1   **Two-tiered SARS-CoV-2 seroconversion screening in the Netherlands and stability of nucleocapsid,**

2   **spike protein domain 1 and neutralizing antibodies**

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4   **Short title**

5   SARS-CoV-2 seroconversion screening and antibody stability

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19   **Abstract**

20   Serological testing in the COVID-19 pandemic is mainly implemented to gain sero-epidemiological

21   data, but can also retrospectively inform about suspected SARS-CoV-2 infection. We verified and

22   applied a two-tiered testing strategy combining a SARS-CoV-2 receptor-binding domain (RBD)-

23   specific lateral flow assay (LFA) with a nucleocapsid protein (NCP) IgG ELISA to assess seroconversion

24   in n=7241 individuals. The majority had experienced symptoms consistent with COVID-19, but had

25   no access to RT-PCR testing. Longitudinal follow-up in n=97 LFA+ individuals was performed up to 20

26   weeks after initial infection using NCP and spike protein S1 domain (S1) IgG ELISAs and a surrogate

27   virus neutralization test (sVNT). Individuals reporting symptoms from January 2020 onwards showed

28   seroconversion, as did a considerable proportion of asymptomatic individuals. Seroconversion for

29   symptomatic and asymptomatic individuals was higher in an area with a known infection cluster

30   compared to a low incidence area. Overall, 94% of individuals with a positive IgG result by LFA were

31   confirmed by NCP ELISA. The proportion of ELISA-confirmed LFA results declined over time, in line

32   with contracting NCP IgG titers during longitudinal follow-up. Neutralizing antibody activity was

33   considerably more stable than S1 and NCP IgG titers, and both reach a plateau after approximately

34   100 days. The sVNT proved to be not only highly specific, but also more sensitive than the specificity-

35   focussed two-tiered serology approach. Our results demonstrate the high specificity of two-tiered

36   serology testing and highlight the sVNT used as a valuable tool to support modelling of SARS-CoV-2

37   transmission dynamics, complement molecular testing and provide relevant information to

38   individuals.

39

Introduction

The novel coronavirus SARS-CoV-2 is the causative agent of the worldwide pandemic of coronavirus disease 2019 (COVID-19), which has led to millions of infections with substantial morbidity and mortality [1]. COVID-19 is characterized by a range of symptoms including cough, fever, pneumonia and a characteristic loss of smell and taste [2, 3]. The clinical manifestations of COVID-19 differ considerably and range from asymptomatic or mild self-limiting disease to severe disease and death. Next to co-morbidities predisposing to severe disease, immune hyperresponsiveness appears to be a critical factor driving COVID-19 disease severity [4, 5].

In the Netherlands, the spread of SARS-CoV-2 started in the Southern provinces, likely exacerbated by regional carnival celebrations following travel to and from Northern Italy during the school holidays [6, 7]. However, due to limited capacity at the time RT-PCR testing for SARS-CoV-2 was largely restricted to hospitalized patients with suspected COVID-19 and symptomatic individuals with moderate disease that had a recent travel history to high risk areas such as Northern Italy. Even household members of RT-PCR positive individuals were advised to self-isolate but not tested. Therefore, a large number of symptomatic individuals in The Netherlands were not tested for SARS-CoV-2 up until the start of July 2020, which left many affected individuals uncertain about whether or not their symptoms were due to COVID-19. Serological testing offers a possibility to abolish this uncertainty. In an early study, it was shown that seroconversion for anti-SARS-CoV-2 IgG and IgM occurred simultaneously or sequentially within 19 days of infection in all symptomatic COVID-19 patients analyzed [8]. The receptor-binding domain (RBD) of the spike protein of coronaviruses is a particularly interesting target for serological testing since it is the target of neutralizing antibodies [9-11], and serology tests based on the detection of these antibodies are being evaluated as indicators of protective immunity.

A crucial requirement when offering individualized serological testing, however, is a very high specificity of the test(s) selected to avoid false positives. At the early stage of the pandemic, the risk and benefit of serological tests and in particular rapid tests such as lateral flow assays (LFAs) was heavily debated [12]. Next to concerns about the performance of such rapid tests, another worry was poor registration and potential misinterpretation of the results of these tests outside controlled laboratory settings [13].

Following careful verification of a range of CE-marked serological test, Innatoss started in April 2020 to offer testing for SARS-CoV-2 antibodies in a mobile lab setting using finger prick blood. LFA results were interpreted by trained staff and applied in the context of a two-tiered testing strategy to maximize specificity. This testing strategy combined an RBD-directed LFA with strong performance

75   characteristics [14] with a highly specific anti-SARS-CoV-2 nucleocapsid protein (NCP) IgG ELISA for

76   confirmation in follow-up serum samples. This dual test approach is in line with the recommended

77   common practice for serologic testing of Lyme Borreliosis, which also serves to enhance specificity

78   [15]. Pre-screening with an LFA reduces pressure on the general health care system since it abolishes

79   the need for venous blood collection, which is then only necessary for LFA positive individuals for

80   confirmatory ELISA.

81   Here, we report the verification, performance and outcomes of this two-tiered serological testing

82   strategy applied in n=7241 individuals from mid-April to mid-August in The Netherlands. The

83   majority of these individuals had experienced symptoms consistent with COVID-19 at least 4 weeks

84   prior to testing. Our results demonstrate the high specificity and feasibility of this testing approach

85   even in times of strict anti-COVID-19 lock-down measures. We further described the kinetics of anti-

86   SARS-CoV-2 NCP and spike protein domain 1 (S1) IgG levels as well neutralizing antibodies measured

87   using a surrogate virus neutralization test (sVNT) [16] in a subgroup of individuals that underwent

88   diagnostic follow-up up to 20 weeks after initial infection. These data demonstrate that neutralizing

89   antibodies are highly stable in the time frame analyzed and that the sVNT used provides not only a

90   very specific but also highly sensitive and scalable assay to follow-up seroconverted individuals to

91   determine functional antibody responses to SARS-CoV-2.

4

92   **Materials and methods**

93   *Sample collection*

94   All blood/serum samples and data reported in this article were submitted to Innatoss for diagnostic

95   purposes. All individuals whose data are reported in this article provided written consent to the use

96   of pseudonymized test outcomes and of surplus serum samples for additional analyses. The Medisch

97   Ethische Toetsingscommissie Brabant, Tilburg, The Netherlands has waived the need for IRB

98   approval of reuse of surplus diagnostics materials (NW2020-77).

