Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Cao B, Wang Y, Wen D, et al. A trial of lopinavir–ritonavir in adults hospitalized with severe Covid-19. N Engl J Med. DOI: 10.1056/NEJMoa2001282

Supplementary Materials

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Supplementary Methods

Quantitative Viral RNA Testing

Throat swab sampling for study-related monitoring was performed following guidance from manufacturer (Copan Diagnostics Inc., Murrieta, CA, USA). Samples were stored and sent for batch testing after all follow-ups were finished. The quantitative RT-PCR testing methods in this study were validated in Teddy Clinical Research Laboratory (Wuhan City, China) as shown below.

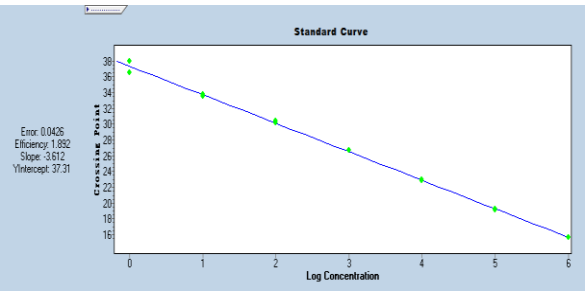
We did method validation including precision, accuracy, specificity and sensitivity following the guidelines of CAP, CLSI and MIQE. Due to the limited number and types of samples, 10 nasopharyngeal swab samples previously tested as SARS-CoV-2 positive using commercial in vitro diagnostic kit were used for the validation tests. The summary results are as follows:

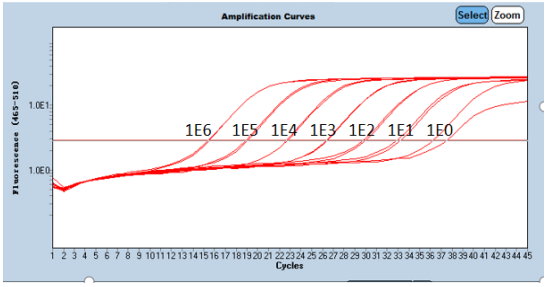
Precision Validation: The coefficient of variation (CV) value of all validation samples was less than 15%, which met the validation criteria and passed the inter and intra batch precision validation.

Accuracy Validation: The CV value of all validation samples was less than 15% and the bias was less than 50%, which met the validation criteria and passed the accuracy verification.

Specificity Validation: The specificity is defined as the consistency between the test results in theory and the actual test results. We used 10 known clinical samples for validation. The CV value of all validation samples was less than 15%. The test results were 100% consistent with the actual test results

Sensitivity Validation: E gene RNA standard provided by Roche Diagnostics was used for sensitivity validation test. LLOQ (lowest limit of quantification) and LOD (Limit of detection) were determined using E gene RNA standard.





The lower limit of quantifcation for this assay is 10 copies/ul and the limit of detection of PCR assay is 1 copy/ul.

The kit contains specific primer and probes designed to detect three genes: RNA dependent RNA polymerase (RdRP), N gene and E gene. E-gene was used for virus screening to eliminate NC_014470Bat SARS-related CoVC; and RdRP and N genes are used for confirmation of SARS-CoV-2 infection.

Although RdRp gene was specific for SARS-CoV-2, we selected E gene to quantify the viral load instead of RdRp gene because the amplification efficiency for RdRp gene was lower than E gene. The quantification was performed by using E-gene RNA standards provided by Roche Diagnostics (Shanghai) Ltd.

E-gene assay in the kit detects both SARS and SARS-CoV-2 as well as other bat-associated SARS-related viruses (Sarbecovirus). There is no cross reactivity found with common human respiratory CoVs (NL63, 229E, HKU, OC43) or MERS-CoV by gene sequence analysis. Since all samples in this study were from patients clinically diagnosed with COVID-19 pneumonia in Wuhan (and no SARS CoV infection has been detected), it is logical that we used the E gene target for the quantification of the viral load of the samples. Detection of RdRp and N genes were also tested for all patient's first visit samples.

Assay controls included the addition of EAV RNA Extraction control (LightMix®, TIB Germany) directly to samples as control to monitor the entire process which is from RNA extraction to gene detection, addition of both negative (water) and positive controls (LightMix®) on different positions of each 96-well PCR plate to monitor the amplification process, standard laboratory processes to mitigate cross contamination

The viral RNA was extracted using MagNA Pure 96 DNA and Viral NA Small Volume kit in MagNA pure 96 system (Roche Diagnostics). 200 ul of each types of samples was added to each wells of 96 deep well plate previously filled with 250 ul of External Lysis buffer (provided in the kit) followed by 60℃ 1h incubation to inactivate the virus before RNA extraction. 10 ul of EAV control was also added to each well. RNA extraction was performed in MagNA Pure 96 System which is a closed system

and fully automated. 10 ul of Purified virus RNA was then added to pre-prepared PCR plate with each well contains 10 ul PCR reaction buffer in biological safety cabinet. qPCR reactions were performed according to instruction. Reagent preparation, sample purification and PCR were performed in separate rooms with passing windows to avoid cross contaminations. All rooms are negative pressured and have buffer rooms for changing. Positive control and standards for quantification were prepared in another room and add into PCR plate only in sample extraction room to avoid contamination.

