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RESEARCH ARTICLE

Impact of Menopausal Hormonal Therapy on Anti-SARS-Cov-2 Antibody Titers in Middle-Aged Women

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ABSTRACT

Aim: **Postmenopausal women often contract the common cold. Following treatment with HRT, symptoms resolve in some women. We hypothesize menopausal hormone therapy (MHT) may increase anti-SARS-Cov-2 IgG antibody titers, leading to greater resilience against COVID-19 infection.**

Methods: **219 patients were enrolled. Anti-SARS-Cov-2 IgG antibody titers were measured, and the number of days since the second SARS-Cov-2 vaccination was recorded. Linear regression analysis was performed to examine the relationship between these two variables for patients receiving MHT, patients with rheumatoid arthritis (RA) receiving methotrexate and non-MHT/non-RA (NMR). Analysis was further divided into groups aged <50, age 50 to 60, and age >60.**

Results: **Anti-SARS-Cov-2 IgG antibody titers in the MHT group were significantly higher than NMR aged >50 ($p < 0.05$). In addition, anti-IgG antibody values in NMR were significantly higher than in patients with RA aged >60 ($p < 0.05$).**

Conclusion: **MHT results in increased production of anti-SARS-Cov-2 IgG antibodies in women aged >50. This may confer additional protection against SARS-Cov-2 infection. Women with RA on methotrexate over 60 have lower antibody titers than NMR aged >60.**

Keywords: Anti-SARS-Cov-2 IgG antibody, menopausal hormone therapy (MHT), non-MHT/non-RA (NMR), vaccine.

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1. Introduction

SARS-CoV-2 infection was first reported in late December 2019 in China, and rapidly spread all over the world. The Receptor Binding Domain (RBD) of the SARS-CoV-2 spike protein binds to the ACE2 receptor in human cells, located on alveolar cells, amongst others. Over the last two years, variations of the spike protein have occurred, including the Alpha (α) and Delta (δ) variants. More recently, in October 2021 the Omicron (o) variant [1] spread explosively across South Africa, Europe and the USA, before arriving in Japan in January 2022. It was suspected that Omicron may escape neutralization by antibodies generated by mRNA COVID-19 vaccination, but recent studies have shown vaccination remains protective against Omicron [2]. detection of antibodies against SARS-Cov-2 was performed using internationally recognized tests provided by Roche, Abbott, and Beckman Coulter [3]–[5].

In Japan, vaccination against SARS-Cov-2 began with medical doctors and healthcare workers from March to April 2021, followed by the elderly and those with significant comorbidities from May to June. Middle-aged and young people followed from July to September.

Despite double vaccination, SARS-Cov-2 infection was noted to occur within two to three months, a so-called *breakthrough infection*. The reason for this is not yet known. Age and male sex are known risk factors for infection and death [6], [7]; obesity and smoking are associated with poor outcomes [8], [9]. In this study, we focus on the influence of methotrexate in patients with rheumatoid arthritis (RA) [10], [11], and menopausal hormone therapy (MHT) in women [12], in order to clarify their influence on the production of anti-SARS-Cov-2 IgG antibodies.

TABLE I: Background of 209 Patients

Age: 50>	**MHT**	**RA**	**NMR**
Case	14	7	11
Male	0	1	1
Mean age	48.7 (47∼49)	48.3 (46∼49)	45.2 (35∼49)
BMI	21.7 (15∼29.4)	24.4 (21.9∼31.6)	20.3 (18.6∼21.8)
CTD	0	1	1
Other/NHS			7/3
Age: 50∼60	**MHT**	**RA**	**NMR**
Case	54	6	13
Male	0	0	1
Mean age	54.4 (50∼59)	54.5 (50∼58)	53.8 (51∼59)
BMI	22.7 (17.3∼34.9)	21.9 (18.4∼27.1)	21.5 (18.9∼23.8)
CTD	3	1	2
Other/NHS			10/1
Age: 60<	**MHT**	**RA**	**NMR**
Case	29	30	43
Male	0	3	12
Mean age	70.5 (60∼82)	73.2 (61∼86)	73.5 (61∼89)
BMI	22.8 (17.1∼28.3)	21.8 (16.0∼30.3)	23.3 (17.2∼30.5)
CTD	2	0	3
Other/NHS			37/3

Note: MHT: menopausal hormone therapy, RA: rheumatoid arthritis, NMR: non-MHT/non-RA, BMI: body mass index, CTD: connective tissue disease, NHS: normal healthy subject.