99   For the verification of the specificity of the serological tests in March and April 2020, up to n=224

100   Dutch negative control serum samples (collected before November 2019) were used. These were

101   derived from subjects tested for different diseases at Innatoss, who had given consent to re-use the

102   sample for quality control purposes. n=85 serum samples were tested in all tests verified. To acquire

103   positive control samples for sensitivity verification, n=21 Dutch individuals with a past positive RT-

104   PCR for SARS-CoV-2 as well as clinical symptoms (fever, cough) were invited to participate in the

105   verification process and donated blood by finger prick for LFA assessment. n=11 of these individuals

106   also donated blood by venipuncture to obtain serum samples for IgG ELISA sensitivity verification;

107   only 6 of these were also available for IgA ELISA verification.

108   Whole blood samples were obtained with a finger prick in Dutch individuals with suspicion of a

109   recently experienced SARS-CoV-2 infection. A mobile laboratory unit enabled the collection of

110   samples in various regions of the Netherlands without violating the COVID-19 behavioural

111   guidelines. To ensure that sufficient time had passed (>4 weeks after symptom onset) and thus that

112   antibody responses were detectable, individuals filled in a questionnaire. This questionnaire further

113   included the onset, end and type of symptoms they experienced prior to applying for the serological

114   test (common cold symptoms, cough, fever, pneumonia, loss of smell or taste). Individuals were

115   encouraged to only get tested at the mobile laboratory when they had experiencing symptoms, and

116   were required to be symptom-free for at least 2 weeks prior to testing. Additionally, some

117   individuals without symptoms requested testing when either a family member had experienced

118   symptoms or when larger groups were tested for screening purposes within a company.

119   When individuals had a positive LFA test, they were invited to undergo a venipuncture to obtain a

120   serum sample. This sample was used for measuring anti-SARS-CoV-2 antibodies by NCP ELISA. A

121   second serum sample was collected from interested individuals for diagnostic follow-up. This

122   included a group of n=97 individuals from the Dutch village of Kessel in the province of Limburg,

123   which experienced a local SARS-CoV-2 outbreak likely in the context of a meeting with hundreds of

124   attendees on 5 March 2020.

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125   To verify potential cross-reactivity of the serological tests, n=23 serum samples were used including

126   13 samples from an internal collection of Dutch donors which were sero-positive one or more of the

127   following pathogens: *C. Pneumoniae, M. Pneumoniae, Coxiella burnetii, Toxoplasma, Legionella*, EBV,

128   HSV-1, CMV, Parvo virus B19). Additionally, n=10 serum samples were purchased from ProMedDx

129   (Norton, Massachusetts, USA; n=4 human anti-mouse antibody (HAMA); n=6 rheumatoid factor

130   (RF)). The negative control serum samples used for initial verification further included n=13 with an

131   old and n=3 with a recent *Borrelia* infection.

132   **Serological tests**

133   Whole blood obtained by finger prick was tested in two commercial CE-marked LFAs: The BIOSYNEX

134   COVID-19 BSS using the SARS-CoV-2 receptor binding domain (RBD) as a target antigen (BIOSYNEX,

135   Fribourg, Switzerland) and the Xiamen Boson rapid 2019-nCOV IgG/IgM Combo test using both the

136   RBD and the SARS-CoV-2 nuclear capsid protein (NCP) (Xiamen Boson Biotech, Xiamen, China). The

137   outcomes of the LFAs were scored positive or negative based on whether the IgG/IgM and control

138   band were visible with the naked eye. Within the visibility, a division was made between 'clearly

139   visible' and "almost invisible" to distinguish in a later phase whether the intensity of the band

140   related to the levels of antibody detected by ELISA.

141   Follow-up serum samples were tested with two commercial CE-marked semi-quantitative ELISAs:

142   The EUROIMMUN SARS-CoV-2 IgG – S1 ELISA, which uses the S1 domain and the EUROIMMUN SARS-

143   CoV-2 IgG – NCP ELISA, modified to only contain diagnostically relevant epitopes (both from

144   EUROIMMUN, Lübeck, Germany). Some samples were additionally tested using the *recom*Well SARS-

145   CoV-2 IgG ELISA detecting antibodies directed against SARS-CoV-2 NCP (Mikrogen, Neuried,

146   Germany). The EUROIMMUN SARS-CoV-2 IgA – S1 ELISA was also tested. However, during

147   verification this test showed a specificity of only 89% and was therefore excluded from further

148   analysis.   The outcomes of the ELISAs were scored as follows according to the manufacturer's

149   guidelines: The EUROIMMUN ELISA results are calculated as a ratio by dividing the optical density

150   (OD) of the sample by the OD of the calibrator (Negative <0.8; 0.8 ≤ Borderline ≤ 1.1; positive >1.1).

151   The Mikrogen ELISA outcomes are expressed in arbitrary units calculated as (OD_{sample} / $OD_{cut-off control}$)

152   × 20, and test results interpreted as: Negative < 20; 20 ≤ Borderline ≤ 24; Positive > 24.

153   A subset of left-over serum samples was additionally tested using a CE-marked surrogate virus

154   neutralization assay (sVNT, cPass[TM], GenScript, Nanjing, China) for verification and comparison

155   purposes. The cPass[TM] sVNT quantifies antibodies of any isotype that interfere with the binding of

156   the SARS-CoV-2 RBD to surface-immobilized angiotensin-converting enzyme (ACE)2. The outcome of

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157 cPass^{TM} was expressed as the relative level of inhibition with a cut-off value of 0.2 (equivalent to 20%

158 inhibition). The relative level of inhibition was calculated as 1-(OD_{sample}/$OD_{negative\ control}$).

159 Finally, some individual serum samples were tested for verification and cross-reactivity using the

160 *recom*Line SARS-CoV-2 IgG immunoblot (Mikrogen, Neuried, Germany) incorporating the SARS-CoV-

161 2 NCP, S1 domain and RBD domain as well as the NCP of the four endemic coronaviruses (CoV) 229E,

162 NL63, OC43 and HKU1. The immunoblot score was evaluated by scoring the intensity of the bands

163 with the naked eye as no reaction, borderline or positive.

164 All of these tests were performed according to the manufacturer's instructions. Claimed and verified

165 characteristics of the assays are summarized in **Supporting Table S1**.

