Presence and strength of binding of IgM, IgG and IgA antibodies against SARS-CoV-2 during CoViD-19 infection

Richard Schasfoort (r.b.m.schasfoort@utwente.nl)
University of Twente
Jos van Weperen
Vysens BV
Margot van Amsterdam
InterFluidics BV
Judicaël Parisot
Carterra
Jan Hendriks
University of Twente
Michelle Koerselman
University of Twente
Marcel Karperien
University of Twente
Anouk Mentink
University of Twente
Martin Bennink
Saxion University of Applied Sciences
Hans Krabbe
Medlon
Leon Terstappen
University of Twente
Leontine Mulder
Medlon, MST, ZGT

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Abstract

Surface Plasmon Resonance imaging (SPRi) was used to determine the presence and strength of binding of IgG, IgM and IgA against the Receptor Binding Domain (RBD) of SARS-CoV-2 in sera of 119 CoViD-19 patients. The high-throughput assay enables to follow the specific immune response of ultimate 384 individuals for these four parameters in one run. The measured IgG, IgM and IgA levels correlated with ELISA (Euroimmun: Anti-SARS-CoV-2, IgG assay, r-0.95, ECLIA: Anti-SARS-CoV-2 Ig electrochemiluminescence r=0.73). During the course of the disease, the IgG levels and strength of binding increased while generally the IgM and IgA levels went down. Recovered patients all show high strength of binding of the IgG type to the RBD protein. The anti-RBD immune globulins SPRi assay provides additional insights in the immune status of patients recovering from CoViD-19 and can be applied for the assessment of the immune reaction of healthy individuals in vaccination programmes.

Introduction

Corona viruses pose serious health threats to humans and animals. SARS-CoV-2 is the third coronavirus that crossed the species barrier and causes serious respiratory infections in humans. In contrast to SARS-CoV that appeared in 2003 and the Middle East respiratory syndrome (MERS) in 2012, SARS-CoV-2 shows an unprecedented pandemic spread. Diagnostic tests of individuals who are suspected of SARS-CoV-2 infection, mainly rely on real-time reverse transcription polymerase chain reaction (rRT-PCR) of viral genetic material collected in nasopharyngeal swabs [1,2]. In contrast to the PCR test, serologic assays demonstrate the presence of an immune reaction against the virus through detection of immunoglobulins directed against SARS-CoV-2 structural proteins [3]. The immune system of a CoViD-19 patient produces antibodies to SARS-CoV-2 within days to a few weeks following viral infection [4]. Antibodies are expected to remain at a high level for months, perhaps years, following infection, as previously shown following the 2003 outbreak of SARS-CoV-1 [5,6]. However, this is still uncertain for SARS-CoV-2 patients. The immune reaction to corona viruses generally provides immunity via neutralizing antibodies [7] in the event of a second exposure to the virus and this also provides the basis for vaccine development. Serological antibody testing [8] is essential to get an indication whether or not an individual has been infected with SARS-CoV-2. The quality of the immune response is not only determined by the quantity of antibodies but also by the overall strength of binding of the pool of potential neutralizing antibodies that binds to the relevant immunogenic proteins of the Corona virus. Recently it was shown that the most potent, highest affinity neutralizing antibodies were directed to the receptor-binding domain (RBD) [9]. This RBD of SARS-CoV-2 domain binds to the angiotensin converting enzyme2 (ACE2) receptor expressed by target cells. The affinity of the RBD of SARS-CoV and SARS-CoV-2 to the ACE2 receptor appeared to be approximately 31 nM and $K_D \sim 5$ nM respectively as determined by surface plasmon resonance [10]. Hence, the quality of the antibodies to SARS-CoV-2 should be more than a factor 6 better than antibodies to SARS-CoV in order to prevent the virus binding to its receptor. In a US study [11] the calculated affinity difference between SARS-CoV and SARS-CoV-2 was even 10-20 times. The two most potent neutralizing monoclonal antibodies against RBD in a Dutch study had an equilibrium dissociation constant K_D of 47

and 60 pM [9]. Generally, an antibody in that range will effectively block the virus monovalently. Polyclonal supporting antibodies preferably of high affinity (<nM) should be raised additionally to block the RBD of SARS-CoV-2 from binding to the ACE2 receptor and allow the removal of the virus via e.g. nucleocapsid domains. Early stages of vaccine development and clinical trials will require assessing antibody titers in combination with detecting the apparent polyclonal affinity of IgG against RBD for SARS-CoV-2.

Antibody detection is typically performed using ELISA or related automated immuno-assays. While ELISA has high-throughput capability when automated, it requires several hour-long steps that lengthen assay time. Testing of IgG, IgM and IgA requires individual assays in series for both ELISA and other immunoassays. Lateral flow assays have often been proposed for antibody detection, but suffer from reliability issues and are not quantitative [12]. An attractive alternative for an antibody detection method is surface plasmon resonance imaging (SPRi). SPRi is a label-free sensing technique that is highly sensitive enabling the quantitative and qualitative interaction between biomolecules, such as the interaction between antibodies and their respective antigen [13]. Additionally, the strength of binding measured by the off-rate can be determined in a single assay to obtain an indication of the quality of the total polyclonal antibody response.

