

SARS-COV-2 NEUTRALIZATION ASSAY AS A KEY PREMISE FOR IMPLEMENTATION OF COVID-19 SEROTHERAPY IN CROATIA

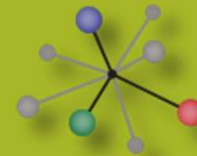
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Sveučilište u
Zagrebu

Centre for Research and Knowledge Transfer in Biotechnology

Centre of Excellence for Virus Immunology and Vaccines



Center of excellence
[for]
Virus Immunology and Vaccines

Passive immunotherapy

a century-old practice (since 1890s) of administering pathogen-specific antibodies to prevent or treat the disease caused by the same pathogen.

Term **SEROTHERAPY**

... either whole serum, containing also antibodies, or purified antibodies originating from serum

the only means of treating certain infection diseases prior to the development of antimicrobial therapy in the 1940s.

the **first** consistently effective **antimicrobial strategy**.

5 Nobel Prizes awarded for discoveries related to treatment of infection diseases with antibodies:

1901 Emil **von Behring** and Shibasaburo **Kitasato**

for discovery of serum therapy

serum from rabbits immunized with tetanus toxin could prevent tetanus in rabbits

1908 Paul **Erlich**

for establishing the field of humoral immunity and concepts of active and passive immunization; he also worked on generating antitoxin immunity in animals

1972 Gerald M **Edelman** and Rodney R **Porter**

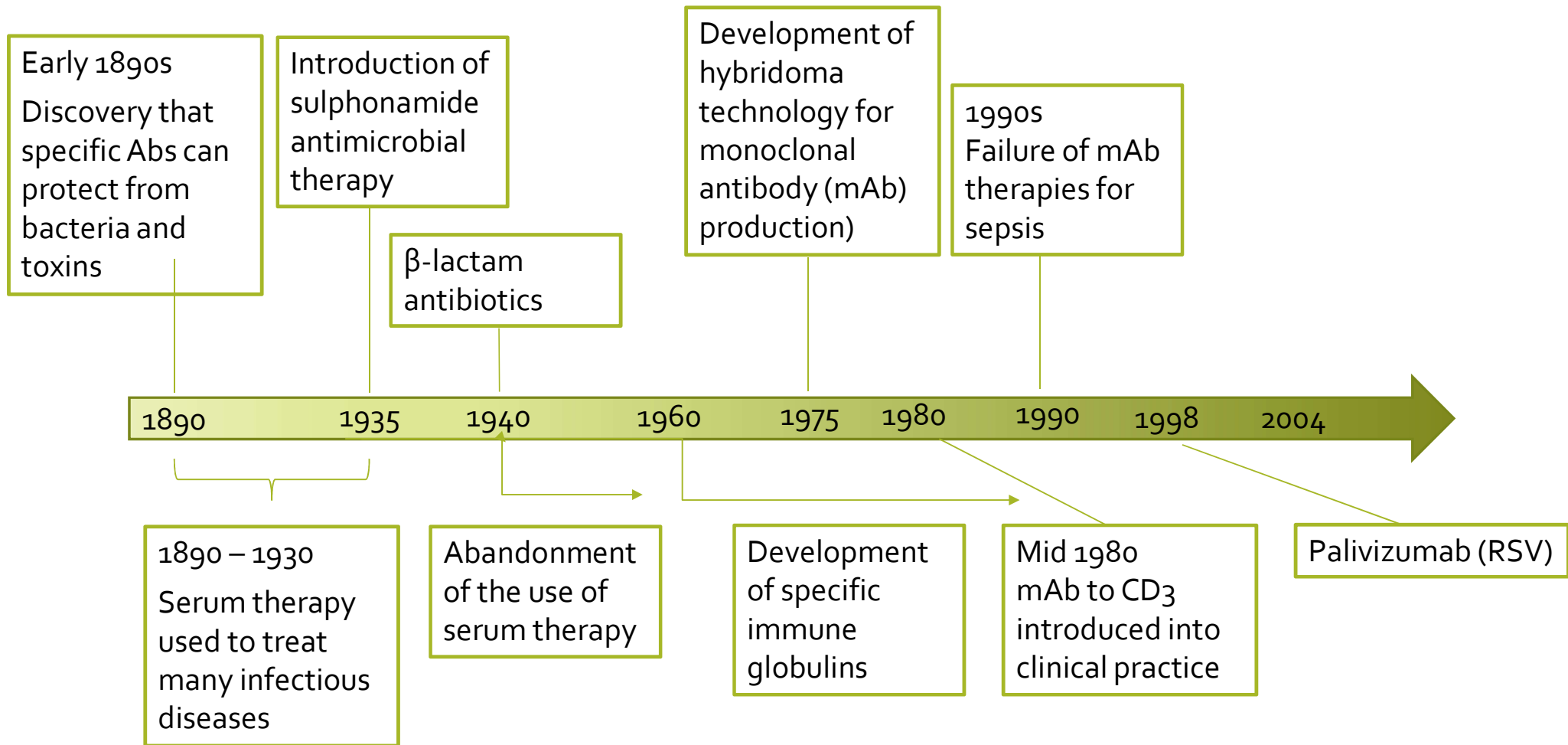
for their discoveries concerning the chemical structure of antibodies

1984 Georges JF **Köhler** and Cesar **Milstein**

for the discovery of the principle for production of monoclonal antibodies

1987 Susumu **Tonegawa**

for his discovery of the genetic principle for generation of antibody diversity



Timeline from Casadevall et al. NatRev2004

General principles of passive antibody therapy (based on a century of its use to combat infectious diseases):

- it is more effective when used for prophylaxis than for treatment of disease;
- when used for therapy, antibody is most effective when administered shortly after the onset of symptoms
- a sufficient (high) amount of antibody must be administered.

Antibody formats in regular times:

specific polyclonals immunoglobulins (purified from vaccinated donors)
monoclonals (less often)

Interest in using antibodies to treat infectious diseases is now being fuelled:

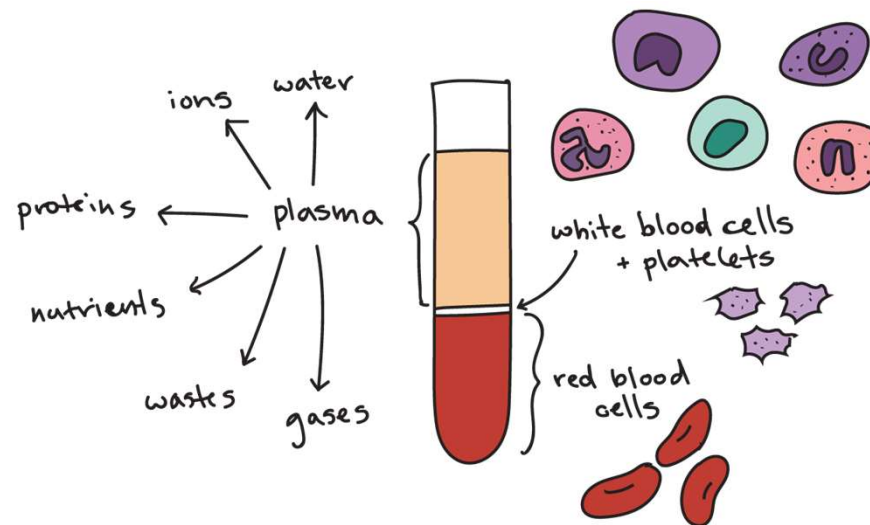
wide dissemination of drug-resistant microorganisms,
the emergence of new microorganisms,
the relative inefficacy of antimicrobial drugs in immunocompromised hosts,
the fact that antibody-based therapies are the only means to provide
immediate immunity against biological weapons.

During emerging infections and pandemics (influenza, SARS-CoV-1, MERS, Ebola)

there is insufficient time or resources to generate immunoglobulin preparations

convalescent plasma is an antibody format used for passive Ab therapy

(Bloch et al., 2020).



Experience from prior outbreaks with other coronaviruses (SARS-CoV-1, MERS) convalescent sera contain neutralising antibodies against relevant virus their use was beneficial in the treated patients.

Casadevall & Pirofski. JCI 2020;130:1545

Cheng et al. EJCMI 2005;24:44

Early-non randomized studies

safety and potential efficacy of CCP treatment in COVID-19 hospitalized patients.

Duan et al. PNAS 2020;117:9490;

Shen et al. JAMA 2020; 323:1582-9;

Joyner et al. JCI2020;130:4791;

Liu et al. NatMed2020;26:1708)

Therapy with antibody-laden blood of those who have survived SARS-CoV-2 infection was used and investigated worldwide in the previous year .

