Serial Viral Load Analysis by Ddpcr to Evaluate Fnc Efficacy and Safety in the Treatment of Moderate Cases of Covid-19

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Abstract

A highly pathogenic coronavirus termed severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in 2019 causing coronavirus disease 2019 (COVID-19). Trials have been carried out to find drugs effective in fighting the disease, as COVID-19 is being considered a treatable disease only after we have antivirals. A clinical candidate originally developed for HIV treatment, AZVUDINE (FNC), is a promising drug in the treatment of COVID-19, being able to reduce the patient's viral load leading to cure. In this study, a randomized clinical trial was performed in moderate COVID-19 patients to evaluate the efficacy of FNC added to standard treatment, compared with placebo group added to standard treatment. RT-qPCR and ddPCR were applied to estimate the viral load in samples from patients, which was performed every 48 hours throughout the treatment. Also, the clinical improvement was evaluated as well as the liver and kidney function. Notably, FNC treatment in moderate COVID-19 patients may shorten the time of nucleic acid negativity conversion versus placebo group, accelerates the elimination of the virus, decreasing the viral load significantly, especially in the first days. Due to the lack of specific antiviral drug, the pandemic is not under the control and resurfaces in different waves of infection, which cause a large cumulative expense of medical resources. Fortunately, FNC could reduce treatment time of moderate COVID-19 patients and save a lot of medical resources, making it a strong candidate for the treatment of COVID-19.

Trial registration number: NCT04668235

https://clinicaltrials.gov/ct2/show/NCT04668235?term=azvudine&cond=COVID-19&draw=2&rank=1

1. Introduction

A novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has been identified as the pathogen of the coronavirus disease 2019 (COVID-19) and rapidly spread around the world, with high rates of transmission and substantial mortality[1;2;3]. COVID-19 symptoms can range from mild, self-limited respiratory disease to severe progressive pneumonia, multiple organ failure, and even death[3;4]. Trials have been carried out to find drugs effective in fighting the disease, as COVID-19 is being considered a treatable disease only after we have antivirals[4].

Many studies have been conducted with antiviral potential presented nephrotoxicity and hepatoxicity as adverse drug events in patients with Covid-19, as remdesivir[5;6]. Other drugs increase the number of mutations in the RNA structure dramatically as favipiravir and molnupiravir[7].

Nucleoside antiviral drugs are characterized by a high antiviral efficacy and a high drug resistance barrier, inhibiting the activity of virus DNA-dependent DNA polymerases (DdDps), RNA-dependent DNA polymerases (RdDps) and RNA-dependent RNA polymerases (RdRps), which can lead to the inhibition of viral replication[8]. FNC (Azvudine) treatment in the mild and common COVID-19 may shorten the NANC

time versus standard antiviral treatment, accelerate the elimination of the virus, maintaining the vital signs of the patients[9].

The evolution of the viral load is an important point in the evaluation of the example, Liu and colleagues[10] showed that the viral load of severe cases was higher than in mild cases, and it's also reported that the risk of incubation and death increased with higher viral loads[11]. Moreover, Fajnzylber and colleagues[12] revealed that viral load was implicated in the severity and mortality of COVID-19. A univariate survival analysis revealed a significant difference in survival probability between those with high viral load and those with low viral load[13].

This study was one of the first studies to quantify viral load (absolute quantification by ddPCR), every 48 hours, establishing information on viral load behavior and course of infection. The mean times of the nucleic acid negative conversion (NANC) was measured in the FNC and placebo groups, and the nephrotoxicity and hepatoxicity were monitored.

2. Methodology

2.1 STUDY DESIGN

This prospective, randomized, double-blind, placebo-controlled clinical trial was performed at Santa Casa de Misericordia de Campos hospital as a strategic decision due to the need to concentrate molecular biology analyzes to maintain their standardization and quality, remembering that each RT-PCR equipment has its own sensitivity and different kits of reagents for RT-PCR have different performances. This trial was approved by the institutional review board of the National Health Surveillance Agency CE 0937457/21-4. The study was approved by the National Council for Research Ethics, CAAE 52176421.8.0000.5244. The study was also published in clinical trials (NCT04668235). All enrolled participants provided written informed consent. Inclusion and exclusion criteria of patients, design, goals and outcomes are found in the supplementary methodology.

Patients in the FNC group were treated with oral AZVUDINE tablets 5 mg (five tablets once a night) and standard treatment. For the 5mg dose of FNC, the mean half-life is 13.8h, with the intact drug and metabolites being excreted in the urine within 24h. Patients in the control group were treated with placebo added standard treatment.

Patients: Patients meeting the following criteria were enrolled in the study: 1) age 18 and over, regardless of gender; 2) respiratory or blood samples that were tested positive for SARS-CoV-2 nucleic acid by RT-PCR, or respiratory or blood samples that were tested highly homologous with the known SARS-CoV-2 by viral gene sequencing; 3) the confirmation of COVID-19 according to the diagnostic criteria of "the latest Clinical guide-lines for novel coronavirus" issued by the World Health Organization (WHO) on 2020 January 28. All enrolled patients signed informed consent forms (ICFs).

