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## List of abbreviations

BAU: Binding antibody unit

CLIA: chemiluminescence immunoassay

D2: second dose

D3: third dose

IGRA: IFN-γ-releasing assay

KTRs: Kidney transplant recipients

RBD: Receptor binding domain

## Abstract

Kidney transplant recipients (KTRs) have reduced ability to mount adequate antibody response after two doses of COVID-19 mRNA vaccine. French health authorities have allowed a third booster dose (D3) for KTRs, but their response is heterogeneous and tools able to discriminate the responders are lacking.

Anti-RBD IgG titers (chemiluminescence immunoassay), spike-specific cellular responses (IFN-γ-releasing assay, IGRA), and *in vitro* serum neutralization of the virus (the best available correlate of protection), were evaluated 7-14 days after the second dose (D2) of BNT162b2 vaccine in ninety-three KTRs. Among the 73 KTRs, whose serum did not neutralize SARS-CoV-2 in vitro after D2, 14 (19%) acquired this capacity after D3, and were considered as “responders”. Exploratory univariate analysis identified short time from transplantation and high maintenance immunosuppression as detrimental factors for the response to D3. In addition, any of the presence of anti-RBD IgGs and/or positive IGRA after D2 was predictive of response to D3. By contrast, none of the KTRs with both a negative serology and IGRA responded to D3. In summary, routinely available bioassays performed after D2 allow identifying KTRs that will respond to a booster D3. These results pave the way for the personalization of vaccination strategy in KTRs.

## Keywords:

Renal transplantation; SARS-CoV-2; COVID-19; mRNA-vaccine

## 1. Introduction

Kidney transplant recipients (KTRs) carry a very high risk of death due to COVID-19 in case of infection by SARS-CoV-2 [1–5]. This vulnerable population has therefore been prioritized for vaccination. However, only 25% (range: 2.5%-48%) of KTRs develop adequate antibody response after the “standard” two doses of COVID-19 mRNA vaccine–[6–14]. These antibodies are responsible for the neutralization of the virus, that can be assessed using *in vitro* functional assay, which currently represents the best available correlate of protection against severe COVID-19 for both the general population[15] and KTRs [16]. Accordingly the lack of adequate antibody response after vaccination in KTRs correlated with the occurrence of (sometime severe forms of) COVID-19 in vaccinated patients[17–19].

Preliminary reports suggest that a third dose (D3) of vaccine improves the humoral response in transplant recipients [20–25]. In this prospective observational study, we aimed at describing the immune response of KTRs to D3 of mRNA vaccine and identifying the variables associated with response to this booster dose.

## 2. Methods

### *2.1. Study population*

The study protocol was approved by Institutional Review Board (approval number: 2020-A02918-31, Comité de Protection des Personnes Sud-Est I). All patients gave signed informed consent for the participation to the study. According to French health authority's recommendations, a third vaccine injection was offered to all KTRs from Lyon University Hospital, whose serum showed no *in vitro* viral neutralization capacity after two doses of BNT162b2 mRNA vaccine (Pfizer-BioNtech).

Blood samples were collected the day of the first vaccine injection and between 7 and 14 days following D2 and D3.

Of note, we verified that none of the 3 assays (described below) that we used in this study was affected by difference in sampling time across patients, as shown in **Supplementary Figure 1.**

### *2.2. Assessment of the tolerability and safety of vaccine injections*

Local and systemic adverse events were collected retrospectively at each follow-up visit. Data collected correspond to adverse events within 7 days after D2 and D3, respectively. Data on allograft dysfunction were collected at the end of the follow-up.

### *2.3. Anti-SARS-CoV-2 humoral response assessment*

#### *2.3.1. Anti S-RBD IgG*

The IgG antibodies directed against the Receptor Binding Domain (RBD) of the spike glycoprotein of the SARS-CoV-2 were detected by a chemiluminescence immunoassay (CLIA), using the Maglumi® SARS-CoV-2 S-RBD IgG test (Snibe Diagnostic, Shenzen, China) on a Maglumi 2000® analyser (Snibe Diagnostic)[26],

according to the manufacturer's instructions. This test displays clinical sensitivity and specificity of 100% and 99.6%, respectively. As recommended by the WHO, the obtained titer was then expressed as binding antibody units/mL (BAU/mL); correction factor for Maglumi®: 4.33.