Barreira et al. FrontMed 2021;8:1

Cohn et al. Transfus 2021;61:44

Katz. NEJM 2021;384:666

Libster et al. NEJM 2021;384:610

Convalescent plasma therapy has been considered generally beneficial due to multiple examples, both historical and recent

Casadevall & Scharff, CID 1995;21:150

Graham & Ambrosino, CurrOppHIV 2015;10:129

Luke et al. AnnalsIntMed 2006;145:599

Definitive proof of its effectiveness coming from carefully designed randomized clinical trials **is lacking.**

Bloch et al. JCI 2020;130:2757

Subbarao et al. EJI 2020;50:1447

When such were undertaken, they have usually not been able to demonstrate the beneficial effect of plasma over placebo.

The reasons lay mostly in the specificity of the circumstances of CP usage:

the time frame of convalescent plasma usage is short (during epidemics;
pathogen-specific therapy and vaccines are lacking)

the new and not sufficiently known pathogen

methods for plasma neutralization potency determination are being developed,
are not standardized, not validated

results of such assays vary within the individual trials,

and are uncomparable between different case studies, case series, trials.

CP is a complex, non-standardized medicine

varying in neutralization antibody titre,

in a content of non-specific immunomodulators

between units collected from different individuals.

Hurt & Wheatley, Viruses 2021;13:1

Joyner et al. NEJM 2021;384:1015

Aim:

to establish COVID-19 convalescent plasma (CCP) characterization in a way
enabling reliable selection of plasma units for therapy
enabling comparison of Croatian to international practice

in circumstances when no concrete directions or methodology existed

Strategy:

development of the assay of wild-type SARS-CoV-2 neutralization

BSL₃ pathogen

Plaque reduction neutralization test (PRNT)
(based on Plaque assay)

Virus neutralization assay – ED₅₀ assay
(based on CCID₅₀ assay)

Lower biosafety risk
Better resolution



No CPE
Smooth, clear layer of cells



CPE
Manifested as holes, clumps,
vacuoles in the cell layer.

SARS-CoV-2 /
Vero E6 cells

I Binary serial dilutions of serum

serum dilution factor	-	2	4	8	16	32	64	128	256	512	1024	2048
undiluted serum volume (μL/well)	-	25	12.5	6.25	3.13	1.56	0.78	0.39	0.2	0.1	0.05	0.03

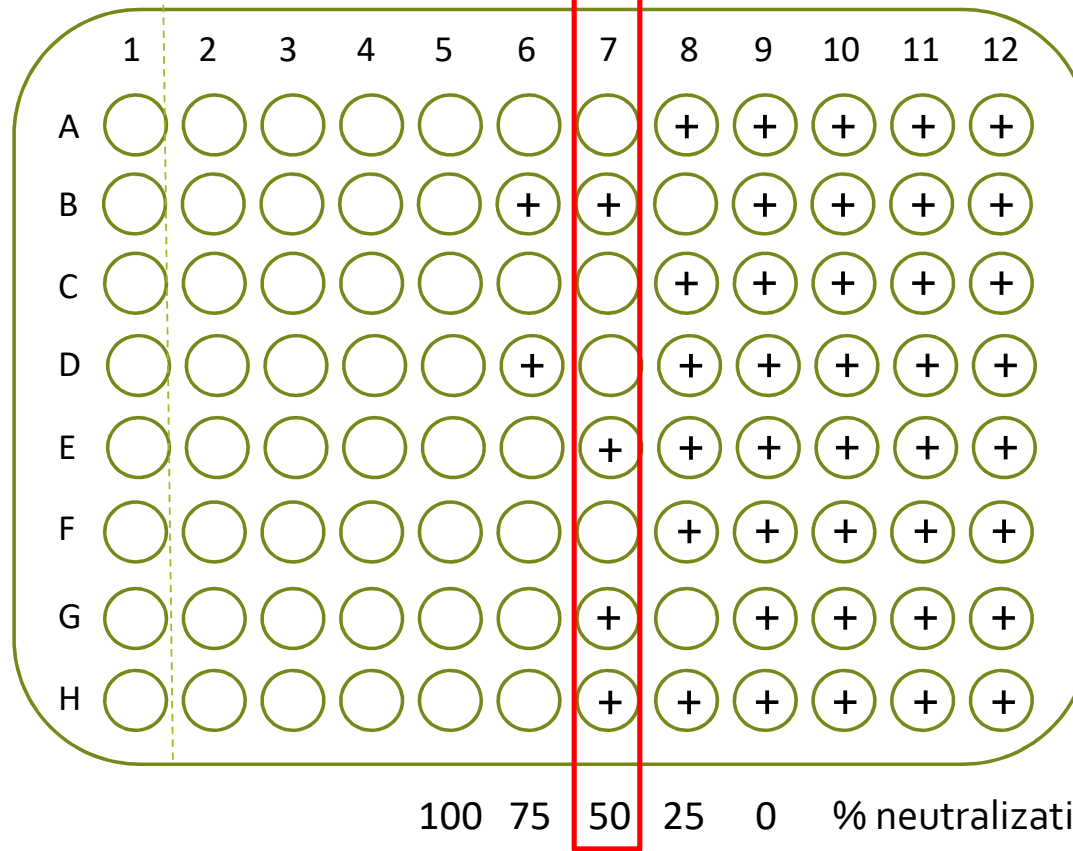
II challenge virus cca 20
CCID₅₀/well

incubation 1.5 hr at 37 °C

III cell suspension (100 μL)

incubation 4 days at 37 °C

IV CPE inspection and counting



$$ED_{50} = 0.78 \mu\text{L}$$

$$NT = \frac{1000 \mu\text{L} / \text{mL}}{0.78 \mu\text{L}}$$

$$NT = 1280 ED_{50} / \text{mL}$$

$$NT = 3,1 \log ED_{50} / \text{mL}$$

+ CPE

Quantal assay



Classical quantitative assay

Results on discontinuous scale

Resolution

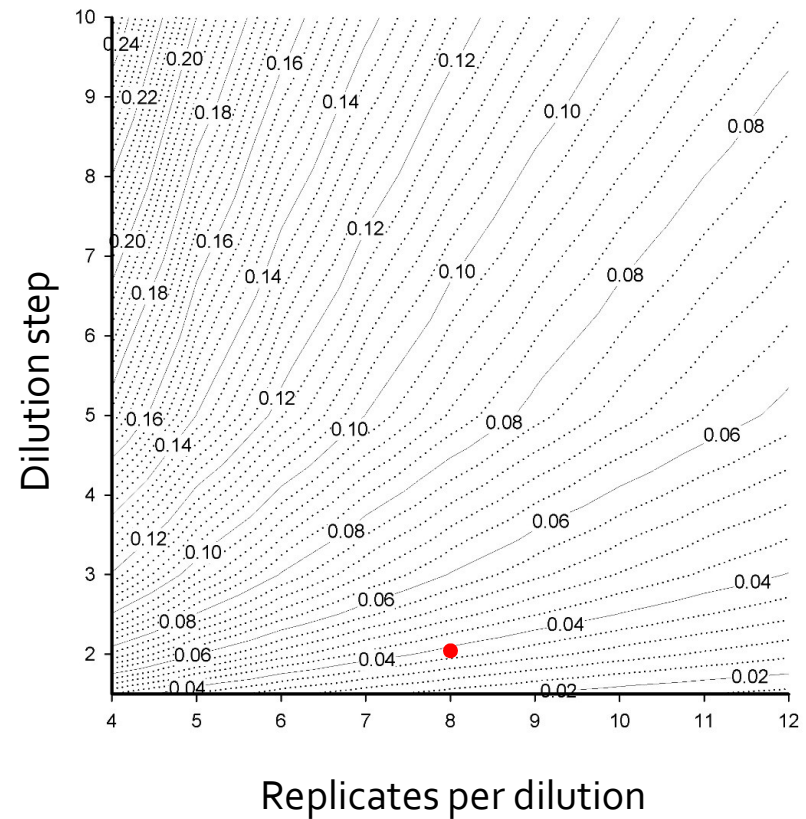
dilution step

number of repetitions in each dilution

Resolution:

ED₅₀ 0.040

PRNT 0.301



To gain reproducible and reliable assay, we established

a unique Laboratory Working bank of **Vero E6 cells** (stored in liquid nitrogen)

a unique Laboratory working **SARS-CoV-2** stock (stored at $\leq -60^{\circ}\text{C}$)

a unique bank of **anti-SARS-CoV-2 antibodies** (in-house standard) (stored at $\leq -16^{\circ}\text{C}$)