Here, we describe a high throughput Surface Plasmon Resonance imaging (SPRi) assay for the quantitative measurement of IgG, IgM and IgA antibodies binding to the RBD spike protein and their apparent polyclonal affinity in sera of CoViD-19 patients.

Materials And Methods

Patient and control serum samples

Residual serum samples (n=70) were obtained from 53 unique CoViD-19 patients confirmed by RT-qPCR and CT-scans. 20 out of 70 serum samples from cases were collected within 10 days after first symptoms (range 4-9 days), 50 were collected 10 or more days after first symptoms (range 10-28 days).From 10 patients more than 1 serum sample obtained at different time points was included.

Control non-SARS-CoV-2 samples (n = 49) were obtained from anonymous stored residual serum samples from healthy pregnant women collected in March 2019 (n=37) and from 12 hospitalized patients with repetitive negative RT-PCR and a non-COVID explanation for their clinical symptoms.

Disease severity of the SARS-CoV2 infection was classified according to the WHO criteria [14] as either mild, moderate, severe or critical. Mild patients did not show abnormal CT imaging. Moderate patients had fever and/or classical respiratory symptoms, and typical CT images of viral pneumonia. Severe patients met at least one of the following additional conditions: (1) Shortness of breath with respiratory rate (RR) ≥30 times/min, (2) Oxygen saturation (SpO2, Resting state) ≤93%; or (3) PaO2/FiO2 ≤ 39.9 kPa. Critically ill patients met at least one of the extra following conditions: (1) Respiratory failure that

required mechanical ventilation; (2) Shock; or (3) Multiple organ failure that required intensive care unit (ICU). Patient characteristics are provided in supplementary table 1.

ELISA

Euroimmun Anti-SARS-CoV-2 ELISA IgG assay (Euroimmun, Luebeck, Germany) was performed on serum samples on a Thunderbolt ELISA robot (Gold standard diagnostics, CA, US) according to the manufacturer's instructions. This ELISA provides a semi-quantitative in vitro determination of human IgG against SARS-CoV-2. The microplate wells are precoated with recombinant S1 structural protein. The results are evaluated by calculation of a ratio of the extinction of samples over the extinction of the calibrator. The sensitivity and specificity of samples > 10 days of disease duration are 80% and 99% respectively, according to the manufacturer (Manual, March 2020).

The anti-SARS-CoV-2 Ig electrochemiluminescence (ECLIA) test (Roche Diagnostics, Rotkreutz, Switzerland), performed on the Cobas e 801 platform, detects antibodies to the recombinant nucleocapsid protein of SARS-CoV-2. The results are evaluated semi-quantitatively by calculation of the chemiluminescence of samples over the extinction of the calibrator. The sensitivity and specificity of samples > 14 days of disease duration according to the manufacturer are 99.5 % and 99.8 % respectively (Manual, May 2020).

Sensor preparation

For SPRi measurements the multiplex SPR imaging instrument (IBIS MX96, IBIS Technologies, Enschede, the Netherlands) and the Carterra LSA platform (Salt Lake City UT, US) were used with an installed sensor prism (HC30M, Xantec Bioanalytics Düsseldorf, Germany). Similar results for both instruments were obtained with this sensor surface. The sensor was prepared by first stabilization and removing the protective layer in water, followed by treatment with a 1:1 aqueous solution of 100 mM N-hydroxysuccinimide (NHS) and 400 mM N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) for 10 minutes. After rinsing with water for 20 seconds, the sensor was exposed for 20 minutes to the Spike RBD his-tag (SINO biological Frankfurt, Germany) in immobilization buffer (50 mM sodium acetate pH 4.8). Coupling with EDC-NHS yielded a reproducible sensor surface. After rinsing the sensor 20 seconds with water the surface was passivated with 1M ethanolamine (pH 8.5) for 10 minutes. The sensor was then equilibrated in the running buffer composed of PBS (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4) supplemented with 1% bovine serum albumin (BSA) and 0.5% Casein and 0.1% Tween 20. Details of the choices made to obtain the best sensor surface are described in the supplementary data.

Spotting sera

A Thunderbolt ELISA robot was used to dilute the serum samples in an optimized dilution ratio of 1:100. Each 2 µl serum was diluted with 198 µl of running buffer and pipetted in a 96 wells plate. For measurements on the IBIS MX96 a Continuous Flow Microfluidic (CFM) system (Carterra Salt Lake City

UT, US) was used to capture 96 sera on a sensor functionalized with RBD-Spike coupled. The first 48 samples were spotted in duplicate for 15 minutes. The Carterra LSA enables printing of 384 spots as 4 nested positions of 96 each. While the operation and injection of sample is similar for both instruments, the spotting process and dissociation rate can be followed in real time on the LSA.