*2.3.2. In-vitro viral neutralization assay*

The test was performed as previously reported[27,28]. SARS-CoV-2 [BetaCoV/France/IDF0571/2020 virus (GISAID Accession ID = EPI_ISL_411218)] was isolated in Vero E6 from a nasal swab of one of the first COVID-19-positive patient in France and was kindly provided by Dr Olivier Terrier and the Virpath lab (CIRI-Lyon). To generate virus stocks, Vero E6 cells (kindly provided by Dr F-L. Cosset, CIRI-Lyon) were inoculated with virus at an MOI of 0.01. Supernatant fluid was harvested at 72 h post-infection, clarified by low-speed centrifugation, aliquoted, and stored at −80°C. Virus stock was quantified by classic limiting dilution plaque assay on Vero E6 cells.

Two-fold dilutions of serum in 50 µl of Dulbecco's modified Eagle medium (DMEM), containing 2X penicillin/streptomycin, were incubated with 200 plaque-forming units (PFU) of SARS-CoV-2 in 50 µl of DMEM for 15 min at room temperature. Aliquots of 100 µl of DMEM + 4% FBS containing $2.5x10^4$ Vero E6 cells were added to achieve a final dilution of sera from 1:100 to 1:12,800 (4 wells per dilution). Cells were incubated for 5 days at 37°C, 5% CO2. After 15min of fixation in PFA4% in PBS1X, cytopathic effect was revealed by crystal violet staining and scored by a researcher (CM) blinded to the study design and sample identity. Neutralization endpoint titers were expressed as the log10 value of the last serum dilution that completely inhibited virus-induced cytopathic effect.

### *2.4. Anti-SARS-CoV-2 Spike cellular response assessment*

Spike specific cellular response was quantified in the circulation of the KTRs using the QuantiFERON® SARS-CoV-2 test (Qiagen, Netherlands), a commercially available Interferon Gamma Releasing Assay (IGRA), according to the manufacturer's instructions [29].

Briefly, one milliliter blood was distributed in each tube of the assay: (i) uncoated tube: negative control/background noise, (ii) tube coated with mitogen: positive control, and (iii) tube coated with 13-mers peptides derived from the SARS-CoV-2 S1-Spike glycoprotein (thereafter designated as Ag1 tube). After 20 hours of culture at 37°C, tubes were centrifugated 15 minutes at 2500g, and stored at 4°C before IFN-γ quantification in the supernatant by ELISA. Although various cell types contribute to the production of IFN-γ in the IGRA, CD4+ T cells is the dominant subset (38% in the Ag1 tube, **Supplementary Figure 2A & 2B** and **Supplementary Methods**). Furthermore, the results of the IGRA correlate with the response of follicular helper T cells (the subset of CD4+ T cells specialized in B cell help for antibody production) evaluated by flow cytometry [30] (**Supplementary Figure 2A to 2D** and **Supplementary Methods**).

To be deemed analyzable, the IFN-γ concentration in the positive control tube had to exceed 0.5 IU/mL. The cellular assay value was the difference between the tube (iii) and the negative control (i). A test was considered positive if the value of IFN-γ concentration exceeded 0.07 IU/mL, a threshold which corresponds to the highest value obtained in a cohort of 13 controls (healthy volunteers naive for SARS-CoV-2, **Supplementary Figure 2E**). To allow for log scale representations, negative and zero values were reported at 0.01 IU/mL.

### *2.5. Statistical analysis*

All the analyses were carried out using R software version 4.0.4 (R Foundation for Statistical Computing, Vienna, Austria, 2021, https://www.R-project.org) and or GraphPad Prism v8.0 (San Diego, California USA).