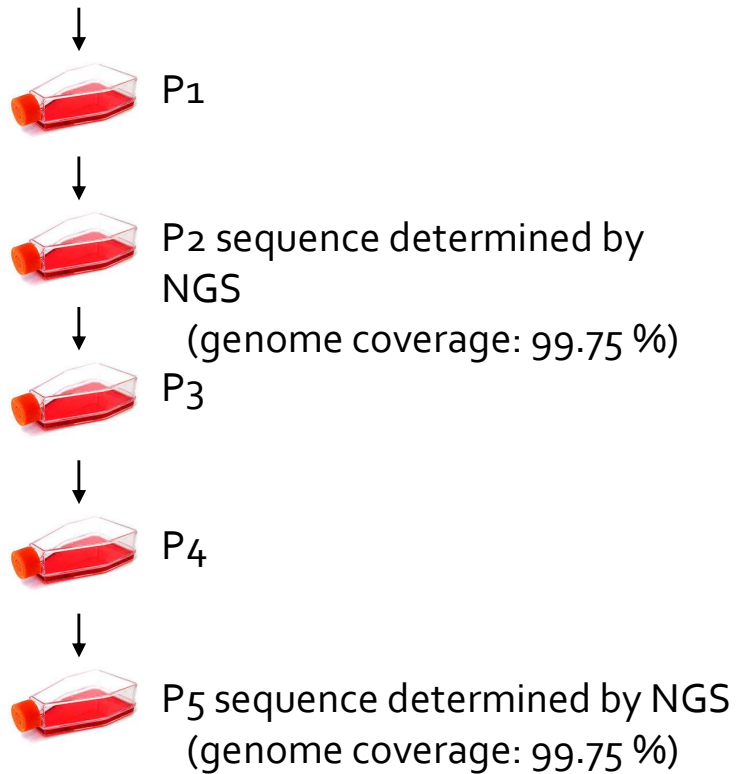
and controlled the assay performance:

Titre of Laboratory working SARS-CoV-2 stock was checked in each assay run

NT of in-house anti-SARS-CoV-2 Ab standard was checked in each assay run

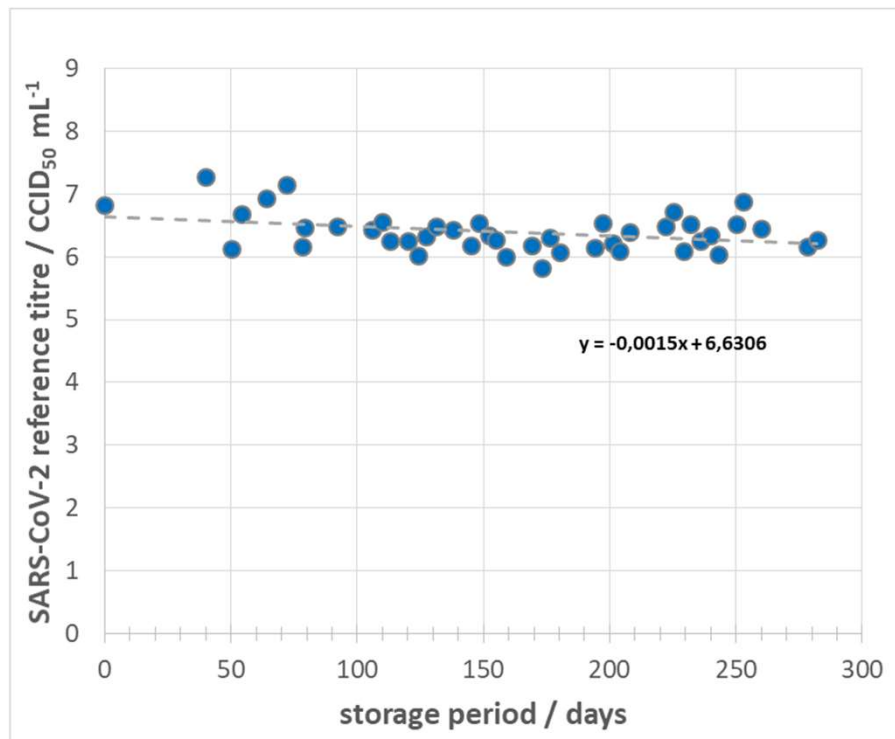
Laboratory working stock preparation and characterization

patient sample 297/20



Virus lineage: B.1.1.1

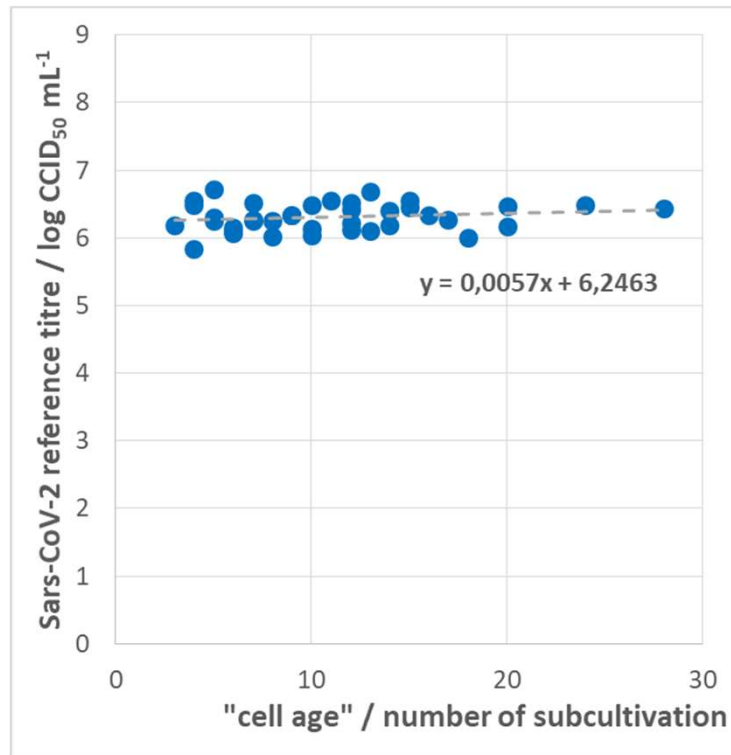
Genomic position (gene)	Nucleotide change (P2→P5)	Protein	Amino acid change
4 402 (ORF1ab)	C→Y	Nsp3	none
23 607 (S)	G→R	Spike	Arg682Arg/Gln



Control of Laboratory working SARS-CoV-2 stock drop in titre during storage

from 6.83 log CCID₅₀ mL⁻¹ to 6.21 log CCID₅₀ mL⁻¹,
cca 0,61 log CCID₅₀ mL⁻¹ for 9.3 months of storage
0,09 log CCID₅₀ mL⁻¹ per month

virus titre after indicated period of storage at -60 °C or bellow

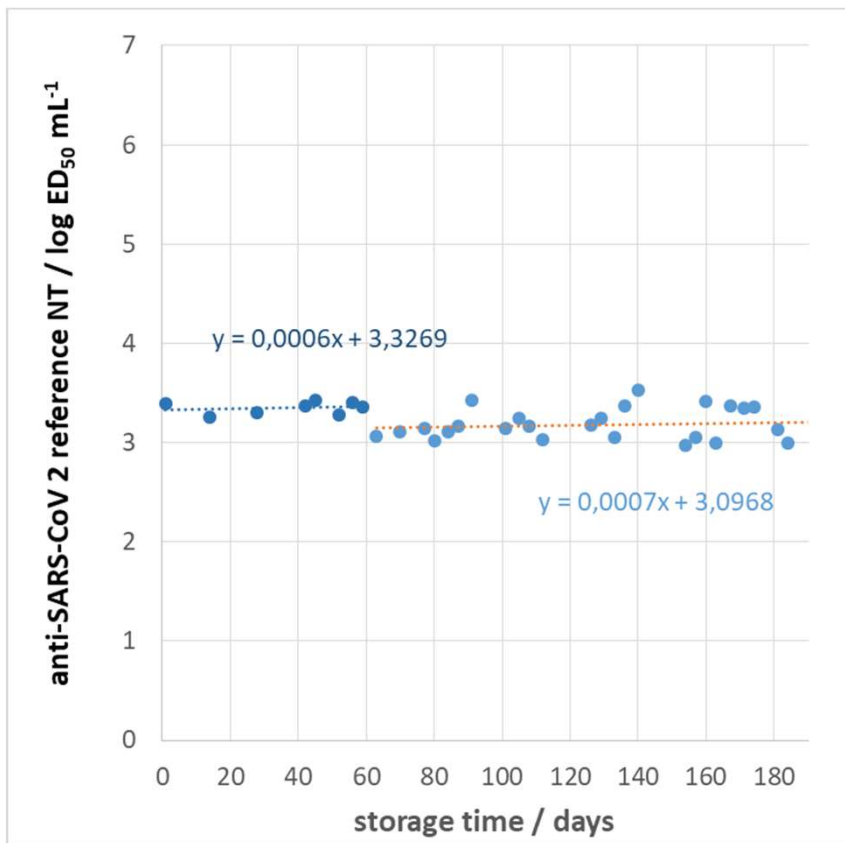


Laboratory Working SARS-CoV-2 titre determined in each assay run using Vero E6 cells from different subcultivations.

Control of Vero E6 cells

"Cell age"

(3 to 28 subcultivation after thawing)
does not influence the titration results.



