ORIGINAL CLINICAL SCIENCE

High torque tenovirus (TTV) load before first vaccine dose is associated with poor serological response to COVID-19 vaccination in lung transplant recipients

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Rogier AS Hoek, MD,[a,1] Erik AM Verschuuren, MD, PhD,[b,1] Rory D de Vries,[c]
Judith M. Vonk,[d] Debbie van Baarle, PhD,[e,f] Marieke van der Heiden,[e]
Johanna P van Gemert, MD, PhD,[b] Edmund J Gore, MSc,[e]
Hubert GM Niesters, PhD,[e] Michiel Erasmus, MD, PhD,[g]
Merel E. Hellemons, MD, PhD,[a] Sandra MJ Scherbeijn, BSc,[c]
Nynke Wijbenga, MSc,[a] Edris A.F. Mahtab, MD, PhD,[h]
Corine H. GeurtsvanKessel, MD, PhD,[c] and Coretta Van Leer Buter, MD, PhD[e]

*From the [a]Department of Pulmonary Medicine, University Medical Center Rotterdam, Rotterdam, the Netherlands;
[b]Department of Pulmonary Diseases, University of Groningen, University Medical Center Groningen, the Netherlands;
[c]Department of Viroscience, Erasmus Medical Center, Rotterdam, the Netherlands; [d]Department of Epidemiology and
Groningen Research Institute for Asthma and COPD (GRIAC), University of Groningen, University Medical Center Gro-
ningen, Groningen, the Netherlands; [e]Department of Medical Microbiology, University of Groningen, University Medical
Center Groningen, the Netherlands; [f]Center for Infectious Disease Control, National Institute for Public Health and the
Environment, Bilthoven, the Netherlands; [g]Department of Thoracic Surgery, University of Groningen, University Medical
Center Groningen, Netherlands; and the [h]Department of Cardiothoracic Surgery, Erasmus Medical Center, Rotterdam,
the Netherlands.*

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BACKGROUND: Serological responses to COVID-19 vaccination are diminished in recipients of solid
organ transplants, especially in lung transplant recipients (LTR), probably as result of immunosuppres-
sive treatment. There is currently no marker of immunosuppression that can be used to predict the
COVID-19 vaccination response. Here, we study whether torque tenovirus (TTV), a highly prevalent
virus can be used as an indicator of immunosuppression.
METHODS: The humoral response to the mRNA 1273 vaccine was assessed in 103 LTR, who received a
transplant between 4 and 237 months prior to vaccination, by measuring Spike (S)-specific IgG levels
at baseline, 28 days after first, and 28 days after the second vaccination. TTV loads were determined
by RT-PCR and Pearson's correlation coefficient was calculated to correlate serological responses to
TTV load.

RESULTS: Humoral responses to COVID-19 vaccination were observed in 41 of 103 (40%) LTR at 28 days after the second vaccination. Sixty-two of 103 (60%) were non-responders. Lower TTV loads at baseline (significantly) correlated with higher S-specific antibodies and a higher percentage of responders. Lower TTV loads also strongly correlated with longer time since transplantation, indicating that participants with lower TTV loads were longer after transplantation.

CONCLUSIONS: This study shows a better humoral response to the SARS-CoV-2 vaccine in subjects with a lower TTV load pre-vaccination. In addition, TTV load correlates with the time after transplantation. Further studies on the use of TTV load in vaccination efficacy studies in immunocompromised cohorts should provide leads for the potential use of this marker for optimizing vaccination response.

