

Immunophenotypes of anti-SARS-CoV-2 responses and fatal COVID-19

ONLINE DATA SUPPLEMENT

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Study participants.

We conducted this study of COVID-19 at the University Clinic of Respiratory and Allergic Diseases Golnik, a quaternary, acute care hospital in Slovenia. The first hospitalized patient was included on September 4th and the last on December 12th, 2020. All information presented in this report is based on a data cut-off of January 9th, 2021. During a follow-up of 3 to 26 days after presentation, 45 patients (18%) died (8 after intubation), with December 16th, 2020, as the time of the last endpoint event. During a follow-up of 3 to 35 days after presentation, 209 patients had survived hospital discharge (7 after successful cessation of invasive mechanical ventilation), with December 17, 2020, as the time of discharge of the last patients. Six of those patients were discharged more than 28 days after the presentation (during a follow-up of 29 to 35 days; one after successful cessation of invasive mechanical ventilation). None of the patients was transferred to another

For non-hospitalized control subjects we included 40 adult individuals with prior COVID-19 confirmed by a nasopharyngeal swab PCR test (Supplement Table E1). In first control subject SARS-CoV-2 infection was confirmed on April 19th, 2021 and in the last on February 18th, 2022. The first blood sampling was done on July 12th, 2021 and the last on March 16th, 2022. Consequently a single post-blood sample was collected median of 60 days (range 20 to 175 days) after infection. None of the controls subjects was vaccinated neither before infection nor before sampling.

Data analysis

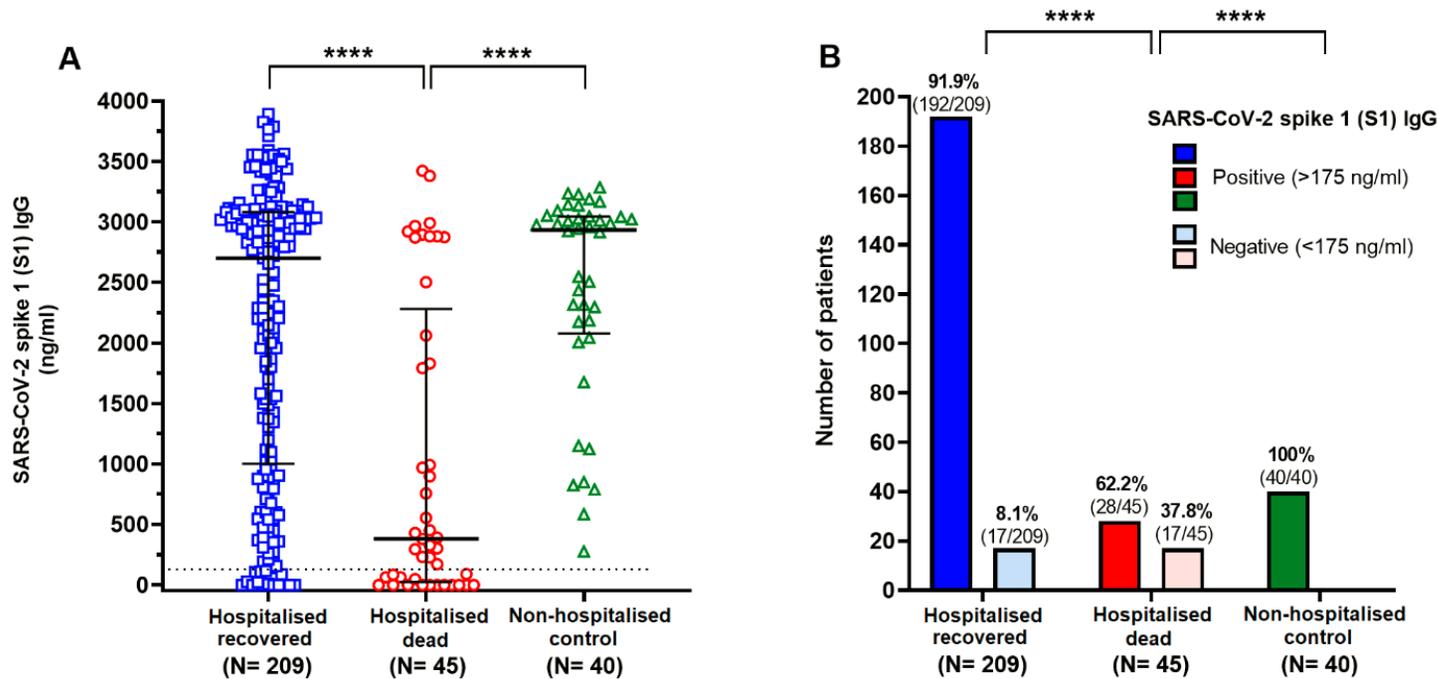
First, we performed a standard Cox proportional hazards analysis. The analysis was done with all the variables and in a stepwise manner. We analysed the variables in an univariate fashion and if the variable correlated significantly with the risk of death in univariate analysis it was further used as predictor in multivariable analysis (Supplement Table E1).