Stability of anti-SARS-CoV-2 internal reference during the period of 6 months of its usage. The break in line indicates the start of double concentrated SARS-CoV-2 challenge virus usage, due to initial drop of its infectivity.

Anti-SARS CoV-2 in-house standard

nominal titre $3,50 \pm 0,04 \log \text{ED}_{50} \text{ mL}^{-1}$ ($n=14$).

stable throughout the 6-month period

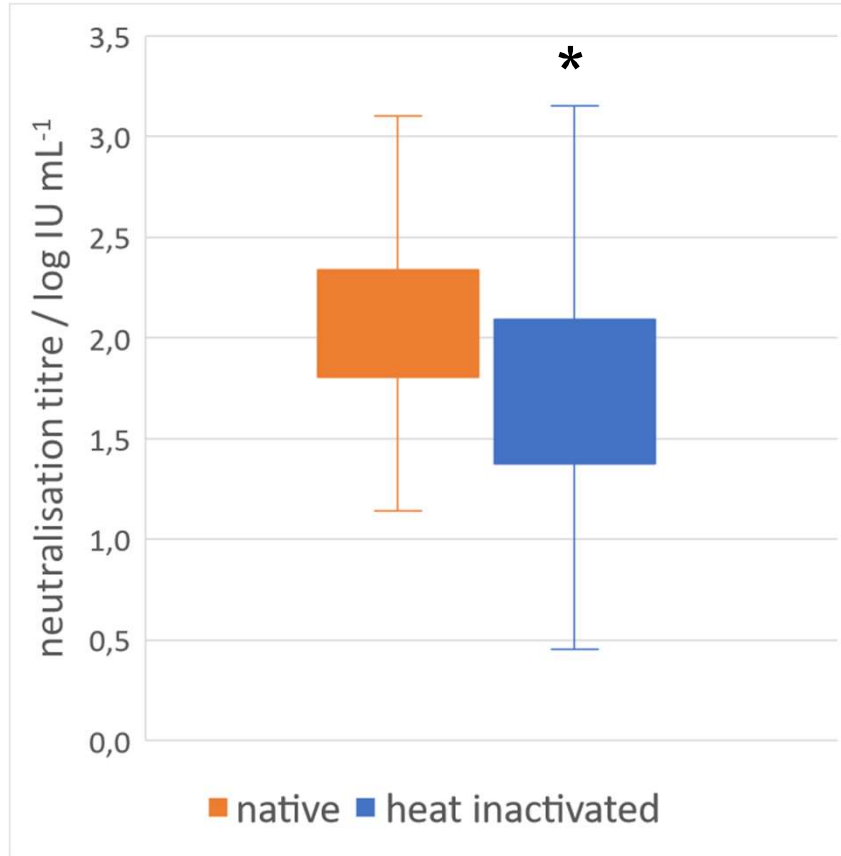
1st WHO International Standard for anti-SARS-CoV-2 (NIBSC, UK) became available in Spring 2021. with nominal neutralizing titre value 1000 IU mL^{-1} ($4,32 \pm 0,11 \log \text{ED}_{50} \text{ mL}^{-1}$ ($n=11$) in our assay)

Calibration of our in-house standard to it and gained the value of **152 IU mL^{-1} ($2,18 \log \text{IU mL}^{-1}$)**

This enabled recalculation of all previously collected results and their expression in IU mL^{-1} , the units of the 1st WHO International Standard.

The role of complement in SARS-CoV-2 neutralization

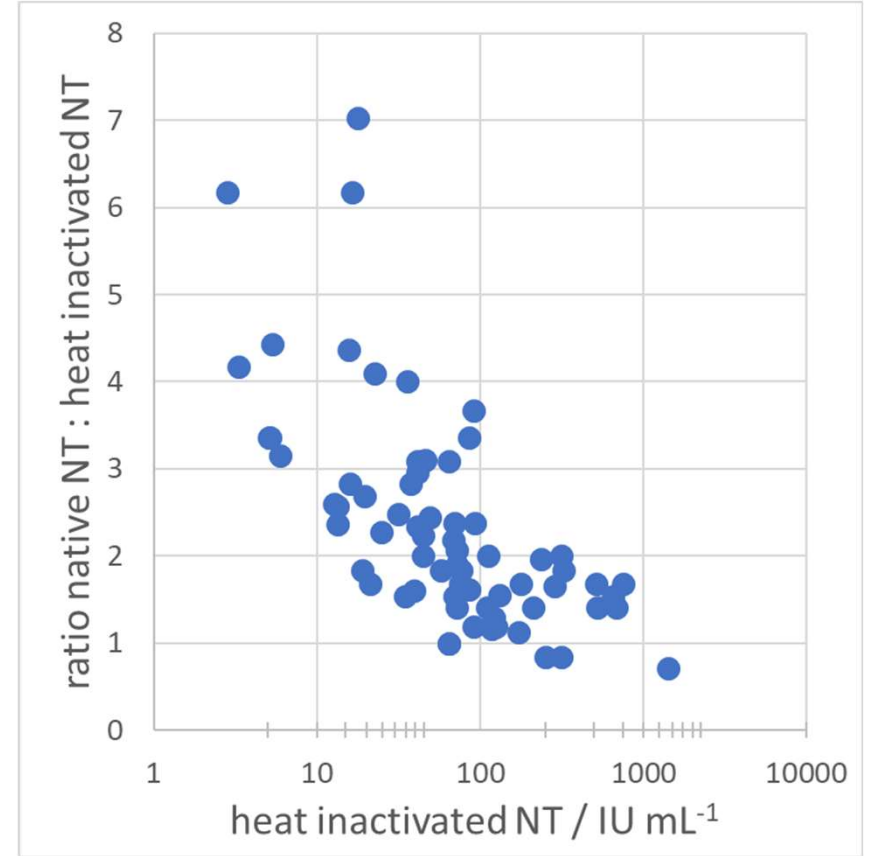
A

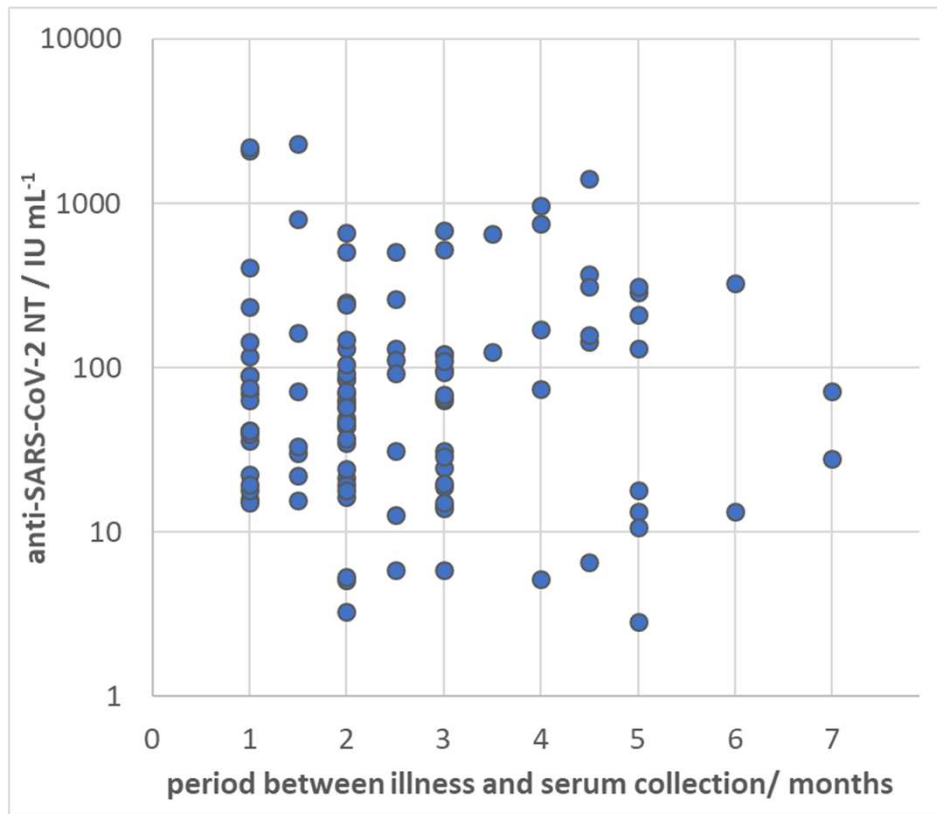


* $p < 0.0001$, $n = 69$.

