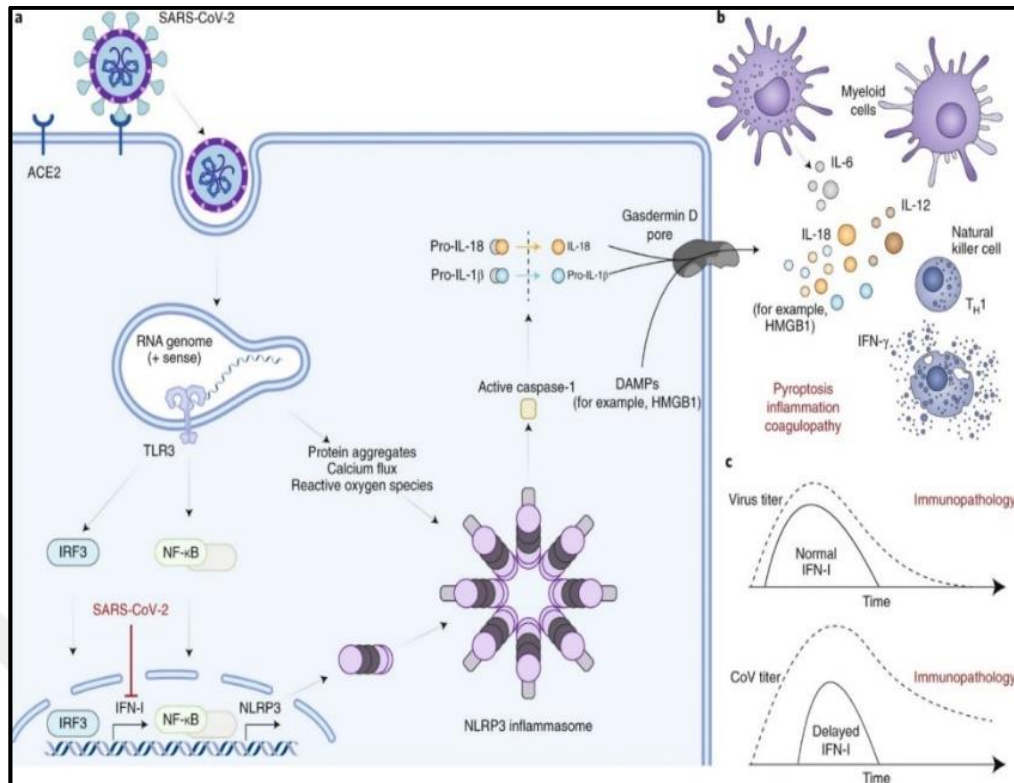


Figure 2.3 Coronavirus recognition and immune response (Brodin 2021)

2.2 Diabetes Mellitus

Based on the WHO, DM is a long-term disease marked by hyperglycemia that eventually cause damage to the eyes, kidneys, nerves, blood vessels, and heart. About 422 million people worldwide have diabetes, the majority living in low and middle-income countries, and 1.5 million deaths are directly attribute to diabetes each year. Both the number of cases and the prevalence of diabetes have been steadily increasing over the past few decades. Over 90% of diabetes mellitus cases are type 2 diabetes, a condition marked by deficient insulin secretion by pancreatic islet cells, tissue insulin resistance (IR) and an inadequate compensatory insulin secretory response. Type 1 diabetes, once known as juvenile diabetes or insulin-dependent diabetes, is a chronic condition in which the pancreas produces little or no insulin by itself. For people living with diabetes, access to affordable treatment, including insulin, is critical to their survival. There is a globally agreed target to halt the rise in diabetes and obesity by 2025. However, diabetes may present with characteristic symptoms such as thirst, polyuria, blurring of vision, and weight loss. Genital yeast infections frequently occur. The most severe clinical

manifestations are ketoacidosis or a non-ketotic hyperosmolar state that may lead to dehydration, coma and, in the absence of effective treatment, death (Sapra *et al.* 2021).

2.2.1 Type one diabetes mellitus

Type 1 DM (T1DM) is an autoimmune condition that damages β cells in the pancreas that secrete insulin. This hormone is important and has diverse effects on lipid, protein, and mineral metabolisms, in addition to glucose homeostasis and growth. Insulin facilitates the entrance of glucose into muscle fibers, fat cells, and helps liver to reserve glucose as glycogen and synthesize of fatty acids. In addition, insulin enhances potassium transport into the cells, increases amino acid intake, and blocks fat breakdown by adipose tissue cells. Patients with T1DM need insulin treatment for the rest of their lives. In addition, in the absence of insulin, patients can develop ketoacidosis and eventually death (Yue *et al.* 2018, Saxby *et al.* 2020).

T1DM defined by the failure of β cells in the pancreas to produce insulin as a result of attacking of pancreas islets by immune system over months or years, resulting in insulin deficiency. Despite the fact that the exact cause is unknown, T1DM thought to have a genetic inclination. The likelihood of developing T1DM is around 4% with no family history of diabetes, compared to 3% to 8% for progeny of a father with T1DM, 1% to 4% for the progeny of mothers with DM, and about 30% in progeny of both fathers with T1DM (Basina and Maahs 2018). Furthermore, the existence of pancreatic antibodies indicates that the person is either developing or has already had T1DM. In individuals who are at risk, it is believed that a viral infection or other environmental factors may trigger the immune attack of β cells in pancreas. Some studies have revealed that children born to mothers who had Coxsackievirus or another Enterovirus during pregnancy are more likely to develop T1DM. Furthermore, ecological toxins have been proposed to be involved. According to the hygiene assumption, increased cleanliness is related with a rise in the prevalence of immune-mediated diseases. Dietary factors have also been explored as potential causes (Redondo *et al.* 2018).

T1DM is one of the most frequent chronic diseases among children, but it can present at any age. The occurrence and ubiquity of T1DM had been gradually increasing, accounting for about 5% to 10% of all diabetics. The most typical age of onset is in early puberty, between the ages of 4 and 6 (10 to 14 years). There is also a lot of variance in incidence across the globe. Finland and other Northern European countries had the highest documented incidences, with rates 400 times higher than China and Venezuela, which have the lowest reported incidences. T1DM in children characterized by hyperglycemic symptoms, with diabetic ketoacidosis accounting for one-third of all cases. The onset of symptoms, particularly in adolescence, might be abrupt at the time of diagnosis. It can turn into a medical emergency if not evaluate and treated immediately (Lee *et al.* 2018).

Adults, on the other hand, may experience higher variability in the development of T1DM symptoms. Diabetes can be diagnosed based on hemoglobin A1C (HbA1c) or glucose of plasma (FPG) levels. If the symptoms were severe, the level sugar plasma should be used to diagnose the patient rather than the HbA1c because the median levels of plasma glucose for two to three month may not be abnormal. In patients with clear symptoms, the random plasma glucose (RPG) higher or close to 200 mg/dL can be utilize as a diagnostic method of T1DM. While FPG higher or close to 126 mg/dL can also be utilize for diagnosis too (fasting means eating nothing for at least 8 hours, except water) (Pozzilli and Pieralice 2018).

Insulin therapy is required for all T1DM individuals. Multiple daily insulin injections using a parietal insulin regimen or continual subcutaneous delivery with an insulin pen, are recommended treatments. In order to calculate the first total daily insulin doses in an adult, it recommended multiplying the patient's bodyweight in kg by 0.5 units. Generally, 40% to 50% of the overall daily dose made up from the long-acting insulin requirements for the patients, while the remaining 50% is made up of daily short-acting insulin necessities, which should be delivered with or before meals. Dosage of insulin are affected by a variety of factors, especially physical activity and food. In addition, modifications also can be performe depending on personal monitoring or persistent glucose measurement (Silver *et al.* 2018).

2.2.2 Type two diabetes mellitus

According to the International Diabetes Federation, diabetes caused 4,2 million deaths in 2019; and 463 million persons aged 20 to 79 were living with diabetes, a number that is expected to reach 700 million by 2045. In 2019, diabetes was the underlying cause of at least \$720 billion in health care costs. In addition, the real disease burden of T2DM has likely underestimated, as 1 in 3 diabetics, or 232 million people, were likely misdiagnosed. The incidence and prevalence of T2DM varies by geographic area, with more than 80% of patients residing in low- to middle-income nations, which presents additional therapeutic issues. When compared to people without DM, people with T2DM have an average mortality risk about 15% greater than those without DM, while cardiovascular disease (CVD) now being the main cause of morbidity and mortality in this population (IDF 2019). In elderly (>40 years) obese people, T2DM appears in a less acute way; many patients have definitely had the condition for some time (even years) before diagnosis. T2DM is uncommon in younger individuals, but is on the rise due to the rising frequency of obesity in this age range. Insulin levels are measurable, and the metabolic deficiency appears to be either inadequate insulin secretion or insulin resistance. Insulin therapy is often unnecessary for the prevention of ketosis in these individuals, since they are resistant to its development. However, insulin may be required to fix blood glucose abnormalities (Beckett *et al.* 2010).

2.2.2.1 T2DM: the lifestyle and genetic factors

Emerging data indicates that a variety of factors including, environment, genetics, ethnicity, and lifestyle, are correlated to the risk of developing T2DM. Particularly, it appears that genomic susceptibility has a crucial impact in the development of T2DM (Hivert *et al.* 2014). It is well recognized that multiple lifestyle factors play a significant role in the onset of T2DM. These include an unhealthy lifestyle, smoking, excessive alcohol intake, and poor physical activity. On the other hand, it has been observed that about 55% of cases of T2DM caused by obesity (Olokoba *et al.* 2012).

Obesity (body-mass index (BMI): 30 kg/m^2) is the strongest risk factor for T2DM and is associated with metabolic abnormalities resulting in IR. There exists an inverse linear relationship between BMI and the age at diagnosis of T2DM. The exact mechanisms by which obesity induces T2DM and IR remain to be elucidated; however, numerous factors have shown a significant role in the development of this pathological process, which involves both cell-autonomous mechanisms and inter-organ communications (Garcia-Garcia *et al.* 2020). Genetic predisposition plays an important part in the risk of developing T2DM. Over the past decade, several T2DM genome-wide association studies have shown the complex polygenic nature of T2DM in which most of these loci increase T2DM risk through primary effects on insulin secretion, and a minority act through reducing insulin action (Fuchsberger *et al.* 2016). Several genes found to be strongly linked with the development of T2DM in many studies, including CDKAL1, FTO, KCNJ11, HHEX, IGF2BP2, JAZF1, NOTCH2, PPARG, SLC30A8, TCF7L2, and WFS1. TCF7L2 promotes the gene expression of pro-glucagon and consequently the generation of glucagon-like peptide-1 and KCNJ11 expresses the ATP-sensitive potassium channel of islet (McCarthy 2010).

2.2.2.2 Insulin therapy during the course of T2DM

Patients with T2DM have insulin resistance and increasing pancreatic β -cell failure, resulting in inadequate insulin production, hyperglycemia, and an elevated level of free fatty acids. In response to hyperglycemia or oral hypoglycemic medications, glucotoxicity and lipotoxicity create a vicious cycle that further impairs the ability of the β -cell to release insulin. In addition, the inevitable decline of pancreatic β -cell activity in T2DM leads eventually to failure in oral agents as a therapeutic modality (Nyenwe *et al.* 2011). Glycemic control is measured by screening HbA1c (A1C indicates average glycaemia over about 3 months), performing continuous glucose monitoring, and self-monitoring blood glucose levels. A1C is the current statistic utilized in clinical trials proving the advantages of better glycemic management (American Diabetes Association 2021). Due to the continuous reduction in β -cell activity throughout the course of the disease, many T2DM patients will ultimately require insulin therapy (Inzucchi *et al.* 2012). Insulin remains the most powerful glucose-lowering drug, particularly for patients

with high HbA1c levels, despite the presence of several challenges to initiating insulin therapy, such as time restrictions, patient discomfort with self-injections, and inadequate knowledge of novel insulin formulations (Thrasher 2017). However, different forms of insulin vary in terms of how fast they act, when they reach their peak, and how long they remain. These include rapid-acting/postprandial (e.g., lispro, aspart, glulisine), short-acting (e.g., human regular), intermediate-acting (e.g., human isophane neutral protamine Haledon), and premixed formulations (American Diabetes Association 2017). When compared to human insulin, quick and long-acting insulin analogs produce less hypoglycemia and weight gain, but are more costly (Nyenwe *et al.* 2011).

The basal insulin alone is the most practical starting insulin regimen, and it can be combined with metformin and other oral medications. Initial dosages can be approximated based on body weight (0.1–0.2 units/kg/day) and the severity of hyperglycemia, with individual titration over days to weeks as necessary. The primary function of basal insulin is to inhibit hepatic glucose synthesis and control hyperglycemia overnight and between meals (American Diabetes Association 2021). However, individuals with T2DM are often more resistant to insulin than individuals with T1DM, require larger daily dosages (1 unit/kg), and have a lower incidence of hypoglycemia. Patients who remain hyperglycemic despite intensified basal insulin treatment may require the addition of prandial insulin based on postprandial glucose levels. Clinical investigations have demonstrated that the administration of prandial insulin in a stepwise fashion reduces HbA1c levels with a minimal risk of hypoglycemia. Alternately, guidelines recommend combining basal insulin with a GLP-1 RA instead of prandial insulin, with studies demonstrating comparable efficacy and the advantages of weight reduction and less hypoglycemia with GLP-1 RA therapy vs prandial insulin therapy (Garber *et al.* 2017).

2.2.2.3 Hyperglycemia and susceptibility to infection

In normal circumstances, the human body uses remarkable mechanisms to protect itself against thousands of viruses, fungi, bacterial infections, parasites, and toxins. In ideal circumstances, microorganisms are unable to cross this protective shield, but the immune

system can fail to do so, and pathogens struggle under normal conditions to penetrate this protective barrier due to the diverse of reasons. Unfortunately, diabetes disrupts the host's immune response. T2DM can impair the cells of the system, along with the possibility of natural protective walls disruption from nerves damages. Insulin insufficiency and hyperglycemia are the prior causes of this condition (Tessaro *et al.* 2017).

According to (Joshi *et al.* 1999), hyperglycemia suppresses neutrophil action to create neutrophil extracellular traps (NETs), making individuals with high blood glucose more vulnerable to infections. Infection is a typical threat for diabetic patients due to the inability of the immune system to combat invading microorganisms (American Diabetes Association 2013). Multiple studies have investigated the mechanism of diabetes by which impair the immune system of the patients and make them unable to fight against infection. These processes include cytokine suppression, phagocytosis defects, immune cell dysfunction, and failure to destroy microbes (Joshi *et al.* 1999).

2.2.2.4 Effect of hyperglycemia on the immune response in patients with diabetes

Generally, in hyperglycemia, immune system changes seem to be connect to a number of mechanisms, such as decreased release of proinflammatory cytokines, reduction of T cell and neutrophil function, in addition to reductions in humoral immunity. Additionally, it has been shown that hyperglycemia may hinder tissue recovery, which could raise the vulnerability of this tissue to develop second infections. Another changes in the immune reaction caused by hyperglycemia can be described by biochemical or/and cellular mechanisms including: (1) reduced migration of polymorphnuclear leukocytes, and/or phagocytosis impairment, chemotaxis production, (2) the formation of advanced glycation end-products, (3) risen apoptosis of polymorphnuclear leukocytes and decreased movement across endothelium, and (4) inhibition of glucose-6-phosphate dehydrogenase (Chávez-Reyes *et al.* 2021) (Figure 2.4).

Patients with T2DM are more likely to get infections that can lead to sepsis. Although a few uncommon infections, such as Klebsiella liver abscesses, malignant otitis externa, and emphysematous cholecystitis closely linked to T2DM, most infections that affect

people with T2DM are also common among the population. In contrast, T2DM, worsens infection prognosis, with these individuals experiencing higher sepsis morbidity and death than the rest of the population (Frydrych *et al.* 2018). Furthermore, respiratory and urinary tract infections are strongly associated to T1DM and T2DM. This vulnerability to infection may lead to a challenges in controlling for diabetes patients, including; sepsis, after operative infections, and chronic periodontitis (Joshi *et al.* 1999, Casqueiro *et al.* 2012).

It is important to note that foot infections are very prominent in diabetic individuals; it has been hypothesized that vascular endothelium damage driven by oxidative stress and inflammation may result in changes in microcirculation and, ultimately, nerve injury. Such infections frequently result in ischemia at wounds, which eventually necessitates the amputations (Eleftheriadou *et al.* 2020).

Additionally, (1) the post-products of advanced glycation can activate inflammatory cells, which in turn can cause a chronic inflammation that impairs the immune system's capacity to fight infections and cope with wound repair. (2) Immunological defects with neutrophil activities such as phagocytic activity, chemotaxis, and intracellular clearance may worsen the infections (Figure 2.5) (Hu *et al.* 2015). Thus, the normal hyperglycemia observed in diabetic individuals may be associated with an elevation in the risk of multiple different form of infections. All of these pieces of evidence obviously indicate that hyperglycemia may create a favorable environment for a number of infectious microorganisms; ultimately, glycemic regulation may lower the likelihood of being infected (Chávez-Reyes *et al.* 2021).

Lastly, it seems that the alterations to the immune system driven by transient hyperglycemia, such as stress, should vary markedly from those driven by chronic hyperglycemia. Nonetheless, patients may be vulnerable to a number of the same clinical consequences in either, a transitory or chronic hyperglycemic scenario, such as greater chance of being infected, and impaired wound healing (McCowen *et al.* 2001) (Figure 2.4) (Figure 2.5).