Exclusion criteria included 1) known or suspected allergy to the com- position of AZVUDINE tablets; 2) patients with malabsorption syndrome or any other condition that affects gastrointestinal absorption, the need for intravenous nutrition or an inability to take oral medication; 3) patients on anti-HIV treatment; 4) patients with one of the following conditions: respiratory failure and the need for mechanical ventilation; shock; intensive care unit (ICU) monitoring and treatment for other organ failures; 5) pregnant women or those who were lactating or may have a birth plan during the trial period and within 6 months after the end of the trial; 6) patients participating in other clinical trials or using experimental drugs within 12 weeks before administration; and 7) patients with other conditions that were not suitable for participating in this experiment according to the judgment of the researcher.

The definition of moderate COVID-19 was patients with fever, poor general condition, severe myalgia, persistent dry cough, diarrhea, moderate dyspnea without hypoxia (SpO2 93-94% / TC <50%) or with hypoxia (SpO2 92 -93% / TC >50%), with hospital admission recommended.

Enrollment: After application and signature of the ICF, the throat swab specimens were collected for nucleic acid testing by RT-PCR confirmation. Then the researcher evaluated the patient whether meeting the criteria and enrolled eligible patients with moderate symptoms, laboratory confirmed COVID-19 transferring them to hospital admission.

Randomization: Patients were randomly assigned in a 1:1 ratio to the FNC group or control group. Randomization was accomplished by using a random table that was generated in the Researcher IGZ v2.0 Software at 1:1. Each enrolled subject was given a number, randomly assigned to the FNC group and control group according to a predetermined random table and received treatment according to the corresponding treatment regimen.

In addition to vital signs and routine hematology and biochemistry exams, SARS-CoV-2 nucleic acids were tested by RT-PCR after the patients began taking their drugs. Nucleic acid detection analyzes were performed every 48hs throughout the treatment period for optimal measurement of participants viral load. Two consecutive negative results configured clinical discharge. These tests were used to obtain the mean times of the nucleic acid negative conversion (NANC).

Outcomes: The primary outcomes: Proportion of participants with improved clinical status. The criterion for a participant to have an improvement in clinical status is a decrease in the WHO Ordinal Scale of Clinical Improvement by at least one category compared to screening.

The secondary outcomes: Proportion of participants with clinical outcome of cure during the study. The clinical outcome of cure is defined in this protocol as the absence of viral RNA in samples collected and clinical conditions for hospital discharge. Time for improvement of diarrhea, myalgia, fatigue, malaise, cough, dyspnea and headache. Baseline changes in liver and kidney function. Evaluation of SARS-CoV-2 viral load negative conversion time by RT-PCR between AZVUDINE group (FNC) and control group. Length of hospital stay. Frequency and intensity of adverse events. Frequency and intensity of unexpected adverse events. Frequency and intensity of serious adverse events. All-cause mortality rate during the

study. Evaluation of the tolerability of the use of AZVUDINE (FNC) in the regimen of 5mg/day for up to 14 days.

Safety was regularly assessed by monitoring vital signs, changes in laboratory values (liver function and renal function), and adverse events (including type, incidence, severity, time and drug correlation, and assessment of severity. Previous studies have already shown that individuals who used FNC did not experience any type of serious adverse event drug related[9].

2.2 STATISTICAL ANALYSIS

The initial sample was 342 participants, however, with the drop in the number of hospitalizations and aggravations at the end of 2021, a review of the sample size was carried out for 180 participants divided randomly and equally in the two study groups (90 practitioners in each group). The sample calculation was performed using the formula of "sample calculation for superiority studies using proportions", described by Chow and colleagues[14]. For the analysis of demographic information and baseline eigenvalues, the mean value, standard deviation, quartiles, minimum and maximum values for numerical variables were calculated. For categorical data, frequency and percentage were calculated. The comparison of the two groups under general conditions was analyzed with appropriate methods according to the types of indicators. The Mann-Whitney test was used to compare the groups regarding quantitative data. Fisher's exact test was used for categorical data. Statistical analyzes were performed using the R-studio software.

2.3 QUANTIFICATION OF SARS-COV-2 VIRAL LOAD BY REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was extracted using the MagMAX™ Viral/Pathogen Nucleic Acid Isolation kit (Applied Biosystems) according to the manufacturer's instructions, using nasal and throat swabs from the clinical study participants.

Once total RNA was extracted, RT-PCRs were performed using the TaqPath™ COVID-19 CE-IVD RT-PCR kit (ANVISA Reg.: 10358940107) according to the manufacturer's instructions, using the QuantStudio5 RT-PCR equipment, Applied Biosystems, (ANVISA Reg: 10358940069). The primers and probes targeted the ORF1ab and N genes.

A standard curve was constructed using serial dilutions of the positive control (TaqPath™ COVID-19 Control), which is SARS-CoV-2 viral RNA at a known concentration of 1×10^4 copies/µL. The CTs obtained from each sample by RT-PCR were plotted on the standard curve to estimate the viral load of each sample.

A positive RT-PCR result occurs in CTs ≤30,5. In this case, when viral RNA is present, the specific probe used to detect SARS-CoV-2 is broken by DNA polymerase, emitting fluorescence. High copy number of viral RNA generates high levels of fluorescence; therefore, the CT value appears earlier during the reaction.