Native : HI NT 2.37 ± 0.30

B



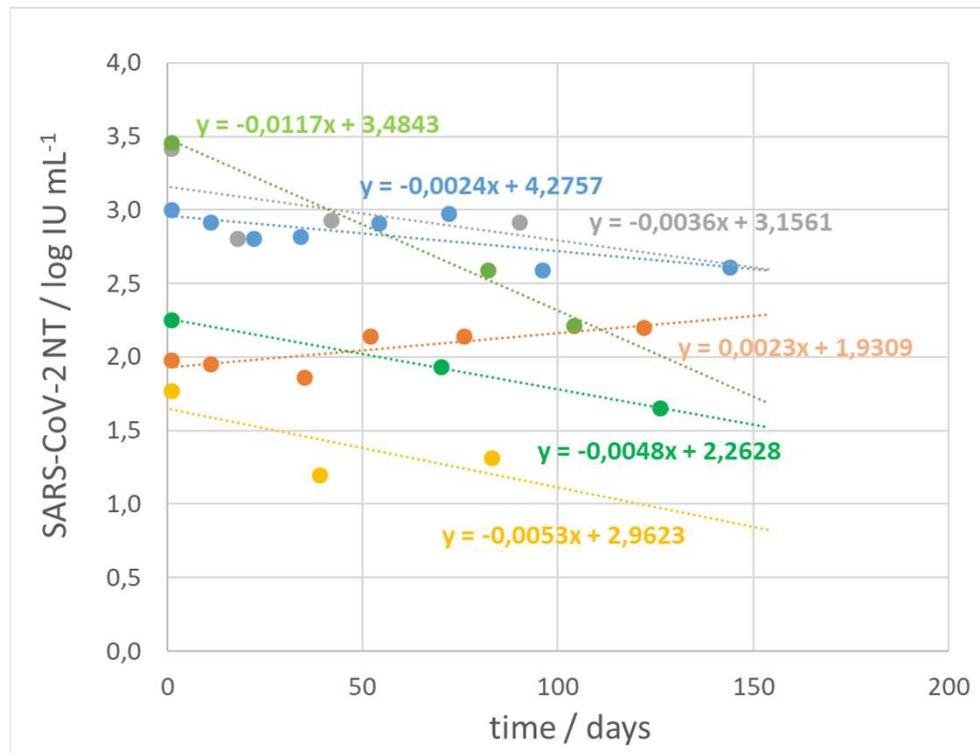


$n=124$

NT range = 3 - 2287 IU mL⁻¹ (0.45 to 3.36 log IU mL⁻¹)

median NT = 63 IU mL⁻¹ (1.80 log IU mL⁻¹).

**SARS-CoV-2 neutralizing titre in convalescent donors' sera
in relation to the time period between the illness and serum collection**

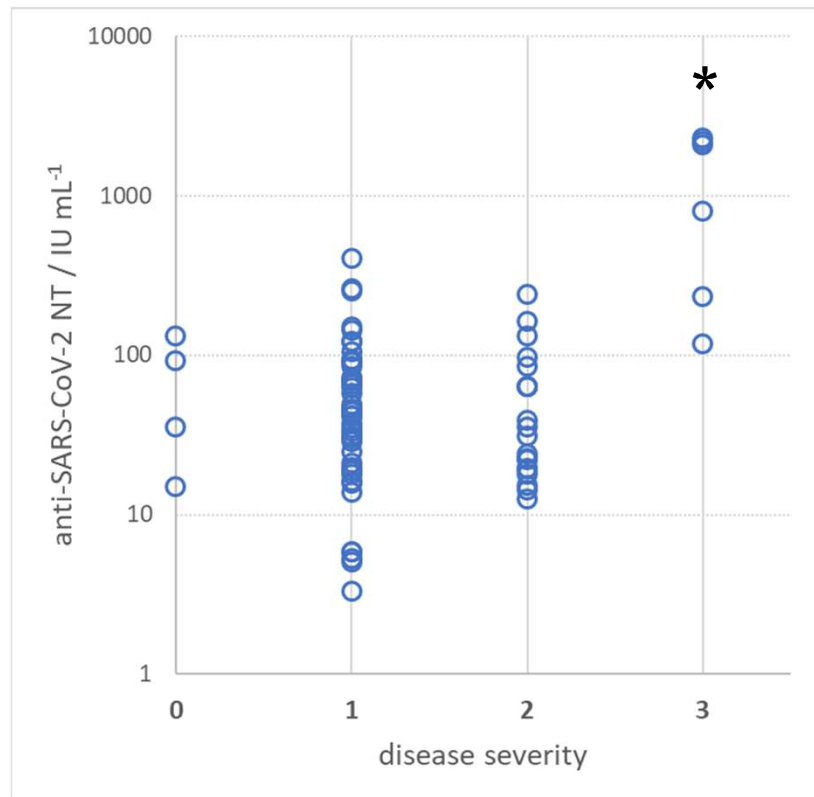


slopes
in range -0.0117 to 0.0023,
average value -0,0042 log IU mL⁻¹ / day

time from the first donation / months	NT / IU mL ⁻¹	NT / log IU mL ⁻¹
	2873	3,46
1	2141	3,33
2	1595	3,20
3	1189	3,08
4	886	2,95

SARS-CoV-2 neutralizing Abs persistence in six individual convalescents

SARS-CoV-2 NT in convalescent donors' sera in relation to disease severity



* $p < 0.002$ in comparison to other groups.

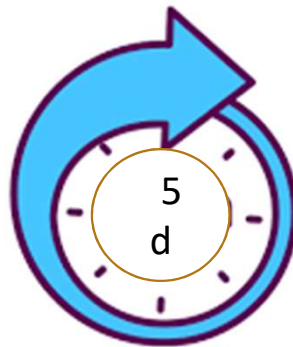
Categories of COVID-19 disease severity

Category	Designation	Symptoms
ASYMPTOMATIC	0	positive PCR test, no symptoms
MILD	1	short-term fever up to 38.5 °C, anosmia, ageusia, runny nose, cough
MODERATE	2	short-term fever over 38.5 °C, accompanied with several or all of the following: headache, mialgia, general weakness, vertigo and anosmia, ageusia, runny nose, cough
SEVERE	3	prolonged, persistent fever over 38.5 °C, accompanied with the most of symptoms 2, involving also pneumonia in some cases; patients that seeked medical help, but without the need for hospitalization

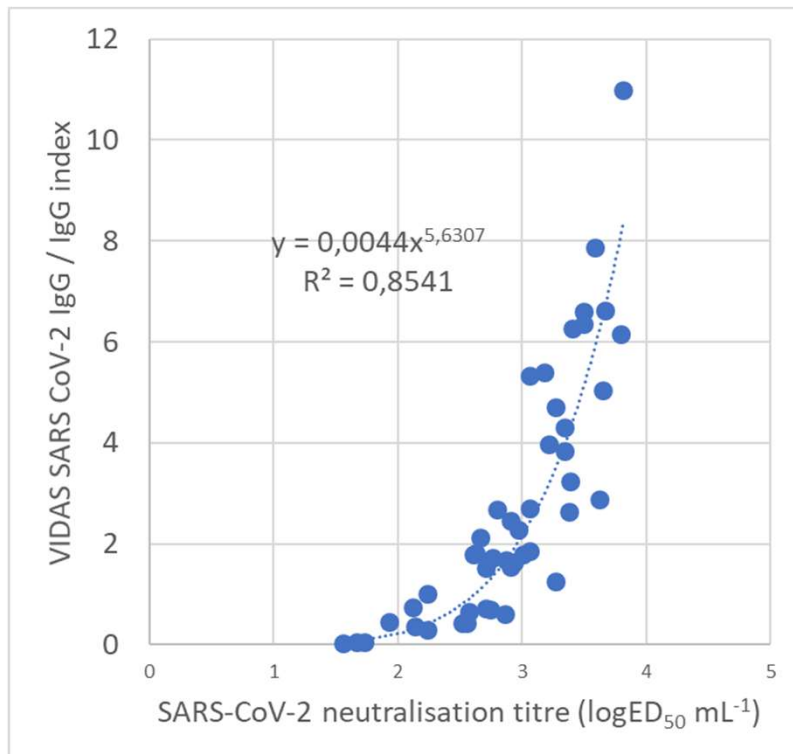
The neutralization test is a very complex and time-consuming procedure !!!



Waiting for results



To avoid and shorten the procedure...