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Since the first reports of infections with SARS-CoV-2 at the end of 2019, a worldwide pandemic of corona virus disease 2019 (COVID-19) has followed affecting millions worldwide.[1] Factors associated with COVID-19 related mortality were older age, obesity, recent malignancy and chronic respiratory, cardiac and renal disease.[2,3] Solid organ transplant recipients (SOTR) are also at increased risk for severe COVID-19 because of their immunosuppressed state.[4-7] Vaccination of transplant recipients against COVID-19 is recommended by international guidelines,[8] although these patients were initially excluded from vaccine trials. Post-licensure vaccination studies in SOTR reported vaccination was safe, but low serological responses were reported. Seroconversion rates range from 0 to 36% in Lung transplant recipients (LTR)[9-11] compared to >90% for the mRNA-vaccines in the general population.[12] This vaccination response in LTR is significantly lower that what was demonstrated for recipients of liver, kidney, and heart transplants,[9] and can be explained by the fact that compared to other SOTR, LTR receive higher levels of immunosuppression. LTR also have higher mortality due to COVID-19 than other SOTR, because of infection of the allograft itself, advanced age of the recipient and increased intensity of immunosuppression.[13-16]

No marker exists which can be used assess the immunosuppressed state of SOTR, but in recent years Torque tenovirus (TTV) has been under investigation as a potential surrogate.[17] TTV is a single stranded DNA virus, with high prevalence worldwide.[18] TTV has not been shown to cause pathology.[18,19] Studies have demonstrated an association between the amount of detectable TTV in blood and graft survival after solid organ transplantation (SOT).[20,21] TTV is currently also being investigated as a marker for immunosuppression and immune reconstitution in stem cell transplantation recipients and in HIV infection.[19,22-24] In SOTR, TTV loads increase rapidly after transplantation with the induction of immunosuppression, reaching a plateau phase after 3 to 6 months.[25,26] The TTV load in this plateau phase is higher for LTR that for recipients of other organs.[27-30] In all SOTR, low TTV levels were shown to predict rejection episodes.[27-29] In contrast, high immunosuppression levels, as reflected in high TTV loads, potentially carry a risk for infection.[20,21,26,27,30]

Here, we hypothesize that the TTV load may be a predictor of the humoral response to the COVID-19 vaccine in LTR. To test this hypothesis, we determined the pre-vaccination TTV load in 103 LTR vaccinated with the mRNA-1273 (Moderna) COVID-19 vaccine, and determined if the serological response at 28 days after the second vaccine dose was associated with the baseline TTV load.

Methods

Study population

The study protocol was approved by the UMCG and the Erasmus MC ethical review boards and was registered in the Dutch clinical trials register under number NL9538. One hundred three adult LTR with a minimum time since transplantation of 4 months were included at 2 transplantation centers in the Netherlands: the Erasmus Medical Center in Rotterdam and the University Medical Center in Groningen. Patients with various times since and reasons for transplantation were included, reflecting the diversity of the entire population of LTR in the Netherlands. Patients with a prior positive PCR test for SARS-CoV-2 were excluded, as were patients with recent rejection episodes of less than 6 months ago. Participants received the mRNA-1273 vaccine in February and March 2021 (Moderna) and blood samples were obtained prevaccination, at the time of the second vaccination (28 days after the first vaccination) and 28 days after the second vaccination. Age, sex, transplantation type and date, medication and reason for transplantation were recorded using a Research Electronics Data Capture (REDcap) database.

Adverse and reactogenicity effects

Participants reported adverse events for seven days after each vaccination dose, noting local symptoms (pain, swelling, and redness), as well as systemic symptoms (fever, gastrointestinal symptoms, headache, fatigue, and myalgias). Participants recorded for how many days the symptoms lasted and how severe they were, ranging from not interfering with daily activities to preventing performing daily activities. Lung function was measured at each study visit and participants measured their lung function using home peak flow measurement or home spirometry at least once after each visit, or more frequently in case of symptoms. Participants were asked if they had been diagnosed with COVID-19 prior to each study visit.