Table S1. Outcomes in the modified intention-to-treat population.

Characteristics	Total (N = 196)	Lopinavir/ritonavir (N = 96)*	Standard Care (N = 100)	Difference[§]
TTCI	16.0 (15.0, 17.0)	15.0 (13.0, 17.0)	16.0 (15.0, 18.0)	1.39 (1.00,1.91)†
Day 28 mortality	41 (20.9)	16 (16.7)	25 (25.0)	-8.3 (-19.6, 3.0)
Early (≤12 days of symptom onset)	19 (21.6)	6 (15.0)	13 (27.1)	-12.0 (-28.8, 4.7)
Late (> 12 days of symptom onset)	22 (20.4)	10 (17.9)	12 (23.1)	-5.2 (-20.4, 10.0)
Day 7, no. (%)	8 (4.1)	6 (6.3)	2 (2.0)	4.3 (-1.3, 9.8)
Day 14, no. (%)	75 (38.3)	45 (46.9)	30 (30.0)	16.9 (3.4, 30.3)
Day 28, no. (%)	148 (75.5)	78 (81.3)	70 (70.0)	11.3 (-0.7, 23.2)
ICU length of stay (days)	11 (5, 17)	8 (4, 11)	11 (7, 17)	-4 (-9, 1)
ICU length of stay of survivors (days)	10 (8, 17)	9 (5, 44)	11 (9, 14)	-1 (-16, 38)
ICU length of stay of non-survivors (days)	11 (5, 17)	6 (2, 11)	12 (7, 17)	-6 (-11, 1)
IMV duration (days)	5 (3, 9)	4 (2, 7)	5 (3, 9)	-1 (-4, 2)
IMV duration of non-survivors (days)	5 (2, 9)	4 (1, 6)	5 (3, 10)	-2 (-5, 1)
IMV duration of survivors (days)	5 (3, 28)	28 (28, 28)	4 (3, 5)	24 (23, 25)
Length of oxygen support (days)	13 (8, 16)	12 (9, 16)	13 (6, 16)	0 (-2, 2)
Hospital length of stay (days)	15 (12, 17)	14 (12, 17)	16 (13, 18)	-1 (-2, 1)
Days from randomization to discharge (days)	13 (10, 16)	12 (10, 16)	14 (11, 16)	-1 (-2, 1)
Days from randomization to death (days)	10 (7, 15)	10 (7, 14)	12 (6, 15)	-2 (-5, 3)
Seven-category scale at day 7				
2 Not hospitalized, but unable to resume normal activities, no. (%)	4 (2.0)	4 (4.2)	0 (0.0)	
3 Hospitalization, not requiring supplemental oxygen, no. (%)	29 (14.8)	12 (12.5)	17 (17.0)	

4 Hospitalization, requiring supplemental oxygen, no. (%)	109 (55.6)	58 (60.4)	51 (51.0)	
5 Hospitalization, requiring HFNC and/or non-IMV, no. (%)	35 (17.9)	14 (14.6)	21 (21.0)	
6 Hospitalization, requiring ECMO and/or IMV, no. (%)	9 (4.6)	5 (5.2)	4 (4.0)	
7 Death, no. (%)	10 (5.1)	3 (3.1)	7 (7.0)	
Seven-category scale at day 14	3 (2, 4)	3 (2, 4)	3 (2, 5)	0 (-1, 0)
2 Not hospitalized, but unable to resume normal activities, no. (%)	71 (36.2)	43 (44.8)	28 (28.0)	
3 Hospitalization, not requiring supplemental oxygen, no. (%)	32 (16.3)	8 (8.3)	24 (24.0)	
4 Hospitalization, requiring supplemental oxygen, no. (%)	45 (23.0)	25 (26.0)	20 (20.0)	
5 Hospitalization, requiring HFNC and/or non-IMV, no. (%)	11 (5.6)	5 (5.2)	6 (6.0)	
6 Hospitalization, requiring ECMO and/or IMV, no. (%)	8 (4.1)	3 (3.1)	5 (5.0)	
7 Death, no. (%)	29 (14.8)	12 (12.5)	17 (17.0)	

Number (percentage) or median (interquartile range) is summarized as appropriate. Abbreviation: ICU = intensive care unit; HFNC = high-flow nasal oxygen therapy; IMV = invasive mechanical ventilation; ECMO = extracorporeal membrane oxygenation; TTCI = time to clinical improvement. Clinical improvement (the event) was defined as a decline of two categories on the modified seven-category ordinal scale of clinical status, or hospital discharge.