166

167 ***Statistical analysis***

168 Statistical analysis was performed using GraphPad Prism v8 (San Diego, CA, US).

169   RESULTS

170   **Selection of tests for two-tiered SARS-CoV-2 serology assessment**

171   To select the most appropriate tests for the two-tiered SARS-CoV-2 serology assessment, a number

172   of CE-marked diagnostic tests including two different LFAs and four different ELISAs were verified for

173   their specificity and sensitivity using pre-corona serum samples and samples from individuals that

174   tested positive by RT-PCR for SARS-CoV-2, respectively (**Supporting Table S1 and Supporting Figure**

175   **S1A**). Based on these data, the BIOSYNEX LFA and the EUROIMMUN NCP IgG ELISA were selected as

176   the most appropriate tests with the highest specificity.

177   A panel of cross-reactivity serum samples known to be sero-positive for other pathogens, human

178   anti-mouse antibodies (HAMA) or rheumatoid factor (RF) was tested using the combination of

179   BIOSYNEX LFA and EUROIMMUN NCP IgG ELISA. All samples were scored negative using this two-

180   tiered approach. The only sample that tested false-positive in the BIOSYNEX LFA with positive IgM

181   band was the one of the six tested RF samples with the highest RF concentration (**Supporting Figure**

182   **S1B**). Of note, the BIOSYNEX LFA has elsewhere also been reported to cross-react with RF [14]. Given

183   that the false positive result in the RF serum sample by LFA was subsequently not confirmed in the

184   NCP ELISA, the combined specificity of these two tests was 100%.

185   Despite the high specificity of the selected tests in negative control samples, a recurring question

186   was whether tests would cross-react with endemic 'common' human coronaviruses (HCoV). Our

187   approach had been to use a large group of sera collected in 2019 prior to the SARS-CoV-2 pandemic,

188   and we assumed that a large proportion would be sero-positive for one or several of the HCoVs. In

189   contrast to other diseases, control sera for common coronaviruses are not easily available. To

190   demonstrate the presence of anti-HCoV antibodies in the collection of negative control sera, we

191   employed an immunoblot that includes NCP antigens from the four common HCoVs as well as the

192   SARS-CoV-2 NCP-antigen, the S1 antigen and the S1-RBD domain. This immunoblot was used to

193   assay any negative control sample that was false positive in one of the tests verified (n=17) as well as

194   n=9 negative control samples that were negative in all other tests. All samples tested were

195   (borderline) sero-positive for at least one of the HCoV, with the exception of a single S1 IgA false-

196   positive sample (**Supporting Figure S1C**).

197

198   *National and regional outcomes of the two-tiered SARS-CoV-2 serology assessment*

199   The two-tiered testing strategy to detect seroconversion for SARS-CoV-2 was offered in a mobile lab

200   setting for finger prick LFA testing, followed by laboratory analysis by ELISA in serum obtained from

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201   those individuals with a positive LFA. In total there were n=7241 consenting individuals who were
202   tested by LFA between 14 April 2020 and 15 August 2020, including n=3156 males (43.6%), n=4028
203   females (55.6%) and n=57 unspecified individuals (0.8%). The age range of subjects was 2-95 years
204   (median 50 years, interquartile range (IQR) 40-59 years).

205   Due to supply chain issues for the BIOSYNEX LFA (the first shipment was confiscated by the French
206   authorities for priority national use), the first n=1611 individuals were tested using the Boson LFA,
207   knowing that the 2nd tier NCP IgG ELISA would filter out false positives (Supporting Figure S1A). Once
208   available in mid-May 2020, the BIOSYNEX LFA was phased in and used to test another n=5630
209   individuals (Supporting Table S2). Out of n=7241 individuals that were tested by LFA, n=1481
210   individuals tested positive by LFA (n=478 by Boson and n=1003 by BIOSYNEX). For both LFAs, 6-7% of
211   individuals failed to provide a follow-up serum sample for ELISA before August 15 (total n=97)
212   (Figure 1). The South of the Netherlands - in particular the province of North Brabant - was the
213   center of the initial spread of infection during the first phase of the SARS-CoV-2 pandemic, mainly
214   linked to travel to and from Northern Italy during the school holidays in the end of February 2020,
215   followed by regional carnival celebrations [6] (Supporting Figure S2). In line with this, the majority of
216   all individuals tested was from North Brabant (4300/7241 individuals; 59,4%). When analyzing the
217   results by area, individuals were more often positive by both LFA and NCP ELISA (35.9% of those with
218   symptoms and 8.9% of those without symptoms) in the hardly hit village of Kessel and surroundings
219   (situated in the municipality Peel en Maas in the province of Limburg) than in the area of Amsterdam
220   (15.4% of those with symptoms and 1.4% of those without; Supporting Table S2 and Figure 1). This
221   is in line with the much higher incidence of notified COVID-19 cases in Peel and Maas, which is
222   particularly striking given the much higher population density in Amsterdam compared to the rural
223   municipality of Peel en Maas (Supporting Figure S2C).

224   Of note, although some individuals who requested serological testing reported symptoms as far back
225   as October 2019, positive LFA results confirmed by ELISA were only found for individuals who
226   reported symptoms from January 2020 onwards (Supporting Figure S3A). The highest proportion of
227   individuals with self-reported symptoms that tested positive by the 2-tired LFA-ELISA strategy
228   reported symptoms in March-May 2020 (Supporting Figure S3B).

229   Focusing on the results of the more specific BIOSYNEX LFA, a change was noted over time from
230   samples being solitary IgM positive or IgG+IgM positive to solitary IgG positives (Figure 2A). LFA
231   IgG+IgM positives could be confirmed in the EUROIMMUN NCP IgG ELISA in 94% of the cases.
232   Confirmation of a solitary IgG positive band was lower (88.7%), except in the presence of a very clear
233   band in the BIOSYNEX LFA (93.7%; Figure 2B). Moreover, the proportion of BIOSYNEX LFA positive
234   samples that could not be confirmed by NCP IgG ELISA increased over time (Figure 2C).

235   When analyzing the data regardless of whether the LFA outcome was IgM+, IgM+ and IgG+, or IgG+

236   and whether a Boson or BIOSYNEX LFA was used for pre-screening, then out of all LFA+, 77.9% were

237   positive by SARS-CoV-2 NCP IgG ELISA (67,6% of Boson LFA+ and 82,8% of BIOSYNEX LFA+) and 3.9%

238   were borderline by NCP IgG ELISA (5.2% of Boson LFA+ and 3.3% of BIOSYNEX LFA+). Moreover, 11%

239   of positive LFA results were tested negative by NCP ELISA (21.1% of Boson LFA+ and 7.2% of

240   BIOSYNEX LFA+; **Supporting Table S2)**. The overall lower percentages (compared to the high

241   confirmation rates of IgG+IgM or solitary IgG+ BIOSYNEX results, **Figure 2**) are partly due to the fact

242   that an IgG ELISA was used also to confirm results with a solitary IgM band. Moreover, these results

243   confirm that the Boson LFA is less specific than the BIOSYNEX LFA and highlight the advantage of the

244   two-tiered strategy of combining an LFA for screening and an ELISA for confirmation of a positive

245   result, especially when used in combination with a less specific LFA. Not surprisingly, positive results

246   were more common in individuals that reported symptoms (18% LFA+ and ELISA+; 0.9% LFA+ and

247   ELISA borderline) than in those that did not (5% LFA+ and ELISA+; 0.5% LFA+ and ELISA borderline).

248   Those who failed to report whether they had experienced symptoms or not were clearly a mixed

249   group with 8.8% LFA+ELISA+ and 0.4% LFA+ELISA borderline.