Low copy number of viral RNA generates low level of fluorescence, and consequently, the CT value appears later. Values of CTs>30,5 are considered negative. By establishing a viral RNA concentration curve (present in the positive control), we will obtain a curve of CTs, from lower values (higher copies of viral RNA) to higher values (lower copies of viral RNA).

2.4 QUANTIFICATION OF SARS-COV-2 VIRAL LOAD BY DROPLET DIGITAL POLYMERASE CHAIN REACTION (DDPCR)

Total RNA was extracted using the MagMAX^{TM} Viral/Pathogen Nucleic Acid Isolation kit (Applied Biosystems) according to the manufacturer's instructions, using nasal and throat swabs from the clinical study participants. Once total RNA was extracted, the ddPCR were performed subsequently.

Reverse transcription–PCR was conducted with primers and probes targeting the ORF1ab and N genes and a positive reference gene. Reaction system and amplification conditions were performed according to the manufacturer’s specifications (Shanghai BioGem Medical Technology Co Ltd, China).

Digital droplet PCR analyzes were performed by the Targeting One Digital PCR System; COVID-19 digital PCR detection kit; droplet generation Kit; Droplet detection kit. The kits allow detection of the ORF1ab gene, N gene and a positive reference gene. The detection limit was 10 copies/test. (Targeting OneTechnology is licensed by the China Food and Drug Administration). A fractional number represents viral fragments that do not constitute a viral unit.

3. Results And Discussion

3.1 DEMOGRAPHIC ANALYSIS

A total of 476 participants were approached for this study between April 2021 and May 2022. Of these, 296 were excluded for not meeting the eligibility criteria, for worsening before being transferred to the ward of the research center or for withdrawal before participating in the clinical trial. A total of 180 participants were randomized, of whom 172 successfully completed treatment, 7 worsened due to natural disease progression and were referred to the ICU, 6 deaths and 1 hospital discharge in total. There was only 1 dropped out before completing treatment and 1 death (during follow-up) due to cardiac surgery refusal detected on admission (Figure 1).

Patient demographics and baseline characteristics were well matched between the FNC group and the control group at enrollment (Supplementary Table 1). The median age was 48 years (IQR 41-58), there was no significant difference between the age of participants who used the FNC and the placebo (p=0.135). The largest number of participants was male, totaling 104 individuals (58%), there were no significant differences in relation to gender (p=0.075), indicating that the results obtained were not influenced by the age of the individuals or by gender (Supplementary Table 1).

3.2 CLINICAL IMPROVIMENT

At the time of clinical discharge, except for one patient with withdrawal or seven patients who worsened, all ended up with a Clinical Score of 0 or 1, according to the WHO Ordinal Scale of Clinical[14] Improvement. Participants who used the FNC had a final score of 0.02 ± 0.15, while those who participated in the control group had 0.11 ± 0.31, with a statistically significant difference between the groups (p=0.024) (Table 1; Supplementary Figure 1 and 2).

3.3 TIME OF THE NUCLEIC ACID NEGATIVE CONVERSION

The results indicate that participants treated with FNC had a significantly shorter time to first negative nucleic acid conversion (6.24 days; p=0.002) compared with participants treated with placebo (7.94 days) (Figure 2). The same was repeated in the results obtained for the second negative nucleic acid conversion, in which subjects treated with FNC had a shorter time to consecutive negative (7.73 days; p=0.028) compared to subjects treated with placebo (8.89 days) (Figure 2).

3.4 DETECTION OF SARS-COV-2 VIRAL LOAD BY RT-PCR AND DDPCR TECHNIQUE

Some reports regarding relationships between viral loads and disease severity have been reported[16; 17; 18]. For example, Liu and colleagues[10] showed that the viral load of severe cases was higher than in mild cases, and it's also reported that the risk of incubation and death increased with higher viral loads[11]. Moreover, Fajnzylber and colleagues[12] revealed that viral load was implicated in the severity and mortality of COVID-19. A univariate survival analysis revealed a significant difference in survival probability between those with high viral load and those with low viral load[13].

Although it was possible to observe a greater tendency of decrease in viral load quantified through the RT-PCR technique of the participants who used the FNC, it was not possible to identify a significant difference between them and the control group. Although this method is regarded as the gold standard for the etiological diagnosis of COVID-19 the sensitivity and reliability of RT-PCR were questioned due to the presence of negative results in some patients who were highly suspected of having the disease based on clinical presentation and exposure history, as well as positive results in some confirmed cases after recovery[19; 2]. The results from RT-PCR testing using primers in the ORF1ab gene and N genes can be affected by the variation of viral RNA sequences.

Although there was a significant reduction (p=0.028) in the viral load conversion time of the FNC group in relation to the PLACEBO group, it was expected to see the same in the quantification of viral load by RT-PCR in the standard curve calculation, however the logarithmic variability, despite demonstrating the course of the disease, is not reliable in quantification. Thus, after quantification of viral load by PCR, there was a significant difference between the two groups. (Table 2, Figure 3B). The high sensitivity of the DDPCR confronts the variability obtained by calculating the viral load by RT-PCR after treatment with FNC, showing a significant reduction in viral load in D3 ($p<0.002$), D5, D7 and D9 ($p<0.001$) and D11 ($p<0.006$). Several studies have shown that droplet digital PCR (ddPCR) has the advantages of absolute quantification and is more sensitive for virus detection than RT-PCR[20,21,22].