*This total excludes 3 patients who died within 24 hours of randomization and did not receive lopinavir/ritonavir.

§ Differences were expressed as rate differences or median difference (Hodges-Lehmann estimate) and 95% confidence intervals.

† The hazard ratio was estimated by Cox proportional risk model.

Table S2. Virological outcomes in the modified intention-to-treat population.

Undetectable proportion of viral RNA	Total (n=130)	Lopinavir/ritonavir (n=59)	Control group (n=71)	Difference[§]
Day 5, no. (%)	43 (33.1)	20 (33.9)	23 (32.4)	1.5 (-14.8, 17.8)
Day 10, no. (%)	63 (48.5)	29 (49.2)	34 (47.9)	1.3 (-16.0, 18.5)
Day 14, no. (%)	72 (55.4)	32 (54.2)	40 (56.3)	-2.1 (-19.3, 15.1)
Day 21, no. (%)	75 (57.7)	34 (57.6)	41 (57.7)	-0.1 (-17.2, 16.9)
Day 28, no. (%)	76 (58.5)	35 (59.3)	41 (57.7)	1.6 (-15.4, 18.6)
Survivors, n	97	48	49	
Day 5, no. (%)	36 (37.1)	17 (35.4)	19 (38.8)	-3.4 (-22.6, 15.9)
Day 10, no. (%)	53 (54.6)	26 (54.2)	27 (55.1)	-0.9 (-20.8, 18.9)
Day 14, no. (%)	61 (62.9)	29 (60.4)	32 (65.3)	-4.9 (-24.1, 14.3)
Day 21, no. (%)	64 (66.0)	31 (64.6)	33 (67.3)	-2.8 (-21.6, 16.1)
Day 28, no. (%)	65 (67.0)	32 (66.7)	33 (67.3)	-0.7 (-19.4, 18.0)
Non-survivors, n	33	11	22	
Day 5, no. (%)	7 (21.2)	3 (27.3)	4 (18.2)	9.1 (-21.8, 40.0)
Day 10, no. (%)	10 (30.3)	3 (27.3)	7 (31.8)	-4.5 (-37.3, 28.2)
Day 14, no. (%)	11 (33.3)	3 (27.3)	8 (36.4)	-9.1 (-42.2, 24.0)
Day 21, no. (%)	11 (33.3)	3 (27.3)	8 (36.4)	-9.1 (-42.2, 24.0)
Day 28, no. (%)	11 (33.3)	3 (27.3)	8 (36.4)	-9.1 (-42.2, 24.0)
AUC, mean (SD)	26.20 ± 22.93	30.16 ± 27.91	22.56 ± 16.54	7.61 (-0.65, 15.86)
⩽12 days	30.78 ± 26.78	38.96 ± 33.35	24.77 ± 19.06	14.18 (0.44, 27.93)
>12 days	21.69 ± 17.46	23.29 ± 20.86	19.86 ± 12.66	3.43 (-5.65, 12.51)
Survivors	27.41 ± 24.61	32.45 ± 29.74	22.47 ± 17.18	9.97 (0.21, 19.74)
Non-survivors	20.87 ± 12.22	17.98 ± 7.81	22.86 ± 14.50	-4.88 (-15.98, 6.22)

AUC in mITT analysis set (excluding patients without any detectable viral RNA), as determined by quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis. Results less than the lower limit of quantification of PCR assay and greater than the limit of qualitative detection are imputed with 1 log10 copies/mL; results of patients with viral negative RNA are imputed with 0 log10 copies/mL. AUC, area under the curve.

§ Differences were expressed as rate differences or Hodges-Lehmann estimator differences and 95% confidence intervals.

Figure S1. Kaplan Meier plot of time-to-clinical improvement in the modified intention-to-treat population.