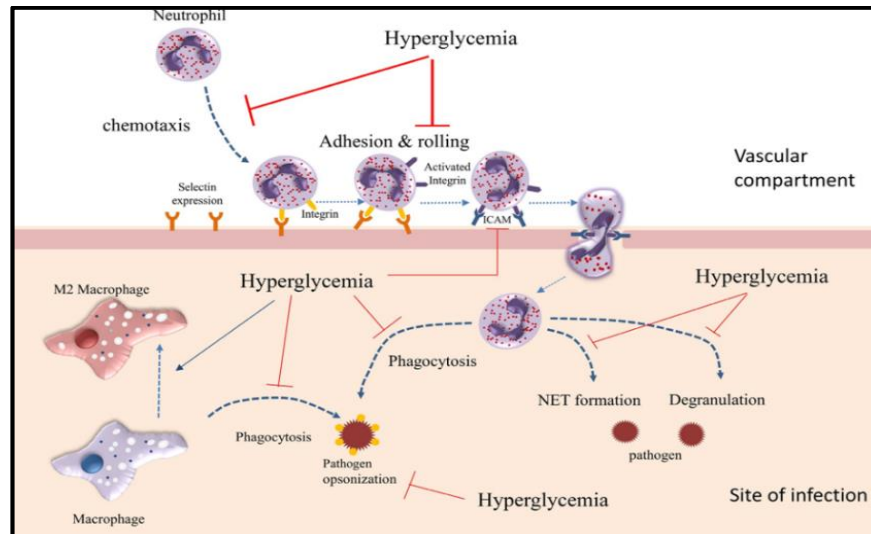


Figure 2.4 Impairment in immune response mechanisms during hyperglycemia (Ley *et al.* 2007)

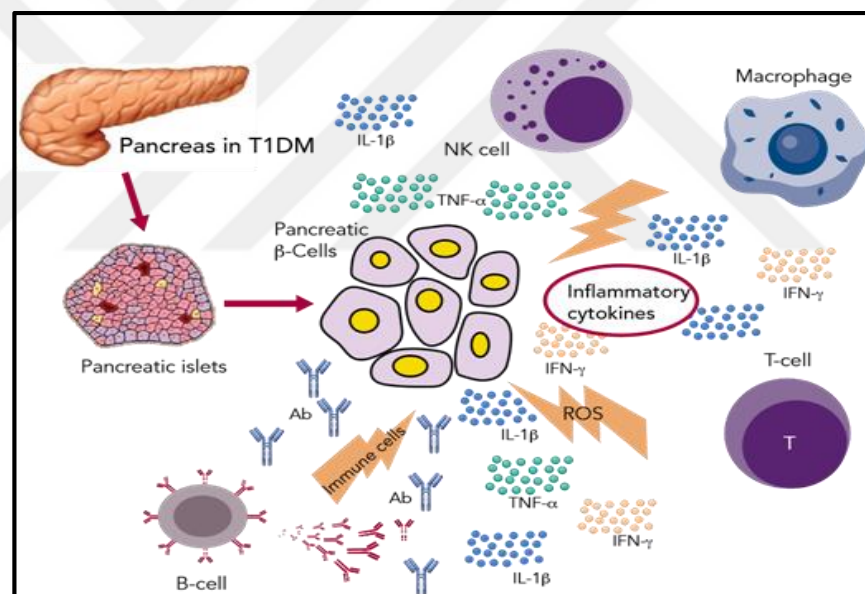


Figure 2.5 Significance role of inflammation in the development of T2DM and its relationship with other metabolic disorders (Tsalamandris *et al.* 2019)

2.2.3 Gestational diabetes

Gestational diabetes mellitus (GDM) characterized as glucose intolerance developing during pregnancy in women without a diabetes background. Thus, by definition, it differs from both T1DM and T2DM in that it is temporary and gender-specific. Moreover, it only happens during pregnancy, a unique physiological situation. However, GDM and T2DM

share many characteristics. These include insulin resistance and metabolic syndrome components. Due to its brief onset during pregnancy, GDM has been hypothesized to represent a pre-diabetic state or a temporary manifestation of a T2DM-like syndrome caused by pregnancy. GDM lacks an auto-immune component, unlike T1DM (Vokalova *et al.* 2018). In many countries, GDM is diagnosed clinically by a variety of methods. In general, the techniques utilize at least one of the following procedures: A) evaluation of clinical risk, B) glucose tolerance screening, and C) formal glucose tolerance testing. The procedures are used on pregnant women who have not been diagnosed with diabetes previously (Buchanan *et al.* 2007).

Glucose intolerance (GI) is dysglycemia, which includes both prediabetes and diabetes. In addition to diabetes, it involves impaired glucose tolerance and impaired fasting glucose. People with these disorders are more likely to develop diabetes and Cardiovascular diseases. To eliminate the stigma associated with diabetes, the WHO suggests employing the term intermediate hyperglycemia rather than pre-diabetes. Moreover, not every case is destined to develop into Diabetes. Women with an intermediate stage should be assessed to see whether home glucose monitoring is necessary. At 24-28 weeks, all women with risk factors should have an oral glucose tolerance test (Beckett *et al.* 2010). According to the American Diabetes Association, normal glucose tolerance declines throughout pregnancy, especially in the third trimester. Fasting plasma glucose levels ≥ 100 mg/dL (5.6 mmol/L) but < 126 mg/dL (7.0 mmol/L) or 2 hour oral glucose tolerance test values ≥ 140 mg/dL (7.8 mmol/L) but < 200 mg/dL (11.1 mmol/L) in GDM women are considered normal (American Diabetes Association 2004).

2.3 SARS-CoV-2 and T2DM

The global outbreak of SARS-CoV-2 has significant implications for the treatment of common metabolic disorders including T2DM. It is well known that diabetic individuals, particularly those with hyperglycemia, glycemic fluctuation, and metformin usage, have an increased risk of infection and T-cell dysfunction in their immune system. This tendency is highly correlated with HbA1C levels. In practical medicine, diabetic

individuals have a higher prevalence of yeast infections, foot infections, urinary tract infections, and infections at the site of operation (Abu-Ashour *et al.* 2018).

Several host factors, spanning from local bacterial invasion to systemic alteration of the immune response, might explain this result. Diabetes, for instance, affects the release of cytokines by macrophages and T-cells, which restricts neutrophil recruitment (Delamaire *et al.* 1997). Both in in vitro and in vitro, alteration seen with humoral innate immunity, and innate immune responses (Mazucanti and Egan 2020). In individuals with either T1DM or T2DM, IFN- α synthesis by dendritic cell reduced. Reduction of NK cell activation and altered dendritic cell activity restrict effective adaptive immune responses. (Figure 2.6) (Summers *et al.* 2006). Moreover, it is recognized that obese individuals are at a higher risk for influenza-related complications, and obesity is emerging as a key comorbidity for illness severity in the context of SARS-CoV-2 (Ryan *et al.* 2020).

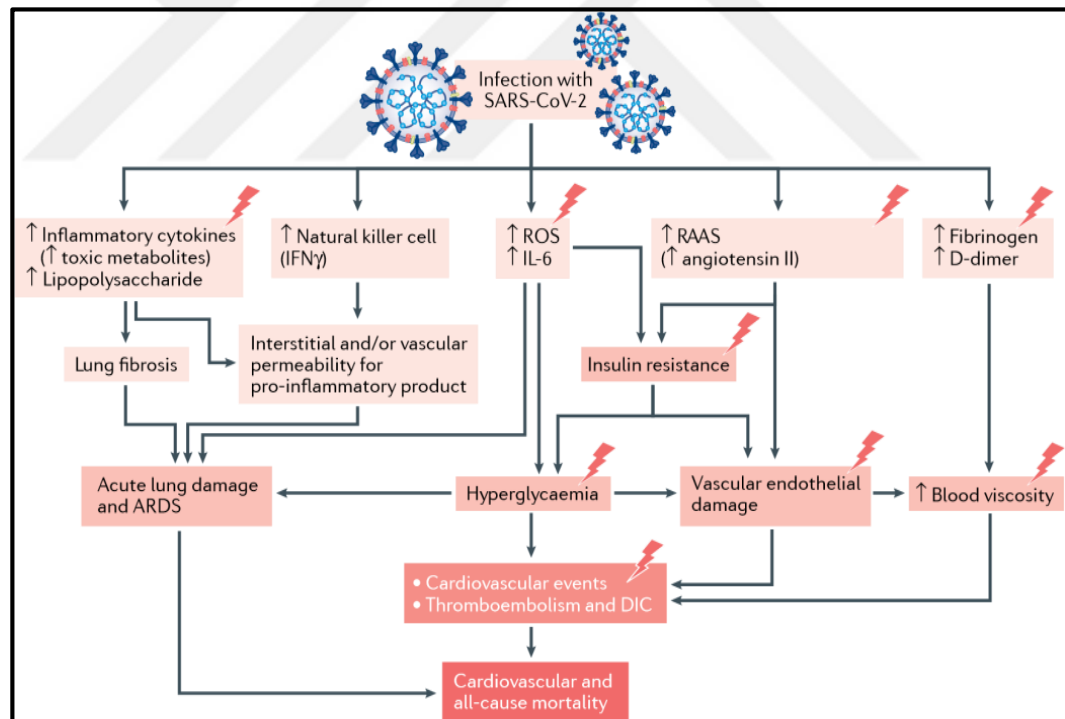


Figure 2.6 Potential pathogenic mechanisms in patients with T2DM and Covid-19 (Lim *et al.* 2021).

The respiratory epithelium is composed of several cell types, including ciliated and non-ciliated epithelial cells, goblet cells, which create mucus that serves as the first line of

defense against invading viruses, and club cells, which produce proteases. When virus particles inhaled or come into direct touch with the nose or throat mucosa, the virus can infect the cells of the respiratory mucosa and cause viral respiratory infections (Subbarao and Mahanty 2020). The lung's cells, including pneumocytes, are important cellular locations for coronavirus entrance and inflammation (Hamming *et al.* 2004). Some of these pulmonary cells may also express vital proteins that facilitate coronavirus entrance into cells, including ACE2, transmembrane protease serine 2 (TMPRSS2), and dipeptidyl peptidase-4 for some viral strains (DPP4). ACE2 and DPP4 have demonstrated pleiotropic metabolic activities that contribute directly to the physiological and pharmacological regulation of cardiovascular and glucose homeostasis, and DPP4 inhibitors are commonly using to treat T2DM (Drucker 2020).

2.3.1 Association between ACE2 receptors and infection by covid-19 in patients with diabetes

SARS-CoV-2 entrance across the membrane of the virus mediated by S glycoprotein, which produces protruding homotrimers on the viral surface. (Figure 2.7). The S glycoprotein is composed of (2) functional subunits involved for either fusion of the viral and cell membranes (S2 subunit) or attachment to the infected cells receptor (S1 subunit such as the RBD). According to latest publications, Angiotensin converting enzyme-2 also serves as a receptor for the novel coronavirus (Yang *et al.* 2020).

Three coronavirus strains (SARS-CoV, NL63, and SARS-CoV-2) enter cells via ACE2 receptors. The heart, arteries, gut, lung (especially in type 2 pneumocytes and macrophages), kidney, testis, and brain all have widespread and widespread expression of ACE2 receptors. Only a little amount of ACE2 found in the circulation in a soluble form since it has mostly linked to cell membranes. Angiotensin II is broken down to angiotensin-1-7 by membrane-bound and soluble ACE2, which is a crucial therapeutic function. Therefore, the negative consequences of angiotensin II binding to AT1 receptors, such as vasoconstriction, increased inflammation, and thrombosis, are limited by ACE2 receptors (Verdecchia *et al.* 2020). ACE2 has been found to be the receptor for Covid-19 proteases, including TMPRSS2, which also cleave the ACE2 C-terminal

region, in particular, compounds 697-716, therefore facilitating S protein-driven viral entry (Yan *et al.* 2020) (Figure 2.7).

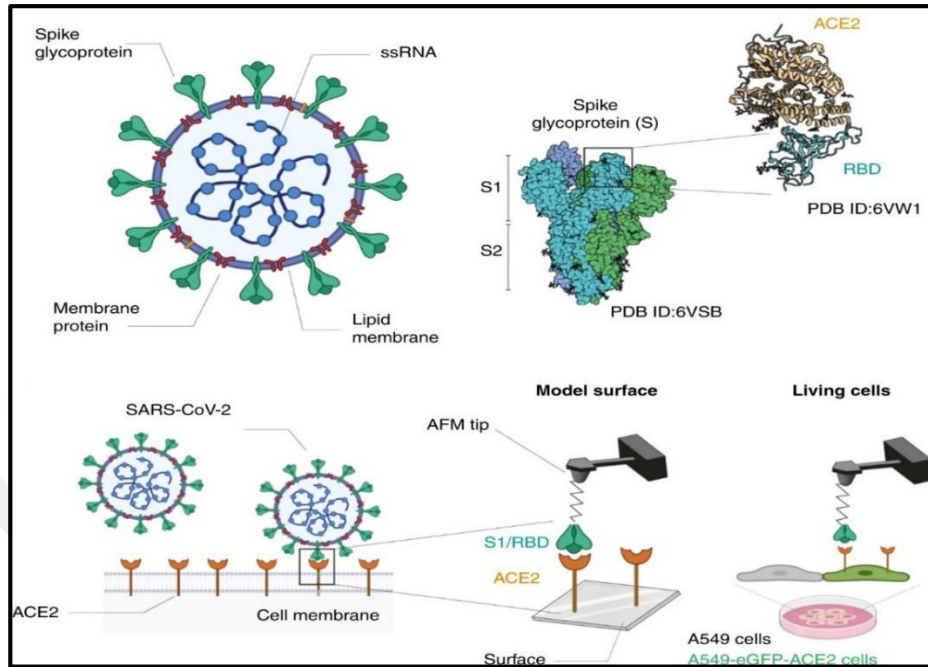


Figure 2.7 Structure of ACE2 as SARS-CoV-2 cell entry receptor (Yang *et al.* 2020)

Because of, it consider the host cellular entry receptor, Covid-19 virus attaches to the ACE2, the expression of which has shown to be greater in smokers and chronic obstructive pulmonary disease (COPD) patients. It has hypothesized that greater pulmonary ACE2 mRNA levels in diabetic individuals lead to increased SARS-CoV-2 infectivity. Despite the fact that they are supported by results in non-obese diabetic mice. There are a little investigations on human lung tissue and ACE2 protein levels (Wijnant *et al.* 2020).

Yan *et al.* 2020 demonstrated that SARS-CoV-2 had an affinity for cellular ACE2 that was 10-20 times greater than SARS-CoV-1's. This is most likely due to the insertion of a furin like cleavage site (682RRAR/S686) at S1 and S2 subunits cleavage site of SARS-CoV-2. Furthermore, plasmin, which is higher in individuals with chronic diseases as with DM, has shown to cleave SARS-CoV (S protein) in vitro. however, additional study is required on SARS-CoV-2 (Ji *et al.* 2020). In addition, diabetes and comorbid diseases

lower the cytosolic pH, which may enable the virus to bind to ACE2, and enter the cell easier. Chronic inflammation of pancreatic β cells may potentially induce β -cell autoimmunity, increasing an already heightened immune response. SARS-CoV-2 enters human cells via surface proteins in human respiratory and lung tissues by binding to ACE2 and induces a series of respiratory events, which might also infect endocrine pancreatic cells due to the presence of these receptors. There appear to be underlying processes that might explain the SARS-CoV-2-induced acute damage to pancreatic islets and subsequent loss of insulin secretion capability. A virus-mediated the immunological response, which includes the secretion of and cytokines and chemokines, has the potential to destroy β cells within the pancreas and decrease their ability to regulate glucose and synthesize insulin. In addition, the immune response to a virus infection may reduce the liver's and muscle cells' capacity to recognize abnormalities (Rubino *et al.* 2020).

The ACE2 dysfunction and inflammatory destruction caused by Covid-19 seem to be increase in all cells with ACE2 expression, including pancreatic islets. The elevated pancreatic enzymes observed in Covid-19 patients are supportive of such damage. An organoid investigation demonstrated that the expression of ACE2 receptors on β cells of the pancreas, induces Covid-19 sensitivity, which triggers β cell apoptosis, cytokine secretion and inflammation, and eventually decreased insulin production (Ardestani and Maedler 2020). Another study that utilized postmortem examinations of Covid-19 infected individuals, and cell cultures of pancreatic islets, revealed the existence and multiplication of the virus in the islets. In addition to decreased insulin that depend of glucose, along with diminution of insulin secretory granules (Müller *et al.* 2021). Two other recent researches using autopsy specimens revealed a dual impact on beta cells, causing apoptosis or trans-differentiation in the case of those that survive. In mice fed a high-fat diet, ACE2 deficiency results in β -cell dedifferentiation, while deletion of ACE2 receptors in mice with DM causes, oxidative stress on β cells, decrease in the synthesis of insulin, and high blood glucose. Preclinical findings also indicate the involvement of ACE2 in β -cell homeostasis. Finally but not least, eradication of ACE2 in fat mice inhibits β -cell proliferation, reduces beta cells density, and causes β -cell malfunction (Kazakou *et al.* 2022). Evidence in mice with DM, ACE2 activity was higher in pancreas cells. While in individuals without DM, the binds of SARS-CoV to ACE2 receptors shows to

damaging islets, induce high blood glucose, and reducing insulin production. Nevertheless, the high blood levels may be transient. Despite the fact that no such impact was documented yet for Covid-19, These data show the significance of glucose monitoring both during the severe phase of illness and throughout follow-up. Additionally, unmanaged blood glucose may result in modifications in glycosylation of the virus (s) protein and ACE2 receptors, which in turn might alter how the S protein binds to ACE2, and the efficacy of the immune response to the virus (Yin *et al.* 2021).

2.3.2 Role of diabetes in severity and susceptibility to infection with covid-19 and other viruses

Diabetes can exacerbate infectious diseases and put people at higher risk of developing serious conditions. In a matched group, recently, research assessed the rates of infection in 306,003 individuals (102,493 patients with T1DM and T2DM) and found that people With DM, especially type one, much more likely to contract infectious diseases (Carey *et al.* 2018). Furthermore, diabetes patients are more vulnerable to bacterial, fungal, and viral infections, than in individuals without DM, and their outcomes are much worse when the infection occurs (Erben *et al.* 2013). One of most prominent comorbidity linked to severe or deadly infections that elevates admissions among individuals with DM is respiratory tract infections (Klekotka *et al.* 2015). Furthermore, patients with DM are at higher risk of hospital admission due to pneumonia, which is the main reason for acute lower respiratory tract disease (Jensen *et al.* 2017). Emerging global health researches indicate that, in addition to pneumonia, viral agents causing additional chest infections in people with DM and higher fatality rate. Viruses such as, MERS, Influenza, SARS-CoV, and, the latest one covid-19 are among them (Chávez-Reyes *et al.* 2021).