Notably, it was possible to observe significant differences in the time of improvement of fever in D1 (p<0,015), in D2 (p<0,040) and in D3 (p<0,026) and Chill (p=0.08) symptoms (Table 1). Other information is found in the supplementary material.

3.5 SEQUENCING OF SARS-COV 2 STRAINS

Here, genetic sequencing was performed to demonstrate the distribution of strains between the FNC and placebo groups.

After the genetic sequencing, it was possible to observe that the volunteers who participated in the study were infected by three strains of SARS-COV-2, namely: ALPHA, DELTA and GAMMA (Table 3 and Figure 4). The strain with the lowest prevalence was ALPHA, which affected 7.8% and 18.8% of the volunteers who used FNC and placebo, respectively (Figure 4). The DELTA strain affected 37.7% of both volunteers who used FNC and placebo (Figure 4). The strain with the highest incidence during the research was GAMMA, which affected 54.5% and 43.8% of the volunteers who used FNC and placebo respectively (Figure 4). The data suggest that there is no difference in the distribution of strains between both groups, FNC and placebo.

This study had only 06 vaccinated participants, 03 from placebo group and 03 from FNC group. This study was carried out in a period when vaccines were not widely available for the population, and therefore vaccine interference may exist in only 03 vaccinated participants, infected by the DELTA strain variants AY.99.1, AY .99.1, AY.99.2, respectively.

3.6 CHANGES IN KIDNEY AND LIVER FUNCTIONS BASELINES

In this study, the treatment with FNC was well tolerated by patients. Vital signs, liver function and kidney function in both groups were normal. The results of the exams referring to the renal function of the individuals distributed in FNC and placebo groups, including creatinine and blood urea nitrogen, showed profiles of similar values, within the normal parameters throughout the treatment and without significant differences between the groups during the days of treatment (Figure 5A and B). These data reinforce what was observed in the pilot clinical trial previously performed with FNC, in which hepatic and renal functions do not change between the FNC and control group, indicating non-toxicity of the drug. This is not the case for many antivirals, in studies with remdesivir, for example, nephrotoxicity and hepatoxicity were reported as adverse drug events in patients with Covid-19[5; 6]. It was reported that similar type of antiviral drugs may cause mitochondrial injury in renal tubular epithelial cells[23; 6]. Therefore, our results highlight the safety of FNC since no changes were observed in markers of kidney and liver damage when the two groups are compared.

Regarding the results of the tests referring to the liver function of the individuals distributed in FNC and placebo groups, including aspartate aminotransferase, alanine aminotransferase, glutamyl transpeptidase and total bilirubin, they presented values within the normal range, with the groups

presenting similar results profiles and without statistically significant changes during the days of treatment, as well as the results observed in exams referring to renal function (Figure 5C, D, E, and F).

3.7 ADVERSE EVENTS AND CLINICAL SAFETY OF FNC

A total of 112 cases of adverse events were observed in this study, of which 105 were considered non-serious adverse events and only 7 were considered serious adverse events. These 7 SAEs were not related to the use of FNC, being caused as a consequence of the rapid progress of the disease (Table 5).

The adverse events observed in this study were mainly related to the increase in ALT (45 cases), GGT (13 cases), AST (10 cases) and GRADE 1 HEADACHE (8 cases), with normalization of these events until the end of treatment (Table 4). Under these conditions, it was possible to observe an increase as well as a reduction in GGT, however the values tended to reduce even under normal conditions. The adverse reactions observed in this study were the same as those related to antiviral drugs, with no unexpected adverse reactions occurring (Table 4).

The phlebitis that occurred during the study is due to the administration of intravenous antibiotic, which was later changed to oral. There was also no significant change in urinary phosphorus. In preliminary studies, vertigo (incidence $\geq$ 5%) is attributed to FNC, however in this study there were only 2 reported cases of dizziness related to Labyrinthitis (Past history) and hypoglycemia (due to loss of taste). It should also be considered that the participants were bedridden, which could potentiate these events.

There were 7 exclusions due to disease worsening, progressing to ICU. There were 6 deaths and 1 recovery where participants received adequate care and support during hospitalization. In the case of deaths, 3 participants arrived at the hospital with a worsening condition, since after admission they were transferred to the ICU within 1 to 3 days (Table 5).

When comparing the adverse events of the FNC and placebo groups, there is an equivalence without predominance in either group, as would be expected, showing that adverse events are also due to the disease.

The analysis of the viral load, every 48 hours, served as a safety examination that could identify how much the individual is infected, being a marker in the prevention of worsening (a condition that, when it occurs, excludes the participant from the study). The management avoided aggravation and allowing the safety parameters to be better evaluated, keeping more participants in the study.

