European Review for Medical and Pharmacological Sciences 2024; 28: 2605-2614

Evaluation of the hematological and immunological markers after the first and second doses of BNT162b2 mRNA vaccine

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Abstract. – OBJECTIVE: Both humoral and cellular immunity can be significantly influenced by the immunological responses to vaccination, and both responses are essential. Vaccination is the most consistent, safe, and cost-efficient practice for controlling the COVID-19 pandemic.

PATIENTS AND METHODS: Blood samples were collected from participants who received two vaccine doses of COVID-19 Pfizer/BioNTech (BNT162b2) before and on days 7 and 10 after the first and second immunization. We evaluated some hematological and immunological markers responses to the 1st and 2nd doses of the BNT162b2 mRNA (Pfizer/BioNtech) vaccine.

RESULTS: In healthy subjects' neutrophil and WBC counts significantly increased compared to those after the first dose. The results of all first-group participant categories demonstrated no discernible variations in lymphocyte counts. There was no change in IgM or IgG in all second-group cohorts, except for a considerable rise in IgG levels in people with a history of coronavirus infection following the second dosage compared to baseline. After the second dose, $CD4^+$ T-cell and $CD8^+$ T-cell levels rose in all groups compared to before the immunization and after the first dosage. Data demonstrated a substantial rise in neutrophil-lymphocyte ratio (NLR) after the second dose of the vaccine. Individuals who had previously had COVID-19 disease experienced a considerable increase in C3 and C4 levels after the first and second dosages compared to baseline. Additionally, compared to their levels after the first dosage, C4 levels increased significantly following the second dosage. Interleukin (IL)-6, IL-15, macrophage colony-stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), interferon gamma-induced protein 10 (IP-10/CXCL10), and macrophage inflammatory protein-1 alpha (MIP-1α/CCL3) levels were increased after boost correlated with Spike antibody levels, supporting their utility as indicators of successful humoral immunity development in response to vaccination.

CONCLUSIONS: We can conclude that the Pfizer/BioNTech vaccine produced a more potent T-cell response than humoral ones.

Key Words:
COVID-19, Pfizer/BioNTech vaccine, Humoral immunity, CBC, CD4, CD8, C3, C, Cytokines, Chemokines.

Introduction

In December 2019, an outbreak of pneumonia of unidentified causes in Wuhan, China, led to a

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global epidemic triggered by an unusual virus now called SARS-CoV-2. This virus is extremely contagious and pathogenic. This infectivity is made worse because asymptomatic and pre-symptomatic individuals can transmit viruses. In contrast, SARS-CoV-1 and MERS-CoV are usually transmitted by patients showing symptoms and, thus, could be contained more efficiently[1]. Additionally, COVID-19 individuals with comorbidities, including diabetes, obesity, and cardiovascular diseases (CVDs), have a greater risk of morbidity and death. An investigation[2] dealing with 1,099 participants having COVID-19 reported that of the 173 people with acute illness, 5.8% also suffered from coronary heart ailment, 2.3% also had cerebrovascular disease, 23.7% also had concomitant hypertension and 16.2% also had diabetes mellitus. When a safe and effective vaccine approach is developed, and a successful worldwide vaccination campaign is implemented, pre-pandemic normalcy is assumed never to return. The Food and Drug Administration (FDA) and the European Medicines Agency (EMA) approved the BNT162b2 mRNA coronavirus disease 2019 (COVID-19) vaccine to be used in emergencies in December 2020[3]. The nanoparticles in the mRNA vaccines include genetic material. The nanoparticles shield the mRNA from the body's enzyme activities, which would otherwise break it down[4]. It facilitates mRNA entry into the muscle cells close to the injection site. The human DNA is not harmed since just a portion of the protein is produced, which combines to form a spike. The leftover and undesired mRNA strand is broken down by human cells after the spike proteins are formed[5,6]. The most favorable tactic for achieving immunity toward COVID-19 is to induce antibodies that can neutralize the virus. Thus, such antibodies can prevent the attachment of the virus with its receptors on the host cell surface (angiotensin-converting enzyme, ACE2). Indeed, the majority of the COVID-19 vaccine candidates depend on this approach. Consequently, the spike glycoproteins of SARS-CoV-2 are essential proteins for constructing multi-epitope vaccines[7]. T-cells could protect against SARS-CoV-2 even without the antibody response. The T-cell response includes $CD4^+$ and $CD8^+$ T-cells that may have an essential role in protecting against SARS-CoV-2, even in convalescent people after asymptomatic or mild infection and in the seronegative exposed family members[8,9]. Evolving proof[10] advocates that T-cells are essential in COVID-19 immunity in the natural infection and/or vaccination. In this study, we investigated the hematological and immunological parameters induced by the first and second doses of BioNTech BNT162b2 mRNA vaccination to find indicators related to vaccination and result in the formation of protective antibodies. BioNtech BNT162b2 mRNA vaccination at a variety of time points following the initial and subsequent doses. The outcome could help in the identification of mechanisms that lead to effective vaccination, and they could be utilized as biomarkers that predict the successful application of mRNA vaccines.