Categorical variables were expressed as percentages and compared with a two-sided chi-square test or a two-sided Fisher's exact test when the conditions for a chi-square were not fulfilled. Since the results of the biological assays did not have a normal distribution, they were expressed as median and interquartile range (IQR) and compared using Mann Whitney test. Wilcoxon test was used for paired data. Other continuous variables were expressed as mean ± SD.

Logistic regression model was used for univariate analyses aiming at identifying potential differences between responders and non-responders to D3. No correction was applied for multiple tests in this exploratory analysis and the threshold for significance was set as $p<0.05$.

# 3. Results

## *3.1. Description of the cohort*

Ninety-nine consecutive kidney transplant recipients (KTRs) from Lyon University Hospital were offered a two-doses scheme of an anti-SARS-CoV-2 mRNA vaccine (BNT162b2, Pfizer-BioNtech). Among them, 2 developed COVID-19 before receiving the second dose and 4 were lost during the follow-up (**Figure 1**).

The clinical characteristics of the 93 remaining patients are presented in **Table 1.** Mean age was 55.7±12.4 years, 54% were male (50/93). Forty-one percent (38/93) of the patients had comorbidities, including 25% (23/93) with a cardiovascular disease and 23% (21/93) with diabetes mellitus. Enrolled patients were transplanted for 9.9±8.8 years in mean. Seventy percent (65/93) were on a triple immunosuppression maintenance regimen (including a calcineurin inhibitors, an anti-proliferative and low-dose steroids).

Before vaccination, 16/93 KTRs (17%) had detectable titers of anti-RBD IgGs (**Figure 2A**), among which only 5 had a past positive SARS-CoV-2 PCR. In the rest of the study, these16 patients were all considered as having a past history of COVID-19.

## *3.2. Spike-specific humoral and cellular responses after two doses of vaccine*

The cellular and humoral responses against the spike protein of SARS-CoV-2 were measured between 7 and 14 days after D2 (mean sampling time: 9.6±3.0 days).

Forty-seven percent (31/77) of naive patients developed detectable anti-RBD IgG after the D2 (**Figure 2A**), but only 3 of them (3%) had a serum with viral neutralization capacity *in vitro* (**Figure 2B**). Patients with a past history of COVID-19

developed higher titers of anti-RBD IgG after D2 (1801 BAU/mL, IQR [90; 3757] in KTRs with a history of COVID-19 vs 2 BAU/mL, IQR [1, 39] in naïve KTRs; p<0.0001) and as expected, a higher proportion (10/16, 62.5%) of the latter had serum with *in vitro* viral neutralization capacity (**Figure 2B**). Thus, a history of COVID-19 appeared to be a major determinant of serum neutralization capacity after 2 doses of vaccine (OR 41.1, 95%CI [8.9; 154.6], p<0.0001).

Analyzing paired data from serology and viral neutralization assay, we confirmed the relation between high titers of anti-RBD IgG and viral neutralization capacity of the serum (Pearson's $R^2$=0.7982, p=0.0002; **Figure 2C**). All sera with ≥ of 1000 BAU/mL of anti-RBD IgG had viral neutralizing capacity *in vitro*. Of note, this relatively high threshold as compared with what published in the literature, is due to the fact that we determined virus neutralization capacity *in vitro* using live virus. This is considered as the gold standard for coronaviruses [31], but requires higher antibody titers than assays relying on pseudotyped virus-like particles or surrogate virus neutralization assays. We believe that this choice is justified by the fact that high antibody titers are necessary to retain neutralizing activity against the various emerging variants, and because of the rapid decay of antibody titers over time [32].

As the humoral response, the spike-specific cellular response of KTRs was also heterogeneous after D2. Only 23/76 (30%) of naive KTRs and 8/16 (50%) of patients with a past history of COVID-19 had a positive IGRA. However, in contrast with the humoral response, no significant difference was observed between these 2 groups regarding spike-specific cellular response (**Figure 2D**).