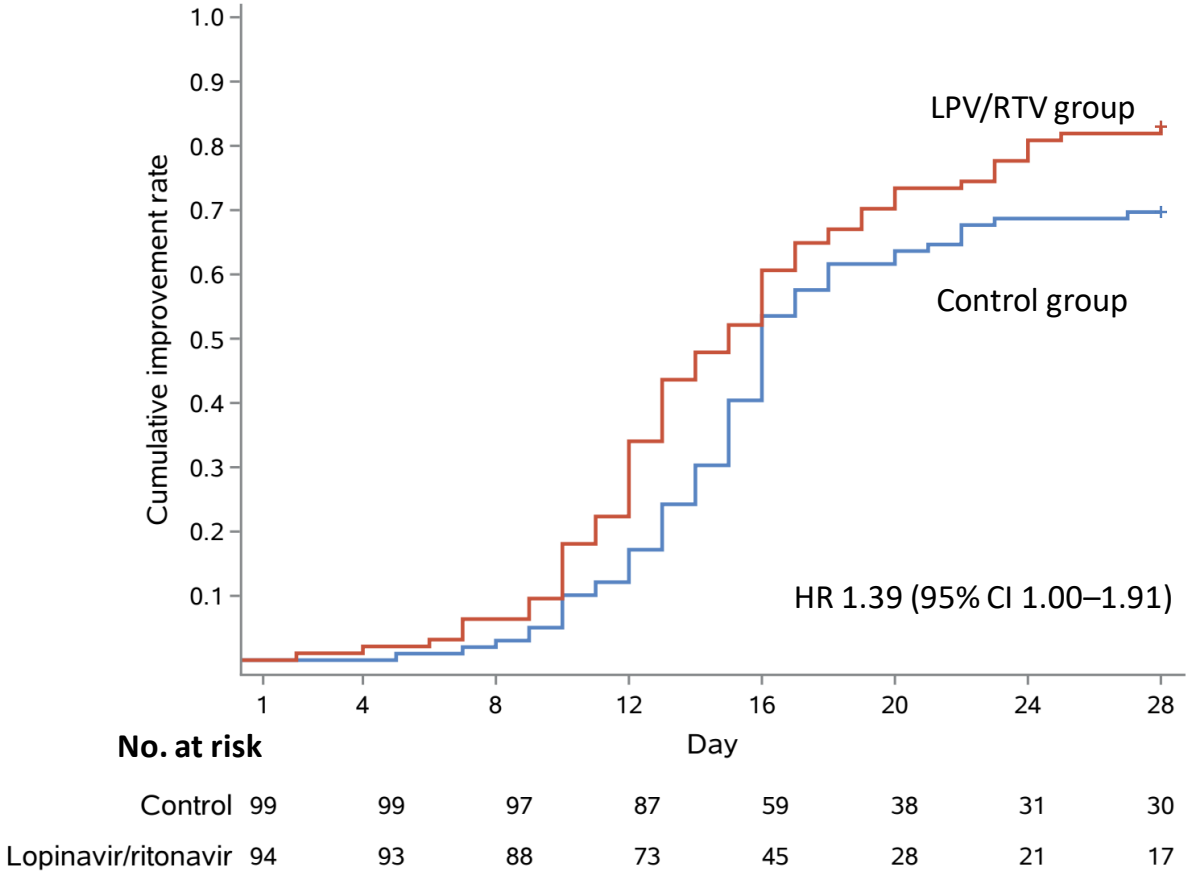


Figure S2. Kaplan Meier plot of time-to-clinical improvement by duration of illness (≤ 12 days [Panel A] vs > 12 days [Panel B]) in the intention-to-treat population.