2. Materials and Methods

2.1. Patients

In this study, testing commenced on October 5, 2021, and concluded in December. Anti-SARS-Cov-2 antibody titers were measured once per patient, and the interval between the measurement date and the date of the second SARS-Cov-2 vaccination was recorded. During this period, the prevalence of the Delta variant was declining in Japan. Therefore, the results are likely to be minimally influenced by the presence of the Delta variant, and more purely reflect anti-SARS-Cov-2 antibodies produced as a result of vaccination.

Anti-SARS-Cov-2 IgG antibody titers were tested in 219 patients, of whom 18 were male and 201 were female. Patients were 11 to 180 days post-second SARS-Cov-2 vaccine injection. Of these, two patients declined vaccination due to concerns regarding adverse effects, and ten had a history of COVID-19 infection prior to vaccination. The remaining 207 included 43 patients with RA receiving methotrexate, 97 receiving MHT, and 67 NMR including 12 with connective tissue disease who were not receiving methotrexate (Table I).

2.2. Vaccination

Patients received Pfizer-BioNTech (COMIRNATY) vaccine (BNT 162b2) 0.3 ml at the author's clinic, or Moderna vaccine (mRNA-1273) 0.5 ml at an alternative vaccination practice. Vaccines were injected into the deltoid muscle. The interval between first and second doses of Pfizer and Moderna was 3 weeks and 4 weeks, respectively.

2.3. Detection of Anti-SARS-Cov-2 IgG Antibodies

Anti-SARS-Cov-2 IgG antibody detection was performed by Access 2 (Beckman Coulter) at the Health Sciences Research Institute in Yokohama. Testing was blinded, with only patient ID and name provided. Less than 10 Arbitrary Units (AU)/ml was considered negative.

2.4. Rheumatoid Arthritis (RA) and Other Connective Tissue Disease (CTD)

The diagnostic criteria for RA were the presence of bone erosion, or a score greater than 6 per the 2010 EULAR/ACR criteria [13]. The diagnostic criteria for CTD is ANA 1:160 titer or greater, and disease-specific antibodies such as anti-SS/A, anti-RNP, anti-Scl 70, anti-Jo-1 antibodies and so on [14]. Patients received up to 5 mg of prednisolone, but additional immunosuppression including methotrexate was not given.

2.5. Menopausal Hormone Therapy (MHT)

MHT was prescribed for menopausal or perimenopausal women. MHT consisted of a 2-day 17β-estradiol (E2) patch (0.72 mg) for 26 days, with dydrogesterone (10 mg) orally for 10 days followed by 4–5 days off. This cycle was repeated every 30 to 31 days. As five or more days off resulted in the recurrence of menopausal symptoms, the drug holiday was omitted.

For women aged over 55, a 2-day E2 patch and dydrogesterone (5 mg) orally every day were prescribed. For women aged over 60, estriol (E3) 2 mg daily was used, with dydrogesterone 10 mg administered every 6 months.

2.6. Statistics

SPSS version 26 (SPSS Inc., Chicago, IL, USA) was used for the statistical analyses.