Other data from the study are described in detail in the supplementary material, such as the presence of comorbidities among the participants (Supplementary Table 5), Demonstration of inflammatory marker (Supplementary Table 9, 10 and 11), Demonstration of lung imaging values (Supplementary Table 12), Demonstration of respiratory symptoms and O2 saturation (Supplementary Table 13, 14 and 15), Demonstration of the distribution of participants in the type of ventilatory support and O2 consumption during ventilatory support (Supplementary Table 16 and 17), Demonstration of the use of mechanical

ventilation (Supplementary Table 18) and other variables during the days of study (Supplementary Table 6, 7 and 8).

4. Conclusion

In summary, moderate COVID-19 patients treated with FNC may shorten the time of nucleic acid negativity conversion versus placebo group and consequently may reduce the hospitalization length of these patients. The FNC treatment can accelerate the elimination of the virus, decreasing the viral load significantly, probably declining infection and fever, consequently. Due to the lack of specific antiviral drug, the pandemic is not under the control and resurfaces in different waves of infection, which cause a large cumulative expense of medical resources. The fact that it is an oral drug, excreted via the kidneys as an intact drug and metabolites within 24 hours, without being incorporated into human genetic material, with good safety and efficacy, makes AZVUDINE an excellent drug option, reducing treatment time of moderate COVID-19 patients and save a lot of medical resources, to contain the pandemic that still causes many deaths/day.

Declarations

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AUTHOR CONTRIBUTIONS

PC coordinated the project and supervised the writing of the manuscript. SBS performed the analysis of the data. ABVJ assisted in the acquisition of statistical data. RMS, RFA, SPFC and ALEM assisted in the acquisition of data. RMS and SBS wrote the manuscript. WMSD and ASM performed the sequencing analyzes. CGS assisted the medical team that conducted the clinical research. JC and PL assisted in reviewing the manuscript. All authors read and approved the final version of the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

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Tables

Tables 1 to 5 are available in the Supplementary Files section.

Figures

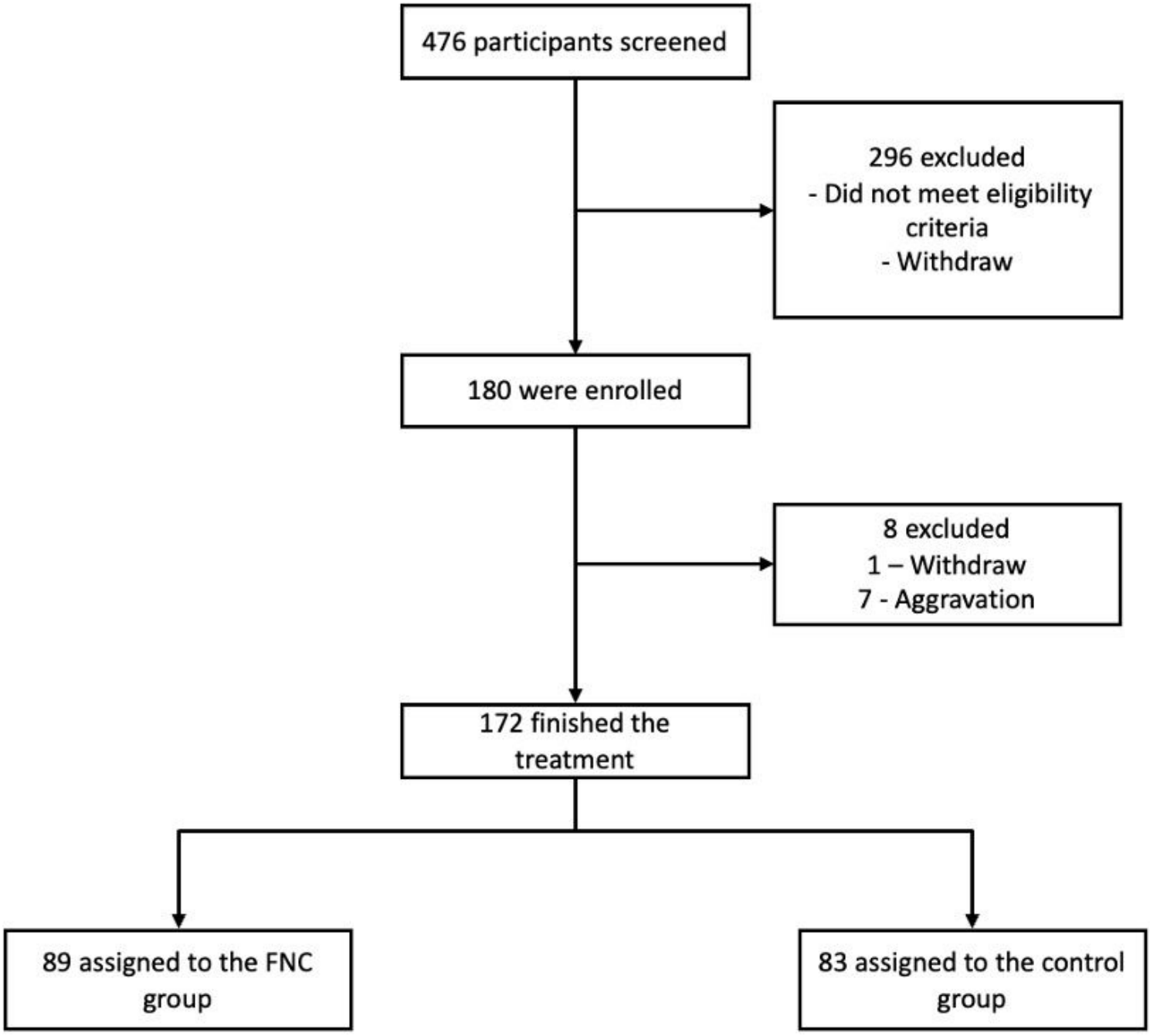

Figure 1

Trial profile.