250

251   *Stability of SARS-CoV-2 specific antibody responses*

252   As outlined earlier, the village of Kessel and its surrounding experienced a local SARS-CoV-2 outbreak

253   likely linked to a meeting with hundreds of participants on 5 March 2020. n=97 individuals from this

254   area who were sero-positive in the first round of serological testing (positive LFA followed by ELISA)

255   returned for diagnostic follow-up 19-93 days after the first measurement, enabling longitudinal

256   analysis of antibody levels. Out of these 97 individuals, 89 reported symptoms, 7 reported no

257   symptoms and 1 made no report. 86/89 individuals with self-reported symptoms specified a date of

258   symptom onset, mostly in calendar weeks 9-12 (24 February – 22 March 2020; **Figure 3A**. The first

259   serological testing was performed 39-144 days (median 65 days, IQR 55-75 days) after the onset of

260   symptoms. There was no correlation between the time lapsed from symptom onset until the first

261   serological test on the one hand, and the level of antibodies or antibody-mediated inhibition on the

262   other hand (**Supporting Figure S4**). Therefore, the absolute level of antibodies and the degree of

263   antibody-mediated inhibition did not depend on how long after the onset of symptoms the first

264   serological test was conducted.

265   Based on this and the fact that the outbreak occurred in a short window of time, changes in antibody

266   levels in the interval from first to second serological assessment were analyzed for the group as a

267   whole, regardless of the exact time of symptom onset or whether individuals reported symptoms or

268   not. While anti-SARS-CoV-2 NCP and S1 IgG levels declined significantly over time between the first

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269   and second measurement, this decline was not observed for the level of antibody-mediated
270   inhibition measured by sVNT (Figure 3B). For further analysis, the change in the level of specific
271   antibodies and antibody-mediated inhibition was calculated as log[test1]-log[test2], and an increase
272   or decrease of 0.1 was considered a substantial change. When focusing on n=85 individuals with an
273   interval of >6 weeks (47-93 days) between the two test time points, it was evident that anti-NCP IgG
274   and anti-S1 IgG levels declined significantly more than the antibody-mediated inhibition of RBD-
275   ACE2 interaction. In contrast, antibody levels measured by all three testes were stable within a
276   shorter time interval of 2-5 weeks (19-35 days) (Figure 3C). In this latter group of n=12 individuals,
277   10 reported symptoms and the first serological test was carried out much later after SARS-CoV-2
278   infection, 102-144 days after symptom onset (median 108; IQR 105-117 days) compared to 39-103
279   days after symptom onset (median 63, IQR 54-69 days) in the former group. Overall, within an
280   interval of >6 weeks there was a decline in SARS-CoV-2 specific IgG antibodies in 65% and 75% of the
281   individuals tested for NCP and S1, respectively. In total, 91% of individuals tested showed a decline
282   in NCP and/or S1 IgG levels (77/85), in contrast to only 31% of the individuals when assessing
283   antibody-mediated inhibition measured by sVNT (Figure 3D). The number of individuals that were
284   scored positive at the first and second measurement was 77/85 versus 71/85 by NCP IgG ELISA,
285   83/85 versus 73/85 by S1 IgG ELISA and 85/85 versus 83/85 by sVNT, stressing the stability of the
286   sVNT results.

287   Already in the verification phase with negative control samples collected in 2019 as well as serum
288   samples from SARS-CoV-2 RT-PCR+ individuals, the sVNT proved to be both highly specific and
289   sensitive. Performance of this functional assay was further evaluated with a random selection of
290   serum samples acquired from the mobile lab setting from n=60 LFA negative and 206 LFA positive
291   individuals in a side-by-side comparison with the BIOSYNEX LFA, EUROIMMUN NCP and S1 IgG
292   ELISAs (Supporting Figure S5). In this analysis, 8/60 LFA negative individuals had a low positive result
293   in the sVNT assay (level of inhibition 20-54%; median 32%). It was already known from the BIOSYNEX
294   LFA verification that this rapid test is only ~90% sensitive, as it misses 2/20 individuals with a prior
295   RT-PCR confirmed SARS-CoV-2 infection. In contrast, the sVNT was able to identify all 11/11 positive
296   control samples tested. Moreover, of the 206 BIOSYNEX LFA positive serum samples, 25 were
297   negative and 6 were borderline by NCP IgG ELISA. In contrast, only 15/206 BIOSYNEX LFA positive
298   serum samples were negative by sVNT, all but one of which were negative by NCP IgG ELISA. These
299   data therefore show that the highly specific and sensitive sVNT assay is able to identify additional
300   samples that contain functional anti-SARS-CoV2 antibodies that are missed when using the
301   BIOSYNEX RBD LFA for pre-screening or the NCP IgG ELISA for confirmation. This confirms the lower
302   sensitivity of those two tests, alone and in combination, which were in fact selected for their

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303   excellent specificity. Not surprisingly, inhibition levels of RBD-ACE2 interaction measured by sVNT

304   correlated more strongly with anti-S1 IgG levels than with NCP IgG level (**Supporting Figure S6**).

305   **Discussion**

306   In this study, we report the verification, performance and outcomes of a two-tiered serological

307   testing strategy combining a SARS-CoV-2 RBD-specific LFA with a confirmatory NCP IgG ELISA to

308   assess seroconversion in n=7241 individuals, the majority of which had experienced symptoms

309   consistent with COVID-19, but had no access to RT-PCR testing. Using this highly specific testing

310   approach that showed no cross-reactivity with endemic HCoVs, we demonstrate that individuals

311   reporting symptoms as far back as January 2020 showed seroconversion to SARS-CoV-2. Although

312   individuals with past symptoms seroconverted more often than those without, a considerable

313   proportion of 5% of asymptomatic individuals also showed seroconversion, and this number was

314   higher in an area with a known infection cluster (8.9%) compared to a low incidence area (1.4%).