### *3.3. Tolerability of a third dose of mRNA vaccine*

Considering that these patients were not adequately protected against symptomatic COVID-19 and following French health authorities' recommendations, a third dose of BNT162b2 mRNA vaccine was offered to all 73 KTRs, whose serum had no viral neutralization capacity after D2 (7 patients were lost from follow-up after D2, **Figure 1**). The mean time between the second and third dose was 34.6±5.3 days. Overall, the clinical tolerance of D3 was excellent and comparable to that of D2 (**Figure 3**). There were no serious adverse events or graft dysfunction reported. The main side-effect was pain at the site of injection, which occurred with the same incidence after the second and the third dose (~50% of patients). Five patients had fever <39°C for a maximum of two days after D3.

### *3.4. Efficacy of a third dose of mRNA vaccine*

Spike-specific humoral and cellular immune responses of KTRs were monitored after D3 (mean sampling time: 12.3±2.1 days). Administration of D3 resulted in a significant increase in anti-RBD IgG titers in non-responders to D2 (median IgG titer: 2.3, IQR [1.0; 40.6] after the second dose vs 82.3, IQR [1.9; 464.9]; p<0.0001, **Figure 4A**). Fourteen patients (14/73, 19%) developed viral neutralizing capacity after D3 (**Figure 4B**). In contrast, spike-specific cellular response after D3 was much more heterogeneous, with some patients increasing IFN-γ secretion in IGRA while the cellular response of others remained stable or even decreased (**Figure 4C**). Overall, spike-specific cellular responses after D2 and D3 were not statistically different (p=0.205; **Figure 4C**).

### *3.5. Clinical and biological variables predictive of response to D3*

In order to identify which clinical and biological variables were associated with response to D3, KTRs were divided into responders and non-responders, according

to whether or not they had acquired viral neutralizing capacity after the booster dose. Regarding clinical parameters, responders and non-responders had the same age and comorbid profile. Baseline biological parameters, including lymphocyte and monocyte counts and creatinine, were also similar (**Table 1)**. In contrast, responders to D3 had been transplanted for longer time than non-responders (17.6±11.3 vs 8.9±6.9 years, p=0.002), and were less frequently exposed to mycophenolate mofetil (5/14 vs 50/59, p=0.001) and tacrolimus (6/14 vs 44/59, p=0.027; **Table 1**).

In addition, the presence of non-neutralizing titers of anti-RBD IgG (**Table 1, Figure 4D**), or a positive IGRA (**Table 1, Figure 4E**) after D2, were both associated with a better response to D3 (OR 10.09, 95%CI [2.46; 68.85], p 0.004 and OR 4.19, 95%CI [1.25; 14.82], p=0.021, respectively). Furthermore, we observed that the probability to respond to D3 in KTRs was the highest in patients positive for both tests (46%, **Figure 4F**). The response rate decreased to 29% in KTRs with only sub-optimal anti-RBD IgG titers after the second dose and 22% in those with only a positive IGRA. Finally, none of the KTRs in whom both assays were negative after D2 did develop a viral neutralization capacity after D3. These results suggest that combining the results of these two assays may allow refining the prediction of the response to D3 in KTRs.

## 4. Discussion

Our monocentric prospective observational study confirms that the “standard” scheme of vaccination, based on two doses of mRNA vaccine, induces a very heterogeneous response in KTRs [6–14]. Administration of a third dose in non-responder patients was well tolerated and induced a significant increase in their anti-RBD IgG titers, allowing 19% (14/73) of them to develop viral neutralization capacity, which is currently considered as the most reliable correlate of protection [15,33]. Our results are in line with recent independent reports[20,21,23–25] and provide original evidence that the response to this third dose of vaccine can be predicted after the second dose combining the results of simple and easily accessible biological assays.

An important finding of our study is the fact that the third dose of vaccine was very well tolerated, with no serious adverse events nor graft rejection reported. At a time when SARS-CoV-2 vaccine regimens are regularly updated with additional booster doses, the information that an additional dose is as well tolerated as the second dose of vaccine is reassuring for KTRs.