2.3.3 Role of hyperglycemia in patients with DM during covid-19 infection

Hyperglycemia is the most prevalent metabolic complication of T2DM and defined as persistently elevated glucose levels in blood. The immune response and high blood glucose seem to have significant overlap, which directly worsens the pathogenesis of the Covid-19 infection. T2DM individuals and SARS-CoV-2 infection have had elevated

serum levels of inflammatory cytokines such as IFN-1, IL-1, 6, 8, and TNF- α . This is so-called cytokine storm and accompanied by other inflammatory markers such as ferritin, CRP, and D-dimer, and is linked to the acuteness of SARS-CoV-2 infection (Viurcos-Sanabria and Escobedo 2021).

Analysis of more than 500 patients hospitalized in China with SARS-CoV found an association between elevated fasting glucose levels and higher mortality rates (Yang *et al.* 2010). In addition, accumulating data suggests that high blood glucose is a poor indicator in SARS-CoV-2 due to, endothelial dysfunction, blood clotting, reactive oxygen species production (ROS), and high inflammatory mediators releasing (Bode *et al.* 2020). Chronic hyperglycemia enhances ROS production and oxidative stress of mitochondria, which ultimately leads to, malfunction of the β -cells, vasculature damages, and reduced insulin releasing (Zhang *et al.* 2019).

Simultaneously, increased lactate production and LDH action enhanced by elevated blood glucose. This occurrence is especially significant inside this scenario of SARS-CoV-2, in which it discovered that high lactate dehydrogenase values accurately predict mortality in individuals who had serious Covid-19 disease. Obviously, LDH levels in T2DM individuals may delay Covid-19 virus removal by suppressing the RIG-1-like receptor through the antiviral-signaling peptide of mitochondria, hence preventing interferon synthesis and reducing antiviral response (Zhang *et al.* 2019).

In T2DM, high blood glucose have a negative influences on a multiple neutrophil functions, such as, phagocytic activity, and the eliminate infection by bacteria, and the migration of cells (Alba-Loureiro *et al.* 2007). Considering the immunological implications of the observed mechanisms, may possibly clarify why T2DM individuals are more vulnerable to acute SARS-CoV-2 infection and negative consequences such sepsis, elevated viral load, and fatality in the course of Covid-19. Therefore, unmanaged high blood glucose should always be considere to play a significant factor in the pathogenesis of Covid-19, in individuals with T2DM (Viurcos-Sanabria and Escobedo 2021).

2.3.4 Role of obesity in DM patients during covid-19 infection

Individuals with DM, hypertension, and extreme obesity (BMI 40 kg/m^2) are higher vulnerability to have Covid-19 infection and have a greater risk of developing complications and mortality (Guan *et al.* 2020). Obesity is also a risk factor for the intensification of SARS-CoV-2-associated disease. Analysis of 124 recurrent Intensive care unit (ICU) admissions at a single institution in Lille, France, between February 27 and April 5, 2020, found higher rates of obesity and severe obesity among SARS-CoV-2 patients compared to non-infected control individuals (Simonnet *et al.* 2020). In this observational investigation, the prevalence of obesity was 47.5%, compared to 25.8% in a historical control group of ICU participants with no infection of Covid-19. Moreover, the need for intubation in addition to mechanical ventilation was greater in obese individuals. In a retrospective review of 3615 SARS-CoV-2 patients who admitted to the emergency room of a single medical hospital in New York from March 4 to April 4, 2020, related observations were reported that 60-year-olds with obesity or severe obesity were more likely to seek emergency medical treatment and ICU hospitalization (Lighter *et al.* 2020).

Obesity is a commonly related with T2DM and may have detrimental influence on T-cell function. Indeed, obese Covid-19 patients had a considerably greater risk of hospitalization, ICU admission, and death rates than in non-obesity Covid-19 patients, highlighting the need to evaluate BMI as a confounding risk factor. Obesity linked to a decreased T-cell response to influenza virus infection, with prolonged T-cell activation resulting in T-cell dysregulation. In particular, increasing adipose tissue in obese patients inhibits T-cell and macrophage function, producing persistent inflammation and reducing antiviral response. A study found that obese patients have impaired dendritic cells, indicating decreased T-cell responses to influenza antigens. Obesity was also associated with increased T-cell and immune cell degeneration (Tong *et al.* 2021).

In adipose tissue of mice models with T2DM and human patients, anti-inflammatory and/or regulatory macrophages “M2” and regulatory “T-cells” converted for Th17 CD4+, Th1 T-cells, and “M1” proinflammatory macrophages. Thus, late hyperinflammatory

response and prolonged Th1 response seen frequently in DM. Those changes to the immunological composition certainly play a role within the occurrence and intensity of infections in diabetic individuals (Mazucanti and Egan 2020).

2.3.5 Innate immunity in T2DM patients with covid-19 infection

Innate immune response is essential for containing and eliminating an invading virus. There is evidence that disorders such as obesity and T2DM modify the innate immune system, leaving patients more vulnerable to infection (Martín-Cordero *et al.* 2011).

Despite substantial production of chemokines such as the chemokine C-C motif ligand (CCL) 2 and IFN-induced protein-10, innate system are unable to initiate a rigorous IFN-III and I, antiviral defence in severe Covid-19 infections. These chemical substances may have the ability to increase the number of activated inflammatory cells within the lungs, which in turn causes an excessive production of pro-inflammatory cytokines including IL-6, 1, 8, and TNF- α , or develop a "cytokine storm". Multiple researches have demonstrated associations between the virus of Covid-19 and lung immune cell overactivation, which can result in systemic hyperinflammation (Otsuka and Seino 2020). The highly inflammatory activation of the mononuclear phagocyte system can induce macrophage activation syndrome (MAS), leading to ARDS (Mauvais-Jarvis 2020). The MAS is activated directly by cytokines such IL-6 and TNF- α , and indirectly by IL-1. The feature of MAS is a macrophage with phagocytic activity can penetrate nearly each organ with coagulation or cytopenia (Otsuka and Seino 2020). It is hypothesized that activation of the ACE2 related to mitochondrial assembly receptor-1 axis within the immune system is downregulated or malfunctioning during SARS-CoV-2 infection, hence altering its downstream signaling pathway (Iannelli *et al.* 2020). This can lead to activation of the MAPK6 pathway, which in turn triggers the inflammatory pathway, resulting in a molecular complex of inadequately regulated pyroptosis, accompanied with a robust IL-1 synthesis and low IFN-1 releasing. Furthermore, the response of IFN gene might downregulated with high IL-1 and IL-6 microenvironment, which increases susceptibility and viral dispersal (Verdecchia *et al.* 2020). While in obese and T2DM people who

infected with SARS-CoV-2, this response seems to be stronger, where multiple inflammatory pathways already weakened.

To conclude that, according to many studies, individuals who have at least one pre-comorbid condition for examples: T2DM, CVD, and obesity are more vulnerable to serious Covid-19 infections and have higher levels of inflammatory mediator's secretion. The majority of authors recommend quick and specialized anti-inflammatory treatment to prevent catastrophic outcomes. An efficient innate response and adequate management of the proinflammatory may limit the infection from continuing to develop, which might explain the limited prevalence of contagion rate in some people, including patients without obvious symptoms (Pérez-Galarza *et al.* 2021).

2.3.6 Adaptive immunity in T2DM patients with covid-19 infection

Adaptive immune system is crucial to eliminating pathogens and clear it from the body, like SARS-CoV-2 virus. If a virus, such as Covid-19 infects obese or T2DM individuals, it is hypothesized that preexisting abnormalities in their immune cells such as B and T cells may increase their chance of experiencing poor clinical outcomes. The association between the activation and inactivation in adaptive immune cells during the infection with Covid-19 virus are yet to be studying. The features T-lymphocytes residents' throughout the medical phase of Covid-19 infection seems to be in a predictable pattern. In the same context, several investigations demonstrated substantial indication of an overall decreases in T-lymphocyte (lymphocytopenia) in T2DM and obese individuals as the infection going to precede (Pérez-Galarza *et al.* 2021). Another study reported that hospitalized individuals with underlying conditions had more severe lymphocytopenia than those who did not have comorbidities (Zhao *et al.* 2020).

In contrast, to previous data, regarding the statistical characteristics of T-lymphocytes number during Covid-19 incubation, it is not well characterized if T-cell response may contribute positively or adversely on to severity of this disease (Chen and John Wherry 2020). In this regard, in a few research, it was observe that the proportion of SARS-CoV-2-specific CD4⁺ to CD8⁺ T-cells was greater with severely affected patients, along with

morphological and functional alterations. Flow cytometry study on Covid-19 specific CD4⁺ T-lymphocyte indicated that the population of lymphocytes capable of generating IFN- γ , TNF- α and IL-2, and seemed to be much lesser in people with acute infection than in convalescent patients. In addition, the CTLA-4 expression values of CD4⁺ T-lymphocyte in Covid-19 infection from critical care unit patients were considerably greater than in convalescent patients. In addition, CD8⁺ T-cells specific for SARS-CoV-2 in UCI patients displayed decreased levels of granzyme B (Schub *et al.* 2020).

Despite the only few B-lymphocyte found in severe cases, it was reported that these patients have higher amounts of specific antibodies such as “IgA” and “IgG” than in recovered patients. Surprisingly, not all infected individuals can have these antibodies during Covid-19 infection. (Schub *et al.* 2020) found IgG-specific antibodies against SARS-CoV-2 in 83 percent of cases, but “IgA”specific antibodies in just 69% of participants. It is, thought that among individuals who are severely ill, the function of CD4⁺ lymphocyte in inducing humoral immunity is changed. The proportion of CD4⁺ T lymphocytes specific for SARS-CoV-2 correlated significantly with both specific IgG and IgA antibodies (Schub *et al.* 2020). This finding is important because it shows a correlation between the synthesis of IL-17A and the initiation of ARDS (Xu *et al.* 2020). Contradictory data has emerged about the absence of Covid-19 antibodies in mild infection. (Ibarrondo *et al.* 2020) showed a significant decline in antibodies which found in people whom have treated from SARS-CoV-2. nevertheless, according to other research, antibodies against Covid-19 were elevate between (35-40) days following the beginning of illness (Wang *et al.* 2020). In agreement, (Yang and Ibarrondo 2020) observed that antibody values against Covid-19 has been raised for 50-60 days following the start of illness, while the “IgG” values of antibodies remains higher with only a modest decline at 120 days after the beginning of symptoms. As a result of these discoveries, it is required to do more research to determine if this occurrence indicates a changed immunomodulation in Covid-19 patients, particularly in individuals with greater danger of a life-threatening disease (Pérez-Galarza *et al.* 2021).

2.4 White Blood Cells (WBC) Overview

2.4.1 Myeloid cells

They constitute the majority of peripheral blood leukocytes. Myeloid cells are a subclass of leukocytes that includes monocytes, dendritic cells, macrophages, and granulocytes. They float within the bloodstream and the lymphoid system, where they quickly attracted towards the areas of injured tissues and inflammation via chemokines. Phagocytosis and the production of proinflammatory cytokines are stimulated inside the tissues, where they play essential activity in host defense. likewise, under steady-state conditions, tissues may contain myeloid cells, which regulate homeostasis, in addition to tissue growth and repair (De Kleer *et al.* 2014).

The hematopoietic stem cell (HSC) is a multipotent stem cell that dwells in the bone marrow and can differentiate into all blood and immune system cells. It is the prototypical stem cell, capable of self-replication and differentiation into progeny of numerous lineages. Hematopoiesis is the process by which HSCs differentiate into mature, functioning cell types of the blood lineages (Weiskopf *et al.* 2017). Myelopoiesis involves a series of maturational steps from HSCs to common myeloid progenitor cells through terminal differentiation leading to the formation of granulocytes, including basophils, neutrophils, eosinophils, and monocytes. This is sometimes further divided into granulopoiesis and monocytopoiesis. In bone marrow, there is typically a spectrum of myeloid differentiation with neutrophils and neutrophil precursors predominating (Treuting *et al.* 2017). The earliest HSCs to develop in the embryo was derive from the anterior wall of the aorta inside aorta-gonad-mesonephros area. During this phase, hematopoietic stem cells also found within the placenta, yolk sac, and vitelline/umbilical arteries. About week five of pregnancy hematopoietic stem cells derived from the yolk sac, the aorta-gonad-mesonephros, implant the fetus liver, which later becomes the main embryonic hematopoietic region. In the liver of fetus, hematopoietic stem cells multiply and produce functional lymphocyte, erythroid, and myeloid cells (De Kleer *et al.* 2014).

2.4.1.1 Granulocytes

Granulocytes are at the forefront of an immune response and form crucial elements in the innate immunity. Granulocytes are WBC that categorized as neutrophils, basophils, mast cells, or eosinophils based on changes in cellular morphology and the staining of their cytoplasmic granules. Granulocytes are physiologically different and easily distinguished from lymphocytes, whose nuclei rounded due to their multilobed nucleus. Granules are abundant in the cytoplasm of all granulocytes and released upon contact with pathogens. These granules include several proteins with diverse functions: Some destroy pathogens directly, while others control the trafficking and activity of other WBCs, including lymphocytes, yet others contribute to tissue remodeling at the site of infection (Owen *et al.* 2013).

- **Neutrophils**

Neutrophils are the most abundant WBC, accounting for 40-70% of all leukocytes in peripheral blood. The presence of neutrophils in a tissue indicates infection and it related to acute inflammation. In the bone marrow, myeloid progenitor cells give rise to neutrophils. These cells have an average diameter of 916 mm with a multilobed nucleus. Neutrophil cytoplasm contains many granules containing degradative enzymes. Neutrophils have a relatively short lifespan of two to three days and migrate rapidly to the site of infection; they are the first cells to be saw in significant numbers at the site of infection. A large number of neutrophils are also retained at a 'marginal pool', which may be mobilized during infection to enhance the amount of cells capable of responding. Neutrophils phagocytose pathogens and have many granules that can integrate with the phagosome to facilitate killing. Once the neutrophil's granules are exhausted, the cell dies and leads to pus production (Todd *et al.* 2015).

In response to several of stimuli, activated neutrophils produce neutrophil extracellular traps (NETs) (Figure 2.8). NETosis is a specific form of cell death that is distinct from necrosis and apoptosis (Zawrotniak and Rapala-Kozik 2013). NETosis process is triggered by protein-arginine deiminase type 4, resulting in the release of intracellular

granule components that capture and eliminate diverse microorganisms. As demonstrated by a number of illnesses, the beneficial effects of pathogen control are countered by pro-inflammatory consequences. Components of NETs are non-specific, and in addition to eliminating microorganisms, they cause damage to the surrounding tissue either directly or indirectly enhancing the pro-inflammatory response. NETs may contribute to the intensification of inflammation in autoimmune conditions including systemic lupus, rheumatoid arthritis, and psoriasis (Mutua and Gershwin 2021).

The antibacterial characteristics of NETs have determined by their primary components, DNA and granular antimicrobial proteins. The microorganisms trapped in NETs are destroyed by oxidative and non-oxidative processes in NETs (Zawrotniak and Rapala-Kozik 2013). Inhibition of NETs may reduce the severity of several diseases, ultimately enhancing survival (Mutua and Gershwin 2021). On the other hand, it was shown that chromatin and proteases released into the circulatory system during NET formation could modulate procoagulant and prothrombotic factors and contribute to blood vessel clot formation (Fuchs *et al.* 2007) (Figure 2.8).

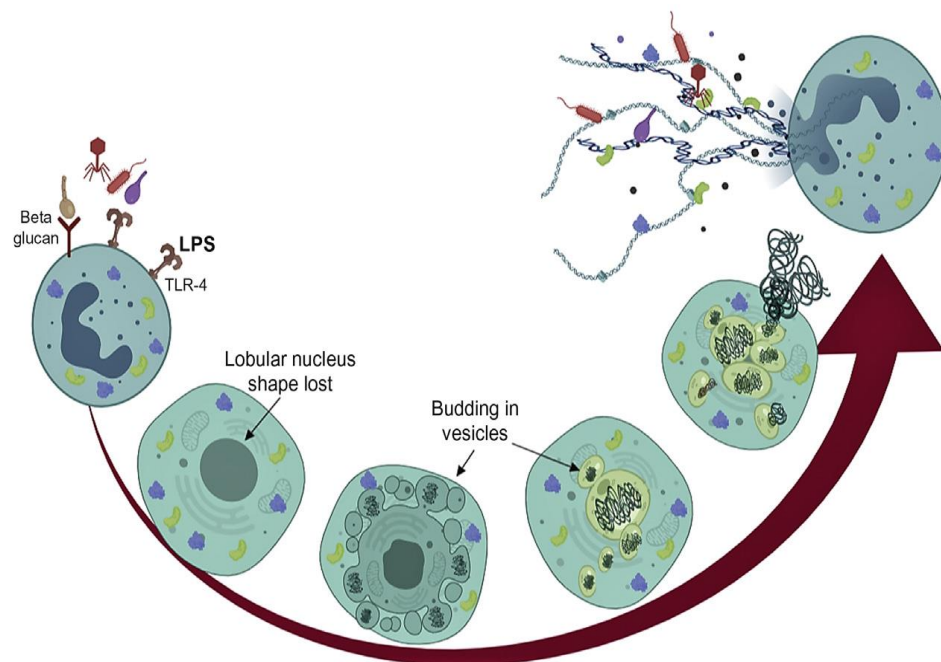


Figure 2.8 Neutrophil and NETosis formation (Rai 2019)

- **Basophils**

The least numerous WBCs are basophils, which are granulocytes with nuclei that are rounded, indented, banded, or segmented. Less than 1% of leukocytes in mice and less than 2% in rats are basophils. Basophils are similar in size to neutrophils in humans. They make about 0–1% of leukocytes and are infrequently observed in peripheral blood. Basophils are named for the noticeable (0.2-1 μ m) cytoplasmic granules in their cells that have a preference for basic dyes. These granules have an irregular distribution, are abundant, and range in hue from deep purplish-blue to dark purple-red. Heparin, histamine, and other substances are present in them. Basophils are not phagocytic; instead, they expel the chemicals in their granules in response to stimulation (Ward *et al.* 2018).