315   Overall, 94% of individuals with a positive IgG result by BIOSYNEX LFA were also confirmed by NCP

316   ELISA. Over time, the proportion of ELISA-confirmed LFA results declined, in line with contracting

317   NCP IgG titers during longitudinal diagnostic follow-up in a subgroup of individuals. We further find

318   that in contrast to S1 and NCP IgG titers, neutralizing antibody activity is a lot more stable, and that

319   both neutralizing antibody levels and S1 and NCP IgG levels reach a plateau after approximately 100

320   days. The sVNT used to assess SARS-CoV-2 neutralizing antibodies proved to be not only highly

321   specific, but also more sensitive to identify individuals with functional anti-SARS-CoV2 antibodies

322   that are missed using the specificity-focussed 2-tiered serology assessment.

323   Overall, our results demonstrate the feasibility of this two-tiered testing approach to retrospectively

324   inform individuals about the likely cause of their symptoms early during the pandemic when limited

325   RT-PCR testing capacities left many affected individuals uncertain about whether or not their

326   symptoms were due to COVID-19. We further demonstrate that this two-tiered strategy of

327   combining an LFA for screening and an ELISA for confirmation of a positive result is perfectly feasible

328   even when used in combination with a slightly less specific LFA such as the Boson Xiamen rapid test,

329   if supplies of a more specific LFA are limited. The high specificity of the BIOSYNEX LFA makes it an

330   interesting candidate rapid test to support molecular diagnosis or help triage suspected cases when

331   there is shortage of RT-PCR capacities to help avoid missing true cases of COVID-19 and imposing

332   unnecessary quarantine [17], as well as for source tracing by identifying seroconverted individuals in

333   transmission chains that have already cleared infection and are hence not anymore RT-PCR positive.

334   In the meantime, the focus has shifted for individuals from the question whether or not they had

335   been infected by SARS-CoV-2, to whether or not this prior infection and the mounted immune

336   response would provide them with protection against future infection and thus the risk for (severe)

337   COVID-19 and transmission of SARS-CoV-2 to others. With a potential second wave of infections

338   coming up, this question is not only relevant on an individual level, but also for policy makers and

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339   health care authorities to determine how to optimally allocate potentially limited capacities for RT-
340   PCR testing and more importantly both source and contact tracing. Identifying individuals with
341   protective immunity using an easily scalable functional serological assay would allow for instance to
342   identify individuals that do not need to undergo repeated RT-PCR testing or self-isolate when
343   experiencing symptoms, and to release individuals from exposed clusters from quarantine measures.

344   Since early on in the COVID-19 pandemic, it has been suggested that serological tests could be used
345   to issue so-called 'immunity passports' [18-20]. This has caused heated discussions about potential
346   ethical, equitable and legal implications as well as public health ramifications due to potentially
347   increased/encouraged risky behavior by 'immunity passport' holders [17, 21]. One often used
348   argument in this context is that it is not yet established whether antibodies to SARS-CoV-2 confer
349   protective immunity to further infection, what amount of antibody is needed for protection or how
350   long any such immunity lasts. In the mean-time, a wealth of data has emerged that strongly supports
351   both the stability (as far as it can be evaluated up to now) and protective efficacy of neutralizing
352   antibodies to SARS-CoV-2.

353   In line with our findings, several studies have demonstrated that neutralizing antibodies as well as
354   anti-RBD IgG+ memory B-cells that can produce SARS-CoV-2 neutralizing antibodies are generated
355   and maintained in an encouragingly stable fashion for at least 3-4 months post-SARS-CoV-2 infection
356   [22-26]. Some studies have noted a contraction in levels of anti-SARS-CoV-2 IgG antibodies including
357   neutralizing SARS-CoV-2 antibody titers in the early convalescent phase up to 3 months after
358   symptom onset and even complete loss in individual cases [27-29]. However, the accumulating
359   evidence indicates that these early antibody dynamics likely only reflect the typical kinetics of a
360   primary immune response. In particular, SARS-CoV-2-specific IgG level described for a range of
361   individual patients show 'text book kinetics', with an initial peak followed by first a steep and then a
362   much more gradual decline [28]. Overall, IgM and IgA antibody responses against SARS-CoV-2 RBD,
363   S1 and NCP appear to contract rapidly until three months post symptom onset of COVID-19, while
364   circulating anti-SARS-CoV-2 IgG directed against these antigens remain much more stable [22, 24,
365   28]. Taking into account that IgA dominates the early neutralizing antibody response to SARS-CoV-2
366   [30] and that neutralizing activity can be observed before an IgG response to S1 and RBD is
367   detectable by ELISA, this strongly suggests that the initial decrease in neutralizing antibody activity
368   simply reflects the natural contraction of the short-lived plasma blast response, while a much
369   smaller population of long-lived plasma blasts is responsible for the continuous production of
370   circulating IgG antibodies [24, 31, 32]. Much in line with this is our observation that RBD-specific
371   responses detected by LFA convert from IgM to IgG over time, and that both anti-S1 and anti-NCP
372   IgG levels decrease when re-tested up to three months after symptom onset, while the levels of

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373   antibody in follow-up samples obtained 102-144 days post symptom onset are stable. This suggests

374   that stable antibody levels are reached within 100 days post infection. That neutralizing antibody

375   levels showed a lesser contraction than NCP and S1 IgG levels might also be due to the affinity and

376   avidity of anti-RBD antibodies. For very high affinity and/or avidity antibodies, a plateau of

377   neutralizing activity may be reached, so that an initial contraction of anti-RBD antibodies is not yet

378   translating in the same degree of decline in neutralizing capacity.