Identification of patients that would benefit from a third dose of vaccine is an important unmet medical need for physicians, who need to simultaneously optimize the protection of this vulnerable population, while avoiding wasting time and precious vaccine doses. As previously reported by others, we observed that both a shorter time from transplantation and a higher level of maintenance immunosuppression were detrimental for the response to D3 [21,23]. However, we did not confirm the negative impact of age or renal function reported by Kamar et al [21]. This discrepancy could be related to the fact that our study only enrolled KTR or explained by a lack of statistical power due to the relatively small number of patients enrolled (n=73). Our

study went deeper in the exploration of the predictive factors associated with the response to D3 and, beyond clinical variables, demonstrated that this prediction could be refined by the use of biological assays. We not only confirmed that a sub-optimal titer of anti-RBD IgG after D2 was a predictive factor for response to D3 [20,23] but provide original evidence that KTRs without antibodies but a detectable spike-specific cellular response in IGRA had similar chances to respond to D3. This is concordant with the fact that the results of the IGRA correlate with the response of follicular helper T cells, the subset specialized in providing help to B cells for antibody production [34] (**Supplementary Figure 2C and 2D**).

An important, yet unsolved, question resulting from the total absence of response to D3 among KTRs with no detectable anti-RBD IgG and negative IGRA after D2 is how the latter vulnerable patients should be protected against COVID-19. Different strategies have been proposed to optimize response to vaccine. Reduction of maintenance immunosuppression[35] in KTRs may result in acute rejection and/or anti-HLA sensitization. Increasing vaccine immunogenicity by the use of heterologous prime-boost vaccination scheme seems an attractive option. However, when applied for the third vaccine dose in patients with immune-mediated inflammatory diseases or treated with Rituximab, this strategy did not increase response rates compared to an homologous 3D mRNA vaccine [36,37]. An alternative option is the passive immunization with anti-SARS-CoV-2 monoclonal antibodies, a primary prevention strategy which was recently successfully tested in people with household exposure to SARS-CoV-2 [38].

Our study has several limitations. First, we did not evaluate hard clinical endpoints (i.e., incidence of symptomatic COVID-19). Second, the analysis of the response to D3 has been performed without control group. This is due to the fact that in France,

health authorities have strongly encouraged the rapid administration of a third dose of vaccine in vulnerable patients (including KTRs), making the randomization against a placebo deemed unethical by regulatory authorities. Although it is theoretically possible that the increase in viral neutralization capacity observed in patients' serum is due to time rather than D3, we consider this possibility as highly unlikely. A recent double-blind, randomized, controlled trial of a third dose of mRNA-1273 vaccine performed in Canada indeed demonstrated that serum neutralization capacity tended to decrease after D2 in patients that received a placebo while it significantly increased in those that received a third dose of vaccine [22]. A third limitation of our study is the fact that the evaluation of the response to D2 and D3 was made at a single time point, 7-14 days after vaccine injection. One can argue that, although this time point is ideal for the assessment of cellular responses, it could be too early to evaluate humoral responses, especially in KTRs in whom these responses may be delayed [39,40]. However, delayed humoral response in vaccinated KTRs has not been observed by all investigators. Rincon-Arevalo et al reported that antibody titers after the second dose of COVID-19 vaccine peaked as early as 7 days and remained stable for several weeks thereafter in a cohort of KTRs [41]. This unique time point had the advantage to allow the simultaneous evaluation of both the cellular and the humoral response of patients and was therefore chosen to optimize the chances that patients would participate to the study.

In conclusion, combining the results of the serology and IGRA after D2 could allow optimizing the personalization of the strategy of vaccination against COVID-19 in KTRs [35], an approach that still requires independent validation.

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## Disclosure:

All authors declared no conflicts of interest.

## Supporting information

Additional supporting information may be found online in the Supporting Information section.

## Figure legends

### *Figure 1 – Flow chart of the study*

Abbreviations are: D2, second dose of BNT162b2 vaccine; D3, third dose of BNT162b2 vaccine.