Serology

Humoral immune responses to vaccination were measured with a quantitative assay directed against the SARS CoV-2 Spike (S) protein (Liaison SARS CoV-2 TrimericS IgG assay, DiaSorin, Italy), with a lower limit of detection of 4.81 BAU/ml. The assay was performed following the manufacturer's instructions. Values >= 10 BAU/ml were considered reactive and values of >= 300 BAU/ml were shown to correlate with presence of neutralizing antibodies, based on in-house validation studies and harmonization between serological methods used in collaborative study teams.[31] Antibodies against the viral Nucleocapsid protein (N) were measured on an Abbott Architect instrument using the Abbott SARS-CoV-2 IgG assay following the manufacturer's instructions.[32] Qualitative results and index values were used for analysis, with the cut-off for positive set at 1.4 S/CO.

Quantitiative TTV PCR

DNA extraction from serum samples, and amplification of DNA was performed as previously described.[21, 28] In brief, DNA extraction was carried out using the eMAG Nucleic Acid Extraction System (BioMerieux, Marcy, France). For DNA amplification and quantification, the Argene R-Gene TTV quantification kit (BioMerieux) was used on an Applied Biosystems 7500 (Thermo fisher, Waltham, MA) according to the manufacturer's instructions. The R gene assay is designed to detect the majority of TTV genotypes (1, 6, 7, 8, 10, 12, 15, 16, 19, 27 and 28). Due to limited sample volumes, 100 μl, a 1 in 2 dilution using DMEM, was performed prior to sample extraction (ThermoFisher,). Results are expressed in log copies/ml.

Statistical analysis

SPSS version 23 was used for statistical analysis. Serological responses below the lower limit of detection were set to 4.81 BAU/ml. Serological responses at different time-points were compared by paired t-tests on log-transformed data. Differences in serological responses among quartiles of TTV load were compared by independent t-tests on log-transformed data. Pearson's correlation coefficient was calculated to correlate serological responses to TTV load and TTV load to time since transplantation (all data were log-transformed). A linear regression line was calculated for both correlations. To compare baseline data among groups, we used Mann-Whitney-U tests for continuous data and chi-square tests for categorical data. Two-sided exact p-values are reported; a p-value < 0.05 was considered to be statistically significant.

To investigate the independent association of TTV load, age and time since transplantation with vaccine response a logistic regression was performed including these 3 variables. Since age was not linearly related to serological response, age was included as a categorical variable (i.e., <35 years, 35-50 years, 50-65 years, ≥65 years). In a second logistic regression model, the use of immunosuppressive medication was added to investigate the predictive value of immunosuppressive medication use on vaccine-response.

Results

Study population (characteristics)

Baseline characteristics of the 103 participants of the study are depicted in Table 1. Median age of the participants was 60 years (IQR 49-66), 54 (52%) were male. Most frequent reasons for transplantation were cystic fibrosis (CF) (17, 16.5%), obstructive lung disease, including COPD and obliterating bronchiolitis (46, 44.7%), pulmonary arterial hypertension (6, 5.8%), interstitial lung diseases and pulmonary fibrosis (24, 23.3%). Ten patients (9.7%) received a transplant for other reasons. Median time since transplantation at time-point of the first vaccination dose ranged from 4 to 250 months, with a median of 55 months (IQR 20-111 months). All participants received immunosuppression

Table 1   Baseline Characteristics

	(Low) Responders (n = 41)	Non-responders (n = 62)	p value
Age (median [IQR])	56.0 (38.0-65.5)	62.0 (54.5-66.0)	0.075
Sex (male)	22 (53.7%)	32 (51.6%)	0.839
Time since transplantation (months) (median IQR)	86.0 (28.0-155.0)	48.0 (14.8-86.0)	0.013
- <36 months	11 (26.8%)	29 (46.8%)	0.069
- 36-120 months	16 (39.0%)	22 (35.5%)	
- >120 months	14 (34.1%)	11 (17.7%)	
Lymphocyte count (median [IQR])*	1.67 (0.76-2.41)	1.29 (0.89-1.84)	0.315
Immunosuppressive treatment			
- Tacrolimus	38 (92.7%)	61 (98.4%)	0.299
- mTor inhibitors	4 (9.8%)	8 (12.9%)	0.759
- Mycophenolate-mofetil	30 (73.2%)	59 (95.2%)	0.001
- Azathioprine	8 (19.5%)	1 (1.6%)	0.003
Reason for transplantation			
- CF	12 (29.3%)	5 (8.1%)	0.022
- Obstructive lung disease	12 (29.3%)	34 (54.8%)	
- Pulmonary Hypertension	3 (7.3%)	3 (4.8%)	
- ILD/ Pulmonary fibrosis	9 (22.0%)	15 (24.2%)	
- Other/unknown	5 (12.2%)	5 (8.1%)	