379   One concern is that on the longer term, antibody responses to SARS-CoV-2 might show a pattern

380   similar to that of the four circulating (endemic) coronaviruses (HCoV-229E, OC43, NL63 and HKU1),

381   to which most individuals are exposed to and seroconvert for the first time during childhood [33,

382   34]. Antibodies to HCoVs return to baseline levels within one year after natural or controlled

383   infection [35-37] and challenge infection showed full homologous protection but only limited

384   heterologous protection after 1 year [37, 38]. As a result, a 2-3 year cyclic re-infection pattern for

385   endemic HCoVs is observed [34, 37, 38], which is consistent with waning neutralizing antibody titers

386   to levels that are no longer protective. For SARS-CoV, it was observed that 50% of former patients

387   lost detectable circulating anti-SARS-CoV antibody responses after 3 years [39]. In contrast to these

388   total opsonizing antibodies, however, serum samples collected from recovered SARS-CoV patients

389   17 years after the original infection still showed neutralizing activity against anti-SARS-CoV (8/10) by

390   sVNT [16]. This indicates that neutralizing antibody levels against this more closely to SARS-CoV-2

391   related coronavirus may be more long-lived. The longevity of the neutralizing antibody response to

392   SARS-CoV-2 will need to be determined by carefully designed longitudinal follow-up studies in

393   cohorts of seroconverted individuals like ours, ideally with prospective analysis of potential re-

394   infections. A modelling study of SARS-CoV-2 transmission has demonstrated how the duration of

395   immunity to SARS-CoV-2 infection determines whether or not SARS-CoV-2 enters into regular

396   circulation after the initial pandemic wave [40]. Of note, even a duration of 'only' two years

397   compared to one year of protection would already positively affect the total incidence of COVID-19

398   over the next five years. On a population level, sero-epidemiological data on neutralizing antibody

399   levels and their longevity are therefore crucial to support modelling the impact of preventative

400   measures and post-pandemic transmission dynamics [40, 41].

401   In regards to protective efficacy, several animal models have provided evidence that SARS-CoV-2

402   infection induces protective immunity against re-challenge in rhesus macaques, Syrian hamsters and

403   ferrets [42-45], and protection from disease could be linked to neutralizing antibodies (either

404   naturally induced or passively transferred) in the Syrian golden hamster model [44, 46, 47]. In

405   humans, neutralizing antibodies were shown to correlate with protection from SARS-CoV-2 during a

406   high attack rate fishery vessel outbreak, in which three sero-positive crew members were amongst

15

407 the few that did not contract infection [48]. And even in the event of re-infection, symptoms are

408 likely to be milder or even absent, as reported for the first documented case for SARS-CoV-2

409 reinfection [49]. Consequently, passive immunization using convalescent plasma or monoclonal

410 antibodies for SARS-CoV and SARS-CoV-2 is also pursued as therapeutic approach to treat critically ill

411 COVID-19 patients [50-57] and antibody neutralization assays are used as a key read-out in SARS-

412 CoV-2 vaccination trials in non-human primates and human clinical trials [58-62].

413 Finally, in hospitalized COVID-19 patients, it was further observed that upon seroconversion,

414 shedding of infectious SARS-CoV-2 dropped rapidly to undetectable levels. Infectious virus could not

415 be isolated from respiratory tract samples once patients had a serum neutralizing antibody titer of at

416 least 1:80 measured by plaque reduction neutralization test (PRNT), which may be due to the fact

417 that infectious virions are still produced but directly neutralized by antibodies in the respiratory

418 tract. Whether the same holds true for individuals with mild disease remains to be determined.

419 Nevertheless, the authors suggested that both quantitative viral RNA load assays and serological

420 assays should be used to monitor individuals to discontinue or de-escalate infection prevention and

421 control precautions [63].

422 While PRNTs are the gold standard for assessing functional antibody activity, these assays are labor-

423 intensive and require a biosafety level 3 laboratory. In contrast, the sVNT assay employed here is

424 scalable to high throughput use and has shown good agreement with PRNT [16], and has hence been

425 highlighted as an interesting alternative to PRNT to quantify functional antibodies [64]. This sVNT

426 also used in a recently published Australian study evaluating a range of serological tests for SARS-

427 CoV-2 [65]. This study reported slightly lower sensitivity values for the sVNT, likely due to the fact

428 that serum samples from convalescent Australian COVID-19 patients were collected at an earlier

429 time point in the convalescent phase. Indeed, they report a considerable increase in sVNT sensitivity

430 depending on whether serum samples were collected within 14 days or after 14 days of symptom

431 onset. In our study, all but 2/235 of the individuals with a reported date of symptom onset that were

432 tested by sVNT provided their serum samples at least 4 weeks after symptom onset (median 62

433 days, IQR 51-86 days). Ultimately, a key question to be answered in future studies is how the level

434 of inhibition measured by sVNT at a given serum dilution compares to the level of neutralizing

435 antibody required at which shedding of infectious virus is reduced to undetectable levels. This

436 information will be highly valuable for the interpretation of the sVNT test outcome in regards to

437 protection from future infection in an 'immunity passport' scenario. Given that it is not yet known

438 how long neutralizing antibodies to SARS-CoV-2 are stable and that this may also differ on an

439 individual level, periodic re-testing will likely be required.

440 There are some limitations to our study. Firstly, it should be highlighted that our data do not reflect
441 the seroconversion dynamics in the general population, since we encouraged individuals only to get
442 tested if they had experienced symptoms consistent with COVID-19. Even amongst this group, the
443 proportions of seroconversions we find are not representative for the whole of the Netherlands:
444 Although tests were performed in almost every region of the Netherlands, the majority of tests were
445 carried out in North Brabant (the most heavily affected area in March/April 2020) and Amsterdam.
446 Secondly, using the specificity-focussed two-tiered serological testing approach applied here, we
447 were bound to miss some individuals that had seroconverted. This is exacerbated over time by the
448 fact that NCP IgG levels contract and thus an increasing proportion of individuals that show a
449 positive result by RBD-LFA cannot be confirmed by NCP IgG ELISA. Using the sVNT in combination
450 with the RBD-LFA for pre-screening may partially alleviate this limitation. However, seeing the high
451 scalability of the sVNT in combination with both its high specificity and sensitivity (provided samples
452 are collected long enough after symptom onset), it can also simply be used as a stand-alone
453 screening tool. Thirdly, as any serological screening approach, the small proportion of individuals
454 which fail to seroconvert [23, 66] and instead only mount a cellular immune response to SARS-CoV-2
455 [67] will logically not be identified. Given that the degree of the antibodies seems to be related also
456 to disease severity, this may be particularly true for individuals with mild or asymptomatic infection
457 [27, 68]. Finally, while our data indicate that stable antibody levels are reached 100 days post
458 infection, the group of individuals that was assessed >100 days post symptom onset consisted of a
459 mere n=12 individuals. Therefore, analysis in larger groups is needed to support this finding.

460 In conclusion, we herein present a careful analysis of the performance of a two-tiered serological
461 testing approach to assess seroconversion and thus retrospectively confirm exposure to SARS-CoV-2.
462 Our data support that the highly specific LFA used in this combination approach may be useful also
463 in other settings, for instance to support or complement molecular testing and source tracing of
464 SARS-CoV-2 infection in transmission chains. Finally, we confirm other studies showing the stability
465 of neutralizing antibodies to SARS-CoV-2, and provide the first longitudinal assessment of these
466 antibodies using a highly scalable sVNT. This high specificity and sensitivity assay could thus be a
467 valuable tool for serological follow-up in large cohorts to support modelling of future SARS-CoV-2
468 transmission dynamics, support health authorities and provide relevant serological information to
469 individuals.