The amount of basophils in the blood increases in proportion to how strongly the adrenocorticotrophic hormone pleads for more glucocorticoids. Innate immunity includes basophils, which are very effective against allergies and parasites. In the event that the cortisol response and eosinophil response are insufficient, basophils may act as a third technique of adjusting the adrenal response to aggressors by sharing receptors with eosinophils, such as eotaxin. Circulating basophile levels drop when cortisol is present. All leukocytes contribute to immune function, tissue repair, and inflammatory processes, particularly during the acute phase of infections (Hedayat and Lapraz 2019).

- **Eosinophils**

Eosinophils similar to neutrophils, they are motile and phagocytic cells (Weller and Spencer 2017), that can migrate from the blood into the tissue space. They assumed to perform their most significant defense against multicellular parasitic species, such as worms, as their phagocytic role is substantially less significant than that of neutrophils. Invading worms, whose membranes were harmed by the activity of proteins produced from eosinophilic granules, can be detected clustered around them. Eosinophils, like neutrophils and basophils, may release cytokines that control T and B-lymphocytes, affecting the cells of the adaptive immunity. Eosinophils are also better understood as

causes of asthma and allergy symptoms (Owen *et al.* 2013). The comparatively large, spherical granules that have a special affinity for stains made of acid eosin are what give eosinophils their name. The cells stain reddish-orange, occasionally with hints of brown (Ward *et al.* 2018).

2.4.1.2 Monocytes

WBCs called monocytes originate in the bone marrow. As a component of the innate immunity, the monocyte regulates cellular homeostasis, especially when there is infection or inflammation. They make up about 5% of the cells with a nucleus that circulate through bloodstream of adult individuals. Circulating monocytes have a half-life of one to three days (Patel *et al.* 2017). Myelodysplastic syndromes frequently exhibit monocytopenia, which is a reduction in the number of circulating monocytes. While “monocytosis” defined as an increase in circulating monocytes and it is common finding in the peripheral blood of people particularly when associated with infection, drug usage, autoimmune diseases, trauma and different types of cancers. If monocytosis persists and remains unknown, the possibility of chronic myelomonocytic leukemia demands further research (Espinoza and Emmady 2022). In different tissues, monocytes finally develop into macrophages, which may exhibit a distinctive, tissue-dependent shape and particular functions. For instance, osteoclasts in the bone, macrophages in the lungs, Kupffer cells in the liver, the mesangial macrophages in kidneys. In lymphoid organs and the skin, monocytes can also develop into DC, which serve as expert antigen-presenting cells (Kawamoto and Minato 2004).

- **Macrophages**

Blood monocytes that leave the bloodstream to differentiate in various organs give rise to macrophages. Macrophages are specialized cells that hunt for, engulf, and eliminate bacteria and other dangerous organisms. They can also activate other cells by releasing substances known as cytokines, which present antigens to T-cells and cause inflammation (He *et al.* 2021). Each macrophage population exhibits significant variety, which most likely reflects the level of specialization necessary for each tissue's environment. They

exhibit variation in their shape, the types of pathogens they can identify, and the quantities of inflammatory cytokines they secrete (i.e. IL-1, IL-6, and TNF- α) (Saldana 2021). These are known as inflammatory macrophages, and they function in the immune system in two different ways: as efficient phagocytes that can help clear infections from a tissue and as antigen-presenting cells that can stimulate T lymphocytes (Owen *et al.* 2013). Instances of tissue-specific macrophages exhibiting these characteristics are osteoclasts within bones, the cells of microglial in nervous system, and macrophages inside lungs (Rich *et al.* 2012). Furthermore, bacteria that phagocytosed by macrophages can be killed by ROS like nitric oxide. These cells' diverse makeup may not only be the outcome of their differentiation process; rather, it is probably a trait they received from their monocyte predecessors (Saldana 2021).

- **Dendritic cells**

Dendritic cells (DCs), so called because of their probing, "tree-like" or dendritic forms, are in charge of the start of adaptive immune responses and serve as the immune system's "sentinels". Leukocytes generated from bone marrow called DCs are the most effective type of antigen-presenting cells (Geissmann *et al.* 2010, Guernonprez *et al.* 2019). DCs have been developed to capture and process antigens, turning proteins into peptides that are displayed on major histocompatibility complex (MHC) molecules recognized by T lymphocytes (Boltjes and Van Wijk 2014). The DC subtypes differ in location, migratory paths, precise immunological function, and dependence on infections or inflammatory triggers for their formation. They also contain distinctive markers (Guernonprez *et al.* 2019). Due to their many cytoplasmic processes, DCs have a vast surface area that enables close interaction with a variety of nearby cells, including neutrophils, epithelial cells, T-cells, and NK cells. When a disease invades a tissue, local DCs use pattern recognition receptors (like TLRs) to identify invaders, collect antigens, and then immediately exit the area (De Kleer *et al.* 2014). In reaction to various chemokines like CCL19 and CCL21, they scurry across the cells, cross the endothelium of lymphatic arteries, and go to the draining lymph nodes (LN). DCs travel to T-cell zones after reaching the subcapsular sinus of the LN. Here, the antigen presentation to T-cells is actively carried out by the interdigitating DCs (Patente *et al.* 2019).

2.4.1.3 Mast cells

Although tissue mast cells resemble basophils, they have a distinct ancestry. Mast cells found in a range of human tissues, including skin, connective tissue, and the mucosal epithelial tissue of the digestive, respiratory, and genitourinary tracts. Mast cells are bigger than basophils and have more granules and a tiny, spherical nucleus. They have a lengthy lifespan of between 9 and 18 months, unlike basophils. Mast cells differ from basophils in that their granules include enzymes such as acid phosphatase, alkaline phosphatase, and protease in addition to histamine. Mast cells can operate as antigen-presenting cells (APCs). In addition, it plays a role in allergic responses. They have the ability to both stimulate and inhibit the immune system (Stevens and Miller 2016).

2.4.2 Lymphoid cells

The lymphoid progenitor cell gives rise to three key cell types: NK cells, which can recognize malignant and virally infected cells, B-cells, which make antibodies that target extracellular antigens, and T-cells, which detect protein antigens displayed on MHC molecules. T and B-cells are small (7 mm), inert cells that, when activated, grow to 9-15 mm in size. They generally circulate through the blood and lymphatics in a latent state or are present in the lymphoid tissues. Genetic recombination used to create the antigen receptors on T and B-cells. Because each T and B-cell may only express one particular antigen receptor, the population as a whole exhibits a tremendous diversity of specificities. A particular receptor that expressed by T-cells enables the identification of a distinctive protein antigen within a particular host cell compartment (Abbas *et al.* 2019).

2.4.2.1 T-lymphocytes

The progenitors of T-lymphocytes leave the bone marrow at a very early stage of their development and move to the thymus, where they become known as thymocytes. T-lymphocyte get their letter identification from their place of maturation in the thymus (Todd *et al.* 2015). They circulate throughout the body until they detect their antigen on

the surface of APCs. The MHC complex, a structure on the surface of the APC, is where the antigen is retained when the T-cell receptor (TCR) attaches to it. This causes the T-cells to first become activated. Three signals are required for T-cell activation and differentiation to occur successfully: engagement between the T-Cell receptors and the protein introduced by MHC complex, signals from co-stimulatory molecules, and involvement of cytokines that trigger clonal proliferation (Kapsenberg 2003). Although the constitution of these granules is quite different from that of myeloid cells, activated T-cells can produce co-stimulatory molecules to stimulate other immune system components or release cytotoxic granules to destroy pathogen-infected cells (Mak *et al.* 2013). On cell membranes, MHC molecules are genetically heterogeneous glycoproteins. MHC molecules' capacity to bind to antigen enables cells to adorn their surfaces with internal (both self and foreign) proteins, exposing them to T-cells that are able to browse them. Almost all nucleated cells of vertebrate species contain class I MHC molecules, whereas class II MHC molecules are expressed by professional APCs and a few additional cell types during inflammation (Owen *et al.* 2013).

T-helper (TH) cells and T-cytotoxic (TC) cells are the two main cell types of T-lymphocytes, and they are identified from one another by having either CD4 or CD8 membrane glycoproteins on their surfaces. In general, CD4⁺ TH lymphocyte cells serve as TH cells and are able to identify exogenous antigens given in conjunction with MHC class II molecules. Unique subsets of cytokines are secreted by various TH cell subclasses to support functional activity. Cytotoxic T-cells, or CD8⁺ T-lymphocytes, distinguish between endogenous antigen presented in the context of MHC class I molecules and those usually functioning as TC cells. Cytotoxic T-cells directly destroy target cells by triggering apoptosis with the help of secreted proteins (Actor 2019). In healthy mouse and human peripheral blood, the ratio of CD4 to CD8 T-cells is around 2:1. A shift in this ratio is frequently a sign of autoimmune illnesses, immunodeficiency diseases (such as HIV), and other conditions (Owen *et al.* 2013).

2.4.2.2 B-lymphocytes

A comparable function carried out by B-lymphocytes, which use their Ig receptor to find free-floating antigens. The earliest B-lymphocyte lineage cells may be seen during the liver development and remains throughout life within red bone marrow. The B-lymphocyte (B-cell) got its name from the bursa fabricius, where it matures in birds. In human, mice, and many other animals, however, bone marrow is where it matures most frequently (Owen *et al.* 2013). The generation of antigen-specific Ig against invading pathogens are mediating by B-cells, which are at the core of the adaptive humoral immune system (typically known as antibodies). Five to twenty percent of the peripheral blood mononuclear cells are B-lymphocytes. An antigen-independent phase of B-lymphocyte development takes place in primary lymphoid tissues, and followed by an antigen-dependent phase in secondary lymphoid tissues. B-lymphocytes are found in secondary lymphoid tissues like the spleen, lymph nodes, tonsils, and Peyer's patches as well as primary lymphoid tissues like the bone marrow and Peyer's patches (a primary lymphoid tissue in some species because it is the site of B-lymphocyte development rather than the bone marrow).

HSCs serve as the starting point for the formation of B-cells. This develops into an early lymphoid progenitor, from which the common lymphoid progenitor, which gives rise to the DC, NK cells, and common lymphoid progenitor-2, which gives rise to the B-cell lineage to be formed. This is thought to be the beginning of the immature B-cells (Nagasawa 2006). “Notch-one” pathway that is required for the maturation of T-lymphocytes, must be absent or suppressed in order for B-cells to grow in bone marrow (Cruickshank and Ulgiati 2010).

A mechanism of gene recombination that firstly encodes for segments “D” divers, “V” variable, and “J” joining of the heavy chain (H-chain) together with segments “V” and “J” of the light chain (L-chain) of Ig membrane bound, has organized during the differentiation of the B-lymphocytes (MIg). The RAG1-RAG2 protein complex, which breaks the double chain of DNA between gene segments, triggers the recombination process. These recognition sites sometimes referred to as patterns of recombination

signals. Such procedures results in developing of B-lymphocytes that exhibit a different types of MIg. As a result, the BCs Receptor, that can distinguish between more than 51013 distinct ages, will be formed (Pieper *et al.* 2013). However, the combining sites of every antigen receptor on a single B-cell are the same. B-cells expand and transform into fast dividing lymphoblasts when stimulated by antigen. A portion of their offspring grow into plasma cells, which lack surface Ig but produce vast amounts of Ig molecules that are released as free antibodies. Plasma cells also contain highly developed arrays of rough endoplasmic reticulum (Mak *et al.* 2013). The five immunoglobulin classes-IgM, IgD, IgG, IgA, and IgE- that make up B-cell antigen receptors and released antibodies differ in the amino acid sequences of their heavy chains (Todd *et al.* 2015). IgM and IgD expressed by naive B-cells; however, after being stimulated by an antigen, surface IgD expression is lost. This category of Ig is not present in memory B-cells. While some of the B-cells that are activated during the main response develop into IgM-secreting plasma cells, other B-cells, including memory cells, alter the class of Ig they synthesis to IgG, IgA, or IgE with little to no change in their antigenic specificity. As a result, classes other than IgM account for the majority of the antibodies generated during a secondary reaction (Todd *et al.* 2015).

2.4.2.3 NK cells

The majority of innate immunity's effector lymphocytes are known as NK cells, and they may be found in both primary and secondary immunological compartments as well as different mucosal tissues (Vivier *et al.* 2008). Less than 5% of the circulating lymphocyte population are NK cells. They express germline-encoded receptors, in contrast to T and B-cells, that recognize changes in the expression of a number of chemicals on the surface of the target cell (Abel *et al.* 2018). Numerous cell surface activating and inhibitory receptors are part of the NK cell detection system, and their activation controls NK cell activity. Therefore, the dynamic equilibrium governing NK cell activation is controlled by the integration of antagonistic pathways upon contact with surrounding cells, and this determines whether or not NK cells are triggered to kill the target cell (Vivier *et al.* 2004). Inhibitory receptors used by NK cells to determine if vulnerable target cells lack constitutively expressed self-molecules. In various in vitro and in vivo models, NK cells

express MHC class I-specific receptors and 'lose' inhibitory signals when they come into contact with hematopoietic cells lacking in MHC class I (Yokoyama 2005). NK cells formerly thought to be big granular lymphocytes that naturally attacked tumor cells with cytotoxicity. Later, it was shown that NK cells have a distinct lymphocyte lineage that have both cytotoxic and cytokine-producing effector activities (Vivier *et al.* 2008).

The cytokine microenvironment, as well as interactions with other immune system cells including T-cells, DCs, and macrophages, determine the strength and quality of NK cell cytotoxic and cytokine responses (Long 2007).

2.5 Phagocytosis

Phagocytosis is a cellular mechanism for consuming and expelling objects with a diameter of more than 0.5 μ m, such as bacteria, foreign objects, and apoptotic cells (Figure 2.9). Since phagocytosis occurs in a wide variety of cell types, it is crucial for maintaining tissue homeostasis. However, only specialized cells known as professional phagocytes are very effective in phagocytosis. These specialized cells include osteoclasts, neutrophils, monocytes, DCs, and macrophages. These expert phagocytes have a variety of phagocytic receptors that trigger signaling cascades that lead to phagocytosis (Uribe-Querol and Rosales 2020).

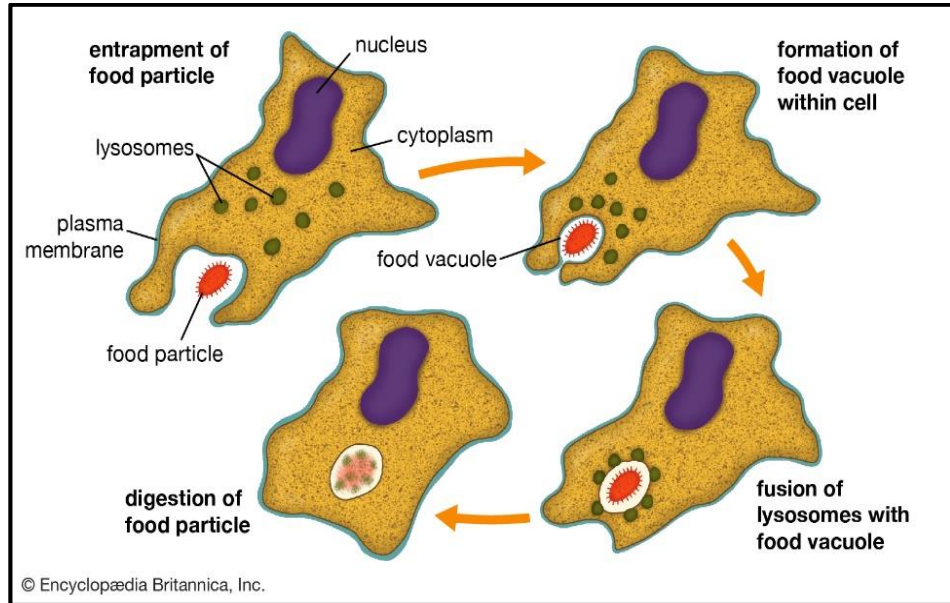


Figure 2.9 Stages of phagocytosis

2.5.1 The mechanism of phagocytosis

2.5.1.1 Identifying the molecules to be ingest

Finding the target particle is the first step in phagocytosis. On phagocytic cells, certain receptors facilitate detection. Pattern recognition receptors (PRRs) are receptors which detect specific patterns on the surface of pathogens. Some of these PRRs are non-opsonic phagocytosis receptors since they can start phagocytosis. TLRs are one kind of PRR that can bind to PAMPs but does not cause phagocytosis. However, these receptors have the ability to stimulate the cell for phagocytosis. Opsonic sensors are also capable of indirectly detecting foreign particles. The most well known opsonic receptors are those for antibodies and complement (Uribe-Querol and Rosales 2020).

2.5.1.2 Triggering of the internalization mechanism

In order to start phagocytosis, many signaling pathways engaged when phagocytic receptors detect a particle. The actin cytoskeleton reorganized, and the membrane undergoes modifications that culminate in the phagocytic cup, a depression of the portion

of the membrane that touches the particle. Once the membrane has completely encircled the particle, pseudopods are created around it to create a new phagosome inside the cell. Fc receptors and complement receptors are the signaling molecules that trigger phagocytosis. Signaling mechanisms for other phagocytic receptors are just being researched (Levin *et al.* 2016).