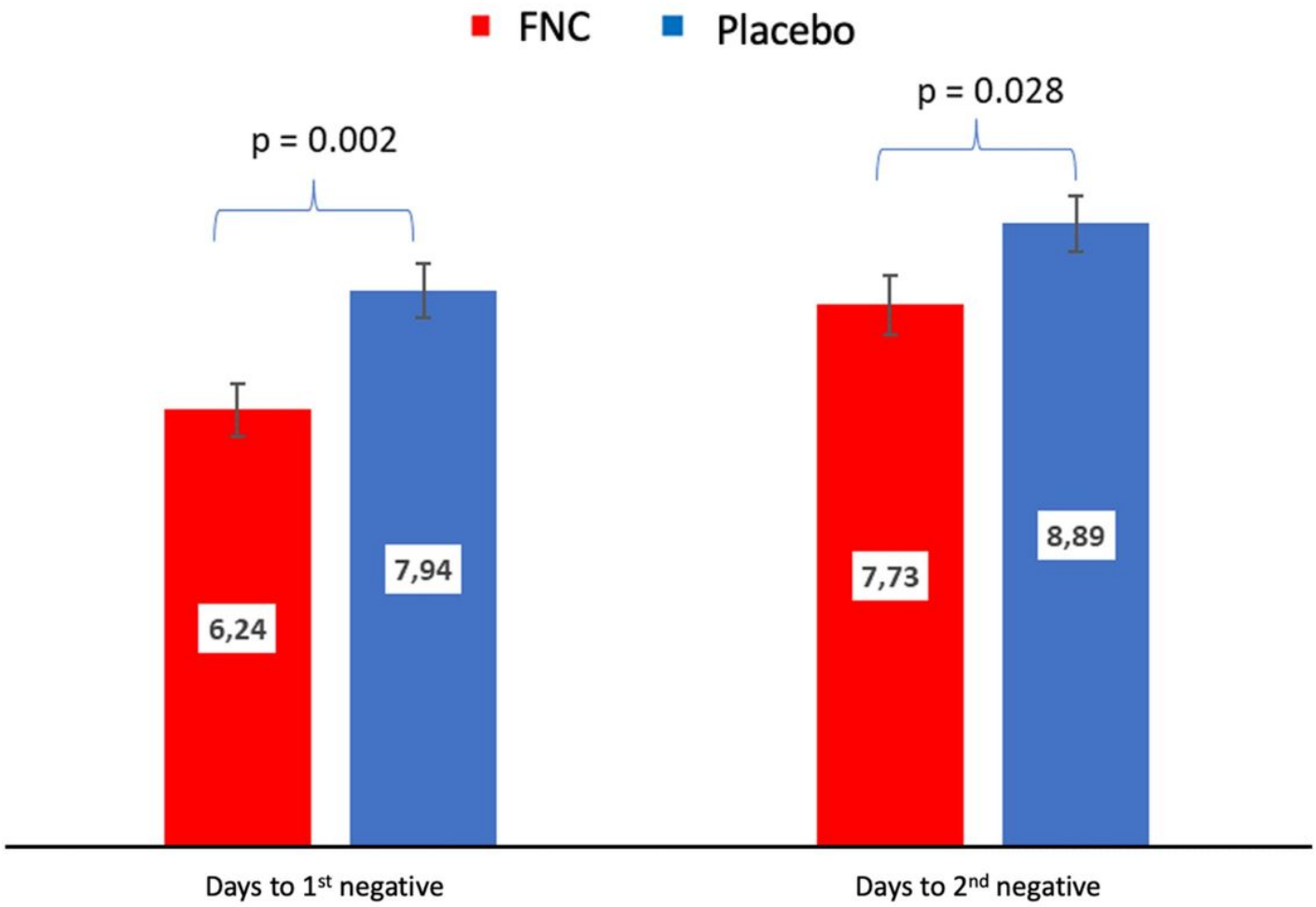

Figure 2

Comparison of the time of the first and the second nucleic acid testing negativity between all subjects in the FNC group and the placebo group. Data are mean (SD). The differences between groups were analyzed using Mann-whitney test (Red bar: FNC; Blue bar: PLACEBO).