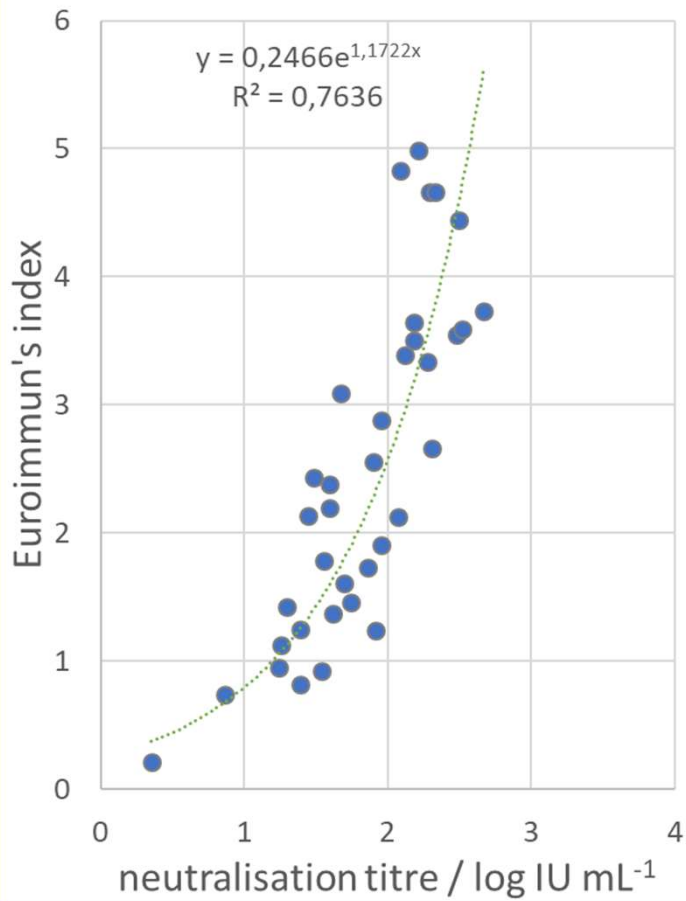


VIDAS SARS CoV₂ IgG (Biomerieux, France)
specific for receptor binding domain of “spike”
automatized Enzyme linked fluorescent assay

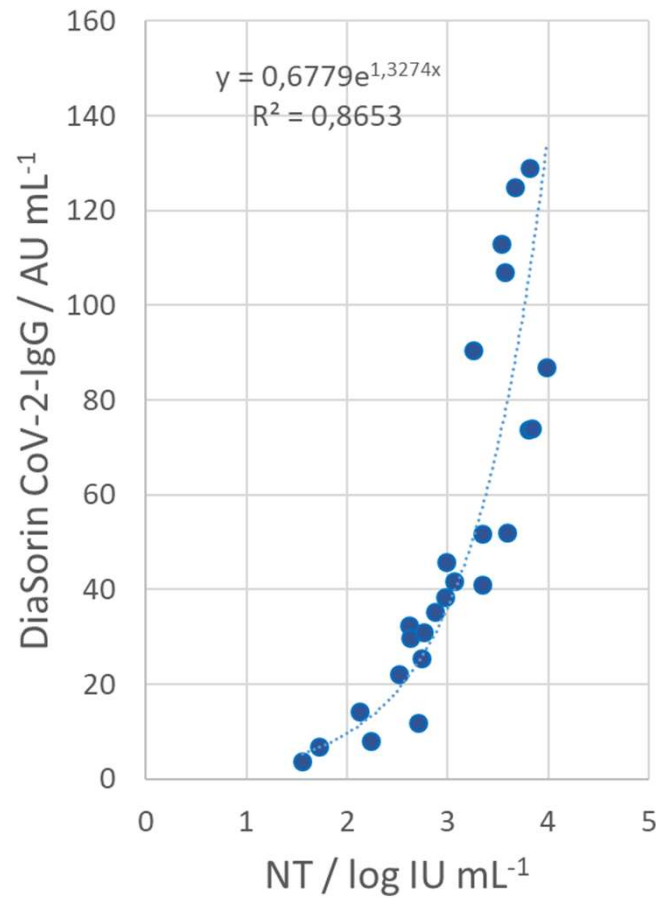
Significant correlation

Pearsons's $r = 0,8738$ ($0,7649 - 0,9342$)

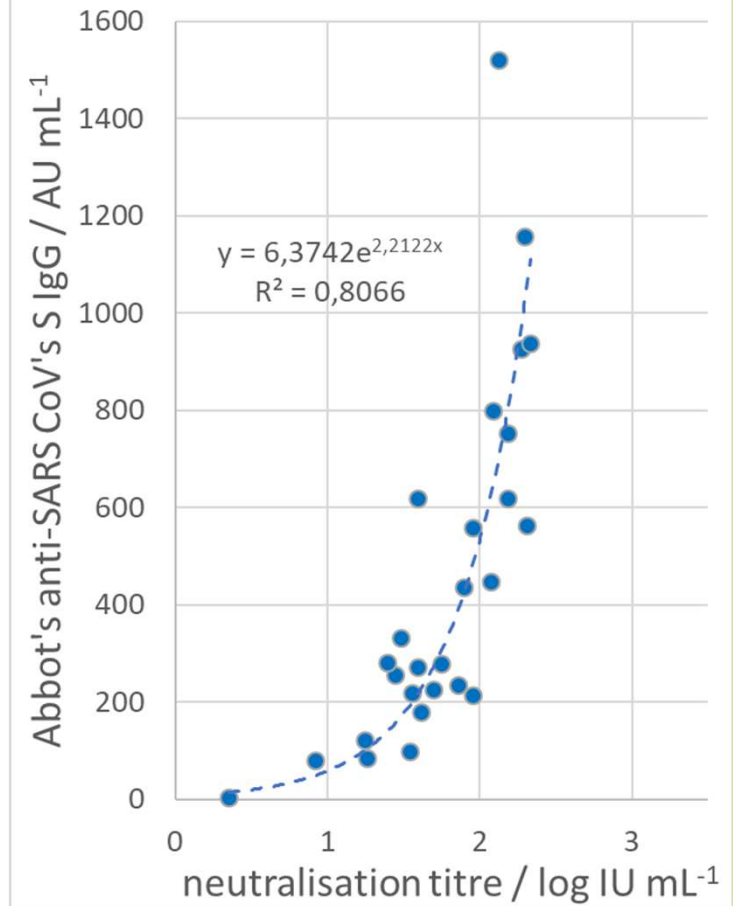
$p < 0,0001$)



Recombinant S1 domain
UHID



Recombinant S1 + S2 domain, CLIA
Osijek, Rijeka

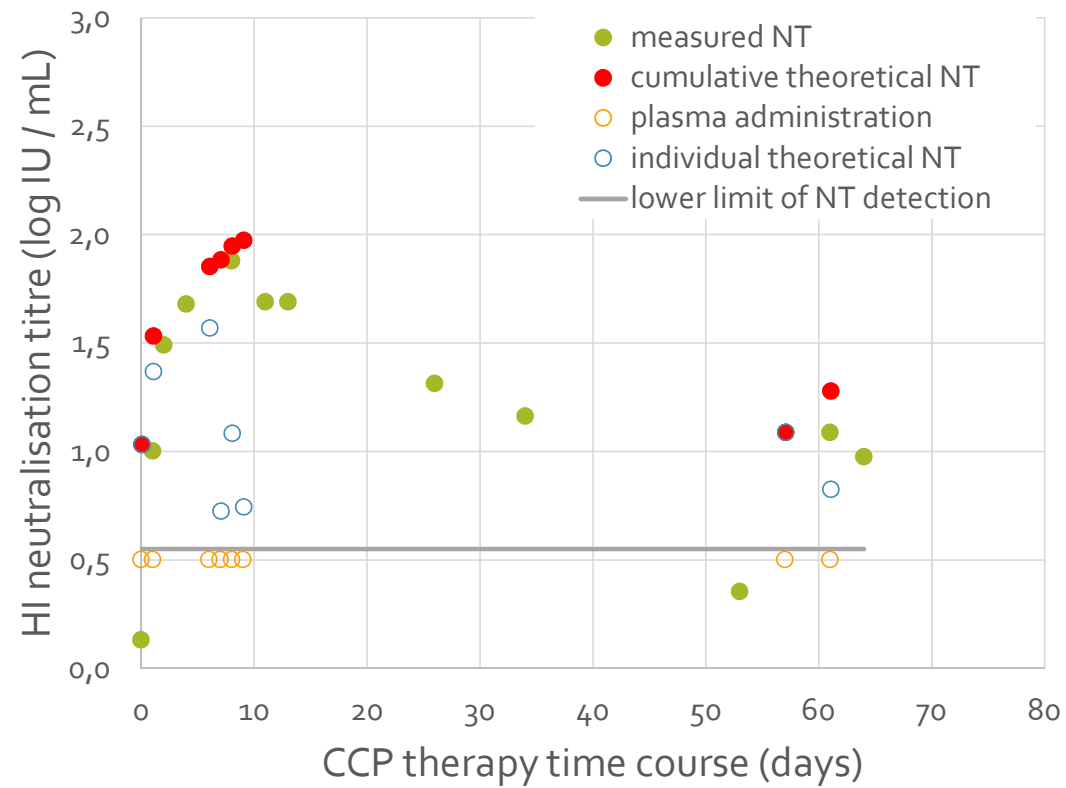


RBD of S1, CLIA
UHID

Decision on cut-off value for the release of plasma unit for transfusion

the lowest neutralization titre measured in sera of COVID-19 convalescents was around 3 IU mL^{-1}
the standard volume of one plasma unit is 200 mL, antibodies will be 25 - 30 times diluted
recruitment of convalescents for plasma donations was slow
plasma's have to be given only to ABO compatible patients

35 IU mL^{-1} and greater as suitable for transfusion,
with recommendation to provide ABO compatible plasma with the highest titre available at the moment and, if plasma units are of lower titres, to provide several units.