2.5.1.3 Creation of a phagosome

When phagocytic receptors bind to ligands on the target particle, phagocytosis begins. The creation of membrane protrusions to cover the particle happens because of receptors activating signaling pathways that modify the membrane's structure and regulate the actin cytoskeleton. Finally, a new vesicle pinches out from the plasma membrane because of the distal fusion of these membrane protrusions. The phagosome is the new vesicle that is holding the ingested particle (Levin *et al.* 2016).

2.5.1.4 Maturation of the phagosome and formation of phagolysosome

Once internalized, the new phagosome changes its membrane structure and its contents to produce the phagolysosome, a new vesicle that may break down the ingested particle. This process, called phagosome maturation, involves interactions between the newly formed phagosome and early endosomes, late endosomes, and eventually lysosomes (Canton 2014).

2.6 Interleukin-1 Family

The immune system responds to adverse stimuli like infections, damaged cells, poisonous substances, or radiation with inflammation, which operates by eliminating the harmful stimuli and starting the healing process. Thus, inflammation is a defensive process that is essential for maintaining health. It has been demonstrated that individuals who are outwardly healthy have a wide range of inflammatory mediators, and some individuals continually fall within the greater range for certain mediators, such as the IL-1 family and

C-reactive protein (Chen *et al.* 2018). Although the inflammation may have initially been a necessary reaction to an infection or tissue injury, it lingers and turns harmful in a number of disorders, impairing tissue function (Sims and Smith 2010).

Inflammation is one of the adaptive reactions to several damage, including physical, chemical, and biological elements, in terms of general pathology. The traditional indications of inflammation are thought to be connected to tissues and cells reacting to pathological cell injury brought on by both internal and external stimuli, such as bacteria and viruses as well as damage-associated products and metabolites (Hotamisligil 2006). Within an hour of the discovery of warning signals, an inflammatory transcriptional pathway so-called "heightened alert" is launched in sentinel cells of the innate immune system, including tissue-resident DCs and macrophages. This program produces an antipathogen state as well as Several inflammatory chemokines, cytokines, eicosanoids, and biogenic amines, that cause nearby tissue cells to enter a similar condition (Cronkite and Strutt 2018).

The IL-1 family is connecting more intimately to innate inflammatory and immunological responses than any other cytokine family. This connection results from the Toll IL-1 receptor domain, which is highly similar to the cytoplasmic domains of all TLR, being present in the cytoplasmic portion of every member of the IL-1 family of TLR (Dinarello 2009). An inflammatory cytokine called IL-1 thought to have a variety of physiological and pathological implications and to be crucial in both health and illness. This decade has seen the expansion of the IL-1 family members, and mounting data emphasizes the significance of IL-1 in tying innate immunity to a wide range of illnesses beyond inflammatory disorders (Kaneko *et al.* 2019).

According to human sequence algorithm technologies, there are already 11 members of the IL-1 family, each of which has unique or comparable biological effects (Garlanda *et al.* 2013). IL-1 β , IL-1 α , IL-1Ra, IL-18, IL-33, IL-36Ra, IL-36 γ IL-36 β , IL-36 α , IL-37, and IL-38 which identified and characterized. They include the receptor-agonistic IL-1 β , IL-1 α , IL-18, IL-33, and IL-36, and the receptor-antagonistic IL-1Ra, IL-36Ra and IL-38. IL-37 exclusively, is the only anti-inflammation cytokine. Despite the fact that the

role of each member of the IL-1 family are currently being studied, IL-1 is the most well-known of these members (Dinarello *et al.* 2010). The IL-1 α and IL-1 β genes, which code for IL-1 α and IL-1 β , respectively, are two related but separate IL-1 genes. Each IL-1 interacts to the same type 1 (IL-1RI) cell surface receptor, which found on almost all cells. When IL-1 binds to its receptor, a series of inflammatory mediators, chemokines, and other cytokines released. Studies of people who received low nano-gram dosages of either IL-1 α or IL-1 β injections have taught us a lot about IL-1-mediated inflammation. Although patients received IL-1 to enhance bone marrow function, these individuals also suffered from severe toxicity, including fever, appetite loss, widespread muscle and joint pains, weariness, gastrointestinal and sleep disorders, and hypotension. Therefore, it was hypothesized that precise pharmaceutical inhibition of IL-1 activity would have positive effects on inflammatory illnesses (Dinarello *et al.* 2012).

2.6.1 Expression of IL-1 in tissues and target cells

Both IL-1 α and IL-1 β are widely expressed in a wide range of tissues and cells, particularly in macrophages in lymphoid organs such the thymus, spleen, lymph nodes, Peyer's patches, as well as bone marrow. IL-1 α and IL-1 β are expressed in tissue macrophages in the liver, gastrointestinal system, and lung in non-lymphoid organs. Additionally, they are expressed in glomeruli, outer cortical regions of the kidney, cellular sub-epithelial endometrial tissue of the uterus, as well as in neutrophils, keratinocytes, epithelial and endothelial cells, lymphocytes, smooth muscle cells, and fibroblasts (Kaneko *et al.* 2019).

IL-1, which is a significant pathogenic mediator of autoimmune, infectious, degenerative, and autoinflammatory illnesses virtually, effect all cells and organs. (Dinarello 2009, Gabay *et al.* 2010, Dinarello *et al.* 2012). The innate and inflammatory responses amplified by the signaling cascade, in which IL-1R complex activates. This cascade causes transcription of numerous genes that code for cytokines, chemokines, and other inflammatory mediators, including IL-1 itself and the rest of the family members, like IL-18 and IL-36 as well as for endothelial adhesion molecules. IL-1 promotes neutrophil and macrophage survival and effector activity (Mantovani *et al.* 2011). Additionally, by

primarily stimulating certain growth factors and receptors, IL-1 orchestrates the development and operation of innate and adaptive lymphoid cells. Fever (IL-1 is the endogenous pyrogen that promotes leukocyte migration), stimulation of the hypothalamus-pituitary-adrenal axis, and synthesis of cortisol, which controls inflammation, are all effects of IL-1 on the central nervous system. Furthermore, IL-1b has a detrimental effect on synaptic plasticity and learning that depends on the hippocampal. The humoral arm of innate immunity, such as C-reactive protein, mannose binding lectin, and complement components, mediates the innate response controlled by IL-1, which in turn causes IL-6, which initiates the acute phase response in the liver and controls tissue damage (e.g., α 1-antitrypsin) (Sims and Smith 2010).

2.6.2 IL-1 β gene and its expression

This gene produces a protein that belongs to the IL-1 cytokine family. On chromosome 2, this gene joins eight other members of the IL-1 family to create a cytokine gene cluster. Monocytes, macrophages, DCs, B-lymphocytes, neutrophils, and NK cells are the main producers of IL-1 β after being stimulated by microbial ligands that act through TLRs, complement elements, other cytokines (like TNF), or IL-1 itself. Contrarily, IL-1 (precursor and mRNA) is present in endothelial cells, skin keratinocytes, fibroblasts, platelets, epithelial cells, and astrocytes and can be elevated by hypoxia. One of the most significant mediators of inflammation and host defenses against infections, such as cell division, proliferation, and apoptosis, is IL-1. This cytokine has reported to induce cyclooxygenase-2 in the central nervous system, which has been proven to contribute to inflammatory pain hypersensitivity. Numerous disorders have been connecte to increased IL-1 β production. Patients with bacterial, fungal, parasitic and viral illnesses has proven to have an increase in IL-1 production. Increased IL-1 β levels also linked to T2DM, atherosclerosis, and a number of autoimmune diseases, including rheumatoid arthritis, multiple sclerosis, and Crohn's disease. An increase in IL-1 β production has also been observed in neurodegenerative illnesses including Alzheimer's and Parkinson's that are accompanied by inflammatory processes (Church *et al.* 2008). Wide ranges of biological actions produced by IL-1 β . IL-1 β can have negative consequences in addition to playing a helpful role in modulating host defenses against microbial invasion. For instance, in the

etiology of chronic inflammatory disorders, IL-1 β can enhance tissue damage and enable tumor invasiveness (Apte *et al.* 2006). Synthetic IL-1 β receptor antagonists found to lessen the severity of a number of auto-inflammatory diseases, including adult-onset Still's disease, lupus arthritis, osteoarthritis, and ankylosing spondylitis. These diseases include acute pancreatitis, systemic onset juvenile arthritis, and several others (Dinarello *et al.* 2012).

When the cytokine's recombinant version became accessible, it was established that IL-1 β played a wide range of roles in both health and sickness. The right acute inflammatory response to an infectious challenge and, consequently, the recovery from infection or damage, depend on the presence of IL-1 β . However, the cytokine also has a harmful function in a number of chronic illnesses, including as cancer, diabetes, and atherosclerosis (Dinarello 2011). Pro-IL-1 β , a (31 kDa) precursor that is inactive, is created in response to "PAMPs", which are molecular patterns carried by infections. PAMPs influence the pathways that govern gene expression by acting through the PRRs on macrophages (Takeuchi and Akira 2010). Caspase-1 is activated by being recruited to the inflammasome, a multi-protein complex (Schroder and Tschopp 2010). In response to inflammasome activators such ATP, nigericin, serum amyloid A, or pneumolysin from *Streptococcus pneumonia*, neutrophils produce massive quantities of bioactive IL-1b (Bakele *et al.* 2014).

The cytosolic PRR NLRP3 creates the inflammasome that has thoroughly studied. A PAMP/DAMP sensing a central nucleotide binding or NACHT domain, an N-terminal pyrin domain (PYD), and C-terminal leucine rich repeat to make up the tripartite structure of NLRP3. Through a homotypic connection with its PYD domain, the PYD domain of tripartite structure binds the adaptor molecule; apoptosis-associated speck-like protein to caspase recruitment domain (Brough and Rothwell 2007) (Figure 2.10).

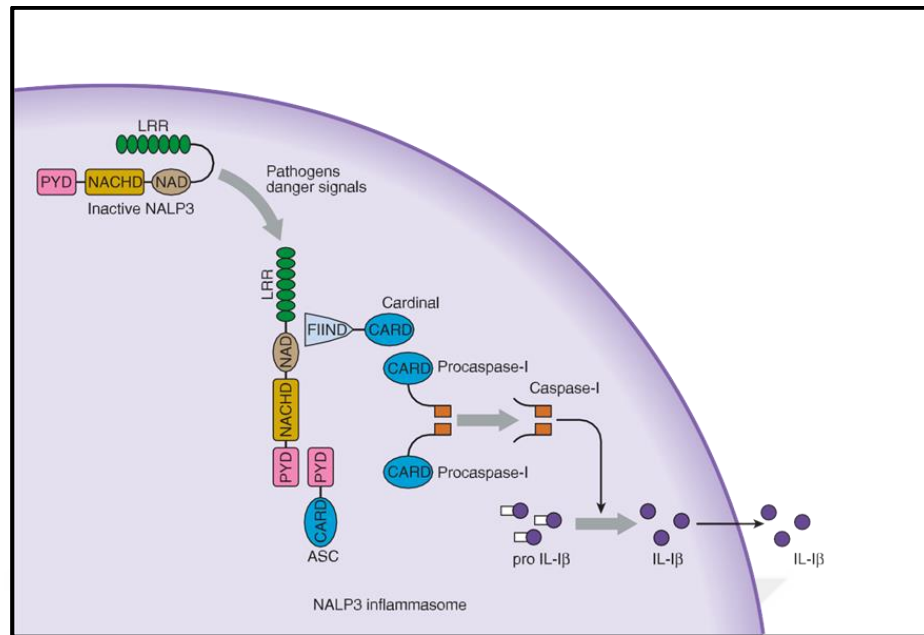


Figure 2.10 The inflammasome; how the pro-IL-1 β converted into active IL-1 β by the NLRP3 inflammasome. Through signaling, different PAMPs or DAMPs cause pro-IL-1 to production (Leung *et al.* 2010).

Numerous cytoplasmic stimuli, such as microbial products, naturally occurring or endogenous crystals, and decreased cytosolic potassium ion concentrations, can cause NLRP-inflammasome reactions. These stimuli are frequently related to infections and cell stress (Abbas *et al.* 2014). How IL-1 β really released from cells is still unknown, despite developments in the mechanism of IL-1 β processing and the finding that additional leaderless proteins are involved in innate immunity. This is mostly because of technical issues, such the challenges involved in reconstructing this secretory route. Additionally, there is no evident shared sequence or other characteristics between IL-1 β and the other leaderless secretory proteins that may be used to pinpoint their binding partners and comprehend the mechanism governing their intracellular pathway from the cytosol to the extracellular space (Lopez-Castejon and Brough 2021).

Additionally, a variety of proteases, including the typical neutrophil protease proteinase-3, elastase, matrix metalloprotease 9, and granzyme B, as well as a mast cell chymase, can convert the IL-1 β (and IL-18) precursor extracellularly into an active cytokine (Afonina *et al.* 2015). In the last 23 years, a number of processes have been put out; some are exclusive to IL-1 β , while others, may be shared by other leaderless secretory proteins,

such as: IL-1 can be externalized directly at the plasma membrane or through vesicles during IL-1 β secretion. In several cell types (primary monocytes, monocyte continuous cell lines, murine macrophages), secretory lysosomes, micro vesicles shed from the plasma membrane, and exosomes have been discovered as vesicles able to transport IL-1 β out of the cell (MacKenzie *et al.* 2001, Piccioli and Rubartelli 2013).

2.6.3 IL-1 β and its role in SARS-CoV-2 and T2DM

T2DM characterized by both insulin resistance and relative insulin insufficiency. While insulin resistance is always present in the early stages, the rate at which the illness manifests itself determined by β -cell failure. The development of medications that target inflammatory cytokines has been sparked by mounting evidence that T2DM is a disease in which the immune system is engaged and plays a significant role (Eguchi and Manabe 2013).

Inflammatory reactions' potential significance in the pathophysiology of T2DM has been supported by growing research in recent years. Failure of β -cells has been linked to IL-1 β . Upon stimulation with glucose, " β -cells" themselves produce IL-1 β . Further, IL-1 β attracts macrophages, which can serve as an additional source of IL-1 β and other cytokines, and increases IL-1 β production in β -cells. Therapies that target IL-1 β have demonstrated significant progress, but with mixed outcomes in various clinical studies, despite the fact that it is still unknown whether inflammatory responses are a fundamental cause or a subsequent impact in the evolution of T2DM (Zhao *et al.* 2014). Additionally, the raised glucose concentrations that result in elevated levels of IL-1 within cells of the pancreas and lower levels of IL-1R antagonist in islets of T2DM individuals leading to inhibiting the cell differentiation, reduced insulin production, and increase β cells demise (Böni-Schnetzler and Donath 2013). An earlier epidemiological research found that the increase of each of cytokines IL-6 and IL-1 was linked to an approximately three-fold greater incidence of T2DM, and furthermore, IL-1 β may cause resistance in body cells to insulin by triggering the IKK- β . (Spranger *et al.* 2003) reported elevated IL-1 β levels significantly correlated with reduced insulin concentration, decreased β -cell activity, and increased fasting hyperglycemia.

On the other hand, they discovered that the C allele of IL-1 β -511 enhanced the the incidence of T2DM in a research that examined the connection of IL-1 β -511 genotypes with T2DM in an endogamous Northern Indian community. The TC and CC genotypes increased T2DM risk as compared to TT, while the T allele had a protective impact. In bronchial alveolar lavage fluid samples from individuals with acute Covid-19, high expression values of cytokines such IL-1 β have seen. Because of the inflammatory response caused by cytokines such IL-1 β , the SARS-CoV-2 mostly causes lung injury (Doody *et al.* 2017).

There is growing evidence with indicate that people during acute Covid-19 infection; experienced cytokine storm. VEGF, TNF- α , PDGF, MIP1-B, MIP-1A, MCP-1, IP-10, IL-1 β , IL-7, IL-8, IL-9, IL-10, IFN- γ , GM-CSF, G-CSF, and FGF, levels were found to be higher in patients admitted to the ICU than in individuals who were not, as compared to healthy people. All trial participants had pneumonia, and 1/3 of these patients died after being brought to the ICU (Huang *et al.* 2020). According to a different research, patients with serious Covid-19 had a tendency to develop a strongly correlated (and extended) cluster of IL-1 cytokines, as well as a tendency for an IFN- γ /IL-6 cluster to develop correlation with TNF- α . Overall, research suggests that the severity of Covid-19 illness may be distinguished by its highly pro-inflammatory cytokine co association profile (Vanderbeke *et al.* 2021). Additionally, IL-1 β , IL-6, TNF- α , and CXCL-10 levels were considerably greater in early-phase infected persons and asymptomatic individuals in an Indian investigation, while they were significantly lower in recovered patients after 45-65 days. The study conducted by (Tripathy *et al.* 2021) suggests that IL-6, IL-1 β , TNF- α , CXCL-10, and decreased antiviral cytokines may employed as biomarkers of Covid-19 marker infection, and immune systems of those individuals with Covid-19 may take longer to normalize.

3. MATERIALS AND METHODS

3.1 Samples Collection

Participants chosen without consideration for sex or race. Participants who attended the (Al-Wafaa Center for Diabetes and Endocrine Disorders at the Ibn-Sina Hospital in Mosul, Iraq), and signed up for the study had a median age of 50 years, ranging from 23-65 years old, and start the collecting at 30 August 2021 until 10 November 2021.

75 non-obese participants were involved in this study and divided into three groups:

- 25 patients with T2DM, insulin-dependent for over 3 years as the only medication with no other comorbidities.
- 25 diabetic patients with the same characteristics to group 1, who recovered from SARS-CoV-2 infection after 8-12 weeks after symptoms onset.
- Healthy control group.

3.2 Blood Glucose Test

Employing an enzymatic colorimetric kit to measure the random glucose concentration during the examination as per the manufacturer's recommendations (Biolabo Kit, French manufacturer of Reagents for Medical Biology). A red quinoneimine created when peroxidase oxidized glucose, interacts with chloro-4-phenol and PAP (4-amino antipyrin). The absorbance of the colored complex, measured by a spectrophotometer at 500 nanometers, related to glucose concentrations in the specimens (Figure 3.1).