Patients and Methods

Study Design and Participants

This ongoing prospective study investigates the role of some immunological markers in volunteers receiving the mRNA-Pfizer/BioNTech (BNT162b2) vaccine against SARS-CoV-2 initiated from October 2021 until February 2022. One hundred and fifty-three (153) people who participated in the study were included. The age ranged from 18 to 66 years. Samples from subjects who had received two doses of the vaccine at a dosage of (30 μg) were taken. This study was approved by the Tanta University Ethical Approval Committee (TP/RE/4/23 p-0016), and informed consent was obtained from all individual participants. All methods were performed following the relevant guidelines and regulations. Blood samples were taken. Analyses were done on samples taken at baseline (before the first vaccine), days 7 and 10 after the first vaccination, and days 7 and 10 after the second vaccination. Samples were collected from Ibn Al-Balady Hospital for Children and Women, the Vaccination Center of the University of Technology, and the Al-Saydiya Health Center, Baghdad, Iraq.

All subjects provided both oral and written informed consent. Analyses were done on samples using full-automated hematology analyzers (Genex, Wayne, Pennsylvania, USA) and High Sensitivity Human ELISA Kit corresponding for each marker (Elabscience, Houston, TX, USA) and a radial immunodiffusion assay plate corresponding to each marker (LTA; Milan, Italy). The participants in this study were divided into two different ways (Figure 1). Individuals were assigned to the first group according to comorbidities; the one hundred and twenty-three (123) people participating were split into four clusters: first, the healthy individuals; second, the subjects with hypertension; third, the subjects with diabetes; and finally, the subjects with hypertension, diabetes, and heart disease. White blood cells (WBC), neutrophil and lymphocyte