Rnjak, Ravlić et al. Transfus Clin Biol 2021; 28:264

Poster No. 12

Current position on the use of CCP for therapy

Prati et al. Blood Transfus 2021; 19:277

2020-2021 Extensive investigation of CCP usage

7 randomised clinical trials have been completed or published interim results

All demonstrate no effectiveness of CCP over placebo in the treatment of immunocompetent hospitalized patients.

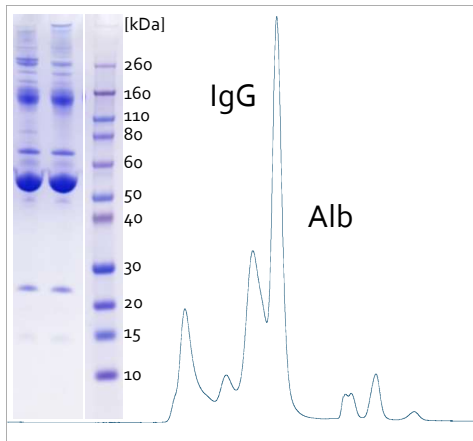
we can not compare the quantity of neutralising antibodies used in each of them.

However, there is a huge amount of case studies or case series reporting the **beneficial effect of CCP usage in the treatment of immunocompromised patients**, and the results of clinical trials are being awaited.

CCP therapy in Croatia

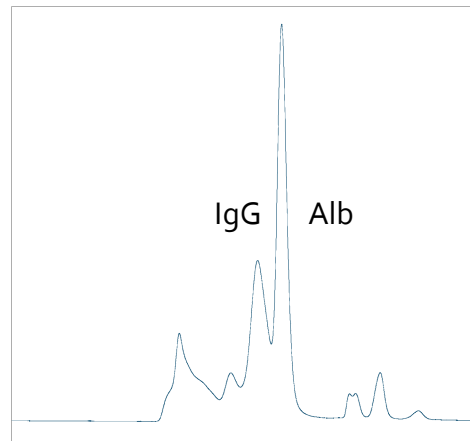
collection of CCP at CITM started in July 2020,
the development of SARS-CoV-2 neutralization assay started in September 2020 and
the first unit for clinical use was issued in December 2020
99% of plasma units was used for COVID-19 patients with hematological malignancies;
until September cca 700 plasma units were issued, and cca 130 patient were treated.

CCP



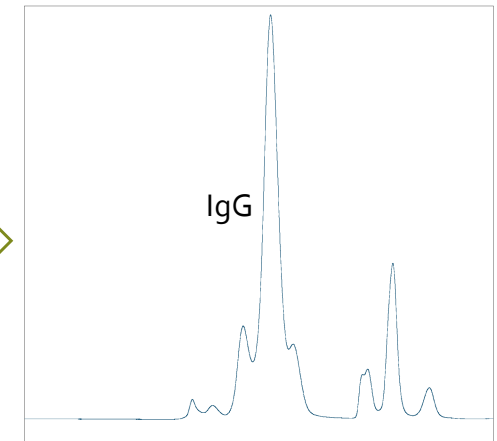
Coagulation
(56 °C / 1 h),
precipitate
removal (3200
× g / 45')

Fibrinogen-depleted CCP



Caprylic acid (5%)
precipitation
(23°C / 1 h),
precipitate
removal (3200 × g
/ 45')

Crude IgG fraction

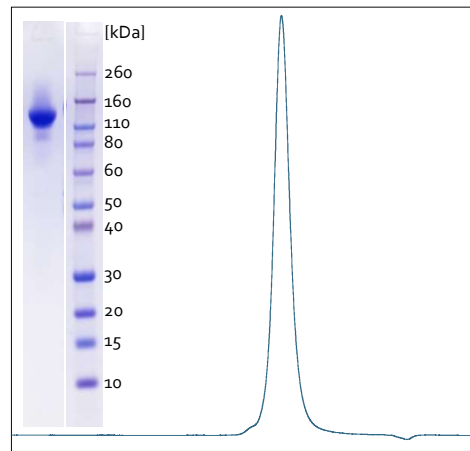


Diafiltration
(MWCO = 100 kDa)

IgG-BASED FINAL PRODUCT'S PROPERTIES

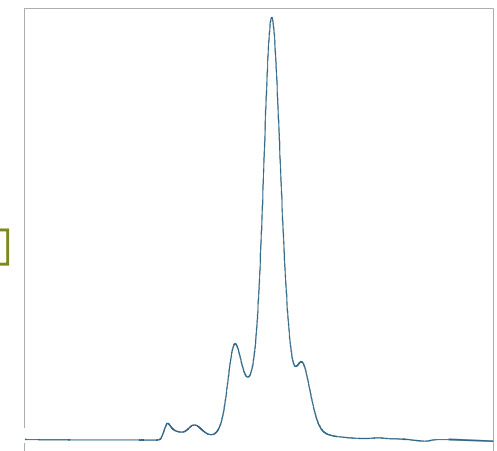
Total IgG recovery [%]	75.3 ± 2.0 (n = 8)
Purity [%]	100 (n = 8)
Aggregate content [%]	0 (n = 8)
Residual IgM [%]	0.3 ± 0.2 (n = 8)
Residual IgA [%]	3.9 ± 2.4 (n = 8)
NT activity recovery	
Protein S-specific IgG recovery [%]	102.3 ± 0.5 (n = 8)

Ultrapure IgG fraction (final product)



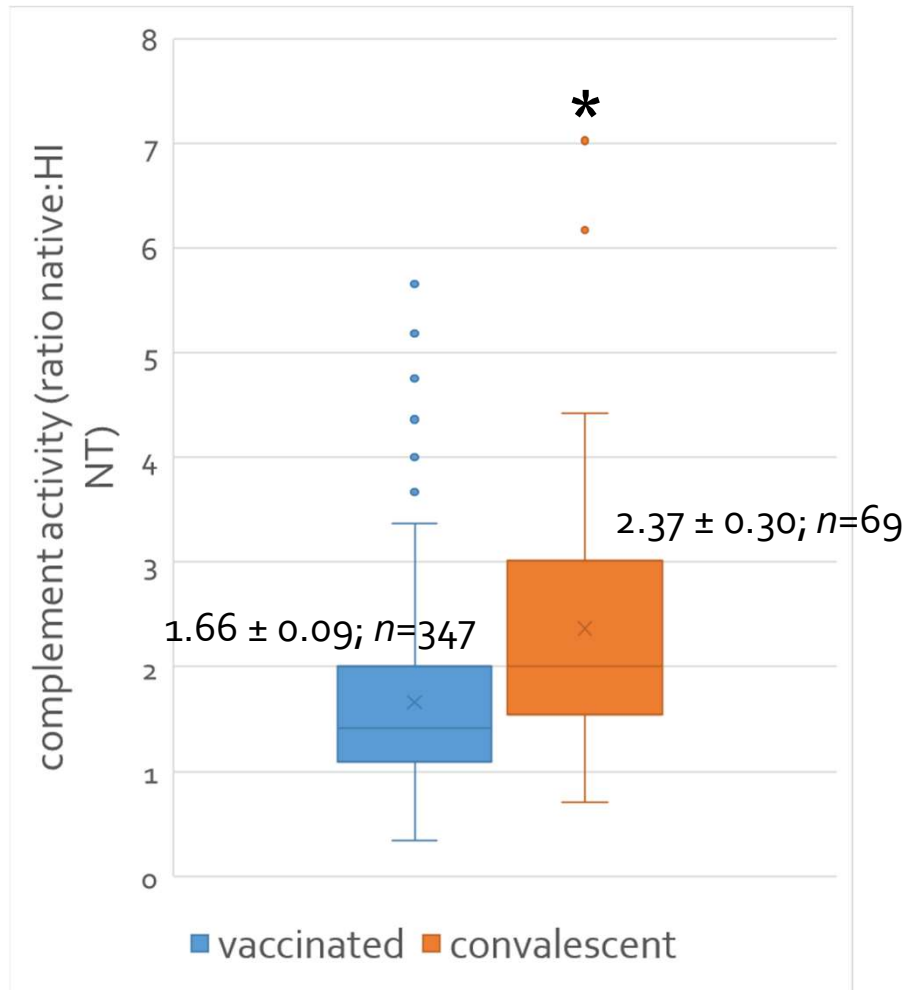
Anion-exchange
HPLC in flow-
through mode
at pH = 5.0

Pure IgG fraction



n denotes the number of independently performed processing procedures on CCP from three donors

Neutralizing antibody response after vaccination



Activation of complement by antibodies (classical pathway) is significantly stronger in convalescent than in vaccinated persons