Linear regression analysis was performed on each of the NMR, MHT and RA on methotrexate groups. First, the gradient and intercept of the linear regression line in each group was obtained. For comparison between groups, analysis of covariance was conducted: F-test of parallelism and F-test of intercept difference were performed. The

TABLE II: PATIENTS WITH A HISTORY OF COVID-19 INFECTION

No	Age	Sex	Diag	BMI	Date of Infection	Type	Date of first shot	Date of second shot	Date of blood drown	Date of duration	SARS-Cov-2 IgG
1	56	f	Menopause	18.5	22.04.2021	Pf	22.8.2021	09.09.2021	01.11.2021	52	86.1
2	53	f	Menopause		23.07.2021	Pf	22.09.2021	03.10.2021	20.10.2021	17	391.6
3	50	m	RA	23.4	16.09.2021	Pf	23.10.2021	13.11.2021	24.11.2021	11	214.4
4	53	f	Menopause	25	25.10.2020	Pf	12.08.2021	02.09.2021	30.09.2021	27	193.1
5	54	f		20.3	02.09.2021	Pf	29.08.2021	23.10.2021	19.11.2021	26	1195.6
6	60	f		27	31.05.2021	Pf	06.09.2021	04.10.2021	10.11.2021	36	554.1
7	74	f		24.1	17.09.2020	Pf	02.06.2021	21.07.2021	03.12.2021	132	195.9
8	60	f	Menopause	20.7	16.12.2020	Mod	12.08.2021	10.09.2021	16.11.2021	66	112
9	46	f	Menopause	21.9	13.01.2021				29.10.2021	316	5.5
10	41	f	RA	21.5	11.06.2021				27.12.2021	196	20.5
11	55	f	Menopause	23.7					08.11.2021		2
12	54	f	Menopause	17.5					05.11.2021		3.6

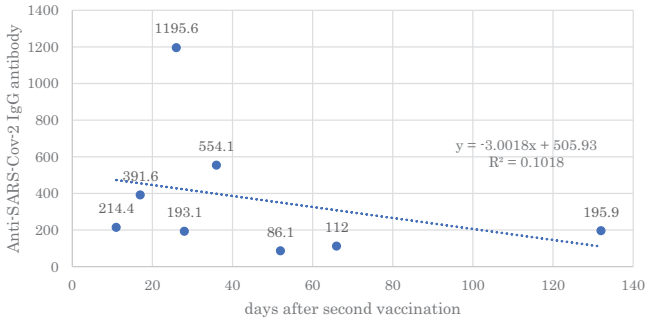

Fig. 1. Anti-SARS-Cov-2 IgG antibody titers: patients with a history of SARS-Cov-2 infection (n = 8).

number of days from vaccination to antibody titer measurement was input as a covariate.

2.7. *Informed Consent*

Written informed consent was obtained from all patients.

3. RESULTS

3.1. *Anti-SARS-Cov-2 Antibody Titers vs. Days Post-Second Vaccination: Patients with a History of SARS-Cov-2 Infection*

12 cases are described in Table II: 10 with a history of SARS-Cov-2 infection and two controls. In the infection group, two unvaccinated patients recorded anti-SARS-Cov-2 IgG antibody titers of 5.5 AU at 316 days post-infection, and 20.5 at 196 days post, respectively. Comparatively, two control unvaccinated patients who had not experienced SARS-Cov-2 infection recorded results of <2 and 3.6 AU. The anti-SARS-Cov-2 IgG antibody titers of the remaining 8 patients, all of whom had experienced both vaccination and infection, are plotted in Fig. 1, with an equation of $y = -3.0018x + 505.93$, $R^2 = 0.1018$. The constant 505.93 was the highest in this study, indicating high antibody titers.