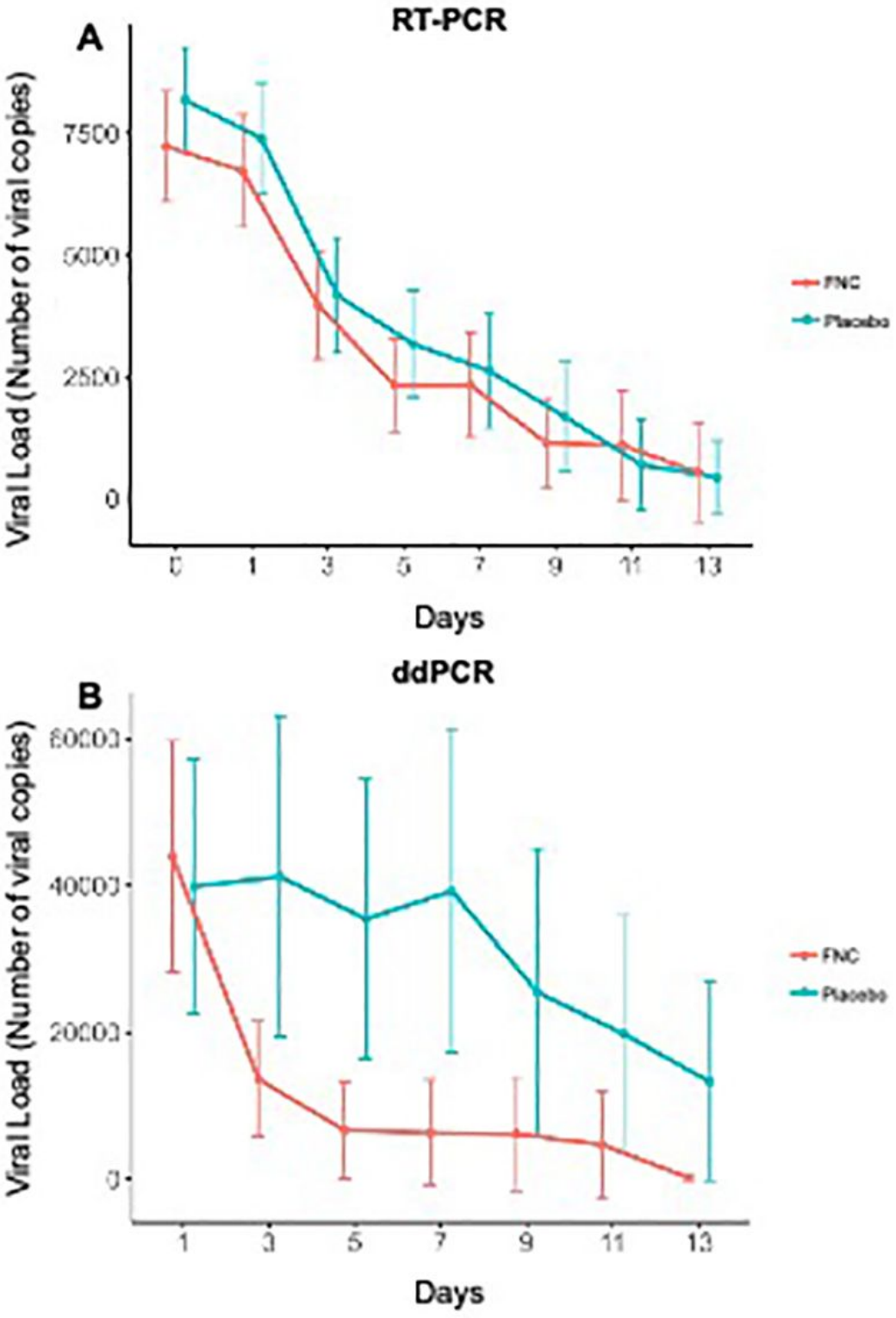

Figure 3

A) Estimated (RT-PCR) and B) Absolute Viral load analysis (ddPCR) of participants in the FNC group and the placebo group during the treatment days. Data are median (SD). (Red line: FNC; Blue line: PLACEBO).