Lymphocyte count was determined for 47 participants.

containing corticosteroids (dosed according to weight, 0.05-0.1 mg/kg), and most received tacrolimus (n = 99, 96%) and Mycophenolate-mofetil (MMF) (89, 86%). Patients receiving the most commonly used regimen of tacrolimus, Azathioprine or MMF, and corticosteroids (n = 85) were assessed by therapeutic drug monitoring for tacrolimus, aiming at through levels between 7 and 10 μg/L. At baseline, trough levels of tacrolimus were a median level of 7.8 μg/L (IQR 6.8-8.7 μg/L). During the study, some patients had slight dose adjustments if the trough levels were outside the target range, but none of the patients required changes in immunosuppression because of rejection, infection or toxicity.

Side effects and reactogenicity of the vaccine

Limited side-effects and reactogenicity of vaccination were observed. The most commonly reported symptom was tiredness for one day only (25% of participants), followed by muscle pain (16%) and headache (13%) for one day. Three individuals experienced local symptoms for 2 days. Symptoms were mild, responsive to simple pain relief, and did not interfere with daily activities in any case. No significant changes in lung function were observed as a result of vaccination. One participant who did experience reduction in lung function during the follow-up period was diagnosed with an unrelated infection.

Serological immune response

At baseline, all participants tested negative for antibodies against N. One of the participants tested low-positive for antibodies against S prior to vaccination, but this participant was included for further analysis. At the time of the second vaccination dose, 28 days after the first dose, seventeen participants (16.5%) had developed detectable antibodies against S (median 20 BAU/ml, range 11-473). At 28 days after the second vaccine dose, 41 participants (40%) had detectable antibodies against S-protein (median 55 BAU/ml, range 10-8540 BAU/ml). These 41 participants were all considered responders. Sixty-two participants who did not develop detectable antibodies above 10 BAU/ml were considered non-responders. None of the participants developed antibodies against N in this period. Only 13 (12.6%) participants had antibody levels exceeding 300 BAU/ml (Figure 1).

Non-responders to the vaccine were more likely to have received immunosuppression containing MMF. In the groups of responders, 71% (n = 29) received MMF and in the non-responder group this was 95% (n = 59; $p = 0.001$; Table 1). Use of azathioprine was associated with good serological response ($p = 0.003$). Seventeen individuals with CF were included, 12 (71%) of whom showed a serological response to the vaccine. In contrast, 46 individuals with obstructive lung disease were included, of whom only 12 (26%) showed serological response. LTR with CF were significantly younger at time of vaccination than patients with obstructive lung disease (41.7 years [IQR 34-49.5] versus 63 years [IQR 57.5-66.3]; $p < 0.001$).
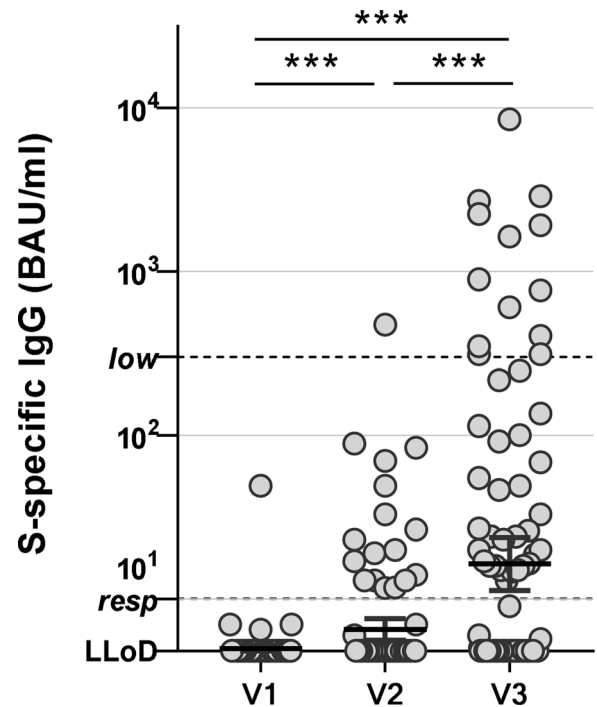