***Figure 2 – Spike-specific humoral and cellular responses after the second dose of BNT162b2 vaccine***

Spike-specific humoral and cellular responses were measured 7-14 days after the D2 in kidney transplant recipients naïve for the SARS-CoV-2 (white circles) or with a previous history of COVID-19 (black circles).

**A.** Histogram showing the titers of anti-receptor binding domain (RBD) IgG before vaccination and after D2 of vaccine. Dashed line represents the limit of positivity of the assay. Wilcoxon test; ***, $p<0.001$; ****, $p<0.0001$.

**B**. Histogram showing the viral neutralization capacity of the serum in *in vitro* functional assay after D2. Mann-Whitney U test. Pie charts represent the proportion of patients with viral neutralization capacity. Fisher test; ****, $p<0.0001$; VNT, viral neutralization titer

**C**. The relationship between the anti RBD IgG titers and viral neutralization capacity was plotted. A linear regression was performed for patients with anti-RBD IgG titer above 1000 BAU/mL. $R^2$: Pearson's coefficient.

**D**. Histogram showing the concentration of interferon gamma (IFN-γ) measured in IGRA (Quantiferon SARS-CoV-2) after D2. Dashed line represents the limit of positivity of the assay. IU, international units

***Figure 3 – Tolerability of the third dose of BNT162b2 vaccine***

The proportion of kidney transplant recipients that developed local or systemic adverse events after the second and after the third dose of vaccine are plotted.

***Figure 4 – Spike-specific humoral and cellular responses after the third dose of BNT162b2 vaccine***

Humoral and cellular responses were measured 7-14 days after the third dose of vaccine in kidney transplant recipients without viral neutralization capacity after D2.

**A.** Histogram showing the individual evolution of anti-receptor binding domain (RBD) IgG tiers (in binding antibody units, B.A.U.) after D2 and D3. Dashed line represents the limit of positivity of the test. Wilcoxon test; ****, p<0.0001

**B.** Histogram showing the viral neutralization capacity of the serum in *in vitro* functional assay after D3. VNT, viral neutralization titer.

**C.** Histogram showing the evolution of the concentration of interferon gamma (IFN-γ) in IGRA (Quantiferon SARS-CoV-2) between D2 and D3. Dashed line represents the limit of positivity of the test. IU, international units

**D-E.** Histogram showing the viral neutralization capacity after D3 according to the absence (neg) or the presence (pos) of anti-RBD IgG (**D**) or IFN-γ in IGRA (**E**) after D2. Mann-Whitney U test. Pie charts represent the proportion of patients with viral neutralization capacity after D3. Fisher test. VNT, viral neutralization titer; *, p<0.05; **, p<0.01; ***, p<0.001

**F.** Histogram showing the proportion of patients with viral neutralization capacity after D3 according to the presence (+) or the absence (-) of anti-RBD IgG and or positive IGRA after D2.