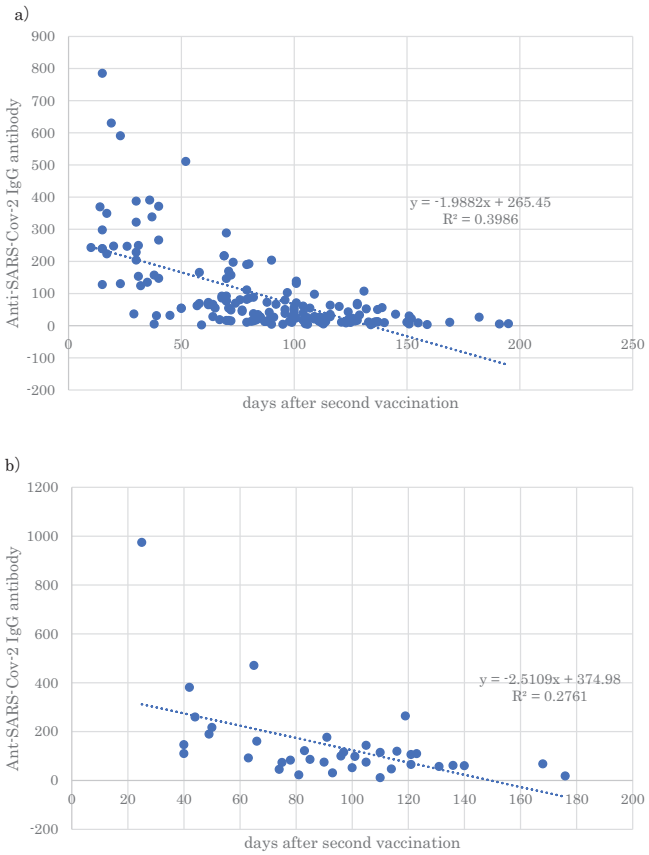

Fig. 2. Anti-SARS-Cov-2 IgG antibody titers: (a) Pfizer vaccination (n = 168); (b) Moderna vaccination (n = 38).

3.2. *Anti-SARS-Cov-2 Antibody Titers vs. Days Post-Second Vaccination: Pfizer vs. Moderna Patients*

Antibody results from 206 patients who had not previously experienced SARS-Cov-2 infection were analyzed. This group included both male and female patients. Anti-SARS-Cov-2 IgG antibody values post Pfizer (n = 168) and Moderna (n = 38) vaccination are shown in Figs. 2a and 2b, respectively. There was no statistically significant difference in antibody titers between these two groups. $F(1,202) = 0.926$ $p = 0.337$. Therefore, these groups were combined in subsequent subgroup analyses.

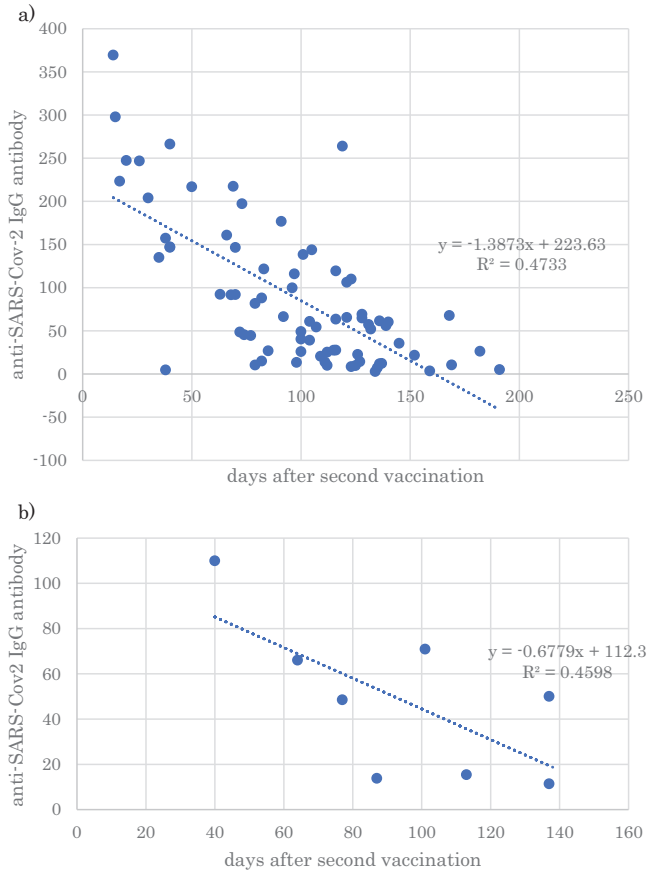

Fig. 3. Anti-SARS-Cov-2 IgG antibody titers: (a) BMI 30 (n = 192); (b) BMI ≧ 30 (n = 8).