Figure 1 Serological responses to mRNA-1273 vaccination in lung transplant recipients. SARS-CoV-2-specific antibodies were measured pre-vaccination (V1), 28 days after the first vaccination (V2) and 28 days after the second vaccination (V3). Individuals with a value of >10 are considered responders. IgG levels >300 BAU/ml are known to correlate with neutralizing antibody activity. All individual values are shown, bars indicate the geometric mean $\pm$ 95% confidence interval. ***$p < 0.001$ as calculated by paired t-test on log-transformed values of the S-specific IgG. LLoD = lower limit of detection.

Time since transplantation was associated with serological response to the vaccination. The median time since transplantation was 86 months (28-155 months) for responders and 48 months (15-86 months) for non-responders ($p = 0.013$). Median age in responders was 56 years (38-65) and in non-responders the median age was 62 (54-66).

TTV

We measured the viral load of TTV in serum to assess its predictive value on the serological response to the COVID-19 vaccine. Six participants were negative for TTV. In 5 of 6 of we confirmed latent infection by positive testing of follow-up blood samples. For further analysis all 6 were considered latently infected with undetectable loads.

SARS-CoV-2-specific antibody titers in response to the mRNA-1273 vaccination correlated with TTV loads at baseline. Antibody levels were higher in patients with lower TTV viral loads (R between log(TTV) and log(SARS-CoV-2 antibodies) = -0.322, $R^2 = 0.104$, $p = 9.05*10^{-4}$; Figure 2A). For analysis of patient characteristics associated with TTV loads, participants were divided in 4 groups of 25 to 26 participants based on TTV load. The percentage of responders was higher in patient groups with lower
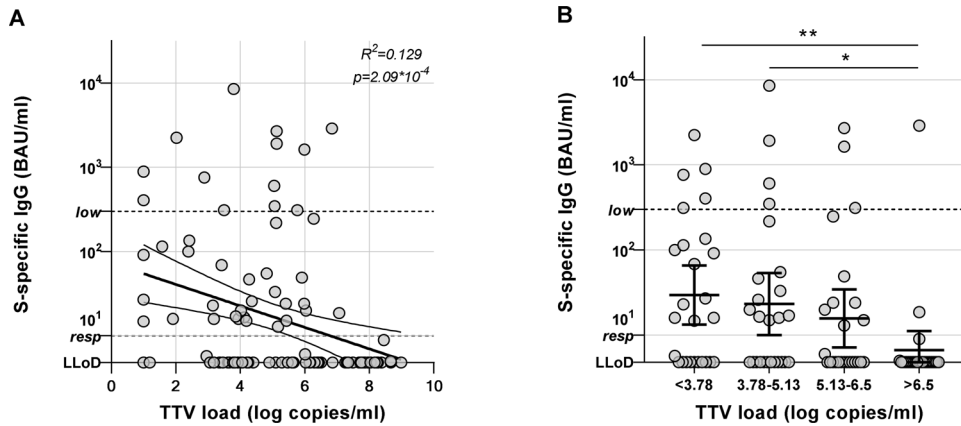
A