Regression analysis (including Cox regression) is considered a supervised machine learning method, since it fits the predictors to the outcome of interest (i.e. fatal COVID-19). To search for patterns in immune response that are not fitted to the outcome of interest and thus possibly reflect inherent biological processes, we next performed cluster analysis (unsupervised machine learning method) of immune variables. We used Gaussian mixture model algorithm as previously described¹. Clustering was performed with variables of anti-SARS CoV-2 immune response for which several studies demonstrated that likely contribute to disease severity. Those variables included antibody^{2,3}, circulating lymphocyte subsets⁴⁻⁹ and serum interleukin-6^{1,3,5,8,13} responses. Before clustering, all variables were normalized. The model was fitted by the expectation-maximization algorithm and the optimal number of clusters was chosen using the Bayesian information criterion (global optimum n=6 clusters; Figure 1).

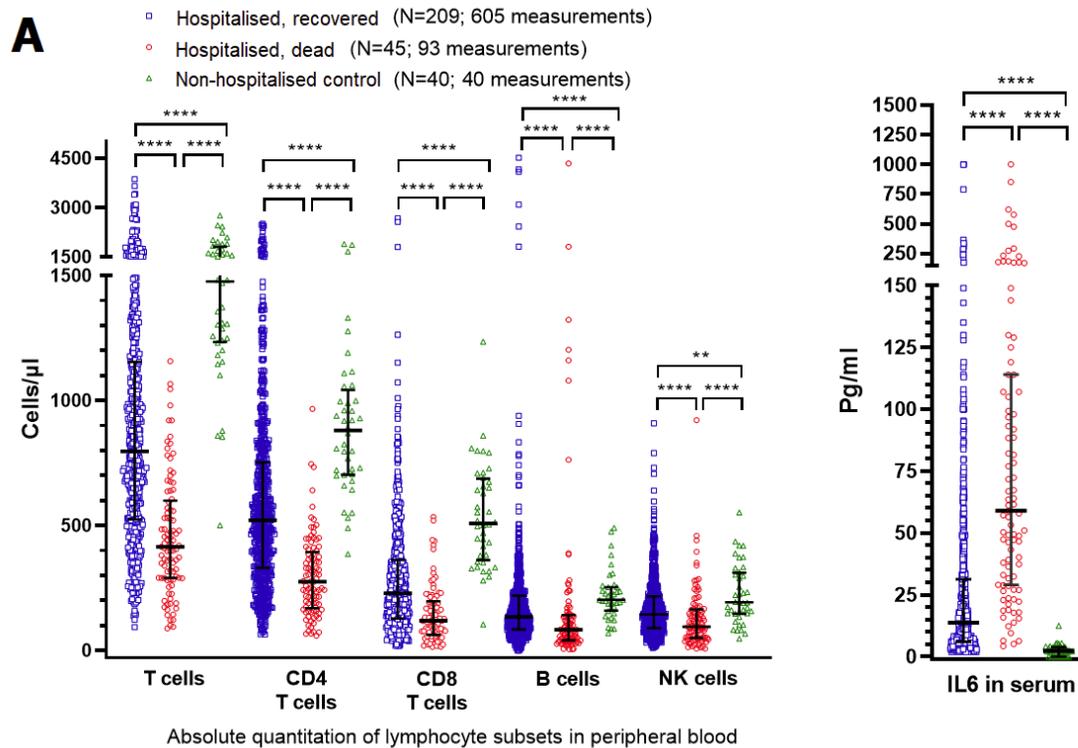
We compared the end point characteristics of each cluster with other clusters, and Kaplan–Meier survival curves were constructed to visualize mortality over the 28 days across clusters. To account for possible differences between clusters in potential confounding variables (i.e. clinical severity factors^{14,15} that could influence the risk of fatal COVID-19), we used multivariable Cox regression with clusters and clinical severity factors serving as predictors of fatal COVID-19. We included only the non-immunological factors that were significant predictors of the fatal COVID-19 in a before constructed (please see above) multivariable adjusted Cox proportional hazard model.

Figure E1A-B. Anti-SARS-CoV-2 spike 1 IgG antibodies levels in 254 hospitalized COVID-19 patients according to whether patients progressed to the primary endpoint of death at 28 days or recovered to hospital discharge. **A** shows individual concentrations and **B** positivity of antibody testing. Non-hospitalized controls represent adult individuals with prior COVID-19 confirmed by a nasopharyngeal swab PCR test, post-sampling median 60 days after infection.