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481

482 **Authors contributions**

483   Conceptualization and design: AG, MLCEK. Acquisition of data: DWAvdN, APFS, ESD, LSvG. Analysis

484   and interpretation of data: AG, AS, DWAvdN, MLCEK. Drafting the article: AS, DWAvdN. Revision of

485   article: AG, MLCEK. All authors have read and approved the final manuscript.

486

487 **Conflict of interest**

488   AG is a senior officer and shareholder, and AS, DWAvdN, APFS, ESD, LSvG and MLCEK are employees

489   of Innatoss Laboratories B.V., which provides diagnostic screening for infectious diseases including

490   SARS-CoV-2.

491   **Figure legends**

492   **Figure 1 – SARS-CoV-2 LFA and NCP IgG ELISA results by area.** LFA and NCP ELISA results are shown

493   for n=7241 individuals that were tested by either Boson or BIOSYNEX LFA. N=97 individuals that

494   tested positive by LFA did not provide a follow-up serum sample for EUROIMMUN NCP IgG ELISA.

495   Data are shown as the proportion of individuals tested in the indicated areas, stratified depending

496   on whether or not symptoms were reported. Symptoms include common cold symptoms, cough,

497   fever, pneumonia, loss of smell or taste.

498   **Figure 2 – Confirmation of BIOSYNEX LFA results by EUROIMMUN NCP IgG ELISA.** BIOSYNEX LFA

499   results per month of testing (n=1003), stratified into individuals with solitary IgM or IgG bands or

500   IgM+IgG+ bands (A). IgG levels of anti-SARS-CoV-2 NCP were determined during 2nd tire serological

501   follow-up in n=935 individuals that were positive by BIOSYNEX LFA. The proportion of samples

502   negative, borderline or positive by NCP IgG ELISA is shown (B) for individuals with solitary IgM or IgG

503   bands, those with both IgM+ and IgG+ bands and separately for those with a strong solitary IgG

504   band, and for (C) all LFA IgM and/or IgG positive individuals per month of testing.

505   **Figure 3 – Stability of anti-NCP, anti-S1 and neutralizing antibodies.** (A) The number of individuals

506   who reported onset of symptoms is shown per calendar week. (B) The change in IgG levels of anti-

507   SARS-CoV-2 NCP and S1 antibodies as well as the level of inhibition conferred by anti-SARS-CoV-2

508   RBD-neutralizing antibodies are plotted against the time between first and second ELISA/sVNT for

509   n=97 SARS-CoV-2 sero-positive individuals regardless of presence or time of onset of reported

510   symptoms. Data were analyzed by Spearman correlation. (C) Change in anti-NCP or anti-S IgG

511   antibodies and anti-RBD neutralizing antibodies measured by sVNT in a time interval of ≤5 weeks

512   (n=12) and >6 weeks (n=85) between first and second ELISA/sVNT. Data were analyzed by Friedman

513   test. *** $p < 0.0001$. (D) Proportion of individuals with declining, increasing or stable antibody levels

514   in the group with >6 weeks (n=85) between first and second ELISA/sVNT. Changes in antibody levels

515   were calculated by subtraction of log values (test at time point 2 minus test at time point 1). An

516   increase or decrease of 0.1 was considered a substantial change. A change of less than 0.1 was

517   considered stable (grey shaded area in B and D).
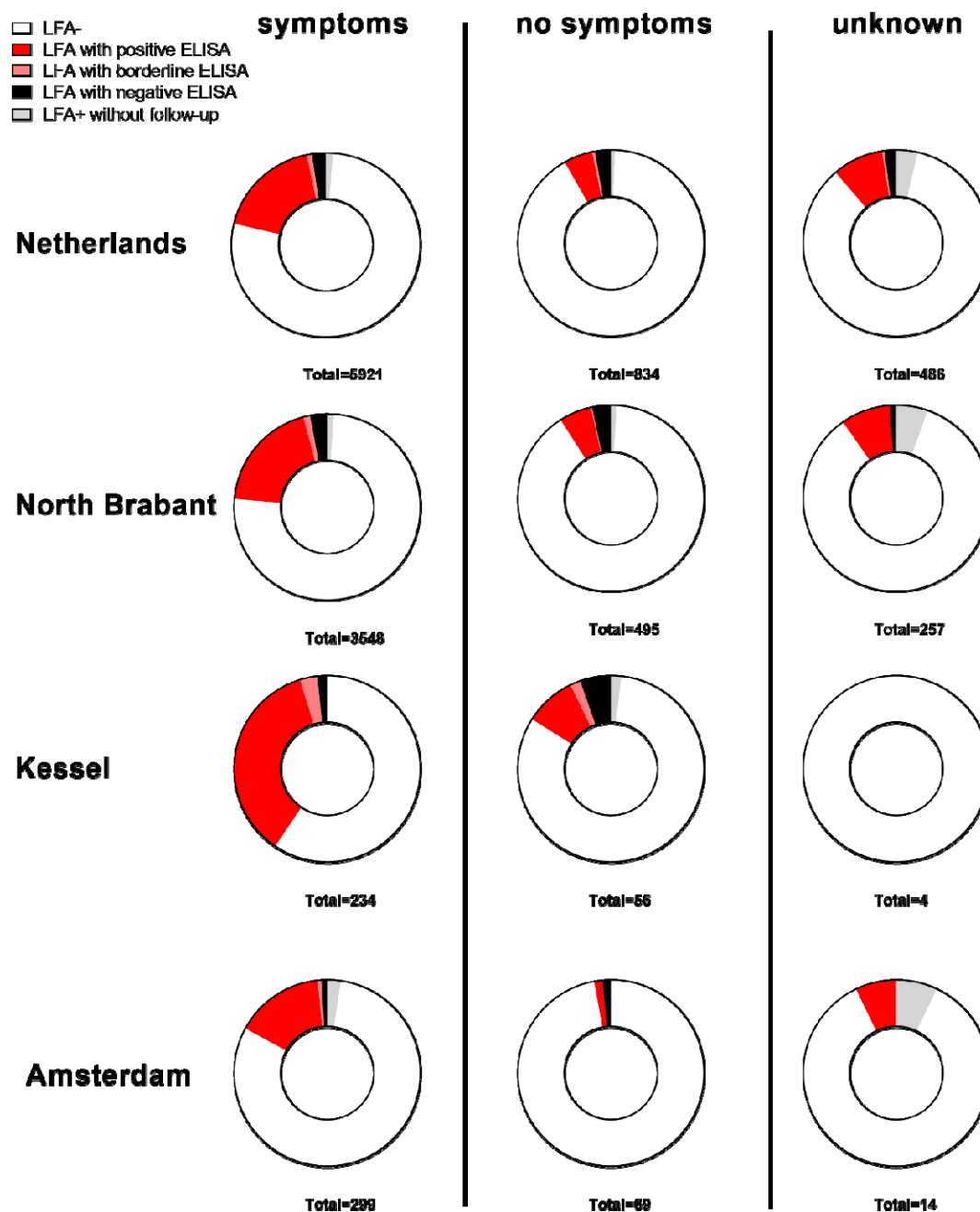
518