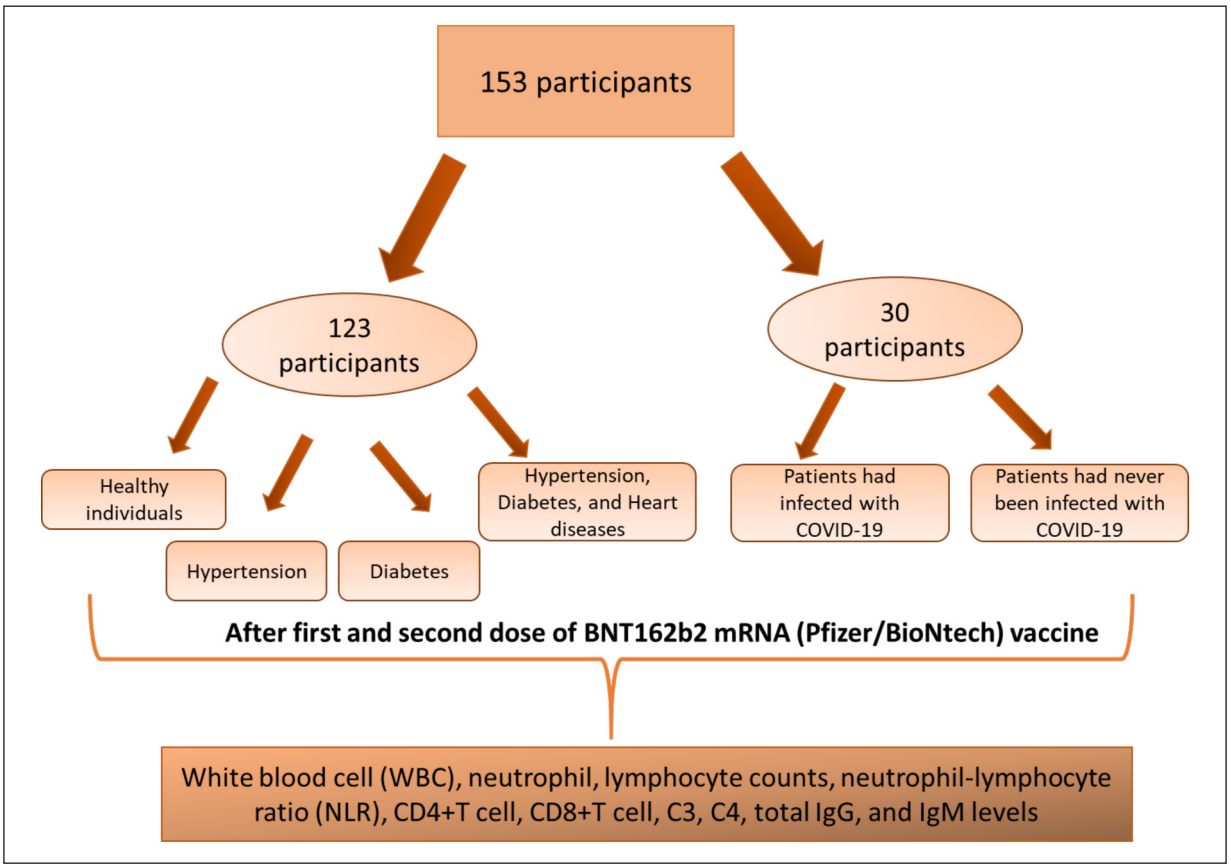

Figure 1. Schematic view of the study protocol.

counts, neutrophil-lymphocyte ratio (NLR), $CD4^+$ T-cell, and $CD8^+$ T-cell were measured for these participants. Individuals were assigned to the second group according to the previous infection. 30 participants were split into two groups: 12 subjects who had never been infected with COVID-19 before and 18 persons who had. C3, C4, total Immunoglobulin G (IgG), immunoglobulin M (IgM), interleukin (IL)-6, IL-15, macrophage colony-stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), interferon gamma-induced protein 10 (IP-10/CXCL10), and macrophage inflammatory protein-1 alpha (MIP-1α/CCL3) were measured for these participants. The participants in this study were divided into two different ways (Figure 1) as follows:

The first group was classified based on the comorbidities; the one hundred and twenty-three (123) subjects participating were split into four clusters: first, the healthy individuals; second, the subjects with hypertension; third, the subjects with diabetes; and finally, the subjects suffering from hypertension, diabetes, and heart disease. In the first group, white blood cell (WBC), neutrophil, lymphocyte counts, and neutrophil-lymphocyte ratio (NLR), $CD4^+$ T-cells, $CD8^+$ T-cells were measured.

The second group was classified based on whether patients had a previous infection of COVID-19 or not: 30 participants were split into two groups: 12 subjects who had never been infected with COVID-19 before and 18 persons who had been previously infected with COVID-19. In the second group, C3, C4, total IgG, and IgM were measured.

Specimen Collection

The blood was collected in (a 3 mL K3 EDTA tube) following standard venipuncture protocol and steps, and the sample volume was 1.5-2 mL of whole blood in the closed-vial mode. Contributors' serum samples were extracted from blood and frozen. Before the required analysis, the frozen samples were thawed at room temperature for one hour and vortexed[11].

Complete Blood Count (CBC) Test

All samples were tested by fully automated hematology analyzers (Genex, Wayne, PA, USA). The sample was mixed gently, the tube

was opened, and the aspirator tip was placed in it before pressing the sample bar.

ELISA Assay

The levels of $CD4^+$ T-cell and $CD8^+$ T-cell were determined using a High Sensitivity Human ELISA kit (Elabscience, Houston, TX, USA), as revealed in the **Supplementary File**. Anti-spike-protein receptor-binding domain (RBD) IgG antibodies against SARS-CoV-2 were measured according to the manufacturer's protocol. Serum cytokine/chemokine concentrations (IL-6, IL-15, M-CSF, G-CSF, IP-10/CXCL10, and MIP-1α/CCL3) were measured with the V-PLEX Human Biomarker Assay kit (Meso Scale Diagnostics, MD, US) according to manufacturer's recommendations.