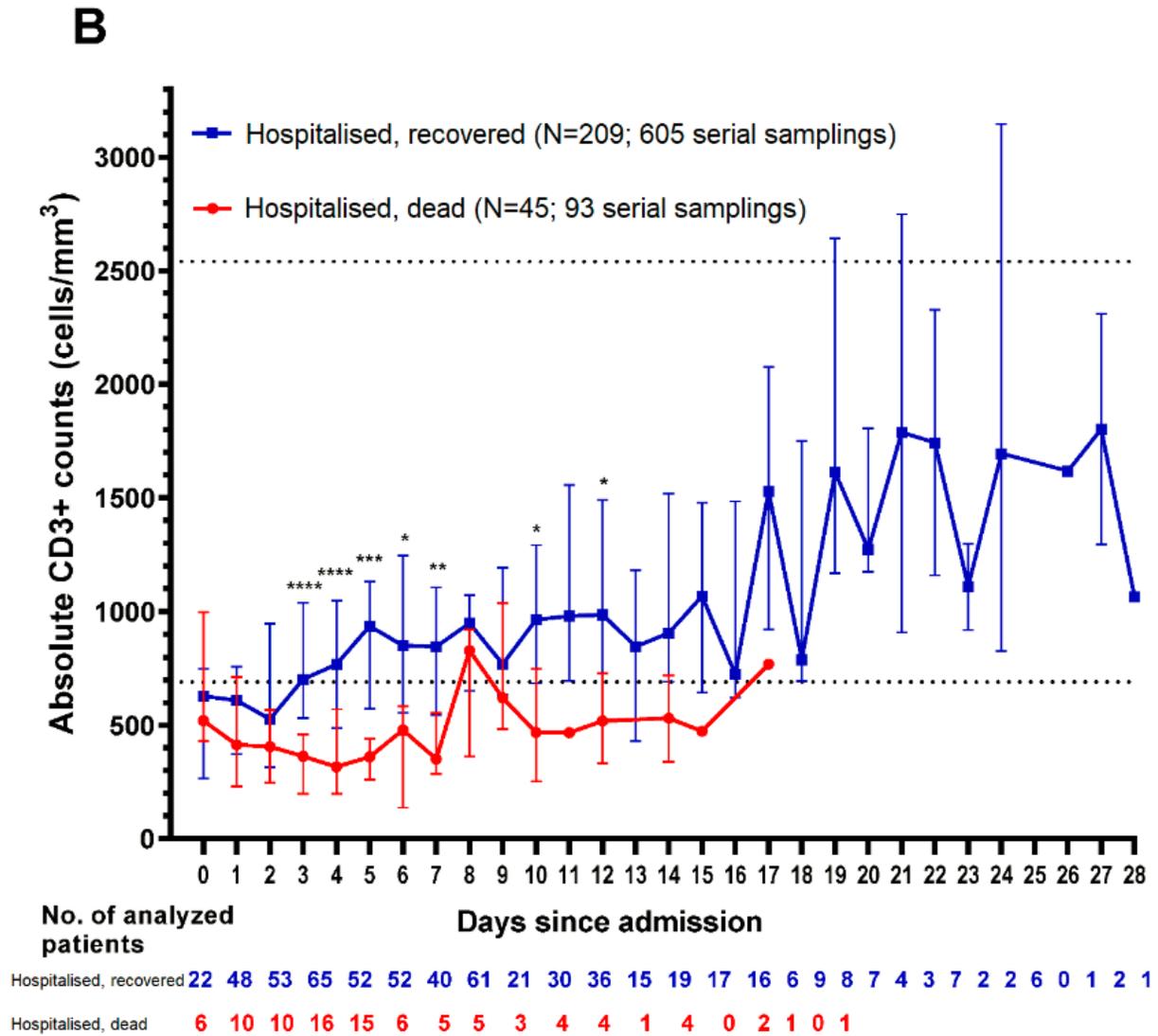
The median time of single blood sampling for both (hospitalized) subgroups was 4 days since hospital admission. A dotted horizontal line represents the cut-off between positive and negative antibody concentrations, assigned according to the manufacturer threshold for positivity (175 ng/ml). Horizontal lines represent the median with IQR. **** P<0.0001.