Figure 1 – SARS-CoV-2 LFA and NCP IgG ELISA results by area.
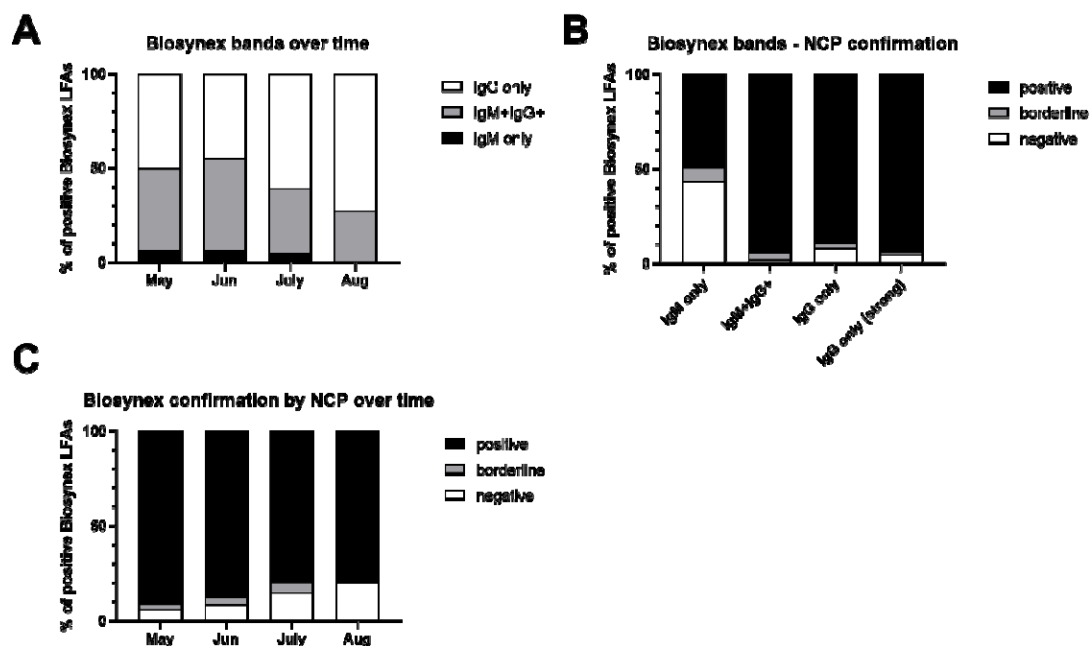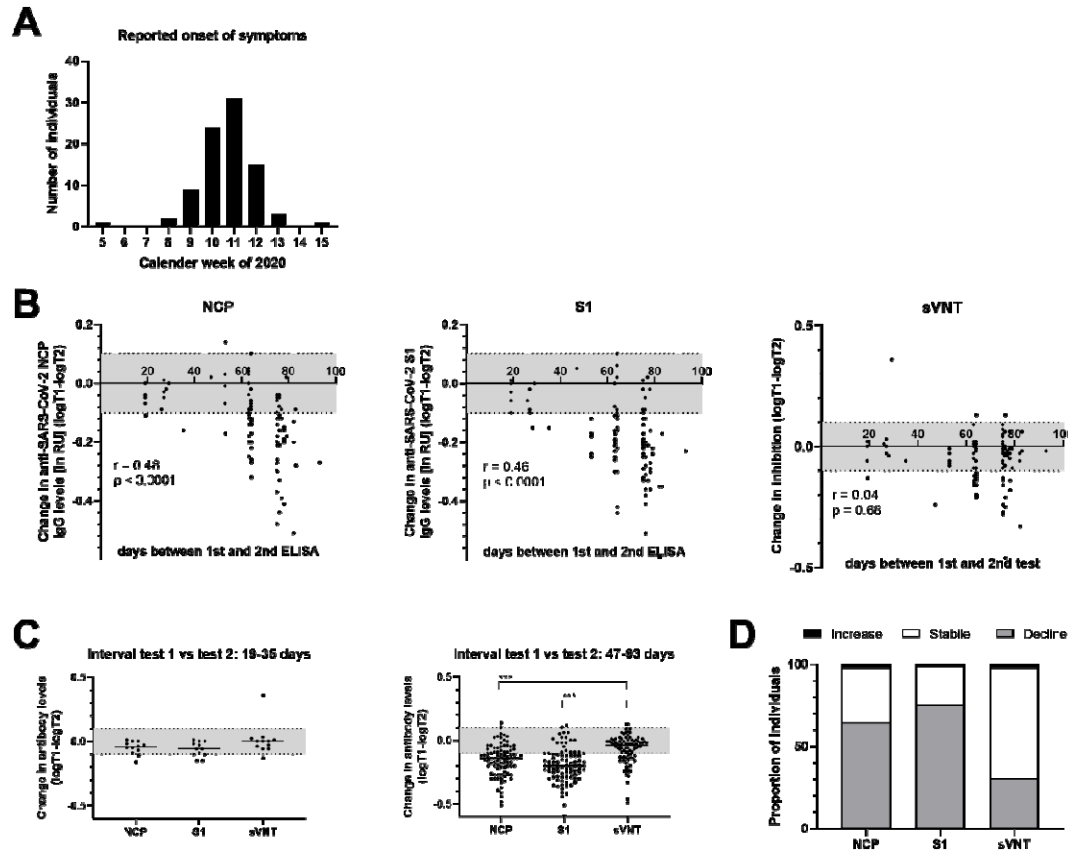
520

521   *Figure 2 – Confirmation of BIOSYNEX LFA results by EUROIMMUN NCP IgG ELISA.*

522

523   *Figure 3 – Stability of anti-NCP, anti-S1 and neutralizing antibodies.*

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