3.3. Anti-SARS-Cov-2 Antibody Titers vs. Days Post-Second Vaccination: Obese vs. Non-Obese Patients

In this study, patients with a BMI ≧ 30 were rare (n = 8). The majority of patients had a BMI <25 (n = 192). Anti-COVID-19 antibody titers in patients with BMI ≧ 30 were not significantly different from patients with BMI < 30 in Figs. 3a and 3b. F (1.194) = 1.321 p = 0.252 Therefore, these groups were also combined in subsequent subgroup analyses.

3.4. Anti-SARS-Cov-2 Antibody Titers vs. Days Post-Second Vaccination: Smokers/Ex-Smokers vs. Non-Smokers

Smoking status was known in 183 patients. Of these, 17 were smokers or ex-smokers, and 167 were never smokers. There was no statistically significant difference in anti-SARS-Cov-2 titers between these two groups in Figs. 4a and 4b [F (1,183) = 3.082, p = 0.081] One ex-smoker who quit 15 years prior was an outlier with a significant antibody titer of 974.5 AU; she was included in the analysis in the smoker/ex-smoker group.

In the following three analyses, groups are divided by age: age <50, age 50–60, and age >60.

3.5. Anti-SARS-Cov-2 Antibody Titers vs. Days Post-Second Vaccination: MHT, NMR and RA on Methotrexate Patients Age <50

There was no statistically significant difference in antibody titers between the MHT (n = 14) and NMR (n = 11) groups [p = 0.646, F(1,21)=0.217] in Figs. 5a and 5b. Similarly, there was no difference between the NMR and RA (n = 7) groups [p = 0.487, F(1,17) = 0.504]; particularly, NMR did not have significantly higher antibody titers.

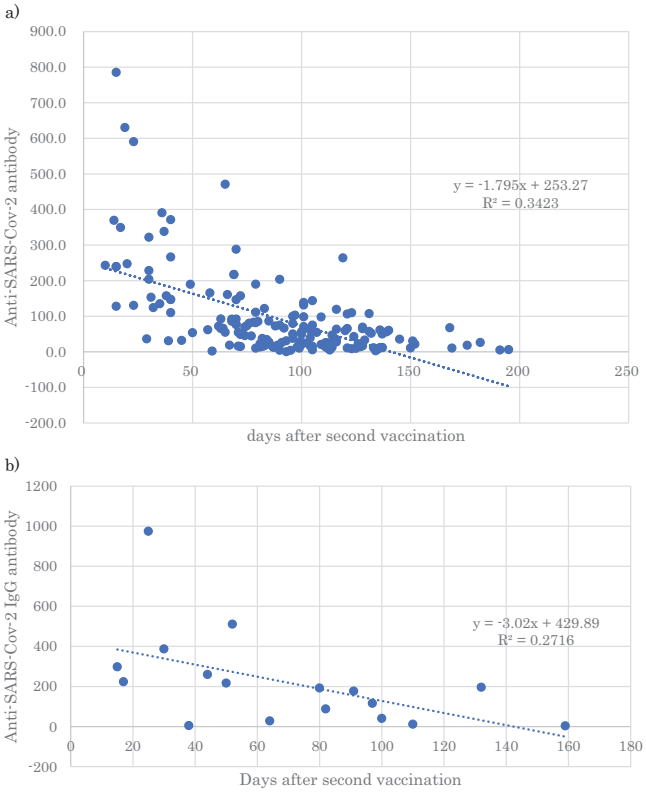

Fig. 4. Anti-SARS-Cov-2 IgG antibody titers: (a) non-smokers (n = 167); (b) smokers (n = 17).