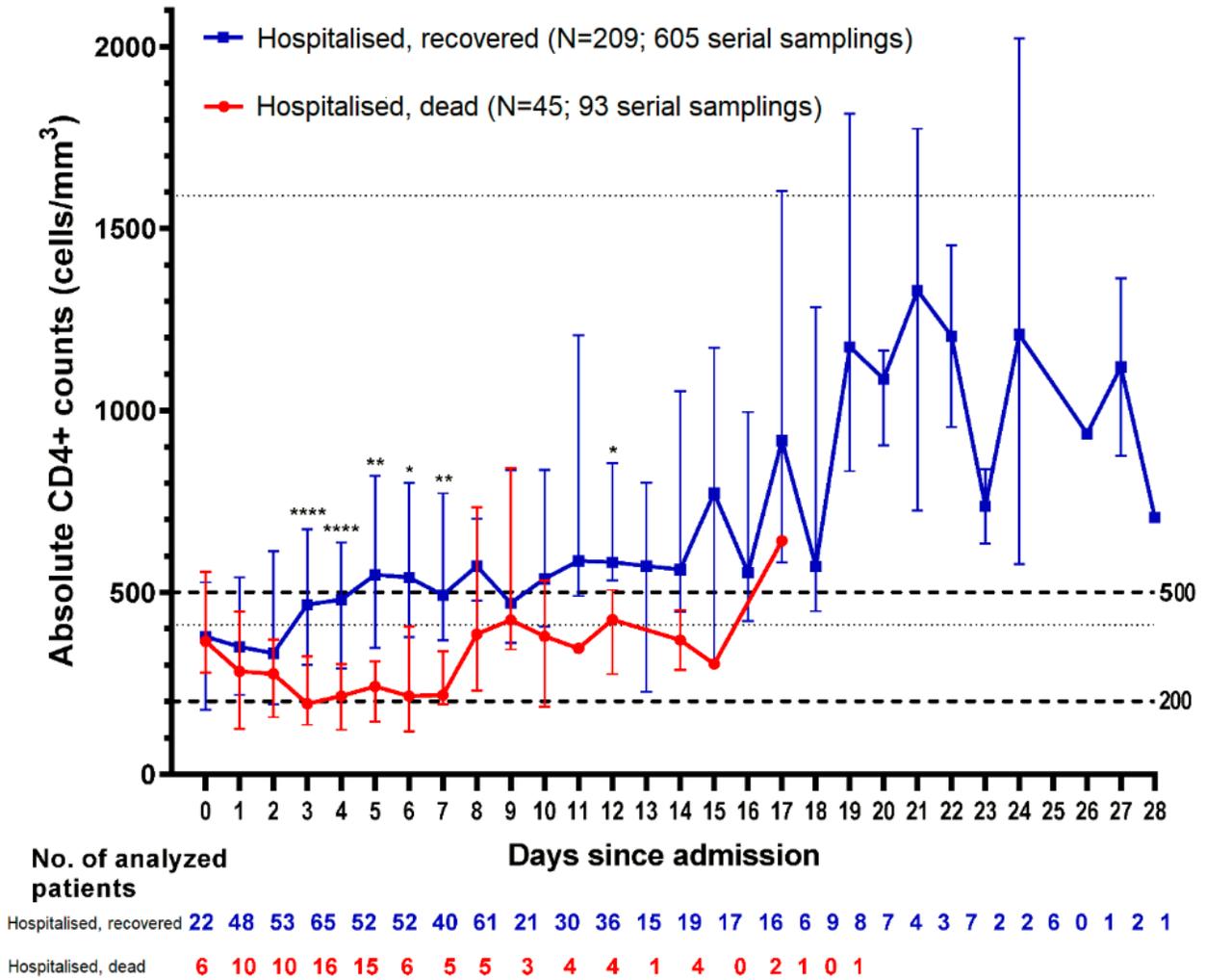
Figures E2A-G. A shows overall absolute counts of circulating T cells, CD4 and CD8 subpopulations of T cells, B and NK cells, and IL-6 measurements in sera, and longitudinal dynamic of circulating **B** T cells, **C** CD4 and **D** CD8 subpopulations of T cells, **E** B and **F** NK cell and **G** IL-6 in sera of hospitalized COVID-19 patients per day after admission according to whether patients progressed to the primary endpoint of death at 28 days or recovered to hospital discharge. Non-hospitalized controls represent adult individuals with prior COVID-19 confirmed by a nasopharyngeal swab PCR test, post-sampling median 60 days after infection.