Figure 3.1 Spectrophotometer/EMC-11D-V/ used in measuring blood glucose of samples

3.3 White Blood Cell Count (WBCc) Test

Auto hematology analyzer (Figure 3.2) has used to identify cell counts in the research participants' blood samples, giving each participant in the study a comprehensive image of their blood. The proportion of each cell type has calculated by dividing the percentage of each cell type results from the test by (100 cell) to find the real number of the cells (Table 3.1).



Figure 3.2 Auto hematology analyzer

Table 3.1 Tools and devices used in the research

Devices	Manufacturer
Auto hematology analyzer (CBC)	Rayto RT-7600/China
qRT-PCR	Stone Staffordshire OSA-UK, ST15
Nanophotometer N50	Implen Gmbh-Germany
Incubator	Syria
mixer Vortex	Gemmy industrial coopration-Taiwan
Centrifuge	Poland- MPW
EMC-11D-V, single beam/spectrophotometer	EMCLAB/Germany
Refrigerator	Whirlpool/USA

3.4 Determination of Phagocytic Activity of Neutrophil

3.4.1 Nitro-blue tetrazolium (NBT) test

To determine how redox potential affects cell viability, utilize nitro-blue tetrazolium (NBT) chloride. The water-soluble NBT transformed to a dark blue precipitate by actively respiring cells, whereas the NBT test assay assesses the ROS produced by different phagocytic cells. Free oxygen radicals (O_2) decrease NBT stain, generating a blue-black compound termed (formazan) (Agarwal *et al.* 2018).

3.4.2 Peripheral blood smear preparation with NBT

- A sterile plastic pipette has used to transfer around 50 μ L of NBT stain into a plastic vial.
- A gentle mixture of NBT solution and 50 μ L of heparinized blood has performed.
- The incubation procedure took place for 30 minutes at 37 °C.
- Five μ L of the liquid were deposited using a plastic pipette onto a spotless glass slide
- To prevent mechanical harm to the more delicate formazan-containing cells, a relatively thick smear has formed.
- Leishman Stain used to treat the smear.

3.4.3 Microscopic examination and counting

A stained smear that examined with an oil immersion objective and had at least 100 neutrophils in it overall. The presence of formazan deposits on neutrophils reported as positive, Light microscopy was used to count the proportion of cells that were positive for formazan under a 100-x magnification lens. NBT penetrates cells without restriction, and phagosomes reduce the dye intracellularly to produce an insoluble blue crystalline form (formazan crystals). Microbicidal action depends on intracellular reduction, which triggered by activation of the hexose monophosphate shunt. This equation used in the study to calculate the proportion of neutrophils Equation (3.1).

$$\% \text{ of neutrophils} = \frac{\text{Number of neutrophils with formazan}}{\text{Total number of neutrophils}} \times 100 \quad (3.1)$$

3.5 Molecular Study

3.5.1 Measuring the expression of IL-1 β

3.5.1.1 Extraction of DNA

The AddPrep genomic extraction kit was used to extract DNA (Figure 3.3) (Table 3.2) as seen below:

- To a 1.5 mL microcentrifuge tube, 20 μ L of proteinase-K-solution equal to 20 mg/mL were added
- Transfer 200 μ L of blood plasma to a 1.5 mL microcentrifuge tube containing Proteinase K solution
- 200 μ L of binding-solution was added to the tube of sample, and fully shuffled with vortex mixer for 15 seconds.
- The tube sample has incubated at 56°C for 10 minutes; a prolonged incubation time would have no impact on quality of purified DNA or efficiency.

- 200 μ L of absolute ethanol has added and mixed for 15 seconds. Then, the sample spin down briefly to allowing the droplets to attach underneath the cap.
- The lysate carefully transferred to the upper reservoir of the spin column in addition to 2.0 mL collection tube with avoiding wetting the margins.
- The fluid placed into and the spin column inserted with a 2.0 mL collection tube and centrifuged for one minute in 13.000 rpm.
- 500 μ L of washing-1-solution added to the spin column with collection tube and centrifuged for one minute at 13,000 rpm; the effluent and mount the spin column with a 2.0 mL collection tube poured.
- 500 μ L of washing-2-solution added to the spin column with collection tube and centrifuged for one minute at 13.000 rpm; the effluent and mount the spin column with a 2.0 mL collection tube poured.
- The sample then centrifuged for an extra minute at 13.000 rpm to remove the remaining ethanol from the spin column to dry it.
- The spin column then transferred to a new 1.5 mL microcentrifuge tube
- 100~200 μ L of elution fluid has added to the spin column with a microcentrifuge tube and left for 1 minute to reset.
- The sample then centrifuged at 13.000 rpm for 1 minute and then the DNA eluted.

Table 3.2 Kits used in the research

Kits	Manufacturer
Blood glucose kit	BIOLABO/French manufacturer
extraction for DNA	(addpreb)-extraction-Korea
qRT-PCR	Promega Company-United State



Figure 3.3 (A) qRT-PCR, (B) kit for PCR

3.5.1.2 Nanodrop-photometer

After the extraction of DNA, Nano drop-photometer device (Figure 3.4) employed to determine DNA concentration in every sample.



Figure 3.4 Implen GmbH-Nano drop

Principle:

Nucleic acids absorb light in the solution with a maximum at 260 nm in the UV region. The Beer-Lambert law used along with absorbance at 260 nm to calculate the concentration of genetic material in samples. Beside of determining nucleic acid contents, absorbance data can be utilized to evaluate nucleic acid pureness in addition to determining nucleic acid by measuring the 260/230 nm and 260/280 nm values. Additionally, probes that contain fluorescent dye could well utilized to assess the level of nucleic acid tagging.

The Procedure

- Selecting the DNA technique from the homescreen.
- The proper amount of blank solution has pipetted onto the illuminated sample window using a micropipette. When the arm dropped, the illumination automatically goes off.
- Drop the specimen arm and press the blank button to start a blank measurement.
- The measuring window and mirror on the sample arm has wiped by ethanol 70% and water.
- The vortex mixer utilized to mix the sample in order to achieve a homogenous state.
- Place the proper amount of sample solution pipetted on the visible sample window. After the measurement is completed, the sample arm was raised, the surfaces wiped, and the next sample added (Nano-Photometer User Guide).

3.5.1.3 Quantitative polymerase chain reaction (qPCR)

PCR allows many copies of a DNA fragment to be obtain from an extract via in vitro replication. It is a method for extracting significant volumes of a particular DNA sequence from a DNA sample. The replication of a double-stranded DNA template used in this amplification. It is divided into three stages: denaturation, primer hybridization, and

elongation. The products of each synthesis step act as templates for subsequent steps, resulting in exponential amplification (Kadri 2019).

In order to measure relative gene expression, Promega's (GoTaq-qPCR) master mix (A6000) was used (Figure 3.3). For each specimen, the reaction repeated for every single gene of interest in addition to maintenance genes. Cts has measured for our samples to compare the variations in gene expression. While, the ΔC_t for each specimen was the difference between the C_t gene of interest and the C_t of internal gene, $\Delta\Delta C_t$ has been estimated as the difference between the ΔC_t values of the test and control samples. The fold differences in gene expression are calculated by $\Delta\Delta C_t$. The specimens prepared by the DNA content adjusting to 10 ng/mL in a final volume of 3.5 μ L of nuclease-free water, and this volume was deposited in each experimental well of a cooled 96 PCR-plate (Sigma, UK) and maintained in ice state until utilized. 6.5 μ L of GoTaq-qPCR, 4 μ L of DNase ultrapure RNase-free distilled water, and 0.5 μ L of each related primer (Table 3.3) were combined to prepare master mix. Mix directly transferred to the PCR machine. 2 μ L of specimens then added to the well-mixed master mix, an optical colloidal coating (Thermofisher, UK) was used to lock the plate, and the plate then transported to the Strata-gene (Mx3005P). Real-Time heat processor (Ecosystem-Germany) utilized qRT-PCR software (MxPro3005P) (Figure 3.5) (Ecological Research Software).

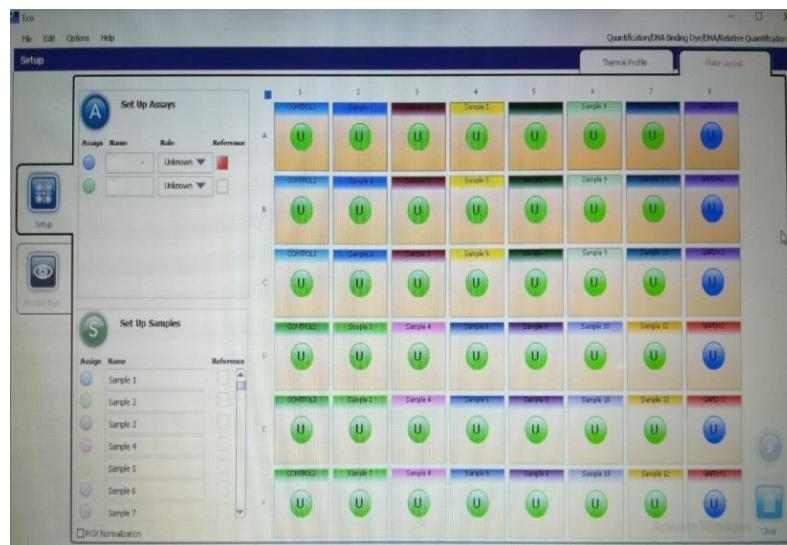


Figure 3.5 qPCR software MxPro3005P (Ecological Research Software)

Table 3.3 IL-1 β Gene primer that used in this study in human

Gene primer	Sequence (5'→3')	Length	Weight	Tempreture
Homo sapiens IL-1 β	Forward- TAAAGGGCTTTGGCTCTGGG Reverse- CTCGGGATTCTTTCAAGCCC	20mer	31 kDa	59.96°C 58.62°C

3.6 Statistical Analysis

- The study used the ready-made software (SPSS version 26) to find the arithmetic means and standard deviations in addition to calculating the minimum and maximum value as well as testing the statistical hypotheses.
- Comparisons of means analyzed statistically using a Kruskal-Wallis Test to perform independent comparisons between the eight parameters for the three groups of study.
- Shapiro-Wilk analysis employed in the study to gauge how closely the probability distribution of the variables and dimensions under consideration matched the normal distribution.
- P value used for probability as $p < 0.05$.

4. RESULTS AND DISCUSSION

4.1 Molecular Study

4.1.1 Investigating of IL-1 β expression

To examine the expression of the IL-1 β gene in the research participants, the study employed five blood samples from each of the three-study groups. The qRT-PCR instrument used to evaluate the expression of the proinflammatory cytokine gene IL-1 β . Interestingly, this study found there were a significant increase in the secretion of the IL-1 β in T2DM.R (28.1 ± 0.6) compared to those in T2DM group (23.8 ± 0.8), while in control group, the mean value was (25.9 ± 2.8) (Figure 4.1) (Figure 4.2).

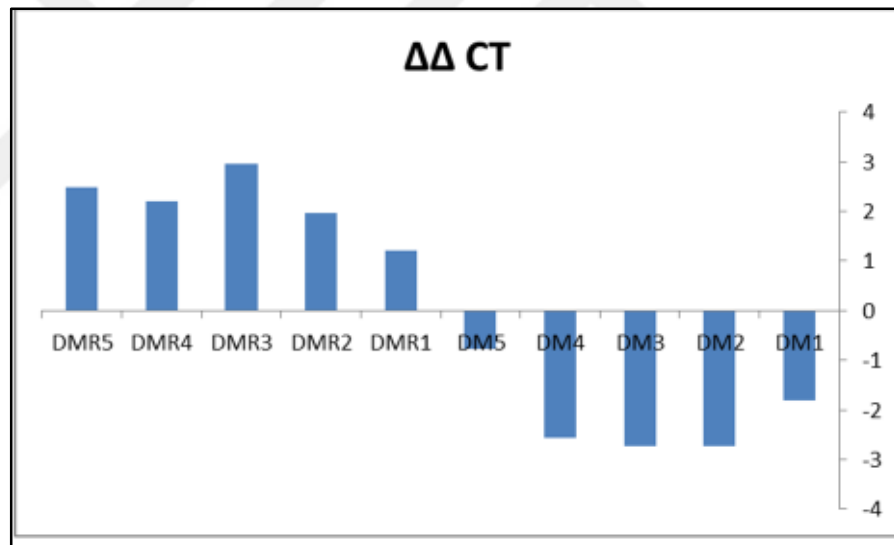


Figure 4.1 Difference in IL-1 β gene expression in monocyte between the diabetic patients groups. The 0 line refer to control group, DM refer to T2DM, and DM.R refer to T2DM.R

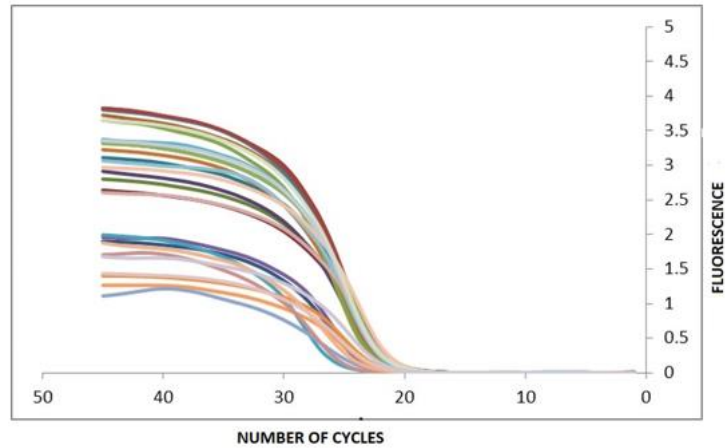


Figure 4.2 The components of amplification curve, this figure represent the increase of fluorescence with the number of cycles for different samples during amplification process.

All cells of the innate immune system express IL-1 family members and/or are affected by them. Moreover, each inflammatory cytokine within the family of IL-1 is essential for the development and function of both innate and adaptive immune cells (Garlanda *et al.* 2013). The results of this study are consistent with a clinical investigation study conducted in Wuhan, China, on 129 diabetic patients who were infected with SARS-CoV-2, which showed a considerable rise in the release of the proinflammatory cytokine IL-1 in the third week following admission (Han *et al.* 2021). According to another in vitro experiment conducted by (Rodrigues *et al.* 2021) on monocytes infected with SARS-CoV-2, they found that the virus induces IL-1 β production by activation of caspase-1-dependent cleavage, releasing of proinflammatory cytokines such as IL-18, and aderman D-mediated pyroptotic cell death. In addition to the degree of NLRP3 activation correlates with the severity of Covid-19 disease.

The link between IL-1 β and T2DM is an additional important factor. Several preclinical and clinical studies supported the assumption that a metabolic stress (such as an elevated glucose concentration) could induce IL-1 β production (resulting in an inflammatory state) from human islets, thereby impairing insulin secretion; This was the first indication that IL-1 cytokine might contribute to the emergence of T2DM (Donath 2014). Another notable observation is that post-meal hyperglycemia activates inflammasomes in macrophages derived from omental fat. This results in the processing of pro-IL-1 β and

the release of IL-1 β , which in turn increases glucose-stimulated insulin secretion, macrophage glucose uptake, and additional IL-1 β production, producing a feedforward loop in which insulin and IL-1 β enhance glucose disposal in concert (Dror *et al.* 2017).

Indeed, several factors found to contribute to the severity of SARS-CoV-2 infections. Diabetes, along with age and CVD, is one of the most consistently related comorbidities with poorer prognosis in Covid-19 patients (Mazucanti and Egan 2020). Recent research on patients with DM revealed an increase in proinflammatory cytokines such as IL-1 β , leading in adverse outcomes and complications. Due to the high prevalence of T2DM, however, the majority of current evidence does not distinguish between the main types of DM associated with the infection (Lim *et al.* 2021).

Finally, it seems that there is a bidirectional association between SARS-CoV-2 infection and DM. Firstly, individuals with DM are more likely to experience significant infection-related outcomes than people who does not live with DM. sccondly, medical care unit studies indicate that Covid-19 virus might induce biochemical imbalance in addition to glucose homeostasis abnormalities. The notion that SARS-CoV-2 has a direct impact on glucose metabolism was further supported by new data on individuals with SARS-CoV-2 who developed diabetes for the first time (Kazakou *et al.* 2022).

4.2 Statistical Analysis

4.2.1 Glucose level

Our study found that there are significant differences between two groups of the three study groups, T2DM, T2DM.R patients, and control in glucose level (Table 4.1) (Figure 4.3). This study also found that glucose level is slightly higher but non-significant in-group of T2DM.R compare with the diabetes group T2DM without infection, with mean values shown in Table 4.2 respectively, and T2DM.R and T2DM were significantly higher in comparison to control group. However, to identify which pair of the three groups

showed significant differences, the study conducted the same test for each case (Table 4.1).

Table 4.1 Independent comparisons between each pair of the study groups in glucose level, (* $P \leq 0.05$ refer to significant differences)

Pairwise Comparisons of Glucose level		
Sample 1-Sample 2	Test Statistic	P-value
Control-T2DM	31.440	0.000*
Control-T2DM.R	38.880	0.000*
T2DM-T2DM.R	-7.440	0.227

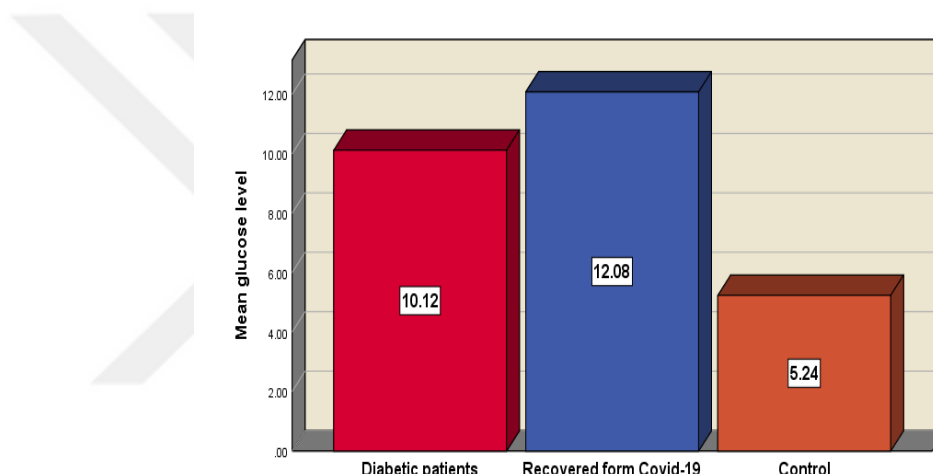


Figure 4.3 Differences in mean glucose levels between the three study groups

Table 4.2 Mean values and standard deviation ($\pm$ Std.) for samples of each study group in glucose level.