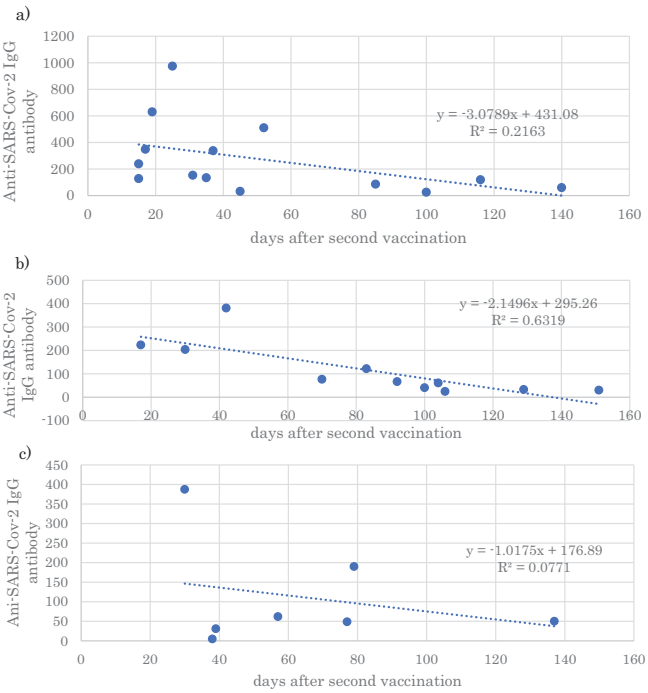

Fig. 5. Anti-SARS-Cov-2 IgG antibody titers: (a) age < 50, MHT (14); (b) age < 50, NMR (n = 11); (c) age < 50, RA on methotrexate (n = 7).

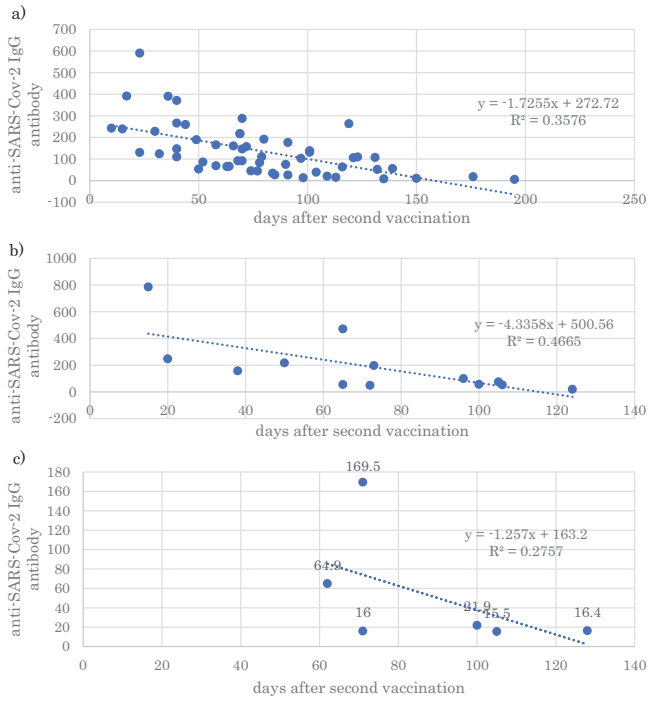

Fig. 6. Anti-SARS-Cov-2 IgG antibody titers: (a) age 50–60, MHT (54); (b) age 50–60, NMR (n = 13); (c) age 50–60, RA on methotrexate (n = 6).