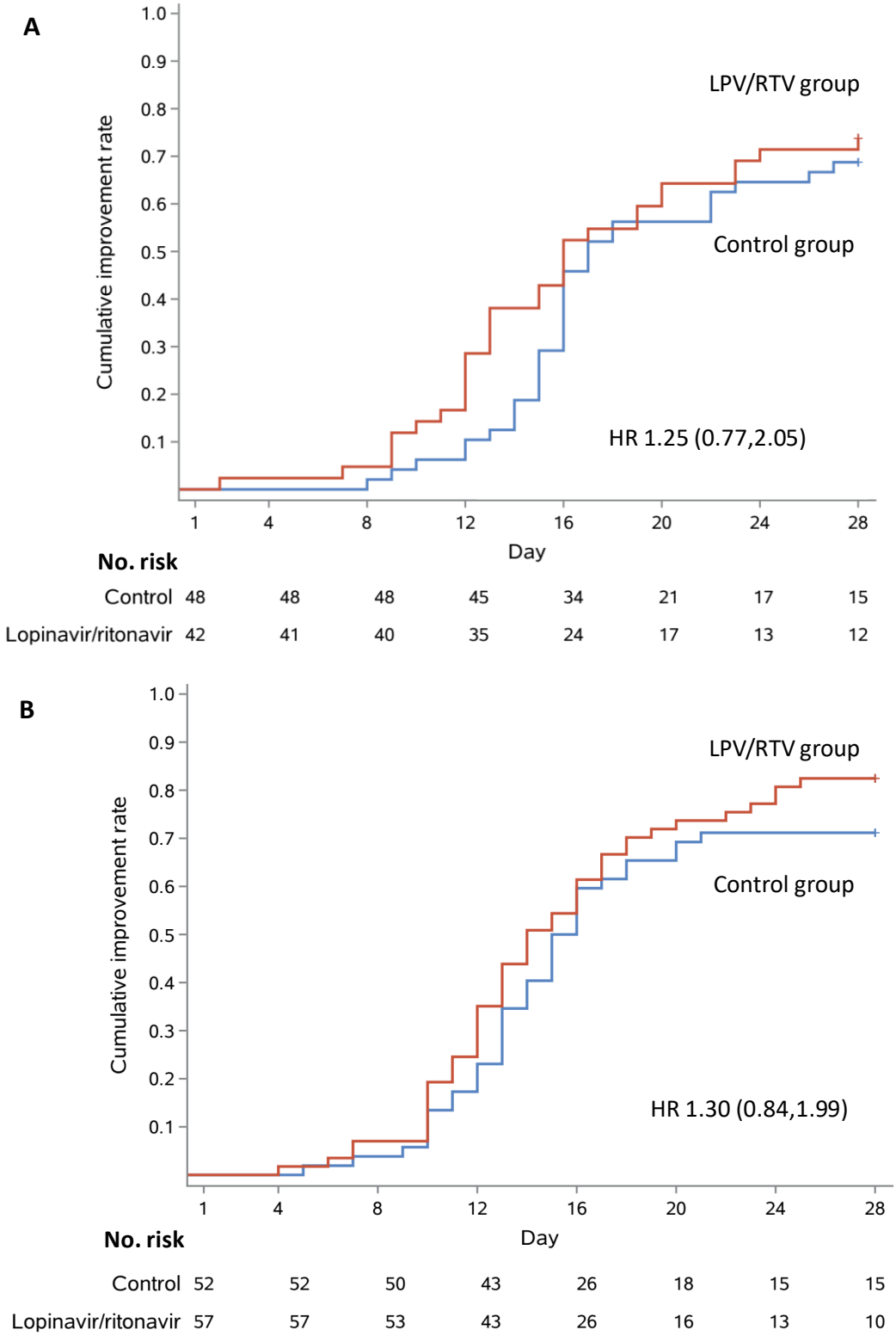
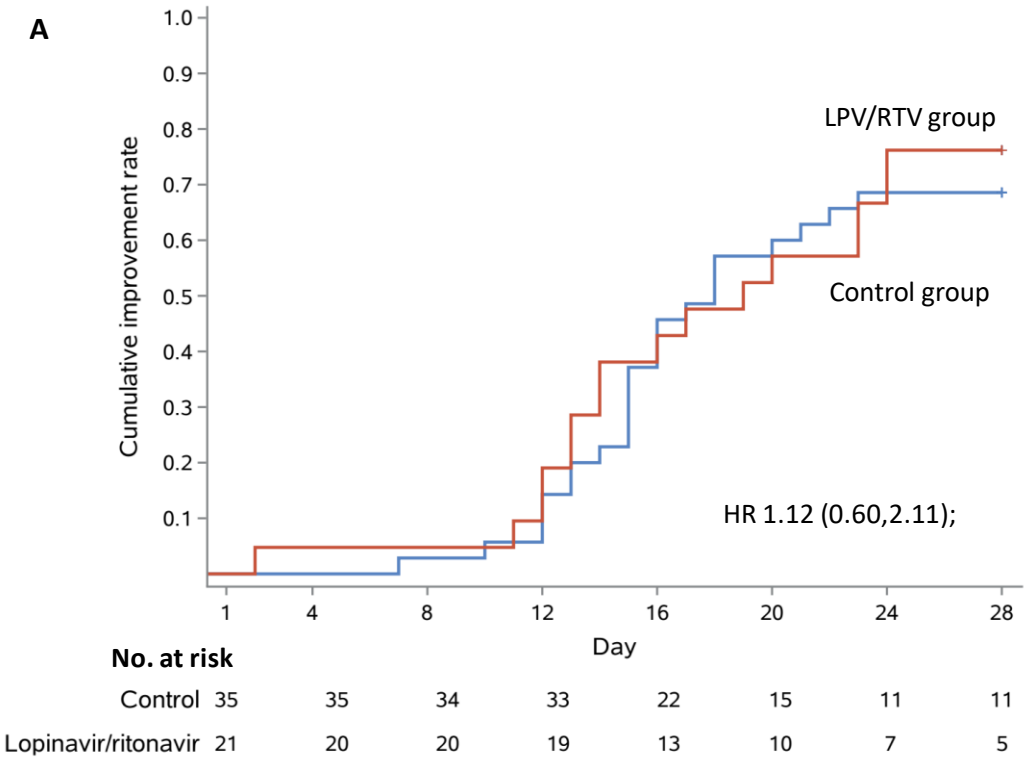


Figure S3. Kaplan Meier of time-to-clinical improvement by severity of illness (NEWS2 score > 5 [Panel A] vs ≤ 5[Panel B]) in the intention-to-treat population.