Radial Immunodiffusion Assay

Sera were analyzed by radial immunodiffusion assay to assess SARS-CoV-2-specific IgG, IgM, C3, and C4 by commercially available tests as described by the manufacturer (LTA, Milan, Italy). The plates were left for a few minutes to evaporate any condensed water in the wells. Then, the wells were filled with 5 µl of samples, and after completely absorbing the samples, the plates were closed and placed in a moist chamber for 72 h, except for IgM, for 96 h.

Statistical Analysis

The one-way ANOVA analysis of variance was applied to perform the statistical analysis using GraphPad Prism 7 (San Diego, CA, USA). If the ANOVA test was significant, then multiple comparison methods were used to compare the three possible pairwise comparisons. *p*-value lower than 0.05 was considered significant.

Results

The recent COVID-19 pandemic put the globe in an emergency, necessitating the development of a scientific and research-based response to the viral epidemic. Hence, creating a COVID-19 vaccine is a required global effort to control such pandemic disease. Many pharmaceutical companies are working to provide an efficient and safe vaccine. Numerous people worldwide are immunized with the ground-breaking anti-SARS CoV-2 mRNA vaccine BNT162b2. Post-vaccination immunity analysis has become essential for determining the effectiveness of the COVID-19 vaccine. Our prospective cohort investigation contributes to discovering a dynamic innate and humoral immune response to BNT162b2 immunization 7 and 10 days after the first and second vaccination doses. Figure 1 represents a schematic view of the study protocol. One hundred and fifty-three (153) individuals aged 18 to 66 years old were administered the mRNA Pfizer/BioNTech (BNT162b2) COVID-19 vaccine.

116 were women, and 37 were men. Fifty-five of them were healthy; 26 had hypertension, 23 had diabetes, and 19 had hypertension, diabetes, and heart disease. Sera were analyzed before and on the 10^{th} day after the vaccination, after the first and second mRNA Pfizer/BioNTech (BNT162b2) COVID-19 vaccine. The results showed that healthy subjects' neutrophil counts significantly increased after the second vaccine dose compared to those after the first dose. In contrast, there was no noticeable increase in the rest of the participants. In healthy subjects, the numbers of WBC following the second dose revealed a rise compared to after the first dose.

Additionally, among the people who had hypertension, there were noticeable changes in WBC counts after the second dose compared to their pre-vaccine values. There was no change in the outcomes for people with diabetes and others with high blood pressure, diabetes, and heart disease, as shown in **Supplementary Figures 1, 2, 3, 4**, respectively. For all participant categories, the results demonstrated no discernible variations in lymphocyte counts before the immunization and seven days after receiving the first and second doses of the vaccine, as shown in **Supplementary Figures 2, 3, 4, 5**, respectively. Our results revealed that CD4 levels rose in all groups after the second dose in relation to that before the immunization and after the first dose.

Supplementary Figure 5 represents the levels of chemokine/cytokine changes. The results demonstrated the cytokine/chemokine profile induced after the 1^{st} and 2^{nd} vaccinations and the comparison between the effects caused by each dose for the individual recipients. These results were correlated with spike antibody levels, as shown in **Supplementary Figure 6**.

According to our investigation, C3 and C4 levels did not significantly rise in people who had not previously contracted COVID-19. At the same time, participants who had previously had the disease experienced a considerable rise in C3 and C4 levels after the first and second dosages compared to baseline. Additionally, compared to their levels after the first dosage, C4 levels increased significantly following the second dose.

Discussion

The recent COVID-19 pandemic put the globe in an emergency, necessitating the development of a scientific and research-based response to the viral epidemic. Hence, creating a COVID-19 vaccine is a required global effort for controlling such pandemic disease. Many pharmaceutical companies are working to provide an efficient and safe vaccine. Numerous people worldwide are immunized with the ground-breaking anti-SARS CoV-2 mRNA vaccine BNT162b212. Post-vaccination immunity analysis has become essential for determining the effectiveness of the COVID-19 vaccine[13]. Our prospective cohort investigation contributes to discovering a dynamic innate and humoral immune response to BNT162b2 immunization.