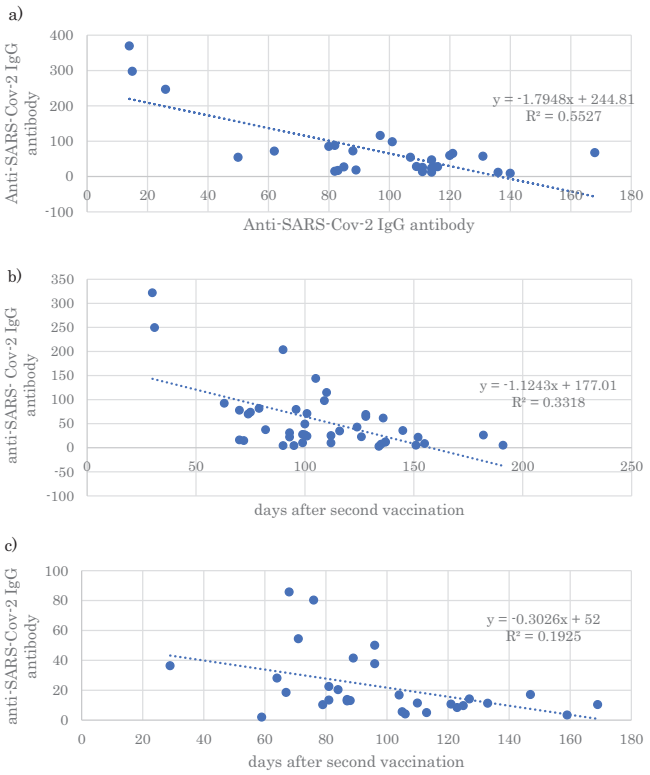

Fig. 7. Anti-SARS-Cov-2 IgG Antibody Titers: (a) age > 60, MHT (n = 29); (b) age > 60, NMR (n = 41); (c) age > 60, RA on methotrexate (n = 29).

3.6. Anti-SARS-Cov-2 Antibody Titers vs. Days Post-Second Vaccination: MHT NMR and RA on Methotrexate Patients Aged 50–60

Anti-SARS-Cov-2 IgG antibody titers were significantly higher in the MHT group (n = 54) than the NHS group (n = 13); [F (1,63)=7.340, p=0.009] in Figs. 6a and 6b.

3.7. Anti-SARS-Cov-2 IgG Antibody Titers vs. Days Post-Second Vaccination: MHT, NMR and RA on Methotrexate Patients Age > 60

Antibody titers were significantly higher in the MHT group (n = 29) than in the NMR group (n = 41); [F (1,53 = 5.893, p = 0.019]. In this study, 15 cases with oral E3 2 mg were included because of the narrowing age difference among the three groups in Figs. 7a and 7b.

Antibody titers were also significantly higher in the NMR group (n = 41) than the RA in the methotrexate group (n = 29); [F (1,69 = 6.100, p = 0.016] in Figs. 7b and 7c.

4. Discussion and Conclusion

A three-month analysis of total humoral response to the Pfizer vaccine in healthcare workers has been reported [15]. This data was obtained using Roche assay; however, this is similar to the Beckman Coulter used in our study. Furthermore, ELISA is also used in testing for anti-antibodies in convalescent and longitudinal vaccinated patients [16].

Third doses of Pfizer (*boosters*) became available in the author's clinic on January 5. My anti-SARS-Cov-2 IgG antibody titers as assessed by CLIA (Beckman Coulter) were 4.9 AU/ml the day before, 26.9 AU/ml after 7 days, 144.5 AU/ml after 10 days, 230.3 AU/ml after 14 days, 206.6 AU/ml after 21 days, 166.3 AU/ml after 56 days and 96.9/ml after 84 days. According to these results, antibody titers may be highest 14 days following booster vaccination. In this study of 219 patients, very few were tested 14 days post-vaccination. It is likely that the highest titers, 1200 AU for Moderna and 800 AU for Pfizer were obtained 14 days post-second vaccination dose. In Japan, three anti-SARS-Cov-2 antibody tests are available, the data from which is not directly comparable. Standard sera obtained from WHO may be 1/8 on Abbott testing, and 4 times on Beckman Coulter (per personal experience at the Health Sciences Research Institute in Yokohama). It is necessary to develop a universal standard. At present, anti-SARS-Cov-2 IgG antibody values obtained on Abbott testing may be equivalent to 32–50 times those detected by Beckman Coulter. Further, according to the author's experience, anti-SARS-Cov-2 IgG antibody titers post-second vaccination return to baseline at 4 months in patients aged over 65, and at 6 months in younger patients.