Measurements on the IBIS MX96

The SUIT (Set Up Ibis Tool), DAX (Data acquisition software) and SPRINTX (Analysis software) software packages on the SPRI MX96 were used and Scrubber (BioLogic Software, Canberra, Australia) was used for the off-rate determination. After washing of the sensor chip spotted with patient sera, the sensor was first incubated with 50x diluted goat-anti-human-IgM (aIgM, 20-S5170 GND1-D0 Fitzgerald) in running buffer (200 µl for one run) and the second a 100x diluted goat-anti-human-IgG (aIgG-Fc, 20-S1211G001-S4 Fitzgerald) in SPRi running buffer. The third injection was with a 100x diluted goat-anti-human-IgA (aIgA, 20-S1111G000-S4 Fitzgerald). After converting the data by local referencing, zeroing the baseline and aligning the injection points of the three injections, the R_{max} value was determined using a special biphasic fit algorithm (InterFluidics, Haaksbergen, The Netherlands). This software tool programmed using Microsoft 'R' Studio allows calculating the data on both SPR imagers. If the curve did not show an exponential behavior (e.g. negative samples) then a linear fit was applied and the average value of the linear fit was determined.

Off-rate measurements on the LSA

In total 48 selected serum samples were spotted in duplicate in a single run on the HC30M RBD coupled sensor prism surface in 4 dilutions (1:50, 1:100, 1:200 and 1:400) to generate a 384-array. During the spotting process, the binding signals are followed for 15 minutes and each serum sample was measured 8 times at 4 dilutions. The signal recorded in RU mirrors the total anti-RBD antibodies bound. Following the spotting process, a 5 min injection of RBD (15 µg/ml) in dilution buffer resulted in sufficient dissociation of the anti-RBD antibodies. For all 384 spots, the global dissociation- or global off-rate constant can be calculated. The final step consists of sequential injections of solutions of anti-IgM, anti-IgG and anti-IgA antibodies. The ratio of bound immunoglobulins can be calculated by determining the R_{max} values from the anti-isotype antibodies binding signals. The R_{max} value has a direct relation with the concentration of anti-RBD antibodies in serum.

Results And Conclusion

Simultaneous measurement of 96 samples for anti-SARS-CoV-2 IgM, IgG and IgA antibodies by SPRi

Figure 1 shows the principle of the SPRi assay for determining the isotype fractions. In Panel A, 96 sera are spotted on an RBD coupled sensor. In Panel B, the sensor is placed in the SPRimager and real-time measurements were performed during three concatenated injections of anti-IgM, anti-IgG and anti-IgA. Panel C shows the SPR reflection image after the injection of the anti-isotype antibodies of the 96 sera. Panel D shows typical sensorgrams of 3 sera (red, blue, green). The red curve represents a serum with

high IgM (R_{max} 1940 RU), very high IgG (5012 RU) and weak IgA (243 RU). The blue curve shows a serum with moderate IgM (845 RU), a moderate IgG (1215 RU) and a weak IgA (464 RU). The green curve shows a serum with a weak IgM (203 RU) a high IgG (3950 RU) and a high IgA (3796 RU). Panel E shows a patient overlay for calculating the R_{max} values of anti-IgM, anti-IgG and anti-IgA which is proportional to the bound IgM, IgG and IgA anti-RBD. The baselines are zeroed and the injections are aligned. This enables the application of a biphasic binding model for calculation of R_{max} for the three isotypes. Sensorgrams were measured for all samples simultaneously and shown as an overlay plot in Figure 2. Repetitive measurements using the same sera showed that the RU level variation was less than 5%.

In Figure 3 the IgM, IgG, IgA and total IgG SPRi RU values are shown and samples are divided into those that are CoViD-19 PCR negative and the CoViD-19 PCR positive. The latter were subdivided into those with onset of disease symptoms of less than 10 days and those with onset of disease symptoms more than 10 days. Differences between the groups were all significant ($p<0.001$ non-parametric Mann Whitney U test). Additional data is shown in supplementary Figure 3, 4 and 5)

Comparison with ELISA and ECLIA

Using ROC curves, the optimal threshold and AUC were calculated for each parameter (IgG,IgA,IgM, total Ig (all SPR), ELISA IgG and ECLIA Ig). The calculations were performed on 42 positive samples from positive patients and 46 samples from controls. The obtained thresholds and AUCs are shown in Figure 4D. The calculated threshold for the ELISA was in line with the recommendations from the manufacturer, the calculated threshold for the ECLIA was lower than the recommendation from the manufacturer. With the thresholds, the sensitivity and specificity could be calculated.