The immune cells which are most plentiful in human blood are neutrophils. During infections, they serve as initial responders and can modify cell-mediated responses. Neutrophils go to a target tissue after activation, where they fight against invasive organisms. Additionally, they can interact with different immune cell types and influence the microenvironment[14]. The vaccine's effectiveness has been enhanced by encapsulating the mRNA into lipid nanoparticles (LNPs), which help to protect the mRNA from RNase destruction. When LNP-formulated mRNA vaccines are administered intramuscularly, a slight local inflammatory response attracts neutrophils to the injection area[15,16]. According to our findings, after getting the second vaccine dose, healthy subjects' neutrophil counts significantly increased compared to those after the first dose.

In contrast, there was no noticeable increase in the rest of the participants. In healthy subjects, the numbers of WBC following the second dose revealed an increase compared to after the first dose. Additionally, among the people who had hypertension, there were noticeable changes in WBC counts after the second dose compared to their pre-vaccine values. There was no change in the outcomes for people with high blood pressure, diabetes, and heart disease, as shown in **Supplementary Figure 1, 2, 3, 4**, respectively.

Patients with COVID-19 commonly exhibit a complete blood count with lymphopenia, either with or without total leukopenia[17]. It is unknown why severe illnesses and lymphopenia are related. T lymphocyte damage is a significant factor contributing to the worsening of the patient's condition, and it has been theorized[18] that COVID-19 may affect T lymphocytes. Nevertheless, one study[17] showed that in patients with mild COVID-19, the lymphocyte count remained within the normal range.

For all participant categories, the results demonstrated no discernible variations in lymphocyte counts before the immunization and seven days after receiving the first and second doses of the vaccine, as shown in **Supplementary Figure 1, 2, 3, 4**, respectively. The lymphocyte count was evaluated 7 and 10 days after vaccination, and within this time, it was restored to normal levels. Therefore, the findings of this study do not conflict with ours.

Even without an antibody response, T-cell responses may offer a defense against SARS-CoV-2[19,20]. According to two minor investigations[21,22], a few people with SARS-CoV-2 may generate specific memory T-cell responses without specific antibodies, demonstrating that cellular immunity may be elicited by SARS-CoV-2 if the humoral immune response is not present.

According to an autopsy report[22] of a patient who passed away due to severe COVID-19, the patient's lungs accumulated mononuclear cells, and the peripheral blood had a low concentration of hyperactive T-cells. These results imply that T-cells are brought into the diseased lung tissues from the circulation to suppress viral infection. Although the cause and mechanism of lymphopenia in COVID-19 patients are unclear, the discovery of SARS-CoV RNA and SARS-like viral particles in T-cells points to a direct impact of the SARS virus on T-cells, perhaps through apoptosis.

A previous study[22] was performed using interferon (IFN)-γ enzyme-linked immunospot and intracellular cytokine staining after induction with overlapping spike glycoprotein peptides to quantify T-cell responses in the 108 vaccine recipients (an adenovirus serotype-5-vectored vaccine expressing the spike glycoprotein) in humans. T-cell responses from $CD4^+$ T-cells and $CD8^+$ T-cells peaked at day 14 post-vaccination. Furthermore, seven days after finishing the BNT162b1 vaccination (day 28), it was found in a previous study[21] that the virus-specific $CD4^+$ and $CD8^+$ T-cell responses were in 94% and 80% of tested individuals. Our results revealed that CD4 levels rose in all groups after the second dose in relation to that before the immunization and after the first dosage[23,24]. In contrast, CD8 levels rose after both doses, with the rise following the second dosage being the greatest, as shown in **Supplementary Figure 1, 2, 3, 4**, respectively.

A greater neutrophil-lymphocyte ratio (NLR) is typically linked to higher death rates and a bad

prognosis. It has been demonstrated[23] that the NLR is a reliable predictor of severe COVID-19. According to the latest report on 61 patients[25,26], the NLR was the most helpful prognostic factor influencing the prognosis for severe COVID-19. Following this, European research[27] in Italy revealed that patients with acute COVID-19 were older and had greater NLR than non-severe patients, indicating that NLR may be a valuable marker for early screening of COVID-19 patients.