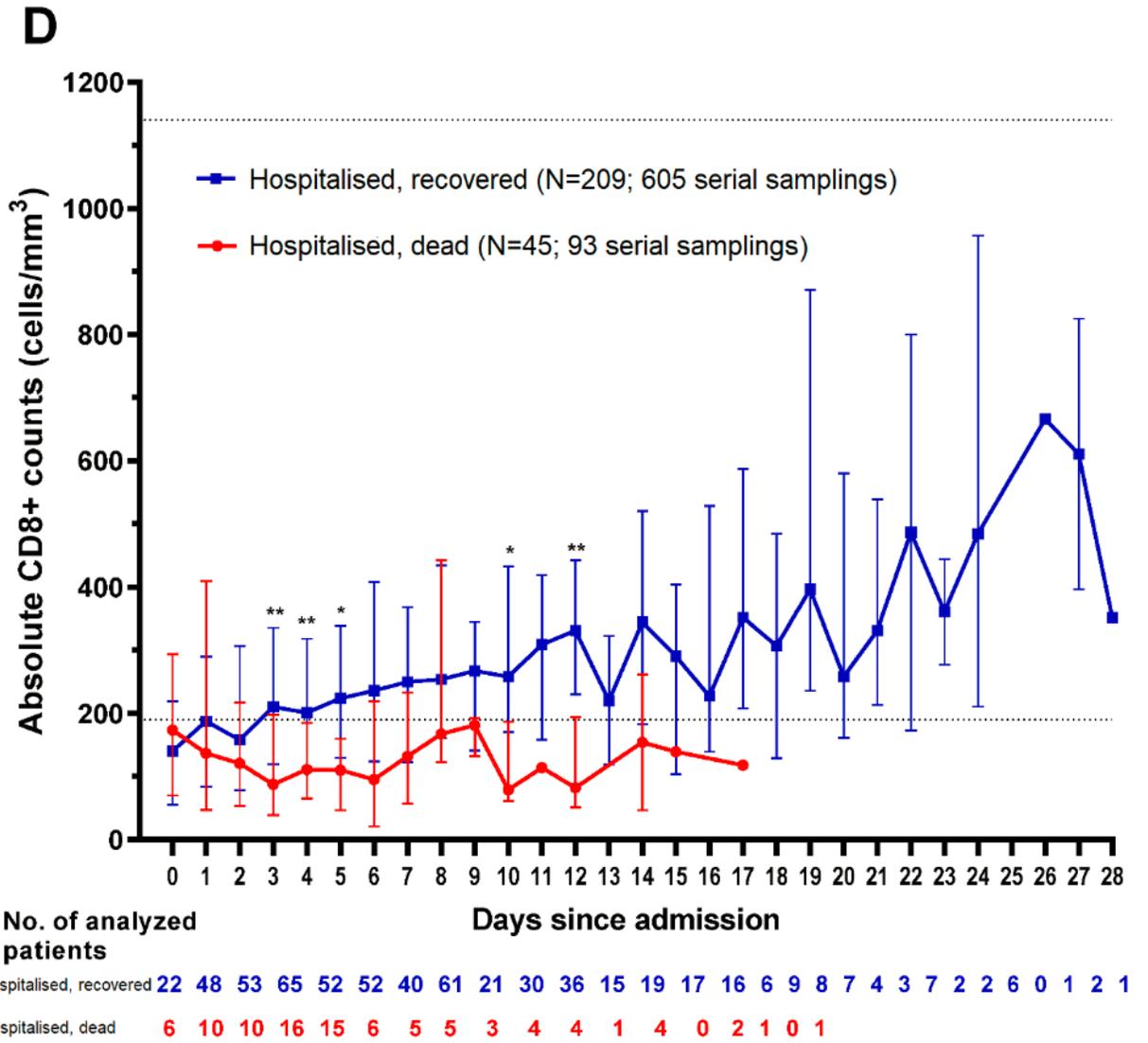


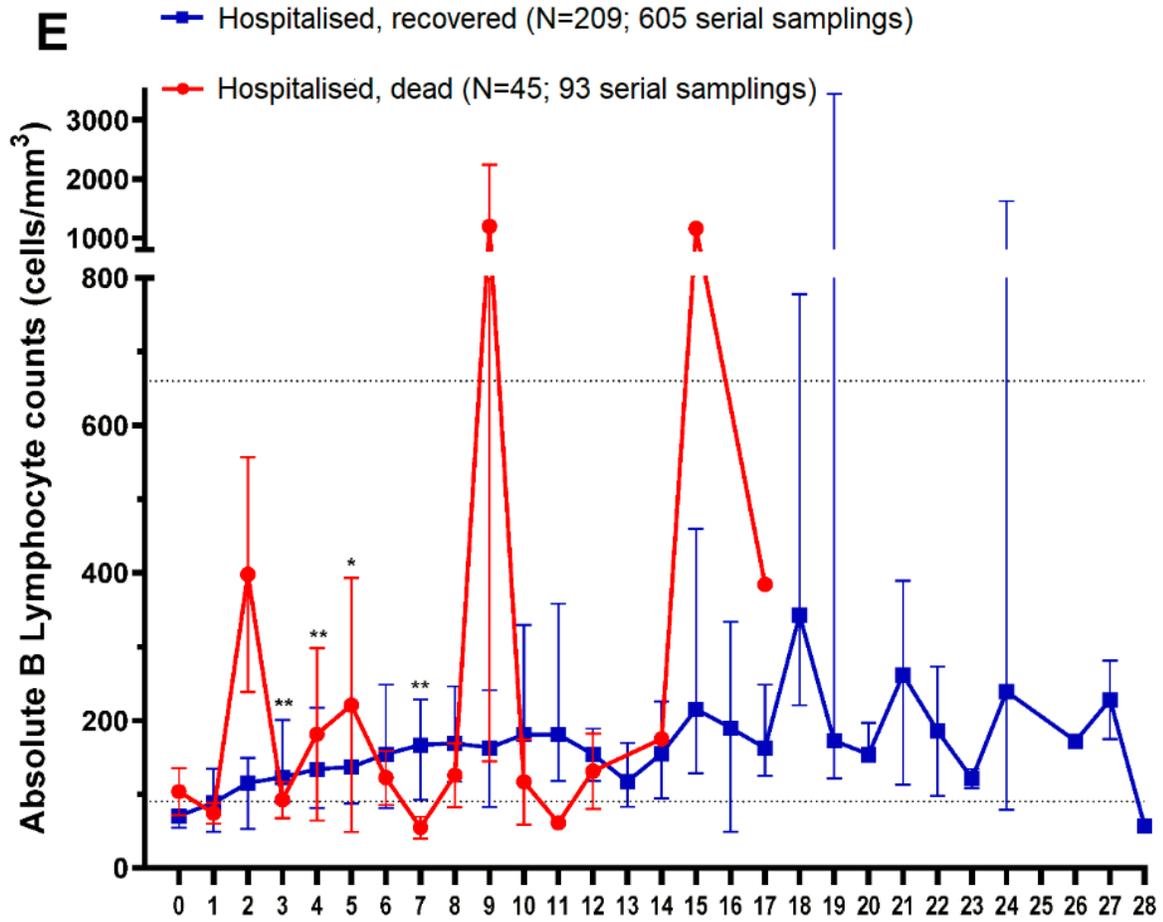
Data are shown as median with interquartile ranges continuously per day. Dotted or dashed horizontal lines are showing reference intervals. * P<0.05; ** P<0.01; *** P<0.001, and **** P<0.0001.