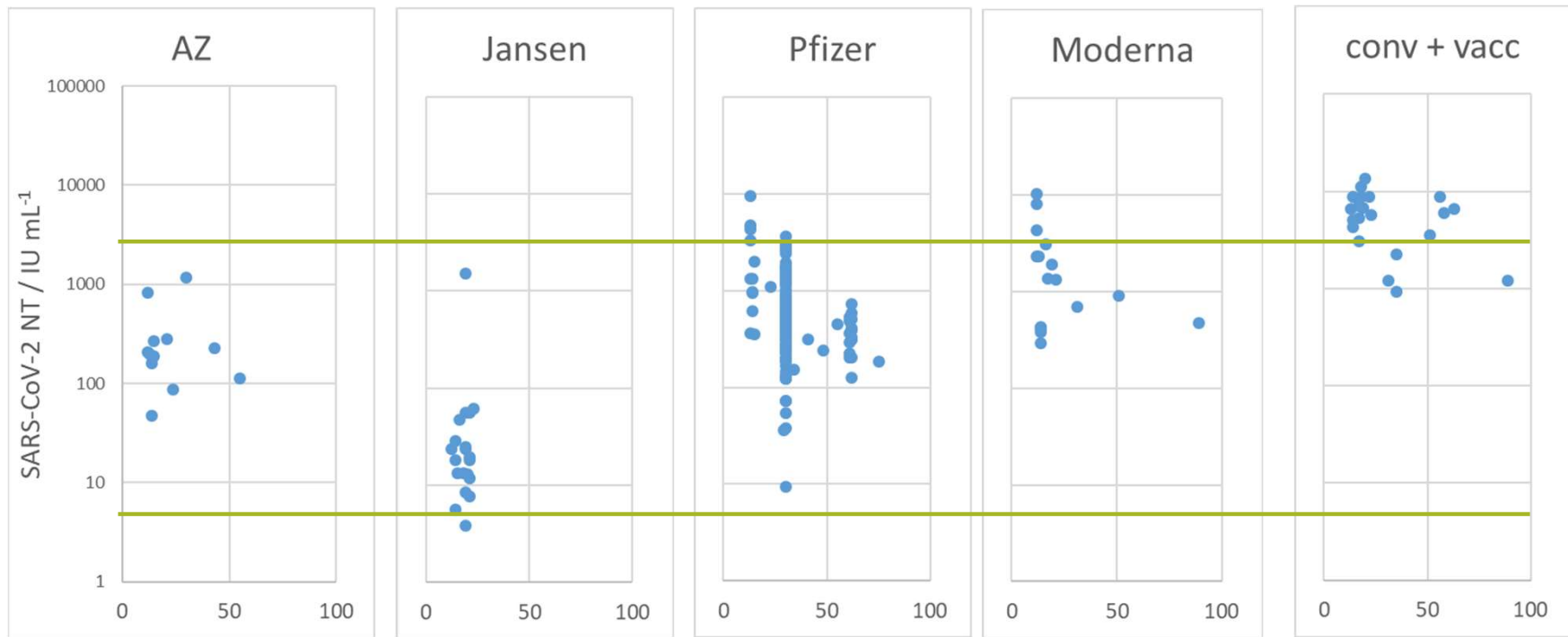
* $p < 0,001$

76%

72%

90%

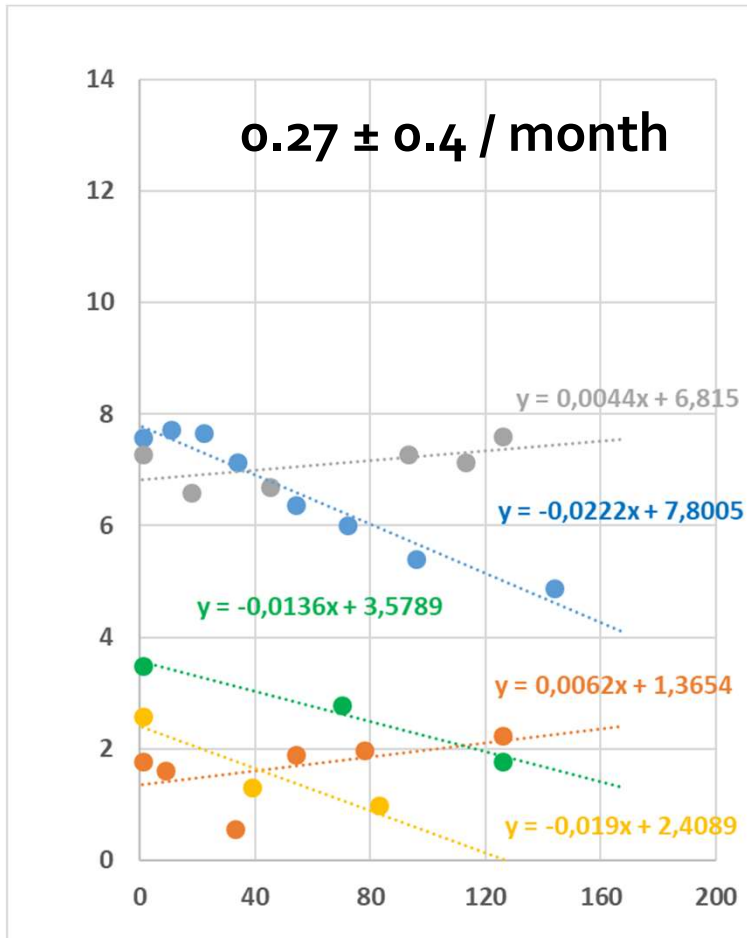
91%



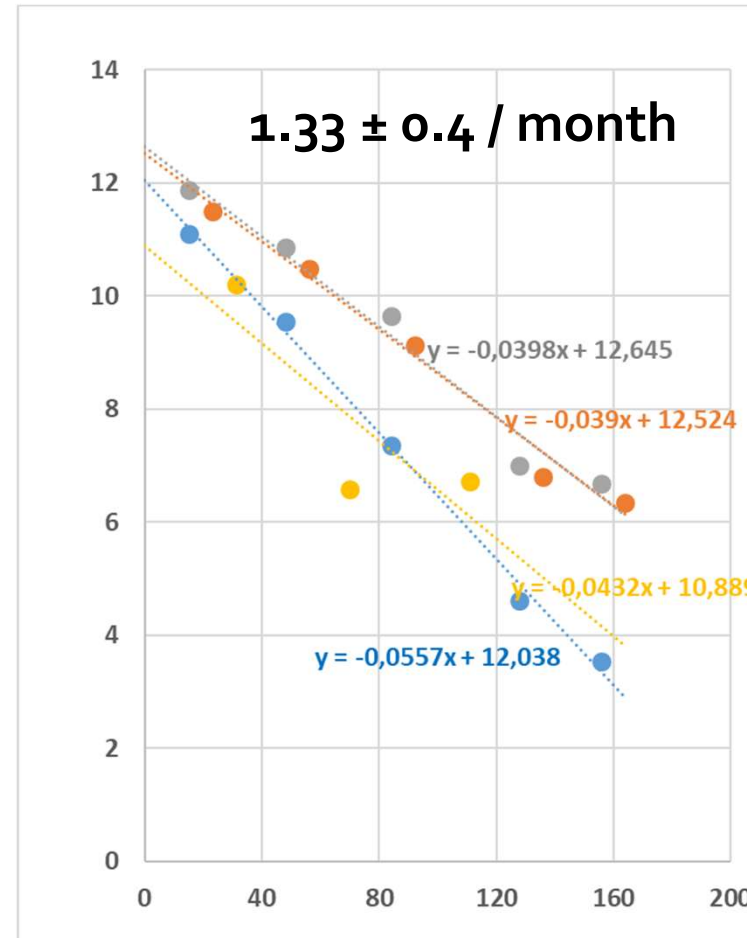
Time after vaccination completion / days

anti-S IgG / Eurimmun's index

convalescents



vaccinees



time after first sampling / days

Conclusions I

reliable and reproducible SARS-CoV-2 neutralization assay calibrated to current international antibody standard is established in Croatia

enabled fast introduction of CCP therapy during COVID-19 pandemics in Croatia

NT in convalescents correlate with disease severity, and are persistent and slowly declining

SARS-CoV-2 – specific antibodies activate complement activity, most probably through classical pathway of activation

complement activating properties are stronger in convalescent than in vaccinated people

Conclusions II

assay of neutralization might serve as an indicator of the strength of induced immunity

the response to Moderna's, Pfizer's, AZ's and Jansen's vaccines correlated to their described efficacies.

this assay could be a valuable complementary tool to evaluate someone's response to disease or to vaccine, and to make decision whether and when additional vaccination is required

ACKNOWLEDGEMENTS



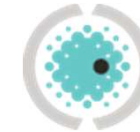
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