B

Figure 2   Serological responses in relation to TTV load. (A) SARS-CoV-2-specific antibody responses to mRNA-1273 vaccination were correlated to TTV viral loads. Pearson's correlation coefficient between log(TTV) and log(S-specific IgG) = −0.359 ($R^2 = 0.129$, $p = 2.09 \times 10^{-4}$). Curve indicates the linear regression line with 95% confidence interval. (B) Participants were grouped in 4 quartiles corresponding to TTV viral load (<3.78, 3.78-5.13, 5.13-6.5, and >6.5 log copies/ml) and serological responses per group are shown. Individuals with a value of >10 are considered responders. IgG levels >300 BAU/ml are known to correlate with neutralizing antibody activity. All individual values are shown, bars indicate the geometric mean ± 95% confidence interval. *$p < 0.05$, **$p < 0.01$ as calculated by t-test on log-transformed values of S-specific IgG. LLoD = lower limit of detection.

TTV loads. In the group with the highest TTV levels (>6.5 log copies/ml [n = 26]), there were only 2 serological responders (7.7%). In the group with the second highest TTV loads (between 5.13 and 6.50 log copies/ml), there were 10 serological responders (40%). In the group with TTV levels between 3.78 and 5.13 log copies/ml, there were 14 responders (53.8%), and in the group with the lowest TTV levels (<3.78 log copies/ml) which included the 6 participants with undetectable TTV, there were 15 responders (57.7%) ($p = 7.76*10^{-4}$; Figure 2B). Five of 6 patients (83%) with undetectable TTV loads at baseline developed an antibody response to the vaccine.

TTV loads correlated strongly with time since transplantation. The TTV loads were lower in individuals who were longer after transplantation (R between log(time since transplantation) and log(TTV) = −0.428, $R^2 = 0.183$, $p < 6.46*10^{-6}$) (Figure 3). Time since transplantation was the shortest 17.5 (11-57.5) in the group with the highest TTV loads (>6.50 log copies/ml). The group with the longest time since transplantation (97 months (57-159)) also had the lowest TTV loads (<3.78 log copies/ml) ($p = 2.86*10^{-5}$). Tacrolimus through levels and age of the participants were not associated with TTV load (Table 2).

Multivariate logistic regression analysis was performed to assess the impact of TTV load at baseline, time since transplantation and age on humoral vaccination response. The results show that both TTV and age ≥50 years are significantly associated with a lower odds of being a (low) responder. Time since transplantation is not independently associated with vaccine-response (Table 3).

Furthermore, logistic regression analysis aiming to investigate the impact of MMF and azathioprine treatment on the responder status of the patient was performed, showing that the use of MMF was associated with a lower odds of being a (low) responder, whereas the use of azathioprine was not (Table 4).

Discussion

Here, we studied the correlation between the serological response to the mRNA 1273 COVID-19 vaccination and baseline TTV loads. We observed that a low number of LTR produce antibodies to the S protein after vaccination (40% after 2 vaccinations), similar to 35% responders that Hallett et al[33] found in a study of equal size. Only 13 participants (13%) developed IgG levels >300 BAU/ml, a level above which neutralizing antibody activity can usually be demonstrated.[31] Several studies have now reported that LTR have lower responses to the COVID-19 vaccine
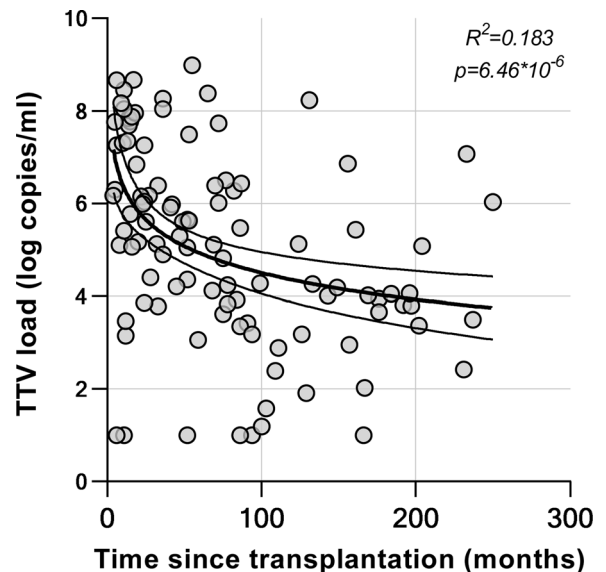