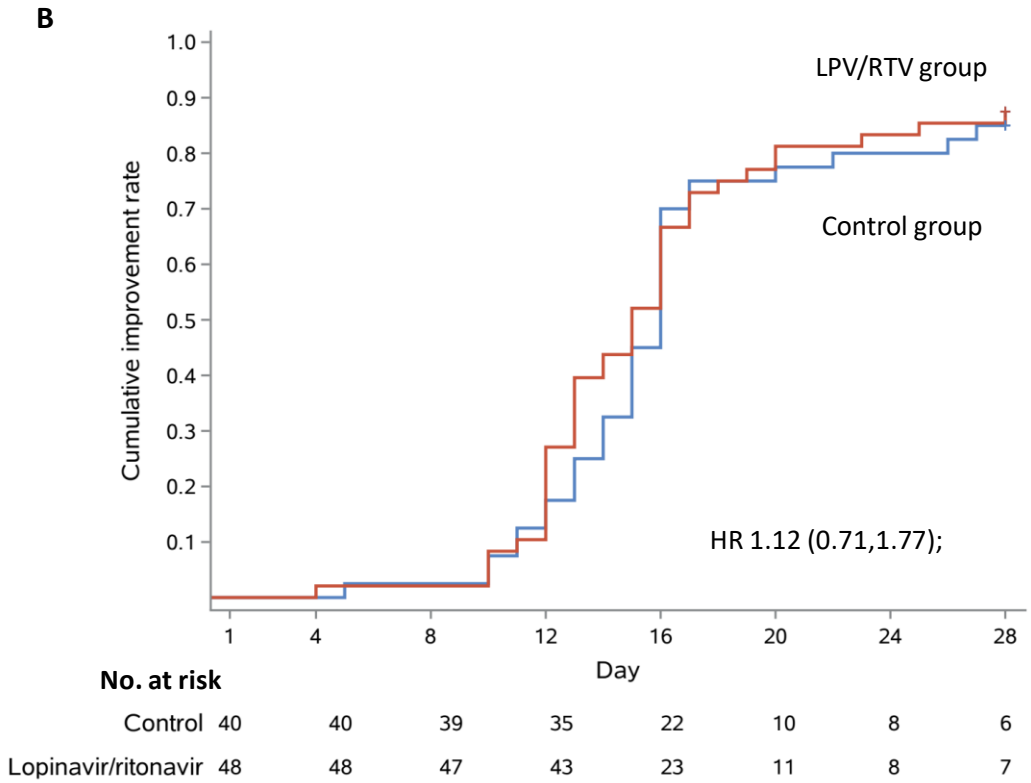
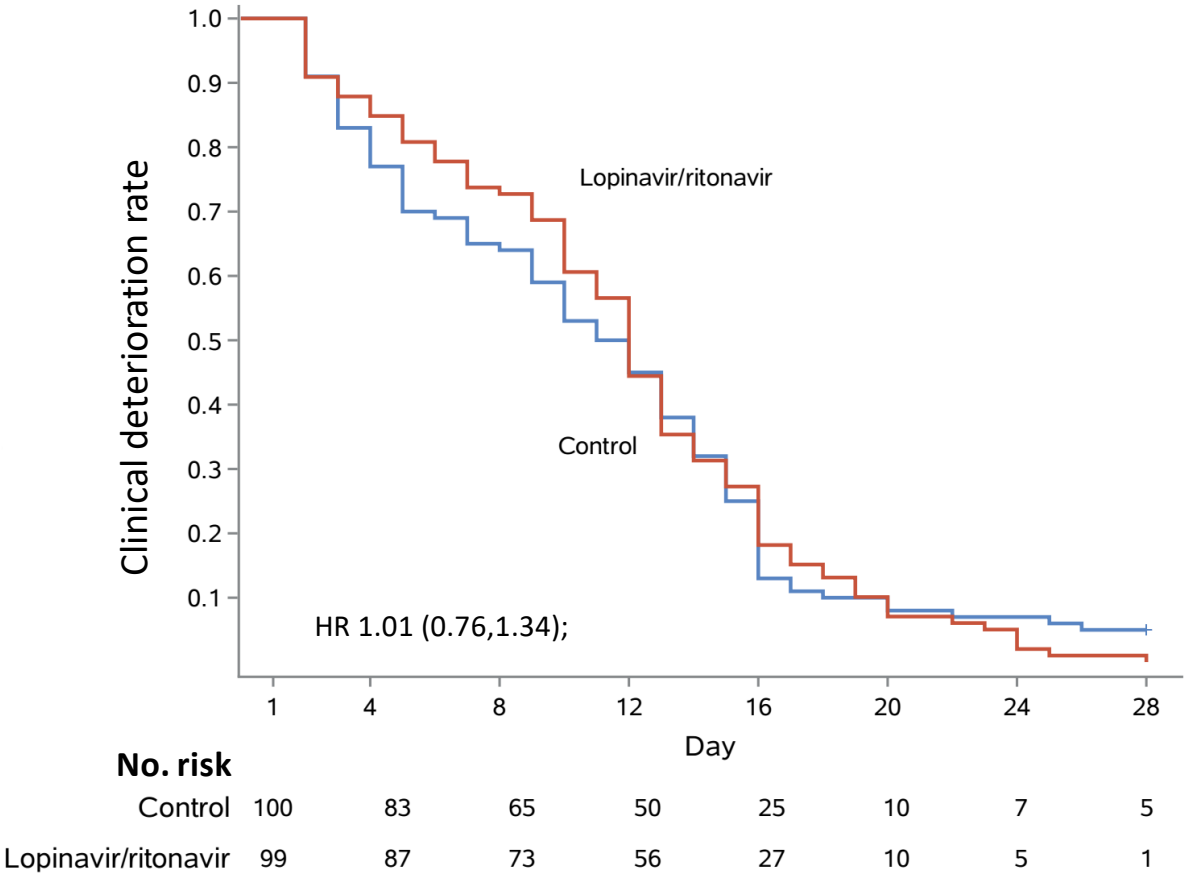