C



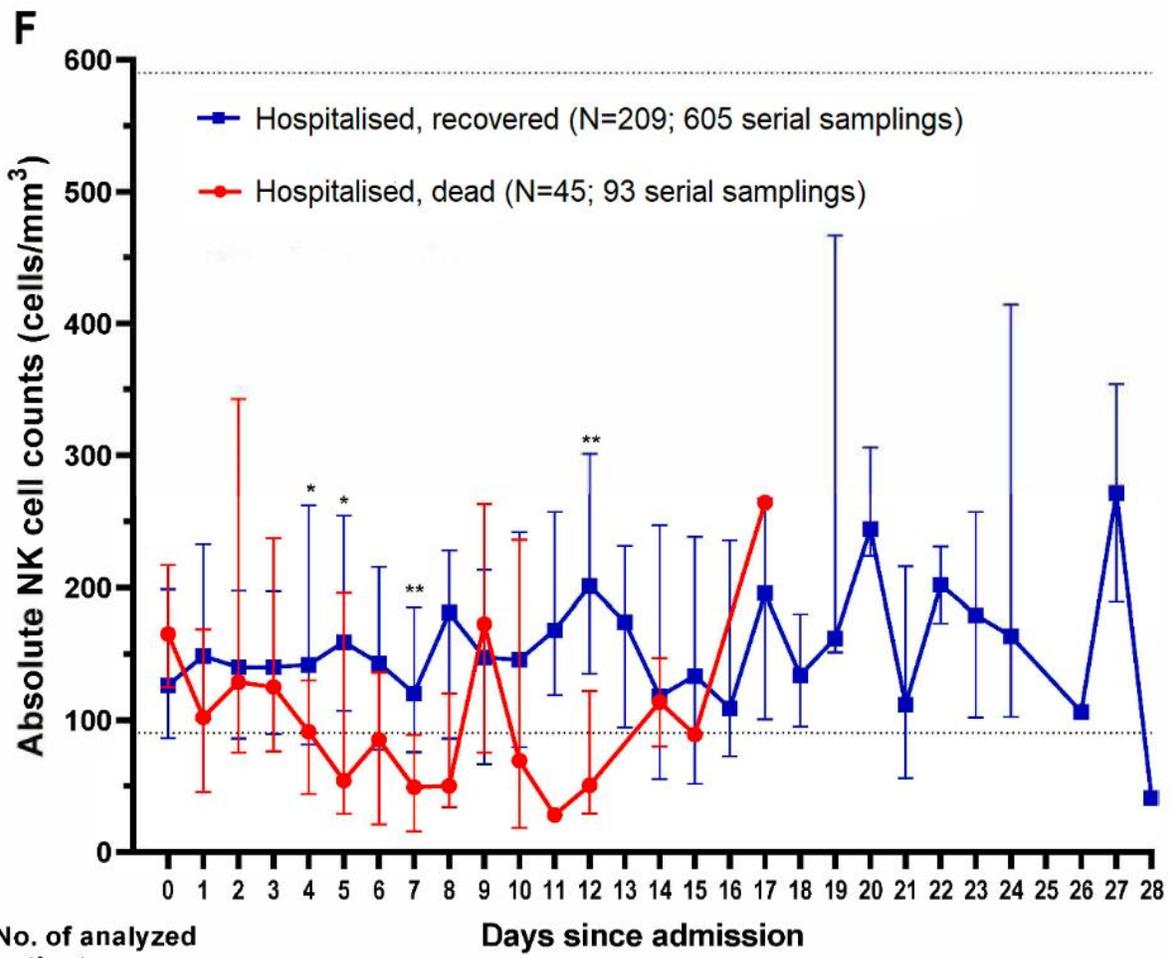




No. of analyzed patients

Days since admission

Hospitalised, recovered	22	48	53	65	52	52	40	61	21	30	36	15	19	17	16	6	9	8	7	4	3	7	2	2	6	0	1	2	1
Hospitalised, dead	6	10	10	16	15	6	5	5	3	4	4	1	4	0	2	1	0	1											



No. of analyzed patients	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	
Hospitalised, recovered	22	48	53	65	52	52	40	61	21	30	36	15	19	17	16	6	9	8	7	4	3	7	2	2	6	0	1	2	1	
Hospitalised, dead	6	10	10	16	15	6	5	5	3	4	4	1	4	0	2	1	0	1												