At the commencement of 2022, a well man who had been infected with Omicron two weeks prior presented to the author's clinic. His anti-SARS-Cov-2 IgG antibody titer was 2526.4 AU/ml. It is suspected that antibody titers in active SARS-Cov-2 infection are twice that of vaccinated patients. However, even following infection, anti-SARS-Cov-2 IgG antibodies do not remain >10 AU/ml for one year, as presented in Table I.

Regarding the production of anti-SARS-Cov-2 IgG antibodies, many factors such as vaccine type, age, gender, BMI, tobacco smoking, and others are implicated. However, there was no significant difference between Moderna and Pfizer in this study, possibly owing to low numbers

of Moderna patients. In addition, BMI and smoking did not have a significant impact on results, though again the small number of patients in the overweight/obese and smoking cohorts makes analysis fraught. Therefore, we have compared anti-SARS-Cov-2 IgG antibody titers in the MHT, NMT, and RA on methotrexate groups.

In general, premenopausal women have higher SARS-Cov-2 infection rates but lower mortality. The reasons are not identified, however, ACE2 receptors increase from childhood to adulthood. Sufficient estradiol prevents the production of inflammatory cytokines [17], leading to the prevention of fatal disease.

17β Estradiol was reported to be protective against SARS-Cov-2 infection in Germany in October 2020 [12]. A cohort of women aged over 50 were divided into estradiol users and non-users, and the 180-day survival probability was compared. For estradiol users, the survival rate of 96.7% (deaths 10/439) was significantly higher than the rate of 84.9% in the non-user group (deaths 1,072/16,278). These results strongly suggested that HRT is protective against SARS-Cov-2 mortality. This result led us to conduct this study into the influence of MHT on antibody titers following SARS-Cov-2 vaccination in menopausal women. As expected, anti-SARS-Cov-2 IgG antibody titers post-second vaccination were significantly higher in the MHT group than the NMR group in patients aged >50; [$F(1,63) = 7.340$, $p = 0.009$]. Furthermore, MHT patients aged >60 also produced higher antibody titers than the corresponding NMR group; [$F(1,53) = 5.893$, $p = 0.019$]. Fifteen patients in the MHT group were treated with oral E3 and were initially excluded from the analysis because E3 is significantly less potent than conventional MHT; however, these were later re-included due to being of a similar age.

Patients in the RA on methotrexate group, regretfully, had lower antibody titers than their NMR comparators in the over-60 age group. The antibodies resulting from vaccination in this group decline and become negative within 3 months.

However, middle-aged women receiving HRT did not respond to the second vaccination. The reason remains clear, but some B cell activation might be concerned [18].

Hopefully, At the onset of SARS-Cov-2 infection determined by PCR, anti-SARS-Cov-2 IgG antibodies were simultaneously measured in vaccinated patients. Sufficient anti-SARS-Cov-2 19 IgG antibodies will be determined to prevent coronavirus.

A recent study showed the existence of HLA A 24 in 60% of Japanese is higher than that in 15% of the USA or Europe, and cytotoxic T lymphocytes having this type of HLA will be more reactive with COVID-19 infection, in previously infected people, than those without HLA A 24 [19]. Cellular immunity is also important to prevent SARS-Cov-2 infection.

In the last, this is the first report on anti-SARS-Cov-2 anti-IgG antibody determined by Beckman Coulter assay. In contrast, the results of the Abbott assay were too high to understand. Under 320 or 500 U/ml is estimated to be negative. It is not necessary to make amplification in clinical study. R2 of linear regression is relatively low because of exponential decline, however, MHT receiving over age 50 is statistically significant than NMR.

The limitations of this study include a small sample size due to being a single-centre trial.

As a control, 67 cases with non-MHT/non-RA (NMR) included patients with hypertension, hypercholesterolemia, and osteoporosis, 12 cases with CTD and 7 normal healthy subjects. It is difficult to enrol normal healthy subjects in clinical study.

Acknowledgment

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Conflict of Interest

Authors declare that they do not have any conflict of interest.

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