Variable	Group	Mean	95% Confidence Interval for Mean		$\pm$ Std.	Minimum	Maximum
			Lower Bound	Upper Bound			
Glucose level	T2DM	10.1200	8.1187	12.1213	4.84845	3.70	24.60
	T2DM.R	12.0800	9.9385	14.2215	5.18805	6.20	22.20
	Control	5.2360	5.1461	5.3259	0.21772	5.00	5.60

Multiple clinical studies have demonstrated that Covid-19 individuals are prone to significant glucose dysregulation. A remarkably high incidence of diabetic ketoacidosis and hyperglycemic hyperosmolar syndrome has been recorded in Covid-19 infected

patients (Cariou *et al.* 2020, Kim *et al.* 2020). (Han *et al.* 2021) demonstrated significant increased levels of fasting plasma glucose (FPG) (9.4 mmol/L) and random plasma glucose (RPG) levels (8.9 mmol/L) in patients with T2DM who infected with Covid-19 virus compare with non-diabetic individuals (5.6 mmol/L) and (6.0 mmol/L) respectively. Another study found that 47.2% of Covid-19 patients (critical and non-critical cases) had increased blood glucose levels at the time of admission (13% had diabetes), and that blood glucose level was an indirect risk element for development to severe conditions or death in mild infection (Wu *et al.* 2020). An increase in blood glucose levels could indicate relative hyperglycemia. In addition, IR may result from a cytokine storm induced by an infection. Moreover, the pancreas could be affected directly by the SARS-CoV-2 infection. These factors collectively may increase the risk of hyperglycemia in Covid-19 patients (Šestan *et al.* 2018). It should be noted that evidence from cases of SARS and MERS indicates that inflammatory cells might have an impact on the liver, inhibiting glucose intake by the cells and resulting in insulin deficiency and hyperglycemia as a result (Channappanavar and Perlman 2017). Consequently, during Covid-19 infection, inflammation and immunological responses can decrease sensitivity to insulin and inhibit glucose metabolism, resulting in a detrimental cycle of high blood glucose and inflammatory response that demolishes tissue integrity and biochemical reactions. In addition, it has been discovered that the pancreas and endothelium tissues that express ACE2 receptors are the virus's primary binding site. To summarize all above, the examination of the impacts of Covid-19 on metabolism and glucose regulation is crucial in avoiding and managing the infection consequences and supporting convalescent individuals (Kazakou *et al.* 2022).

On the other hand, glycemic decline is a common consequence of Covid-19 in patients with impaired glycemic control or diabetes (Wu *et al.* 2020). The results of this study contradicting those of previous studies that suggested that blood glucose levels below the median of 6.1 mmol/L would increase the risk of hypoglycemia and mortality in diabetic individuals (Yamada *et al.* 2017). One explanation could be because diabetic individuals were the focus of earlier research, and hypoglycemia is more likely to result in cardiovascular events, which raise mortality. However, compared to individuals with diabetes, those with Covid-19 had a decreased frequency of cardiovascular comorbidities.

Therefore, the survival advantages of lowering blood glucose level does not exceeded by a modest increase in the risk of hypoglycemia. This finding supports the significance of thorough blood glucose monitoring and regulation throughout the management of Covid-19 (Wu *et al.* 2020).

4.2.1.1 Correlation coefficient

Correlation coefficient between glucose level and other parameters in T2DM.R group:

- The statistical analysis of our study shows that there was a positive and significant correlation between elevated glucose level in group of T2DM.R and the increase of absolute number of neutrophil and absolute number of phagocytic activity, which appeared equal to p-value (0.049), (0.001) respectively Table 4.3 and Figure 4.4. While in the same group, there is a positive and an inverse correlation but it was non-significant.
- There is a direct, but non-significant relationship between glucose level and WBC count, hemoglobin, absolute number of lymphocyte and absolute number of eosinophil ($P > 0.05$).
- There is an inverse correlation, but it is not significant between glucose level and absolute number of monocyte ($P > 0.05$) (Table 4.3) (Figure 4.4).

Table 4.3 Correlation between glucose level and other parameters in T2DM.R group

Correlations								
		WBC count	Hemoglobin	Absolute No. of Neutrophil	Absolute No. of Lymphocyte	Absolute No. of Monocyte	Absolute No. of Eosinophil	Absolute No. of phagocytic cells
Glucose level	Pearson Correlation	0.328	0.019	0.397*	0.048	-0.146	0.328	0.605**
	P-value	0.110	0.926	0.049	0.820	0.486	0.110	0.001
	N	25	25	25	25	25	25	25
*. Correlation is significant at the 0.05 level (2-tailed).								
**. Correlation is significant at the 0.01 level (2-tailed).								

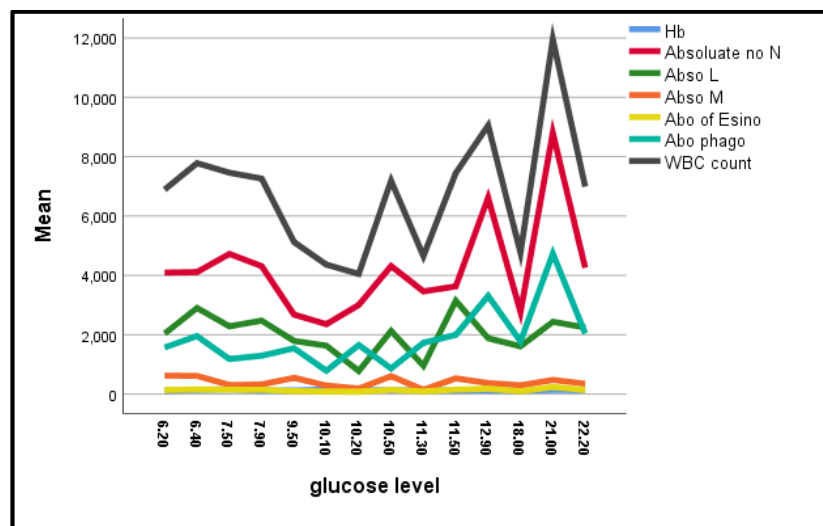


Figure 4.4 Correlation between glucose level and other parameters in T2DM.R group

Correlation coefficient between glucose level and other parameters in T2DM group:

- In the group of T2DM the statistical analysis shows an inverse correlation, but non-significant between glucose level and WBC count, hemoglobin, absolute number of neutrophil, absolute number of monocyte, absolute number of eosinophil and absolute number of phagocytic activity ($P > 0.05$).
- There is a direct, but non-significant correlation between glucose level and absolute number of lymphocyte ($P > 0.05$) as shown in Table 4.4 and Figure 4.5.

Table 4.4 Correlation between glucose level and other parameters in T2DM

Correlations								
		WBC count	Hemoglobin	Absolute No. of Neutrophil	Absolute No. of Lymphocyte	Absolute No. of Monocyte	Absolute No. of Eosinophil	Absolute No. of phagocytic cells
Glucose level	Pearson Correlation	-0.093	-0.051	-0.064	0.109	-0.259	-0.093	-0.288
	P-value	0.659	0.809	0.763	0.603	0.211	0.659	0.163
	N	25	25	25	25	25	25	25
**, Correlation is significant at the 0.01 level (2-tailed).								
*, Correlation is significant at the 0.05 level (2-tailed).								

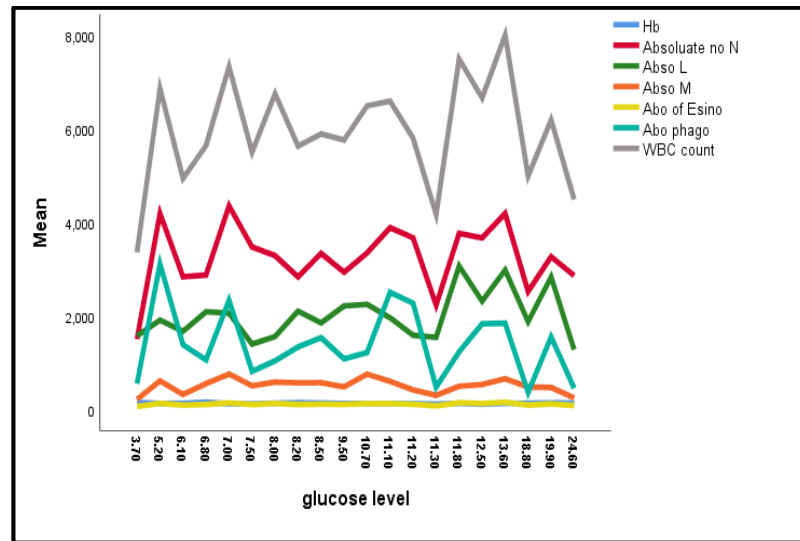


Figure 4.5 Correlation between glucose level and other parameters in T2DM group

Infection with the SARS-CoV-2 virus found to have a notable influence on both impaired immunological function and glucose homeostasis. The study discovered that infection with SARS-CoV-2 increased glucose level in comparison to those without infection, even though the patients in this study had a moderate illness and were already receiving insulin injections as a treatment for lowering blood sugar. Indeed, the cause of hyperglycemia in Covid-19 individuals is under investigation, and it most likely has a variety of causes. In fact, SARS-CoV-2 may induce-cell dysfunction, either directly by encouraging a damage to this cell type or indirectly by increasing the levels of circulating pro-inflammatory cytokines that drive cell death. Furthermore, because soluble cytokines known to interfere with the insulin pathway, the induced inflammatory response may promote or aggravate insulin resistance (Ceriello *et al.* 2020).

Diabetes is widely recognized to be related with dysfunctional innate and adaptive immunity; hence, it is hypothesized that decreased immune function in diabetes patients may exacerbate SARS-CoV-2 infection and its detrimental role (Donath *et al.* 2019). Hyperglycemia and IR in DM can stimulate the releasing of a several inflammatory cytokines in addition to adhesion molecules, exacerbating the inflammation state and oxidative stress in the body (Petrie *et al.* 2018). The mean of neutrophil-to-lymphocyte ratio (NLR) and neutrophil-to-monocyte ratio (NMR) were higher in diabetic patients

than in non-diabetic individuals. Furthermore, the NLR and NMR found to be positively linked with Covid-19 disease severity (Anurag *et al.* 2020).

According to data collected in a laboratory, (Shang *et al.* 2021) revealed that diabetic individuals had lower lymphocyte counts and greater levels of neutrophils, C-reactive protein, and procalcitonin than patients without diabetes. Additionally, it had been observe that high glucose levels facilitate the multiplication of SARS-CoV-2 in monocytes, which inhibits the T-cell response (Codo *et al.* 2020).

The present study discovered that in diabetic individuals with SARS-CoV-2 infection, the glucose level substantially linked with the number of neutrophil and their phagocytic activity. Neutrophils are innate immune cells that exist in a latent, primed, or active condition after leaving the bone marrow. Because they are among the first innate leukocytes recruited during infections, these leukocytes are key participants in innate immunity. The fundamental function of neutrophils is the phagocytosis of microorganisms and debris. They also play a variety of other immunological roles, including as the releasing of NETs for viral infection clearance and the secretion of cytokines to limit virus replication (Borges *et al.* 2020).

In line with the findings of this investigation, (Sun *et al.* 2020) tested 116 Covid-19 patients and observed a higher NLR. The researchers compared severe Covid-19 patients hospitalized to the ICU with others severe patients not admitted to the ICU, According to their findings, Covid-19 patients have the lowest lymphocyte counts and the greatest neutrophil counts and NLR (Sun *et al.* 2020). (Wang *et al.* 2020) similarly reported that during the severe state, several SARS-CoV-2 infected individuals had higher neutrophil cells and a dropped lymphocyte levels. In individuals with severe SARS-CoV-2 infection, an elevated total leukocyte count and a differential neutrophil count frequently observed. In addition, (Yuan *et al.* 2020) found comparable results in severe Covid-19 cases.

Furthermore, (Anurag *et al.* 2020) revealed that lymphopenia was substantially associated with severe infection, and that differential monocyte count and differential eosinophil count were adversely correlated with disease severity. There was a strong positive link

between NLR and neutrophil count with disease severity, ICU admission, and death; this aligns with (Liu *et al.* 2020)'s findings that NLR is a good indicator of Covid-19 serious illness in the early stages of the disease.

In addition, Decreased cell count or reduced leukocyte function, on the other hand, can have a role in the progression of mild to severe clinical illness states (Lamichhane and Samarasinghe 2019).

In Covid-19 patients, the NLR, a well-known indicator of infection and systemic inflammation, showed an increased inflammatory response (Qin *et al.* 2020). Because ARDS is the major reason for mortality in Covid-19 individuals, increased NLR values indicate a bad prognosis in SARS-CoV-2 infection, particularly severe illness compared to mild patients (Lagunas-Rangel 2020).

The current research reveals that SARS-CoV-2 infection raised glucose levels, NLR, and IL-1 β production in diabetic individuals compared to non-infected patients. These findings are consistent with those of (Zhu *et al.* 2020) who discovered that despite getting more intensive Covid-19 and comorbidity treatment, the diabetic group had higher rates of low lymphocyte counts and higher neutrophil counts. As well as increased serum IL-6, CRP, and LDH in addition to greater BG levels when compared to the non-diabetic group. As a result, early glycemic regulation has proposed as a useful therapeutic approach for improving outcomes in hospitalized hyperglycemic patients, Covid-19 patients with or without a history of diabetes (Zhu *et al.* 2020).

4.2.2 WBC count

The results of this study showed significant differences between T2DM, T2DM.R and control in WBC count ($P \leq 0.05$) (Table 4.5).

Table 4.5 Pairwise comparisons of WBC count between the three groups (*P≤0.05)

Pairwise Comparisons of WBC count		
Sample 1-Sample 2	Test Statistic	P-value
T2DM-T2DM.R	-7.420	0.229
T2DM-Control	-26.240	0.000*
T2DM.R-Control	-18.820	0.002*

From the observation of the results of Table 4.6, the study found that there is a significant difference in the total numbers of WBC count between T2DM, T2DM.R in comparison with control (P=0.000 and P=0.002, respectively). There were no significant differences between T2DM and T2DM.R (P>0.05).

In more details, T2DM and T2DM.R shows significant decreases in absolute number of WBC count compared with control group, while the group of T2DM showed non-significant decreases compared with T2DM.R as shown in Table 4.6.

Table 4.6 Mean values and standard deviation (±Std.) for samples of each group in WBC count

Variable	Group	Mean cell/μL	95% Confidence Interval for Mean		±Std.	Minimum	Maximum
			Lower Bound	Upper Bound			
Absolut No: of WBC count	T2DM	6048.00	5572.94	6523.06	1150.884	3360	8000
	T2DM.R	6700.00	5792.19	7607.81	2199.271	4050	11950
	Control	8272.00	7568.50	8975.50	1704.289	5200	12000

WBC counts determine the number of leukocytes in the blood. In a study, the proportion of circulating WBCs was somewhat higher in the early phase of an infection and in patients who had recovered, but it decreased in healthy blood donor controls (Manunta *et al.* 2021). In contrast, a study conducted on a convalescent patient to test the immunological response to SARS-CoV-2 after recovery revealed that there were no significant variations in WBC counts, T-cells, or B-cells between the groups during the course of the investigation (Pan *et al.* 2021). Additionally, (Files *et al.* 2021) discovered

that hospitalized SARS-CoV-2-infected participants in their study had normal WBC counts but markedly decreased numbers of lymphocytes.

4.2.3 Monocytes

Our results showed that there is a significant differences between T2DM and T2DM.R ($P=0.009$), while there were no significant differences between T2DM.R and T2DM compared with control ($P>0.05$) (Table 4.7). Indeed, the results showed that absolute number of total monocyte are significantly decreases in T2DM.R compare with the T2DM (Table 4.8). On the other hand, the group of T2DM of our study showed increased mean value but non-significant in total number of monocyte than in both T2DM.R and control groups (Table 4.8).

Table 4.7 Pairwise comparisons of absolut number of monocyte between groups as pairs (* $P\leq 0.05$ =significant)

Pairwise Comparisons of Absolute No: of Monocyte		
Sample 1-Sample 2	Test Statistic	P-value
T2DM.R-Control	-8.160	0.557
T2DM.R-T2DM	18.240	0.009*
Control-T2DM	10.080	0.306

Table 4.8 Mean values of absolute number of monocyte in the three study groups

Variable	Group	Mean Cell/ μ L	95% Confidence Interval for Mean		$\pm$ Std.	Minimum	Maximum
			Lower Bound	Upper Bound			
Absolute No: of Monocyte	T2DM	539.7100	469.7416	609.6784	169.50553	221.76	919.62
	T2DM.R	382.8916	320.1524	445.6308	151.99201	144.15	620.10
	Control	461.2000	380.2706	542.1294	196.05951	208	1080

These results are consistent with a study on SARS-CoV-2-infected people (hospitalized and non-hospitalized groups), which revealed decreased CD14+ monocyte frequencies in hospitalized people compared to healthy and non-hospitalized people, as well as decreased CD16+ monocyte frequencies in hospitalized people over non-hospitalized

people. In contrast, the non-hospitalized group in the same study had more double-positive CD16⁺ and CD14⁺ monocytes than healthy controls did (Files *et al.* 2021). While (Manunta *et al.* 2021) indicated that the whole blood monocytes exhibited fewer fluctuations than the enriched fractions and that the populations were more compact in all three cohorts (early phase infection, after discharge, and healthy). The number of total monocytes reduced in infected individuals with a slight increase in discharged recovered patients. (Kwiecień *et al.* 2021) demonstrated that Covid-19 patients had lower absolute monocyte numbers than convalescent patients, also the Covid-19 patients had a lower proportion of monocytes than convalescent patients.