The IgG SPR results were correlated with the S1 domain ELISA results and the total Ig SPR results were correlated with the ECLIA total Ig results. As can be seen in Figure 4, the correlation between IgG SPR and ELISA SPR was superior (n= 101, Pearson's r 0.95) compared to the total Ig SPR and ECLIA Ig (n= 116, Pearson's r 0.73). This can be explained by the fact that the ECLIA contains the nucleocapsid protein as antigen, whereas the ELISA uses the S1 domain protein, which contains the RBD antigen used in the SPRi. The SPRi results were correlated with clinical and laboratory parameters known to have a relation with disease activity and/or severity as CRP, ferritin, procalcitonin, lymphocyte count, lactate dehydrogenase (LD) and d-dimer. Only a slight correlation was found between the IgG SPR results and the D-dimer level. (n= 22 , Pearson's r =0.77). Although the mean level of IgG SPR was higher in patients with pulmonary embolism compared to patients without, this did not reach significance (p=0.17)

Strength of binding measurement of anti-SARS-CoV-2 Spike RBD IgG, IgA and IgM

Ligand density can cause analyte to rebind during its dissociation. Rebinding results in an overestimation of the off-rate value. [15]. To reduce the rebinding effect of dissociating molecules, we added free RBD in a concentration of 15 µg /ml to the running buffer. In 5 minutes, we observed a mixed degree of dissociation of the various and longitudinal samples (see supplementary Figure 2) and the dissociation or off-rate constant can be calculated and plotted as a function of the days of symptoms onset (see

Figure 5). During the development of the disease, we observed a smaller off-rate indicating that the avidity or quality of the antibodies improves. So, the patients are producing a better-quality repertoire of polyclonal anti-RBD antibodies over time. For all longitudinal samples this trend in off-rate is observed (strength of binding becomes better). The method is reliable, independent of concentration, high throughput and accurate for profiling the immunity of patients. Our method revealed the trends of maturation of the overall quality of the antibodies.

SARS-CoV-2 binds the ACE2 receptor stronger in comparison to SARS-CoV. This implies that antibodies need to have high affinity to compete and neutralize the virus. Our method for profiling the immunity in terms of isotype concentration and strength of binding enables to reveal this effect in a high-throughput manner. High avidity anti-RBD antibodies at low concentration are perhaps more effective for neutralizing the SARS-CoV2 than a higher concentration anti-RBD antibodies with lower affinity.

It is also worthy of mention that this approach can be readily applied to monitoring immune response in other types of disease as well. Any protein targeted by an immune response can be immobilized to the sensor surface allowing for a very high-throughput, quantitative, and reproducible means of characterizing immunity. The real-time monitoring of signals in SPRi makes it well suited too for rapid deployment and optimization. This feature is valuable for instances where screening must be done against evolving forms of antigen, such as in the case of mutations.

In conclusion, we demonstrate a high-throughput SPR imaging platform for 384 sera suited for the rapid detection of the strength of binding of SARS-CoV-2-associated antibodies of isotypes IgG, IgM and IgA. We measured 119 sera including longitudinal samples obtained from 53 unique positively PCR-tested patients with critical, severe, moderate and mild symptoms and control sera. Although the patients show a high variation in immune response composition; generally, the strength of binding showed an affinity maturation over time.

In this workflow, the maturation effect of the affinity of antibodies can be ranked and quantified precisely with the goal of improving clinical outcomes. In addition to following the strength of binding and concentration of anti-RBD antibodies for CoViD-19 patients, this assay is ideally suited for monitoring of healthy people who will be vaccinated against SARS-CoV-2 in upcoming clinical trials. The SPRi assay described here can provide critical insights in determining if the final quality of the IgG response after vaccination is adequate to generate neutralizing antibodies with sufficient affinity for clearing the virus.

Additionally, in order to gain the highest success rate in developing therapeutic neutralizing mAb’s, individuals and donors for passive immunization programs should be screened for the highest strength of binding immune response against the immunogenic proteins of SARS-Cov-2.

Declarations

Acknowledgements

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Author Contributions

RS performed the SPRi study, analyzed the data and wrote the manuscript with input from all authors. RS, MKa, HK, LM and LT designed the study. JvW contributed to the IgA implementation and daily discussions with RS. MvA wrote the biphasic software program that was essential for calculating the antibody R_{max} values. JP performed the data analysis and measurements on the LSA equipment of Carterra. JH and MKo contributed to details of spotting and buffer compositions in the SPRi test. MKa helped to organize the manuscript and edited it extensively. AM performed the MGA-experiments with the patients in the hospital. MB supported with additional information and changed the focus of the TFF project. HK supported with patient data collected at the hospital. LT supervised and edited extensively and the clinical diagnostic study was performed under supervision of LM.