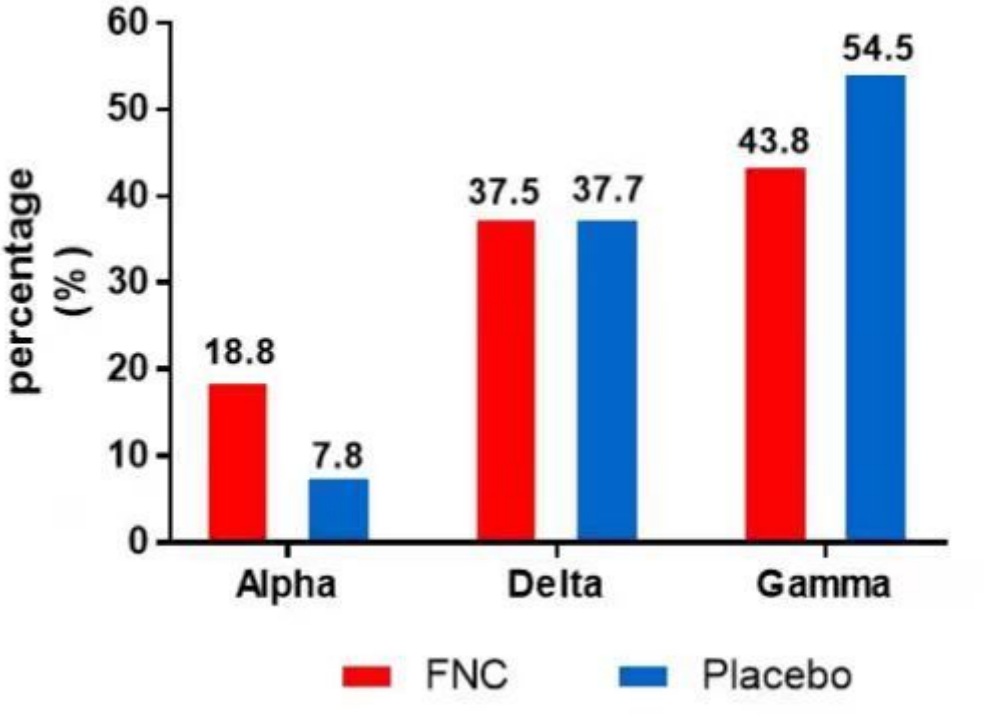

Figure 4

Percentage of volunteers infected by the different strains of SARS-COV-2 distributed among the treatments. (Red bar: FNC; Blue bar: PLACEBO).

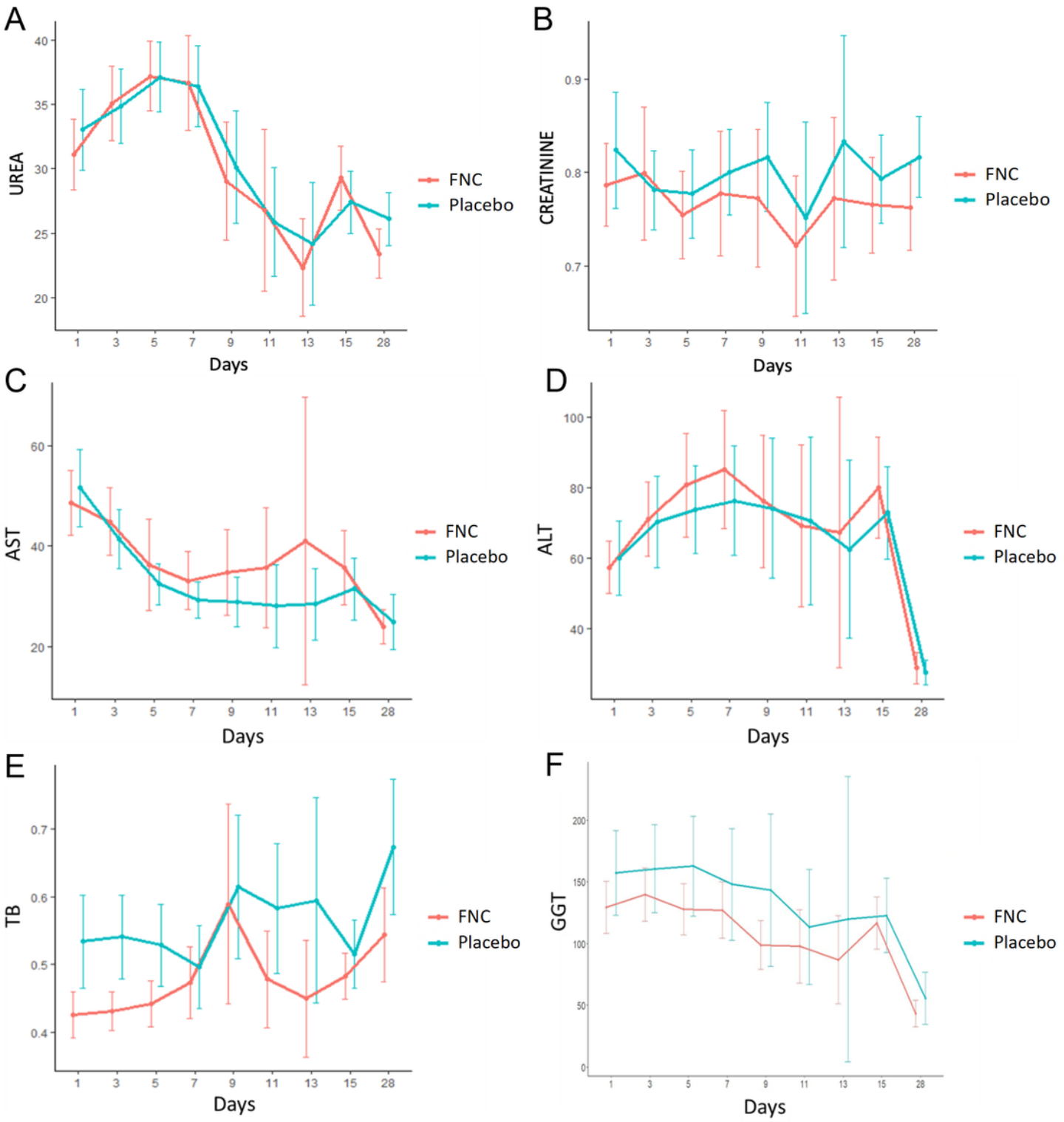

Figure 5

During the treatment, the dynamic changes in kidney and liver markers: a) Creatinine, b) Urea, c) ALT, d) AST, e) BT and F) GGT of the patients in the FNC group and patients in the placebo group. Data are median (SD). (Red line: FNC; Blue line: PLACEBO).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- ProtocolandSAP.pdf
- Supplementarymaterial.docx
- Tables.docx