Figure 3   TTV load in relation to time since transplantation. TTV viral loads were correlated to the time since lung transplantation. Pearson's correlation coefficient between log(time since transplantation) and log(TTV) = −0.428 ($R^2 = 0.183$, $p < 6.46*10^{-6}$). Curve indicates the linear regression line with 95% confidence interval for log(time since transplantation) on log (TTV).

Table 2 Patient Groups According to TTV Levels and Associations With Baseline Characteristics

	TTV ≥ 6.5 log copies/ml (n = 26)	TTV 5.13-6.5 log copies/ml (n = 25)	TTV 3.78-5.13 log copies/ml (n = 26)	TTV < 3.78 log copies/ml (n = 26)	p value
% (low) responders	7.7 % (n = 2)	40.0% (n = 10)	53.8% (n = 14)	57.7% (n = 15)	$p = 2.86 \times 10^{-5}$
Age (median IQR)	61.5 (51.0-65.0)	59.0 (38.5-63.5)	61.0 (39.5-66.3)	62.0 (50.8-67.3)	$p = 0.657$
Time from transplant (median, IQR)	17.5 (11.0-57.5)	41.0 (22.5-71.0)	81.0 (50.3-170.8)	97.0 (57.3-159.3)	$p = 2.86 \times 10^{-5}$
Tacrolimus trough levels (median, IQR) (μg/L)*	8.20 (6.80-9.55)	8.40 (7.40-9.70)	7.40 (6.98-8.50)	7.45 (6.60-8.55)	0.542
MMF-free treatment (n = 13)	7.7% (n = 2)	20.0% (n = 5)	11.5% (n = 3)	15.4% (n = 4)	0.581

*Tacrolimus trough levels are only given for the 85 participants who received the standard triple therapy regimen with Tacrolimus, MMF and prednisone.

Table 3 Logistic Regression Including TTV Load, Age (in categories) and Time Since Transplantation on Vaccine Response

		OR	95% CI	p-value
Log(TTV load at baseline)		0.62	0.47-0.80	3.40×10^{-4}
Age	<35	reference		
	35-50	0.49	0.08-3.09	0.45
	50-65	0.14	0.03-0.78	0.03
	≥65	0.15	0.03-0.87	0.04
Time since transplantation		1.00	1.00-1.01	0.31

The results show that both TTV and age >50 years are significantly associated with a lower odds of being a (low) responder. time since transplantation is not associated with vaccine-response.

compared to recipients of liver, kidney and heart transplants.[9,34] This can be explained by the fact that compared to other SOTR, LTR receive higher levels of immunosuppression. No marker exists which can be used assess the immunosuppressed state of SOTR, but in recent years TTV has been under investigation as a potential surrogate. As such, studies have found higher TTV loads in LTR than in recipients of other SOTs.[27-30]

In this study we found that poor serological responses to COVID-19 vaccination are significantly associated with baseline TTV loads. Our study shows a remarkably wide range of TTV loads, from negative to 10^9 copies/ml. Higher S-specific antibody titers and responder rates are found in individuals with lower TTV loads. However, even in the lowest TTV level group of < 3.78 log copies/ml there were

only 57.7% responders, which is a response rate far below that in populations with no underlying illness.[12] When comparing immunosuppression, we found that the use of MMF was associated with less serological responses, which was already shown by several other studies.[31,33] However, the use of MMF was not clearly associated with TTV load. After adjustment for MMF use, we did not find that azathioprine was associated with response rates, although both MMF and azathioprine belong to the same class of antirejection drugs. However, the number of patients receiving azathioprine was small in our cohort and azathioprine was only used in patients not receiving MMF. Possibly, immunosuppressive agents, even those with a comparable effect on antirejection activity, have diverse effects on the vaccination response. Furthermore, factors unrelated to