Competing Interests statement

The authors declare no competing interests

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Figures

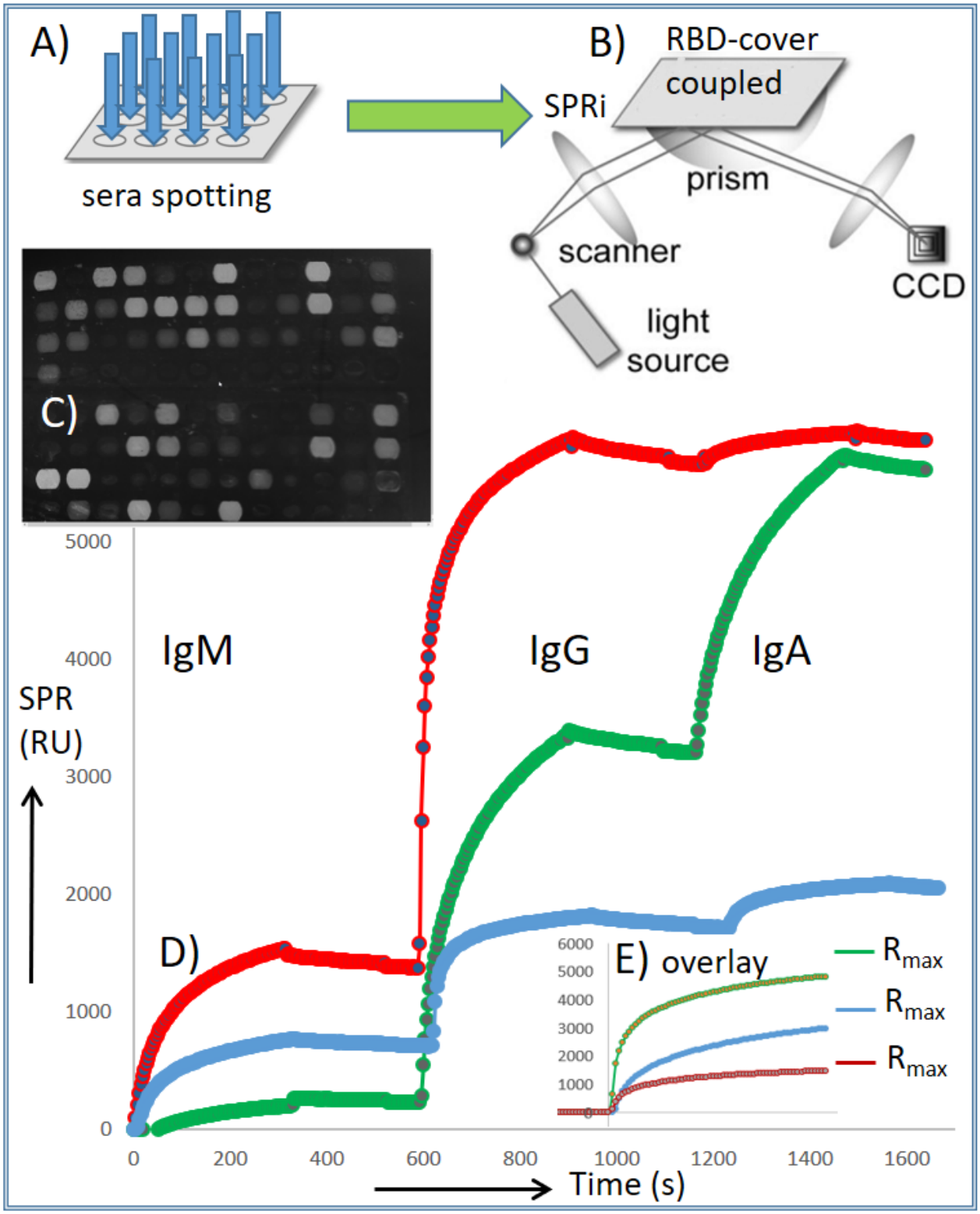


Figure 1

Principle of the anti-SARS-CoV-2 immune globulins SPRi assay. The process of spotting the sera (A) to an RBD coupled surface in the MX96 SPRi instrument (B) resulted in a SPRi reflectivity image (C). In (D) the sensorgram is shown of injections of three antisera to determine the response of IgM, IgG and IgA antibodies. In panel (E) an overlay of the injections of the anti-IgM, IgA and IgG antibodies of a single

spotted serum is presented for calculating the Rmax values of the IgM, IgG and IgA binding, after zeroing, aligning the sensorgram.