The circulating leukocyte count and, consequently, the NLR may be affected by several chronic illnesses. 44% of COVID-19-infected patients had at least one comorbidity, primarily hypertension, diabetes, cardiovascular illness, or chronic obstructive pulmonary disease[28,29]. These negative correlations might result from how these diseases develop mainly due to acute inflammation and compromised immune systems[30,31].

Furthermore, NLR may predict death in the population and, consequently, the general effect of immunity as well as inflammation on health[32,33]. Conversely, individuals with chronic conditions, including diabetes and renal disease, have already been shown[34] to have a decreased risk of hospitalization when their NLR is reduced. Compared with NLR before the vaccination and after the first dosage, our data demonstrated a substantial rise in NLR after the second dose in healthy individuals and in those with hypertension, diabetes, and heart disease. The rest of the participants showed no noticeable increase, as shown in **Supplementary Figure 1, 2, 3, 4**.

After the initial dosage of the Pfizer-BioNTech or Moderna SARS-CoV-2 mRNA vaccines, a few rare cases of anaphylaxis have been described[35]. These mRNA vaccines' polyethylene glycol (PEG) or lipid moieties probably cause these allergic responses[34,35]. They directly reflect the complement activation-related pseudoallergy reaction (CARPA) observed[36,37] in the case of liposomal carriers and include complement and mast cell activation in an IgE-independent way. In this instance, IgG or IgM directed toward the PEGylated lipids of these vaccination formulations may be the first to activate the complement[38,39].

Hypocomplementemia (complement 3, C3 0.096, normal range 0.8-1.6 g/dL; and C4 0.067, normal range 1.3-7.5 g/dL) was also observed. According to our research, C3 and C4 levels did not significantly rise in people who had not previously contracted COVID-19. At the same time, participants who had previously had the disease experienced a considerable rise in C3 and C4 levels after the first and second doses compared to baseline. Additionally, compared to their levels after the first dosage, C4 levels increased significantly following the second dose, as shown in **Supplementary Figure 5 and 6**, respectively.

IgM antibodies are generated early in the humoral immune response to viral infections and quickly offer protective immunity. Isotype class switching and maturation are followed by producing memory IgG antibodies with higher affinity[40,41]. Strong correlations between falling neutralizing antibody responses and dropped anti-spike (S) protein and anti-receptor binding domain (RBD) IgM levels highlighted the role of IgM in COVID-19 protective immunity. Investigation on the humoral response to the SARS-CoV-2 vaccine is ongoing since it is still unclear what role pre-existing immunity plays in the body's response to the vaccine[42]. According to research[42], previously infected (PI) people have been demonstrated to produce a more effective antibody response to COVID-19 vaccinations than immunologically naive individuals (IN).

Interestingly, the neutralizing activity shown in PI vaccines seven days after the first dose of the vaccination did not vary substantially from that seen in IN vaccines seven days after the second dosage. There is data41 on the kinetics of IgM emergence following vaccination and its correlation with virus-neutralizing activity. According to one research[43], after receiving the first dose of the BNT162b2 vaccination, just around 50% of IN vaccine recipients did not develop IgM.

According to a Fraussen investigation[43], pre-existing immunity to cross-reactive human coronaviruses may explain why recipients of the BNT162b2 vaccination failed to produce anti-S IgM in vaccine-naive people. However, this agrees with the predictable decay of a primary immune response to the virus in prior SARS-CoV-2-infected vaccines. The same study[43] demonstrated that the persistence of virus-specific IgM responses might indicate the persistence of IgM+ memory B cells that are not switched classes. Although the production of anti-S IgM following vaccination in people with a history of prior infection is unforeseen, it may indicate that these participants could not develop an adequate antibody response due to transient or asymptomatic prior infections. As a result, these people can have an IgM and IgG response to the vaccine similar to a prime immunological response. No noticeable rise in IgM and IgG levels was seen in all the cohorts in our study, except for a conside-

rable rise in IgG levels in people with a history of coronavirus infection following the second dosage compared to baseline, as shown in **Supplementary Figure 5 and 6**, respectively.