Table 4 Logistic Regression Model in which the Significant Variables from Table 3 (i.e., Age and TTV Load) are Included and the 2 Immunosuppressive Medications that are Significantly Associated with Vaccine-Response in the Univariate Analysis in Table 1 (Azathioprine and MMF)

		OR	95% CI	p-value
Log(TTV load at baseline)		0.54	0.40-0.73	7.95×10^{-5}
Age	<35	reference		
	35-50	0.79	0.12-5.46	0.81
	50-65	0.17	0.03-1.02	0.05
	≥65	0.21	0.03-1.30	0.09
Azathioprine		2.20	0.16-29.57	0.55
MMF		0.09	0.01-0.62	0.02

The use of MMF is associated with a lower odds of being a (low) responder.

immunosuppression such as age, may also influence the immune response. In the logistic regression analysis we show that higher age correlated with a lower response to the vaccine. In our study, we show that patients with CF had higher response rates, and patients with obstructive lung diseases had lower response rates to vaccination. However, LTR who had CF were significantly younger than those with obstructive lung disease, which suggests that the differential responses may be attributable to the age difference between the groups.

Interestingly, TTV loads correlate with time since transplantation. We show that a longer time since transplantation was associated with both better serological response to the COVID-19 vaccine, and with lower TTV load. The association between time after transplantation and vaccination response was shown by others in LTR as well as in recipients of other types of organs.[9,31] We now found that that time after transplantation correlates strongly with TTV load. By logistic regression analysis we show that the TTV load is an important predictor of serological response, but that time since transplantation is not independently associated with vaccination response. High TTV loads in this study nevertheless are found predominantly in the first months to years after transplantation, and patients who have received lung transplants several years before, tend to have lower TTV.

There are only few studies which have investigated the kinetics of TTV loads. TTV levels are found to increase after transplantation, and a plateau phase is reached after 3 to 6 months in these studies.[25,26] If no acute rejection events occur, immunosuppression is stable after this period, and the thought is that TTV levels are stable as well. However, the existing data investigating TTV levels years after transplantation is not based on longitudinal observations and is inevitably biased toward SOTR who are still alive at that time. Although our study was not set up as a long-term/longitudinal TTV study, our data may suggest that TTV levels vary over time. The high TTV loads observed in the first few years after transplantation might result from long-term effects of induction therapy, and TTV load may decrease over time. Another explanation for the finding of lower TTV loads years after transplantation is that patients with lower TTV loads are more likely to be alive at that time. Although this has not been established for LTR, in renal transplant recipients it was shown that survival was lower in patients with high TTV loads.[21] If indeed LTR with high TTV loads have lower survival rates, this could mean that the favorable vaccination response in patients longer after transplantation is not necessarily associated with time, but that patients who are more affected by the immunosuppression and respond less well to vaccination have a lower chance of survival beyond a certain number of years.

In conclusion, this study investigated the serological response to the mRNA1273 vaccine in lung transplant recipients and shows a serological response rate of 40% after 2 vaccinations. This study is the first to investigate the association between TTV load and vaccination response. We show a significant association between a lower TTV load and a better serological vaccination response. This finding sheds a different light on the more favorable response found in SOT patients who are longer after transplantation which is consistently found also in COVID-19 vaccine efficacy studies in different transplantation patients. All these studies, including this present study, may be biased toward inclusion of patients with more optimal immunosuppression, resulting in better vaccine response as well as longer survival. Longitudinal, prospective studies investigating TTV loads in transplant recipients looking into graft survival and overall survival in all SOT patients, are therefore recommended. Moreover, the association between TTV load and vaccine response should be investigated in other cohorts of immunocompromised individuals. If the TTV load prior to vaccination is indeed a representative predictor of vaccine response, this could be used as a potential guidance for optimizing vaccination regimens.

Author contributions

Disclosure statements

Acknowledgments

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