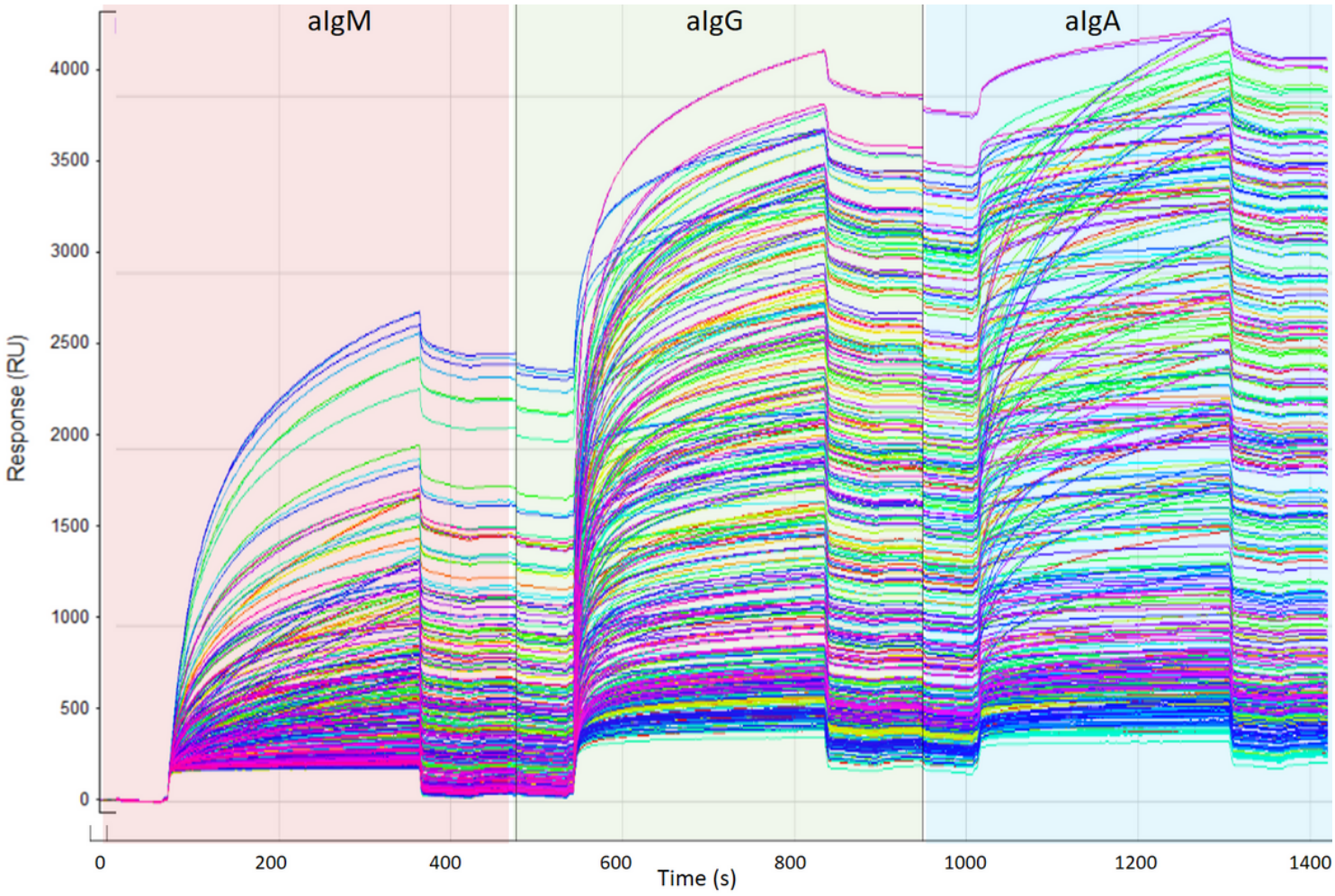


Figure 2

Raw non-referenced, non-zeroed sensorgrams of 384 spots after concatenated injections of anti-IgM, anti-IgG and anti-IgA antibodies using the LSA SPR imager. Clearly the various responses of the spotted sera can be observed.