G

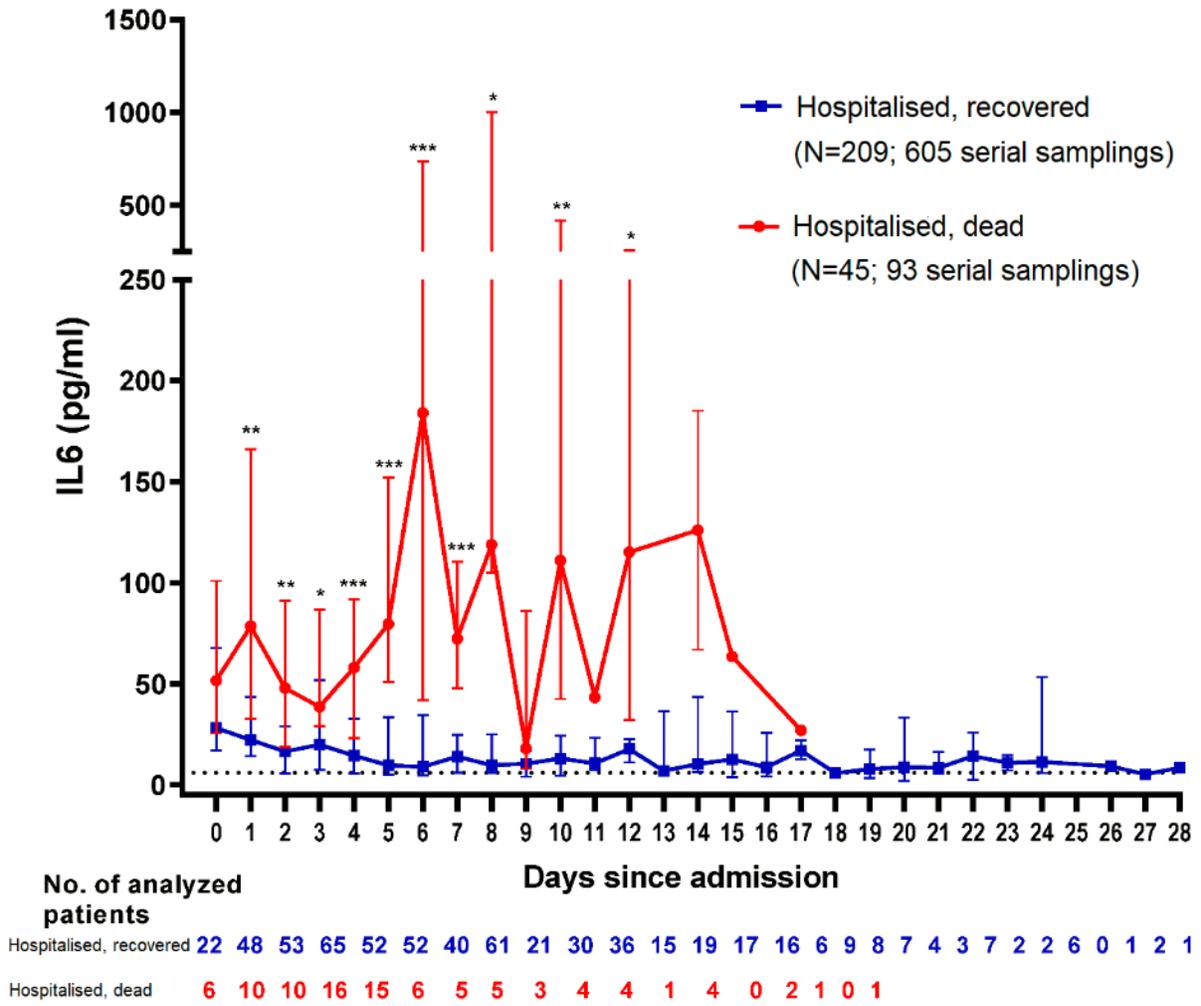


Figure E3. The lowest T cells, CD4 and CD8 subpopulations of T cells, B and NK cells absolute counts, and the highest serum IL-6 after admission to the hospital according to whether COVID-19 patients progressed to the primary endpoint of death at 28 days or recovered to hospital discharge. Non-hospitalized controls represent adult individuals with prior COVID-19 confirmed by a nasopharyngeal swab PCR test, post-sampling median 60 days after infection.