The likelihood that Ig levels may rise and peak a month after the second dosage may help to explain why there was no rise in Ig levels 7 and 10 days after immunization. According to prior research[44], the maximum levels of anti-spike immunoglobulin G are attained 2-3 weeks after receiving the second dosage of the Pfizer/BioNTech vaccine. According to the manufacturer's specifications, the Pfizer/BioNTech vaccine is around 90% efficient against illnesses with high viral loads, but only one month after the second dose.

Additionally, most of our study's participants were over 50 years old and had chronic illnesses such as high blood pressure, diabetes, and heart disease. The anti-spike IgG level peaked one month following the second immunization, according to research by Ikezaki et al[45]. Additionally, older Japanese subjects (60 years or older) showed lower anti-spike IgG levels than younger Japanese people. After the second BNT162b2 vaccination, the anti-spike IgG level was consequently shown[44] to be significantly inversely correlated with age. IgG levels induced by BNT162b2 were lower by day 21 than those induced by mRNA-1273 following the second immunization, according to research by Keshavarz et al[46]. In BNT162b2 recipients, age significantly influenced IgG levels but not mRNA-1273. Regardless of chronic medical problems, age has a significant role in the humoral response caused by vaccination, according to the investigation[45]. It has been established[44,45] that individuals older than 60, particularly those with underlying chronic diseases, are at a greater risk of developing severe illness and dying from COVID-19. In reality, because of immunological senescence, the response to vaccinations typically decreases in older persons.

Despite receiving the vaccine's initial dosage, four previously infected participants (20, 24, 35, and 39) years old, did not see an elevation in Ig levels, which may be explained by the fact that these individuals had a mild illness and had been infected more than five months prior to getting the vaccination, with the potential for a swab result that is falsely positive. Since there is a correlation between the level of neutralizing antibodies and the acuteness of COVID-19 illness, people with mild or no symptoms are less likely to produce a neutralizing response[47,48]. Severe hospitalized patients had more significant IgG titers than favorable situations[49], whose lower or undetectable antibody levels have been recorded[50,51]. Previous studies[49,50] have found that RBD-specific IgG titers rapidly decrease within 2-4 months in patients with mild COVID-19. This shows that humoral immunity elicited by SARS-CoV-2 may not be long-lasting in those with mild illness. In contrast to symptomatic patients, the neutralizing antibody response decreases and declines more quickly in asymptomatic individuals.

Conclusions

The present study revealed that individuals from all categories, except people with diabetes, showed increases in WBC, neutrophils, lymphocyte count, and NLR. The Pfizer/BioNTech vaccine caused T-cell solid responses compared to humoral responses. Because viral alterations may reduce the effectiveness of neutralizing antibodies (NAbs), T-cells are a crucial part of anti-SARS-CoV-2 immunity. Therefore, to limit infection, a robust cellular response would be essential. For various reasons, humoral immunity showed a weak response to the vaccine during the 7th and 10th days after the two doses. As for C3 and C4, they showed an increase in people who were previously infected. This study focused on the association between several immunoregulatory molecules induced by vaccination and innate and adaptive immune responses elicited by an mRNA-based vaccine. The results could be used to evaluate the vaccination activity and as a guide to improve the efficacy of mRNA vaccination approaches.

Data Availability

All data are available in the manuscript and in supporting materials.

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Ethics Approval

This study was approved by the Tanta University Ethical Committee (TP/RE/4/23 p-0016).

Informed Consent

Informed consent was obtained from all individual participants.

Conflict of Interest
The authors have no relevant financial or non-financial interests to disclose.

Authors' Contributions
All authors contributed to the study's conception and design. Data collection and analysis were performed by M.S.J, HMA, M.R, S.F.J, M.A.A.N, A.F.A, EE, and GEB. The first draft of the manuscript was written and reviewed by M.S.J, HMA, M.R, S.F.J, M.A.A.N, A.F.A, EE, and G.E.B. J.Z.T, A.T, and B.A.A have revised the manuscript. All authors read and approved the final manuscript.

Acknowledgments
This research was funded by Princess Nourah bint Abdulrahman University Researchers Supporting Project number (PNURSP2024R142), Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia.

Funding
This research was funded by Princess Nourah bint Abdulrahman University Researchers Supporting Project number (PNURSP2024R142), Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia.

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