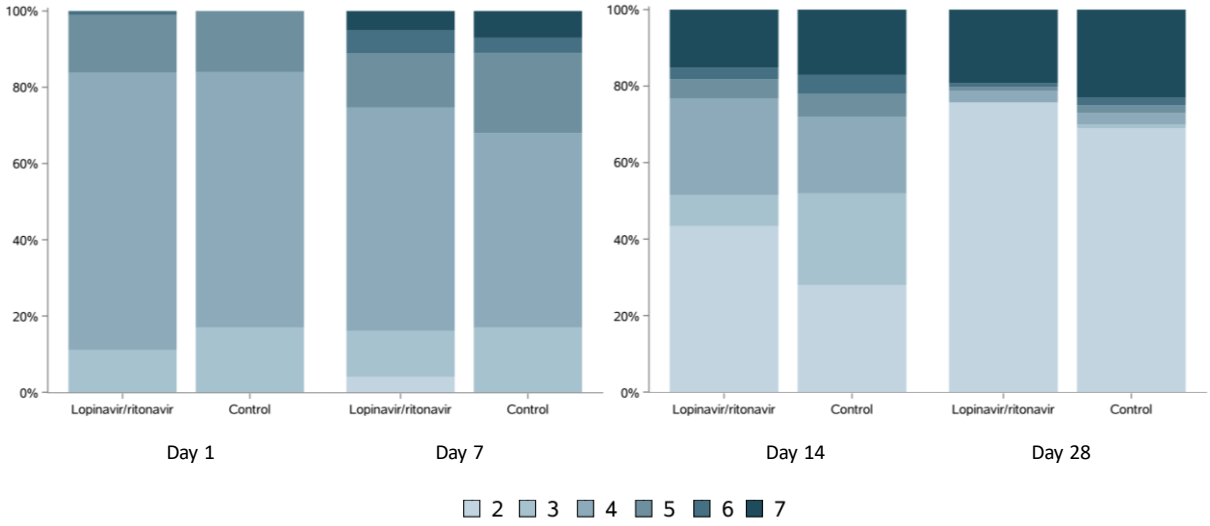
Figure S4. Kaplan Meier of time-to-clinical deterioration in the intention-to-treat population.



No. risk

Control	100	83	65	50	25	10	7	5
Lopinavir/ritonavir	99	87	73	56	27	10	5	1

Figure S5. Proportional distribution of primary endpoint categories at day1, 7, 14 and 28 in the intention-to-treat population



Proportion of severe outcomes according to 7-category ordinal scale that ranges from 1 (discharged with normal activity) to 7 (death). 2 Not hospitalized, but unable to resume normal activities; 3 Hospitalized, not requiring supplemental oxygen; 4 Hospitalized, requiring supplemental oxygen; 5 Hospitalized, requiring high-flow nasal oxygen (HFNC)and/or non-invasive mechanical ventilation (IMV); 6 Hospitalized, requiring ECMO and/or IMV; 7 Death.