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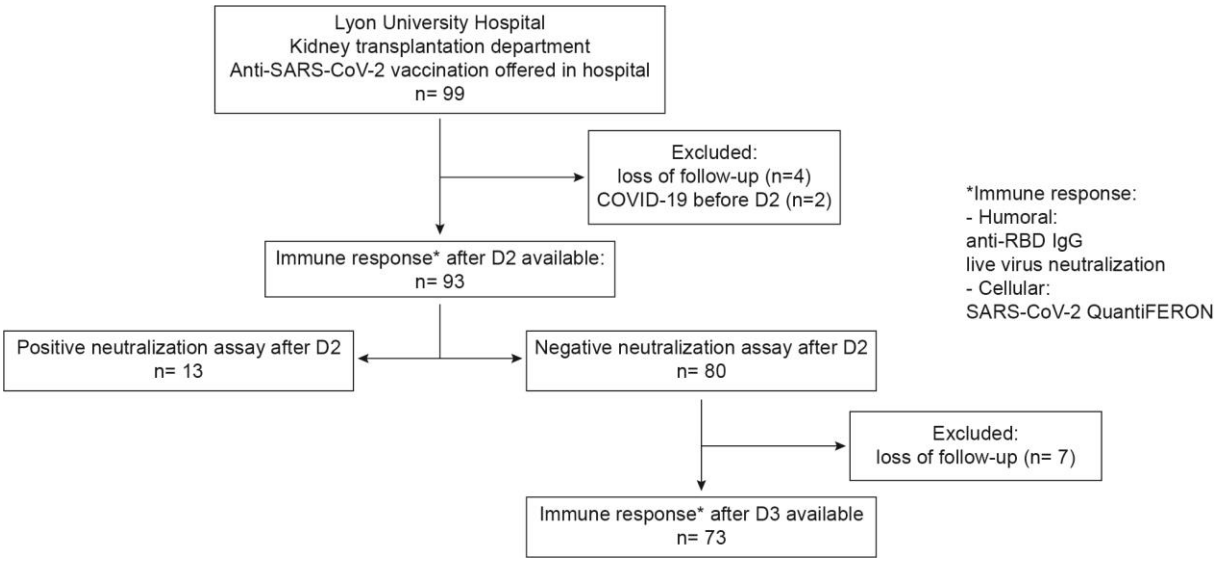
## Table. Characteristics of patients from Lyon University Hospital cohort

| n (%) or mean +/- SD | Initial cohort (2 doses) n = 93 | KTRs who received D3 (n=73) | | p* |
|---|---|---|---|---|
| | | Non-responders to D3 n = 59 | Responders to D3 n = 14 | |
| **Age (y)** | 55.7 ± 12.4 | 57.7 ± 12.6 | 55.0 ± 12.2 | 0.455 |
| **Male** | 50 (54) | 33 (56) | 7 (50) | 0.514 |
| **Comorbidities** | | | | |
| Cardiovascular disease | 23 (25) | 17 (29) | 3 (21) | 0.579 |
| Diabetes mellitus | 21 (23) | 11 (19) | 4 (29) | 0.412 |
| **Time from transplantation (y)** | 9.9 ± 8.8 | 8.9 ± 6.9 | 17.6 ± 11.3 | **0.002** |
| **Induction treatment** | | | | 0.374 |
| Anti-thymocyte globulins | 55 (59) | 38 (64) | 8 (57) | |
| Anti-CD25 | 32 (34) | 20 (34) | 2 (14) | |
| **Maintenance immunosuppression** | | | | |
| Tacrolimus (vs no) | 65 (70) | 44 (75) | 6 (43) | **0.027** |
| MMF/MPA | 71 (76) | 50 (85) | 5 (36) | **0.001** |
| Steroids | 80 (86) | 50 (85) | 12 (86) | 0.927 |
| imTOR | 8 (9) | 6 (10) | 3 (21) | 0.260 |
| **Biological data** | | | | |
| Lymphocytes (G/L) | 1.6 ± 0.8 | 1.6 ± 0.8 | 1.6 ± 0.8 | 0.859 |
| Monocytes (G/L) | 0.7 ± 0.2 | 0.6 ± 0.2 | 0.6 ± 0.1 | 0.827 |
| Creatinine (µmol/L) | 126 ± 46 | 133 ± 48 | 117 ± 37 | 0.241 |
| **COVID-19 history** | 16 (17) | 5 (8) | 1 (7) | 0.870 |
| **Biological results after the second dose** | | | | |
| Anti-RBD IgG | - | 22 (35) | 12 (86) | **0.004** |
| IGRA | - | 14 (24) | 8 (57) | **0.021** |