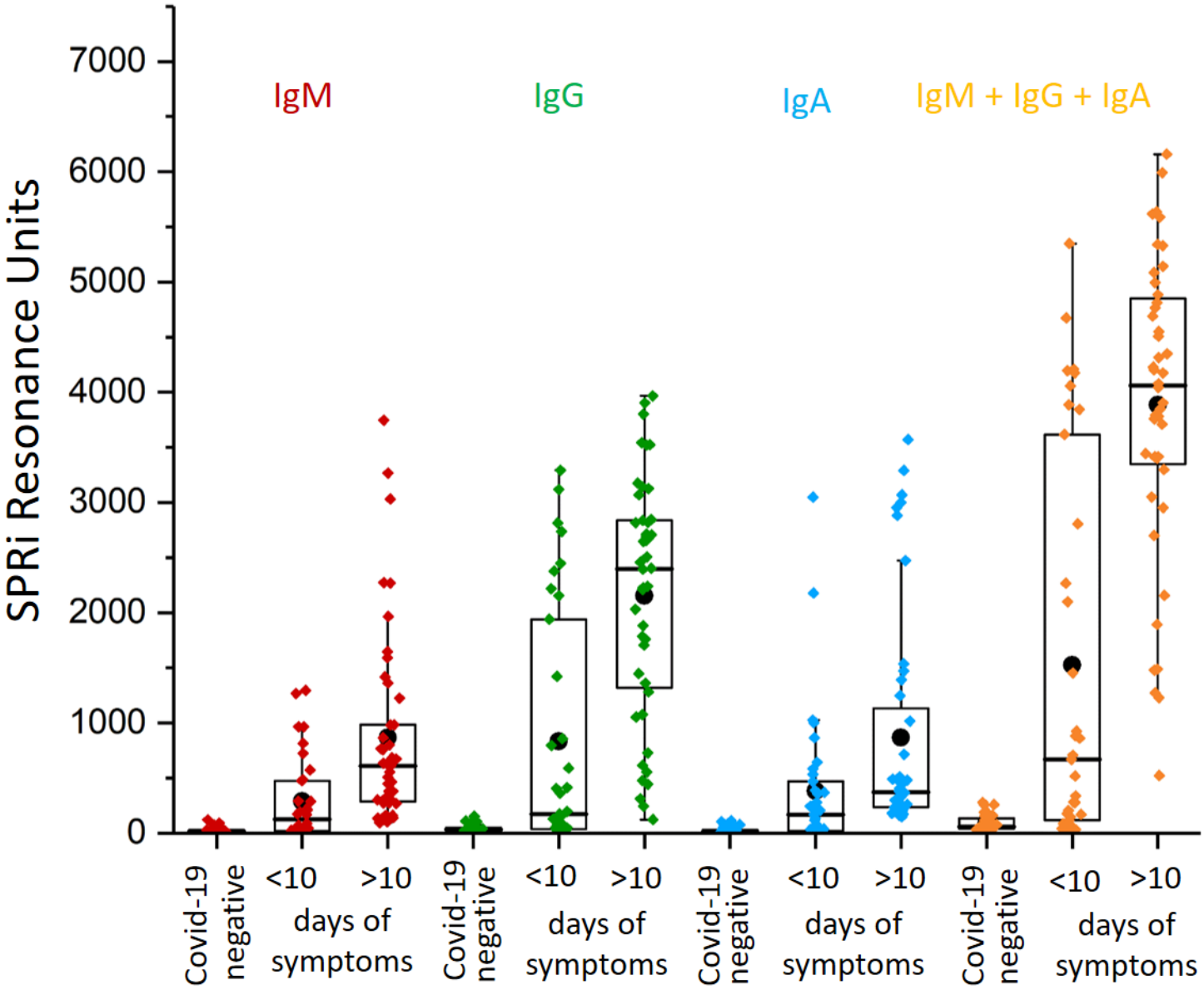
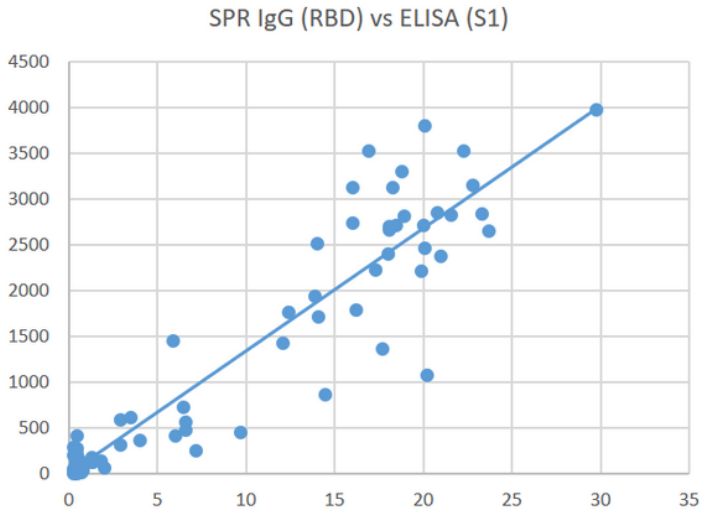
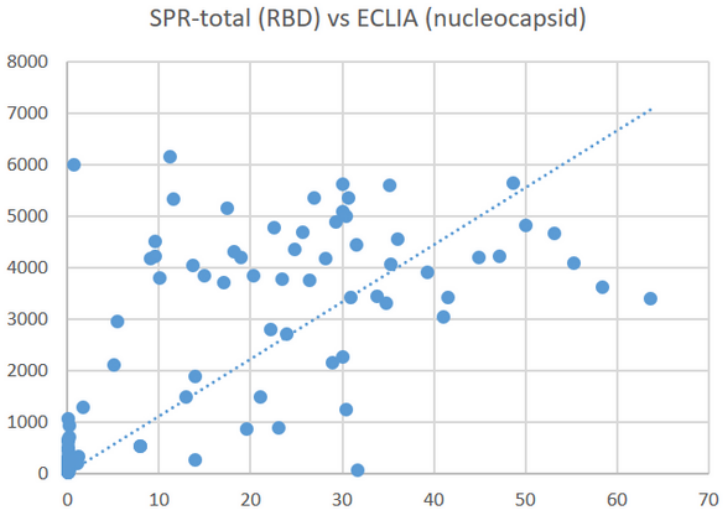
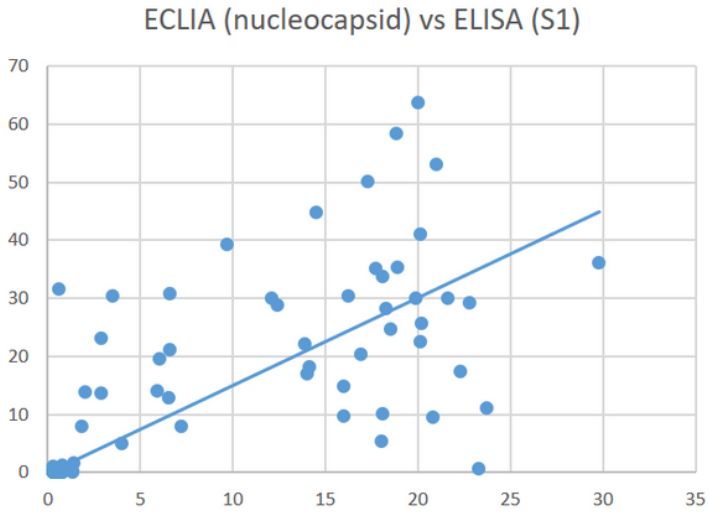


Figure 3

The IgM, IgG, IgA and total Ig values of 36 CoViD-19 PCR negative sera, 32 CoViD-19 PCR positive sera with onset of disease symptoms of less than 10 days and 45 CoViD-19 PCR positive sera with onset of disease symptoms of more than 10 days . The box plot represents the median, p25 and p75 values and the black circle the median SPRi RU value.