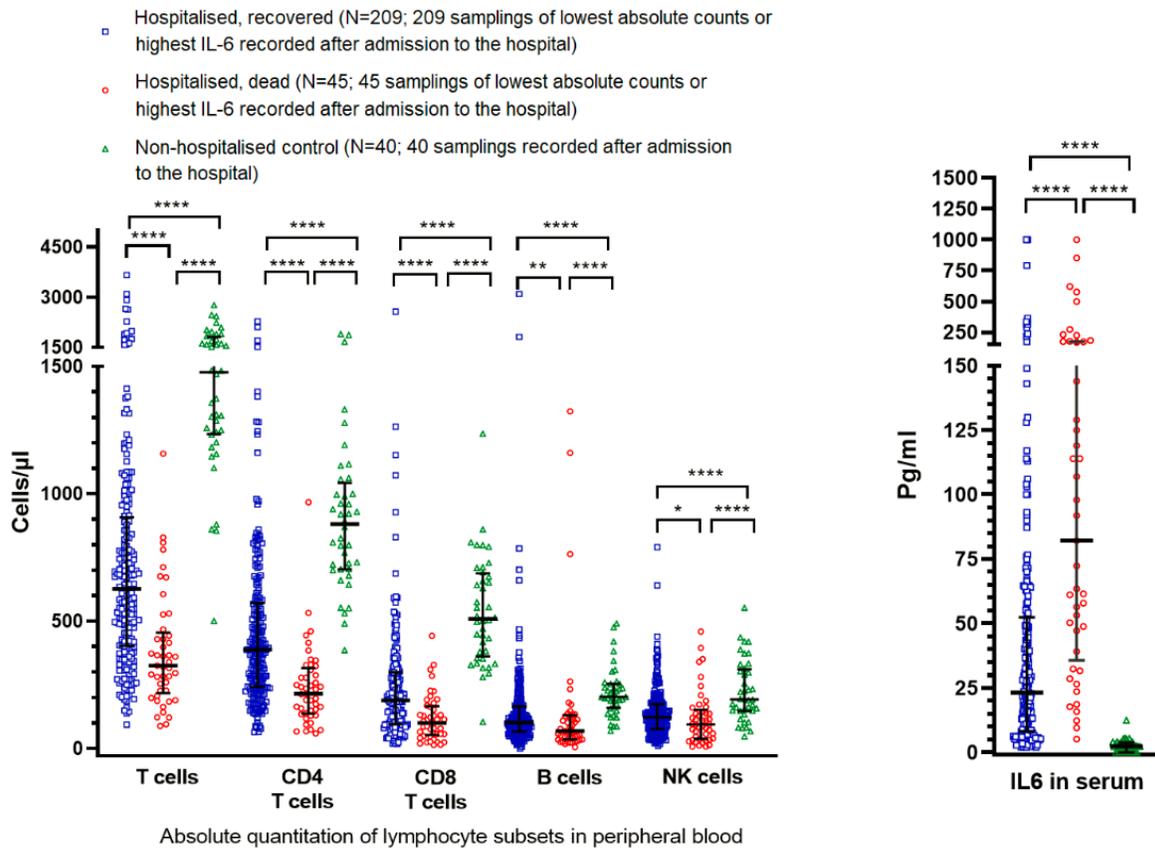


Table E1. Demographic characteristics, antibody response, lymphocytes, and IL-6 in non-hospitalized control subjects.

Age – mean ± SD	45.6 ± 12.1
Male sex — no. (%)	22 (55%)
Anti-SARS-CoV-2 S1 IgG antibodies (pg/ml)	
Minimum	277.2
25%	2077
Median	2935
75%	3046
Maximum	3286
T cells/mm³	
Minimum	501
25%	1234
Median	1477
75%	1814
Maximum	2763
B cells/mm³	
Minimum	69
25%	159
Median	202
75%	253
Maximum	491
NK cells/mm³	
Minimum	47
25%	146.5
Median	192.5
75%	310.5
Maximum	552
IL-6 (pg/ml)	
Minimum	0
25%	0
Median	2.425
75%	3.853
Maximum	12.50

Table E2. Associations between demographic and clinical factors, antibody response, lymphocyte subsets, IL-6, and the endpoint of death at 28 days in 254 hospitalized subjects.

UNIVARIATE COX ANALYSIS				
	Hazard ratio	Lower 95% CI	Upper 95% CI	P-value
Age — yr	1.0887	1.0538	1.1247	<0.0001
Sex (female)	0.7072	0.3871	1.2920	0.26
Respiratory support received at the time of the admission (WHO severity ordinal scale 3) ¹⁶	5.7008	2.5448	12.771	<0.0001
Type 2 diabetes	1.2478	0.6713	2.3195	0.484
Hypertension	1.0620	0.5813	1.9402	0.845
No. of coexisting diseases (sum)	1.2775	0.8435	1.9348	0.247
Body mass index (≥ 30.0) [§]	0.9399	0.8701	1.0154	0.116
Glucocorticoid use (Yes)	0.6615	0.3682	1.1884	0.167
Anti-SARS-CoV-2 S1 IgG antibodies*	0.9993	0.9990	0.9996	<0.0001
T cells/mm ³ †	0.9965	0.9951	0.9979	<0.0001
CD4+ T cells/mm ³ †	0.9948	0.9926	0.9971	<0.0001
<500 CD4+ cells/mm ³	8.9102	2.1583	36.7840	0.0025
<200 CD4+cells/mm ³	3.8183	2.1246	6.8623	<0.0001
CD8+ T cells/mm ³ †	0.9936	0.9903	0.9969	0.0002
B Lymphocyte cells/mm ³ †	0.9998	0.9986	1.0011	0.808
Natural killer cells/mm ³ †	0.9977	0.9941	1.0014	0.219
IL-6‡	1.0022	1.0012	1.0032	<0.0001
MULTIVARIABLE COX ANALYSIS				
Age— yr	1.0652	1.0294	1.1023	0.0003
Respiratory support received at the time of admission	4.5264	1.9594	10.4563	0.0004
Anti-SARS-CoV-2 spike 1 IgG antibodies*	0.9994	0.9991	0.9996	<0.0001
T cells/mm ³ † or	0.9977	0.9963	0.9991	0.0015
CD4+ T cells/mm ³ † or	0.9965	0.9943	0.9988	0.0024
CD8+ T cells/mm ³ †	0.9960	0.9929	0.9991	0.012
IL-6‡	1.0014	1.0001	1.0027	0.031

*SARS-CoV-2 anti S1 IgG antibodies were measured median 4 days after admission to the hospital. †Decreases in circulating lymphocytes were defined as the lowest T cell, CD4, and CD8 subpopulations of T cells, B and NK cell absolute counts recorded after admission. ‡IL-6 increase was defined as the maximal serum level recorded after admission. §BMI data were missing for 34 patients. The decision to prescribe glucocorticoids was at the discretion of the treatment team for each patient. In the multivariable Cox model, only significant predictors (P<0.05) from univariate Cox analysis were retained and only CD3 T-cell counts were used (since they reflect the presence of CD4 as well as CD8 cells). HR-hazard ratio.

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