Figure S6. SARS-CoV-2 viral RNA load over time from baseline by qPCR on throat swabs (viral positive population) by duration of illness (≤ 12 days [Panel A] vs > 12 days [Panel B]) in the modified intention-to-treat population.

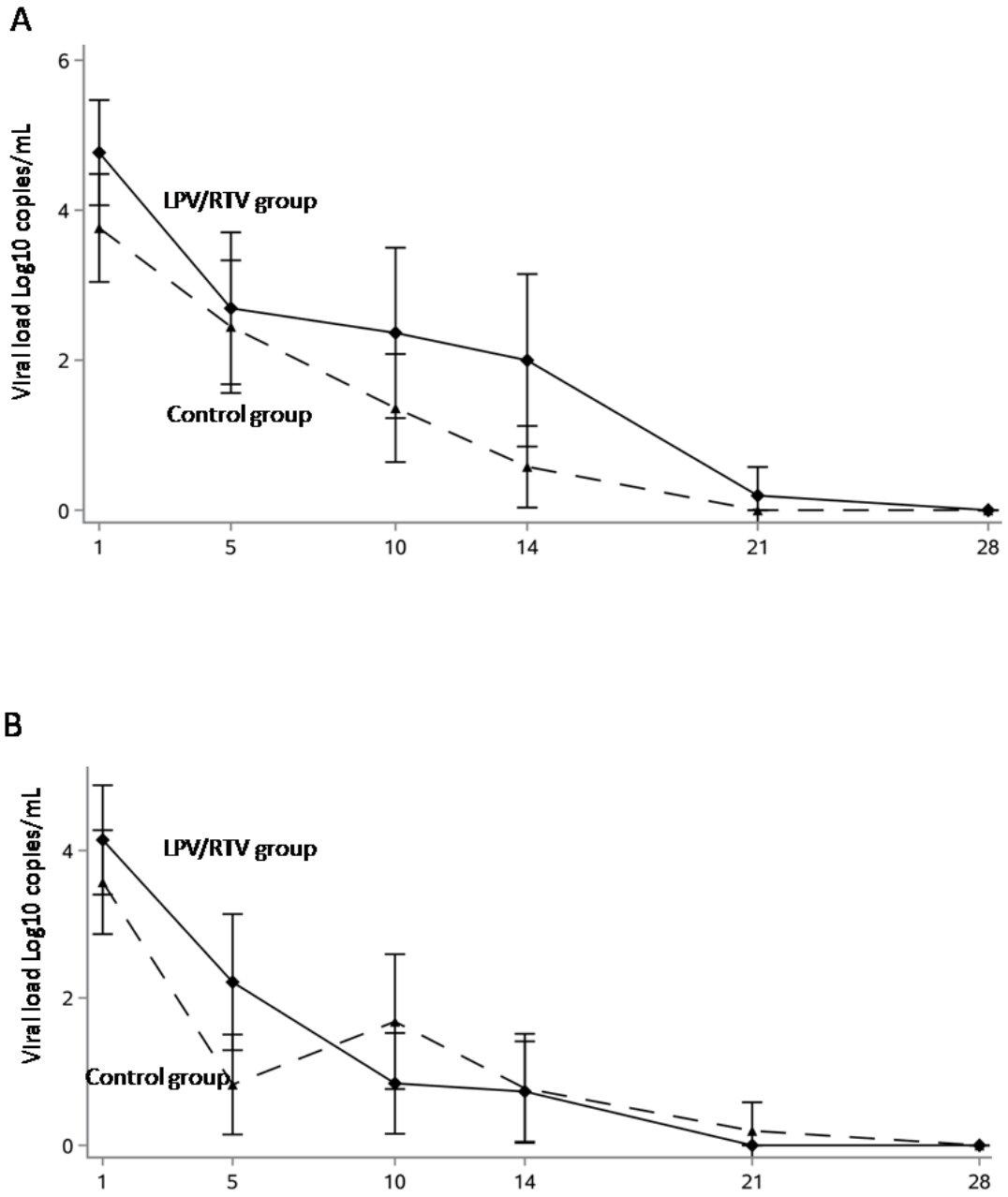


Figure S7: Summary of Protocol Violations.

Protocol Violations	Lopinavir/ritonavir (n=99)	Control group (n=100)
Major Violations		
Intervention violation	2 (2.0) *	1 (1.0)
Minor violation		
Wrong dosage	1 (10.1)	0
Exceeding sampling time window	20 (20.2)	15 (15.0)
No sample	3 (3.0)	3 (3.0)

Denominator of the percentage is the total number of patients in each group. * The attending physician refused to prescribe lopinavir–ritonavir after randomization