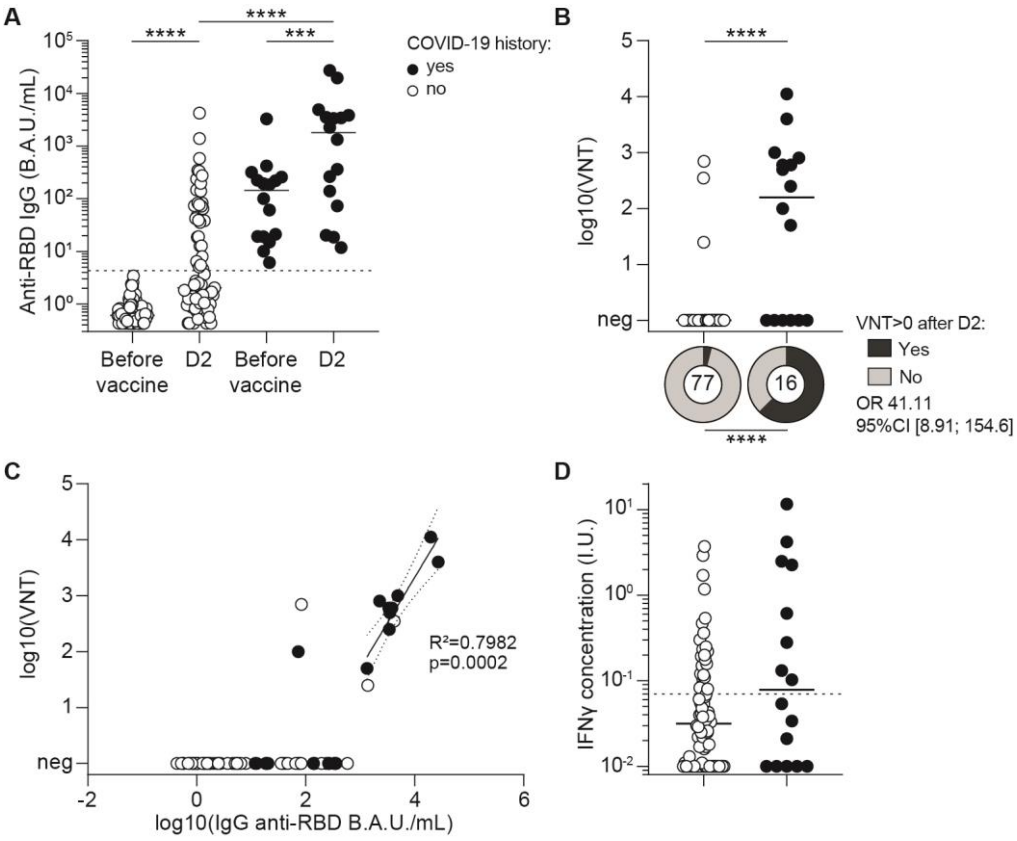
*: univariate logistic regression.

**Abbreviations are**: y, years; CNI, calcineurin inhibitor; MMF/MPA, mycophenolate mofetil/mycophenolic acid; imTOR, inhibitor of the mechanistic target of rapamycin; RBD, receptor-binding domain; IGRA, interferon γ releasing assay.

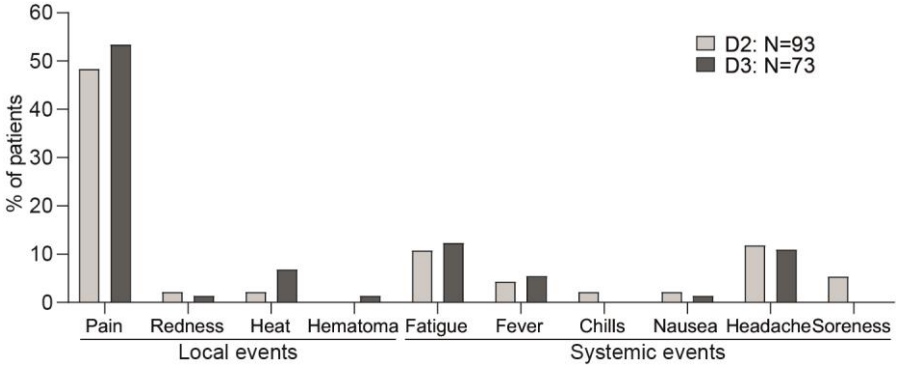
Figure 1-




## Figure 2-



## Figure 3-




## Figure 4-