In contrast, (Wen *et al.* 2020) discovered an increase in monocytes among Covid-19 patients in the ERS. In addition, the ERS had a bigger proportion of classical CD14⁺⁺ monocytes with high inflammatory gene expression and a larger number of CD14⁺⁺IL-1⁺ monocytes.

In mild and asymptomatic cases of SARS-CoV-2 infection, monocytes increased, although they were higher in severe cases, as demonstrated by (Carsetti *et al.* 2020). Additionally, the results of a study on SARS-CoV-2 convalescent patients after a two-month recovery period revealed that both intermediate monocytes and NK cells had already recovered at the two-month evaluation and were stable (Kostopoulos *et al.* 2021). (Manunta *et al.* 2021) also discovered that the number of lymphocytes and monocytes in circulation was decreased in early phase Covid-19 patients.

Currently, the pathogenesis of T2DM believed to be associated with both innate and adaptive immunological mechanisms, which identified as key pathogenic contributors in the development of insulin resistance. Moreover, the abnormalities of innate immunity, adaptive immunity, or both, were implicated in the progression of T2DM, and interacted with each other (Zhou *et al.* 2018). Several studies have demonstrated higher monocyte activation in T2DM patients relative to healthy controls (Yang *et al.* 2012, Dai *et al.* 2015, Malandrino *et al.* 2015). Others, however, have shown no significant changes in monocyte levels between T2DM and control groups (Gacka *et al.* 2010). The exact role of monocytes in the pathogenesis of T2DM remains elusive. In addition, (Adrover *et al.*

2020) found a correlation between an increase in monocyte activation in T2DM patients and an increased risk of developing CVD compared to the control group. TNF- α and IL-6 produced from monocytes were similar between T2DM patients and healthy controls. Changes in TNF- α metabolism may trigger the onset and progression of T2DM (Adrover *et al.* 2020). These results may indicate that patients with T2DM have a decreasing or altered number of monocytes, that the SARS-CoV-2 infection may cause further depletion in the total number of cells, and the number will be restored during the recovery phase and remain stable to the level before infection.

4.2.4 Neutrophil and their phagocytic activity

Neutrophil and their phagocytic activities were seen in Figure 4.6. There were significant differences between T2DM and T2DM.R compared with control in the levels of absolute number of neutrophil ($P \leq 0.05$) (Table 4.9). In contrast, the results showed that there were no significant differences between T2DM and T2DM.R ($P > 0.05$).

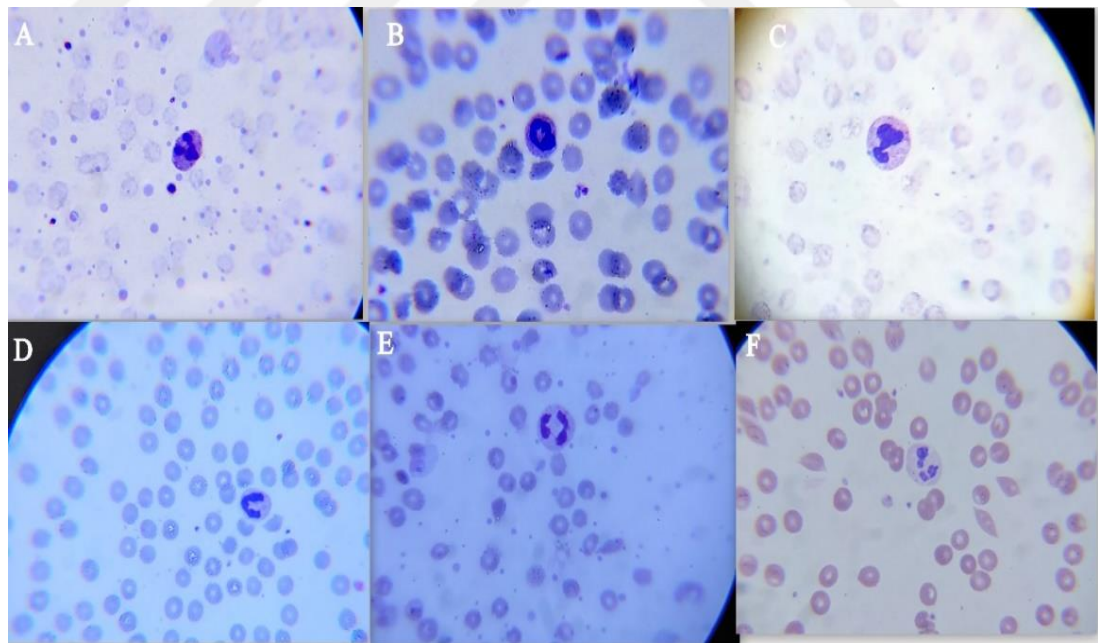


Figure 4.6 Phagocytic activity of neutrophil for different sample of the study (A, B and C), neutrophil in phagocytosis state with the presence of formazan crystals deposits on neutrophils, compare with healthy control cells in D, E, and F

Table 4.9 Pairwise comparisons of absolute number of neutrophil between groups (* $P \leq 0.05$ =significant)

Pairwise comparisons of absolute numbers of neutrophils		
Sample 1-Sample 2	Test Statistic	P-value
T2DM-T2DM.R	-11.880	0.054
T2DM-Control	-33.600	0.000*
T2DM.R-Control	-21.720	0.000*

On the other hand, the results of this study showed that there were significant differences in the levels of absolute number phagocytic activity between control and T2DM and T2DM.R ($P \leq 0.05$) (Table 4.10). While the results showed that there were no significant differences between T2DM and T2DM.R ($P > 0.05$).

Table 4.10 Pairwise comparisons of absolute number of phagocytic cell between groups (* $P \leq 0.05$ =significant)

Pairwise comparisons of absolute number of phagocytic cell		
Sample 1-Sample 2	Test Statistic	P-value
T2DM-T2DM.R	-8.360	0.175
T2DM-Control	-38.560	0.000*
T2DM.R-Control	-30.200	0.000*

In T2DM and T2DM.R showed that the value of neutrophil and their phagocytic activity are significantly decreased than in control group Table 4.11 and Table 4.12. This could explained due to hyperglycemia or dysregulation in glucose level, which cause chronic inflammation and impairment of immune system whether it was because diabetes or SARS-CoV-2-related inflammation. While, between T2DM and T2DM.R in neutrophil and phagocytic activity the data showed decreasing in absolute number but non-significant (Table 4.11) (Table 4.12).

Table 4.11 Mean values of absolute number of neutrophil in groups

Variable	Group	Mean/ Cell/ μ L	95% Confidence Interval for Mean		$\pm$ Std.	Minimum	Maximum
			Lower Bound	Upper Bound			
Absolute No: of Neutrophil	T2DM	3311.8048	3009.5929	3614.0167	732.13857	1508.64	4488.06
	T2DM.R	4246.2076	3519.7357	4972.6795	1759.95053	2354.40	8795.20
	Control	5387.9600	4903.9086	5872.0114	1172.66287	2808.00	7315.00

Table 4.12 Mean values of absolute number of phagocytic cell activity in groups

Variable	Group	Mean Cell/ μ L	95% Confidence Interval for Mean		$\pm$ Std.	Minimum	Maximum
			Lower Bound	Upper Bound			
Phagocytic cell	T2DM	1448.4809	1150.6964	1746.2654	721.41258	371.17	3114.25
	T2DM.R	1921.5757	1491.4372	2351.7142	1042.05343	784.02	4731.82
	Control	4138.3200	3718.7920	4557.8480	1016.34835	2106	6291

A study using the Sysmex XN-1500 analyzer technology on 71 convalescent SARS-CoV-2 patients with diabetes and other comorbidities found that Covid-19 patients had higher neutrophil proportions than convalescent patients and control; neutrophil proportions were significantly different between Covid-19 patients and control, as well as between convalescent patients and control. They discovered variations in neutrophil morphology and used the same technology to measure neutrophil reactivity, which they discovered was higher in Covid-19 patients than in Covid-19 convalescent patients and lower in Covid-19 patients than in control. In Covid-19 convalescent patients, a reduced proportion of the neutrophil reactivity parameter was observed (Kwiecień *et al.* 2021).

Another study, using an automated hematology cell analyzer, discovered that the population of neutrophils displayed in peripheral blood mononuclear cells isolated by polysaccharide density gradient centrifugation was less distributed in convalescent individuals after 2 weeks from hospital discharge than in SARS-CoV-2 patients after 24 hour hospitalization, with p-values (P 0.0001/24h, P = 0.0469/convalescent) (Manunta *et al.* 2021).

Indeed, there are lacks of information regarding convalescent patients, who have recovered from SARS-CoV-2, because all literatures designed to investigate innate and adaptive immunity in general, and the rise in vaccination reduces the likelihood of examining pure cases of diabetes and their relationship to SARS-CoV-2 infection. This study attempted to evaluate neutrophil activity, by analyzing phagocytic activity under a microscope to determine the impact of SARS-CoV-2 on neutrophil activity to phagocytes using the NBT. In addition, measuring the number of neutrophils alone in patients with T2DM.R and T2DM is insufficient to determine if SARS-CoV-2 infection has an effect on the activity of these cells in phagocytes in the two research groups. Thus, employing phagocytic activity as a proponent research test appears to be reasonable to quantify not only the level of neutrophils, but also their functions in the study cohorts during the recovery phase.

4.2.5 Lymphocytes

The results of this work showed that there were no significant differences between the all groups in terms of the absolute numbers of lymphocytes ($P>0.05$). Our results shows that there are a non-significant decreasing in total absolute number of lymphocyte in T2DM.R patients than in group of T2DM and control, while the group of T2DM had slightly decreasing than in control healthy (Table 4.13).

Table 4.13 Mean values of absolute number of lymphocyte in the three study groups

Variable	Group	Mean Cell/ μ L	95% Confidence Interval for Mean		Std.	Minimum	Maximum
			Lower Bound	Upper Bound			
Absolut no: of Lymphocyte	T2DM	2003.0388	1735.2652	2270.8124	648.70814	23.63	3104.70
	T2DM.R	1936.9008	1683.7116	2190.0900	613.37602	777.60	3143.90
	Control	2351.8800	2050.2977	2653.4623	730.61312	1152	3740

Consistent with these findings, a study found a lower absolute lymphocyte count in SARS-CoV-2 patients than in a group of recovered patients with comorbidities such as (diabetes, hypertension, and obesity), but no significant difference between the

convalescent and control groups (Kwiecień *et al.* 2021). Another study comparing individuals with T2DM and those without diabetes who have infected by Covid-19 virus found that people with DM had a significantly lower peripheral lymphocyte count than without diabetes (Han *et al.* 2021). Indeed, lymphocytopenia correlates with the severity of SARS-CoV-2 infection (Guan *et al.* 2020). In addition, the ratio of neutrophils to lymphocytes in T2DM patients with Covid-19 revealed an increase in neutrophil levels and a decrease in lymphocyte proportion (Yang *et al.* 2021). Moreover, a study related to SARS-CoV-2 revealed that T-lymphocytes numbers was dramatically decreased in individuals with SARS-CoV-2 virus, and the remaining were immunologically fatigued (Diao *et al.* 2020).

These data support the hypothesis of this study, that infection with SARS-CoV-2 depletes the lymphocyte population in infected patients, particularly those with DM, and that the population begins to expand again during the recovery period.

4.2.6 Eosinophils

The results of this study showed that there was a significant difference between control and other two groups in the levels of absolute number eosinophil ($P \leq 0.05$, Table 4.14). In addition, there were no significant differences between T2DM.R and T2DM.

Table 4.14 Pairwise comparisons of absolute number of eosinophil between groups (* $P \leq 0.05$ is significant)

Pairwise Comparisons of Eosinophil		
Sample 1-Sample 2	Test Statistic	P-value
Control-T2DM	24.480	0.000*
Control-T2DM.R	27.960	0.000*
T2DM - T2DM.R	-3.480	0.572

In fact, the absolute number of eosinophil in T2DM and T2DM.R were significantly higher compared to control group, while the number is lower but non-significant in T2DM compared with T2DM.R (Table 4.15).

Table 4.15 Mean values of absolute number of eosinophil in the three study groups

Variable	Group	Mean Cell/ μ L	95% Confidence Interval for Mean		$\pm$ Std.	Minimum	Maximum
			Lower Bound	Upper Bound			
Absolute No of Eosinophil	T2DM	120.9600	111.4588	130.4612	23.01767	67.20	160
	T2DM.R	134.0000	115.8437	152.1563	43.98541	81	239
	Control	70.9600	46.1395	95.7805	60.13005	0.00	220

It should be noted that diabetes individuals also experience changes in their myeloid cells, including macrophages, monocytes, neutrophils, eosinophils, and basophils. Furthermore, the increase in monocytes' capacity to act as effective cells on the numbers of neutrophils and eosinophils as well as on the emergence of inflammation in diabetics (Rahmani-Kukia and Abbasi 2021). In contrast, most Covid-19 patients had a low amount of eosinophils in the early stages of infection. However, eosinophil counts rise later in the infection, coinciding with improving viral load and radiographic imaging. Furthermore, this improvement occurs faster in persons with mild symptoms (Hasan *et al.* 2021). However, several investigations demonstrated that the number of eosinophils decreased during SARS-CoV-2 infection and began to improve during the convalescent phase (Kwiecień *et al.* 2021, Neeland *et al.* 2021).

4.2.7 Hemoglobin

The results showed a significant difference T2DM.R and control in the levels of hemoglobin ($P \leq 0.05$, Table 4.16). In addition, there were no significant differences between T2DM and control and T2DM and T2DM.R ($P > 0.05$).

Table 4.16 Pairwise comparisons of hemoglobin between groups (* $P \leq 0.05$ is significant)

Pairwise Comparisons of Hemoglobin		
Sample 1-Sample 2	Test Statistic	P-value
T2DM.R-T2DM	10.720	0.245
T2DM.R-Control	-24.320	0.000*
T2DM-Control	-13.600	0.082

According to the findings of this study, hemoglobin levels in patients with T2DM.R who have recovered from SARS-CoV-2 and compared to the control cohort, markedly decreased. (Table 4.17). On the other hand, although the results showed a decrease in hemoglobin level in T2DM than in T2DM.R and control, it is still statistically non-significant (Table 4.16). However, other studies found that hemoglobin levels decreased with age, as did the proportion of participants with diabetes, hypertension, and total comorbidities (Taneri *et al.* 2020, Benoit *et al.* 2021, Faghih Dinevari *et al.* 2021). Moreover, in a study comparing patients infected with SARS-CoV-2 to those with the same clinical characteristics and showed negative Covid-19 test, 61% of participants were anemic, compared to 45% in control group (Bergamaschi *et al.* 2021).

Table 4.17 Mean values of hemoglobin in the three study groups

Variable	Group	Mean	95% Confidence Interval for Mean		±Std.	Minimum	Maximum
			Lower Bound	Upper Bound			
Hemoglobin	T2DM	138.6400	133.4080	143.8720	12.67504	11	164
	T2DM.R	130.1200	123.3245	136.9155	16.46289	109	162
	Control	148.6800	143.0193	154.3407	13.71350	121	170

Iron metabolism and anemia may play a key role in the multiple organ failure syndrome associated with Covid-19. Covid-19 are frequently linked to a blood clotting disorders that may contribute to anemia through intra-vascular hemolysis and has been hypothesized to represent a blend of low-grade distributed of intra-arterial thrombosis and regional pulmonary thrombotic microangiopathy (Levi *et al.* 2020).

5. CONCLUSIONS AND RECOMMENDATION

5.1 Conclusions

During SARS-CoV-2 infection, T2DM or hyperglycemia reduced the number of immune cells, such as granulocytes, monocytes, and phagocytic activity, which might exacerbate the intensity of Covid-19, particularly among T2DM people who had the impairment of immune response to infections. This study may include a piece on the immune system strategies, in addition to association of T2DM with novel Covid-19 infection in recovered individuals. This study examined the impact of Covid-19 infection on the immune response of T2DM patients in the recovery phase and provided immunological insight into the relationship between SARS-CoV-2 infection and T2DM. The results revealed that infection with SARS-CoV-2 in T2DM.R affected glucose homeostasis, altered cellular immunological status, hemoglobin, and increased IL-1 β expression from monocyte cells compared with diabetic individuals without infection T2DM, and controls. However, it is still unknown how long immune dysregulation continues following Covid-19 infection and if this may change the immunological response to future infections. Similarly, persistent neutrophil and monocyte activation may have a role in the development or worsening of other inflammatory diseases, such as cardiovascular disease and rheumatoid arthritis.

5.2 Recommendations

- A prolonged hyper-inflammatory response for more than seven days after hospital discharge recommended that patients should medically monitored after hospital discharge.
- Longitudinal investigations of recovered patients in a broader cohort may help in understanding the disease's repercussions.
- The findings highlight the importance for more research on T2DM patients infected with Covid-19 in order to better manage glucose levels and identify the immunological system of these individuals.

- A deeper examination of the role of cytokines in aggravating the inflammatory state or their use for therapeutic purposes recommended.
- Additional study about the effects of Covid-19 infection to the adaptive immune system for patients with T2DM will contribute to minimization infection-related consequences and the implementation of advanced treatment approaches.
- Because the samples in this study were restricted owing to the appearance of vaccination during sample collection and are from a specific geographic region, it would be preferred to conduct the study in a broader geographical area with the potential of utilizing more samples.



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