Parameter	Optimal threshold	AUC	Sensitivity (%)	Specificity (%)
IgM SPR	91	0.96	98	89
IgG SPR	284	0.98	90	98
IgA SPR	113	0.96	95	91
Totaal SPR	1062	0.98	93	100
IgG ELISA	0.80	1.0	100	100
Ig ECLIA	0.53	0.98	98	100

Figure 4

Correlation plot of the SPRi (in RU), ELISA (optical density, OD) and ECLIA (OD) assays including the table of optimal threshold, AUC and sensitivity and specificity of the test.

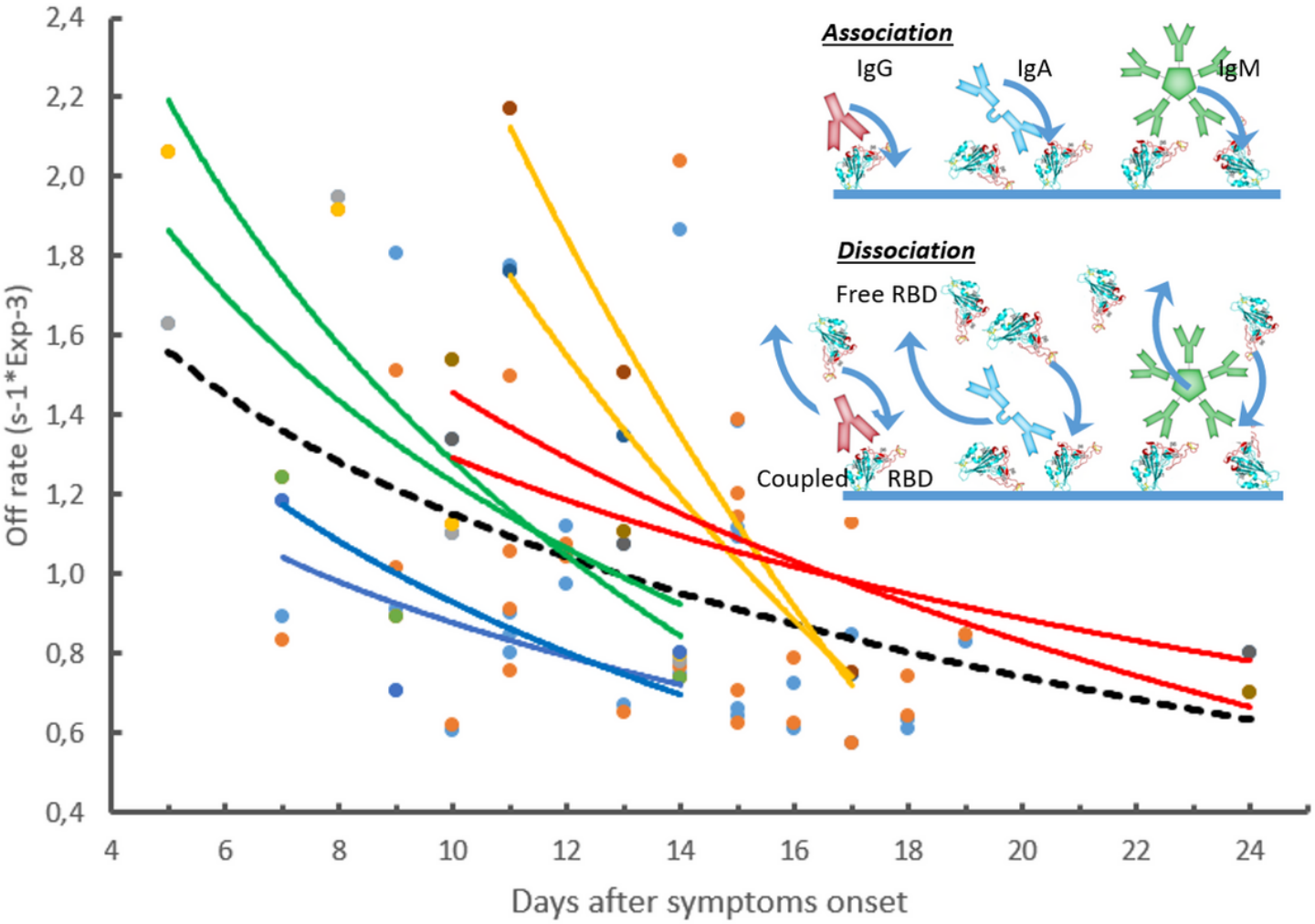


Figure 5

The dissociation constant of the anti-RBD antibodies as function of the days after symptoms onset. The black dashed line is the overall trend line of all measured samples. The colored lines connect four longitudinal samples in duplicate.

Supplementary Files

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- 20200710Supplementarydatafinal.docx
- FigureS1.jpg
- FigureS2.jpg
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- FigureS4.jpg
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- FigureS6.jpg